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Production, Purification and Characterization of Protease by *Aspergillus flavus* under Solid State Fermentation

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Abstract

Protease production under solid state fermentation (SSF) was investigated using isolated *Aspergillus flavus*. Different agro-industrial waste products were evaluated to check the possibility of potential utilization of substrates in SSF for protease production by *Aspergillus flavus* using wheat bran as a substrate. The results showed that the optimum conditions for maximum protease production were found to be 7^{th} day of incubation at pH 5.0, temperature 30^{o}C; inoculum size 3%; substrate concentration 3% and 3% KNO_3 as nitrogen source. The purified enzyme produced 5.8 fold with recovery of 3.2% by DEAE-column chromatography and the molecular weight was estimated to be 46kDa by SDS-PAGE. It has a V_{max} value of 60.0 U/mg and K_m value of 0.6 mg/ml at pH of 7. The enzyme activity was found to be stable at 50^0 C and it was stimulated by metal ions like Cu^{2+} and Zn^{2+} and inhibited by Ca^{2+} and Mg $^{2+}$.

Keywords: protease, *Aspergillus flavus*, purification, SDS-PAGE, wheat bran, metal ions, precipitation.

1. Introduction

Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are (physiologically) necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale (Godfrey and West, 1996; Chouyyok *et al.*, 2005). They are generally used in detergents (Barindra *et al.*, 2006), food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Rao *et al.*, 1998; Paranthaman *et al.*, 2009). They also have medical and pharmaceutical applications.

Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins (Raju *et al.*, 1994; Haq *et al.*, 2006). The molecular weight of proteases ranges from 18 – 90 kDa (Sidney and Lester, 1972). These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi. Microbial proteases are predominantly extracellular and can be secreted in the fermentation medium.

Solid state fermentation (SSF) was chosen for the present research because it has been reported to be of more grated productivity than that of submerged fermentation (Ghildyal *et al.*, 1985; Hesseltine, 1972). Economically, SSF offers many advantages including superior volumetric productivity, use of simpler machinery, use of inexpensive substrates, simpler downstream processing, and lower energy requirements when compared with submerged fermentation (Paranthaman *et al.*, 2009). Fungi elaborate a wide variety of proteolytic enzymes than bacteria. The filamentous fungi have a potential to grow under varying environmental conditions such as time course, pH and temperature, utilizing a wide variety of substrates as nutrients (Haq *et al.*, 2006). Several species of strains including fungi (*Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratinophilum, Fusarium graminarum, Penicillium griseofulvin, Scedosporium apiosermum*) and bacteria (*Bacillus licheniformis, Bacillus firmus, Bacillus alcalophilus, Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus subtilis, Bacillus thuringiensis*) are reported to produce proteases (Ellaiah *et al.*, 2002).

The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of a microbial population. The most important among these are the medium, incubation temperature and pH. The pH of the fermentation medium is reported to have substantial effect on the production of proteases (Al-Shehri, 2004). It can be affect growth of the microorganisms either indirectly by affecting the availability of nutrients or directly by action on the cell surfaces. Another important environmental factor is the

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incubation temperature, which is important to the production of proteases by microorganisms. Higher temperature is found to have some adverse effects on metabolic activities of microorganisms producing proteolytic enzymes (Tunga, 1995). However, some microorganisms produce heat stable proteases which are active at higher temperatures. The thermal stability of the enzymes may be due to the presence of some metal ions or adaptability to carry out their biological activity at higher temperature (Al-Shehri, 2004; Haq *et al.*, 2006).

In the production of protease, it has been shown to be inducible and was affected by the nature of the substrate used in fermentation. Therefore, the choice of an appropriate inducing substrate is of great importance. Different carbon sources such as wheat bran, rice straw, rice bran, cotton and bagasse have been studied for the induction and biosynthesis of protease. However, wheat bran is a superior carbon source for the production of protease by *Aspergillus flavus*. So the further studies were carried out by using wheat bran as carbon source.

The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling (Singh *et al.*, 2009). Major impediments to the exploitation of commercial enzymes are their yield, stability, specificity and the cost of production. New enzymes for use in commercial applications with desirable biochemical and physiochemical characteristics and low production cost have been focus of much research (Kabli, 2007).

The purpose of this study was to isolate, produce and purify protease from *Aspergillus flavus*, and to characterize some properties of the isolated enzymes using wheat bran as a substrate under SSF.

2. Materials and Method

2.1. Cellulosic material

In our preliminary studies, various agro wastes were used as a carbon source, and, hence, it could reduce the cost of enzyme production, which is collected in dried form from cattle shop, Coimbatore. Substrates, like wheat bran, cotton seed, rice bran, rice straw and sugarcane bagasse, were screened for enzyme production, in which wheat bran showed higher protease production, so it was used for the further studies.

2.2. Organism and inoculum preparation

Fungal strains were isolated from soil of sugarcane field Coimbatore, India by serial dilution plate method (Waksman, 1922). Fungus were isolated from 10^{-3} - 10^{-4} dilutions by plating into Potato Dextrose Agar (PDA) medium. Isolated fungal cultures were screened for protease enzyme production. The organisms were identified using lacto phenol cotton blue mounting method (Konemann *et al.*, 1997). The isolated culture (*Aspergillus flavus*) was purified by routine sub-culturing and stored at 4°C for further use.

2.3. Fermentation condition

SSF was carried out in 250ml conical flask contains 10g of substrate with 10 ml of salt solution (g/l). KNO_3 2.0, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 1.0, $ZnSO_4.7H_2O$ 0.437, $FeSO_4.7H_2O$ 1.116, $MnSO_4.7H_2O$ 0.203, pH 7.0 and it was autoclaved at 121°C for 30 min. After sterilization, the flasks were inoculated with 1.0ml of spore solution (10^6 spores/ ml) and incubated at 30°C for eight days in an incubator shaker at 125rpm. At the end of fermentation, cultures were extracted with 100ml of distilled water by shaking for 2hr. The filtrate obtained was centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant used as crude enzyme extract.

2.4. Protease assay

Protease activity was determined according to the modified Anson's method. 1.0 ml of the culture broth was taken in a 100 ml flask and 1.0 ml of pH 7.0 phosphate buffer added to it. One ml of the substrate (2% Hammersten's casein pH 7.0) was added to the buffer-enzyme solution and incubated at 37°C for 10 minutes in a water bath. At the end of 10 minutes, 10.0 ml of 5N TCA (trichloroacetic acid) was added to stop the reaction. The precipitated casein was then filtered off and 5.0 ml of the filtrate were taken in a test tube. To this 10.0 ml of 0.5N NaOH solution and then 3.0 ml of the folin ciocalteu reagent (one ml diluted with 2 ml of distilled water) were added. Final readings were taken in a spectrophotometer at 750 nm. Blanks of the samples were prepared by adding the TCA before the addition of substrate. The effect of various factors like noculums size, carbon source, nitrogen sources, pH and temperature on the production of protease was studied.

2.5. Purification and characterization of Protease

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 50mM phosphate buffer (pH 7.0) for 24 hours at 40°C. The filtrate was loaded onto a DEAE-Cellulose chromatographic column equilibrated with phosphate buffer, 50mM, pH 7.0.The enzyme was eluted with a linear salt concentration gradient (Na Cl, 0-0.4 M) in the same buffer and 3.0 ml fractions were collected at a flow rate of 20 ml per hour.

SDS-PAGE electrophoresis was carried out and molecular weight was determined. The protein content was estimated by the method of Lowry *et al.* (1951). The kinetic parameter of the purified protease enzyme was determined and the optimum pH 3.0 -8.0 [The pH was adjusted using, the following buffers: 50 mM sodium citrate (pH 3.0-6.0) and 50 mM sodium phosphate (pH 7.0 & 8.0)] and temperature (30-70°C) on the activity of the enzyme was also assayed. All experiments were conducted in triplicates and their mean values represented.

3. Results

Enzyme production by micro organisms is greatly influenced by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, incubation time and inoculum density. It is important to produce the enzyme in inexpensive and optimized media on a large scale for the process to be commercially viable; hence the studies on the influence of various physico-chemical parameters such as incubation periods, inoculum size, temperature, pH, carbon, and nitrogen

sources. Agricultural byproducts rich in cellulosic biomass can be exploited as cheap raw material for the industrially important enzymes and chemicals (Bigelow and Wyman, 2004). The fermentation medium was inoculated with the fungal strain and incubated for various time intervals (1-8 days). The enzyme production was gradually increased with the passage of time and highest enzyme activity (49.3 U mL^{-1}) was obtained on 7th day of incubation (Fig.1). It was also observed that prolonged incubation decreased the enzyme activity. However the growth of the microorganism was not significantly affected.

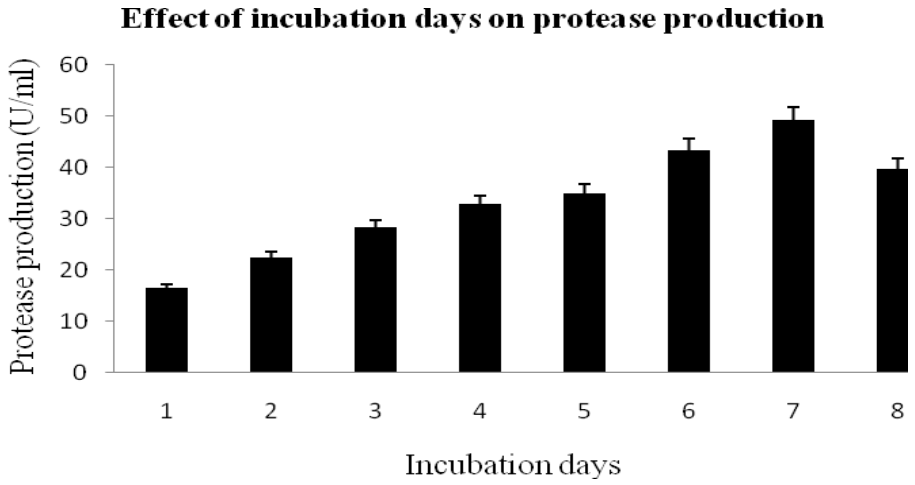


Figure1.Results are mean of three independent determinations. Bars correspond to standard deviation.

Productivity of the enzyme by culture is greatly dependant on pH of the fermentation medium. Therefore, the effect of pH (3.0 - 8.0) was studied for the production of protease by *Aspergillus flavus*. There was a gradual increase in protease synthesis from pH 3.0 to 5.0, and a maximum production of enzyme was observed at pH 4.0 i.e. 48.6 U mL^{-1} (Fig. 2).

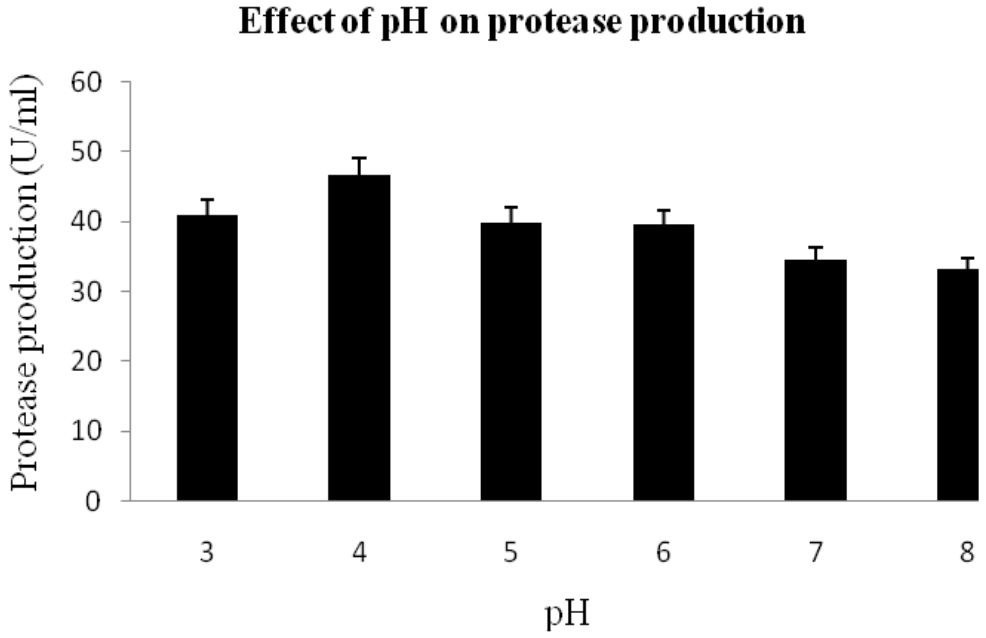


Figure 2. Results are mean of three independent determinations. Bars correspond to standard deviation.

The enzyme production by *Aspergillus flavus* at 20 - 70°C temperature range revealed that there was a sudden increase in protease production when the incubation temperature was increased from 20°C to 30°C. The enzyme production was slightly decreased up to 40°C. So the optimum incubation temperature for the production of protease was found as 30°C (Fig. 3).

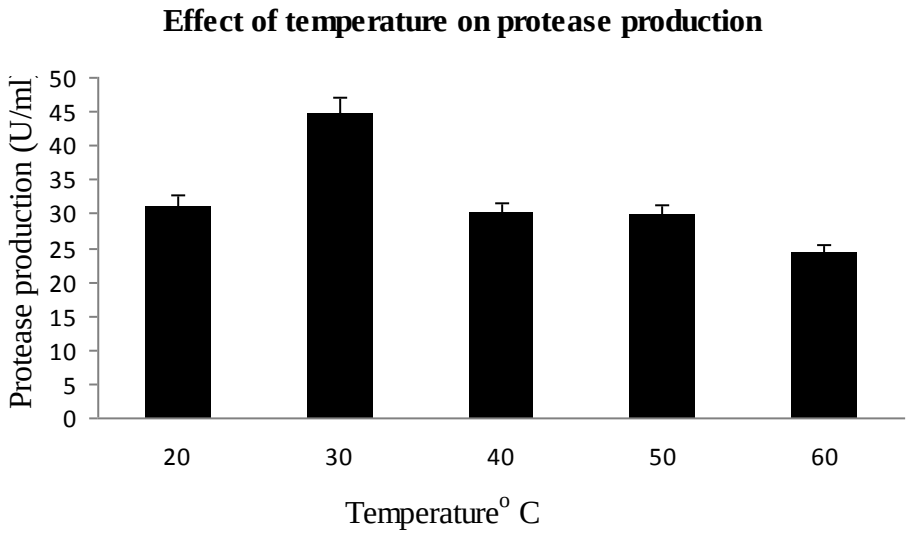


Figure 3. Results are mean of three independent determinations. Bars correspond to standard deviation.

Size of inoculum is an important biological factor in the production of the enzyme. Maximum enzyme production (Fig. 4) was obtained when SSF medium was inoculated with 3.0 ml of inoculum.

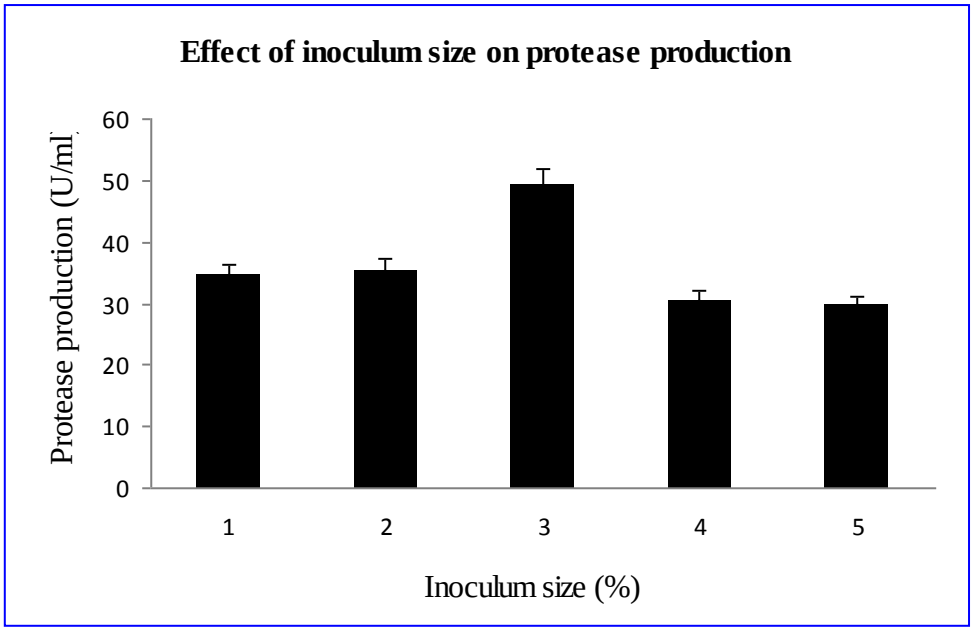


Figure 4. Results are mean of three independent determinations. Bars correspond to standard deviation.

Different agricultural byproducts such as wheat bran, rice bran, rice straw, cotton seed and sugarcane bagasse were tested for the production of enzyme (Fig. 5). Of all the substrates tested, wheat bran was found to be the best substrate for the production of protease. The other substrates gave comparatively less production of protease.

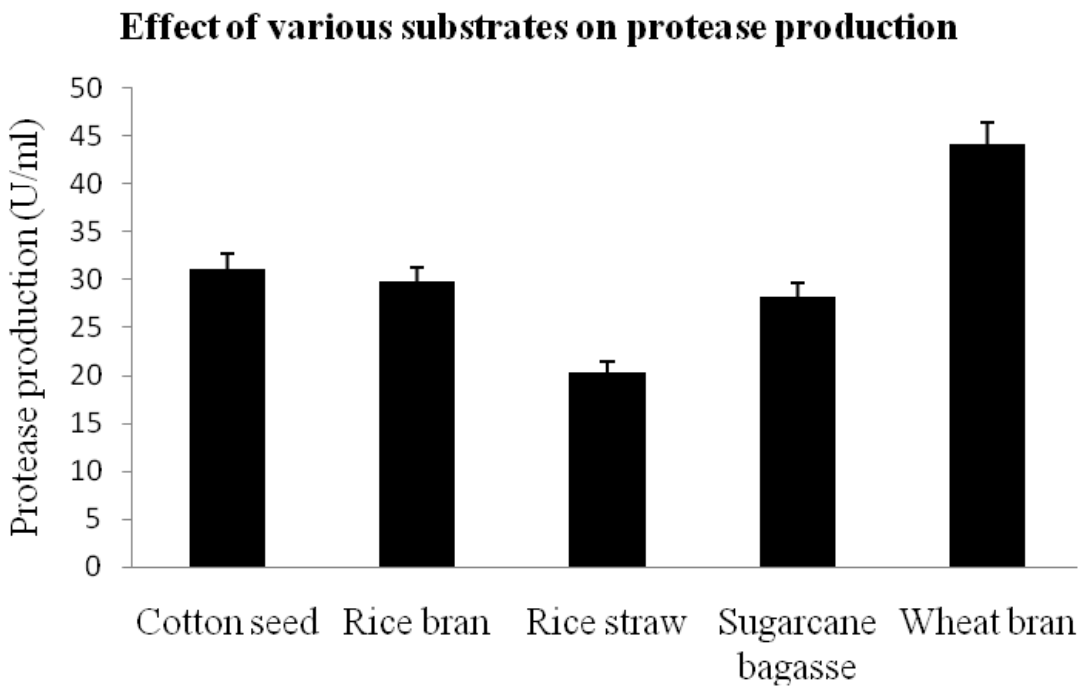


Figure 5. Results are mean of three independent determinations. Bars correspond to standard deviation.

Protease production in fermentation medium was found to be maximal when 3.0% of wheat bran was used (Fig. 6). A further increase in the amount of wheat bran resulted in a decreased production of the enzyme.

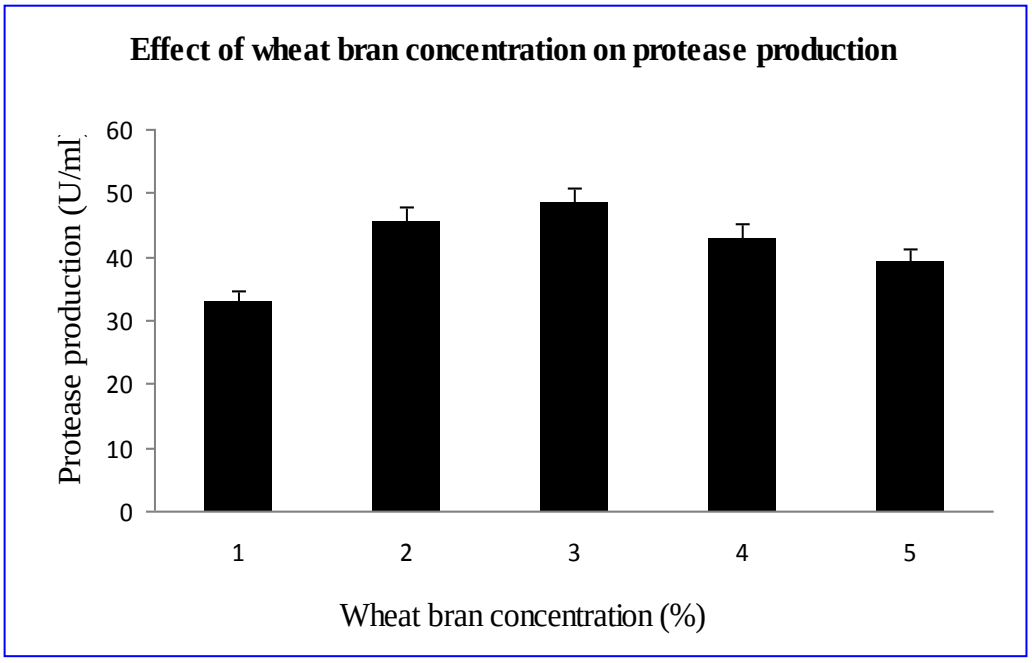


Figure 6. Results are mean of three independent determinations. Bars correspond to standard deviation.

The nitrogen sources beef extract, KNO_3, peptone, yeast extract and urea were screened for synthesis of protease (Fig 7). In that, KNO_3 (at 3% concentration) was found to be the most suitable nitrogen sources for protease production (Fig. 8).

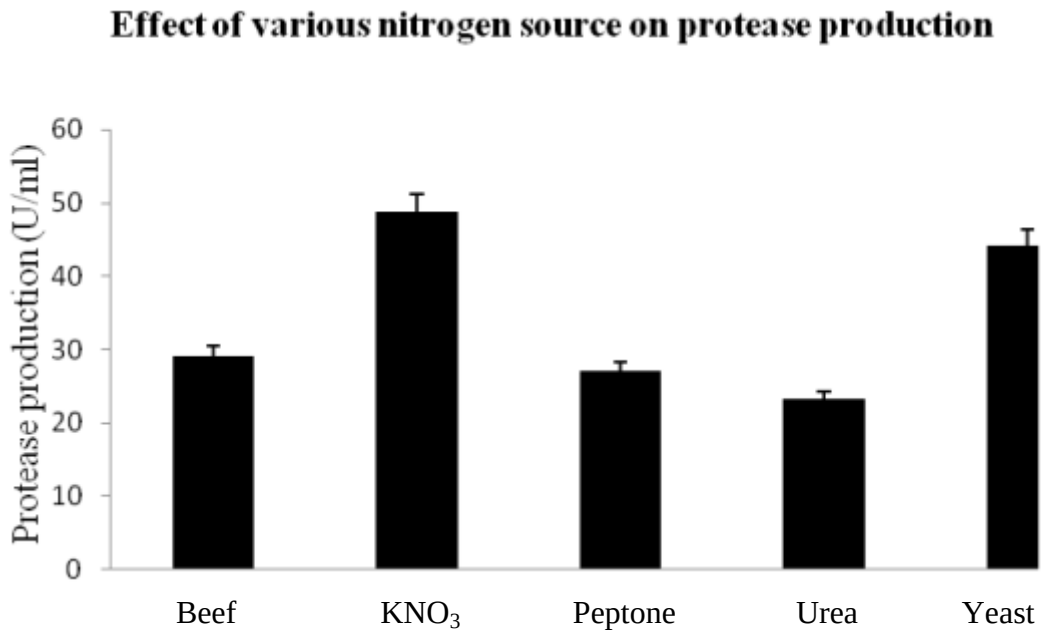


Figure 7. Results are mean of three independent determinations. Bars correspond to standard deviation.

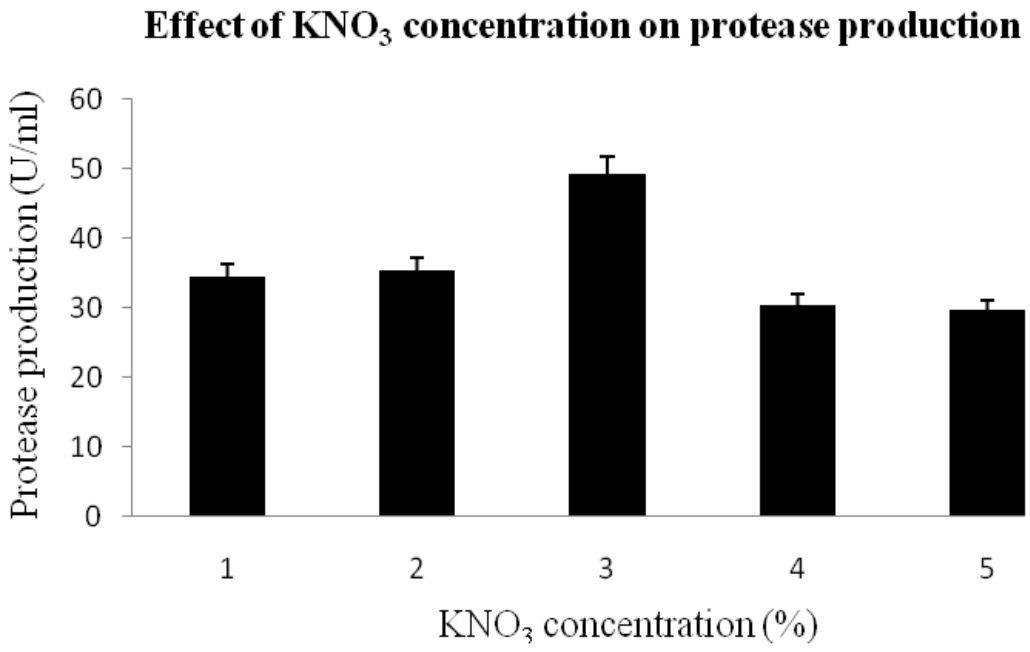


Figure 8. Results are mean of three independent determinations. Bars correspond to standard deviation.

Purification steps for protease production from *A.flavus* are given in Table 1 and Figure 9. The purification of protease resulted in 2 fold purification with 66% of recovery by ammonium sulphate precipitation. The purification of crude enzyme through DEAE cellulose column chromatography gave 5.8 fold increases in purity with 3.2% recovery of protease from *A. flavus*.

Table 1. Purification and recovery of protease from A. *flavus*

Steps	Protease production (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	8670	295.0	29.3	1	100
70% Ammonium sulphate precipitation	5750	124.0	46.3	1.58	66.32
Dialysis	323	1.9	104.17	2.53	3.72
DEAE Cellulose Column Chromatography	284	0.37	170.0	5.8	3.2

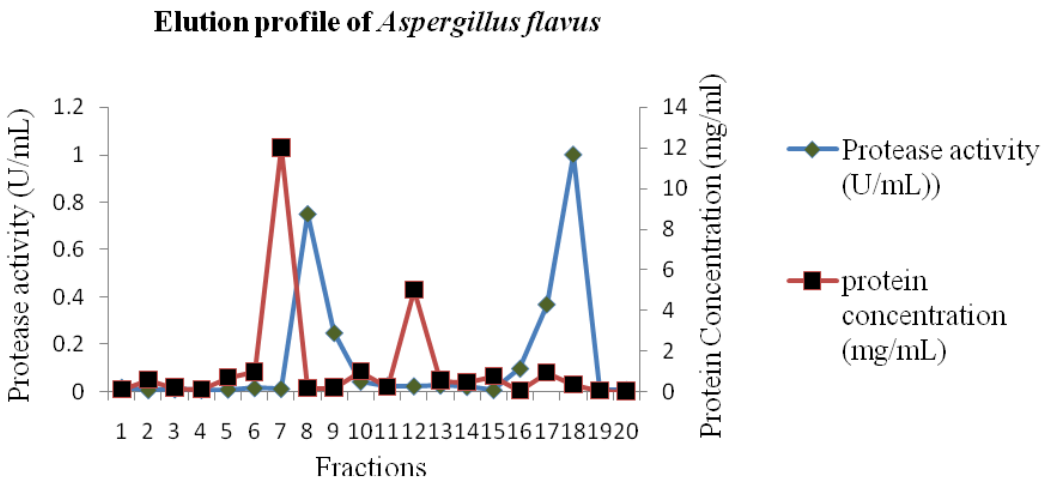


Figure 9. Results are mean of three independent determinations.

Using SDS-PAGE, the partially purified enzyme from *A.flavus* showed a single band (Fig. 10), to confirm it is an enzyme protein band, the protease activity of purified enzyme was also observed and apparent molecular weight of the purified protease was 46KDa.

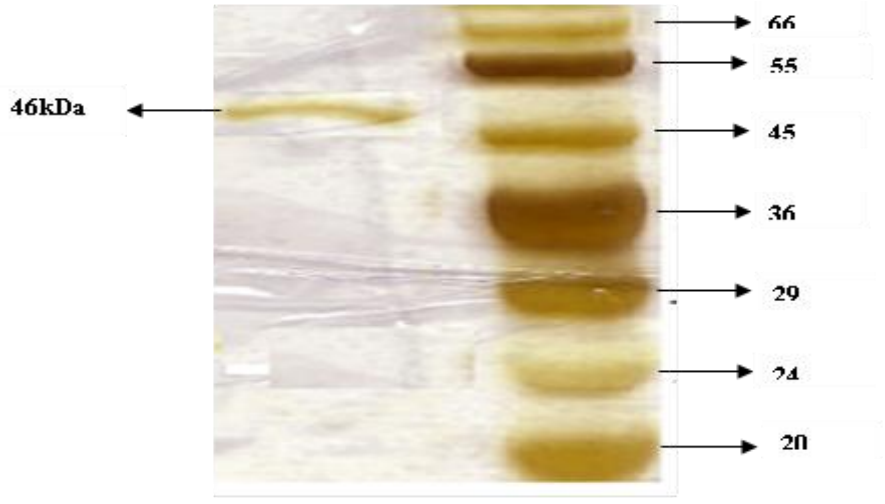


Figure 10. SDS-PAGE analysis of protease purified from *Aspergillus flavus*.

SDS-PAGE analysis of *Aspergillus flavus* strain protease. M-indicates molecular weight markers and P-shows purified protease band of molecular weight approximately 46 kDa.

Thermo stability and stability at wide range of pH are desirable properties of any enzyme for industrial applications. In our case the optimum temperature of enzyme activity was 50°C, while the optimum pH for its activity was recorded as pH 7.0 (Fig. 11 & 12).

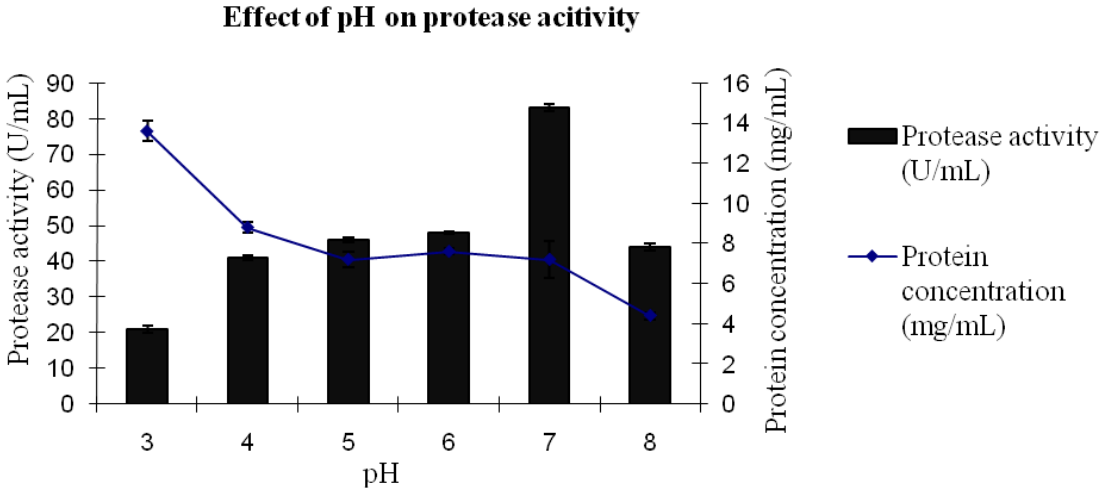


Figure 11. Results are mean of three independent determinations. Bars correspond to standard deviation.

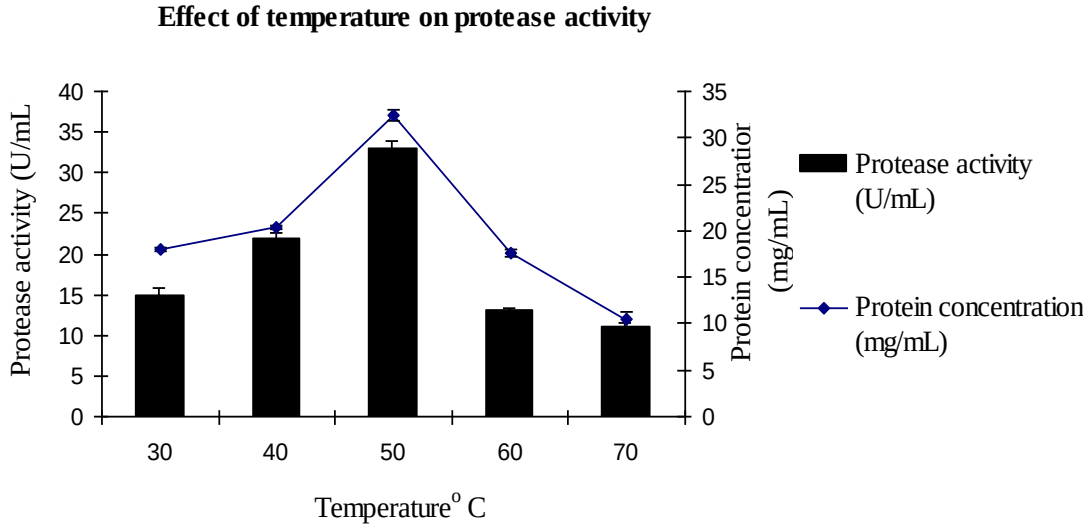
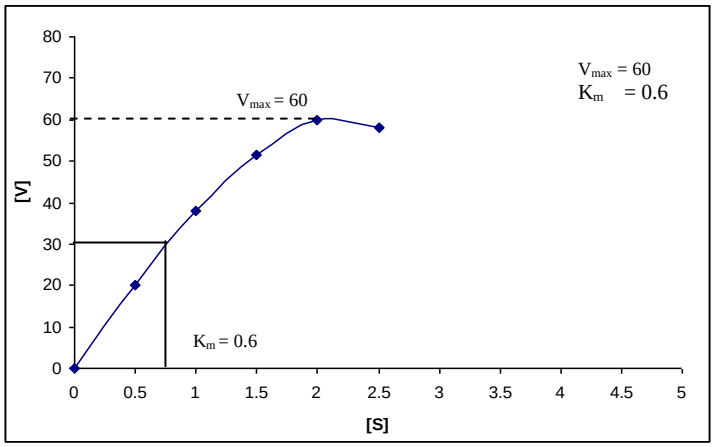


Figure 12. Results are mean of three independent determinations. Bars correspond to standard deviation.

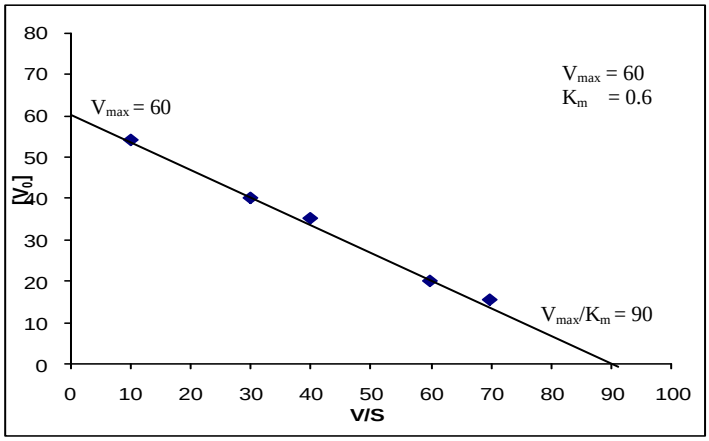
In order to study the effect of substrate concentration various substrate concentrations ranging from 0.5-2.5 % were used. Reaction rate verses substrate concentration curve was plotted to determine whether the enzyme obeys Michaelis-Menten kinetics, and constants were determined from a Line Weaver–Burk plot. The results are presented in Fig. 13.

Figure 13 **Enzyme kinetics of *A. flavus***

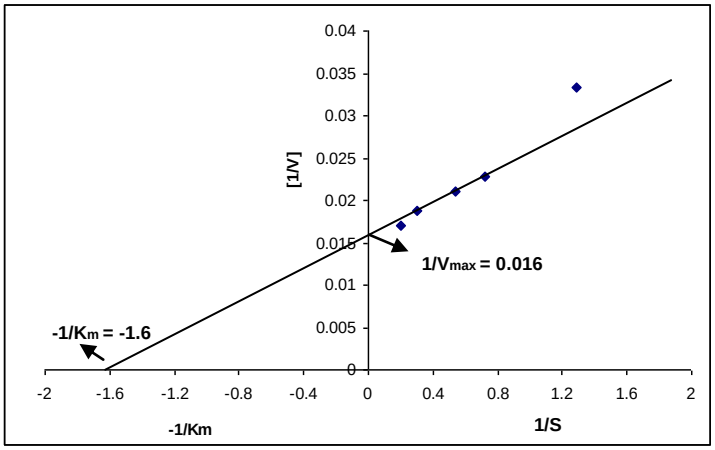
Michaelis-Menten equation



Lineweaver Burk plot



Eadie-Hofstee plot



From Fig.13, it can be seen the V_{max} and K_m values were determined against wheat bran as substrate. *A. flavus* had a higher V_{max} of 60.0 U/mg and K_m of 0.6 mg/ml.

The effects of various metal ions (Zn^{2+}, Mg^{2+}, Ca^{2+}, Na^{+} and Cu^{2+}) on activity of the protease from *Aspergillus flavus* is shown in Fig. 14. It can be shown that the metal ions Zn^{2+} and CU^{2+}supported the maximum enzyme activity whereas Na^{2+} and Ca^{2+} drastically inhibited the protease activity particularly Mg^{2+} was found to be the potent inhibitor of protease.

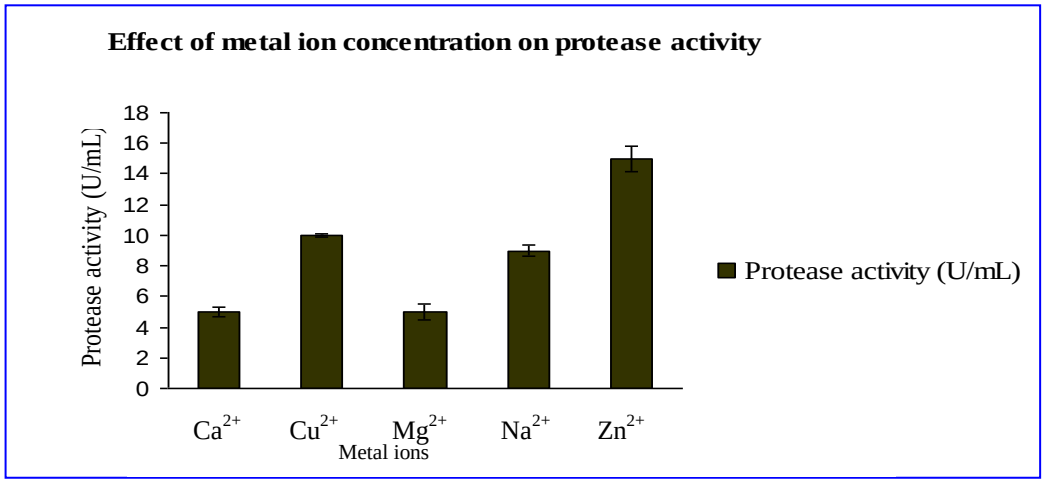


Figure 14. Results are mean of three independent determinations.

4. DISCUSSION

Microbial proteases have a number of commercial applications in industries like food, leather, meat processing and cheese making. A major commercial use is the addition of microbial proteases to domestic detergents for the digestion of pertinacious stains of fabrics (Sharma *et al.*, 1980). It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions. So, it becomes necessary to understand the nature of proteases and their catalytic potentiality under different conditions.

Time course for the production of protease by *A.flavus* was studied at 7th day. These results are supported by Johnvesly *et al.* (2002) and Impoolsup *et al.* (1981) who reported the maximum protease enzyme production, occurred during 7th day of incubation by using *A. flavus.* The incubation period is directly related to production of enzymes and other metabolites to a certain extent. After that, the enzyme production and the growth of the microorganism decreases; this can be attributed to the reduced availability of nutrients and the production of toxic metabolites (Romero *et al.*, 1998).

Protease production by microbial strains strongly depends on the extra-cellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production (Elliah *et al.*, 2002). *Aspergillus flavus* showed maximal protease production at pH - 4 (Fig. 2). Identical observations were earlier recorded in *A. flavus, A. oryzae* and *A. candidus* at pH 4.0 (Nasuno and Onara, 1972; Dworschack *et al.*, 1952).

The higher enzyme production was found to be at 30°C. Earlier studies report that different species of *Penicillium* including *P. citrinum, P. perpurogerum* and *P. funculosum* gave highest yield of protease when incubated at 30°C (Sharma *et al.*, 1980). Haq *et al.* (2004) have also reported that maximum production of protease by *P. griseoroseum* was obtained at an incubation temperature of 30°C and the enzyme production was reduced when the incubation temperature was increased above 30°C. Fungal proteases are usually thermolabile and show reduced activities at high temperatures (Sharma *et al.*, 1980). Higher temperature is found to have some adverse effects on metabolic activities of microorganism (Tunga, 1995) and cause inhibition of the growth of the fungus. The enzyme is denatured by losing its catalytic properties at high temperature due to stretching and breaking of weak hydrogen bonds within enzyme structure (Conn *et al.*, 1987)

At lower inoculums levels, the yield was very low. The decrease seen with large inoculums size could be due to the shortage of the nutrients available for the large biomass and faster growth of the culture (Hesseltine *et al.*, 1976). In our study, the maximum protease synthesis was noticed with 3% inoculum size whereas at higher concentration there was a decrease, it might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also enzyme release. From the survey of literature it can be seen that the 3% of inoculum size gave maximum production reported by Haq *et al.* (2003) and Kalisz (1988).

There are general reports showing that different carbon sources have different influences on extra cellular enzyme production by different strains. Among the various substrates (cotton waste, sugarcane bagasse, rice bran, rice straw and wheat bran) tested wheat bran was found to be the most effective substrate for the production of protease with the concentration of 3% (Fig. 6). Further increase in this carbon source adversely affected protease production in this *A. flavus* under SSF environment. These results were in accordance with reported protease production in presence of different substrates (Elliah *et al.*, 2002); and different carbon sources have different influences on enzyme production by different strains (Wang & Lee, 1996; Nehra *et al.*, 2002). It might be due to the fact that increase level of substrates decreases the aeration and porosity of the medium, which were very essential for the proper growth of the organism.

In the present study the various nitrogen sources like beef extract, KNO_3, peptone, yeast extract and urea were also studied. In that, KNO_3 showed the maximum protease production with the concentration of 1%. Certain nitrogenous salts tend to decrease the pH of the culture medium and had the adverse effect on enzyme production although they supported the growth of the organism (Wang *et al.*, 1974).

A summary of purification steps for protease from *A. flavus* is given in Table 9. The purification of protease resulted in 2 fold purification with 66% of recovery by ammonium sulphate precipitation. The purification of crude enzyme through DEAE cellulose column chromatography gave 5.8 folds increase in purity with 3.2% recovery of protease from *A. flavus*. The similar observation was reported by Ogundero and Osunlaja (1986) for *A. clavatus.*

Fractions from DEAE-Cellulose column which showed the highest activity were pooled and subjected to SDS-PAGE for determination of molecular weight of the protein. Purified enzyme preparation showed only one band corresponding to molecular weight of approximately 46 kDa (Fig. 10). Our results are more or less similar to that of Akel *et al.* (2009) who reported that the purified protease enzyme revealed a molecular mass of 49 kDa.

The maximum enzyme activity was found to be pH 7.0. Similar results were obtained for the optimum pH for enzymatic activity of other *Bacillus* species: pH 7.5 for *Bacillus subtilis* ITBCCB 148 (Yandri *et al.*, 2008), *Bacillus* sp. HS08 (Huang *et al.*, 2006) and *Bacillus* sp. S17110 (Jung *et al.*, 2007); pH 8.0 for *Bacillus cereus* KCTC 3674 (Kim *et al.*, 2001), Thermophilic *Bacillus* SMIA2 (Nascimento and Martins, 2004) and *B. cereus* BG1 (Ghorbel-Frikha *et al.*, 2005).

The maximum enzyme activity was found to be 50°C. This was supported by Li *et al.* (1997) who reported that alkaline protease isolated from *Thermomyces lanuginose* P134 had a broad temperature optimum of 50°C. Samal *et al.* (1991) also reported an alkaline protease from *Tritirachium albumlimber* to be quite thermostable even up to 50°C. The protease activity was accelerated by Zn^{2+} and it was inhibited by Mg^{2+} and Ca^{2+}. In contrast, Nehra *et al.* (2004) reported that Mg^{2+} was found to be an activator of the alkaline protease enzyme produced by *Aspergillus* sp. suggesting these metal ions had a capability to protect enzyme against denaturation.

V_{max} and K_m values for protease enzyme from *Aspergillus flavus* were determined from Line Weaver and Eadie-Hofstee plots. The results revealed that alkaline protease from *A. flavus* had a V_{max} of 60.0 U/mg of protein and K_m value of 0.6mg/ml. Matta *et al.* (1994) has reported proteases with lower K_m values with casein substrate from *Bacillus alkalophilus* and *Pseudomonas* species, which showed K_m values of 0.4 and 2.5 mg/ml, respectively. A slightly higher K_m value of 3.7 mg/ml has been reported for the enzyme from *B. polymyxa* strain indicating higher affinity of the enzyme towards casein (Kaur *et al.*, 1998).

We have characterized protease from a locally isolated fungus *Aspergillus flavus*. Its desirable characters such as broad substrate specificity, stability at high pH, stability at high temperature are significant characteristics of any enzyme for industrial application. Overall, the study provides that the wheat bran has a good potential to be used as solid state fermentation for protease production using *A. flavus*. The lab-scale study on protease production from wheat bran as major substrate might give the basic information of further development for large scale production.

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