Biochemical and thermodynamic characteristics of a new serine protease from *Mucor subtilissimus* URM 4133

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ABSTRACT

A protease from the fungus *Mucor subtilissimus* URM 4133, capable of producing bioactive peptides from goat casein, was purified. SDS-PAGE and zymography showed a molecular mass of 30 kDa. The enzyme was active and stable in a wide pH range (6.0–10.5) and (5.0–10.5), respectively. Optimum temperature was at 45–50 °C and stability was above 80 % (40 °C/2 h). Activity was not influenced by ions or organic substances (Triton, Tween, SDS and DMSO), but was completely inhibited by PMSF, suggesting that it belongs to the serine protease family. The Km and Vmax were 2.35 mg azocasein.mL-1 and 333.33 U.mg protein-1, respectively. Thermodynamic parameters of irreversible denaturation (40–60 °C) were enthalpy 123.63 – 123.46 kJ.mol-1, entropy 120.24–122.28 kJ.mol-1 and Gibbs free energy 85.97 – 82.45 kJ.mol-1. Any peptide sequences compatible with this protease were found after analysis by MALDI-TOF, which suggests that it is a new serine protease.

1. Introduction

One of the main areas of research in current biotechnology is related to the production of enzymes. These molecules are considered biological catalysts that have a high specificity of reaction with respect to the substrate. Its performance is also associated with factors such as temperature, pH and the presence of metal ions. Proteases or peptidases are responsible for catalyzing the cleavage of peptide bonds of proteins and peptides and, in some cases, may also have self-proteolytic activity [1].

Serine proteases correspond to the largest family of proteases in existence and are involved in several physiological processes. Their active site presents serine as a nucleophile, in addition to histidine and aspartic acid residues that complete the catalytic triad. However, in some serine proteases the nucleophile is combined with only a lysine or histidine residue, forming a catalytic dyad. Or, in some cases, serine can be combined with a pair of histidine residues to form a new catalytic triad [2]. In this class of enzymes, the nucleophile attacks the carbonyl portion of the peptide bond and forms an acyl-enzyme intermediate. In addition, specific inhibitors such as phenylmethanesulfonyl fluoride (PMSF) can inactivate the catalytic site. Functionally, these enzymes participate in post-transduction reactions of the polypeptide chains and act mostly as endopeptidases in the activation of zymogens, as is the case of trypsin [3].

Proteases have a wide range of uses in different industries, such as pharmaceuticals, detergents, food processing, dairy production, beer, leather, textiles, bioremediation and bio-control [4–6]. Thus, proteases are considered to be the main commercially produced

enzymes in the world. About two thirds of these enzymes are produced by microorganisms such as fungi, bacteria and yeast [6,7] Since fungal proteases are of commercial importance they have been described also in the patent literature, so it is important to look at such literature as well. Related to serine protease of fungus of the genus *Mucor*, the theme of this work, there is, for example, a patent for the *Mucor pusillus* protease as a source of milk-clotting enzymes, which was requested by Cornelius et al. [8]. Patents on serine proteases obtainable from *Fusarium acuminatum* and from *Fusarium equiseti* for different uses were deposited by Juntunen et al. [9] and Juntunen at al. [10], respectively. An interesting review of protease patents for the dairy industry was published by Feijoo-Siota et al. [11].

The advantage of using microorganisms as a source of protease production is due to the higher yield in the purification processes, together with a diversity of biochemical and catalytic properties. For this reason, filamentous fungi belonging to the genus *Mucor* are widely used in various industrial food processes, especially in the processing of dairy products. These fungi are considered as essential technological microorganisms in producing specific characteristics in these products, such as appearance, texture and sensory qualities [12,13].

The extensive use of proteases by different industries stimulates research to find this kind of enzyme with new properties and enables significant advances in the processing, preparation, storage and application of products. Within this scenario, the possibility of producing and applying proteases at room temperature without the need to increase energy production costs to generate heat is of extreme industrial importance [1]. Thus, studies on enzymatic thermostability are important to assess the economic viability of industrial processes that use enzymes as biological catalysts. A high enzymatic stability guarantees a reduction in the cost of the process, since there is little loss of these molecules. The analysis of kinetics and thermodynamics provides necessary information about enzymatic thermostability in relation to a specific operating temperature. In addition, to describe the thermodynamics of enzymatic denaturation, the parameters that are reported in the scientific literature correspond to activation energy and changes in Gibbs free energy, enthalpy and entropy between the stable enzyme and its activated state [14].

In previous work it was shown that the fungus *Mucor subtilissimus* URM 4133, a non-mycotoxin-producing microorganism, was capable of hydrolyzing goat casein and releasing different bioactive peptides [15]. Therefore, the objective of the present work was to purify an extracellular serine protease produced by the filamentous fungus *Mucor subtilissimus* URM 4133 and to analyze the biochemical characteristics and the kinetic and thermodynamic parameters of the denaturation, with a view to its later application for the release of bioactive peptides. As far as we know, no studies have been published on the serine protease produced by the filamentous fungus *Mucor subtilissimus*.

2. Material and methods

2.1. Microorganism and cultivation conditions

The filamentous fungus *Mucor subtilissimus* URM 4133 used in this study is deposited and stored in Micoteca – URM from Mycology Department/Center of Biosciences of the Federal University of Pernambuco - UFPE, Recife - PE / Brazil. This strain was preserved in mineral oil and maintained on Potato Dextrose Agar (PDA) at 30 °C for 7 days. This fungus was chosen for study because its protease was able to hydrolyze goat casein and produce biologically active peptides acting as antimicrobial and antihypertensive agents [15].

Protease production was carried out by submerged fermentation in 125 mL Erlenmeyer flasks containing 25 mL of fermentation medium, consisting of wheat bran (0.25 g), soybean meal (0.2 g) and 0.2 g of potassium monobasic phosphate (KH$_2$PO$_4$), sterilized at 121 °C for 20 min. The inoculum was standardized at 10^6 spores. mL^{-1} and triplicate assays performed in submerged fermentation systems for 96 h at 35 °C and 150 rpm. Enzyme production conditions were established using a full factorial design 2^4 according to [15]. At the end of the fermentation, the crude enzyme extract was obtained by filtration and centrifugation (4.000 x g at 4 °C) and stored (−20 °C) for further analysis.

2.2. Proteolytic activity and protein determination

Proteolytic activity was measured according to Leighton et al. [16], using azocasein (10 mg.mL^{-1}) as substrate. One unit of enzymatic activity (U) was defined as the 0.01 change in absorbance at 440 nm over one hour. The optical density of samples was taken on a Beckman spectrophotometer (model DU-800) against appropriate substrate and enzyme blank. The control of enzyme was performed by replacing the sample with deionized water. Enzyme dosages were expressed as U.mL^{-1}, and all assays performed in triplicate. The protein concentration of the samples was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). A curve was plotted to correlate optical density using bovine serum albumin (BSA) at different concentrations (0 and 2000 µg.mL^{-1}) as standard.

2.3. Protease purification

The serine protease was purified by two steps using ion exchange and gel filtration chromatographic techniques according to Darwesh et al. [17], with modifications. An Äkta Purifier FPLC system (GE Healthcare) was used. In the first step, a HiTrap DEAE FF 1 mL column (from the HiTrap IEX Selection Kit) was coupled to the FPLC using Tris-HCl buffer (20 mM) pH 8.5 as eluent A and Tris-HCl buffer (20 mM) pH 8.5, with 1 M NaCl, as eluent B. A linear gradient was applied, with a flow of 1 mL.min^{-1} and fractionation of 1 mL per microtube. Enzymatic activities and protein dosage of each tube were measured. The partially purified enzyme was lyophilized, and then resuspended in Tris-HCl buffer (20 mM) pH 8.5, with 100 mM glycine, to obtain twice the enzyme concentration for use in the next purification step. For the second purification step, a Superdex 75 10/300 G L molecular exclusion column was used with the Tris-HCl buffer (20 mM) pH 8.5, containing 100 mM glycine, as the only eluent with a flow rate of 0.5 mL.min^{-1} and a fractionation of 0.5 mL per microtube. Enzymatic and protein dosage from each tube was measured.

2.4. Sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) and Protein Zymography

SDS-PAGE electrophoresis analyses of the crude extract and purified aliquots of the enzyme were carried out using a 10 % polyacrylamide running gel in accordance with the method described by Laemmli [18] under denaturing conditions. The molecular standard (SDS-PAGE Molecular Weight Standards, Broad Range) from Bio-Rad Laboratories (Santo Amaro, Brazil) was composed of myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), albumin serum (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). Gel samples run at 150 V for 100 min. The bands were developed by the silver nitrate staining process.

The proteolytic activity of the purified enzyme was confirmed by zymography analysis according to the method described by

Egito et al. [19] under non-denaturing conditions. SDS-PAGE electrophoresis was performed on polyacrylamide gels containing casein (10 mg.mL^{-1}) as substrate. The purified sample was lyophilized and resuspended in Zymography Sample Buffer (Bio-Rad Laboratories, Santo Amaro, Brazil). After electrophoretic migration, the gel was washed with 2.5 % Triton X-100 for 30 min. at 4 °C. The hydrolysis reaction was performed inside the gel during incubation at 37 °C for 48 h in a 0.05 M Tris−HCl buffer pH 7.5. Subsequently, the protease were detected by the dye solution composed of ethanol 40 % (v/v), acetic acid 10 % (v/v) and 1 mg.mL^{-1} of Coomassie Briliant Blue (Sigma Aldrich, St.Louis, USA) for 60 min. Soon after, a bleach solution composed of ethanol 30 % (v/v) and acetic acid 7.5 % (v/v) was added until translucent bands appeared.

2.5. Protease biochemical characterization

2.5.1. Optimal pH and temperature
For optimum pH assays, azocasein (10 mg.mL^{-1}) was diluted in the following buffers: 0.2 M citrate - phosphate (pH: 5.0; 5.5; 6.0; 6.5; 7.0; 7.5 and 8.0); 0.2 M Tris −HCl (pH: 7.5; 8.0; 8.5 and 9.0) and 0.2 M carbonate - bicarbonate (pH: 8.5; 9.0; 9.5; 10.0 and 10.5). Proteolytic activity was performed at each pH. Subsequently, to study the purified serine protease optimal temperature, the proteolytic activity was performed at the pH determined as optimal and in a temperature range that ranged from 30 to 80 °C. The optical density of samples was taken on a Beckman spectrophotometer (model DU-800) against appropriate substrate and enzyme blank. The activity of non-heated enzyme, which was cooled on ice, was considered as a control (100 %). The control of enzyme was performed by replacing the sample with deionized water. Enzymatic activities were expressed as relative activity (%).

2.5.2. Temperature and pH stability
To verify the thermal stability of the serine protease, the purified enzyme was incubated in the absence of substrate at temperatures between 30 and 70 °C. For pH stability, the enzyme was diluted (1:1 v/v) in the same buffers used for the optimal pH study without substrate. Protease stability was assessed over the incubation time, so aliquots were taken at 0, 1, 2, 8 and 24 h. The optical density of samples was taken on a Beckman spectrophotometer (model DU-800) against appropriate substrate and enzyme blank. In both cases the stability was evaluated by measuring the residual activities of the aliquots taken at their respective times, under the conditions of temperature and optimum pH obtained previously. The activity of non-heated enzyme, which was cooled on ice, was considered as a control (100 %). The control of enzyme was performed by replacing the sample with deionized water. Enzymatic activities were expressed as relative activity (%).

2.5.3. Effect of metal ions, organic compounds and inhibitors on protease activity
To evaluate the interference of metal ions on protease activity, salts at the final concentrations of 1 and 5 mM containing the following metal ions were used: K$^+$, Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, Hg^{2+}, Ag$^+$, Co^{2+}. The following protease inhibitors were used at the final concentrations of 0.5 and 1 mM: phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor; ethylenediamine tetraacetic acid (EDTA), metallo protease inhibitor; pepstatin-A, aspartic protease inhibitor and inhibitor E-64, used for inhibition of cysteine proteases. In addition, other substances such as sodium dodecyl sulphate (SDS), Triton X-100, Tween-20, Tween-80, β-mercaptoethanol and DMSO at final concentrations of 1 and 2.5 mg.mL^{-1}, were

also evaluated for interference with enzymatic activity. The control sample was dialyzed against the buffer used and the activity of the enzyme without any additives (metal, inhibitors or ther substances) was considered to be 100 %.

2.6. Determination of kinetic parameters k_m and v_{max}

Azocasein (0.2 M Tris−HCl buffer, pH 8.5) was used as a substrate over a concentration range from 1 to 20 mg.mL^{-1} to determine the purified serine protease kinetics parameters: V_{max} and K_m. Proteolytic activity assays were performed at 45 °C. Both the maximum velocity (V_{max}) and the Michaelis-Menten constant (K_m) were obtained through the Lineweaver-Burk equation (Eq. 1), plotting a linear regression graph from the reaction velocities at different substrate concentrations.

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \tag{1}$$

2.7. Determination of kinetic and thermodynamic parameters of thermal inactivation

The thermal inactivation study of the purified serine protease was performed in the absence of substrate. The enzyme was incubated in Tris−HCl buffer (20 mM, pH 8.5) at various temperatures (40–60 °C) and aliquots taken at times 0, 1, 2, 8 and 24 h, and immediately cooled in an ice bath for 30 min. Residual sample activity was determined with the standard substrate (azocasein) under optimal pH and temperature conditions.

The value of the deactivation velocity constant (k_d) was expressed as an exponential decay and found by plotting ln (A/A$_0$) versus time. Denaturation energy (E_d) was determined by the Arrhenius plot, plotting ln (k_d) against 1000/T in degrees Kelvin [20].

The apparent half-life of the enzyme was defined as the time when the residual activity falls to 50 %, being estimated by the ratio of 0.693/k_d. Decimal reduction time (D value) was defined as the time required for 90 % reduction in activity or 1-log10 initial enzyme activity at a given temperature. This parameter is related to the deactivation constant (k_d) and was calculated by Eq. 2:

$$D = 2,303/k_d \tag{2}$$

The enzymatic denaturation thermodynamic was determined by rearrangement the Eyring absolute rate equation [21], derived from the transition state theory, as shown in Eq. 3:

$$k_d = \left(\frac{k_b T}{h}\right) \times\ e^{\left(\frac{-\Delta H_d}{RT}\right)} \times\ e^{\left(\frac{\Delta S_d}{R}\right)} \tag{3}$$

Where, k_b corresponds to the Boltzmann constant (1.38 × 10^{-23} J. K^{-1}); T is equivalent to absolute temperature in Kelvin (K); h is the Planck constant (6.626 × 10^{-34} Js); R is the universal gas constant (8.314 J.K^{-1}. mol^{-1}); ΔH_d^* is the denaturation enthalpy and ΔS_d^* the denaturation entropy.

For calculations of denaturation enthalpy (ΔH_d^*), denaturation entropy (ΔS_d^*) and denaturation Gibbs free energy (ΔG_d^*) from the enzymatic reaction system, the following equations were used:

$$\Delta H_d^* = E_d - RT \tag{4}$$

$$\Delta G_d^* = -RT ln^{\left(\frac{k_d h}{k_b \times T}\right)} \tag{5}$$

$$\Delta S_d^* = \frac{(\Delta H^* - \Delta G^*)}{T} \tag{6}$$

2.8. Peptide prospecting by mass spectrometry (MALDI-TOF)

The analysis of peptide sequences of the serine protease was performed using a 4800 Plus MALDI-TOF/TOF mass spectrometer (ABI/MDS Sciex, Foster City, USA). For this, the sample was prepared according to the methodology proposed by Osório and Reis [22]. The gel band containing the purified enzyme was subjected to the trypsin digestion process. Subsequently, the sample was enriched using Zip Tip C18 reverse phase chromatography ZipTips, (Merck Millipore, Burlington, USA) following the manufacturer's instructions and eluted on the MALDI plate with alpha-cyano-4-hydroxycinnamic acid elution solution (8 mg. mL^{-1}) of the MALDI matrix in 50 % (v/v) acetonitrile (ACN), 0.1 % (v/v) trifluoroacetic acid (TFA) and 6 mM ammonium phosphate. The mass spectra of the sequences were acquired in positive reflective mode in the m/z 700–5000 mass range. The highest intensity peaks were selected for MS/MS peptide sequencing. The proteins were identified by the Peptide Mass Fingerprint + MS/MS approach with Mascot software (v2.5.1, Matrix Science) using the UniProt protein sequence database for Mucoromycota taxonomic selection (2017_08 version). Protein screening settings were methionine oxidation (variable modification), zero lost cleavages, and maximum error tolerance of 50 ppm (MS) and 0.5 Da (MS/MS). Protein scores greater than 67 were considered significant (p < 0.05).

3. Results and discussion

3.1. Protease production

Experimental results obtained using a full factorial design 2^4 [15] revealed a minimum production of 0.814 ± 0.070 U.mL^{-1} at 45 °C, with 0.25 g of wheat bran, 0.4 g of soybean meal and 0, 1 g of monobasic potassium phosphate (KH$_2$PO$_4$). Moreover, that the best production conditions were found at 35 °C using 0.25 g of wheat bran, 0.2 g of soybean meal and 0.2 g of monobasic potassium phosphate (KH$_2$PO$_4$), obtaining a maximum proteolytic activity of 8.943 ± 0.220 U.mL^{-1}. Nascimento et al. [23], working with statistical planning for the optimization of extracellular serine protease production by *Acremonium* sp. L1−4B, used a factorial 2^3 as the first-order planning and obtained quite different results between assays, with proteolytic activities ranging from 0.07 ± 0.01–191.32 ± 0.01 U.mL^{-1}. This shows that such a planning already results in significant influences on enzyme production, since it allows the observation of which variables have a direct influence on enzyme synthesis.

It is difficult to compare the results from different studies even though the techniques are identical. Conditions of work in the lab, variations in experimental parameters such as composition and activity of individual proteases in the enzyme solution, the unit of activity for protease etc. are parameters that can contribute to different results. Furthermore, the proteases are influenced by the nature of the substrate, such as natural or chemical substrates. However, the results obtained in this work were compared with other fungal species under similar reaction conditions due to the lack of studies in the scientific literature using the same fungus species as the present work. Even the commercial proteases available are from different fungal species. A search on the MEROPS website (https://www.ebi.ac.uk/merops/index.shtml) the most complete protease/peptidase website in the world, reveals that no protease of *Mucor subtilissimus* fungus origin has yet been reported in the literature. Although performed under different experimental conditions, the results of the present work (8.943 ± 0.220 U.mL^{-1}) were higher than those found by Yegin et al. [24], who worked with the production of aspartyl protease from *Mucor mucedo* DSM 809 by submerged fermentation in a medium composed of casein, glucose and a mineral solution with monobasic potassium phosphate and reached a production of 5.4 ± 0.8 U.mL^{-1}. It was also larger than Merheb-Dini et al. [25], who worked with the production of a coagulant aspartic protease from the fungus *Thermomucor indicae-seudaticae* N31 and obtained a maximum proteolytic activity of 2.6 ± 0.1 U.mL^{-1}. However, Daudi et al. [26], worked with several strains of *Mucor* sp. and from a screening, they obtained maximum activity for *Mucor pusillus*, a fungus known for the production of acidic proteases with coagulant activity, of 75 U. mL^{-1}.

3.2. Protease purification

The SDS-PAGE electrophoretic profile of proteins present in the crude extract of the fungus *Mucor subtilissimus* URM 4133 revealed the presence of dense bands with molecular mass ranging from approximately 6.5–31 kDa (Fig. 1A). The FPLC purification procedures were efficient as to the purity obtained, as can be seen in Fig. 1B and Table 1 (62 % recovery and a purification factor of 22). Zymography confirmed the result of SDS-PAGE electrophoresis and also revealed that the serine protease of the fungus URM 4133 has a molecular mass of approximately 30 kDa (Fig. 1C).
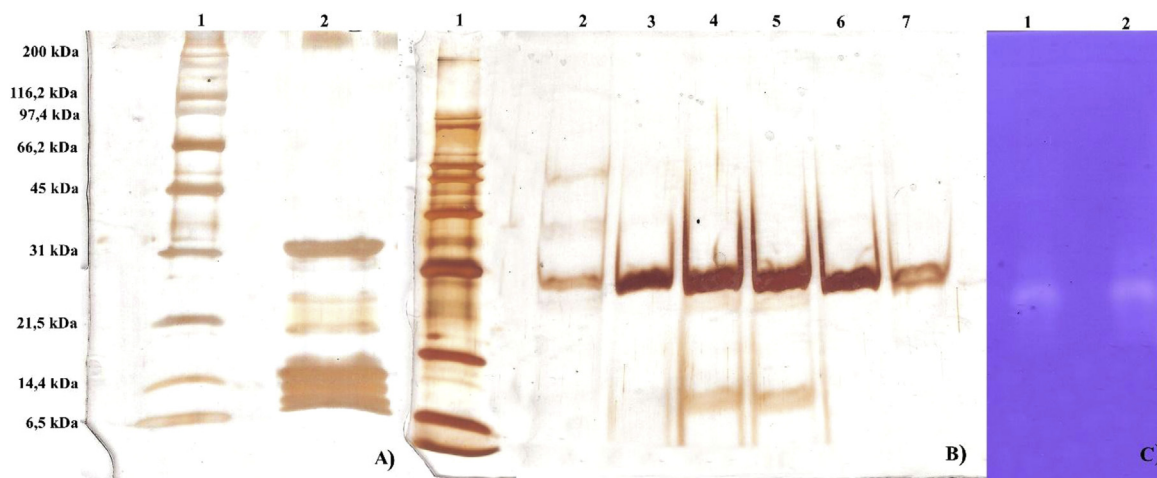

Fig. 1. SDS-PAGE electrophoresis of crude extract and purified fractions and zymography of serine protease produced by *Mucor subtilissimus* URM 4133.
(A). SDS-PAGE: Lane 1, protein molecular marker; Lane 2, crude extract. (B). SDS-PAGE: Lane 1, protein molecular marker; Lanes 2–7, fractions collected, with high purity in Lanes 6 and 7. (C). Zymography (10 %) of purified serine protease (Lanes 1 and 2).

Table 1
Purification steps of *Mucor subtilissimus* URM 4133 serine protease.

URM 4133	Total activity (U. mL^{-1})	Total protein (mg. mL^{-1})	Specific activity (U. mg^{-1})	Yield (%)	Purification (x)
Crude extract	4.95	0.628	7.88	100	1.0
DEAE FF	4.85	0.404	12.00	98	1.5
DEAE FF [2X][a]	8.92	0.615	14.50	90	1.8
Molecular Exclusion	5.56	0.020	278.00	62	35

[a] The partially purified enzyme was concentrated twice by lyophilization; yield was divided by 2, as total activity was increased twice by lyophilization.

The diversity of proteolytic enzymes present in fungal extracts is a stimulating factor in the search for new proteases from these microorganisms. Nascimento et al. [27], worked with the fungus *Mucor subtilissimus* UCP 1262 and reported the production of a 97 kDa molecular mass fibrinolytic protease. Whereas, Yadav et al. [28], working with the fungus *Aspergillus flavus* MTCC 9952, observed the presence of a serine protease with a molecular mass of 38 kDa. These results are different from those obtained in this work and demonstrate the variety of proteolytic enzymes that can be obtained from different fungal species and possibly with different properties and applications.

3.3. Biochemical characterization of purified protease

3.3.1. Effect of pH and temperature on enzymatic activity and stability

The enzyme showed a broad range of action at different pH values, having good performance over the entire pH range studied (Fig. 2A). The highest relative activity value was obtained under optimal conditions of pH 8.5 in carbonate-bicarbonate buffer. In addition, as shown in Fig. 2B, the serine protease showed stability over the entire pH range tested (5.0–10.5), with a slight decrease above pH 8.5.

There are few studies in the literature on biochemical, kinetic and thermodynamic characteristics using proteolytic enzymes from fungus of the *Mucor* genus. Therefore, reports with other species of filamentous fungus were also used for comparison. Yegin et al. [24] working with the fungus *Mucor mucedo* DSM 809 and Fernandez-Lahore et al. [29] working with different species of

Mucor sp., reported results of maximum proteolytic activity only in a small range of acidic pH. These enzymes did not show stability under alkaline pH conditions. However, the fungi of genera *Aspergillus* [30–32] and *Purpureocillium* [32] are reported to produce alkaline proteases with pH stability in the range of 8.0–10.0. The effect of pH on the protease obtained in this work shows a typical profile of serine proteases (broad pH action). In addition, its stability appears to be greater than that of other enzymes purified from different filamentous fungi, since few studies report considerable activity after 24 h. Thus, the results in the present study suggest that the enzyme may be a good candidate for alkaline process applications such as use in detergents [34] or bio-control agent against nematodes [5].

The optimum temperature of the purified serine protease was 45 °C, with the activity remaining virtually unchanged at 50 °C. However, even above 55 °C, the enzyme activity was greater than 50 % (Fig. 2C). The serine protease obtained in this study showed a maximum activity temperature higher than the enzyme from a different strain of *Mucor subtilissimus*, reported by Nascimento et. al [27], from a fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262, which presented an ideal temperature of 35 °C. Other species of filamentous fungi produce proteases with optimum temperatures similar to that found in this study, as is the case of the synthesized protease by *Aspergillus flavus* MTCC 9952 [28].

Regarding the thermal stability of the purified serine protease, the enzyme activity started to decline at 35 °C after 1 h of incubation and the enzyme lost 20 % of its activity after 24 h at that
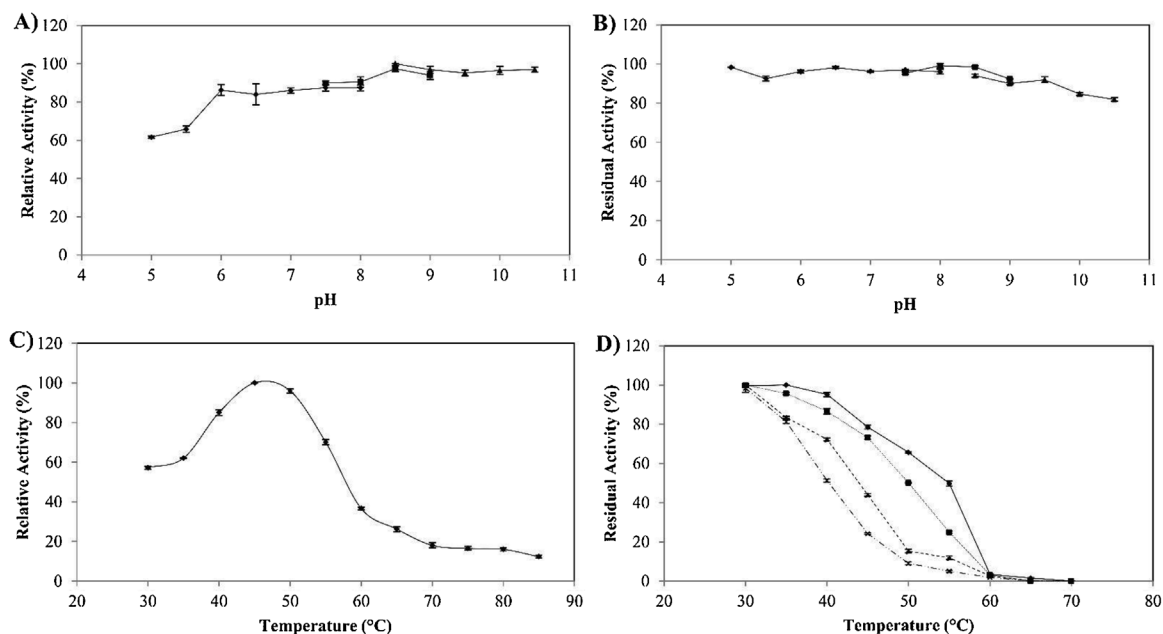

Fig. 2. Biochemical characterization by *Mucor subtilissimus* URM 4133 purified serine protease.
(A). Optimum pH in citrate - phosphate (◆), Tris −HCl (■) and carbonate - bicarbonate (▲) buffers. (B). pH stability in buffers after 24 h of incubation. (C). Optimum temperature (D). Thermal stability for 1 h (◆); 2 h (■); 8 h (▲) and 24 h (×). All assays were performed in triplicate.

same temperature (Fig. 2D). This drop in activity is accentuated at higher incubation temperatures. However, even after the 24 h incubation period, the enzyme remained above 50 % of activity at 40 °C. Proteases produced by mesophilic fungi are generally denatured at temperatures above 50 °C [23,35]. Similar results were obtained by Areces et al. [36], for the protease produced by *Mucor bacilliformes*, a mesophilic species, with residual activity of 29 % at 55 °C and only 8% after 30 min. incubation at 60 °C. An advantage can be seen in the serine protease of *Mucor subtilissimus* URM 4133, since this enzyme was only denatured at 65 °C after 2 h of incubation and at 70 °C after 1 h.

3.3.2. Effect of metal ions, organic compounds and inhibitors

The effect of various ions, inhibitors and other substances on the activity of the purified enzyme was evaluated and the results are shown in Table 2. The concentration of 5 mM of the ions Mn^{2+}, Mg^{2+} and K^+ resulted in greater activation of the serine protease, while in Hg^{2+}, Ag^+ and Co^{2+} ions there was a marked inhibition of activity at a concentration of 5 mM. Similar results were found by Yadav et al. [28], who also observed a positive effect of the divalent ions Mn^{2+} and Mg^{2+} on the increase of proteolytic activity (154.1 % and 108.1 %, respectively), as well as inhibition of this activity by mercury at higher concentrations. Positive effects for Mg^{2+} and K^+ were also found by Farhadian et al. [7], in the synthesized serine protease from *Bacillus subtilis* DR8806, as well as in the inhibition by Hg^{2+} and Co^{2+} ions. This suggests that cations with a positive effect on proteolytic activity may contribute to the maintenance of the active site of the enzyme and may improve its thermostability.

Among the substances evaluated, the anionic detergent sodium dodecyl sulphate (SDS) partially inhibited the enzymatic activity of the serine protease only in its highest concentration (2.5 mg.mL^{-1}). The nonionic surfactants Triton X-100, Tween-20 and Tween-80 did not show considerable reduction on residual enzyme activity, while β-mercaptoethanol, a disulfide bridge reducer, partially inhibited protease at both concentrations evaluated. The polar dimethyl sulfoxide solvent (DMSO) did not able to interfere with the catalytic performance of the purified serine protease, even at the 15 % concentration [37], in which about 97 % of the activity was maintained (data not shown). These results were better than those found for a serine protease produced by the fungus *Myceliophthora* sp., which showed a 12 % reduction in enzymatic activity for Triton X-100 and an 80 % reduction in proteolytic activity for Tween-80, both non-ionic surfactants, and was denatured in the SDS [38]. This demonstrates the ability of the serine protease produced by the fungus *Mucor subtilissimus* URM 4133 to maintain its activity in the presence of different substances, with surfactant or reducing

characteristics, within the reaction medium, being useful for several industrial processes.

In the study of protease enzyme inhibitors, the enzyme was completely inhibited by PMSF, an irreversible serine protease inhibitor, showing the involvement of a serine residue in its active site, which suggests that this protease belongs to the serine protease family. Other types of *Mucor* species that secrete serine proteases are portrayed in the literature, such as the protease excreted by the fungus *Mucor racemosus* [39] and the fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262 [27]. Despite few reports of serine protease from fungi of the *Mucor* genus, recent research of Muszewska et al. [40] has suggested that serine proteases are more ubiquitous for fungal kingdom than expected.

In addition to the inhibition by PMSF the serine protease was also inhibited by Hg^{2+} (∼ 62 %), which could indicate a possible thiol group dependence, similar to the result of Matkawala et al. [41]. However, it was not stimulated by β-mercaptoethanol, in fact it showed some inhibition in the presence of this reagent (∼ 32 % inhibition), differing from the results of these authors. Others authors as Beg and Gupta [42] and Moradian et al. [43] also reported a thiol-dependent serine protease from *Bacillus mojavensis* and from *Bacillus* sp., respectively. However, on these works 2-mercaptoethanol has also increased the enzyme activity. In this sense, we cannot affirm that the serine protease of this work is a thiol group-dependent serine protease.

3.4. Kinetic parameters k_m and V_{max}

Kinetic parameters V_{max} and K_m of the purified serine protease were measured at 45 °C and pH 8.5 and substrate concentration ranging from 1 to 20 mg.mL^{-1}. The results were plotted according to the double reciprocal graph (Lineweaver-Burk) presented in Fig. 3.The data showed a good correlation (R^2 = 0.994) with Michaelis-Menten kinetics with K_m = 2.35 mg azocasein.mL^{-1} and V_{max} = 333.33 U.mg protein^{-1} values. Until the writing of this article, no reports of kinetic parameters were found for *Mucor* fungus proteases with the use of azocasein as substrate. However, Castro et al. [44] evaluated the application of different substrates for protease production by *Aspergillus niger* and obtained K_m values ranging between 0.44 and 1.92 mg azocasein.mL^{-1} and V_{max} between 42.74 and 344.83 U. g^{-1}, values that do not differ much from the results obtained in this work.

3.5. Kinetic and thermal inactivation thermodynamics parameters

To determine the kinetic and thermodynamic parameters of irreversible thermal inactivation of purified serine protease, data

Table 2
Effect of metal ions, inhibitors and other substances on purified serine protease activity.

| Residual Activity (%)					
Metal ions			**Inhibitors**		
	1 mM	**5 mM**		**0.5 mM**	**1 mM**
CaCl$_2$	109.32 ± 1.21	100.93 ± 2.18	EDTA	101.55 ± 2.55	101.97 ± 0.95
MnCl$_2$	124.35 ± 2.91	128.45 ± 1.78	Pepstatin-A	89.57 ± 1.72	88.15 ± 1.99
MgCl$_2$	105.80 ± 1.54	114.30 ± 0.67	PMSF	2.38 ± 0.49	1.14 ± 0.46
FeCl$_3$	105.28 ± 1.93	93.89 ± 0.91	E-64	103.32 ± 0.21	101.97 ± 1.02
CdCl$_2$	106.84 ± 1.97	104.14 ± 1.28			
HgCl$_2$	93.06 ± 2.20	38.76 ± 0.87	**Other substances (mg. mL^{-1})**		
AgNO$_3$	86.63 ± 0.72	28.60 ± 1.87		**1 mg.mL^{-1}**	**2.5 mg.mL^{-1}**
CuCl$_2$	98.55 ± 2.17	79.59 ± 2.43	Triton X-100	93.16 ± 2.11	91.60 ± 2.07
CoCl$_2$	61.14 ± 0.43	60.62 ± 0.69	Tween-20	104.04 ± 1.92	94.92 ± 1.20
KCl$_2$	111.40 ± 2.11	112.64 ± 1.65	Tween-80	100.62 ± 2.66	99.17 ± 2.17
			SDS	101.11 ± 1.93	68.19 ± 1.96
Control	100 %		DMSO	106.42 ± 3.33	103.83 ± 1.43
			β-Mercaptoethanol	65.91 ± 0.73	68.39 ± 2.04
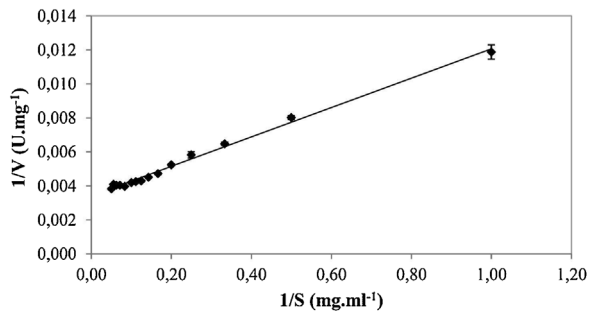
Fig. 3. Determination K_m and V_{max} of purified serine protease produced by *Mucor subtilissimus* URM 4133. Double reciprocal (Lineweaver-Burk) plot of initial azocasein hydrolysis rate by purified serine protease versus azocasein concentration. Error bars represent standard deviation.

from the enzymatic thermal stability study were used. In this case, the Arrhenius graph (Fig. 4A) was plotted between temperatures of 40 and 60 °C. The half-life ($t_{1/2}$) corresponds to the time required for enzymatic activity to reduce to 50 % of its initial catalytic activity when subjected to a given temperature. This parameter is very important for the use of an enzyme at an industrial level, since the higher its value, the higher the enzymatic thermostability. Table 3 shows that $t_{1/2}$ decreased progressively, associated with the increase of the first-order specific rate of thermal protease inactivation (k_d), as a function of the gradual temperature increase, which means that irreversible denaturation is increasingly expressive. In the present work, the lowest value of k_d was obtained for the temperature of 40 °C (0.0297 h^{-1}) and the highest value at 60 °C (0.7916 h^{-1}), presenting half-life times ($t_{1/2}$) of 23.34 and 0.88 h, respectively. Similar results were obtained in a study of the protease produced by *Aspergillus niger*, which presented $t_{1/2}$ values =1386.29 min., or 23.1 h [44]. Hernández-Martínez et al. [12], observed a $t_{1/2}$ of only 34 min. for the serine protease produced by *Aspergillus fumigatus*. Therefore, the purified serine protease in the present work presented thermostability similar to other proteases cited in the literature.
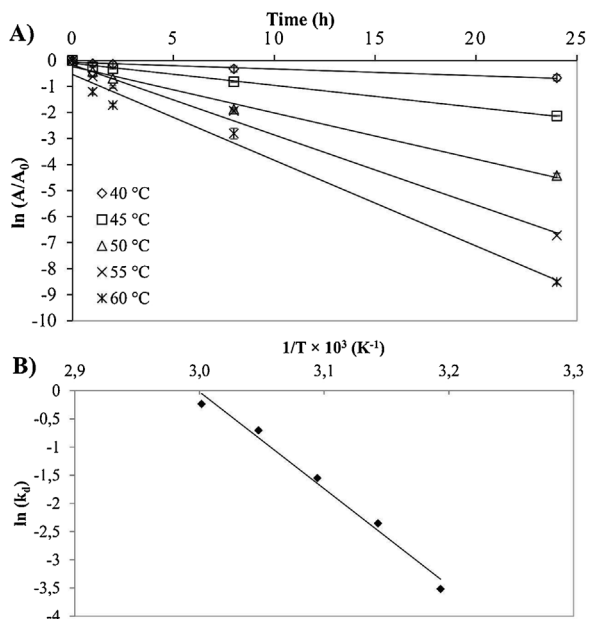

Fig. 4. Determination of the thermal denaturation kinetic and thermodynamic parameters of the purified serine protease by *Mucor subtilissimus* URM 4133. (A) First-order graph of irreversible thermal denaturation. (B) Arrhenius graph to calculate the activation energy (E_d) of irreversible thermal inactivation/denaturation..

Decimal reduction time (*D* value), defined as the time required for the 90 % reduction in initial enzyme activity, corresponds to another parameter that can be used to ascertain the enzyme's resistance to thermal inactivation and to provide essential information for industrial use. It can be seen from Table 4 that at 40 °C the enzyme showed good stability, since it takes 77.54 h to reduce its activity to 10 % of the initial value.

The Arrhenius graph (Fig. 4B) of $\ln(k_d)$ *vs* 1/T allowed to estimate an activation energy for thermal denaturation (E_d) of 123.26 kJ.mol^{-1} (R^2 = 0.9844). This value is higher than those found by Silva et al. [15] who had an E_d =49.7 kJ.mol^{-1} for the protease present in the crude extract produced by the fungus *Aspergillus tamarii* URM 4634 and E_d = 28.8 kJ.mol^{-1} for its purified form. And by Lima et al. [45] who obtained an E_d =107.4 kJ.mol^{-1} for a collagenolytic protease produced by the fungus *Penicillium aurantiogriseum* URM 4622. This indicates a higher thermostability of the purified serine protease studied in the present work. Furthermore, it can be seen from these comparisons that the variability of E_d data is very large, probably due to large differences in the source and purity of the enzymes along with the substrates used.

Moreover, the stability of a protein at a given temperature corresponds to the result of the balance between stabilizing and destabilizing forces, which are influenced by hydrophobic and electrostatic interaction, hydrogen and disulfide bonding and the degree of folding of the molecule [46]. Thus, the investigation of thermodynamic parameters such as enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) of the purified serine protease of the fungus *Mucor subtilissimus* URM 4133 was performed to understand the behavior of this molecule under different conditions and the results are presented. in Table 3. The ΔH is seen as a measure of the number of broken non-covalent bonds forming a transition state for enzymatic inactivation. In general, higher ΔH values are associated with greater enzyme stability [47,48]. The ΔH_d was 123.63 kJ.mol^{-1} at 40 °C and was higher than that found by Melikoglu et al. [48] for protease synthesized by the fungus *Aspergillus awamori* that had a ΔH_d =80.4 kJ.mol^{-1} at the same temperature. Souza et al. [49], from a thermophilic protease from *Aspergillus foetidus*, obtained ΔH_d =311.39 kJ.mol^{-1} at 55 °C. This, therefore, agrees with the fact that the higher the ΔH, the higher the thermal stability of the enzyme.

The extent of the enzyme's thermal denaturation process also depends on the denaturation entropy (ΔS_d) which expresses the amount of energy per degree involved in the transition from a native to a denatured state. The ΔS represents the variation in the extent of local disorder between transition state and ground state. Thus, a higher ΔS implies an increase in the number of protein molecules in the transitional active state and an increase in the disorder that may be from the enzyme's active site or structure, which equals the main driving force in the heat denaturation process [48,50,51]. The ΔS_d values were positive at all temperatures at which the *Mucor subtilissimus* URM 4133 serine protease was tested and ranged from 120.24–124.82 kJ.mol^{-1}. This suggests a high state of disorder in the transition process. Positive ΔS_d values were also found for *Aspergillus niger* protease [44] (222.47 $\leq \Delta S_d \leq$ 227.58 J.mol^{-1}. K^{-1}) and for the protease secreted by *Aspergillus foetidus* [49] (599.59 $\leq \Delta S_d \leq$ 610.49 J.mol^{-1}. K^{-1}). Olusesan et al. [46] indicate that positive values for ΔS_d are found if the limiting reaction rate is protein folding, with moderately high results for ΔH and lower for ΔG, which is in accordance with the present work.

Gibbs free energy (ΔG) is another important thermodynamic parameter and includes both previously reported contributions (enthalpy and entropy). Thus, it can be considered as a more accurate and reliable tool for assessing the stability of an enzyme. The lower or negative its value, the more spontaneous the process,

Table 3
Kinetic and thermodynamic parameters of irreversible thermal denaturation of purified serine protease.

T (°C)	k_d (h^{-1})	$t_{1/2}$ (h)	D (h)	R^2	ΔH_d (kJ. mol^{-1})	ΔG_d (kJ. mol^{-1})	ΔS_d (J. mol^{-1}. K^{-1})	E_d (kJ. mol^{-1})
40	0.0297	23.34	77.54	0.9681	123.63	85.97	120.24	126.23
45	0.0951	7.29	24.22	0.9942	123.59	84.31	123.45	
50	0.2127	3.26	10.83	0.9905	123.54	83.51	123.87	
55	0.4952	1.40	4.65	0.9784	123.50	82.54	124.82	
60	0.7916	0.88	2.90	0.9594	123.46	82.45	122.28	

i.e. the enzyme becomes less stable and denaturation occurs more easily [50,52]. Consistent with the other parameters previously discussed, serine protease purified from *Mucor subtilissimus* URM 4133 showed positive values for ΔG_d (Table 3), which ranged from 82.45–85.97 kJ.mol^{-1} in the temperature range studied. The decrease in G*, according to the increase in temperature, revealed that the thermal destabilization of the enzyme was due to the drop in free energy (functional energy) that induced the enzyme to unfold its transition state. Similar values were obtained by Silva et al. [14] for the purified protease from *Aspergillus tamarii* URM 4634 (91.8 ≤ ΔG_d ≤ 98.0 kJ.mol^{-1}) and by Hernández-Martínez et al. [12] for the purified *Aspergillus fumigatus* serine protease (89.2 ≤ ΔG_d ≤ 91.4 kJ.mol^{-1}).

3.6. Peptide prospecting by mass spectrometry (MALDI-TOF)

Mass spectrometry using matrix assisted laser desorption ionization coupled to time of flight analysers, MALDI-TOF-MS, has become popular during the last decade due to its high speed and sensitivity for detecting proteins and peptides [53]. The tool used to sequence the purified serine protease produced by the fungus *Mucor subtilissimus* URM 4133 was based on the peptide mass fingerprinting (PMF) technique using a MALDI-TOF/TOF mass spectrometer. The mass of trypsin-digested peptides was determined and searched in databases at the National Center for Biotechnology Information (NCBI), more specifically at the Universal Protein Resource (UniProt), and was not matched with any proteases present in this database (Fig. 5). However, the sequence obtained was combined with many hypothetical and uncharacterized proteins from *Mucoromycota* division fungi (Fig. 6), such as an uncharacterized *Rhizopus delemar* protein (Accession No: I1CSQ0, Mass: 167476, Score: 59, Matches: 66), another unclassified *Lichtheimia ramosa* protein (Accession N°: A0A077 × 1Z2, Mass: 170271, Score: 57, Matches: 64) and a fragment of a *Rhizopus stolonifer* fungus heat shock protein (Accession No: Q8J1X9, Mass: 55014, Score: 54, Matches: 28). In addition, the peptide mass derived from *Mucor subtilissimus* URM 4133 serine protease was also combined with other non-
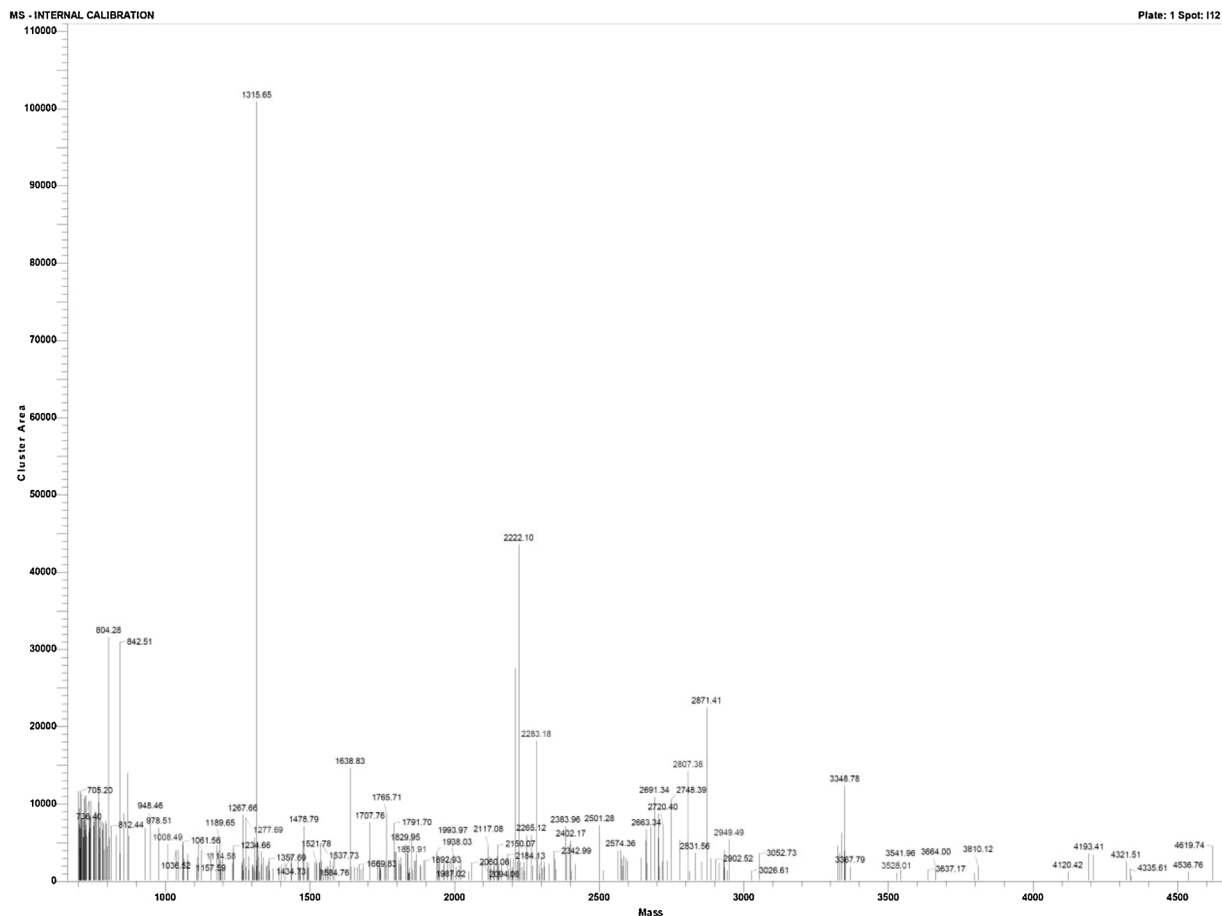

Fig. 5. MALDI-TOF/TOF – MS/MS mass spectrum of the purified serine protease produced by Mucor subtilissimus URM 4133. The observed mass PMF was performed using the MASCOT search system.

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I1CSQ0     Mass: 167476   Score: 59     Expect: 0.36  Matches: 66
Uncharacterized protein OS=Rhizopus delemar (strain RA 99-880 / ATCC MYA-4621 / FGSC 9543 / NRRL 43880)

A0A077X1Z2  Mass: 170271   Score: 57     Expect: 0.63  Matches: 64
Uncharacterized protein OS=Lichtheimia ramosa GN=LRAMOSA05466 PE=4 SV=1

Q8J1X9     Mass: 55014    Score: 54     Expect: 1.3   Matches: 28
70 kDa heat shock protein 1 (Fragment) OS=Rhizopus stolonifer PE=3 SV=1

S2JX18     Mass: 30010    Score: 50     Expect: 2.8   Matches: 18
Uncharacterized protein OS=Mucor circinelloides f. circinelloides

A0A1X2G5U6  Mass: 21602    Score: 48     Expect: 4.9   Matches: 14
Uncharacterized protein OS=Hesseltinella vesiculosa GN=DM01DRAFT_264443 PE=4 SV=1

A0A1C7MWK9  Mass: 69744    Score: 48     Expect: 5.3   Matches: 30
Iron-sulfur clusters transporter atm1, mitochondrial (Fragment) OS=Choanephora cucurbitarum
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Fig. 6. Some corresponding proteins from the UniProt database with the observed mass of Mucor subtilissimos URM 4133 purified serine protease.

characterized fungi proteins of the same species and with molecular mass similar to that found in the present work, such as a non-characterized protein from *Mucor circinelloides* (Accession No: S2JX18, Mass: 30010, Score: 50, Matches: 18).

There are various reports about other kind of proteases from fungus of genera Mucor. However, this genera secretes mostly aspartic proteases, also known as mucor rennins [49,54,55]. For this specific fungus, Mucor subtilissimus, the authors found only a protease with fibrinolytic activity described in the literature, reported by Nascimento et al. [27].

4. Conclusion

The extracellular serine protease produced by *Mucor subtilissimus* URM 4133 was efficiently purified to homogeneity, and was biochemically characterized. The purified serine protease had a molecular weight of 30 kDa. The enzyme demonstrated high stability over a wide pH range (5–10) and at temperatures up to 40 °C for 24 h which allows its application in processes that do not require energy costs with heating. In addition, the use of metal ions and other substances, for the most part, were not able to generate major interference in its proteolytic activity. The thermodynamic parameters of thermal denaturation k_d, $t_{1/2}$, ΔH, ΔS and ΔG supported thermal stability reflected in the temperature of stability assays and show that the enzyme is compatible with use in different industrial or biological processes at medium temperatures (≈ 40 °C). After analysis in MALDI-TOF, no compatible sequences were found, indicating that this enzyme was not yet cataloged in specific databases, such as NCBI and UniProt. In conclusion, the results obtained in the present work, mainly for stability to pH, temperature and presence of most metal ions and organic substances; demonstrate that this enzyme is potentially useful for industrial and biotechnological applications.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Biotechnology Reports.

Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020. e00552.

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