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# Production of Bleach-Stable and Halo-Tolerant Alkaline Protease by an Alkalophilic *Bacillus pumilus* JB 05 Isolated from Cement Industry Effluents

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**Abstract** A new alkalophilic strain of *Bacillus pumilus* JB-05 producing bleach-stable and halo-tolerant alkaline protease was isolated from cement industry effluents in Karnataka, India. The effects of carbon and nitrogen sources on protease production by this alkalophilic strain were observed after a 30-h incubation. A high level of alkaline protease activity was obtained in the presence of starch as the carbon and peptone as the nitrogen sources. The partially purified enzyme showed an optimum temperature and pH activity at 58 °C and 10.5, respectively. The enzyme was completely inhibited by PMSF (95.0%) indicating it as a serine protease. It is bleach-stable as it retained 35% original activity in the presence of 10% (v/v) hydrogen peroxide at 30°C after 2 h and is halo-tolerant as it retained 70% original activity in the presence of 2.5 M sodium chloride at 30°C after 2 h incubation.

**Key words:** Alkaline protease, bleach-stable, halo-tolerant, *Bacillus pumilus*

Alkalophilic *Bacilli* have been the subject of attention for its application in biotechnology, because of their relative ease of isolation from a diverse range of environments. Bacteria of the genus *Bacillus* are known to produce a group of commercially important enzymes including proteolytic enzymes. Proteolytic enzymes constitute more than 65% of the total industrial enzymes market [15]. Alkaline protease represent one of the largest groups of industrial proteases useful in the detergent industry which contribute to more than 30% of the total global enzyme market. Alkaline proteases found extensive applications in the detergent, leather tanning, pharmaceutical, and food industries [7, 11, 13, 18].

Naturally occurring and man-made alkaline inhabitats provide excellent sources of microorganisms for research programmes in innovative biotechnology. The isolation and screening of microorganisms from different origins and the nature of alkaline sources for alkaline proteases have been reported by various investigators [1, 3, 8]. Production and characterization of alkaline protease by an alkalophilic and neutral *Bacillus* sp. have been reported by various investigators [4, 5, 9, 10, 12, 13]. In the present investigation, a bacterial strain has been isolated from cement industry effluents, a potentially rich source of alkalophiles, and methodology has been standardized for the production and partial characterization of the industrially important novel alkaline protease from this alkalophilic *Bacillus pumilus* JB 05.

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## MATERIALS AND METHODS

All analytical reagents and media components were purchased from Merck and Hi Media Mumbai, India.

### Culture Media for Isolation and Characterization of Alkalophilic *Bacillus pumilus* JB 05

Peptone yeast extract glucose (PYG) medium was used for the isolation and maintenance of the alkalophilic bacteria, with the following composition (g/l). Part A: Peptone, 10.00; yeast extract, 2.00; glucose, 10.00; $K_2HPO_4$, 2.00; NaCl, 5.00; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.1, and Part B: $Na_2CO_3$, 20.00. The solutions of Part A and Part B were sterilized separately by autoclaving. *Bacillus pumilus* JB 05 was isolated from an alkaline cement industry affluent sample of pH 10.7, collected from three different industries located in and around Gulbarga city, Karnataka, India. The organisms were isolated at 37°C by enrichment techniques in PYG medium followed by plating on PYG agar plates containing 0.5% (w/v) skimmed milk powder. The cement industry effluent (1.0 ml) was directly added into 50 ml of sterilized PYG medium in a 250-ml Erlenmeyer flask. The flasks were kept on an incubator shaker at 37°C for 24 h. The culture isolate was again streaked on PYG agar plates containing 0.5% (w/v) skimmed milked powder.

The plates were incubated at 37°C for up to 36 h. After 36 h incubation, more than 75–100 colonies were observed on PYG plates. Among 100 colonies of alkalophilic bacteria, only 4 strains were proteolytic in action. *Bacillus pumilus* JB 05 producing high level of alkaline protease was selected from the 4 isolates. The identification and biochemical characterization of the organisms were carried out at Institute of Microbial Technology (IMTECH), Chandighar, India.

**Fermentation Conditions**

The new strain *Bacillus pumilus* JB 05 was cultured for enzyme production in 250-ml Erlenmeyer flasks containing 50 ml of fermentation medium consisted of (g/l) starch, 10.0; peptone, 5.0; yeast extract, 2.0; NaCl, 10.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.1; $K_2HPO_4$, 1.0; and $Na_2CO_3$, 10.0; pH 10.2. Sodium carbonate was separately autoclaved and added to the rest of the medium after cooling. The Erlenmeyer flasks were inoculated with 3 ml of 14-h-old culture (OD 0.8). The flasks were incubated on a rotary incubator shaker at 36°C at 180 rpm upto 30 h. After 30 h of submerged fermentation, the growth medium was centrifuged at 15,000×g for 20 min at 4°C and the supernatant collected was used as a source of enzyme.

**Partial Purification of Alkaline Protease**

The culture filtrate was precipitated with solid ammonium sulfate to 80% saturation and the resulting precipitate was dissolved in a minimum volume of 50 mM glycine NaOH buffer (pH 10.5) and dialyzed against 3 changes of the same buffer. The dialyzed enzyme was used for characterization studies.

**Protease Assay**

The reaction mixture in a total volume of 2 ml was composed of 1 ml of 1% (w/v) casein in 50 mM glycine NaOH buffer (pH 10.5), 100 μl of enzyme, and 0.9 ml of 50 mM glycine NaOH as buffer. The assay was carried out at 50°C for 20 min and the reaction arrested with addition of 10% trichloro acetic acid and the contents were centrifuged. To 0.5 ml of supernatant, 2.5 ml of 0.5 M sodium carbonate was added followed by 0.5 ml (1:2 diluted) Folin-Ciocalteus reagent. The reaction mixture was allowed to stand for 30 min at room temperature and absorbance was recorded at 660 nm. One unit of alkaline protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per min under the assay condition.

**Effect of Carbon and Nitrogen Sources on Enzyme Production**

Different carbon sources like glucose, maltose, lactose, arabinose starch, sucrose xylose, and cellulose and nitrogen sources like peptone, yeast extract, tryptone, meat extract, and casein (0.5–2%) were employed in the production of alkaline protease.

**Effect of pH on Activity and Stability of Alkaline Protease**

The optimal pH of alkaline protease was determined with 1% (w/v) casein as substrate dissolved in different buffers (citrate phosphate, pH 4–6; phosphate buffer, pH 7.0; tris HCl, pH 8.0; and glycine NaOH, pH 9–12). The pH stability of alkaline protease was determined by incubating enzyme in different buffers pH (6–12) at 40°C for 4 h.

**Effect of Temperature on Activity and Stability of Alkaline Protease**

The optimum temperature of protease activity was determined with reaction mixture incubated at different temperatures ranging from 30°C–70°C for 20 min in the presence and absence of 10 mM $Ca^{2+}$. For determination of thermostability, 100 ml of enzyme was preincubated with 0.9 ml of glycine NaOH buffer, pH 10.5, at different temperatures ranging between 30–70°C for 10 min. After a 10 min incubation, 1 ml of 1% casein was added and the mixture was incubated at 50°C for 20 min.

**Comparision of Bleaching Agents on Stability of *Bacillus pumilus* JB05 Alkaline Protease and Subtilisin Carlsberg Protease**

One hundred U of partially purified enzyme from *Bacillus pumilus* JB 05 alkaline protease and subtilisin carlsberg protease (Sigma, U.S.A.) was preincubated with 0.5–1% (w/v) sodium perborate, sodium hypochlorite (5% v/v), and 5% to 15% (v/v) hydrogen peroxide for about 2 h. After incubation, 1 ml of 1% casein was added and the mixture was incubated at 50°C for 20 min.

**Effect of Inhibitors on Alkaline Protease Activity**

Partially purified enzyme (100 μl) was preincubated with 1 mM PMSF (Phenyl Methyl Sulfonyl Fluoride), 10 mM 1,10-phenanthroline, 10 mM EDTA, and 0.5% (w/v) SDS at 30°C for 2 h. After incubation, enzyme activity was determined as per the method described earlier.

## RESULTS AND DISCUSSION

An alkalophilic *Bacillus pumilus* JB 05 producing a novel alkaline protease was isolated from cement industry effluents. Cells are Gram-positive, nonmotile aerobic, regular rods, nonpigmented, and spore forming. The detail morphological, physiological, and biochemical characteristics of the *Bacillus pumilus* JB 05 are shown in Table 1. On the basis of these characters the new bacterial strain was identified as *Bacillus pumilus* JB 05 according to *Bergey's Manual of Determinative Bacteriology* [2].

The growth profile of *Bacillus pumilus* JB 05 is shown in Fig. 1. The figure shows specific growth of the isolated organism and the time dependent increase of extracellular

**Table 1.** Characteristics of *Bacillus pumilus* JB 05.

| | |
|---|---|
| Cell morphology | Nonmotile, rods, round, single, flat, shinny surface, opaque, nonpigmented |
| Grams Reaction | Gram-positive |
| Spore | Endospore central in position and ellipsoidal in shape |
| **Physiological tests** | |
| **Growth at temperature** | |
| 15°C | No growth |
| 20°C | No growth |
| 25°C | Slow growth |
| 30°C | Moderate growth |
| 37°C | Excellent growth |
| 40°C | Moderate growth |
| 45°C | Slow growth |
| 50°C | No growth |
| **Growth at pH** | |
| pH 5 | No growth |
| pH 6 | No growth |
| pH 6.8 | Slow growth |
| pH 8 | Moderate growth |
| pH 9 | Excellent growth |
| pH 10 | Moderate growth |
| pH 11 | Slow growth |
| pH 12 | No growth |
| **Growth on NaCl (%)** | |
| 2.5 | Excellent growth |
| 5 | Excellent growth |
| 7 | Moderate growth |
| 8.5 | Moderate growth |
| 10 | Slow growth |
| 15 | No growth |
| **Biochemical test** | |
| Indole test | Negative |
| Methyl Red test | Negative |
| Voges-Proskauer test | Negative |
| Citrate utilization | Positive |
| Casein hydrolysis | Positive |
| Starch hydrolysis | Positive |
| Urea hydrolysis | Negative |
| Fat hydrolysis | Negative |
| Nitrate reduction | Negative |
| $H_2S$ production | Negative |
| Cytochrome oxidase | Negative |
| Catalase | Positive |
| Arginine dihydrolase | Negative |
| Lysine decarboxylase | Negative |
| Ornithine decarboxylase | Negative |
| DNase test | Negative |
| Phenylalanine deamination | Negative |

Acid production from various carbohydrates rendered negative results for all sugars.

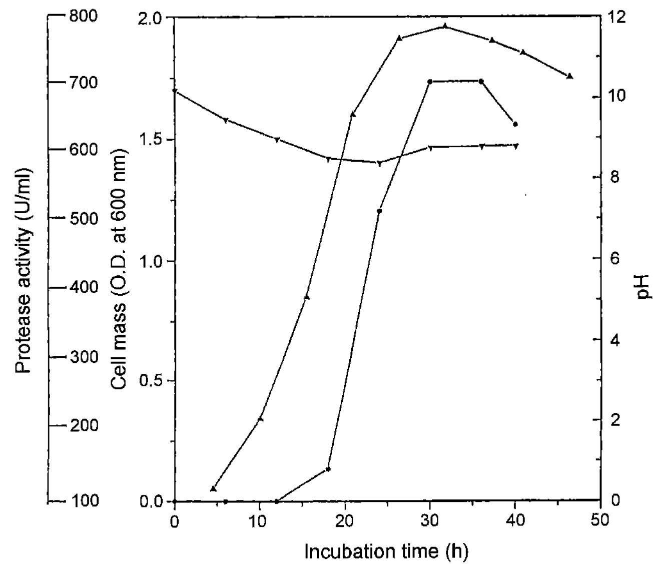


**Fig. 1.** Typical growth profile and fermentation of *Bacillus pumilus* JB 05.
Cell mass (▲), protease activity (●), and pH (◄).

proteolytic activity. When the isolate was grown in a medium without casein, it also secrets extracellular protease and is a constitutive enzyme. The proteolytic activity was noticed in the fermentation broth as soon as the bacteria entered the end of the exponential phase (~18 h), and the activity reached the maximum level in the stationary growth phase (~32 h). Thereafter, the proteolytic activity remained constant throughout the stationary phase. During fermentation, the pH of the fermentation broth decreased from 10.2 to 8.6 and then increased to 9.0, and remained constant during prolonged fermentation. The time at which the pH reached its minimum value during batch submerged fermentation coincided with the time at which maximum cell density and enzyme concentration could be used to monitor enzyme production.

The maximum proteolytic activity of 700 U/ml occurred when the cells were grown in 1% (w/v) starch as the carbon source. The optimum concentration of starch for protease production was in the range of 1–3%. The proteolytic production was markedly low, being 100 and 120 U/ml in the presence of 1% (w/v) xylose and 1% (w/v) cellulose as carbon sources, respectively.

*Microbacterium* sp., isolated from an alkaline soda lake by Gessesse and Gashe [5], showed maximum protease production on glucose. When glucose, maltose, or xylose was incorporated in media, they repressed the protease production of *Bacillus licheniformis* [4]. When the isolate was grown on a medium containing nitrogen source, there was no proteolytic activity in spite of increase in the biomass, however, organic nitrogen sources had profound effects on protease production. Among the organic nitrogen sources tested, the maximum proteolytic activity was observed

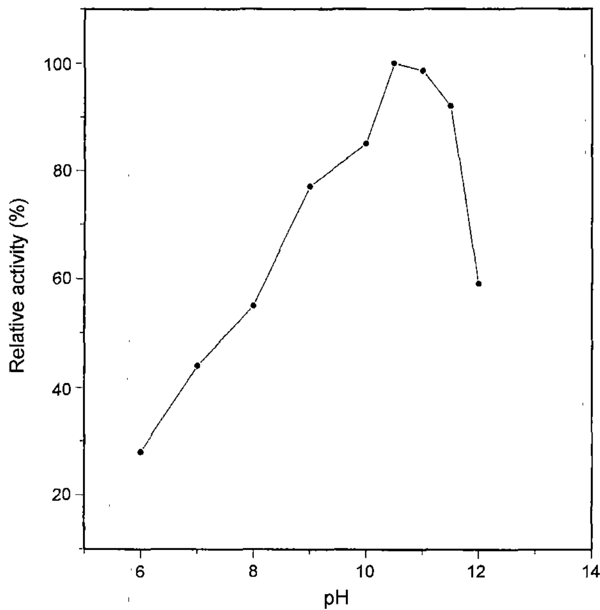


**Fig. 2.** Effect of pH on *Bacillus pumilus* JB 05 alkaline protease.
The 1% (w/v) casein substrate was dissolved at each pH buffer and other conditions were the same as in Materials and Methods (100% activity considered at pH 10.5).

when the cells were grown on peptone. The optimum concentration of peptone for protease production was in the range of 0.5–1.25%. Casein has been shown to act as an inducer of protease in *Bacillus licheniformis* [4] *Microbacterium* sp. [5].

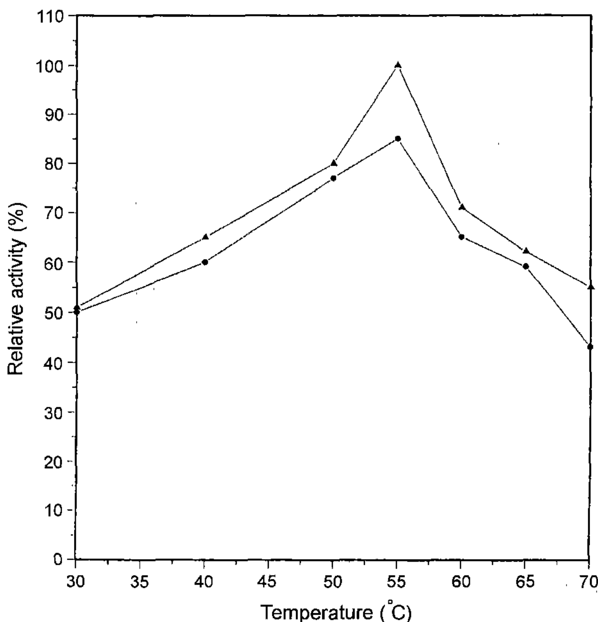


**Fig. 3.** Effect of temperature on *Bacillus pumilus* JB 05 alkaline protease activity.
The reaction was carried out at each temperature for 30 min at pH 10.5 with 10 mM $Ca^{2+}$ (▲) and without 10 mM $Ca^{2+}$ (●).

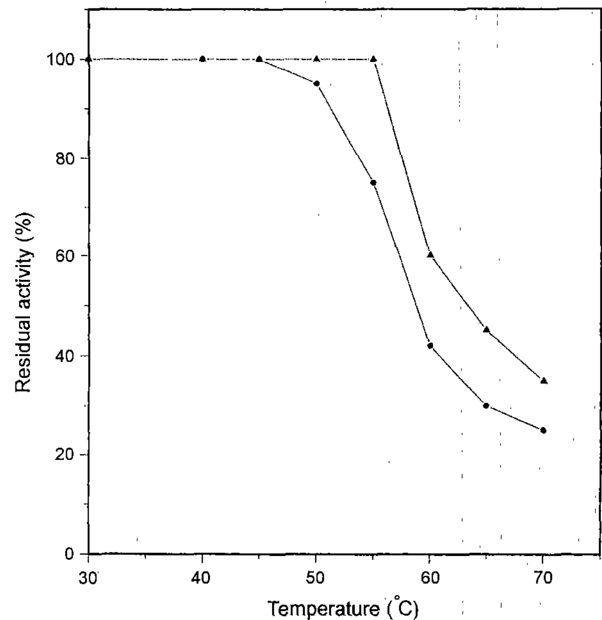


**Fig. 4.** Effect of temperature on *Bacillus pumilus* JB 05 alkaline protease stability.
The enzyme was preincubated for 15 min at different temperatures, without $Ca^{2+}$ (●) and with $Ca^{2+}$ (▲).

The partially purified enzyme from the isolate showed optimum pH and temperature at pH 10.5 and 55°C (Fig. 2). The enzyme is stable in a wide pH (7–12) and temperature (50–55°C) range. It is almost 90% stable at pH 9–12 and 70% stable at pH 9.0 after 4 h incubation at 40°C. The pH stability showed that the enzyme was stable as alkalinity increases. The optimum temperature for protease activity was at 55°C in the presence of 10 mM $Ca^{2+}$ (Fig. 3). However, $Ca^{2+}$ was required for enzyme stability at temperature values above 55°C (Fig. 4). At 60°C, the enzyme retained 60% of its original activity after 15 min in the presence of 10 mM $Ca^{2+}$, which stabilized the alkaline protease activity [5, 17]. The proteolytic activity was inhibited by 1 mM PMSF. The inhibition by PMSF on protease activity showed that it is a serine protease. The

**Table 2.** Comparison of *Bacillus pumilus* JB05 and subtilisin carlsberg protease for bleach stability.

| Components | AP* Residual activity (%) | SC• Residual activity (%) |
|---|---|---|
| Control | 100.00 | 100.00 |
| Sodium perborate (0.5%) | 85.00 | 43.00 |
| Sodium perborate (1%) | 60.00 | 26.00 |
| Sodium hypochlorite (5%) | 76.53 | 17.20 |
| Hydrogen peroxide (5%) | 100.00 | 9.50 |
| Hydrogen peroxide (10%) | 85.00 | 00.00 |
| Hydrogen peroxide (15%) | 30.00 | 00.00 |

*AP - Alkaline protease from *Bacillus pumilus* JB 05.
•SC - Subtilisin carlsberg protease (Sigma).

**Table 3.** The effect of enzyme inhibitors on *Bacillus pumilus* JB 05 alkaline protease.

| Inhibitor | Concentration (mM) | Residual activity (%) |
|---|---|---|
| Control | 00.0 | 100.0 |
| PMSF | 1.0 | 5.0 |
| EDTA | 10.0 | 90.0 |
| 1,10-phenanthroline | 10.0 | 95.0 |
| SDS | 0.5% | 55.0 |

enzyme was resistant to inhibition by EDTA and 1,10-phenanthroline which indicates that the enzyme is not a metalloprotease (Table 2). Protease from *Bacillus licheniformis* was inhibited by PMSF but not by EDTA [4].

In addition to pH and temperature stability, most of the detergent proteases are also stable in the presence of surfactants, detergents, and oxidizing agents. In the present study, we compared the *Bacillus pumilus* protease with commercially available subtilisin carlsberg protease (Sigma, U.S.A.) for bleach stability. *Bacillus pumilus* JB 05 showed good stability in the presence of some detergents and bleaching agents (Tables 2 and 3). The bleach stability of the enzyme was further tested in the presence of hydrogen peroxide. At 5% hydrogen peroxide, the enzyme retained 100% activity, whereas subtilisin carlsberg retained 9.5% activity. In the presence of 10% hydrogen peroxide, subtilisin carlsberg lost all activity (0%) while the *Bacillus pumilus* JB 05 protease retained 85% residual activity after 2 h of incubation (Table 3). The bleach stability of alkaline protease is an important property which is relevant to detergent formulations. The reports on bleach-stable alkaline protease is limited in the literature [6, 14]. The bleach stability for the protease could be achieved by site-directed mutagenesis or by modifying amino acid residues by protein engineering [19] or by using traditional screening methods to isolate thermo-alkali-stable and bleach-stable proteases from extremophiles.

In order to evaluate the importance of protease for industrial applications, we could also demonstrate that alkaline protease from *Bacillus pumilus* JB 05 is also salt-tolerant. The salt-tolerant enzymes are important in surfactant and detergent formulations. The reports on salt-tolerant protease are limited in the literature [10, 14, 20]. Alkaline protease was resistant to NaCl in a reaction mixture of up to 1.0 M concentration. It retained 95% of its original activity after 2 h at 40°C while it retained 50% of its original activity at 2.5 M NaCl. Considering these properties, *Bacillus pumilus* JB 05 alkaline protease may find potential application in the detergent and leather tanning industries. The experiments for scale-up criteria for commercialization are in progress.

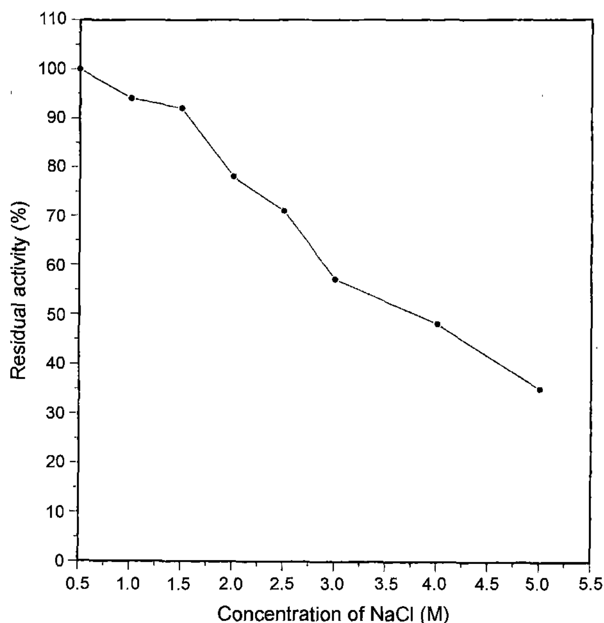


**Fig. 5.** Effect of sodium chloride on *Bacillus pumilus* JB 05 alkaline protease activity.
The enzyme was preincubated with sodium chloride (0.5 M to 5 M).

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