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A Thermotolerant Protease from *Bacillus* sp– Isolation, Characterization, Optimization and Purification

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Protease producing bacteria was isolated from soil of Tatapani area of Mandi district of Himachal Pradesh. These bacteria were screened in skim milk agar medium using skim milk as the substrate. The highest clear zone producing bacterial isolate P5 was selected for further optimization studies. The isolate was identified as *Bacillus subtilis* based on morphological, biochemical and molecular characterizations. The isolate was able to grow under alkaline conditions at pH 9.0 and a temperature of 50°C. 1.9 fold purification of enzyme following ammonium sulphate precipitation and DEAE-cellulose chromatography was achieved. The molecular weight of the enzyme was estimated to be approximately 29 kDa as shown by electrophoresis. Interestingly ca^{2+} (5mM) activated enzyme activity, while $Mg^{2+,}$ Mn^{2+} Co^{2+} moderately activated enzyme activity, where as Fe^{2+} and Cu^{2+} inhibited the activity of enzyme.

Key words: *Bacillus subtilis*, alkaline protease, chromatography, Electrophoresis

The various benefits of thermostable protease have been increasingly known for use in industrial applications. Thus, the aim of this research was to isolate, screen, and identify bacteria that produce thermostable protease. Proteases are the enzymes of utmost biotechnological interest and are present in all living organisms but bacterial proteases are the most preferred group of industrial enzymes as compared to animal and fungal proteases, because of their ability to grow in simple culture medium with minimum space requirement, faster growth rate, higher productivity and low production cost. Among various types of proteases, alkaline proteases have extensive applications in industries like laundry detergents, pharmaceutical, food industry, leather processing and proteinaceous waste bioremediation[1]. Particularly, extracellular thermoalkaline bacterial proteases are important for the hydrolysis of waste proteins and enable the bacteria to absorb and utilize hydrolytic products by growing easily under extreme pH and temperature conditions[2, 3]. Recently, bacterial alkaline proteases have received attention as a viable alternative for bioremediation of protein rich tannery waste and their use in treatment of raw hide by replacing the hazardous chemicals especially involved in soaking, dehairing and bating of hides prior to tanning to produce quality leather without causing environmental pollution [4,5]. The present work describes the isolation and purification of alkaline thermotolerant protease from soil.

MATERIALS AND METHODS

Chemicals

The media components were purchased from Hi Media Laboratories (Mumbai, India). Casein (Hammarsten) was a product of SISCO Research Laboratories (Mumbai, India). Other

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chemicals used in present study were of analytical grade procured from various commercial sources.

Experimental methods

Isolation of extracellular protease producing bacteria

Soil samples were collected using sterile flasks from Tatapani (H.P.) Samples were transported under refrigerated condition to the laboratory and isolation was done by using serial dilution method and streak plate method using 5% skim milk agar. Plates were incubated at 50°C. The strains were purified by repeated streaking on the same medium. Proteolytic microorganisms were identified by streaking pure cultures on agar plates containing production medium with 5-g/l skim milk. The plates were incubated at 50°C and observed daily for signs of clearing of the agar around the colonies, indicating proteolytic activity.

Identification of Protease Producing Bacteria

The isolate showing maximum clearance zone was selected for further studies and the isolate was identified based on morphological, Physical and biochemical characteristics [6]. The culture of selected isolate was sent for identification till species level to Bangalore GeneI India by partial 16S r DNA sequence analysis. The partial 16S r DNA sequence of the efficient isolate was deposited in the GenBank

Production of Enzyme in Cultivation Media

Protease production was carried out in media (pH 9) with the following composition (g $^{L-1}$): Peptone 0.5%; Glucose 1%; NaCl, 0.5%; $CaCl_2.2H_2O$, 0.05%; $MgSO_4 \cdot 7H_2O$, 0.025; and yeast extract 0.5%. Cultures were incubated on a shaker (120 rpm) for 24 hrs at a temperature of 50°C.

Growth rate and protease production at different temperatures

The ability of the proteolytic isolates to grow at different temperatures (25°C,30°C,40°C,50°C,60°C) were analyzed in flasks filled with 50 ml of Glucose, yeast and skimmed milk medium with constant shaking (200 rpm). Extracellular protease production was measured and observed daily until clarification of the medium was evident (milk coagulation; caseinolytic activity). At this time colony forming units (CFU) were determined, and cultures were centrifuged (10, 000 rpm, 4 °C for 10 min) and the proteolytic activity was determined in the cell-free supernatant using skim milk as a substrate [7] In brief, reaction mixtures containing 250 ml of 1% (w/v) skim milk, 250 ml of 0.5-M Tris buffer (pH 9.0) and 250 ml of culture supernatant were incubated at 50°C for 10 min. The reaction was stopped by the addition of 3 ml of 5% (w/v) trichloroacetic acid; then each tube was centrifuged at 10, 000 rpm and the absorbance of the supernatant was read at 275 nm. One unit of enzyme activity (U) was defined as the amount of cell-free supernatant required to increase absorbance at 275 nm by one unit under the assay conditions. Protein content was estimated by method of Lowery [8] by using bovine serum albumin as the standard.

Optimization of production conditions of isolate *Bacillus* sp

Using one parameter variant at a time (OVAT) approach, the culture conditions for the production of protease by the selected isolate were optimized. The optimization of different production conditions were done in triplicate.

Media composition

Different media were optimized for the production of protease such as GYC (glucose yeast casein agar) and skim milk agar and MGYP media (maltose, glucose, yeast and peptone).

Effect of media pH, temperature and incubation time on protease production

The selected isolate *B. subtilis* was cultured in the media with pH varying from 5.0-10.0 and the protease activity of the supernatant was determined. The effect of incubation temperature and time on the production of protease by *B. subtilis* was studied. The microorganism was grown at various temperatures ranging from 30-60°C for 24 hrs.

Optimization of reaction conditions for the assay of extracellular protease

The reaction conditions for the estimation of protease produced by the bacterial isolate were optimized by using various buffer systems [phosphate buffer, acetate buffer, sodium citrate buffer, borate buffer and Tris- HCl buffer of 0.1 M strength], buffer pH (5.0-10), temperature (30-65°C), reaction time (5-40 min), substrate specificity [Casein, Gelatin, Skimmed milk, Azocasein] and metal ions(Ca^{2+},Mg^{2+},Mn^{2+},Zn^{2+},Fe^{2+},Cu^{2+}).

Purification and ultrafilteration of protease

All steps of purification were performed at 4°C and 0.1 M Tris HCl buffer pH 9.0 was used in

the purification of protease enzyme from isolate ATP-P5. Centrifugation was carried out at 10,000 g at 4°C. The enzyme preparations of various stages of purification were subjected to SDS-PAGE and analyzed for protein concentration and enzyme activity using casein (0.2% w/v) as substrate. The enzyme was concentrated by using ultralfilteration membrane.

Ammonium sulfate precipitation

The cell free concentrated crude enzyme was subjected to various percent saturation concentration of ammonium sulfate (0-80%). The fraction exhibiting maximum activity of protease was termed as ASF and was dialysed over night and taken for further purification of protease.

DEAE -Anion-exchange chromatography

The dialyzed ammonium sulfate fractionation was filtered through 0.45 -µm filter and loaded on DEAE cellulose anion exchange column (16mm diameter X 100 mm length) equilibrated with 0.05M, Tris-HCl buffer pH 9.0. The column was eluted with a linear gradient of NaCl from 0.1 M to 0.5 M in 0.05M Tris-HCl buffer. The protease activity and protein concentration were estimated in each fraction. The fractions inhibited single band on SDS PAGE were polled and termed as DEAE (i.e purified protease).

RESULTS AND DISCUSSION

Isolation and Identification of Protease-Producing Bacterial Strains

Total of 10 isolates were obtained from soil sample (Table 3a) and it is clear that the isolate P5 showed highest zone of clearance(Fig 3a) so it was selected for further studies. Isolate P5 grew aerobically and formed opaque, round, undulated, raised and rough colony. Microscopic observation of the isolate showed it as gram positive rods after Gram's staining. These phenotypic characteristics based on Bergey's Manual of Determinative Bacteriology confirmed the isolate P5 as *Bacillus* sp.

Strain Identification by 16S rDNA Sequencing

Analysis of 16SrDNA sequence of isolate ATP-P5 with available sequence in the NCBI database revealed 99% sequence similarity with published 16SrDNA sequence of *Bacillus subtilis* and assigned GenBank accesession number (KF550056). (Fig 3b).

Optimization of cultural conditions for production of Protease from isolate ATP-P5

The isolate (P5) *B.subtilis* exhibited maximum growth and protease activity in glucose, yeast, skim milk broth and resulted in maximum extracellular enzyme production (2.6 IU/ml) (Table

Table 3a. Screening of alkaliphilic proteolytic bacteria isolated from soil samples

S. No.	Code	Casein hydrolysis	Protease (IU/ml)
1	ATP-P1	+	2.4
2	ATP-P2	+	1.0
3	ATP-P3	+	2.1
4	ATP-P4	+	1.8
5	ATP-P5	++	2.6
6	ATP-P6	–	0.5
7	ATP-P7	–	0.2
8	ATP-P8	–	0.01
9	ATP-P9	–	0.3
10	ATP-P10	–	0.10

+ (Zone of hydrolysis) ; - (No hydrolysis zone)

Table 3b. Media optimization

Sr.No	Name of Media	Activity (IU/ml)
1	GYC (glucose yeast casein agar)	2.6
2	Skim milk agar	2.5
3	MGYP media (maltose, glucose, yeast and peptone)	2.3
4	GYP(Glucose, yeast, peptone)	1.6

Table 3c. Purification of extra-cellular protease

Steps	Total protein(mg/ml)	Specific activity(IU/mg)	Total activity(IU/ml)	Yield %	Purification fold
Crude	55	5.6	308	100	-
ASF	37	7.2	266	86	1.2
DEAE	15	13.4	201	75	1.9

3b) whereas least enzyme production (1.5 IU/ml) was recorded in the GYP broth (glucose, yeast extract and peptone ,skim milk agar and MGYP media (maltose, glucose, Yeast and peptone) (Table 3b). Several workers have reported the production of thermostable alkaline proteases from alkaliphilic and neutrophilic *B.subtilis* in a complex media [14,15]. However, there are very few reports on production of alkaline protease by *Bacillus* sp. in a synthetic medium. The pH of the media exerted significant effect on growth. When *B.subtilis* was grown on skim milk media at various pH ranging from 6.0-12.0 and the maximum growth (2.9 IU/ml) was recorded in alkaline pH (9.0) of the medium (Figure 3c), below and above this pH, the growth and production of extracellular protease by *B.subtilis* was markedly declined, whereas the organism failed to grow in the medium at pH 10-12. Majority of alkaliphilic *Bacilli* have been found to grow at pH 5.8-8.0 [10]. Maximum growth and production of protease by this organism was observed at 50° C (3.2 IU/ml) and minimum activity was recorded at 30° C (1.5 IU/ml) (Fig 3d). There was a sharp decline in growth and enzyme production when the temperature was kept higher and lowers than the 50° C. While studying the production of a thermostable alkaline protease by *Bacillus* sp. at 50° C, any deviation from 5° C sharply decreased the enzyme production by the organism [16]. Many mesophilic bacteria produce extracellular protease at 37° C [11, 12, 15].

Optimization of reaction conditions for maximum Protease activity

Among the four different buffer systems the protease from *B.subtilis* exhibited maximum activity in Tris HCl buffer (3.8 IU/ml) (fig 3e). The protease was stable in Tris HCl buffer pH 8.0 (Fig 3f) and exhibited maximum protease activity 4.7 IU/ml. These finding are in accordance with several earlier reports showing pH optima of 10.0-10.5 for proteases from *Bacillus* sp., *Thermus aquaticus*, *Xanthomonas maltophila* and *Vibrio metscnikovii*

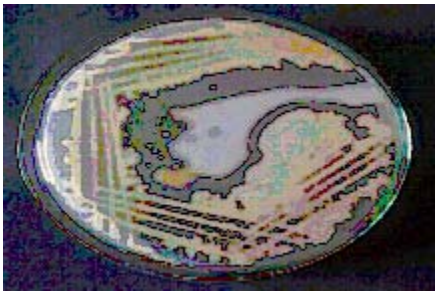

Fig 3a. Zone of hydrolysis around the colony

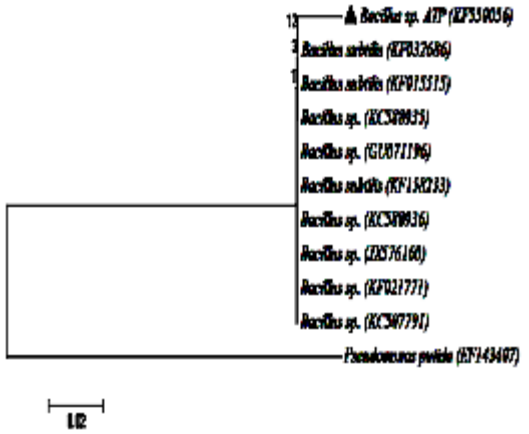

Fig 3b. Dendrogram

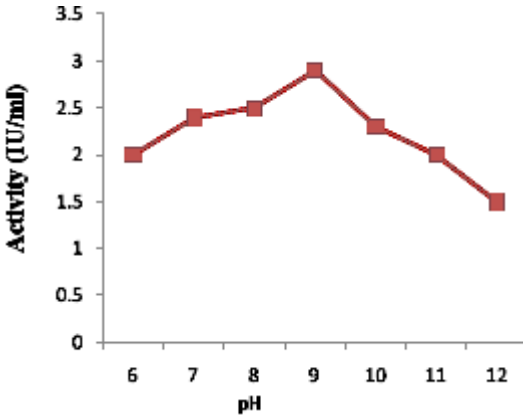

Fig 3c. Buffer pH optimization for protease production

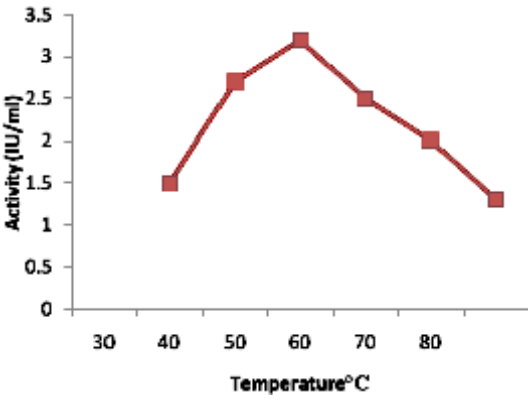

Fig 3d. Effect of temperature for protease production

[17]. The important detergent enzymes Subtilisin Carlberg and Subtilisin Novo [18] also show maximum activity at pH 10.5.

The protease enzyme was found stable at the ion concentration of 0.1 M of Tris HCl buffer (4.7 IU/ml) at pH 8.0. As the ionic concentration of the buffer rises, the protease activity goes on decreasing. The protease enzyme also exhibited maximum activity (5.4 IU/ml) at 50°C (Fig 3g) maximum enzyme activity of protease (5.5 IU/ml) from *B. subtilis* was observed after 10 min of incubation at 50°C (Fig 3h). With a further increase in time of incubation, the enzyme activity decreased thereof. This decrease in activity may be due to denaturation of protease after prolonged incubation at high temperature that is likely to alter the tertiary structure of the enzyme that shall affect the proteolytic activity of the enzyme. Approximately, 60% activity remained after 20 min of incubation of reaction mixture at 50° C. While studying the effect of reaction temperature, the alkaline proteases produced by *Actinomycetes* and *Bacillus* sp. were found to be unstable at high temperatures [19, 17]. Moreover, this protease exhibited high activity in the presence of skimmed milk (5.2 IU/ml) (Fig 3i). These results are conformity with the the earlier workers who used the skim milk for maximum protease activity[20]. All metal ions used in this study showed an enhancing effect on the activity of the enzyme.. In the presence of 5 mM $Ca^{2+,}$ enzymatic activity increased (5.0 IU/ml) (Fig 3j). Whereas maximum protease activity in the presence of Ca^{2+} ions with 5 mM concentration has been reported[21].

Protein Purification

The purification of protease cell free extract (CFE) involved ammonium sulphate fractionation (ASF), DEAE - ion exchange chromatography (DEAEF). Protease protein was

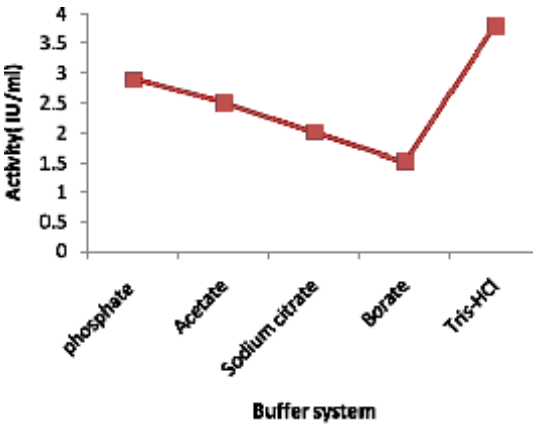

Fig 3e. Buffer optimization for protease activity

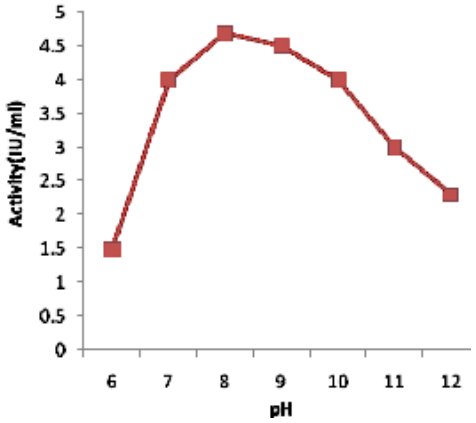

Fig. 3f. Optimization of buffer pH

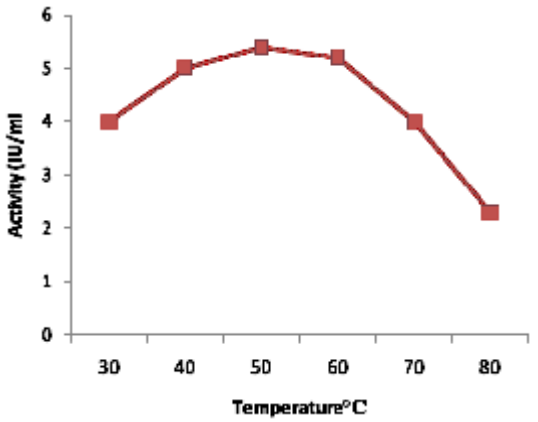

Fig 3g. Effect of temperature for protease activity

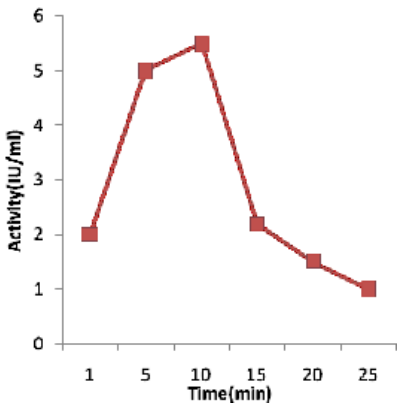

Fig 3h. Effect of incubation time

precipitated at 60-70 % saturation of ammonium sulphate fractionation (ASF), and it contained 37 mg protein with specific activity of 7.2 U/mg proteins (Table 3b). Ammonium sulphate fractionation (60-70 % saturation cut) resulted in 1.2 fold purification of enzyme (AFS) with a yield of 86 % of protease activity (Table 3c). The protease of *B.altitudinis* [22], *B.subtilis* (low and high molecular weight both) [23]. *Pseudomonan thermaerum*) [24], *Pleurotus sajor- cagu* [22] and *Alternaria alternata*) [25] were precipitated in the 80%, and 60% to 90% ammonium sulphate respectively. The dialysed protein was further loaded to the DEAE ion exchange column which resulted to the 1.9 fold purification of enzyme with a yield of 75% with specific activity of 13.4 U/mg proteins (fig 3k). Earlier, the protease of *B. Cereus*, *Vibrio parahaemolyticus*, *B. Laterosporus*, *B. subtilus* were purified by employing gel permeation chromatography techniques [26, 27, 28, 29].

Determination of molecular weight of the protease of *Bacillus* sp.

The heat-denatured protein fraction was loaded onto the SDS-PAGE gel along an appropriate range of molecular weight marker proteins. The appearance of a single band of protein in SDS-PAGE indicated the purity of the major protease that had a molecular weight of 29 kDa. Previously, a low molecular weight (24.5 kDa) protease was reported from *Bacillus circulance* [30]. A *Bacillus* sp. B001 exhibited a protease of 28 kDa [31], while the enzyme isolated in the present study exhibited a molecular weight of 29 kDa.(fig 3l)

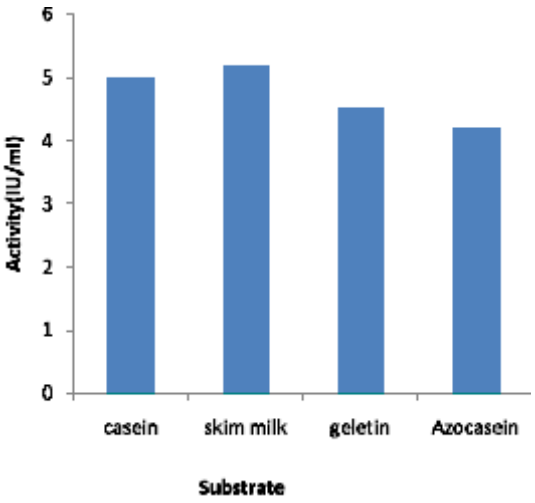

Fig 3i. Effect of substrate

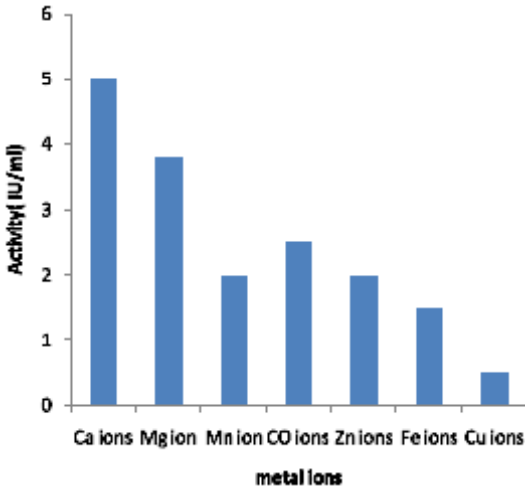

Fig 3j. Effect of metal ions

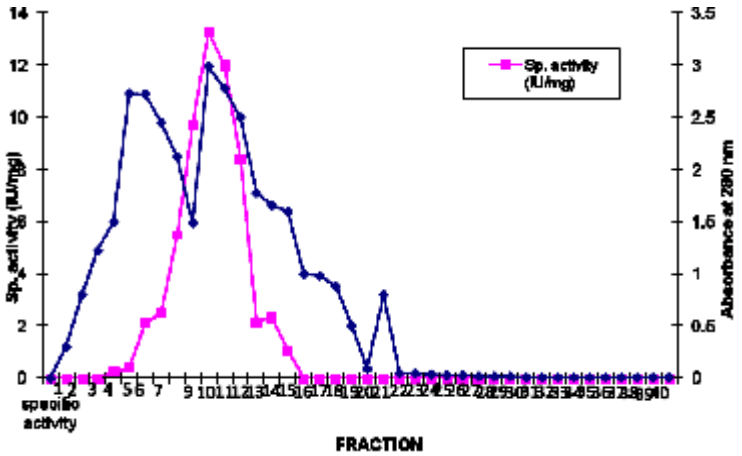

Fig 3k. Protein and protease activity profile during Anion Exchange chromatography

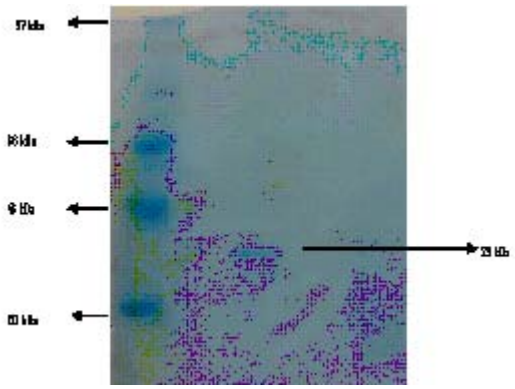

Fig. 3l. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of protease

CONCLUSION

The aim of this study was to optimize the physical factors affecting the productions of thermostable and alkaline protease. For this purpose, the alkalophilic and thermostabe protease were isolated from a newly isolated bacterium that was identified, based on the 16S rDNA analysis, biochemical tests and morphological studies as *Bacillus subtilis*. It can be concluded that the maximum bacterial growth and production of protease were achieved under optimized conditions. The extra-cellular protease was found to exhibit a remarkable stability towards high pH range. In more specific, it was found to retain 100% and 80% activity at 55 and 60°C, respectively, after 10 min of incubation.

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