Bioscouring refers to the enzymatic removal of impurities from fibres/fabrics, which endows it with improved hydrophilicity for further wet processes. Enzymatic scouring preserves the fibre’s structure and strength, avoids high energy consumption and severe pollution problems that are associated with conventional alkaline treatments. In the present study, protease enzyme was extracted from Bacillus subtilis which was isolated from abattoir (slaughter house) waste. Different medium parameters were optimized for maximal enzyme production. The enzyme was partially purified using ammonium sulphate precipitation followed by dialysis bag method. Partially purified protease enzyme was used in the bioscouring of wool fibres. Different parameters such as pH, temperature, time, enzyme concentration were optimized to achieve an efficient scouring. Comparison of enzymatic process for wool fibre with conventional alkaline soap process in terms of weight loss, whiteness index, tensile strength and FTIR studies confirmed that bioscouring could be as effective as the conventional process.

Keywords: Bioscouring, Protease, Bacillus subtilis, Abattoir waste, Wettability, Alkali solubility, Whiteness index
MATERIAL AND METHODS

Raw Materials

The raw greasy merino wool fibres of Australian origin were procured from (WRA) Wool Research Association, Thane, Mumbai, India for the scouring experiments being difficult to scour as they contain the high amount of contaminants such as wool wax, dirt, dust and sunt. Protease enzyme used for bioscouring was extracted from B. subtilis obtained from abattoir waste at our laboratory. Identification of the bacteria was carried out by 16S rRNA sequencing method. Casein was purchased from Sigma Chemicals. Chemicals for microbiological experiments were supplied by Himedia, Mumbai and all other chemicals were procured from S.D. Fine chemicals, Mumbai.

Microorganism and enzyme production

The B. subtilis used in this study was isolated from degraded abattoir (slaughter house waste), collected from local market, Mumbai, India. For production of protease enzyme, 100 ml of nutrient medium containing 1% gelatin w/v (as protein source), 0.5% yeast extract and 100 ml distilled water at pH 14 was inoculated with 1% inoculums of 24 h old B. subtilis culture (1 × 10^14 CFU/ml). As reported earlier, Bacillus species has optimum temperature of 40ºC for its growth and its metabolic activity is at highest peak during its growth phase due to which the enzyme works efficiently (Badhe et al. 2016). The flasks were incubated at 40ºC for 24 h on a rotary shaker maintained at 150 rpm. After 24 h, the broth was centrifuged at 5000 rpm at 4ºC for 20 min and the cell free supernatant was collected. The supernatant was used as crude enzyme extract. The application of enzyme in the textile industry does not require high grade purity for enzymes and generally requires use of the crude or the partially purified enzyme preparation (Nerurkar et al. 2013). Thus, partial purification of the protease was carried out by ammonium sulphate precipitation since ammonium sulphate is highly soluble in water, cheap, and has no deleterious effect on the structure of protein. Ammonium sulphate was added to the crude enzyme sample to get 30% saturation and was kept overnight at 4ºC. It was then centrifuged at 6000 rpm for 20 min. The precipitate was suspended in phosphate buffer, pH 7.0 and was dialyzed against the same buffer to carry out desalting process.

Protease assay

Protease activity was measured by caseinolytic method. (Walter, 1984). One unit of enzyme was defined as the amount of enzyme that liberates peptide fragments equivalent to 1 mg of bovine serum albumin (BSA) under the assay conditions.

Conventional scouring

Conventional scouring was carried out at pH 10 for 2 h at 35ºC to 40ºC. The solution used for scouring contained 2 g/L Auxipon NP solution (non-ionic surfactant) and 3 g/L sodium carbonate solution (alkali). MLR was kept at 1:30. Sample was given hot wash. The fibres were firstly air dried and then conditioned at 20 ± 1 ºC and 65 ± 2% RH for at least 24 h in desiccator containing calcium chloride.

Bioscouring

Different parameters of bioscouring namely buffer pH, enzyme concentration, temperature and time period were optimized to examine the efficiency of protease from B.subtilis and to further obtain efficient removal of impurities. The raw wool fibres were subjected to four different treatment solutions stated below:

1. Phosphate buffer at pH 7.0 and enzyme (B+E)
2. Phosphate buffer, soap and enzyme(B+S+E)
3. Phosphate buffer, alkali and enzyme (B+A+E)
4. Phosphate buffer, soap, alkali and enzyme (B+S+A+E)

The raw wool fibre samples were subsequently suspended in buffer of pH ranging from 3.0 to 11.0. The material to liquor ratio was set at 1:30 with varying dosage of protease in the range of 2% to 14% on the weight of fabric for treatment time of 30, 60, 90, 120, 150 and 180 min at different temperatures ranging from 30 °C to 80 °C. The bioscouring process optimization was carried out by varying one parameter at a time, keeping other factors constant. The experiments were performed in the water bath (Bio Technics India). After the enzymatic treatment; the fibres were given hot wash at boiling temperature for 30 min, air dried and dried in desiccator for 24 h. Efficiency of bioscouring for each parameter was evaluated by monitoring weight loss of the fibres as the difference before and after the bioscouring treatment. Wool sample, bioscouring used protease enzyme under optimized parameters, was used for further analysis.

Evaluation of fibre properties

Weight loss determination

Enzyme-treated and untreated wool fibers were conditioned at 20 ± 1ºC and 65 ± 2% RH for at least 24 h before being weighed. Weight loss was expressed as:

\[
\text{Weight loss \%} = \frac{W_1 - W_2}{W_1} \times 100
\]

Where, W₁ is the conditioned weight of the sample before enzymatic treatment, and W₂ is the conditioned weight of the sample after enzymatic treatment respectively.

Wettability (Drop test)

The wettability of raw wool and enzyme treated wool fibres was measured using standard test. BS 4554:1970. According to the method, the time (sec) required by a drop of water to be absorbed by the fibre is defined as the ability of the fibre to get wet.

Sinking time

Sinking time test AATCC 17-2005 was modified for being used for fibre. Instead of fabric, a bundle of fires was used as indicative test for evaluating absorbency.

Alkali solubility

To quantify the damage to the epicuticle of wool, alkali solubility in terms of weight loss of raw wool and enzyme treated fibres was measured using IWTOTM-4-00 standard test method. The values were calculated as a percentage of the original mass, according to the equation given below,

\[
\text{Alkali solubility (\%)} = \frac{M_1 - M_2}{M_1} \times 100
\]

Where, M₁ is the mass of oven dry sample before sodium hydroxide treatment, and M₂ is the mass of oven dry sample after sodium hydroxide treatment.

Residual grease content

Efficiency of removal of grease content of conventional scoured and enzyme scoured wool sample was evaluated by using the following formula according to standard method IWTO1903:

\[
\text{Grease Content} = \frac{W_1 - W_2}{W_1} \times 100
\]

Where, W₁ is the weight before removal of grease from wool sample and W₂ is the weight after removal of grease from wool sample.

Determination of Moisture Regain

Moisture regain was calculated using the following equation according to ASTM method 2654-76:

\[
\text{Moisture regain (\%)} = \frac{W_1 - W_2}{W_2} \times 100
\]

Where, W₁ is the weight of the conditioned sample at standard humidity and W₂ is the weight of the sample dried to constant weight.

Determination of Moisture Content

The samples were preconditioned in a desiccator for 24 hr at 65 ± 2 % RH and 27 ± 2 ºC. The moisture content was determined after obtaining the weight of wool dried at 105ºC for 3 hr. The oven dry mass was determined according to standard IWTO-34-85-E method.

\[
\text{Moisture Content (\%)} = \frac{W_1 - W_2}{W_1} \times 100
\]

Where, W₁ is the weight of the conditioned sample at standard humidity and W₂ is the weight of the sample dried to constant weight.

Single Fiber Strength Test

The single fibre strength of raw and scoured wool was measured on Universal Tensile Machine supplied by Ainil Limited, Navi Mumbai according to ASTM D 3822 standard test method. The instrument was based on constant rate of
elopment (CRE) principle. The distance between jaws was 10 mm and the travel rate was 6 mm/min.

Fiber Mean Diameter Test

Fiber diameter measurement was carried out using OFDA 100 as per the standard IWTO47:2011. The fiber samples were cut into 2 mm snippets and spread on a 70 mm square glass slide. The whole slide was scanned with a minimum of 6000 fibers. For each sample, three measurements were taken. The mean diameter and standard deviation of the sample were then calculated.

Whiteness & Yellowness measurement

The ASTM whiteness Index (WI) and yellowness Index (YI) of samples, before and after scouring were determined by using Computer Colour Matching (CCM) System (Spectrascan5100+) according to IWTO35:03 standard test method. The Improvement in whiteness and reduction in yellowness are expressed as the percentage change relatively to the original whiteness and yellowness respectively.

FTIR analysis

FTIR study was carried out to analyze the changes in structural groups and impurities on the wool surface of raw wool and enzyme treated wool fibres.

SEM

The surface morphologies of the wool samples were visualized using a JSM-6380L, an analytical scanning electron microscope (JEOL Company, Japan), operated at a typical accelerating voltage of 5 kV. The samples were sputter-coated with Platinum for 30 s at 15 mA prior to the observation.

Statistical Analysis

All the experiments were conducted in triplicate and results were expressed as mean±standard deviation. Student’s t-test was used to analyze data, and statistical significance was declared at p<0.05.

RESULTS AND DISCUSSION

Protease production in submerged fermentation

Glucose was used as sole carbon source for protease production using B. subtilis in submerged fermentation. Gelatin was used as protein source as a substrate. Protease so produced showed maximum protease activity of 63 U/ml after optimizing various physical, chemical parameters of fermentation and partial purification using Ammonium sulphate.

Bioscouring

Raw wool fibres were subjected to four different treatment solutions as reported above. It was seen from (Figure.1) that there was maximum weight loss and a less than 2 sec wetting time when raw wool was treated with the solution containing buffer and enzyme (B+E). Solution B+E was found to be optimum and was used for further studies, due to that protease enzyme is stable in buffer solution (Joshi et al. 2013).

In order to explore the potential of our protease enzyme in bioscouring of wool, various factors like pH, enzyme concentration, temperature and time period were optimized. As seen from (Figures. 2–5), the optimal conditions for the bioscouring of wool sample were 4% protease dosage on the weight of the fibre at 60 ºC, Phosphate buffer pH 7.0 and a treatment time of 120 min.

Effect of Time period

Effective bioscouring using protease was optimized for 120 min in terms of weight loss and wettability. From the (Figure.2), it can be seen that the weight loss remained steady upto 60 min, after that there was a steep rise and the maximum weight loss was observed at 120 min. After that there was a sudden decline in weight loss which can be attributed to protein specific action of protease such that within treatment time of 120 min itself, protein and hence the other loosened impurities get removed.

Effect of temperature

Effect of temperature on protease scouring can be seen from (Figure. 3). As compared to 20% weight loss obtained in alkaline scouring, sufficient weight loss is achieved when scouring is carried out between temperature ranges of 30ºC and 80ºC, where as scouring efficiency decreases drastically as temperature of scouring bath is raised to 80ºC. This is because the protease enzyme must be showing less activity at 80ºC as compared to lower temperatures mentioned earlier. Optimum temperature for scouring of wool using protease was observed as 60ºC.

Figure 2 Effect of time period on protease bioscouring. Protease scouring was carried out at protease dose of 2 % (owf), pH 7.0, at 40ºC for varying period of time keeping MLR at 1:30. Filled squares indicate percentage weight loss of the wool fibre and filled diamonds indicate wetting time of wool fibres in second.

Figure 3 Effect of temperature on protease bioscouring. Protease scouring was carried out at protease dose of 10% (owf), pH 7.0, for 120min at various temperatures keeping MLR at 1:30. Filled squares indicate percentage weight loss of the wool fibre and filled diamonds indicate wetting time of wool fibres in second.

Effect of enzyme concentration

As can be seen from (Figure. 4), 4 % of protease enzyme (owf) is optimum for scouring as further increase in the protease concentration showed more or less similar weight loss as compared to weight loss achieved when 4% of protease enzyme was used for scouring.
Residual grease content

Efficiency of removal of grease content of raw wool which was 11.90% for conventional scoured, (B+S+A+E) scoured was 0.16% and enzyme scoured (B+E) wool sample was 0.36%.

Moisture Regain and moisture content

Removal of hydrophobic greasy substances from the surfaces of the raw wool is expected to increase the moisture regain values and higher weight loss in the scouring treatment translates to higher moisture regain values. The highest moisture regain values were observed in the case of enzyme scoured i.e. B+E samples at 42.85 %, while the moisture regain values of conventional i.e. B+S+A+E scoured, B+S+E scoured samples, or B+A+E scoured wool samples were in the range of 25 %. According to standard method, enzyme and buffer showed maximum moisture regain and content as compared to other.

Effect on Physical properties

Fiber Mean Diameter Test and Single Fiber Strength Test

There was no significant effect on mechanical properties of wool fibers on bioscouring. Little difference in the tenacity and elongation of wool fibers is attributing of high degree of variability in fiber dimension and non uniformity in wool. It has also been found that there is no difference in fiber diameter of all the samples as shown in (Table 1). The fiber diameter ranges between 19.2-19.5 micron (Table 2) (Kalantzis et al., 2008).

<p>| Table 1 Effect of Protease and alkaline scouring on tensile strength of wool fibers |</p>
<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Sample</th>
<th>Breaking strength (%)</th>
<th>Extension (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Raw wool</td>
<td>5.80</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>Alkaline scouring (B+S+A+E)</td>
<td>6.00</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>Protease Scouring (B+E)</td>
<td>4.10</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>B+S+E</td>
<td>5.30</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>B+A+E</td>
<td>4.80</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>B+S+A+E</td>
<td>4.10</td>
<td>50</td>
</tr>
</tbody>
</table>

<p>| Table 2 Effect of protease scouring on the properties of wool fiber. |</p>
<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Fibre sample</th>
<th>Raw wool</th>
<th>Alkaline scoured (B+S+A+E)</th>
<th>Protease Scoured (B+E)</th>
<th>(B+S+E)</th>
<th>(B+A+E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Average fibre fineness (micron) (IWTO 47)</td>
<td>19.3</td>
<td>19.2</td>
<td>19.2</td>
<td>19.2</td>
<td>19.2</td>
</tr>
<tr>
<td>2</td>
<td>SD</td>
<td>1.13</td>
<td>1.27</td>
<td>1.59</td>
<td>1.11</td>
<td>1.33</td>
</tr>
<tr>
<td>3</td>
<td>CV%</td>
<td>21</td>
<td>31</td>
<td>27</td>
<td>26</td>
<td>22</td>
</tr>
</tbody>
</table>

SD- Standard Deviation, CV- Coefficient Variation.

Effect on Whiteness and yellowness

Enzymatic removal of wool impurities by the process of bioscouring resulted in whiteness improvement. Though, compared to alkaline scouring yellowness index of the bioscour ed wool was less, whiteness index of both alkaline (B+S+A+E) scoured and protease (B+E) scoured wool was almost similar (Table 3). Protease being substrate specific, it only attacks peptide bonds and does not remove colouring matter in wool whereas caustic soda removes colouring matter being non-specific in action.

<p>| Table 3 Wool fiber properties at different stages of textile wet processing |</p>
<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Fibre sample</th>
<th>WI</th>
<th>VI</th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Raw wool</td>
<td>8.71</td>
<td>10.31</td>
<td>38.51</td>
</tr>
<tr>
<td>2</td>
<td>Alkaline scouring</td>
<td>21.29</td>
<td>20.46</td>
<td>49.57</td>
</tr>
<tr>
<td>3</td>
<td>Protease Scouring (B+E)</td>
<td>20.76</td>
<td>11.37</td>
<td>48.27</td>
</tr>
<tr>
<td>4</td>
<td>B+S+E</td>
<td>12.19</td>
<td>16.96</td>
<td>50.96</td>
</tr>
<tr>
<td>5</td>
<td>B+A+E</td>
<td>16.29</td>
<td>15.56</td>
<td>52.43</td>
</tr>
</tbody>
</table>
FTIR Spectroscopic Analysis

The FTIR spectra of the raw wool, alkaline-scoured, protease-scoured fibers were shown in (Figure 6 to 10) indicating characteristic absorption bands assigned mainly to the peptide bonds (CONH) which represent the fundamental structural unit of the polypeptide chain (Zoccola et al., 2009). The results confirm that no new chemical bonds are produced in wool fibre. The two sharp peaks in the range of 2935–2915/2865–2845 cm\(^{-1}\) for raw wool fibre samples corresponds to Methylene C-H asymmetric/symmetric stretch and similarly at 1485–1445 cm\(^{-1}\) for Methylene C-H bend (Meyers, 2000). Intensity of these peaks in raw wool fibre is high, while after protease treatment the intensity of all these peaks (Methylene C-H stretch and bend) reduces, indicating the removal of 18-MEA acid covalently bonded to epicuticle. The peak at 1737 cm\(^{-1}\) for raw wool fibre corresponds to covalent bond of 18-MEA acid to epicuticle through the (C=O) sulfoester. After B+E solution (protease) treatment, this bond breaks and it can be seen from Fig.7 that, peak at 1737 cm\(^{-1}\) disappears. The five spectra reveal that the bands near 1600 cm\(^{-1}\) assigned to amide I and II vibrations are shifted. They reveal a combination of amide C=O and N-H modes. The frequency is sensitive to protein conformation, i.e., alpha helix, random, beta-sheet, etc. The intensity is proportional to the concentration of amide linkage, i.e., C(=O)-N(-H). Yet in this case, it is suspected that the differences are ascribed to the differences in the water content of fibers. There is an H-O-H bending mode at 1635 cm\(^{-1}\). This is supposed to push up the intensity of the amide I peak after B+E solution (protease) treatment (Mori and Inagaki, 2006). After protease treatment the additional peak appears at 1076 cm\(^{-1}\) which corresponds to the S-S oxidation in the surface of wool after treatment (Meyers, 2000; Hocker, 2002).

**Figure 6** FTIR spectra of raw wool fibre. Analysis of functional group of raw wool fibre at wavelength ranging from 750 cm\(^{-1}\) to 3600 cm\(^{-1}\).

**Figure 7** FTIR spectra of protease scoured (B+E) wool fibre. Analysis of functional group of protease scoured wool fibre at wavelength ranging from 750 cm\(^{-1}\) to 3600 cm\(^{-1}\).

**Figure 8** FTIR spectra of buffer, soap, soda ash and enzyme (B+S+S+E) scoured wool fibre. Analysis of functional group of B+S+S+E scoured wool fibre at wavelength ranging from 750 cm\(^{-1}\) to 3600 cm\(^{-1}\).

**Figure 9** FTIR spectra of buffer, soda ash and enzyme (B+S+A+E) scoured wool fibre. Analysis of functional group of B+S+A+E scoured wool fibre at wavelength ranging from 750 cm\(^{-1}\) to 3600 cm\(^{-1}\).

**Figure 10** FTIR spectra of buffer, soap and enzyme (B+S+E) scoured wool fibre. Analysis of functional group of B+S+E scoured wool fibre at wavelength ranging from 750 cm\(^{-1}\) to 3600 cm\(^{-1}\).

SEM ANALYSIS

The clarity of wool surface was observed with scanning electronic microscope (SEM), as shown in (Figures 11 to 15). It could be seen that, the wool surface scoured using protease enzyme (B+E solution) was very clean and smooth as compared with those scoured using conventional process, B+S+E solution, B+A+E solution and B+S+A+E solution. There was partial removal of cuticles.
from wool fibre due to substrate specificity of protease enzyme. Prominent scales were observed on wool fibre surface when treated with B+E, as it got damaged with protease enzyme treatment. There was though a rise in surface performance and capillarity effect of wool fibre under this bio-scouring.

**Figure 11** SEM images of raw wool fibre (1000x). Surface analysis of raw wool fibre.

**Figure 12** SEM images of protease (B+E) scoured wool fibre (2000x). Surface analysis of protease (B+E)scoured wool fibre.

**Figure 13** SEM images of alkaline(B+A+S+E) scoured wool fibre (2000x). Surface analysis of alkaline (B+A+S+E)scoured wool fibre.

**CONCLUSION**

The bioscouring studies indicated that protease from *B. subtilis* is capable of removing waxes from the raw wool fibre and impart hydrophilicity to the fibre. In addition, the experimental studies of bioscouring parameters clearly states that under alkaline conditions at 60ºC for 120 min with a protease dosage of 10% on the weight of fibre, better than required hydrophilicity is achieved. The sharp peaks obtained from the FTIR data and smooth surface of wool revealed by the SEM images show the efficiency of protease in the removal of waxes from wool fibre indicating no damage to the wool fibre. Thus, the protease is a potential candidate to be used in bioscouring of wool. The approach described in the present work seems to be convincingly reproducible and environment friendly which can be easily adapted by the textile industry. The further work can be undertaken to check dyability of such bioscourred wool fibers vis-a-vis conventionally scoured wool and also combine bioscoring and dyeing to achieve shortening of overall processing cycle for dyed wool.

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**REFERENCES**


