

Full Length Research Paper

Screening of alkaline proteases in *Phanerochaete chrysosporium* grown in non-lignocellulose medium

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Accepted 28 July, 2013

Phanerochaete chrysosporium is a well known organism which produces extracellular proteases. In this study, the two principal extracellular alkaline proteases produced by submerged batch cultures of *P. chrysosporium* were characterized by SDS-PAGE and zymography. The protease activity was observed from the third day of incubation consisted of two protein bands ranging in size of 33 and 40 KDa, respectively. The activity of these alkaline proteases in presence of casein substrate at pH 7.5 was found to be 86 units/ml. The cultures were grown in presence of basal media supplemented with selected agrowastes namely, peanut oil cake, cotton seed oil cake, almond oil cake, oat and corn flour. Among them, a combination of oat flour and peanut oil cake (10:1) showed maximum activity of 46 units/ml on 7th day of incubation. The results obtained were remarkable characterizing specific alkaline proteases which can be explored for novel properties and industrial applications.

Key words: *Phanerochaete chrysosporium*, wood rot fungi, alkaline proteases, SDS-PAGE, zymogram, agrowastes.

INTRODUCTION

Proteases represent an important group of industrially produced enzymes and account for 60% of the worldwide revenue of the industrial enzymes (Godfrey and West, 1996). They are often characterized based on several factors like optimum pH (acid, neutral or alkaline proteases), optimum temperature, ability to hydrolyze specific proteins (casein, collagen, keratin, etc.), and their stability. Proteases of microbial origin have long been used in industry and most of them were derived from bacteria. Recently, there is a renewed interest on fungal

proteases as they are extracellular, easy to extract and hence, replacing bacterial proteases. The significant use of proteases includes biotechnological production of detergents (Bailey et al., 1993), industries as milk-clotting agents (Shalabi and Fox, 1983; Shanley et al., 1993) and for meat tenderization (Bernholdt, 1975). These are also been implicated in clinical and medical diagnostics for therapeutic purposes especially in the reduction of tissue inflammation (Bailey et al., 1993) and this particular aspect attracted many scientists to screen various

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organisms for potential proteases. *Phanerochaete chrysosporium* (*P. chrysosporium*) is known to secrete a large number of hydrolytic and oxidative enzymes for degradation of natural lignocellulosic material, which includes cellulases, hemicellulases, lignin peroxidases, manganese peroxidases (Kirk and Farrel, 1987, Kirk et al., 1990; Shin Sato et al., 2007; Tien, 1987; Wymelenberg et al., 2005, 2006) and pectinases (Farrell et al., 1989). It also produces acid proteinase, glutaminase and five kinds of peptidases which are related to aspartyl protease family (Rajkumar et al., 2011). The protease activity investigated in the batch cultures of *P. chrysosporium* (Carlos et al., 1990a) had showed both primary (81.5, 71.5, 52, 28, 24 and 22 KDa) and secondary activity (75.5 and 25 KDa).

To the best of our knowledge, there has been no published report on medium range molecular weight includes alkaline proteases from this organism. Hence, the present study was undertaken to identify the novel alkaline proteases by inducing the secretion of proteases under submerged fermentation of *P. chrysosporium*. Further, the information procured might give an insight into how the naturally available agroproducts could act as better substrates for protease production and therefore, we here in, present the results of our investigative study.

MATERIALS AND METHODS

Organism

P. chrysosporium BW808 (MTCC 787) was obtained from IMTECH, Chandigarh India. It was maintained at 37°C on 1% malt extract agar slants and subcultured every week.

Chemicals

All the chemicals were procured from Sigma-Aldrich, India. The peanut oil, cotton seed oil and almond seed oil cakes; oat and corn flours were procured from the local market.

Inoculation and media preparation

The inoculum was taken from stationary cultures containing fresh conidial suspension with a conidium concentration of 5×10^5 spores per ml. The inoculation ratio of the experimental cultures was 2% (v/v), and the incubation temperature was maintained at 37°C in all cases. The growth and maintenance medium used was YMD broth medium [1% (w/v) malt extract, 1% (w/v) yeast extract, 0.1% (w/v) KH_2PO_4 , 2% (w/v) dextrose and YMD agar contain 1.5% agar] at pH 6.5. Basal medium used for protease production contained 3% (w/v) starch (carbon source), 0.3% (w/v) NaNO_3 (nitrogen source), 0.05% (w/v) KCl, 0.1% (w/v) KH_2PO_4 and 0.01% (w/v) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and maintained at 37°C in an orbital shaker (120 rpm) for 24 to 96 h. At the end of incubation, the whole fermentation medium was filtered, centrifuged at 5000 rpm for 15 min and the clear supernatant was used as crude enzyme preparation.

Selection of carbon and nitrogen sources for production medium

Selection of carbon source was done by consideration of one variable at a time. In the production medium, starch was replaced by various cheap carbon sources namely, corn and oat flours. All carbon sources were used at a final concentration of 3% (w/v) in the medium. To study the effect of different nitrogen sources on protease production, the inorganic nitrogen source (0.3% sodium nitrate) in basal medium was replaced by various cheap nitrogen sources such as peanut oil, cottonseed oil and almond seed oil cakes.

Caseinolytic and gelatinolytic protease activity

Caseinolytic and gelatinolytic activities were determined by plate assay as described by Frazier (1926). The agar plates supplemented with 0.2% casein and 0.2% gelatin were incubated separately with fungi for 36 to 72 h. The plates were then flooded with 20 ml of 6% HgCl_2 in 1M HCl and left for 15 min. The plates were decanted and subsequently observed for the clear zones. All the experiments were performed in quintuplicate.

Enzyme extraction

Enzyme was extracted from the YMD broth by filtering through whatman filter paper and centrifuging at 5000 rpm for 30 min. The clear supernatant was used for enzyme assay.

Determination of optimum pH

The optimum pH was determined by monitoring protease activity (45°C) at pH values ranging from 5 to 9, using citrate buffer (pH 5.0 to 6.0), phosphate buffer (pH 7.0) and Tris-HCl buffer (pH 8.0 to 9.2).

Alkaline protease activity

Protease activity was determined by modified Anson's method (Anson, 1938), where 2% casein in 0.1M phosphate buffer served as a substrate. Casein solution (0.5 ml) with an equal volume of diluted enzyme (1:5 with double distilled water) was incubated at room temperature. After 30 min, the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The mixture was filtered and to 1 ml of filtrate, 5 ml of 0.5M Na_2CO_3 and 1 ml of 1:1 diluted Folin ciocalteu reagent were added. The color developed after 30 min of the reaction was read at 660 nm against reagent blank prepared in the same manner where tyrosine served as the reference standard. The enzyme activity was expressed in units where one unit is the amount of enzyme that releases 1 μg of tyrosine per minute. All the experiments were performed in quintuplicate.

SDS-PAGE and molecular mass determination

SDS-PAGE was carried out as described by Laemmli (1970). The crude enzyme extract maintained at 0°C was added to acetone containing 10% (w/v) trichloroacetic acid, 2% (w/v) β -mercaptoethanol

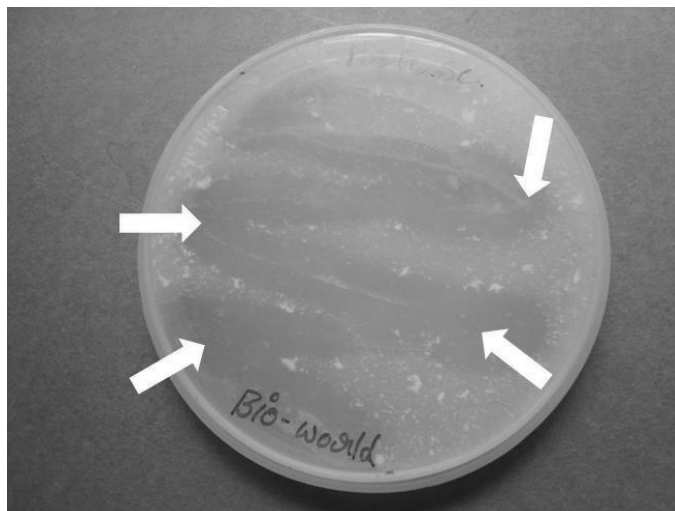


Figure 1. Gelatinolytic activity of *P. chrysosporium*; The plates were incubated for 5 days and treated for proteolytic activity. Clear zones indicative of gelatinolytic activity are shown by arrows.

and mixed well. It was left in refrigerator at 10°C overnight for proteins to precipitate. The solution was centrifuged at 5000 rpm for 30 min and supernatant was decanted. The pellet was washed with ice cold trichloroacetic acid solution (10% w/v in acetone), centrifuged and air dried. The process was repeated thrice. The protein pellet was then resuspended in sample buffer for SDS-PAGE. The electrophoresis was performed on 12% (w/v) acrylamide slab gel with 25 mM Tris in 192 mM Tris-glycinate tank buffer (pH 8.3) containing 0.1% (w/v) SDS as the running buffer. The gel was then stained by commassive blue (0.25% in methanol, acetic acid and water mixture taken in the ratio 4:1:5), destained (to remove excess dye) and subsequently observed for bands. The molecular mass of the proteases were determined by interpolating logarithmic value of the molecular mass against R_f value (relative mobility) using medium molecular mass proteins standards (Bangalore Genei, India). The standard proteins used were phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa).

Gelatin zymography

Polyacrylamide gel electrophoresis was performed on 12% (w/v) acrylamide slab gel with 1% gelatin and 25 mM Tris in 192 mM Tris-Glycinate tank buffer (pH 8.3). Then the gel was placed in incubation buffer (Tris-HCl buffer, pH 7.5) for period of 12 h. The gel thus obtained was subjected to staining, followed by destaining for observation of clear zones.

Statistical analysis

The data was subjected to statistical analysis using Sigma plot (v.12) software.

RESULTS AND DISCUSSION

P. chrysosporium was initially cultured in YMD agar medium for 5 days and subsequently transferred into liquid media containing protein substrates for induction of proteolytic enzymes.

Detection of protease activities

The protease activities against casein and gelatin substrates were checked on 5 day old YMD agar cultures. The clear zones around organism indicated the depletion of protein in the media (Figures 1 and 2) which clearly shows the production of the active proteolytic enzymes with both gelatinolytic and caseinolytic properties.

Assay of caseinolytic and gelatinolytic activities

Casein and gelatin substrates at 1% were used to determine the elicitation capability of substrates to induce higher proteolytic activity. Aliquots of fermented broth containing crude enzyme were taken on 3rd, 5th and 7th day and subsequently the proteolytic activity was determined. It is evident from Figure 3, the activity was found to be the higher with casein (84 units/ml) when compared to that of gelatin (54 units/ml) on the 7th day followed by 5th and 3rd day of incubation. Apparently, casein is more easily degraded than gelatin (Hoffman and Falvo, 2004).

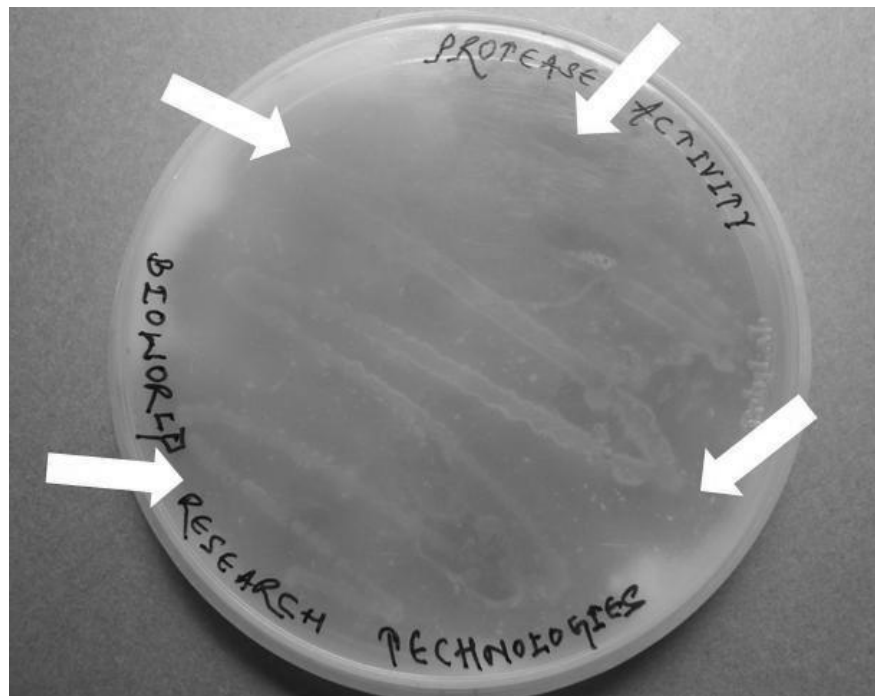


Figure 2. Caseinolytic activity of *P. chrysosporium*; The plates were incubated for 5 days and treated for proteolytic activity. Clear zones indicative of caseinolytic activity are shown by arrows.

Determination of optimum pH

The caseinolytic enzyme activity was found to be higher over a broad range of pH 6.5 to 8. Within this range, the highest caseinolytic activity of 88 units/ml was observed at pH 7.5 indicating the presence of extracellular alkaline protease(s) in the culture medium (Figure 4). Such proteases with broad pH range are also found in other polyporales members and most of these proteases tend to be serine alkaline proteases (Sabotić, 2007).

Optimization of incubation time for production of alkaline protease production

Taking casein as substrate, the crude enzyme extracts were obtained on 3rd, 5th, 6th, 7th and 8th day from YMD broth cultures at pH 7.5. With increase in the day of incubation, there was noticeable increase in the activity with a maximum of 86 units/ml on 7th day and thereafter decreased (Figure 5). Most of the proteases reported in this organism were acidic proteases (Sato et al., 2007) which are mostly produced in early growth phase that is, 2 to 4 days after incubation; whereas, protease(s) in the present study reached to the maximum level on 7th day. This organism is known to exhibit two kinds of protease

activities where the first activity is seen during active growth phase and the second activity during late phase and the later has the ability to degrade lignin peroxidases (Carlos, 1990b). The organism in the present study showed only secondary activity till 7th day after which it gradually declines. The absence of first activity can be due to lack of ligno-cellulose material in the media. Presence of saw dust (ligno-cellulose) in the media induces the organism to create acidic environment initially for the easy degradation of cellulose and acidic proteases (first activity) are released during this period and after which alkaline proteases predominates (that is, secondary activity) (Carlos et al., 1990a; Xiong et al., 2008).

The studies carried out in the present investigation, in absence of such ligno-cellulose substrate, might have led to the production of only alkaline proteases where the exact mechanism is not known and has to be elucidated.

Natural substrates as carbon and nitrogen substrates

The suitability of natural substrates like peanut oil, cotton seed oil and almond oil cakes as a sole source of nitrogen for the production of alkaline protease was determined and the results were depicted (Figure 6). Among all the natural substrates tested, peanut oil cake

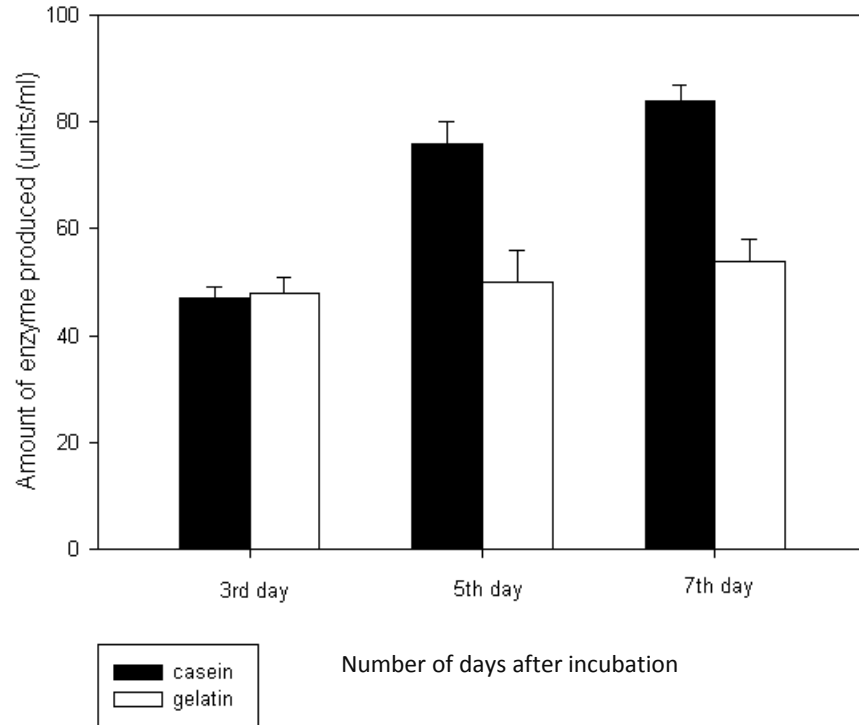


Figure 3. Caseinolytic and gelatinolytic activities shown on 3rd, 5th and 7th days by *P. chrysosporium*. Inoculated broth supplemented with protein source was incubated for various days with samples taken at regular intervals and tested for the protease activity.

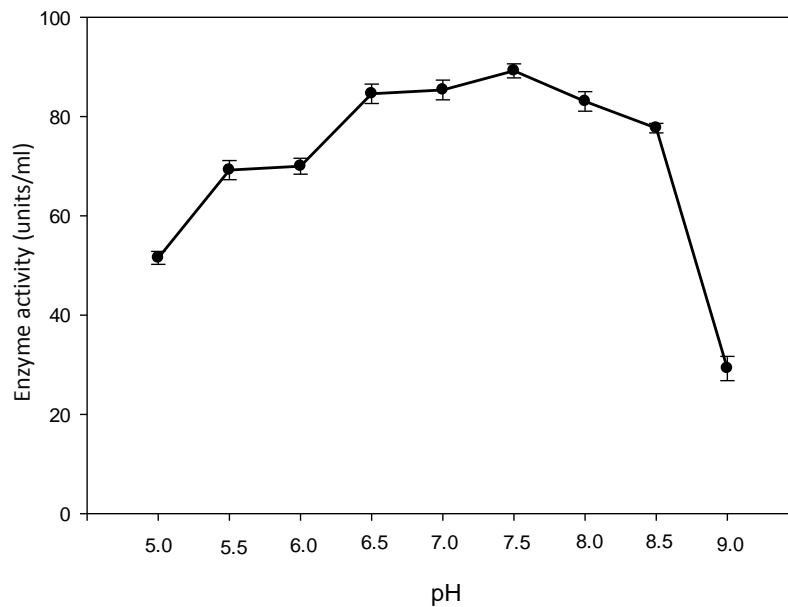


Figure 4. Caseinolytic activity profile shown by *P. chrysosporium* over a pH range of 5 to 9. Inoculated broths (only varying in pH) supplemented with casein were incubated for several days with samples taken on 7th day and tested for their protease activities.

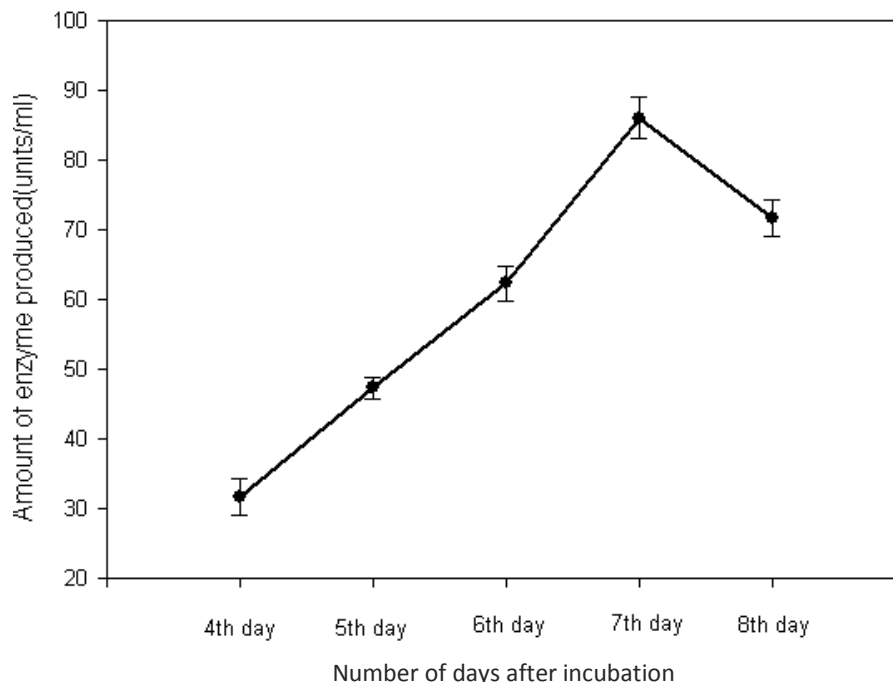


Figure 5. Levels of caseinolytic protease(s) produced by the organism on 3 to 8 days of incubation. Inoculated broth (at 7.5 pH) supplemented with casein was incubated for several days with samples taken on various days and tested for their protease activities.

was found to exhibit maximum protease activity of 30 units/ml followed by cotton seed oil cake (26 units/ml) and almond oil cake (14 units/ml). The utilization of oat and corn flours as a sole source of carbon was determined in presence of peanut oil cake as nitrogen source and the results were presented in Figure 7. The enzyme activity with oat flour was found to be higher (46 units/ml) than the corn flour (42 units/ml) which clearly shows that a combination of peanut oil cake and oat flour was a suitable option for the production of alkaline proteases. The commercial production of any enzyme does not always depend upon the organism but also on the cost effective methods of production (Rajkumar et al., 2011). However, the protease activity produced by the fungi in presence of basal media with natural substrates is found to be lower than basal media with casein.

It could be due to the fact that casein is a milk protein which might be easily digested than plant proteins present in natural substrates.

SDS PAGE and Gelatin Zymography

SDS- PAGE results showed two prominent alkaline proteases which weigh 33 and 40 KDa on 3rd, 5th and 7th

days of growth in basal media supplemented with casein as well as natural substrates (Figure 8). Zymography was performed and the results are presented in Figure 9. Two clear zones were observed in all lanes indicating the presence of active alkaline protease in all the samples. This indicates that these two proteases were produced throughout the growth phase which have broad substrate specificity and evidently degrade not only casein but also the protein residues present in the natural substrates that were used in the study. This is the first report of medium molecular weight alkaline proteases in *P. chrysosporium* which are maintained remarkably in an invariable condition throughout the entire growth period.

Conclusion

In the present study, the two principal extracellular medium molecular weight alkaline proteases produced by *P. chrysosporium* in the presence of casein were partially characterized by SDS PAGE and zymography. A combination of selected agrowastes consisting of oat flour and peanut oil cake was found to be effective in inducing the production of proteases similar to that of casein and gelatin. Further work is underway to clearly establish the

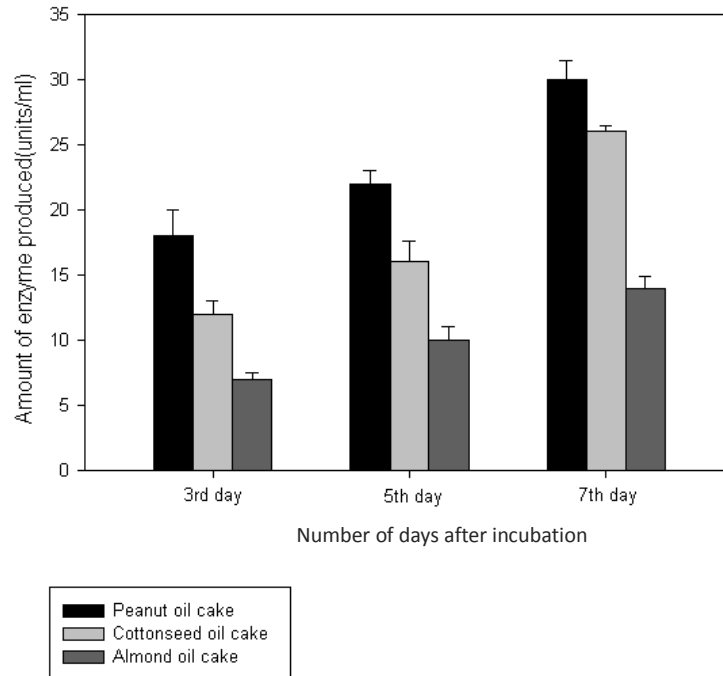


Figure 6. Assay of alkaline protease activity on 3rd, 5th, 7th days with peanut, cotton seed and almond oil cakes as nitrogen sources. Inoculated broths (at 7.5 pH) supplemented with different nitrogen sources were incubated for several days with samples taken on various days and tested for their protease activities.

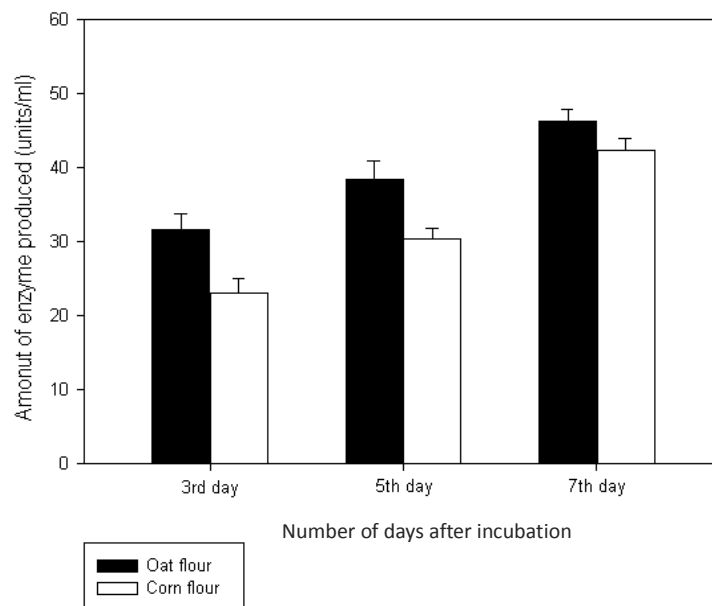


Figure 7. Assay of alkaline protease activity on 3rd, 5th, 7th days with oat and corn flours as carbon sources. Inoculated broths (at 7.5 pH and peanut oil cake as nitrogen source) supplemented with different carbon sources were incubated for several days with samples taken on various days and tested for their protease activities.

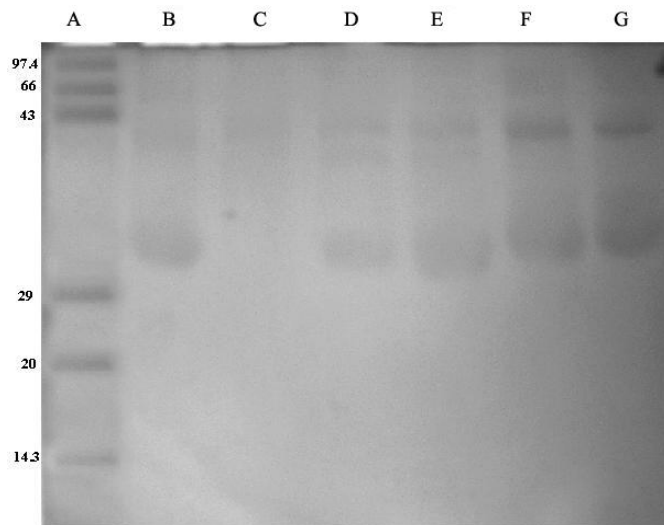


Figure 8. SDS PAGE of proteases from *P. chrysosporium*; Lane A – medium molecular weight protein markers: 97.4 KDa phosphorylase, 66 KDa bovine serum albumin, 43 KDa ovalbumin, 29 KDa carbonic anhydrase, 20 KDa soyabean trypsin inhibitor, 14.3 KDa lysozyme; Lane B – 3rd day sample from BMC; Lane C – 3rd day sample from BMPO; Lane D – 5th day sample from BMC; Lane E – 5th day sample from BMPO; Lane F – 7th day sample from BMC; Lane G – 7th day sample from BMPO; BMC- Basal Media with Casein grown cultures, BMPO- Basal media with peanut oil cake and oat flour grown cultures.

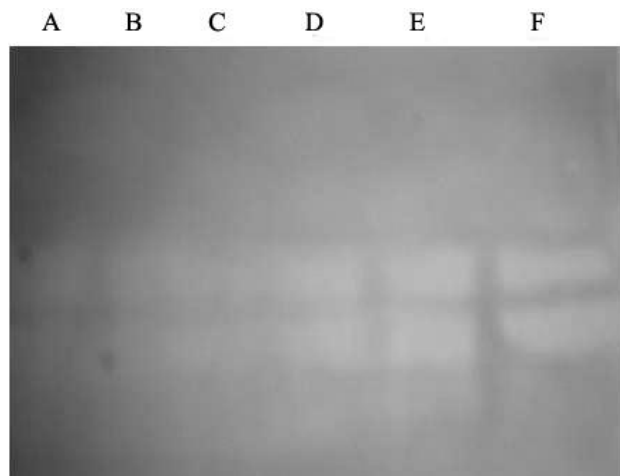


Figure 9. Gelatin zymogram of proteases from *P. chrysosporium*; Lane A – 3rd day sample from BMC; Lane B – 3rd day sample from BMPO; Lane C – 5th day sample from BMC; Lane D – 5th day sample from BMPO; Lane E – 7th day sample from BMC; Lane F – 7th day sample from BMPO.

overall structural and functional aspects of these enzymes to unravel the mechanism of induction and exploit their potential for different industrial applications.

ACKNOWLEDGEMENTS

The authors would like to thank the Director, Sreenidhi Degree and PG College for his constant support and encouragement. The support rendered by Mr. D.Sarath Chandra (PhD), Max Planck Institute for Biophysical Chemistry, Gottingen is gratefully acknowledged.

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