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RESEARCH ARTICLE

Production, purification and characterization of alkaline protease from *Trichoderma viride* N9 by using agro industrial wastes under solid state fermentation

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Abstract

The production of enzymes by bioprocesses is a good value added to agro industry residues. A comparative study was carried out on the production of protease using agro industrial wastes (Wheat bran, rice bran, green gram husk and black gram husk) as substrates in solid-state fermentation (SSF) by *Trichoderma viride* N9. Among the all tested varieties, wheat bran produced the highest activity as 59.6 U/mg of protein, while black gram produced lowest protease as 56.1 U/mg of protein under solid state fermentation conditions. The purified alkaline protease from *Trichoderma viride* N9 obtained from all samples appeared as a single protein band with a molecular weight of 23KDa The optimum pH for the protease production was found to be 8 and optimum temperature at 35^{o}C. All the data suggest that the selected strain of *Trichoderma viride* N9 can significantly produce protease enzyme from wheat bran substrate.

INTRODUCTION

Proteolytic enzymes cause breakdown of proteins into smaller peptides and amino acids by catalyzing the breakdown of peptide bonds and are included in a class of the enzymes hydrolases. Proteases refer to a mixture of enzymes which include proteinases, peptidases and amidases [1]. Proteases represent one of the three largest groups among industrial enzymes and find wide applications in food industry, detergents, pharmaceutical industry, leather industry and bioremediation processes [2]. Proteases are widely present in nature and microbes are considered as a preferred source because of their rapid growth, the lesser space required for cultivation and can easily manipulate genetically to generate new enzymes with altered properties that are desirable for their various applications. Culture conditions play significant role on growth and production of protease by micro organisms. For protease production from micro-organisms, both solid state and submerged fermentation techniques are employed [3;4]. However, Solid-substrate fermentation (SSF) was chosen for the present research work, because it has been reported for much greater productivity than submerged fermentation [5; 6]. The microorganisms which are most adapted to growth under these conditions of low water activity and presence of relatively intractable solid substrates are the fungi. The filamentous fungus *Trichoderma viride* is well known to produce wide variety of extracellular enzymes. Some of these enzymes play a role in degradation of fungal cells, while others take part in decomposition of plant litter which is responsible for survival of the strain in the soil [7;8].This study was taken up with the objective for the production and purification of protease enzyme from various agro-industrial wastes by using fungal isolate *Trichoderma viride* N9 (AB646476).

MATERIALS AND METHODS

Micro- organism and substrates:

Trichoderma viride N9 (AB646476) culture was obtained from department of Microbiology, Global Institute of Biotechnology, Hyderabad. The stock culture was maintained on potato dextrose agar slants at 4°C and sub culturing was done once in fortnight. Agro industrial wastes (wheat bran, rice bran, green gram husk and black gram husk) were obtained from the local market, Pulivendula. The substrates were washed with several changes of sterile water and the substrates were cleaned initially with 2% solution of H_2SO_4. They were allowed to dried off and ground with sterile mortar and pestle. Thus, obtained powder was used as the substrate for solid-state fermentation (SSF).

Fermentation conditions and protease extraction:

In the present study, fermentation media was prepared according to Paranthaman et al., 2009[9]. Each substrate weighing 5 grams was taken in 250 ml Erlenmeyer flask separately and 10 ml of salt solution was added to moisten (the salt solution composition 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), sterilized at 121^0C for 15 min and after cooling 1ml of fungal spore suspension was added (10^6 spores/ml) and incubated for 120hrs at 30^0C. After 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 4^0C to get clear supernatant containing enzyme solution; this clear supernatant was used for further studies.

Alkaline protease activity and protein estimation:

To determine alkaline protease activity, Niyonzima and More (2013) method was employed using casein as substrate. Protein concentration was estimated as per Bradford et al., 1976.

Purification of alkaline protease:

All the purification steps were performed at 4^0C. Initially the crude enzyme samples were subjected to ammonium sulphate precipitation and precipitated protein was collected by centrifugation at 20,000rpm for 30 min [10]. The precipitated protein was dissolved in Tris-HCl buffer (25mM, pH 8.0), dialyzed against same buffer overnight at 4^0C and concentrated [10]. The dialyzed concentrated sample was 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)]. All the fraction showing protease activity were pooled out, combined and lyophilized to concentrate.

SDS- PAGE and molecular weight determination:

To determine molecular weight and purity of protease, Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed under non reducing conditions [11]. For stacking gel (4%), electrophoresis was performed at 50V and 100V for resolving gel (12%). After electrophoresis, the protein bands are fixed in fixation dye and the gels were stained by coomassie brilliant blue staining (coomassie 0.25%, methanol 15%, acetic acid 7.5%) for 2hrs and destained overnight with the stain solution excluding the dye. Based on the protein markers, the molecular weight of protease was calculated.

Characterization of purified protease:

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

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.

Effect of temperature on enzyme activity:

The optimum temperature for the protease activity was determined by performing the standard assay in the range of 25- 60^oc. Thermal stability was determined by assaying the residual protease activity after incubation for 1 hr at the previous mentioned temperatures without substrate.

RESULT AND DISCUSSION

The alkaline protease from *Trichoderma viride* N9 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.

Purification of alkaline protease from *Trichoderma viride* N9 using wheat bran as substrate:

The alkaline protease present in crude sample was concentrated by ammonium sulfate (60%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 7.82-fold with a recovery of 12.7% and specific activity of 42.5 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 pooled 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 18.94 fold with a recovery of 5.27% and specific activity of 59.6 U/mg of protein. Summary of purification steps was given in Table.1.

Table.1.Purification steps of alkaline protease from *T.viride* N9 using wheat bran as substrate

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	36	200.1	5.56	1.0	100
Ammonium sulfate	18.4	204.6	11.1	1.96	51.1
Dialysis	4.6	195.6	42.5	7.82	12.7
Sephadex G-100	1.90	113.24	59.6	18.94	5.27

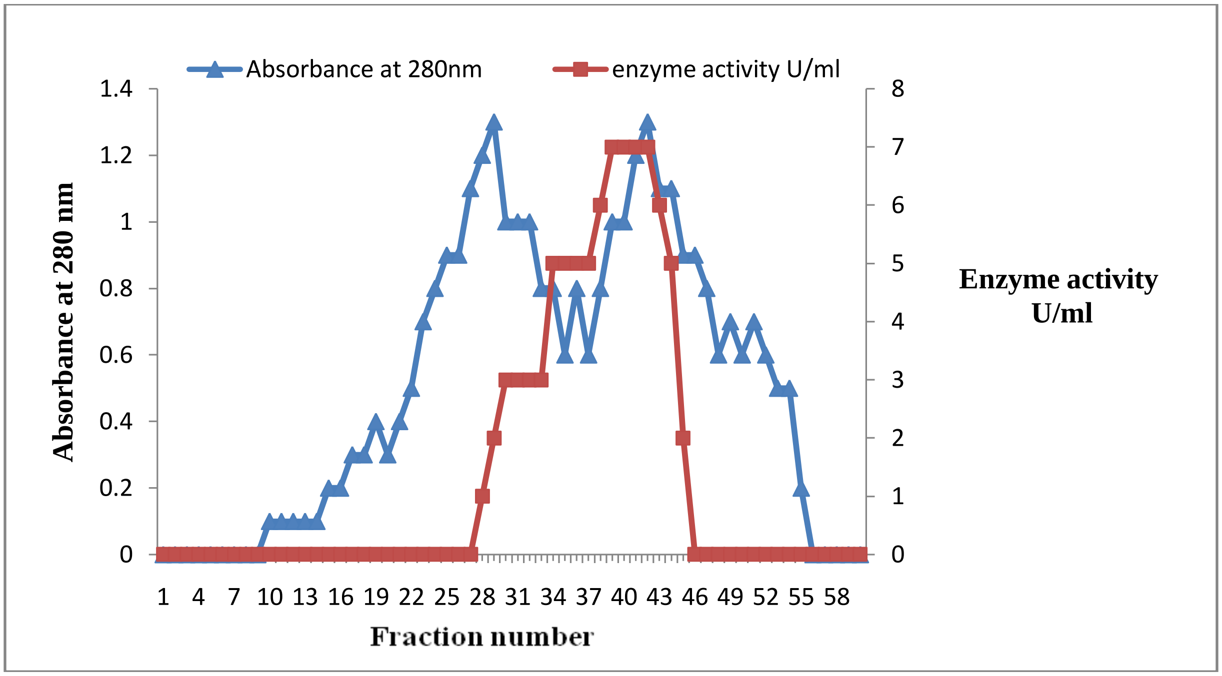

Fig.1 Chromatogram of the alkaline protease from *T.viride* N9 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Purification of alkaline protease from *T.viride* N9 using green gram husk as substrate:

The alkaline protease present in crude sample was concentrated by ammonium sulfate (60%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 7.7-fold with a recovery of 12.9% and specific activity of 40.5 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 pooled 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 18.2-fold with a recovery of 5.47% and specific activity of 58.1 U/mg of protein. Summary of purification steps was given in Table.2.

Table.2 Purification steps of alkaline protease from *T.viride* N9 using green gram husk as substrate

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	34	176.8	5.2	1.0	100
Ammonium sulphate	16.2	176.56	10.9	2.09	47.6
Dialysis	4.4	178.2	40.5	7.7	12.9
Sephadex G-100	1.86	108.6	58.1	18.2	5.47

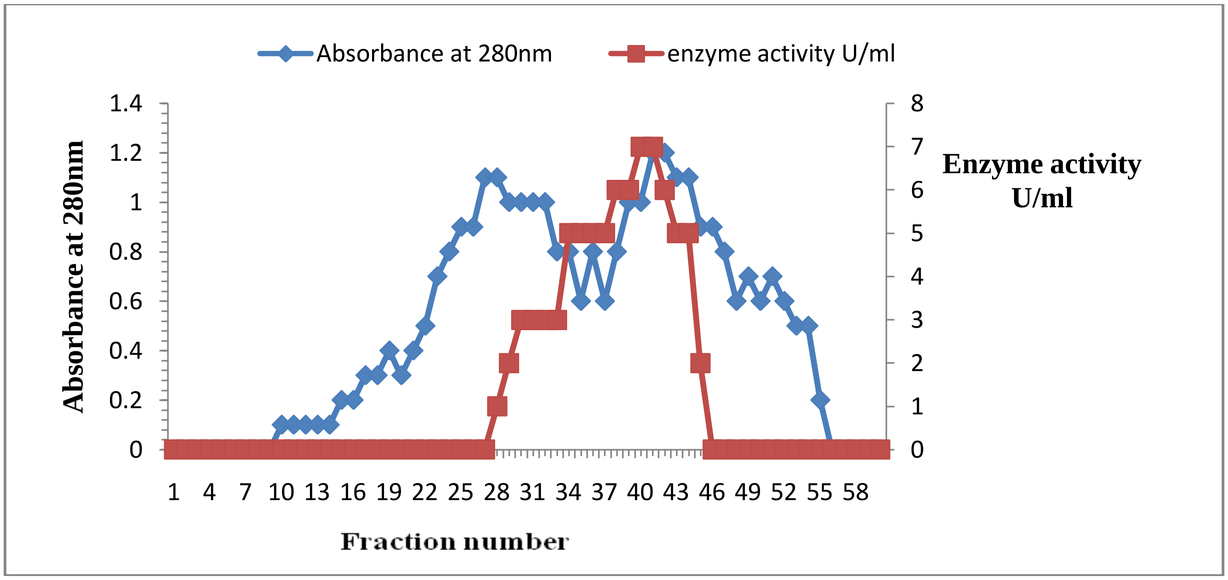

Fig.2 Chromatogram of the alkaline protease from *T.viride* N9 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Purification of alkaline protease from *T.viride* N9 using rice bran as substrate:

The alkaline protease present in crude sample was concentrated by ammonium sulfate (60%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 8.68-fold with a recovery of 11.5% and specific activity of 39.5 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 pooled 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 19.5 fold with a recovery of 5.1% and specific activity of56.8 U/mg of protein. Summary of purification steps was given in Table.3.

Table 3. Purification steps of alkaline protease from *T.viride* N9 using rice bran as substrate

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	33	161.7	4.9	1.0	100
Ammonium sulphate	14.9	135.8	9.12	2.21	45.1
Dialysis	3.8	150.1	39.5	8.68	11.5
Sephadex G-100	1.69	95.99	56.8	19.5	5.1

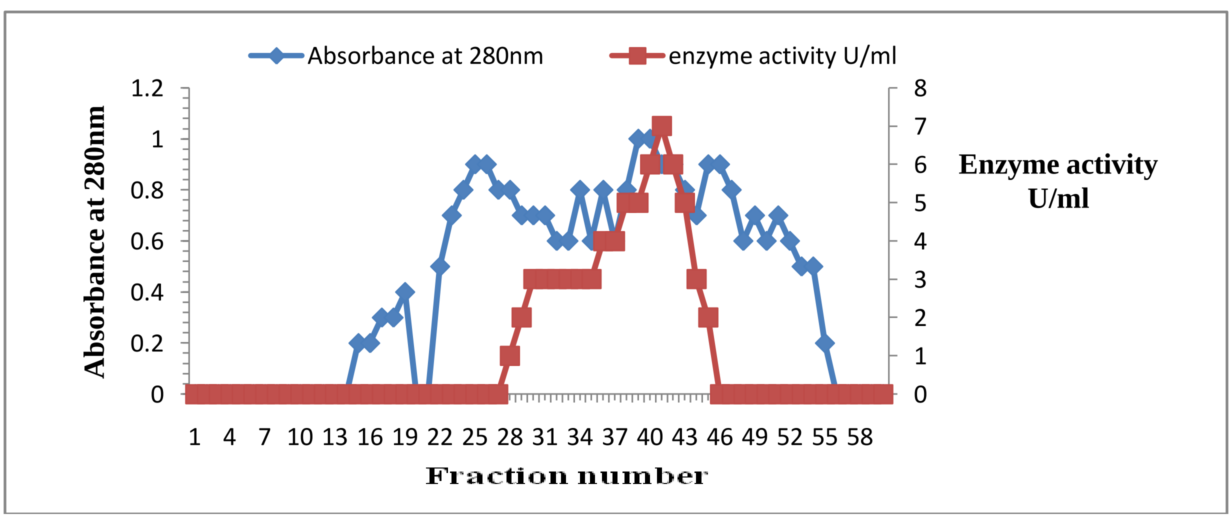

Fig.3 Chromatogram of the alkaline protease from *T.viride* N9 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Purification of alkaline protease from *T.viride* N9 using black gram husk as substrate:

The alkaline protease present in sample was concentrated by ammonium sulfate (60%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 7.78-fold with a recovery of 12.85% and specific activity of 35.1 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 pooled 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 21.5-fold with a recovery of 4.6% and specific activity of 56.1 U/mg of protein. Summary of purification steps was given in Table.4.

Table 4. Purification steps of alkaline protease from *T.viride* N9 using black gram husk as substrate:

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	31.9	142.24	4.46	1.0	100
Ammonium sulphate	16.4	141.69	8.65	1.94	51.4
Dialysis	4.1	143.8	35.1	7.78	12.85
Sephadex G-100	1.48	83.02	56.1	21.55	4.6

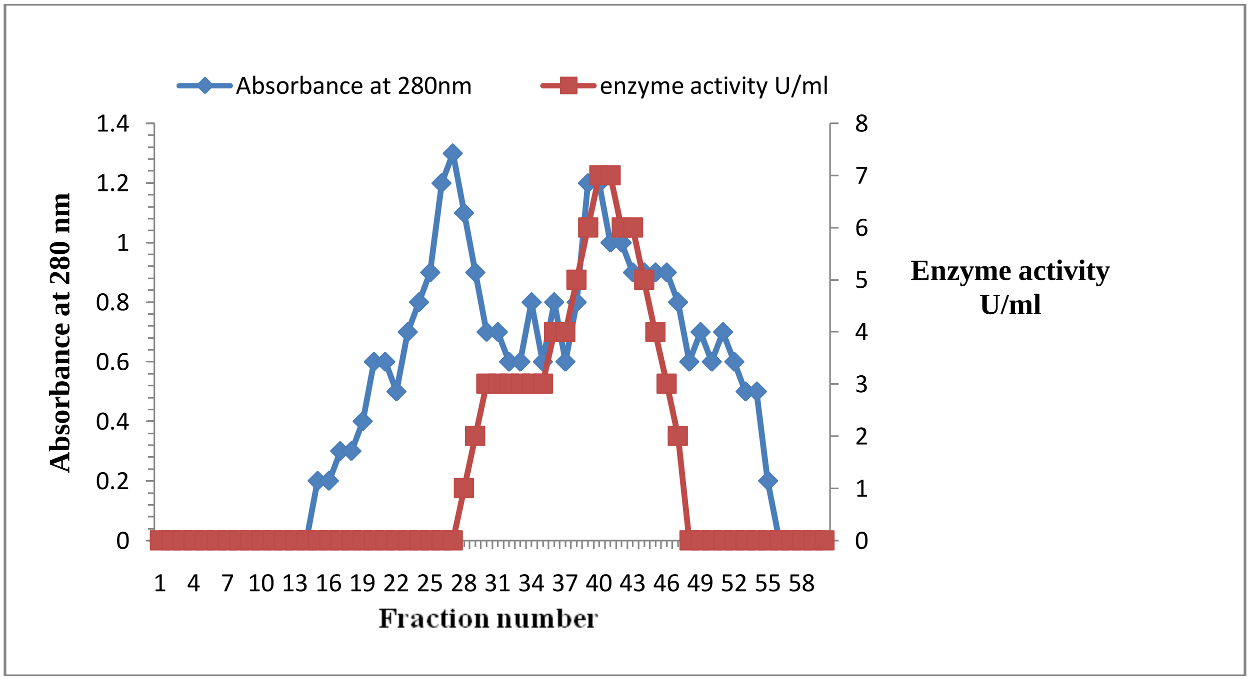

Fig.4 Chromatogram of the alkaline protease from *T.viride* N9 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Knudsen, G.R. and Bin, L. (1990) reported similar results from *Trichoderma rharzianum* using casein as substrate[12].

Table.5 Summary of purified alkaline protease from *T.viride* N9 using four different substrates:

Source	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (Fold)	Yield (%)
Wheat bran	1.9	113.24	59.6	18.94	5.27
Green gram husk	1.86	108.6	58.1	18.2	5.47
Rice bran	1.69	95.99	56.8	19.5	5.1
Black gram husk	1.48	83.02	56.1	21.55	4.6

Molecular weight of alkaline protease:

Purified alkaline protease from *Trichoderma viride* N9 obtained from all samples show a molecular weight of 23,000 daltons (Fig.5). Similar Kredics et al., (2005) reported the molecular weight of protease from *Trichoderma atroviride* as 24KDa[13]. Most of the protease from *Trichoderma* was reported as single band and have a molecular weight from 19 to 43 KDa [14].

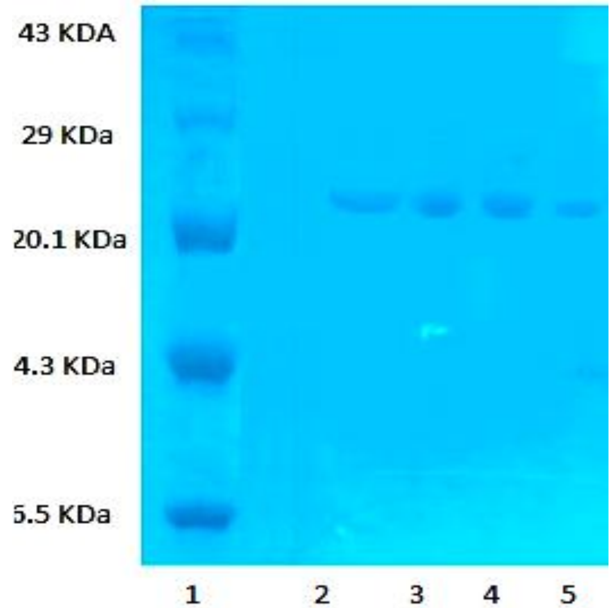

Fig.5. **SDS-PAGE of purified enzyme. Lane 1: Marker protein, Lane 2: Alkaline protease from green gram husk, Lane 3: Alkaline protease from black gram husk, Lane 4: Alkaline protease from wheat bran, Lane 5: Alkaline protease from rice bran.**

Characterization of purified alkaline protease:

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

Effect of pH on protease activity:

The enzyme was active at alkaline pH and the optimum pH for alkaline protease was 8.0 (Fig. 6). Further upon increase of pH, enzyme activity was decreased. Similar results were reported by Martin et al., (2008) from *Trichoderma viride*. Janice et al., (2002) reported similar results in *Trichoderma harzianum*.

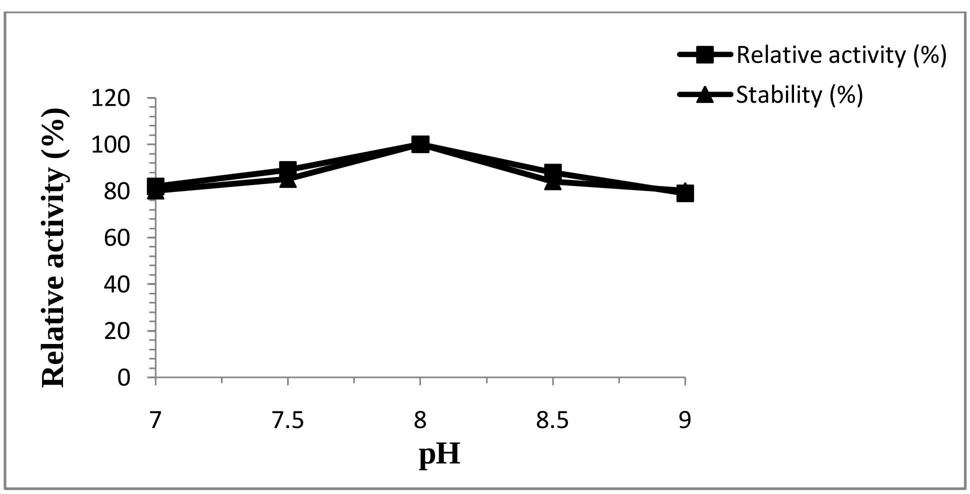

Fig. 6. Optimal pH (▪) and stability of pH (∆) of purified alkaline protease from *Trichoderma viride* N9.

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. Martin et al., (2008) reported the maximum protease activity at 37°C from *Trichoderma viride.* Janice *et al.*, (2002) reported similar results in *Trichoderma harzianum.*

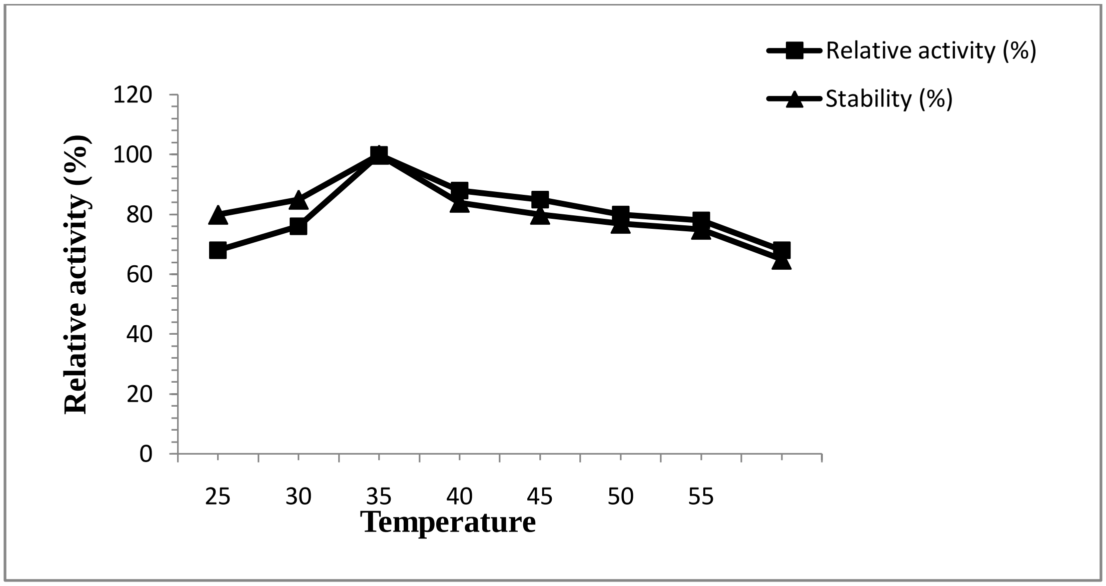

Fig.7 Optimal temperature (∆) and stability of temperature (▪) of purified alkaline protease from *Trichoderma viride.*

CONCLUSION

In the present study, the extracellular alkaline protease produced by *Trichoderma viride* N9 using four sources as substrate under solid state fermentation was purified, characterized. The protease enzyme was purified by ammonium sulphate precipitation, dialysis, and sephadex G-100 gel filtration from four different fermentation media. The alkaline protease from wheat bran substrate was produced in abundant and the enzyme was purified 18.94 fold and the apparent molecular weight of the enzyme was found to be 23 kDa by SDS-PAGE. All these data suggest that the selected strain of *Trichoderma viride* N9 can significantly produce protease enzyme from wheat bran substrate.

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