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Aspergillus flavo furcatis: Aflatoxin test and milk-clotting protease production in submerged and solid state fermentation

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Proteolytic enzymes are metabolites that can be produced by microbial sources and develop important functions in food industry as in cheese manufacturing. However, it is necessary to ensure the final product safety by testing toxins production by microorganisms. As a result of this, the aim of this study was to investigate the production of proteases by *Aspergillus flavo furcatis* DPUA 1608 in submerged and solid state fermentation and also certify the non-production of aflatoxin by this species. The aflatoxin test was carried out using the method of ammonia vapor. In this test, *A. flavo furcatis* DPUA 1608 was inoculated in seven different media cultures (COA, YES, CZ, CYA, GMS, PMS and PDA) and the production of toxins was confirmed by the color change of culture reverse after adding a 25% (w/v) ammonia solution. The protease production was conducted using four inoculums (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC) in three media cultures of submerged fermentation obtained by a base mineral solution (MA01, MAGli and MASac) and in solid state fermentation using açai seeds and rice bran as substrate (SAFA). According to ammonia vapor test, *A. flavo furcatis* is not an aflatoxin producer. There was no color change in the colonies reverse of any culture media. All crude extracts obtained in both fermentations were tested for protease production. The best protease activity was observed in the medium MA01 (inoculums SAB+SAC, BDA+GLI and BDA+SAC). Milk clotting activity was determined in all crude extracts of submerged and solid fermentation. However, the clot formed was considered as strong milk coagulation only in the media MA01 of submerged fermentation (inoculums SAB+SAC, BDA+GLI and BDA+SAC) and in all inoculums of solid state fermentation (SAFA). *A. flavo furcatis* DPUA 1608 showed potential milk-clotting protease production in both fermentations media used.

Key words: Ammonia vapor test, *Aspergillus*, milk-clotting enzymes, liquid fermentation, solid fermentation.

INTRODUCTION

Proteases are enzymes capable of hydrolyzing the peptide bond in a protein molecule. They represent one of the largest groups of industrial enzymes with increasing market demands due to their applications

in industrial process like in cheese manufacturing using rennet enzymes (Mandujano-González et al., 2016; Sandhya et al., 2005). Since the limited availability of mammalian rennet and most rennet of plants are unsuitable because they impart a bitter taste to cheese, microbial sources can be potential as substitute to animal rennet. Among the microbial sources, fungi as enzyme producers have many advantages, since they are normally generally regarded as safe (GRAS) strains and the produced enzymes are extracellular which makes its easy recuperation from fermentation broth (Ayana et al., 2015; Sandhya et al., 2005).

Aflatoxins are carcinogenic fungal metabolites commonly produced by some species of *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus* (Blankson and Mill-Robertson, 2016; Kamika et al., 2016). These fungi can infect a number of foods and feeds such like peanuts and maize and cause contamination (Saito and Machida, 1999).

Aspergillus flavo furcatis is anamorphic fungi that have shown potential as enzyme producer (Teixeira et al., 2012). A recent study reported the production of milk-clotting enzymes by the species in submerged fermentation (Alecrim et al., 2015). On Czapek's solution agar, its colony spread rapidly, attaining a diameter of 6.0 to 7.0 cm in 10 to 12 days at room temperature (24-26°C) and showing dark olive-buff through brownish olive color when young, becoming sepia to mummy brown (Raper and Fennell, 1977).

Submerged fermentation (SmF) is a well-known process to produce enzymes by the growing of microorganisms in liquid substrate or in the presence of excess water while solid-state fermentation (SSF) involves the growth of microorganisms on solid substrates in the absence or near absence of free water in the space between particles (Bensmail et al., 2015; Singhania et al., 2010). Because of need for new microbial milk-clotting proteases sources due the shortage of rennet and the increase demand for cheese, the aim of this study was to investigate the protease production by *A. flavo furcatis* DPUA 1608 in submerged and solid state fermentation and also certify the non-production of aflatoxin by this species.

MATERIALS AND METHODS

Microorganisms

The culture of *A. flavo furcatis* DPUA 1608 (Culture Collection DPUA/Federal University of Amazonas-UFAM) was inoculated in test tubes containing (w/v): 1.0% sucrose, 1.0% meat peptone and 1.5% Agar and maintained at 30°C for seven days (Klich and Pitt, 1988).

Aflatoxin test by ammonia vapor

The aflatoxin test was carried out using the method reported by Saito and Machida (1999). The toxin production was confirmed by color change at colony reverse in the media used. Seven different media were used to determine the color change: Coconut (COA), yeast extract-sucrose (YES), Czapek (CZ), Czapek-yeast extract (CYA), glucose-mineral salts (GMS), peptone-mineral mix (PMS) and potato-dextrose agar (PDA). The strain (4 days growth) was inoculated at the center of solidified agar medium in Petri dishes and incubated at room temperature (25°C). To observe the color change of colony reverse, the dishes were placed upside down and a drop (0.2 ml) of 25% (w/v) ammonia solution was put into the lid of the Petri dish. The samples were prepared in triplicate.

Inoculum

Four different inoculums of *A. flavo furcatis* DPUA 1608 were used to select the best one after the fermentation processes. The strain was inoculated in test tubes containing: [1] glucose+meat peptone (SAB+GLI) [2] sucrose+meat peptone (SAB+SAC) [3] potato+glucose (BDA+GLI) and [4] potato+sucrose (BDA+SAC). The cultures were incubated at 30°C for 7 days and after this period, solutions of 10^5 mL^{-1} spores were prepared for use in each fermentation medium.

Fermentation media and culture conditions

The production of milk-clotting enzymes was made by submerged and solid state fermentation. The submerged fermentation was conducted by using three media cultures (MA01, MAGli and MASac) based on a mineral solution (MA01) ($g.L^{-1}$): [KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); yeast extract (1.0) and gelatin (5.0)]. In MAGli medium was added 1% (w/v) glucose in the mineral solution (MA01) and in MASac medium was added 1% (w/v) sucrose in the mineral solution (MA01) (Table 1). The fermentation was performed in 125 mL Erlenmeyer flasks containing 50 ml of sterilized medium with the spore solution (10^5 mL^{-1} spores) of all inoculums (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC), incubated at 30°C, and 180 rpm for 72 h. The biomass was separated from the crude extract by vacuum filtration on Whatman filter paper number 1. The solid-state fermentation was carried out using two local agro industrial residues: açai seeds and rice bran (SAFA) [9:1]. The mixture (50 g, 60% humidity and pH 6.0) was distributed in 500 mL Erlenmeyer flasks and sterilized at 121°C for 15 min. After cooling, the flasks were inoculated using the same spore solutions (inoculums) of the submerged fermentation. The flasks were maintained at 30°C for 5 days. The enzymes were extracted by adding 250 mL of sterilized distilled water and submitted to agitation (180 rpm) for one hour. The biomass was separated from the crude extract by vacuum filtration on Whatman filter paper number 1.

Proteolytic activity assay

The proteolytic activity was determined according to the

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Table 1. Submerged fermentation media based on a mineral solution (MA01).

Medium	Medium composition (g.L $^{-1}$)
MA01	KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); Yeast extract (1.0), Gelatin (5.0)
MAGli	KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); Yeast extract (1.0), Gelatin (5.0), Glucose (10)
MASac	KH_2PO_4 (2.0); $(NH_4)SO_4$ (1.0); $MgSO_4.7H_2O$ (0.1); $Na_2HPO_4.7H_2O$ (0.9); Yeast extract (1.0), Gelatin (5.0), Sucrose (10)

MA01 = Mineral soution base medium; MAGli = Mineral solution base medium+glucose; MASac = Mineral solution base medium+sucrose.

method described by Leighton et al. (1973). The crude extracts (0.15 mL) were mixed with 1.0% (w/v) azocasein (0.25 mL) in 0.2 M Tris-HCl buffer, pH 7.0. The incubation was made in absence of light for one hour. The reaction was stopped by adding 1.2 mL of 10% (w/v) tricloroacetic acid (TCA), centrifuged at 10 000 rpm and the supernatant (0.8 mL) was transferred to 1.4 mL of 1 M NaOH. One unit of proteolytic enzyme was defined as the amount of enzyme that produces a 0.1 increase of absorbance in 1 h at 440 nm. All samples were prepared in triplicate.

Milk-clotting protease assay

Milk-clotting activity was determined according to Alecrim et al. (2015) using 10% (w/v) skimmed milk powder in 0.05 M $CaCl_2$, as substrate. The milk solution (5 mL) were distributed in test tubes and pre-incubated in water bath at 50°C for 15 min. The enzyme extract (0.5 mL) was added to the milk solution and the clot formation was observed while manually rotating the test tube. The time, in seconds, at which the first particles were formed was measured. All samples were prepared in triplicate.

The milk-clotting activity unit (U) was defined as the amount of enzyme required to coagulate 1 mL of substrate in 40 min at 50°C. Milk-clotting activity (U) (Equation 1) and the ratio (R) (Equation 2) were calculated using the following equations:

$$U = \frac{2400 \text{ x } S}{T \text{ x } E} \quad (1)$$

$$R = \frac{\text{Milk} - \text{clotting activity}}{\text{Proteolytic activity}} \quad (2)$$

Where, 2400 is the total time of milk-clotting activity (s), S is the milk volume (mL), E is the enzyme volume (mL) and T is the time of clotting formation (s). The samples were grouped into two classes according to the formation of compact milk clot and milk whey separation in the test tube: strong milk coagulation (distinct clot and abundant whey) and weak milk coagulation (clot formation without clear separation of the whey).

Statistical analysis

In all experiments, the data were subjected to descriptive statistical analysis of variance and the averages were compared by Tukey's test ($p<0.05$) using Minitab program, version 17.0. (Minitab, 2010).

RESULTS AND DISCUSSION

Aflatoxin test by ammonia vapor

A. flavo furcatis DPUA 1608 did not produce aflatoxin in any of the media tested (Figure 1). In the colony reverse, the change of color induced by ammonia vapor was not observed. Saito and Machida (1999) observed the change of color in 13 strains of *A. parasiticus* and in 55 strains from 83 of *A. flavus* while the strains of *A. oryzae* (19 strains) and *Aspergillus sojae* (5 strains) did not produce aflatoxin.

Aflatoxins have been studied because of the possible hazards to human health. They commonly are produced by *A. flavus* and *A. parasiticus*. In the study of Kulkarni and Chavan (2015), nine of twelve isolates of *A. flavus* (three maize varieties), showed the aflatoxin production represented (75%) by thin layer chromatography (TLC) method. However, in the same study, the aflatoxin production was also detected by the ammonia vapor test. In this test, only one isolate was found to be non-toxigenic.

Nair et al. (2014) also reported aflatoxin production by the ammonia vapor test in *Aspergillus* strains isolated from spice samples that previously were considered positive by HPLC analysis. The ammonia vapor test was also effective and considered easy and not expensive in the study of Zrari (2013). The author isolated strains of *Aspergillus* spp. showed both aflatoxigenic and non-aflatoxigenic classifications.

The result of the present study in respect to *A. flavo furcatis* DPUA 1608 is important due the intention to implicate the enzymes of this fungus in food industry. Another test might be done to consider it as safe or GRAS as citotoxic tests *in vitro* (*Artemia salina* and hemolysis, for example).

"GRAS" is an acronym for the phrase generally recognized as safe. Under sections 201(s) and 409 of the Federal Food, Drug, and Cosmetic Act (the Act), any substance that is intentionally added to food is a food additive, that is subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excluded from the definition of a food additive (FDA, 2016).

Protease production and milk-clotting activity

In this study, *A. flavo furcatis* synthesized and excreted

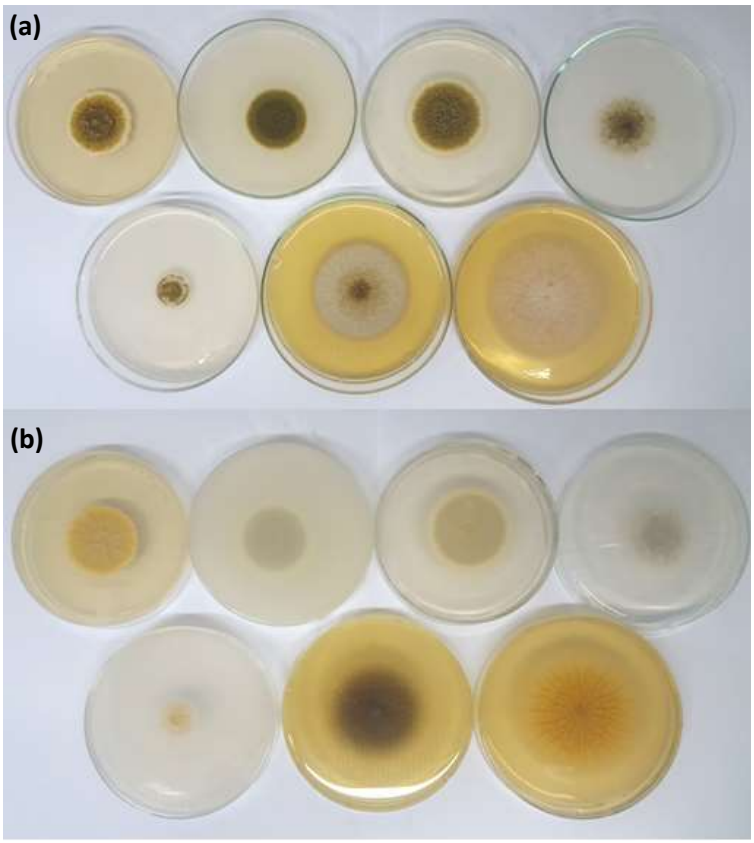

Figure 1. Results of aflatoxin production by ammonia vapor test. Front (a) and reverse (b) of *A. flavo furcatis* in BDA, COA, CYA, CZ, GMS, PMS and YES plates, respectively (from the top to the bottom).

proteases from all media of submerged and solid-state fermentation (Table 2). Comparing the media and inoculums used in submerged fermentation, the proteolytic activity was higher in the media supported with mineral solution base (MA01) in the inoculums SAB+SAC, BDA+SAC and BDA+GLI (53.00, 52.40 and 50. 66 U/mL, respectively). In the inoculum supported with SAB+GLI, the activity was 0.49 U/mL. In the other two media of submerged fermentation (MAGli and MASAc), the activity was low in all inoculums. In MAGLi, the protease activity were 0.75, 0.75, 0.88 and 0.93 U/mL (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC, respectively) and in MASAc, the proteases activity were 0.67, 0.84, 0.71 and 1.53 U/mL (SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC, respectively). In the media of solid fermentation, the proteolitic activity was determined in all crude extracts from the inoculums used: SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC (17.31, 16.97, 13.27 and 13.60 U/mL, respectively).

The milk-clotting activity was determined in all crude extracts for the submerged and solid fermentation. However, in the submerged fermentation, according to the classification of coagulation established, the milk strong coagulation was observed only in the mineral solution base media (MA01) (Figure 2A) (inoculums SAB+SAC, BDA+GLI and BDA+SAC). The other crude extracts of submerged fermentation (MAGli and MASac) also promoted milk clotting activity but it was considered as weak milk coagulation (Figure 2B). In the solid-state fermentation, the milk coagulation was observed in all crude extracts using açai seeds and rice bran (inoculums SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC).

Alecrim et al. (2015) observed similar results of milk coagulation using the same strain in submerged fermentation with agro industrial residues as substrates. The authors reported three classifications of milk-clotting: strong milk coagulation, weak milk coagulation and milk without coagulation. The statistical analysis of milk-clotting activity and coagulant ratio were made only with the results of the crude extracts (submerged and solid fermentation) that showed milk strong coagulation in the visual classification: MA01 (Inoculums SAB+SAC, BDA+GLI and BDA+SAC) and SAFA (Inoculums SAB+GLI, SAB+SAC, BDA+GLI and BDA+SAC).

Table 2. Protease activity (U/mL), milk-clotting activity (U), coagulant ratio (R) and clot classification of *A. flavo furcatis* DPUA 1608 enzymes produced in submerged and solid-state fermentation.

Media / Inoculum		Proteolytic activity (U/mL)	Milk-clotting activity (U)	Coagulant Ratio (R)	Clot Classification
Submerged fermentation	MA01 (SAB+GLI)	0.49±0.10^{f}	45.80	93.7	Weak
	MA01 (SAB+SAC)	53.00±0.07^{a}	470.71±9.23^{c}	8.90±0.20^{e}	Strong
	MA01 (BDA+GLI)	50.66±0.11	507.09±6.14^{b}	10.03±0.11^{d,e}	Strong
	MA01 (BDA+SAC)	52.40±0.35^{a}	595.12±8.45^{a}	11.33±0.21^{d}	Strong
	MAGli (SAB+GLI)	0.75±0.04^{e,f}	122.11	161.60	Weak
	MAGli (SAB+SAC)	0.76±0.75^{e,f}	107.35	142.10	Weak
	MAGli (BDA+GLI)	0.89±0.07^{e,f}	199.21	224.10	Weak
	MAGli (BDA+SAC)	0.93±0.00^{e,f}	188.25	201.70	Weak
	MASac (SAB+GLI)	0.67±0.00^{e,f}	110.31	165.50	Weak
	MASac (SAB+SAC)	0.84±0.07^{e,f}	195.95	232.00	Weak
	MASac (BDA+GLI)	0.71±0.03^{e,f}	192.80	271.10	Weak
	MASac (BDA+SAC)	1.54±0.11^{e}	254.17	165.80	Weak
Solid-state fermentation	SAFA (SAB+GLI)	17.31±0.19^{c}	389.70±17.8^{e}	22.53±0.93^{b}	Strong
	SAFA (SAB+SAC)	16.98±0.27^{c}	428.66±7.66^{d}	25.27±0.59^{a}	Strong
	SAFA (BDA+GLI)	13.27±1.04^{d}	247.63±8.67^{f}	18.70±0.87^{c}	Strong
	SAFA (BDA+SAC)	13.60±0.35^{d}	273.00±10.5^{f}	20.07±0.66^{c}	Strong

Means followed by the same letters in the columns did not differ from one another by the Tukey's test ($p < 0.05$)/ (±) = st. dev./ DPUA= Code of Culture Collection from Federal University of Amazonas/MA01 = Mineral solution base medium; MAGli = Mineral solution base medium+glucose; MASac = Mineral solution base medium+sucrose; SAFA= Açai seeds+rice bran/ Inoculums: SAB+GLI= Glucose+Peptone; SAB+SAC= Sucrose+Peptone; BDA+GLI= Potato+Glucose; BDA+SAC= Potato+Sucrose; NMCA= No milk-clotting activity.

Figure 2. Classification of the samples according to clot and whey formation: (A) Strong milk coagulation (B) Weak milk coagulation.

The milk-clotting activity of *A. flavo furcatis* crude extracts were more significant in the submerged fermentation as compared to the solid state fermentation. From the inoculums used in the submerged fermentation, the most significant milk-clotting activity was BDA+SAC (595.12 U) followed by BDA+GLI and SAB+SAC (507.09 and 470.71 U, respectively). In the solid-state fermentation, the most significant inoculums to milk-clotting activity was SAB+SAC (428.66 U), followed by SAB+GLI, BDA+SAC and BDA+GLI (389.70 U, 273.00 U and 247.63 U) (Table 2).

Ayana et al. (2015) reported the production of milk-clotting enzymes by *Mucor mucedo* in liquid medium containing glucose, peptone, casein, KHPO, olive cake and corn steep liquor. *Aspergillus tamari* and *Penicillium pinophillum* also demonstrated great milk-clotting protease production in liquid medium containing whey as one of the main substrates (Benlounissi et al., 2014). In the study of Bensmail et al. (2015), *A. niger* produced proteolytic enzymes with wheat bran as substrate in submerged and solid-state fermentation. Castro et al. (2014) also studied milk-clotting enzymes secreted by *A. niger*. The authors used wheat bran, soybean meal and cottonseed meal as substrates in solid-state fermentation.

Solid state fermentation an appropriate method

because solid substrates resemble the natural habitat of fungi improving their growth and enzymes production. The simplicity, low cost, high yields and concentrations of the enzyme sand are some of the advantages of this process (Castro et al., 2014).

However, in submerged fermentation, the conditions are monitored with greater accuracy as compared to solid state fermentation. Almost all the large-scale enzyme producing facilities are using the proven technology of submerged fermentation due to better monitoring and ease of handling (Singhania et al., 2010). Both submerged and solid-state fermentation are suitable for use of inexpensive and widely agricultural residues as substrates (Sumantha et al., 2006)

According to coagulant ratio (R), there was significant difference between the inoculums used on submerged fermentation and solid state fermentation. In the solid state fermentation, the most significant value (25.27) was observed in the inoculums supported with SAB+SAC followed by SAB+GLI, BDA+SAC and BDA+GLI (22.53, 20.06 and 18.70, respectively) (Table 2). In the submerged fermentation inoculums, the highest value of coagulant ratio was observed in BDA+SAC (11.33), followed by BDA+GLI (10.03) and SAB+SAC (8.90).

The coagulant ratio (R) demonstrates the potential commercial suitability of milk-clotting enzyme in cheese manufacturing.

Enzymatic preparations used for milk clotting usually exhibit proteolytic action, but is important that the enzymes have specificity of cleavage (Phe_{105}-$Met1_{06}$ connection of casein). It defines a good coagulant (Perry, 2004; Visser, 1993; Barros et al., 2001; Hashem, 1999; Merheb-Dini et al., 2010; Yegin et al., 2011).

Conclusions

The strain of *A. flavo furcatis* does not produce aflatoxin in the media tested by the ammonia vapor method. The color change in the reverse of colonies was not observed. Proteolytic milk-cloltting enzymes were produced by *A. flavo furcatis* in submerged and solid fermentation media. In submerged fermentation, MA01 was the medium that promoted the highest activity of these enzymes in three of the inoculums used. In solid fermentation, açai seeds and rice bran showed potential as substrates to produce proteolytic milk-clotting enzymes by the microorganism.

The coagulant ratio was higher in crude extracts from solid fermentation than the submerged fermentation. The inoculums used in the process promoted the production of milk-clotting proteases. In submerged fermentation, the best inoculum was BDA+SAC (MA01) and in solid fermentation, the best inoculum was SAB+SAC.

The properties of *A. flavo furcatis* enzymes in this study encourage future milk-clotting characterization and cheese production experiments to check its potential as microbial coagulant source.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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