

# ENZYMATIC AND TOXIGENIC POTENTIAL OF FUNGI ISOLATED FROM COFFEE BEANS

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**ABSTRACT:** The presence of some species of filamentous fungi in coffee beans may indicate reduced quality and risks of mycotoxins. Moreover, other species may be bioprotective of the bean integrity, indicators of environmental changes and informative. The objective of this study was to evaluate the enzymatic activity and toxigenic potential of filamentous fungi isolated from 12 samples of coffee beans (11 samples of *Coffea arabica* and 1 sample of *Coffea canephora*). 182 fungi were isolated and identified belonging to two genera: *Aspergillus* and *Penicillium*. Of the 138 fungi from the genus *Aspergillus* belonging to the Section Nigri and Section Circumdati tested, 28.3 % were producers of ochratoxin A, particularly for the species *A. ochraceus* and *A. ostianus*. Of the 14 isolates of *Aspergillus flavus* tested, 78.6 % were producers of aflatoxin B1 and B2. *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Penicillium roqueforti* and *Penicillium sp.* showed an enzymatic index greater than 2 (IE > 2) for polygalacturonase and *Penicillium funiculosum*, *Penicillium* and *Aspergillus sclerotiorum aurantiogriseum* showed pectate lyase activity above 2 (IE > 2). Species of *Penicillium brevicompactum* showed potential pectinolytic for the two enzymes tested. These results demonstrate that coffee beans can be an important source of fungi with biotechnological potential and potentially toxigenic fungi have limited enzyme capacity of degrading pectin-rich substrates such as mucilage and produce mycotoxins.

**Index terms:** *Aspergillus*, *Penicillium*, coffee, mycotoxins, biotechnological potential.

## POTENCIAL ENZIMÁTICO E TOXIGÊNICO DE FUNGOS ISOLADOS DE GRÃOS DE CAFÉ

**RESUMO:** A presença de algumas espécies de fungos filamentosos em grãos de café pode indicar redução da qualidade e riscos de micotoxinas. Por outro lado, outras espécies podem ser bioprotetoras da integridade dos grãos, indicadores de alterações ambientais e indicativo. Objetivou-se, neste estudo, avaliar a atividade enzimática e o potencial toxigênico de fungos filamentosos isolados de 12 amostras de grãos de café (11 amostras de *Coffea arabica* e uma amostra de *Coffea canephora*). Foram isolados e identificados 182 fungos pertencentes a dois gêneros: *Aspergillus* e *Penicillium*. Dos 138 fungos do gênero *Aspergillus* pertencentes à Seção Circumdati e Seção Nigri testados, 28,3% foram produtores de ocratoxina A, com destaque para as espécies *A. ochraceus* e *A. ostianus*. Dos 14 isolados de *A. flavus* testados, 78,6% foram produtores de aflatoxina B1 e B2. *A. versicolor*, *Cladosporium cladosporioides*, *P. roqueforti* e *Penicillium sp.* apresentaram índice enzimático superior a 2 (IE > 2) para poligalacturonase e *P. funiculosum*, *P. aurantiogriseum* e *Aspergillus sclerotiorum* apresentaram atividade pectato liase acima de 2 (IE > 2). Espécies de *P. brevicompactum* apresentaram potencial pectinolítico para as duas enzimas testadas. Esses resultados demonstram que os grãos de café podem ser importante fonte de fungos com potencial biotecnológico e que os fungos potencialmente toxigênicos apresentam limitada capacidade enzimática de degradar substratos ricos em pectina como a mucilagem e produzir micotoxinas.

**Termos para indexação:** *Aspergillus*, *Penicillium*, café, micotoxinas, potencial biotecnológico.

### 1 INTRODUCTION

Coffee is one of the agricultural products that yields more riches in the planet (ESPADALÉ; LAMPURLANÉS; AUBERT, 2008). It is responsible for a large number of jobs in all sectors of economy, directly and indirectly employing half a billion people around the world (PASIN; ALMEIDA; ABREU, 2009).

Brazil leads the world's coffee production ranking, responsible for more than one third of all the production, followed by Vietnam and Colombia and, altogether, these countries are responsible for half the world's production (MONTEIRO et al., 2010). Minas Gerais state produces half of the national coffee (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2010).

The fruits and beans, as well as in other

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cultures, can be contaminated by microorganisms during different stages of growth, processing, transportation and storage (BATISTA et al., 2009; SILVA; BATISTA; SCHWAN, 2008). The diversity of fungi found in coffee beans depends on many factors, such as coffee variety, geographic region, weather and processing method (PERRONE et al., 2007). In cultivation system, the lack of good agricultural practices increases contaminations and development of microorganisms, among them, toxigenic fungi (BATISTA et al., 2009; DUARTE; PENA; LINO, 2010).

Presence of toxigenic fungi in coffee beans affects not only quality, as it also jeopardizes the safety of the final product due to the production of mycotoxins, which could be harmful to consumers (BERNET; KLICH, 2003; VILELA et al., 2010). Fungi associated to coffee fruits and beans can, under specific conditions, cause quality losses, producing unpleasant tastes and smells (VILELA et al., 2010). Such degradation occurs due to the complex enzymatic system produced by filamentous fungi, such as Tal cellulases, hemicellulases, xylanases, pectinases, proteases and others. Pectinases are used to degrade the pulp and mucilage of coffee and favour the development of fungi. This development could come together with the production of mycotoxins. The most common mycotoxin present in coffee is ochratoxin A (OTA) (FERRAZ et al., 2010; GIL-SERNA et al., 2011). OTA is produced, mostly, by fungi species of the genus *Aspergillus* belonging to the sections *Circumdati* and *Nigri* and *Penicillium* (*P. nordicum* and *P. verrucosum*) (GIL-SERNA et al., 2011). OTA has nephrotoxic, teratogenic and, possibly, carcinogenic actions (INTERNATIONAL AGENCY FOR RESEARCH ON CANCER - IARC, 1993), being detected in many food products, such as cereals, nuts, coffee beans, cocoa, dried fruits, spices, wine and beer (BATISTA et al., 2009; PERRONE et al., 2007; SILVA et al., 2007).

Besides secondary toxic metabolites, filamentous fungi could be enzyme producers of biotechnological importance. The pectinases produced by fungi show important characteristics for application in bioprocesses (MALVESSI; SILVEIRA, 2004). Pectinolytic enzymes are responsible for the degradation of a complex pectine molecule present in all of the young vegetal tissues. Among the species of fungi capable of producing pectinolytic enzymes are: *Alternaria mali*, *Aspergillus aculeatus*, *A. awamori*, *A.*

*japonicus*, *A. nidulans*, *A. niger*, *A. oryzae*, *A. tubingensis*, *Colletotrichum lindemuthium*, *Fusarium moniliforme*, *F. oxysporum*, *Neurospora crassa*, *Rhizopus stolonifer* (JAYANI; SAXENA; GUPTA, 2005). The use and treatment of agroindustrial residues by enzymatic bioprocesses not only provide alternative substratum, which will be converted in products of high commercial value, as also help lower environmental problems (SOCCOL; VANDENBERG, 2003). The objective of this work is to study the biodiversity and analyse, semiquantitatively, the pectinolytic and toxicogenic enzymatic activity of different species of filamentous fungi isolated in coffee beans.

## 2 MATERIALS AND METHODS

### Samples

Twelve samples of green beans (500g) were studied, obtained at the Cooperativa Alto Rio Grande Cafés, in Lavras, MG, from which eleven samples were of *Coffea arabica* L. and one sample of *Coffea canephora* Pierre ex. A. Froehner.

### Isolation of filamentous fungi

For the isolation of fungi, the technique used was direct plating in mean of culture Dicloram Bengal Rose Chloramphenicol (DRBC) (10g glucose; bacteriological peptone 5g, 1g KH<sub>2</sub>PO<sub>4</sub>; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.5 g, agar 15g, distilled water 1L; rose bengal 15mg; dichloran 2 mg, chloramphenicol 100g), being used, for each sample, 100 superficially disinfected beans (alcohol 70% and NaCl 1%) and 100 beans without disinfection, as in Samson et al. (2000). Isolated fungi were purified in malt extract (MA) 2% and kept at 25°C, during seven days. After that, the isolated were cultivated in specific means for identification as in Klich (2002), Pitt (2000), Pitt and Hocking (1997) and Samson et al. (2000). Purification and identification of filamentous fungi were performed in the Mycotoxins and Mycology of Foods Laboratory, at the Department of Food Science of the Lavras Federal University.

### Ochratoxigenic and aflatoxigenic Potential of isolated fungi of coffee beans

To determine toxigenic potential of the species was used the methodology of Plug Agar, described by Filtenborg and Frisvad (1980).

### Biotechnological potential

To detect the pectinolytic activity, we used the culture medium described by Hankin et al. (1971), containing citrus pectin as a substrate.

The medium at pH 7.0 was used to detect the production of pectate lyase at pH 5.0 and to evaluate the activity of polygalacturonase. To visualize the pectinolytic activity was added to the culture medium a solution of 1% hexadecyltrimethylammonium bromide (cetrimide 1%).

The ability to degrade pectin was determined by the production of pectinolytic enzymes: pectate lyase and polygalacturonase in media with pH optimum for each enzyme, and pH 7 and pH 5, respectively. Enzyme activity was detected by the formation of a clear halo around the colony, viewed with the addition of reagent 1% cetrimide (Figure 2a and 2b). This reagent precipitates intact pectin in the middle, allowing the formation of clear zones around the colonies, where there was degradation of pectin. Thus, as the ratio of halo to colony size, it was possible to quantify (semiquantitatively) the activity of pectinolytic fungi analyzed. The rate of enzyme activity is the parameter semiquantitative more used to evaluate enzyme production by microorganisms in solid medium. Microorganisms considered enzyme producers have direct correlation between the diameters of degradation and ability degrading microorganisms (LIN; CHANG; SHEN, 1991). Filamentous fungi considered potentially useful for biotechnological use have an index assay (IE - the ratio of the diameter of the halo by the diameter of the colony) above 2.0 (LEALEM; GASHE, 1994; STAMFORD; ARAÚJO; STAMFORD, 1998).

### 3 RESULTS AND DISCUSSION

The average level of contamination of the samples was 62% with superficial disinfection and 100% without surface disinfection. These values were expected because, with the superficial disinfection, only the fungi inside the grains are detected. All the samples showed contamination by several species of fungi. However, when it held the disinfection of surface grains, reduction in the rate of contamination by filamentous fungi and higher presence of yeasts (Figures 1a and 1b) as already observed by Batista et al. (2003).

In all samples, showed the highest contamination rate was *Coffea canephora* sfor surface disinfection. Species of filamentous fungi isolates, 50.5% belonged to genus *Aspergillus* Section *Nigri* and *A. niger* was more frequent. In this study was not detected the species *A. carbonarius* which is described as a species of the genus *Aspergillus* Section *Nigri* with greater production potential of ochratoxin A (PARDO et al., 2004).

The contamination with this species is slightly higher in Conillon than in coffee arabica (NOONIM et al., 2008; PARDO et al., 2004). This statement was reinforced by the studies of Taniwaki et al. (2003), that found 62,95% *A. niger* and 6,19% *A. carbonarius* in coffee beans and identified as ochratoxigenic only 3% of the isolated *A. niger* while the isolated *A. carbonarius* this proportion was 77%. Fungi of the genus *Aspergillus* Section *Nigri* are economically important due to the production of ochratoxin A, and production of enzymes that are used in industry, for example those produced by *A. niger* (PERRONE et al., 2007; SAMSON et al., 2007; SAMSON; HONG; FRISVAD, 2006).

The main species identified belong to the genus *Aspergillus* and *Penicillium*. The isolated species of the genus *Aspergillus* were: *Aspergillus flavus*, *A. foetidus*, *A. lacticoffeatus*, *A. niger*, *A. niger* Agregado, *A. ochraceus*, *A. oryzae*, *A. ostianus*, *A. sydowi*, *A. tubingensis*, *A. versicolor*, *A. wentii*, *A. westerdijkiae* and *Aspergillus* sp. The isolated species of the genus *Penicillium* were: *Penicillium brevicompactum*, *P. funiculosum*, *P. hirsutum*, *P. roquefortii* and *Penicillium* sp, these isolated were also reported by other authors (BATISTA et al., 2003, 2009; GIL-SERNA et al., 2011; SILVA; BATISTA; SCHWAN, 2008).

One hundred and thirty-eight isolated of genus *Aspergillus*, Section *Nigri* (n = 99) and *Aspergillus* Section *Circumdati* (n=39) were tested for production of ochratoxin A. Only one isolate of the species *A. niger* was producer of ochratoxin A. 97.4% of isolates belonging to Section *Circumdati* were potentially ochratoxigenic, highlighting *A. ochraceus*, *A. ostianus* and *A. westerdijkiae* that 100% of the isolates were OTA producers. These results are similar to those found by Batista et al. (2009) which indicated the species *A. ochraceus* as the main ochratoxigenic species isolated in coffee fruits and beans. *A. ostianus* and *A. westerdijkiae* can be ochratoxin A producers and be isolated in coffee beans (BATISTA et al., 2003; FRISVAD et al., 2004; GIL-SERNA et al., 2011). From the total of 14 isolates of *Aspergillus* Section *Flavi*, 78,6% were aflatoxin producers. Similar results were obtained by Batista et al. (2003) and the species tested was *A. flavus* (Table 1).

The presence of these species does not necessarily indicate the presence of ochratoxin A and aflatoxin B1 and B2 in samples of coffee beans. A number of factors are involved in the synthesis of secondary metabolites of filamentous fungi, such as the chemical composition of coffee, water activity, environmental factors such as temperature and humidity (PATERSON; LIMA, 2010).



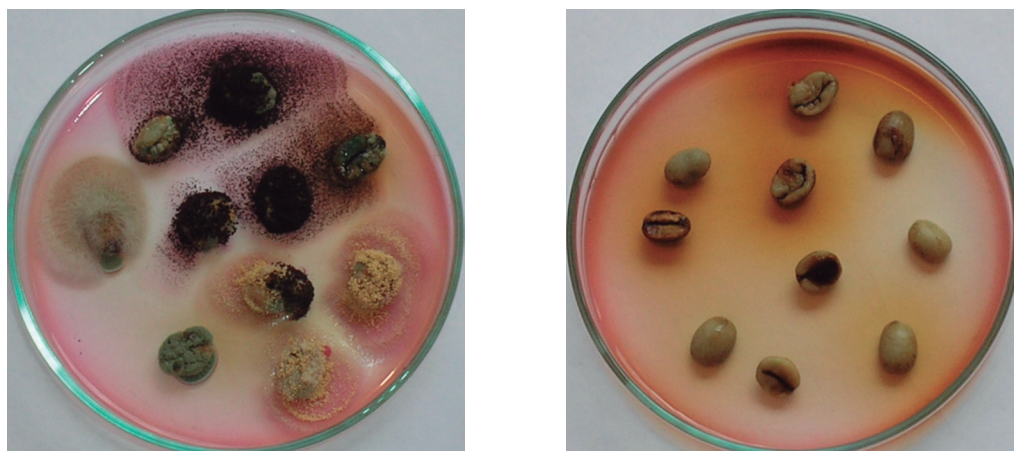


FIGURE 1 - (a) Coffee beans without the disinfection process, (b) Coffee beans with the disinfection process.

TABLE 1 - Ochratoxin A producing fungi species.

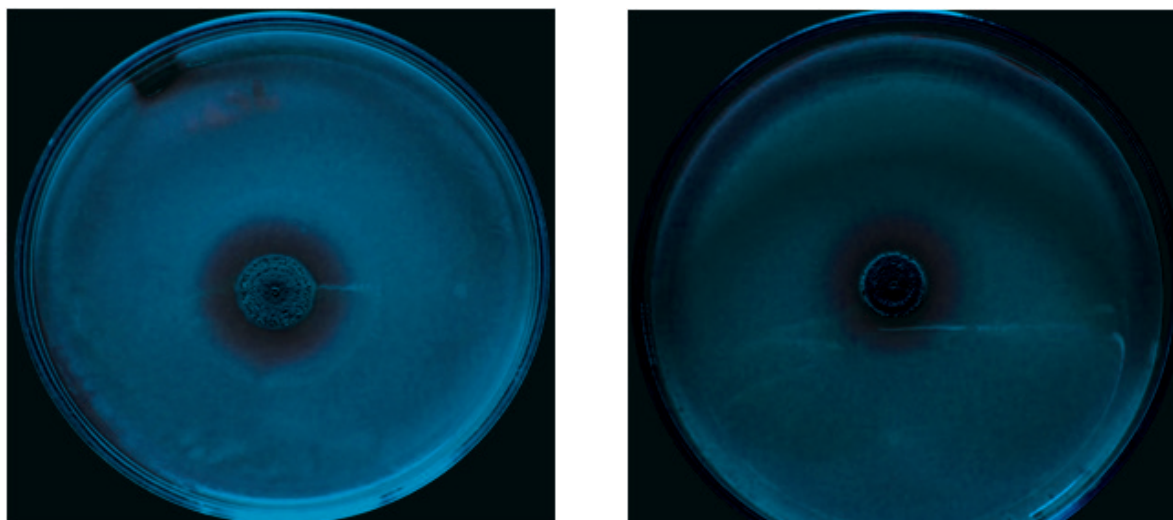
Species	N° of isolates tested	N° of potentially toxigenic isolates
		OTA
<i>Aspergillus aculeatus</i>	1	-
<i>Aspergillus foetidus</i>	44	-
<i>Aspergillus lacticoffeatus</i>	1	-
<i>Aspergillus niger</i>	24	1 (4,17%)
<i>Aspergillus niger Agregado</i>	2	-
<i>Aspergillus ochraceus</i>	27	27 (100%)
<i>Aspergillus ostianis</i>	7	7 (100%)
<i>Aspergillus sclerotiorum</i>	1	-
<i>Aspergillus sp.</i>	1	-
<i>Aspergillus tubingensis</i>	26	-
<i>Aspergillus westerdijkiae</i>	4	4 (100%)

The species producing mycotoxins is essential for adoption of phytosanitary control measures and ensure the safety of products (SILVA; BATISTA; SCHWAN, 2008).

Of the 182 analyzed for fungal enzyme potential, 42 showed pectinolytic activity  $\geq 2$ . From 32 *P. brevicompactum* isolates tested, 18 showed pectinolytic potential for both the enzymes tested, pectate lyase and polygalacturonase (Figure 2a and 2b) the 4 isolates of *A. versicolor*, 75%, showed potential for polygalacturonase. *Cladosporium cladosporioides*, *P. roqueforti* and *Penicillium sp.* showed good activity to polygalacturonase and *P. funiculosum*, *P. aurantiogriseum* and *A. sclerotiorum* showed pectate lyase activity  $\geq 2$  (Table 2).

Forty-seven percent of the isolates were producers of pectinases, although none of them presented activity exceeding 2. The species showing Enzyme Index less than 2 were: *Aspergillus aculeatus*, *A. flavus*, *A. foetidus*, *A. fumigatus*, *A. lacticoffeatus*, *A. niger*, *A. niger Agregado*, *A. ochraceus*, *A. oryzae*, *A. ostianus*, *A. sydowii*, *A. tubingensis*, *A. versicolor*, *A. wentii*, *A. westerdijkiae*, *Aspergillus sp.*, *Eurotium chevalieri*, *Penicillium citrinum*, *P. hirsutum*, *P. pinophilum*, *P. hirsutum* and *Penicillium sp.*

The species of *Penicillium brevicompactum* were producers of pectate lyase and polygalacturonase.



**FIGURE 2** - Enzyme potential of tested fungi. (a) *Penicillium brevicompactum* pH 5; (b) *Penicillium brevicompactum* pH 7.

**TABLE 2** - Species of fungi producers of pectate lyase and polygalacturonase.

Identification	Species	Pectate lyase	Polygalacturonase
DCA01	<i>Penicillium brevicompactum</i>	2,10	3,20
DCA02	<i>Penicillium brevicompactum</i>	2,20	2,90
DCA03	<i>Penicillium brevicompactum</i>	2,80	2,40
DCA04	<i>Penicillium brevicompactum</i>	2,00	2,50
DCA05	<i>Penicillium brevicompactum</i>	2,00	2,40
DCA06	<i>Penicillium brevicompactum</i>	1,50	3,60
DCA07	<i>Penicillium brevicompactum</i>	1,90	2,70
DCA08	<i>Penicillium brevicompactum</i>	1,70	2,70
DCA09	<i>Penicillium brevicompactum</i>	2,60	1,80
DCA10	<i>Cladosporium cladosporioides</i>	1,80	3,40
DCA11	<i>Aspergillus versicolor</i>	1,30	2,20
DCA12	<i>Aspergillus versicolor</i>	1,10	2,50
DCA13	<i>Aspergillus versicolor</i>	1,60	2,50
DCA14	<i>Penicillium roqueforti</i>	1,10	3,20
DCA15	<i>Penicillium</i> sp.	1,10	2,00
DCA16	<i>Penicillium funiculosum</i>	2,00	1,40
DCA17	<i>Penicillium aurantiogriseum</i>	2,00	1,60
DCA18	<i>Aspergillus sclerotiorum</i>	2,00	1,20

This species has been cited as producing pectinases (PIANZZOLA; MOSCATELLI; VERO, 2004). According to Varavallo et al. (2005), *P. brevicompactum* has potential for industrial application due to their efficient production of pectinolytic complex. *Cladosporium cladosporioides* has the potential to produce polygalacturonase. The presence of this species in the fruits may be related to the good quality of coffee (PEREIRA; PFENNING; CASTRO, 2005). *Cladosporium* growth acts as a barrier to the entry of other harmful fungi quality of the coffee, so the presence of *C. cladosporioides* can be considered positive for the safety of the coffee (CHALFOUN, 2010; CHALFOUN et al., 2009; MARTINS; SILVEIRA; SILVA, 2001). *C. cladosporioides* characterized by being a saprophyte fungus found in maximum intensity when the fruits are in the maturation stage, but you can find it at other stages of fruit (PEREIRA; PFENNING; CASTRO, 2005). Being a fungus often present in coffee, it can have a big role biotechnology to treat waste from coffee production. It is also suggested that further research be conducted in order to use *P. brevicompactum* for biotechnological processes, mainly for the degradation of pectin in the agro-industrial coffee residues (pulped and dismucilaged), since this is a microorganism present in the environment in large quantities.

#### 4 CONCLUSIONS

The results of this study demonstrate that there is great diversity of filamentous fungi in coffee beans (*Coffea arabica* and *Coffea canephora*). Were identified as species producing ochratoxin A, *Aspergillus ochraceus*, *A. ostianus*, *A. westerdijkiae* and *A. niger* and, as a producer of aflatoxin B1 and B2, *A. flavus*. The mycobiota studied had several species with enzymatic activity, confirming the potential of biotechnology for future studies aiming to use these filamentous fungi in the food industry and agribusiness. These results also demonstrate that the potentially toxigenic fungi have limited enzymatic capacity to degrade coffee fruits substrates rich in pectin.

#### 5 THANKS

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