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Immobilization of Lipase From *Candida rugosa* on Layered Double Hydroxides for Esterification Reaction

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Abstract

Synthesis of layered double hydroxides (LDHs) of Zn/Al-NO_3^- hydrotalcite (HIZAN) and Zn/Al-diocytyl sodium sulfosuccinate (DSS) nanocomposite (NAZAD) with a molar ratio of Zn/Al of 4:1 were carried out by coprecipitation through continuous agitation. Their structures were determined using X-ray diffractometer spectra, which showed that basal spacing for LDH synthesized by both methods was about 8.89 Å. An expansion of layered structure of about 27.9 Å was observed to accommodate the surfactant anion between the interlayer. This phenomenon showed that the intercalation process took place between the LDH interlayer. Lipase from *Candida rugosa* was immobilized onto these materials by physical adsorption method. It was found that the protein loading onto NAZAD is higher than HIZAN. The activity of immobilized lipase was investigated through esterification of oleic acid and 1-butanol in hexane. The effects of pore size, surface area, reaction temperature, thermostability of the immobilized lipases, storage stability in organic solvent, and leaching studies were investigated. Stability was found to be the highest in the nanocomposite NAZAD.

Index Entries: Immobilization; lipase; layered double hydroxides; esterification; *Candida rugosa*.

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Introduction

Layered double hydroxides (LDHs) have been receiving considerable attention owing to their potential application as adsorbents, ion exchangers, detergents, polymer stabilizers, and catalysts *(1)*. LDHs can be produced at a low temperature, which keeps the precipitated particles at the smallest size and their surface area and reactivity to a maximum. Ions or molecules such as enzymes can be introduced into the interlayer space, thereby generating a diverse group of materials with various applications in biotechnology.

Immobilization of enzyme by means of localization or confinement of enzyme in a certain defined region of space with retention of its catalytic activities will protect the enzyme against changes in reaction parameters. Pure enzymes are sensitive and not suitable in many catalytic reactions owing to their unstable nature, and they are also easily denatured at extreme conditions. This can be reflected by the enhanced activity and stability after the enzymes are immobilized. Physical methods such as adsorption of enzyme on a support are relatively easier and cheaper compared with chemical methods such as covalent attachment of the enzyme and support *(2)*. Primary considerations for the selection of supports applied are hydrophilic characteristics, high surface area, high porosity, and surface chemical variety.

In this article, we report on the immobilization of lipase from *Candida rugosa* onto LDHs. Lipase sources from *C. rugosa* have been widely investigated owing to their ability to catalyze stereoselective esterification and transesterification *(3)*.

Materials and Methods

Chemicals

Lipase from *C. rugosa* (EC 3.1.1.3, type VII) was purchased from Sigma (St. Louis, MO) and water extracted as described later. All other chemicals were of analytical grade.

Synthesis of Zn-Al-NO_3^- Hydrotalcite (HIZAN)

HIZAN was prepared at a ratio of Zn:Al of 4:1 (w/w). A solution of 2*M* NaOH was added to 100 mL of solution containing 0.2 *M* $Zn(NO_3)_2$ and 0.8*M* $Al(NO_3)_3$. The dropwise addition was completed until the pH of the solution reached 10.0. The mixture was stirred and heated in an oil bath at 70°C for 18 h. The cooled mixture was then centrifuged and washed several times with distilled water and dried in an oven at 120°C.

Synthesis of Zn-Al-Diocytyl Sodium Sulfosuccinate Nanocomposite (NAZAD)

A 50-mL solution 0.1*M* diocytyl sodium sulfosuccinate (DSS) was added to 100 mL of solution containing 0.2*M* $Zn(NO_3)_2$ and 0.8*M* $Al(NO_3)_3$.

The dropwise addition was completed until the pH of the solution reached 10.0. The mixture was stirred and heated in an oil bath at 70°C for 3–5 d, cooled, centrifuged, and washed several times with distilled water. The sample was dried in an oven at 120°C.

Characterization of LDHs

LDHs were characterized using X-ray diffractometry (XRD), Brunauer, Emmet, and Teller (BET) technique, and scanning electron microscopy (SEM).

Water Extraction of Lipase

Crude lipase from *C. rugosa* (1.5 g) was added to distilled water (15 mL). The mixture was stirred for 1 h, and followed by a 15-min centrifugation at 10,000 rpm. The supernatant was used as partially purified lipase.

Immobilization of Lipase

Immobilization was carried out by continuously stirring at 100 rpm LDHs (2.0 g) with purified free lipase supernatant (15 mL) for 1 h at room temperature. The immobilized lipase was filtered, and the supernatant was kept for protein assay. The amount of protein was determined by the Bradford method *(4)*.

Characterization of Immobilized Lipase

Thermal Stability

The immobilized enzymes were incubated at 30, 40, 50, 60, and 70°C in sealed vials for 1 h. The enzymes were left to cool before esterification assay. The relative activities were determined as compared to activity of the untreated lipase with an esterification assay. All experiments were done in triplicate. Control experiments were carried out with native lipase.

Leaching Study

The immobilized enzymes (0.3 g) were washed five times with 4 mL of hexane each time. The relative activities were determined as compared to the activity of unwashed immobilized lipase with an esterification assay. All experiments were done in triplicate.

Stability in Hexane

The immobilized enzymes were incubated in hexane without shaking for 1–10 d at room temperature. The relative activities were determined as compared to activity of the first day with an esterification assay. All experiments were done in triplicate. Control experiments were carried out with native lipase.

Storage Stability

The immobilized enzymes were stored in hexane for 30 d at –20, 0, and 4°C and at room temperature. The relative activities were determined as compared to the activity of d 1 with an esterification assay. All experiments

were done in triplicate. Control experiments were carried out with native lipase.

Esterification Assay

The esterification reaction consisted of 1-butanol (4.0 mmol), oleic acid (2.0 mmol), immobilized enzyme (0.3 g), and hexane (2 mL). The reaction mixture was incubated at 30°C for 5 h with continuous shaking at 150 rpm in a horizontal shaker water bath. The reaction was terminated by the addition of 3.5 mL of acetone:ethanol (50:50 [v/v]). The remaining free fatty acid in the reaction was determined by titration with 0.15*M* NaOH using an automatic titrator to an end point at pH 10.0. The activities were expressed as specific activity ([μmol of free fatty acid/min/mg of protein]). All experiments were done in triplicate. Control experiments were carried out with the untreated enzyme.

Results and Discussion

Both layered double hydroxides, HIZAN and NAZAD, formed as white powder were synthesized with a ratio of $R = 4$ and pH 10.0 by direct hydrolysis and were characterized by XRD, BET, and SEM techniques. Analysis of the XRD diffractogram showed that the basal spacing for HIZAN was 8.9 Å compared with 27.9 Å for the nanocomposite NAZAD. This indicates that the surfactant of dodecyl sodium sulfoacetate was successfully intercalated between the interlayer of inorganic LDHs to form a layered nanocomposite of organic-inorganic hybrid type. The analysis of BET surface area and micropore volume is summarized in Table 1.

The nitrogen adsorption/desorption isotherms of HIZAN and NAZAD are typical of type II based on the Brunauer, Demming, Demming, and Teller technique. The isotherm with this type of hysteresis loop, which is of type B, is the result of open, slit-shaped capillaries with parallel walls or capillaries with very wide bodies and a narrow, slit neck. Pore size distribution of the resulting materials shows that both LDHs are of mesoporous range. The SEM morphology of HIZAN was found to be of picked platelike nonporous structure and more compact in comparison to NAZAD (Fig. 1). Generally, both of the morphologies shown were of nonporous structure.

Immobilization of Lipase

Immobilization of lipase onto hydrotalcites by physical adsorption was carried out via extraction, centrifugation, homogenization, filtration, and drying according to industrial need. The percentage of protein adsorbed on HIZAN was 63% and on NAZAD was 78.5%, as shown in Fig. 2. The higher amount of protein adsorbed on NAZAD may be owing to the large surface area of NAZAD compared with HIZAN. During adsorption, the protein molecules (from lipase) were randomly scattered on the surface. This situation leads to a better protein adsorption by NAZAD.

Table 1
BET Surface Area and Micropore Volume of HIZAN and NAZAD

LDHs	BET surface area (m^2/g)	Micropore volume ($\times 10^{-4}$ cc/g)
HIZAN	10.9 ± 0.5	4.37 ± 0.05
NAZAD	21.6 ± 0.5	43.64 ± 0.05

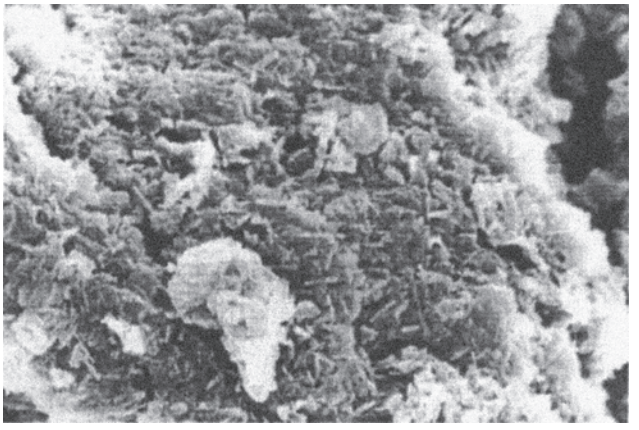

Fig. 1. SEM morphology of NAZAD (×4000).

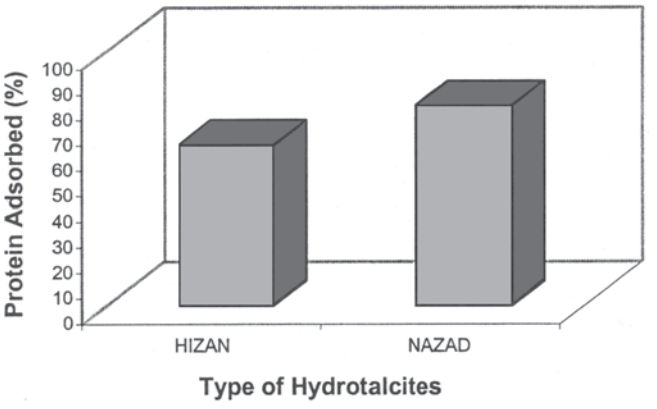

Fig. 2. Percentage of protein adsorbed on lipase immobilization.

Thermal Stability

The thermal stability of the immobilized lipases after 1 h of incubation at temperatures ranging from 30 to 70°C is shown in Fig. 3. Both immobilized lipases were significantly more thermostable than the native lipase, which easily underwent aggregation, leading to loss of enzymatic activity. The thermal stability started to decrease with an increase in temperature from 40 to 70°C owing to denaturation of the protein molecule because of

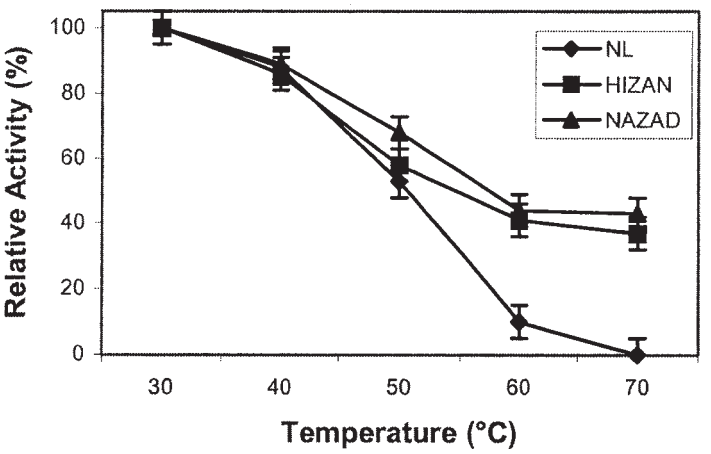

Fig. 3. Thermal stability of immobilized lipases compared with native lipase (NL) after incubation for 1 h at temperatures ranging from 30 to 70°C.

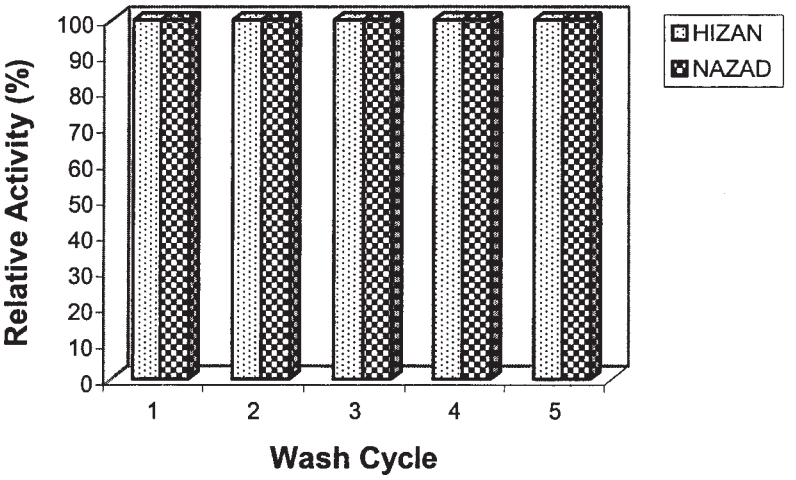

Fig. 4. Leaching study of immobilized lipases with 4 mL of hexane each cycle.

the heat capacity, which caused protein unfolding. Enzymatic reaction decreased with the increase in temperature owing to thermal instability of the protein molecules. However, the immobilized lipase had greater thermal resistance than the native lipase and, hence, greater esterification activity.

Leaching Study

Figure 4 shows that the immobilized lipase retained 100% of its catalytic activity even after five cycles of washing. This indicated that the lipase from *C. rugosa* remained immobilized on the support even after washing with 20 mL of hexane. These results prove that hydrotalcite and nanocomposite are suitable supports for lipase immobilization. This support

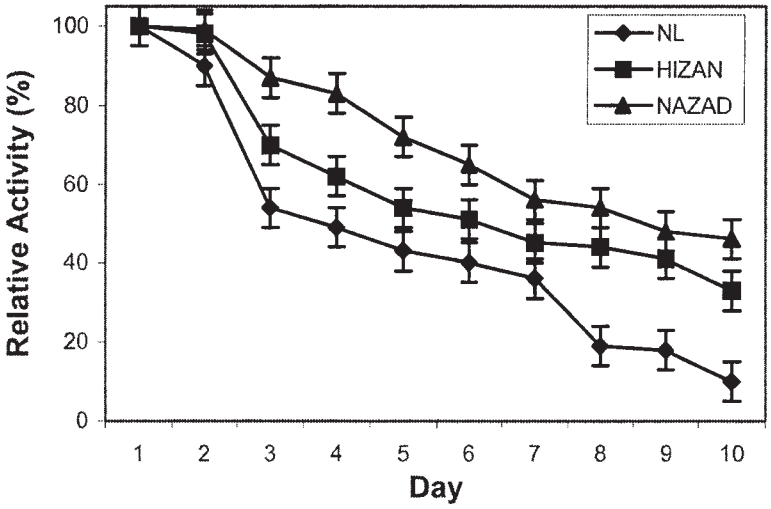

Fig. 5. Stability of immobilized lipases at room temperature in presence of hexane. NL, native lipase.

has the ability to prevent the lipase from being leached out from the support. Furthermore, the results also proved that the adsorption method used in our study is a suitable method of immobilization and was successfully done. The loss of some enzyme during washing might reflect the inherent consequence of its immobilization on each support surface *(4)*.

Stability in Hexane

Figure 5 presents the stabilities of the immobilized and native lipase in hexane. It clearly shows that the stabilities of the immobilized enzymes were found to be higher up to 10 d compared to native lipase. The nanocomposite exhibited the best stability by retaining 50% of its activities. This indicates that immobilizing the lipase can prevent the lipase from being destroyed by the organic solvent.

Storage Stability

The ability to be stored for a period of time with activity is one of the key factors to be considered when using immobilized lipases. Table 2 summarizes the effect of temperature on storage stability for 30 d. At –20°C, all experiments retained full esterification activity. Both immobilized lipases retained 100% enzymatic activities when stored at 0°C, but there was a slight decrease in activity when the storage temperature was increased to 4°C and room temperature. When stored at higher temperature, immobilized lipases showed increased storage stability compared with native lipase. At lower storage temperatures, the lipase is probably locked in its native, catalytically active conformation. At these temperatures, the stability may be owing to multipoint attachment of the enzyme to the support,

Table 2
Storage Stability of Immobilized Lipase at Different Temperatures

	Activity (100%) (±5%)			
Lipase	Room temperature	4°C	0°C	–20°C
Native	33	67	69	100
HIZAN	53	74	100	100
NAZAD	86	76	100	100

creating a more rigid enzyme molecule. Hence, disruption of the active center becomes less likely to occur *(5)*.

Conclusion

LDHs from hydrotalcite (HIZAN) and nanocomposite (NAZAD) were successfully synthesized and used as supports for immobilization of lipase from *C. rugosa*. The activity and stability of the lipase after immobilization on both LDHs were found to be increased compared to native lipase. NAZAD, with higher porosity and surface area, adsorbed higher protein and thus gave better activities compared to HIZAN and native lipase. The immobilized enzymes can therefore be used as biocatalysts in synthetic reactions.

Acknowledgment

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References

1. Rives, V., Labajos, F. M., and Ulibari, M. A. (1993), *J. Inorg. Chem.* **32,** 5000–5001.
2. Basri, M., Ampon, K., Razak, C. N. A., Salleh, A. B., and Yunus, W. M. Z. (1994), *Appl. Biochem. Biotechnol.* **48,** 173–183.
3. Aldercreutz, P., Triantafyllou, A. O., and Mattiasson, B. (1992), *J. Mol. Catal. B: Enzymat.* 167–178.
4 Bradford, M. M. (1976), *Anal. Biochem*. **72,** 248–254.
5. Basri, M., Ampon, K., Razak, C. N. A., Salleh, A. B., Yoong, W. S., and Yunus, W. M. Z. (1997), *J. Mol. Catal. B: Enzymat.* **3,** 171–176.