Journal of Materials Chemistry B

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Controlling performance of lipase immobilised on bioinspired silica†

Cite this: *J. Mater. Chem. B*, 2013, **1**, 1164

Claire Forsyth and Siddharth V. Patwardhan*

Lipase (*Candida antarctica lipase B*) was immobilised on silica that was produced *via* a mild route using a recently developed biologically inspired silica formation method. This route of immobilisation was favourable compared to traditional methods due to its simplicity, mild conditions, low cost, one-step procedure and short preparation time. Lipase was chosen as the enzyme due to its wide implementation in industry, particularly in the food and pharmaceutical industries. Compared to other methods using bioinspired silica entrapment of enzymes, we demonstrate several significant improvements in the performance of the biocatalysts produced. Very high immobilisation efficiencies (close to 100%) were achieved under optimised conditions. The immobilised enzymes also displayed high levels of activity, which exceeded those reported in previous studies of lipase that was immobilised on bioinspired silica. Furthermore, we demonstrate the control over enzyme activity which was achieved through the entrapment conditions by regulating surface area, the average pore diameter and the mean particle size of the silica support. The immobilised enzymes also had very good reuse potential, and showed improved thermal and pH stability. The biocatalysts also performed well in turbulent conditions, showed good storage properties and were successfully used in bench-top reactors, therefore strongly supporting their use in commercial applications. The immobilised enzymes also performed comparably to or better than the industrial benchmark, Novozym® 435. The bioinspired method of immobilisation used has significant benefits over current methods, and these factors could increase potential applications in industry.

Received 27th August 2012
Accepted 21st December 2012

DOI: 10.1039/c2tb00462c

www.rsc.org/MaterialsB

1 Introduction

In recent years, it has become necessary to investigate the feasibility of more environmentally friendly, or 'green' process routes, that make use of mild processing conditions. This is largely due to increasingly stringent environmental regulations, environmental concerns, and drives for greater efficiency.[1] These can greatly reduce operating costs (such as handling and waste treatment), and by making an efficient use of resources, process efficiency – and therefore economics – can be improved.[2]

A key area of green route investigation is in the application of biocatalysts. Enzymes are examples of biocatalysts, and are advantageous since they are able to catalyse reactions at near ambient temperatures, they are highly specific and can therefore be used to reduce by-product formation, and in some cases, enzymes can even make otherwise non-attainable reactions feasible.[2] Enzymes do, however, have several inherent problems that restricts their use. One such problem is their soluble nature; this hinders the ease of enzyme recovery and reusability potential, and can also lead to instability. Because of this, expensive and energy intensive separations are often necessary, and product contamination can be problematic. The molecular structure of enzymes is also prone to destruction through non-benign conditions, such as high temperatures, extreme pH conditions and the presence of organic solvents.[2,3]

To overcome such problems, considerable research is being carried out in enzyme immobilisation techniques, whereby the enzymes are effectively supported or confined for the duration of the reaction.[4,5] This allows their catalytic properties to be preserved, while assisting with repeated and continuous use. Effective immobilisation has also been shown to result in greater enzyme stability over more intense process conditions, therefore increasing potential enzyme applications.[3,6] Recently, additional advantages of enzyme immobilisation have been reported and include possibility of using multi-enzyme systems, improved activity and modification of substrate selectivity.[4] There are various enzyme immobilisation techniques and these include covalent attachment, adsorption,[3,7] cross-linked enzyme aggregates (CLEAs), entrapment and encapsulation.[2]

Many of the current immobilisation approaches suffer from problems, such as long preparation times, tedious multi-step procedures, loss of enzyme activity during immobilisation and poor reusability. Within the entrapment/encapsulation

Department of Chemical and Process Engineering, University of Strathclyde, 75 Montrose Street, Glasgow G1 1XJ, UK. E-mail: Siddharth.Patwardhan@strath.ac.uk; Tel: +44 (0)141-548-5786

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c2tb00462c

approaches of immobilisation, the sol–gel method is by far the most common approach and this involves immobilising the enzyme within a silica gel. Although maintaining high levels of enzyme activity, a major disadvantage of the sol–gel route is its long preparation time; it can take several hours to days and industrially this is impractical (Table S1†).[6,8,9] Due to the materials and techniques involved, many could also not be classed as 'green'. These drawbacks restrict their industrial applications.[3]

The enzyme lipase – in particular the strain *Candida antarctica lipase B* (CALB, simply referred to as "lipase" herein) – forms the cornerstone of this study, as it is a technologically important enzyme. Lipase is primarily associated with catalysing the hydrolysis of triglycerides (fats) into fatty acids and glycerol.[7,10] CALB is one of the more notable enzymes because of its high degree of selectivity, and importance in a range of industrial applications.[10] Due to the ability to produce it on a large scale through micro-organism proliferation, it has been widely implemented in industry, including the food processing industries (particularly dairy), pharmaceuticals industries and chemicals industries (with significant use seen in producing synthetic polymers and detergents),[7] and more recently, in the renewable energy industry in biodiesel production.[11,12] Its use on an industrial scale is, however, often limited due to reasons mentioned, such as instability at higher temperatures and pH extremes, and the difficulty to recover it. These drawbacks are particularly important for the foods and pharmaceutical industries, where the production of pure, non-contaminated (enzyme-free) products is a necessity. As a result, finding efficient and cost-effective means of immobilising lipase, in such a way that its inherent problems may be alleviated or overcome, is of interest.

There are number of lipase immobilisation methods that have been tested. Perhaps the most common form of immobilised CALB is the commercially available Novozym® 435, which is produced by Novozymes. It consists of CALB immobilised onto a macroporous acrylic resin. Covalent binding of lipase onto commercial Eupergit® supports and glyoxyl agarose beads have also been reported.[3,13–15] Other examples of lipase immobilisation include adsorption onto silica nanoparticles,[10,16,17] cross-linking using glutaraldehyde[18,19] and sol–gel entrapment.[20,21] Many of these methods, however, suffer from tedious and expensive preparation methods, poor stability, or the use of toxic or hazardous chemicals.

There appears to be considerable scope for improved enzyme immobilisation techniques through the use of bioinspired silica. Due to its mild, rapid, low cost and controlled protocol, biologically inspired silica has recently been demonstrated as a green method for enzyme immobilisation *via in situ* entrapment of enzymes.[2] Bioinspired silica routes aim to mimic nature's *in vivo* approach to producing silica which is termed 'biosilicification'. In nature $\sim 6.7 \times 10^9$ tonnes of silicon are estimated to be processed per year by marine biological systems such as diatoms.[22,23] The understanding of this biological silica formation has resulted in silica being produced *in vitro* under mild conditions (near neutral pH and $\sim$20 °C), while still maintaining high levels of control, resulting in high quality silica formation.[9] It is known from studies carried out on silica producing species that biological molecules are involved in controlling biosilicification. Further consideration of these biomolecules has been used to establish synthetic "additives" which have proven to be effective for producing silica on a laboratory scale.[2,24] Additives can greatly affect the silica production process and often act as catalysts, therefore allowing the process to be carried out rapidly, and under mild conditions.[9] We have reported previously that a key advantage of using such bioinspired methods is that the properties of silica (surface area, particle size, porosity, *etc.*) can be fine-tuned by carefully chosen synthetic conditions.[24]

A comparison between bioinspired silica synthesis and existing methods for enzyme immobilisation reveals that our method is rapid, easy and environmentally friendly (Table S1†). Previous studies have reported immobilisation of a variety of enzyme using bioinspired approaches,[2] however, the success of these for lipase immobilisation has been limited, with problems such as low activity being reported. Lipase immobilised on bioinspired silica has been achieved by using polyethyleneimine (PEI),[25] polyallylamine (PAH)[26] or the R5 peptide[27] as additives, however, these suffer from a few problems discussed in Results and discussion section.

In this work, dilute aqueous solutions of various additives (ethyleneamines) were used as additives along with sodium metasilicate as a silica precursor, to immobilise the enzyme lipase (CALB) *via* an entrapment method. These additives were chosen due to the rapid silica formation they induce[28,29] and their low cost. There are no previous reports of using these combinations for enzyme immobilisation, making the route highly novel. Furthermore, varying the chemistry of the additives allowed a systematic study to be performed and the properties of the biocatalyst to be controlled. In this study, we aim to address following questions. (i) Is it possible to achieve significant improvements in loading and activity of lipase using bioinspired silica entrapment method? (ii) Can we control the performance of biocatalyst through the synthetic conditions of entrapment? (iii) Can this method produce stable biocatalysts that would be suitable for use in industrially relevant continuous reactors?

2 Experimental

Chemical reagents

All chemicals were purchased from Sigma-Aldrich and used as received. Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4), sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), sodium metasilicate ($Na_2O_3Si \cdot 5H_2O$), ethanol (99.5% v/v), hydrochloric acid (1 M), lipase (CALB), *p*-nitrophenol, *p*-nitrophenyl acetate, pentaethylenehexamine (PEHA), tetraethylenepentamine (TEPA), triethylenetetramine (TETA), diethylenetriamine (DETA), Novozym® 435. All experiments were performed at 20 °C, unless stated otherwise. Unless otherwise specified, the experiments were performed in a 0.1 M pH 7 phosphate buffer. Experiments were carried out in triplicate, and Minitab 16 was used to evaluate errors.

Enzyme immobilisation

Silica was prepared using sodium metasilicate as the silica precursor, and the amines PEHA, TEPA, TETA or DETA as the

additives. Lipase (CALB) was added during silica condensation, producing immobilised enzyme. Separate solutions of sodium metasilicate and amine were prepared using deionised water, and were mixed to obtain a 30 mM concentration of silicic acid, and maintain a molar ratio of [Si] : [N] = 1 : 1 in the final solution. A pre-determined volume of 1 M hydrochloric acid was added to this so that the pH was 7 ± 0.10. A known mass of enzyme was added immediately, and the resulting solution was given a gentle mix. This was then left unstirred for 5 minutes, before being centrifuged for 15 minutes at 8000 rpm, to allow the immobilised enzyme to be separated. The supernatant was removed and stored (for further testing), and deionised water was added to make up the volume again. This process of centrifugation and washing was repeated another two times. The immobilised enzyme was then dried at 40 °C in a vacuum oven for 5 hours. Control experiments were carried out where silica was prepared without enzyme being added.

It was necessary to deduce how much lipase had become entrapped within the silica. This allowed the immobilisation efficiency to be determined (and compared for the various amines), and was also necessary to ensure that the same concentrations of enzyme were used for comparing the free and immobilised enzyme systems. This was achieved by measuring the protein content in the supernatant produced during enzyme immobilisation, using UV-spectroscopy (Shimadzu UVmini-1240) at 283 nm with a quartz cuvette. A lipase calibration curve (Fig. S1†) was produced to allow the relationship between lipase concentration and absorbance to be deduced.

Materials characterisation

Lipase immobilised on silica were dried and mounted on SEM sample holders with double sided sticky carbon tape. Upon gold coating by sputtering, samples were analysed on a HITACHI SU-6600 Field Emission Scanning Electron Microscope (FE-SEM) at 10 kV. For particle size measurements, sizes of a number of particles from selected high resolution images were measured manually. Fourier Transform Infra-Red (FTIR) spectroscopy was used to analyse the dried immobilised enzyme samples, in order to detect the formation of silica and the presence of lipase. Attenuated Total Reflectance-FTIR analyses (ABB Miracle MB 3000) of dried silica (without lipase) and dried immobilised enzyme samples were performed, with 32 scans at a resolution of 4. Prior to centrifugation during enzyme immobilisation (*i.e.* after leaving the samples unstirred for 5 minutes), dynamic and static light scattering (DLS/SLS) techniques were used to estimate average particle/aggregate sizes. Measurements took place at 90° (ALV/CG3 Compact Goniometer, ALV/LSE-5004 Electronic System and 50 mW He–Ne laser (λ = 632 nm)). Immobilised enzyme samples underwent N_2 adsorption (Micromeritics ASAP 2420); these gave details of surface area, pore volume and pore width.

Enzyme activity assay

The enzyme assay used involved the hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol (and acetate acid). At pH ≥ 7, the *p*-nitrophenol mainly exists as a yellow anion. UV-vis spectroscopy was therefore used to measure the absorbance of the samples at 405 nm, and the corresponding *p*-nitrophenol product concentration was deduced using a calibration curve (Fig. S2†). This procedure was repeated for the various temperatures and pH conditions involved in the entirety of the experiment, since it was found that calibration did not remain unchanged for different temperature and pH conditions.

Since the assay reaction can occur even without the presence of lipase (although to a lesser extent), it was necessary to determine non-enzymatic reaction rates in order to allow the true increase in reaction rate, due the presence of lipase, to be determined. When enzymes were present, the rate calculated was the sum of both the enzymatic and non-enzymatic reaction rates. The non-enzymatic rate was then subtracted, giving the true enzymatic rate. A 1.0 M stock solution of *p*-nitrophenyl acetate was prepared by dissolving the necessary mass in ethanol (99.5% v/v). This was diluted into 5, 10, 20, 40, 80 and 100 mM solutions using ethanol. The assay mixture was then prepared and consisted of: 3 mL 0.1 M, pH 7 phosphate buffer; 15 μL 0.1 M, pH 7 phosphate buffer (when free enzyme was present, 15 μL of enzyme solution was added, hence buffer of this volume was added to maintain concentrations) and 30 μL *p*-nitrophenyl acetate (in ethanol) substrate solution. This was prepared for all *p*-nitrophenyl acetate concentrations, as well as a 0 mM solution as a control. The assays were kept gently stirred, and the absorbance was measured at 405 nm, every minute for 10 minutes. The absorbance was then measured at various intervals after, until there was no further change. This indicated that the reaction was complete. The *p*-nitrophenol calibration curve equation was then used to convert the absorbance into product concentrations. Linear equations were found to describe the data for the first 10 minutes, and these were used to estimate the non-enzymatic reaction rate for each substrate concentration. A first order reaction model was then used to estimate the rate constants.

The kinetic parameters and activity of free lipase were estimated. This was necessary to allow a comparison between free and immobilised systems, and hence allowed the impact of immobilisation on lipase to be evaluated. The procedure was similar to that used to deduce the non-enzymatic reaction rates, however, 15 μL of 1 mg mL^{-1} free lipase in buffer solution (producing a 0.005 mg mL^{-1} enzyme concentration overall in the assay) was added, rather than the additional 15 μL of buffer added previously.

The corresponding non-enzymatic reaction rates were subtracted from the total reaction rates deduced, giving the enzymatic reaction rates. The enzymatic reaction rates were then plotted against substrate concentration, to produce a Michaelis–Menten model. Through non-linear regression techniques, the kinetic parameters were deduced; for this, the Hill function in Origin 8.5 was used to describe the following equation:

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad (1)$$

Where: v = reaction rate; V_{max} = maximum rate; [S] = substrate concentration; K_M = Michaelis–Menten constant. The kinetic parameter V_{max} was used to estimate the specific enzyme activity of the free enzyme. An enzyme activity unit (U) was defined as

the amount of enzyme activity that caused the disappearance of 1 μmol of substrate, or formation of 1 μmol of product, per minute. The specific activity (U mg^{-1}) was defined as the activity of the enzyme per mg of enzyme.

The kinetic parameters and activity of the immobilised enzymes were also determined. This allowed the impact of immobilisation and the effectiveness of the different amines to be considered. The performance of Novozym® 435, the industry standard immobilised CALB, was also evaluated. The procedure was similar to that used for free enzyme, however, rather than adding free enzyme, an equivalent volume of buffer was added, and an appropriate mass of immobilised enzyme was added to ensure a 0.005 mg mL^{-1} enzyme concentration, as before. According to Novozyme, Novozym® 435 is 21% w/w enzyme.[30] Therefore, since this range was similar to that achieved with the immobilised lipase produced herein, a fair comparison between lipase supported on silica and that in Novozym® 435 was possible.

Leaching and reuse

A known mass of immobilised enzyme (whose lipase content had been determined) was placed in 5 mL of 0.1 M pH 7 phosphate buffer. The absorbance of the surrounding buffer at 283 nm (using a quartz cuvette) was measured at various time intervals. The lipase calibration curve was then used to obtain an enzyme concentration from this, and the loss of activity was deduced. This procedure was carried out for lipase immobilised using bioinspired silica, as well as Novozym® 435.

Immobilised enzyme that had been used in a prior reaction (and was present in the reaction medium for 1 hour) was separated from the surrounding liquid by centrifugation, and washed. The material was then reused in an assay, as described for the immobilised enzyme. The product formation after 10 minutes was measured, and compared to its initial use. This procedure was repeated for additional cycles, and was carried out for lipase immobilised using bioinspired silica, as well as Novozym® 435.

Enzyme stability

The performance of the immobilised enzymes (including Novozym® 435) was tested under various temperatures to study thermal stability. For all stability tests, it was necessary to perform the assay without enzyme, with free enzyme, and with immobilised enzyme, at each temperature chosen. The assay compositions used were identical to those described previously, with the *p*-nitrophenyl acetate substrate concentration at 10 mM. Thermal stability experiments were carried out between 20 °C and 70 °C in 10° increments; these temperatures were established through the use of a water bath. The product formation after 10 minutes was measured, which allowed a comparison of activity of the systems at various temperatures.

The procedure used for pH stability was similar to that used in the temperature tests, however, rather than varying the temperature, the pH of the assay was varied by altering the pH of a 0.1 M phosphate buffer. Since the direct assay procedure only works well at pH $\geq$ 7 (it is only at these conditions that the *p*-nitrophenol exists mainly as a yellow anion), only pH 7, 8, 9, 10 and 11 tests were performed using this method (all at 20 °C).

Attempts were also made to evaluate performance in acidic conditions (pH 4), by adjusting the pH of the buffer. Since the *p*-nitrophenol was not in neutral or basic conditions, it remained colourless. Therefore, at a time of zero and a time of 10 minutes, a known volume of the assay solution was taken and added to the pH 7 buffer, in order to produce the yellow anion. The absorbance was measured and adjusted for the corresponding dilutions; this allowed the product formation after ten minutes to be deduced, allowing a comparison of the systems.

The effectiveness of the immobilised enzymes was tested under extreme physical conditions, to simulate the turbulent flow conditions which may be encountered in an industrial situation, rather than the benign, gently stirred assay conditions. It also allowed an insight into the mass transfer limitations of the systems. The procedure was similar to that used for the temperature tests, however, all tests took place at 20 °C, during which the samples were subjected to shaking, vorticing or the gentle stirring. Product formation after at a short fixed time was measured, which produced initial rates of reaction. These rates were compared for the same sample but when measured without any mixing and reported as "relative activity". The procedure was performed without enzyme, with free enzyme, with the bio-inspired immobilised enzymes and with Novozym® 435. The storage stability of the enzyme systems was also investigated. After vacuum drying, the immobilised enzymes were stored at −18 °C, 4 °C or 20 °C, and their performance after 5 weeks under these conditions was evaluated.

Bench-top reactors

The performance of supported lipase was evaluated in two simple bench-top reactors – continuous stirred tank reactor (CSTR) and plug flow reactor with a fixed catalyst bed (PFR). As given in Section 3.8, the reactors were designed and dried powder of supported enzyme catalyst was loaded. Special care was taken in the PFR by loading the immobilised enzyme into the tubular reactor and keeping it in place with glass-wool and filter paper to prevent any enzyme loss. The reactors were operated for several hours while monitoring product concentration and a steady state was attained.

3 Results and discussion

In order to address the questions given above, a complete analysis of the immobilised enzymes produced was necessary. A colorimetric enzyme assay scheme was used to assess the activity of the immobilised enzymes compared to free enzyme; the kinetic parameters were determined through non-linear regression techniques. A comparison of the performance of the immobilised enzymes and Novozym® 435 (N-435), the industry benchmark CALB, was also performed.

Improved loading and activity of immobilised lipase

An optimisation procedure took place by altering the mass of enzyme added during immobilisation to see if a maximum

immobilisation efficiency and w/w enzyme was reached: an example for PEHA is shown in Fig. 1a. There appeared to be a maximum enzyme capacity in the silica, with silica produced using TETA having the greatest capacity, followed by DETA, PEHA, and TEPA. The use of TETA and DETA were found to produce the highest mass of immobilised enzyme, followed by PEHA and TEPA (Fig. 1b).

When low masses of enzyme were added, protein in the supernatant was undeterminable, suggesting very high levels of immobilisation. The *p*-nitrophenyl acetate assay was used to confirm this and it was found that immobilisation efficiencies were close to 100%. The immobilisation efficiencies and w/w enzyme of the samples compared well with many bioinspired enzyme immobilisation routes that used similar techniques,[2,25–27,31–33] and exceeded many too.[26,27,34–39] Previously reported immobilisation efficiencies using lipase and bioinspired silica approaches included 12%,[27] 90%[25] and 96%,[26] therefore suggesting that the use of PEHA, TEPA, TETA and DETA as additives was at least as efficient as these, or perhaps better.

The morphologies and particle sizes of lipase–silica biocatalysts were investigated using Scanning Electron Microscopy (SEM) and the data is shown in Fig. 2a–i. From the SEM data it is evident that the lipase–silica samples formed spherical or pseudo-spherical nanoparticles, which were found to aggregate. Particle size analysis revealed that the particle were roughly around 250 nm in size with broad size distributions (Fig. 2i). SEM results along with the gas adsorption and light scattering data (discussed below) suggest that the lipase–silica particles exhibited features at three length-scales. The aggregation of primary particles (not shown) allowed the formation of pores (<20 nm, see discussion below for Fig. 5), the formation of secondary particles (~250 nm, SEM data) and larger aggregates of these particles (1–2 μm, see discussion below for Fig. 5). Although the exact location of enzyme in silica is not clear at this point in time, it is reasonable to assume that the enzyme may reside in the pores, as demonstrated for a related system in the literature.[40] FTIR spectra (Fig. 2j) confirmed successful immobilisation of the enzyme; this was evident from amide peaks at 1650 cm^{-1} and 1450 cm^{-1} (marked as dashed lines in the Fig. 2j) that were not detected with control silica. A comparison with free lipase also confirmed successful enzyme immobilisation. Strong bands in the region of 950–1150 cm^{-1} and 800 cm^{-1} confirmed silica formation.

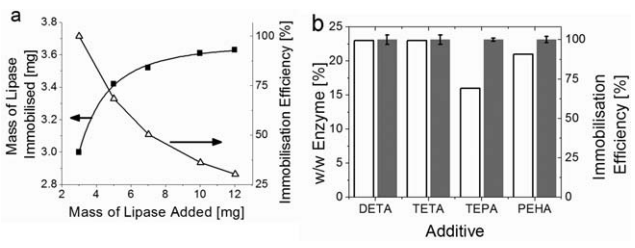

Fig. 1 Enzyme immobilisation efficiency and amount of enzyme immobilised using PEHA (a) and all additives (b). In (b) □ shows w/w enzyme (left axis) and ■ shows immobilisation efficiency (right axis).

The enzymatic activities of various biocatalysts produced herein, free enzyme and Novozym®-435 were investigated using protocol detailed in the Experimental section. In each assay, the total amount of enzyme used was kept constant irrespective of their immobilisation method. The experimental data conformed well to the Michaelis–Menten model (Fig. 3a). The kinetic parameters determined (Table 1 and Fig. 3b) showed that V_{max} was smaller and K_M was larger for the bioinspired immobilised systems, compared to free enzyme. Smaller V_{max} for silica immobilised enzymes, when compared with Novozym®-435, suggests that silica bound enzyme is less active. Since K_M is effectively a dissociation constant, the larger value suggested that the substrate had less of an affinity for the enzyme in the immobilised systems and that a higher concentration of the substrate is required to saturate the enzyme. These trends have been observed in many studies of enzyme immobilisation techniques.[33,36,39,41] The immobilised enzymes produced showed very good potential. Almost 90% of free enzyme activity was preserved during immobilisation using DETA, and this was better than that achieved through alternative bioinspired lipase immobilisation approaches, where 51%,[26] 10%[25] and 14%[27] of activity being maintained have been reported. Even PEHA, the poorest performer herein, maintained high levels of activity, exceeding previous approaches. The results suggested that the amine used during immobilisation ultimately affected the performance of the immobilised enzyme, particularly in terms of reaction rates and activity. On average, enzyme immobilised using DETA had the highest V_{max}, followed by TETA, TEPA and PEHA. K_M for PEHA and TETA were the same, while those for DETA and TEPA were higher (Table 1 and Fig. 3). Novozym® 435 compared very well to the free enzyme. Although slightly lower on average, V_{max} for Novozym® 435 and the free enzyme were very similar, and K_M was almost identical. Novozym® 435 also performed better than the lipase immobilised *via* bioinspired silica.

It is known that immobilisation of enzymes can cause several effects leading to reduction in the enzyme activity. These effects include enzyme distortion, blockage of enzyme active centre, diffusion limitations and hyperactivation.[5] Since the activities of the biocatalysts reported herein are comparable with the free enzyme activity, hyperactivation can be ruled out. The findings suggested that immobilisation may have restricted the enzyme's overall activity. Active sites may have become impaired during immobilisation, or the silica support may have made the sites more difficult to access, possibly due to the porous nature of the silica support, therefore reducing mass transfer, and ultimately the reaction rate and activity of the enzyme. Due to the wide pore sizes in silica (discussed below), compared to the size of enzyme, it is also likely that enzyme agglomerates were formed within these pores, attributing to reduced enzyme activity.

In order to probe whether the reduced activity upon immobilisation was caused by enzyme deactivation or diffusion limitation, the enzyme activity was measured by inducing more turbulent conditions. Although, extensive calculations and analyses on diffusion can be performed by incorporating internal and external diffusion models, the focus here was to

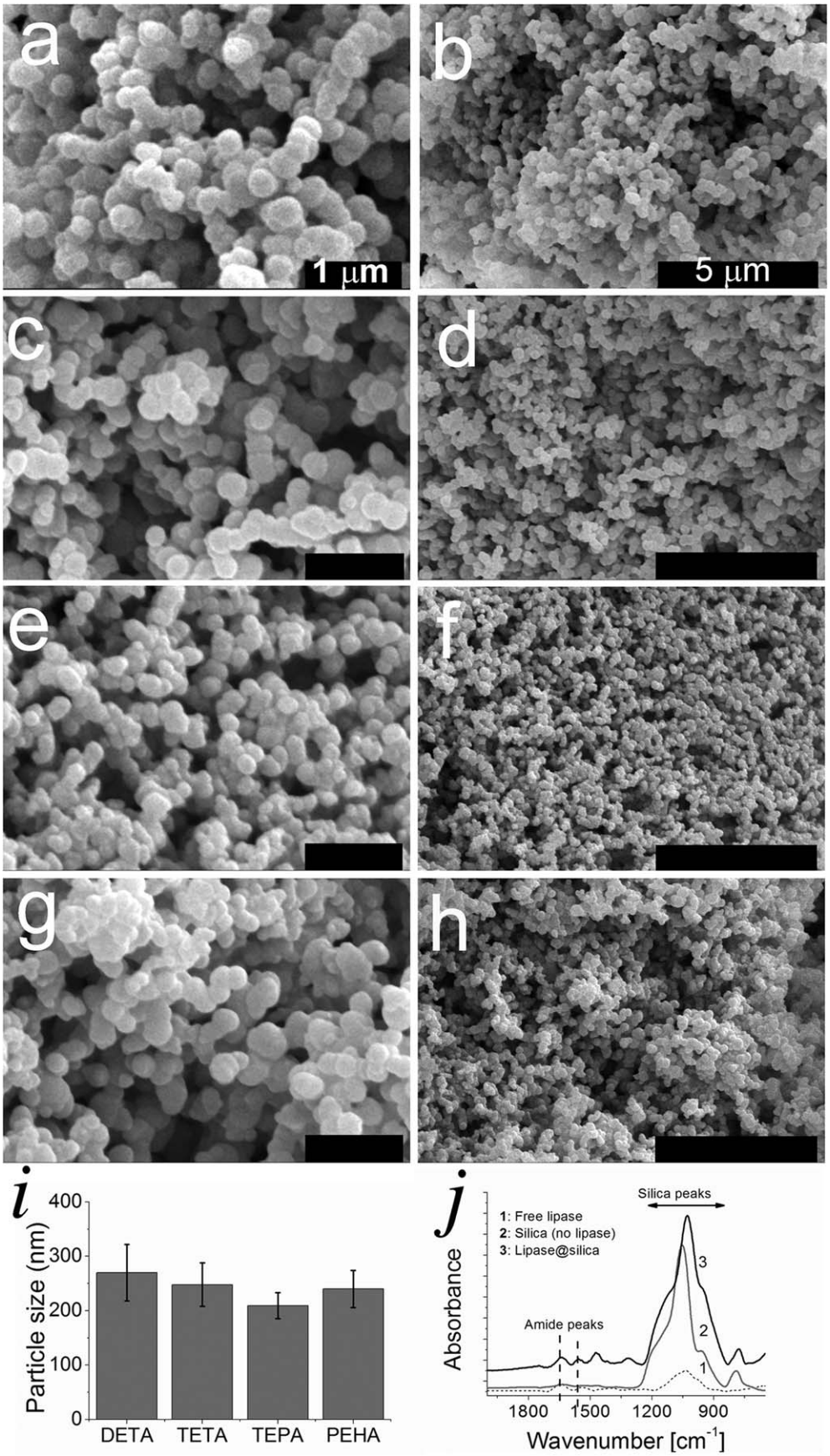

Fig. 2 SEM images for lipase immobilised in silica using (a and b) DETA, (c and d) TETA, (e and f) TEPA and (g and h) PEHA. Scale bars are 1 μm for images on left and 5 μm for images on right. (i) Particle size measurements from SEM images. (j) ATR-FTIR spectra for free lipase, silica without lipase and lipase immobilised on silica.

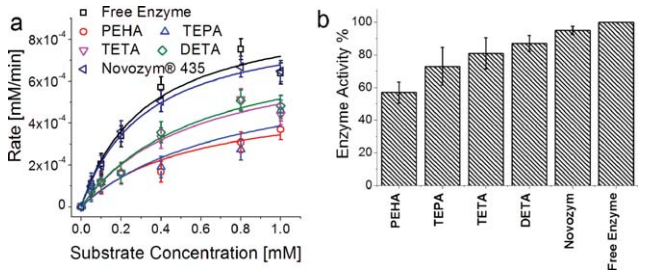

Fig. 3 (a) Michaelis–Menten model of enzymatic rate data for immobilised enzymes (including Novozym® 435) pH 7, 20 °C. (b) Enzymatic activity after immobilisation compared with that of the free enzyme.

simply check if the immobilised enzyme was still active (*i.e.* whether lower conversions were diffusion limited). It was observed that significant increases in reaction rate in the initial stages of the reactions were seen for all immobilised enzyme systems (Fig. 4). This was likely to have been due to the more turbulent conditions increasing rates of mass transfer, since the mass transfer coefficient is a function of the Reynolds number. For example, enzyme immobilised using TETA had 25 times faster initial reaction rates when shaken, compared to free enzyme being just 3 times faster and Novozym® 435 being 11 times faster. Although Novozym® 435 showed increased rates of product formation upon mixing, the synthesised immobilised enzymes showed notably enhanced activities compared to Novozym® 435. Free enzyme, on the other hand, did not show notable increase in activity under these harsher conditions.

This suggested that mass transfer during the assays was limiting the immobilised enzymes' performance. It was also possible that in turbulent conditions, shearing of the free enzyme led to conformational deformation, resulting in deactivation, while due to large particles of Novozym® 435, mixing did not influence reaction rates. This may explain why the activities of Novozym® 435 and free enzyme were not as greatly enhanced as the synthesised immobilised enzymes: the silica supports may have been more effective at protecting the enzyme from these conditions than Novozym® 435, while free enzyme had no protection at all.[7]

Controlling enzyme performance through entrapment conditions

Based on the structure of the amines, the data suggested that there may have been some correlation between performance of the immobilised enzymes, and the number of amines in a molecular unit. DETA, which resulted in the best performance, has fewest amines (3), while PEHA, which resulted in the poorest performance, has most (6).

From porosity measurements, although surface areas and pore volumes remained unchanged, the average pore diameter was found to correlate with the enzyme activity (Fig. 5 and Table S3†). All samples showed mesoporous structures, and TETA immobilised enzyme was found to have the largest average pore diameter (22 nm), while PEHA immobilised enzyme, the poorest performer, was found to have the smallest (13 nm). Smaller pores were likely to have reduced mass transfer, resulting in the lower rates and poorer performance which were observed. The DLS/SLS results (Fig. 5, Table S2 and Fig. S3†) also suggested a correlation between the number of amines and performance. In the PEHA immobilised enzyme system, aggregates were shown to have the largest mean radii (2400 nm) after 5 minutes of reaction and aggregation, while DETA immobilised enzyme aggregates were the smallest (1200 nm). The larger particle sizes suggested higher levels of aggregation, since it is known that for these additives, after 5 minutes of reaction, micron sized aggregates are formed that are composed of 200–400 nm silica particles.[28,29] This may have inhibited mass transfer; larger particle sizes would have made it more difficult for reactants to penetrate further into the particle.[42]

The effect of the additive chemistry on the improved enzyme activity can be explained through the relation between mass transfer and pore size of the support. Theory for liquid diffusion, particularly in pores, is not as advanced as gaseous

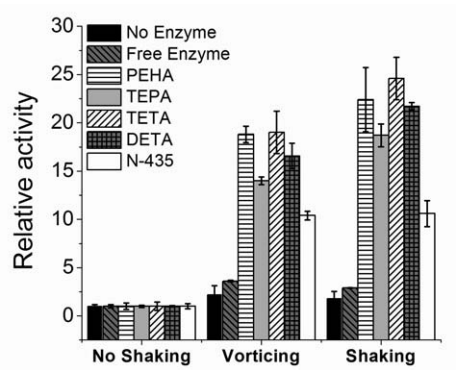

Fig. 4 Relative enzymatic activity compared to non-shaken equivalent. The activity reported here is a measure of initial rate of the product formation.

Table 1 Comparison of enzyme performance (pH 7, 20 °C)

Method	V_{max} [mM min^{-1}]	K_M [mM]	Specific activity [U mg^{-1}]	% Activity *cf.* free enzyme
PEHA	$5.4 \times 10^{-4} \pm 5 \times 10^{-5}$	0.57 ± 0.1	0.11 ± 0.01	57 ± 7
TEPA	$6.9 \times 10^{-4} \pm 1 \times 10^{-4}$	0.77 ± 0.2	0.14 ± 0.02	73 ± 12
TETA	$7.7 \times 10^{-4} \pm 1 \times 10^{-4}$	0.57 ± 0.1	0.16 ± 0.02	81 ± 10
DETA	$8.3 \times 10^{-4} \pm 1 \times 10^{-4}$	0.61 ± 0.1	0.17 ± 0.01	87 ± 5
Novozym® 435	$9.0 \times 10^{-4} \pm 1 \times 10^{-4}$	0.32 ± 0.02	0.18 ± 0.01	95 ± 3
Free enzyme	$9.5 \times 10^{-4} \pm 5 \times 10^{-5}$	0.33 ± 0.02	0.19 ± 0.02	100

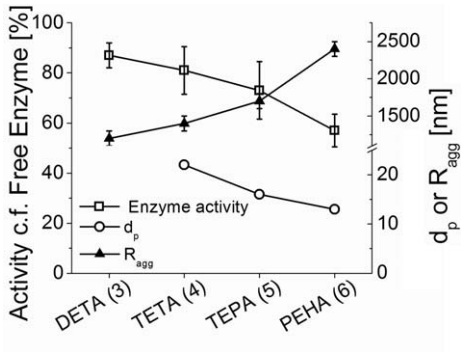

Fig. 5 Effect of number of nitrogen atoms per amine molecule on activity, pore diameter (d_p) and aggregate size in solution (R_{agg}).

diffusion. As a result, evaluation of porous liquid systems typically relies on approximations and empirical equations. A pore can be approximated as a straight, cylindrical pipe. Darcy's law (eqn (2)) and the Hagen–Poiseuille equation (eqn (3)) can be used to show the relationship between diameter and pressure drop. This is therefore analogous to the relationship between the pore diameter and the ease of mass transfer through the pore.[43,44]

$$\Delta P = 2f\rho v^2 \frac{l}{d} \tag{2}$$

$$\Delta P = \frac{32\mu l v}{d^2} \tag{3}$$

where: ΔP = pressure drop; f = fanning friction factor; ρ = density; v = velocity; μ = viscosity; l = pore length; d = pore diameter.

Both of these equations clearly show that bigger diameters result in less resistance to flow. This suggests better mass transfer with larger pore diameters, as suggested by the experimental results. This idea is further reinforced with the following adaption of Fick's law for liquid flowing through pores:[45]

$$N_i = D_i\left(\frac{\varepsilon}{\tau}\right)(\Delta C)K \tag{4}$$

$$K = \left(1 - \frac{d_m}{d_p}\right)^4 \tag{5}$$

where: N_i = flux of component i; D_i = diffusivity of solute i; ε = porosity of material; τ = tortuosity of material; ΔC = concentration driving force; K = geometry factor to account for effect of pore diameter; d_m = diffusing molecule diameter; d_p = pore diameter.

The above interpretation strongly supports the experimental results (Fig. 5) that as the pore diameter of the support is increased, the geometry factor is larger, resulting in a higher flux, hence better mass transfer (*i.e.* enzymatic activity). The results presented herein and their interpretation is corroborated with a previous study immobilising lipase on SBA-15 silica nanoparticles where it was shown that significant improvements in activity (three times more) were observed by increasing the pore sizes of the silica from 5 nm to 24 nm. This highlighted the effect of poor mass transfer on enzyme activity, and the influence of pore diameter.[16]

Improving biocatalyst stability and its use in continuous reactors

For the free and immobilised enzyme systems, a clear reaction optimum at 40 °C was observed which compared well with literature, where optimum conditions between 40 and 50 °C were generally reported.[46–49] Importantly, at 60 °C, the immobilised enzymes (PEHA, TEPA, TETA, DETA and Novozym® 435) were still having an enzymatic effect, whereas the free enzyme had become denatured (Fig. 6 and S4†). This strongly suggested that immobilisation successfully improved the stability of the enzyme, with the synthesised immobilised enzymes' stability comparing very well to Novozym® 435.

The pH for optimum activity appeared to be pH 8, which was consistent with literature where pH values of 6–8 were typically stated as optimums.[48] As with the temperature tests, an increase in stability of the immobilised enzymes was observed, and the immobilised enzymes prepared using PEHA, TEPA, TETA and DETA appeared to show better stability than Novozym® 435 (Fig. 7 and S5†). At pH 10, the immobilised enzymes prepared were still having an enzymatic effect, whereas the free enzyme and Novozym® 435 were no longer effective. The test in acidic conditions also suggested an increased stability of the immobilised enzymes; at pH 4, the immobilised enzymes were shown to have more of an enzymatic effect than the free enzyme and Novozym® 435 (Fig. S5†). These results suggest improved thermal and pH stability, which are consistent with advantages identified from enzyme immobilisation.[4]

The improved thermal and pH stabilities upon entrapment of CALB in silica, which is well documented, in particular for silica supports,[50] is attributed to the steric constraints the support imposes on the enzyme structure, making denaturation more difficult. Improved stability is also indicative of strong and favourable enzyme–support interactions, which could arise from the *in situ* enzyme entrapment that was only possible due to the one-step green approach. In the case of pH stability, the

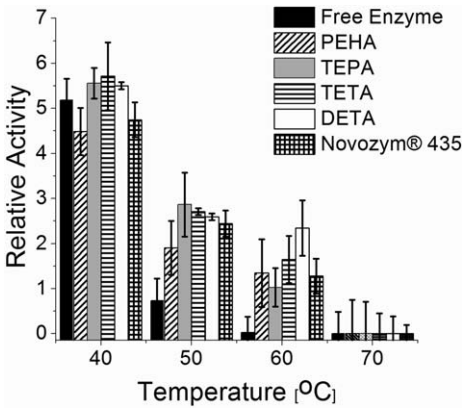

Fig. 6 Thermal stability of biocatalysts reported as activity relative to that at 20 °C.

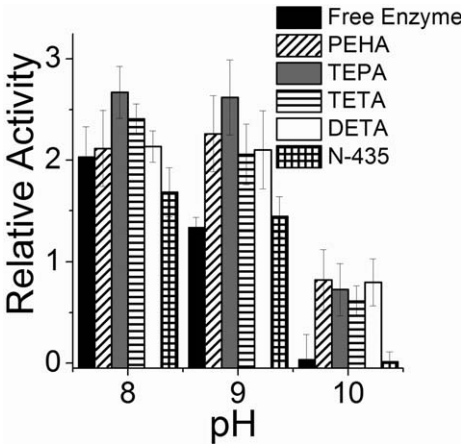

Fig. 7 Relative activity at various pH conditions reported as activity compared to that at pH 7.

interactions of CALB with the silica (which is known to vary surface chemistry with changes in pH)[51] as well as with amines incorporated within silica may well be providing favourable micro-environments for CALB and minimising detrimental effects of high and low pH conditions.

The immobilised enzymes also retained very high residual activity (>90%) after leaching experiments, which highlights stability of the enzyme over time as well as demonstrates negligible levels of leaching (Fig. 8a). These results suggested that the silica supports had strong entrapment of the enzyme, while the small loss of activity is attributed to experimental errors and loss of sample during recovery by centrifugation after the leaching experiments but prior to activity measurements. Novozym® 435 displayed no apparent leaching. On average, DETA showed most loss in activity, still below 10%. This may be for similar reasons to those mentioned previously such as pore diameter; PEHA, with the smallest average pore diameter, displayed the lowest levels of loss of activity. When the immobilised enzymes were recovered from the reaction mixtures, they exhibited excellent reuse potential: this was comparable to Novozym® 435 (Fig. 8b). They showed only a very slight reduction in activity from their primary use, with ~5% reduction in activity being observed between any consecutive cycles. The small drop in activity from both the leaching and reuse experiments was likely to be due to silica nanoparticles with immobilised enzyme being lost during centrifugation and transfer. The leaching and reuse results suggest that there may be a strong and beneficial interaction between the enzyme and silica. Furthermore, enzyme performance upon storage was investigated. Storage at −18 °C appeared to have the most detrimental effect on the immobilised enzymes. Storage at 4 °C appeared most effective, and loss of activity due to storage at ambient did not appear to be a significant problem (Fig. 9).

Due to their improved stability and reuse potential, the effectiveness of the immobilised enzymes on a larger scale was tested by assembling a bench-top continuous stirred tank reactor (CSTR) and a bench-top tubular plug flow reactor (PFR). These reactor types were chosen due to their simplicity to construct and operate, their compatibility with liquids and suspended particles, and their popularity in industry.[7] Material balances were carried out across the systems to produce design equations that allowed the volume of the reactors to be determined based on known parameters for CSTR (eqn (6)) and PFR (eqn (7)).

$$V_{\mathrm{CSTR}} = \frac{F[\mathrm{S_{in}}] - F[\mathrm{S}]}{r_{\mathrm{S}}} \tag{6}$$

$$V_{\mathrm{PFR}} = F \int_{S_{\mathrm{in}}}^{S_{\mathrm{out}}} \frac{\mathrm{d}[\mathrm{S}]}{-r_{\mathrm{S}}} \tag{7}$$

where: F = volumetric flow rate of inlet and outlet stream; S_{in} = inlet substrate concentration; S = reactor substrate concentration (*N.B.* this varies along the reactor length in a tubular reactor); S_{out} = outlet substrate concentration; P = product concentration; r_{s} = rate of substrate consumption. Despite several assumptions made in eqn (6) and (7), and their implementation, these equations served as good first approximations.

The rate of substrate consumption was the sum of the non-enzymatic and enzymatic reaction rates. Therefore, the Michaelis–Menten rate equation was substituted in for the enzymatic reaction rate using the kinetic parameters deduced for the immobilised systems, and a simple first order rate law was used for the non-enzymatic rate, using the rate constant

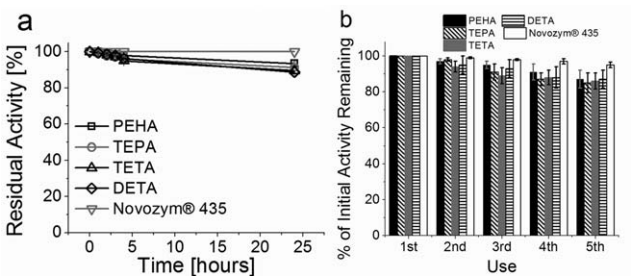

Fig. 8 (a) Residual activity of immobilised enzymes after leaching experiments and (b) reuse potential after up to five uses.

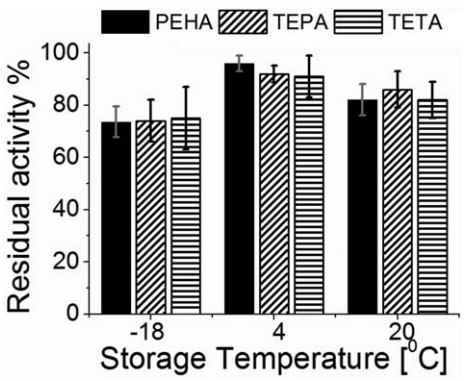

Fig. 9 Percentage of activity remaining after 5 weeks storage at various conditions.

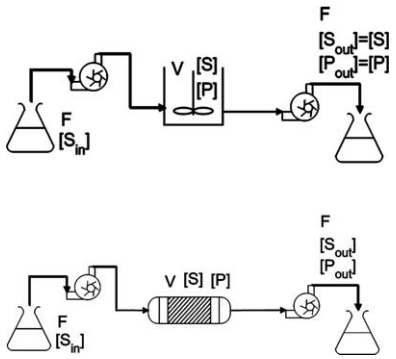

Fig. 10 Schematic representation of the CSTR (top) and the tubular reactor set-up.

deduced for the non-enzymatic reactions as given in eqn (8) and (9) below.

$$V_{\mathrm{CSTR}} = \frac{F[\mathrm{S_{in}}] - F[\mathrm{S}]}{\frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{M}} + [\mathrm{S}]} + k[\mathrm{S}]} \quad (8)$$

$$V_{\mathrm{PFR}} = F \int_{S_{\mathrm{in}}}^{S_{\mathrm{out}}} \frac{\mathrm{d}[\mathrm{S}]}{-\left(\frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{M}} + [\mathrm{S}]} + k[\mathrm{S}]\right)} \quad (9)$$

Suitable feed flow rates, substrate concentrations and conversions (X) were chosen to ensure practical designs, and this allowed the required reactor volumes to be estimated (Fig. 10). The enzyme concentrations used were kept identical to those used in the free and immobilised enzyme experiments. To prevent enzyme being lost in the product, a filter was placed at the entrance of the outlet pipes. This method of minimising enzyme loss is common in industry.[7]

After leaving the systems to reach a steady-state, product formation was measured, allowing the conversions achieved to be calculated, and compared to the theoretical values. All conversions achieved experimentally compared very well to the theoretical values (within 15%). The reactors were operated for several hours and no loss of enzyme activity was observed after the steady state was reached (Fig. S6†). The immobilised enzymes produced therefore have the potential to be effective on a larger scale.

4 Conclusion

Lipase immobilised on silica that was produced *via* a bio-inspired route clearly has significant potential, with main advantages being its mild processing conditions, the simplicity at which it can be produced (one-step procedure), its low cost and its short preparation time compared with current methods, in addition to high activity and stability. Very high immobilisation efficiencies (close to 100%) were achieved through optimisation; these compared well with, or exceeded, those achieved with many bioinspired enzyme immobilisation routes which used similar techniques. Some loss of activity due to immobilisation was observed as expected, however, it was revealed that this loss was due to mass transfer limitations, and not enzyme deactivation. The activities of all immobilised enzymes produced exceeded prior studies of lipase immobilised using bioinspired routes. The synthetic conditions were found to control physical properties of the biocatalysts, which in turn regulated the biocatalyst performance.

The immobilised enzymes showed excellent reuse potential (comparable to Novozym® 435), maintaining high levels of activity after repeated use. They also showed very low levels of leaching, making them particularly suitable for the food and pharmaceuticals industries. Importantly, the immobilised enzymes were shown to perform better over a wider range of temperatures and pH conditions than free enzyme; this was indicative of improved enzyme stability. The immobilised enzymes also showed better stability than Novozym® 435, particularly over the pH range studied. The immobilised enzymes showed good storage potential, performed well in turbulent conditions and were shown to perform well in bench-top reactors, therefore taking a step forward in their commercial utilisation.

The bioinspired method of immobilisation used has significant benefits over current methods, largely due to its mild, rapid, low-cost and simple preparation. These factors could therefore increase potential applications in industry. Future work, utilising the results presented herein, is directed towards exploring other benefits from the immobilisation method by developing lipase based biocatalysts for applications in enantioselective reactions, for example, and also demonstrating the versatility of this method for other industrially relevant enzymatic processes.[52]

Acknowledgements

We thank funding from Royal Society (Grant no. TG090299) for the initiation of this project, the Department of Chemical and Process Engineering and Carnegie Undergraduate Vacation Scholarship. The authors also thank Professor Stephen Clarson and Professor Peter Halling for various helpful discussions and Carol Forsyth for help with the light scattering analysis.

References

1 P. T. Anastas and J. B. Zimmerman, *Environ. Sci. Technol.*, 2003, **37**, 94A–101A.
2 L. Betancor and H. R. Luckarift, *Trends Biotechnol.*, 2008, **26**, 566–572.
3 C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2007, **40**, 1451–1463.
4 D. Brady and J. Jordaan, *Biotechnol. Lett.*, 2009, **31**, 1639–1650.
5 C. Garcia-Galan, A. Berenguer-Murcia, R. Fernandez-Lafuente and R. C. Rodrigues, *Adv. Synth. Catal.*, 2011, **353**, 2885–2904.
6 U. Hanefeld, L. Gardossi and E. Magner, *Chem. Soc. Rev.*, 2009, **38**, 453–468.

7 V. M. Balcao, A. L. Paiva and F. X. Malcata, *Enzyme Microb. Technol.*, 1996, **18**, 392–416.
8 J. Brinker and G. Scherer, *Sol–Gel Science: The Physics and Chemistry of Sol–Gel Processing*, Academic Press, 1990.
9 S. V. Patwardhan, *Chem. Commun.*, 2011, **47**, 7567–7582.
10 J. C. Cruz, P. H. Pfromm and M. E. Rezac, *Process Biochem.*, 2009, **44**, 62–69.
11 K. Ban, M. Kaieda, T. Matsumoto, A. Kondo and H. Fukuda, *Biochem. Eng. J.*, 2001, **8**, 39–43.
12 U. Schoerken and P. Kempers, *Eur. J. Lipid Sci. Technol.*, 2009, **111**, 627–645.
13 E. Katchalski-Katzir and D. M. Kraemer, *J. Mol. Catal. B: Enzym.*, 2000, **10**, 157–176.
14 O. Barbosa, C. Ortiz, R. Torres and R. Fernandez-Lafuente, *J. Mol. Catal. B: Enzym.*, 2011, **71**, 124–132.
15 D. S. Rodrigues, A. A. Mendes, W. S. Adriano, L. R. B. Goncalves and R. L. C. Giordano, *J. Mol. Catal. B: Enzym.*, 2008, **51**, 100–109.
16 H. Jaladi, A. Katiyar, S. W. Thiel, V. V. Guliants and N. G. Pinto, *Chem. Eng. Sci.*, 2009, **64**, 1474–1479.
17 S. Zheng, *Effect of Pore Curvature and Surface Chemistry of Model Silica Hosts on Biocatalytic Activity of Immobilised Lipase*, Department of Chemical Engineering, University of Cincinnati, 2005.
18 R. A. Sheldon, *Biochem. Soc. Trans.*, 2007, **35**, 1583–1587.
19 B. Devi, Z. Guo and X. B. Xu, *J. Am. Oil Chem. Soc.*, 2009, **86**, 637–642.
20 M. T. Reetz, A. Zonta and J. Simpelkamp, *Biotechnol. Bioeng.*, 1996, **49**, 527–534.
21 A. Tomin, D. Weiser, G. Hellner, Z. Bata, L. Corici, F. Peter, B. Koczka and L. Poppe, *Process Biochem.*, 2011, **46**, 52–58.
22 P. Treguer, D. M. Nelson, A. J. Vanbennekom, D. J. Demaster, A. Leynaert and B. Queguiner, *Science*, 1995, **268**, 375–379.
23 R. Tacke, *Angew. Chem., Int. Ed.*, 1999, **38**, 3015–3018.
24 S. V. Patwardhan, S. J. Clarson and C. C. Perry, *Chem. Commun.*, 2005, 1113–1121.
25 J. C. McAuliffe, W. C. Smith, R. Bond, J. Zimmerman, D. E. Ward, K. Sanford and T. H. Lane, *Abstr. Pap. Am. Chem. Soc.*, 2005, **229**, U1159.
26 G.-C. Chen, I. C. Kuan, J.-R. Hong, B.-H. Tsai, S.-L. Lee and C.-Y. Yu, *Biotechnol. Lett.*, 2011, **33**, 525–529.
27 S. Emond, D. Guieysse, S. Lechevallier, J. Dexpert-Ghys, P. Monsan and M. Remaud-Simeon, *Chem. Commun.*, 2012, **48**, 1314–1316.
28 D. J. Belton, S. V. Patwardhan, V. V. Annenkov, E. N. Danilovtseva and C. C. Perry, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 5963–5968.
29 D. J. Belton, S. V. Patwardhan and C. C. Perry, *J. Mater. Chem.*, 2005, **15**, 4629–4638.
30 Novozymes, private communication.
31 H. R. Luckarift, J. C. Spain, R. R. Naik and M. O. Stone, *Nat. Biotechnol.*, 2004, **22**, 211–213.
32 L. Li, Z. Jiang, H. Wu, Y. Feng and J. Li, *Mater. Sci. Eng., C*, 2009, **29**, 2029–2035.
33 S. A. Miller, E. D. Hong and D. Wright, *Macromol. Biosci.*, 2006, **6**, 839–845.
34 J. K. Lai, T. H. Chuang, J. S. Jan and S. S. S. Wang, *Colloids Surf., B*, 2010, **80**, 51–58.
35 L. Betancor, H. R. Luckarift, J. H. Seo, O. Brand and J. C. Spain, *Biotechnol. Bioeng.*, 2008, **99**, 261–267.
36 I. C. Kuan, J.-C. Wu, S.-L. Lee, C.-W. Tsai, C.-A. Chuang and C.-Y. Yu, *Biochem. Eng. J.*, 2010, **49**, 408–413.
37 M. Ramanathan, H. R. Luckarift, A. Sarsenova, J. R. Wild, E. K. Ramanculov, E. V. Olsen and A. L. Simonian, *Colloids Surf., B*, 2009, **73**, 58–64.
38 S. V. Patwardhan and C. C. Perry, *Silicon*, 2010, **2**, 33–39.
39 J. S. Edwards, A. Kumbhar, A. Roberts, A. C. Hemmert, C. C. Edwards, P. M. Potter and M. R. Redinbo, *Biotechnol. Prog.*, 2011, **27**, 863–869.
40 M. B. Cardoso, H. R. Luckarift, V. S. Urban, H. O'Neill and G. R. Johnson, *Adv. Funct. Mater.*, 2010, **20**, 3031–3038.
41 F. Neville, M. J. F. Broderick, T. Gibson and P. A. Millner, *Langmuir*, 2011, **27**, 279–285.
42 S. Fogler, *Elements of Chemical Reaction Engineering*, Pearson, 2004.
43 E. M. Renkin, *J. Gen. Physiol.*, 1954, **38**, 225–243.
44 U. Heinbuch and J. Fischer, *Phys. Rev. A*, 1989, **40**, 1144–1146.
45 M. Peters, T. Klaus and R. West, *Plant Design and Economics for Chemical Engineers*, McGraw Hill, 2002.
46 J. Pfeffer, S. Richter, J. Nieveler, C.-E. Hansen, R. B. Rhlid, R. D. Schmid and M. Rusnak, *Appl. Microbiol. Biotechnol.*, 2006, **72**, 931–938.
47 A. P. A. Sujith, T. V. Hymavathi and P. Y. Devi, *Int. J. Biol. Life Sci.*, 2010, **6**, 67–76.
48 BRENDA Comprehesive Enzyme Information System.
49 A. Proskova, Z. ZKopicova, J. Kucera and L. Skarkova, *Res. Agric. Eng.*, 2010, **56**, 122–125.
50 D. Avnir, S. Braun, O. Lev and M. Ottolenghi, *Chem. Mater.*, 1994, **6**, 1605–1614.
51 S. V. Patwardhan, F. S. Emami, R. J. Berry, S. E. Jones, R. R. Naik, O. Deschaume, H. Heinz and C. C. Perry, *J. Am. Chem. Soc.*, 2012, **134**, 6244–6256.
52 C. Forsyth, T. W. S. Yip and S. V. Patwardhan, *Chem. Commun.*, 2013, DOI: 10.1039/c2cc38225c.

