IMMOBILIZATION OF LIPASE ON WOOLLEN FABRICS: ENHANCED EFFECTIVENESS IN STAIN REMOVAL

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ABSTRACT

The aim of this research was to examine the effectiveness of an enzyme in enhancing the cleaning effectiveness of woollen fabric without addition of any detergent. As a model enzyme, lipase from *Pseudomonas fluorescens* was immobilized onto a woollen cloth using a unique protocol that involved: chlorination of the wool, adsorbing a polyethyleneimine (PEI) spacer, adsorbing and cross-linking with glutaraldehyde (GA) followed by adsorption of the lipase. It was determined that for this protocol, the immobilized activity was dependent on the GA solution pH and not on its concentration.

The cloth exhibited excellent oily stain removal ability: after being stained with olive oil and stored for one day in air at room temperature, the oily stain could be easily removed by 0.05 M pH 8.5 Tris buffer without any detergent addition. This enhanced cleaning was stable also over a period of one month. The activity of the cloth (based on activity assay) dropped considerably over just 15 days storage in air. This therefore likely indicates that the enhanced cleaning seen over an extended storage period may not require as high an enzyme activity. The activity of the immobilized lipase was also very stable when stored under near ideal conditions: when the immobilized cloth was stored in 0.05 M Tris buffer (pH 8.5) for more than 80 days in a refrigerator, more than 80% of the lipase activity remained.

Overall, results indicate that this immobilization protocol is a promising step towards producing a woollen fabric with enhanced cleaning properties.

1. Introduction

Although enzyme immobilization techniques have been extensively researched for many years (*1-4*), industrial utilization is rare, due to the high preparation and processing costs and the lack of stability of the products for long-term industrial application. A range of support materials have been investigated and used for enzyme immobilizations. Among the support matrix materials, fabrics have many advantages, such as: availability in bulk, low-pressure drop and mature processing technologies. The most popular fabric used for enzymes is cotton cloth (*5-7*). Some studies have also been carried out on silk (*8*). However, very few studies have been published establishing enzyme immobilization on woollen cloth. As a keratin type protein fibre, wool possesses rich reactive residues (such as lysine, serine and glutamic acid), which might be used in surface activation in the immobilization process. Furthermore, it's rigid structure and wide availability further makes wool an attractive candidate as an immobilization matrix. Therefore, the possibility of developing a cheap and efficient lipase immobilization on woollen cloth was investigated.

Woollen fabric processing techniques have been well studied. The surface of untreated wool is quite hydrophobic (*9*). To facilitate chemical accessibility, normally this chemical bound hydrophobic layer is removed. The most popular processing technique is the Hercosett chlorination process (*10*), in which wool fibre was first chlorinated in a solution containing active chlorine, followed by cross-linking of a layer of polymer onto the surface to provide the wool with a superior washing ability (compared to untreated wool). Enzyme immobilization onto this surface can then achieved by using a glutaraldehyde (GA) cross-linking agent. GA has widely been used as a cross-linking agent in enzyme immobilizations due to its fast reaction and low toxicity to enzymes (*11-13*). The most widely accepted theory on the GA cross-linking mechanism is that the aldehyde groups in GA react with the lysine residues in the protein through a Schiff base reaction (*14*). To stabilize the product, treatment with a reductive agent, such as sodium borohydride, is required after GA coupling.

Furthermore, in this work, a polyethyleneimine (PEI) arm spacer is used to bridge the enzyme and wool matrix and allow the enzyme activity to be less spatially restricted by the matrix it is bound to. Utilization of PEI as an arm spacer in enzyme immobilization is a widely accepted method (*15-16)*. As a highly positively charged water-soluble polycation, PEI is able to interact with the negatively charged enzymes, giving them more protection. Moreover, the pending primary amine groups of the PEI could potentially react with enzyme in the presence of a cross-linking agent, forming immobilization through a stable chemical bond. (*17*) The most immediate application of a woollen fabric or cloth with immobilized enzymes is in self-cleaning and/or enhanced-cleaning of this woollen fabric. It has been established that oily stains on a woollen cloth/fabric are quite difficult to remove using detergents only (*18*). In laundry washing ingredients, most of the fatty stain removal capacity originates from the alkaline additives: the fatty acid component of fatty stains forms water-soluble soaps in the alkaline solution, which can easily be removed during washing process. But this cleaning ability is only limited to the fatty acid component of the dirt and most of the triglycerides still remain on the cloth due to their hydrophobicity. The enzyme additive in laundry ingredients can however promote this cleaning process. However, it has been found the lipase possesses only limited cleaning efficiency in water solutions, because of the mass transfer barriers between free enzymes and the stains on the cloth (*18*, *19*). Therefore, it has been suggested that if the lipase was adsorbed onto cloth surface, the lipid stain hydrolysis could either occur during storage *(20)* and/or the cleaning

efficiency in water would be enhanced by removing the aforementioned mass transfer resistances. Furthermore, immobilization of lipase onto a surface retains the enzyme, so that the same enzyme can be used again and again over multiple wash cycles and after periods of storage on the same material (i.e. not used for a extended period for enhanced stain removal), and so long as the enzyme activity can be retained, this reduces the need to use additional fresh (and potentially expensive) enzymes in each wash cycle.

Consequently, the main aim of this research is to determine if an immobilized enzyme can enhance the removal of an oily stain on a woollen cloth surface in aqueous solution compared to non-treated (enzyme bare/lipase free) cloth alone and if this activity is effective over multiple wash cycles and after periods of storage with the same immobilized enzymes. A thermophilic enzyme, lipase from *Pseudomonas fluoresces*, was selected as the model enzyme. Note that there is other work in the literature looking at an immobilisation protocol for wool with this enzyme (*21*), however this work was not optimised towards a specific application (such as here). The other differences between this work and ref. (*21*) will be addressed throughout this manuscript. This work therefore will be the first to investigate developing a cheap and effective method of immobilizing lipase on a woollen cloth in order to produce an enhanced cleaning effect and to test this by comparing the oily stain removal capacity of the lipase immobilised woollen cloth to enzyme bare cloths.

2. Materials and Methods

2.1. Materials.

Enzymes used in the research are the lipase derived from *Pseudomonas fluoresces* (*PFL*, Sigma-Aldrich New Zealand (NZ), product code 39,044-5). Note that lipase was not in a pure form - the protein concentration was only 18.5%. PEI was purchased from Sigma-Aldrich NZ (product code 25987-06-8). The buffer used in this study was prepared with trizma-hydrochloride (Sigma-Aldrich NZ, T-2253) and di-sodium tetraborate (BDH, product code 102674E) as well as a phosphate buffer detailed in Section 2.2.1.2. p-nitrophenyl palmitate (pNPP, Sigma-Aldrich NZ, N-2752) was used the substrate for the lipase activity assay. Triton X-100 was used as a surfactant in the preparation of the pNPP assay solution (BDH NZ, product code 306324N). Dichlorocyanuric acid (DCCA) was purchased from the local market (swimming pool bleaching agent). 100% woollen cloth without bleaching was provided by AgResearch Limited (NZ) – note that this differs from the pure organic wool cloth used in ref (*21*). All aqueous solutions were prepared with deionised water (produced from a Milli-Q Gradient A10 made by Millipore). All other chemicals used were of analytical grade.

2.2. Experimental techniques

2.2.1 Immobilization by a covalent binding method

The immobilization protocol developed and used involved five steps: chlorination of the wool, adsorbing a PEI spacer, adsorbing and cross-linking with GA, followed by adsorption (and potentially cross-linking via residual GA) of the lipase. This differs from the protocol in ref. (*21*), the major difference being that in this work lipase is adsorbed onto the woollen cloth after GA treatment, whilst in the above reference the GA treatment is done after lipase adsorption. This is a significant difference in protocol order that should affect the activity of the enzyme and the properties of the lipase immobilised cloth (as discussed below).

2.2.1.1 Woollen Cloth Chlorination

Wool fibres are hydrophobic and chemically resistant, due to a covalently bound lipid layer and sulfur-rich cortex layer on the surface (*9, 22*). Therefore, the surface was modified using DCCA chlorination, which is considered effective and is acceptable to the wool industry: *22-23)*. The chlorine concentration was quantified by 'on-weight-fabric' (owf; % chlorine equivalent to the weight of the cloth used) as used by the wool industry.

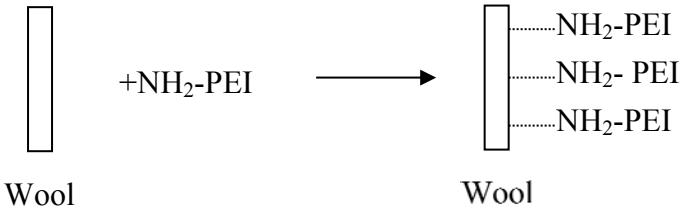
In this paper a 'low pH' DCCA chlorination process was used - the procedure in ref. (*22*) was used for wool chlorination with some modifications. Cloth was treated for between 30 seconds and 3 minutes in a beaker containing 2.5% owf of DCCA. The solution pH was adjusted to 1.5-2.0 by using solutions of sulphuric acid and acetic acid. Then the cloth was soaked in the solution containing 10 $g.l^{-1}$sodium carbonate and 4 $g.l^{-1}$ sodium sulfite for 30 sec, then rinsed with water and dried.

2.2.1.2 Immobilization Buffer selection and preparation

Several different buffers were tried in initial work (e.g. Tris buffer, phosphate buffer, acetate buffer, and carbonate buffer). A 0.1 M phosphate buffer was selected as the main immobilization buffer because of its inertness to the chemicals involved, in particular GA (*24*). Buffers were prepared by methods described elsewhere (*25*). The optimal pH for lipase from *PFL* was found to be around 8.5 (see Section 3.2) and therefore this pH was selected for the storage buffer (Tris buffer). Any other buffers used are stated where appropriate.

2.2.1.3 Immobilization Protocol Step 1: Immersion in PEI solution

The first step of the method is to physically adsorb the PEI arm spacer onto the wool:



(1)

PEI is needed, since the isoelectric point of lipase from *PFL* is 4.46 (*26*) and the isoelectric point of wool is approximately 2.5-3.5 (*27*). The optimum pH for this lipase is reported by the supplier to be between pH 5 to pH 9. To directly apply lipase to wool, the immobilization pH has to be between 3.5 and 4.46, which is not conducive for lipase stability. Thus a positively charged arm spacer between the wool and lipase was imperative in order to conduct this immobilization. PEI is a charged water soluble polymer, possessing a primary, secondary and tertiary structure, which is in the ratio of 1:1:1. Wool protein carries a negative charge in the pH used during immobilization, so when the PEI and lipase is brought into contact, a layer (assumed to be a mono layer) of PEI is established on the woollen cloth. Furthermore, lipase also adheres to PEI through electrostatic interactions and can be cross-linked to PEI (for example by using GA), making it an ideal arm spacer.

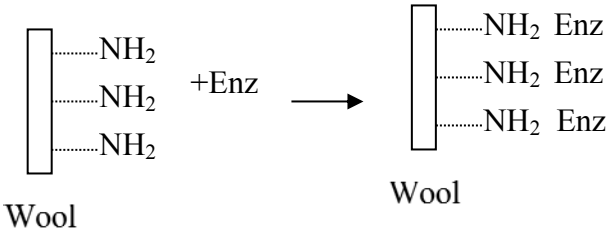
2.2.1.4 Immobilization Protocol Step 2: Soaking in GA solution – Investigation of the Cross-linker

A cross-linker is needed to fix the PEI structure to the wool (and also to bind the enzyme to the wool after Step 3). GA was used for this purpose, based on success in other work (*28, 29*).

To evaluate the effect of GA on the immobilized lipase activity via this immobilization method, the GA concentration was varied between 0.1% and 12%. Chlorinated cloth, was firstly soaked in 2% PEI solution for 2 hours, then treated in GA solution (pH 8) for 10 min.

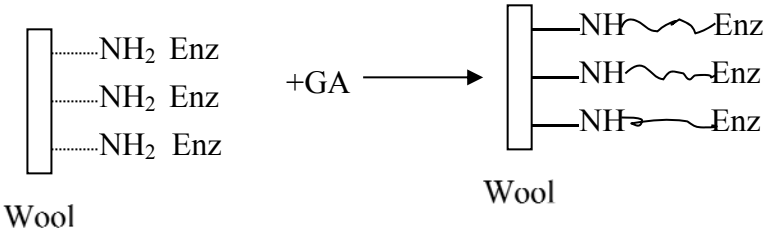
2.2.1.5 Immobilization Protocol Step 3: Addition of lipase solution

In the final step, enzyme is adsorbed onto PEI coated woollen cloth, and through shaking the PEI and GA coated woollen cloth in lipase solution (1 mg ml^{-1}) overnight:



(2)

This step was done after the GA soak to minimise the amount of GA the enzymes are exposed to, since excess GA exposure to enzymes may potentially cause deactivation, such as through excess GA cross-linking. The disadvantage however is that since the enzyme is exposed to lower concentrations of GA compared to other methods (such as ref. (*21*)), this will most likely mean that the enzyme will not be as completely cross-linked as in those methods. The consequence however is that in this step, enzyme is adsorbed and some may also be potentially cross-linked to the PEI and the wool by any residual GA:



(3)

Experiments were conducted investigating the effect of lipase solution immersion times. The conditions used were: after chlorination, 0.5 g woollen cloth was treated in 0.1% GA solution at pH 9.0 for 5 min, and then the cloth was treated in 1 mg ml^{-1} lipase solution for a range of different immersion times.

2.2.2 Lipase Activity Assay

2.2.2.1 pNPP assay solution preparation

The lipase-catalysed conversion of pNPP to p-nitrophenol (pNP) was used as the activity assay due to its convenience and rapid response. Note that this means a direct comparison to the nearest wool immobilisation method to that developed and tested in this paper (*21*) cannot (and therefore will not) be made, as a very different tributyrin pH-stat activity method was used in that work.

The pNPP assay solution was prepared by a method taken from the literature (*30*) with some modifications. This was because it was found that the colour of 0.4 mM pNPP solution turned yellow within a couple of hours even without lipase addition. The yellow colour developed is ascribed to the pNPP hydrolysis in the alkaline solution used, which accelerates with increased pNPP concentration, pH and temperature. This process slows down in a neutral or acidic solution and/or at a low temperature.

Therefore, the pNPP stock solution was prepared in DI water and stored in a fridge (at 4 ^{o}C), and then diluted with the buffer solution before use. The preparation steps were as follows:

2.2.2.2 Preparation of the stock solution

The pNPP concentration in the stock solution was 0.8 mM. pNPP was firstly dissolved in 3% (wt %) isopropanol and 1% (wt%) Triton X-100. After pNPP was completely dissolved, this solution was diluted with DI water to the wanted concentration. This stock solution was stable for up to two days in a fridge (at 4 ^{o}C), after which it gradually increased in turbidity. However, this turbidity could be removed (and therefore the stock solution recovered), as the solution could be made clear again by heating to 70-80 ºC until the turbidity disappeared.

2.2.2.3 Preparation of the working solution

Before use, this stock solution was diluted with 0.1 M Tris buffer at pH 8.5. The accuracy and freshness of the dilute solution was checked: if the stock solution was stored for too long, it would turn a yellow colour directly after dilution. If this occurred, the stock solution was discarded and fresh pNPP stock solution prepared.

pNPP concentration values were obtained by using an UV-Vis spectrometer (Agilent 8453). The UV adsorption from the now yellow coloured reaction mixture was then recorded at 410 nm. This yellow colour is generated by pNP, which is hydrolysed from the pNPP by the lipase. To calculate the lipase activity, the UV-Vis absorbance has to be converted into μmol of pNP by the means of a standard curve of pNP concentration versus UV adsorption (i.e. and external calibration). Three independent replicates were taken for every measurement and the results presented are the mean of these three repeats; the standard deviation was also calculated.

2.2.2.4 Activity of free lipase

0.1 ml of 1 mg.ml^{-1} lipase solution (i.e. 0.1 mg of lipase) was mixed with 4.9 ml of 0.4 mM pNPP solution. After hand shaking for 10 seconds, its colour change at 410 nm was recorded by UV-Vis spectrometry for 30 seconds. One unit of free enzyme is defined as the amount of enzyme, which catalyses one μmol of pNPP to pNP per minute at the testing conditions (25 ^{o}C, at 0.05 M pH 8.5 Tris buffer). According to the definition in *(31)*, the lipase specific activity is then expressed as amount of pNPP (in μmol) converted into pNP per minute by one mg of lipase (giving activity units of μmol pNPP.min^{-1}.(mg lipase)$^{-1}$ or μmol pNP.min^{-1}.(mg lipase)$^{-1}$).

The effect of pH on the activity of the free lipase was measured in 0.1 M buffer, set at different pHs. To accomplish this, pHs between 6 and 8.0 were prepared with the phosphate buffer and pHs between 8 and 10.5 were prepared with an ammonia/ammonium sulphate buffer. The activity of 1 mg/ml lipase prepared in the different buffers was tested using the free lipase activity assay.

2.2.2.5 Activity of immobilized lipase

The pNPP (0.4 mM) assay was also utilized for the immobilized lipase. Lipase immobilized cloth (0.1 g) was shaken in 5 ml of 0.4 mm pNPP assay solution over a recorded time period. The UV absorbance change at 410 nm in that period of time was recorded by UV spectrophotometry. Similarly to the free lipase, one unit of immobilized lipase is defined as the amount of immobilized lipase that catalyses one μmol of pNPP to pNP per minute at the test conditions (25 ^{o}C at 0.05 M pH 8.5 Tris buffer). Then in accord with Warmuth *et al. (32)*, the specific immobilized activity is then expressed as the amount of pNPP (μmol) converted into pNP per minute per gram

of lipase-immobilized cloth (giving activity units of μmol pNP.min^{-1}.(g cloth)$^{-1}$ or μmol pNPP.min^{-1}.(g cloth)$^{-1}$).

The effect of pH on the activity of the immobilized lipase was also determined. The same buffers as used with the free lipase were also used. The activity of 0.1 g of lipase immobilized woollen cloth was tested in 5 ml of 0.4 mM pNPP assay solution, in which the assay solution was prepared with different pH buffers. The stability of the immobilized lipase was sufficient for this assay - there is negligible activity change throughout the whole assay.

2.2.2.6 Activity of lipase leaked from immobilization

In addition to the two types of lipase activity defined above (both of which commonly appear in the published literature), another new type of lipase activity is defined in this research: the 'leaked activity'. This is the activity of the lipase leaked out (lost) from the cloth during activity determination (where lipase lost is most likely originates from that just absorbed/adsorbed onto the cloth and/or poorly GA cross-linked lipases). One unit of leaked activity is defined as the amount of lipase that produces one μmol of pNPP to pNP per minute at the testing conditions (25 ^{o} C, at 0.05 M pH 8.5 Tris buffer) and the specific leaked activity is expressed as the amount of pNPP (μmol) converted into pNP per minute caused by the lipase leaked from per gram of lipase-immobilized cloth (with activity units of μmol pNP.min^{-1}.(g cloth)$^{-1}$ or μmol pNPP.min^{-1}.(g cloth)$^{-1}$).

2.2.2.7 Enzyme storage stability

The free and immobilized lipases were stored either at room temperature (approximately 25 ^{o}C) or in a refrigerator at 4 ºC, either in tris buffer solution or dry. Their activity was monitored periodically over time and the trend of activity change versus time was used as an indication of enzyme storage stability.

2.3 Determination of Protein Load

2.3.1. Determination of protein concentration

Protein concentration of lipase was determined in order to calculate the amount of lipase immobilized on cloth and the lipase purity. The protein concentration in solution was determined by the micro bicinchoninic acid (BCA) method, based on the Sigma-Aldrich QuantiPro BCA Assay Kit. The principle is based on the alkaline reduction of Cu^{2+} to Cu^{1+} by proteins, and the formation of BCA: Cu^{1+} complex having a maximum absorbance at a wavelength of 562 nm.

2.3.2 Determination of protein load on the lipase immobilized cloth

Two methods were used for protein load estimation: pNPP activity assay and a QuantiPro BCA protein concentration assay kit.

The amount of lipase adsorbed onto cloth was first estimated from the lipase activity difference in the free lipase solution before and after cloth adsorption. The amount of lipase leak-out was estimated from the leaked activity tested by pNPP assay. The protein load can then be calculated using equations 4 to 6:

$$\text{Lipase adsorbed} = \frac{\text{Activity before - Activity after}}{\text{Specific activity} \times \text{cloth weight}} \qquad (4)$$

$$\text{Lipase leaked} = \frac{\text{Leaked activity}}{\text{Cloth} \times \text{specific activity}} \quad (5)$$

$$\text{Protein load} = \text{Lipase adsorbed} - \text{Lipase leaked} \quad (6)$$

In equations 4 to 6, "Lipase adsorbed" is the amount of lipase (in mg/g cloth) adsorbed onto cloth during immobilization. "Activity before" is the free lipase activity in the lipase solution before cloth soaking. "Activity after" is the free lipase activity in the lipase solution after cloth soaking. "Specific activity" is the free lipase activity in one mg of lipase at the testing condition. "Cloth weight" is the weight of woollen cloth used in immobilization. "Leaked activity" is the leaked activity in the first activity determination with the lipase-immobilized cloth.

Protein concentration before and after cloth adsorption was again determined using the QuantiPro BCA Assay Kit.

2.4 Oily Stain Removal of Lipase Immobilized Cloth

To determine the potential improvement in washing performance compared to enzyme bare cloth, the stain removal capacity of lipase immobilized woollen cloths were compared to the enzyme bare woollen cloths in a washing test. The washing test is similar to the methods in the literature (*33, 34*) with some modifications. The test was conducted as follows:

2.4.1. Preparation of fabric strips

The lipase-immobilized cloth was soiled with olive oil, and then dried with paper towels to remove the excess olive oil. Immediately after drying with silica gel for 4 hours, the cloth weight before and after soiling was measured and recorded as "cloth weight" and "soiled cloth weight". On average, 0.3 g of oil was adsorbed onto the first soaking of the cloth strips (and varied thereafter depending on the removal effectiveness).

2.4.2. Cloth washing

After storing for 1 day in air, the soiled cloth strips were washed with pH 8.5 Tris buffer in an incubator at 100 rpm for 30 min at ambient temperature. Immediately after drying with silica gel for 4 hours, the weight of the cloth strip was measured and recorded as "washed cloth weight". For comparison, the washing data of the untreated woollen cloth with pH 8.5 Tris buffer were also measured.

2.4.3. Determination of cleaning efficiency

The effectiveness of washing was quantified using the percentage of oil remaining on cloth, as defined in equation 7:

$$\text{Percentage of oil remaining} = \frac{\text{Weight after washing} - \text{Original cloth weight}}{\text{Original cloth weight}} \times 100\% \quad (7)$$

Values are the mean of 3 independent replicates. Error bars represent ± one standard deviation.

2.5 ESEM imaging

An environmental scanning electron microscope (ESEM) was used to image the enzyme-immobilized supports. A thin layer of gold was sputtered onto the samples prior to imaging. These samples were mounted on aluminium stubs and powder coated with platinum (10 nm coating thickness) for 7 min at 10 mA using a high resolution Polaron

SC7640 sputter coater. Images were taken using a FEI QUANTA 200 FEG ESEM (FEI Ltd, Eindhoven, Netherlands), operating at 0.8 Torr with accelerating voltage of 5 kV.

3. Results

3.1 Effects of preparation parameters on immobilized activity

The effects of each step in the immobilization protocol are examined in turn, and so the results are presented in the order: (i) chlorination of the wool, (ii) soaking in GA solution (examining the effect of concentration, reaction time and GA solution pH), and (iii) soaking time in the lipase solution.

3.1.1 The effect of chlorination on the wool

ESEM images of the untreated and chlorinated (by the low pH process) woollen cloth are shown in Figure 1A and 1B respectively. Comparison of the two shows that the chlorination process has the following effects:

- Almost all the scales on the wool fibre (on the surface layer) are removed.
- The degree of chlorination across the entire cloth is not even, with the surface layer receiving more chlorine attack than the inner layer, suggesting that more time was needed for the chlorine to distribute into the inner layer (perhaps indicative of mass transfer resistances being present).

Since it appears that the effects of the low pH chlorination process used here is both too aggressive and non-uniform, it is recommended that in future work a more moderate chlorination process is used, such as that detailed in reference (*22*). The low pH chlorination process was however still adopted for the rest of this work. A study of the effect of chlorination process on immobilization and cloth performance is the subject of a future paper.

3.1.2 Effect of GA solution: concentration, pH and exposure time

The effect of GA on the immobilized lipase activity was examined by varying GA concentration within 0.1% and 12% and is presented in Figure 2.

Figure 2 indicates that an increase in GA concentration does not substantially improve the activity of the immobilized enzymes. Therefore a GA concentration of 0.1% (w/w) was used in the experiments following this.

Figure 3 shows the effect of GA pH and exposure time on immobilized activity: the pH of the GA solution was varied between 6 and 11 and various reaction times were studied (1-15 min). By comparing the profiles from A to D, it can be observed that the reaction time in the GA solution does not affect the immobilized activity to a great extent. Based on this, it can be deduced that the reaction between PEI and GA is most likely a fast process, suggesting that one or two minutes is enough to complete the reaction. Hence, in all following experiments, the following immobilization conditions were used (unless otherwise stated): GA pH at 9.0, 5 min incubation time and 0.1 % GA concentration.

The independence of the immobilized activity to an increase in reaction time in GA solution and the strong dependency on GA solution pH corresponds well to the results in the literature, such as that in reference (*35*) on *Aspergillus oryzae* galactosidase immobilized on PEI coated cotton cloth using GA as cross-linking agent.

The optimal pH of 9.0 is most likely the result of two opposing factors that influence the immobilization process:

- PEI polymer must be first physically adsorbed onto woollen cloth through electrical interaction, before fixing to wool via GA cross-linking. As the majority of amino groups in PEI are protonated when the solution pH is less than 10, the degree of interaction between the wool and PEI will decrease with increased pH.
- GA shows better reactivity in alkaline solutions, which might result in more reactive binding sites present on the cloth surface and subsequently lead to a better immobilized-activity at a higher pH (*19*).

Therefore, considering these two opposing factors, an optimal pH of around 9.0 seems reasonable.

Overall, the findings that increased GA concentration and reaction time in GA solution does not greatly improve the activity of the immobilized lipase, indicates that the aldehyde density on wool surface may not be related to the amount of GA present in aqueous solution. This result differs from other similar studies (*28,29,36*), where a change in GA concentration either increases or decreases the immobilized activity. However, the immobilization support used in the aforementioned literature was silica and alumina ceramic, instead of wool. The biggest difference between wool and those inorganic supports is that no GA reactive groups are present on them and so they must be created before reacting with GA. For wool, the situation is different: a number of GA reactive groups (such as lysine residues, cysteine residues and terminal amino groups) are present. Therefore, the finding that an increase in the GA concentration and reaction time in GA solution does not improve the immobilized lipase activity to a significant extent may be related to the surface chemistry of wool. Further work is required to confirm this however.

3.1.3 Lipase addition - the effect of time in lipase solution on immobilized activity

It can be observed from Figure 4 that immobilized activity increased with time spent in the lipase solution until reaching a limit of around 3.6 μmol pNPP.min^{-1}.(g cloth)$^{-1}$. Further extending the time in the lipase solution does not improve the immobilized activity significantly.

The dependence of immobilized activity on the immersion time in the lipase solution could be ascribed to the lipase adsorption on the PEI-coated cloth. It is noted that PEI was used as spacer arm in this immobilization. In the pH range used in immobilization, PEI was able to adsorb lipase. GA modification did not significantly change its adsorption ability to lipases if the GA concentration used and treatment time in GA is taken into account. Thus, lipase was firstly adsorbed onto PEI, followed by reaction with the amine groups on the PEI. The more lipase that is adsorbed, the greater the chance that this lipase can be chemically immobilized until all the available PEI is occupied. When the cloth is only treated in lipase solution for a short time, this PEI coated cloth is potentially not saturated with lipase. Therefore an extended soaking time in the lipase solution acts to enhance adsorption coverage, increase immobilized lipase load and (if this lipase remains active) improve the immobilized activity. However, when the adsorption reaches an equilibrium limit or saturation, the immobilization becomes independent of these factors.

Overall, and most importantly, these results indicate that lipase adsorption is most likely the rate-determining step in this process.

3.2 Changes in activity of the lipase with pH; pH profile of free lipase and immobilized lipase

Figure 5 shows that the optimum pH for both free and immobilized enzyme is approximately the same, at approximately a pH of 8.5 to 8.8.

A spacer-arm (i.e. the PEI) immobilization technique has been used to keep the immobilized enzyme away from the surface in order to try to prevent the enzyme activity from being too affected by immobilization (where, for example, steric hindrance from a surface can inhibit activity and prevent access to the active site of the enzyme). The fact that the optimum pH is approximately the same may indicate that the immobilized enzymes are not too affected by the surface constraints.

3.3. Protein Load on Immobilized Support

Based on the literature *(13, 15, 16)*, immobilization by adsorption normally produces protein loadings ranging from 200 $mg.g^{-1}$ support to 8 $mg.g^{-1}$ support. For chemical immobilization (such as the protocol developed in this work), the variation of protein load data is even larger - from 250 mg /g support to only 0.109 mg/g support. However, these assays do not indicate if this protein loading has enzyme in the active or deactivated form – clearly only the active form is of any use for a reaction such as cleaning, as studied in this work. Therefore, the amount of enzyme in the active form must be considered if the protein load is to be compared between different immobilization methods and enzyme species.

Using the protein loading assay (Section 2.3), the amount of lipase immobilized onto cloth was found to be 4.29 mg/(g cloth). Comparing this to the protein load from the other studies in the literature *(37-40)*, the amount of enzyme immobilized is low. This may be due to the wool itself as an enzyme immobilization support. Wool possesses a number of reactive surface groups liable to immobilization, such as lysine residue, cysteine residue, and terminal amino groups. In contrast, most of the matrices reported in the literature are inert to GA (activation steps are necessary for some of the supports, such as silk, silica) or less reactive (cotton, for example). In addition, wool is more electrostatically charged than these support matrices (*41*). This means that many of the reactions occurring during enzyme immobilization on woollen cloth are different from the support matrices examined in the literature, which may lead to competing reactions that lower the overall enzyme that will be immobilized on the wool.

Furthermore, knowing the amount of enzyme immobilized now allows a comparison between the optimal activities of the free and immobilized systems in Figure 5. Since the approximate amount of enzyme in the free enzyme system is 0.1 mg (but only 18.5% pure) and in the immobilized system is 0.4 mg, and under optimal conditions the specific activity of the free enzyme is 8 U/mg and that of the immobilized enzyme is <2 U/g, there is a greater than 16 fold reduction (and up to a 86 fold reduction if accounting for enzyme purity) in activity after immobilization. There are several possible reasons for this, including:

- Deactivation of the enzymes during immobilization.

- Mass transfer limitations to the enzymes when immobilized on a surface – it is well known that free catalysts have less mass transfer limitations compared to surface immobilized catalysts, which also have the friction of the surface causing a stagnant layer of fluid to overcome as well as the inherent mass transfer limitations of the reactant contacting the enzymes (free or not).
- Availability of the active site may be less due to the fact that the enzyme is now immobilized, slowing activity and/or mass transfer rate to the active sites.

This therefore indicates that further work is needed to examine all of the aforementioned phenomena and effects and therefore optimise the immobilization of lipase onto wool outlined in this work.

3.4 ESEM Images of Immobilized Lipase

Now that some of the immobilization parameters have been investigated, the overall morphology of a typical lipase immobilized woollen cloth can be examined. ESEM images of a typical lipase immobilized on wool are presented in Figure 6. The immobilization procedure and preparation procedure for this sample was: low pH chlorination, temperature 25 °C, cloth was first treated in pH 9.0 phosphate buffer for 10 min, followed by soaking in 1 mg ml^{-1} lipase solution prepared in 0.1 M pH 8 phosphate buffer for 1 day.

Figure 6 reveals some further characteristics of the immobilization. Firstly, from Figures 6A and B it can be seen that the immobilization on chlorinated wool fibre was successful – there appears to be agglomerations of immobilized chemicals, which are suspected to contain the lipase attached to the fibres, since the images differ from the wool fibres in Figure 1. However, it cannot be definitely determined if this aggregate is from the immobilized lipase. This is because it could also be from the protein impurity, as the protein concentration for the lipase we used is only 18.5%. What can be said however is that the presence of the aggregate is related to the lipase immobilization process and could be used as a reference to visualize the immobilization.

3.5 Oily stain removal capacity

To study the oil cleaning capacity of the lipase immobilized woollen cloth, the cloth was stained with olive oil and stored at room temperature for 1 day, then washed in pH 8.5 Tris buffer solution and compared under the equivalent tests for an enzyme bare woollen cloth. Note that the aim of this part of the work was not to compare to free enzyme as in conventional washing (which may well be more active, based on the results in Sections 3.2 and 3.3), but to show that immobilised enzymes has a retained activity that means it can be reused without sending it to waste (as free enzyme is commonly sent).

Observations of the cleaning showed that the woollen cloth with lipase has enhanced cleaning ability: when the stained lipase immobilized woollen cloth was washed with buffer, a milky white emulsion very quickly appeared in the buffer solution and the stained cloth became clean. In comparison, the enzyme bare (lipase free) woollen cloth was still heavily stained with oil. This cleaning effect was quantified using the weight difference before and after buffer cleaning, and repeated periodically over one month, storing the cloth in air, as illustrated in Figure 7. The effect of storage on the activity is detailed in Section 3.6 and needs to be considered in parallel to these results. The

percentage of oil remaining in the cloth was used as the standard basis to exam stain removal ability over the multiple washes and between the lipase-immobilized woollen cloths and the enzyme bare (lipase free) cloths.

As shown in Figure 7, the lipase-immobilized cloth demonstrates an excellent stain removal capacity, even without any detergent addition. This is because, as outlined in the Section 1, the detergent used in laundry ingredient does not effectively remove the oily stain on fabrics. For woollen products, a special wash agent is required, which must be mild in pH and contain non-aggressive detergents, since the existence of alkaline chemicals would lead to structure damage in the woollen fabrics. Furthermore, although this cloth was stored in room temperature in air (which is non-ideal storage conditions for an enzyme as shown in Section 3.6, but realistic for a cloth that is to be used as an enhanced cleaning fabric) and there is still an enhanced stain-removal ability (compared to unmodified woollen cloth) even after storage for 1 month in air. This stability and enhanced cleaning ability indicates that this immobilization protocol is a promising step towards producing an enhanced cleaning and even self-cleaning woollen fabric and that this route should be pursued further in future work.

3.6 Storage stability of free and immobilized lipase

The stability of the activity of the lipase on the immobilized cloth over extended periods is key to the success of any lipase-immobilized cloth for enhanced and/or self-cleaning. Two types of stability are often used in literature: storage stability and operational stability. Operational stability means the ability of the immobilized enzyme to retain activity after repeated substrate exposure. Storage stability is the activity change during enzyme storage, i.e. the storage efficiency defined as the ratio of free or immobilized enzyme activity after storage to their initial activity. Since the application of the immobilized lipase in this project is for enhanced laundry cleaning, the storage stability is more meaningful and directly relates to the experiments conducted in Section 3.5.

3.6.1 Storage Stability of immobilized lipase in buffer

To investigate the stability of this immobilized preparation, lipase immobilized cloth was first stored in pH 8.5, 50 mM Tris buffer at 4 °C as a control (as these are ideal conditions which to store and retain activity of the lipase and therefore an excellent condition to benchmark against). The activity change of the same samples were monitored periodically over 80 days. The results are illustrated in Figure 8A. From Figure 8A, it can be seen that the activity of the immobilized lipase did not drop significantly compared to the spread of the data. Consequently, it can be concluded that the lipase immobilized on wool is stable when stored in Tris buffer and at low temperature (4 ^{o}C) – it is therefore stable when stored under ideal conditions.

3.6.2 Storage stability of immobilized lipase in air

Woollen garments, carpets and other items are obviously not going to be stored in a buffer solution. Consequently, to model the real situation that they will be used if the immobilized lipase cloth is used in woollen products, the storage stability of immobilized lipase in air was also tested. The results are illustrated in Figure 8B. The activity of immobilized lipase decreased considerably over a period of 2 weeks. This drop is much greater than the immobilized samples stored in Tris buffer in 4 °C (Section 3.6.1). As enzymes normally lose their activity quickly in unfavourable environments, it

is reasonable to assume that the immobilized lipase deactivated rapidly in dry air and at temperature of 25 ºC. Possible reasons for this are outlined in Section 3.6.3.

These results also indicate that the enhanced cleaning seen over an extended storage period (Figure 7) may not require as high an enzyme activity to be maintained as the pNPP assay reaction over the same period (since the drop is less significant compared to the drop in activity measured). This may imply that the lipase is better adapted to oil hydrolyse compared to the pNPP reaction and is better suited for the cleaning application intended here. This also indicates that a different activity test should be used in future work so that the activity in the cleaning application is comparable to the activity measured by the enzyme activity test.

3.6.3 Comparison of activity in 4°C Tris Buffer and 25°C air

The immobilized lipase maintained 80% of its activity when stored in Tris buffer at 4ºC for more than 70 days while almost 80% activity was lost when stored in air at room temperature only within 15 days. This different in stability may be due to two reasons:

Firstly, enzymes normally demonstrate higher stability at their optimal pH and at low temperature, whilst the immobilized lipase stored in air cannot maintain a favourable pH. Instead, the pH surrounding the immobilized lipase will change gradually during the dehydration of the cloth.

Secondly, the free aldehyde groups, which still remain on the lipase-immobilized cloth when stored in air, may be a key part of the mechanism of activity loss. When the immobilized lipases are stored in Tris buffer solution, the active aldehyde groups on woollen cloth could be quenched by the amine groups contained in Tris buffer (*42*) and slow the enzyme deactivation, giving the stability shown in Figure 8A. In dry air, free aldehyde on the surface of woollen cloth are not quenched and their reaction may be another reason for the enzyme activity loss.

3.6.4 Storage stability of free lipase stored in buffer

It is also of interest to compare the storage stability of the immobilized-lipase to that of the free lipase. However, it is difficult to compare the storage stability of lipase powder in air (lipase powder stored in air) at room temperature (25°C) with the immobilized lipase. The surrounding environment around the lipase powder and the immobilized lipase is considerably different, as stabiliser is contained inside the lipase powder. Also, it has no practical significance for the purpose of the current research. So this comparison was omitted. However, to determine if the activity loss in Section 3.6.2 is acceptable or not, the activity change of 1 mg/ml of lipase solution stored in pH 8.5 buffer at the higher temperature of 25 ˚C was monitored continuously for nearly one month. This experiment was done to compare the higher activity system (free enzymes) in the more ideal storage medium (tris buffer) but at the less ideal temperature (room temperature) in order to compare to the activity loss of the immobilized lipase at this temperature, whilst being able to benchmark against existing literature. The results are shown in Figure 8C. This shows that around 40% activity was lost during an almost one-month storage period in Tris buffer at room temperature (25 °C), if the experimental error (data scatter) is considered. In previous studies, the storage stability of free Amano lipase from *Pseudomonas fluorescens* (AK *PFL*, same lipase as the one used in this research) was reported, but the experimental conditions are slightly different from the one used in the current research. Koki Itoyama et al. (*43*) reported that free AK *PFL* lost more than 50% of its initial activity after storing in 0.05 M phosphate buffer (pH 7.4) at 4 °C within half a year. In the literature (*44*) the value reported was approx. 80% of AK *PFL* activity and was possible to maintain this activity after heat treatment

at 45 °C in pH 7.0 for 3 days. Therefore, if the different experimental conditions are taken into account, the storage stability data obtained in this study is acceptable.

Note that although the authors have done so in this work, it is complicated to compare the stability between the free and immobilized lipase by the CVB protocol, since the lipase from *PFL* has a strong tendency to form a bi-molecular aggregate, meaning that its stability is different at low and high lipase concentration. Prior research has shown that bimolecular *PFL* is much more stable than the unimolecular *PFL*, with the bimolecular *PFL* maintaining over 80% of the initial activity after 72 hours at 45°C, while the unimolecular structure retained only around 30% of initial activity after 4 hours of incubation under the same experimental conditions (*44*). It was also reported that the *PFL* bimolecular structure appeared when the enzyme concentration was higher than 160 μ g ml^{-1} in the absence of Triton X-100 (lipase AK from Amano enzyme Inc, purified lipase was applied) (*44*). The lipase concentration used in this research is mostly higher than 1 mg ml^{-1}. Furthermore, considering that the purity of lipase AK used in this research, the free lipase used here should be in the bimolecular form. The immobilized lipase may also be either unimolecular or bimolecular. Triton X-100 was reported to be a retardant for bimolecular aggregate formation (*44*). Consequently, no bimolecular aggregate of lipase can form in the presence of Triton X-100. This was evident from the experiments where the immobilized lipases on woollen cloth had been repeatedly tested by the pNPP assay solution containing Triton X-100. The bimolecular form of lipase on cloth may have been converted into monomolecular status in the presence of Triton X-100 surfactant. Therefore, compared to the free lipase stability in the unimolecular structure reported in literature (70% activity loss within 4 hours (44) at 45°C), it can concluded that enhanced stability was present for the immobilized lipase by the protocol presented herein.

4. Conclusions

Lipase has been immobilized onto woollen cloth successfully by a new immobilisation protocol, where lipase was cross-linked onto PEI coated woollen cloth. The immobilization protocol was: wool was firstly chlorinated, PEI was then adsorbed onto cloth, the PEI coated woollen cloth was soaked in GA solution and finally lipase was adsorbed and bound (and potentially cross-linked by any residual GA). Lipase adsorption is thought to be the rate-determining step in the immobilization. The optimum GA pH used for immobilization was around a pH of 6, with a short treatment time (1 minute) in GA solution preferred to avoid deactivation from excess GA cross-linking.

The optimum pH for both free and immobilized enzyme was found to be approximately the same, however the activity of the enzyme dropped by at least an order of magnitude when immobilised, indicating that further optimisation of this immobilization protocol is required. The cloth also exhibited excellent oily stain removal ability: after being stained with olive oil and stored for one day in air at room temperature, the oily stain could be easily removed by 0.05 M pH 8.5 Tris buffer without any detergent addition. This enhanced cleaning was stable also, since after cloth was stored in air for almost one month, similar cleaning performance was observed. This preparation also demonstrates excellent storage stability in Tris buffer at 4ºC and acceptable storage stability at room temperature. The storage stability is only acceptable at room temperature in air since the activity of the cloth (as measured by a p-nitrophenyl palmitate (pNPP) based activity assay) dropped from 1.4 to nearly 0.4 μmol $pNPP.min^{-1}.(g\ cloth)^{-1}$ over just 15 days

storage in air. This therefore likely indicates that the enhanced cleaning seen over an extended storage period may not require as high an enzyme activity to be maintained as for the pNPP reaction over the same period. This indicates that a different activity test should be used in future work so that the activity in the cleaning application is comparable to the activity measured by the enzyme activity test.

As a whole, these stability and enhanced cleaning ability results indicate that this immobilization protocol is a promising step towards producing an enhanced cleaning and even self-cleaning woollen fabric and that this route should be pursued further in future work.

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Figure Captions

Figure 1: A: ESEM picture of untreated woollen cloth, original magnifications: upper 1000×, lower 5000×. B: ESEM picture woollen cloth after low pH chlorination, DCCA chlorination and sodium bisulphite reduction, original magnifications: upper 1000×, lower 5000×.

Figure 2: Effect of GA concentrations on immobilized activity.

Figure 3: Effect of GA pH and reaction time on immobilized activity. Unit of specific activity: μmol pNP.min^{-1}. (g cloth)$^{-1}$. A: 1 min in 0.1% GA solution; B: 5 min in 0.1% GA solution; C: 10 min in 0.1% of GA solution, D: 15 min in 0.1% of GA solution. Values are the mean of *three* independent *replicates, error bar represents mean* ± one standard deviation.

Figure 4: The effect of treatment time in lipase solution on immobilized activity (Values are the mean of *3* independent *replicates*. *Error* bars represent ± one standard deviation).

Figure 5: A: The effect of pH on the activity of the free lipase. B: The effect of pH on the activity of immobilized lipase.

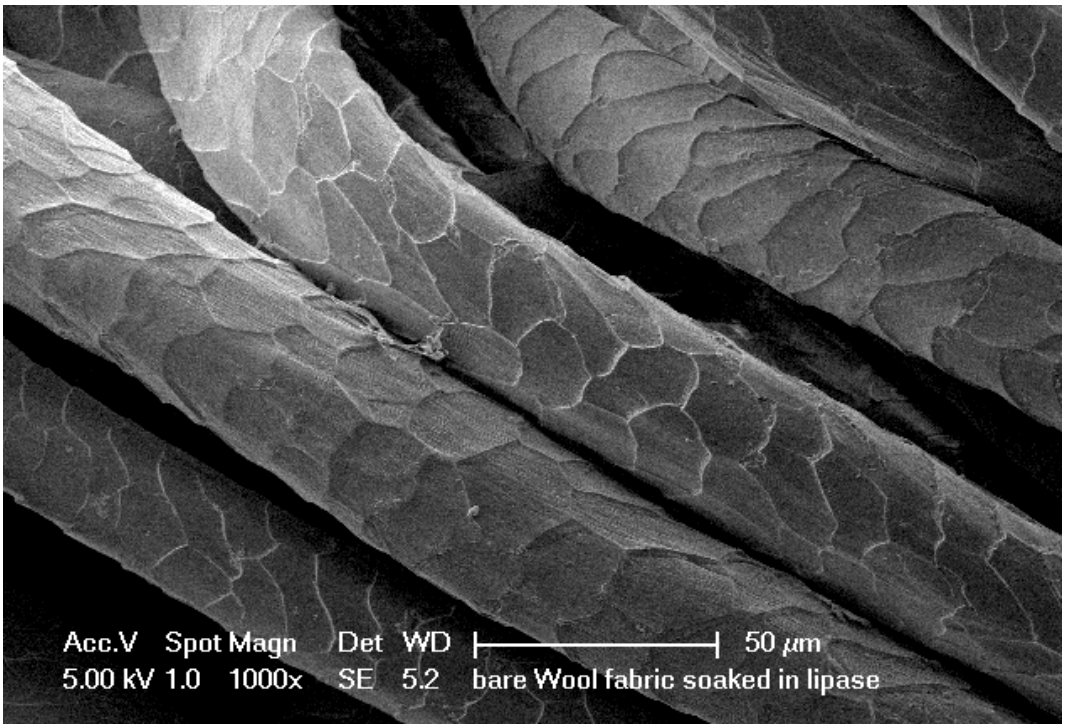
Figure 6: ESEM images of the woollen cloth with lipase immobilized by the CVB protocol. The immobilization conditions adopted for this cloth was: temperature 25 °C, cloth was first treated in pH 9.0 phosphate buffer for 10 min, followed by soaking in 1 mg. ml^{-1} lipase solution prepared in 0.1 M pH 8 phosphate buffer for 1 day.

A: ESEM image at 1000× original magnification. The agglomerations of immobilized chemicals, which are suspected to contain the lipase attached to the fibres, can be observed on the wool fibre. It can also been seen that distribution of this aggregate is not even across the fibre surface. Instead, they concentrate on discrete areas of the fibre surface. In addition, the fibres in the deeper layers of the cloth seem have no aggregate coverage.

B: ESEM image at 5000× original magnification. The appearance of aggregate can be seen more clearly in this image.

Figure 7: Comparison of the capacity of the cloth with immobilized lipase to remove an olive oil stain (in percentage mass of olive oil remaining) compared to a enzyme bare (lipase free) woollen cloth at various times in the storage of the cloths over a month.

Figure 8: A: The stability of immobilized lipase on wool cloth stored in tris buffer, measured as activity of lipase over 80 days at 4 ºC. B: Storage stability of immobilized lipase in air at room temperature (approximately 25 ºC). C: Specific activity change of free lipase stored in Tris buffer pH 8.5 at 25 ˚C. Values are the mean of three replicates; error bar represents the mean ± one standard deviation.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5.00 kV 1.0 1000x SE 5.2 bare Wool fabric soaked in lipase

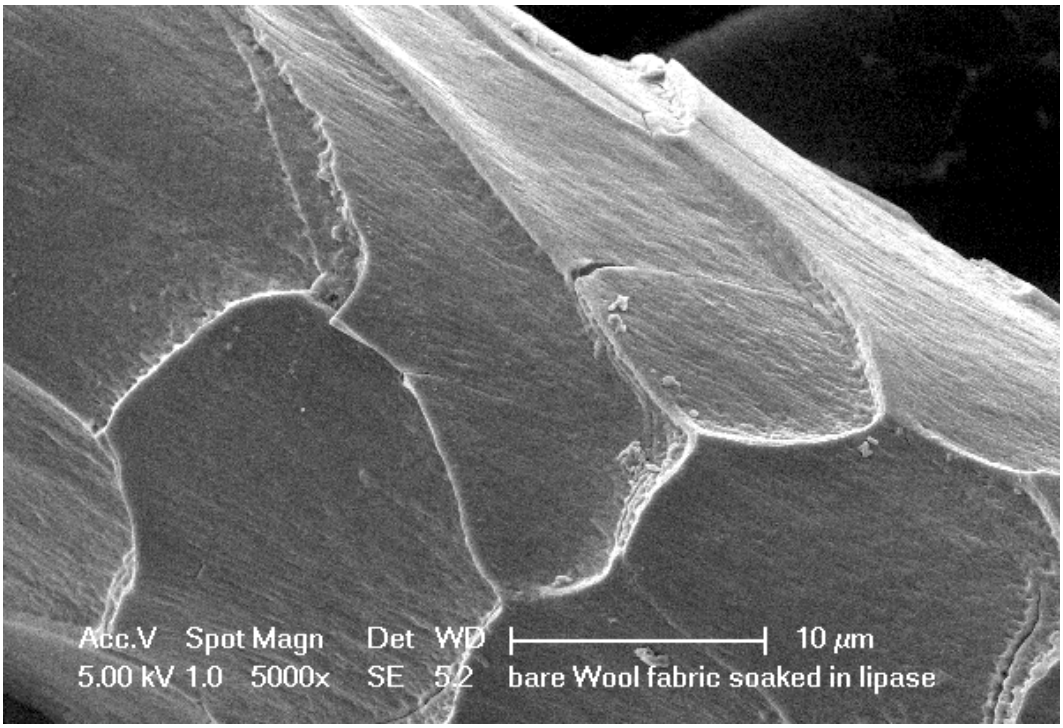
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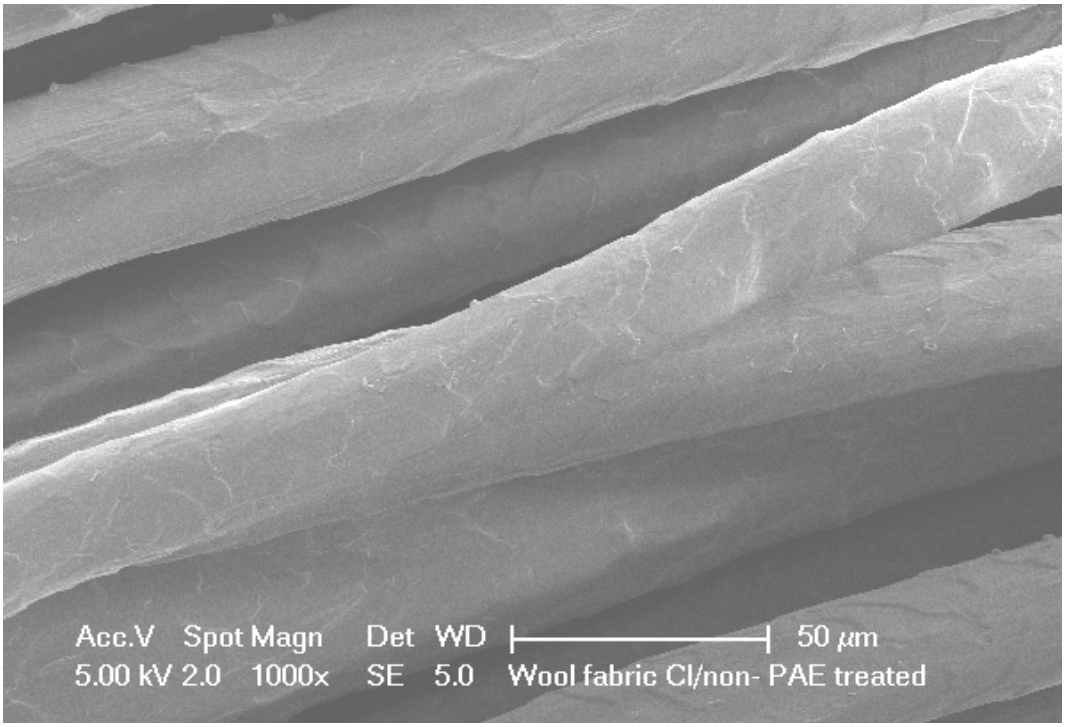
Figure 1A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5.00 kV 2.0 1000x SE 5.0 Wool fabric Cl/non- PAE treated

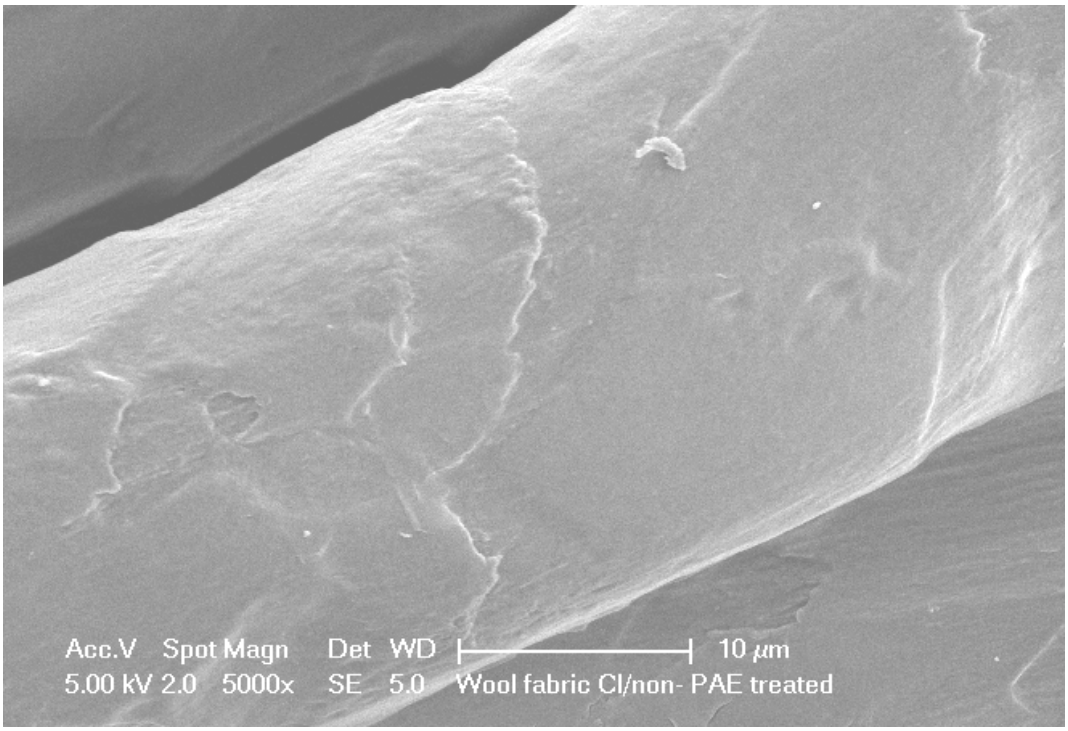
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Figure 1B

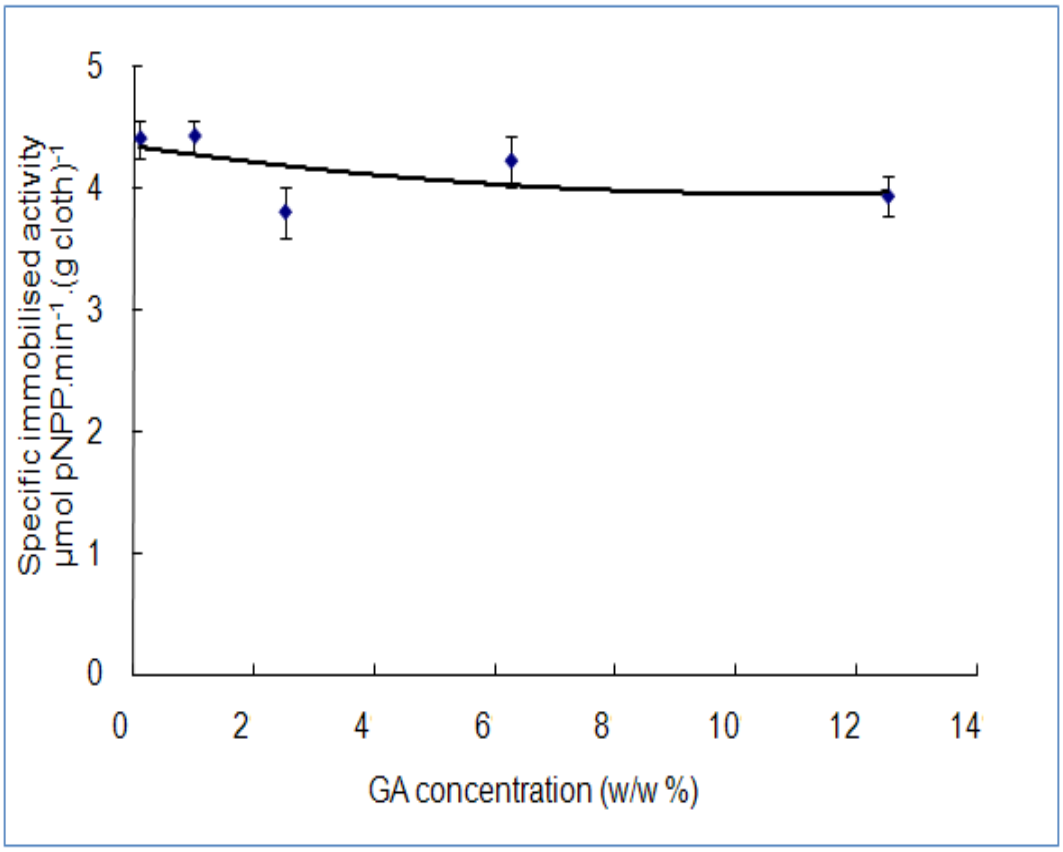
Specific immobilised activity
µmol pNPP.min-1.(g cloth)-1
0
1
2
3
4
5
0
2
4
6
8
10
12
14
GA concentration (w/w %)

Figure 2

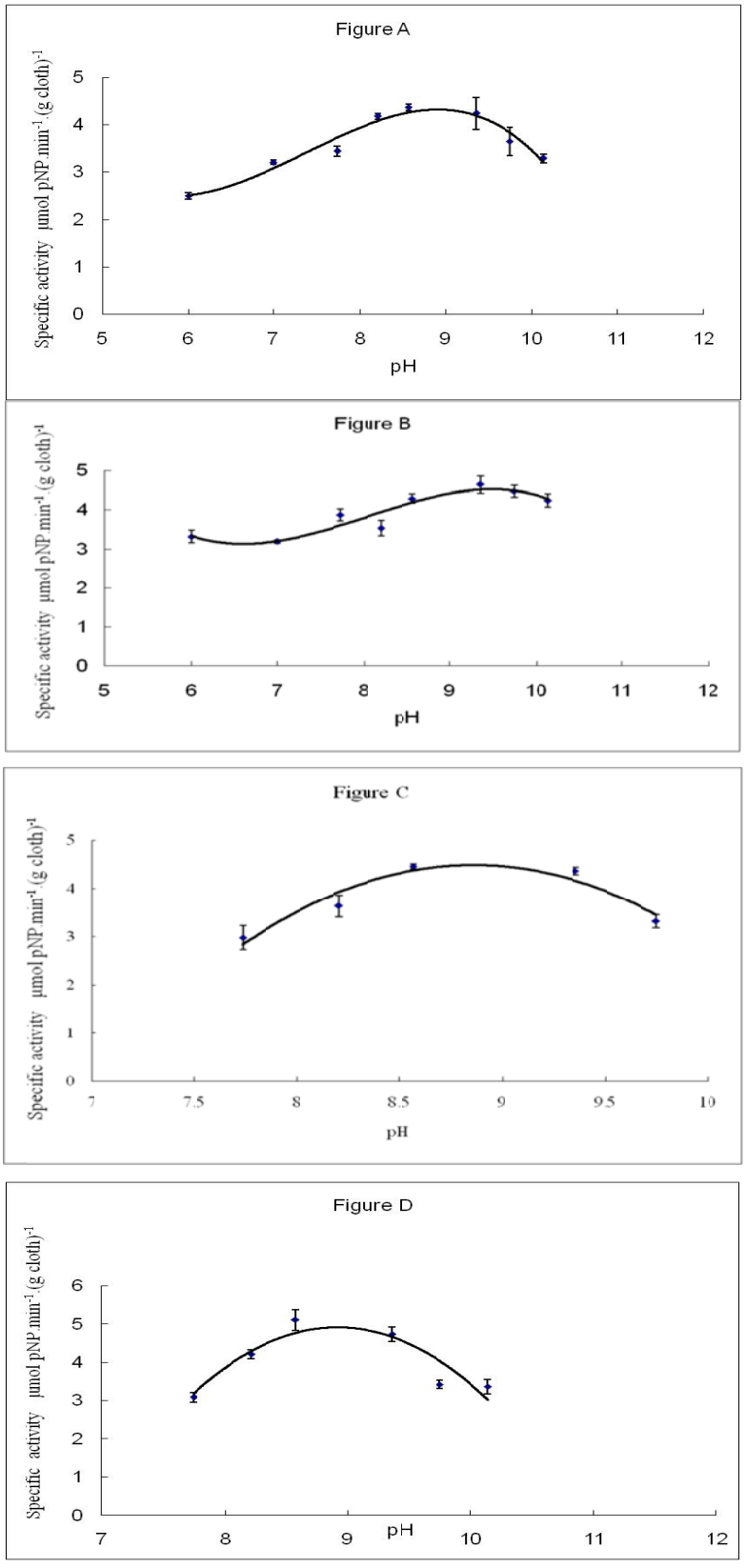
Figure A
Specific activity μmol pNP min-1 (g cloth)-1
pH
Figure B
Specific activity μmol pNP min-1 (g cloth)-1
pH
Figure C
Specific activity μmol pNP min-1 (g cloth)-1
pH
Figure D
Specific activity μmol pNP min-1 (g cloth)-1
pH

Figure 3

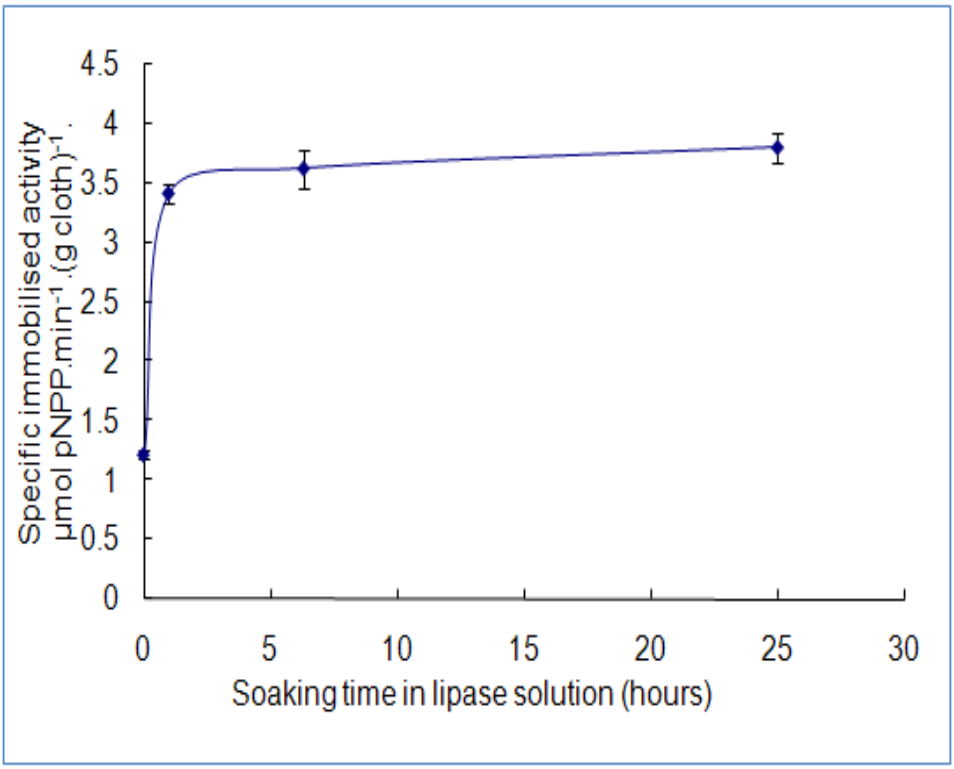
Specific immobilised activity
µmol pNPP.min-1.(g cloth)-1.
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
Soaking time in lipase solution (hours)

Figure 4

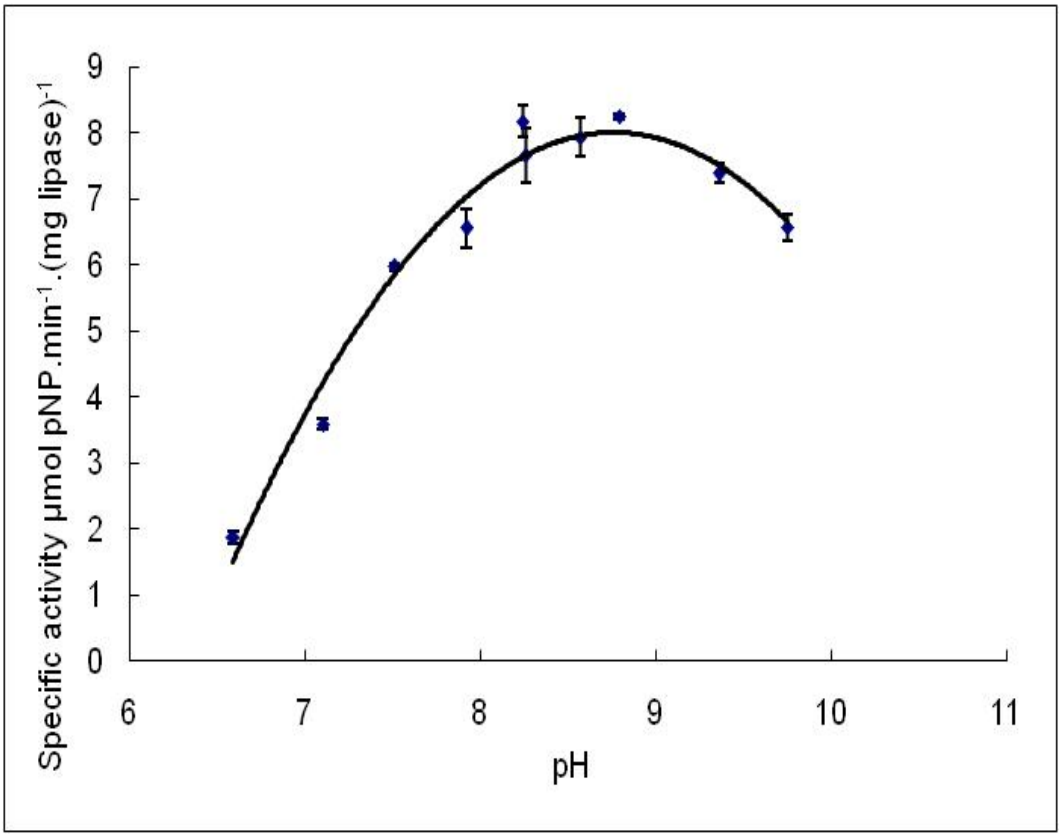
Specific activity µmol pNP.min-1.(mg lipase)-1
0
1
2
3
4
5
6
7
8
9
6
7
8
9
10
11
pH

Figure 5 A

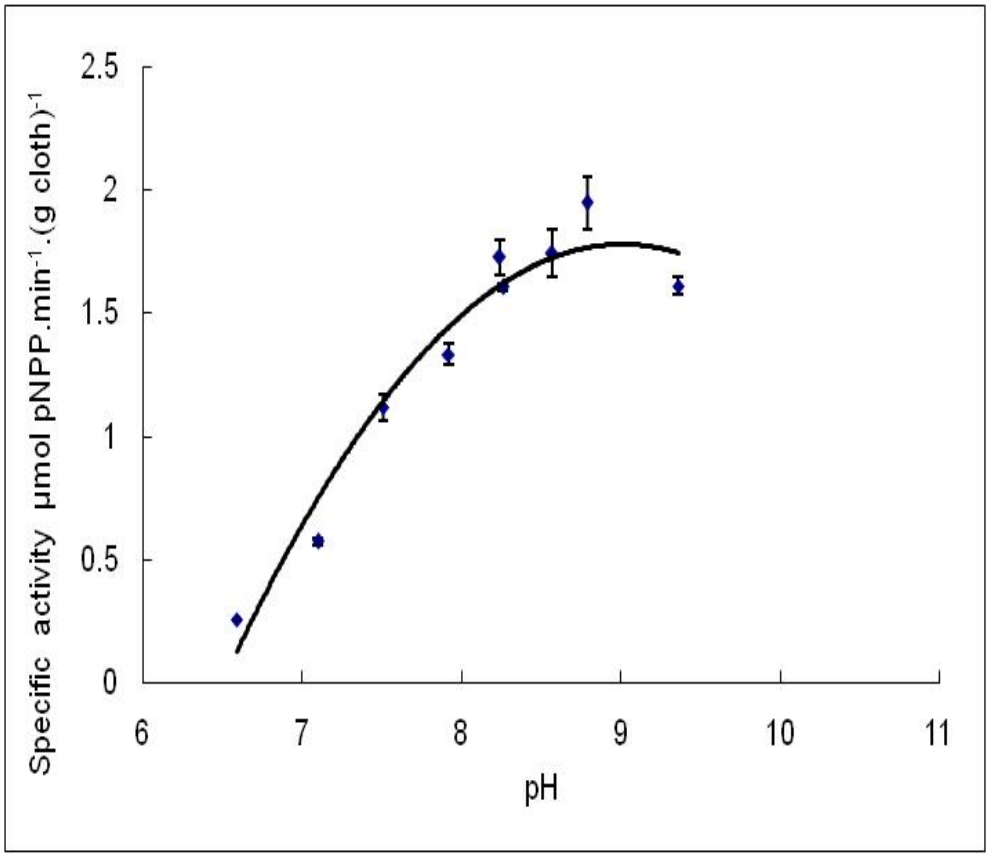
Specific activity µmol pNPP.min-1.(g cloth)-1
2.5
2
1.5
1
0.5
0
6
7
8
9
10
11
pH

Figure 5 B

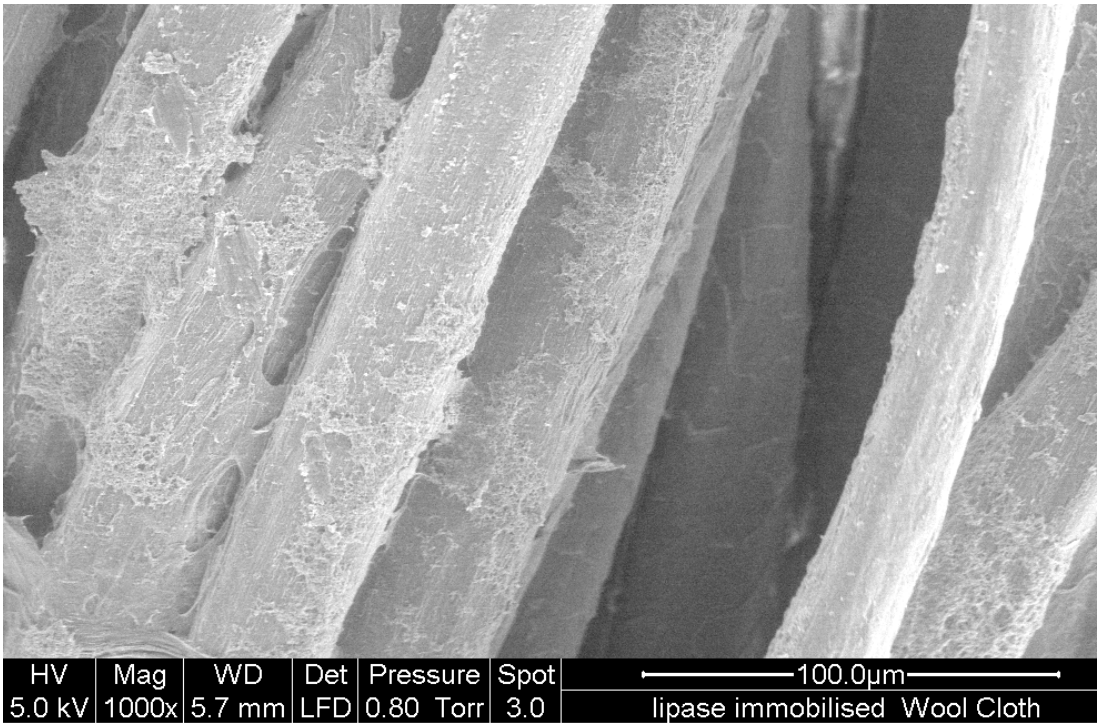
HV
5.0 kV
Mag
1000x
WD
5.7 mm
Det
LFD
Pressure
0.80 Torr
Spot
3.0
100.0µm
lipase immobilised Wool Cloth

Figure 6 A

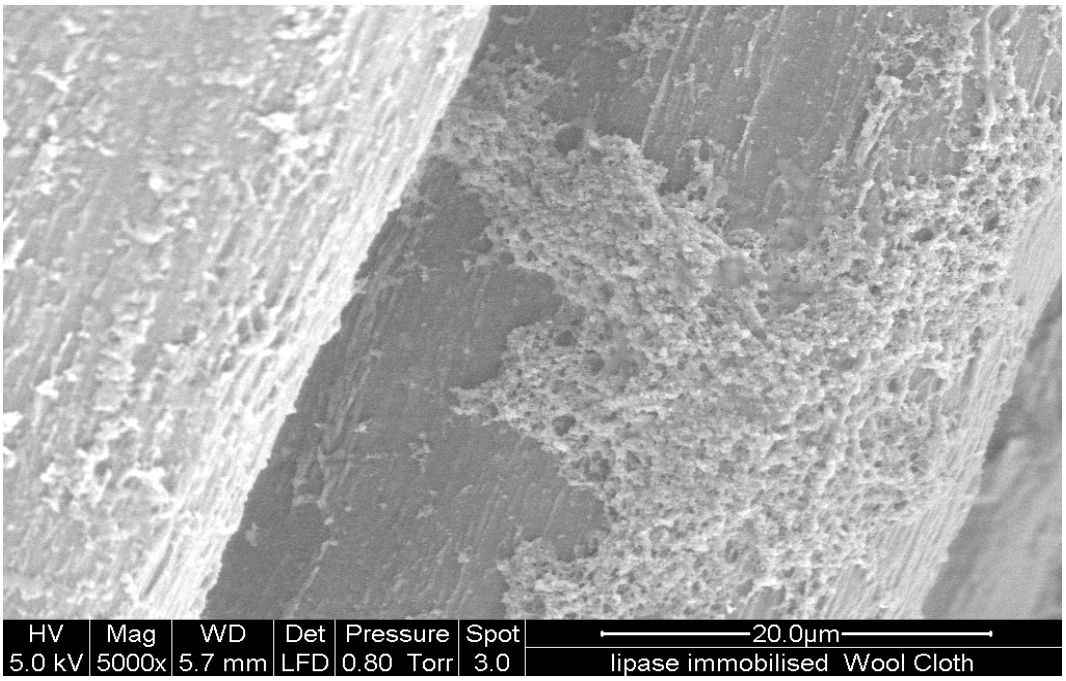
HV
5.0 kV
Mag
5000x
WD
5.7 mm
Det
LFD
Pressure
0.80 Torr
Spot
3.0
20.0µm
lipase immobilised Wool Cloth

Figure 6 B

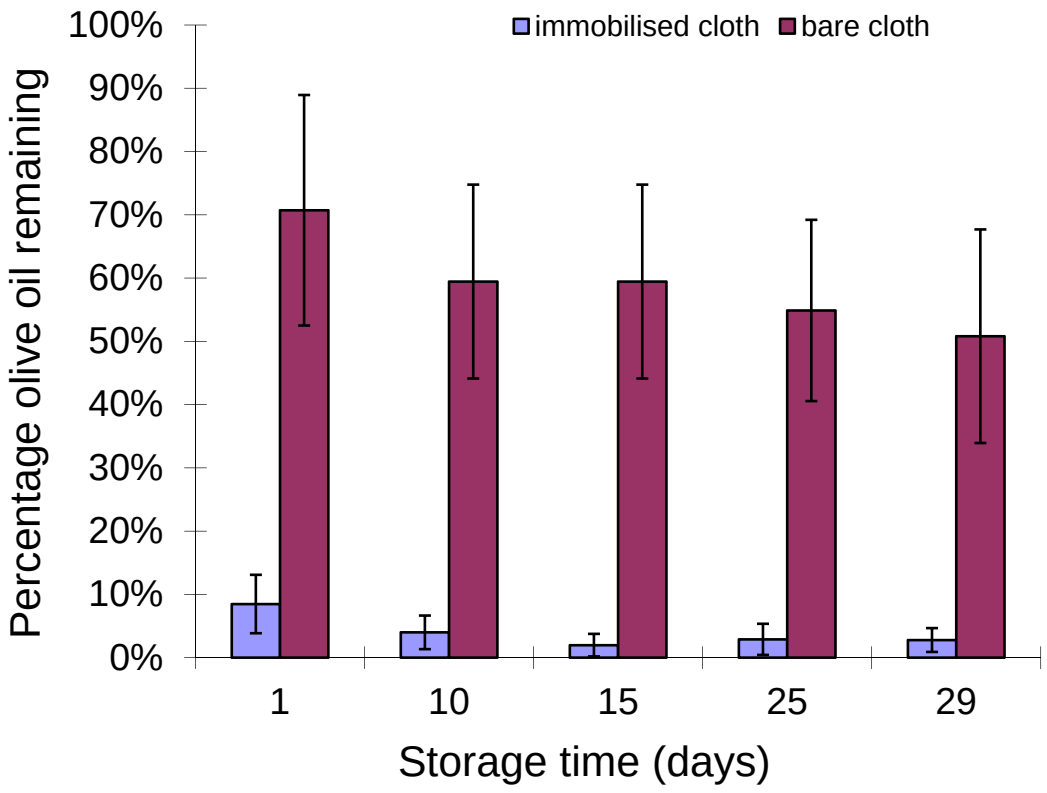
immobilised cloth
bare cloth
Percentage olive oil remaining
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
10
15
25
29
Storage time (days)

Figure 7

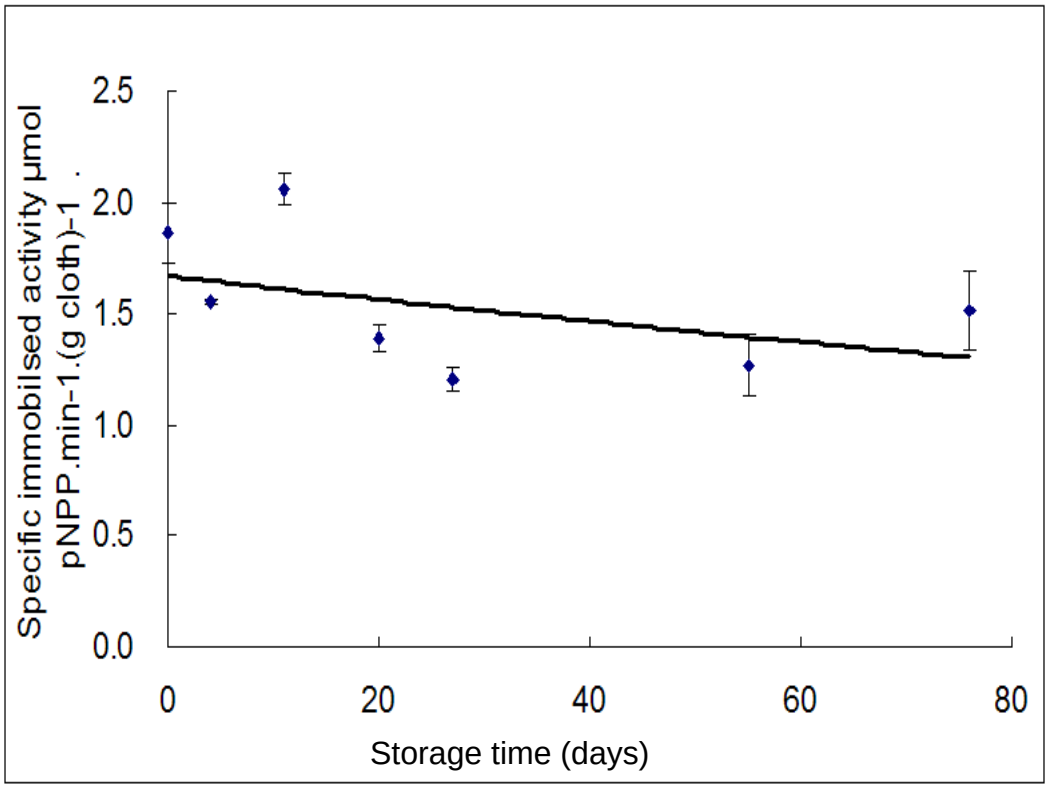
Specific immobilsed activity µmol pNPP.min-1.(g cloth)-1 .
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
Storage time (days)

Figure 8A

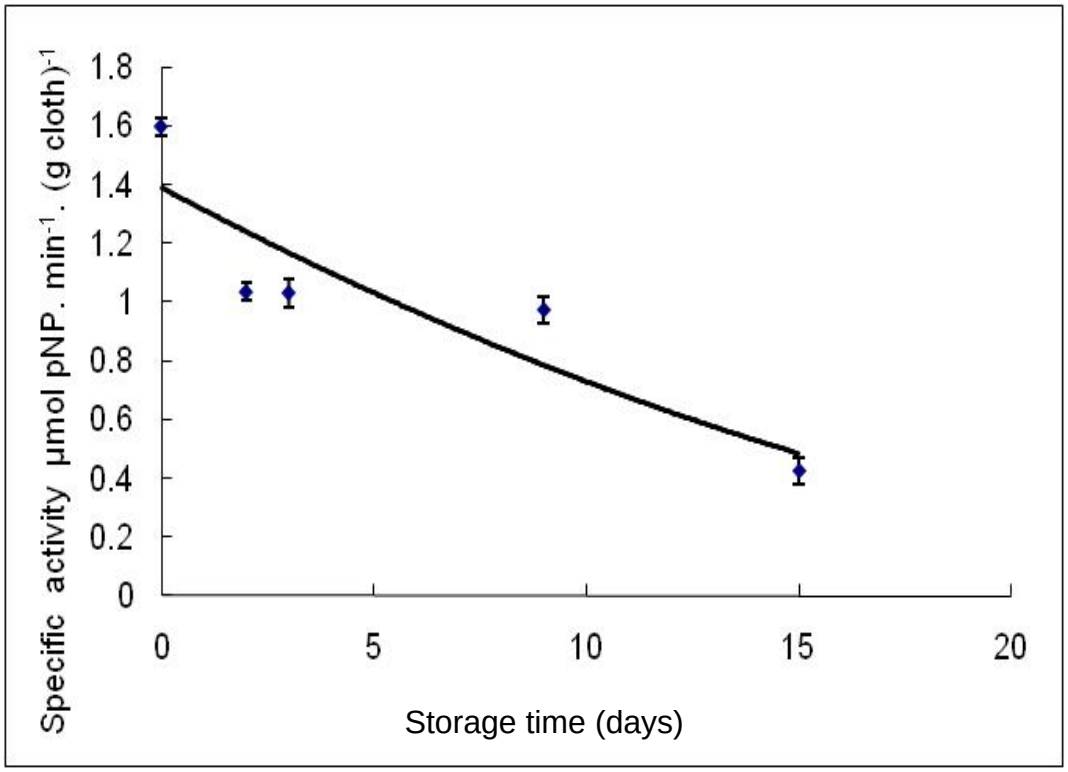
Specific activity µmol pNP. min-1. (g cloth)-1
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
Storage time (days)

Figure 8B

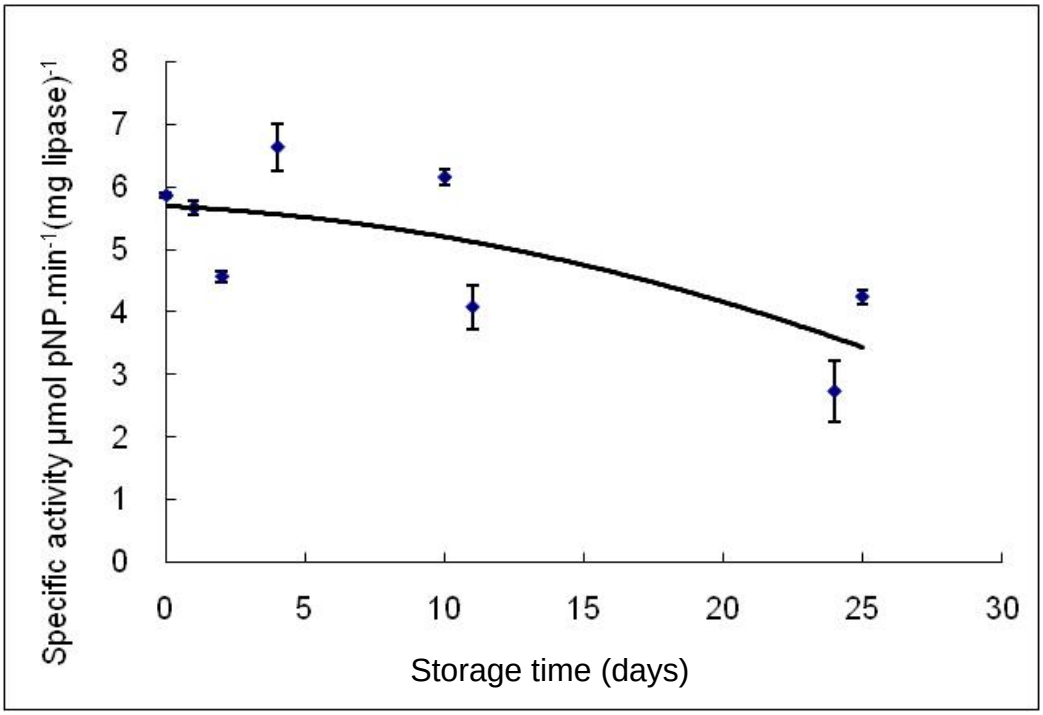
Specific activity µmol pNP.min-1(mg lipase)-1
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
Storage time (days)

Figure 8C