PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASE FROM AGRO INDUSTRIAL WASTES BY USING *ASPERGILLUS TERREUS* (AB661667) UNDER SOLID STATE FERMENTATION

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Abstract: *Proteases are the most important industrial enzymes and accounts at least 25% of global enzyme production. In the present work, Aspergillus terreus(AB661667) was used for the production of proteases by solid state fermentation using varieties of agro industrial waste (wheat bran, rice bran, green gram husk and black gram husk). Among the all tested varieties, wheat bran produced the highest activity in 67.4U/mg while black gram husk produced lowest protease as 59.1 U/mg under solid state fermentation conditions. All the purified alkaline protease from Aspergillus terreus(AB661667) showed a single protein band with a molecular weight of 38 KDa on SDS-PAGE. The optimum pH for the protease production was found to be 8.5 and optimum temperature at 35°C. All the data suggest that the selected strain of Aspergillus terreus (AB661667) can significantly produce a protease enzyme from the wheat bran substrate.*

Keywords: *Solid state fermentation, Aspergillus terreus (AB661667), Agroindustrial waste, Alkaline protease, SDS-PAGE*

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1. INTRODUCTION

Proteolytic enzymes are included in a sub-class of the enzymes hydrolases. These enzymes cause breakdown of proteins into smaller peptides and amino acids by catalyzing the breakdown of peptide bonds. Proteases refer not to a single enzyme, but a mixture of enzymes, which include proteinases, peptidases and amidases. The proteinases hydrolyze intact protein molecules to proteoses, peptones and some amino acids. Peptidases hydrolyze peptones to amino acids while amidases hydrolyze amino acids and release ammonia. Proteases represent one of the three largest groups of industrial enzymes and find application in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes. Proteases belong to the class of enzymes, which have been most extensively studied by scientists from various aspects (Beg and Gupta, 2003; Takami et al, 1989). Culture conditions play a significant role on growth and production of protease by fungi. For protease production from micro-organisms, both solid state and submerged fermentation techniques are employed (Pandey et al., 2001; Mukhtar and Haq, 2008). However, solid-state fermentation is a preferred method for the production of acid proteases by the fungi (Tremacoldi et al., 2004). The essential feature of solid-substrate fermentation (SSF) is the growth of micro-organisms on a pre-dominantly insoluble substrate without a free liquid phase. The substrates used in (SSF) are usually Rice bran and wheat bran.The moisture level in solid-state fermentation is the most important factor. It may be between 30 to 80% and for enzyme production is typically in the range of 60% (Tsuchiya et al., 1998). The organisms which are most adapted to grow under these conditions of low water activity and presence of relatively intractable solid substrates are the fungi.The present study was aimed at the production of protease enzyme from various Agro-industrial wastes such as wheat bran, rice bran, green gram husk and black gram husk by *Aspergillus terreus* (AB661667)**.**

2. MATERIALS AND METHODS

2.1. Micro- organism

Aspergillus terreus (AB661667) culture was procured from the department of Microbiology, Global Institute of Biotechnology, Hyderabad. The culture was grown on Potato Dextrose Agar (PDA) slants at 30 °C with fortnightly transfer to fresh medium.

2.2 Substrates for fermentation

Different agro industrial wastes (wheat bran, rice bran, green gram and black gram) were obtained from the local market, Pulivendula, Andhra Pradesh. These substrates were washed with several changes of sterile water and were cleaned with a 2 % solution of H_2SO_4 initially. After drying, they were powdered and were used as the substrate for solid-state fermentation (SSF).

2.3. Fermentation conditions and protease extraction

In the present study, Solid Surface Fermentation was carried out according to Paranthaman *et al.,* 2009. Five grams of each substrate were weighed and taken in a four different 250ml conical flask contains 10 ml of salt solution (g/l) each. The composition of salt solution was as follows (%w/v: g/100ml) ammonium nitrate 0.5, potassium dihydrogen orthophosphate 0.2, sodium chloride 0.1 and magnesium sulphate 0.1. The media was autoclaved at 121°C for 20 min. After cooling, 1 ml of fungal spore solution (10^6 spores/ ml) was inoculated and incubated at 30°C for seven days in an incubator shaker at 125 RPM. After the end of incubation, 25 ml of 0.1% Tween-80 was added to each flask and was homogenized in a rotary shaker at 180rpm for 1 hour. The media were then centrifuged at 8000xg for 10 min at 4^{0}C to get clear supernatant containing enzyme. The resultant enzyme solution was used as a crude enzyme for further studies.

2.4. Alkaline protease activity and protein estimation

The alkaline protease activity of samples was determined according to Niyonzima and More (2013) method using casein as substrate. Total protein concentration was estimated according to Bradford *et al.,* 1976 method.

2.5. Purification of alkaline protease

All the purification steps were performed at 4^{0}C. The crude enzyme samples were mixed with ammonium sulphate to precipitate proteins which were later collected by centrifugation at 20,000rpm for 30 min (Charles *et al.,* 2008) initially. The precipitated proteins were dissolved in Tris-HCl buffer (25mM, pH 8.0), dialyzed against the same buffer overnight at 4^{0}C and concentrated by lyophilization (Charles *et al.,* 2008). The dialyzed concentrated samples were purified by Sephadex G-100 gel filtration column chromatography. The column (1.6 x 36 cm) was equilibrated with the same buffer [Tris-HCl

buffer (25mM, pH 8.0)]. Finally, all the fractions showing protease activity were pooled out, combined and lyophilized to concentrate.

2.6. SDS- PAGE and molecular weight determination

The molecular weight of purified protease produced by *Aspergillus terreus* from four different substrates was estimated by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis under non reducing conditions (Laemmli, 1970). Electrophoresis was performed at 50V for stacking gel (4%) and 100V for resolving gel (12%). After electrophoresis, the protein bands are fixed in fixation dye for overnight and the gels were stained with coomassie brilliant blue staining (coomassie 0.25%, methanol 45%, acetic acid 10%) for 20 min. The gels are destined with the stain solution, excluding the dye until clear background appears. The molecular weight of protease was calculated based on the protein markers.

2.7. Characterization of purified protease

The highest activity shown protease was used for determining the effect of pH, temperature.

2.7.1. Effect of pH on enzyme activity

The effect of pH on protease activity was determined by incubating purified protease (10 µg protein) at different pH levels under standard assay conditions using casein as substrate and appropriate buffer (pH 7-8 phosphate buffer; pH 8-9 Tris-HCl buffer). After pre-incubation at different pH without substrate for 12 hrs, the enzyme stability was determined.

2.7.2. Effect of temperature on enzyme activity

The optimum temperature for the protease activity was determined by performing the standard assay in the range of 20- 60°c. Thermal stability was determined by assaying the residual protease activity after incubation for 1 hr at the previous mentioned temperatures without substrate.

3. RESULT AND DISCUSSION

The production of enzymes by microorganisms is influenced greatly by the composition of the media, especially carbon and nitrogen sources. Production of the enzyme in an inexpensive medium on a large scale is a very important process to be commercially viable. The alkaline protease from *Aspergillus terreus* was purified from the culture filtrate by

ammonium sulphate precipitation, dialysis and Sephadex G-100 column chromatography. The purification details from each substrate are summaries below.

3.1. Purification of alkaline protease from *Aspergillus terreus* using wheat bran as substrate

The alkaline protease produced by Aspergillus terreus grown in wheat bran substrate was concentrated by ammonium sulfate (70%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 7.88-fold with a recovery of 12.6% and specific activity of 47.3 U/mg of protein. The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The active fractions with alkaline protease activity was pulled out and collected separately. The elution profiles of protein and alkaline protease activity are shown in Fig. 1. After the final purification step, the enzyme was purified 17.08-fold with a recovery of 5.08% and specific activity of 67.4 U/mg of protein. Summary of purification steps was given in Table.1.

Table 1. Purification steps of alkaline protease from *A.terreus* using wheat bran as substrate.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	41	287.82	7.02	1.0	100
Ammonium sulfate	21	266.70	12.7	1.95	51.2
Dialysis	5.2	245.96	47.3	7.88	12.6
Sephadex G-100	2.4	161.76	67.4	17.08	5.8

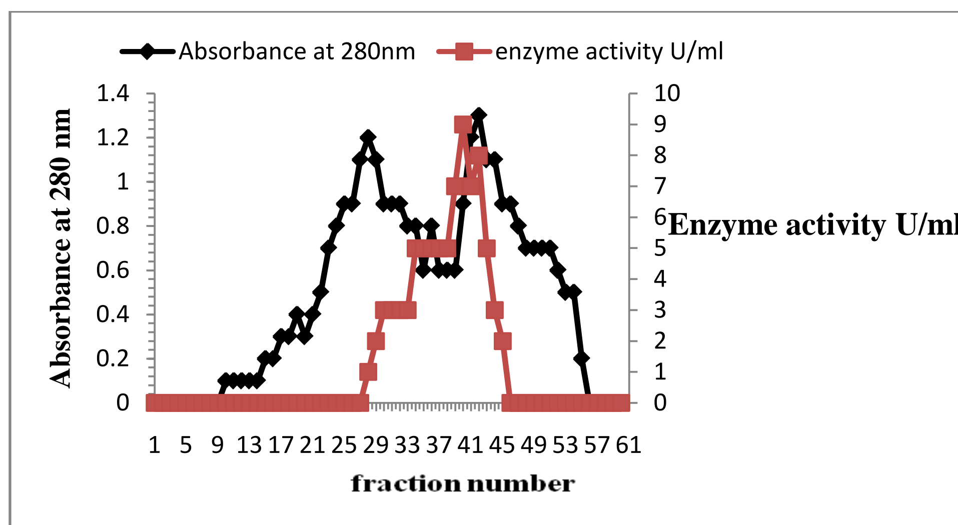

Fig. 1. Chromatogram of the alkaline protease from *A.terreus* on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

3.2. Purification of alkaline protease from *Aspergillus terreus* using the green gram husk as substrate

The alkaline protease produced by *Aspergillus terreus* in green gram husk substrate was concentrated by ammonium sulfate (70%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 8.26-fold with a recovery of 12.09% and specific activity of 42.65 U/mg of protein. The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The active fractions with alkaline protease activity was pulled out and collected separately. The elution profiles of protein and alkaline protease activity are shown in Fig. 2. After the final purification step, the enzyme was purified 17.68-fold with a recovery of 5.92% and specific activity of 66.9 U/mg of protein. Summary of purification steps was given in Table.2.

Table.2 Purification steps of alkaline protease from *A. terreus* using the green gram husk as substrate.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	38.9	264.52	6.8	1.0	100
Ammonium sulfate	18.6	221.34	11.9	2.09	48.9
Dialysis	4.7	190.35	40.5	8.27	12.09
Sephadex G-100	2.2	147.2	66.9	17.68	5.92

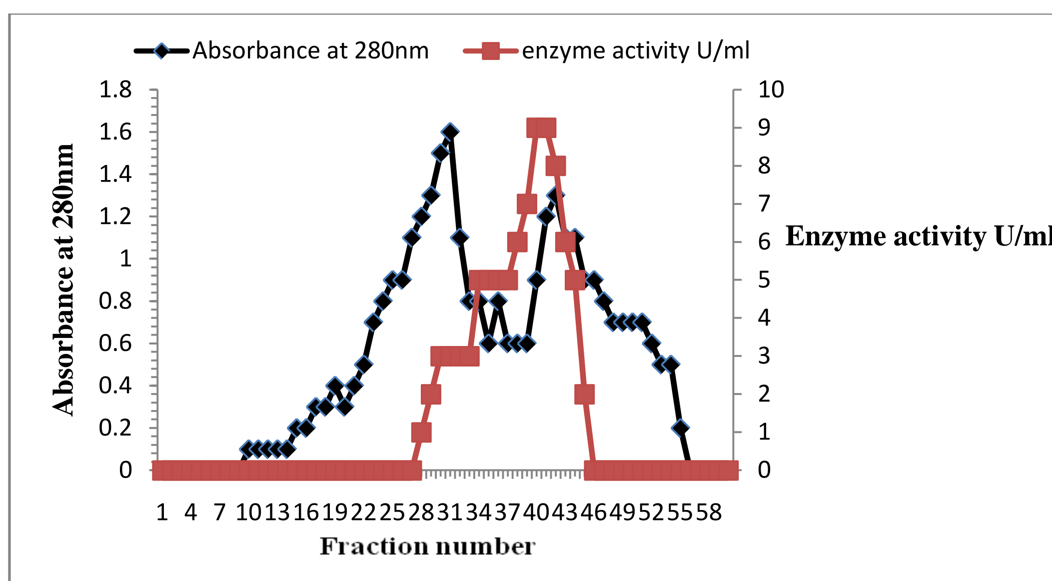

Fig. 2. Chromatogram of the alkaline protease from *A.terreus* on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

3.3. Purification of alkaline protease from *Aspergillus terreus* using rice bran as substrate

The alkaline protease produced by *Aspergillus terreus* in rice bran substrate was concentrated by ammonium sulfate (70%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 11.6-fold with a recovery of 8.57% and specific activity of 30.40 U/mg of protein. The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The active fractions with alkaline protease activity was pulled out and collected separately. The elution profiles of protein and alkaline protease activity are shown in Fig. 3. After the final purification step, the enzyme was purified 44.7-fold with a recovery of 2.23% and specific activity of 61.8 U/mg of protein. Summary of purification steps was given in Table.3.

Table.3 Purification steps of alkaline protease from *Aspergillus terreus* using rice bran as substrate.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	80.5	266.7	3.30	1.0	100
Ammonium sulfate	29.8	256.0	8.58	2.69	37.0
Dialysis	6.9	209.8	30.40	11.6	8.57
Sephadex G-100	1.8	111.3	61.8	44.7	2.23

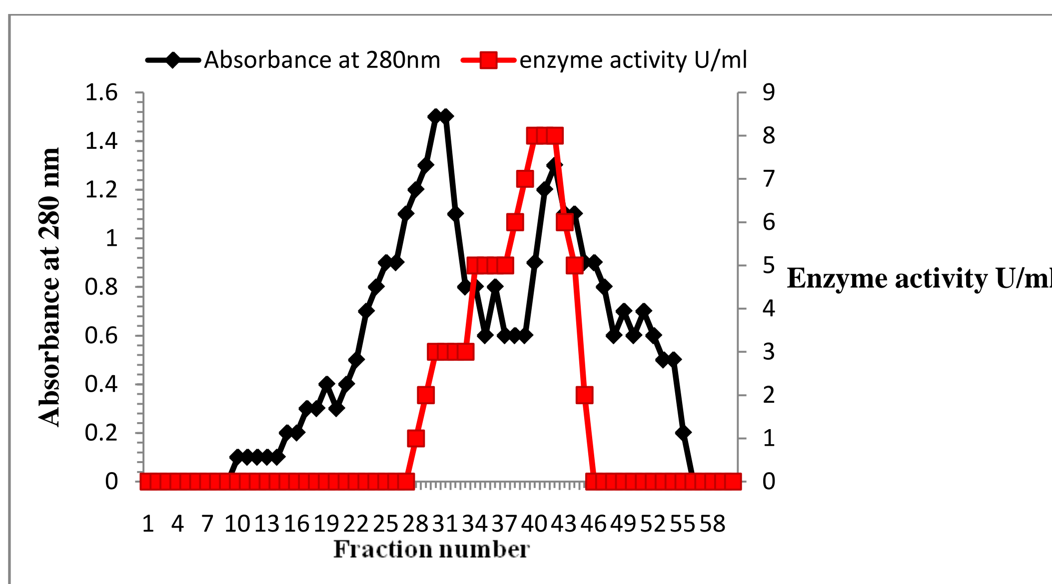

Fig. 3. Chromatogram of the alkaline protease from *A.terreus* on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

3.4. Purification of alkaline protease from *Aspergillus terreus* using black gram husk as substrate

The alkaline protease produced by *Aspergillus terreus* in black gram husk substrate was concentrated by ammonium sulfate (70%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 6.32 -fold with a recovery of 15.8% and specific activity of 29.1 U/mg of protein. The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The active fractions with alkaline protease activity was pulled out and collected separately. The elution profiles of protein and alkaline protease activity are shown in Fig. 4. After the final purification step, the enzyme was purified 25.28-fold with a recovery of 3.92% and specific activity of 59.1 U/mg of protein. Summary of purification steps was given in Table.4.

Table.4 Purification steps of alkaline protease from *A.terreus* using black gram husk as substrate

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	35.4	109.7	3.10	1.0	100
Ammonium sulfate	19.2	149.0	7.8	2.05	54.23
Dialysis	5.6	162.9	29.10	6.32	15.8
Sephadex G-100	1.4	82.7	59.1	25.28	3.9

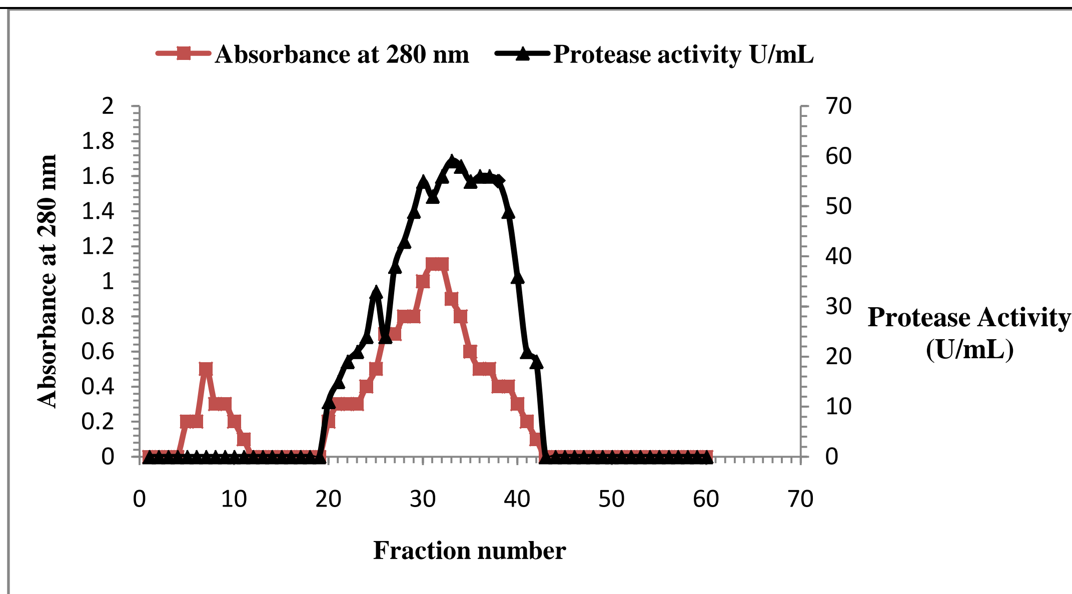

Fig. 4. Chromatogram of the alkaline protease from *A.terreus* on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Similarly, Sumantha *et al.,* (2006) and Benazir *et al.,* (2011) reported maximum protease activity from *Aspergillus niger* using wheat bran as substrate.

Table.5 Summary of purified alkaline protease from *Aspergillus terreus* using four different substrates

Source	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (Fold)	Yield (%)
Wheat bran	2.4	161.76	67.4	17.08	5.8
Green gram husk	2.2	147.2	66.9	17.68	5.92
Rice bran	1.8	111.3	61.8	44.7	2.23
Black gram husk	1.4	82.7	59.1	25.28	3.9

3.6. Molecular weight of purified alkaline protease from four sources

The purified alkaline protease from *A.terreus* using wheat bran (lane 4) and rice bran (lane 5) appeared as a single protein band in SDS-PAGE and with a molecular weight of approximately 45 kDa. In case of green gram (lane 2) and black gram (lane 3) it appeared as a single protein with a molecular weight of 38 KDa (Fig. 5). Similarly Chakrabarti et al (2000) reported the molecular weight of alkaline protease from *Aspergillus terreus* as 37 KDA. Most of the protease from *Aspergillus* was reported as single band and have a molecular weight from 30 to 130 KDa (Gimenez *et al.,* 2000; Studdert *et al* 2001).

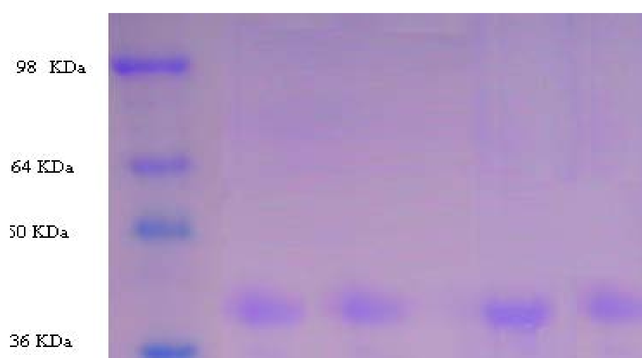

Fig. 5. SDS-PAGE of purified enzyme. Lane 1: Marker protein, Lane 2: Alkaline protease from green gram husk, Lane 3: Alkaline protease from black gram husk, Lane 4: Alkaline protease from wheat bran, Lane 5: Alkaline protease from rice bran.

3.7. Characterization of purified protease

Alkaline protease produced from wheat bran showed maximum activity, was used for further characterization of optimum temperature and optimum pH.

3.7.1. Effect of pH on protease activity

The enzyme was active at alkaline pH and the optimum pH for alkaline protease was 8.5 (Fig.6). An increase of pH resulted in decrease of enzyme activity. Similar results were reported by Chakrabarti *et al.,* (2000) from *Aspergillus terreus.* Francois *et al.,* (2014) reported similar results in *Aspergillus terreus.*

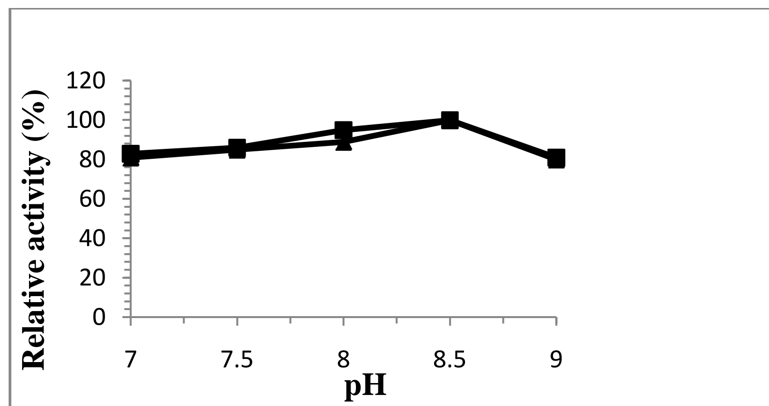

Fig. 6. Optimal pH (■) and stability of pH (Δ) of purified alkaline protease from *Aspergillus terreus.*

3.7.2. Effect of temperature on protease activity

The enzyme was most active at 35°C (Fig.7) and above increase in temperature, the activity was decreased. At 60°C, the activity was significantly inactivated and completely lost beyond this temperature. Chakrabarti *et al.,* (2000) from *Aspergillus terreus.* Francois *et al.,* (2014) reported similar results in *Aspergillus terreus.*

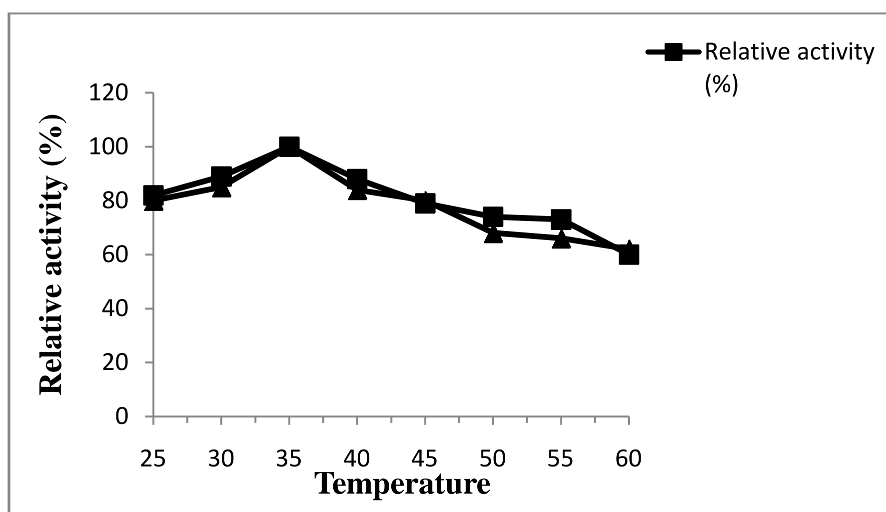

Fig. 7. Optimal temperature (Δ) and stability of temperature (■) of purified alkaline protease from *Aspergillus terreus.*

4. CONCLUSION

In the present study, the extracellular alkaline protease produced by *Aspergillus terreus* (*AB661667)* using four sources as a substrate under solid state fermentation was purified, characterized. The protease enzyme was purified by ammonium sulfate precipitation, dialysis, and sephadex G-100 gel filtration from four different fermentation media. The alkaline protease from wheat bran substrate was produced in abundance and the enzyme was purified 17.08 fold and the apparent molecular weight of the enzyme was found to be 38 KDa by SDS-PAGE. All these data suggest that the selected strain of *Aspergillus terreus* (*AB661667*) can significantly produce a protease enzyme from the wheat bran substrate.

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