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Production and Characterization of New Fibrinolytic Protease from *Mucor subtillissimus* UCP 1262 in Solid-State Fermentation

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Abstract

Fibrinolytic enzymes have received attention regarding their medicinal potential for thrombolytic diseases, a leading cause of morbidity and mortality worldwide. Various natural enzymes purified from animal, plant and microbial sources have been extensively studied. The aim of this work was to produce fibrinolytic protease by solid state fermentation using agro industrial substrates. *Rhizopus arrhizus* var. *arrhizus* UCP 1295 and *Mucor subtillissimus* UCP 1262 filamentous fungi species isolated from soil of Caatinga-PE, Brasil, were used as producer microorganisms. Wheat bran was shown to be the best substrate for the production of the enzyme and by using a 2^3 full factorial design the main effects and interactions of the quantity of the substrate wheat bran, moisture and temperature on the fibrinolytic enzyme production and protease were evaluated. The best results for fibrinolytic and protease activities, 144.58 U/mL and 48.33 U/mL, respectively, were obtained with *Mucor subtillissimus* UCP 1262 using as culture medium 3 g wheat bran, 50% moisture at a temperature of 25˚C for 72 hours. The optimum temperature for the produced enzyme was 45˚C and most of its original activity was retained after being subjected to 80˚C for 120 min. The pro-

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tease activity was enhanced by K^+, Ca^+ and Mn^+; but with Cu^+ there was an inhibition. The specificity to chromogenic substrate and the inhibition by PMSF indicates that it is a chymotrypsin-like serine protease. Presented results suggest that this enzyme produced by solid-state fermentation is an interesting alternative as a candidate for thrombolytic therapy.

Keywords

***Mucor*, Enzyme, Protease, Fibrinolytic, Wheat**

1. Introduction

About 17.3 million people die each year of cardiovascular disease. It is estimated that in 2030 the number of deaths could reach 23.6 million [1]. Cardiovascular diseases include acute myocardial infarction, ischemic heart disease, valvular heart disease, peripheral vascular disease, arrhythmias, high blood pressure and stroke [2] [3]. These problems are usually caused by accumulation of fibrin (blood clots) that adhere to the blood vessels wall leading to thrombosis [4].

Various thrombolytic agents have been used in the therapeutic treatment of thrombosis, but due to their high cost and hemorrhagic side effects, new sources of these agents have been sought after. Fibrinolytic enzymes produced by microorganisms, have the potential to inhibit blood coagulation and are able to degrade the fibrin. Some potential microorganisms like bacteria of the genus *Bacillus*, cyanobacteria, fungi, and *Streptomyces* have been described as sources of fibrinolytic agents [5]-[9].

Filamentous fungi have been shown to be a good choice for the production of fibrinolytic enzymes due to the good production values and easy separation of the enzymes, since most of them are extracellular [10]. The order Mucorales comprising the genus *Rhizopus*, *Mucor*, *Rhizo-Mucor*, *Cunninghamella* and *Absidia* is known to demonstrate a potential for production of proteinases with milk clotting activity. The genus *Mucor* is known as producer of extracellular proteases, amylases and lipases [11].

Among the fermentation processes available for enzyme production by filamentous fungi, the solid-state fermentation (SSF) is highly suitable since the microorganisms grow naturally in solid substrates such as wood, roots, plant leaves, and in the absence of free water. SSF is gaining an increasing interest for enzyme production because it is a low-cost technology; it allows an easy recovery of biomolecules and presents higher yields when compared to submerged fermentation. Also, SSF is an alternative to the use of agro industrial residues as substrate avoiding negative impacts to the environment [12]-[14].

Another advantage to using SSF is preventing negative effect phenomena in the biosynthesis of the enzyme such as catabolite repression by glucose or inhibition of the biosynthesis of amino acids and ammonia [11] [15] [16]. The present study evaluates the production of fibrinolytic protease by *Rhizopus arrhizus* var. *arrhizus* UCP 1295 and *Mucor subtillissimus* UCP 1262 in solid-state fermentation using agroindustrial residues.

2. Material and Methods

2.1. Microorganisms

Rhizopus arrhizus var. *arrhizus* UCP 1295 and *Mucor subtillissimus* UCP 1262 were used. Both were isolated from Caatinga soil, Serra Talhada, PE-Brazil and deposited in the culture collection of the Catholic University of Pernambuco-Brazil. These microorganisms were maintained on Czapek medium (Sucrose 30 g/L; Sodium nitrate 2 g/L; Dipotassium phosphate 1 g/L; Magnesium sulphate 0.5 g/L; Potassium Chloride 0.01 g/L; Agar 15.0 g/L; final pH (at 25˚C) 7.3 ± 0.2; HIMEDIA M075-500G) at 30˚C for 7 days.

2.2. Preparation of Inoculum

The spores were collected of microorganisms by nutrient solution comprised of 0.5% (w/v) yeast extract, 1% (w/v) glucose and 0.01% Tween 80 (w/v) diluted in sodium phosphate buffer 245 mM and pH 7.0 previously sterilized: The spores were counted in Neubauer chamber to a final concentration of 10^7 spores/mL.

2.3. Selection of the Substrate(s) for the Production of Fibrinolytic Protease by SSF

Rhizopus arrizus var. *arrizus* UCP 1295 and *Mucor subtillissimus* UCP 1262 were inoculated to a final concentration of 10^7 spores/mL in 125 mL Erlenmeyer flasks, containing 5 g of substrate: passion fruit peel, corncob, cassava peel, soybeans, *Malpighia emarginata* seed, wheat bran and citrus pulp with a granulometry between 0.6 to 2.0 mm. (moisture of 40%) at 30˚C for 72 hours. The substrates were dried at 65˚C until complete dehydration and then stored in plastic containers for further use. For each situation, the amount of produced fibrinolytic protease was evaluated by Fibrinolytic activity.

2.4. SSF Optimization for the Production of Fibrinolytic Protease

Experiments were carried out in 125 mL Erlenmeyer flasks containing the selected substrate(s) autoclaved for 20 min at 121˚C. The substrate(s) were moistened as needed with nutrient solution and water to reach the desired moisture. A full factorial design (2^3) was used to determine the influence of the parameters: mass of substrate, moisture and temperature (**Table 1**) on the production of the enzyme. After inoculation, the flasks were incubated (10^7 spores/mL) for 72, 96 and 120 hours of fermentation. These parameters were selected according to pre-test.

2.5. Extract Fibrinolytic Enzymes and Proteases

Extraction of the enzyme was performed after 72 hours of fermentation. 7.5 mL of sodium phosphate buffer pH 7 (245 mM) per 1 g of substrate were added and the flasks were placed in an orbital shaker at 150 rpm for 90 min at room temperature. After this period, the contents were centrifuged at 3500 rpm for 10 min, and the supernatant was used for determination of the enzyme activity.

2.6. Protease Activity

The determination of protease activity was done by the method of Ginther [17]. One unit of enzyme activity was defined as the amount of enzyme that produced an increase in the optical density of 0.1 per hour at 420 nm. The experiments were done in duplicate.

2.7. Fibrinolytic Activity

The fibrinolytic activity was determined using the spectrophotometric method described by Wang *et al*. [18]. First, 0.4 mL of 0.72% fibrinogen was placed in a test tube with 0.1 mL of 245 mM phosphate buffer (pH 7) and incubated at 37˚C for 5 min. Then, 0.1 mL of a 20 U/mL thrombin solution was added. The solution was incubated at 37˚C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation continued at 37˚C. This solution was again mixed after 20 and 40 min. At 60 min, 0.7 mL of 0.2 M trichloroacetic acid (TCA) was added, and mixed. The reaction mixture was centrifuged at 15,000 × g for 10 min. Then, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01-per-minute increase in absorbance at 275 nm of the reaction solution.

2.8. Protein Determination

The protein content was determined by the method described by Bradford [19] using bovine serum albumin (BSA) as a standard. Each experiment was performed in triplicate and the average value was then calculated after correction with the corresponding blank.

Table 1. Levels of the variables studied in a 2^3 complete factorial design for the production of the fibrinolytic proteases.

Variables	Levels		
	Low (−1)	Central (0)	High (+1)
Substrate amount (g)	3	5	7
Temperature (˚C)	25	30	35
Moisture (%)	30	40	50

2.9. Substrate Specificity

Amidolytic activity was measured spectrophotometrically using the chromogenic substrates: N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (S7388 Sigma) and Gly-Arg-*p*-nitroanilide dihydrochloride (G8148 Sigma). The reaction mixture (0.8 mL) contained 30 µL of enzyme solution, 30 µL of chromogenic substrate and 140 µL of 20 mM Tris-HCl (pH 7.4). After incubation for 15 min at 37˚C, the amount of liberated *p*-nitroaniline (pNA) was calculated by spectrophotometric absorption at 405 nm. One unit of amidolytic activity (AU) was expressed as the number of micro moles of substrate hydrolyzed per minute and per milliliter by the enzyme Kim *et al*. [20].

2.10. Effect of Temperature on Protease Activity and Stability

The temperature effect was determined by incubating the crude extract at temperatures ranging between 10 and 100˚C for 60 min. To determine the stability to temperature, aliquots were withdrawn every 30 min for a period of 3 hours. These aliquots were submitted to the determination of protease activity.

2.11. Effect of Metal Ions on Protease Activity

The protease activity of the crude extract was evaluated in the presence of metal ions. These ions are described as inhibitors or activators of protease activity. The effect of ionic solutions was evaluated at concentrations of 2.5 mM; 5 mM and 10 mM. The crude extract was exposed to the following ions: zinc (Zn^{+}), magnesium (Mg^{+}), copper (Cu^{+}), ferrous (Fe^{+}), calcium (Ca^{+}), magnesium (Mg^{+}), sodium (Na^{+}), potassium (K^{+}), and cobalt (Co^{+})·and incubated at 37˚C for 60 min. The ions were dissolved in Tris-HCl pH 7.75 with 150 mM NaCl.

2.12. Effect of Inhibitors in Protease Activity

To evaluate the effect of inhibitors on enzyme activity, the crude extract was exposed to the following inhibitors: PMSF (fluoride-methylphenylsulfonyl $C_7H_7FO_2S$), 2-mercaptoethanol (2-hydroxy1-ethanethiol-C_2H_6SO), ethylenediaminetetraacetic-acid (EDTA-acetic-$C_{10}H_{16}N_2O_8$), Pepstatin A and Iodoacetic Acid. Incubations were done for 60 min at 37˚C; the inhibitors were dissolved in according to the protocol provided by Sigma, and the concentration of the solutions was standardized at 5 mM.

2.13. Statistical Analysis

The effects were evaluated by an analysis of variance with a significance level of 95% to make estimates of the main and second-order effects a linear, absolute value of the factors in relation to the response variables studied. Statistical analysis of the experimental design was performed using the software Statistical 8.0 [21]. For data analysis, we used the Student t test for independent samples; For the purpose of compare the means of the effect of the inhibitors in the enzymatic activity of the protease. The results were considered statistically significant at $p \leq 0.05$ and was used excel software 2007.

3. Results and Discussion

3.1. Selection of the Substrate for Production of Fibrinolytic Protease

The production of fibrinolytic protease by *Rhizopus arrizus* var. *arrizus* UCP 1295 and *Mucor subtillissimus* UCP 1262 was carried out using 7 agroindustrial substrates. No growth was observed when passion fruit peel, citrus pulp and *Malpighia emarginata* seed were used. This phenomenon may be due to low content of protein in these substrates, as described by [22]-[24]. The substrates with a higher protein such as soybeans and wheat bran were those with a higher protease production as can be seen in **Table 2**. Fibrinolytic activities of 47 U/mL and 78 U/mL were obtained using soybeans and wheat bran, respectively, by *Mucor subtillissimus* UCP 1262; in the *Rhizopus arrhizus* var. *arrhizus* UCP 1295 strain were obtained values 39 U/mL and 58 U/mL for soybean and wheat bran, respectively.

The maximum protease production was observed when wheat bran was used as substrate. These results are similar to those reported by Ravikumar *et al.* [25] when producing protease by SSF using *Pleurotus sajor-caju* with different agricultural products, wheat bran was the best for the production of protease with an activity of 35 U/mL.

Table 2. Production of fibrinolytic protease by *Rhizopus arrizus* var. *arrizus* UCP 1295 and *Mucor subtillissimus* UCP 1262 using different substrates in SSF.

Substrates	FA (U/mL)		U/gdb (FA)		PA (U/mL)	
	Mucor	*Rhizopus*	*Mucor*	*Rhizopus*	*Mucor*	*Rhizopus*
Corncob	10.81	10.47	162.15	157.05	9.87	11.76
Cassava peel	18.24	16.42	273.60	246.30	16.10	14.54
Soybean	47.00	39.03	705.01	585.06	27.06	23.87
Wheat bran	78.00	58.05	1170.05	870.07	38.08	30.77

FA: Fibrinolytic Activity; U/gdb: Unit/grams of substrate on a dry basis; PA: Protease activity.

3.2. Production of Fibrinolytic Protease by SSF

The results presented for the production fibrinolytic protease by *M. subtillissimus* UCP 1262 using wheat bran as substrate was performed according to the **Table 1**. The highest enzyme production was obtained by using wheat bran as a result of the availability of nutrients for microbial growth. These grains are rich in nutrients such as proteins, lipids, vitamins and functional compounds—wheat brains are not used as food due to its biological unavailability and unpleasant organoleptic nature [26].

Wheat bran also provides compounds, which can induce the production of determined biomolecules [14] [27]. Besides, wheat bran substrate and a simple, consisting of co product of agricultural origin unrefined that contain all the nutrients required for the microorganism growth. Studies by Alves [28] [29] showed protease production by genus *Mucor* and from the 12 species evaluated; the largest producer was *Mucor racemosus* Fres. f. *chibinensis*. In the second study, with 56 isolates of *Mucor* originating from dung of herbivores, 82% showed production of enzymes with protease activity. Sathya [30] also obtained extracellular protease production by *Mucor circinelloides* using agroindustrial substrate by SSF.

Soares *et al*. [31] using wheat bran as substrate for protease production by the nematophagous fungus *Monacrosporium sinense* (SF53) by solid-state fermentation, obtained an activity of 38.0 U/mL at 211 hours of incubation. The highest values of activity protease and fibrinolytic by *M. subtillissimus* UCP 1262 occurred with 72 hours of fermentation using wheat bran as substrate. These results were obtained with a less fermentation time (72 hours) and greater protease activity (48.33 U/mL).

Table 3 shows the values of the protease activity and fibrinolytic activity obtained using factorial design, being determined that the best conditions for the production of the enzyme were 3 g of substrate, 50% moisture and temperature of 25˚C. Other authors reported enzyme production by *Mucor* spp. in SSF using wheat bran mixture was optimal at 30˚C [11] [32]. Agrawal *et al.* [33] showed that most fungi have an optimum temperature for protease production by SSF between 28˚C to 30˚C unlike the optimal conditions obtained in this work show a better enzyme production at 25˚C. The temperature in the solid state fermentation is an important factor for this type of fermentation is characterized by being exothermic, or large amounts of heat are released, which are directly proportional to the metabolic activity of the micro-organism, especially fermentations by filamentous fungi where the temperature directly influences the spore germination, growth and product formation.

The statistical analysis showed that all variables were significant with variance analysis ANOVA with confidence level 95% ($p \leq 0.05$) (**Figure 1**). However, the variable temperature was the statistically most significant showing a negative effect. The decrease of temperature indicated a greater enzyme production by the microorganism. The temperature is essential in metabolic regulation and in the enzyme synthesis [34]-[36].

The moisture demonstrated a positive effect (**Figure 1**). The moisture studies in this type of fermentation allow studying the reduction of the risk of contamination. Furthermore, a study with adequate moisture for its substrate allows the formation of a water film on the surface, to facilitate dissolution and transfer of nutrients and oxygen. High moisture level tends to increased enzyme production, which may be due a rise in the substrate porosity that allows for a better oxygen transfer favoring the development of the microorganism [37]. The optimal value for moisture (50%) was in accordance with that reported by Chutmanop *et al*. [38] for the production of proteases by *Aspergillus oryzae* (Ozykat-1) using wheat bran and rice bran as substrate. Thanapimmetha *et al.* [39] also showed similar results, with an optimum value of 45% for the production of protease in solid-state fermentation using *Jatropha curcas* as residue.

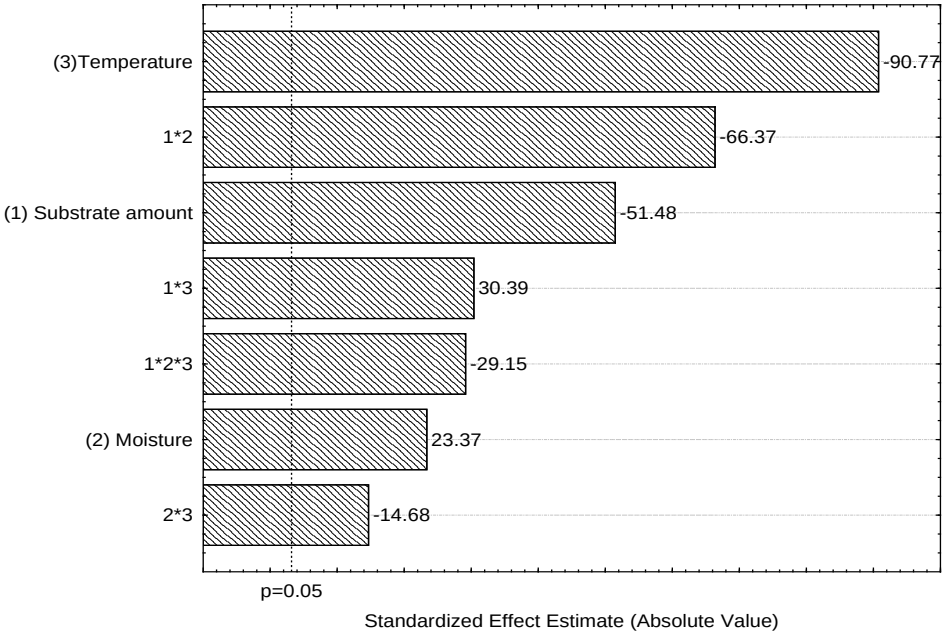

Figure 1. Pareto chart of variables effects in fibrinolytic activity (U/mL) produced by *Mucor subtillissimus* UCP 1262 in solid-state fermentation using wheat bran as substrate after 72 hours fermentation. PA: Protease activity.

Table 3. Production of fibrinolytic proteases by *Mucor subtillissimus* UCP 1262 using a 2^3 factorial design.

Runs	Variables			Wheat bran			
	Substrate amount (g)	Moisture (%)	Temperature (˚C)	PA (U/mL)	U/gds	FA (U/mL)	U/gds
1	3	30	25	36.20	440.74	106.66	799.95
2	7	30	25	27.23	316.62	84.16	631.20
3	3	50	25	48.33	801.93	144.58	1084.35
4	7	50	25	47.90	644.97	84.58	634.35
5	3	30	35	26.26	223.29	38.33	287.47
6	7	30	35	23.36	218.72	75.83	568.72
7	3	50	35	45.83	668.72	90.83	681.22
8	7	50	35	38.66	569.64	32.08	240.60
9 C	5	40	30	35.56	456.67	79.16	593.70
10 C	5	40	30	36.20	465.69	80.41	603.07
11 C	5	40	30	22.43	253.73	80.00	600.00
12 C	5	40	30	29.16	367.56	80.83	606.22

U/gdb: Unit/gram of substrate on a dry basis; PA: Protease activity; FA: Fibrinolytic activity; C: Central points.

3.3. Amidolytic Activity

The crude extract produced by *M. subtilissimus* exhibited the highest activity against N-succinyl-Ala-Ala-Pro-Phe-pNa, a substrate for chymotrypsin, suggesting the presence of a chymotrypsin-like protease. The protease with fibrinolytic activity produced by Sugimato *et al*. [40] using *Fusarium* sp. BLB was also characterized as serine protease. Similar results were presented by *Cordiceps militaris* [41], *Perennipiria fraxinea* [41] and *Armillaria mella* [43].

3.4. Effect of Temperature on Protease Activity and Stability

The temperature is one of the most critical parameters to be controlled in bioprocesses [30]. As shown in **Figure 2** *M. subtillissimus* UCP 1262 extract exhibited an optimum temperature for protease activity at 45˚C. A similar value 45˚C was obtained for proteases from *Colletotrichum gloeosporioides* [44], *Rhizopus chinenesis* 12 [45], *Schizophyllum commune* [7] and *Myceliophthora* sp. [46] while 50˚C was the optimal for *Trichoderma reesei* QM9414 [47] and *Fusarium culmorum* [48]. The protease from *M. subtillissimus* UCP 1262 *M.* retained 75.52% of its original activity after being subjected to 45˚C for 120 min (**Figure 3**), presenting a higher stability than the enzyme reported by Zanphorlin *et al*. [49]. That maintained 95 % of the maximum activity at 60˚C. No protease activity was detected after heating the enzyme at 100˚C for 15 min.

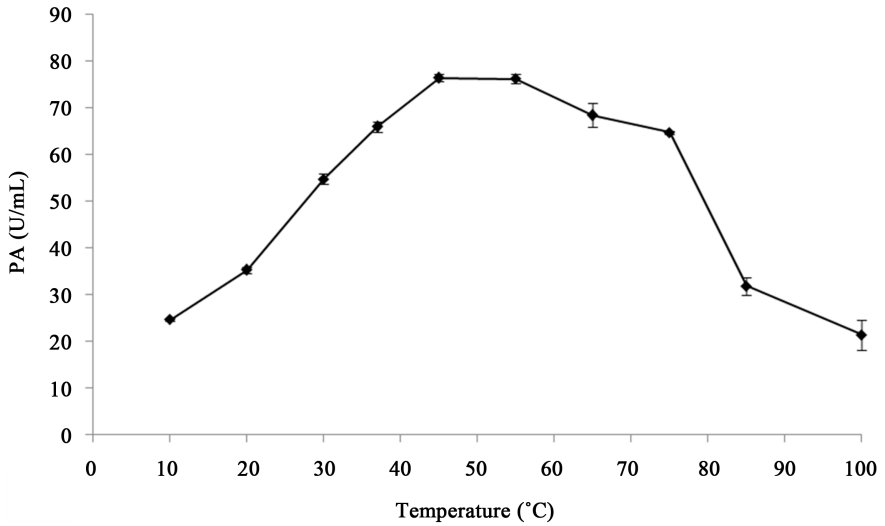

Figure 2. Effect of temperature on protease produced by *Mucor subtillissimus* UCP 1262 in solid-state fermentation using wheat bran as substrate after 72 hours fermentation. PA: Protease activity.

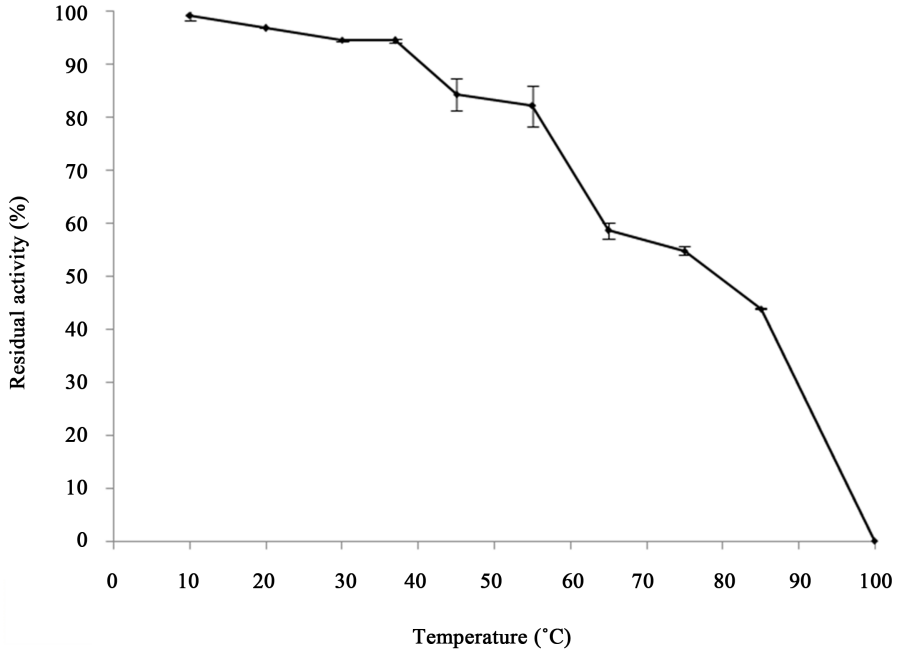

Figure 3. Stability of protease produced by *Mucor subtillissimus* UCP 1262 by solid-state fermentation at 45˚C after 120 min.

3.5. Effect of Metal Ions on Protease Activity

It was found that the addition of the ions K^+, Mn^{2+}, Ca^{2+}, increased protease activity at all concentrations used, while a reduction was observed when Zn^{2+} and Co^{2+} were added at concentrations of 5 mM and 10 mM, Cu^{2+} ions had a negative effect on enzyme activity for all concentrations tested (**Table 4**).

The activity of protease produced by *Ulocladium botrytis* using solid-state fermentation [50] was inhibited in the presence of Zn^+ or Cu^{2+} and increased significantly in the presence of Ca^{2+}, Mg^{2+}, and K^+. The increase of protease activity may be due metallic ions that link the substrate and enzyme, and that ion keep the substrate in active site of the enzyme [50] [51].

3.6. Effect of Inhibitors in Protease Activity

The enzyme activity was significantly inhibited by PMSF (81%) and not by any other inhibitor (**Table 5**) which was validated by the Student's t-test. These results demonstrate that the enzyme is a serine protease. Also, the activity of the protease produced by *Penicillium* sp. using SSF was 93% inhibited by PMSF [10]. Zanphorlin *et al*. [46] also obtained through SSF by the thermophilic fungus *Myceliophthora* sp. a protease that was completely inhibited by PMSF.

4. Conclusion

In conclusion, a new protease was produced by *Mucor subtillissimus* UCP 1262 using wheat bran as substrate in SSF. Inhibition studies and high specificity to N-succinyl-Ala-Ala-Pro-Phe-*p*Na indicate that it is a chymotrypsin-like serine protease. The enzyme exhibited stability over a wide range of temperature values. Further studies will be made to the purification of the enzyme.

Table 4. Effect of metal ions on protease activity.

Ions	Residual activity (%)		
	2.5 mM	5 mM	10 mM
Control	100.0	100.0	100.0
K^+	130.0*	133.9*	106.7
Ca^+	123.1*	124.8*	116.1
Mn^+	109.3	104.3	110.4
Zn^+	108.4*	98.7	85.8
Mg^+	103.8	102.9	96.5
Co+	101.1	91.9	84.5
Cu^+	85.7*	78.2*	64.5*
Fe^+	101.3	80.7	107.1
Na^+	89.8	94.4	115.7

*Student t test for independent samples; $^*p \leq 0.05$.

Table 5. Effect of inhibitors on protease activity.

Inhibitors	Residual activity (%)
Control	100.0
EDTA	90.9*
β-Mercaptoethanol	93.4*
PMSF	18.4*
Pepstatin A	98.9
Iodoacetic acid	97.6

*Student t test for independent samples; $^*p \leq 0.05$.

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