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Research Article

Effect of Different Carbon and Nitrogen Sources on Protease Production and Partial Characterization from *Bacillus* sp. DK1

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Abstract

Alkalophilic bacterial strains (15) isolated from soil samples of compost, flour mill and milk processing industry were screened on skim milk agar medium for their ability to produce alkaline protease. The most potent producer was identified as *Bacillus* sp. DK1. Basic culture parameters influencing the enzyme synthesis in shaker flask and bioreactor cultures were evaluated to improve the yields of the process. The addition of glucose 0.7% (w/v) and beef extract 1.25% (w/v) enhanced protease production. The strain was able to produce protease at 45°C at pH 9.0. The optimized culture conditions for the production of protease were increased up to 142.5±1.2 U/mL. The molecular weight of partially purified protease was found to be ca 67 kDa. The protease enzyme had an optimum pH of 9.0 and exhibited highest activity at 65°C. The purified protease showed a maximum relative activity on casein substrate. Enzyme activity was strongly enhanced by the metal ions Ca^{2+} and Mg^{2+} and was strongly inhibited by PMSF, suggesting that it belongs to the family of serine proteases. The purified protease remove bloodstain from cotton cloths completely when used with Power Active detergent.

Keywords: Alkaline protease, *Bacillus* sp., caseinolytic activity, purification, characterization.

Introduction

Protease is one of the most important industrial enzymes and accounts for at least a quarter of the total global enzyme production (Han *et al.,* 2005). These proteases are being widely used as industrial enzymes in the detergent industry, since their introduction in the 1914 as detergent additive. Alkaline proteases secreted by both neutrophilic and alkalophilic bacilli are of particular interest due to their wide applications in laundry detergents, leather processing, protein recovery or solubilization, organic synthesis, meat tenderization, detergents, food industry, photography and pharmaceuticals etc. (Cowan 1996; Quasim and Rani, 2003). The use of alkaline proteases has increased significantly in various industrial processes such as detergent and feed additives, dehairing, decomposition of gelatin on X-ray films and peptide synthesis (Layman 1986; Kembhavi *et al.,*, 1993; Gessesse, 1997; Stevenson *et al.,*, 1998; Masui *et al.,*, 1999). A solvent stable metalloprotease produced by *Bacillus* sp. TKU004 is used in the deproteinization of liquid pen for β-chitin preparation (San *et al.,*, 2006). Many researchers have examined various habitats for the production of industrially suitable alkaline proteases (Steele *et al.,*, 1992; Sen and Satyanarayana 1993; Paliwal *et al.,* 1994; Gajju *et al.,* 1996). These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion (Kumar *et al.,* 1999).

Proteases are among the commercially most viable enzymes (Rajesh *et al.,* 2006) and currently a large proportion of these are derived from *Bacillus* strains (Genckal and Tari, 2006). The performance of protease is influenced by several factors, such as pH of industrial process, ionic strength, temperature and mechanical handling. Therefore, extensive studies were made on various nutritional and environmental factors influencing the optimum production. Newer enzymes with novel properties that can further enhance the industrial process using the current enzyme is always in demand. The major use of detergent-compatible proteases is in laundry detergent formulations (Vantilburg, 1984; Anstrup and Andersen, 1974). Detergents available on the international market such as Dynamo, Era Plus and Tide (Procter and Gamble), contain proteolytic enzymes, the majority of which are produced by members of the genus *Bacillus*. Thus it was considered of interest to investigate further the potential of the *Bacillus* protease, in particular as an additive in various bio-formulations such as biological detergents, enzymic debriders and contact-lens cleansing agents. For the production of enzyme for industrial use, it is essential to isolate and characterize the promising strains using cheap carbon and nitrogen sources (Parekh *et al.,* 2000).

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The present work was carried out to optimize the culture conditions for the alkaline protease production by *Bacillus* sp. DK1 isolated from milk processing industry samples and the alkaline proteases was partially purified, characterized and studied for its compatibility with various commercially available detergents.

Materials and methods

Isolation and screening: Samples of fresh compost, flourmill and milk processing industries, collected in sterile bags, were brought to the laboratory and processed for analysis within 6 h of collection. Smashed samples were serially diluted up to 10 fold. The diluted samples were plated onto skim milk agar plates containing peptone (0.1% w/v), NaCl (0.5% w/v), agar (2.0% w/v), and skim milk (10% v/v). Plates were incubated at 37°C for 24 h. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms. Bacterial colonies exhibiting the larger zone were quantitatively determined for protease activity by spectrophotometric analysis. The potential protease producing strain was selected and designated as DK1. This strain was selected for further experimental studies. The bacterium was identified as *Bacillus* sp. DK1 by 16S rRNA gene sequencing. The culture was maintained on Luria Bertani agar slants and stored at 4°C. PCR amplification of the 16S rDNA gene and sequence determination Chromosomal DNA was isolated by a versatile quickprep method for genomic DNA of Gram-positive bacteria with some modifications (Pospiech and Neumann 1995). A PCR was performed in order to amplify the 16S ribosomal DNA of isolate. The primers used were 63f 5`-CAGGCCTAACACATGCAAGTC-3` 1387r 5`-GGGCGGTGTGTACAAGGC-3` (Julian *et al.*, 1998). The DNA sequence of the PCR products was determined by using DNA thermal cycler. Sequencing reaction products were analyzed with automated DNA sequencer (Applied Biosystems). The sequence was deposited in NCBI Gene bank with an accession No. FJ827134.

Enzyme production: Production of protease from *Bacillus* sp. DK1 was carried out in a medium containing the following (w/v): glucose, 0.5%; peptone, 0.75%; FeSO4, 0.01%; $MgSO_4$.$7H_2O$, 0.5% and KH_2PO_4, 0.5%. The initial pH was adjusted to 9.0 and autoclaved at 120°C for 20min. The Erlenmeyer flasks were inoculated with 5% (v/v) of 24h old *Bacillus* sp. DK1 culture and flasks were kept on a rotary incubator shaker at 37°C with agitation 150 rpm for 48 h. After the complete fermentation the broth was centrifuged at 10000 rpm 4°C and clear supernatant was recovered. The crude enzyme was subjected to estimation of protease activity.

Effect of culture conditions of enzyme production: In order to optimize the protease production from *Bacillus* sp. DK1 various culture parameters were employed. To find out the best carbons source, glucose was replaced in the above medium with different carbon sources 0.5% (w/v) like, galactose, maltose, citric acid, lactose, sucrose, starch, fructose, mannose and sorbitol. The effects of nitrogen sources on extracellular protease production was studied by replacing peptone in the medium namely urea, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, KNO_3, beef extract, yeast extract, NH_4NO_3 and gelatin. The effect of different concentrations of the best carbon (glucose) and nitrogen sources (beef extract) on the protease production was studied. In a medium amended with different concentrations of glucose viz., 0.1, 0.3, 0.5, 0.7, and 0.9% and beef extract viz., 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5%. The effect of temperature on enzyme secretion was investigated by incubating culture flasks at various temperatures ranging from 30-60°C in a rotary incubator shaker. For the effect or pH, the culture medium was adjusted to pH range from 5-10 by adding 1% Na_2CO_3.

Enzyme assay and protein determination: The alkaline protease activity was determined by a method of Tsuchida *et al.* (1986). One unit of the protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the experimental conditions used. The amount of protein content of the sample was determined by the dye binding method of Bradford (1976) using bovine serum albumin as standard.

Enzyme production in bioreactor: Protease production from *Bacillus* sp. DK1 was carried out in a lab scale bioreactor, which had provision for 1.5 L working volume (B Braun Biotech International, Germany). One and half liters of optimized medium containing the following (w/v): glucose, 0.7%; beef extract, 1.25%; KH_2PO_4, 0.5%; $MgSO_4.7H_2O$, 0.5%; and $FeSO_4$; 0.01%, pH 9.0 were added to the reactor and sterilized for 30 min at 121°C at 15 lbs. After cooling, 24h old cultures were transferred into bioreactor (2% inoculum per liter). The temperature was kept constant at 45°C±0.05 and the pH was maintained at 9.0±0.05 by the addition of 0.5M NaOH (or) 0.5M H_2SO_4. The agitator and flow rate of filter sterilized air were set at 150 rpm and 8.01/min, respectively. The internal temperature of the bioreactor was maintained at 45°C.

Partial purification: The optimized medium was used for enzyme production in Bioreactor. Unless otherwise stated, all procedures were performed at 4°C. The bacterium *Bacillus* sp. DK1 was grown for 36h and the cells were separated by centrifugation at 10,000 x g, 15 min. The protein in the supernatant was precipitated with ammonium sulfate fractions of 0-40, 40-50 and 50-70% (w/v) was collected by centrifugation at 10,000 x g. The pellet obtained in each fraction was suspended in a minimum

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volume of 50 mM Tris-HCl, pH 9.0 and dialyzed extensively against Tris-HCl buffer (50 mM; 9.0). The dialyzed protein was concentrated by lyophilization and used for further purification steps. The precipitated 50-70% (w/v) fraction was subjected to gel filtration column Sephadex G-100 (1.5 cm x 24 cm) equilibrated with Tris-HCl buffer (50 mM, pH 9.0). The protein was eluted at a flow rate of 30 mL/h with same buffer using an automatic fraction collector. Each fraction was assayed for protein (A280 nm) and protease activity. Protease active fractions were pooled, desalted, filter sterilized, and stored at -20°C.

Characterization of partially purified protease: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the protocol of Laemmeli (1970) with 5% w/v stacking gel and 10% w/v resolving gel at 15 mA in Broviga electrophoresis kit (Broviga, India). Protein bands were stained with silver nitrate and molecular mass of the purified protease was determined by calculating the relative mobility of standard protein markers (Life Tech, India) run alongside.

Effect of temperature and pH on protease activity and stability: The optimum temperature of protease activity was determined with reaction mixture incubated at different temperature ranging from 20 to 90°C in 50 mM Tris-HCl (pH 9.0) buffer for 30 min. For determination of the pH optimum, the enzyme was assayed with different buffers like Phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0-9.0) and glycine-NaOH (pH 10-12) at 45°C. The thermal stability of the enzyme was measured after pre-incubation of the enzyme in 50 mM Tris-HCl (pH 9.0) buffer for 30 min at various temperatures at 20-90°C. The pH stability was determined by measuring the residual activity of the enzyme after 60 min of pre-incubation in the buffers of different pH values (5.0-12.0). The residual activities were measured under standard assay conditions.

Effect of metal ions and inhibitors on enzyme activity: To determine the inhibitory and stimulatory activity, the protease enzyme was pre-incubated with final concentration of 5 mM in magnesium chloride, mercury chloride, calcium chloride, sodium chloride, cobalt chloride, and zinc chloride were investigated by adding them to the reaction mixture at 50 mM Tris-HCl buffer (pH 9.0) at 45°C for 60 min. After 60 min pre-incubation, the reaction was initiated by addition of 1% (w/v) casein and kept at 45°C for 30 min. After 30 min the protease activity was assayed as described earlier. The effect of various protease inhibitors (2 mM) namely phenyl methyl sulphonyl fluoride (PMSF), ethylene diamine tetra acetic acid (EDTA), 2-mercaptoethanol, pepstatin and iodoacetate was determined by pre-incubation with the enzyme solution for 60 min at 45°C before the addition of substrate.

Substrate specificity: Protease activity with different protein substrates such as casein, gelatin, BSA, hemoglobin and egg albumin was assayed by mixing 10 µg of the enzyme and 200 µL of assay buffer containing the protein substrate (2 mg/mL). The reaction mixture was incubated at 45°C for 30 min. After 30 min the protease activity was assayed as described above.

Compatibility with detergents and washing test with protease preparation: The compatibility of *Bacillus* sp. DK1 protease with commercial laundry detergents was studied. Detergents were used namely Power Active (RKN Pvt. Ltd, Puducherry, India), Ariel (Procter and Gamble, India), Surf Excel (Hindustan Lever Ltd., India), and Kite (Dynavista Industries Pvt. Ltd, Puducherry, India). The detergents were diluted to a final concentration of 7 mg/mL in distilled water to give washing condition according to Phadtrae *et al.* (1993) and boiled for 10 min to denature the enzymes present in the detergent solution. The detergent solution was then incubated with protease (1:1) for 1 h at 60°C, and every 10 min interval the residual activity was determined. The enzyme activity of control sample (without any detergent) was taken as100%. Efficiency of the purified protease (1200 U/mL) was studied by mixing with a commercial detergent, Power Active used on a white cotton cloth pieces (4 x 4 cm) stained with blood. They were kept immersed in (a) Distilled water (100 mL) – control, (b) Distilled water (100 mL) + 1 mL detergent (7 mg/mL) (C) Distilled water (100 mL) + 1 mL of detergent (7 mg/mL) + 2 mL enzyme solutions. Then the samples were incubated at 60°C for 15 min and rinsed thoroughly with distilled water and dried. Visual examination of various pieces was made.

Results

Isolation and identification of bacterial strains: Three different samples were analyzed for isolation of proteolytic bacterial cultures. Around 15 morphologically distinct bacterial colonies were isolated from each sample. Isolated cultures were separately screened for their proteolytic activity. Out of total isolates tested, 50% isolates exhibited proteolytic activity at various extents. Four efficient bacterial isolates were selected for qualitative and quantitative assay at different pH (Table 1).

Table 1. Screening of proteolytic bacterial isolates at different pH.

Isolate	Diameter of zone (mm) at different pH			Enzyme activity at pH 9.0 (U mL^{-1})
	7.0	8.0	9.0	
PTK1-G	1.0	0.2	0.4	10.8
DK1	0.5	0.6	14.5	50.2
PTK3-G	0.3	0.2	0.6	14.0
PTK4-G	0.8	0.3	0.3	7.5

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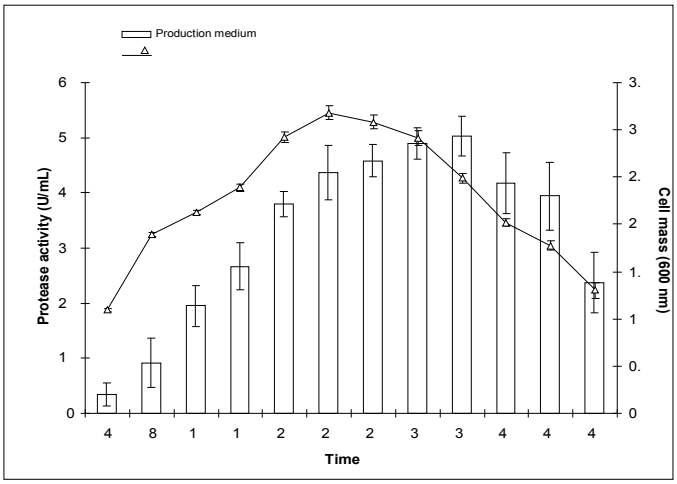
Table 2. Effect of various Temperatures on protease production of *Bacillus* sp. DK1.

Temperature (°C)	Protease activity (U mL^{-1})
30	38
35	41
40	50
45	60.5
50	55.8
55	30
60	22

Fig. 1. Time-course of alkaline protease production and growth of *Bacillus* sp. DK1. Agitation: 150 rpm. Cultivation period: 36 h. pH: 9.0.



Values represent the mean±standard deviations of triplicate sample.

Among the different isolates tested, the isolate DK1 showed highest zone of clearance 14.5 mm at pH 9.0 with proteolytic activity i.e., 50.2±2.4 U/mL, followed by isolate PTK3-G 14.0 U/mL. Other isolates showed maximum activity at pH 7.0. The bacterial isolate was identified as *Bacillus* sp. DK1 based on 16S rRNA partial sequence. Hence, it was selected for further studies. In order to assess potential of the *Bacillus* sp. DK1 as protease producer, protease activities obtained in this work was compared with those reported in literature (Gessesse 1997; Mabrouk *et al.*, 1999; Hamid *et al.*, 2007).

Optimization of culture conditions: At room temperature, while the maximum growth of strain *Bacillus* DK1 observed at 24 h the maximum protease production was recorded at 36 h (Fig. 1). Low amount of protease was produced during the early log phase. However, a rapid increase in protease production was recorded in the late log phase and declining phase of the growth. The results of present study were supported by the findings of Kim *et al.* (2001) and Arulmani *et al.* (2007) where the maximum protease production usually occurs at the late log-phase to the beginning of stationary phase of the growth.

Borris (1987) reported that the alkaline protease production was found to be the maximum at pH 9.0-13.0. Depending upon the strain used, different ranges of protease activities could be obtained varying from 8.4 to 1517 U/mL. Temperature dependent growth studies revealed that *Bacillus* sp. DK1 was able to grow over a wide range of temperature 30-60°C. The maximum protease secretion was observed at 45°C, while at low (30°C) and high (60°C) temperatures it was not significant (Table 2). In the present work protease activity showed of 145.4 U/mL when compared standard medium resulting in 3 fold increase the protease production. In the present work, glucose was found to be a good carbon source for enzyme production.

Role of carbon and nitrogen sources for enzyme production: Among the various carbon sources tested, glucose was found to be the best source for efficient protease production (60.5±0.65 U/mL) (Fig. 2). The addition of sucrose and sorbitol in the production medium declined the protease production. Owing to a substantial increase in the presence of 0.7% of glucose amended medium supported maximum protease production 128±3.6 U/mL (Fig. 4). Figure 3 depicts the effect of nitrogen sources on protease production. Organic nitrogen sources such as beef extract gave maximum protease production of 71.2±0.1 U/mL, followed by Urea 57.0±0.6 U/mL. On using beef extract at graded concentration, 1.25% induced maximum protease production of 142.5±1.2 U/mL (Fig. 4). Hence, beef extract was selected as the best nitrogen source. The protease production in bioreactor 142.5±1.2 U/mL (Fig. 5) was observed in optimized medium when compared with standard medium 3 fold increase of the production. Some workers have also reported that the presence of glucose as a carbon source enhances the maximum enzyme production (Sinha and Satyanarayana, 1991; Gajju *et al.*, 1996). From the observations made, it can be said that the addition of organic nitrogen sources especially beef extract 1.25% seems to be necessary for protease production when compared with other inorganic substituents. Several reports have demonstrated the use of organic nitrogen sources leads to higher enzyme production (Fujiwara and Yamamoto 1987; Gajju *et al.*, 1996). However, addition of inorganic nitrogen sources resulted in decreased production of enzyme.

Purification and characterization of purified enzyme: The 50-70% (w/v) ammonium sulfate fraction showed higher protease activity (3023 U/mL) than 40-50% (w/v). No activity was observed in 0 to 40%. The 50-70% (w/v) ammonium sulfate fraction was subjected to gel filtration chromatography on Sephadex -100 column. A total of 100 fractions were collected. Of them fractions from 38 to 58 showed a single peak of protease activity (data not shown). The results of purification step are summarized in Table 3.

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Fig. 2. Effect of different carbon sources on alkaline protease production. Production medium with beef extract 0.75% as nitrogen source and varying carbon sources (0.5%); agitation: 150 rpm; cultivation period: 36 h, pH: 9.0; by *Bacillus* sp. DK1.

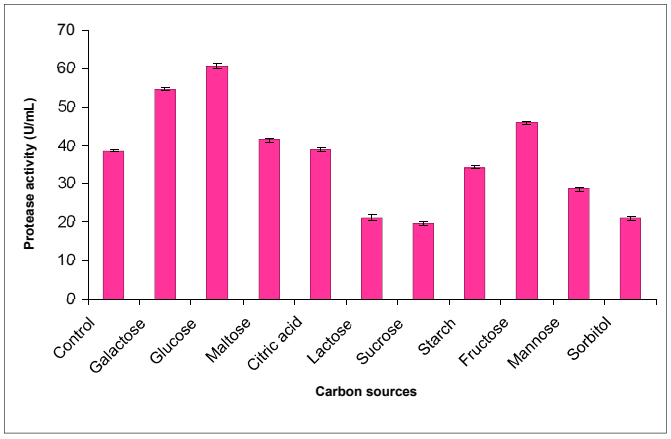

Fig. 3. Effect of nitrogen sources on alkaline protease production. Production medium with beef extract 0.75% as nitrogen source and varying carbon sources (0.5%); agitation: 150 rpm; cultivation period: 36h, pH: 9.0; by Bacillus sp. DK1.

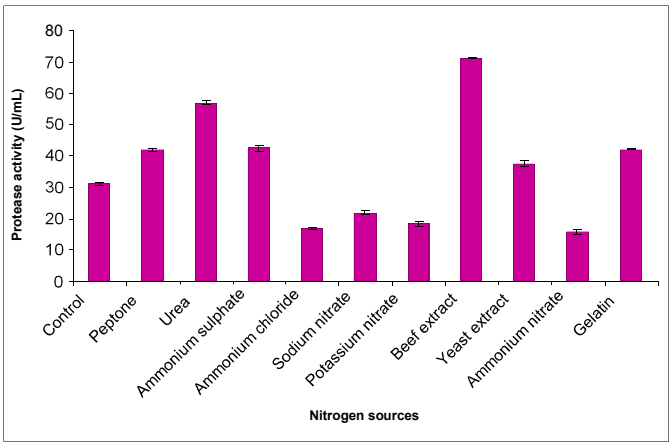

Fig. 4. Effect of different initial concentrations of glucose and beef extract on protease production. Production medium with beef extract 0.75% as nitrogen source and varying carbon sources (0.1- 1.2%); and production medium with glucose 0.7% as carbon source and varying nitrogen sources (0.25-1.5); agitation: 150 rpm; cultivation period: 36 h, pH: 9.0; by Bacillus sp. DK1.

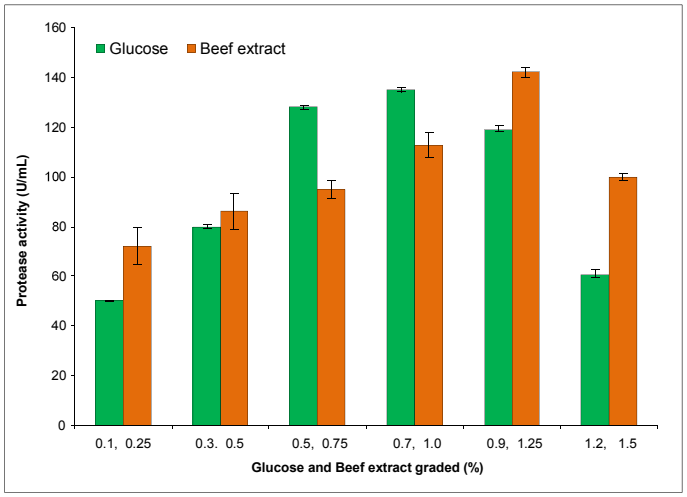

Fig. 5. Time course of alkaline protease production on Bioreactor by *Bacillus* sp. DK1. Agitation: 150 rpm; cultivation period: 36 h, pH: 9.0; by *Bacillus* sp. DK1.

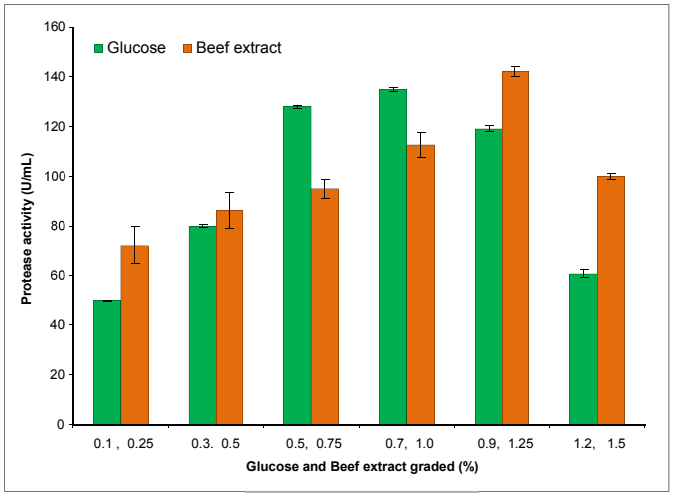

Table 3. Summary of partial purification procedure of alkaline protease from *Bacillus* sp.DK1.

Purification steps	Total activity (U)	Total Protein (mg)	Specific activity (U/mg protein)	Purification fold	Recovery (%)
Culture supernatant	58000	488	118.85	1	100
$(NH_4)_2SO_4$ Fraction 50-70% (w/v)	30230	134	225.59	1.9	52.1
Sephadex G-100	9250	28	330	2.77	15.9

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The purified protease had a specific activity of 330 U/mg of protein, purification fold of 2.77 and recovery of 15.9%. The purified protease showed a single band in SDS-PAGE indicating the homogeneity of purified enzyme. The molecular weight of the protease was found to be 67 kDa as determined by comparison of the migration distances of standard markers (Fig. 6). However Singh *et al.* (2001) reported a serine protease from *Bacillus sphaericus* had a molecular mass of 68 kDa.

Characterization of protease

Effect of temperature and pH on protease activity and stability: The purified protease showed a maximum activity at 60°C and good stability at temperature ranges of 50-70°C. The purified enzyme exhibited optimum activity at pH 9.0 and good stability in the pH range of 7.0-10.0 (Fig. 8). The optimum temperature was 60°C and the enzyme relatively stable at 50-70°C (Fig. 7). According to Genckal and Tari (2006), an alkaline protease from alkalophilic Bacillus sp. showed stability at 60°C. Comparing the present results with those of Nilegaonkar *et al.* (2002) it could be concluded that the optimum temperature of proteolytic activity frequently exceeded the optimum temperature for enzyme production. It was also suggested that the stability of protease enzyme could be due to their genetic adaptability to carry out their biological activity at a higher temperature (Whittle and Bloomfield, 1999; Kanekar *et al.*, 2002). The purified enzyme exhibited optimum activity at pH 9.0 and relatively stable in the range 7.0-10.0 (Fig. 8). Han-Seung *et al.* (2002) has reported that alkaline protease optimum pH at 9.0. The optimum pH range of alkaline proteases was generally at pH 9.0 and with a few exceptions of higher pH optima of 11.5 (Adinarayana *et al.*, 2003).

Effect of metal ions, inhibitors and Substrate specificity on protease activity: Most of the metal ions tested showed a stimulatory effect. Among the tested metal ions Ca^{2+} and Co^{2+} increased and stabilized the protease activity whereas, other metal ions Na^{+}, Hg^{2+} resulted in slightly inhibited. Among the different inhibitors, PMSF completely inhibited the protease activity while others showed only partial or slight inhibition (Table 4). Protease was resistant or partially resistant to several cations like Magnesium chloride, Mercury chloride, Calcium chloride, Sodium chloride, Cobalt chloride and Zinc chloride. Cations are known to increase the thermal stability of other *Bacillus* alkaline proteases (Rahman *et al.*, 1994). These results suggest that concerned metal ions apparently protect the enzyme against thermal denaturation and play a vital role in maintaining the active confirmation of the enzyme at high temperatures (Pan and Lin 1991; Steele *et al.*, 1992). Among the different inhibitors, PMSF completely inhibited the protease activity while others showed only partial or slight inhibition (Table 4).

Table 4. Effect of different metal ions and inhibitors on the activity of the purified alkaline protease from *Bacillus* sp. DK1.

Metal ions & inhibitors	Concentration (mM)	Relative activity (%)
Control	-	100
Mg^{+} ($MgCl_2$)	5	50
Hg^{+} ($HgCl_2$)	5	92
Ca^{+} ($CaCl_2$)	5	120
Na^{+} (NaCl)	5	96
Co^{+} ($CoCl_2$)	5	108
Zn^{+}($ZnCl_2$)	5	60
PMSF	2	0
EDTA	2	80
2-mercaptoethanol	2	45
Pepstatin	2	30
Iodoacetate	2	85

Fig. 6. Molecular mass determination of purified protease on SDS-PAGE. Silver staining, lanes 1&4; standard marker proteins consist of phosphorylase B (97,400D), bovine serum albumin (66,000 D), ovalbumin (43,000 D), carbonic anhydrase (29,000 D) and lactoglobulin (18,400 D).

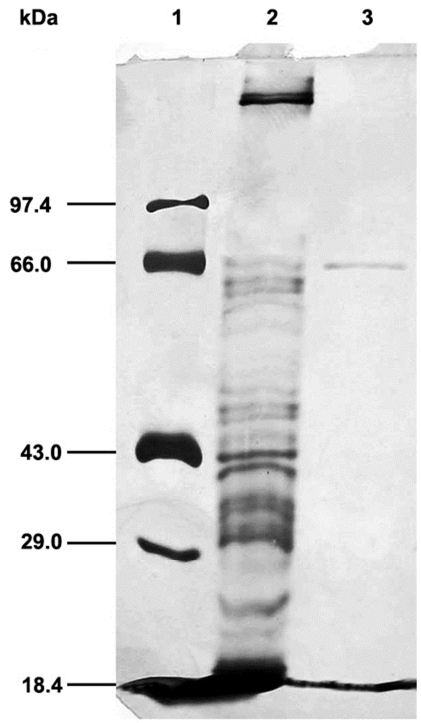

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Fig. 7. Enzyme activity and stability of purified protease from Bacillus sp. PTKG2 at various temperatures. The relative activity (%) was calculated relative to the case of reaction at which maximum activity was taken as 100%.

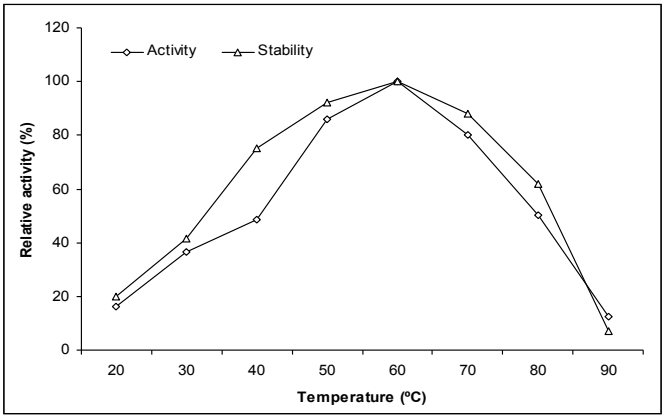


Fig. 8. Enzyme activity and stability of purified protease from Bacillus sp.PTKG2 at various pH. The enzyme activity was assayed in Phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0-9.0), and glycine-NaOH (pH 10-12). The relative activity (%) was calculated relative to the case of reaction at which maximum activity was taken as 100%.

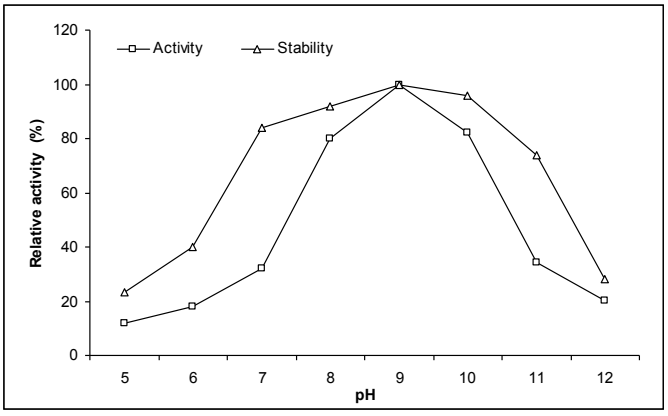


Fig. 9. Stability and compatibility of alkaline protease of *Bacillus* sp. DK1 in the presence of various commercial detergents.

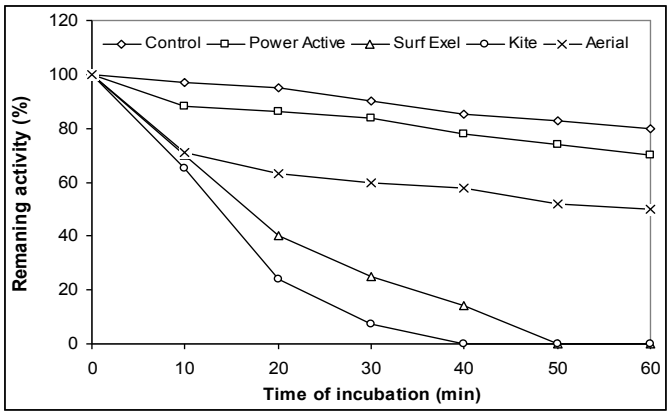
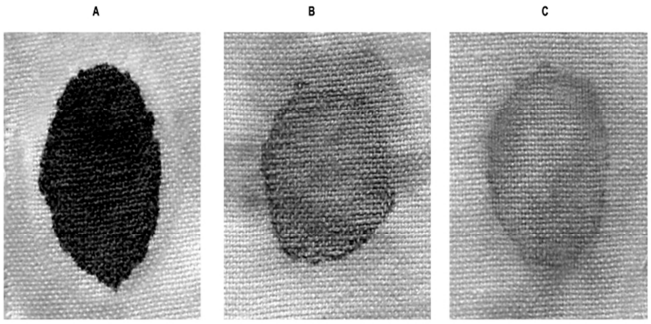


Fig. 10. Washing performance of alkaline protease from *Bacillus* sp. DK1 in the presence of detergent (Power Active). (A) Cloth stained with blood (B) Blood stained cloth washed with detergent only; and (C) blood stained cloth washed with detergent and enzyme



Sigma and Mooser (1975) reported that the inhibition studies give insight into the nature of enzyme, its cofactor requirements, and the nature of active site. However, PMSF sulphonates the essential serine residue in the active site and completely inhibited the enzyme activity, which is characteristic for serine protease as already observed by Keay *et al.* (1970) and Thangam and Rajkumar (2002). The protease exhibited highest activity towards casein and lowest towards haemoglobin. Similarly, alkaline protease obtained from *B. subtilis* PE-11 and *B. laterophorus* were reported to exhibit highest activity towards casein (Kobayashi *et al.*, 1995; Arulmani *et al.*, 2007).

Compatibility with detergents and washing test with protease preparation: Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. Protease from *Bacillus* sp. DK1 showed stability and compatibility with a wide range of commercial detergents at 60°C. Protease showed good stability and compatibility in the presence of Power Active followed by Ariel retained 50% activity even after 1 h incubation (Fig. 9). The supplementation of the enzyme preparation with Power Active detergent could significantly improve cleansing of blood stains (Fig. 10). The supplementation of the enzyme preparation in Power Active detergent could significantly improve cleansing of blood stains (Fig. 10). Earlier reports made on addition of enzyme preparation with commercial wheel detergent significantly enhanced the washing performances and removal of blood stains (Adinarayana *et al.*, 2003; Arulmani *et al.*, 2007). The data obtained from the experiments carried out in presence of commercial detergents, the broader substrate specificity of protease in cleaving most of the proteins suggest strongly that the proteolytic enzyme from *Bacillus* sp. DK1 has all the potential to be used as a laundry detergent additive, in order to improve the performance of heavy-duty laundry detergents.

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It is now well established that proteases exhibiting activity in the high-alkaline range have potential in detergent and stain removing formulations. Their utility can be significant only if they also exhibit compatibility with various detergents (Phadtare *et al.,* 1993; Anwar and Saleemuddin, 1997).

Conclusion

In this study, the bacterium was isolated from milk processing industry found to be an excellent protease producer. High protease yield was obtained with glucose and beef extract amended medium enhanced protease production at pH 9.0 over 36 h of incubation. Protease activities achieved by using isolated bacterium are significantly higher than most of the reported strains under similar conditions. Collectively, these results may justify the selectivity and suitability of the bacterial strain *Bacillus* sp. DK1 for commercial production of alkaline protease. Further studies could reveal better utilization of protease in industries for commercial purposes. Therefore, this strain could be an attractive source as protease producer, an enzyme that has widespread industrial applications.

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