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Full Length Research Paper

Optimization and characterization of an extracellular proteases from *Aspergillus flavus* "MTCC 277"

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The present study was undertaken to describe the optimization and characterization of proteases produced by, *Aspergillus flavus* MTCC 277. This strain exhibited the highest protease production after 4 days of incubation when grown on casein-containing basal salt medium. The optimum temperature of proteases was recorded at 35°C whereas optimum pH was 5 and 9. During the studies on the activity of enzyme with metal ions at 50 and 100 mM, Zn^{2+}, $Co^{2+}$and Fe^{2+} enhanced the enzyme activity and the rest ions showed inhibitory effect. Amongst twelve solvents, when used at the rate of 50 mM, formic acid showed maximum inhibition (0.00 U/ml) followed by formaldehyde (1.45 U/ml) and acetic acid (4.40 U/ml). Where as when the concentration was increased to 100 mM of all solvents, it greatly decreased the activity. A total of eight inhibitors were studied and reported that EDTA highly inhibited (4.15 U/ml) followed by urea (8.25 U/ml) and H_2O_2 (15.60 U/ml) at 50 mM concentration. When the concentration was increased to 100 mM all the inhibitors greatly repressed the activity. Zymographical analysis of this enzyme indicated that there are two alleles/loci responsible for proteases production and their relative mobility was $P1_{5.8}$ and $P2_{14.4}$.

Key words: Inhibitors, optimization, proteases, zymography.

INTRODUCTION

The proteolytic enzymes from micro-organisms have many physiological functions, ranging from the generalized protein digestion to the more specific regulated processes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell. Proteases are one of the industrially most important enzymes, which cover approximately 60% of all enzyme market (Rao et al., 1998), and are utilized extensively in a variety of industries, including detergents (Bailey and Ollis, 1977), meat tenderization, cheese-making, dehairing, baking, brewery, the production of digestive aids. In view of the recent trend of developing eco friendly technologies, it replaced chemical hydrolysis of protein used in various industries especially in leather treatment, bioremediation processes, and in pharmaceutical industry for preparation medicines. In recent years the potential of using microorganisms as biotechnological sources of Industrially relevant enzymes has stimulated interest in the of exploitation of extracellular enzymatic activity in several microorganisms (Godfrey and West, 1996; Kumar and Takagi, 1999).

Although protease can be obtained from several sources, such as plant and animals, microbial origin especially from fungal origin have long been used in industry. Among fungi, the proteases from Aspergillus spp. are widely used in various industries because of its activity at various pH and temperature (Haq et al., 2006; Impoolsup et al., 1981). Therefore, the present study discusses the optimization and characterizations of proteases produced from high selected isolates of *Aspergillus flavus* MTCC 277.

MATERIALS AND METHODS

Fungal isolates

In the present study, a high protease producing fungal culture *A. flavus* MTCC 277 was taken from Microbial Type Culture Collections, IMTECH, Chandigarh, India. One set of these isolates

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was incubated at 28°C for experiment and other set was stored at 4°C for further uses.

Screening of isolates for protease production

Screening of protease production was done on gelatin plate assay as per Upadhyay et al. (2002). Fungal isolate was inoculated in Petri plate containing NAM with 1% gelatin and incubated at 280°C for 5 days. The plate was flooded with 15% of $HgCl_2$ and the clear zone around the colony was observed

Extraction of enzyme

For the extraction of proteases, 15 ml of basal salt medium with 1 g of solid protein sources was autoclaved. Seven days old *A. flavus* MTCC 277 culture was inoculated and incubated at 28°C for 5 days. Crude enzyme extract (CEE) was prepared from the culture supernatant which was centrifuged at 10,000 rpm for 5 min at 4°C and crude extract was stored at - 40°C for further use (Charles et al., 2008).

Determination of enzyme activity

Protease activity was determined as per Shimogaki et al., (1991), with slight modification using casein as the substrate. The reaction mixture comprise of crude enzyme extract (1.0 ml) and 1.0 ml of substrate solution (1.0% casein in 0.1 M Sodium phosphate buffer, pH 7.4). The mixture was incubated at 37ºC for 60 min. The reaction was terminated by the addition of 3.0 ml of 5% Tri chloro-acetic acid, and the mixture was kept at room temperature for 10 min followed by filtration through Whatman filter paper No. 1. The absorbance of the filtrate was measured at 280 nm against the blank. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine/ml/min under assay conditions.

Effect of various parameters on the protease activity

Effect of incubation periods, pH and temperatures

The enzyme activity was observed from 1 to 10 days of incubation. 1.0 ml of CEE along with 1.0 ml substrate solution (1.0% casein in 0.1 M Sodium phosphate buffer, pH 7.4) was incubated for 60 min at 37°C as described above and protease activity was observed spectrophotometrically against the blank. The effect of the pH on the activity of protease were determined in the pH range of 3 to 11 where as optimum temperature was estimated by incubation of reaction mixtures at the various temperatures viz; 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60°C.

Effect of metal ions

The effects of metal ions on the activity of enzyme were studied to understand the nature of enzyme active site. During this study the following salts of ions Na^+ (NaCl), K^+ (KCl), Mg^{+2} ($MgCl_2$), Ca^{+2} ($CaCl_2$), Mn^{+2} ($MnCl_2$), Fe^{+2} ($FeCl_3$), Co^{+2} ($CoCl_2$), Cu^{+2} ($CuSo_4$), Zn^{+2} ($ZnSO_4$), Cd^{+2} ($CdCl_2$), Hg^{+2} ($HgCl_2$), Ag^+ ($AgNO_3$) were taken at the rate of 50 and 100 mM of reaction mixture.

Effect of the solvents

The effect of various solvents on enzyme activity was studied in order to obtain some valuable information about catalytic site of enzyme. The following solvents, Acetic acid, Ethanol, Formic acid, Amyl alcohol, Carbon tetra chloride, Formaldehyde, Toluene, Diethyl ether, Aniline, Benzene, Chloroform, Acetaldehyde, were added to the reaction mixture at the rate of 50 and 100 mM concentration.

Effect of inhibitors

Enzyme inhibitors viz; EDTA, Sodium nitrite, DTT, SDS, Sodium meta bisulphate, Hydrogen peroxide, β- mercaptoethanol, Urea were added to the reaction mixture at the rate of 50 and 100 mM per ml of mixture.

Immobilization

For industrial exploitation of enzyme, it was immobilized by Ca-alginate and Polyacrylamide gel entrapment methods (Mosbach, 2005). Enzyme leakage was checked by taking 1 ml of water in which immobilized beads were dipped for 24 h at 4°C, in place of enzyme the water in which beads were kept was taken and activity was determined by standard assay procedure.

Protease zymography

The enzymes were separated by SDS polyacrylamide gel electrophoresis in a discontinuous buffer system. The resolving gel contained 10% acrylamide with substrate and stacking gel was made 5% acrylamide (Sambrook et al., 1989). 15 µl enzyme samples were loaded in the wells along with gel loading dye. The gel was run at 100 V and 15 mA current at 40°C for 4 h. The gel was washed for 10 min each with 1st and 2nd washing buffer in shaking condition (Upadhyay et al., 2005) and then it was incubated in substrate buffer ($CaCl_2$ – 0.367 g , NaN_3 – 0.457 g , Triton- X- 100 – 0.5 ml, 50 mM Tris buffer – 50 ml) for 12 h. The bands were visualized by staining the gel in staining solution (0.25 g Coomassie-Brilliant Blue R-250, 45 ml methanol, 5 ml Acetic acid, 50 ml distilled water) for 30 min. at room temperature. The stained gel was destained with destaining solution (45 ml methanol, 5 ml Acetic acid, 50 ml distilled water) for 15 min and photograph was taken. The relative position of bands was calculated as per Upadhyay et al (2010).

RESULTS AND DISCUSSION

The time course of protease secretion and casein degradation by *A. flavus* MTCC 277 in a basal salt medium containing casein was determined by batch culture. After complete incubation extract was prepared from culture supernatant, which acts as crude protease enzyme. The extracellular protease activity of crude enzyme extract increased during cell growth and reached maximum value 85.30 U/ml at 3rd day of incubation, thereafter decrease slowly at periodic incubation (Figure 1). Similar observation was recorded Haq et al. (2006) while studying optimization of protease production in *Penicillium chrysogenum*.

The effect of pH on the protease activity

pH is the most important factor, which markedly influence

Figure 1. Effect of various incubation periods on protease production of *A. flavus* MTCC 277.

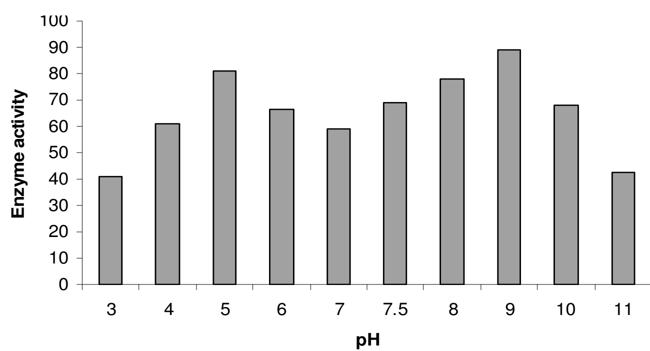

Figure 2. Effect of pH on protease activity of *A. flavus* MTCC 277.

the enzyme activity. Enzymes are affected by changes in pH. Extremely high or low pH values generally result in complete loss of activity of enzymes. From Figure 2 it is clear that the protease produced by this strain is active in the pH range 3.0 -11.0 with two optimal peaks that is, one acidic (pH 5.0) and other is alkaline (pH 9.0). The multiple pH optima observed suggests that the presence of at least two proteolytic activities in the crude protease (Impoolsup et al., 1981). This property makes the enzyme suitable for leather treatment and industrial production of detergents.

The effect of temperature on the protease activity

Morimura et al. (1994) have reported that the rate of enzyme catalyzed reactions increases with temperature up to a certain limit. Above a certain temperature, enzyme activity decreases with temperature because of enzyme denaturation. Maximum protease activity was recorded at 35°C in *A. flavus* MTCC 277. Further increase in temperature resulted in decrease in the activity of protease (Figure 3). Similar observation was shown by Morimura et al. (1994) for *Aspergillus usami*. It

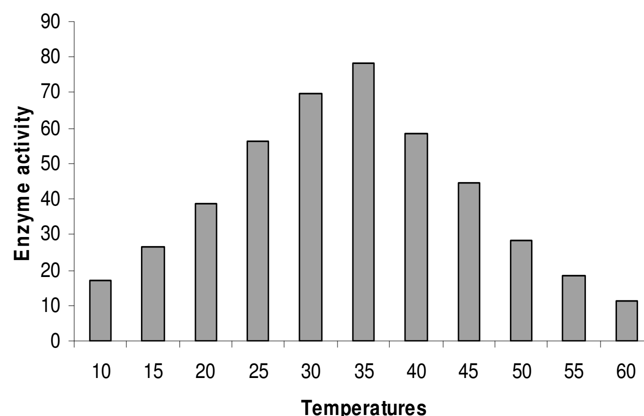

Figure 3. Effect of temperatures on protease activity of *A. flavus* MTCC 277.

was revealed that environmental temperature not only affects growth rates of organism but also exhibit marked influence on the levels of protease production.

Effects of various metal ions

The influence of various metal ions on enzyme activity was studied. As reported from studies on protease a concentration as low as 50 mM of some metal ions could affect enzyme activity. Zn^{2+}, Co^{2+}, Fe^{2+} enhanced the activity of enzyme whereas metal ions viz. Na^{+}, Mg^{2+}, Ca^{2+}, Mn^{2+} , Hg^{+2} and Cu^{2+} reduced the enzyme action and showed approximately 60% relative activity. When the concentration was increased at 100 mM of mixture all the ions highly inhibited the activity, except Zn^{2+} and Na^{+} (Figure 4). Inhibition by Hg^{2+} may indicate the importance of indole amino acid residue in enzyme function as has been demonstrated for other microbial enzymes (Gupta et al., 2002) more or less similar observation by Kalpana et al. (2008) while studying effect of metal ion on *A. niger.*

Effects of various inhibitors on enzyme activity

A total of eight inhibitors were studied with crude enzyme extract at the concentration of 50 and 100 mM of reaction mixture. It was reported from the Figure 5 that EDTA is greatly inhibited (4.15 U/ml) followed by urea (8.25 U/ml) and H_2O_2 (15.60 U/ml) whereas DTT, sodium bisulphate, β-mercaptoethanol showed more than 80% relative activity. When the concentration was increased up to 100 mM of mixture all the inhibitors except DTT and β-mercaptoethanol greatly inhibited the activity. Similar observation was recorded by Monod et al. (1991) while studying characterization of an extracellular protease of *A. fumigatus*.

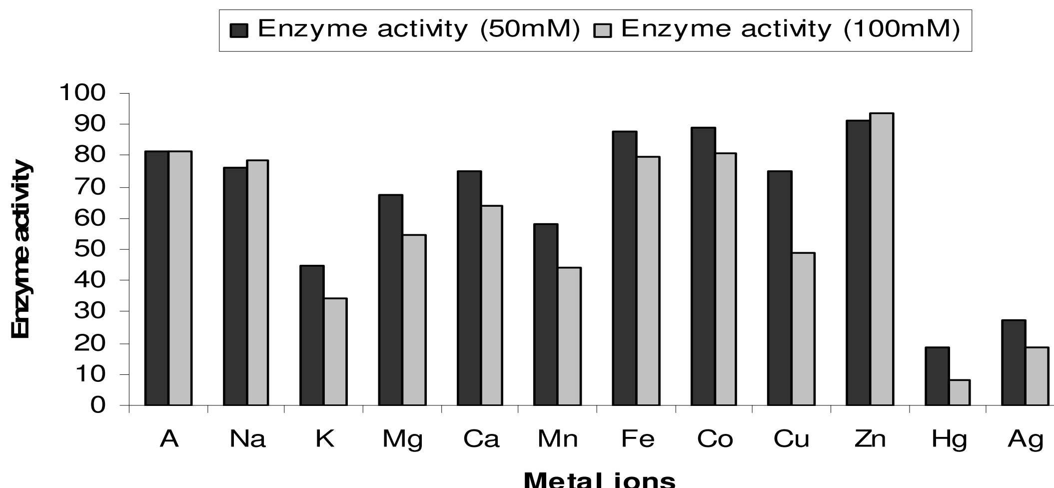

Figure 4. Effect of metal ions on protease activity of *A. flavus* MTCC 277. A- Control.

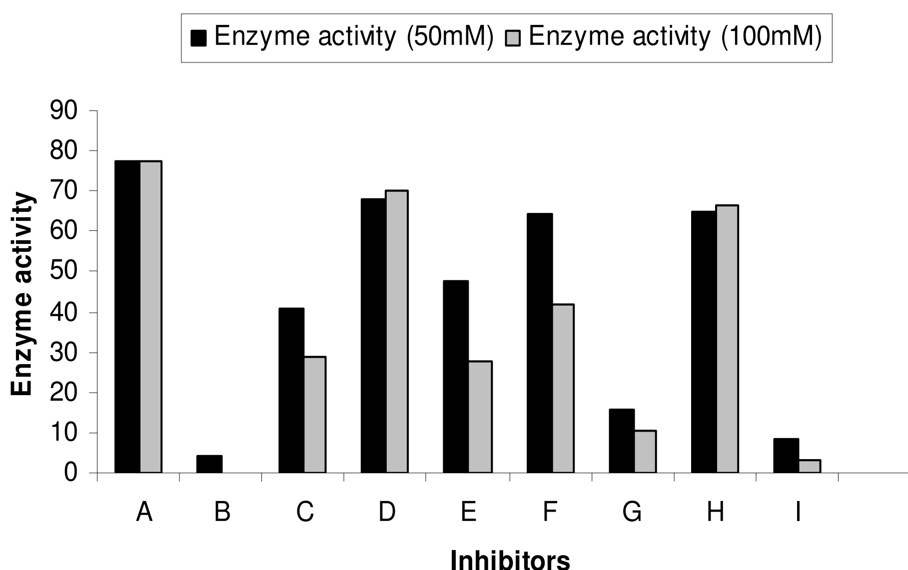

Figure 5. Effect of inhibitors on protease activity of *A. flavus* MTCC 277. Control (A); EDTA (B); Sodium nitrite (C); DTT (D); SDS (E); Na meta bisulphate (F); Hydrogen peroxide (G); Hydrogen peroxide (G); β-mercaptoethanol (H); Urea (H).

Effects of solvents on enzyme activity

Amongst twelve studied at the concentration of 50 mM, formic acid showed maximum inhibition (0.00 U/ml) followed by formaldehyde (1.45 U/ml) and acetic acid (4.40 U/ml). At this concentration (50 mM) ethanol and toluene slightly affected the activity and their relative activity was more than 85% followed by acetaldehyde (relative activity 68%) As the concentration was increased 100 mM of all solvents the activity was greatly decreased by all (Figure 6). There are few reports on the stability of protease in organic solvents. The reasons for low activity of protease in organic solvents may be due to limited dispersion of enzymes, the partial denaturation of enzymes and the reduced flexibility of proteins in anhydrous solvents. Most of the enzymes are easily inactivated or denatured in organic solvents and proteases, which are active in organic solvent, have potential application in catalyzing synthetic reactions.

For the industrial exploitation of this enzyme, it was immobilized by Na - alginate and poly acrylamide gel method. The immobilized Na alginate beads showed less leakage than acryl amide gel method (Figure 7). Therefore, Ca-alginate method can be used for industrial

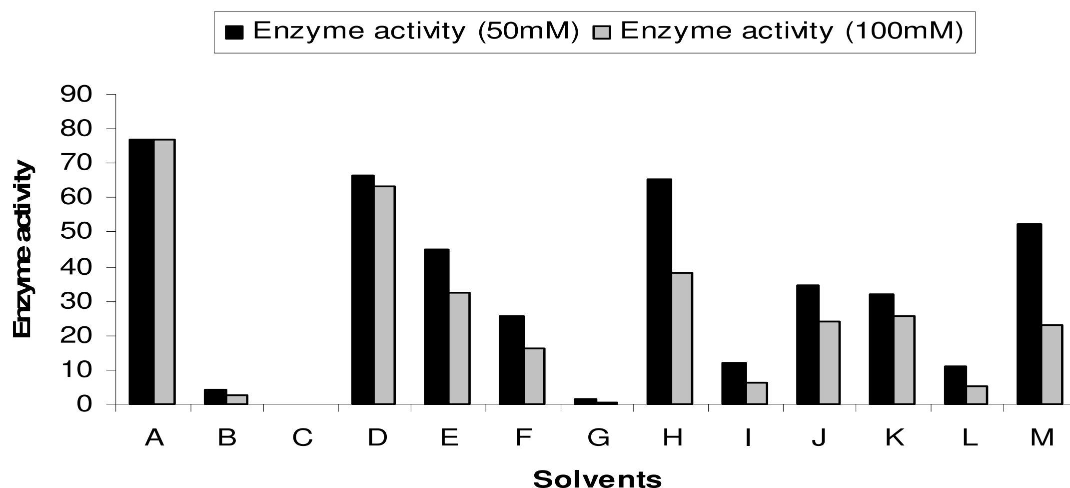

Figure 6. Effect of solvents on protease activity of *A. flavus* MTCC 277. Control (A); Acetic acid (B); Formic acid (C); Ethanol (D); Amyl alcohol (E); Carbon tetra chloride (F); Formaldehyde (G); Toluene (H); Diethyl Ether (I); Aniline (J); Benzene (K); Chloroform (L); Acetaldehyde (M).

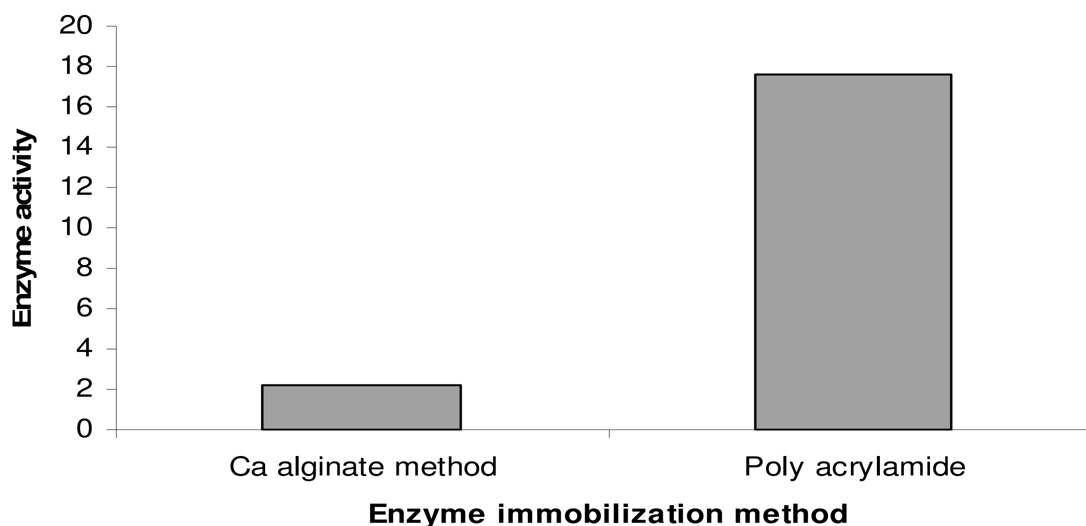

Figure 7. Leakage of protease from immobilized enzyme beads of *A. flavus* MTCC 277.

purpose. This work was supported by Farag (2004) while studying immobilization of a keratinase of *A. oryzae*. The zymographical studies showed that a total of two proteases designated as P15.8, P214.4 were produced by *A. flavus* MTCC 277 and it is also possible to interpret that there are two loci or alleles which are responsible for the production of same isozymes (Figure 8).

Conclusion

The present investigation is on proteases produced by *A. flavus* MTCC 277 which clearly shows that this fungus is potent producer of proteases by batch culture, which are active at different pH ranges. The enzyme production was considerably enhanced under the set of conditions optimized in this study. These findings have great industrial implications. It is clear from the results that the protease produced by *A. flavus* is active in the pH range 3.0 -11.0 with two peaks, one acidic (pH 5.0) and the other basic (pH 9.0). This suggests that the enzyme would be useful from slightly acidic to alkaline range and vice-versa. The multiple pH optima observed suggests the presence of at least two proteolytic activities in the crude protease. This property makes the enzyme suitable for leather treatment and industrial production of detergents.

Studies with regard to temperature optimization show that the optimum temperature for *A. flavus* MTCC 277

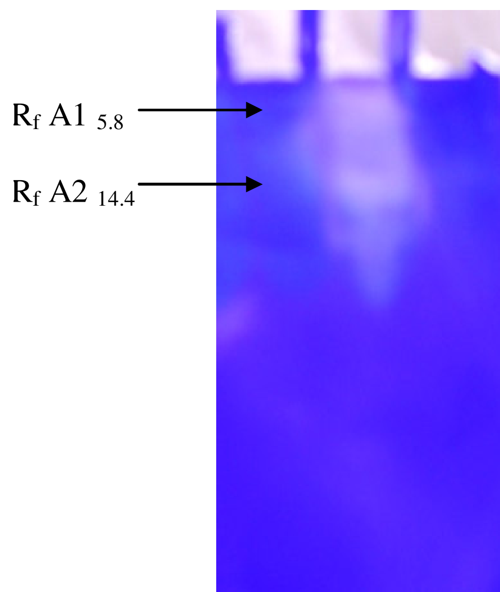

Figure 8. *A. flavus* MTCC 277: Poly acrylamide gel stained for proteases.

was 35°C. Optimum temperature for the proteases is considerably at a mesophilic range, and thereby proves to be advantageous in industrial applications. Protease activity was inhibited by EDTA but active in the presence of Zn^{++}, Co^{++}, Fe^{++}, even though it was only marginally inhibited by other chemicals. Further it was concluded that the protease would be metalloprotease. Moreover, the physicochemical properties of protease produced by *A. flavus* MTCC 277 makes it a suitable candidate for detergent formulation.

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