Characterization and immobilization of protease secreted by the fungus *Moorella speciosa*

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Abstract

Protease was extracellularly produced in submerged fermentation by the fungus *Moorella speciosa* with maximum activity of 8.6×10^3 U/mL. The optimal pH and temperature for enzyme activity were 6.78 and 60.88 °C, respectively. The enzyme was incubated in the presence of several ions at concentrations of 0.1 M and 0.01 M to address the effect on enzyme activity. Enzyme activity was increased by 56% and 130% in the presence of 0.1 M BaCl₂ and of 0.01 M Na₂SO₄, respectively. The $V_{\max}$ and K_m values were 0.01474 U/min/mg protein and 0.04190 mg/mL, respectively. The enzyme retained about 90% of enzymatic activity at 90 °C. Among the methods tested for enzyme immobilization, adsorption onto MAT540 carrier led to the most promising results, since after 15 reuse cycles up to 60% of the initial catalytic activity was retained. Entrapment in calcium alginate matrix allowed to retain up to 51% of the initial catalytic activity after 8 reuse cycles. This protease from *M. speciosa*, in either free or immobilized form, can be foreseen as a useful biocatalytic tool in process design by reducing operating costs, decreasing the use of chemical processing and, consequently, meeting the global demand for clean technologies.

Keywords Protease · Immobilization · Matrim 540 · Enzyme · Purification

Introduction

Since the advent of enzymology, enzymes have been widely used in industrial activities such as textiles, pharmaceuticals, leather, food and detergents (Razzaq et al. 2019). According to estimates, the global industrial enzyme market will grow by 4.9% and reach US \$ 7.0 billion—by 2023 (Dewan 2018). This growth can be associated with the gradual adaptation of industrial-scale production processes over the years, based on the shift from chemical to enzymatic processes to manufacture goods and commodities (Vashist et al. 2019). The wide biochemical diversity of microorganisms,

in association with large-scale production feasibility, are the main factors turning microorganisms into the most used source to produce industrial enzymes (Dewan 2018). Besides, enzymes have been increasingly used in several industries; thus, it is important finding new microbial isolates capable of producing enzymes to meet this growing demand (Banerjee and Kumar 2017).

Proteolytic enzymes rank second in the global market, since proteolysis is a powerful tool used to modify protein properties. However, the use of these enzymes faces limitations in some processes, such as enzyme chain instability, low reaction rate or even low affinity to the substrate (Rubin-Pitel and Zhao 2006; Tavano 2013; Dewan 2018).

It is essential preserving the properties of products, peptides and amino acids generated in some protein hydrolysis processes, which, in their turn, must be carefully controlled. Thus, protease application is an important tool that should be taken into consideration in these cases (Tavano 2013).

As the growing application of these enzymes depends on constant innovation studies focused on finding new protease sources, Brazilian researchers should further explore the potential of microbial sources to generate new enzymes, since the country has a vast continental area comprising a wide diversity of environments (Orlandelli et al. 2015).

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Different studies have established the association between the niche occupied by a given microorganism and the features of its enzymes. Thus, microorganisms capable of proliferating in environments subjected to high temperatures produce extracellular enzymes capable of enduring temperatures corresponding to, at least, the optimum temperature necessary for their growth (Gomes et al. 2007; Egorova and Antranikian 2005).

Brazilian Northeastern semiarid region presents high solar irradiance rates, high temperature and low monthly temperature variations (Zanella 2014); thus, microorganisms found in this region are expected to have a metabolic apparatus to help them survive in hot and dry environments for most of the year. Fungal species *M. speciosa* (Rao and Rao 1964) is a saprophytic ascomycete isolated from the semi-arid region of Bahia State, in the biome called Caatinga (Cruz and Gusmão 2009). Studies about this species remain scarce in the literature; some of them focus on bioprospecting saprobic fungi to control plant diseases (Resende et al. 2015; Botrel et al. 2018).

However, several studies with ascomycete fungi have been carried out to produce different enzymes, with emphasis on genera *Aspergillus* and *Penicillium* (Zhu et al. 2009; Morya and Yadav 2010; Yadav et al. 2011; Lanka et al. 2017). The proteolytic enzyme produced by these fungi has the ability to degrade proteolytic materials; thus, it is applicable to many fields (Chandrasekaran et al. 2015). Thus, the aim of the current study was to investigate protease production by fungal species *M. speciose* with emphasis on its characterization and purification to tolerate temperature variations.

Material and methods

Microorganism

Moorella speciosa was obtained from the Culture Collection of Microorganisms from Bahia, of the State University of Feira de Santana (UEFS). This collection is certified by the Council of Management of the Genetic Heritage (CGEN).

Inoculum preparation and fermentation

Moorella speciosa grown in Potato-dextrose-Agar (PDA) plates, at 28 °C for 10 days and after growth, was used in inoculum preparation. Subsequently, mycelial discs of 1.5 cm diameter (10^6 spores) of the isolate were taken from the solid culture and transferred to 250-mL-Erlenmeyer flasks containing 100 mL liquid culture.

Cultures were kept under orbital agitation (100 rpm) at 28 °C for 10 days. After, culture medium was centrifuged at 10,000g, at 4 °C, for 10 min, to remove the cells; cell-free

enzyme extract was used to measure proteolytic activity. All experiments were performed in triplicate.

Proteolytic activity determination

The casein digestion method was used to determine proteolytic activity (Kunitz 1947). The reaction mixture contained 100 µL of enzyme extract and 1 mL of 0.5% casein, it was prepared in sodium phosphate buffer (0.2 M, pH 6.0), at 35 °C for 20 min. The aliquot of 100 µL of mixture was collected at the end of the reaction process, added with 5 mL of Bradford reagent and subjected to spectrophotometry reading at 595 nm. With this value, was calculated the amount of casein consumed using the linear equation from a previously determined protein standard curve. One activity unit (U) is defined as the amount of enzyme activity that converts one mg of casein per minute. Specific activity (U/mg) was also determined, based on total protein. All experiments were conducted in triplicate.

Purification

The enzyme was purified from the culture filtrate using 70% ethanol. Ethanol (70%) was added to 3 ml of crude enzyme extract at the ratio of 2:1 v/v (at 4 °C) and the whole mixture was centrifuged at 10,000g, 4 °C, for 15 min. The supernatant was discarded; phosphate buffer was added to the mixture in the same amount as the initial extract used in precipitate resuspension.

Total protein determination

The total protein concentration throughout the different steps in protease purification was determined using the Bradford method (1976), using bovine serum albumin (BSA) as reference protein for the standard curve.

Kinetic characterization

Optimum pH and temperature determination

Doehlert experimental design comprised two variables—activity's temperature and pH—and three replicates in the center of the domain, which totaled 9 experiments. Casein (0.5%) was prepared in sodium phosphate buffer at pH values of 5.5, 6.0, 6.5, 7.0 and 7.5. Experiments were carried out at different temperatures for 20 min; specific enzyme activity (U/mg) was quantified for each condition.

The data obtained were transformed into relative proteolytic activity for plotting the graphs. Experimental errors were evaluated based on the replication of the central point. Experimental data were processed in STATISTICA software version 7.0; data were also tested through analysis of variance (ANOVA) when the model did not fit them.

Effect of ions on enzyme activity

The following salts were added to the reaction medium, using casein as substrate: magnesium chloride (MgCl_2), calcium chloride (CaCl_2), ammonium chloride (NH_4Cl), barium chloride (BaCl_2), aluminum sulfate ($\text{Al}_2(\text{SO}_4)_3$), magnesium sulfate (MgSO_4), ferrous sulfate (FeSO_4), manganese sulfate (MnSO_4), ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$), potassium sulfate (K_2SO_4), and sodium sulfate (Na_2SO_4) at concentrations of 0.1 M and 0.01 M. Enzyme activity without ion addition reached 100%. All experiments were conducted in triplicate. Enzyme activity was measured as described in item 2.4.

Enzyme kinetics

Casein substrate at different concentrations (0.04–0.2%) was used to investigate the enzymatic kinetics of protease produced by *M. speciosa*. Enzymatic kinetics was determined by incubating the enzymatic reaction at 60° C, pH 6.7, for 20 min. Enzymatic kinetics parameters K_m and $V_{\max}$ —were determined based on the Lineweaver–Burk double-reciprocal plot.

Thermostability

Thermostability was assessed by incubating samples of the enzyme at different temperatures (50, 60, 70, 80 and 90 °C) and times (0, 10, 20, 30, 40, 50 and 60 min). Thermostability was assessed by incubating enzyme samples at different temperatures (50, 60, 70, 80 and 90 °C) and times (0, 10, 20, 30, 40, 50 and 60 min). After the incubation period was over, test tubes enzymes were cooled in ice bath. The enzymatic tests were performed as described in item 2.4. All experiments were conducted in triplicate.

Enzyme immobilization

Eight commercial supports were tested for enzyme immobilization: MAT 540 (Materium 540, Silica microspheres), CPC 27709 (CPC-Silica Carrier Silane Coated, Ref. No. 27709), Purolite Lifetech ECR8806F (methacrylic polymer), ECR8204F (hydrophilic methacrylate Polymer), ECR1090F (porous styrene copolymer), silica gel, Celite 545 and chitosan; 0.5 mL of phosphate buffer at pH 6.5 and 0.10 g of each support were added to 1 mL of crude enzyme extract of the protease to be tested.

Samples added with the afore mentioned reagents were incubated under stirring (100 rpm) at 25 °C for 120 min. Particles were allowed to settle to enable supernatant separation. The immobilized enzyme was washed twice in 2.5 mL of the same buffer. Supernatant was removed after sedimentation with the aid of a micropipette. The amount of protein

in the supernatant and the enzyme activity in the immobilized crude extract were evaluated.

Calculation for evaluation of the immobilization:

* Yield of the immobilization process (%) :

YP : Activity of the immobilized CE $\times$ 100 / Activity of the CE

* Protein retention (%) (amount of immobilized enzyme) :

PR (%) : (1 – Protein of the supernatant /total protein of the CE) $\times$ 100)

Reuse of the immobilized enzyme

The runs used to determine the cycles of use of the immobilized enzyme were carried out at 20 min of reaction. Between each cycle, the immobilized enzyme was washed twice with 2.5 mL of phosphate buffer 50 mM, pH 6.5. After sedimentation, the supernatant was removed with a micropipette and the enzymatic activity of the immobilized crude extract was performed according to item 2.4. The result was presented in relative activity (%), and the activity in the first cycle was set at 100%. All runs were performed in triplicate.

Determination of the optimum temperature and pH of the immobilized enzyme

The proteolytic activity of the immobilized enzyme was evaluated at different temperatures (50, 70 and 90 °C) and at different pH values (5.5, 6.0, 6.5, 7.0, 7.5) to establish the effect of these variables in enzyme activity and to identify the conditions that maximize biocatalyst activity. Casein (0.5%) was prepared 0.2 M sodium phosphate with pH 5.5, 6.0, 6.5, 7.0 and 7.5. The experiments were carried out at different temperatures, for 20 min, with the enzymatic activity quantified at each condition in U/mg. The data obtained were transformed into relative proteolytic activity for plotting the graphs.

Immobilized enzyme kinetics

Casein substrate was used to establish the kinetics of the selected immobilized protease at different concentrations (0.04–0.2%), based on results presented in item 2.9.1. Enzyme formulation enabled the largest number of cycles. Enzymatic kinetics was determined by incubating the enzymatic reaction at 49° C and pH 5.7, for 20 min. Parameters such as K_m and $V_{\max}$ were calculated based on the Lineweaver–Burk double-reciprocal plot. An affinity constant was defined as $V_{\max}/K_m$.

Immobilized enzyme thermostability

Immobilized enzyme thermostability was evaluated by incubating enzyme samples at different time intervals (0, 10,

20, 30, 40, 50 and 60 min) at different temperatures (50, 60, 70, 80 and 90 °C). After the incubation period, enzymatic assays were performed as described in item 2.4. All tests were performed in triplicate.

Results

Protease growth and production by fungal species *Moorella speciosa* was studied for the first time. Microbial cells were incubated at 28 °C in medium added with 2% peptone (w/v), which was used as substrate to induce protease

production. The investigated fungal species was considered a good enzyme producer, with specific volumetric activity of 8.6×10^3 U/mL.

pH and temperature optimization in free-protease activity

The enzymatic assay conducted at different incubation pH and temperatures allowed establishing that the *M. speciosa* protease activity had its peak at 60 °C and pH 6.5 (Table 1).

The dataset was also used to generate response surface graphs (Fig. 1). Results have shown optimal proteolytic activity at 60.88 °C and pH of 6.78.

Analysis of variance was applied to data deriving from the experimental method to evaluate the quadratic model (Table 2). A test based on Fisher's distribution (*F* test) has indicated significantly adjusted equation ($F = 78.66 > 9.01$). Lack of squares' adjustment ($F = 0.090966 < 18.81$) has indicated good agreement between the expected response of the model and experimental values recorded for each variable.

Statistica 7.0 software was used to statistically evaluate the effects of pH and temperature on enzyme activity. Results have shown that these variables had positive influence on enzyme activity at 5% significance level ($p < 0.5$), as well as that there was interaction between pH and temperature in enzyme activity, as seen in the Pareto Chart (Fig. 2).

Table 1 Doehlert matrix used in the optimization of for *M. speciosa* protease characterization

Experiment	Temperature (°C)	pH	Enzyme activity (U/mg)
1	80	6.0	5.457143×10^{-3}
2	80	7.0	7.442857×10^{-3}
3	60	5.5	4.340476×10^{-3}
4 (C)	60 (C)	6.5 (C)	7.2857×10^{-3}
5 (C)	60 (C)	6.5 (C)	7.6214×10^{-3}
6 (C)	60 (C)	6.5 (C)	7.8214×10^{-3}
7	40	7.5	6.385714×10^{-3}
8	40	6.0	7.147619×10^{-3}
9	40	7.0	3.8×10^{-3}

C central point

Fig. 1 Response surface for optimization of protease characterization showing the effects of pH and temperature (°C). Enzymatic casein hydrolysis (20 min runs) was performed with an initial substrate concentration of 0.5%

Table 2 Analysis of variance of the optimization of pH and Temperature °C in the protease secreted by *M. speciosa*

Variation source	SQ	DF	QM	F calculated	F tabulated (95% CI)	R ²
Regression	0.03	5	0.005664	78.66788	9.01	0.992431
Residual	0.00	3	7.2E-05			
Lack-of-fit	0.00	1	9.4E-06	0.090966	18.81	
Pure error	0.00	2	0.000103			
SQ total	0.03					

QS quadratic sum; DF degrees of freedom; QM quadratic mean; CI confidence interval

Fig. 2 Pareto chart of standardized effects of the variables studied in the planning. Influence of pH and temperature on the proteolytic activity of *M. speciosa* protease

Ion effect on *M. speciosa* protease catalytic activity

Among all evaluated ions, sodium sulfate and potassium sulfate (both 0.01 M) were capable of increasing enzyme activity to 230% and 206%, respectively, in comparison to the control (no ion addition). Magnesium sulfate and barium chloride also had positive effect on enzyme activity, which increased to 168% and 151%, respectively (Table 3).

Free protease thermostability investigation

Enzyme thermostability in buffer at pH 6.5 was noteworthy, since it maintained more than 90% activity for up to 60 incubation minutes at temperature ranging from 50 to 90 °C. This outcome has indicated the thermostable nature of protease secreted by *M. speciosa* (Fig. 3).

Table 3 Evaluation of enzymatic activity under the effect of different ions at a concentration of 0.1 M and 0.01 M. The control activity was taken as 100%

Salt	Residual activity % (average + SD)	
	0.01 M	0.1 M
Control	100	100
Na ₂ SO ₄	230 ± 0.0008	108 ± 0.0031
K ₂ SO ₄	206 ± 0.0028	105 ± 0.002
(NH ₄) ₂ SO ₄	121 ± 0.0014	114 ± 0.0024
MnSO ₄	126 ± 0.0026	118 ± 0.0018
FeSO ₄	120 ± 0.0037	109 ± 0.0034
MgSO ₄	168 ± 0.0016	136 ± 0.0029
Al ₂ (SO ₄) ₃	81 ± 0.0014	91 ± 0.0002
CaCl ₂	125 ± 0.0028	34 ± 0.0028
MgCl ₂	114 ± 0.018	122 ± 0.0001
NH ₄ Cl	131 ± 0.0002	42 ± 0.001
BaCl ₂	151 ± 0.0017	156 ± 0.005

SD standard deviation

Kinetic parameters of free protease

The kinetics of the protease reaction was determined using the Lineweaver–Burk plot (Fig. 4), with $V_{\max}$ and K_m ($R^2 = 0.9705$) of 0.01474 U/mL and 0.04190 mM, respectively, as kinetic parameters. The affinity constant was 0.35 U/mL/mM.

Enzyme immobilization through adsorption

Enzyme activity was measured in all carriers used for immobilization purposes. MAT540 and Celite 545 were the most efficient supports used in the enzyme immobilization. They enabled proteolytic activity much higher than that of free enzyme and recorded casein hydrolysis rate of 0.0310 and 0.0197 $\mu\text{g/mL/min}$, respectively. The other carriers allowed immobilization, however with lower enzymatic activity than MAT540 and Celite 545 (Table 4). The adsorption technique

is easy to perform and reproducible, increasing the stability and useful life of the enzyme.

MAT540 and Celite 545 supports presented 591 and 374% yield in the immobilization process, respectively, much higher than in the remaining carriers (Table 5).

Reusability of Materium 540-immobilized enzyme adsorption

Although all tested carriers have effectively immobilized protease, the enzyme immobilized on MAT540 support was selected to be tested for reuse cycles, since it was the one showing the best performance in the enzyme immobilization process (Fig. 5). The biocatalyst has shown catalytic activity higher than 90% until the second reuse; then, its enzyme activity decreased to 60% and remained stable until the 15th reuse.

Fig. 3 Variation in the enzymatic activity of the protease of *M. speciosa* as related to the temperature of the reaction medium within 50–90 °C. Catalytic activity was expressed as residual activity

Fig. 4 Lineweaver–Burk plot aiming at the determination of the enzymatic kinetic parameters $V_{\max}$ and K_m for casein hydrolysis by free *M. speciosa* protease

Table 4 Total protein concentration in the supernatant after immobilization and enzyme activity of free and immobilized protease by *M. speciosa* adsorption

Sample	Total protein	Enzymatic activity (U/mg) + SD
Free enzyme	10.06	$5.2 \times 10^{-3} \pm 7 \times 10^{-4}$
MAT 540	0.53	$3.1 \times 10^{-2} \pm 1 \times 10^{-3}$
ECR 8806F	9.18	$4.0 \times 10^{-3} \pm 2.0 \times 10^{-4}$
ECR 8204F	7.34	$9.9 \times 10^{-3} \pm 1 \times 10^{-4}$
ECR 1090	7.41	$5.8 \times 10^{-3} \pm 1.0 \times 10^{-4}$
CPC 27709	8.25	$7.3 \times 10^{-3} \pm 2.0 \times 10^{-4}$
SILICA	8.86	$5.1 \times 10^{-3} \pm 7.0 \times 10^{-4}$
CELITE 545	8.28	$1.97 \times 10^{-2} \pm 3 \times 10^{-4}$

SD Standard deviation

Table 5 Yield of immobilization of *M. speciosa* protease by adsorption on different inorganic supports

Support	Process yield %	Protein retention %
MAT 540	591	95
ECR 8806F	75	9
ECR 8204F	188	27
ECR 1090	111	26
CPC 27709	139	18
SILICA	96	12
CELITE 545	374	18

Temperature and pH optimization in the catalytic activity of immobilized protease

The pH and temperature investigations were performed based on Doehlert matrix (Table 6); maximum proteolytic activity was observed at the optimum temperature of 49.15 °C (Fig. 6). Immobilized protease has shown optimum activity at pH 5.74. Temperature and optimum pH found for the immobilized enzyme were lower than parameters optimized for free enzyme (Table 7). This pattern may have resulted either from the microenvironment surrounding the immobilized enzyme or from conformational changes that may have during protease immobilization. Figure 6 shows the response surface plot of pH and temperature effects on the protease activity of *M. speciosa* immobilized on MAT 540 support.

ANOVA was applied to evaluate the quadratic model based on data deriving from the experimental method. A test based on Fisher's distribution (F test) has indicated significantly adjusted equation ($F = 43.11 > 0.512$). Lack of squares' adjustment ($F = 9.01 < 18.81$) has indicated good agreement between the expected response of the model and experimental values recorded for each variable (Table 8).

Statistica 7.0 software was used to statistically evaluate the effects of pH and temperature on enzyme activity. Results enabled inferring that temperature/pH interaction had positive effect on enzyme activity at 5% significance level ($p < 0.5$), as seen in the Pareto Chart (Fig. 7).

Immobilized protease thermostability

The operating temperature of enzymes can substantially influence their yield and activity; besides, it plays an essential role in the application of natural-origin biocatalysts. The enzyme immobilized on MAT540 microspheres was tested

Fig. 5 Consecutive batch runs (cycles) for the hydrolysis of 0.5% casein solution with *M. speciosa* protease immobilized in MAT540

Table 6 Doehlert matrix used for the optimization of the enzymatic kinetics of immobilized protease of *M. speciosa*

Experiment	Temperature (°C)	pH	Enzyme activity (U/mg)
1	80	6.0	1.74×10^{-2}
2	80	7.0	2.06×10^{-2}
3	60	5.5	1.78×10^{-2}
4 (C)	60 (C)	6.5 (C)	1.82×10^{-2}
5 (C)	60 (C)	6.5 (C)	2.01×10^{-2}
6 (C)	60 (C)	6.5 (C)	1.97×10^{-2}
7	40	7.5	2.05×10^{-2}
8	40	6.0	1.85×10^{-2}
9	40	7.0	1.11×10^{-2}

at five different temperatures; it was capable of maintaining 100% of its residual activity at 50 °C. In addition, it maintained over 80% of residual activity at operating temperatures of 60 °C and 70 °C. No catalytic activity was observed at temperatures higher than 70 °C (Fig. 8).

Kinetic parameters of immobilized enzyme

Kinetic parameters of the protease produced by *M. speciosa* immobilized on MAT540 support were evaluated. Data were plotted using the Lineweaver–Burk double-reciprocal plot, as shown in Fig. 9.

K_m of 0.0796 mM and V_{max} of 0.0831 U/mL were obtained with a correlation R^2 of 0.9922, corresponding to an affinity constant of 1.04 U/mL/mM, while for the free enzyme V_{max} of 0.01474 U/mL and K_m of 0.04190 mM were found. Different concentrations of casein were used as a substrate and, as with the free enzyme, in concentrations above 2% of the substrate, a decrease in the immobilized protease activity was observed.

Reusability of alginate capsule-immobilized protease through encapsulation

Protease immobilized in alginate capsules was used in nine successive cycles, at pH 6.5 and 37 °C. Figure 10 shows the enzyme activity behavior in each reuse. The immobilized enzyme maintained 53% of its initial activity in the second cycle and reached 51% of it in the eighth use, whereas the encapsulated enzyme presented full activity loss during the ninth reuse cycle.

Table 7 Comparison of pH and Temperature (°C) parameters optimized for free and immobilized *M. speciosa* protease in MAT540

	pH	Temperature °C
Free enzyme	6.78	60.88
Immobilized enzyme	5.74	49.15

Fig. 6 Response surface for optimization of immobilized protease characterization showing the effects of pH and temperature (°C). Enzymatic casein hydrolysis (20 min runs) was performed with an initial substrate concentration of 0.5%

Table 8 Analysis of variance of the optimization of pH and Temperature (°C) in the protease secreted by *M. speciosa* immobilized on MAT540 support

Variation source	SQ	DF	QM	F calculated	F tabulated (95% CI)	R ²
Regression	0.00	5	3.52E-05	43.11083	9.01	0.986273
Residual	0.00	3	8.17E-07			
Lack-of-fit	0.00	1	5E-07	0.512991	18.81	
Pure error	0.00	2	9.75E-07			
SQ total	0.00					

QS quadratic sum; DF degrees of freedom; QM quadratic mean; CI confidence interval

Fig. 7 Pareto diagram for interaction of the variables analyzed using the Doehlert matrix. Influence of pH and temperature on the proteolytic activity of the *M. speciosa* immobilized enzyme **Fig. 8** Variation in the enzymatic activity of the protease of *M. speciosa* immobilized in MAT540 against variations in the reaction temperature from 50 to 90° C. Catalytic activity was expressed as residual activity

Discussion

Enzymes are highly specialized catalysts used to accelerate the pace of chemical reactions; thus, the production, purification and characterization of extracellular protease obtained from fungal species *M. speciosa* was evaluated in the current study. The investigated fungus was capable of producing protease with specific activity of $8.2 \times 10^{-3} \mu\text{g/mL/min}$. The requirement for specific nitrogen supplements differs from organism to organism (Sharma et al. 2017). In this study, peptone was chosen for use in the fermentation medium, because, among the nitrogen sources used for the composition of the media, this was considered the best source for the production of protease by *Pseudomonas fluorescens* (Kalaiarasi and Sunitha 2009), *Aspergillus niger* (Kalaskar et al. 2014), *Bacillus safensis* (Shakir et al. 2019).

Fig. 9 Lineweaver–Burk plot aiming at the determination of the enzymatic kinetic parameters $V_{\max}$ and K_m for casein hydrolysis by *M. speciosa* protease immobilized on MAT540

Fig. 10 Consecutive batch runs (cycles) for the hydrolysis of 0.5% casein solution with *M. speciosa* protease immobilized in calcium alginate by encapsulation

Species such as *Rhizopus oryzae*, *Aspergillus niger*, *A. oryzae*, *A. terreus* and *Cellulomonas bogoriensis* have shown optimal activity at 60 °C (Kumar et al. 2005; Siala et al. 2009; Castro and Sato 2014; Li et al. 2016; Raol et al. 2016), which was very close to the optimal temperature for *M. speciosa* proteolytic activity. According to Li et al. (2012), both the optimum temperature and pH of enzyme activity vary among different proteases produced by a single fungus, and it makes these values very specific to each enzyme.

Some ions manage to maintain the active conformation of the protein through the formation of either ionic bond or salt bridges between the residues, increasing the activity of the enzyme (Gupta 2003). The use of bivalent ions in low concentrations promotes the stabilization of some enzymes, as it stimulates the formation of cross-links, which stabilize the tertiary structure of the protein. Some cations can reduce enzymatic stability by destabilizing negative charges,

resulting in repulsion in the protein molecule, which can lead to loss of enzymatic activity (Tomazic 1991; Ghorbel et al. 2003; Pannkuk et al. 2015; Verma and Pandey 2019).

The identification of thermostable proteases has received considerable attention by the industry (Delatorre et al. 2010). Although fungi have moderate thermophilic nature, they are good thermostable protease producers. Protease produced by *Pleurotus sajor-caju* showed to be thermostable for up to 8 h at 80 °C and for up to 3 h at 90 °C (Benmradi et al. 2019). Enzymes produced by non-thermophilic fungi have shown thermostability up to 70 °C (Benmradi et al. 2016). Protease produced by *M. speciosa* has shown higher stability than the ones produced by different fungal species when it was incubated at high temperatures, although this behavior may be related to the cooling of the enzyme right after the incubation period at high temperatures. Thus, according to Jaenicke (1999) and Gomes et al. (2007) some proteins recover their active conformation after cooling. The results are interesting

for the industrial application of these enzymes, since thermostable proteases are widely used in the detergent and cosmetics industries.

Casein concentration in excess of 2%, led to a decrease in enzyme activity, probably due to inhibition by the high concentration of the substrate, which may have bound to the enzyme in an incorrect orientation (Barata et al. 2002; Schmidt and Salas-Mellado 2009). It was possible to compare these results with those reported in the literature regarding the proteolytic activity of enzymes produced by different microbial sources with casein as substrate, with K_m and V_{max} as reference. Thus, for *Rhizopus oryzae*, 7.0 mg/mL and 54.30 $\mu\text{mol}/\text{min}$ (Mushtaq et al. 2015); for *Bacillus mojavensis* 0.0357 mg/mL and 74.07 $\mu\text{g}/\text{mL}/\text{min}$ (Beg et al. 2002); for *Aspergillus niger* II acid protease 1.02 mM and 2.2 $\mu\text{mol}/\text{min}$ (Siala et al. 2009); and for *Aspergillus foetidus* 1.92 mg/mL and 357.14 U/mL (Souza et al. 2015). It can be concluded that the protease described in this work has high affinity for casein substrate, due to the low K_m .

The literature presents different reports about enzyme immobilization performance, as the one by Demirkan et al. (2017), who recorded yield of approximately 83%. Jing et al. (2017) reported increase to 927% and 201% in the enzyme activity of an alkaline protease immobilized on different supports. These values were much higher than the activity recorded for free protease in solution.

The use of immobilized enzymes implies that inactivation of the enzyme is not necessary at the end of the process, because the catalyst can be easily removed from the reaction medium, which is especially important in the limited hydrolysis of food protein (Tavano 2013). From the point of view of reducing the cost of the enzyme, the reuse of the immobilized enzyme is very important when considering its suitability for commercial applications (Tanksale et al. 2001).

Results in the present study have shown that, after fifteen reuses, enzyme retained 60% catalytic activity in comparison to the first catalytic activity cycle of the immobilized protease. The non-activated MAT540 support establishes only ionic bonds and physical interactions, attaching the enzyme molecules to its surface, allowing a general and smooth immobilization with high operational stability (Olah et al. 2018). Activated MAT540 as enzyme carrier has been considered to display significant operational stability, as it allowed to retain, above 60% of its initial activity after 5 cycles (Nagy et al. 2019) and 54% of its initial activity after 10 cycles (Olah et al. 2018). These data corroborate previous studies that have suggested that MAT540 is an efficient carrier in the enzyme immobilization processes and in the reuse of different enzymes such as protease and lipase (Olah et al. 2018; Nagy et al. 2019).

According to Bezerra et al. (2017) the operational half-life of the immobilized enzyme must be sufficiently to

allow reusing its derivative for several cycles; thus, it is an important operational parameter. According to Kumari et al. (2015) the strength of the interaction between the carrier and the enzyme is weakened during repeated use, leading to leaching of the enzyme from the matrix and loss of activity, which may explain the loss of enzyme activity during reuse cycles.

Olah et al. (2018) used the MAT540 support in immobilizing lipase at temperatures from 0 to 90 °C, and according to the results presented, the most productive and selective operations took place at a 60 °C. Nagy et al. (2019) tested the activity of the lipase immobilized in MAT540 up to 100 °C, and observed that the enzyme remained stable until the temperature of 70 °C and showed up to 20% of residual activity at the temperature of 100 °C. These results indicate that this support allows the use of different enzymes at high temperatures, thus maintaining the stability of the enzyme. This fact was corroborated by this work, in which the enzyme maintained up to 80% of initial catalytic activity at 70 °C, indicating that this is a good support for enzyme immobilization.

The apparent K_m increase upon immobilization is likely to be caused by mass transfer limitations that hamper the access of the enzyme to the substrate. Still, this effect was quite mild in the present system. The increased in V_{max} may reflect conformational changes upon immobilization favoring enzyme turnover (Anwar et al. 2009; Homaei et al. 2010; Homaei and Samari 2017; Moslemi et al. 2018). Moreover, the affinity constant increased about threefold upon immobilization, suggesting a positive effect on enzyme kinetics upon adsorption in MAT540.

The use of proteases immobilized in alginate capsules is already well reported in the literature (Anwar et al. 2009; Sharmila et al. 2012; Rezakhani et al. 2014; Thu and Krasae-koopt 2016), however studies have few cycles reuse. Zaphorlin et al. (2010), considered the *Myceliophthora* sp. protease to be stable for 7 cycles of use. Anwar et al. (2009) reported the total loss of catalytic activity in the 4th cycle of protease use. Sharma and Tripathi (2013), tested only three cycles of enzyme use, with loss of catalytic activity at each cycle. Both studies reported that the decrease in activity was due to the leakage of enzyme from the capsules, due to the washing process that occurs at the end of each cycle. This loss of activity was also observed in this study, and we can corroborate with the authors, taking into account that the enzymes were leached in the washing process.

Conclusions

The present study reported the production of proteases by the fungus *M. speciosa* with important characteristics for the world scenario of enzymes. The results revealed that

the free and immobilized enzyme showed an optimum temperature above 40 °C, suggesting its thermotolerant nature. However, the optimum pH of the free enzyme differs from the immobilized enzyme indicating structural changes in the three-dimensional structure of the protease. The maximum enzymatic activity was observed when the protease was immobilized in the Materium 540 carrier compared to the calcium alginate and free enzyme system. The enzyme immobilized in MAT540 retained 60% of the initial catalytic activity after 15 cycles of reuse, suggesting that this enzyme formulation has high operational stability. The results achieved hint that the protease from *M. speciosa* can be used either free or immobilized in bioconversion processes, reducing operating costs, decreasing the use of chemical processing, and consequently meeting the global demand for clean technologies.

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Compliance with ethical standards

Conflict of interest We declare no conflicts of interests.

References

- Anwar A, Qader SAU, Raiz A, Iqbal S, Azhar A (2009) Calcium alginate: a support material for immobilization of proteases from newly isolated strain of *Bacillus subtilis* KIBGE-HAS. *World Appl Sci* 7(10):1281–1286. [https://www.idosi.org/wasj/wasj7\(10\)/11.pdf](https://www.idosi.org/wasj/wasj7(10)/11.pdf)
- Banerjee G, Ray AK (2017) Impact of microbial proteases on biotechnological industries. *Biotechnol Genet Eng Rev* 33(2):119–143. <https://doi.org/10.1080/02648725.2017.1408256>
- Barata RA, Andrade MHG, Rodrigues RD, Castro IM (2002) Purification and characterization of an extracellular trypsin-like protease of *Fusarium oxysporum* var. lini. *J Biosci Bioeng* 94:304–308. <https://doi.org/10.1263/jbb.94.304>
- Beg QK, Saxena RK, Gupta R (2002) Kinetic constants determination for an alkaline protease from *Bacillus mojavensis* using response surface methodology. *Biotechnol Bioeng* 78(3):289–295. <https://doi.org/10.1002/bit.10203>
- Benmrad MO, Moujehed E, Elhoul MB, Jaouadi ND, Mechri S, Rekik H, Kourdali S, Hattab ME, Badis A, Sayadi S, Bejar B (2016) A novel organic solvent- and detergent-stable serine alkaline protease from *Trametes cingulata* strain CTM10101. *Int J Biol Macromol* 91:961–972. <https://doi.org/10.1016/j.ijbmac.2016.06.025>
- Benmrad MO, Mechri S, Jaouadi NZ, Elhoul MB, Rekik H, Sayadi S, Bejar S, Kechaou N, Jaouadi B (2019) Purification and biochemical characterization of a novel thermostable protease from the oyster mushroom *Pleurotus sajor-caju* strain CTM10057 with industrial interest. *BMC Biotechnol* 19(1):43. <https://doi.org/10.1186/s12896-019-0536-4>
- Bezerra TMS, Bassan JC, Bertazzoli RM (2017) Processo de obtenção de um suporte sólido para imobilização de enzimas, suporte sólido de fibra de coco verde, processo de imobilização de enzimas e derivado obtido. <https://hdl.handle.net/11449/148934>
- Botrel A, Laborde MCF, Medeiros FHV, Resende MLV, Ribeiro PM Jr, Pascholati SF, Gusmão LFP (2018) Saprobic fungi as biocontrol agents of halo blight (*Pseudomonas syringae* pv. garcae) in coffee clones. *Coffee Sci* 13(3):283–291. <https://doi.org/10.25186/cs.v13i3.1438>
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254. <https://doi.org/10.1006/abio.1976.9999>
- Castro RJS, Sato HH (2014) Production and biochemical characterization of protease from *Aspergillus oryzae*: Na evaluation of the physical-chemical parameters using agroindustrial wastes as supports. *Biocatal Agric Biotechnol* 3:20–25. <https://doi.org/10.1016/j.bcab.2013.12.002>
- Chandrasekaran S, Kumaresan SSP, Manavalan M (2015) Production and optimization of protease by filamentous fungus isolated from paddy soil in thiruvavur district tamilnadu. *J App Biol Biotech* 3:066–069. <https://doi.org/10.7324/JABB.2015.3610>
- Cruz ACR, Gusmão LFP (2009) Conidial fungi from Caatinga: lignicolous species. *Acta Bot Bras* 23:1133–1144. <https://doi.org/10.1590/S0102-33062009000400023>
- Delatorre AB, Ladeira AS, Andrade MVV, Barbosa JB, Martins MLL (2010) Microrganismos termofílicos e enzimas termoestáveis de importância comercial. *Perspectivas* 4:132–144
- Demirkan E, Avci T, Aykut Y (2017) Protease immobilization on cellulose monoacetate/chitosan-blended nanofibers. *J Ind Text* 47:2092–2111. <https://doi.org/10.1177/1528083717720205>
- Dewan SS (2018) Global markets for enzymes in industrial applications. Report overview, BIO030J. BCC Research, Wellesley
- Egorova K, Antranikian G (2005) Industrial relevance of thermophilic Archaea. *Curr Opin Microbiol* 8(6):649–655. <https://doi.org/10.1016/j.mib.2005.10.015>
- Ghorbel B, Kamoun AS, Nasri M (2003) Stability studies of protease from *Bacillus cereus* BG1. *Enzym Microb Technol* 32(5):513–518. [https://doi.org/10.1016/S0141-0229\(03\)00004-8](https://doi.org/10.1016/S0141-0229(03)00004-8)
- Gomes E, Guez MAU, Martin N, Silva R (2007) Enzimas termoeestáveis: fontes, produção e aplicação industrial. *Quím Nova* 30(1):136–145. <https://doi.org/10.1590/S0100-40422007000100025>
- Gupta R, Beg QK, Khan S, Chauhan B (2003) An overview on fermentation, downstream processing and properties of alkaline protease. *Appl Microbiol Biotechnol* 114(1):328–333. <https://doi.org/10.1007/s00253-002-1142-1>
- Homaei A, Samari F (2017) Investigation of activity and stability of papain by adsorption on multi-wall carbon nanotubes. *Int J Biol Macromol* 105:1630–1635. <https://doi.org/10.1016/j.ijbmac.2017.02.038>
- Homaei AA, Sajedi RH, Sariri R, Seyfzadeh S, Stevanato R (2010) Cysteine enhances activity and stability of immobilized papain. *Amino Acids* 38(3):937–942. <https://doi.org/10.1007/s00726-009-0302-3>
- Jaenicke R (1999) Stability and folding of domain proteins. *Prog Biophys Mol Biol* 8(6):738–748. [https://doi.org/10.1016/S0079-6107\(98\)00032-7](https://doi.org/10.1016/S0079-6107(98)00032-7)
- Jing M, Fei X, Ren W, Tian J, Zhi H, Xu L, Wang X, Wang Y (2017) Self-assembled hybrid nanomaterials with alkaline protease and a variety of metal ions. *RSC Adv* 7(76):48360–48367. <https://doi.org/10.1039/C7RA10597E>
- Kalaiarasi K, Sunitha PU (2009) Optimization of alkaline protease production from *Pseudomonas fluorescens* isolated from meat waste contaminated soil. *Afr J Biotechnol* 8:7035–7041. <https://www.ajol.info/index.php/ajb/article/view/68791>
- Kalaskar VV, Narayanan K, Subrahmanyam VM, Rao JV (2014) Optimization of extracellular acid protease production from

- Aspergillus niger* by factorial design. J Microbiol Biotechnol Food Sci 4(2):132–136. <https://doi.org/10.15414/jmbfs.2014.4.2.132-136>
- Kumar S, Sharma NS, Saharan MR, Singh R (2005) Extracellular acid protease from *Rhizopus oryzae*: Purification and characterization. Process Biochem 17:89–94. <https://doi.org/10.1016/j.procbio.2004.06.047>
- Kumari A, Kaur B, Srivastava R, Sangwan RS (2015) Isolation and immobilization of alkaline protease on mesoporous silica and mesoporous ZSM-5 zeolite materials for improved catalytic properties. Biochem Biophys Rep 2:108–114. <https://doi.org/10.1016/j.bbrep.2015.05.009>
- Kunitz M (1947) Crystalline trypsin inhibitor. II: general properties. J Gen Physiol 30:291–310. <https://doi.org/10.1085/jgp.30.4.291>
- Lanka S, Anjali CH, Pydipalli M (2017) Enhanced production of alkaline protease by *Aspergillus niger* DEF 1 isolated from dairy form effluent and determination of its fibrinolytic ability. Afr J Microbiol Res 11:440–449. <https://doi.org/10.5897/ajmr2016-8379>
- Li S, Yang X, Yang S, Zhu M, Wang X (2012) Technology prospecting on enzymes: application, marketing and engineering. Comput Struct Biotechnol J. 2:e201209017. <https://doi.org/10.5936/csbj.201209017>
- Li F, Yang L, Lv X, Liu D, Xia H, Chen S (2016) Purification and characterization of a novel extracellular alkaline protease from *Cellulomonas bogoriensis*. Protein Expr Purif 121:125–132. <https://doi.org/10.1016/j.pep.2016.01.019>
- Morya V, Yadav D (2010) Production and partial characterization of neutral protease by an indigenously isolated strain of *Aspergillus tubingensis* NIICC-08155. Internet J Microbiol. <https://doi.org/10.5580/1b13>
- Moslemi M, Homae A, Toiserkani H (2018) Aspartic acid introduce the functional amine groups on the surface of superparamagnetic Fe(OH)3@Fe3O4 nanoparticles for efficient immobilization of *Pancreas vannamei* protease. Bioprocess Biosyst Eng 202:110–115. <https://doi.org/10.1007/s00449-018-1908-1>
- Mushtaq Z, Irfan M, Nadeem M, Naz M, Syed Q (2015) Kinetics study of extracellular detergent stable alkaline protease from *Rhizopus oryzae*. Braz Arch Biol Technol 58(2):175–184. <https://doi.org/10.1590/S1516-8913201400071>
- Nagy F, Szabó K, Bugovics P, Hornyánszky G (2019) Bisepoxide-activated hollow silica microspheres for covalent immobilization of lipase from *Burkholderia cepacia*. Period Polytech Chem Eng. <https://doi.org/10.3311/PPch.12665>
- Olah M, Suba S, Boros Z, Kovács P, Gosselin M, Gaudreault C, Hornyánszky G (2018) Lipase B from *Candida antarctica* immobilized on epoxy functionalized hollow silica microspheres: efficient biocatalysts for enantiomer selective acylation of alcohols and amines. Period Polytech Chem Eng 62(4):519–532. <https://doi.org/10.3311/PPch.12517>
- Orlandelli RC, Almeida TT, Alberto RN, Polonio JC, Azevedo JL, Pamphile JA (2015) Antifungal and proteolytic activities of endophytic fungi isolated from *Piper hispidum*. Braz J Microbiol 46:359–366. <https://doi.org/10.1590/S1517-838246220131042>
- Pannkuk EL, Risch TS, Savary BJ (2015) Isolation and identification of an extracellular subtilisin-like serine protease secreted by the bat pathogen *Pseudogymnoascus destructans*. PLoS ONE 10(3):e0120508. <https://doi.org/10.1371/journal.pone.0120508>
- Rao PR, Rao D (1964) Some Helicosporae from hyderabad-I. Mycopathol Mycol Appl 22:47–54. <https://doi.org/10.1007/BF02058732>
- Raol GG, Patel PM, Raol BV, Panchal RR (2016) Alkaline protease production by *Aspergillus terreus* BAB-346 using poultry litter waste. Int J Curr Microbiol Appl Sci 5:174–184. <https://doi.org/10.20546/ijcmas.2016.510.020>
- Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A, Ashraf M (2019) Microbial proteases applications. Front Bioeng Biotechnol. <https://doi.org/10.3389/fbioe.2019.00110>
- Resende RS, Milagres CA, Rezende D, Aucique-Perez CE, Rodrigues FA (2015) Bioprospecting of saprobe fungi from the semi-arid north-east of Brazil for the control of anthracnose on sorghum. J Phytopathol 63:787–794. <https://doi.org/10.1111/jph.12376>
- Rezakhani N, Molaei A, Parivar K, Khayati M, Etemadzade S (2014) Immobilization of protease in biopolymers (mixture of alginate-chitosan). J Paramed Sci 5:108–113. <https://doi.org/10.22037/jps.v5i4.7858>
- Rubin-Pitel SB, Zhao H (2006) Recent advances in biocatalysis by directed enzyme evolution. Comb Chem High Throughput Screen 11:5898–5903. <https://doi.org/10.2174/138620706776843183>
- Schmidt CG, Salas-Mellado M (2009) Influence of alcalase and flavourzyme performance on the degree of hydrolysis of the proteins of chicken meat. Quím Nova 32(5):1144–1150. <https://doi.org/10.1590/S0100-40422009000500012>
- Shakir H, Mahmood R, Irfan M, Deebea F, Javed I, Qazi J (2019) Protease production from *Bacillus safensis* in submerged fermentation using response surface methodology. Rev Mex Ing Quim 18(1):375–385. <https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2019v18n1/Shakir>
- Sharma N, Tripathi S (2013) Kinetic study of free and immobilized protease from *Aspergillus* sp. J Pharm Biol Sci 7:86–96. <https://doi.org/10.9790/3008-0728696>
- Sharma KM, Kumar R, Panwar S, Kumar A (2017) Microbial alkaline proteases: optimization of production parameters and their properties. J Genet Eng Biotechnol 15:115–126. <https://doi.org/10.1016/j.jgeb.2017.02.00>
- Sharmila S, Rebecca LJ, Saduzzaman MD (2012) Immobilization of plant protease using calcium alginate beads. J Chem Pharm Res 4:4484–4488
- Siala R, Sellami-Kamoun A, Hajji M, Abid I, Gharsallah N, Nasri M (2009) Extracellular acid protease from *Aspergillus niger* 11: purification and characterization. Afr J Biotechnol 8:4582–4589
- Souza PM, Aliakbarian B, Filho EXF, Magalhães PO, Pessoa A Jr, Converti A, Perego P (2015) Kinetic and thermodynamic studies of a novel acid protease from *Aspergillus foetidus*. Int J Biol Macromol 81:17–21. <https://doi.org/10.1016/j.ijbiomac.2015.07.043>
- Tanksale A, Chandra PM, Rao M, Deshpande V (2001) Immobilization of alkaline protease from *Conidiobolus macrospores* for reuse and improved thermal stability. Biotechnol Lett 23(1):51–54. <https://doi.org/10.1023/A:1026782206945>
- Tavano OL (2013) Protein hydrolysis using proteases: an important tool for food biotechnology. J Mol Catal B Enzym 90:1–11. <https://doi.org/10.1016/j.molcatb.2013.01.011>
- Thu TTM, Krasakoopt W (2016) Encapsulation of protease from *Aspergillus oryzae* and lipase from *Thermomyces lanuginosus* using alginate and different copolymer types. Agric Natl Resour 32(5):1144–1150. <https://doi.org/10.1016/j.anres.2016.06.002>
- Tomazic SJ (1991) Protein stabilization. In: Dordick JS (ed) Biocatalysts for industry. Topics in applied chemistry. Springer, Boston, pp 241–255
- Vashist P, Kanchana R, Devasia VLA, Shirodkar PV, Muraleedharan UD (2019) Chapter 7-Biotechnological implications of hydrolytic enzymes from marine microbes. Advances in biological science research a practical approach. Academic Press, London, pp 103–118
- Verma J, Pandey S (2019) Characterization of partially purified alkaline protease secreted by halophilic bacterium *Citricoccus* sp. isolated from agricultural soil of northern India. Biocatal Agric Biotechnol 17(2):605–612. <https://doi.org/10.1016/j.bcab.2019.01.020>
- Yadav SK, Bisht D, Shikha S, Darmwal NS (2011) Oxidant and solvent stable alkaline protease from *Aspergillus flavus* and its

- characterization. Afr J Biotechnol 10:8630–8640. <https://doi.org/10.5897/AJB10.1611>
- Zanella ME (2014) Considerations on climate and water resources of the northeastern semiarid. Cad Prudent Geogr 36:126–142
- Zanphorlin LM, Facchini FDA, Vasconcelos F, Bonugli-Santos RC et al (2010) Production, partial characterization, and immobilization in alginate beads of an alkaline protease from a new thermophilic fungus *Myceliophthora* sp. J Microbiol 48:331–336. <https://doi.org/10.1007/s12275-010-9269-8>
- Zhu HY, Tian Y, Hou YH, Wang TH (2009) Purification and characterization of the cold-active alkaline protease from marine cold-adaptive *Penicillium chrysogenum* FS010. Mol Biol Rep 36:2169–2174. <https://doi.org/10.1007/s11033-008-9431-0>