

# Immobilization of horseradish peroxidase in cross-linked phyllosilicates: conditions and characterizations

Siyuan Shen and Shu-I Tu<sup>1</sup>

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, PA 19038, U.S.A.

**An innovative immobilization procedure was developed for intercalation of enzymes into dispersed phyllosilicates which were cross-linked with silicates resulting from the hydrolysis of tetramethyl orthosilicate. Donor:hydrogen-peroxide oxidoreductase intercalative immobilized in the cross-linked phyllosilicate exhibited a similar or higher activity than the free enzyme. The kinetic properties of peroxidase were unaffected by intercalative immobilization. Different factors, including drying methods, particle size, surface cations of the phyllosilicate and ratio of phyllosilicate to tetramethyl orthosilicate, were investigated to optimize immobilization conditions. The immobilized peroxidase exhibited similar kinetic properties to the free enzyme and good storage stability.**

## Introduction

The immobilization of enzyme at the interface in a heterogeneous system is currently an active research area in biotechnology. Immobilized enzymes exhibit increased stability and enable a continuous conversion process with good product recovery and minimal loss of enzyme activity. The conventional methods of enzyme immobilization include adsorption (electrostatic and hydrophobic adhesion) and covalent binding (internal cross-linkage) of enzymes on a solid support [1]. Unfortunately, covalent or ionic bonds formed by these methods usually increase the stability but decrease the activity of the enzymes [2]. Immobilization of enzymes by entrapment has been achieved by encapsulating enzymes through sol-gel processes. Sol-gel materials for enzyme entrapment are usually prepared by mixing a silane, e.g. tetramethyl orthosilicate (TMOS), buffer solution and methanol in different ratios [3]. The entrapped enzyme retains much of its activity and gains better stability in sol-gel matrices [4]. But the extension of this technique has been limited by two shortcomings of sol-gel materials; their brittleness and narrow pore network [5]. Efforts were made to improve the activity of immobilized enzymes by mixing alkyl-substituted silanes in specific ratios, and introducing polymers or phyllosilicates into sol-gel matrices [5-8].

As layered silicates with large surface area, phyllosilicates possess both hydrophilic and hydrophobic sites on

their layer surfaces and edges. Their layered structures can be broken down into nanoscale building blocks, which makes them good matrices for intercalation. Metal hydroxyl polymeric cations, alkylammonium ions, polymers and combinations of these have been intercalated into phyllosilicates to form a broad spectrum of materials ranging from pillared clays and organoclays to polymer-clay nanocomposites [9-12]. Linoleate:oxygen 13-oxidoreductase (lipoxygenase) immobilized into cross-linked phyllosilicates exhibited high enzymic activity and retained the characteristics of free lipoxygenase [8,13]. The activities of immobilized enzymes are sensitive to immobilization conditions and factors, which deserve further study. Donor:hydrogen-peroxide oxidoreductase (EC 1.1.1.7, peroxidase) is a hydrogen peroxide-specific oxidoreductase with many applications in medical, environmental and industrial processes [14,15]. In this study, with horseradish peroxidase as a model protein, several factors affecting the activity of intercalatively immobilized enzymes, such as drying methods and ratio of volume of phyllosilicate suspension to TMOS, were tested. The particle sizes and surface cations were evaluated as they are major factors for phyllosilicate dispersion, which determines the space for enzyme intercalation. The properties of the phyllosilicate-immobilized enzymes, including their kinetic properties, storage stability and reusability, were also characterized.

## Materials and methods

A phyllosilicate of 2:1 layer type (montmorillonite from WY, U.S.A.; SWy-1) was obtained from the Source Clay Minerals Repository (Clay Mineral Society, Columbia, MO, U.S.A.). The cation-exchange capacity (CEC) of this phyllosilicate was  $0.764 \text{ mol} \cdot \text{kg}^{-1}$  and the surface area was  $756 \times 10^3 \text{ m}^2 \cdot \text{kg}^{-1}$  [16,17]. The phyllosilicate was sodium-saturated by three washes with 1 M NaCl solution, followed with three washes with deionized water to remove excess salt. The saturation of sodium was checked by determination of sodium content

Abbreviations used: TMOS, tetramethyl orthosilicate; CEC, cation-exchange capacity; TMA, trimethylammonium chloride; HDTMA, cetyltrimethylammonium chloride.

<sup>1</sup> To whom correspondence should be addressed.

on phyllosilicate surfaces and in the washing solutions [18]. The sodium-saturated SWy-1 was fractionated for particle size  $\leq 2$  or  $\leq 0.5 \mu\text{m}$  and dispersed in water. The concentration of SWy-1 in the suspension was 3.3% (w/v).

A portion of phyllosilicate suspension was added to a glass tube. In some experiments,  $\text{Na}^+$  ions were exchanged with alkylammonium ions by treatment of the Na-phyllosilicate with trimethylammonium chloride (TMA, Aldrich) or cetyltrimethylammonium chloride (HDTMA, Aldrich). The intercalation of horseradish peroxidase (EC 1.11.1.7, type I from Sigma) into the galleries (i.e. interlayers) of phyllosilicates was accomplished by mixing the enzyme with the dispersed phyllosilicate in buffer solution (0.1 M sodium citrate/phosphate, pH 7.0). The cross-linking of enzyme-phyllosilicate mixture was initiated at 23 °C by adding TMOS (Aldrich) to the mixture, and vortexing the tube for 1 min to obtain a uniform mixture for silicate polymerization. A small amount of sodium fluoride or other salts was added as a catalyst for TMOS hydrolysis. The volume ratio of buffer solution to phyllosilicate suspension was 1:1 (v/v). The weight ratio of the horseradish peroxidase to phyllosilicate was 0.006 (w/w). The volume ratios of phyllosilicate suspension to TMOS are listed in the Figures and Tables. For comparison, the simple sol-gel immobilization preparation [3] was conducted by mixing TMOS with buffer solution (volume ratio, 1:1) containing the same amount of the peroxidase as other preparations. The cross-linked enzyme-phyllosilicate complex was left to stand at 23 °C overnight for completion of the polymerization reaction. To remove unbound peroxidase, the enzyme-phyllosilicate composite was shaken with 10 ml of sodium citrate/phosphate solution (0.1 M, pH 7.0) for 24 h, then centrifuged and washed with deionized water. Three drying methods for the composite were used: (i) freeze-drying, the composite was first frozen and then kept in a freeze-drier (-35 °C) for 24 h; (ii) vacuum drying, the composite was kept in a desiccator (23 °C) at 101 kPa (1 atm) suction for 24 h; and (iii) air drying, the composite was left in a fume hood (23 °C) for 48 h. After drying, the immobilized peroxidase was ready for use. The residual water content of the dried composite was determined by measuring a small portion of the composite before and after oven drying (105 °C) for 48 h. The enzyme and reagents were used as received without further purification.

Activity of free and immobilized peroxidase was determined by oxidation of guaiacol [19]. The reaction mixture (2 ml) contained 0.5 mM guaiacol (Aldrich), 0.5 mM  $\text{H}_2\text{O}_2$ , 0.1 M sodium citrate/phosphate buffer (pH 7.0) and a suitable amount of free or immobilized peroxidase. The reaction was followed by absorbance increase at 436 nm ( $\epsilon_{436} = 25.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ ): 1 unit was defined as the amount of peroxidase that oxidized 1  $\mu\text{mol}$  of guaiacol in 1 min at 24 °C and pH 7.0. Relative activity was compared on the

basis of the same protein amount for both free and immobilized peroxidase. Protein concentration of peroxidase in solution was measured by Lowry assay and the protein content of immobilized peroxidase was calculated as the difference between the total added enzyme protein and the protein content in solutions after immobilization. All measurements were in replicates of three or five.

## Results and discussion

Intercalative immobilization of horseradish peroxidase was very effective as the activity of peroxidase immobilized in the cross-linked phyllosilicate was similar to that of the free enzyme. The vacuum-dried samples exhibited higher activity than freeze-dried samples for the intercalatively immobilized peroxidase (Figure 1). Vacuum drying only removed excess water and methanol produced from the hydrolysis of TMOS, and still kept the samples in a hydrated state, which preserved the original structure of the enzyme-phyllosilicate composite. Freeze drying removed most of the water and caused shrinkage of the sample framework. The shrinkage of the silicate framework may have denatured the immobilized peroxidase. The air-dried samples showed similar properties to the vacuum-dried samples, but air drying required a longer time than vacuum drying. Accordingly, all samples were vacuum dried in the subsequent experiments.

Figure 2 shows the activity of peroxidase immobilized in differently treated phyllosilicates. For Na-phyllosilicate, the activity of peroxidase immobilized in the fine-particle fraction ( $\leq 0.5 \mu\text{m}$ ) was higher than that in the larger-particle fraction ( $\leq 2 \mu\text{m}$ ). The particle size of phyllosilicates affects their dispersion in aqueous media [20]. The fraction of particle size  $\leq 0.5 \mu\text{m}$  was more dispersed than the  $\leq 2 \mu\text{m}$

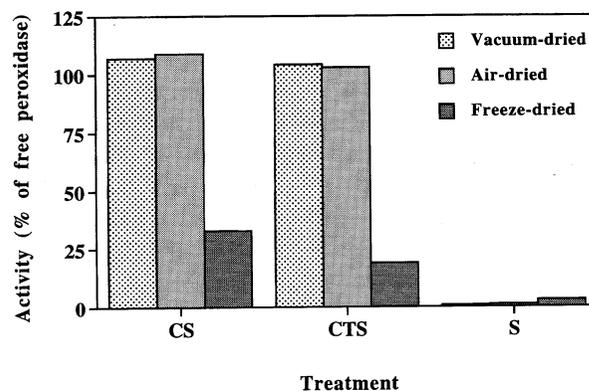


Figure 1 Activity of immobilized peroxidase after vacuum drying, air drying, or freeze-drying

The activity of free peroxidase was measured as  $98.2 \mu\text{mol}$  of guaiacol  $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ . Peroxidase was intercalatively immobilized in cross-linked Na-phyllosilicate (CT), TMA-phyllosilicate (CTS), or simple sol-gel material (S). The ratio of phyllosilicate suspension to TMOS was 5:1 (v/v).

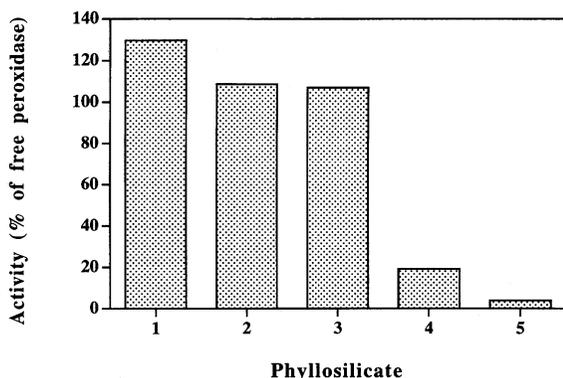


Figure 2 Activity of peroxidase immobilized in cross-linked phyllosilicates

1, Na-phyllsilicate,  $\leq 0.5 \mu\text{m}$  fraction; 2, Na-phyllsilicate,  $\leq 2 \mu\text{m}$  fraction; 3, TMA-phyllsilicate,  $\leq 2 \mu\text{m}$  fraction; 4, HDTMA-phyllsilicate,  $\leq 2 \mu\text{m}$  fraction; 5, peroxidase immobilized in simple sol-gel matrix. The ratio of phyllosilicate suspension to TMOS was 5:1 (v/v). The activity of free peroxidase was measured as  $98.2 \mu\text{mol of guaiacol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ .

fraction in the solutions under the same conditions, which benefited enzyme intercalation.

For the same particle-size ( $\leq 2 \mu\text{m}$ ) fraction, surface cations showed significant effect on the activity of intercalatively immobilized peroxidase (Figure 2). The activity of peroxidase immobilized in TMA-phyllsilicate was similar to that in Na-phyllsilicate, whereas the activity of peroxidase immobilized in HDTMA-phyllsilicate was much lower. One of the explanations may be the effect of surface cations on the dispersion of phyllosilicates in water. With TMA replacing  $\text{Na}^+$  on the surface, phyllosilicate dispersion might be decreased because TMA is less hydrated than  $\text{Na}^+$ . The hydrophobicity of phyllosilicate increased because of TMA on the surface. As a result, the activity of immobilized peroxidase was little affected by TMA replacement of  $\text{Na}^+$  on phyllosilicate surfaces. HDTMA contains far more hydrophobic groups than TMA. The replacement of  $\text{Na}^+$  by HDTMA on the surface caused a great reduction in phyllosilicate dispersion because of strong hydrophobic attraction. X-ray diffraction indicated that average interlayer distance decreased from 4.9 nm for TMA-phyllsilicate to 3.2 nm for HDTMA-phyllsilicate. The decrease in interlayer spacing of the phyllosilicate reduced the galleries for enzyme intercalation and, thus, the activity of immobilized peroxidase.

Various amounts of TMA or HDTMA were used to occupy exchange sites of the phyllosilicate. The amounts of TMA and HDTMA used were smaller than, equal to or larger than the CEC of SWy-1. The activity of immobilized peroxidase increased with the increasing amount of TMA on the phyllosilicate surface (Figure 3). The activity of immobilized peroxidase was the lowest when the amount of HDTMA added was equal to the CEC of SWy-1. HDTMA contains a long carbon chain and behaves similarly to

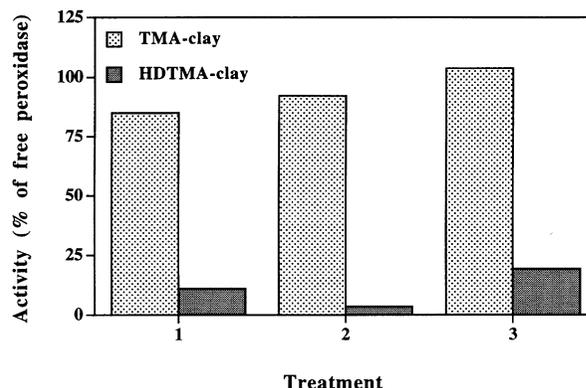


Figure 3 Activity of peroxidase immobilized in cross-linked phyllosilicate SWy-1 with different amounts of TMA or HDTMA

1, Less than the CEC of SWy-1; 2, equal to the CEC of SWy-1; 3, greater than the CEC of SWy-1. The ratio of phyllosilicate suspension to TMOS was 5:1 (v/v). The activity of free peroxidase was measured as  $98.2 \mu\text{mol of guaiacol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ .

polymers on the phyllosilicate surface. Added in moderate amounts, HDTMA caused phyllosilicate flocculation, whereas large amounts of HDTMA caused steric stabilization of phyllosilicate suspension and small amounts of HDTMA caused sensitization [21]. The amount of TMA or HDTMA added affected phyllosilicate dispersion, which is one of the important factors in intercalative immobilization of enzymes.

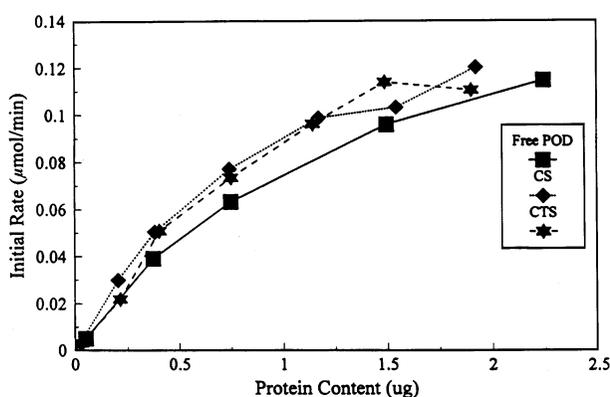
As shown in Table 1, another important factor influencing the activity of intercalatively immobilized peroxidase was the volumetric ratio ( $R_v$ ) of phyllosilicate suspension and TMOS used in the cross-linking of phyllosilicates. With increasing  $R_v$ , the activity of immobilized peroxidase increases. This trend was the same for all phyllosilicates with different surface cations. An increase in  $R_v$  means a decrease in amount of TMOS used, for a given amount of phyllosilicate, from 62.5 to 6.25, and an increase in the stoichiometric ratio of water to silane from 8.5 to 84 (Table 1). When immobilizing lipases in methyltrimethoxysilane-derived gels, Reetz et al. [7] found that the optimal stoichiometric ratio of water to silane was 8–10. Below and above this region, the activity of immobilized lipases decreased. In our study, phyllosilicates provided the framework for the immobilizing matrix. TMOS was used only as a cross-linking agent. With  $R_v \leq 10$ , increasing the amount of phyllosilicate resulted in increasing porosity of the cross-linked phyllosilicate [8], which reduced the limitation on the substrate diffusion and increased the activity of immobilized peroxidase. The attempt to use  $R_v = 20$  for intercalative immobilization failed because the amount of TMOS fell short of the minimum requirement for matrix solidification.

In the kinetic studies, free and immobilized peroxidases were assayed at pH 7.0 with the concentration of guaiacol ranging from 0.1 to 8 mM. The immobilized peroxi-

**Table 1** Relative activity (percentage of free enzyme) of peroxidase immobilized in the cross-linked phyllosilicates at various volume ratios ( $R_v$ ) of phyllosilicate (SWy-1) suspension to TMOS (v/v)

The activity of free horseradish peroxidase was measured as  $98.2 \mu\text{mol of guaiacol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ . Peroxidase was immobilized in cross-linked Na-phyllosilicate (CS), TMA-phyllosilicate (CTS) or HDTMA-phyllosilicate (CHS).

Treatment	$R_v$	$R_v$			
		10	5	2.5	1
Weight ratio of TMOS to SWy-1 ...	6.25	12.5	25	62.5	
Molar ratio of water to silane (TMOS) ...	84	42	21	8.4	
CS	128.5	109.7	84.2	14.3	
CTS	136.7	104.9	67.5	17.7	
CHS	42.5	19.6	8.2	2.2	



**Figure 4** Activity (initial transformation rate) of free and immobilized peroxidase (POD) at different enzyme amounts

Peroxidase was immobilized in cross-linked Na-phyllosilicate (CS), or in TMA-phyllosilicate (CTS). The ratio of phyllosilicate suspension to TMOS was 5:1 (v/v).

dase exhibited similar a  $K_m$  (0.29 mM) to free peroxidase (0.24 mM). The  $V_{max}$  of the immobilized peroxidase increased from  $103 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  for free peroxidase to  $192 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  for CTS (peroxidase immobilized cross-linked in TMA-phyllosilicate) and  $218 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  for CS (peroxidase immobilized in cross-linked Na-phyllosilicate) treatments. The activities of both immobilized and free peroxidase increased in a similar way to the enzyme amount (Figure 4). The kinetic properties of the peroxidase were unaffected by intercalative immobilization in the cross-linked phyllosilicates.

The reusability of intercalatively immobilized peroxidase was evaluated by repeating incubation cycles with the buffer solution (0.1 M sodium citrate/phosphate buffer, pH 7.0). After each cycle, the reaction mixture was removed and the immobilized peroxidase samples were washed with deionized water and incubated in fresh buffer solution. Table 2 lists the activity of the immobilized peroxidase at different incubation cycles (cycles 1–7). The activity of peroxidase immobilized in Na-phyllosilicate showed an almost linear decrease from cycle to cycle. The activities of peroxidase immobilized in TMA- or HDTMA-phyllosilicate showed a pronounced decrease during the second incubation cycle, after which the decrease in activities were similar for peroxidase immobilized in either Na-phyllosilicate or (HD)TMA-phyllosilicate. At the seventh incubation cycle, the residual activity of the immobilized peroxidase was about 20% of the original activity.

The leaking of immobilized peroxidase during each incubation and wash may be a major reason for the activity decrease with the increasing number of cycles. Leaching tests were conducted by incubating enzyme–phyllosilicate composites in deionized water or 0.1 M sodium citrate-phosphate solution and shaking at 70 rev./min for 24 h. After centrifugation, the protein content of the incubation solution was measured by Lowry assay. Protein leaking from the enzyme–phyllosilicate composite was 5.5% when incubated in water, and 9.8% in the citrate-phosphate solution. As the decrease in percentage of activity from one cycle to the next (Table 2) was larger than the amount of protein leaking in one incubation, other factors must also have contributed to the decrease of immobilized peroxidase

**Table 2** Relative activity (percentage of 1st incubation) of immobilized peroxidase at different incubation cycles

Peroxidase was immobilized in cross-linked Na-phyllosilicate (CS), TMA-phyllosilicate (CTS) or HDTMA-phyllosilicate (CHS). The ratio of phyllosilicate suspension to TMOS was 5:1 (v/v). The peroxidase activity in the first incubation was 107.7, 103.1, and  $19.3 \mu\text{mol of guaiacol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  for CS, CTS and CHS, respectively.

Treatment	Cycle ...	$R_v$						
		1	2	3	4	5	6	7
CS	100	86.2	78.0	60.1	48.5	36.8	20.1	
CTS	100	65.3	51.9	35.9	29.3	12.8	14.2	
CHS	100	56.6	46.7	46.2	43.1	29.9	22.1	

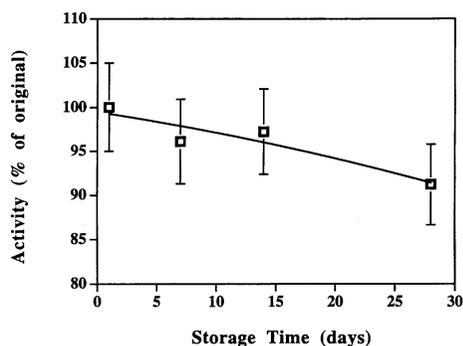


Figure 5 Storage stability of peroxidase immobilized in Na-phyllosilicate

The volumetric ratio of phyllosilicate suspension to TMOS was 5:1 (v/v). The original activity of immobilized peroxidase was  $107.7 \mu\text{mol}$  of guaiacol  $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ .

activity with each cycle. The possible factors include the adsorption of reaction products in the cross-linked phyllosilicates and the denaturation of immobilized peroxidase.

The intercalatively immobilized peroxidases were stored without buffer solution at  $23^\circ\text{C}$  for 4 weeks. The activity of intercalatively immobilized peroxidase decreased 5% in the first week and remained similar in the second week (Figure 5). After 4 weeks of storage, the residual activity of the immobilized peroxidase was about 90% of the original activity. The half-life of intercalatively immobilized peroxidase was estimated to be 169 days by extrapolating the results using regression analysis.

The intercalative immobilization of enzymes prevents the diffusion of the protein from the interlayer space of cross-linked phyllosilicates to the bulk solution space. This physical arrangement does not hinder the free diffusion of the enzyme substrate and the reaction product through the cross-linked interlayers. Thus, the intercalative immobilization does not significantly change the peroxidase activities. Within the interlayer space, the enzyme presumably retains its preferred conformation. The cross-linking of phyllosilicates also prevents the possible contact between the enzyme and microbes in the solution. These two factors may attribute to the long shelf life obtained for the immobilized enzyme. The possibility of extending the described immobilization approach to other enzymes is currently being evaluated.

## Acknowledgment

Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

## References

- 1 Nilson, K. and Mosbach, K. (ed.) (1987) *Methods Enzymol.* **135**, 65–78
- 2 Janecek, S. (1993) *Process Biochem.* **28**, 435–445
- 3 Dave, B. C., Soye, H. and Miller, J. M. (1995) *Chem. Mater.* **7**, 1431–1434
- 4 Avnir, D., Braun, S., Lev, O. and Ottolenghi, M. (1994) *Chem. Mater.* **6**, 1605–1614
- 5 Heichal-Segal, O., Rappoport, S. and Braun, S. (1995) *Biotechnology* **13**, 798–800
- 6 Shtelzer, S., Rappoport, S., Avnir, D., Ottolenghi, M. and Braun, S. (1992) *Biotechnol. Appl. Biochem.* **15**, 227–235
- 7 Reetz, M. T., Zonta, A. and Simpelkamp, J. (1996) *Biotechnol. Bioeng.* **49**, 527–534
- 8 Shen, S., Hsu, A. F., Foglia, T. A. and Tu, S.-I. (1998) *Appl. Biochem. Biotechnol.* **62**, 79–89
- 9 Pinnavia, T. J. (1983) *Science* **220**, 365–371
- 10 Vaia, R. A., Teukolsky, R. K. and Pinnavia, T. J. (1983) *Chem. Mater.* **6**, 1017–1022
- 11 Srinivasan, K. R. and Fogler, H. S. (1990) *Clay Clay Miner.* **38**, 277–286
- 12 Yan, Y. and Bein, T. (1993) *Chem. Mater.* **5**, 905–907
- 13 Hsu, A. F., Shen, S., Wu, E. and Foglia, T. A. (1998) *Biotechnol. Appl. Biochem.* **28**, 55–59
- 14 Klibanov, A. M., Tu, T.-M. and Scott, K. P. (1983) *Science* **221**, 259–261
- 15 Sandberg, R. G., Van Houten, L. J., Schwartz, J. L., Bigliano, R. P., Dallas, S. M., Cabelli, M. A. and Narayanswamy, V. (1992) In *Biosensor Design and Application* (Mathewson, P. R. and Finley, J. W., eds.), pp. 81–88, ACS, Washington
- 16 Van Olphen, H. and Fripiat, J. J. (1979) *Data Handbook for Clay Materials and other Non-Metallic Minerals*, p. 19, Pergamon Press, Oxford
- 17 Rytwo, G., Nir, S. and Margulis, L. (1995) *Soil Sci. Soc. Am. J.* **59**, 554–564
- 18 Shen, S., Stucki, J. W. and Boast, C. W. (1992) *Clays Clay Minerals* **40**, 381–386
- 19 Bergmeyer, H. U., Grassl, M. and Walter, H.-E. (1983) *Methods of Enzymatic Analysis*, 3rd edn., vol. 2, pp. 267–268, Verlag Chemie, Weinheim
- 20 Van Olphen, H. (1977) *An Introduction to Clay Colloid Chemistry*, 2nd edn., pp. 1–56, John Wiley and Sons, New York
- 21 Theng, B. K. G. (1979) *Formation and Properties of Clay-Polymer Complexes*, pp. 37–61, Elsevier, New York