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Silica-Gelatin Nanocomposite as a Carrier for Enzyme Immobilization

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ABSTRACT

Silica-gelatin nanocomposite of W_0 = 30 (W_0 = $[H_2O]/[AOT]$), r = 4 (r = $[H_2O]/[TEOS]$), and 8%w/v gelatin, synthesized using hydrochloric acid catalyst followed by pH adjustment before enzyme addition, was investigated for *Candida cylindracea* lipase immobilization. Transesterification of racemic menthol and hyxyl acetate was selected as a model reaction for this study. The material synthesized was believed to partly contain microemulsion-based organogels (MBGs) while mostly consisted of a lightly cross-linked silica network structure. The system could immobilize up to 7.1 mg enzyme/mL solution without enzyme leakage. The immobilized enzyme reaction was found to be controlled by intra-particle diffusion, however, free enzyme activity appeared to be 34.0-61.8% less than that of immobilized enzyme due to free enzyme agglomeration in isooctane. Moreover, immobilized enzyme exhibited a storage stability with surprisingly 134% higher activity than freshly immobilized ones after 15 days of storage at room temperature. Altered nanocomposite structure due to aging might be the cause of this phenomenon.

Keywords: biocatalysis; enzyme technology; immobilized enzyme; reverse micelles; organogels; nanocomposite

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INTRODUCTION

Reverse micelles, thermodynamically stable nanometer sized water dispersion in organic solvent stabilized by amphiphilic molecules, have been applied for enzyme encapsulation for over the past twenty years (Zhou, *et al.* 2001; Bru, *et al.* 1989; Luisi, 1985; Sebastiao, *et al.* 1993; and Walde, 1989). This is due to their numerous advantages: high interfacial area (approximated at 100 m^2/mL; Verhaert and Hilhorst (1991), low mass transfer limitation, and enhancement of catalytic activity and stability in some cases (Castro and Cabral, 1989). However, problems could still arise due to scaling-up the process and difficulties in product recovery caused by surfactant contamination. Consequently, microemulsion-based organogels (MBGs), a gelation system of reverse micelles and semisolid carriers for enzyme immobilization, were applied to solve mentioned complications (Rees, *et al.* 1991; Nascimento, *et al.* 1992; and Jenta, *et al.* 1992). Nonetheless, usage of MBGs is limited due to their poor mechanical and thermal stability especially in an aqueous environment. Recently, however, Schuleit and Luisi (2001) were the first to demonstrate that *Chromobacterium viscosum* lipase could be successfully immobilized in novel silica-gelatin nanocomposite in which gelatin organogels were hardened by the in situ polymerization of tetraethoxysilane (TEOS) demonstrated in reaction steps as follows:

Hydrolysis: $\equiv Si\text{-}OR + H_2O \leftrightarrow \equiv Si\text{-}OH + ROH$

Alcohol Condensation:

$\equiv Si\text{-}OR + \equiv Si\text{-}OH \leftrightarrow \equiv Si\text{-}O\text{-}Si\equiv + ROH$

Water Condensation:

$\equiv Si\text{-}OH + \equiv Si\text{-}OH \leftrightarrow \equiv Si\text{-}O\text{-}Si\equiv + H_2O$

(where R represents an alkyl group)

Using this method, Schuleit and Luisi (2001) found that matrix stability could be tremendously improved while conserving advantageous microemulsion characteristics. In addition, immobilized lipase was found to give a high production yield (80-85%) as well as operating stability up to seven months.

Inspired by the previous work of Shuleit and Luisi (2001), we demonstrate, in this work, different preparation method for lipase immobilization in silica-gelatin nanocomposite. Hydrochloric acid of 0.1 M was used as a catalyst for hydrolysis and condensation of TEOS, while 1.0 M ammonium hydroxide solution was later included for pH adjustment before the addition of enzyme. Analyses of synthesized nanocomposite namely; MAS ^{29}Si NMR, BET, and SEM are discussed. Moreover, enzyme loading capacity and leakage tests as well as comparisons of residual activities and storage stability between free and immobilized enzymes are considered. Transesterification of racemic menthol and hexyl acetate in isooctane was chosen to test enzyme activity.

MATERIAL AND METHODS

Chemicals

Tetraethoxysilane (TEOS) (98%), sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) (98%), gelatin type A 300 Bloom, racemic menthol (99%), hexylacetate (99%), and *Candida cylindracea* lipase (EC 3.1.1.3) were purchased from Aldrich Chemicals (USA). Isooctane was from Ajax Chemicals (Australia), hydrochloric acid (35.4%) was from BDH (UK), while ammonium hydroxide (30%) was from Carlo Erba (Italy).

Preparation of silica-gelatin nanocomposite

Silica-gelatin nanocomposite was prepared according to the following procedure. 250 mM AOT/isooctane water-in-oil microemulsion was initially prepared before addition of 0.1 M HCl to the microemulsion system to accomplish the W_0 of 30 (W_0 = $[H_2O]/[AOT]$). Gelatin was next added to make up to 8% (w/v) while the solution was continuously stirred at 50°C until very viscous and homogeneous solution was obtained. The system was then quenched down to room temperature (32±2°C) and heated up again to 50°C to obtain, once again, viscous homogeneous gel. The silica sol-gel process

was subsequently initiated by the addition of TEOS to the value of r = 4 (r = $[H_2O]/[TEOS]$) under stirring at room temperature for one hour. The sample was then left in a closed container as before for the next 24 hours before being air-dried. The surface characteristic of the obtained material was analysed using SEM (Jeol JSM 5400, Japan) while porosity determination was carried out using BET (Micromeritics ASAP 2000, USA). MAS ^{29}Si NMR (Bruker DPX-300, Switzerland) was performed in order to follow the condensation reaction of TEOS.

Enzyme immobilization in silica-gelatin nanocomposite

Silica-gelatin nanocomposite was initially prepared according to as above mentioned, however, after one hour of silica sol-gel process 1.0 M ammonium hydroxide solution was then included until the pH was adjusted to the value of 7. This resulted in a much slower TEOS polymerization as well as appropriate condition for enzyme immobilization. *Candida cylindracea* lipase was subsequently added under stirring to the amount that a homogeneous system could still be obtained. The sample was then left in a closed container as before for the next 24 hours before being cut into small cubic pieces of 2 mm in diameter and subsequently air-dried.

Enzyme activity test

Transesterification activity of free or immobilized lipase was examined using racemic menthol (73 mM) and hexyl acetate (360 mM) as substrates in 150 mL isooctane. The reaction was carried out in a 200 mL stirred tank with 110 rpm stirring speed and temperature controlled at 30 °C. Samples were taken at appropriate time intervals and the amount of (-)menthyl acetate was quantified using gas chromatography (Shimadzu 7AG, Japan).

Enzyme leakage test

In order to test for enzyme leakage, the immobilized material was separated out of the reacting solution after the appropriate reaction time. The solution was then left stirred as before and samples were withdrawn for (-)menthyl acetate analyses at appropriate time intervals.

Enzyme storage stability test

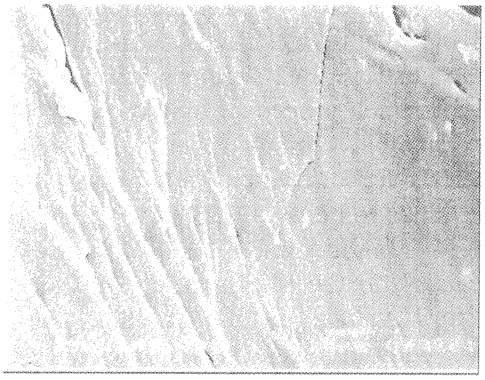
Lipase immobilized in silica-gelatin nanocomposite was left at room temperature (32±2°C) for 15 and 35 consecutive days before being tested for transesterification activity.

RESULTS AND DISCUSSION

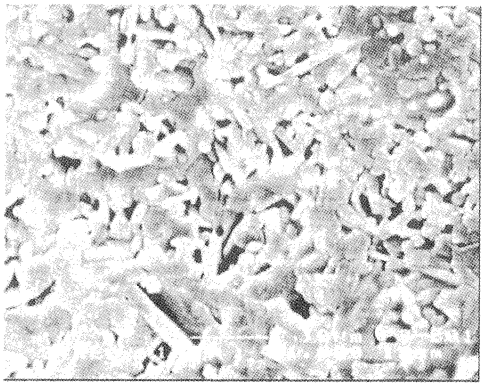
Characteristics of synthesized silica-gelatin nanocomposite

Silica-gelatin nanocomposite synthesized in this work was primarily prepared from a network of nanogel droplets where gelatin was believed to be helically solubilized and acted as a linking agent between micelles. The following sol-gel process initiated by TEOS addition most likely took place in the water-pool of the micelles and in the gelatin/water channels according to the MBG model proposed by Atkinson (1991). The suitable compositions of synthesized material for enzyme immobilization were determined to be W_0=30, 8% (w/v) gelatin, and r = 4 (Julpharpimon, 2000). The surface characteristic of this material is shown in Figure 1(A) and is found to be smooth and dense without phase separation indicating a homogeneous blend between the silica compound and the gelatin organogel. The nanocomposite material was then calcined at 1,200°C which revealed a silica network of clusters of small particles interconnected by rod-shaped materials (See Figure 1(B)). This silica network was believed to be mostly formed from silica nanoparticles synthesized inside the micelles. The SEM picture (Figure 1(B)) obtained interestingly coincides with the MBG model developed on the basis of neutron scattering and conductivity data proposed by (Atkinson, *et al.* 1988 and 1989) See Figure 1(C)), though of a different scale of magnitude.

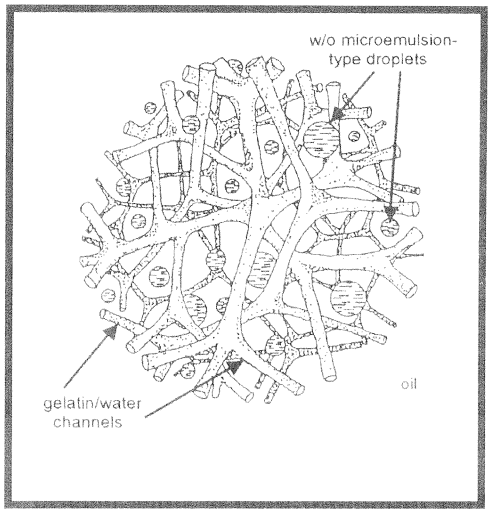
The BET result of this particular silica-gelatin nanocomposite reveals a low surface area of 0.29 m^2/g which was probably due to a surface covering of MBGs according to the SEM evidence. MAS ^{29}Si-NMR spectrum of silica gelatin nanocomposite demonstrated in Figure 2 evidences that a silica sol-gel process did take place. Moreover, the dominant Q^3 species (Q^2:Q^3:Q^4 = 1:4:2) indicates incomplete condensation resulting in lightly cross-linked silica network structure possibly via polymeric gel formation which Watzke and Dieschbourg (1994) suggested a porous gel structure of very small pores. This result is in contrast to densely cross-linked silica network (dominating Q^4 peak) found in case of Schuleit and Luisi (2001) where no catalyst was used for TEOS polymerization. However, a higher r ratio in their case (r = 5) is likely one of the reasons for a larger extent of hydrolysis as already suggested by our previous work (Julpharpimon, 2000).



(A)



(B)



(C)

Figure 1 SEM images of silica-gelatin nanocomposite of W_0=30, 8% (w/v) gelatin, and r = 4 (A) air-dried sample; (B) 1,200°C calcited sample; (C) schematic of MBG structure proposed by (Atkinson, *et al.* 1988 and 1989 Reed, *et al.* 1991; Sebastial, *et al.* 1993; Stamatis, *et al.* 1993).

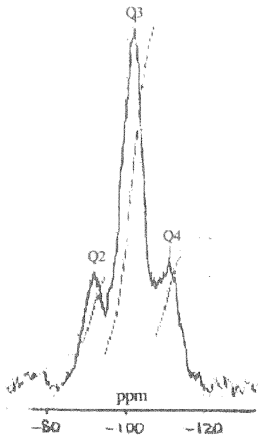


Figure 2 MAS ^{29}Si-NMR spectrum of silica-gelatin nanocomposite derived from TEOS. (Q^n represents the extent of crosslinking through siloxan bonds; n = 4 designates completely crosslinked silicon atoms with four siloxan bonds while the reduced number of superscript n indicates more silanol groups at eh silicon atoms.)

Enzyme loading and leakage

Up to 7.1 mg of *Candida cylindracea* lipase could be loaded in 1 mL solution of silica-gelatin nanocomposite of 8% (w/v) gelatin. This loading capacity is considered reasonably high in comparison to 0.4 mg/mL Jenta, *et al.* (1997) and Stamatis, *et al.* (1999) 0.36 mg/mL Jenta, *et al.* (1997), and 10 mg/mL Zhou, *et al.* (2001), and 10 mg/mL (Zhou, *et al.,* 2001) for AOT organogel systems. Higher gelatin concentrations resulted in lower enzyme loading (results not shown) due to higher extent of water required for gelatin hydration leaving less amount of water for TEOS polymerization and enzyme solubilization. The immobilized enzyme system of silica-gelatin nanocomposite appeared transparent and of the same yellowish color as of the entrapped enzyme.

Due to its insolubility in isooctane, lipase is believed to be located in the water pool of the micelles which was heretofore partially transformed into silica-gelatin nanocomposite. Enzyme leakage was not detected after 25-200 hours of reaction in any cases. This was in accordance to Schuleit and Luisi (2001) who suggested that a silica hardened organogel system was proved to be impossible for the immobilized enzyme to leak out from.

Transesterification of immobilized enzymes

Candida cylindracea lipase was immobilized in the very last step of composite synthesis before the sample was left standing in a closed container and subsequently air dried. Nonetheless, pH adjustment was required prior to enzyme inclusion due to a hostile environment from acid catalyst. Transesterification activities were tested with a carrier of similar specific surface area with particle sizes of 1 and 2 mm in diameters, and it was found that smaller particles gave a 1.73 times higher initial rate than that of the larger ones. This clearly indicates intra-particle diffusion resistance and that substrate and/or product mass transfer was a rate limiting step. This is in contrast to reverse micelle based systems, of silica-gelatin nanocomposite, where mass transport process would not be expected to influence the rate of reaction (Stamatis, *et al.* 1993; Stamatis, *et al.* 1995). However, substrate and/or product mass transfer limitation in MBGs is considered rather common Stamatis, *et al.* (1999), Pizarro, *et al.* (1997) and Nagayama, *et al.* 1998) due to higher rigidity of MBGs in comparison to their non-gel counterparts. Hence, it is not surprising that diffusion resistance was observed in silica-gelatin nanocomposite which is an even more rigid system than MBGs.

Transesterification activities of free and immobilized enzymes were tested and initial rates determined in the first 25 reaction hours revealed 100, and 173 μmol/L-hr-g.enz. for entrapped enzyme activities in 2 and 1 mm particles, respectively. While that of free enzyme was measured at 66 μmol/L-hr-g.enz or 34.0-61.8% less activity than immobilized enzyme. Although mass transfer limitation was recognized for entrapped enzyme activity in silica-gelatin nanocomposite, distinctly higher rates obtained in comparison to that of free enzyme indicates advantageous microemulsion characteristics in which the enzyme was dispersed to a molecular level and not in an aggregated form as in the case of free enzyme. Results in Figure 3. It was markedly showed that immobilized enzyme activity was higher than that of free enzyme throughout 225 hours of the reaction.

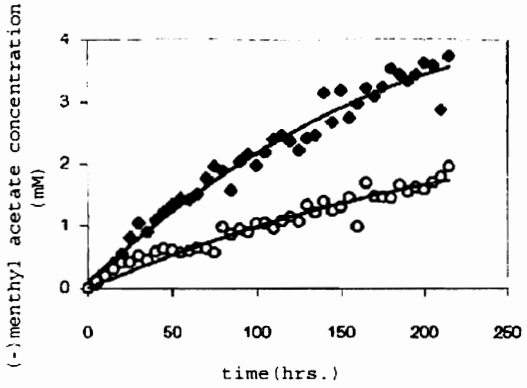


Figure 3 Time course of lipase catalyzed (-)menthyl acetate production. (°)

free enzyme (◆) immobilized enzyme in 2 mm nanocomposite particles reacting conditions: 73 mM racemic menthol, 360 mJ hexyl acetate, 110 rpm, 30°C, and same enzyme loading for both cases.

Storage stability of immobilized enzyme

Table 1 demonstrates initial transesterification activities of immobilized lipase in silica-gelatin nanocomposite comparing to those of free enzyme after being left at room temperature (32±2°C) for 0, 15, and 35 consecutive days, respectively. The results evidently suggest a loss of free enzyme activity with storage time, since activity reduction was up to 55% after 35 days of storage. On the contrary, this phenomenon was not observed for immobilized enzyme. To our surprise, stored immobilized enzyme showed even higher activity in comparison to freshly immobilized enzyme which resulted in a 134% activity increase after 15 days of storage. It might be that altered nanocomposite structure due to aging helped facilitating molecular diffusion. However, a longer storage time did not cause higher activity since a storage time of 35 days resulted in a lower transesterification activity than that of 15 days, although still culminated in a 70% higher activity than freshly immobilized enzyme.

Table 1 Initial transesterification activities of immobilized and free enzyme after 0, 15, and 35 days of storage.

Storage Time (days)	Immobilized Enzyme Activity (μmol/l-hr-g.enz)	Free Enzyme Activity (μmol/l-hr-g.enz)
0	100	66
15	234	32
30	170	30

CONCLUSIONS

Candida cylindracea lipase was immobilized in silica-gelatin nanocomposite with W_0 = 30, r = 4, and 8%w/v gelatin, and was tested for transesterification activity with no leakage detected. The material synthesized was believed to partly contained MBGs and mostly consisted of a lightly cross-linked silica network structure. The reaction was found to be controlled by intra-particle diffusion, however, free enzyme activity appeared to be 34.0-61.8% less than that of immobilized ones which probably resulted from free enzyme agglomeration in isooctane. Moreover, immobilized enzyme exhibited storage stability with surprisingly 134% higher activity than freshly immobilized enzyme after 15 days of storage at room temperature. An altered nanocomposite structure due to aging might be the cause of this phenomenon.

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