ISOLATION, PARTIAL PURIFICATION, AND APPLICATION OF FUNGAL PROTEASES FROM BEAN CROP SOIL

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Abstract

The present study is based on the characterization of 11 and 16 isolated fungi from the bean crop soil in the Vegetable and Fruit Research Development Centre (Hlegu Township) and Myaungtagar Village, respectively. In a total of 27 isolated fungi, 13 fungi showed a clear zone around the colony to determine the preliminary protease-producing fungi. Still, the two selected fungi (V2 and M16) were found to be the highest protease producers. The two selected fungi (V2 and M16) were found as the genus *Penicillium* sp. The two selected proteolytic fungi were purified using 50% ammonium sulfate precipitation and then dialysis to obtain a partially purified enzyme. When compared with standard molecular weight markers, the relative molecular weight of *Penicillium* sp. (16) was found to be approximately ≈ < 40kDa, but *Penicillium* sp. (2) was estimated to be 70kDa. The partial purification fold was 2.16% for *Penicillium* sp. (2), which will recover 13.05% by dialysis, but *Penicillium* sp. (16) had a fold of 2.05% which will recover 23.14% by dialysis. In the Lineweaver-Burk plot, the K_m values of *Penicillium* sp. (2 and 16) were shown to be 1.04 g%. V_{max} values were 102.04 $mMmin^{-1}$ of *Penicillium* sp. (16) and 100.00 $mMmin^{-1}$ of *Penicillium* sp. (2). In the removal of blood stain, *Penicillium* sp. (2 and 16) exhibited complete clearance of the removal of blood stain compared with the control. In the milk clotting test, two selected, partially purified proteases exhibited strong milk coagulation within about 30 minutes. In the degradation of gelatin using waste X-ray films, it was observed that 70 minutes for *Penicillium* sp. (16) and 120 minutes for Penicillium sp. (2) were required for complete degradation.

Keyword: Protease Fungi, *Penicillium* sp., Partial Purification, K_m and V_{max} Values, Milk Clotting, Removal of Blood Stain, Degradation of Gelatin

Introduction

Proteases (EC: 3.4.21-24 and 99; peptidyl peptide hydrolases) are enzymes that catalyze the cleavage of the peptide bond in proteins (CO-NH), liberating small chains of amino acids called peptides (Godfrey and West, 1996). Enzymes are very effective biological catalysts that accelerate almost all metabolic reactions in living organisms. Proteases are a group of enzymes whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids.

Microbial proteases are degradative enzymes that catalyze the total hydrolysis of proteins (Haq *et al*., 2006). The molecular weight of proteases ranges from 18-90kDa (Sidney and Lester, 1972). Microbial proteases are an important family of proteases and have an advantage over animal and plant proteases, as microbes can be cultured on a large scale in less time, and the growth conditions can easily be optimized in lab conditions. Fungi elaborate a wider variety of enzymes than do bacteria, and proteases are among the most important enzymes produced by fungi. Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites (Adrio and Demair 2003).

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Proteases, one among the three largest groups of industrial enzymes, account for about 60% of the worldwide sales of enzymes (Nurullal and Fikret, 2011). The majority of the total sales (89%) of proteases are used in detergent formulation. Historically, proteases were the first to be used in laundry detergents, about two-thirds share of this industry (Mienda and yahya, 2014). They are generally used in detergents, food industries, leather, meat processing, milk clotting, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Paranthaman et al., 2009). The specific objectives are to determine the enzyme activity and protein content of crude protease enzyme at the optimal experimental conditions, kinetic parameters, and to investigate of protease enzyme activities and their usages.

Materials and Methods

Sample collection, isolation, and purification of fungi

Soil samples used from different environmental sources, like two different places of bean crop soil in Vegetable and Fruit Research Development Centre, Hlegu Township, and Myaungtagar Village, were collected aseptically from a 6-inch depth in sterile zip-lock plastic bags. One gram of each soil sample was serially diluted under sterile conditions. From the diluted suspension, 100µL was spread on potato dextrose agar (added 1% casein) medium, which was incubated at room temperature for three to five days and monitored every day for the growth of the fungal colony. Further, these fungi were sub-cultured on potato dextrose agar (added 1% casein) to obtain the pure strain (Sharma *et al*., 2015). All isolated fungi were designated V1 to V11 for bean crop soil in the Vegetable and Fruit Research Development Centre and M1 to M16 for bean crop soil in Myaungtagar Village.

Preliminary production of proteolytic enzyme on skim milk agar (SMA) medium (Sethis and Gupta, 2015)

Preliminary screening was done by inoculating the fungal isolates on skim milk agar medium containing casein as a protein source at room temperature for one to seven days. After incubation, the protease was exhibited by a zone of proteolysis which was exhibited by a zone of proteolysis. The zone diameter was measured in cm, and the results are recorded (Abe *et al*., 2015). The enzyme index (EI) is expressed as R/r, where R is the degradation zone diameter and r is the colony diameter (Abe *et al*., 2015). The species that exhibits the maximum clear zone was selected for further identification.

Identification and characterization of protease fungi

The two selected fungi were examined to the genus level with taxonomic keys under macroscopic and microscopic field according to the standard method Barnett and Hunter (1960); Dube (1983).

Fermentation condition (Anson, 1938)

The 100mL of synthetic media contained casein 0.5g, dextrose 0.5g, yeast extract 0.5g, magnesium sulfate 0.02g, dipotassium hydrogen phosphate 0.1g, and sodium carbonate 0.01g at pH 8.0. After autoclaving, the respective fungi of fermentation broth were operated on a rotary

shaker at 250 rpm for 4 days at room temperature. At the end of the fermentation period, the contents were centrifuged at 10000 rpm (4°C) for 10 minutes at room temperature, and the cell-free supernatant was used as crude enzyme for the enzyme activity assay.

Protease-specific activity assay

Protease-specific activity assay was performed according to the method of Colowick and Kaplan (1955). Briefly, 1.3mL of 1% casein dissolved in 50mM potassium phosphate buffer (pH 8) was added to both sample and blank tubes and incubated at room temperature for 30 minutes. Subsequently, 0.7mL of crude protease extract was added to only the sample tube and incubated for 30 minutes at room temperature. The reaction was stopped with the addition of 3mL of 5% trichloroacetic acid (TCA) solution in both the sample and blank tubes. The mixture was then incubated for 30 minutes at room temperature. A UV-visible spectrophotometer was used to determine the absorbance value at 275nm. From the standard curve, the activity of protease samples can be determined in terms of Units, which is the amount in microgram of tyrosine equivalents released from casein per mL minute under assay conditions described by Abdilbar *et al*. (2021).

Construction of a standard curve for the determination of protein

Protein contents were determined using standard bovine serum albumin according to the method of Lowry *et al*. (1951).

Partially purified the crude enzyme

Extraction of crude enzyme from the optimum fermentation medium

The selected optimum production medium (medium II) was sterilized and then inoculated with one loop of selected protease on a shake at 200 rpm using the best fermentation period of each selected protease fungi. Extraction of each enzyme was done by simple filtration of medium using Whatman filter paper for the separation of fungal mycelia (Karthik *et al*., 2014). The filtrate was centrifuged at 10,000 rpm (4°C) for 10 minutes. Clear supernatant was taken as crude enzyme (Sind and Hamid, 2013).

Ammonium sulfate precipitation

After centrifugation of the filtrate, the cell-free solution of the enzyme was precipitated by the addition of various ammonium sulfate $(NH_4)_2SO_4$ (30%, 50%, 60%, 70% and 80%) by continuous mixing on an ice bag using magnetic stirring. Then it was kept for an overnight period at 4°C for precipitation of protein. Again, it was centrifuged at 10,000 rpm at 4°C for 10 minutes. The precipitate in pellets was used as the crude extract of protease and examined for each protease using an enzyme assay according to the method of Huang *et al*. (2017).

Dialysis (Desalting of enzymes)

The Procedure of dialysis (Spectra/Pro®3 Dialysis Membrane, 3500 Dalton; Flat width 18mm and Length 16ft) by Anustrup (1980) is like that, firstly put the submerged fermentation under optimal conditions in centrifuged at 4,000 rpm, 15 minutes. Then, 50% ammonium sulfate was added and stirred continuously with a magnetic stirrer for 4 hours at room temperature. After 4 hours, put into the freezer for one hour to receive pellet precipitate after centrifuging at 4,000 rpm, 15 minutes. Then re-dissolved received pallet precipitate was dissolved with a minimum amount of water. After that added this solution into a dialysis bag and added the received dialysis

bag to distilled water. Then continuously stirred with a magnetic stirrer for 2 hours, every 2 hours, and distilled water 5 times. And then dialysis was continued overnight at room temperature.

Determination of the molecular weight of the partially purified protease enzyme

The molecular mass of the enzyme was determined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), which was carried out as described by Laemmli (1970). The supernatants of protease enzyme *Penicillium* sp. (2 and 16) were partially purified with 50% ammonium sulfate precipitation by using dialysis. The molecular weight of the enzyme was estimated using a low molecular weight marker (Broad Range Unstained Protein Ladder, cat no. 26630).

Determination of the kinetic parameter

Casein was used over the concentration range 0.25% to 2% to determine the kinetic parameters K_m and V_{max} of the partially purified protease enzyme. To investigate the kinetic parameters, the best concentration of enzyme 0.7mL was added with different concentrations of casein (0.25% to 2%) as substrate. The reaction mixture was incubated at predetermined conditions (pH 8, 37°C, and within 30 to 40minutes). The Michaelis constants (K_m) and maximal velocity (V_{max}) values were determined as reciprocal absolute values of the intercepts on the X- and Y-axis, respectively, of the linear regression curve (Monika Karas and Barbara Baraniak, 2006). The measurement was made at 37°C for 30 minutes as previously determined.

Applications of the partially purified proteases

Blood stain removal activity

Activity of protease enzyme as a detergent additive was analyzed through different combinations of treatment on blood sample stain cotton fabric according to Choudhari (2012). This was studied by a washing test, seven white cotton fabric pieces (5×5cm) were stained by 60µL (0.06mL) of blood. After the dryness and stains at room temperature for seven days, they were immersed in a solution for one set of the experiments. The sets were (1) 100mL distilled water (DW) as a control, (2) 1mL partially purified protease plus 99mL DW, (3) 1mL detergent solution plus 99mL DW, (4) 1mL detergent solution plus 98mL DW and 1mL partially purified protease, (5) 1mL detergent solution plus 97.5mL DW and 1.5mL partially purified protease and (6) 1mL detergent solution plus 97mL DW and 2mL partially purified protease at room temperature for one hour. By the end of incubation, fabrics were rinsed with tap water, and the stain elimination effectiveness was determined by visual inspection of the color intensity of the cloth.

Milk clotting test

The assay of milk-clotting enzyme activity was carried out according to the standard procedure described by Alecrim *et al*. (2015). In test tubes of equal volume, cleaned of any trace of fat, 10mL of milk containing 2.6% fat and 1mL of enzyme solution were added. The test mixtures were thoroughly shaken and incubated at room temperature, and the clotting times and visual inspection of clotting were recorded at regular intervals and times.

Removal of gelatinous coating from waste X-ray film

The waste of X-ray film was done by taking (2cm by 2cm strip) 2×2cm pieces, and then these were cleaned with distilled water and wiped with a cotton swab soaked in ethanol. After drying in an oven at 40°C for 30 minutes, each of the weighty films was immersed in a flask containing 1mL of the partially purified protease in 9mL of phosphate buffer, and subsequently incubation was carried out at room temperature for three hours with a shaking speed of 200 rpm. The film was incubated in a 10mL phosphate buffer served as a control. Turbidity of the reaction mixture increased with time by visual inspection of the color. So, the changing color of each film was removed and recorded at regular intervals and time (Kumar and Dwivedi, 2019).

Results

Sample collection, isolation, and purification of fungi

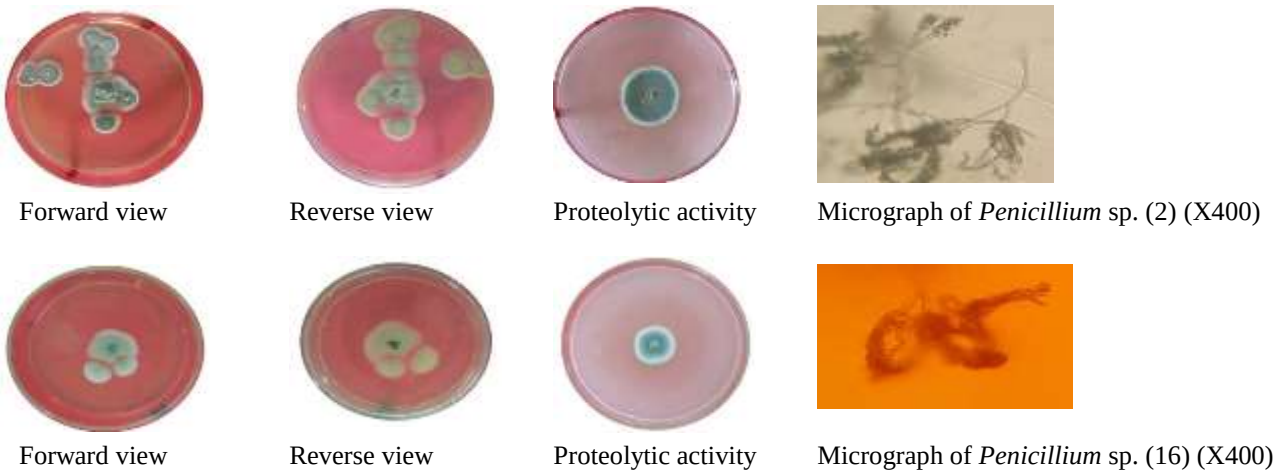
Eleven and sixteen soil fungal isolates were isolated on potato dextrose agar (added 1% casein) medium from bean crop soil in Vegetable and Fruit Research Development Centre, Hlegu Township, and Myaungtagar Village, respectively. These isolated fungi were used by using serial dilution of the pour plate method at room temperature for three to five days.

Preliminary production of proteolytic enzyme on skim milk agar (SMA) medium

The purified fungal isolates were screened for the ability to produce protease enzyme on skim milk agar (SMA) medium by the streak plate method. Among all isolated fungi, 13 isolated fungi showed clear zone formations around the fungal growth, indicating the property of protease. Although only two fungi, V2 and M16, showed maximum proteolytic activity with a zone of about 2.5cm for five days and 2.9cm for six days, respectively, as shown in Figures 1 and 2.

Identification and characterization of protease fungi

In the present investigations, the selected fungal strains were characterized by the use of macroscopic and microscopic characters using standard methods. The microscopic observation based on the two selected protease fungi (V2 and M16) was identified as *Penicillium* sp. (2 and 16), as shown in Figure 1.



Forward view | Reverse view | Proteolytic activity | Micrograph of *Penicillium* sp. (2) (X400)

Forward view | Reverse view | Proteolytic activity | Micrograph of *Penicillium* sp. (16) (X400)

Figure 1. Morphological and microscopical characters of isolated fungi from V2 and M16

Construction of a standard curve for the determination of protein

The determination of protein by using bovine serum albumin (BSA) as a standard is a modification of Lowry's method. Before determining the protein content in the sample solution, it is necessary to construct a calibration curve by using standard protein concentration and their absorbance at 540nm. At this fixed wavelength of 540nm, the calibration curve of absorbance against the various concentrations of bovine serum albumin (BSA) was drawn as shown in Figure 3. The nature of the curve was a straight line passing through the origin, showing that Beer's law was obeyed (Mikkelsen and Corton, 2004).

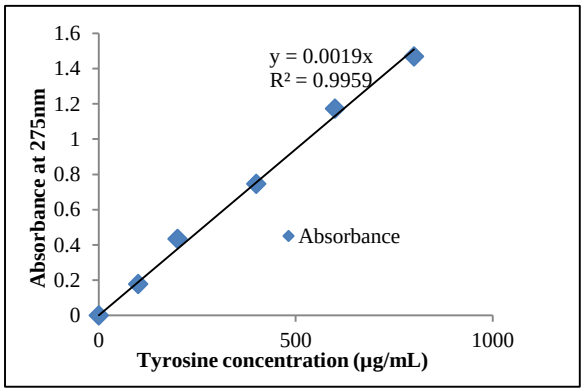


Figure 2. Plot of absorbance as a function of standard tyrosine concentration

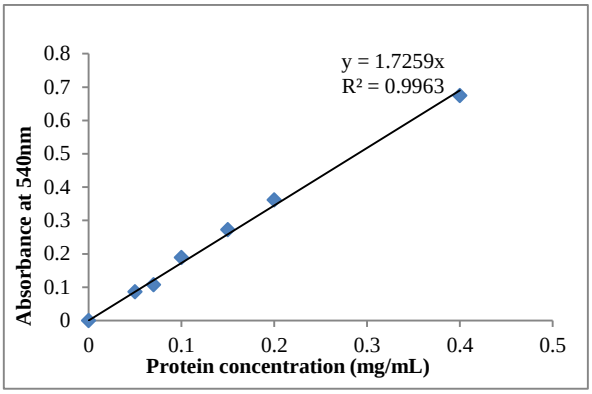


Figure 3. Calibration curve for standard protein solution

Partially purified the crude enzyme

The supernatants of two selected protease enzymes, *Penicillium* sp. (2 and 16), were partially purified with 50% ammonium sulfate precipitation followed by dialysis, as shown in Figure 4. Among the two selected proteases, the purification step for maximum protease production from *Penicillium* sp. (2) was observed in a 2-fold purification with 13.05% recovery by dialysis, and their specific activity was increased from 45.27U/mL. The *Penicillium* sp. (16) was exhibited in a 2-fold purification with 23.14% recovery, and its specific activity was increased from 32.97U/mL. Purification fold results of two selected proteases are summarized in Tables 1 and 2.

Penicillium sp. (2) *Penicillium* sp. (16)

Figure 4. Purification of extracted enzymes by dialysis

Table 1. Partial purification of protease from *Penicillium* sp. (2)

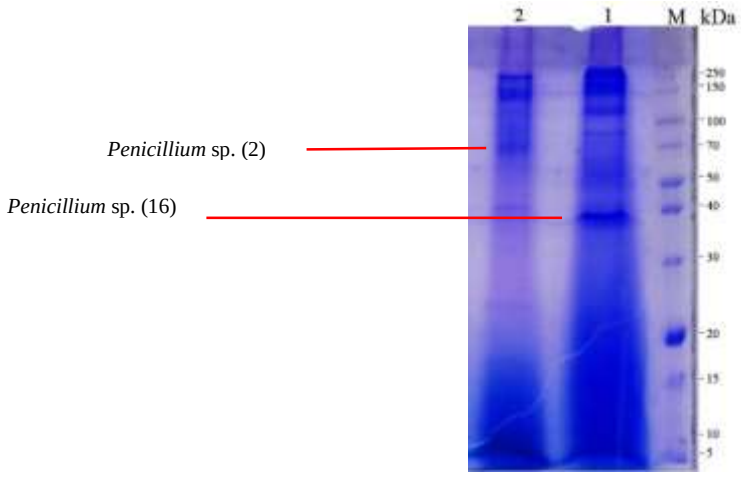
Purification steps	Total protease activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mL)	Purification (fold)	Recovery (%)
Crude protease	3113.27	149.39	20.98	1	100
50% of $(NH_4)_2SO_4$ and Dialysis	876.53	19.36	45.27	2.16	13.05

Table 2. Partial purification of protease from *Penicillium* sp. (16)

Purification steps	Total protease activity (U/mL)	Total protein (mg/mL)	specific activity(U/mL)	Purification (fold)	Recovery (%)
Crude protease	1901.02	118.19	16.08	1	100
50% of $(NH_4)_2SO_4$ and Dialysis	902.04	27.36	32.97	2.05	23.14

Determination of the molecular weight of the partially purified protease enzyme

The molecular weight of partially purified protease was determined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), using low molecular weight when compared with standard molecular weight markers. The molecular mass from *Penicillium* sp. (16) was found ≈ < 40kDa, and *Penicillium* sp. (2) was estimated to be 70kDa, as shown in Figure 5.



M - Molecular weight markers

Lane 1 - Purified protease from *Penicillium* sp. (16)

Lane 2 - Purified protease from *Penicillium* sp. (2)

Figure 5. Determination of molecular mass of partially purified protease by SDS-PAGE

Determination of the kinetic parameter

In order to study the effect of various casein substrate concentrations ranging from 0.25% to 2% was conducted. The reaction rate versus casein substrate concentration curve was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics, and constants were

determined from a Lineweaver-Burk plot. From the Lineweaver-Burk plot, the K_m values were found to be 1.04g% *Penicillium* sp. (2 and 16). While the V_{max} value was observed as 100.00 $mMmin^{-1}$ and 102.04 $mMmin^{-1}$ for *Penicillium* sp. (2 and 16). The results are presented in Figures 6 and 7.

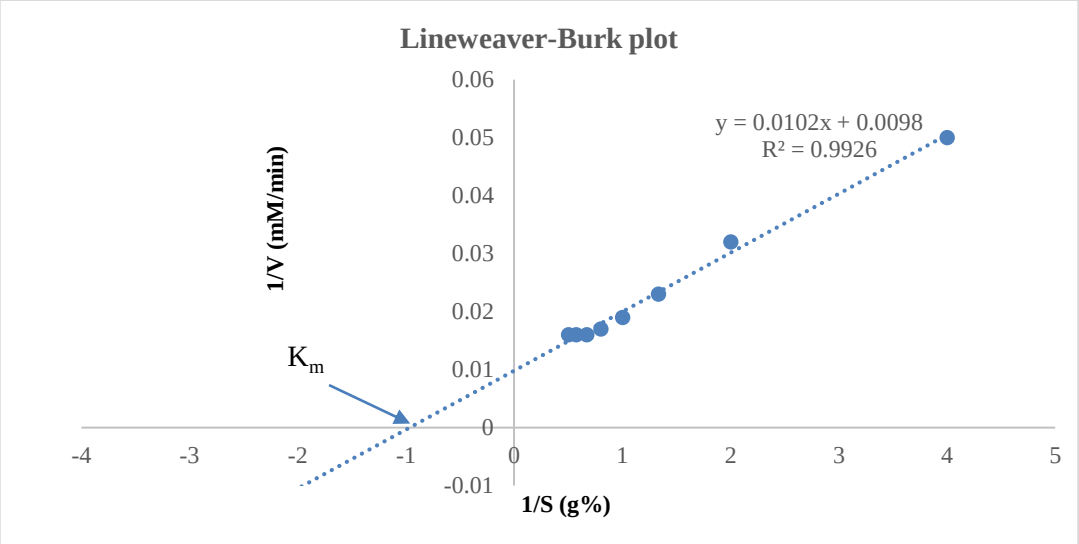


Figure 6. Kinetic plot based on the specific substrate for partially purified protease produced by *Penicillium* sp. (2) using the Lineweaver-Burk plot

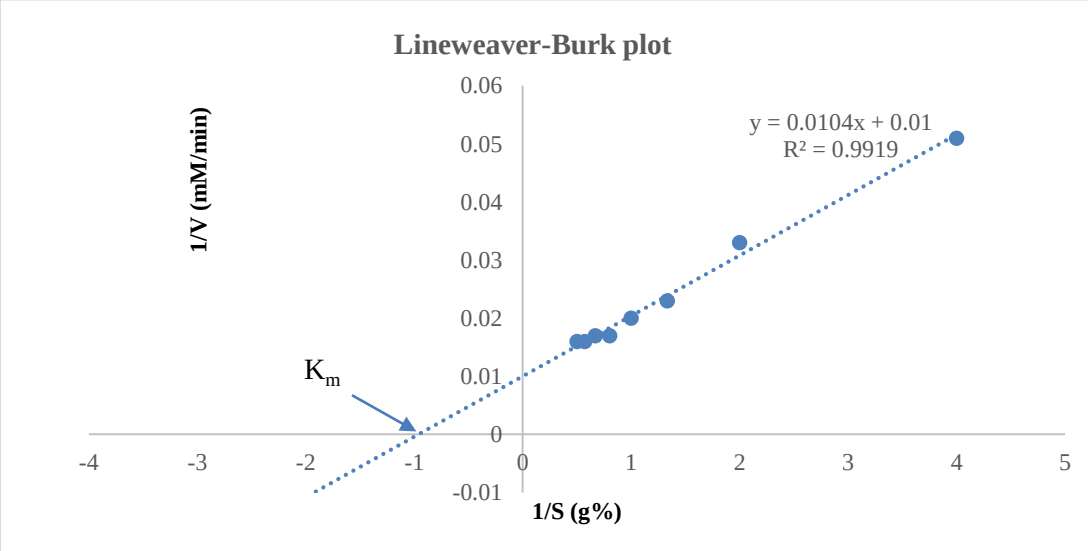


Figure 7. Kinetic plot based on the specific substrate for partially purified protease produced by *Penicillium* sp. (16) using the Lineweaver-Burk plot

Applications of the partially purified proteases

Blood stain removal activity

The washing effect of different concentrations of partially purified protease (1mL, 1.5mL, and 2mL) and cotton fabrics stained with blood was analyzed. After one hour of incubation, the blood stain cloth in 1mL of detergent solution plus 2mL of protease enzyme, for *Penicillium* sp. (2 and 16), was completely removed blood stain and *Penicillium* sp. (16) was removed blood stain by visual inspection. Only water, enzyme solution, and detergent solution were observed to have poor cleaning for protease, as shown in Table 3 and Figures 8 and 9.

Table 3. Blood stain removal activity

No.	Blood removal stain	Water	Water + protease	Water + detergent	Water + Detergent +enzyme (1mL)	Water + Detergent + enzyme (2mL)
1	*Penicillium* sp. (2)	+	++	++	+++	+++
2	*Penicillium* sp. (16)	+	++	++	++ +	++++

+ = poor stain removal, ++ = medium stain removal, +++ = good stain removal, ++++ = very good stain removal

(A) Cotton pieces stained with blood (0.06mL) and dried

(B) Washed with distilled water (100mL)

(C) Washed with distilled water (99mL) + protease (1mL)

(D) Washed with distilled water (99mL) + detergent (1mL)

(E) Washed with distilled water (98mL) + detergent (1mL) + protease (1mL)

(F) Washed with distilled water (97.5mL) + detergent (1mL) + protease (1.5mL)

(G) Washed with distilled water (97mL) + detergent (1mL) + protease (2mL)

Figure 8. Result of blood stain removal activity from *Penicillium* sp. (2)

(A) Cotton pieces stained with blood (0.06mL) and dried

(C) Washed with distilled water (100mL)

(B) Washed with distilled water (99mL) + protease (1mL)

(D) Washed with distilled water (99mL) + detergent (1mL)

(E) Washed with distilled water (98mL) + detergent (1mL) + protease (1mL)

(F) Washed with distilled water (97.5mL) + detergent (1mL) + protease (1.5mL)

(G) Washed with distilled water (97mL) + detergent (1mL) + protease (2mL)

Figure 9. Result of blood stain removal activity from *Penicillium* sp. (16)

Milk clotting test

In the determination of milk clotting enzyme, *Penicillium* sp. (2 and 16) were observed most active milk clotting enzyme within about 30minutes, as shown in Figure 10.

Penicillium sp. (2) *Penicillium* sp. (16)

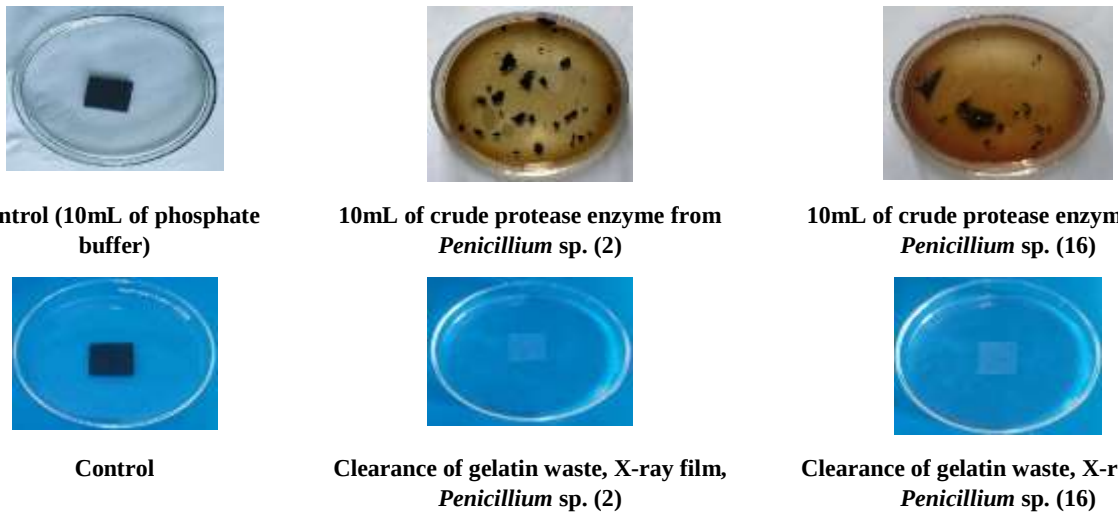
Figure 10. Milk clotting activity of the protease enzyme

Removal of gelatinous coating from waste X-ray film

In visual examination of the waste X-ray films, 1mL of partially purified enzymes of *Penicillium* sp. (2 and 16) was used at different time intervals to give efficient in-gelatin hydrolysis from waste X-ray film. As a result, the weight loss of the film was noticed after cleaning the waste X-ray film. Firstly, *Penicillium* sp. (2 and 16) were revealed to clear after 120minutes and 70 minutes, respectively, compared with the control, as shown in Table 4 and Figure 11.

Table 4. Recovery of time for gelatin hydrolysis and weight loss

No.	Strains	Duration of time at room temperature	Initial weight of X-ray sheet in grams (2 × 2) cm	Weight of X-ray film after treatment	Weight loss in grams
1	*Penicillium* sp. (2)	120 mins	0.1028	0.1005	0.0023
2	*Penicillium* sp. (16)	70 mins	0.1028	0.1005	0.0023



Control (10mL of phosphate buffer) **10mL of crude protease enzyme from *Penicillium* sp. (2)** **10mL of crude protease enzyme from *Penicillium* sp. (16)**

Control **Clearance of gelatin waste, X-ray film, *Penicillium* sp. (2)** **Clearance of gelatin waste, X-ray film, *Penicillium* sp. (16)**

Figure 11. Weight loss of the film during gelatin removal from used waste X-ray film

Discussion and Conclusion

The soil samples were collected from two different sites of bean crop soil from Hlegu Township and Myaungtagar Village. A total of 27 strains were screened on potato dextrose agar (added 1% casein) medium from bean crop soil. Among the 27 isolated fungi on skim milk agar medium, 13 fungi showed positive results of clear zone as protease activity, but the selected 2

fungi were found to be the high protease producers. These were subsequently identified to the genus level as two *Penicillium* sp. (V2 and M16) were observed based on their morphological characters and spore structure. The two selected proteolytic fungi, *Penicillium* sp. (2) and *Penicillium* sp. (16), were used for protease production via submerged fermentation (SmF). Casein was decomposed by the protease to produce degraded stages of protein products, which include tyrosine, among them Haldane (1965). In the present work, the collected clear filtrate (crude enzyme) was initially concentrated from 30% to 80% using ammonium sulfate. The crude enzyme formed from the use of 50% ammonium sulfate showed the best concentration with the highest activity. The molecular weight of partially purified proteases was revealed by SDS-PAGE when compared with standard molecular weight markers; the relative molecular weight of *Penicillium* sp. (16) was approximately ≈ < 40kDa, and *Penicillium* sp. (2) was approximately 70kDa, as shown in Figure 5. This result is similar to the protease purified from *Penicillium janthinellum*, as reported 33kDa Abirami *et al*. (2011).

The specific activity of the partially purified protease increased from 20.98μgmL^{-1}min^{-1} to 45.27μgmL^{-1}min^{-1} for *Penicillium* sp. (2) and 16.08μgmL^{-1}min^{-1} to 32.97μgmL^{-1}min^{-1} for *Penicillium* sp. (16), while the protease was purified up to 13.05% for *Penicillium* sp. (2) and 23.14% for *Penicillium* sp. (16), respectively, as shown in Tables 1 and 2. Devi *et al*. (2022) reported that the 2.89-fold increase with 26.28% recovery by ammonium sulphate precipitation of protease from *Penicillium chrysogenum*.

Owing to its significant commercial application, it is important to investigate the kinetic characterization of the protease enzyme. The value of Michaelis-Menten constant (K_m) for *Penicillium* (2 and 16) was 1.04g%. The results of higher V_{max} revealed 100.00 mMmin^{-1} for *Penicillium* sp. (2) and 102.04 mMmin^{-1} for *Penicillium* sp. (16) as shown in Figures 6 and 7.

A good protease must be stable and active in detergents so that the efficiency of the enzyme can be achieved. So, in the exploitation of industrial applications, the partial purification of the protease was used to check the detergent compatibility. In the blood stain removal studies, *Penicillium* (2 and 16) obtained from a partially purified source effectively removed blood stains from cotton fabric pieces when added along with a detergent solution. Although *Penicillium* sp. (16) gives more satisfactory results than *Penicillium* sp. (2), as shown in Table 3 and Figures 8 and 9. Benluvankar *et al*. (2016) reported that the usefulness of the protease from *Penicillium* sp. LCJ228 for removal of blood stains from cotton cloth in the presence and absence of detergents. In the presence of commercial detergents, its cleaning property indicates the possibilities to utilize these proteases in the detergent industry. The supplementation of the partially purified protease in detergent improved the cleaning of blood according to Choudhari (2012) and Suryawanshi and Pandya, (2017).

Variation in the production of milk-clotting enzyme by *Penicillium* sp. (2 and 16) was found 30 minutes, as shown in Figure 10. The filtrate possessed high general proteolytic activity because of peptonization of the curd formed. Aicha *et al*. (2012) reported that the *Penicillium* sp. obtained the best data in milk-clotting fungus enzymes. Partially purified enzyme of each protease was used at different time intervals to give efficient gelatin hydrolysis from waste X-ray film. Under the obtained condition, from protease enzyme *Penicillium* sp. (16) effectively hydrolyzed gelatin after 70 minutes from waste X-ray film. Although *Penicillium* sp. (2) revealed complete clearance of waste X-ray film after 120minutes, as shown in Table 4 and Figure 11. Abrusci *et al*. (2007) and Biswajit *et al*. (2021) reported that the gelatin layer was completely

removed, leaving the polyester film clean, and the silver was recovered in the hydrolysate. The usage of proteases has increased as detergent additives and several other applications, which include radioactive wastes like used X-ray film and the removal of gelatinous strips from film. The present isolation of radioactive wastes needs to be decomposed so that they produce less harm to living welfare (Chung *et al*., 2019). The study of two selected enzymes can be thought of as a potential commercial good protease, and will also yield good results for future applications.

Acknowledgements

We would like to express our sincere gratitude to Professor Dr. Thida Oo (Professor and Head), Department of Botany, University of Yangon, for their permission to provide the research facilities. We would like to express our deep appreciation also to Dr. Bay Dar (Professor and Head) (Retired), Department of Botany, Sittwe University, for her helpful advice and suggestions.

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