Keratin is fibrous protein which imparts tensile strength to the cells. Keratin constitutes 90% of the chicken feather and 10% of the body weight of chicken. Keratinase are mostly alkaline protease involved in the breakdown of keratin protein, which is mechanically tough and chemically stable. Keratinase find application in detergent, leather, and textile, pharmaceutical, cosmetic and agricultural sectors. Due to the elevated costs of the medium and substrates involved in the production of industrial enzymes, the costs of these enzymes are on hike. Chicken feathers generated in huge pool, as unutilized waste from the poultry farms and slaughter houses, dictates the use of feathers as cheap substrate to lower the cost of production of the valuable market enzymes such as keratinase. Due to the health hazards associated with chemical detergents, the use of keratinase from microbial origin is on demand in detergent based formulations.

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Currently, most of the detergent in use contains hazardous chemical components like bleaching agents, optical fibre brighteners, anti re-deposition agents, foam regulators, organic sequestering agents, perfumers and surfactants. One of the major groups of anionic surfactant is linear alkyl benzene, that contaminate drinking water and is reported to be highly toxic towards aquatic faunas, soil biota etc. Moreover, in India, phosphate based detergents are most widely used, and can cause serious problem of eutrophication. However, the best way to remove these serious environmental problems is to use protease enzyme based detergents. This does not leave any toxic residue (unlike chemicals) and is also reported to improve appearance and feel of fabric. The prerequisite criteria, set up by laundry detergent manufacturers warrants need for a suitable protease that has efficient wash performance at wide range of temperature, pH and must be good at removing proteinaceous dirt or stain, must have stability with other chemical components of detergent formulations. In developed countries more than half of the branded detergents incorporate protease to get excellent performance. Proteases are effective in removing stains of milk, egg, fish and meat. The criterion for an enzyme to show better efficacy as detergent additive is its synergy with detergent at high temperature. Some of the bacterial keratinases reported for fulfilling such criteria and having potential to be used in detergent formulations are reported from Bacillus pumilus, B. thuringiensis, B. licheniformis, B. subtilis, B. taquilienis; Brevibacillus, Paenibacillus woosongensis and Streptomyces. Over the years keratinases has evolved as the protease of choice for detergent industries. This is primarily due to the fact that there are broad range proteases having better substrate specificity towards both soluble and insoluble dirt/stain substrates as well as better compatibility with different chemical additives used for detergent formulations.

In this paper we demonstrate the detergent additive functional activities by crude keratinase enzyme from the cell free culture extract of a Stenotrophomonas sp. strain Norja-1 and explore its wash performance and possible applications in combination with existing detergent.
MATERIAL AND METHODS

Isolation and identification of keratinase producing bacterium: The crude keratinase enzyme extract used in this study was obtained from keratinase producing bacterial isolate designated Norja-1 isolated from a soil sample by enrichment techniques using a minimal medium (Feather Meal Media) supplemented with chicken feather as sole source of carbon and nitrogen. The isolate Norja-1 was identified as Stenotrophomonas sp. 16S rRNA gene sequence based analyses carried out at EzTaxon server and RDP site. The strain was deposited at MCC, NCCS Pune, India and its 16S rRNA gene sequence accession number assigned by GenBank was KM924018. The strain was regularly maintained on skim milk agar plates at 4°C.

Production of crude enzyme: The keratinolytic strain Norja-1 was inoculated in Feather Meal Media FMM broth (composition, (g/l): NaCl, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.4; MgCl₂.₆H₂O, 0.1; chicken feather, 5.0; pH 7.5) incubated at 37°C under shaking conditions (120 rpm) for 4 days. After complete degradation of feathers, the broth culture was centrifuged at 10,000 rpm for 15 min. The supernatant obtained was used as crude enzyme extract and stored at 4°C until used for further experiments.

Effect of surfactant on enzyme activity: The effect of anionic detergent SDS and non-ionic detergent Triton X-100 on keratinase enzyme activity was determined by measuring the enzyme activity in the presence of different detergents at 0.1% and 0.5% concentrations under standard assay conditions. Keratinase activity was measured at 595nm. Positive result was indicated by the release of blue colour azo dye. The untreated enzyme was considered to be control having 100% residual activity.

Detergent application: Destaining of various stains on fabric: In order to check possible application of crude enzyme extract (obtained from culture supernatant, mentioned before) as detergent additives with ability to remove dirt and stain, white cotton cloth of specific size (4cm x 4cm) were taken. The crude enzyme extract having keratinase activity of 5.72 KU/ml/hr, was taken. Market available commercial detergent was diluted to final concentration of (10 mg/ml) to enhance the washing. Commercial detergent was heated at 100°C for 30 mins to inactivate the endogenous enzymes present in the detergent. Three separate pieces of clothes were prestained with goat blood smear. The ability to remove other stains such as egg yolk and tea was also tested. Three types of treatments were given: first with commercial detergent protease free alone, second with enzyme and detergent protease free in the ratio 1:1, while, the third one was with only crude enzymatic treatment. In the three experimental set up, the pieces of clothes were immersed in 30ml of a) detergent solution, b) enzyme+ detergent solution and c) solely crude enzyme extract. The clothes were incubated at 55°C for 30 minutes. After incubation, the pieces of clothes were taken out of solution, washed under running tap water, allowed to dry, followed by comparative evaluation to check how much of the stain was removed/retained on cloth following each treatment.

Parameters checked to increase the efficacy of washing: Temperature: Different temperatures of water were tested, viz, 10°C, 37°C and 55°C to evaluate the efficacy of crude enzyme for cloth washing.

Enzyme-Detergent ratio: The crude enzyme and detergent ratio were varied as 1:2, 1:1 and 2:1 to determine which combination works better and improves the wash performance.

Soaking time: Soaking time was varied as 15 mins, 30 mins and 45 mins to test the optimum time required for soaking.

Evaluation of wash performance: The pieces of small cloth were washed extensively under tap water and then dried. In order to evaluate wash performance, the brightness of the cloth was measured by measuring the % transmission using a UV-Vis spectrophotometer (Cary 50, Varian) as reported earlier. The dried clothes were cut into small pieces, grinded in mortar pestle, dissolved in minimal volume of water and scan was performed in the range 300-800nm. Baseline correction was carried out with clean cloth used as control.

Scanning Electron Microscopy of washed clothes: To study the effect of three treatments (i.e. detergent, enzyme+ detergent and enzyme) on the fibre texture of the clothes, scanning electron microscopic study was carried out. The clean dry clothes without gold coating were observed at 50X.
Fig. -2.: Effect of temperature on wash performance

Fig. -3.: Effect of enzyme and detergent ratio on wash performance.

Fig. -4.: Effect of soaking time ratio on wash performance.
Fig. -5. : Scanning electron microscopic examination of washed clothes at 50X magnification. Control a) white cloth without any treatment. The texture of the cloth after treatment with b) detergent c) enzyme+ detergent and d) enzyme.

Table 1: Effect of surfactant on enzyme activity

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Concentration</th>
<th>Residual activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sodium Dodecyl Sulphate (SDS)</td>
<td>0.1%</td>
<td>103.94±1.62</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>110.59±1.95</td>
</tr>
<tr>
<td>Triton-X-100</td>
<td>0.1%</td>
<td>107.62±0.55</td>
</tr>
<tr>
<td></td>
<td>0.5%</td>
<td>114.20±2.79</td>
</tr>
</tbody>
</table>

magnification. A clean white cloth without any treatment was used as control.

RESULTS AND DISCUSSION
Enzyme based, especially protease based additives in laundry detergents are considered superior, eco-friendly, cost effective, energy saving, smarter alternative to conventional chemical based formulations and processes. Annual detergent consumption in India was estimated to be 2.8 kg/capita in 1994. The detergent consumption in rural areas is expected to grow 7 to 8% annually. Most of these detergents are phosphate based and use sodium triphosphates. These phosphates can cause serious problems of eutrophication making most of the water bodies absolutely non-functional. The situation is grim as the latter not only serves as primary source of drinking water but also is the source of their livelihood. As per World Bank reports detergent washing effluents are responsible for 17 to 20% of water pollution and in India 69.90% area is represented by rural area. The chemical based detergents are therefore very dangerous source of water pollution. Since, pure water is becoming more and rarer essential life throbbing commodity; it is right and urgent stage that we take measures to stop this water pollution. Therefore, it is very essential to look for a greener, safer eco-friendly alternative as possible replacements for these toxic detergent...
Table 2: Evaluation of wash performance of crude keratinase at different temperature, enzyme and detergent ratio and soaking time.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>%Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme + Detergent</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
<tr>
<td>10°C</td>
<td>25.65 ± 2.05</td>
</tr>
<tr>
<td>37 ºC</td>
<td>62.54 ± 7.67</td>
</tr>
<tr>
<td>55 ºC</td>
<td>66.27 ± 3.61</td>
</tr>
<tr>
<td>Enzyme: Detergent ratio</td>
<td></td>
</tr>
<tr>
<td>1:2</td>
<td>53.46 ± 4.28</td>
</tr>
<tr>
<td>1:1</td>
<td>74.88 ± 4.61</td>
</tr>
<tr>
<td>2:1</td>
<td>83.36 ± 4.87</td>
</tr>
<tr>
<td>Soaking time</td>
<td></td>
</tr>
<tr>
<td>15 mins</td>
<td>50.75 ± 2.93</td>
</tr>
<tr>
<td>30 mins</td>
<td>65.79 ± 6.38</td>
</tr>
<tr>
<td>45 mins</td>
<td>83.58 ± 3.94</td>
</tr>
</tbody>
</table>

additives. In fact, keratinolytic proteases are such alternatives. These enzymes are non-toxic, biodegradable, do not generate any toxic residues and are very efficient in break down as well as removal of various kind of stains and soils. Being bio-based they take care of the cotton quality and also improve the feel and appearance of the fabric as well. Although, this aspect of application for keratinase enzyme has been reported from several bacteria, such as *Bacillus subtilis*, no demonstrative evidence for the same is reported from *Stenotrophomonas*. There are reports highlighting the role of proteases (and not keratinases) as detergent additives from psychro-tolerant *Stenotrophomonas maltophilia* strain MTCC 752821 and cold active protease from *Stenotrophomonas sp*.

**Stability of the enzyme in the presence of Detergent:**
The crude keratinase enzyme from strain Norja-1 is activated in the presence of anionic detergent Sodium dodecyl sulphate (SDS) and non-ionic detergent Triton X-100, compared to control. The % residual activity is slightly higher in presence of 0.5% concentration as compared to 0.1% in case of both the detergents. The results are indicated in Table-1.

**Detergent additive property of crude enzyme:** The white pieces of cotton clothes which were pre stained with blood smear (Fig.-1a); egg yolk (Fig.-1b) and tea (Fig.-1c) were washed with commercial detergent alone, enzyme alone as well as enzyme and detergent mixture. In the clothes washed with detergent, traces of stain remained, while, comparative more fresh white texture of the cloth with no stain was recorded for the clothes washed with enzyme and detergent as well as with enzyme alone. The crude keratinase enzyme was found to be potential in removing blood smear even in the absence of detergent.

Study of evaluation and efficacy of wash treatments (under different conditions using combination of crude culture free enzyme extract and detergents) carried out spectrophotometrically (Table-2) revealed that the % transmission was maximum (and this maximum brightness of the cloth) at 55 ºC as compared to 37 ºC and 10 ºC (Fig.-2). The % transmission was maximum for clothes washed with enzyme + detergent (66.27) followed by enzyme alone (47.33) and detergent (31.54) at 55 ºC. The effect of enzyme and detergent ratio (Fig.-3) on wash performance showed that 2:1 is more effective (yielded brightest cloths upon wash) than 1:1 and 1:2. The % transmission for clothes treated with enzyme and detergent in 2:1 ratio was 83.36 followed by clothes treated with enzyme alone is 74.25 and for clothes treated with detergent alone is 66.78. The effect of soaking time (Fig.-4) showed that 45 mins of soaking increased the % transmission to 83.58 for cloth treated with enzyme and detergent. For clothes treated with enzyme alone it was recorded to be 68.11 while for clothes treated with detergent.
alone the % transmission was recorded to be 64.97. Clothes treated with enzyme and detergent showed more whiteness than the one treated with only enzyme extract. Similar evaluative performance studies were earlier reported for *Paenibacillus woosongensis* TKB218, however it has never been studied for any *Stenotrophomonas* strains.

In order to evaluate the role of crude enzyme extract in maintaining the quality of fabric, scanning electron microscopic studies were undertaken. As evident from the Fig.-5, compared to the control (Fig.-5a), the washed clothes after treatment with detergent alone (Fig.-5b) showed damaged structure of cloth fibre. On the other hand, enzymatic wash (when carried out in combination with detergent) showed that the texture of the cloth was smooth, fine with relatively less or little fibre damage. While, the texture of the cloth washed with enzyme alone showed even better results (no fibre damage and very smooth texture) as compared to that treated with enzyme and detergent mixture (Fig.-5c, 5d). It was therefore concluded that in terms of cloth fibre protection, washing with crude enzyme alone was best.

Literature survey revealed that although several cold active alkaline proteases are known from various strains of *Stenotrophomonas maltophilia* none are reported to have keratinase activity with illustrative detergent property. Very recently, improved detergent tolerance of keratinase KerSMD from *Stenotrophomonas maltophilia* by partial truncation of PPC domain has been documented. Since, keratinases are involved in degradation of almost recalcitrant keratin protein, it is currently considered more advantageous over other alkaline proteases especially in detergent industry. In this context, the strain Norja-1, isolated in this study, that produced extracellular crude keratinase in the culture filtrate which showed remarkable stability and activity makes it a potential candidate for biotechnological applications. These characteristics would allow the crude enzyme to be used as a possible effective detergent additive for smart cleanup process as already has been documented for similar keratinases from *Paenibacillus woosongensis*. However, same for *Stenotrophomonas* was never documented in literature to the best of our knowledge.

**ACKNOWLEDGMENTS**

The authors are grateful to the University of Burdwan, for infrastructural support. NS is the recipient of a state fellowship through Burdwan University.

**REFERENCES**

Fig.-1. Wash performance of crude Keratinase on cloth stained with
a) blood smear, b) egg yolk and c) Tea