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Isolation and Characterization of Alkalophilic *Pseudomonas* sp. and Optimization of Culture Conditions for Alkaline Protease Production

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Abstract: Proteases are well known enzymes for their wide range application in food industry, detergent industry and pharmaceuticals industry. They are also widely used in leather industry for dehairing and bating of hides as an alternative for toxic chemicals which in turn hamper the environment. In the present investigation a total of 20 bacteria were isolated from the leather industry plant effluent. All isolates were screened for proteolytic activity using alkaline skim milk agar plate, among 20 bacterial isolates only 3 isolates showed alkaline proteolytic activity, one isolate was selected for further study. The effect of carbon sources, nitrogen sources, pH and temperature on production of alkaline protease was investigated by one factor at a time method. The best enzyme activity was observed at pH 8 and temperature 37°C. Protease activity was enhanced by Cu^{2+} and Mg^{2+}. Hg^{2+} and Zn^{2+} slightly activated the protease activity. Fe^{2+} slightly inhibited the proteolytic activity. Extracellular alkaline protease was purified from Pseudomonas isolate in a two-step procedure involving ammonium sulphate precipitation and Sephadex G-100 column chromatography. The molecular size of the alkaline protease enzyme was determined to be 43 kDa by SDS-PAGE.

Key words: Pseudomonas, alkaline protease, leather industry effluents, ammonium sulphate, India

INTRODUCTION

Microbial proteases play an important role in biotechnological processes and they account for approximately 59% of the total enzymes used (Spinosaa *et al*., 2000). Proteases are having wide range of application in food, meat and leather processing industries as well as pharmaceutical industries (Gupta *et al*., 2002). Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions (Maurer, 2004; Saeki *et al*., 2007). These enzymes find applications in detergents, feather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation (Bhaskar *et al*., 2007; Gupta *et al*., 2002; Jellouli *et al*., 2009; Sareen and Mishra, 2008). The majority of commercial alkaline proteases are produced by bacteria, especially *Bacillus* sp. (Jellouli *et al*., 2009). Since, the first alkaline protease Carlsberg from *Bacillus licheniformis* was commercialized as an additive in detergents in the 1960s (Saeki *et al*., 2007), a number of Bacillus-derived alkaline proteases have been purified and characterized and significant proteolytic activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification and low cost have been demonstrated (Haddar *et al*., 2009; Maurer, 2004). Alkaline protease production has been reported in both *Pseudomonas* and *Bacillus* sp. (Najafi *et al*., 2005). The growth and enzyme production of the organism are strongly influenced by medium components like carbon and nitrogen sources. Besides the nutritional factors the cultural parameters like temperature, pH, incubation time were also plays a major role in enzyme production (Jameel and Mazharuddin, 2011) and as so the optimization of media components and cultural parameters is the primary task in a biological process. So, the media components and cultural conditions were optimized. However, very few reports exist on the optimization and production alkaline protease from *Pseudomonas* sp. *P. aeruginosa* ATCC 27853 was declared as a strain producing elastase and alkaline protease both having a molecular mass of about 30 kDa (O'Callaghan *et al*., 1996). In the present study, isolate of *Pseudomonas* sp. producing high levels of extracellular proteases was isolated and an alkaline protease was purified and characterized from leather industry effluents.

MATERIALS AND METHODS

Isolation of protease producing microorganisms: Samples were collected from leather industry at Common Effluent Treatment Plant, Varadayapalam and transported to laboratory under sterile conditions. Bacteria were isolated from leather effluent by serial dilution technique on

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nutrient agar medium. The 1 mL of effluent sample was taken and was serially diluted up to 10^{-7} dilution. The 0.1 mL aliquots of 10^{-4}, 10^{-5}, 10^{-6} dilutions was spread onto the medium and incubated at room temperature for 24 h. A total of 20 bacterial isolates from enriched sample was plated over nutrient agar medium containing 0.4% gelatin. After 24 h of incubation, plates were flooded with 1% tannic acid. Isolates having a higher ratio of clearing zone to colony size were grown in liquid broth and the amount of protease production was determined from culture filtrate. The isolate which showed higher protease activity was selected and maintained on nutrient agar slants and subcultured after every 15th day. Selected isolates were identified based on morphological and biochemical characteristics.

Screening for alkaline protease activity: The purified cultures were screened for the production of alkaline protease by streaking them on alkaline skim milk agar plate (Skim milk 1.0%, Peptone 0.1%, NaCl 0.5%, Agar 2.0% and pH 8). The alkaline protease production was confirmed by the formation of clear zones around the colonies. The organism with maximum zone formation was further analysed.

Enzyme assay by plate zone method: Activity of alkaline protease was determined by Casein Plate Method. The casein solution [2.5 mL of 2% (w/v) casein in 0.1 M glycine NaOH buffer, pH 8] was added into skim milk agar medium and after sterilization, poured into the petriplates. The holes were made on a plate by suction by using steel gel puncture (0.5 cm). The 50 and 100 μL enzyme solution was dropped into each hole and incubated at 30°C for 24 h. After incubation, plates were observed for clear zones.

Alkaline protease production medium: The pure cultures of test organisms were maintained in nutrient agar slants for further studies. For the production of alkaline protease enzyme, three isolates were inoculated in production medium. The culture medium used in this work for protease production contained 0.5% glucose (w/v), 0.75% peptone (w/v), 0.5% (w/v), 1% casein (w/v), $MgSO_4.7H_2O$, 0.5% (w/v) KH_2PO_4 and 0.5% (w/v) K_2HPO_4 maintained at 37°C for 24-72 h in a shaking incubator (140 rpm). At the end of each fermentation period, the whole fermentation broth was centrifuged at 10,000 rpm at 4°C for 15 min and the clear supernatant was used as crude enzyme preparation.

Alkaline protease activity assay: Alkaline protease activity was determined by applying the method given by Meyers and Ahearn (1977) with some modifications. According to this procedure 0.5 mL of glycine NaOH buffer (0.2M, pH 10) was added to 0.5 mL of appropriately diluted enzyme and was incubated with 1 mL of 1% casein solution (prepared in glycine NaOH buffer, pH 10) for 15 min at 60°C. The reaction was stopped by addition of 4 mL of 5% (v/v) trichloro acetic acid. The contents were centrifuged after 1 h at 3000×g for 10 min and the filtrate was used for measuring protease activity on the basis of color change. The 5 mL of 0.4 M Na_2CO_3 solution was added to 1 mL of the filtrate and was kept for 10 min. The 0.5 mL of Folin-Ciocalteu's Phenol reagent of 1:1 dilution was added and kept in dark for 30 min at room temperature. The optical densities of the solutions were read against the sample blanks at 660 nm using spectrophotometer. One alkaline protease unit (U) was equivalent to the amount of enzyme required to release 1 mg/mL/min of tyrosine under the defined assay conditions.

Optimization of culture conditions for enzyme production: The cultural conditions (pH, temperature, incubation period and different sources of C and N) were optimized for maximum enzyme production using yeast extract casein medium (Tsujibo *et al*., 1990) containing (g/L) glucose (10), casein (5), yeast extract (5), KH_2PO_4 (2) and Na_2CO_3 (2). The medium was incubated for 48-72 h on incubator shaker.

Alkaline protease production and alkaline protease activity was measured and monitored at 24 h intervals over 96 h fermentation period. To study the effect of pH on alkaline protease production, fermentation experiments were carried out at initial pH varying from 8-11 using appropriate buffers, Tris-HCl buffer (pH 8-11). To study the effect of temperature on alkaline protease production, fermentation experiments were carried out at temperature varying from 30, 37, 40 and 45°C. Alkaline protease production was monitored at 6 h intervals over a 72 h fermentation period through assay of alkaline protease activity. To check the effect of different carbon sources on the production of alkaline protease, wheat bran was substituted with different carbon sources, viz., glucose, fructose, dextrin, wheat ban and starch at 10 g L^{-1}. To study the effects of different nitrogen sources on alkaline protease production, nitrogen sources like peptone, yeast extract, casein, soyabean meal and urea were incorporated in to the medium at 5.0 g L^{-1}.

Effect of various metal ions on alkaline protease activity: The effects of metal ions on enzyme activity (e.g.,) $HgCl_2$, $MgSO_4$, $CuSO_4$, $FeCl_2$ and $ZnCl_2$ (50 mM) were determined by adding them to the reaction mixture and pre incubated

for 30 min at 45°C, pH 10.0. The activity of the enzyme was measured. The activity was expressed as a percentage of the activity level in the absence of metal ion. The enzyme was pre incubated with metal ion (45°C, 5 min). Separate blanks with individual metal ions were prepared.

Characterization of alkaline protease: After fermentation process, proteases were separated out by using filtration, centrifugation and ammonium sulfate salt precipitation for partially purification then protease enzyme sent for dialysis, dialysed enzyme were used for characterization studies.

Partial purification by precipitation and dialysis: The partial purification of enzyme has been carried out as per the method described by Sadashivam and Manicum (1996). The cell free supernatant was precipitated with different concentrations of ammonium sulphate, i.e. from 10-90%. The precipitate was dissolved in small amount of 0.5 M glycine-NaOH buffer (pH 10) and dialyzed over night against the same buffer. Dialysis is a very simple technique used extensively to separate macromolecules from smaller molecules. The dialyzed enzyme was used for following studies.

RESULTS

Isolation of protease producing microorganisms: In the present study, 20 isolates were isolated from tannery effluent and was screened for alkaline protease production by inoculating them in gelatin agar and skim milk agar. Among which three *Pseudomonas* sp. showing skim milk hydrolysis were selected for the production of alkaline protease and were named as ABP 4, ABP7 and ABP10 (Fig. 1). The zone of skim milk agar hydrolysis was found to be 0.9 mm by ABP 4 followed by 0.8 mm by ABP7 and 0.6 mm by ABP10 (Fig. 2).

Enzyme assay: Alkaline protease production by selected three *Pseudomonas* species ABP4, ABP7 and ABP10 was determined. Among the three isolates, Pseudomonas isolate ABP4 had maximum enzyme activity than other two isolates. Pseudomonas isolate ABP4 exhibited the highest alkaline protease activity (1.56 U/mL/min) on submerged fermentation (Fig. 3).

Optimization of culture conditions for enzyme production: The enzyme activity was increased from 2nd day of incubation to 4th day and it is optimum (1.56 U/mL/min) on 72 h of fermentation at regular temperature 37°C, pH 8 and it is gradually decreased on 96 h of incubation in pseudomonas ABP4 inoculated media (Fig. 4).

Fig. 1: Casein hydrolysis by pseudomonas

Fig. 2: Enzyme assay by Plate Zone Method

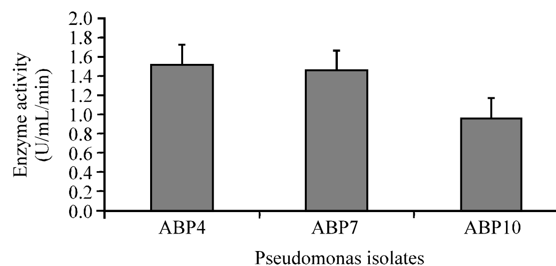

Fig. 3: Alkaline protease production. Each value is an average of 3 replicate samples. ±Standard error

The production of alkaline protease enzyme was affected with pH change of the medium. The alkaline protease enzyme activity was found maximum at pH 8 (1.9 U/mL/min). The enzyme activity was decreased slowly above pH 8 (Fig. 5). The production of alkaline protease

enzyme was highest at 37°C of 1.65 U/mL/min for Pseudomonas ABP4, the alkaline protease production decreased significantly at 40°C (Fig. 6). The study of alkaline protease production with various carbon sources in the medium showed that the enzyme production was more with glucose (4.5 U/mL/min) (Fig. 7). The remaining isolates exhibited less enzyme activity than pseudomonas ABP4 in medium supplemented with different carbon sources. The selected pseudomonas ABP4 grown well with all nitrogen sources tested. Soyabean meal was the best among all nitrogen sources tested with 4.7 U/mL/min alkaline protease activities (Fig. 8). The remaining isolates exhibited less enzyme activity than pseudomonas ABP4 in medium supplemented with different nitrogen sources.

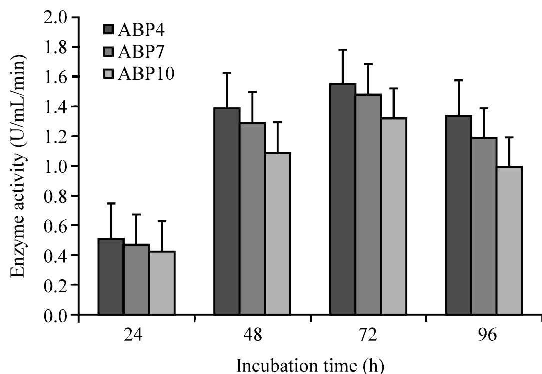

Fig. 4: Effect of incubation period on alkaline protease production. Each value is an average of 3 replicate samples. ±Standard error

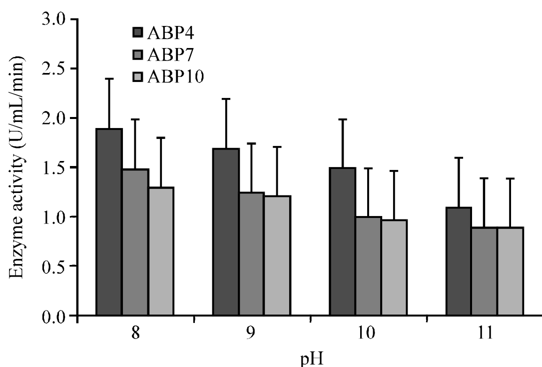

Fig. 5: Effect of pH on alkaline protease production. Each value is an average of 3 replicate samples. ±Standard error

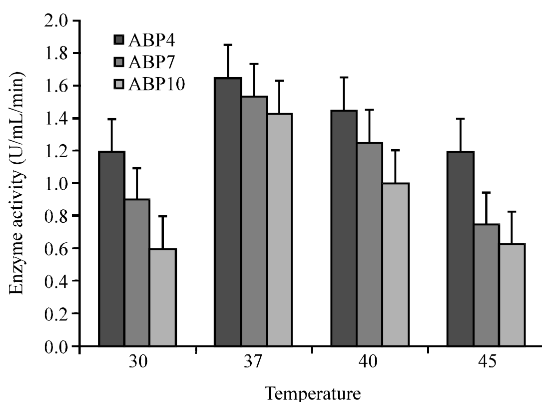

Fig. 6: Effect of temperature on alkaline protease production. Each value is an average of 3 replicate samples. ±Standard error

Effect of various metal ions on alkaline protease activity: A metal ion in media is an important factor that affects enzyme production. Most of the metal ions tested had a stimulatory effect (Cu^{2+}, Mg^{2+}) on enzyme activity. These metal ions protected the enzyme from thermal denaturation and maintained its active conformation at the high temperature. In addition, alkaline protease required a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations for its maximum activity

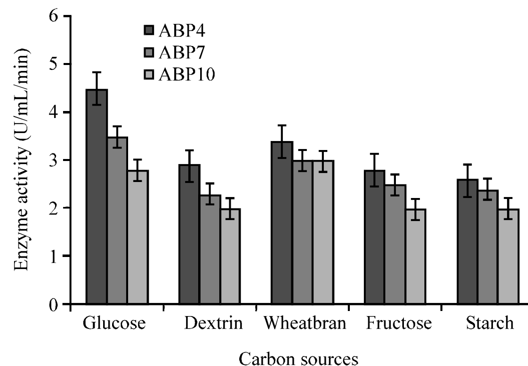

Fig. 7: Effect of different carbon sources on alkaline protease activity. Each value is an average of 3 replicate samples. ±Standard error

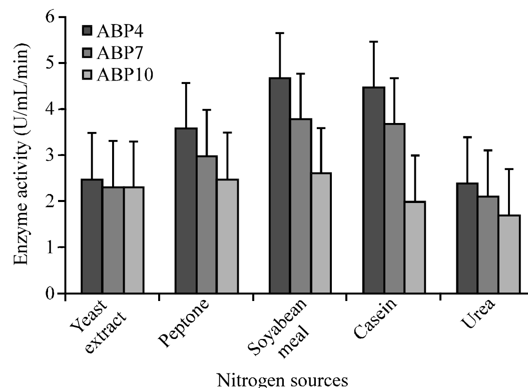

Fig. 8: Effect of different nitrogen sources on alkaline protease activity. Each value is an average of 3 replicate samples. ±Standard error

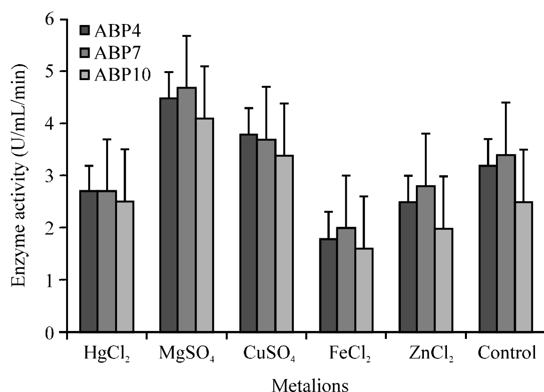

Fig. 9: Effect of various metal ions on alkaline protease activity. Each value is an average of 3 replicate samples. ±Standard error

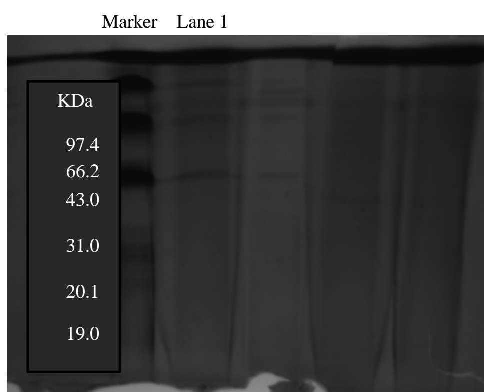

Fig. 10: SDS-PAGE of the alkaline protease from Pseudomonas isolate. Lane M: Molecular mass markers; Lane 1: Partially purified alkaline protease

(Kumar and Takagi, 1999). $FeCl_2$ has a strong inhibitory effect where as $ZnCl_2$, $HgCl_2$ have mild effects on alkaline protease activity (Fig. 9). Interestingly $MgSO_4$, $CuSO_4$ strongly activated enzyme activity at 50 mM concentration.

Partial purification and characterization of alkaline protease: Molecular weight of the partially purified alkaline protease from pseudomonas isolate ABP4 was found to be 43 kDa on SDS-PAGE (Fig. 10).

DISCUSSION

Alkaline protease producing bacteria were isolated from leather industry effluents and identified as *Pseudomonas* species. The identified bacterial isolates were plated on the skim milk agar plates and incubated at 37°C for 24 h until a clear zone of skim milk hydrolysis gave an indication of alkaline protease producing organism at pH 8. Among the three isolates, pseudomonas isolate ABP4 had maximum enzyme activity than other two isolates. Maximum growth and maximum enzyme production was observed at 96 h when cultured in casein containing medium (pH 8) under shaking condition (~150 rpm) at 37°C. The addition of carbon source in the form of either monosaccharides or polysaccharides could influence the production of enzyme (Saxena *et al*., 2007). Among the carbon sources, glucose and wheat bran were found to support protease production. The best nitrogen source for the pseudomonas ABP4 was soyabean meal and casein, respectively. However, production medium enriched with soyabean meal has been reported as best nitrogen source for protease production as stated by Sinha and Satyanarayana (1999). Protease production was found to be maximum at 72 h. The enzyme activity gradually decreases from 72 h. This finding is in partial agreement with findings of Kumar (2002) who reported that *Pseudomonas* sp. S22 showed a peak for protease production at 24 h of incubation and again peaks at 108 h which was against the present study. In order to determine the influence of pH and temperature the production medium was adjusted to various levels of pH. The organism was found to grow over a wide range of pH and temperatures. Pseudomonas isolate ABP4 showed enzyme production at pH 8. Similar findings were reported by Shimogaki *et al*. (1991) and Kaur *et al*. (1998) from some thermophilic *Bacillus* sp. the maximum of enzyme production was observed at pH 8. Initial pH of the production medium is the most important factor that significantly influences the production of proteases (Da Silva *et al*., 2007). Proteases that having optimum pH between 8 and 12 are having potential applications in the fields of detergent application, dehairing of hides and silver recovery from waste X-ray and photographic films (Rao *et al*., 1998). Majority of *Bacillus* sp. showed growth and enzyme production under alkaline conditions. The optimum temperature for alkaline protease production by Pseudomonas isolates was found to be 37°C (although it grows and produces alkaline protease in the range of 30-40°C). The results show that the bacterium cannot be assumed as a thermophile species because no growth and enzyme production were detected at temperatures above 40°C. Cu^{2+}, Mg^{2+} strongly activated enzyme activity. The relative activity of Mg^{2+} was 125% compared to control. The relative activity of Cu^{2+} was 105% compared to control. Abu Sayem *et al*. (2006) reported that enzyme activity was accelerated by the addition of Mg^{2+}, Ca^{2+} and Mn^{2+} where as it was inhibited by Hg^{2+}. On the other hand, Yossan *et al*. (2006) reported

that Mn^{2+}, Ca^{2+} and Mg^{2+} ions have been described to increase the relative protease activity produced by *Bacillus megatarium* isolated from Thai fish sauce. These ions also have been reported to increase the thermal stability of proteases (Paliwal *et al*., 1994). Thus, concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperature (Donaghy and Mckay, 1993). Molecular mass of the partially purified alkaline protease from pseudomonas isolate ABP4 was found to be 43 kDa on SDS-PAGE. Najafi *et al*. (2005) described thermostable alkaline proteases of size 36 and 38 KDa from *Pseudomonas aeruginosa* strain PD100.

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