

Full Length Research Paper

# Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme

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Soil samples from different habitats including tanneries, soap industries, garden soil and soil compost were screened for the presence of alkalophilic *Bacillus* isolates capable of producing alkaline protease in large quantities. One hundred and eighteen (118) isolates were found having proteolytic activity on skim milk agar plates. Isolates forming larger zones, as a result of casein hydrolysis were further studied for quantitative production of extracellular alkaline protease activity in the shake flask studies. Isolate CEMB10370 gave maximum activity. Time course studies indicated that strain CEMB10370 had the highest protease activity (380 APU/mL) after 48 h of fermentation. The wild type enzyme was biochemically characterized. The enzyme exhibits optimal activity at 50°C and pH 11.5. The protease enzyme was completely inhibited by phenylmethylsulfonyl (PMSF, serine protease inhibitor) and its isoelectric point was ~9.5. The enzyme was purified by ion-exchange chromatography using CM-Sepharose column as a ~29 Kilo Dalton (kDa) protein.

**Key words:** Alkaline protease, alkalophilic, *Bacillus subtilis*

## INTRODUCTION

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which is expected to exceed \$ 2.9 Billion by 2012 (Jon, 2008). They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar et al., 2007; Deng et al., 2010; Jellouli et al., 2009). Sources of proteases include all forms of life, that is, plants, animals and microorganisms.

Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases perform best at pH range of 2.0-5.0 and are mostly produced by fungi.

Proteases having pH optima in the range of 7.0 or around are called neutral proteases. Neutral proteases are mainly of plant origin. While proteases having optimum activity at pH range of 8 and above are classified as alkaline proteases. Alkaline proteases produced from microorganisms play important role in several industries example detergent, tanning, photographic industries, pharmaceutical and waste treatment etc. (Gupta et al., 2002).

The genus "*Bacillus*" is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production (Ferrari et al., 1993; Kocher and Mishra, 2009). They are widely distributed in soil and water, and certain strains tolerate extreme environmental conditions including highly alkaline conditions. Isolation of alkaline protease producing *Bacillus* spp. has been reported from a variety of sources including soils characterized by high pH and/or the presence of detergent contamination (Singh et al., 1999; Hsiao et al., 1994), dried fish (Centeno et al.,

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1996), sand soil, milk processing plant, slaughterhouses (Adinarayana et al., 2003). Screening of alkaline proteases producing *Bacillus* spp. from different ecological environments can result in isolation of new alkaline proteases with unique physio-chemical characteristics (Shumi et al., 2004; Singh et al., 1999). One of the most important characteristics that determine the industrial suitability of proteases is their requirement of high pH for optimum enzyme activity. This study reports isolation of a new bacillus isolate CEMB 10370 capable of producing higher amount of alkaline protease. The extracellular enzyme of CEMB 10370 was identified as serine protease with isoelectric point of ~9.5 and optimal activity at 50°C and pH 11.5.

## MATERIALS AND METHODS

### Isolation and screening of alkaline protease producing *Bacillus* spp

Soil samples were collected from different habitats including, tanneries, soap factory, garden soil, soil composite and waste dumping areas. One gram of soil sample was added to a glass tube containing 10 mL sterilized distilled water. The test tube containing the soil suspension was placed in a water bath at 80°C for 15 min. After 15 min they were immediately cooled in ice-cold water. Samples of 50 to 100 µL were spread on the S.K agar plate (Bacto tryptone 1%, NaCl 0.5%, BactoYeast Extract 0.5% and Skim milk 1%). These plates were incubated at 30°C for 48 h. Colonies forming transparent zones, because of partial hydrolysis of milk casein, were selected. Purified cultures of selected isolates were streaked on LB agar slants and stored at 4°C.

### Shake flask studies for screening of isolates

A loop full of the culture from fresh slants was inoculated in 25 mL of autoclaved S.K medium in a 250 mL conical flask. The flasks were incubated at 30°C for 18 h and agitated at 250 rpm in a shaker.

Overnight grown bacterial starter cultures (1%, v/v) were transferred to 50 mL S.K medium in 250 mL flasks. The flasks were incubated at 30°C for 48 h and agitated at 180 rpm in a shaker. After 48 h of incubation, 1 mL of broth culture was transferred to 1.5 mL sterilized tubes and centrifuged at 14,000 rpm for 5 min. Supernatant was used to determine proteolytic activity present in the culture supernatant.

### Protease assay

Quantitative analysis of alkaline protease activity in the supernatant was determined by the modified method of Horikoshi (1971). In this method, 200 µl of enzyme solution (5 X diluted) was added in 2.5 ml of 0.6% casein solution prepared in 0.05M Glycine-NaOH buffer pH 11.0 and incubated at 30°C for 20 min. To stop the reaction, 2.5 ml of T.C.A mixture (0.11M T.C.A, 0.22M CH<sub>3</sub>COONa, 0.33M CH<sub>3</sub>COOH) was added to the reaction mixture. The mixture was again incubated at 30°C for 20 min and then centrifuges at 10,000 rpm for 5 min. Absorbance of the clear supernatant was measured at 275 nm. The blank was prepared by mixing the buffer instead of

enzyme and performing the rest of the steps similar to the experimental samples.

One unit of protease activity was defined as “that quantity of enzyme, which liberates amino acids and non precipitated peptides equivalent to 1 µg/mL of tyrosine per minute under assay conditions”.

### Characterization of selected isolate

Morphological and physiological properties of selected isolate CEMB No. 10370 were investigated according to the methods described in “Bergey’s Manual of Systematic Bacteriology 1986”.

### Physio-chemical characterization of the CEMB10370 crude extracellular protease

#### Time course studies

Three flasks (1 L) containing 200 mL of S.K medium were inoculated with 1% overnight starter culture of CEMB10370 prepared in L.B broth (5 mL). Quantitative analysis of protease activity present in the culture supernatant was determined till the 68<sup>th</sup> h of fermentation at different time intervals.

#### Determination of optimum pH

The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (pH 5-12). For that purpose, 0.6% casein (substrate) used in the assay was dissolved in the following buffers: Acetate buffer (pH3.5-4.5), Citrate-phosphate buffer (pH5-6), Phosphate Buffer (pH 7-8), Glycine-NaOH buffer (pH 8.6-11.5), KCl-NaOH buffer (pH 12).

#### Determination of optimum temperature

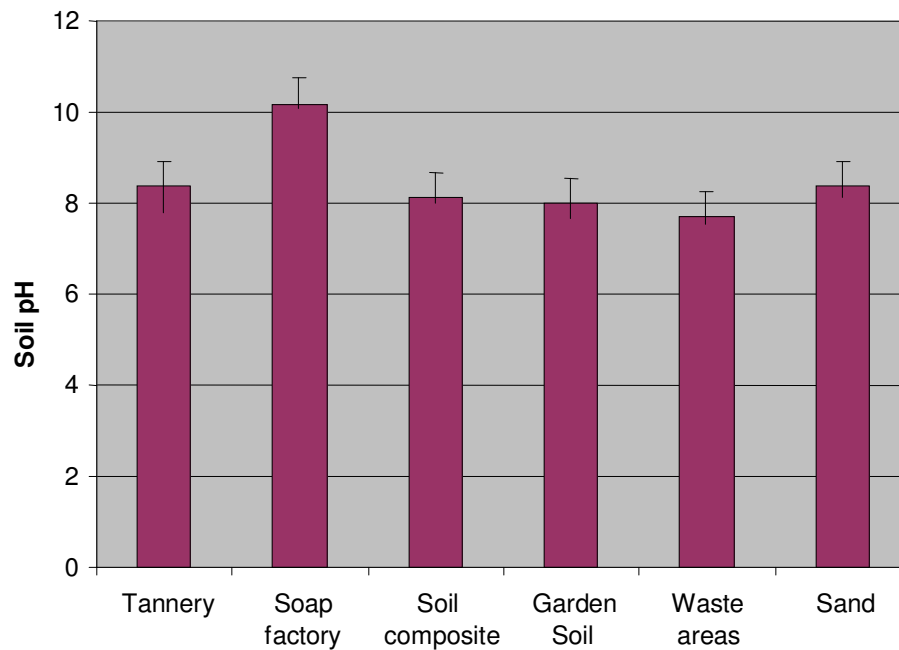
Protease activity was determined by incubating reaction mixtures containing enzyme at different temperatures, that is, 20, 30, 40, 50, 60 and 70°C. Substrate solution was prewarmed at the required temperatures before the addition of enzyme. After incubation, the enzyme activity was assayed.

#### Determination of thermal stability

Prior to conducting protease assay, 5X diluted crude enzyme was incubated at different temperatures (30, 40, 50, 60 and 70°C) for 10 min with and without 0.5 mM CaCl<sub>2</sub>. After incubation at different temperatures, enzyme was shifted immediately to ice and used for the determination of residual protease activity.

#### Protease inhibition studies

Effects of protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and Ethylenediaminetetraacetic acid (EDTA) were determined by incubating 5X diluted extracellular crude protease with different concentrations of PMSF (1, 5 and 10 mM) and 10mM EDTA, separately, at 35°C for 30 min. Residual protease activity was



**Figure 1.** pH value of soil samples collected from different habitat.

determined.

#### Determination of isoelectric point

Isoelectric focusing of the protein present in the culture supernatants was conducted by performing preparative IEF (Isoelectric focusing) using the Biorad rotore electro-focusing cell according to manufacturer instruction. The pH and proteolytic activity present in each fraction was determined.

To generate profiles of different proteins present in each fraction with better resolution of protein bands, SDS-PAGE (12%) was carried according to Laemmli (1970) on 1.5 mm thick and 15 cm long gel, silver staining of the gel was carried out according to Bloom et al. (1987).

#### Purification of alkaline protease

Partial purification of the alkaline protease was obtained with ammonium sulphate precipitation (80% saturation). The partially purified protease was further purified by ion-exchange chromatography using CM-Sepharose column. The column equilibrated with 50 mM phosphate buffer (pH 7.4) was eluted with linear salt gradient of NaCl (0.0 to 1 M) to collect different proteins fractions. The proteolytic activities of fractions obtained were compared by hydrolyzing 0.6% casein substrate with 200  $\mu$ l of each fraction for 30 min at 30°C as described previously, absorbance of the clear supernatant was measured at 275 nm.

## RESULTS AND DISCUSSION

The pH values of all the soil samples were in alkaline region ( $\geq$ pH8), the highest pH values (pH 10.5) were

observed for the soil samples isolated from soap factories, followed by samples from tanneries (Figure 1). As a result of initial screening of the soil samples, 118 isolates were found having proteolytic activity on S.K agar plates (Figure 2). Extracellular proteolytic activity of various isolates resulted in partial hydrolysis of milk casein present in the S.K agar medium, resulting in the formation of clear zones of different sizes. Isolates forming larger zones were further screened quantitatively for the production of alkaline protease in the shake flask containing 50 mL of S.K medium (Figure 3a). Isolate S-10 gave maximum activity (Figure 3b), and was deposited in strain CEMB collection center under the accession number strain CEMB 10370. The morphological and physiological properties of the strain CEMB 10370 were determined and it was observed that the isolate strain CEMB 10370 belonged to the genus *Bacillus* (Table 1).

Proteases are largely produced during the onset of stationary phase and thus are generally regulated by carbon and nitrogen stress (Gupta et al., 2002). Big variation is reported between the incubation periods for attaining optimal alkaline protease production by different *Bacillus* spp., with some *Bacillus* spp. reaching optimal production just after 6 to 8 h of fermentation (Longo et al., 1999) and other's reaching after 18 to 96 h of incubation (Beg et al., 2002; Mabrouk et al., 1999; Morita et al., 1998; Oberoi et al., 2001; Puri et al., 2002; Singh et al., 2001). In this study, maximum activity of 372 APU/mL was attained after 48 h of fermentation, after which the activity started to decline (Figure 4).

Physio-chemical characterization of extracellular

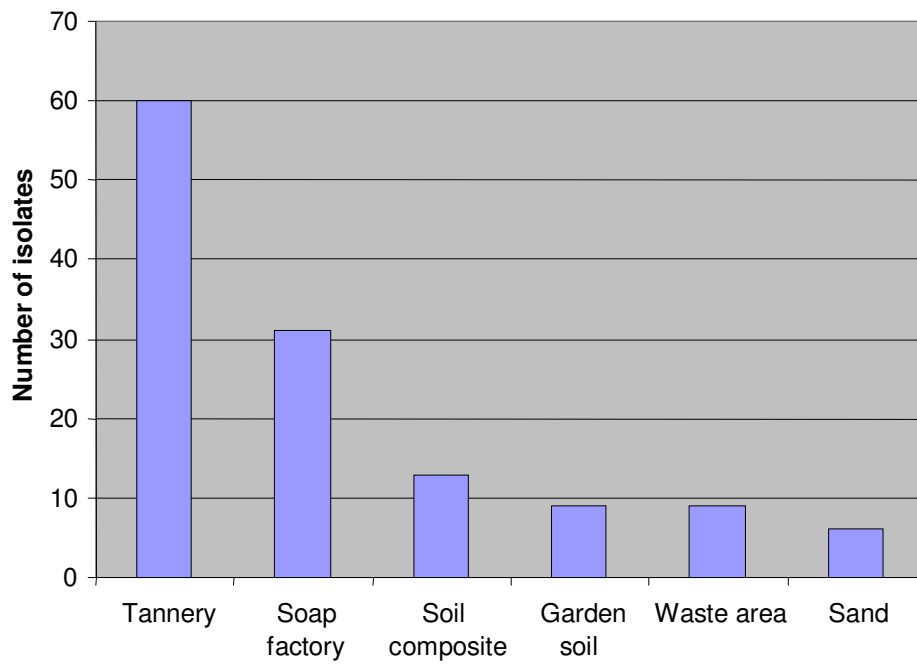
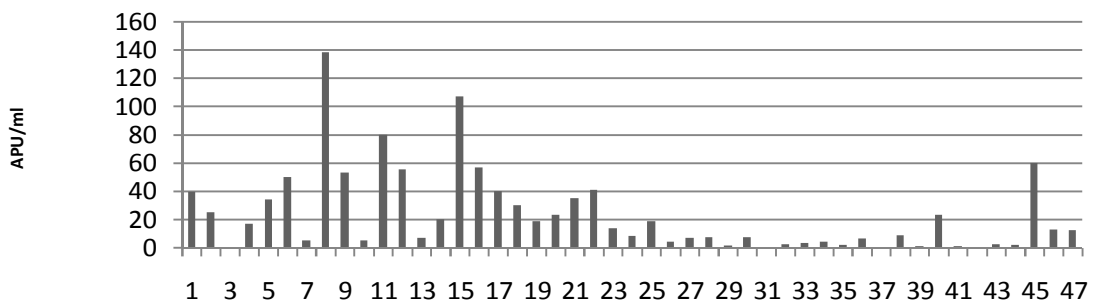
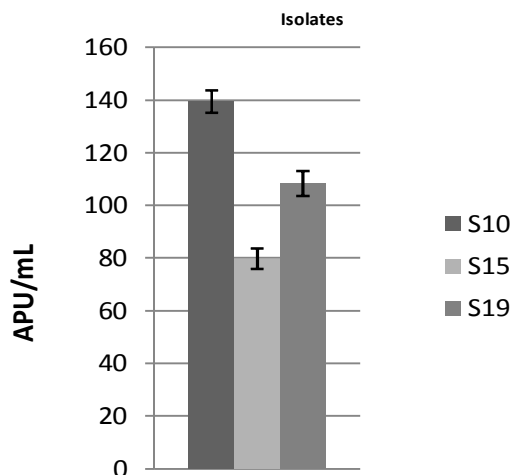


Figure 2. Isolation of bacterial cultures from different habitats

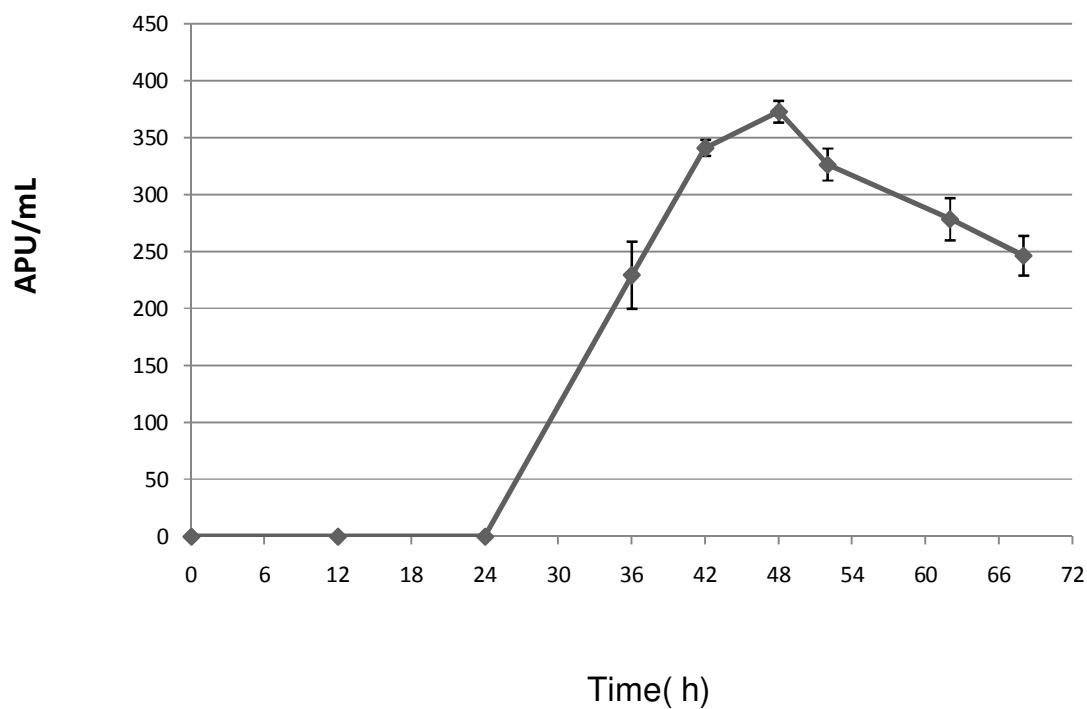


(a)

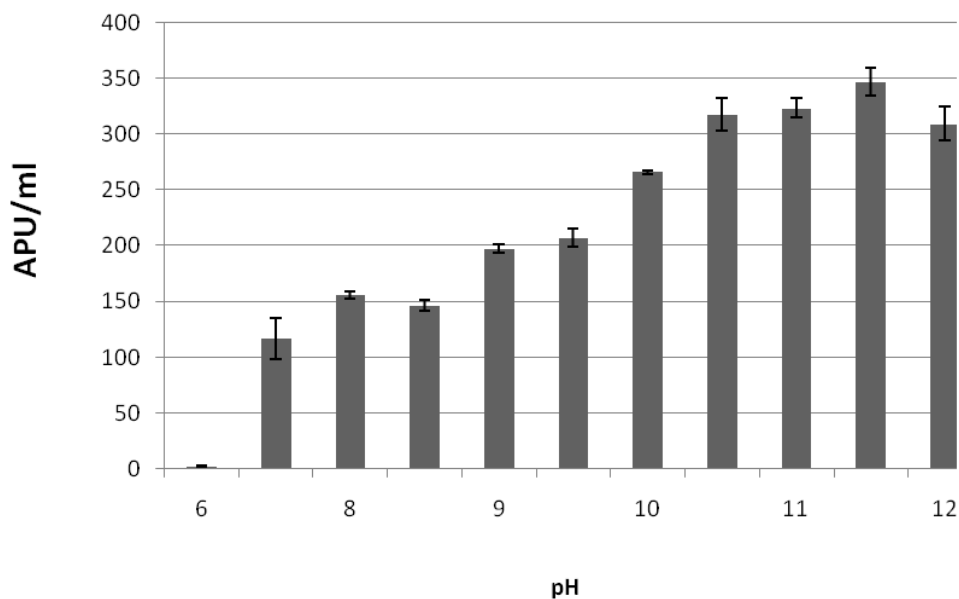


(b)

Figure 3. (a) Proteolytic activity of isolates in shake flask studies, (b). Isolates showing higher activity in shake flask studies.



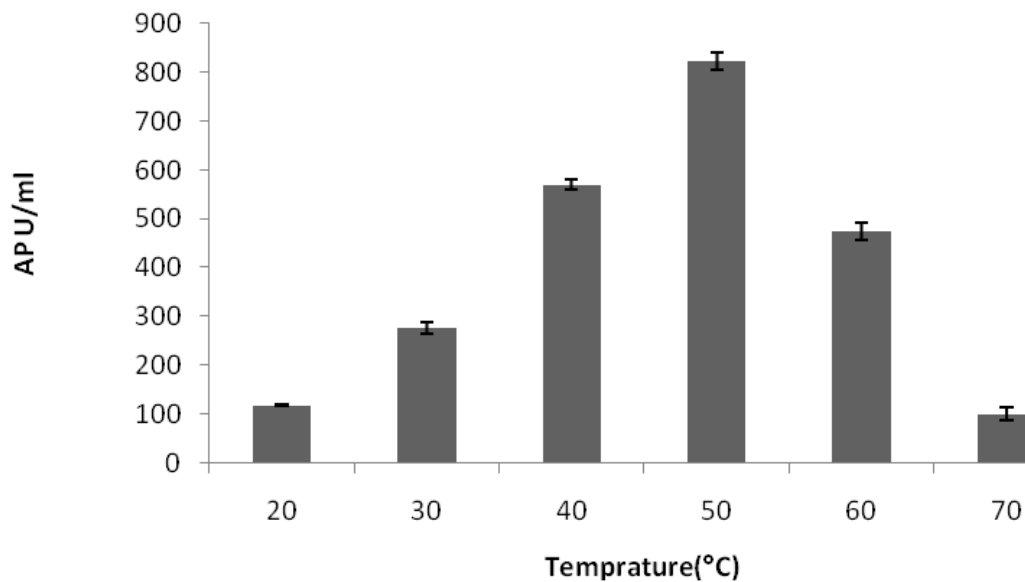
**Figure 4.** Time course studies of the extracellular protease activity of strain CEMB 10370.



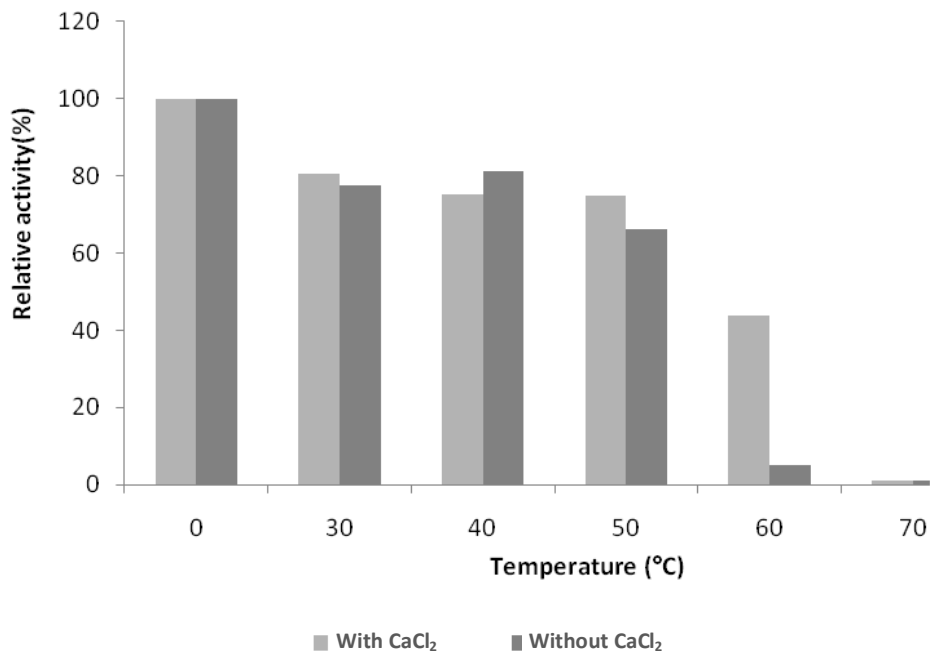
**Figure 5.** Determination of the optimum pH for maximum alkaline protease activity of CEMB 10370.

alkaline protease present in the culture supernatant of strain CEMB 10370 showed that very high pH value of 11.5 was required for its optimal activity (Figure 5), which is a very suitable characteristic for its industrial accept-

ability and a common feature of most of commercialized alkaline proteases from *Bacillus* spp. While conducting studies on optimal temperature requirement, maximum activity was observed at 50°C (Figure 6). While



**Figure 6.** Determination of the optimum temperature for maximum alkaline protease activity of CEMB 10370.

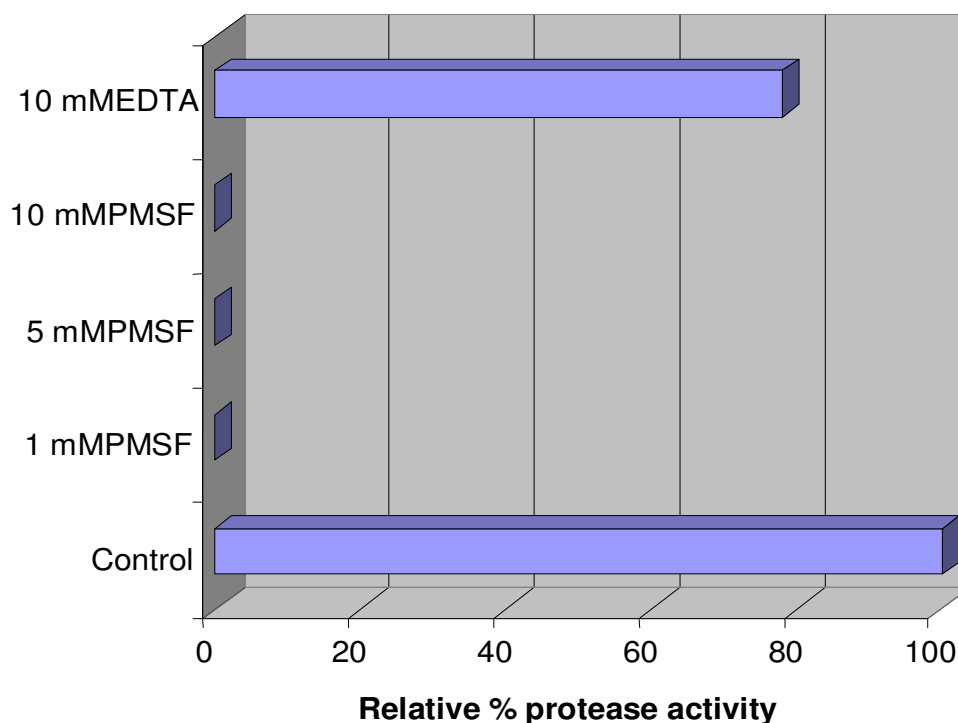


**Figure 7.** Determination of the heat stability of the extracellular proteases of CEMB 10370.

conducting thermal stability studies with and without CaCl<sub>2</sub>, complete abolishment of proteolytic activity was observed at 60°C in the case of enzyme without 0.5M CaCl<sub>2</sub>, while 40% of enzyme activity was retained by enzyme containing 0.5M CaCl<sub>2</sub>. But no activity was observed for both enzyme mixed at 70°C, indicating complete denaturation of enzyme (Figure 7). The physio-

chemical characterization of the protease indicates its suitability as a potential additive in detergents.

*Bacillus* are known to secrete two major types of proteases, that is, serine and metallo protease, both having application in industries. Yet serine alkaline protease dominates the industry due to its widely suitable physio-chemical features needed for industrial consump-



**Figure 8.** Effect of protease inhibitors on CEMB 10370 extracellular proteases.

tions. Protease can easily be characterized based on their inhibition on exposure to different protease inhibitors.

We used PMSF (serine protease inhibitor) and EDTA (metallo protease inhibitor) for determining the nature of protease present in the culture supernatant. Complete inhibition was observed with 1 to 10 mM PMSF, while 28% proteolytic activity was reduced by the crude protease treated with 10 mM EDTA (Figure 8), indicating small percentage of metallo protease present in the culture supernatant and serine protease being the major protease in the culture.

Alkaline proteases mostly have their isoelectric points near to their pH optimum in the range of 8 to 11 (Gupta et al., 2002). It is also known that proteases perform best when the pH value of the solution in which it is working example detergent solution, is approximately the same as the pI value (isoelectric point) for the enzyme (Poza et al., 2001; Urtz and Rice, 2000). Another reason for determination of pI value of the extracellular proteolytic enzyme present in culture supernatant of strain CEMB 10370 was to estimate pH conditions requirements, for further purification of protease using the ion exchange chromatography.

Maximum activity was observed in fraction with pH 9.8 (Figure 9), which was the maximum pH limit of the ampholytes used, since no single band was observed in the fraction with maximum activity (Figure 10), further

purification was performed using separation of CM-Sepharose columns. Alkaline proteases are generally positively charged and they are not bound to anion exchangers (Kumar and Takagi, 1999). Therefore, cation exchangers are the method of choice in most of the cases (Kumar, 2002).

Ammonium sulphate precipitated (80% saturation) and dialyzed, partially purified protease was further purified by ion-exchange chromatography using CM-Sepharose column. The proteolytic activities of fractions obtained were compared (Figure 10). The protein in the active fractions as analyzed by SDS-PAGE, showed presence of a single band just below 29 kDa marker protein ((Figure 11), which lies within the molecular weight range reported for most of the alkaline proteases isolated from *Bacillus* spp. (Johnvesly and Naik, 2001; Kumar et al., 1999; Singh et al., 2001; Beg et al., 2002; Gupta et al., 1999).

The separated fractions had different proteolytic activities (Figure 11), a single band 29 kDa was obtained in active fraction (Figure 12), which lies within the molecular weight range reported for most of the alkaline proteases isolated from *Bacillus* spp. (Beg et al., 2002; Gupta et al., 1999; Johnvesly and Naik, 2001; Kumar et al., 1999; Singh et al., 2001).

It is concluded that as a result of this studies, a new *Bacillus* isolate CEMB 10370 was isolated, capable of producing proteolytic enzyme, giving optimal performance

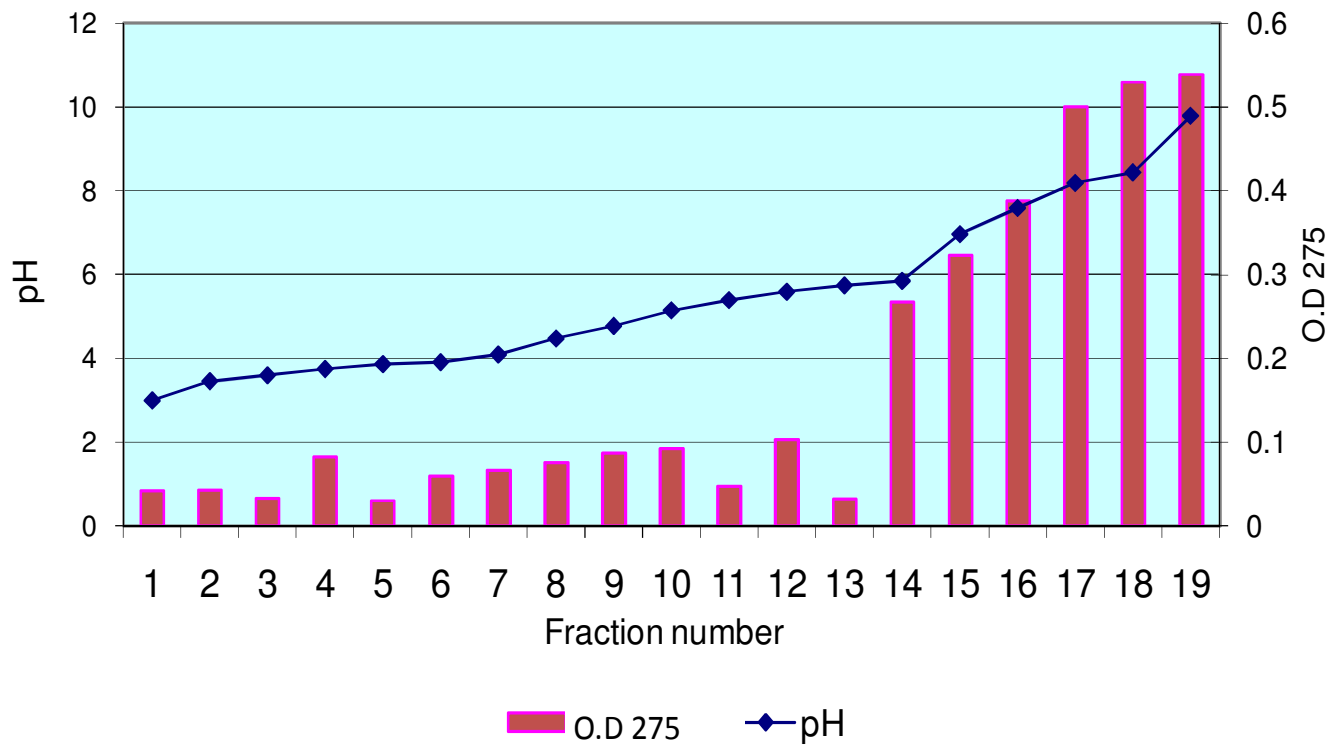


Figure 9. Protease activity and pH values of CEMB 10370 extracellular proteins in Rotofor-separated fractions.

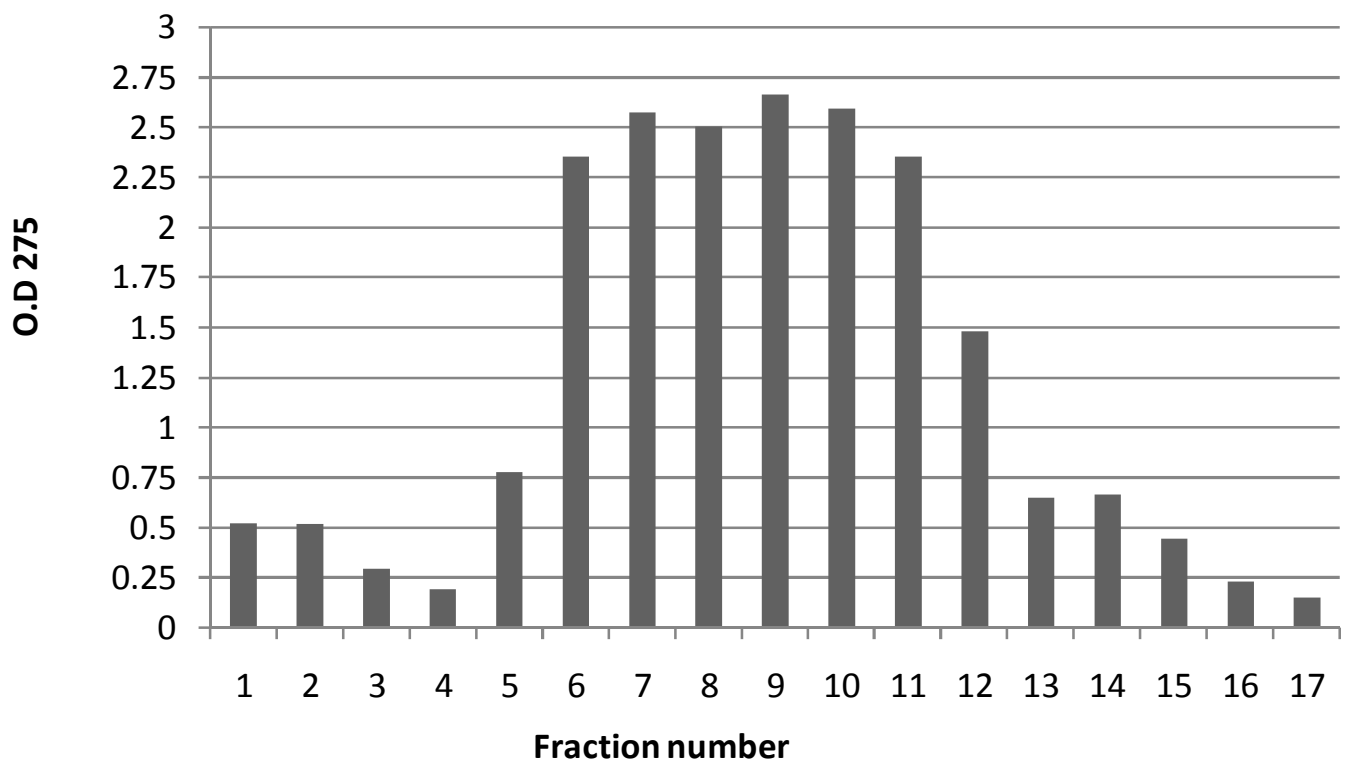
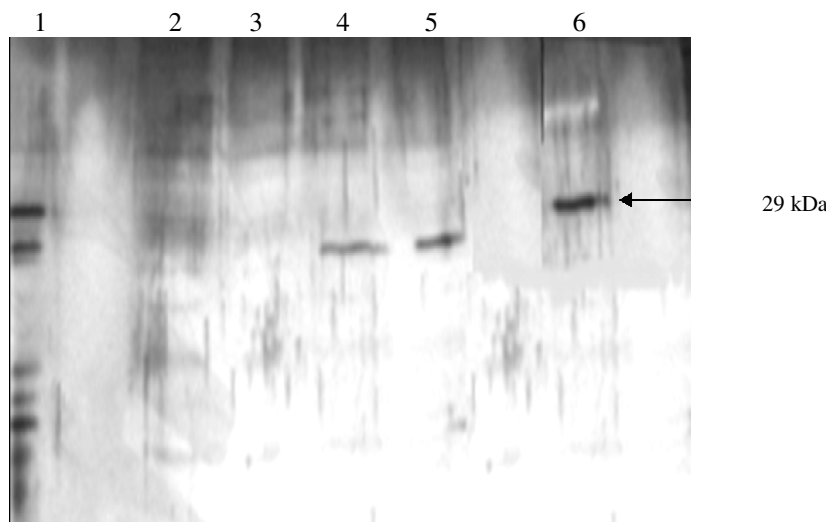
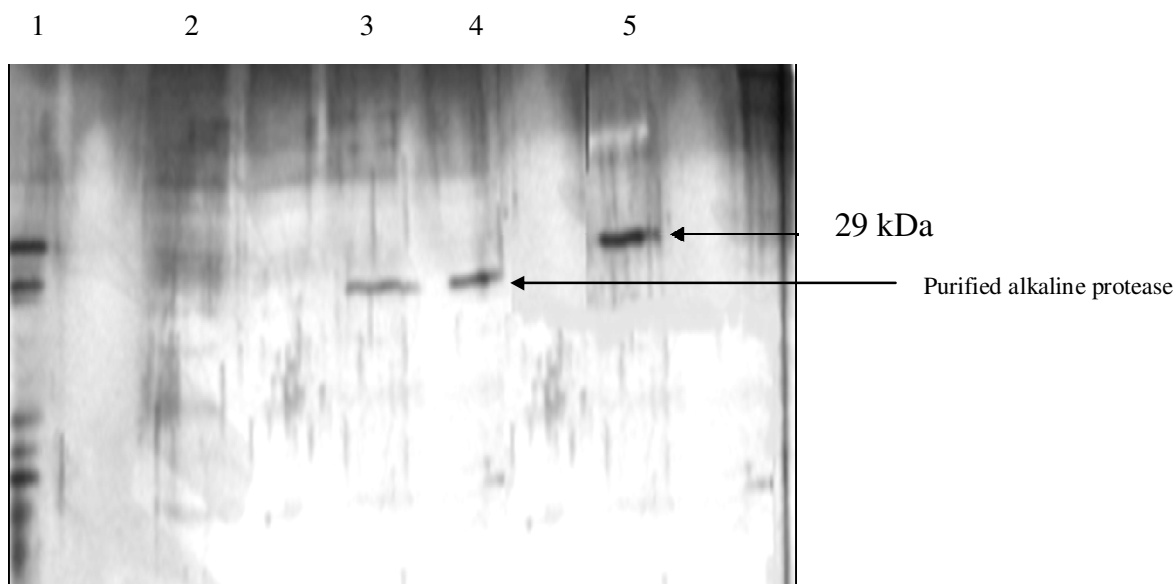


Figure 10. Proteolytic activity in different fractions of CEMB 10370 extracellular proteins, separated by CE-Sepharose





**Figure 11.** SDS -PAGE profiles of the purified alkaline protease. Lane: 1, Flow through; lanes 2 and 3, inactive fraction; lanes 4 and 5, fractions with high protease activity; lane 6, purified proteinase K (29kDa) as marker.



**Figure 12.** SDS -PAGE profiles of proteins present in different fractions of CEMB 10370 using CE-Sepharose columns. Lane 1: Flow through; lane 2, Fraction 5; lane 3, Fraction 6; Lane 4, proteinase K (As marker).

under highly alkaline conditions, making it a potential candidate for industrial applications where proteolytic activity is required under highly alkaline conditions.

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