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**Characterization of tributyrin hydrolysis by immobilized lipase
on woolen cloth using conventional batch and novel spinning cloth
disc reactors**

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Abstract

Optimal loading and operating conditions for a new, superior immobilization of amano lipase from *P. fluorescens* on woolen cloth were determined. The optimal enzyme loading was 46.8 mg g dry cloth⁻¹ with activity of 200 U. A batch reactor was used to characterize process conditions important to industrial application of the wool immobilized lipase. The optimal pH for immobilized lipase in tributyrin hydrolysis was 7, slightly lower than that of free lipase (pH 8). The optimal temperature for both free and immobilized lipase was 45 °C. The immobilized lipase was more stable to reuse than some other lipase immobilizations, maintaining 85% of its activity after 6 long term runs and 75.8% of the original activity after storage of 40 weeks at 4 °C. The thermal stability of lipase was improved by 2.4 times after immobilization. The thermal deactivation rate of immobilized lipase followed the Arrhenius law with $E_d=199 \text{ kJ mol}^{-1}$. The Michaelis-Menten constant (K_m) of the lipase increased from 1.63 mM to 4.48 mM after immobilization. The immobilized lipase was also successfully applied for tributyrin hydrolysis in a novel enzyme process intensification technology – the spinning cloth disc reactor (SCDR): conversion increased by around 13% under similar conditions compared to a conventional batch stirred tank reactor. The SCDR is therefore key to exploiting the advantages of the wool immobilized lipase developed in this work.

Keywords: enzyme immobilization; lipase; woolen cloth support; thermal deactivation; tributyrin hydrolysis; spinning cloth disc reactor.

1. Introduction

Enzymes have advantages over most non-biological catalysts such as: high efficiency, specificity and selectivity, and ability to function under mild conditions (Klibanov, 1983). However, the main disadvantages of free enzymes are insufficient stability and difficulty of recycling, restricting their wider industrial application compared to chemical catalysts (Klibanov, 1983; Sheldon, 2007). Immobilization of enzymes has been shown to help overcome these disadvantages; however the properties of the support and immobilization methods have a significant effect on the activity of immobilized enzymes (Yemul and Imae, 2005). The properties of the support materials can affect the adsorption, conformation and expressed activity of immobilized enzymes. In addition, the surface chemistry and size can influence the behavior of enzymes and substrate at the support interfaces (Talbert and Goddard, 2012). Although various immobilization protocols have been reported in the literature, many of them include complex immobilization procedures as well as expensive materials. Simple, effective and non-expensive immobilization methods are still very desirable from both an academic and industrial/application point-of-view.

Lipases, known as triacylglycerol acylhydrolases, catalyze the hydrolysis of triglycerides to fatty acids and glycerol over an oil-water interface (Treichel et al., 2010). In addition, in the presence of lipases, the reverse esterification and transesterification reactions can occur in water restricted environments (Al-Zuhair, 2005). Due to their ability to catalyze reactions with high specificity and selectivity, lipases have been widely used in food (Othman et al., 2008), organic synthesis (Gomes et al., 2004) and dairy industry (Ren et al., 2008). Several methods have been reported for the immobilization of lipases on different supports, including covalent binding (Ye et al., 2005), encapsulation (Yang et al., 2009) and adsorption (Mateo et al., 2000). However, it is still a challenge to obtain high protein loading and enzyme activity for practical applications. A variety of materials have been used as the support for enzyme

immobilization. Among them, fibers have been increasingly investigated due to their low price, large specific surface and excellent mechanical properties. For example, cotton, silk and nylon have been reported to be successfully employed for enzyme immobilization (Albayrak and Yang, 2002; Chatterjee et al., 2009; Isgrove et al., 2001). Wool, as a complex and highly cross-linked protein fiber, consists of keratin type proteins that have rich reactive residues thus giving it great potential to be used as immobilization support. Although wool is a very important biomaterial, it has not been well studied like other fabrics in enzyme immobilization. To date, lipase has been reported to be immobilized on to wool in a small number of studies (An et al., 2008a; An et al., 2008b; Monier et al., 2010). However, several drawbacks to these techniques limited its practical application, including low stability and the necessary use of complex immobilization procedures. Recently, a simple and effective protocol has been reported by the authors to immobilize lipase onto woolen cloth (Feng et al., 2013a). Lipase was immobilized on polyethyleneimine (PEI) modified woolen cloth with glutaraldehyde (GA) as a cross linker. The success of the immobilization was verified by means of zeta potential, FTIR and confocal laser scanning microscope. A chemical analysis of immobilized lipase was thoroughly performed, showing that the enzyme loading is mainly determined by the electrostatic interaction between lipase and woolen cloth, and the optimal pH for immobilization is around 6. The current paper will focus on investigating the performance of immobilized lipase in reactions to determine its suitability for industrial application.

For immobilized enzymes to be adopted by industry, they must also be applied in a reactor, which can produce a stable, high yield and fast reaction. There are many reactors available, but this paper will look at applying the immobilized lipase on wool to two types only: (1) a conventional batch stirred tank reactor (BSTR) to determine the conventional performance of the immobilized lipase and, (2) a new innovation in enzyme reactor technology – the

spinning cloth disc reactor (SCDR) - which is a variant on the more common spinning disc reactor (SDR) used for process intensification. In the SCDR (Fig. 1), a liquid stream is fed onto the top of a spinning disc which holds the lipase immobilized onto wool on top. The centrifugal force of the spinning disc forces this liquid onto and into the wool, forming a highly sheared thin film on top of and within the rotating cloth. Research has shown for conventional SDRs that the heat and mass transfer can be significantly enhanced by the fluid dynamics within these films (Jachuck and Ramshaw, 1994; Meeuwse et al., 2012; Visscher et al., 2012) resulting in process intensification, where the reaction rates are much higher than conventional reactors operated under comparable conditions (Boodhoo and Jachuck, 2000). Therefore it would be interesting to investigate if such advantages can be extended into a cloth immobilized enzyme system.

Consequently, the aims of this study are:

1. To determine the optimal enzyme loading (in terms of activity) for the wool immobilized lipase.
2. To characterize and compare key performance characteristics of the wool immobilized lipase and free lipase: thermal stability, pH stability, and reaction kinetics.
3. To determine the operational stability of the wool immobilized lipase by evaluating the impact of reuse on activity.
4. To compare the performance of this immobilized lipase in two types of enzyme reactors: a conventional BSTR and a novel SCDR.

2. Materials and Methods

2.1. Materials

Unbleached organic woolen cloth was bought from Treliske (Otago, New Zealand). Amano lipase from *P. fluorescens*, polyethyleneimine (PEI), tributyrin (98%), triton X-100, Coomassie brilliant blue G 250, sodium bicarbonate and sodium carbonate were obtained from Sigma-Aldrich (New Zealand). Glutaraldehyde (GA) 25% (w/v), sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Unilab (ECP, New Zealand). Hydrogen peroxide 30% (v/v) was obtained from Scharlau (Thermofisher, New Zealand). Bovine serum albumin (BSA) was obtained from Gibcobl (Life Technologies, New Zealand). All chemicals were used as received. All solutions were prepared using deionized water (produced from a Milli-Q Gradient A10, Millipore).

2.2. Pretreatment of the woolen cloth

The woolen cloths were cut into 4×4 cm squares and treated with a solution containing 30 mL L⁻¹ hydrogen peroxide (30%) and 2 g L⁻¹ sodium silicate at pH 9 (0.1 M Na₂CO₃, NaHCO₃ buffer) at 55 °C for 70 min. The treated cloths were thereafter thoroughly rinsed with deionized water three times and air dried.

2.3. Enzyme immobilization

The detailed procedure of enzyme immobilization has been described in the authors' recent publication (Feng et al., 2013a). The main process was as follows: the bleached woolen cloth was firstly immersed in 2% PEI solution at pH 8 for 2 h at room temperature and then rinsed with deionized water. The resulting cloth was soaked in lipase solution (0.1 M phosphate buffer, pH 6) with different concentrations for 24 h, followed by immersion in 0.5% (w/v) GA solution (0.1 M phosphate buffer, pH 6) for 10 min for cross-linking, unless stated

otherwise. The cloth was then washed with deionized water until no free enzyme was detected in the washed solution. The immobilized lipase was stored in a pH 7, 0.1 M phosphate buffer solution at 4 °C until further use.

2.4. Enzyme loading determination

The enzyme loading on woolen cloth was determined by measuring the protein content of the enzyme solution after immobilization and in the washed solution using the Bradford method at 595 nm using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.5. Application of wool immobilized lipase in the BSTR and lipase activity assay

Tributyrin hydrolysis in a conventional BSTR with a pH stat (807 Dosing unit, Metrohm, Switzerland) was used as the benchmark reactor for this work. All lipase activity measurements were performed in this system except for the SCDR experiments. The activity of free and immobilized lipase were both determined. This system is the same as that in Fig. 1, except there was no spinning cloth disc reactor and the pump inlet and outlet, and cloth was placed directly in the reactant vessel. In a typical experiment, 0.33 g tributyrin and 0.15 g triton X-100 were added to phosphate buffer solution (pH 7) to make a final volume of 85 mL. This substrate solution was agitated with a magnetic stirrer at 25 °C at 600 rpm for 30 min to form a homogenous emulsion. The reaction was then started by adding either free or immobilized lipase and monitored for 10 min in a water bath at 45 °C. During the hydrolysis, sodium hydroxide was added into the reactor by the pH stat to neutralize the produced fatty acid. Each reaction was repeated at least 3 times and error bars were calculated as $\pm$ one standard deviation. One enzyme unit (U) was defined as the amount of lipase which catalyzes the release of 1 μ mol butyric acid per minute under the specified conditions. Reaction conversion was correlated to moles of sodium hydroxide consumed by the reaction according to Eq. 1:

$$Conversion(\%) = \frac{\text{moles of free butyric acids}}{\text{moles of original esters in tributyrin}} \times 100 \quad (1)$$

2.6. Thermal stability of free and immobilized lipase

Free and immobilized lipase preparations were incubated in pH 7 phosphate buffer solution at 60°C and sampled periodically. The residual activities were evaluated by the tributyrin emulsion method as described in Section 2.5. The hydrolytic activity of the non-incubated enzyme was taken as 100%.

2.7. Thermal deactivation of immobilized lipase

Heat is one of the most important causes of enzyme deactivation in industrial reactors (Klibanov, 1983; Luo and Zhang, 2010). Therefore, it is of vital importance to investigate the deactivation kinetics of the immobilized lipase to provide valuable information for further industrial application. Many theoretical mechanisms and mathematical models have been proposed to describe the thermal deactivation of enzymes. Among them, a general scheme applicable to the deactivation of most enzymes was developed by Henley and Sadana (1986). A first order process has been most widely used in enzyme thermal deactivation due to its simplicity (Peterson et al., 1989):

Where E is the active enzyme; E_d is the deactivated enzyme; k_d is the deactivation rate constant.

Following this process, the thermal deactivation rate equation can be obtained:

$$\frac{da}{dt} = -k_d a \quad (3)$$

Where a is the enzyme activity at time t during the thermal deactivation process.

Eq. 3 can be integrated to Eq. 4:

$$\ln(a/a_0) = -k_d t \quad (4)$$

Where a_0 is the initial activity.

Therefore, at a given temperature, a semi natural logarithm plot of residual activity versus time should give a straight line where the negative slope is the deactivation rate constant k_d .

The Arrhenius equation has been widely used to describe the relationship between the reaction rate constant and temperature. Therefore, k_d can then be expressed as follows:

$$k_d = A_d e^{-E_d/RT} \quad (5)$$

Where E_d is the thermal deactivation energy; A_d is the pre-exponential factor.

The natural logarithm of k_d versus reciprocal absolute temperature should give a straight line, where E_d and A_d can be determined from the slope and intercept.

To characterize the thermal deactivation of the wool immobilized lipase, the immobilized lipase was incubated in a water bath at 60, 65 and 70 °C respectively. Samples were taken periodically and the activity was tested as described in Section 2.5.

2.8. Application of wool immobilized lipase in the SCDR

A schematic diagram of the batch SCDR process used in this study is shown in Fig. 1a. The SCDR consisted of the pH stat, a liquid feeding system, an overhead stirrer connected to a disc, a liquid funneling vessel around the disc, and a reactant solution storage vessel. The critical spinning surface in this SCDR was a Perspex disc 250 mm in diameter, driven by a variable speed motor (Glas-Gol, US). This spinning disc was enclosed in a steel funnel-shaped chamber 300 mm in diameter and 210 mm deep. Woolen cloth was cut into circular pieces 250 mm in diameter and used as support for lipase immobilization according to the procedure described in Section 2.3. Then this woolen cloth with immobilized lipase was fixed on the disc as shown in Fig. 1b. Further details can be obtained from Feng et al., (2013b). In a

typical experiment, firstly, the disc with immobilized lipase woolen cloth was connected to the driving motor and spun to the desired rotational speed. Then, the reaction was launched by pumping the tributyrin emulsion to the center of the spinning disc. As a result, the solution was spread over the spinning cloth surface (and within the volume of the cloth due to wetting) by the centrifugal force. The tributyrin was hydrolyzed by the immobilized lipase on the cloth, and thereafter returned to the feed vessel. During the hydrolysis, the volume of sodium hydroxide added into the feed vessel by the pH stat to keep a constant pH was measured. All results were repeated three times and error bars were calculated as $\pm$ one standard deviation.

3. Results and Discussion

3.1. Optimizing immobilized enzyme loading and activity: the effect of lipase concentration during immobilization

For a successful enzyme process at industrial scale, obtaining the highest reaction rate per area of support used in a reactor is key to obtaining an economically feasible reaction. Consequently, the enzyme loading and associated activity on the wool support needs to be maximized. Fig. 2 shows the enzyme loading and activity as a function of the amount of lipase in the immobilization solution ranging from 0.5 to 5 g L⁻¹, with lipase activity measured in the BSTR system as described in Section 2.5. It can be seen that both the enzyme loading and activity increased with the increase of lipase provided. The maximum activity (215.6 U g cloth⁻¹) and enzyme loading (61.98 mg g cloth⁻¹) were obtained with a lipase concentration of 5 g L⁻¹. When the lipase concentration was increased from 0.5 to 2 g L⁻¹, the activity and enzyme loading showed a dramatic increase of 90.6% and 214.4% respectively. Thereafter (between 2 and 5 g L⁻¹), the increase of activity was less significant (increased by 7.8%). However, the enzyme loading kept increasing until the lipase concentration reached 3

g L⁻¹ (increased by 25.5% from 2 to 3 g L⁻¹), indicating that the activity did not correspond strictly to enzyme loading on wool and instead it may also be dependent on steric effects. In view of these results, 2 g L⁻¹ of lipase (producing an enzyme loading of 46.8 mg g cloth⁻¹) was used in the immobilization protocol for all the following experiments due to this being around the optimum, giving high activity and immobilization efficiency.

3.2. Free vs. wool immobilized lipase 1: Effect of pH and temperature on activity

The operating envelope of the immobilized enzyme needs to be well defined so that a well-controlled and robust reaction process can be designed and used in practice. Two key variables during the operation of any enzyme reaction and reactor are pH and temperature, as adverse values of these will deactivate the enzymes. Consequently, the pH and temperature profiles of activities of both free and immobilized lipase were quantified.

Fig. 3 depicts the pH profile of the activity of both the free and immobilized lipase. The free lipase displayed a maximum activity at around pH 8, which is consistent with data from the product supplier (Sigma-Aldrich). The optimum pH for immobilized lipase was reduced approximately 1 pH unit from 8 to 7 compared to the free lipase. This change in optimum pH might be due to a change in the charge distribution of the functional amino acids of the lipase after immobilization. The modification with PEI introduces more alkaline groups to the lipase surface during immobilization and provides an alkaline environment which is more favorable for tributyrin hydrolysis, shifting the optimum pH to a more acidic value. In earlier studies where PEI was also used for enzyme immobilization, similar changes in optimum pH were observed, validating the results in this study (Gao et al., 2006; Kamath et al., 1988; Yun et al., 2000).

The effect of temperature on the activity of both free and immobilized lipase is given in Fig. 4. The results demonstrated that the wool immobilization of lipase did not change its activity

vs temperature profile. The activity of both lipase forms increased dramatically as the temperature increased from 25 °C to 45 °C, because the higher temperature was able to not only accelerate the diffusion of lipase and substrate (thus leading to a shortening of the contact time required for reaction to take place), but also overcome the activation energy barriers allowing a higher enzyme activity. After the temperature reached 45-55 °C, the activity decreased with a further increase in temperature due to the thermal deactivation of the lipase. Therefore the immobilized enzymes should be used at 55 °C and below to avoid decreased activity, defining the top end of the thermal operating envelope in the BSTR and SCDR.

3.3. Free vs. wool immobilized lipase 2: thermal stability over time

The thermal stability with time of both free and immobilized lipase were investigated at 60°C. The free lipase lost most of its activity more rapidly than the immobilized lipase: a relative activity of 29% remained for free lipase after 300 min but 70% for immobilized lipase after 390 min (Fig. 5). It has been reported that the thermal stability could be largely improved by immobilization due to the enhanced tertiary structure stability upon the covalent binding of enzymes to the support (Bai et al., 2006; Bayramoglu et al., 2005; Ghamgui et al., 2007; Yemul and Imae, 2005).

3.4. Thermal deactivation kinetics of the wool immobilized lipase

Thermal deactivation rates at 60, 65 and 70 °C for the wool immobilized lipase were quantified to better understand the stability phenomena presented in Fig. 4 and Fig. 5, and to estimate deactivation kinetics. As shown in Fig. 6, the residual activity of the immobilized lipase decreased with incubation time at all three temperatures, further confirming that thermal deactivation occurred. The semi-log plot of residual activity (Fig. 6) showed a reasonably good linear relation with incubation time at various temperatures, indicating that the deactivation of this immobilized lipase on woolen cloth can be described by first order

kinetics. This means that from Eq. 4, the half-life $t_{1/2}$ for the immobilized lipase can also be calculated (using $\ln 2/k_d$). The deactivation rate constants at various temperatures and their corresponding half-life are given in Table 1. The deactivation rate was faster at a higher temperature. This is also clear from the data in Fig. 6: at 60 °C, only 30.2% of the original activity was lost after 390 min, however at 70 °C, 79.7% of the initial activity was lost after 200 min. To determine the relationship between temperature and rate, the data was plotted to determine a fit to Eq. 5, as shown in Fig. 7. This indicates that the deactivation kinetics for the wool immobilized lipase follows an Arrhenius type relationship with temperature, and the deactivation energy is approximately 199 kJ mol⁻¹. There are no comparable values for the amano lipase used in this study, however the deactivation energy was 114.3 to 143.6 kJ mol⁻¹ for the lipase from *C. rugosa* immobilized on six different supports (Shaw et al., 1990) – lower than in this study. The difference may just be due to the fact that the lipases are from different microbial sources (so naturally have different deactivation energies). Indeed, the deactivation energy value varies widely between different lipases: for example, 93.8 kJ mol⁻¹ for lipase from *P. citrinum* (Pimentel et al., 1997), 228.8 kJ mol⁻¹ for lipase from *M. javanicus* (Balcao et al., 1998), and 304 kJ mol⁻¹ for lipase from *R. miehei* (Noel and Combes, 2003). Our value is within the overall range reported.

3.5. Free vs. wool immobilized lipase 3: initial rate kinetics

Quantifying reaction kinetics is a key step in understanding how the reaction mechanism may have been affected by the immobilization process, and reaction kinetics can also provide a means and basis from which reactor sizing and design can be calculated. The Michaelis-Menten initial rate kinetics of both free and immobilized lipase for tributyrin emulsion hydrolysis were compared in the BSTR at different concentrations, ranging from 5 to 40 mM. The initial rate was estimated by means of the slope of the hydrolysis curve (produced by the pH stat) at the beginning of the reaction: this method has been shown to accurately quantify

the initial rate (Jurado et al., 2006). Since the pH stat provides a large continuous data set (logging pH and base addition every two seconds), a large number of experimental points were incorporated into a least squares fitting method for a straight line, to provide an accurate estimate of the initial rate. All data points from the pH stat were included from the start of the reaction until the point at which the slope of the fitted straight line began to decrease, which has previously been shown to be where the initial rate period ends (Haas et al., 1995).

The kinetic constants were evaluated using a Lineweaver-Burk plot, as shown in Fig. 8. The Michaelis–Menten constant K_m of immobilized lipase was estimated to be 4.48 mM, which was nearly threefold higher than that of the free lipase, which was 1.63 mM. An increase in K_m after immobilization has been seen in other research: for example, Ye et al. (2005) found that K_m of lipase increased from 0.45 to 1.36–1.43 mM after immobilization on a membrane (Ye et al., 2005). Yiğitoğlu et al. (2010) immobilized lipase on polyester fibers and found that the K_m increased from 47.2 to 151.6 mg mL⁻¹ after immobilization (Yigitoglu and Temoçin, 2010). K_m is described as an inverse binding constant: an increase in the K_m value indicates that immobilized lipase has a lower affinity to the substrate. This is likely due to either a distortion of tertiary structure caused by immobilization and/or the expected mass transfer resistances caused mainly by the larger stagnant film around the larger woolen cloth surface compared to the free lipase.

3.6. Storage stability of immobilized lipase

Storage stability is another critical factor to be considered for the industrial application of these wool immobilized enzymes. Enzymes must be able to retain their activity after transportation and storage so that they can be used at near maximum (fresh) activity in the industrial process. In this study, storage stability was investigated by storing the immobilized lipase on woolen cloth in phosphate buffer (0.1 M, pH 7) for an extended period at two different temperatures: 4°C and 25°C. As shown in Fig. 9, the immobilized lipase maintained

75.8% and 55.3% of its original activity after storage of 40 weeks at 4°C and 25 °C, respectively. It is well accepted that a lower temperature is more favorable for enzymes to maintain the tertiary structure, thus keeping a high activity. This result indicates that it is possible to store the immobilized lipase for an acceptable period for industrial application (i.e. for transportation and storage before application) with high residual activity.

3.7. Operational stability of the wool immobilized lipase

One advantage of immobilized enzyme over its free form is the reusability, so a successful immobilized enzyme system should be reusable with both good stability and high activity. Therefore, the loss of enzymatic activity during repeated use was investigated over 6 batches of tributyrin hydrolyses by reusing the same cloth. After each consecutive 4 h run, the woolen cloth with immobilized lipase was washed with phosphate buffer (pH 7, 0.1 M) and reintroduced to the fresh tributyrin emulsion (13 mM) at 45 °C. Fig. 10 presents the operational stability of the immobilized lipase over the 6 runs.

It can be seen that the tributyrin conversion was reduced 3% between the first and second run which accounted for 32% of the total activity loss, and this can most likely be attributed to the release of free lipase, which was most likely adsorbed onto the wool surface rather than more strongly bound via the intended covalent binding (Feng et al., 2013a). The tributyrin conversion decreased from 62.3% to 52.7% after 6 runs, which means that the immobilized lipase maintained 85% of its original activity. This reusability is superior to previous studies of wool in lipase immobilization, where, for example, there was less than 70% retained activity after 6 cycles of 5 min each (Monier et al., 2010). This result is also better than that seen with some other lipase immobilization methods. For example, it was reported that lipase immobilized on insoluble yeast β -glucan maintained around 50% of its original activity after 6 reuses (Vaidya and Singhal, 2008). Özmen and Yilmaz (2009) immobilized lipase on β -cyclodextrin-based polymer and retained 80% activity after 6 runs (Ozmen and Yilmaz,

2009). This result demonstrates that the protocol developed for wool in this study can achieve a superior immobilization, and has good potential for application in a continuous lipase reactor, such as for the continuous hydrolysis of tributyrin.

3.8. Comparison of BSTR and SCDR performance

All of the results above show that the wool immobilized lipase provides a highly active and stable performance in a conventional BSTR. However, for wool to be considered a versatile support material for lipase immobilizations, it should be able to function well in a range of different reactors. Therefore the performance of the wool immobilized lipase was quantified in a new type of enzyme process intensification reactor – the SCDR. Operation in the SCDR is expected to be more testing than in a BSTR: the high shear forces produced by the centrifugal movement of the reactant/product solution on the spinning disc are a more adverse environment for immobilized lipase than a BSTR as they can potentially deactivate under high hydraulic shear.

Fig. 11 shows the conversion of tributyrin with time in both the SCDR and BSTR at two initial tributyrin concentrations (20 and 40 g L⁻¹). Under comparable reaction conditions, tributyrin hydrolysis in the SCDR proceeded at a higher rate than in the BSTR, giving a higher conversion over the entire hydrolysis process at all the investigated concentrations: for example, for 20 g L⁻¹ tributyrin, the final conversion was 52.2% in the BSTR and 65.4% in the SCDR after 240 min. This result indicates that both the reaction rate and yield are improved in the SCDR, which is most likely attributed to the more rapid mixing between substrate and immobilized lipase, and enhanced mass transfer in the thin film on top of and within the spinning cloth. This confirms that the wool immobilized lipase is a robust immobilization system for this type of lipase and that the SCDR can intensify enzyme reactions. This combined system is therefore worthy of a more detailed study and so a full characterization of the SCDR will be performed.

4. Conclusions

Amano lipase from *P. fluorescens* has been successfully immobilized on woolen cloth using polyethyleneimine (PEI) with glutaraldehyde (GA) cross-linking. The enzyme immobilized on one gram cloth was 46.8 mg with activity of 200 U. The protocol developed for wool in this study can achieve a superior immobilization, where the wool immobilized lipase potentially has sufficient activity and stability to be an effective enzyme system for industrial enzyme processes. A number of different parameters were optimized and quantified, primarily in a conventional BSTR system:

- The optimal pH for immobilized lipase in tributyrin hydrolysis was 7 which was slightly lower than that of the free lipase (pH 8), implying the introduction of PEI provides an alkaline environment which is more favorable for tributyrin hydrolysis in a more acidic value.
- The optimal temperature for both free and immobilized lipase was 45 °C.
- The thermal stability of lipase was significantly improved after immobilization. The thermal deactivation rate of immobilized lipase was found to follow the Arrhenius law with the thermal deactivation energy of 199 kJ mol⁻¹.
- Kinetic studies showed that the K_m of lipase increased from 1.63 mM to 4.48 mM after immobilization.
- The immobilized lipase maintained 85% of its original activity after the same wool immobilized lipase was used in six consecutive tributyrin hydrolysis reactions in the BSTR. This result is superior to previous wool immobilized enzyme systems, therefore taking immobilized lipase systems one step closer to implementation in continuous enzyme reaction technologies. Further studies are needed to determine the effect of truly continuous operation.
- The immobilized lipase displayed good storage stability, maintaining 75.8% of the

initial activity after storage of 40 weeks in buffer at 4 °C.

These results show that the wool immobilized lipase can produce a highly active and stable performance for tributyrin hydrolysis in a conventional BSTR. To extend these results to another reactor system, the immobilized lipase was successfully applied in tributyrin emulsion hydrolysis in an innovative enzyme process intensification technology: the SCDR. This reactor appears to intensify the reaction compared to the BSTR results (under comparable conditions), indicating that the combination of mesh and/or cloth immobilized enzymes and SCDRs is a very promising system for improving enzyme reactions in future applications.

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535 Figure 1 (a) Schematic diagram of the enzymatic reactor system with the SCDR. (b) Top
536 view of a woolen cloth with immobilized lipase on the disc of the SCDR.

537 Figure 2 Effect of lipase amount in the immobilization solution on the enzyme loading and
538 activity of the immobilized lipase. The immobilized lipase was cross-linked with 0.1% GA.
539 Values are the average of three independent replicates; error bars represent average $\pm$ one
540 standard deviation.

541 Figure 3 Effect of reaction pH on the activity of free and immobilized lipase. Values are the
542 average of three independent replicates; error bars represent average $\pm$ one standard
543 deviation.

544 Figure 4 Effect of reaction temperature on the activity of free and immobilized lipase. Values
545 are the average of three independent replicates; error bars represent average $\pm$ one standard
546 deviation.

547 Figure 5 Thermal stability of free and immobilized lipase at 60°C. Values are the average of
548 three independent replicates; error bars represent average $\pm$ one standard deviation.

549 Figure 6 Thermal deactivation of immobilized lipase at various temperatures. Values are the
550 average of three independent replicates; error bars represent average $\pm$ one standard
551 deviation.

552 Figure 7 Arrhenius plot of deactivation rate constant (k_d)

553 Figure 8 Lineweaver-Burk plots of free and immobilized lipase in tributyrin hydrolysis
554 showing that Michaelis-Menton kinetics fit the initial rate data. Values are the average of
555 three independent replicates; error bars represent average $\pm$ one standard deviation.

556 Figure 9 Storage stability of the immobilized lipase on woolen cloth: the effect of
557 temperature on the residual activity when stored in phosphate buffer (pH 7, 0.1 M). Values

558 are the average of three independent replicates; error bars represent average $\pm$ one standard
559 deviation.

560 Figure 10 Operational stability of the immobilized lipase on woolen cloth: results for
561 repeated use of wool immobilized lipase in six consecutive tributyrin hydrolysis reactions in
562 the BSTR. Values are the average of three independent replicates.

563 Figure 11 Time course reaction data from the pH stat comparing tributyrin emulsion
564 hydrolysis in the BSTR and SCDR at different concentrations. Operational conditions of
565 SCDR are as follows: reactant volume of 1 L, reaction temperature of 45 °C, flow rate of 5
566 mL s⁻¹ and spinning speed of 350 rpm. The same enzyme to substrate ratio was maintained in
567 comparing the SCDR and BSTR. Values are the mean of three independent replicates.

568

Figure 1

Figure 2

Figure 3

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Figure 10

Figure 11

Table 1 The deactivation rate constant (k_d) and half-life ($t_{1/2}$) of immobilized lipase at various temperatures. Error bars are calculated as $\pm$ one standard deviation.

T (°C)	k_d (min ⁻¹)	$t_{1/2}$ (min)
60	0.000985 $\pm$ 0.00015	703.7 $\pm$ 79.8
65	0.00335 $\pm$ 0.0003	206.9 $\pm$ 10.5
70	0.00834 $\pm$ 0.00045	83.1 $\pm$ 4.3