Production, Optimization and Characterization of an Acid Protease From Filamentous Fungi by Solid-state Fermentation

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Abstract

Acid proteases represent an important group of enzymes, extensively used in food and beverage industries. There are a diversification of food industries and thus an increasing demand for biocatalysts capable of adapting the industrial extreme environments. These demands can be covered by a plant and animal proteases; however there is a shortage to meet the present industrial demands. This necessitates the search for an alternative acid protease sources from fungi. The fungal isolates were recovered from grape and dairy farm soil using potato-dextrose Agar. The fungi were further screened for protease production based on the hydrolysis of clear-zone on skim-milk agar media. The potential fungi were then subjected to secondary screening under solid-state fermentation. After primary and secondary screening, the potential fungus (isolate Z1BL1) was identified to the genus level by combination of macroscopic and microscopic morphological study. The growth condition and media composition for potential fungal isolate (Z1BL1) was further optimized under solid-state fermentation. The crude enzyme produced from isolate Z1BL1 was characterized after partial purification by acetone and ammonium sulphate precipitation. A total of 9 fungal isolates were showed protease production in primary and secondary screening, however 1 potential isolate (Z1BL1) was selected for further study based on its protease activity. The potential fungus, isolate Z1BL1 was identified to the genus *Aspergillus* based on their morphological features. The optimization of media composition and growth conditions for acid protease production from Z1BL1 were slightly increased the protease activity. The acetone precipitated enzyme exhibited the maximum activity at 50 °C and pH 5 with stability at pH 4-6 and temperature 40-60 °C. Thus based on the above findings, the acid protease produced from *Aspergillus* was shown suitable enzyme characteristics required in industry and could be a candidate to be applicable in food industry after further purification by high resolution techniques.

Introduction

Aspartic proteases (EC3.4.23), also known as acidic proteases, are a subfamily of endopeptidases that have been isolated from diverse sources, including viruses, bacteria, fungi, plants, and animals. Several fungal aspartic proteases have been purified and characterized as rennin-like and pepsin-like enzymes (1). The rennin-like enzymes are produced by *Endothia parasitica* (endothiapepsin, EC 3.4.23.22), *Mucor*, and *Rhizomucor* species (mucorpepsin, EC 3.4.23.23). The pepsin-like enzymes include aspergillopepsin (aspergillopepsin I, EC 3.4.23.18) from *Aspergillus* species (2) and rhizopuspepsin (EC 3.4.23.21) from *Rhizopus* species (3).

Most of the aspartic proteases (Aps) show the best activity at low pH (pH 3 to 4) and have isoelectric points in the pH range of 3 to 4.5. They are inhibited by a hexapeptide from *Streptomyces* that contains two statin residues called pepstatin. Aspartic proteases are also sensitive to diazoacetyl-DL-norleucine methyl ester (DAN) and 1, 2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin (4).

Acid protease has a wide range of application in various industries to make a change in product taste, texture, and appearance and in waste recovery. Besides this, they have extensive applications in food industry, beverage industry, and pharmaceutical industry (4). The increasing importance of these enzymes and their numerous applications in different industries made us to investigate acid protease production from a new source. The isolation and characterization of new promising strains are possible ways to increase the yield of such enzymes. The aim of the present study was to isolate the fungi from the soil, identification of the culture, screen the protease producing culture, optimize of cultural conditions for the production of acid protease and partial characterization of the enzyme.

Materials And Methods

2.1. Description of study Area

Adequate soil samples were collected from dairy farms and grape farm at three regions of Ethiopia, i.e, Amhara Region (Shewa Robit), Oromia Region (Ziway) and Southern Nations Nationalities People Region of Ethiopia (Halaba). Shewa Robit is located at 100 06' 650" - 090 57'957" N, 0390 54'37"-0390 56'579" E, in North Showa Zone of Amhara Regional State, 225 km North of Addis Ababa, Ethiopia. The agro-ecology of the study site is low-land or Erteb Kola (sub-moist warm) with altitude ranging between 1120 and 1350 m above sea level. The climate data of the study area recorded for the last ten years shows average annual maximum and minimum temperature and precipitation of 32.1, 16.1 °C, and 968 mm, respectively. The most dominant soil type in Shewa Robit is vertisols, which have clay texture with clay content of 56%, sand 10%, silt 34% and a pH value of 8.02 (5).

The second study area, Ziway is located at 7.58° N latitude and 38.43° E longitude in the Southern part of Oromia regional state situated in mid rift valley, 160 Kms South of Addis Ababa. The agro ecologically area is categorized under the semiarid, with minimum mean temperature of 12.7°C and maximum mean temperature of 27.2°C with relative humidity of 60%. The area has an altitude ranging between 1500 and 2000 meter above sea level. The average annual rainfall ranges from 650-750mm and the distribution is highly variable between and within years. Vertisol is the predominant soil type with sand-silt-clay in the portion of 33:48:18; respectively, and has a pH of 7.88 (6).

2.2. Sample collection

Five hundred grams of soil (5-10 cm below the surface) , were collected aseptically from grape farm and dairy farm areas located at Showa Robit, Ziway and Halaba, Ethiopia in September 2019. Soil samples were sieved (3–4 mm mesh), homogenized and stored at 4 °C at Microbiology laboratory of Biology Department for further use as a protocol described by (7).

2.3. Isolation of Fungi

The fungi were isolated by serial dilution technique as described by (8). Thus, ten grams of each soil samples were mixed with a 90 mL of distilled water and homogenized by agitation for 20 min. They were prepared to appropriate dilutions from which 0.1 mL of each sample suspension was plated on Potato Dextrose Agar (PDA) (Microgen, India) plates containing (0.05 g/L) chloramphenicol. The plates were incubated at 30 °C for 5-7 days to isolate distinctive colonies. Each colony was then re-streaked on the same medium to purity and preserved at 4 °C.

2.4. Primary Screening for protease producing Fungi

Primary screening for protease production was tested using skim-milk agar (Nestle TM, Frankfurt, Germany) medium for the production of the clear zone (9). The detection medium (Skim-Milk Agar, SMA) was prepared using 20 g of skim milk, 20 g of agar-agar each dissolved in 200 mL distilled water and 600 mL of 0.2 M Phosphate buffer (K_2HPO_4 and KH_2PO_4 , pH 5.0). All the three media components were autoclaved separately to avoid coagulation and charring of milk due to the presence of buffer salts and later mixed under sterile conditions. The plates were then subsequently inoculated with previously purified fungal isolates and incubated at 30 °C for 2 days. The plates were examined for the formation of the clearing zone by flooding them with a solution of 10% Trichloroacetic acid (TCA) or 10% tannic acid. The relative enzyme activity was calculated using the inhibition zone diameter and colony diameter.

$$REA = CZD / CD$$

Where; REA: relative enzyme activity, CZD: Clear Zone Diameter, CD: Colony diameter (9:10).

2.5 Secondary screening for Acid protease under Solid-State fermentation

2.5.1. Inoculum preparation

Fungal isolates were grown on potato dextrose agar (PDA) and incubated at 30 °C for 5 days. They were scrapped using 10 mL of sterile distilled water to prepare spore suspension. 1 mL of spore solution (10^6 spores/mL) was used according to (11).

2.5.2. Medium and cultural conditions for solid-state fermentation for fungi (M1)

For solid-state fermentation, 0.5 mL of spore suspension (10^6 spores/mL) was transferred into 250 mL Erlenmeyer flasks containing wheat bran (10 g), skim milk powder (2.0 g) and 10 mL of salt solution (g/L: 2.0, KNO_3 ; 0.5, $MgSO_4 \cdot 7H_2O$; 1.0, K_2HPO_4 ; 0.439, $ZnSO_4 \cdot 7H_2O$; 1.116, $FeSO_4 \cdot 7H_2O$; 0.203, $MnSO_4 \cdot 7H_2O$ and pH 5 (11). The flasks were incubated at 30 °C for 6 days under static conditions.

2.5.3. Medium and cultural conditions for solid-state fermentation for Fungi (M2)

The solid-state substrate was also prepared in 250 mL Erlenmeyer flask containing 10 g of wheat bran (Durum wheat bran) moistened by adding 12 mL of HCl (0.2 M) by mixing thoroughly. Then after, the flasks were autoclaved at 121 °C for 30 min. Then 0.5 mL of (10⁶ spores/ml) suspension were inoculated into SSF media and incubated at 30 °C for 6 days (12).

2.6 Enzyme extraction

The enzyme was extracted according to the method of (13). Thus, the fermented substrates were dispersed in 100 mL of distilled water (1:10 ratio of Bran - solvent w/v), and vigorously shaken on a rotary shaker at 240 rpm at room temperature for 40 min and filtered by cotton cloth. The filtrate was then centrifuged (NF200, year, 2009, SN, 02-4005) at 10000 x g, 4 °C for 10 min. The supernatant was used as a crude enzyme.

2.7. Assay for acid protease activity

Protease activity was assayed according to the method of (14) using hemoglobin as a substrate. Enzyme preparation (0.5 mL), suitably diluted, was mixed with 1 mL of 2% (w/v) hemoglobin in 100 mM glycine-HCl (pH 3.0) and the mixture was incubated in a water bath at 50°C for 10 min . The reaction was terminated by adding 2 mL trichloroacetic acid 5% (w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged (NF200, year, 2009, SN, 02-4005) at 10,000×g for 15 min to remove the precipitate. The absorbance of the soluble fraction was measured at 280 nm. A standard curve was generated using tyrosine solutions at 0–50 mg/L. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under the experimental conditions. Tyrosine standard curve and protease activity of the enzyme was calculated.

2.8. Tyrosine standard curve

Tyrosine standard curve was used to quantify the amount of enzyme produced under SSF. Determination of protein concentration by measuring absorbance at 280 nm (A₂₈₀) was based on the absorbance of UV light by the aromatic amino acids tryptophan and tyrosine. The measured absorbance of a protein sample solution was used to calculate the concentration by comparison with a calibration curve prepared from measurements with standard protein solutions. This assay was used to quantitate solutions with protein concentrations of 20 to 3000 µg/ml. Protease enzyme produced by fungal isolate under SSF was quantified by using tyrosine standard curve. The absorbance of the soluble fraction was estimated at 280 nm. A standard curve was generated using tyrosine solutions at 0–50 µg /ml. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per min under the experimental conditions. According Protease Colorimetric Detection Kit (sigma)

$$PA \text{ (U/mL)} = \frac{\mu\text{Tyr} \cdot V_t}{V_s \cdot T \cdot V_a}$$

Whereas PA; Protease activity, μTyr : μg of tyrosine equivalent released, Vt; Total volume of assay in ml, Vs; Sample volume, T ; Reaction time in water bath, Va; Volume of assay for absorbance.

2.9. Assay for milk-clotting activity

Milk clotting activity was determined according to the method of (15), which is based on the visual evaluation of the appearance of the first clotting flakes, and expressed in terms of Soxhlet units (SU). One Soxhlet unit is defined as the amount of enzyme that clots 1 ml of substrate in 40 min at 35°C. In order to perform the assay, 0.1 ml of the sample was added to a glass test tube containing 1 ml of reconstituted skim milk solution (10 g skim-milk powder dissolved in 100 ml of 0.01 M CaCl_2 solution) pre-incubated at 35°C for 10 min. The mixture was mixed well and the clotting time t (s) was measured with a chronometer. The clotting activity was calculated using the following formula: $\text{SU} = (2400 \times 1 \times D) / 0.1 \times t$

Where, D is the dilution factor and t is the clotting time in seconds

2.10. Morphological characterization

The cultural characteristics (colony growth rate, colony texture, colony color, colony size and degree of sporulation) of the fungal isolates were studied by inoculating them on Czapek Dox agar (CDA), Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) as described (16). The micro morphological characteristics of the isolates were observed under the microscope after having prepared them on a slide culture.

2.11. Optimization of cultural conditions and media composition for production of acid protease under SSF

1.1.1. Experimental set-up for preliminary screening for production of acid protease in SSF

The experimental set up for solid-state fermentation was according to (12), with slight modification. The inoculum (0.5 mL of 10^6 spores/mL) was transferred into 250 mL Erlenmeyer flasks containing 10 g of wheat bran (WB) and 2 g of skim-milk powder moistened by 12 mL HCL (0.2 M) / 10 ml of mineral solutions. The flasks were incubated at 30 °C for six days.

2.11.2. Effect of substrate on acid protease production

Screening of the media composition for acid protease production was performed by one-variable-at-a-time approach (17;18). Thus, 0.5 mL of spore suspension (1×10^6 spore/mL) from potential fungi isolate were inoculated into flask containing ten grams of each substrate (wheat bran, Rice Bran) in 250 Erlenmeyer flasks moistened by 12 mL HCL (0.2M)/salt solution and incubated at 30 °C for 144 h. The crude enzyme was extracted as previously described and assayed for acid protease activity according to

(14). After having tested the effect of substrates on enzyme production, the highest enzyme-producing substrate was selected and tested for further optimization.

2.11.3. Effect of incubation time

The effect of incubation time on acid protease production was studied by inoculating the flasks containing the best substrates with 0.5 mL of spore suspension (1×10^6 spore/mL) and incubated at 30 °C for different time periods ranging from 24 h to 144 h (18). The acid protease activity was monitored as previously described.

2.11.4. Effect of incubation temperature

The fungal spores inoculated into SSF medium in 250 mL Erlenmeyer flask was incubated at a temperature of 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C for 120 h to determine the optimum temperature. Then, the obtained optimum temperature used for further study (18). The acid protease activity was determined as described previously.

2.11.5. Effect of inoculum size

The effect of inoculum size on acid protease production was studied by inoculating 0.2 mL, 0.5 mL, 1 mL, 1.5 mL and 2 mL of (3.2×10^6 spores/mL) spore suspension in to SSF media. Then the inoculated flasks were incubated at optimum temperature for 120 h (18). Acid protease activity was assayed as described previously.

2.11.6. Effect of moisture content

The effect of initial moisture content for enzyme production were tested by moistening substrate with distilled water at different percentages of moisture content; 45%, 50%, 55%, 60%, 65%, and 70% to find out the best percentage for enzyme production (19). The acid protease activity was assayed as described previously.

2.11.7. Effect initial media pH

The effect of initial media pH on acid protease production was optimized by adjusting the SSF medium to pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 using HCL or diluted NaOH (18). The acid protease activity was assayed as described previously.

2.11. Partial purification

2.12. Acetone precipitation

The crude enzyme extract was precipitated with chilled acetone (acetone or 75% acetone). Two volumes of chilled acetone were slowly added to the extract and the precipitate was allowed to settle for 1h at -18 °C to permit complete precipitation. The precipitated protein was then separated by centrifuging at 10000

g and 4 °C for 10 min. The pellet was dried in the open air for 30 min to remove trace amounts of acetone and dissolved in 0.02 M phosphate buffer, pH 6.0 (20).

3.9.2. Ammonium sulfate precipitation

The crude enzyme extract was precipitated with ammonium sulfate according to (21). Accordingly, 300 mL of the crude extract was added to 1200 ml saturated ammonium sulfate (100%) to precipitate. Then, the enzymatic solution will be decanted for one night at 4 °C and centrifuged at 10000 x g for 10 min at 4 °C and the pellet was suspended in the phosphate buffer (0.02 M; pH 6).

3.10. Enzyme characterization

3.10.1. Determination of optimum pH and its stability

The optimum pH of the enzyme preparation was studied over a pH range of 2.0–7.0 at 50 °C using 2% (w/v) hemoglobin as described previously. For studying pH stability, the crude enzyme was incubated in buffers (pH 3 and 3.5, Glycine HCL, 4-6 by sodium acetate, 6.5 and 7 with potassium phosphate) of different pH values in the range of pH 3.0–7.0 for 1 h at 4 °C. Residual proteolytic activity was then determined under standard assay conditions. The following buffer systems were used: 100 mM glycine-HCl buffer for pH 3.0, 100mM sodium acetate buffer for pH 4.0–6.0, 100mM potassium phosphate buffer for pH 7.0 (17).

3.10.2. Determination of optimum temperature and its stability

To investigate the effect of temperature, proteolytic activity was tested at different temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60 and 65) using hemoglobin as a substrate for 5 min at pH 5.0. Thermal stability was examined by incubating the enzyme for 60min at 30, 40, 50, 60 and 70 °C. Aliquots were withdrawn at desired time intervals to test the remaining activity at optimum conditions of pH and temperature. The non-heated enzyme was considered as the 100% control (17).

3.9. Data Analysis

The data analyses were performed by using SPSS software version 23 (Inc. Cary NC USA). All experiments were carried out in triplicate. Analysis of variance (ANOVA) and means comparisons will be done by Sigma plot version 12.

Results

4.1. Primary screening of protease producing fungi

A total of thirty two (32) fungi isolates were recovered from the soil samples, of which 12 isolates (38%) were shown clear zone hydrolysis on Skim-milk Agar media up on primary screening. The fungi isolates were shown significant clear-zone diameter and relative enzyme activity (REA) ranging from 6.7 mm and

21.3 mm and 0.4 and 0.74, respectively (Table 1). Among the isolates, the highest REA were recorded by isolate H1W1 (0.8) and Z1BL1 (0.74) (Table 1). The spore concentration of all the 12 isolates was determined using the Neubauer chamber and was in the range between 1.2×10^6 and 4.4×10^8 spores/mL (Table 1).

4.2. Secondary screening of Acid protease under Solid-State fermentation (SSF)

All the twelve isolates that showed a relative protease activity ($\text{REA} = \text{CZD}/\text{CD}$) ≥ 0.4 were subjected for further screening in solid-state fermentation. Out of the twelve isolates, 9 isolates showed an acid protease activity under SSF (Table 2). The highest protease activity (PA) (78.2 U/mL) was recorded from isolate Z1BL1 whereas the lowest PA (31.7U/mL) was noticed from isolate Z1Y2 under SSF. Hence, isolate Z1BL1 that showed the highest acid protease activity was selected for further study.

Table 1: Clear zone diameter of fungi isolates on Skim-milk Agar media

No	Isolates	Spore concentration/mL	Colony diameter	Clear zone diameter	REA CZD/CD
			Mean $\pm$ SEM)	Mean $\pm$ SEM	
1	Z ₁ BL ₁	6.54×10^7	28.7 $\pm$ 1.5 ^b	21.3 $\pm$ 1.5 ^a	0.74
2	H ₁ BL ₁	3.6×10^6	14.7 $\pm$.8 ^a	9.0 $\pm$.6 ^{cd}	0.61
3	Z ₁ BL ₃	2.64×10^8	19.0 $\pm$ 1.5 ^a	11.7 $\pm$ 1.2 ^c	0.61
4	SW ₂ W ₁	1.81×10^7	19.0 $\pm$ 2.0 ^a	12.3 $\pm$.3 ^c	0.65
5	SW ₁ BBr	8.12×10^7	14.3 $\pm$ 1.5 ^a	6.7 $\pm$ 1.5 ^d	0.46
6	SW ₁ YW ₁ ⁺	1.4×10^6	15.7 $\pm$ 1.2 ^a	8.3 $\pm$.3 ^{cd}	0.52
7	Code 3	2.6×10^6	15.7 $\pm$.3 ^a	10.0 $\pm$.6 ^{cd}	0.63
8	Z ₁ Y ₂	1.5×10^6	17.3 $\pm$.8 ^a	7.0 $\pm$.5 ^d	0.40
9	H ₁ W ₁	1.2×10^6	20.3 $\pm$.6 ^a	16.3 $\pm$ 1.2 ^b	0.8
10	SW ₁ WY ₁	2.2×10^7	18.3 $\pm$ 2.4 ^a	11.7 $\pm$.3 ^c	0.63
11	Z ₁ BL ₄	4.4×10^8	16.7 $\pm$.6 ^a	10.0 $\pm$.6 ^{cd}	0.60
12	H ₁ Gr ₁	2.6×10^6	15.3 $\pm$ 1.5 ^a	9.3 $\pm$.3 ^{cd}	0.60

Mean: is average of three measurements, SEM: standard error of the mean, REA: relative enzyme activity, CD: colony diameter, CZD: clear zone hydrolysis diameter, Different letters (a, b, c, d,) designate significantly different means as determined by SPSS test (P<0.05)

Table 2: Acid protease activity of fungal isolates under SSF

No	Isolates	Adjusted Spore concentration/mL	PA (U/mL) Mean±SEM
1	Z ₁ BL ₄	3.6 x 10 ⁶	54.9±1.7 ^a
2	H ₁ Gr ₁	1.3x 10 ⁶	38.8±2.2 ^b
3	Z ₁ BL ₁	3.2 x 10 ⁶	78.2±1.5 ^a
4	Z ₁ BL ₃	3.1 x 10 ⁶	49.2±1.7 ^{ab}
5	SW ₂ W ₁	2.2 x 10 ⁶	70.1±2.8 ^a
6	H ₁ BL ₁	1.8 x 10 ⁶	46.6±4 ^b
7	Z ₁ Y ₂	1.5 x 10 ⁶	31.7±1.7 ^b
8	H ₁ W ₁	1.2 x 10 ⁶	47.2±2.8 ^{ab}
9	SW ₁ WY ₁	2 x 10 ⁶	51.7±1.7 ^{ab}

SEM: standard error of the mean, Mean: is average of three measurements, PA: protease activity Different letters (a, b,) designate significantly different means as determined by SPSS test (P<0.05).

4.3. Morphological characterization of potential fungi isolates

The potential fungal isolate were identified to the genus level based on species descriptions (16 ; 22) (Table 3). The potential fungi isolate Z1BL1 was identified and categorized under the genus *Aspergillus* whereas isolate Z1BL1 based micro morphological characteristics.

Table 3: Macroscopic Characteristics of Potential Fungal Isolates

Isolates	Media	Colony growth	Colony texture	CD, Mean $\pm$ SEM	Obverse Colony color	Reverse colony color	Degree of sporulation
Z1BL1	MEA	Medium	Floccose (cottony)	28.3 $\pm$ 1.5	Black	Dark black	High
	CDA	Low	Floccose (cottony)	21 $\pm$.5	Dark white to gray	Pink	High
	PDA	High	Floccose (cottony)	40.3 $\pm$ 1.2	Dark black	Blue black	High
H ₁ Gr ₁	MEA	Medium	Rough	30.7 $\pm$ 2.2	Green	White gray	Low
	CDA	Medium	Rough	29.6 $\pm$.8	White gray	Green	Low
	PDA	High	Rough	43.0 $\pm$ 1	Green	White to gray	Medium
H ₁ W ₁	MEA	Medium	Floccose (cottony)	31.3 $\pm$ 2	white to brown	Brown	Low
	CDA	Low	Floccose (cottony)	24.5 $\pm$.6	white to brown	Dark brown	Low
	PDA	High	Floccose (cottony)	38.1 $\pm$ 1.1	Yellow	White to yellow	Low
SW ₂ W ₁	MEA	High	Floccose (cottony)	40.4 $\pm$ 3	Blue	Brown	Medium
	CDA	Low	Floccose (cottony)	23.9 $\pm$ 5	Brown	white to brown	Medium
	PDA	High	Floccose (cottony)	45.0 $\pm$.5	Brown	Brown with white	High
SW ₁ WY ₁	MEA	Medium	Rough	25.5 $\pm$ 2.3	White to yellow	white and brown	Medium
	CDA	Medium	Rough	33.5 $\pm$ 1	yellow	yellow at white	Medium
	PDA	High	Rough	38.7 $\pm$ 1.2	White yellow	Light yellow	High
Z ₁ BL ₃	MEA	High	Floccose(cottony)	41.6 $\pm$ 1.4	Blue black	Black	High
	CDA	Medium	Floccose (cottony)	30.0 $\pm$ 2	Black	Dark	Medium

						black	
	PDA	High	Floccose (cottony)	43.0±1.5	Dark black	Blue black	High
Z ₁ Y ₂	MEA	Medium	Rough	29.4±.5	Yellow	White	Low
	CDA	V.Low	Rough	13.7±2.3	Yellow	Brown	Low
	PDA	Medium	Rough	31.5±1.5	White	White	Low
H ₁ BL ₁	MEA	Low	Floccose (cottony)	20.1±1.9	Blue black	Dark black	Low
	CDA	High	Floccose (cottony)	36.6±1.4	Black	Black	Low
	PDA	High	Floccose (cottony)	40.3±.8	Dark black	Blue black	High
Z ₁ BL ₄	MEA	High	Floccose (cottony)	36.0±2	Blue black	Black	Medium
	CDA	Low	Floccose (cottony)	21.0 ±1	Black	Dark black	Low
	PDA	High	Floccose (cottony)	36.2±1.7	Black	Blue black	High

CD, Colony diameter ,MEA: Malt Extract Agar; CDA: Czapek Dox Agar; PDA: Potato Dextrose Agar; Very low :< 15mm; Low: 15-25mm; Medium: 25-35mm, High: 35-45mm; Very high :> 45mm([Perkhofer et al., 2008](#))

4.4. Optimization of media composition and growth conditions for production acid protease by isolate Z1BL1

4.4.1. Effect of Substrate Types for Acid Protease Production

In the present study, two solid substrates (wheat bran and rice bran) moistened by acid/mineral salt solution were used for acid protease production. All the substrates treated by acid and mineral solution were produced significant protease activity. However, the maximum protease activity (107.4 U/mL) was obtained from the potential isolates (Z1BL1) using wheat bran treated with minerals solution whereas the least protease activity (48.5 (U/mL) was recorded using rice bran treated with mineral solution (Fig 2).

4.4.2. Effect of incubation period

The effect of incubation time on the production of acid protease activity was shown in Table 4. The highest protease activity (95.2 U/mL) was recorded at 120 h. Further increase the incubation temperature reduced the enzyme activity.

Table 4: Effect of incubation period on acid protease production by isolate Z1LB1

No	Incubation period (in hour)	PA(U/ml) Mean $\pm$ SEM
1	24	-
2	48	36.8 $\pm$.5 ^a
3	72	56.3 $\pm$ 7 ^b
4	96	88.0 $\pm$ 5.2 ^c
5	120	95.2 $\pm$ 7 ^c
6	144	71.8 $\pm$ 4.9 ^{bc}

PA: protease activity, Mean: is average of three measurements: SEM: standard error of the mean, Different letters (a, b, c,) designate significantly different means as determined by SPSS test ($P < 0.001$).

4.4.3. Effect of moisture content

The effect of moisture content on acid protease production was shown Fig. 3. Accordingly, the maximum enzyme (87.4 U/ml) production was obtained at 50% moisture content where the lowest (44.7 U/mL) was recorded at a moisture content of 65%.

4.4.4. The effect of incubation temperature

In the present study, the highest protease activity (106.1 U/mL) was obtained when SSF carried out at a temperature of 30 °C, while the lowest protease activity was achieved at 45 °C (Fig. 4).

4.4.5. Effect of initial media pH

The optimum pH for acid protease production by fungi isolates Z₁BL₁ was found at pH 4.6. The protease activity was sharply decreased on both side of pH 4.5 (Fig 5).

4.4.6. Effect of inoculum size

The effect of inoculum size on acid protease production by isolate Z1BL1 was shown in fig 6. The maximum protease activity was obtained at inoculum size of 1 mL (3.2×10^6) whereas the lowest activity was recorded at 0.2 mL (3.2×10^5).

4.5. Enzyme characterization

The crude enzyme was partially purified by acetone and ammonium sulfate precipitation and the partially purified enzyme was used for characterization. Partial purification of the crude enzyme extract from isolate Z1BL1 using chilled acetone and 100% $(\text{NH}_4)_2\text{SO}_4$ was increased by the ratio of MCA/PA while reducing the MCA and PA (Table 5). The enzymes were also tested for milk-clotting activity and the ratio of MCA/PA was calculated. The highest ratio was recorded from acetone precipitated enzyme. Thus, based on the protease activity and ratio (MCA/PA) recorded from the enzymes, acetone precipitated enzymes were subjected for further enzyme characterization.

Table 5: Protease activity of crude enzyme and partially purified enzyme

Sample	MCA	PA	Ratio
	Mean $\pm$ SEM	Mean $\pm$ SEM	MCA/PA
Crude enzyme	99.2 $\pm$ 1.7	107.6 $\pm$ 1	0.92
Acetone precipitated enzyme	75.7 $\pm$ 1	50.4 $\pm$ 1.1	1.50
$(\text{NH}_4)_2\text{SO}_4$ precipitated enzyme	64.7 $\pm$ 2.3	48.2 $\pm$ 4	1.34

MCA: milk clotting activity, PA: protease activity, Mean: is average of three measurements, SEM: standard error of the mean

4.5.1. Effect of temperature on enzyme activity and stability

As shown in figure 4.8, the crude enzyme precipitated with chilled acetone showed significant protease activity at a temperature ranges from 40°C to 60°C. However, the highest activity was recorded at a temperature of 50°C. The enzyme activity gradually increased with increasing temperature, followed by a steep decrease at temperatures above 50°C (Fig. 7). The enzyme retained 79% of its activity up on its exposure at a temperature between 40 °C- 60 °C (Fig.8) for 1 h.

4.5.2. Effect of pH on enzyme activity

In the present study, the optimum pH for the activity of acetone precipitated enzyme was obtained at pH 5.0 which is highest protease activity 97.1 U/ml as shown (Fig 9).

4.5.3. pH stability of acetone precipitated enzyme

The pH stability test for acetone precipitated enzyme from isolate Z₁BL₁ was stable from pH 4-6 as shown in (Fig.10). The enzyme retained 83% of its activity up on its exposure at a pH ranges from 4 to 6. The activity of the enzyme at optimum pH was taken as 100%.

Discussion

In the current study, 12 (38%) fungi isolates were shown clear-zone hydrolysis on Skim-milk Agar media during primary screening. Casein or skimmed milk agar plate assays allow qualitative determinations of protease activity, the hydrolysis zone produced on the agar could be related to the amount of protease produced by the fungus (9-23-24). The fungi isolates produced a clear zone diameter and relative enzyme activity between 6.7 mm to 21.3 mm and 0.40 to 0.80 in 48 hour respectively. Similar to the present study, substantial clear zone been hydrolysis were reported by *Mucor sp.* (25) and *Aspergillus niger* FFB1 (19) skim-milk agar and agar media, respectively. Whereas the clear zone diameter (8mm-19.25 mm) and relative enzyme activity (1.07-2.09) recorded in 48 hours from various filamentous fungi using skim milk agar media were higher than this study (10). The variation in inhibition zone diameter and relative enzyme activity could be attributed to the low media pH used in this study.

Out of the 12 isolates subjected to secondary screening, 9 isolates were shown significant protease activity (between 31.7 U/mL and 78.2 U/mL) under SSF. This could be due to filamentous fungi preferred SSF for production of enzymes (18). Similar protease activities were recorded by *Aspergillus* species (26-29) and *Mucor* species under SSF.

The potential fungal isolate Z1BL1 that exhibited the highest protease activity in SSF was identified and categorized under genus *Aspergillus* based on macroscopic and microscopic characteristics (Table 4.1). In comparison to the present study, filamentous fungi isolates collected from Petaling Jaya region, Malaysia identified into different species under the genus *Aspergillus* (10). In other study, (30) reported that identification of filamentous fungi into genus *Aspergillus* based on morphological feature.

The selection of best substrate for enzyme production in a SSF process governed by several factors, mainly related with cost and availability of the substrate material, and thus may involve screening of agricultural waste products (31). In the present study, the suitability of two substrates (wheat bran and rice bran) moistened with acid/mineral solution was verified for protease production in SSF. The highest enzyme activity was obtained from wheat bran moistened with mineral solution. The variation in protease activity between the solid substrates could be due to differences in particle size which is partly related to porosity even though the particle size of each substrate was not determined (32). In other study, wheat bran is considered as the best substrate for production of acid protease from *A. oryzae* MTCC 5341 (33), other *Aspergillus* species (19;34) and *Mucor* species (35).

In this study, the solid state fermentation carried out at 30 °C was the most suitable temperature for acid protease production with protease activity of (106.1 U/mL) (Fig. 4.5) and any fluctuation from this optimum temperatures significantly reduced the enzyme activity. The production of enzyme directly related to the biomass of isolate fungi so this implies optimum growth temperature for isolate Z₁BL₁ was 30 °C. Similar to the current study, optimum acid protease production by *Aspergillus* spp. was obtained at 30 °C (27-29; 33). In other study, (37) also reported maximum milk-clotting proteases production by *Amylomyces rouxii*, *Mucor pusillus* and *Mucor J20* at 30 °C. A maximum acid protease activity was also recorded from *A. oryzae* HG76 at 30 °C and showed significant decrease when the temperature got higher or lower (34).

The protease activity was started from 24 h and reached maximum value (95.2 U/mL) at 120 h incubation period (Table 4.3). Further incubation significantly reduced the enzyme production. The reduction in enzyme yield after optimum incubation period was probably due to depletion of nutrients available to microbial growth (11). The maximum enzyme activity recorded from *M. circinelloides*, *A. oryzae* MTCC 5341 and *Aspergillus sp* on 5th day of fermentation time under SSF was comparable with the present study (11;33;38).

The optimum media pH for acid protease production by isolate Z1BL1 was found at pH 4.5 (Fig.4.6). Further increasing of the media pH significantly reduced enzyme production and this implies that fungi prefer an acidic media pH for inducing acid protease enzyme. Similar observation was reported by (39) on production of aspartic protease from *Mucor mucedo*. In other study, maximum acid protease activity from *Rhizopus oligosporus* HIS13 (40) and *Aspergillus species* (28) under SSF was also obtained at initial media pH 5. Generally, changes of pH during SSF procedure are almost never controlled, except the initial pH of the substrate which is adjusted before inoculation (29).

Size of inoculum is an important biological factor that determines biomass production during fermentation. A highly concentrated inoculum may produce excessive biomass leading to the rapid depletion of nutrients needed for rapid growth of the culture and production of metabolites, while a lower inoculum density may give insufficient biomass inducing low yields of products (29-41-42). Thus determining an appropriate inoculum size in SSF is vital for optimum biomass growth and hence maximum enzyme production. In the present study, the highest protease activity (107.4 U/mL) of the crude enzyme produced from Z1BL was obtained using an inoculum size of 1mL (3.2×10^6 spores/mL) (Fig. 4.7). Correspondingly, maximum protease production from *A. oryzae* NRRL 1808 (41) and *Mucor circinelloides* (11) and was reported at 1mL inoculum size.

Extra addition of inoculum size to 2 mL (3.2×10^6 spores/mL) substantial decreased acid protease activity. The decrease in enzyme activity that has been observed with higher inoculum size could be due to the shortage of the nutrients available for the larger biomass and faster growth of the culture. Hence, a balance between the proliferating biomass and available material is vital for maximum enzyme production (11).

Moisture content is one of a significant factor in enzyme production in solid state fermentation (43). Since microbial growth and product formation occurs at or near the surface particle with optimized water level that controls the water activity (aW) (11). In this study, the highest protease activity was observed from crude enzyme produced at 50% moisture content (Fig 4.4). A similar findings were reported at 55% moisture content for maximum milk-clotting enzyme production from *Aspergillus spp* under SSF (26-44). The decrease in enzyme production at lower moisture content could be due to the non-availability of nutrients. On the other hand, the high moisture content of the fermentation media decreased the porosity and low oxygen transfer that may affect enzyme production (45).

Characterization of protease is an important practice to determine the optimum temperature and pH of the enzyme for its application. In this study, the crude enzyme precipitated using acetone was used for characterization. The partially purified enzyme showed maximum activity at temperature 50 °C and stable at temperature between 40°C – 60 °C. The thermo stability of the enzyme showed in this study, corresponds with the potential applications of the enzyme in food industries such as baking, brewing, dairy (28), was also reported the highest activity of acid protease from *Aspergillus oryzae* MTCC 5341 at 55° C and its stability between 40 to 57 °C. The acid protease most active at pH 5.0 and protease activity decreased significantly below and above pH 5.0. The enzyme was active between pH 4 up to 6 as shown (Fig. 4.10). This suggests that the enzyme is active at acidic pH and appropriate for food industry and beverage industry (4). Similarly, (46), reported the highest activity of Acid protease from *Aspergillus species* result at pH 5. Optimum pH values between 3.0 and 5.5 have been reported for protease activities of other fungi, such as *Penicillium camembertii*, pH 3.5 (47) and *Rhizopus oryzae*, pH 5.5 (48). The optimum pH of *A. niger* 11 protease was lower than that of *A. niger* NRRL 1785 protease which exhibited an optimum at pH 4.0 (49). Thus, the stability of the enzyme in present study corresponds with pH used processing food in food and beverage industries (28).

Conclusions

Based on the results from this study, we can make a conclusion as follows.

Filamentous fungi isolated from dairy and grape farm soil have a potential to produce acid protease. Production of acid protease enzyme from filamentous fungi under SSF was good strategy. From primary and secondary screening, isolate Z1BL1 was selected as a potential fungus for acid protease production. The potential isolate Z1BL1 was successfully identified and categorized under genus *Aspergillus* using macroscopic and microscopic features. Optimization of media composition and growth condition slightly increased the acid protease production by isolate Z1BL1. The characterization study of the enzyme confirms that the enzyme works best at pH 5 and 50 °C. Thus based on the above findings, the acid protease produced in the current study is a candidate to be applicable in food industry.

Declarations

Ethics approval and consent to participate

Non-applicable

Consent for publication

Non-applicable

Availability of data and materials

All data generated or analyzed during this study are publicly available and included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors'contributions

JM: Contributes to conception, acquisition and analysis, interpretation of data, and revised the work.

AU: Contributes to conception, acquisition and analysis, interpretation of data, and drafted the work.

SM: analysis and substantively revised the work.

All authors have approved the submitted version and the modified version that involves the author's contribution to the study; and have agreed both to be personally accountable for the author's own contributions and ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved and the resolution documented in the literature.

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References

1. Hassan N, Rafiq M, Rehman M, Sajjad W, Hasan F, Abdullah S. Fungi in acidic fire: A potential source of industrially important enzymes. *Fungal Biology Reviews*. 2018.
2. Vishwanatha K, Rao AA, Singh SA. Characterisation of acid protease expressed from *Aspergillus oryzae* MTCC 5341. *Food Chemistry*. 2009;114(2):402-7.
3. Hsiao N-W, Chen Y, Kuan Y-C, Lee Y-C, Lee S-K, Chan H-H, et al. Purification and characterization of an aspartic protease from the *Rhizopus oryzae* protease extract, Peptidase R. *Electronic Journal of Biotechnology*. 2014;17(2):89-94.
4. Jermen Mamo, Fassil Assefa. The role of microbial aspartic protease enzyme in food and beverage industries. *Journal of Food Quality*. 2018;2018.
5. Abdissa Y, Tekalign T, Pant L. Growth, bulb yield and quality of onion (*Allium cepa* L.) as influenced by nitrogen and phosphorus fertilization on vertisol I. growth attributes, biomass production and bulb

- yield. African Journal of Agricultural Research. 2011;6(14):3252-8.
6. Bedu H, Tafess K, Shelima B, Woldeyohannes D, Amare B, Kassu A. Bovine cysticercosis in cattle slaughtered at Zeway municipal abattoir: Prevalence and its public health importance. Journal of Veterinary Science and Technology. 2011;2(108):2157-7579.
 7. Li SL, Feng SL, Li ZT, Xu H, Yu YP, Qiao DR, et al. Isolation, identification and characterization of oleaginous fungi from the soil of Qinghai Plateau that utilize D-xylose. African Journal of Microbiology Research. 2011;5(15):2075-81.
 8. Oyeleke S, Egwim EC, Auta S. Screening of *Aspergillus flavus* and *Aspergillus fumigatus* strains for extracellular protease enzyme production. Journal of Microbiology and Antimicrobials. 2010;2(7):83-7.
 9. Saran S, Isar J, Saxena RK. A modified method for the detection of microbial proteases on agar plates using tannic acid. Journal of biochemical and biophysical methods. 2007;70(4):697-9.
 10. Maitig AMA, Alhoot MA, Tiwari K. Isolation and Screening of Extracellular Protease Enzyme from Fungal Isolates of Soil. Journal of Pure and Applied Microbiology. 2018;12(4):2059-68.
 11. Sathya R, Pradeep B, Angayarkanni J, Palaniswamy M. Production of milk clotting protease by a local isolate of *Mucor circinelloides* under SSF using agro-industrial wastes. Biotechnology and Bioprocess Engineering. 2009;14(6):788-94.
 12. Fernández-Lahore HM, Fraile ER, Cascone O. Acid protease recovery from a solid-state fermentation system. Journal of Biotechnology. 1998;62(2):83-93.
 13. Silveira GGd, Oliveira GMd, Ribeiro EJ, Monti R, Contiero J. Microbial rennet produced by *Mucor miehei* in solid-state and submerged fermentation. Brazilian Archives of Biology and Technology. 2005;48(6):931-7.
 14. Kembhavi AA, A. Kulkarni, Pant A. "Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM Applied Biochemistry and Biotechnology. 1993;38:83–92.
 15. Arima K, Yu J, Iwaski S, editors. Milk clotting enzyme from *Mucor pusillus*. In: Perlman New York, : Academic Press; 1970.
 16. Zulkifli NA, Zakaria L. Morphological and molecular diversity of *Aspergillus* from corn grain used as livestock feed. HAYATI Journal of Biosciences. 2017;24(1):26-34.
 17. Siala R, Frikha F, Mhamdi S, Nasri M, Sellami Kamoun A. Optimization of acid protease production by *Aspergillus niger* I1 on shrimp peptone using statistical experimental design. The Scientific World Journal. 2012;2012.
 18. Vishwanatha K, Rao AA, Singh SA. Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters. Journal of industrial microbiology & biotechnology. 2010;37(2):129-38.
 19. Bensmail S, Mechakra A, Fazouane-Naimi F. OPTIMIZATION OF MILK-CLOTTING PROTEASE PRODUCTION BY A LOCAL ISOLATE OF *ASPERGILLUS NIGER* FFB1 IN SOLID-STATE FERMENTATION. The Journal of Microbiology, Biotechnology and Food Sciences. 2015;4(5):467.

20. Palpperumal S, Sankaralingam S, Kathiresan D, Harinathan B, Shankar T, Prabhu D. Partial purification and characterization of a haloalkaline protease from *Pseudomonas aeruginosa*. BMRJ. 2016;15:1-7.
21. Nouani A, Moulti-Mati F, Belbraouet S, Bellal M. Purification and characterization of a milk-clotting protease from *Mucor pusillus*: method comparison. African Journal of Biotechnology. 2011;10(9):1655-65.
22. Ellis DH, Davis S, Alexiou H, Handke R, Bartley R. Descriptions of medical fungi: University of Adelaide Adelaide; 2007.
23. Gnanadoss J, Robert R, Jebapriya R. Production of protease from *Aspergillus niger* and *Mucor mucedo* under submerged and solid state fermentation. Int J Cur Res. 2011;3:75-8.
24. Suganthi R, Benazir J, Santhi R, Ramesh Kumar V, Hari A, Meenakshi N, et al. Amylase production by *Aspergillus niger* under solid state fermentation using agroindustrial wastes. International Journal of Engineering Science and Technology. 2011;3(2):1756-63.
25. Ayana I, Ibrahim AE, Saber WI. Statistical optimization of milk clotting enzyme biosynthesis by *Mucor mucedo* KP736529 and its further application in cheese production. International Journal of Dairy Science. 2015;10(2):61-76.
26. Chutmanop J, Chuichulcherm S, Chisti Y, Srinophakun P. Protease production by *Aspergillus oryzae* in solid-state fermentation using agroindustrial substrates. Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology. 2008;83(7):1012-8.
27. Mukhtar H. Production of acid protease by *Aspergillus niger* using solid state fermentation. Pakistan Journal of Zoology. 2009;41(4).
28. Shivakumar S. Production and characterization of an acid protease from a local *Aspergillus* sp. by Solid substrate fermentation. Archives of Applied Science Research. 2012;4(1):188-99.
29. Bensmail S, Mechakra A, Fazouane-Naimi F. Optimization of milk-clotting protease production by a local isolate of *Aspergillus niger* FFB1 in solid-state fermentation. Journal of Microbiology, Biotechnology and Food Sciences. 2019;2019:467-72.
30. Afzal H, Shazad S, Qamar S, Nisa S. Morphological identification of *Aspergillus* species from the soil of Larkana District (Sindh, Pakistan). Asian J Agric Sci. 2013;1(105):e17.
31. Foda MS, Moharam ME, Ramadan A, El-Bendary MA. Over production of milk clotting enzyme from *Rhizomucor miehei* through adjustment of growth under solid state fermentation conditions. Australian Journal of Basic and Applied Sciences. 2012;6(8):579-89.
32. de Castro RJS, Sato HH. Production and biochemical characterization of protease from *Aspergillus oryzae*: an evaluation of the physical–chemical parameters using agroindustrial wastes as supports. Biocatalysis and Agricultural Biotechnology. 2014;3(3):20-5.
33. Vishwanatha KS, Rao AA, Singh SA. Production and characterization of a milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341. Applied microbiology and biotechnology. 2010;85(6):1849-59.

34. Li C, Xu D, Zhao M, Sun L, Wang Y. Production optimization, purification, and characterization of a novel acid protease from a fusant by *Aspergillus oryzae* and *Aspergillus niger*. *European Food Research and Technology*. 2014;238(6):905-17.
35. Patil P, Kulkarni A, Kininge P. Production of milk clotting enzyme from *Aspergillus oryzae* under solid-state fermentation using mixture of wheat bran and rice bran. *IJSRP*. 2012;2(10):1.
36. Khademi F, Abachi S, Mortazavi A, Ehsani M, Tabatabaei M, Malekzadeh F. Optimization of Fungal Rennet Production by local isolate of *Rhizomucor nainitalensis*. Under Solid Substrate Fermentation system. *IOSR Journal of Pharmacy and Biological Sciences*. 2013;5(2):115-21.
37. Shieh C-J, Thi L-AP, Shih L. Milk-clotting enzymes produced by culture of *Bacillus subtilis natto*. *Biochemical Engineering Journal*. 2009;43(1):85-91.
38. Radha S, Sridevi A, HimakiranBabu R, Nithya V, Prasad N, Narasimha G. Medium optimization for acid protease production from *Aspergillus* sp. under solid state fermentation and mathematical modelling of protease activity. *J Microbiol Biotech Res*. 2012;2(1):6-16.
39. Yegin S, Fernandez-Lahore M, Ullrich M, Polidori M. Cloning and expression of the aspartic proteinase from *Mucor mucedo*: structural characterization and technological properties in comparison with the native enzyme: Jacobs University Bremen; 2011.
40. Ikramul H, Hamid M, Sunila D, Sikander A, Qadeer MA. Production of proteases by a locally isolated mould culture under lab conditions. *Biotechnology*. 2003;2:30-6.
41. Sandhya C, Sumantha A, Szakacs G, Pandey A. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. *Process biochemistry*. 2005;40(8):2689-94.
42. Sharma AK, Sharma V, Saxena J, Yadav B, Alam A, Prakash A. Isolation and screening of extracellular protease enzyme from bacterial and fungal isolates of soil. *Int J Sci Res Environ Sci*. 2015;3(9):0334-40.
43. Hashemi M, Shojaosadati SA, Razavi SH, Mousavi SM, Khajeh K, Safari M. The efficiency of temperature-shift strategy to improve the production of α -amylase by *Bacillus* sp. in a solid-state fermentation system. *Food and Bioprocess Technology*. 2012;5(3):1093-9.
44. Jermen M, Kangwa M, Fernandez-Lahore HM, Fassil A. Optimization of media composition and growth conditions for production of milk-clotting protease (MCP) from *Aspergillus oryzae* DRDFS13 under solid-state fermentation. *Brazilian Journal of Microbiology*. 2020:1-14.
45. Soccol CR, da Costa ESF, Letti LAJ, Karp SG, Woiciechowski AL, de Souza Vandenberghe LP. Recent developments and innovations in solid state fermentation. *Biotechnology Research and Innovation*. 2017;1(1):52-71.
46. Oseni O. Production of microbial protease from selected soil fungal isolates. *Nigerian Journal of Biotechnology*. 2011;23.
47. Chrzanowska J, Kolaczowska M, Dryjański M, Stachowiak D, Polanowski A. Aspartic proteinase from *Penicillium camemberti*: purification, properties, and substrate specificity. *Enzyme and microbial technology*. 1995;17(8):719-24.

48. Kumar DM, Premavathi V, Govindarajan N, Balakumaran M, Kalaichelvan P. Production and purification of alkaline protease from *Bacillus* sp. MPTK 712 isolated from dairy sludge. *Global Veterinaria*. 2012;8(5):433-9.
49. Olajuyigbe F, Ajele J, Olawoye T. Some physicochemical properties of acid protease produced during growth of *Aspergillus niger* (NRRL 1785). *Global Journal of Pure and Applied Sciences*. 2003;9(4):523-8.

Figures

Figure 1

Tyrosine standard curve for acid protease activity

Figure 2

Screening of two agro-industrial residues for acid protease production from Z1BL1 PA : protease activity , Mean is average of three measurements Different letters (a, b, c, d,) designate significantly different means as determined by SPSS test ($P < 0.001$).

Figure 3

Effect of moisture content PA: protease activity, Different letters (a, b, c, d, e) designate significantly different means as determined by SPSS test ($P < 0.001$).

Figure 4

Effect of incubation temperature PA: Protease activity, Different letters (a, b, c, d,) designate significantly different means as determined by SPSS test ($P < 0.001$).

Figure 5

Effect of initial media pH PA: protease activity, Different letters (a, b, c, d, e) designate significantly different means as determined by SPSS test ($P < 0.001$).

Figure 6

Effect of inoculum size PA: protease activity, Different letters (a, b, c, d,) designate significantly different means as determined by SPSS test ($P < 0.001$).

Figure 7

Temperature optimization test for acetone precipitated enzyme

Figure 8

Temperature stability test for acetone precipitated enzyme

Figure 9

pH optimization test for acetone precipitated enzyme

Figure 10

pH stability test for acetone precipitated enzyme

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