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Partial purification and immobilization of a new (R)-hydroxynitrile lyase from seeds of *Prunus pseudoarmeniaca*

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ABSTRACT

Hydroxynitrile lyase (HNL) from seeds of *Prunus pseudoarmeniaca* was partially purified by $(NH_4)_2SO_4$ fractionation and covalently immobilized onto Eupergit C and Eupergit C 250 L. The percentages of bound protein per gram of Eupergit C and Eupergit C 250 L were about 81 and 98 of the initial amount of protein, respectively. K_m and V_{max} values were determined 2.23 mM and 0.54 U/mg prot. for the free HNL, 1.60 mM and 0.87 U/mg prot. for the immobilized HNL onto Eupergit C and 1.03 mM and 0.35 U/mg prot. for the immobilized HNL onto Eupergit C 250 L, respectively at optimized reaction conditions. The half lives ($t_{1/2}$) and the thermal inactivation rate constants (k_i) of free and immobilized HNLs were determined at 25 and 50 °C, immobilized HNLs displayed higher thermal stability. Carboligation activities of free and immobilized HNLs for (R)-mandelonitrile (R-MN) synthesis were also determined. Besides, reusabilities of immobilized HNLs for both lyase and carboligation activities were investigated by using batch type reactors.

1. Introduction

Hydroxynitrile lyases (HNLs; EC 4.1.2.x) are a group of enzymes (mainly plant enzymes) that *in vivo* promote the reversible cleavage of cyanohydrins into hydrocyanic acid (HCN) and aldehyde (or ketone). The release of HCN by the plant – a process called cyanogenesis – presumably serves as a defense mechanism against herbivores and microbial attack [1]. Despite the natural function of HNLs as metabolic enzymes-cleavage of C–C bond – they are powerful *in vitro* catalysts for the preparation of optically active cyanohydrins (hydroxynitriles) – a carboligation process. Optically active (chiral) cyanohydrins are important building blocks for preparing the fine chemicals, pharmaceuticals, agrochemicals, veterinary products, vitamins and food additives. The preparation of hydroxynitriles with high optical purity necessitates a very fast enzymatic reaction to ensure that the rate of the spontaneous reaction can be neglected and the enzymatic reaction is the dominant factor in the formation of hydroxynitrile (Fig. 1).

Recently, HNLs have become a highly appealing tool in the synthesis of chiral cyanohydrins, because they are cofactor-independent enzymes, usually show high enantio-, chemo- and regioselectivities, have wide range of substrates specifities, work under mild conditions and generate less waste than conventional organic synthesis. However, lack of the operational stability and high price of enzymes are main problem in commercial production. On the other hand, enzyme immobilization techniques generally offer several advantages for industrial and biotechnological applications, including repeated use, ease of separation of reaction products from the biocatalyst, improvement of enzyme stability, continuous operation in a packed-bed reactor and the alteration of the properties of the enzyme.

Eupergit supports were used by many researchers as carriers for immobilization of various enzymes. It was reported that these carriers were very stable and had good chemical and mechanical properties (simple immobilization procedure, high binding capacity, low water uptake, high flow rate in column procedures, excellent performance in stirred bath reactors, etc.) [2–4]. Eupergits were made by copolymerization of N,N-methylene-bis-methacrylamide, glycidyl methacrylate, allyl glycidyl ether and methacrylamide. Both Eupergit C and Eupergit C 250 L are microporous, epoxy-activated acrylic beads with a diameter of 100–250 μm. They differ in the content of oxirane groups and in their porosity. While Eupergit C has an average pore size of $r = 10$ nm and an oxirane density of 600 μmol/g dry beads, Eupergit C 250 L has larger pores ($r = 100$ nm) and a lower oxirane density (300 μmol/g dry beads) [3]. Because of their structure, Eupergit C and Eupergit C 250 L are stable, both chemically and mechanically, over a pH range from 0 to 14, and do not swell or shrink even upon drastic pH changes in this range. Thus, they have been described as a suitable carriers for enzymes in enzyme immobilization [2–6]. In

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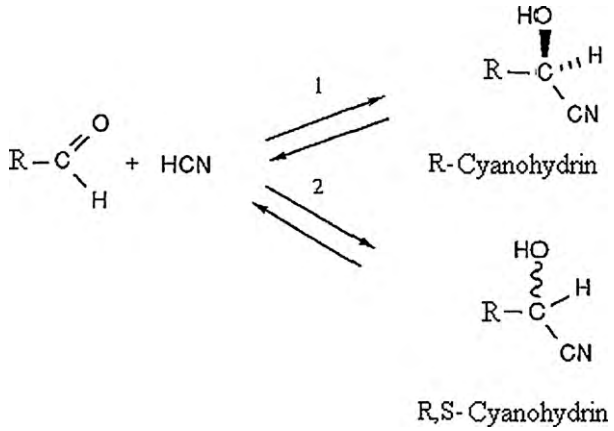

Fig. 1. General synthesis of cyanohydrins: 1, enzymatic reaction; 2, spontaneous reaction.

this study, we attempted the isolation, partial purification, characterization of HNL from seeds of *Prunus pseudoarmeniaca* and then immobilization of HNL onto Eupergit C and Eupergit C 250 L. Also, we investigated the synthesis of (R)-mandelonitrile by using free and immobilized HNL preparations.

2. Materials and methods

2.1. Materials

Ripened *P. pseudoarmeniaca* fruits (a wild apricot) were obtained from Çukurova University, Agricultural Department. Eupergit C and Eupergit C 250 L, racemic mandelonitrile (rac-MN), benzaldehyde, sodium cyanide (NaCN), dimethylsulfoxide (DMSO), tert-buthylmethylether (TBME), acetone, pyridine, acetic anhydride, bovine serum albumin (BSA) and all other reagents and solvents were purchased from Sigma (St. Louis, MO).

2.2. Methods

2.2.1. Partial purification of HNL

Ripened wild apricot fruits were taken and the fleshy covers of its were removed. The upper layer of seeds was cracked with a hammer to obtain soft kernel inside. About 200 g of kernels were homogenized in 200 mL of cold acetone (−20 °C) by using a prechilled Waring blender for 2 min at maximum speed. The slurry was filtered and the residue was extracted with 200 mL of cold acetone. After discarding the solvent each time this procedure was repeated until a white powder was obtained. The resulting powder was dried overnight at room temperature and stored at −20 °C until use [7]. In order to obtain enzyme extract, 5 g of the powder was suspended in 50 mL of prechilled 50 mM phosphate buffer (pH 6.2) and then stirred for 1 h at 4 °C. The suspension was centrifuged at 10,000 rpm for 15 min at 4 °C. After that, the supernatant as crude enzyme extract was fractionated with $(NH_4)_2SO_4$. The precipitate fractionated at 40% saturation was collected by centrifugation at 13,000 rpm for 20 min at 4 °C. The resulting precipitate was dissolved in minimum volume of 50 mM phosphate buffer (pH 6.2), dialyzed overnight against an excess volume of the same buffer and the solution was stored at 4 °C and used as HNL solution. The protein contents of the solutions were determined by the method of Bradford [8]. BSA was used as a standard protein.

2.2.2. Immobilization of HNL

The immobilization procedure has been described earlier by Mateo et al. [6]. Briefly, 1 g of Eupergit C or Eupergit C 250 L was mixed with 9 mL of HNL solution (1 mg prot./mL) prepared in 1 M phosphate buffer (pH 7.0). The reaction was allowed to continue for 24 h at 25 °C. The mixture was shaken gently during immobilization period in a shaken water bath. After that, the resulting immobilized HNL was washed extensively with distilled water until no protein was detected in the filtrate, then stored at 5 °C. The protein contents of the solutions were determined by the method of Lowry et al. [9]. BSA was used as a standard protein. The amount of immobilized enzyme protein was estimated by subtracting the amount of protein determined in the filtrate from the total amount of protein used in immobilization procedure.

2.2.3. Lyase activity of HNL

2.2.3.1. Lyase activity assay of HNL. Lyase activity of HNL was determined spectrophotometrically by measuring the amount of benzaldehyde from rac-MN. Briefly, 2.85 mL of acetate buffer (50 mM, pH 6.0) and 0.1 mL of 1 mg/mL free enzyme or 10 mg immobilized HNL were incubated at room temperature for 2 min. The reaction was started by the addition of 0.05 mL of 30 mM rac-MN solution as substrate prepared in 95% of ethanol. At the end of 10 min, 0.5 mL of aliquots taken from the reaction mixture was diluted to 3 mL with 50 mM acetate buffer (pH 4.0) and its absorbance at 250 nm was measured immediately against blank solution containing 3 mL of 50 mM acetate buffer (OD_e).

Two controls were run in parallel one 2.95 mL acetate buffer + 0.05 mL substrate solution (OD_b) and another 2.90 mL acetate buffer + 0.10 mL enzyme solution (OD_p) and their absorbances were measured at 250 nm described above.

HNL activity was calculated from the equation,

$$\text{Activity (U/ml)} = \frac{\Delta OD}{\varepsilon \times t} \times \frac{V_t}{V_e} \qquad \Delta OD = OD_e - (OD_b + OD_p)$$

where V_t and V_e are total volume of the reaction solution and enzyme volume used in the reaction, respectively. ε is absorption coefficiency of benzaldehyde (18,777 M^{-1} cm^{-1}) at 250 nm, t is the reaction time (min).

2.2.3.2. Effect of pH onto lyase activity of HNL. The effect of pH on the activities of free and immobilized HNL preparations was determined in 50 mM acetate buffer (pH 5.0 and 5.5), citrate buffer (pH 6.0) and phosphate buffer (pH 6.5 and 7.0).

2.2.3.3. Effect of buffer concentration onto lyase activity of HNL. The effect of buffer concentration on the activities of free and immobilized HNL preparations was studied in 25, 50, 75 and 100 mM buffer solutions at predetermined optimum pH.

2.2.3.4. Effect of temperature onto lyase activity of HNL. The effect of temperature on enzyme activity was investigated in the range of 15–50 °C at their optimum pH and buffer concentrations for both free and immobilized HNLs.

2.2.3.5. Kinetic parameters for lyase activity of HNL. The enzyme activity was measured at different substrate concentrations ranging from 0.5 to 5.0 mM at predetermined optimum conditions. V_{max} and K_m values of free and immobilized HNLs were calculated from double reciprocal plot of Lineweaver–Burk.

2.2.3.6. Thermal stability for lyase activity of HNL. To investigate the thermal stabilities of free and immobilized HNLs, HNL samples were incubated at 25 and 50 °C for different incubation times (1, 2, 4, 8 and 15 h) and then their residual activities were measured as described in Section 2.2.3.1.

2.2.3.7. Operational stability of immobilized HNL for the lyase activity. Operational stability of each immobilized HNL was investigated by

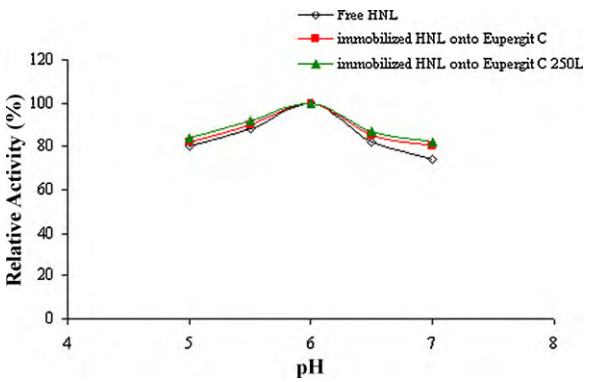

Fig. 2. The effect of pH on the lyase activities of free and immobilized HNLs.

using a batch type column reactor (1.1 × 5 cm). Twenty-five milligrams of immobilized HNL sample was loaded into the column reactor and 3 mL substrate solution was added. The reaction solution was allowed to continue for 10 min. The reaction solution was then immediately separated from the immobilized HNL sample and the amount of benzaldehyde produced was determined by the method as described before. The same experiment using the same immobilized HNL sample was repeated 20 times in every 10 min.

2.2.4. Carboligation activity of HNL

2.2.4.1. Enantioselective synthesis of mandelonitrile by HNL. Two hundred microliters of free HNL or 50 mg of immobilized HNLs were mixed with 600 μL of 400 mM citrate buffer (pH 4.0), 100 μL of 1.0 M benzaldehyde in DMSO and 1.0 mL of TBME. The reaction was started by the addition of 200 μL 1.0 M HCN solution in TBME prepared according to Bhunya et al. [10] and performed on a shaker for 24 h at 5 °C. During the reaction, the reaction mixture was agitated at 100 rpm. After completion of the reaction, 100 μL of samples were withdrawn and diluted to 0.5 mL with acylation solution containing 25 μL pyridine and 25 μL acetic anhydride in 10 mL CH_2Cl_2 and then was heated at 50 °C for 30 min. A hundred microliters of samples were withdrawn and diluted to 0.5 mL with mobile phase. The yield and ee of samples were analyzed by HPLC equipped with a UV detector and ORPak CDC 453-HQ chiral column (4.6 × 150 mm) at 220 nm. Mobile phase was ammonium acetate buffer:acetonitrile (60:40, v/v; pH 4.0), flow rate was 0.250 mL/min. The spontaneous reaction rate was also determined by carrying out the reaction under identical conditions without enzyme.

2.2.4.2. Effect of pH onto carboligation activity of HNL. The effect of pH on the carboligation activities of free and immobilized HNLs was determined in 400 mM citrate buffer at different pH values (4.0–6.0) at 5 °C.

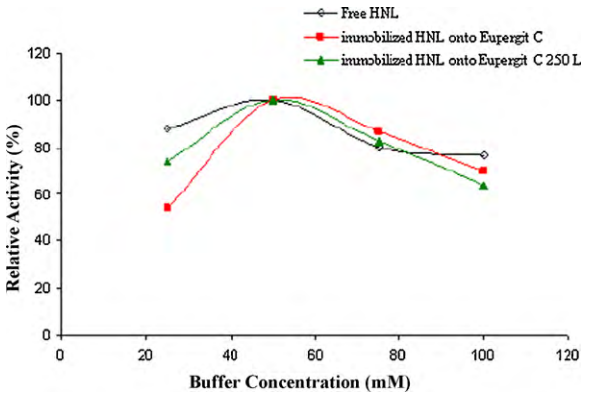

Fig. 3. The effect of buffer concentration on the lyase activities of free and immobilized HNLs.

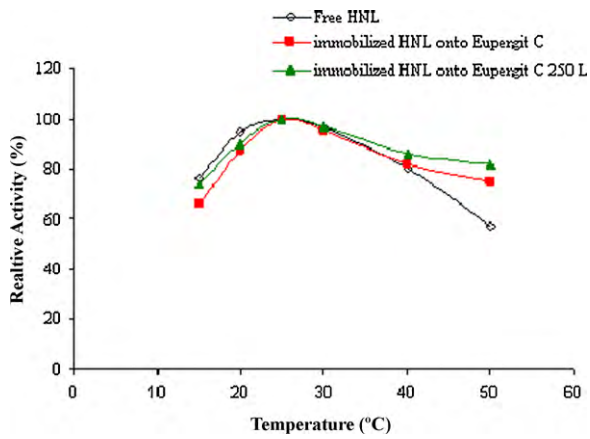

Fig. 4. The effect of temperature on the lyase activities of free and immobilized HNLs.

2.2.4.3. Effect of temperature onto carboligation activity of HNL. To investigate the optimum temperature, carboligation activities of free and immobilized HNLs were measured in the range of 5–20 °C in 400 mM citrate buffer (pH 4.0).

2.2.4.4. Operational stability of immobilized HNL for the carboligation activity. Operational stabilities of immobilized HNLs in terms of lyase activity were determined by using the same immobilized HNL sample by 10 successive 24 h reactions in standard assay conditions.

3. Results and discussions

In this study, HNL was partially purified from seeds of *P. pseudoarmeniaca*. HNL was obtained as 4.4-fold purity extract after 40% $(NH_4)_2SO_4$ precipitation and immobilized onto Eupergit supports. The amounts of bound protein onto per gram of Eupergit C and Eupergit C 250 L were determined as 7.3 and 8.8 mg of the initial total amount of protein (9 mg), respectively after immobilization.

The effect of pH on the lyase activity of free and immobilized HNLs was studied in the pH range of 5.0–7.0 because of the base-catalyzed decomposition of MN, pH dependence was only determined below pH 7.5. As shown in Fig. 2, free and immobilized HNLs both showed their maximum activities at the same pH value as 6.0. Lyase activities of free and immobilized HNLs were measured at buffer concentration range of 25–100 mM at pH 6.0 and the results were shown in Fig. 3. The optimum buffer concentration of all enzyme preparations were determined as 50 mM. In the previous studies, the optimum pH values for free HNLs isolated from other *Prunus* species were reported in the range of 5.0–7.0 [11–14].

Lyase activities of free and immobilized HNLs were measured at 15–50 °C. The optimum temperature of all enzyme preparations were determined as 25 °C (Fig. 4). Free and immobilized HNLs showed similar behaviour at 15–40 °C. However, free HNL retained about 57% of its maximum activity at 50 °C whereas immobilized HNLs onto Eupergit C and Eupergit C 250 L were retained about 75 and 82% of their maximum activities, respectively at 50 °C. In the literature, the optimum temperature for *Eriobotrya japonica* (loquat) HNL [15] and *Phlebodium aureum* HNL [14] were both reported as 40 °C by using acetone cyanohydrin as substrate.

The kinetic parameters were determined in 50 mM citrate buffer, pH 6.0 at 25 °C for all enzyme preparations. The maximum

Table 1
Effect of pH onto carboligation activity.

	pH 4.0		pH 5.0		pH 6.0	
	Yield (%)	ee (%)	Yield (%)	ee (%)	Yield (%)	ee (%)
Free HNL	100	99	63	90	30	80
Immobilized HNL onto Eupergit C	100	99	74	90	32	80
Immobilized HNL onto Eupergit C 250 L	100	99	72	90	25	80

Table 2
Effect of temperature onto carboligation activity.

	5 °C		10 °C		20 °C	
	Yield (%)	ee (%)	Yield (%)	ee (%)	Yield (%)	ee (%)
Free HNL	100	99	96	99	74	99
Immobilized HNL onto Eupergit C	100	99	89	99	87	99
Immobilized HNL onto Eupergit C 250 L	100	99	85	99	83	99

activity of free HNL was determined as 0.54 U/mg prot. and maximum activities of immobilized HNLs onto Eupergit C and Eupergit C 250 L were determined as 0.87 and 0.35 U/mg prot., respectively. K_m values were found as 2.23, 1.60 and 1.03 mM for free HNL, immobilized HNL onto Eupergit C and immobilized HNL onto Eupergit C 250 L, respectively. In the previous studies, the K_m values of different purity HNLs were reported as 0.172 mM (for *Prunus serotina* seeds), 0.290 mM (for *Prunus dilcus* seeds), 0.790 mM (for *Sorghum bicolor* seeds) and 0.093 mM (for *Prunus lyonii* seeds) for mandelonitrile [12,13].

The thermal stability of enzymes is very important parameter in industrial process. It is often observed that immobilized enzyme has higher thermal stability than the corresponding free enzyme because of the reduction of conformational flexibility in the immobilized enzyme. The half lives ($t_{1/2}$) of free HNL at 25 and 50 °C were 49.9 and 30.5 h, respectively and these correspondingly were 96.3 and 43.6 h for immobilized HNL onto Eupergit C; 138.6 and 50.2 h for immobilized HNL onto Eupergit C 250 L. The thermal inactivation rate constants (k_i) at 25 and 50 °C were calculated as 1.4×10^{-2} and 2.3×10^{-2} h^{-1}, respectively for free HNL; as 7.2×10^{-3} and 1.6×10^{-2} h^{-1}, respectively for immobilized HNL onto Eupergit C and; as 5.0×10^{-3} and 1.4×10^{-2} h^{-1}, respectively for immobilized HNL onto Eupergit C 250 L. These results showed that the thermal stabilities of immobilized HNLs were comparably higher than that of the free HNL at both temperatures. The high thermal stabilities of immobilized HNLs onto Eupergit C and Eupergit C 250 L as compared to that of free HNL may suggest the formation of multipoint covalent attachments between a high proportion of the enzyme molecule and Eupergit supports.

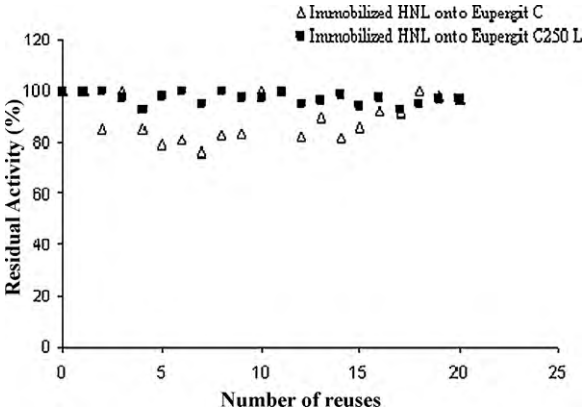

Fig. 5. The operational stabilities of immobilized HNLs for lyase activity.

The reuse numbers of immobilized enzymes are one of the most important aspects for industrial application. An increased stability could make the immobilized enzymes more advantageous than their free form. Operational stabilities of immobilized HNLs for lyase activity were determined in batch type column reactor. As shown in Fig. 5, both of immobilized HNLs were used repeatedly 20 times and the residual activities were about 97% of their initial activities.

The carboligation activities of free and immobilized HNLs were also evaluated for (R)-mandelonitrile (R-MN) synthesis from benzaldehyde and HCN. The optical configuration of synthesized R-MN was identified by comparing the retention time of synthesized R-MN with that of the optically active standard compounds. The retention times of S-MN and R-MN were 4.82 and 6.22 min, respectively.

The pH profiles of the carboligation activities of free and immobilized HNLs were investigated at different pHs and the results were

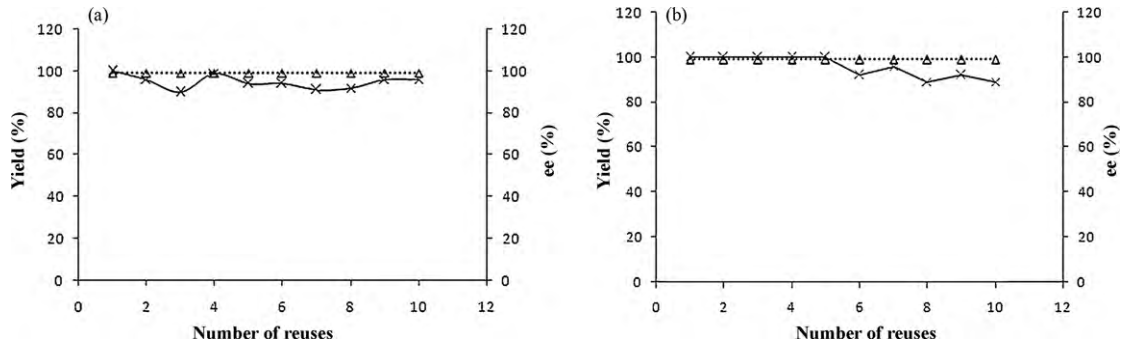

Fig. 6. The operational stabilities of immobilized HNLs for carboligation activity (×): yield %, (Δ): ee%. (a) Immobilized HNL onto Eupergit C. (b) Immobilized HNL onto Eupergit C 250 L.

given in Table 1. The maximum yield and ee% were determined at pH 4.0 for all HNL preparations. It was found that increasing the pH value above 4.0, the yield and ee were dramatically decreased due to the spontaneous chemical decomposition of the product.

As shown from Table 2, all HNL preparations showed optimal carboligation activity at 5 °C. As the temperature was increased from 5 to 20 °C the yields decreased however ee values (99%) unchanged. In the literature, Nanda et al. [16] investigated the enantioselective synthesis of cyanohydrins using partially purified free HNL from *Prunus mume* and the yield and ee of R-MN were determined as 65 and 95%, respectively. Ueatrongchit et al. [17] reported that the optimum temperature was 10 °C for R-MN synthesis by using *Passiflora edulis* HNL, increasing temperature accelerated the nonenzymatic reaction, leading to low ee of product.

The operational stabilities of immobilized HNLs in terms of carboligation activity were investigated and the results were presented in Fig. 6. The residual activities of HNLs immobilized onto Eupergit C and Eupergit C 250 L in the 10th batch were 96 and 90% of the initial batch, respectively and both of immobilized HNLs showed 99% ee after 10 batches.

4. Conclusions

The present study demonstrates that

(a) The *P. pseudoarmeniaca* is a good source of HNL and *P. pseudoarmeniaca* HNL is very powerful tool in the synthesis of (R)-mandelonitrile.

(b) Eupergit C and Eupergit C 250 L are suitable carriers for HNL immobilization. The thermal stability and reusability of HNL were improved upon immobilization onto Eupergit C and Eupergit C 250 L.

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References

[1] A. Nahrstedt, Plant Syst. Evol. 150 (1985) 35–47.
[2] S. Tükel, D. Alagöz, Food Chem. 111 (2008) 658–662.
[3] T. Boller, C. Meier, S. Menzler, Org. Process. Res. Dev. 6 (2002) 509–519.
[4] E. Katchalski-Katzir, D.M. Kraemer, J. Mol. Catal. B Enzyme 10 (2000) 157–176.
[5] M.J. Hernaiz, D.H.G. Crout, Enzyme Microb. Technol. 27 (2000) 26–32.
[6] C. Mateo, G. Fernandez-Lorente, O. Abian, R. Fernandez-Lafuente, J.M. Guisan, Biomacromolecules 1 (2000) 739–745.
[7] M.Y. Coseteng, C.Y. Lee, J. Food Sci. 52 (1987) 985–989.
[8] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[9] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[10] R. Bhunya, T. Mahapatra, S. Nanda, Tetrahedron: Asymmetry 20 (2009) 1526–1530.
[11] A. Hickel, M. Hasslacher, H. Griengl, Physiol. Plant 98 (1996) 891–898.
[12] L.L. Xu, B.K. Singh, E.E. Conn, Arch. Biochem. Biophys. 250 (1986) 322–328.
[13] R.S. Yemm, J.E. Poulton, Arch. Biochem. Biophys. 247 (1986) 440–445.
[14] H. Wajant, S. Forster, D. Selmar, F. Effenberger, K. Pfizenmaier, Plant Physiol. 109 (1995) 1231–1238.
[15] T. Ueatrongchit, A. Kayo, H. Komeda, Y. Asano, A. H-Kittikun, Biosci. Biotechnol. Biochem. 72 (2008) 1513–1522.
[16] S. Nanda, Y. Kato, Y. Asano, Tetrahedron 61 (2005) 10908–10916.
[17] T. Ueatrongchit, K. Tamura, T. Ohmiya, A. H-Kittikun, Y. Asano, Enzyme Microb. Technol. 46 (2010) 456–465.