BIODEGRADATION OF WOOL BY BACTERIA AND FUNGI AND ENHANCEMENT OF WOOL QUALITY BY BIOSURFACTANT WASHING

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Abstract

Biodegradation of Ryeland and Shetland wool by *Bacillus subtilis* W3 and *Streptomyces albidoflavus* were investigated. The effect of treating raw wool with Rhamnolipid was also studied. It is shown that the wool surface morphology is improved with effective displacement of surface contaminants revealing a smooth outer cuticle layer after just 2 days. These results have important practical implications for the establishment of a quick and easy biodegradable process for wool scouring finishing in textile industry or for the pre-treatment of keratinous waste materials before degradation by bacteria or fungi. This methodology provides an environmentally friendly alternative to conventional chemical pre-treatments.

Introduction

The role of microbial biodegradation has increased as sustainable methodologies and treatments are thought to help clean the environment. These biodegradation and biotransformation methods utilise the amazing, naturally occurring ability of microorganisms to degrade. Microorganisms have the ability to breakdown, modify or store almost all the compounds that occur in nature. In the recent years there has been a methodological breakthrough which has enabled the proteomic, genomic, bioinformatics and various other

analyses of environmentally important microorganisms that have provided new insights into important biodegradative pathways and the ability of the microorganisms to adapt to that environment (Saber et al., 2010).

The global production of wool is approximately 1.1 million tons per year but a large quantity of the wool is wasted annually (Kabir et al. 2013). Wool is considered as keratin waste that contributes to the accumulation of environmental waste worldwide and significant amount of keratin waste are produced across the world (Korniłłowicz-Kowalska and Bohacz 2011). Keratin waste from wool is generated in a number of different industries including tanning and meat processing and this is believed to be the cause of a lot of environmental problems (Aluigi et al. 2011; Brebu and Spiridon 2011). Seeking to develop environmentally friendly and efficient ways to utilise waste wool are therefore needed to avoid environmental contamination by microorganisms. An efficient and effective method to treat or purify raw waste wool might hold part of the answer. Queiroga et al. (2012) reported those materials that have high levels of keratin do not accumulate and this highlights that there are natural degraders of keratin. Moreover wool has a very limited use because of its lack of solubility and because of its resistance to degradation by the various proteolysis enzymes (Meng et al. 2013). Wool is composed of proteins and amino acids and α-keratin are the backbone of wool. Keratin is characterised as an insoluble and hard to degrade animal protein. It has a high resistance to enzymatic hydrolysis because of its unique complex structure (Saber 2010). Raw wool can contain large amounts of surface impurities made up of wool wax and grease. In addition to the fatty acids in wool wax and wool grease, raw wool also contains water soluble materials such as suint (formed from dried perspiration), inorganic mineral dirt and vegetable matter (Dominguez et al. 2003). Wool scouring or cleaning is required to prepare raw wool in the textile industry resulting in better dye uptake and polymer adhesion used in shrink resistant treatments (Silva et al. 2005). Commonly used scouring agents include the use of sodium hydroxide in combination with ionic and non-ionic detergents (Long et al. 2013; Raza et al. 2014). Chemical treatment of wool and the subsequent release of contaminated waste water by the textile industry have come under scrutiny in recent years and alternative environmentally friendly alternatives are being sought (Silva et al. 2005; Long et al. 2013). Physical and chemical methods can be challenging, with various drawbacks such as the loss of energy and destruction of key amino acids responsible for the nutritional value of the

products obtained. In order to avoid compromising the quality of the amino acids, the use of modified enzymes, proteases and pectins to alter the surface of wool have attracted attention in recent years particularly with respect to shrink resistance and softening treatments in the textile industry (Hutchison et al., 2007; Silva et al. 2005). Biosurfactants such as Rhamnolipid for textile surface modification have also been considered for cotton fabric (Raza et al. 2014). They concluded that rhamnolipid in combination with pectinase provided a greener and less toxic alternative to conventional chemical scouring agents. The supplement of keratinous waste to nutrient agar improves the nutritional content compared to unmodified one (Zheljazkov et al. 2009).

Keratinolytic microorganisms possess specific unique hydrolytic enzymes: keratinolytic serine proteases and keratinolytic metallo-proteases comprise the majority of these enzymes and have the ability to degrade the keratin that is present in the wool. A variety of keratinolytic microorganisms have been employed in the past for the biodegradation of wool namely *Bacillus*, *Actinomyctes* and other fungi (Zaghloul et al. 2010). The special feature of these kinds of prokaryotic microorganisms is that they possess extracellular proteases that have the ability to break down large polypeptide substrates into smaller entities. This allows for the isolation and characterisation of compounds which have a varied range of applications (Queiroga et al. 2012). Keratinase has found application in the textile industry (Fang et al. 2013), it has been increasingly in demand in the pharmaceutical industry and keratinises have also played a key role in making biological H_2 and eco-friendly materials (Brandelli et al. 2015). Some research have shown that *Bacillus* spp. are abundantly rich in enzymes that degrade wool (Brandelli et al. 2010), however, the amount of enzymes synthesized by the five *Bacillus* isolates in the study by Brandelli et al. (2010) changes depending on the presence or absence of their substrate. So many researchers have agreed that *Bacillus* strains produce wool degrading enzymes constantly whether the substrate is present or not, however, some scientists believe that majority of microorganisms that break down wool produce wool degrading enzymes when the wool is supplied as the main source of nutrient (Kim et al. 2001; Cai et al. 2008 and El-Refai et al. 2005).

Materials containing keratin are more easily degraded when cut and crush in to pieces than intact ones. This could be due to heat from the crusher that causes some changes from the natural structure of the keratin thereby increasing the surface area for more enzymatic attack.

Bacillus sp. HTS 85 and HTS 126 specifically can break down intact wool and feather thereby cutting cost in textile industries (Queiroga et al. 2012). Chopping both sheep's wool and feathers into small pieces prior to inoculation for bacterial degradation has been shown by Zaghloul et al. (2010) to enhance the rate of solubilisation of the wool by increasing the surface area exposed to the bacteria.

In this study, autoclaving was used as the pre-treatment process for biodegradation to remove the indigenous microbial flora in the wool and bioaugmentation by pure cultures of *Bacillus subtilis* was employed to activate the biodegradation process. The bioprocess was monitored for a period of 12 days and the texture of wool was monitored at 3 days intervals. The morphological changes on the wool fibres were observed using scanning electron microscope (SEM) and a significant amount of keratin denaturation was observed during this study.

Materials and Methods

Isolation of wool degrading bacteria

The microorganism used in the present study was isolated from wool by soaking in water for several days and using a sterile metal loop to obtain microbial samples from different areas in the flask. A series of quadrant streaking were carried out in order to identify the organisms that were capable of protease activity. The strains with protease activity were purified by series of streaking on a fresh agar plates to obtain pure and non-contaminated strains. The strain with highest protease activity was isolated. This strain was identified by biochemical tests, morphological tests as well as 16S rRNA sequence and was recognised as *Bacillus subtilis.*

Characterization of *Bacillus subtilis*

In this research, sequencing of 16S rRNA gene of *Bacillus subtilis* was conducted by Deutsche Sammlung Mikroorganismen und Zellkulturen (http://www.dsmz.de/) and the partial sequence of the 16S rRNA gene. The comparison of the nucleotide sequence was done with already identified genes in EMBL and NCBI databases using bioinformatics tool know as Basic

Local Alignment Search Tool. The graphical representation of closely related sequence was done by pairwise comparison using K-2-P model. A phylogenetic tree was obtained with the partial 16S rRNA gene sequences using general purpose computer software called CLUSTALW by the neighbour-joining method (NJ). The data were then treated using the dendrogram-visualization program TreeView. The obtained tree as shown in figure 1 was shortened to save space and only similar species were considered.

1% Rhamnolipid solution

The commercially obtained Rhamnolipid was prepared at a concentration of 1% (v/v) using distilled water**.**

Wool preparation

The two commercially available sheep's wool samples (Ryeland and Shetland) were obtained from Garthenor Organic Pure Wool. The wool was cut into small lengths (approximately 1cm) using scissors.

Defatting of wool using Rhamnolipid

Both wool types (~3 g) were soaked in 1% solution of Rhamnolipid (prepared as above) and distilled water to assess the effect of the solution on wool surface texture. The wool was retained at room temperature for periods up to 7 days.

Autoclaving

Autoclaved wool samples were made as required by taking wool (approximately 3g) prepared as above and placing in 250 ml conical flasks and autoclaved at 121 °C for 20 minutes.

Degradation of wool using *Bacillus Subtilis*

Both wool types were washed thoroughly with a 1% solution of Rhamnolipid and rinsed with distilled water. Prepared wool (~3 g) was placed in 250 ml Erlenmeyer glassware holding100 ml distilled water and introduced either 1 ml or 5 ml of a 1% (v/v) bacterial suspension or 5 ml of a 1% (v/v) fungal suspension both in nutrient broth prepared as described above. Samples were incubated at 37 °C, maintained at pH 7 and rotated at 150 rpm using an orbital shaker at 150 rpm for periods up to 12 days.

Assessment of wool degradation

The extent of degradation and appearance of the wool samples were determined using scanning electron microscopy (SEM). Wool samples were collected at the indicated times, centrifuged for 20 minutes at 4000 rpm and allowed to air dry at room temperature overnight prior to analysis using SEM (Hitachi S-3400 N) at an acceleration voltage of 10-15 kV and a pressure of 50 pa .

Results and Discussion

Effects of autoclaving and inoculum volume (Bacillus subtilis* W3*)

The ability of the *Bacillus subtilis* W3 to degrade autoclaved and non-autoclaved wool was assessed by incubation for periods up to 12 days in distilled water inoculated with either 1 ml or 5 ml of a 1% suspension of bacteria in nutrient broth. Control samples were 3g wool in distilled water stored for a period of 12 days at 37 °C with rotation as above.

Onset of degradation in both the Ryeland and Shetland wool was apparent at 3 days in the autoclaved samples, with a steady increase in wool fibre breakdown over the remainder of the study. It can be seen from Figure 2 that the extent of degradation of the Ryeland wool is much more pronounced in the autoclaved sample inoculated with 5 ml of the bacterial inoculum. The non-autoclaved samples exhibit some surface texture differences resulting in a smoother appearance through partial removal of the wool fibre surface cuticles. Figures 3 show the extent of degradation of the autoclaved and non-autoclaved Shetland wool inoculated with 1 ml and 5 ml of *B. subtilis* W3 following sampling at 6 and 12 days. The non-autoclaved Shetland wool did not show significant degradation however, the autoclaved Shetland wool inoculated with 5ml inoculum of *B. subtilis* W3 showed a pronounced level of degradation with complete removal of the wool cuticles exposing inner wool fibres.

When considering inoculation volume and pre-treatment, the data demonstrates that there is a significant difference in the extent of degradation when 1ml and 5ml inoculum volumes were used, as well as a significant effect associated with the process of autoclaving the wool samples. In all of the cases examined, degradation of the autoclaved wool was much more

pronounced than with the non-autoclaved wool. Likewise, the inoculum volume of 5ml showed enhanced levels of degradation over the 1ml inoculation volume. The findings in this study are similar to those presented by Zaghloul et al. (2010) who reported enhanced solubilisation of wool in cultures containing recombinant *Bacillus subtilis* as a result of autoclaving the wool samples. It is believed (Queiroga et al. 2012) that autoclaving makes the wool more susceptible to degradation by keratinase through unfolding of the polypeptide chains thus exposing the wool cortex. The rapid onset of degradation in the autoclaved Ryland and Shetland wool can be attributed to the fact that the nutrient broth provides a food source to the bacteria enabling them to grow at a faster rate thus become active before they started the degradation of wool.

Effects of autoclaving *(Streptomyces albidoflavus)*

The ability of *Streptomyces albidoflavus* to degrade autoclaved and non-autoclaved wool was assessed in nutrient broth in the volume of inoculum applied (5 ml). Control samples were 3 g wool in distilled water stored for a period of 12 days at 37°C with rotation as above. Onset of degradation in both the Ryeland and Shetland wool was apparent within 48 hours in both the autoclaved and non-autoclaved wool samples, with a steady increase in wool fibre breakdown over the remainder of the study. It can be seen from Figures 4 that the extent of degradation for both wool types is significant after a period of 7 days. There are clear surface texture differences resulting in a smoother appearance through removal of the wool fibre surface cuticles and signs of breakdown of the wool cortex exposing cortical cellular material. *Streptomyces albidoflavus* appears to degrade the wool samples regardless of whether or not the samples are autoclaved and may be considered more effective than *Bacillus subtilis* W3 in the short term.

Effects of defatting

The effect of storing wool samples in a solution of 1% Rhamnolipid was assessed over a period of 28 days. Control samples were stored in distilled water over the same period. It can be seen from Figure 5 that Rhamnolipid removes the naturally occurring lanolin and other fatty deposits from the surface of the wool, highlighting the effectiveness of the biosurfactant. The Rhamnolipid 6 day samples demonstrated surface enhancements without apparent

disruption to the fibre's cortex structural integrity. This applied to both the Shetland and Ryeland wool types. According to Hutchison et al. (2007) the surface of wool is coated in a layer of covalently bonded lipids that represents a complex mixture of polar and non-polar long chain fatty acids. These fatty acids are bound to the fibre matrix through the formation of thioester bonds to cysteine residues resulting in the formation of a cross linked hydrophobic layer (Heine and Hocker 1995; Hutcheson et al. 2007). The highly hydrophobic surface of the wool fibre acts to restrict attack from enzymes like keratinise, therefore, effective removal of surface bound fatty acids through cleavage of the cysteine cross-linkages not only enhances the appearance of the wool, but subsequently exposes the surface of the wool fibre making it more susceptible to enzymatic hydrolysis (Eslahi et al. 2013). Penetration into the fibre cortex as a result of hydrolytic attack, while not advantageous in many applications (Du et al. 2007), is, in this case, desirable in order to cause degradation at macrofibril level allowing the process of wool degradation to get underway. In addition to the fatty acids in wool wax and wool grease, raw wool also contains water soluble materials such as suint (formed from dried perspiration) and inorganic mineral dirt (Dominguez et al. 2003). From the study conducted, the Rhamnolipid appears to effectively remove hydrophobic and hydrophilic surface contaminants from the raw wool in preparation of degradation of the wool. In addition to the above mechanisms for effective enzymatic action, Wen et al. (2010) conclude that Rhamnolipid acts to soften the membranes of bacteria which in turn influence the release and activity of the enzymes making the wool more vulnerable to degradation by microorganisms. Figure 5 shows SEM images for untreated wool and both wool types treated with 1% Rhamnolipid. The 7 day samples treated with 1% Rhamnolipid demonstrated further surface enhancements without apparent disruption to the fibre's cortex structural integrity, this applied to both the Shetland and Ryeland wool types.

Conclusions

Ryeland and Shetland wool can be degraded by both *Bacillus subtilis* W3 and *Streptomyces albidoflavus* as shown by SEM. *Streptomyces albidoflavus* appears to be the more effective strain for rapid degradation of both Ryeland and Shetland wool. However, Rhamnolipid is an effective biosurfactant when used to treat and defat samples of wool. The wool takes on a smoother appearance and becomes more susceptible to attack and degradation by enzymes produced by both the *Bacillus subtilis* and *Streptomyces albidoflavus* strains. Further work will

include assessment of wool quality following treatment with 1% Rhamnolipid and more advanced studies to assess the level of degradation of different wool types over a longer time period.

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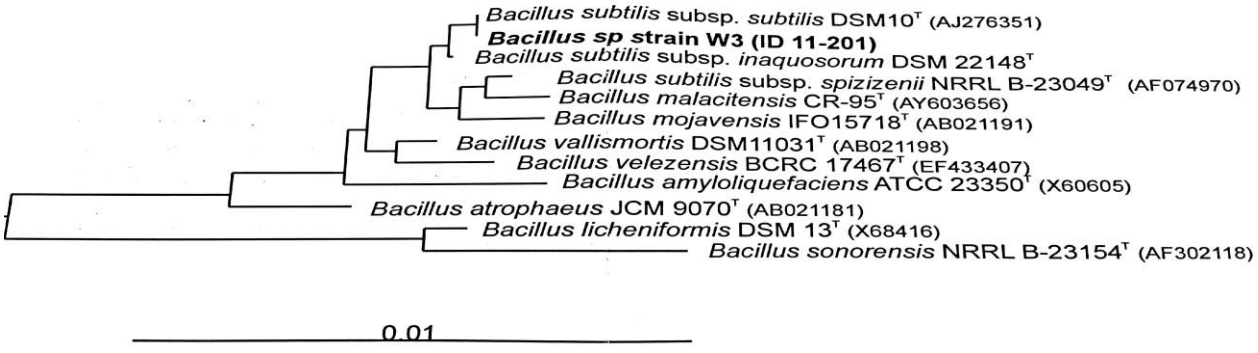


Figure 1: Phylogenetic tree showing relationship between *Bacillus subtilis* W3 and other members of genus *Bacillus* with their accession number in brackets

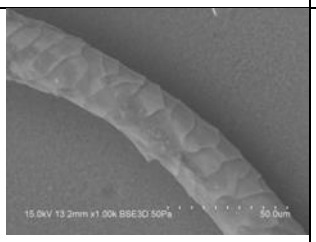
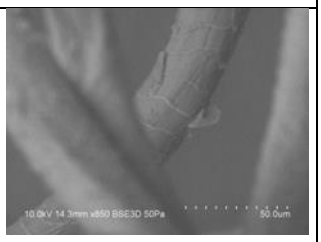
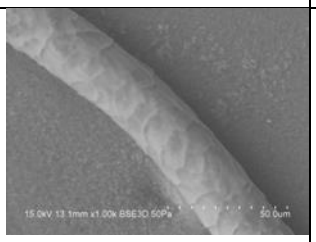
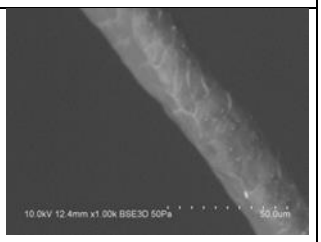
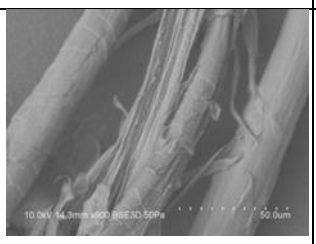
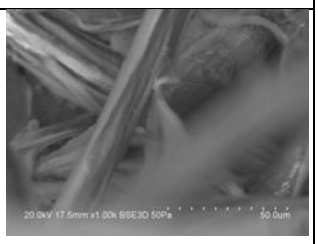
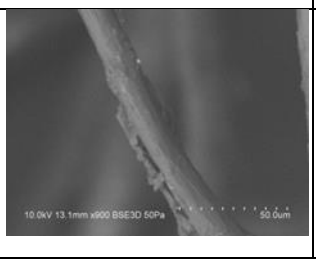
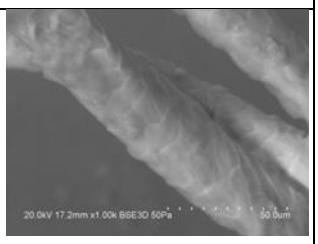
Ryeland 1ml	6 days	12 days
Autoclaved		
Non-autoclaved		
Ryeland 5ml	6 days	12 days
Autoclaved		
Non-autoclaved		

Figure 2: SEM images of Ryeland wool inoculated with 1 ml & 5ml *Bacillus subtilis* W3 for both autoclaved and non-autoclaved samples over a period of 12 days

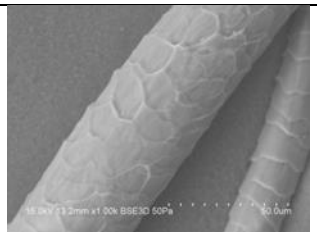
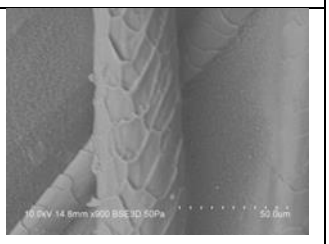
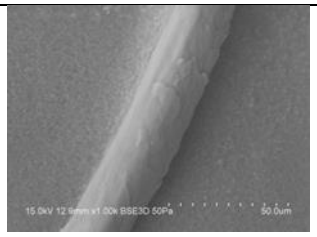
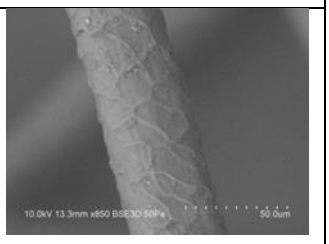
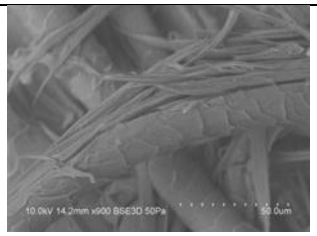
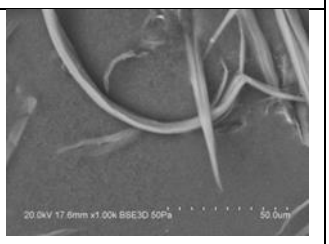
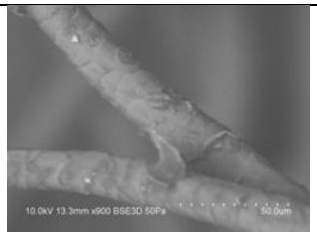
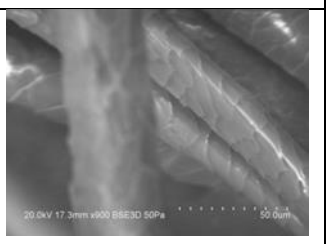
Shetland 1ml	6 days	12 days
Autoclaved		
Non-autoclaved		
Shetland 5ml	6 days	12 days
Autoclaved		
Non-autoclaved		

Figure 3: SEM images of Shetland wool inoculated with 1 ml & 5ml *Bacillus subtilis* W3 for both autoclaved and non-autoclaved samples over a period of 12 days

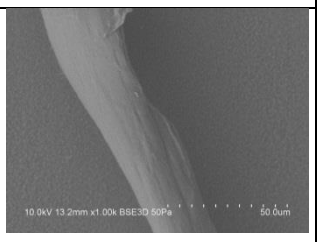
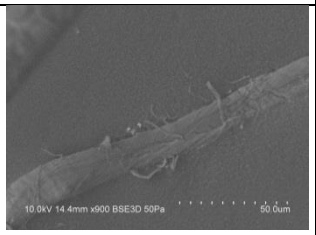
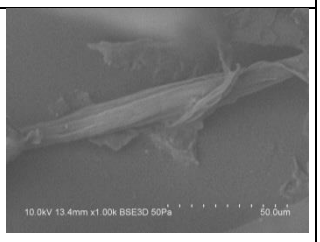
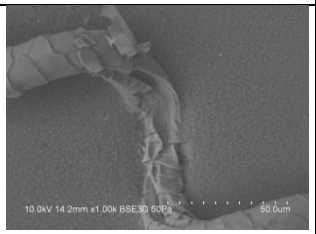
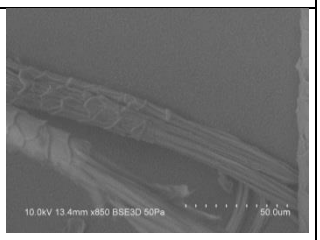
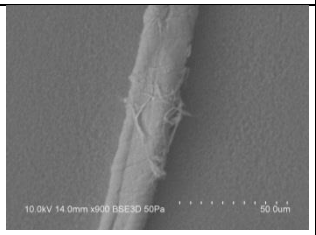
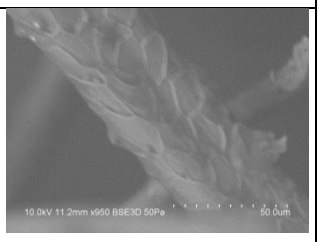
Ryeland Wool	2 days	7 days
Autoclaved		
Non-autoclaved		
Shetland Wool	2 days	7 days
Autoclaved		
Non-autoclaved		

Figure 4: SEM images of autoclaved and non-autoclaved Ryeland & Shetland wools inoculated with 1ml *Streptomyces albidoflavus* over a period of 7 days

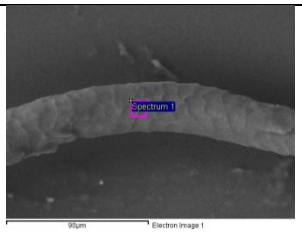
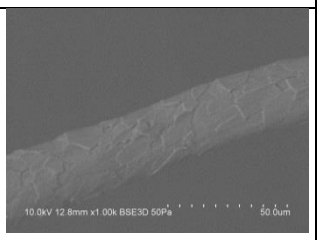
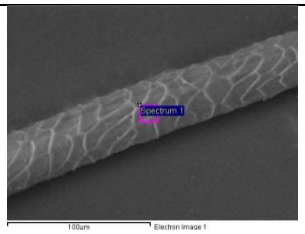
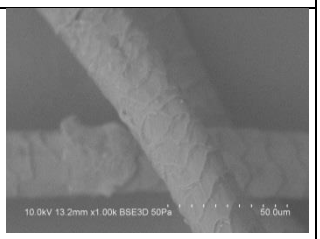
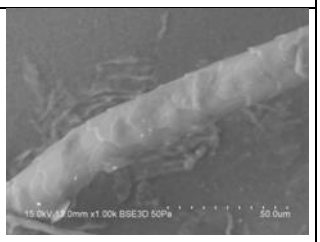
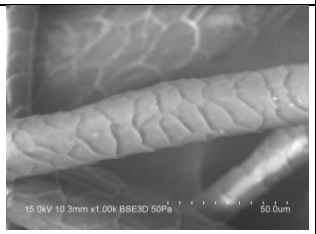
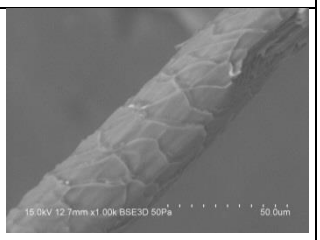
1% Rhamnolipid	Washed only	7 days
Ryeland		
Shetland		
1% Rhamnolipid	3 days	6 days
Ryeland		
Shetland		

Figure 5: SEM images of Ryeland and Shetland wool washed and soaked with 1% Rhamnolipid over a period of 7 days