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RESEARCH ARTICLE

Production, purification and characterization of alkaline protease from *Aspergillus niger* MTCC281 by using agro industrial wastes under solid state Fermentation

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Abstract

A comparative study was carried out on the production of protease using wheat bran, rice bran, green gram husk and black gram husk as substrates in solid-state fermentation (SSF) by *Aspergillus niger* MTCC281. Among the all tested varieties, wheat bran showed the highest activity of 69.1 U/mg of protein, while black gram showed lowest activity of 59 U/mg of protein under solid state fermentation conditions. The alkaline protease from wheat bran substrate was produced was purified 16.28 fold and the apparent molecular weight of the enzyme was found to be 45 kDa by SDS-PAGE. The optimal pH and temperature of the enzyme were 8.0 and 40°C, respectively. The enzyme was more stable at the alkaline pH than at the acidic one and it retained 80% of the activity at 50^{o}C for 60 min. All these data suggest that the selected strain of *Aspergillus niger* MTCC281 can significantly produce protease enzyme from the wheat bran substrate.

INTRODUCTION

Proteases are one of the most important classes of enzymes and are expressed throughout the animal kingdom, plant and as well as microbes [1]. Due to the low production of proteases from plants and animals, they are mainly produced by microorganisms which involve fungi, bacteria and some other microorganisms [2]. The enzymes of fungi are largely alkaline proteases however; many fungi have also been reported to produce neutral and acid proteases [3].Alkaline proteases of microbial origin posses a considerable industrial potential due to wide applications in food, textile industries, medicinal formulations, detergents and processes like waste water treatment and silver recovery [4].This enzyme accounts for nearly 60% of the total worldwide enzyme sales [5;6]. Proteases produced from molds of the genera *Aspergillus, Penicillium and Rhizopus* are generally regarded as safe. Among the *Aspergillus* genus, *Aspergillus niger* is mainly concern for protease production because of its cosmopolitan, ubiquitous nature and non-fastidious nutritional requirement. The hyphal mode of growth and tolerance to low water activity and high osmotic conditions make *Aspergillus niger* suitable for bioconversion of solid substrates, providing an increased product concentration, lower costs of enzyme recovery, less amounts of liquid residues produced and lower energy requirements [7;8]. Hence it is imperative to study the production of protease by *Aspergillus niger* through optimization of various agro-industrial wastes like wheat bran, Rice bran, green gram husk and Bengal gram husk as substrates.

This Present study was taken up with the objective for the production, purification of protease enzyme from various agro-industrial wastes by using *Aspergillus niger* MTCC281 and screening for factors affecting the enzyme production such as incubation temperature and pH was studied and optimized.

MATERIALS AND METHODS:

Micro- organism and substrates

The *Aspergillus niger* MTCC281 strain was obtained from the Microbial Type Culture Collection & Gene Bank (MTCC), Chandigarh, India. The strain was maintained on potato dextrose agar slants at 4^{0}C and sub cultured every

month. Wheat bran, rice bran, green gram husk and black gram husk were collected from different local markets in Pulivendula, A.P, India. The substrates were sun dried, powdered and stored in moisture free bags.

Fermentation conditions and protease extraction:

Fermentation media were prepared according to Paranthaman et al., 2009[9]. Five grams of each substrate mentioned above were taken in 250 ml Erlenmeyer flask separately and moistened with 10 ml of salt solution (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 sulfate 0.1), sterilized at 121^0C for 15 min and inoculated with 1ml of fungal spore suspension (10^6 spores/ml) after cooling and incubated for 120 hrs at 30^0C. 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:

The alkaline protease activity was determined according to Niyonzima and More 2013, 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. The enzyme was precipitated from the clear supernatant (crude enzyme) by gradual addition of solid ammonium sulphate with gentle stirring to 70% saturation and precipitated protein was collected by centrifugation at 20,000 rpm for 30 min [10]. The pellet was dissolved in Tris-HCl buffer (25mM, pH 8.0), dialyzed against the same buffer overnight at 4^0C and concentrated [11]. The concentrated sample was applied to Sephadex G-100 gel filtration column (1.6 x 36 cm) and the column was equilibrated with the same buffer. The protease activity showing fractions was pooled out, combined and lyophilized to concentrate.

SDS- PAGE and molecular weight determination:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed under non reducing conditions [12]. Electrophoresis was performed at 50V in stacking gel (4%) and for resolving gel (12%) at 100V. The gels were stained with 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. The molecular weight of protease was determined with the help of protein markers.

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 (for pH 5-6 acetate buffer; pH 7 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 20- 80^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

An extracellular alkaline protease was purified from the culture filtrate of from *Aspergillus niger* MTCC281 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 *A.niger* MTCC281 using wheat bran as substrate:

The alkaline protease produced by *Aspergillus niger* MTCC281 grown in wheat bran substrate was concentrated by ammonium sulfate (70%) precipitation and purified consecutively by dialysis. The dialyzed sample was purified 7.3-fold with a recovery of 13.6% and specific activity of 49.15 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 16.28-fold with a recovery of 6.13% and specific activity of 69.1 U/mg of protein. Summary of purification steps was given in Table.1.

Table 1. Purification steps of alkaline protease from *A.niger* MTCC281 using wheat bran as substrate.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	42.35	306.61	7.24	1.0	100
Ammonium sulfate	21.82	288.02	13.2	1.9	51.5
Dialysis	5.8	285.0	49.15	7.3	13.6
Sephadex G-100	2.6	179.6	69.1	16.28	6.13

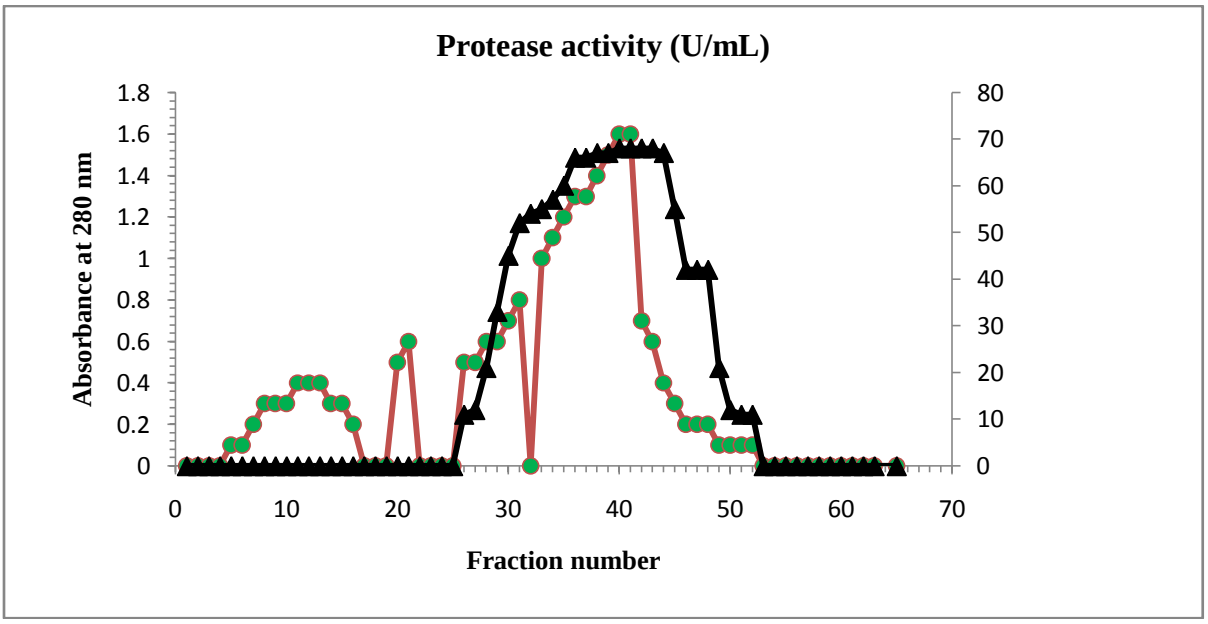

Fig. 1. Chromatogram of the alkaline protease from *A.niger* MTCC281 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Purification of alkaline protease from *A.niger* MTCC281 using green gram husk as substrate:
The alkaline protease produced by *Aspergillus niger* MTCC281 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 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 16.87-fold with a recovery of 5.92% and specific activity of 68 U/mg of protein. Summary of purification steps was given in Table.2

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Table.2 Purification steps of alkaline protease from *A.niger* MTCC281 using green gram husk as substrate.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	40.5	286.61	7.04	1.0	100
Ammonium sulfate	19.82	260.0	13.11	2.04	48.9
Dialysis	4.9	205.9	42.65	8.26	12.09
Sephadex G-100	2.4	163.2	68	16.87	5.92

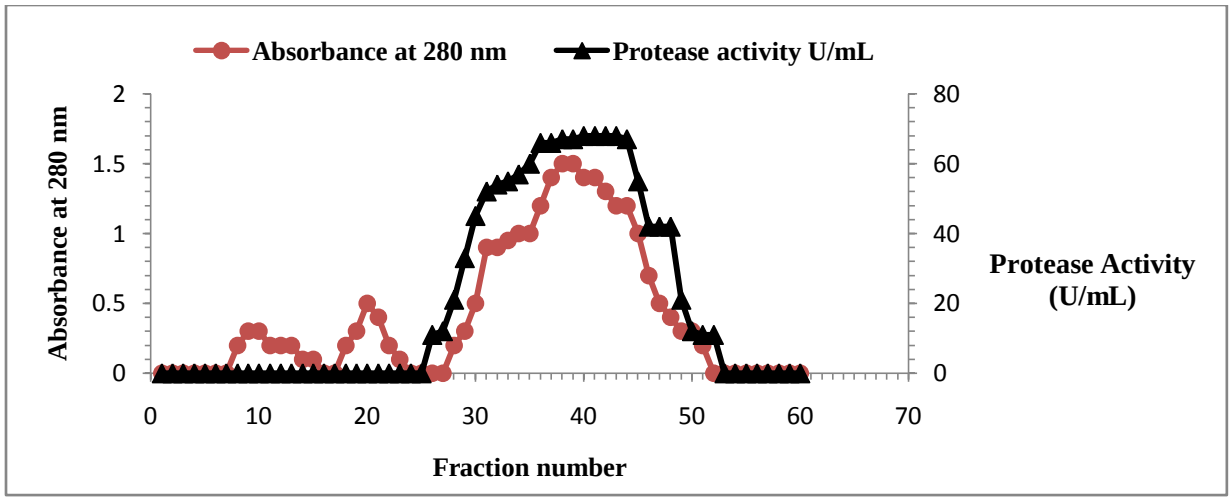

Fig. 2. Chromatogram of the alkaline protease from *A.niger* MTCC281 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Purification of alkaline protease from *A.niger* MTCC281 using rice bran as substrate:

The alkaline protease produced by *Aspergillus niger* MTCC281 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 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 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 *A. niger* MTCC281 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	29.82	256.0	8.58	2.69	37.04
Dialysis	6.9	209.8	30.40	11.6	8.57
Sephadex G-100	1.8	111.3	61.8	44.7	2.23

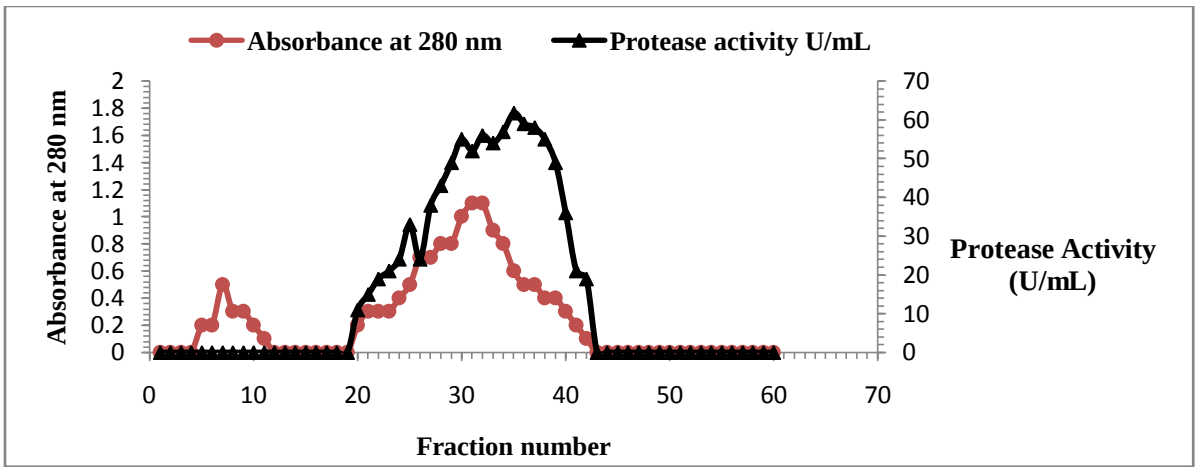

Fig. 3. Chromatogram of the alkaline protease from *A.niger* MTCC281 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Purification of alkaline protease from *A.niger* MTCC281 using black gram husk as substrate:

The alkaline protease produced by *Aspergillus niger* MTCC281 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 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 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.niger* MTCC281 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

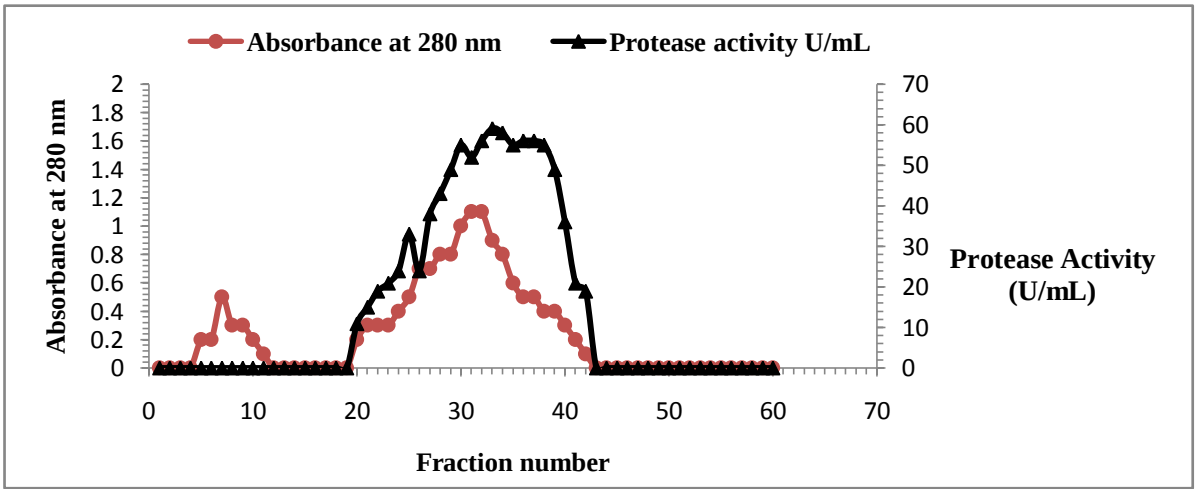

Fig. 4. Chromatogram of the alkaline protease from *A.niger* MTCC281 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 25mM Tris-HCl buffer (pH 8) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

Similarly, Sumantha et al., (2006) and Benazir et al., (2011) reported maximum protease activity from Aspergillus niger using wheat bran as substrate[12;13].

Table.5 Summary of purified alkaline protease from *Aspergillus niger* MTCC281 using four different substrates

Source	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (Fold)	Yield (%)
Wheat bran	2.6	179.6	69.1	16.28	6.13
Green gram Husk	2.4	163.2	68	16.87	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

Molecular weight of purified alkaline protease from four sources:

The purified alkaline protease from A.niger MTCC281 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 44KDa (Fig. 5). Most of the protease from *Aspergillus* was reported as single band and have a molecular weight from 40 to 130 KDa [14;15].

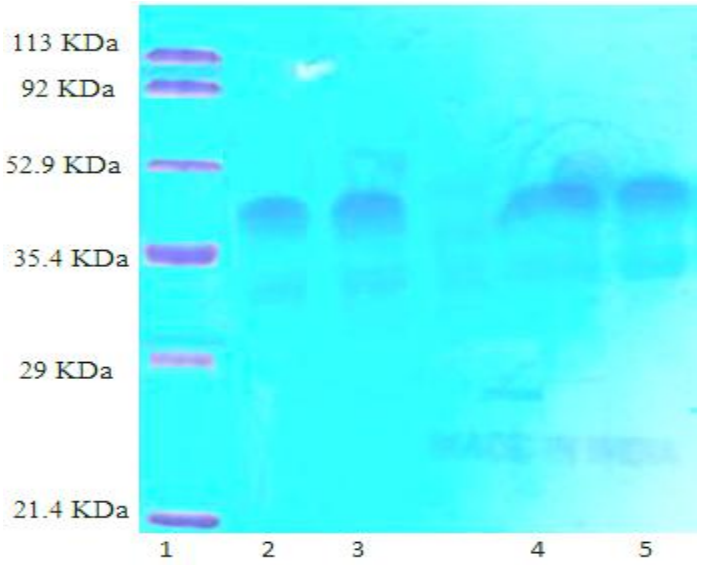

Fig. 5. SDS-PAGE of purified enzyme. Lane 1: Marker protein, Lane 2: Alkaline protease from green gramhusk, Lane 3: Alkaline protease from black gram husk, Lane 4: Alkaline protease from wheat bran, Lane 5: Alkaline protease from rice bran.

Characterization of purified alkaline protease:

Alkaline protease which was purified from wheat bran, shown maximum activity, was used to study the effects of pH and temperature.

Effect of pH on protease activity:

The optimum pH for alkaline protease was 8.0 (fig. 6) The enzyme activity was declined at pH higher than 8. 85% of maximal enzyme activity was observed at pH 9.0. The enzyme was most active between pH 8 and 9 and relatively stable at pH between 7 to 9. Similar results were reported by Kalpana devi et al., (2008) from *Aspergillus niger*[16]. Coral et al (2003) also reported similar results as pH 9 for *Aspergillus niger* Z1[17].

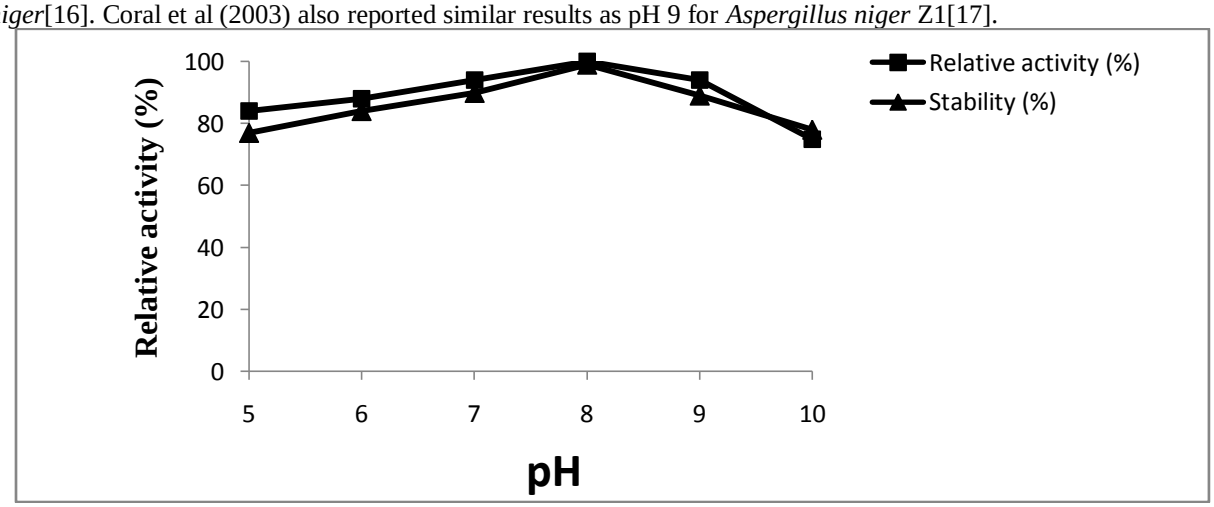

Fig. 6. Optimal pH (•) and stability of pH (■) of purified alkaline protease from *Aspergillus niger* MTCC281

Effect of temperature on protease activity:

The protease enzyme was most active at 40^0C (Fig.7), similar to most of the other fungal protease. Above 40^0C, the activity decrease and completely lost at 70^0C. When the enzyme was kept at various temperatures, it was significantly inactivated above 50^0C and completely lost at 80^0C.

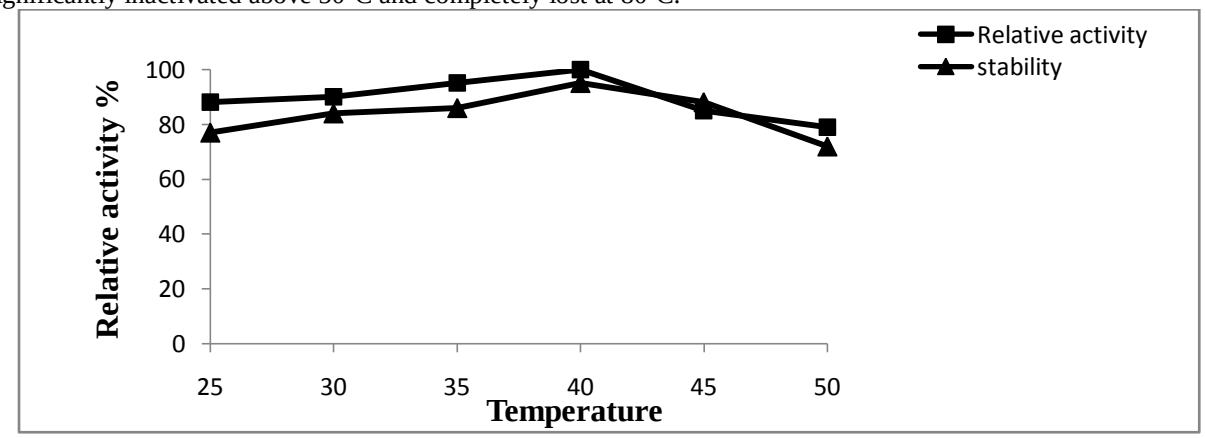

Fig. 7. Optimal temperature (•) and stability of temperature (■) of purified alkaline protease from *Aspergillus niger* MTCC281

CONCLUSION

In the present study, the extracellular alkaline protease produced by *Aspergillus niger* MTCC281 using four sources as a substrate under solid state fermentation was purified, characterized. The enzyme was purified by ammonium sulphate precipitation, dialysis, and sephadex G-100 gel filtration. The alkaline protease from wheat bran substrate was produced in abundance and the enzyme was purified 16.28 fold and the apparent molecular weight of the enzyme was found to be 45 kDa by SDS-PAGE. The optimal pH and temperature of the enzyme were 8.0 and 40°C, respectively. The enzyme was more stable at the alkaline pH than at the acidic one and it retained 80% of the activity at 50^oC for 60 min. All these data suggest that the selected strain of *Aspergillus niger* MTCC281 can significantly produce protease enzyme from wheat bran substrate.

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