Bacillus sp. APR-4 protease as a laundry additive

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A protease from a new isolate of Bacillus sp. APR-4 with optimum activity at pH 9.0 and 65-70°C was found stable in 5% detergents (Farishta®, Fena®) at 50°C and also in 500 mg/l of sodium hypochlorite. It retained 78% activity even after 24 hrs of incubation with detergents (Farishta®, Fena®) at 30°C. The protein stains (egg yolk) were removed within 10 min from test fabric (cotton) in 100 U/ml enzyme at pH 9.0 with 1% detergent (Farishta®) and it took 30 min to remove blood stains with 100 U/ml enzyme only.

Keywords: Bacillus sp. APR-4, alkaline protease, detergent compatibility, stain removal

IPC Code: Int. Cl. A 01 N 63/00; C 11 D 1/32; C 12 S

Introduction
Proteases are used in laundry detergents for over 50 years to facilitate release of proteinaceous materials in stains (blood and milk) and account for approx 25% of total worldwide sales of enzymes1,2. The suitability of an enzyme preparation for use in detergents depends on its compatibility with the detergents at a high temperature. An ideal detergent enzyme should be stable and active in the detergent solution and should have adequate temperature stability to be effective in a wide range of washing temperatures3.

Detergents such as Era Plus® (Procter and Gamble), Tide® (Colgate Palmolive) and Dynamo® (Procter and Gamble) contain proteolytic enzymes, majority of which are produced by Bacillus spp4. In certain cleansing formulations, oxidizing agents may be added to enhance the brightness of or sterilize the washed material5,6. Proteases, used as cleaning agents, should be stable in the presence of chlorine as water supplies are chlorinated in many parts of world including India and some detergent compositions may include bleaching agents7. Few Bacillus spp. produce proteases, which are stable in the presence of ionic, non-ionic detergents, surfactants, bleach, chlorine, commercial detergents and oxidizing agents4,7-11. Protease performance in laundry detergents is evaluated by using soiled test fabrics and the efficiency is measured either visually or by measuring the reflectance of light under standard conditions12,13. Present communication reports stability of a protease from a new isolate Bacillus sp. APR-4 in detergents/bleach and its wash performance.

Materials and Methods

Bacterial Strain and Seed Culture
Bacillus sp. APR-4 was earlier isolated in this laboratory at 50°C from dung compost. The seed culture was prepared by growing this organism on medium containing: glucose, 10; yeast extract, 5; peptone, 5; NaCl, 5; MgSO4.7H2O, 0.25; and CaCl2.2H2O, 0.5 g/l, pH 7.0 at 50°C and 165 rev/min in an incubator shaker for 24 hrs.

Enzyme Production
The seed culture (5% v/v) was added to the production medium (composition same as above) and incubated at 50°C for 40 hrs at 165 rev/min in shaker. The culture was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was used as a source of crude enzyme.

Protease Assay
The protease activity was determined14. The assay mixture contained 4 ml of casein solution (2.5% w/v in 50 mM Tris HCl, pH 9.0) and 1 ml of enzyme. Following incubation for 5 min at 65°C, 5 ml of trichloroacetic acid (5%) was added and vortexed

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after cooling in ice for 5 min. This mixture was filtered through Whatman 1 and the optical density was measured at 275 nm against the control. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate one μg of tyrosine/min under assay conditions.

**Stability of Bacillus sp. APR-4 Protease in Detergents/Surfactants**

The protease was pre-incubated in 5% (v/v of 10 mg/ml) detergents (Wheel®, Surf®, Fena®, Farishta®, Tween-20, Tween-80 and Triton X-100) at 50°C. The residual activity was assayed against enzyme control after 30 and 60 min at 65°C. In the next experiment, the stability of enzyme was studied in varying conc (3-20% v/v of 15 mg/ml) of Fena® and Farishta® after 90 min incubation at 30°C and also the stability of enzyme over a period of 24 hrs at 30°C in 5% (v/v of 15 mg/ml) of Fena® and Farishta®.

**Effect of Bleach and Oxidizing Agent**

The enzyme was incubated with different concentrations of sodium hypochlorite (50-500 mg/l of available chlorine) and hydrogen peroxide (5-20%) at 50°C for 1 hr and the residual activity was assayed.

**Wash Performance of Bacillus sp. APR-4 Protease**

The test fabric (cotton) pieces (1.5” × 1.5”) stained with egg yolk were taken in 250 ml Erlenmeyer flasks and subjected to different temperature (40, 50, 65°C) treatments in 50 ml of reaction mixture under different sets as: i) water only, ii) detergent (1% of 15 mg/ml) in water, iii) enzyme (100 U/ml) in water, and iv) detergent (1% of 15 mg/ml) + enzyme (100 U/ml) in water. The test fabric with bloodstains was also treated under similar conditions at 65°C. Stain removal was checked qualitatively by visualization.

**Results and Discussion**

The optimum temperature (65°C) and pH (9.0) for the activity of Bacillus sp. APR-4 were reported earlier. The enzyme was stable at 40, 50, 65, 70 and 80°C for 24, 5, 5, 1 hrs and 30 min, respectively and half-life of 150 and 90 min, respectively at 70 and 80°C.

The enzyme activity of Bacillus sp. APR-4 was stimulated in the presence of Fena and Farishta after 30 min of incubation (Table 1). After 1 hr of incubation at 50°C, no stimulation in activity was observed with Fena, while 8% higher activity was observed in Farishta in comparison to control. The enzyme retained 99% residual activity in the presence of Wheel and it decreased to 6% with Surf after 1 hr incubation. However, the enzyme activity decreased slightly in the presence of non-ionic detergents (Triton X-100, Tween-20, Tween-80) after 1 hr incubation at 50°C. Non-ionic detergents usually have no influence on the stability of enzymes but the reason for decrease in activity in the present study is not known.

The stability of enzyme was checked in different conc (3-20% of 15 mg/ml) of selected detergents (Fena, Farishta) after 90 min incubation at 30°C. The enzyme was very stable in 3% (v/v) and 6% (v/v) of

<table>
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<tr>
<th>Detergents/surfactants (5% v/v of 10 mg/ml)</th>
<th>Relative activity (%)</th>
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<tr>
<td>After 30 min</td>
<td>After 60 min</td>
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<td>Surf®</td>
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<td>Triton X-100</td>
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Protease activity measured using casein (2.5% in 50 mM Tris HCl, pH 9.0) at 65°C after 5 min of incubation. Relative activity (%) is calculated against enzyme control incubated in buffer (100% activity = 3567 U/ml). All readings were taken in triplicate.

<table>
<thead>
<tr>
<th>Detergent concentration (%) v/v of 15 mg/ml</th>
<th>Relative activity (%)</th>
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<tr>
<td>Farishta®</td>
<td>Fena®</td>
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<tr>
<td>6</td>
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<td>20</td>
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Protease activity measured using casein (2.5% in 50 mM Tris HCl, pH 9.0) at 65°C after 5 min of incubation. Relative activity (%) is calculated against enzyme control incubated in buffer (100% activity = 1556 U/ml for Farishta® and 1678 U/ml for Fena®). All readings were taken in triplicate.
Fena and Farishta, respectively (Table 2). When the detergent conc was increased to 20% (v/v) the residual activity decreased in Fena (7%) and Farishta (38%) and this might be due to denaturation of enzyme at higher conc of detergents.

The stability profile of protease of Bacillus APR-4 in 24 hrs in 5% (v/v of 15 mg/ml) of Fena and Farishta revealed 91% residual activity after 3 and 4 hrs of incubation with both detergents (Fig. 1). However, 78% residual activity was exhibited by enzyme even after 24 hrs of incubation in these detergents. There are very few reports on detergent/bleach stable enzymes. An alkaline protease from B. sphaericus has shown compatibility with a variety of commercial detergents (5 mg/l) for 1 hr at 37°C. Comparing the results with this the protease of Bacillus sp. APR-4 was significantly more stable in some commercial non-enzyme detergents.

Effect of Bleach and Oxidizing Agent

The enzyme was preincubated in 50-500 mg/l of sodium hypochlorite at 50°C for 1 hr. The enzyme was very stable in this bleaching agent and retained 96, 93, 86% of the residual activity in 50, 100 and 200 mg/l, respectively. Whereas, 71% of the activity was retained even at higher conc of sodium hypochlorite (500 mg/l). The use of enzymes in laundry products has become limited because enzymes get deactivated by chlorine bleach.

However, the enzyme retained only 15% of its initial activity at 50°C up to 15% hydrogen peroxide (H₂O₂) and was completely inhibited at 20% H₂O₂. Detergents contain oxidizing and chelating agents, which may hinder the activity of certain enzymes and hence bleach stable enzymes are required for detergent industry.

Effect of Protease on Stain Removal

The efficiency of Bacillus sp. APR-4 protease was tested for removing egg yolk stain from test fabric (cotton) at different temperatures (40, 55, 65°C) with 100 U/ml enzymes and 1% (v/v of 15 mg/ml) detergent (Farishta). It took 2 hrs to completely remove the egg yolk stain at 40°C whereas at 55 and 65°C, the stains were removed in 1 hr and 30 min respectively. However, in the absence of enzyme, the time taken to remove stain was very long.

As water was replaced with buffer (50 mM Tris HCl, pH 9.0), the stain removal was in 10 min at 65°C with 100 U/ml enzyme and 1% (v/v of 15 mg/ml) detergent (Farishta). The enzyme was also efficient in removing bloodstains from test fabric (cotton) at 65°C with enzyme (100 U/ml) only and took 30 min to completely remove stains (Fig. 3). Use of detergent stable proteases from Arthrobacter ramosus and B. alcalophilus in removing blood stains.

![Fig. 1—Stability of Bacillus sp. APR-4 protease after incubation at 30°C over a period of 24 hrs in 5% (v/v of 15 mg/ml) of Farishta® and Fena®. Protease activity measured using casein (2.5% in 50 mM Tris HCl, pH 9.0) after 5 min of incubation at 65°C. Relative activity (%) is calculated against enzyme control incubated in buffer (100% activity = 2337 U/ml).](image1)

![Fig. 2—Effect of Bacillus sp. APR-4 protease on egg yolk stain removal at 65°C: a) buffer (Tris HCl, pH 9.0) only, b) detergent (1% of 15 mg/ml) in buffer (Tris HCl, pH 9.0), c) enzyme (100 U/ml) in buffer (Tris HCl, pH 9.0), and d) detergent (1% of 15 mg/ml) + enzyme (100 U/ml) in buffer (Tris HCl, pH 9.0).](image2)
Proteinaceous materials in stained clothes \(^{20,21}\). The incorporation of proteases has long been incorporated as biobuilders into heavy-duty detergents to hydrolyse and remove proteinaceous materials in stained clothes \(^{5,22,23}\). Tests involving both ordinary clothing and standard conditions and detergent compositions by numerous tests involving both ordinary clothing and standard wash performance analysis of an SDS-stable alkaline protease from \(Bacillus\) sp., its production and use as detergent additive, Indian Patent No. 197/NE/2002, 2002. 

The protease of \(Bacillus\) sp. APR-4 was efficient in removing the gelatin from used X-ray films \(^{24}\) and in peptide synthesis \(^{25}\). \(Bacillus\) sp. APR-4, with its stability in detergents and hypochlorite and wash performance, appears to be a potential enzyme for laundry additive/detergent formulations.

**Acknowledgement**

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**References**


