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SCREENING OF *MUCOR* SPP. FOR THE PRODUCTION OF AMYLASE, LIPASE, POLYGALACTURONASE AND PROTEASE

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ABSTRACT

Fungi are well known by their ability to excrete enzymes into the environment. Among them, representatives of *Mucor* Fresen. have important biotechnological potential and some of them produce industrial enzymes. This work studied amylase, lipase, polygalacturonase and protease production by fifty-six isolates of *Mucor* belonging to 11 different taxa, selected from herbivores dung using solid media. The results showed that the majority of the isolates presented several enzymatic activities with predominance of polygalacturonase (96%), followed by amylase (84%), protease (82%) and lipase (66%).

Key words: Fungal enzymes, *Mucor*, amylase, lipase, polygalacturonase, protease, herbivorous dung

The enzymes are essential proteins for the metabolic system of all living organisms and have an important role in the degradation of organic matter, in host infection and food spoilage. In the metabolic pathways, they act in organized sequences of catabolic and anabolic routes (12). Enzymes may also act in the control of biochemical processes in the living cells. They may be isolated from animals, plants and microorganisms. The last ones are considered good sources of industrial enzymes for the great diversity of enzymes that have been found (13). The enzymes are used in large scale in the textile (amylase, cellulase, oxidoreductase); detergents (protease, lipase, cellulase, oxidoreductase); food (pectinase, protease, cellulase, oxidoreductase); paper (xylanase, oxidoreductase and lipase) and leather (protease, lipase) industries (14).

Extracellular enzymes may be produced in liquid or solid media. The use of solid media permits a fast screening of large populations of fungi, allowing the detection of specific enzymes (3,8,10,17,19,20) and helping in the chemotaxonomical differentiation of many microorganisms (10). The production of enzymes by microorganisms assures a potential and unlimited supply and also makes it possible the genesis of new enzymatic systems that cannot be obtained from plant or animal sources (2,13).

The species of the genus *Mucor* constitute a group of microorganisms responsible for the production of several enzymes such as amylases, lipases, pectinases and proteases (5,16,20). *Mucor hiemalis*, *M. racemosus* (15), *M. bacilliformis* (7) and *M. miehei* (6) present protease activity of commercial value and *M. miehei* is the most studied specie concerning the production of lipase (14).

The objective of this work was to detect the presence of the aforementioned enzymes by taxa of *Mucor* isolated from herbivores dung, using solid culture media.

The 11 taxa of *Mucor* isolated from dung of herbivores animals, from two locations in Recife, PE (1), are shown in the Table 1. These microorganisms have been preserved by the Castelani method (4) in culture collections of the following institutions: Catholic University of Pernambuco (UCP-

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Table 1. Taxa and identification number of the isolates in the Culture Collections of UCP, URM and SPC, indicating the herbivores animal origin.

TAXA	Culture Collection			Dung/Animal	Origin/Animal
	UCP	URM	SPC		
Mucor circinelloides f. ***circinelloides***	6	4136	1768	*Bos indicus* Linnaeus	ZOO
	36	4140		*Bison bonasus* H. Smith	PDI
	37			*Bison bonasus* H. Smith	PDI
	53			*Bison bonasus* H. Smith	PDI
M. circinelloides f. ***griseo-cyanus***	1	4183	1769	*Bison bonasus* H. Smith	PDI
	20	4182		*Mazama gouazoubira* Fischer	PDI
	42			*Mazama gouazoubira* Fischer	PDI
	47	4192		*Ovis aries* Linnaeus	ZOO
	46			*Bison bonasus* H. Smith	PDI
	54	4184		*Capra hircus* Linnaeus	ZOO
	55	4185		*Oryctolagus cuniculus* Lilljeborg	ZOO
	57	4160		*Taurotragus oryx* Wagner	PDI
	58			*Ovis aries* Linnaeus	ZOO
M. circinelloides f. ***janssenii***	8	4139		*Taurotragus oryx* Wagner	PDI
	9	4148		*Mazama gouazoubira* Fischer	PDI
	10			*Oryctolagus cuniculus* Lilljeborg	ZOO
	19	4141	1770	*Oryctolagus cuniculus* Lilljeborg	ZOO
M. circinelloides f. ***lusitanicus***	51	4137	1771	*Mazama gouazoubira* Fischer	PDI
M. genevensis	7	4188	1772	*Dasyprocta fuliginosa* Wagler	PDI
	15			*Dasyprocta fuliginosa* Wagler	PDI
	23	4187		*Ovis aries* Linnaeus	ZOO
	24			*Ovis aries* Linnaeus	ZOO
M. hiemalis f. ***hiemalis***	12			*Equus caballus* Linnaeus	ZOO
	13			*Capra hircus* Linnaeus	ZOO
	14			*Oryctolagus cuniculus* Lilljeborg	ZOO
	18	4190		*Mazama gouazoubira* Fischer	PDI
	28	4193	1773	*Dasyprocta fuliginosa* Wagler	PDI
	30			*Mazama gouazoubira* Fischer	PDI
	31			*Mazama gouazoubira* Fischer	PDI
	52			*Bison bonasus* H. Smith	PDI
M. hiemalis f. ***luteus***	5	4186	1774	*Ovis aries* Linnaeus	ZOO
	11	4191		*Dasyprocta fuliginosa* Wagler	PDI
	17			*Dasyprocta fuliginosa* Wagler	PDI
	2	4142		*Oryctolagus cuniculus* Lilljeborg	ZOO
	35			*Ovis aries* Linnaeus	ZOO
	44	4144		*Equus caballus* Linnaeus	ZOO
	45			*Ovis aries* Linnaeus	ZOO
	48	4147		*Capra hircus* Linnaeus	ZOO
	50			*Ovis aries* Linnaeus	ZOO
M. piriformis	41	4145	1775	*Equus caballus* Linnaeus	ZOO
M. racemosus f. ***chibinensis***	2	4149	1777	*Capra hircus* Linnaeus	ZOO
	3			*Dasyprocta fuliginosa* Wagler	PDI
	4			*Dasyprocta fuliginosa* Wagler	PDI
	16			*Dasyprocta fuliginosa* Wagler	PDI
	21			*Dasyprocta fuliginosa* Wagler	PDI
	26	4135		*Bison bonasus* H. Smith	PDI
	27			*Dasyprocta fuliginosa* Wagler	PDI
	33			*Dasyprocta fuliginosa* Wagler	PDI
	34			*Dasyprocta fuliginosa* Wagler	PDI
	39			*Oryctolagus cuniculus* Lilljeborg	ZOO
	40			*Oryctolagus cuniculus* Lilljeborg	ZOO
	43	4143		*Dasyprocta fuliginosa* Wagler	ZOO
	49			*Dasyprocta fuliginosa* Wagler	PDI
	56			*Dasyprocta fuliginosa* Wagler	PDI
M. subtilissimus	29	4133	1778	*Equus caballus* Linnaeus	ZOO
M. variosporus	25	4219	1779	*Mazama gouazoubira* Fischer	PDI

PDI=Parque Dois Irmãos, Recife, Pernambuco; Zoo=Department of Zootecny of the University Federal Rural of Pernambuco. Font: (1) modified.

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The isolates were reactivated in Petri dishes containing Synthetic *Mucor* Agar (SMA) (Hesseltine and Anderson (11) and/or Potato Dextrose Agar (PDA) and submitted to monosporic cultivation according to the methodology proposed by Gams *et al*. (9) in order to obtain pure cultures and to diminish variability at the morpho-physiologic, biochemical and genetic levels when submitted to successive cultivations.

A suspension of spores was prepared from the monosporic colonies. The mycelium was washed with sterilized distilled water with the aid of glass beads, and the suspension aseptically transferred to a test tube. An aliquot of this suspension was removed for counting in a Newbauer Camera, under optical microscope, and diluted whenever necessary to obtain 10^6 spores/mL.

For detection of enzymes, the methodology used was the one proposed by Hankin and Anagnostakis (10), modified with the substitution of the "hexadecyltrimethylammonium bromide" by hydrochloric acid (HCl) 5N. To verify the activity of the enzymes amylases, lipases, pectinases (polygalacturonases) and proteases, soluble starch (Merck), Tween 20 (Merck), citric pectin (INLAB) and jelly (Difco) were used as substrate, respectively. In a Petri dish containing 15 mL of the relevant substratum, a disk of 5mm of diameter was removed from the central part of the medium, and the hole filled in with 50 µL of 10^6 spores/mL suspension, and incubated at 28ºC. All the tests were performed in triplicate.

The level of enzyme production was evaluated by the halo diameter, measured in centimeters, in the reverse of the Petri dish. The halo produced by amylase, lipase and polygalacturonase activity was measured within 72 hours and for protease within 96 hours.

Data of the halo diameter averages were analyzed according to Fisher's protected LSD test ($P < 0.05$).

According to data presented in Table 2, higher amylases activities (halo ≥ 8 cm) were presented by isolates 37 and 53 (*M. circinelloides* f. *circinelloides*), 47 (*M. circinelloides* f. *griseo-cyanus*), 30 and 31 (*M. hiemalis* f. *hiemalis*), 48 (*M. hiemalis* f. *luteus*), 21 and 43 (*M. racemosus* f. *chibinensis*) and 25 (*M. variosporus*), while the isolates of *M. circinelloides* f. *lusitanicus* and *M. piriformis* were negative for this activity. Isolates *M. circinelloides* f. *circinelloides*, *M. circinelloides* f. *griseo-cyanus*, *M. hiemalis* f. *hiemalis*, *M. racemosus* f. *chibinensis* and *M. variosporus* were also good producers amylase (halo with average ≥ 7cm) Fig. 1A.

Thompson and Eribo (17) and Petruccioli and Federici (16) detected amylase activity in species of *Mucor*. However, the degradation halo presented by these authors (1-2 mm and up to 2 mm, respectively) were lower than the ones verified in this work. For *M. piriformis*, Thompson and Eribo (17) mentioned the presence of amylase activity, which was not observed in the isolate used in our experiment. On the other hand, amylases activity was observed in the isolate of *M. genevensis*, while Petruciolli and Federici (16) did not detect this activity in the isolate used in their work (Table 2).

All taxa studied showed lipases activity with predominance of *M. genevensis*, *M. circinelloides* f. *griseo-cyanus and M. circinelloides* f. *janssenii* species (halo with average > 6 cm). The isolates numbered 1, 46 and 47 (*M. circinelloides* f. *griseo-cyanus*), 10 (*M. circinelloides* f. *janssenii*) and 23 (*M. variosporus*) were the ones that presented the highest lipases activity (halo ≥ 7 cm) (Table 2), while for the averages presented *M. circinelloides* f. *lusitanicus* showed the lowest lipases activities (Fig. 1B).

These results are in accordance with the data presented by Vágvölgyi *et al*. (18) that detected lipase activity in 27 isolates of 10 different species of *Mucor*. They do not agree with the results obtained by Thompson and Eribo (17) that did not detect lipase activity in *M. hiemalis*, *M. mucedo*, *M. piriformis* and *M. racemosus* f. *racemosus*.

In Table 2 it is shown that a large number of isolates (54) presented polygalacturonase activity, mainly the ones numbered 5, 32 and 44 (*M. hiemalis* f. *luteus*), 15 and 24 (*M. genevensis*), 34, 39 and 56 (*M. racemosus* f. *chibinensis*) and 53 (*M. circinelloides* f. *circinelloides*) with halo ≥ 8 cm. Except for *M. subtilissimus*, all taxa presented polygalacturonase activity. *M. circinelloides* f. *lusitanicus*, followed by *M. hiemalis* f. *luteus*, *M. piriformis* and *M. genevensis* (average end of halo > 7 cm) showed the bests halo average for this enzyme (Fig. 1C). These data were similar to the ones presented by Thompson and Eribo (17) that observed polygalacturonase activity in three of the four species of *Mucor* studied. In this work, among the 56 isolates studied, only two isolates did not produced polygacturonase. These results agree with Petruccioli and Federici (16) that detected polygalacturonase in *M. genevensis*, *M. racemosus* and *M. ramannianus*, but the degradation halo (1-9.5 mm and 2-8 mm) produced by *M. genevensis* and *M. racemosus*, respectively, were smaller than the ones obtained in this study.

In Table 2 it can be observed that the majority of the isolates presented protease activity and that the isolates numbered 3, 4, 26, 33 and 40 (*M. racemosus* f. *chibinensis*), 6 (*M. circinelloides* f. *circinelloides*), 7 and 23 (*M. genevensis*), 8 (*M. circinelloides* f. *janssenii*), 11 and 32 (*M. hiemalis* f. *luteus*), 18 (*M. hiemalis* f. *hiemalis*) and 57, 58 (*M. circinelloides* f. *griseo-cyanus*) were the ones that demonstrated the biggest halo (≥ 5.5 cm). It may be observed that the majority showed proteases activity without significant differences among them (Fig. 1D).

The results obtained with the proteases activity agree with the data observed by Thompson and Eribo (17) that obtained this enzymatic activity in four species of *Mucor*. Petruccioli and Federici (16) also detected proteases activity in *M. racemosus* and *M. ramannianus*. Hankin and Anagnostakis (10) observed

Table 2. Averages diameters halo (cm) for the enzymatic activities in taxa of *Mucor* and respective isolates.

TAXA	ISOLATE	ENZYMATIC ATIVITY			
		Amylase	Lipase	Pectinase (polygalacturonase)	Protease
Mucor circinelloides f. ***circinelloides***	6	7.2c	0.0	6.1c	5.7a
	36	7.8b	0.0	5.6d	5.2ab
	37	8.4a	6.5a	6.8b	4.0b
	53	8.5a	5.3a	8.8a	0.0
M. circinelloides f. ***griseo-cyanus***	1	5.7d	7.9a	6.3b	5.0a
	20	5.9d	0.0	6.1bc	3.8b
	42	7.2bc	5.5b	5.8bcd	4.7ab
	46	6.8c	7.0a	0.0	0.0
	47	8.2a	7.1a	5.4de	5.0a
	54	6.2d	0.0	5.2e	5.0a
	55	7.5b	4.7bc	7.9a	5.3a
	57	7.6ab	4.3c	5.6cde	5.7a
	58	7.7ab	6.8a	5.3de	5.7a
M. circinelloides f. ***janssenii***	8	5.7b	4.6c	6.5a	6.2a
	9	4.6c	5.7b	7.0a	4.1c
	10	6.2a	7.3a	6.5a	4.9b
	19	5.7b	0.0	6.5a	4.2c
M. circinelloides f. ***lusitanicus***	51	0.0	2.3	7.9	0.0
M. genevensis	7	6.4	5.3b	5.0c	6.8a
	15	0.0	0.0	8.8a	2.7c
	23	0.0	8.1a	7.2b	5.6b
	24	0.0	0.0	8a	0.0
M. hiemalis f. ***hiemalis***	12	5.0e	4.8ab	5.7a	4.5cd
	13	6.4d	5.7ab	6.2a	4.9bc
	14	6.6d	0.0	6.2a	4.2de
	18	5.5e	4.3b	5.5ab	5.5a
	28	7.7b	0.0	3.7b	3.8e
	30	8.4a	6.1a	6.6a	4.7bc
	31	8.4a	4.3b	6.7a	5.0ab
	52	7.2c	4.9ab	6.7a	0.0
M. hiemalis f. ***luteus***	5	4.9c	0.0	8.0b	3.6e
	11	5.7b	2.3c	7.5b	6.2a
	17	5.7b	5.9a	6.6c	4.8bcd
	32	0.0	0.0	8.5a	5.5ab
	35	5.9b	0.0	7.4b	5.2bc
	44	0.0	5.5a	8.1a	0.0
	45	0.0	0.0	7.3b	0.0
	48	8.0a	4.2b	6.8c	4.2de
	50	0.0	5.0ab	7.6b	4.5cd
M. piriformis	41	0.0	3.6	7.4	0.0
M. racemosus f. ***chibinensis***	2	5.3e	5.0ab	6.6c	4.6de
	3	6.6cd	6.0a	6.7c	6.3a
	4	6.0de	4.2b	6.6c	6.0ab
	16	6.9c	5.8a	6.6c	3.2f
	21	8.1ab	3.0c	5.1e	4.1e
	26	7.3c	2.0c	5.6de	5.9ab
	27	6.6cd	5.0ab	6.1cd	4.4de
	33	7.0c	0.0	6.3c	6.4a
	34	7.1c	2.7c	8.5a	4.9cd
	39	7.3bc	0.0	8.5a	3.0f
	40	7.1c	0.0	5.5de	5.6bc
	43	8.2a	0.0	7.7b	0.0
	49	7.2c	0.0	7.8b	4.1e
	56	7.4bc	0.0	8.4ab	5.0cd
M. subtilissimus	29	4.7	2.5	0.0	0.0
M. variosporus	25	8.3	2.9	5.6	5.3

Averages equal to zero were not included in the statistical analysis. Numbers followed by the same letter among the isolates group of each species were not significantly different according to Fischer's protected LSD test.

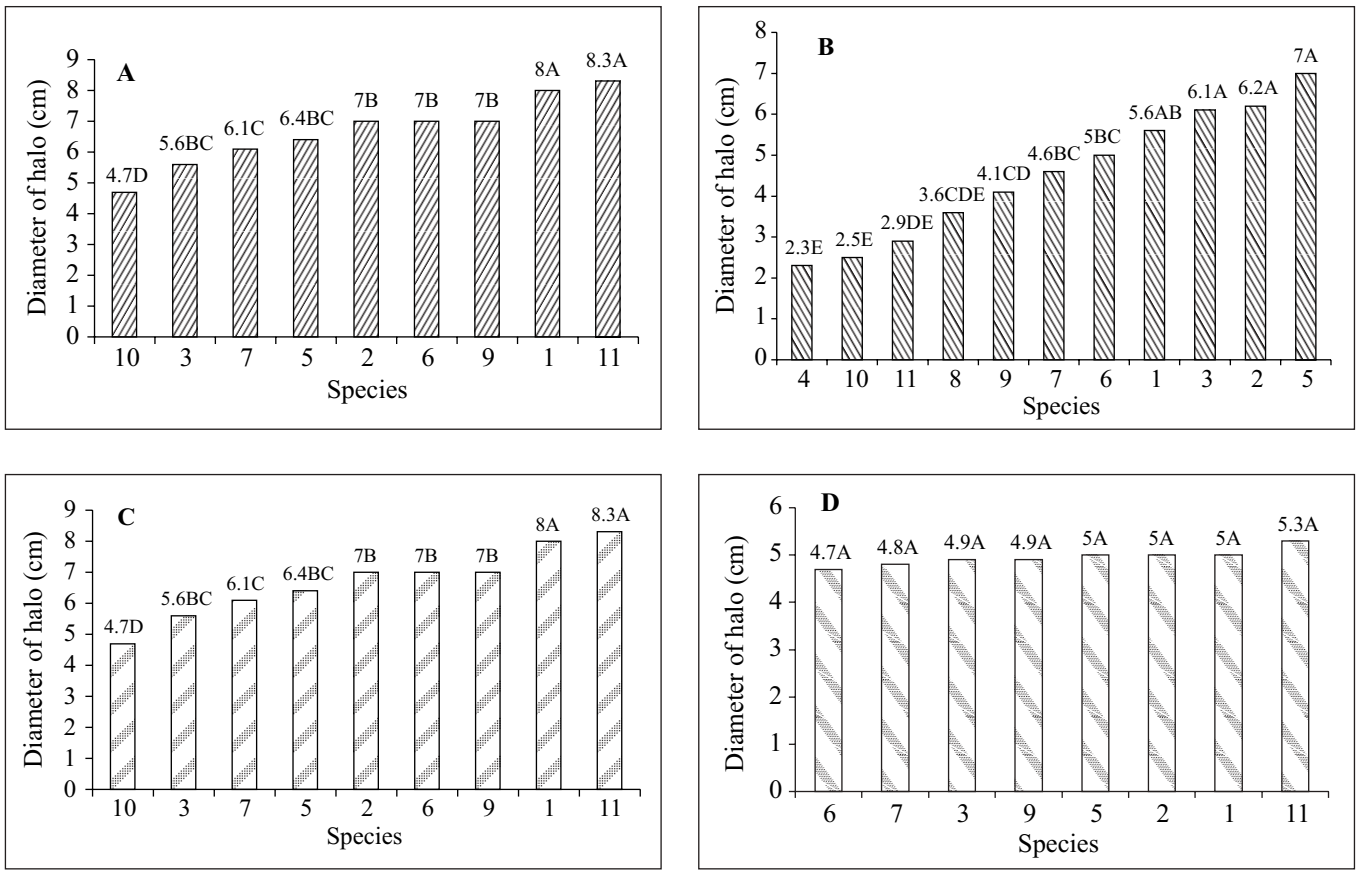

Figure 1. Average diameter of halo (cm) for the activities of amylase (A), lipase (B), polygalacturonase (C) and protease (D) in species of *Mucor*: 1 - *M. circinelloides* f. *circinelloides*; 2 - *M. circinelloides* f. *griseo-cyanus*; 3 - *M. circinelloides* f. *janssenii*; 4 - *M. circinelloides* f. *lusitanicus*; 5 - *M. genevensis*; 6 - *M. hiemalis* f. *hiemalis*; 7 - *M. hiemalis* f. *luteus*; 8 - *M. piriformis*; 9 - *M. racemosus* f. *chibinensis*; 10 - *M. subtilissimus* and 11 - *M. variosporus*. Numbers followed by the same letter are not significantly different according to Fischer's protected LSD test.

protease activity in *Mucor* sp, although the degradation halo diameters mentioned by these authors were smaller than the ones obtained in this work.

The results show that all *Mucor* isolates possessed a high potential for enzyme production, especially lipase, which was present in the majority of the taxa studied. It was observed that enzymatic activity does not establish true standards for separation of the taxa at a specific level since it varied in different isolates belonging to the same taxon.

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RESUMO

Screening de *Mucor* spp. para produção de amilase, lipase, poligalacturonase e protease

Os fungos apresentam a capacidade de produzir e secretar enzimas para o meio ambiente. Entre esses, representantes de *Mucor* Fresen constituem um grupo de microrganismos com importante potencial biotecnológico, sendo responsáveis pela produção de várias enzimas usadas em processos industriais. Foi observado que 56 isolados do gênero *Mucor*, totalizando 11 táxons, obtidos de fezes de herbívoros são capazes de produzir amilase, lipase, polygalacturonase e protease em meios sólidos. Os resultados demonstraram que 96% dos isolados produziram poligalacturonase, (84%) amilase, (82%) protease e (66%) lipase.

Palavras-chave: Enzimas fúngicas, *Mucor*, amilase, lipase, poligalacturonase, protease, fezes de herbívoros.

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