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Screening Brazilian *Macrophomina phaseolina* isolates for alkaline lipases and other extracellular hydrolases

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Summary. *Macrophomina phaseolina*, phylum Ascomycota, is a phytopathogenic fungus distributed worldwide in hot dry areas. There are few studies on its secreted lipases and none on its colony radial growth rate, an indicator of fungal ability to use nutrients for growth, on media other than potato-dextrose agar. In this study, 13 *M. phaseolina* isolates collected in different Brazilian regions were screened for fast-growth and the production of hydrolases of industrial interest, especially alkaline lipases. Hydrolase detection and growth rate determination were done on citric pectin, gelatin, casein, soluble starch, and olive oil as substrates. Ten isolates were found to be active on all substrates tested. The most commonly detected enzymes were pectinases, amylases, and lipases. The growth rate on pectin was significantly higher ($P < 0.05$), while the growth rates on the different media identified CMM 2105, CMM 1091, and PEL as the fastest-growing isolates. The lipase activity of four isolates grown on olive oil was followed for 4 days by measuring the activity in the cultivation broth. The specific lipolytic activity of isolate PEL was significantly higher at 96 h (130 mU mg protein^{-1}). The broth was active at 37 °C, pH 8, indicating the potential utility of the lipases of this isolate in mild alkaline detergents. There was a strong and positive correlation (0.86) between radial growth rate and specific lipolytic activity. [**Int Microbiol** 2012; 15(1):1-7]

Keywords: *Macrophomina phaseolina* · pectinases · amylases · proteases · lipolytic activity · radial growth rate

Introduction

Enzymes are an important group of biological products used in several processes in the food industry and in environmental and industrial biotechnological applications [23]. As biocatalysts, they have many advantages over chemical catalysts: the ability to function under relatively mild conditions of temperature, pH, and pressure; their specificity, and in some cases, their stereoselectivity. In addition, they produce no unwanted by-products [40]. Lipases are of particular interest because of their many applications in oleochemistry, organic synthesis, the detergent industry, and nutrition [30]. Indeed, the single biggest market for enzymes is in detergent formulations [27].

Fungi are excellent sources of enzymes as they produce these biocatalysts in great variety [8,31]. *Macrophomina phaseolina* (Tassi) Goid. [http://nt.ars-grin.gov/fungaldatabases, accessed Feb. 24, 2012] is a phytopathogenic filamentous fungus belonging to the anamorphic Ascomycota,

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Botryosphaeriaceae family [5,10], producing both sclerotia and pycnidia. It is responsible for the plant disease called charcoal rot, which affects both the roots and the stems. There are also reports of the fungus causing human ocular infection, skin infection in an immunocompromised child, and granuloma in a cat [6,14,37]. *Macrophomina phaseolina* is widely distributed in tropical regions and specifically in areas subjected to water stress, where it infects hundreds of different hosts [36] and causes severe economic losses [34]. In Brazil, *M. phaseolina* is found from the Northeastern region, where the climate is mostly hot and dry, to the South, where the humidity is high and temperatures range from 30 °C in the summer to 5 °C in winter. The microorganism penetrates host tissues through mechanical pressure exerted by the spore germ tube and the sclerotia hyphae, as well as through dissolution of the cell wall through processes mediated by secreted enzymes [4].

The plant cell wall is a complex structure of polymers that surrounds the cell. *M. phaseolina* produces cellulolytic, hemicellulolytic, pectolytic, and proteolytic extracellular enzymes [2], as well as lipases. Their concerted action results in the breakdown of the main polymeric components of the cell wall and the cell membrane. Studies on extracellular enzymes produced by *M. phaseolina* are few, and the most recent ones focused almost exclusively on cellulases and endoglucanases [1,7,29,41–43]. The colony radial growth rate reflects the ability of the fungus to use a particular substrate for growth, by secreting the necessary enzymes and thus enabling nutrient uptake for fungal metabolism and cell multiplication. Thus far, only one study examined the relative growth rate of this phytopathogen, on potato-dextrose agar [18], whereas radial growth rates on other substrates have not been reported. The lipolytic activity of *M. phaseolina*, involving one or two isolates, has been described in only a few studies [2,16,28].

The objective of the present work was to screen wild-type *M. phaseolina* collected in Brazil for fast growing isolates that produce hydrolases of industrial interest, and especially to select those producing alkaline lipases in large amounts.

Materials and methods

Equipment and reagents. Reagents and cultivation media were of the purest grade available, bought from Himedia (India), Merck (Germany), Vetec and Nuclear (Brazil). Extra-virgin olive oil was of commercial grade. A Minisart (Sartorius) filter, porosity 0.2 µm, was used for filter sterilizations. The rotatory shaker was from Oxylab (Brazil), and the spectrophotometer was from Shimadzu UV Mini-1240.

***Macrophomina phaseolina* isolates.** Isolates CMM 527, CMM 932, CMM 979, CMM 1048, CMM 1091, CMM 2100, CMM 2105, collected in the northeastern region of Brazil, were provided by the Phytopathogenic Fungi Culture Collection Prof. Maria Menezes of the Federal Rural University of Pernambuco (UFRPe). Isolates MMBF 564, MMBF 16–98, collected in the northeastern region, and MMBF 808, MMBF 04–10, collected in the southeastern region, were from the Fungi Collection Mário Barreto Figueiredo of the Biological Institute of the Department of Agriculture and Supply of the State of São Paulo (IB-SP). Isolate PEL, collected in the southern region, was obtained from the Phytosanitary Department of the Federal University of Pelotas (UFPel). Isolate AJAM, collected in the southeastern region, was from the Phytopathology Department of the Federal University of Viçosa.

Isolates maintenance. Isolates were cultivated on potato dextrose agar (PDA) at 24±1 °C until colonies covered approximately two-thirds of the area of the Petri dishes. Discs of 0.5 cm in diameter were collected from the actively growing regions of the colonies and kept in sterile distilled water, pH 6.5, at 6–8 °C, as stock for future inoculations.

Production of extracellular hydrolases. Petri dishes containing Pontecorvo's minimal medium agar [24], pH 6.8, and 0.2 % glucose [33], with the addition of 1 % (w/v) citric pectin, 4 % (w/v) gelatin (sodium nitrate reduced to 3 mM), or 1 % (w/v) soluble starch, was used to detect pectinases, proteases on gelatin, and amylases, respectively. After the incubation period, substrate hydrolysis was detected by covering the plate with 1 % (w/v) hexadecyltrimethylammonium bromide (CTAB) [13] for pectinases, saturated solution of ammonium sulfate for proteases, or 1 % Lugol solution for amylases. A clear halo around the colony against an opaque surrounding, indicated pectin or gelatin hydrolysis. A reddish or yellowish halo around the colony on a dark background indicated starch hydrolysis.

Extracellular proteolytic enzymes on casein were detected with skim milk agar [19]. After incubation of the plates, a transparent halo around the colony against an opaque background indicated casein hydrolysis. Lipases were detected with sterilized rhodamine B agar [39] added of previously filter-sterilized olive oil at a 1 % (v/v) concentration. A yellow-orange color around the colony, detected using 350 nm UV light, indicated fungal production of lipases.

Each hydrolase assay was done in triplicate per isolate. A mycelium disc in the center of each 9-cm Petri dish was inoculated and the plates were then incubated at 30 °C in the dark for variable periods, until colonies covered 60–75% of the plate area.

Colony radial growth rate. The growth rate was determined using the media and the incubation conditions described above, as well as PDA (pH 6.8). All assays were done in triplicate. Colony size was assessed at regular intervals by measuring the colony diameter along two axes at a right angle to the inoculation point, using a Vernier caliper. Measurements were done until the colonies reached the sides of the plate. Radial growth rate on PDA was determined in 9-cm diameter plates with all isolates, and in 20-cm diameter plates with isolates MMBF 04-10, MMBF 808, PEL, and CMM 2105. The radial growth rate (mm/h), expressed as the angle of the linear portion of the regression line, was calculated based on the radius of the colony vs. incubation time.

Lipolytic activity. Erlenmeyer flasks (250 ml) containing 100 ml of a minimal salts broth [9], pH 6.8, and 1 % (v/v) previously filter-sterilized olive oil were inoculated with three mycelium discs of isolates MMBF 04-10, CMM 2105, PEL, and MMBF 808, one flask per isolate. The flasks were incubated at 30 °C in a rotary shaker (160 rpm). Every 24 h for 4 days, 5-ml samples were collected, filtered through Whatmann paper, and frozen at –17 °C until analysis. The lipolytic activity of the cultivation broths was

assayed by using 4-nitrophenyl palmitate (pNPP) as substrate [22] in Tris-HCl buffer, pH 8, and 15-min incubation at 37 °C (ε = 13,300 M^{-1} cm^{-1}). Absorbance was read at 410 nm by using heat-inactivated cultivation broth as blank. One unit (U) of lipolytic activity is defined as the amount of enzyme that liberates 1 µmol of 4-nitrophenol (pNP) per minute per ml of cultivation broth. The protein content of the samples was determined according to Lowry's method, and the specific lipolytic activity (U mg $protein^{-1}$) was calculated.

Statistical analysis. Assistat. Statistical Assistance software was used for ANOVA, Tukey test, and Scott-Knott test [http://www.assistat.com/indexp.html].

Results and Discussion

Production of extracellular hydrolases. Table 1 shows the hydrolases produced by each isolate. Consistent with previous reports of pectin hydrolysis with enzymatic extracts of *M. phaseolina* [26], all 13 isolates hydrolyzed pectin. Pectinases were also detected in another study of three isolates [3], one of which was more efficient in causing stem rot. Dhingra et al. [11] demonstrated differentiated pectolytic and cellulolytic activity in vitro and in vivo between a virulent and an avirulent isolate. Maximum pectinase activity was detected at 48 h when this phytopathogen was grown in submerged cultivation [2], with a rapid decrease in activity after 96 h. By contrast, weak pectinase activity was reported for *Macrophomina* sp. MS 139 [35].

All 13 isolates secreted amylases. Onilude and Oso [21] also obtained amylases from *M. phaseolina* and used them, either as crude or partially purified preparations, in feed diet to improve the weight gain of broiler chicken. Another study [35], testing for extracellular hydrolases in several fungi, failed to detect amylases in *Macrophomina* sp. MS 139. However, according to one report [12] an isolate of this fungus showed good dextrinizing and saccharizing specific activities on carbon sources such as starch, jackfruit seed flour, and rice flour.

In the present study, 11 isolates produced proteases able to hydrolyze gelatin. Proteases active on casein were also frequently detected, except in two (AJAM and MMBF 564) of the 13 isolates. The sparse references on the proteases produced by *M. phaseolina* also mention variability in their detection. Ahmad et al. [2] examined two strains of this fungus but did not detect proteolysis on casein either in solid medium in Petri dishes or in submerged culture. However, in their study of several fungi, Sohail et al. [35] reported the detection of proteolytic enzymes produced by *Macrophomina* sp. MS 139 in mineral medium with casein. The authors concluded that proteases are the most common hydrolases in filamentous fungi. Kakde and Chavan [15] also observed the ability of this fungus to use casein as source of nitrogen.

Table 1. Detection of extracellular hydrolases of *Macrophomina phaseolina* isolates grown on different substrates

	Pec[a]	Amy[b]	Prot gel[c]	Prot cas[d]	Lip[e]
PEL	+	+	+	+	+
AJAM	+	+	–	–	–
CMM 527	+	+	–	+	+
CMM 932	+	+	+	+	+
CMM 979	+	+	+	+	+
CMM1048	+	+	+	+	+
CMM1091	+	+	+	+	+
CMM2100	+	+	+	+	+
CMM2105	+	+	+	+	+
MMBF564	+	+	+	–	+
MMBF808	+	+	+	+	+
MMBF16-98	+	+	+	+	+
MMBF04-10	+	+	+	+	+

Hydrolases detection: (+) hydrolases detected, (–) negative for substrate hydrolysis on the triplicates.
[a]Pectinases. [b]Amylases. [c]Proteases on gelatin. [d]Proteases on casein.
[e]Lipases on olive oil.

In this study, all isolates showed lipase activity when induced with olive oil. The exception was isolate AJAM, which showed very restricted growth and no lipolysis even after 6 days of cultivation. Other studies also reported the production of lipolytic enzymes by this pathogen. In an experiment comparing lipase production by *M. phaseolina* and *Phoma nebulosa* [28], enzyme production was shown to depend on the culture medium used. In that work, *M. phaseolina* produced higher amounts of lipases when stimulated by the addition of sesame flour to the medium. Another work [38] examined the *M. phaseolina*-induced deterioration of peanuts, specifically, the changes in moisture content, fatty acids, and proteins. A decrease in oil content and an increase in free fatty acids was noted, demonstrating the lipolytic action of this fungus. In the above-mentioned study by Ahmad et al. [2], the two *M. phaseolina* isolates also produced lipases.

Ten of our 13 isolates showed hydrolytic activity on all substrates tested. In contrast to some studies on filamentous fungi, among the hydrolases, proteases were less frequently detected.

Table 2. Radial growth rate (RGR)of *Macrophomina phaseolina* on different substrates

Substrate	RGR[a] (mm/h)
Pectin	0.90±0.44 a
Soluble starch	0.60±0.22 b
Gelatin	0.77±0.27 a
Casein	0.55±0.25 b
Olive oil	0.62±0.28 b

[a]Mean radial growth rate from triplicates of thirteen isolates on the same medium. Means followed by the same letter do not differ statistically from each other (Scott-Knott test, $P < 0.05$).

Colony radial growth rate. The radial growth rates (mm/h) of each isolate grown on the different media were calculated from the radius of the colony and the incubation time. The overall radial growth rate achieved on a medium was determined by taking the individual rates of the triplicates of the 13 isolates on the same medium and calculating their mean (Table 2). The several culture media resulted in two distinct growth rates, showing that *M. phaseolina* grew significantly better ($P < 0.05$) on pectin and gelatin. Radial growth rates were quite variable among isolates grown on the same medium, and also varied for the same isolate on the different culture media (Table 3). The linear correlation coefficient (Pearson's r) of the regression lines remained between 0.99 and 0.83 for all media, except gelatin (r = 0.75).

Although cultivated under the same conditions, isolates of *M. phaseolina* in the present study showed radial growth rates on PDA well above those determined by Mayek-Pérez et al. [18] with Mexican isolates from different hosts and different regions of the country. In that study, the rates ranged from 0.45 to 0.50 mm/h, and the authors related the heterokaryotic nature of the mycelium of *M. phaseolina* to the variability of its morphological characteristics, development in vitro, and virulence. Also mentioned as sources of variability were the geographical origin of the isolate, host type, cultivation time, and culture medium employed. Although in our study isolate MMBF 04-10 showed the highest growth rate on PDA, the other isolates grew faster on several media. Of all substrates tested, pectin yielded the highest growth rates,

Table 3. Radial growth rate of *Macrophomina phaseolina* isolates grown on different substrates[a]

Substrates	Pectin	Soluble starch	Gelatin	Casein	Olive oil	PDA
PEL	0.64±0.09 e	0.68±0.02 b	1.06±0.04 a	0.96±0.02 a	0.98±0.03 a	1.05±0.05 b
AJAM	0.22±0.01 g	0.19±0.05 c	0.28±0.01 d	0.04±0.01 h	0.07±0.01 h	0.06±0.01 i
CMM 527	0.62±0.08 e	0.72±0.04 b	0.72±0.20 b	0.83±0.03 b	0.74±0.01 b	0.61±0.02 f
CMM 932	0.45±0.06 f	0.26±0.18 c	0.35±0.17 d	0.24±0.02 g	0.34±0.02 g	0.45±0.01 g
CMM 979	1.05±0.06 d	0.67±0.02 b	0.98±0.03 a	0.63±0.05 c	0.78±0.01 b	0.74±0.01 d
CMM1048	1.38±0.05 b	0.58±0.01 b	0.84±0.06 b	0.55±0.08 d	0.76±0.01 b	0.49±0.02 g
CMM1091	1.62±0.07 a	0.72±0.02 b	1.06±0.03 a	0.89±0.01 b	0.98±0.05 a	0.61±0.06 f
CMM2100	1.21±0.22 c	0.62±0.04 b	0.91±0.03 a	0.58±0.03 d	0.65±0.03 c	0.41±0.01 g
CMM2105	1.25±0.08 c	1.02±0.01 a	0.98±0.03 a	0.69±0.08 c	1.00±0.01 a	1.02±0.01 b
MMBF564	0.78±0.03 e	0.68±0.11 b	0.78±0.03 b	0.45±0.01 e	0.30±0.01 g	0.69±0.02 e
MMBF808	0.97±0.03 d	0.56±0.03 b	0.57±0.04 c	0.36±0.04 f	0.52±0.02 e	0.85±0.03 c
MMBF16-98	0.26±0.04 g	0.38±0.01 c	0.47±0.09 c	0.47±0.02 e	0.41±0.08 f	0.21±0.06 h
MMBF04-10	1.28±0.10 c	0.60±0.27 b	0.93±0.07 a	0.49±0.03 e	0.59±0.04 d	1.33±0.07 a

[a]Mean radial growth rate (mm/h) and standard error of three determinations. Means followed by the same letter do not differ statistically from each other (Scott-Knott test, $P < 0.05$).

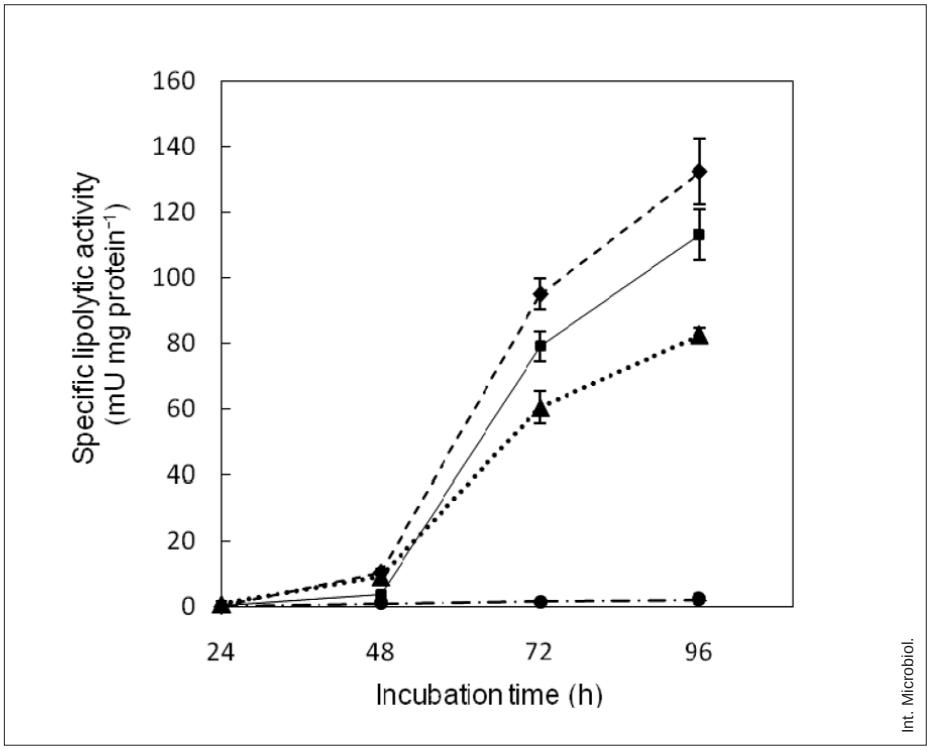

Fig. 1. Extracellular lipase production by *M. phaseolina* isolates PEL (diamonds), CMM 2105 (squares), MMBF 04-10 (triangles), and MMBF 808 (circles) in minimal mineral salts medium with olive oil as inducer. Values represent the means and standard deviation of three determinations.

with isolate CMM 1091 growing significantly faster than the other isolates (1.61 ± 0.06 mm/h, $P < 0.05$). On starch, isolate CMM 2105 showed a significantly higher growth rate ($P < 0.05$). Several isolates grew rapidly on gelatin but not on casein, although both are protein substrates; in fact, casein yielded the lowest growth rates among all substrates. Similar results were obtained with the filamentous fungi *Batrachochytrium dendrobatidis* [25] and *Aspergillus sydowii* [32], which also showed different growth patterns on gelatin than on casein, with both developing a higher mycelium mass in media containing the latter. On olive oil, isolates PEL, CMM 1091, and CMM 2105 showed significantly faster growth ($P < 0.05$) than the other isolates.

The variability in both the production of several extracellular hydrolases by *M. phaseolina* and the fungus' rate of radial growth on PDA, as described in the present study, confirms the diversity reported by other authors. To our knowledge, ours is the first report on *M. phaseolina* radial growth rates on media other than PDA.

A high growth rate and the production of specific enzymes are features that allow the selection of isolates with specific characteristics. CMM 1091 grew rapidly on all substrates tested for the production of hydrolases, while isolates PEL and CMM 2105 grew quickly on most media, producing the corresponding hydrolase. Thus, CMM 1091, PEL, and CMM 2105 are isolates that produce enzymes of potential industrial interest and therefore merit further research.

Lipolytic activity. To verify the production of lipases by four isolates using olive oil as sole source of carbon, the lipolytic activity of their cultivation broths was tested against 4-nitrophenyl palmitate. Figure 1 shows the specific lipolytic activity (U mg protein^{-1}) of isolates PEL, CMM 2105, MMBF 04-10, and MMBF 808 during 4 days of cultivation. The activities of three isolates (PEL, CMM 2105, and MMBF 04-10) increased with cultivation time and in each case reached a maximum at 96 h: PEL 130 mU mg protein^{-1}, CMM 2105 110 mU mg protein^{-1}, and MMBF 04-10 80 mU mg protein^{-1}. The activity of the fourth isolate, MMBF 808, was minimal (2 mU mg protein^{-1} in 96 h). The ANOVA and Tukey test ($P < 0.05$) of the specific lipolytic activities of PEL, CMM 2105, and MMBF 04-10 indicated that they were significantly different, with PEL showing the highest activity throughout the cultivation period.

Ahmad et al. [2] cultivated two strains of *M. phaseolina* in minimal salts medium with olive oil for 96 h, while measuring the lipolytic activity of the broth at regular intervals. The activity of strain 1 peaked at 24 h and reached a minimum at 96 h, while strain 2 activity, which was only 40 % of that of strain 1, peaked at 24 h and reached a minimum at 48 h. Kakde and Chavan [16] cultivated an isolate of *M. phaseolina* for 25 days in submerged cultivation in minimal salts medium containing oil and tested the lipolytic activity of the broth every 5 days for 25 days. Maximum activity was reached on day 25, and half-maximal activity already on day 5. Since no other studies were found using spectrophotometry to determine the lipolytic activity of *M. phaseolina*, our results cannot be compared with those of other researches. However, it is clear that the incubation time necessary for *M. phaseolina* to reach peak lipolytic activity in submerged culture is quite variable, depending on culture conditions and the particular isolate.

A comparison of the radial growth rate on olive oil medium with the specific lipolytic activity of the four isolates tested showed that both CMM 2105 and PEL had high growth rates and produced the highest lipase activities. Studies of other fungi have shown that growth rate and lipolytic activity are regulated by the cAMP/PKA (cyclic AMP-dependent protein kinase A) signaling pathway. In a study by Ocampo et al. [20], a mutant of *Mucor circinelloides* lacking the gene for one of the regulatory subunits of PKA (and thus exhibiting high PKA activity) exhibited a decrease in growth and alterations in germination rates, cell volume, germ tube length, and asexual sporulation. Klose et al. [17] found that cAMP/PKA signaling regulates the morphological growth of *Ustilago maydis*, whether filamentous or budding, and that higher amounts of lipase are secreted in the presence of triglycerides only by strains showing filamentous growth. They speculated that cAMP signaling is involved in the ability of the fungus to use oils as carbon source and that the gene(s) encoding the lipase activity is regulated by PKA. Our findings suggest that this is also the case for *Macrophomina phaseolina*, as the correlation coefficient between radial growth rate on olive oil and lipolytic activity was strong and positive (0.86), indicating that faster filamentous growth was associated with the higher production of lipolytic enzymes.

In summary, *Macrophomina phaseolina* was shown to produce extracellular pectinases, amylases, proteases, and lipases. The isolates, however, varied in their abilities to produce these enzymes, as some did not produce all the hydrolases tested, and proteases were less commonly detected on both gelatin and casein.

The determination of radial growth rates on different substrates together with the detection of the corresponding extracellular hydrolases identified CMM 1091, CMM 2105, and PEL as fast-growing isolates with great diversity in the production of extracellular hydrolases of industrial interest. Among the isolates tested, PEL produced the highest lipase activity, and the enzyme was active at 37 °C, pH 8, with potential use in mild alkaline detergents. It is also reasonable to suggest that, as in other fungi, the radial growth rate and lipolytic activity of *M. phaseolina* are probably regulated by the cAMP/PKA pathway, although this remains to be demonstrated in further studies.

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Competing interests. None declared.

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