CHARACTERIZATION AND DETERGENT COMPATIBILITY OF PURIFIED PROTEASE PRODUCED FROM *Aspergillus niger* BY UTILIZING AGRO WASTES

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A study was conducted to purify and characterize a novel protease produced from *Aspergillus niger* using different lignocellulosic agro-based by-products including corncobs, wheat bran, and rice bran as substrates under SSF. Maximum protease activity was recorded on wheat bran fermented culture media after the 3rd day of incubation. The optimal conditions found for protease production using wheat bran were cultivation period (3 days), substrate concentration (10 g), pH (7), incubation temperature (45°C), inoculum size (4 mL), and 3% surfactant Tween-80 (2 mL). A purification fold of 2.41 with 29 U/mg specific activity and 70.73 % recovery was achieved after purification. Purified protease from *A. niger* had a molecular weight of 47 kDa on SDS-PAGE. The enzyme activity profile showed that purified protease was optimally active at pH 7 and 45°C as optimum values. *A. niger* protease was reasonably stable in the pH range 5-8 and 35-60°C for up to 1 h incubation. Protease was activated by various metal ions/inhibitors tested, Mn^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, PMSF, Pepstatin and Iodoacetic acid at 1 mM, proving the enzyme as metalloprotease, whereas an inhibitory effect was shown by certain agents including EDTA and SDS. The purified protease was compatible with five local detergents with up to 25 days of shelf life at room temperature. The maximum production of protease in the presence of a cheaper substrate at low concentration and its potential as a detergent additive for improved washing makes the strain and its enzymes potentially useful for industrial purposes, especially for the detergent and laundry industry.

Keywords: Aspergillus niger; Protease; SSF; Purification; SDS-PAGE; Characterization; Detergent compatibility

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INTRODUCTION

Proteases (EC 3.4.21-24) form an enlarged cluster of the enzymes that are ubiquitous in nature and the most central category of enzymes from an industrial point of view. Proteases are protein-digesting enzymes that are mainly classified by optimal pH in which they thrive; the types are: acidic, neutral, and alkaline proteases (Rauf et al. 2010; Ahmed et al. 2011; Iqbal et al. 2011). Neutral proteases are important to the food industry because they possess a specific function in hydrolyzing hydrophobic amino acid bonds at a neutral pH, thereby reducing the bitterness of food protein hydrolysates. The vast

diversity of proteases clearly illustrates the influence of these enzymes in the biosphere (Sandhya et al. 2005).

A wide series of micro-organisms including *Rhizopus oligosporous* 1HS13, *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, and *Conidiobolus spp* (Tunga et al. 2001; Papagiani et al. 2001; Sahoo et al. 2003; Ikram and Hamid 2004) have the ability to produce proteases under suitable growth conditions (Arulmani et al. 2007). Among these organisms, *Aspergillus* and *Mucor* have been studied intensively as protease producers. The vast diversity of proteases, in contrast to their mode of action and specificity, has engrossed wide-reaching attention to exploit their physiological and biotechnological applications (Rao et al. 1998). The worldwide requirement for these enzymes, with specific applications, is increasing rapidly (Vishwanatha et al. 2009). Proteases are among the most important groups of industrial enzymes used in the pharmaceutical, food, detergent, protein, brewing, meat, dairy, and leather industries (Agarwal et al. 2004; Joo and Chang 2004; Basu et al. 2007; Merheb-Dini et al. 2009; Paranthaman et al. 2009) to substitute currently used toxic chemicals. This is a comparatively new development and has conferred added biotechnological importance (Rao et al. 1998).

There are few reports on neutral proteases from *Aspergillus* sp. (Paranthaman et al. 2009), although considerable work has been reported on alkaline and acidic proteases from different fungal species. This paper reports the results of a study carried out to investigate the protease production potential of *Aspergillus niger* and the optimization of growth conditions. With respect to the factors affecting culture conditions, productivity and properties of protease, it was considered significant to purify and characterize this enzyme to explore the factors affecting their activity thus, we aimed to purify and characterize protease from *A. niger* to present potential and possible applications for industrial purposes.

EXPERIMENTAL

Materials and Methods

Chemicals and substrates

All the chemicals were of analytical grade unless otherwise stated and used in the present form without further purification. Corncobs, wheat bran, and rice bran were used as growth-supporting substrates and were collected from different localities in Faisalabad, Pakistan. The substrates were sun dried, crushed, and stored in moisture-free bags for the use of the whole experiment.

Micro-organism and culture condition

The strain *Aspergillus niger* was obtained from Enzyme Biotechnology Laboratory, Department of Chemistry & Biochemistry, University of Agriculture Faisalabad, Pakistan, grown on Vogel's agar slants at 30^{o}C for 3 days and stored at 4^{o}C for the whole experiment to prepare the fungal spore suspension.

Fungal spore suspension

Fungal spore suspension was prepared by transferring a loopful of the *A. niger* culture into 100 mL of Vogel's medium additionally supplemented with trace elements. The solution contains $ZnSO_4.7H_2O$, 1g; $CuSO_4.5H_2O$, .50g; $MnSO_4.H_2O$, .50g; and $Na_2MoO_4.2H_2O$, .50g and was kept in a continuous shaking position at 140 rpm to obtain homogeneous spore suspension (1×10^6-10^8 spores/mL).

Fermentation methodology & protease extraction

During the initial substrate screening trial, 5 g of each of the above mentioned substrates was taken in a 250 mL flask, 60% moistened with Vogel's fermentation media, and inoculated with the freshly prepared spore suspension of *A. niger*. All the inoculated experimental flasks were incubated at 30±1°C for 48h. After the stipulated fermentation time period 100 mL of distilled water was added to the fermented substrate and was then homogenized in a rotary shaker at 180 rpm for half an hour. The media were then centrifuged at 9000×g for 10 min at 4°C to get clear supernatant containing enzyme solution; the resultant clear supernatant was used for analytical studies.

Optimization of Production Parameters

Effect of substrate concentration

After selecting the best yielded substrate (wheat bran) from the screening trial another experiment was conducted to investigate the effect of different concentrations (2.5-15g) of the growth substrate (wheat bran) on the production of protease enzyme. A set of three flasks (250 mL) was inoculated with freshly prepared homogenous spore suspension of *A. niger* and incubated at 30°C for the optimum fermentation time period.

Effect of initial medium pH

To optimize pH for maximum protease enzyme activity, wheat bran fermentation media of different pH's ranging from 3 to 9 was used to check the effect of pH on growth media and the enzyme activity profile of protease.

Effect of incubation temperature

To investigate the effect of temperature on protease production by *A. niger,* all the duplicate experimental flasks were incubated in a temperature controlled incubator at different temperatures ranging from 20 to 40ºC.

Effect of Tween 80

To investigate the effect of surfactant (Tween 80) on the protease activity profile, experiments were conducted in duplicate flasks. Varying concentrations (1-5%) of Tween 80 was studied by incubating the wheat bran fermentation media of pH 7 at 30°C with 4 mL inoculum size for the optimum fermentation time period.

Determination of Protease Activity & Protein Contents

To determine the protease activity, the method of McDonald and Chen (1965) was used as described earlier (Rauf et al. 2010), where one unit enzyme activity was

defined as the amount of enzyme that releases 1 µg of tyrosine per mL per min. The amount of protein in the enzyme extract before and after purification was measured according to the method of Bradford (1976) with Bovine serum albumin as standard.

Determination of Biomass Dry Weight

Fungal biomass was determined by direct method at the end of each experiment, the fermented biomass was transferred to a pre-weighed filter paper and dried in a hot air oven for 72 h at 80°C, and the weights (g) were recorded.

Partial Purification Procedure

Crude enzyme concentrate was placed in an ice bath after centrifugation at 9000×g for 15 min at 4°C. Crystals of solid ammonium sulfate were added to attain 70% saturation. The mixture was kept for 24 h at 4°C and was centrifuged as done previously. Pellets were suspended in 50 mM phosphate buffer (pH 6.0) and were dialyzed against the same buffer. Enzyme activity was determined before and after dialysis of ammonium sulfate, precipitated, and finally freeze dried. The partially purified protease was lyophilized and used for further studies related to gel filtration & ion exchange chromatography and SDS-PAGE for further purification and molecular weight determination, respectively.

Gel Filtration Chromatography

Crude enzyme extract obtained after dialysis was loaded on Sephadex G–100 column (2×25 cm) to get further purification of protease enzyme. 50 mM phosphate buffer having 0.15M NaCl was used as elution buffer. The protein fractions were eluted at a flow rate of 0.5 $mLmin^{-1}$. 1mL size fractions were collected and each fraction was assayed for protease activity with the same procedure as described earlier.

Ion Exchange Chromatography

To attain further purification, lyophilized fractions collected after gel filtration chromatography were subjected to ion exchange chromatography using a DEAE cellulose column (2×20 cm) equilibrated with 0.5 M potassium phosphate buffer (pH 6.5). A total of 25 fractions of 2 mL each were collected at a flow rate of 1 mL/2 min; both the enzyme activity and protein contents were determined for each separate fraction, as mentioned in the previous section.

SDS–PAGE

To determine the molecular weight of purified protease, Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970).

Characterization of Protease

The purified protease obtained from the DEAE-cellulose column was subjected to characterization through kinetic studies by studying the effect of different pH values (3-9), effect of different incubation temperatures (30 to 60ºC), and effect of using various compounds as activators and inhibitors on purified protease produced from *A. niger*.

Using casein as substrate, the enzyme activities for each case were determined as described earlier. Stability experiments were performed under an optimum pH (7)/temperature (50°C) conditions for the period of 1 h incubation.

Detergent Compatibility of Protease

Locally accessible detergent brands, namely: Surf excel, Bonus, Ariel, Wheel, and Bright Total were used to investigate the compatibility of *A. niger* protease. Directions as per given on their respective sache were used to prepare detergent solutions. Casein was used as substrate and prepared in phosphate buffer of pH 6. A reaction mixture comprising 2.5 mL of substrate solution, 1.5 mL detergent solution, and 1 mL purified protease was incubated at 55°C for 15 minutes, followed by normal enzyme assay as described earlier.

Shelf Life

Shelf life is an important and significant variable tool for commercial utilization of industrial products. To check the effect of storage of *A. niger* protease on its activity, purified enzyme extract was stored at room temperature (30°C) for 30 days.

RESULTS AND DISCUSSION

Screening of Waste Materials

The results of the screening trial experiment showed that protease production increased as incubation time period increased up to certain limits and then started to decrease after its optimum time period. The results of the initial experimental trial showed that the maximum production of protease (92U/mL) was achieved on wheat bran as a substrate after the 3rd day of incubation, as compared to the other two materials (rice bran and corncobs) (Fig.1).

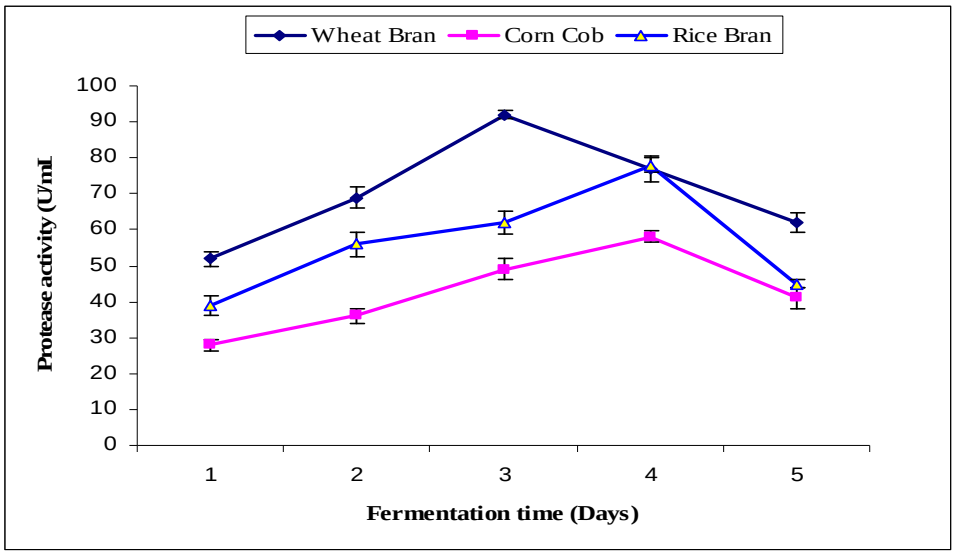

Fig. 1. Effect of fermentation time period on the production of protease from different waste materials by *A. niger*

Sumantha et al. (2006) reported maximum protease activity after 3 days of fermentation with *A. niger*. Benazir et al. (2011) reported a maximum protease specific activity of 33.64 U/mg at pH 7 and 40°C after 7 days of incubation of wheat bran fermentation media.

Effect of Substrate Concentration

The maximum protease production (119U/mL) was found at 10 g concentration of wheat bran with 10.8 g dry biomass. As the substrate concentration was increased from 10 g, the enzyme activity tended to decrease (Fig. 2). Similar results were reported by Haq et al. (2006) by using different substrates such as sunflower meal, soybean meal, cotton seed meal, and wheat bran. Iksari and Mitchell (1994) reported protease production with an activity of 3.9 PU/g from *A. niger* on rice husk as a growth substrate, while Sumantha et al. (2006) reported maximum activity of 3.6 PU/g using the micro-organism *A. niger* with 20 g of the substrate. This little variation may be due the genetic variability among species and as well as due to the uses of different substrates in different concentrations.

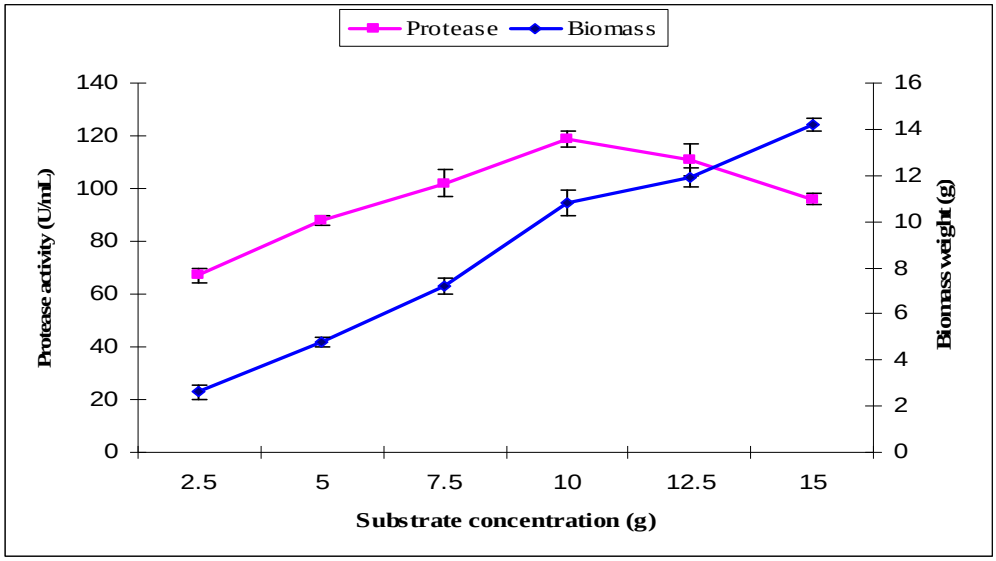

Fig. 2. Effect of different substrate concentrations on the production of protease from wheat bran by *A. niger*

Effect of Initial Medium pH

The enzyme profiles of the experiment revealed that after 3 days at pH 7, protease showed the maximum activity (132U/mL), indicating the role of media pH in enzyme action. Comparable protease activity was present over a wide range of pHs (5-8) in the sample tubes containing the filtrated culture supernatant (Fig. 3).

Extra-cellular culture pH strongly influences the microbial growth and many enzymatic processes, which in turn support the cell growth and product formation. The optimal pH ranges for different fungal neutral or alkaline proteases are from 7-11 (Coral et al. 2002; Benazir et al. 2011).

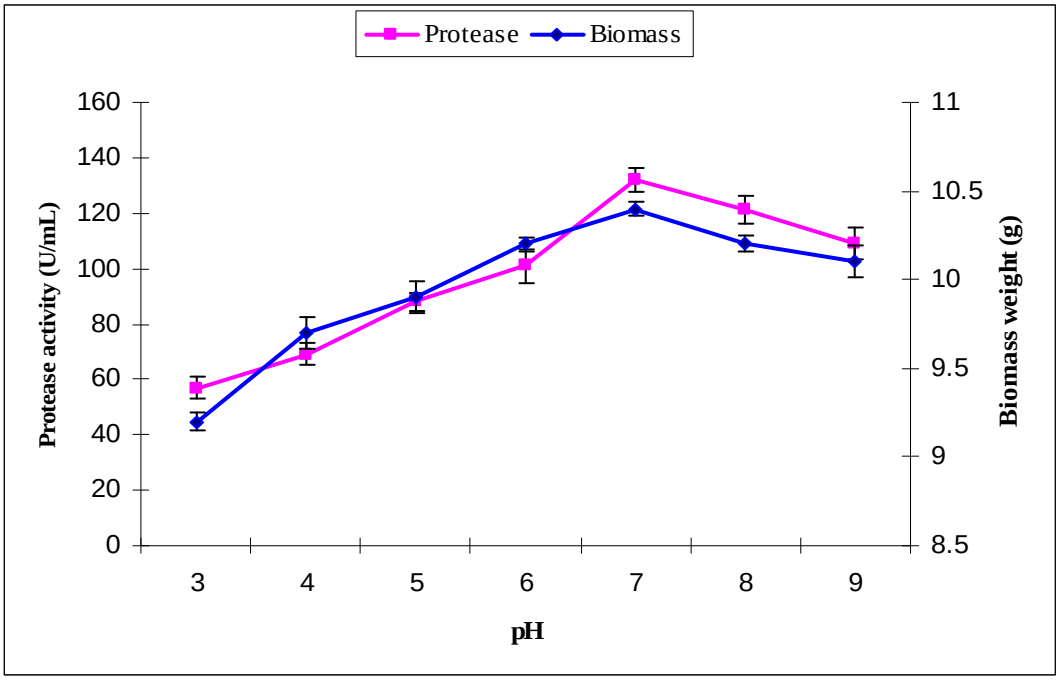

Fig. 3. Effect of different pH values on the production of protease from wheat bran by *A. niger*

Effect of Incubation Temperature

Figure 4 shows the effect of incubation temperature on protease production. The results showed that maximum enzyme production was achieved at a temperature of 45°C (156 U/mL). Higher temperatures have an adverse effect on the metabolic activities and growth pattern of the micro-organism. Temperature higher than the optimal level causes fungus growth inhibition and denaturing of enzymes (Haq et al. 2006).

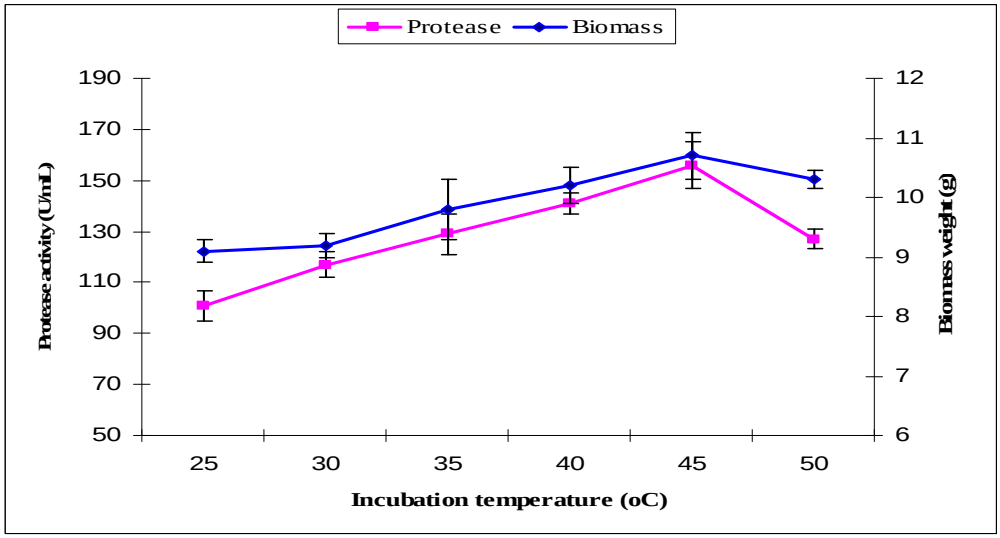

Fig. 4. Effect of different incubation temperatures on the production of protease from wheat bran by *A. niger*

Optimum temperature is different for different *Aspergillus* species (*A. saitoi, A. tamari, A. nidulans and A. awamori*) due to genetic variability and range from 30 to 40°C (Coral et al. 2002; Charles et al. 2008; Negi and Banerjee 2010). Sumantha et al. (2006) reported similar results by using the micro-organism *R. microsporus* NRRL 3671. The thermophilic culture was found to be highly sensitive to temperature changes for both enzyme production and growth. Due to the change of micro-organisms, substrate variation and some other environmental factors, Paranthaman et al. (2009) reported slightly different results.

Effect of Inoculum Size

All the experimental flasks containing 10 g substrate were incubated for 3 days of fermentation time period in duplicate at pH 7 and 30°C. The maximum activity of protease (177 U/mL) was observed in culture filtrate obtained from Solid State Fermentation medium of wheat bran inoculated with 4 mL inoculums; results thus obtained are discussed next (Fig. 5), while any further increase in an inoculum size showed a decrease in the enzyme activity profile. The maximum amount of enzyme (4.8 U mL) was produced when 1.0 mL was added to the flask (Haq et al. 2004). Further increase in inoculum volume resulted in the decrease of protease production. Because a large increase in inoculum volume caused overcrowding of spores, the enzyme activity was decreased (Ahmed et al. 2010). In fermentation, size of inoculum is an important biological factor (Norliza and Ibrahim 2005; Sandhya et al. 2005).

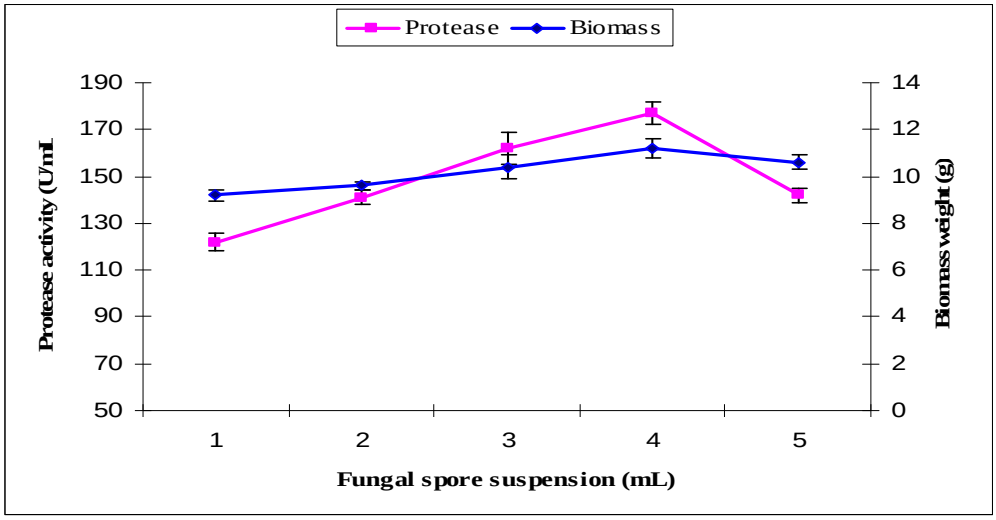

Fig. 5. Effect varying levels of fungal inoculum on the production of protease from wheat bran by *A. niger*

Effect of Tween 80

Varying concentrations (1-5%) of surfactant were studied by incubating the wheat bran fermentation media of pH 7 at 30°C with 4 mL inoculum size. Maximum production was observed at the concentration of 3% of Tween 80 (Fig. 6). The study reveals that the enzyme secretion is greatly influenced by the change in the concentration of Tween 80. Higher concentration of Tween 80 affects the enzyme production because the substrates

also have an adequate supply of nutrients. Similar results were reported by Haq et al. (2004) by using the micro-organism *Penicillium griseoroseum* with solid state fermentation at 4 mL (220.45 PU/g).

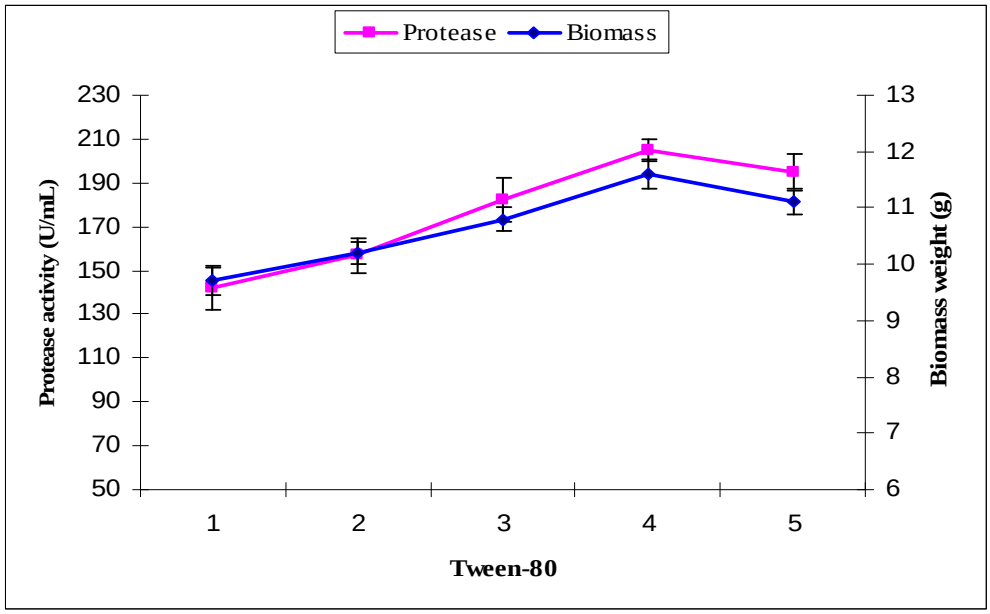

Fig. 6. Effect different concentration of Tween 80 on the production of protease from wheat bran by *A. niger*

Purification and SDS-PAGE

The crude protease enzyme was precipitated at 70 % ammonium sulfate saturation with specific activity of 14 U/mg and 1.16-fold purification. After ion exchange column chromatography, the enzyme purity was increased up to 2.41-fold with a specific activity of 29 U/mg (Table 1).

Table 1. Purification Summary of Protease Produced from *A. niger* using Wheat Bran

Purification Steps	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification fold	Percent Recovery
Crude Enzyme	205	17	12	1.0	100
$(NH_4)_2SO_4$ Precipitation	190	14	14	1.16	92.68
Sephadex G –100	172	9	19	1.58	83.90
DEAE Cellulose	145	5	29	2.41	70.73

A homogenous monomeric protease from *A. niger* was found on SDS-PAGE, as was made evident by a single band corresponding to 47 kDa (Fig. 7). Most of the halophilic and acidic proteases reported as a single band and have a molecular weight in range from 40 to 130 kDa (Gimenez et al. 2000; Studdert et al. 2001). In comparison to molecular weight, the present reported protease of *A. niger* (47 kDa) was different from other reported fungal proteases such as that from *R. oryzae* (31 kDa) (Kumar et al. 2005), *R. oligosporus* (43 kDa) (Iqbal et al. 2011), *A. niger* I1 (50 kDa) (Siala et al. 2009), *A. niger* Z1 (60 kDa) (Coral et al. 2003), *P. ostreatus* (75 kDa) (Palmieri et al. 2001), and *A. fumigatus* (124 kDa) (Wang et al. 2005).

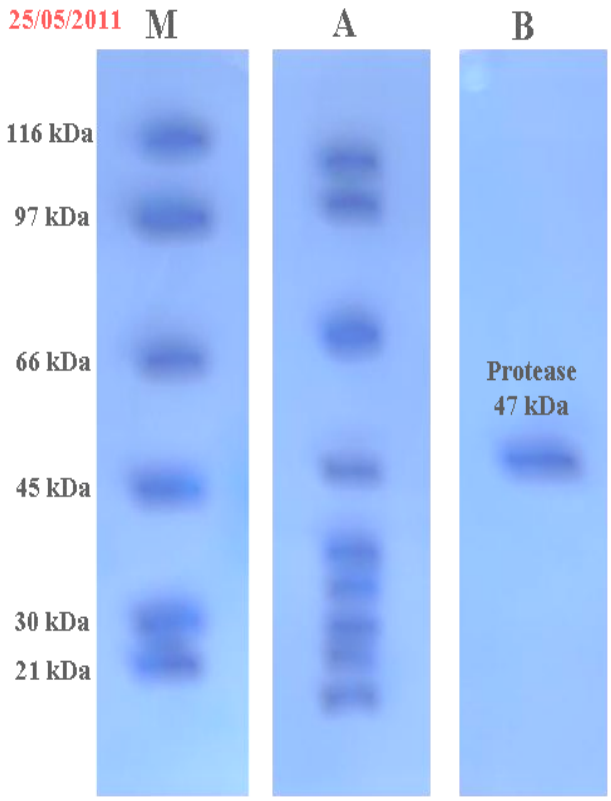

Fig. 7. Molecular mass determination of *A. niger* protease by SDS-PAGE

[Lane M, Molecular weights in kDa of standard marker; lane A, Crude protease; lane B, Purified protease]

Characterization of Purified Protease

Effect of pH on protease activity and stability

Results of the enzyme assay showed that the neutral *A. niger* protease was completely stable within a large pH range (5 to 8) and presented an optimum activity (190 U/mL) at a pH of 7 (Fig. 8), whereas any further increase in pH up to 7 showed a decreasing trend in activity. The pH optimum of the present protease was different from other fungal species like *Penicillium camembertii*, pH 3.5 (Chrzanowska et al.1995), *Rhizopus oryzae*, pH 5.5 (Kumar et al. 2005), and *A. niger* Z1, pH 9 (Coral et al. 2003).

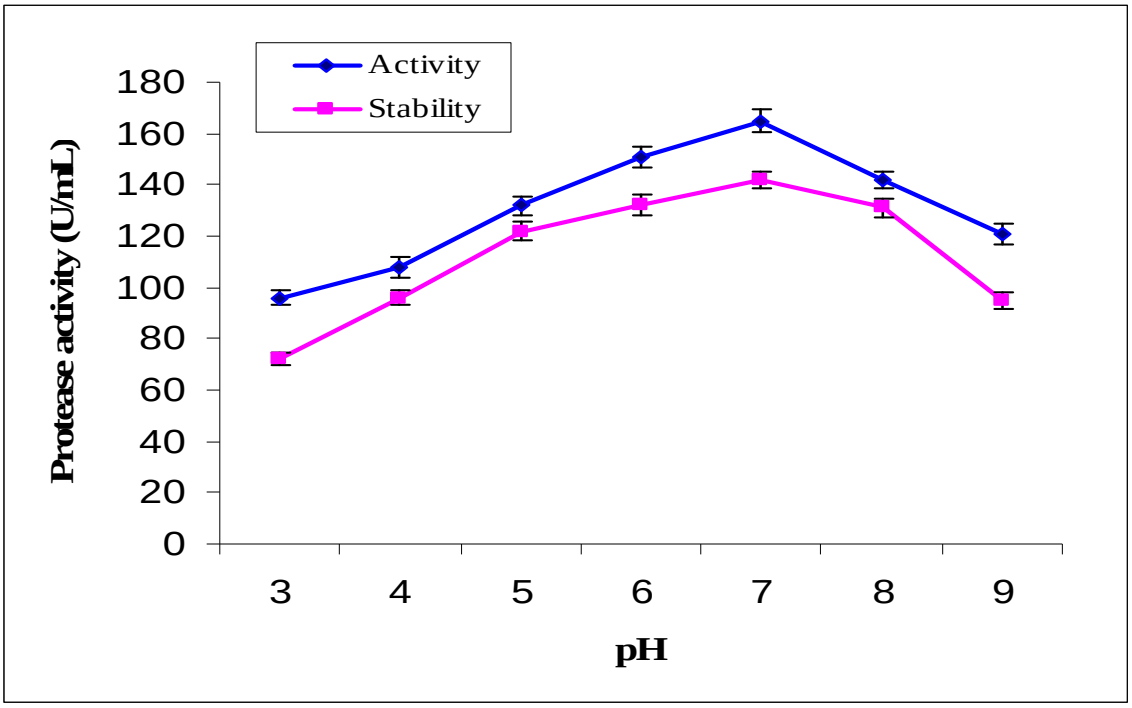

Fig. 8. Effect of different pH values on purified protease produced from *A. niger* using wheat bran

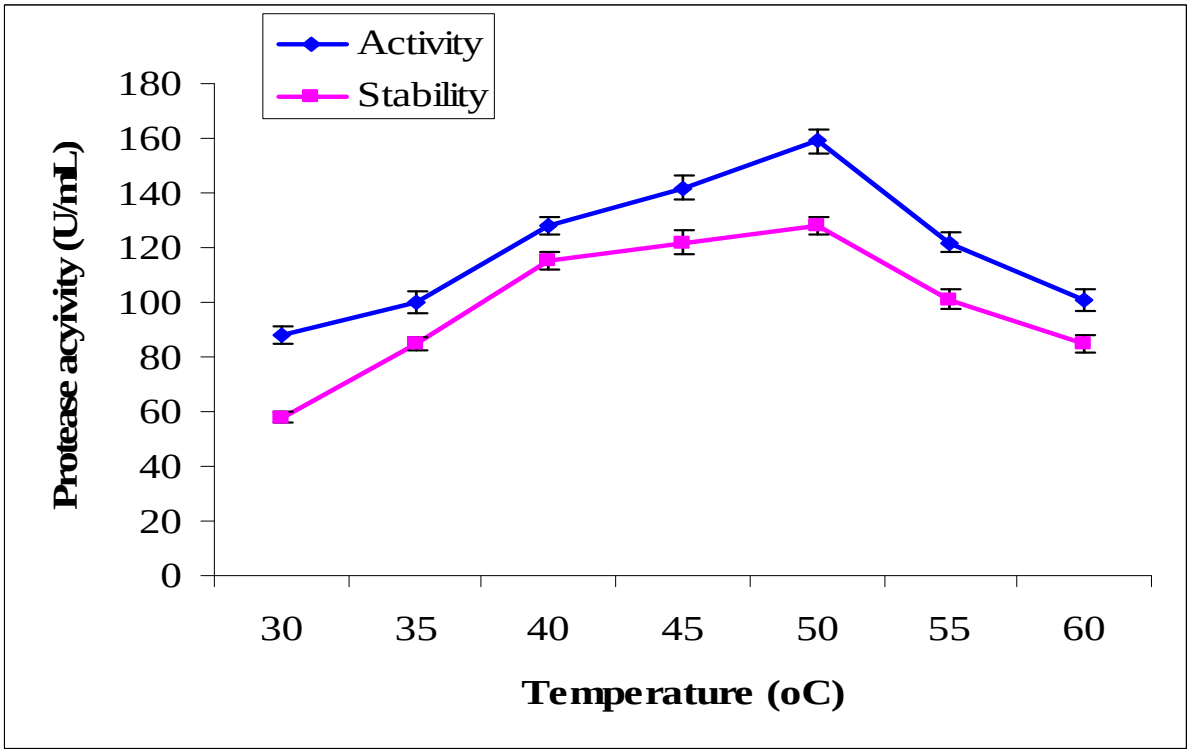

Fig. 9. Effect of different temperatures on purified protease produced from *A. niger* using wheat bran

Effect of temperature on protease activity and stability

To investigate the thermal stability of protease, enzyme was incubated at different temperatures ranging from 30 to 60°C. The optimum temperature for purified acidic protease was observed at 50°C. Results in Fig. 9 showed that at temperatures higher than 50 °C, enzyme started to lose its activity rapidly. Thermo-stability is an attractive and advantageous characteristic of an enzyme for a variety of biotechnological applications (Haddar et al. 2009; Joo et al. 2003). Hussain et al. (2010) reported maximum proteolytic activity at 40°C, while a further increase decreases the activity and shows an 80% loss in activity at 70°C. The present reported protease from *A. niger* is heat stable with optimum in activity at 50 °C and is different from other reported fungal proteases like that from *A. niger* (32°C) (Aalbaek et al. 2002), *P. roqueforti* P2 (25°C) (Durand-Poussereau and Fevre 1996) *Thaumatococcus daniellii* (35°C) (Raimi et al. 2011), and *A. niger* Z1, (40°C) (Coral et al. 2003).

Effect of metal ions and inhibitors

Various metal ions and inhibitors effects on protease activity were studied. Among the metal ions/inhibitors tested, Mn^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, PMSF, Pepstatin, and Iodoacetic acid were observed as being insignificant towards inhibition, as they do not influence the enzyme activity at a concentration of 1 mM. These agents activated the enzyme to a variable extent, proving that the enzyme was a metalloprotease due the inhibition by EDTA. Certain agents including EDTA, and SDS showed significant inhibitory effect on the purified protease (Fig. 10).

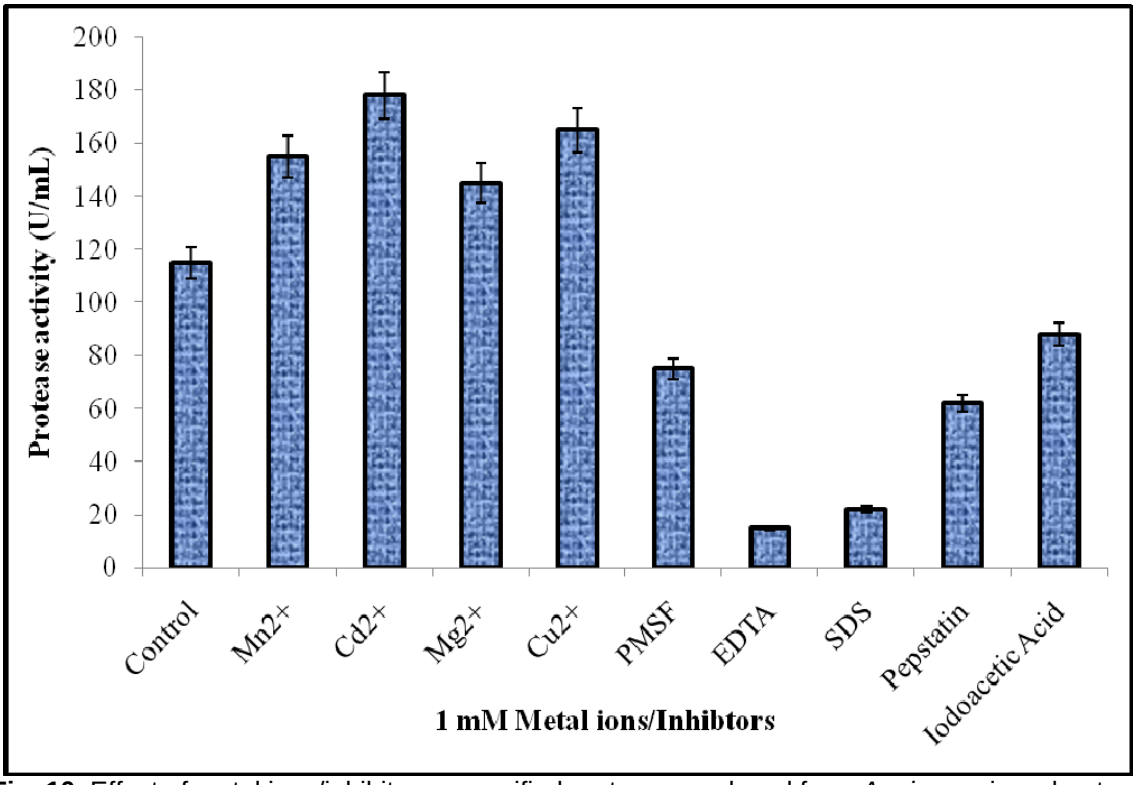

Fig. 10. Effect of metal ions/inhibitors on purified protease produced from *A. niger* using wheat bran

As reported in the literature (Basu et al. 2007; Merheb-Dini et al. 2009), protease enzyme retained its activity in the presence of PMSF and SDS, while it was completely inhibited by 1 mM EDTA. In this present study EDTA showed mild inhibitory effect on the activity of protease, whereas Ramakrishna et al. (2010) reported that EDTA slightly activated the protease activity of *Bacillus Subtilis*.

Industrial Application

Detergent compatibility of purified protease

The purified protease was tested for its compatibility with different locally available detergent brands for its possible commercial utilization in the detergent industry. Enzyme activity assay profile showed that enzyme incubated at 55°C with detergent solution revealed maximum compatibility with Ariel, followed by Surf Excel (Fig. 11). This revealed the compatibility of present *A. niger* neutral metalloprotease with local detergents and suggests its potential as suitable additive to detergents.

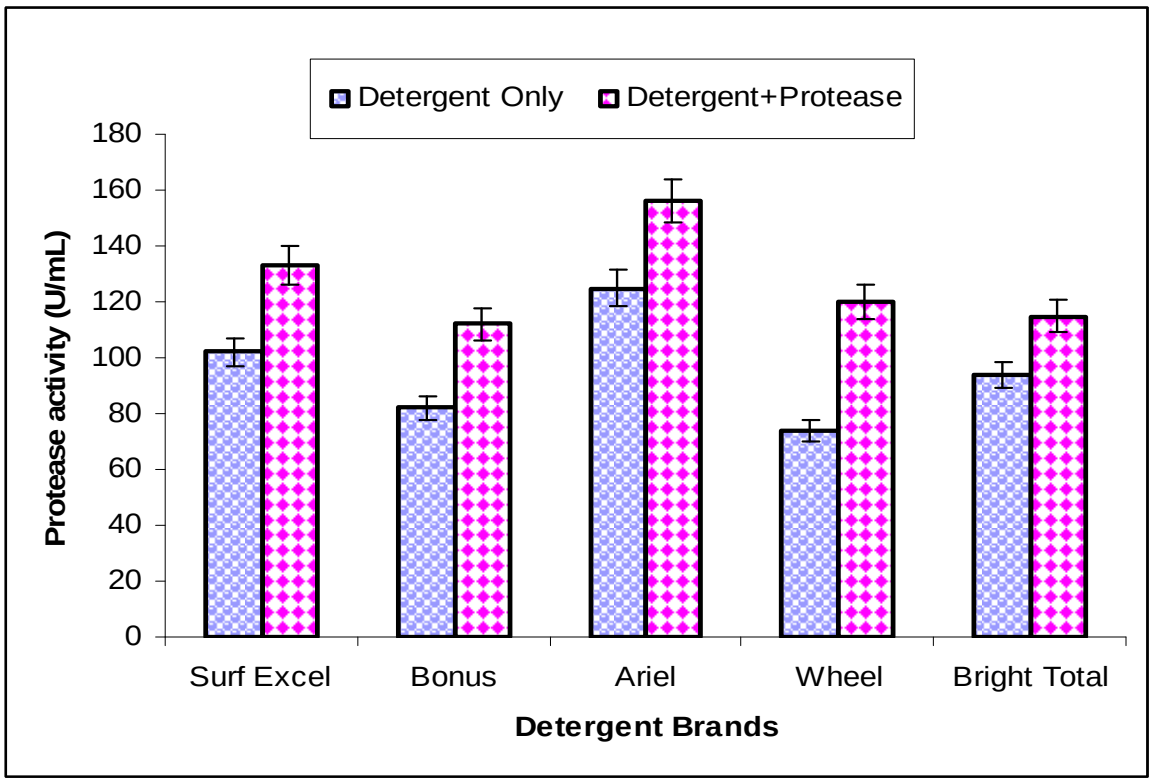

Fig. 11. Detergent compatibility of purified *A. niger* protease with different detergent brands

Shelf life

The effect of storage of purified protease on its activity was determined. The enzyme stored at room temperature (30°C) for up to 30 days (Table 2) revealed that the enzyme was 85% active for 20 days and its activity was reduced to 70 and 38% after the

25th and 30th days, respectively. The enzyme may be stored for up to 25 days at room temperature without losing much of protease activity.

Table 2. Effect of Storage (Shelf Life) on Purified Protease Activity at Room Temperature*

Shelf Life (Days)	Protease relative activity (%)
0	100
15	91
20	85
25	70
30	38

* 30°C

CONCLUSIONS

1. *Aspergillus niger*, used in the present study, showed extraordinary capability to utilize wheat bran (a by-product) as a cost effective substrate for the production of metallo-protease.
2. An attempt was made towards finding the best growth conditions for successful cultivation of *A. niger*, and production of neutral protease enzyme.
3. The purified metalloprotease was found to have a molecular weight of 47 kDa with an optimum activity at pH 7 and 50°C.
4. The purified protease was also compatible with different local detergent brands with up to 25 days shelf life at room temperature, suggesting its potential as a valuable detergent additive for improved washing. It can be concluded that the protease may be potentially useful for industrial purposes, especially for the detergent and laundry industry.

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