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Comparison of mesoporous silicate supports for the immobilisation and activity of cytochrome c and lipase

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ABSTRACT

The activity and stability of *Candida antartica* lipase B (CALB) and cytochrome c immobilised onto SBA-15 and a porous spherical silicate material (PPS) were examined. The materials possess similar pore diameters but have different morphologies, pore volumes and surface areas. CALB exhibited higher catalytic activity and stability on SBA-15 when compared to PPS, while cytochrome c showed similar catalytic activity on both materials. The activity of CALB immobilised on SBA-15 was retained (95%) after 7 uses, while CALB immobilised on PPS retained only 43% activity. Such changes can be mainly ascribed to the different physical properties (pore volume, surface area and pore shape) of the supports.

1. Introduction

Immobilisation of enzymes can confer a number of advantages [1,2] including enhanced stability, ease of recovery and re-use and the capability of using the enzyme in solutions such as in nonaqueous solvents, where the enzyme is insoluble. The main disadvantages of immobilisation are that the activity of the enzyme is usually lowered and the process of immobilisation can add significant extra costs to the process. In addition, immobilisation methods tend to be non-specific and typically the process of immobilisation of a specific enzyme on a support is optimised and developed on a case-by-case approach.

Ideally, support materials for the immobilisation of an enzyme should be mechanically and chemically stable, have high surface areas, be easily made at low cost and display low non-specific protein adsorption properties [2,3]. Immobilisation should occur in a manner which does not compromise the conformation or activity of the enzyme, while diffusion of the substrate and product to and from the active site should not be hindered. Mesoporous silicates (MPS) have been widely used as supports for enzymes and in particular with the view of utilising them as supports for biocatalysis [2–5]. MPS have ordered porous structures with pore diameters in the range 2–30 nm, a size that is suitable for the immobilisation of a wide range of enzymes. The pore diameter of the support can be tailored by altering the reaction conditions (type of silica source and template material used, solution pH, temperature, and the amount of excipients). MPS have large surface areas, are mechanically stable and resistant to microbial attack. The surface of MPS can be altered by direct functionalisation, post-synthesis or by using organo-silane precursors.

Enzyme immobilisation is usually carried out in an unselective manner by either physisorption or by chemical modification. A detailed description of the parameters that influence the adsorption of an enzyme on a porous support has been described [1,2,6]. These factors include the size of the enzyme, the pore diameter, pore size distribution and surface area of the silicate, pH, ionic strength, the isoelectric point, the hydrophilic/hydrophobic, nature of the enzyme, and the surface properties of the support. Physisorption is a relatively straightforward method to use but suffers from the disadvantage of leaching of the enzyme from the support. Covalent attachment can remedy this disadvantage but the process involved may significantly reduce the activity of the enzyme, particularly if attachment occurs adjacent to or at the active site of the enzyme. In addition, it can confer increased stability on the enzyme.

Immobilisation of enzymes can occur both within the pores and on the externally addressable surface area of MPS. In the case of cytochrome c, adsorption on the external surface can constitute up to 10% of the total protein loading [7]. Immobilisation of enzymes within the pores has largely been inferred by comparing the properties of enzymes adsorbed on MPS, with pore diameters

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smaller than that of the enzyme to the properties on MPS with pore diameters larger than the enzyme. The presence of trypsin in the pores of MPS was demonstrated by the far more rapid digestion of proteins by MPS immobilised trypsin in comparison to the solution-based enzyme [8]. Small-angle neutron scattering demonstrated that cross-linked chloroperoxidase was present in the pores of the support [9]. Direct observation of an enzyme within the pores was recently demonstrated in transmission electron microscopy studies of lipase immobilised onto SBA-12 [10] and lysozyme onto SBA-15 [11]. These studies provided direct evidence that the enzyme is adsorbed along the length of the mesopores, the length of which can extend to over several hundred nanometre. The accessibility and catalytic efficiency of enzyme molecules adsorbed deeply within such pores has not been clearly examined, with it being likely that the catalytic efficiency of the enzyme will decrease due to diffusion constraints, and in particular at high enzyme loadings when blockage of the pores is more likely.

In order to investigate this effect, the adsorption and catalytic efficiency of cytochrome c and CALB on MPS materials with different porosities and surface areas have been examined. Cytochrome c is a small redox protein (12.4 kDa) [12] and has been widely used as a model system to investigate the adsorption of proteins on MPS [13]. CALB is a hydrolase enzyme (33 kDa) [14] that is widely used in biocatalysis [15–17] due to its broad substrate range, high activity and stability. CALB has been successfully immobilised by several methods [18–20] onto various solid supports [21–24]. SBA-15 possesses a hexagonal structure with pore diameters of ca. 7.5 nm which are sufficiently large to accommodate lipase and cytochrome c. PPS are monodispersed micron sized porous silica spheres, with an average pore diameter of 7.5 nm. The pore morphology of PPS is continuous and sponge-like. PPS can be utilised in applications where the facile mass transfer of analytes in to and out of the pores is required [25]. Both materials possess average pore diameters (7.5 nm) that are sufficiently large to accommodate lipase or cytochrome c. In this study, the immobilisation of CALB and cytochrome c is examined to determine the influence of differences in the support on factors such as loading, activity and stability. The immobilisation of CALB on SBA-15 results in higher activity and stability when compared to PPS.

2. Experimental

2.1. Materials

CALB was a gift from Novozyme. Pluronic P123 (EO20PO70EO20) was donated from BASF. The following chemicals were obtained from Sigma–Aldrich and used as received without further purification: cytochrome c (horse heart type VI, >97% purity, HCl, tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), KH_2PO_4, K_2HPO_4, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonis acid) (ABTS), ammonium hydroxide solution (32.66%, NH_4OH), methanol, ethanol, glutaraldehyde (25%), 4-nitrophenylbutyrate, Bradford assay and 2-propanol. De-ionised water (18.2 MΩ cm) was used for all aqueous solutions.

2.2. Methods

Nitrogen gas sorption isotherms were measured at 77 K using a Quantachrome Autosorb AS1 system. Samples were pre-treated by heating under vacuum at varying temperatures until the samples were no longer out-gassing. The surface area was measured using the Brunauer–Emmett–Teller (BET) method. The pore size data was calculated using the Barrett, Joyner and Halenda (BJH) method. Mesoporous volumes were estimated from the volume of nitrogen adsorbed after the micropores have been filled until after condensation into the mesopores was complete. UV–visible spectroscopy was performed on a Shimadzu UV1800 spectrophotometer.

Transmission electron microscopy (TEM) was conducted at an accelerating voltage of 200 kV using a JEOL JEM-2011 microscope. The sample was placed directly on a formvar-backed carbon-coated copper grid. Scanning electron microscopy (SEM) was carried out on a FEI Inspect F instrument operating at 10 kV. Silica samples were placed on conductive carbon tape prior to analysis. Focussed ion beam (FIB) was performed using a FEI Helios Nanolab 600 dual-beam FIB. The electron beam was operated at 5 kV with the ion beam operating at 30 kV for Pt deposition and thinning. The cross sections were prepared using a focussed ion beam method [26].

2.3. Synthesis of mesoporous silica

SBA-15 was prepared using a published procedure [27]. PPS was prepared using an adaptation of a previously published methods [25,28], Tetraethyl orthosilicate (TEOS) was used as the silica source and cetyltrimethylammonium bromide (CTAB) acted as the structure directing agent for pore formation and methanol (MeOH) was used as a co-solvent. CTAB (1.2 g) was dissolved in deionised water (88 mL) and methanol (MeOH, 500 mL); the solution was stirred for 2 h. Ammonium hydroxide (32 mL, 32.66% w/w in H_2O) and TEOS (8 mL) were then added to the solution, the temperature was maintained at room temperature and the mixture was stirred for 24 h. The silica precipitate was separated by centrifugation and dried at room temperature. The sample was then calcined at 550 °C for 8 h.

2.4. Protein immobilisation

The adsorption of cytochrome c was performed in 25 mM potassium phosphate buffer at pH 7.0 (2 mg/mL silicate). The concentration of protein was determined using an extinction coefficient of 100,000 M^{-1} cm^{-1} at 407 nm [12]. The concentration of lipase was determined using the Bradford method [29]. The immobilisation of lipase was conducted in phosphate buffer (10 mM, pH 7.0). A stock solution of lipase was prepared by 1:4 dilution of the as received enzyme. Varying concentrations were prepared from the stock solution in 10 mM phosphate buffer at pH 7.0. Adsorption of the enzyme on MPS was then allowed to proceed for a period of 18 h at 25 °C. Lipase loading was calculated by taking 1 mL from the reaction vessel, centrifuging (3000 rpm), measuring the lipase concentration of the supernatant, and subtracting this value from the initial concentration.

2.5. Catalytic activity

The catalytic activity of cytochrome c was determined according to published reports using ABTS as substrate [7,30]. Lipase activity was determined by measuring the rate of hydrolysis of 4-nitrophenyl butyrate (4-NPB); typically, a solution containing 1.9 mL of phosphate buffer (10 mM, pH 7.0), 0.05 mL lipase (either in solution or as a suspension) and 0.05 mL of 4-NPB (1 mM) in 2-propanol was prepared and the increase in absorbance at 410 nm was recorded (ε_{4NP} = 14,775 M^{-1} cm^{-1}) [31]. Recycling experiments were performed with a higher concentration of immobilised lipase (8 mg/mL of enzyme). After incubation with 4-NPB (1 min), the sample was centrifuged (1 min at 3000 rpm) and the absorbance of an aliquot (0.5 mL diluted to 2 mL with buffer solution) measured. The immobilised lipase sample was washed twice with buffer solution (1 mL) and the assay procedure repeated. Stability tests were performed by measured the catalytic activity of immobilised lipase on a weekly basis after storage in phosphate

Table 1
Physical properties of MPS materials.

Material	Surface area (m^2/g)	Mean pore diameter (nm)	Peak width at half height (nm)	Pore volume (cm^3/g)
SBA-15	682	7.5	1.0	1.3
PPS	267	7.5	3.5	0.6

buffer (10 mM, pH 7) at 5 °C. All adsorption and activity measurements were performed in triplicate and the average value reported.

3. Results and discussion

3.1. Characterisation of SBA-15 and PPS

The physicochemical properties of the materials are summarised in Table 1. The adsorption/desorption isotherm of SBA-15 displayed a type IV isotherm with a steep H1 or type A hysteresis loop, indicative of cylindrical shaped pores [32], and the steep slope of the hysteresis loop is evident of a narrow pore size distribution. The average pore diameter (7.5 nm) and surface area (682 m^2/g) (Fig. 1) are in good agreement with literature values [27]. The adsorption/desorption isotherm for PPS also showed a type IV isotherm, with capillary condensation taking place over a wide pressure range 0.6–1.0 indicative of disordered materials and H4 or Type D hysteresis indicative of slit-like pores, with broader pore size distribution [32,33]. In comparison to SBA-15, the total volume of nitrogen adsorbed has reduced, resulting a lower surface area (267 m^2/g) and a similar average pore diameter (~7.5 nm) were observed; while the pore volume was ca. 50% lower (1.3 vs 0.6 cm^3/g). The PPS particles are monodisperse with smooth surfaces (Fig. 2A and B). A cross sectional image of the particle shows the sponge like interior of the particles, while the SBA-15 particles are cylindrical in shape (Fig. 2C) with long-range order (Fig. 2D). Both materials possess the same average pore diameter (7.5 nm), but the differences in pore size distributions, pore shapes, pore volumes and surface areas make these materials useful for comparison of their ability to act as enzyme supports.

3.2. Adsorption isotherms

3.2.1. Cytochrome c

The isoelectric point (PI) of supports and enzymes are important factors to consider when undertaking physisorption studies. The PI of silicate supports are in the range 2.8–3.7 [7], while that of cytochrome c is 10.6 [7]. The adsorption isotherms for cytochrome c were conducted at pH 7. At this pH both silicates surfaces are negatively charged and can interact with positively charged amino acid residues on the surface of cytochrome c. The loading of cytochrome c on SBA-15 was higher when compared to PPS (15.6 versus 6.7 μmol g^{-1}) (Fig. 3). The loading obtained on SBA-15 compares well with values described in previous reports

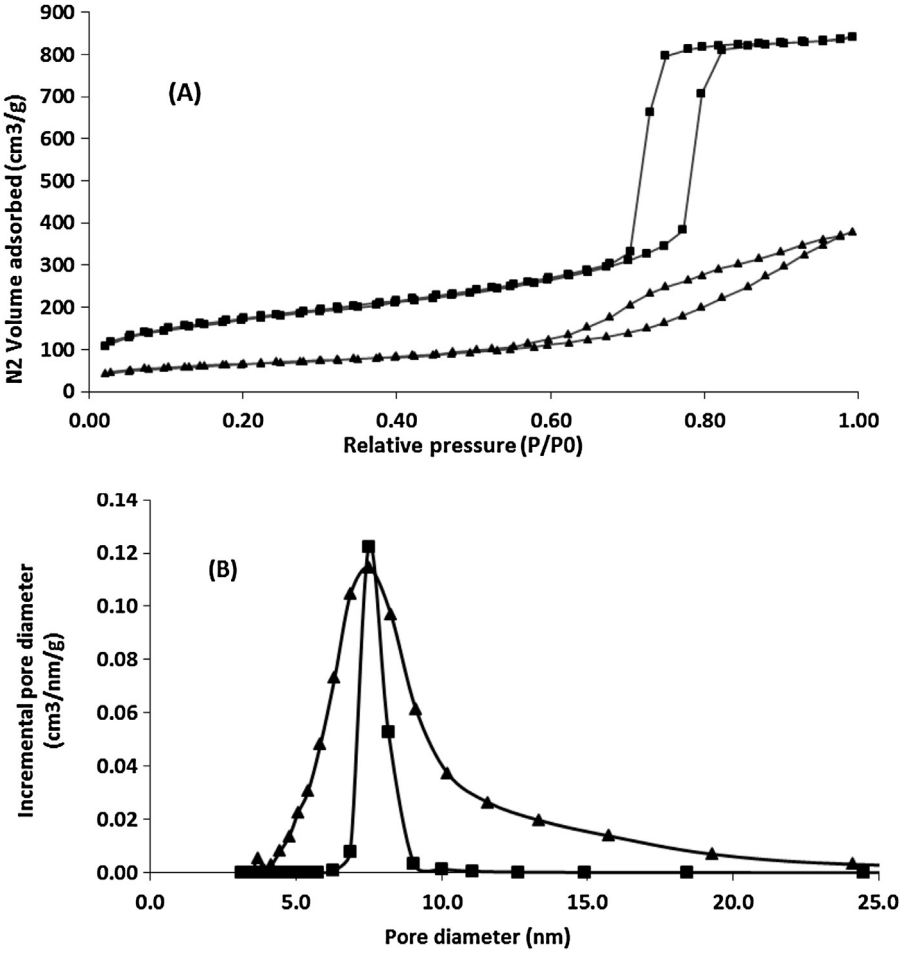

Fig. 1. (A) Nitrogen adsorption isotherms and (B) pore size distributions for SBA-15 (■) and PPS (▲). The scale used for SBA-15 is ×1/10 for purposes of comparison.

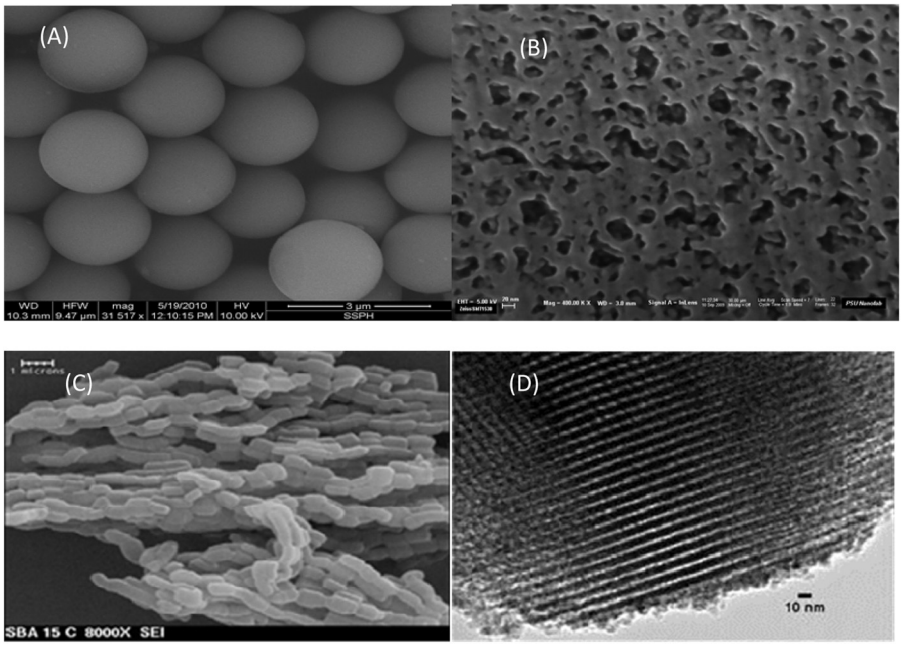

Fig. 2. SEM images of (A) PPS (scale bar 3 μm); (B) cross section of a PPS particle (scale bar 60 nm); (C) SBA-15 (scale bar 1 μm); (D) TEM image of SBA-15.

(19 μmol g^{-1}) [7,30]. As the pore diameters of both materials are larger than that of cytochrome c (ca. 4 nm) [30], these results clearly indicate that the amount of adsorbed protein is a reflection of the different surface areas, pore shapes and pore volumes of the two materials, parameters that are known to influence the loading of enzymes [6,34]. It is also likely that some blocking of the pores occurred, particularly with PPS which has a broader pore size distribution and a larger proportion of channels with smaller pore diameters.

Although its physiological role is that of a redox shuttle, cytochrome c also displays peroxidative behaviour [35]. Using ABTS as a substrate in the presence of H_2O_2 at pH 7, the turnover frequency (TOF, μmol of oxidised ABTS produced per second per μmol of cytochrome c present) of immobilised cytochrome c was significantly higher than that of the free protein (Fig. 4). The TOF of the protein on SBA-15 was 10–20-fold higher than that of the free protein at low concentrations in agreement with previous reports [13,30,36]. As the loading of protein on the supports was increased, the activity of the protein decreased to that of the free protein at high concentrations. This decrease in activity is likely due to the fact that some of the protein becomes inaccessible to the substrate. The increased activity of the protein at low loadings described here has been reported previously. Raman spectra of the adsorbed protein were indicative of a high spin state for cytochrome c at the surface of MPS which could account for the enhanced activity at low protein loadings [7]. As the loading of protein increased, the activity decreased as the surface of the MPS was filled with protein, reducing the relative amount of protein at the silicate surface and in addition, introducing diffusional constraints as the protein loading increased.

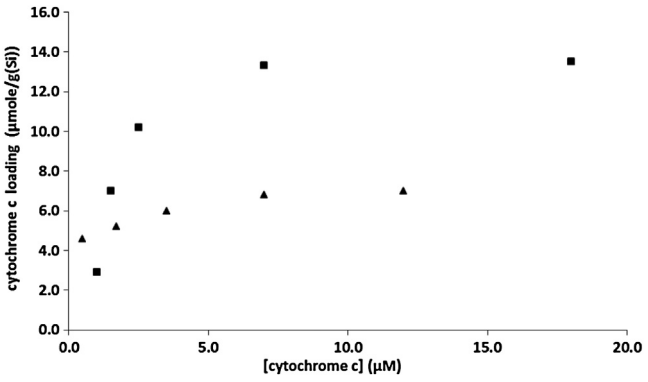

Fig. 3. Adsorption isotherm for cytochrome c on SBA-15 (■) and PPS (▲).

3.2.2. Lipase

The adsorption of lipase onto SBA-15 and PPS silica was performed at pH 7.0, where both supports (SBA-15, PPS) and lipase (PI of 6) [14] are negatively charged. At the pH used for immobilisation, the adsorption process will be aided by hydrophobic interactions between the enzyme and the support. Calcination of the support results in removal of surface hydroxyl groups (as demonstrated by the weak intensity of the band at 3400 cm^{-1} in the FT-IR spectra (Fig. S1)). Since the pH (7.0) is close that of the PI of CALB (6.0) (the net charge of the surface of CALB is small, minimising electrostatic repulsions) high loadings of enzyme can be expected [6,37]. The loading of lipase increased with increasing concentrations of lipase for both materials (Fig. 5); the maximum observed loading of lipase under the conditions used was 2.0 and 0.9 μmol g^{-1} for SBA-15 and PPS, respectively. The results obtained with SBA-15 are consistent with previous reports of 2.8 [38] and 1.5 μmol g^{-1} [39].

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2014.06.007.

As was observed with cytochrome c, the amount of CALB adsorbed on SBA-15 was much higher than on PPS. This difference in loading can be attributed to the more than the 2-fold differences in pore volume and surface area (Table 1). In addition, as described earlier (Section 3.1), the broader pore size distribution of PPS may reduce the amount of immobilised lipase due to the larger proportion of pores with smaller diameters. Differences in morphology may also contribute to the lower loadings of enzyme, (Fig. 2) with more facile access of the enzyme molecules into the cylindrical pores of SBA-15 in comparison to the slit shaped pores of PPS. Such differences have been previously reported to influence the degree of enzyme loading [40,41]. It should be noted that the amounts of cytochrome c and of lipase adsorbed on PPS was lower

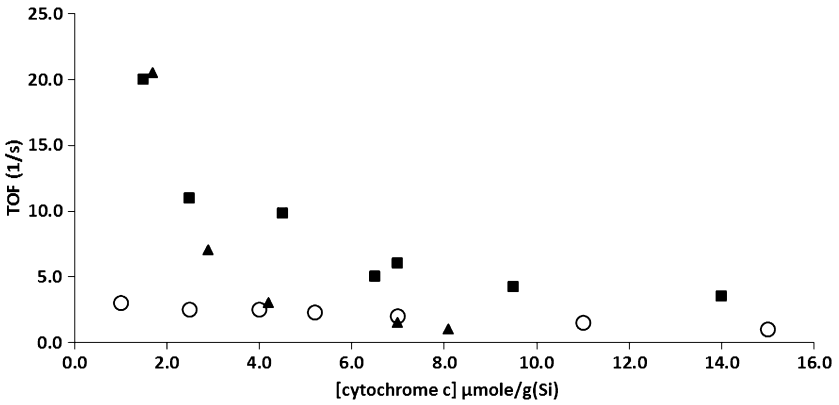

Fig. 4. Activity profiles for cytochrome c immobilised on SBA-15 (■) and PPS (▲) and for aqueous cytochrome c (o).

than on SBA-15. Given that the two proteins have markedly different surface characteristics, this further suggests that differences in the physical properties of the two supports affect the extent of adsorption.

The catalytic activity of lipase was assayed by using the rate of hydrolysis of 4-nitrophenyl-butyrate to 4-nitrophenol. The turnover frequency (TOF, µmol of 4-nitrophenol produced per second per µmol of lipase present) of lipase immobilised on SBA-15 and PPS was 7.5 and 3.6 s^{-1}, respectively, which compares to a value of 7.1 s^{-1} for the free enzyme (Fig. 6). CALB immobilised on SBA-15 had a more than 2- fold higher TOF compared to lipase immobilised onto PPS, a value that was also slightly higher than of the free enzyme, which in agreement with previous reports [42]. The data obtained with SBA-15 indicate that the active site of the immobilised lipase was available to the substrate, while lipase immobilised on PPS may have been in a conformation where a degree of hindrance of substrate to the active site of the enzyme is occurring. As the data obtained was similar to that of the free enzyme, it is likely that internal substrate diffusion is not a limiting step.

3.3. Recycling studies

As the ability to recycle immobilised enzymes is one of the main advantages of immobilisation, the reusability of the immobilised enzyme was examined for both supports. After 7 reaction cycles, the activity of lipase immobilised on SBA-15 was 95% of that observed initially (Fig. 7), a higher level of retention than that reported previously (80% after 6 cycles) [39]. This excellent reusability together with the actual activity obtained indicates that the enzyme is immobilised in an active form with little leaching occurring. In contrast, only 43% of the activity remained for CALB immobilised on PPS silica. This decrease in activity likely arises from leaching of enzyme from the support. Also, it is likely that a significant fraction of the PPS pores are not sufficiently large for CALB to enter the pores, with more enzymes on the surface where it could be removed on washing.

3.4. Stability studies

The stability of lipase to denaturation by high temperature was studied previously for immobilised lipase on mesoporous surfaces [19,43]; however, less attention has been given to storage stability. Here, the storage stability of the immobilised enzyme was examined after incubation at 5 °C in phosphate buffer pH 7. On both materials, 84% of the initial activity of lipase was retained (Fig. 8) after one week of storage. The loss in activity of the immobilised enzyme mainly arises from leaching of the enzyme. For CALB immobilised on PPS, leaching accounted for 17 (1 week) and 27% (2 weeks) of the loss in activity, compared to 13 and 18% on SBA-15, respectively. After three weeks, 50% of the initial activity was observed for lipase immobilised on SBA-15, and 43% of initial activity was retained for lipase-PPS. Similar reductions in activity were observed with both materials after three weeks, and likely arise from the fact that the enzyme is not covalently attached to either support.

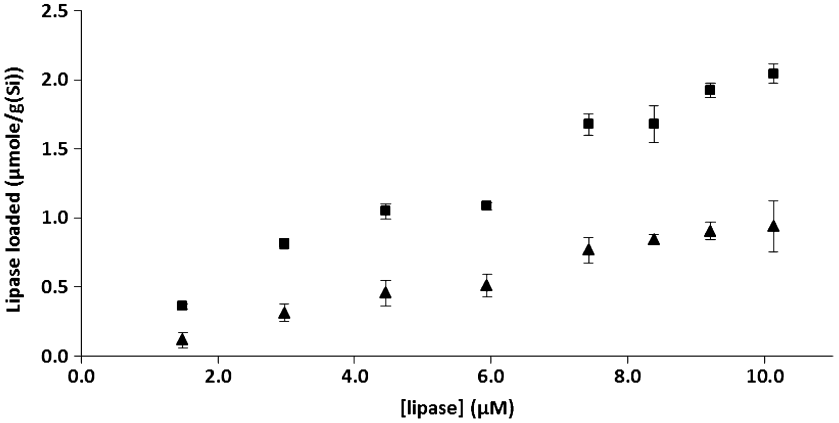

Fig. 5. Adsorption isotherm for CALB on SBA-15 (■) and PPS (▲). The error bars represent the standard deviation.

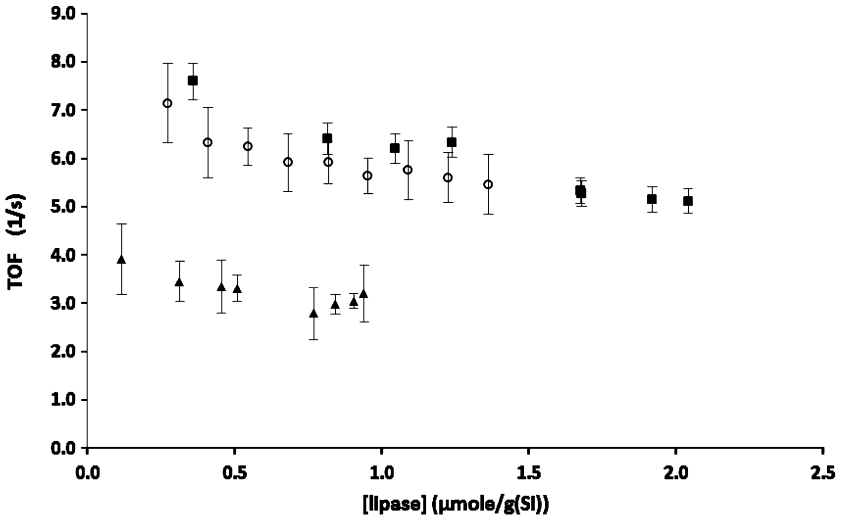

Fig. 6. Activity profile for CALB in solution (o) and immobilised on SBA-15 (■) and PPS (▲). The error bars represent the standard deviation.

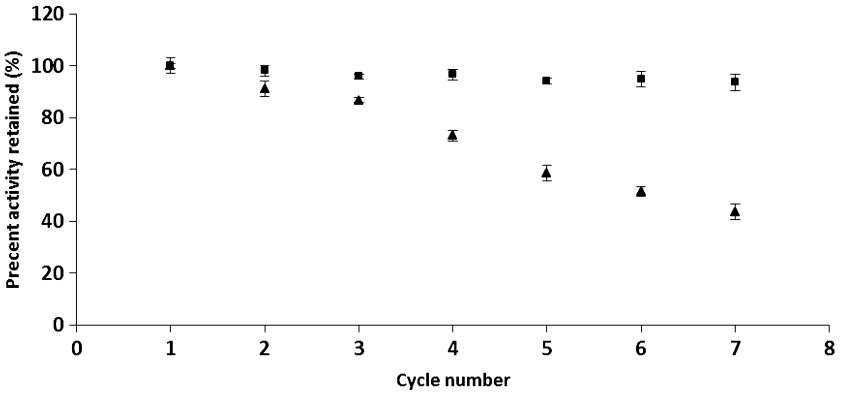

Fig. 7. Activity profile for CALB immobilised on SBA-15 (■) and PPS (▲) as a function of number of cycles.

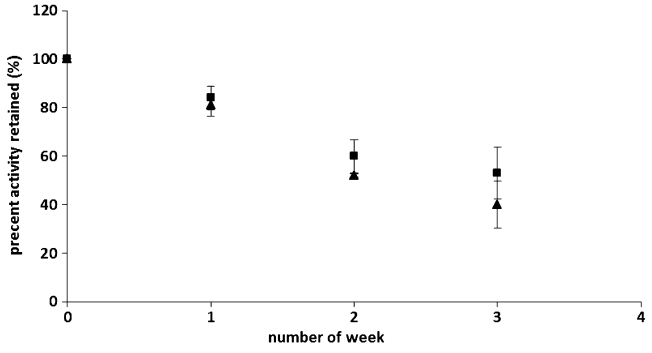

Fig. 8. Plot of activity of lipase immobilised on SBA-15 (■) and PPS (▲) with time.

4. Conclusions

The adsorption and activity of cytochrome c and lipase on two silicate supports (SBA-15 and PPS) with the same average pore diameter but with different pore volumes and surface areas has been examined. On SBA-15 loadings of cytochrome c and CALB of 15.6 and 2.04 μmol g^{-1} were obtained in comparison to loadings of 0.94 and 6.7 μmol g^{-1} on PPS, respectively. The differences in loading can be ascribed to differences in the properties of the supports (pore volume, surface area and morphology). The catalytic activity of cytochrome c was similar on both supports, while the activity of CALB was higher on SBA-15 in comparison to PPS (7.8 vs 4 s^{-1}). These differences in activity for CALB likely arise from the pore morphology and the physical properties of the supports, a hypothesis that was supported by the higher recyclability obtained with SBA-15 as a support. The data indicates that the physical properties of silicate supports can significantly alter the activity and stability of enzymes.

Acknowledgements

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References

[1] L.Q. Cao, Curr. Opin. Chem. Biol. 9 (2005) 217–226.
[2] U. Hanefeld, L. Gardossi, E. Magner, Chem. Soc. Rev. 38 (2009) 453–468.
[3] M. Hartmann, D. Jung, J. Mater. Chem. 20 (2010) 844–857.
[4] S. Hudson, J. Cooney, E. Magner, Angew. Chem. Int. Edit. 47 (2008) 8582–8594.
[5] L. Bayne, R.V. Ulijn, P.J. Halling, Chem. Soc. Rev. 42 (2013) 9000–9010.
[6] E. Magner, Chem. Soc. Rev. 42 (2013) 6213–6222.
[7] J. Deere, E. Magner, J.G. Wall, B.K. Hodnett, J. Phys. Chem. B 106 (2002) 7340–7347.
[8] D. Goradia, J. Cooney, B. Hodnett, E. Magner, J. Mol. Catal. B: Enzym. 32 (2005) 231–239.
[9] D. Jung, M. Paradiso, D. Wallacher, A. Brandt, M. Hartmann, ChemSusChem 2 (2009) 161–164.
[10] A. Mayoral, R.M. Blanco, I. Diaz, J. Mol. Catal. B: Enzym. 90 (2013) 23–25.
[11] M. Piras, A. Salis, M. Piludu, D. Steri, M. Monduzzi, Chem. Commun. 47 (2011) 7338–7340.

[12] R.A. Scott, A.G. Mauk, Cytochrome-c: a multidisciplinary approach, University Science Books, Sausalito, California, 1996.
[13] J. Deere, E. Magner, J. Wall, B. Hodnett, Chem. Commun. (2001) 465–466.
[14] J. Uppenberg, M.T. Hansen, S. Patkar, T.A. Jones, Structure 2 (1994) 293–308.
[15] P. Adlercreutz, Chem. Soc. Rev. 42 (2013) 6406–6436.
[16] E.M. Anderson, K.M. Larsson, O. Kirk, Biocatal. Biotransfor. 16 (1998) 181–204.
[17] M.B. Ansorge-Schumacher, O. Thum, Chem. Soc. Rev. 42 (2013) 6475–6490.
[18] M.I. Kim, J. Kim, J. Lee, H. Jia, H. Bin Na, J.K. Youn, J.H. Kwak, A. Dohnalkova, J.W. Grate, P. Wang, T. Hyeon, H.G. Park, H.N. Chang, Biotechnol. Bioeng. 96 (2007) 210–218.
[19] R.M. Blanco, P. Terreros, M. Fernandez-Perez, C. Otero, G. Diaz-Gonzalez, J. Mol. Catal. B: Enzym. 30 (2004) 83–93.
[20] A. Basso, P. Braiuca, S. Cantone, C. Ebert, P. Linda, P. Spizzo, P. Caimi, U. Hanefeld, G. Degrassi, L. Gardossi, Adv. Synth. Catal. 349 (2007) 877–886.
[21] J. Forde, A. Vakurov, T.D. Gibson, P. Millner, M. Whelehan, I.W. Marison, C. O'Fagain, J. Mol. Catal. B: Enzym. 66 (2010) 203–209.
[22] K. Gawlitza, C. Wu, R. Georgieva, D. Wang, M.B. Ansorge-Schumacher, R. von Klitzing, Phys. Chem. Chem. Phys. 14 (2012) 9594–9600.
[23] N.G. Graebin, A.B. Martins, A.S. Lorenzoni, C. Garcia Galan, R. Fernandez Lafuente, M.A. Ayub, R.C. Rodrigues, Biotechnol. Progr. 28 (2012) 406–412.
[24] E. Serra, A. Mayoral, Y. Sakamoto, R.M. Blanco, I. Diaz, Microporous Mesoporous Mater. 114 (2008) 201–213.
[25] P. Delaney, R.M. Healy, J.P. Hanrahan, L.T. Gibson, J.C. Wenger, M.A. Morris, J.D. Holmes, J. Environ. Monitor. 12 (2010) 2244–2251.
[26] L. Giannuzzi, F. Stevie, Micron 30 (1999) 197–204.
[27] D. Zhao, Q. Huo, J. Feng, B.F. Chmelka, G.D. Stucky, J. Am. Chem. Soc. 120 (1998) 6024–6036.
[28] N. Shimura, M. Ogawa, Bull. Chem. Soc. Jpn. 78 (2005) 1154–1159.
[29] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[30] S. Hudson, E. Magner, J. Cooney, B.K. Hodnett, J. Phys. Chem. B 109 (2005) 19496–19506.
[31] D.M. Quinn, K. Shirai, R.L. Jackson, J.A. Harmony, Biochemistry 21 (1982) 6872–6879.
[32] R. Pierotti, J. Rouquerol, Pure Appl. Chem. 57 (1985) 603–619.
[33] D. Keane, J. Hanrahan, M. Copley, J. Holmes, M. Morris, J. Porous Mater. 17 (2010) 145–152.
[34] M. Hartmann, X. Kostrov, Chem. Soc. Rev. 42 (2013) 6277–6289.
[35] J. Deere, E. Magner, J.G. Wall, B.K. Hodnett, Biotechnol. Progr. 19 (2003) 1238–1243.
[36] J. Deere, E. Magner, J.G. Wall, B.K. Hodnett, Catal. Lett. 85 (2003) 19–23.
[37] A. Vinu, V. Murugesan, M. Hartmann, J. Phys. Chem. B 108 (2004) 7323–7330.
[38] Z. Kai, W. Jianqiang, W. Yan-Hua, L. Hui, H. Ping-Fang, W. Ping, J. Nanosci. Nanotechnol. 11 (2011) 7593–7602.
[39] S. Gao, Y. Wang, X. Diao, G. Luo, Y. Dai, Bioresour. Technol. 101 (2010) 3830–3837.
[40] J. Fan, J. Lei, L. Wang, C. Yu, B. Tu, D. Zhao, Chem. Commun. (2003) 2140–2141.
[41] H. Gustafsson, E.M. Johansson, A. Barrabino, M. Odén, K. Holmberg, Colloids Surf. B: Biointerfaces 100 (2012) 22–30.
[42] E. Dumitriu, F. Secundo, J. Patarin, I. Fechete, J. Mol. Catal. B: Enzym. 22 (2003) 119–133.
[43] Y. Han, S.S. Lee, J.Y. Ying, Chem. Mater. 18 (2006) 643–649.