Enantioselective Esterification Reaction Using Immobilized *Candida rugosa* Lipase on Poly(*N*-vinyl-2-pyrrolidone-*co*-styrene) Hydrogel

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ABSTRACT: Lipase from *Candida rugosa* was immobilized on poly(*N*-vinyl-2-pyrrolidone-*co*-styrene) hydrogel (poly-(VP-*co*-ST)) with ethylene dimethacrylate and α,α'-azoisobutyronitrile, which act as crosslinker and initiator, respectively. Three different compositions of monomers were used, namely VP(%):ST(%), 10:90, 50:50, and 70:30 (wt(%)/wt(%)). The immobilized lipases were used in the enantioselective esterification of (*R*,*S*)-2-(4-chlorophenoxy)-propanoic acid with *n*-tetradecanol. The optimum reaction condition of the enantioselective esterification for the native lipase and the poly(VP-*co*-ST) hydrogel immobilized lipases was determined with respect to temperature, solvents, and initial water activity (a_w). The optimum temperature obtained was 40°C, with the poly(VP-*co*-ST) hydrogel immobilized lipase VP(%)/ST(%):10:90 showing the highest enantiomeric excess. In the solvent effect studies, the best solvents for high enantioselectivity were chloroform and carbon tetrachloride. In the a_w studies, optimum α_w for NL, VP(%):ST(%), 10:90, and 50:50 was 0.328, while for VP(%): ST(%), 70:30, it was 0.55. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 92: 3381–3386, 2004

Key words: lipase; hydrogels; esterification; enantioselectivity

INTRODUCTION

It is well known that the immobilization process is one of the alternative approaches for yielding a better enzyme that is active in organic solvents and at high temperature. This technique makes enzyme use in industries more attractive because it offers certain processing advantages over free enzymes, for example, ease of separation from the product, improved stability, and continuous operation. There are many immobilization techniques available,[1] but one of the popular methods used is entrapment. Generally, this method is done either by the inclusion of enzymes within polymeric matrices or by separation from the bulk phase by semipermeable microcapsules. Hydrogel can be used as a polymeric matrix to entrap the enzymes, where it is made from hydrophilic and/or hydrophobic monomers that can be a copolymer or homopolymer. The hydrophobicity/hydrophilicity of the polymer can be chosen in such a way that it can form an open cross-linked hydrogel so as to allow the solvent to move freely; this enables the substrate to interact with the enzyme easily. The most remarkable aspect of this method is that the enzymes are not actually attached to anything.[2] Therefore, no stearic problems are encountered and the active site, which is the most important part of the catalyst for the reaction to take place, is not obstructed by a portion of the polymer matrix.

Lately, the production of pure chiral compounds, which are known as optically active materials, has been very important in the medical and agricultural fields because these compounds have unique properties and thus are used as anti-inflammatory agents, chiral drug intermediates, hormones, and herbicides. Usually only one of these enantiomers is active while the other may be toxic, and thus the production of pure enantiomer is very important. The pure enantiomer can be prepared by separation of the racemic compounds by chemical methods. However, the laborious steps and the expensive and sometimes toxic chemicals needed to produce the enantiomers mean much higher production costs. An alternative method, which uses enzymes as catalyst to produce enantiomers via enantioselective synthesis, can be carried out, because enzymes are known to be more enantioselective compared to chemical catalysts. Furthermore, the enantioselectivity of the enzyme can be further enhanced by enzyme modification techniques such as immobilization on various matrices.

In this work, immobilization of the enzyme *Candida rugosa* lipase by entrapment on a poly(*N*-vinyl-2-pyr-

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rolidone-*co*-styrene) (poly(VP–*co*-ST)) hydrogel was carried out. The immobilized lipases were used in the enantioselective esterification of (*R,S*)-2-(4-chlorophenoxy)-propanoic acid and 1-tetradecanol. The *R*-enantiomer of this compound is an important intermediate for the synthesis of herbicide.[3]

EXPERIMENTAL

Materials

Lipase from *C. rugosa* (Type VII), the monomers, *N*-vinyl-2-pyrrolidone (VP) and styrene (ST), and the crosslinker, ethylene dimethacrylate (EDMA) were obtained from Sigma Chemical Co. (St. Louis, MO). The initiator, α,α'-azoisobutyronitrile (AIBN) and the substrate, 1-tetradecanol, were purchased from Fluka Chemical (Buchs, Switzerland). The substrate, (*R,S*)-2-(4-chlorophenoxy)-propanoic acid, was from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were of analytical grade.

Purification of monomers

The monomers VP and ST were purified by filtering them through a column containing aluminum oxide (2.5 × 10.0 cm) until colorless products were obtained. EDMA was used as purchased.

Preparation of lipase solution

C. rugosa lipase (0.5 g) was dissolved in distilled water (10.0 mL). This mixture was agitated on a vortex mixer and was centrifuged at 13,000 rpm for 10 min. The solid suspension was removed and the supernatant was collected and used for lipase immobilization.

Immobilization of lipase

The purified monomers VP and ST, of varying weight percentage composition, were mixed together with 1% EDMA (wt %) in a clean, dry flask. The composition of the hydrogels prepared were VP(%):ST(%) (% = wt % of the monomer in total weight of VP + ST) 10:90, 50:50, and 70:30. To these mixtures a dry initiator, AIBN (10^{-4} mol), was added and the flasks were shaken until the AIBN was dissolved. The mixtures were then transferred to a polymerization tube and the solutions were degassed with nitrogen for 15 min to remove any oxygen present. The mixtures were incubated to polymerize in a 55–60°C water bath. After the polymer solution became viscous (1–4 h), the polymers were cooled to 50°C, the lipase solution (1.0 mL), which was previously degassed with nitrogen, was added, and the polymer solution was shaken until a homogeneous solution was obtained. The solution in the polymerization tube was sealed with a rubber stopper and further polymerized at 50°C for about 5 h. The solid polymerized rods were removed from the polymerization tubes. These rods were cut into small pieces (0.2–0.4 cm^3) and stored at 0°C prior to use.

Protein assay

The protein content of hydrogel-immobilized lipases was determined using the method of Bradford[4] with bovine serum albumin as the standard. For the blank determination, a poly(VP-*co*-ST) hydrogel without lipase was used.

Enantioselectivite reaction

The reaction system consisted of native lipase (lyophilized, water extracted *C. rugosa* lipase) (30mg) or hydrogel-immobilized lipase (0.5 g), (*R,S*)-2-(4-chlorophenoxy)-propanoic acid (200 mM), 1-tetradecanol (200 mM), and carbon tetrachloride (10 mL). The mixture was incubated at 30°C (or as otherwise stated) and shaken in a horizontal water-bath shaker at 120 rpm. Aliquots of the sample were taken periodically for gas chromatography analysis. The reaction was terminated by separating the mixture from hydrogel-immobilized lipase by filtration. The solvent in the sample mixture containing the products was removed by evaporation with a rotary evaporator (Eyela SB-650, Japan) under vacuum (Heto Sue-3e, Sweden). The product containing the ester produced and the remaining acid and alcohol was then obtained.

Purification of the ester formed and the remaining acid and alcohol

The product containing the ester, remaining acid, and alcohol was separated into single components through column chromatography (silica gel 60, 70–230 mesh, 2.5 × 10 cm). Eluent solvents used were *n*-hexane/ethyl acetate, 10:1 (v/v) for esters; *n*-hexane/ethyl acetate, 4;1 (v/v) for alcohols, and *n*-hexane/ethyl acetate/acetic acid, 20:5:1 (v/v) for (*R,S*)-2-(4-chlorophenoxy)-propanoic acid. The solvent in each component was again removed using rotary evaporator.

Analysis of (S)-acid, alcohol, and ester

The optical purity of the remaining (*S*)-acid was determined from optical rotation (α) readings using a Jasco digital polarimeter Model DIP-370 (Japan) at 25°C. Enantiomeric excess (% ee) of the remaining (*S*)-acid was determined from the specific rotation, $[\alpha]$, where $[\alpha] = \alpha/(C\ X\ L)$ where C = mg/mL, L = length of cell (cm), compared with the value of $[\alpha]^{25}$ = -40.1^o (specific rotation of pure (*S*)-acid at C = 1, 25°C in ethanol) obtained from Beilsteins Handbuch (Beilstein Institut, Frankfurt, FRG).

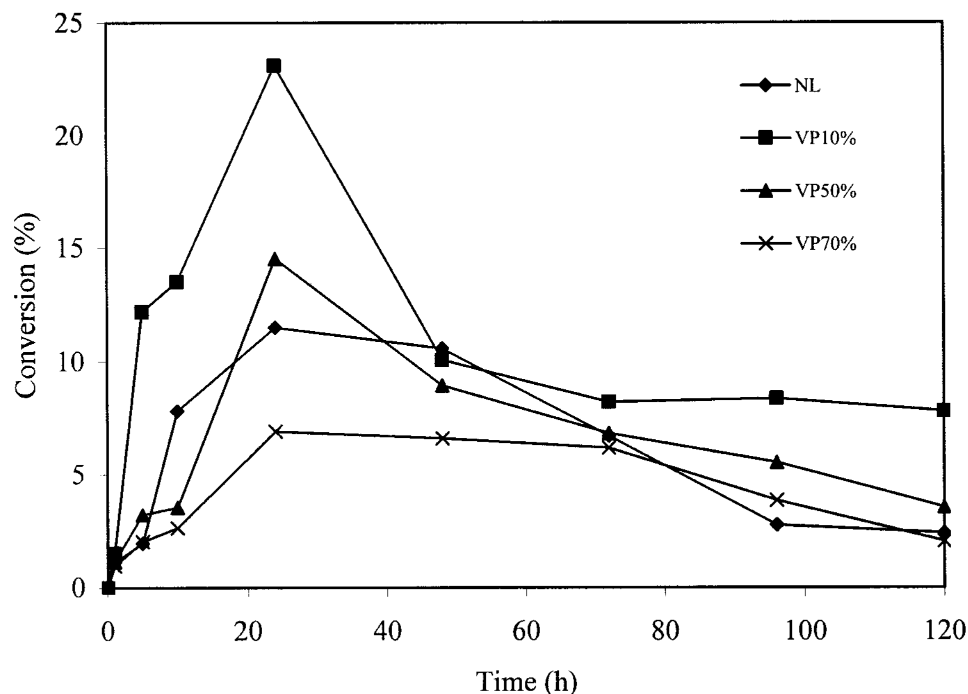

Figure 1 Time course study on the percentage of ester conversion of (VP-*co*-ST) hydrogel-immobilized lipase reaction at 30°C in CCl_4. NL, native lipase; VP10%, VP50%, VP70%, hydrogels with compositions VP(%)/ST(%), 10:90, 50:50, and 70:30 [% = % of monomer in total weight of (VP + ST)].

The amounts of the remaining alcohol and ester produced were determined by gas chromatography (Hitachi G3000, Japan) with a flame-ionization detector using a 30-m very polar capillary column, AT-SILAR (0.32 mm, id), from Alltech (Australia). The injector and the detector were set at 250°C. The initial column temperature was at 150°C. The temperature was increased at 8°C/min to 200°C. *n*-Heptadecane was used as internal standard. The percentage of ester conversion was calculated using the Internal Standard method.

Effect reaction time

The reaction mixture was shaken in a horizontal water-bath shaker at 120 rpm for 1, 2, 3, 4, 5,10, 24, 48, 72, 96, 120, 144, 168, 192, and 240 h. The percentage yield of the ester produced was determined as described earlier.

Effect of temperature

The reaction mixture was incubated at 30, 40, 50, 60, and 70°C for 24 h with continuous shaking in horizontal water-bath shaker at 120 rpm. The % ee of the remaining acid was determined as described earlier.

Effect of different organic solvents

The reaction system consisted of native lipase or hydrogel-immobilized lipase and the substrates and solvents (10 mL) with different log P values; benzene (log P = 2.0), chloroform (log P = 2.0), toluene (log P = 2.5), carbon tetrachloride (log P = 3.0), hexane (log P = 3.5), heptane (log P = 4.0), and isooctane (log P = 4.7). The reaction was incubated at 40°C. The % ee of the remaining acid was determined as described earlier.

Effect of initial water activity (a_w)

The immobilized lipase and the substrates were pre-equilibrated separately in containers containing different saturated salt solutions; LiCl, $MgCl_2 \cdot 6H_2O$, $Mg(NO_3)_2.6H_2O$, KI, KCl, and KNO_3 with a_w's of 0.12, 0.328, 0.55, 0.689, 0.869, and 0.960, respectively. The pre-equilibration process was carried out overnight. The reaction was incubated at 40°C. The % ee of the remaining acid was determined as described earlier.

RESULTS AND DISCUSSION

Effect of reaction time

The effect of reaction time on the percentage of ester conversion of the native lipase and hydrogel-immobilized lipases is shown in Figure 1. Generally, the percentage conversion of 2-(4-chlorophenoxy)-propanoate increases with time for native lipase and all the hydrogel-immobilized lipases. However, after 24 h, the conversion decreased gradually until it reached 120 h. Hence, 24 h was the optimum reaction time for

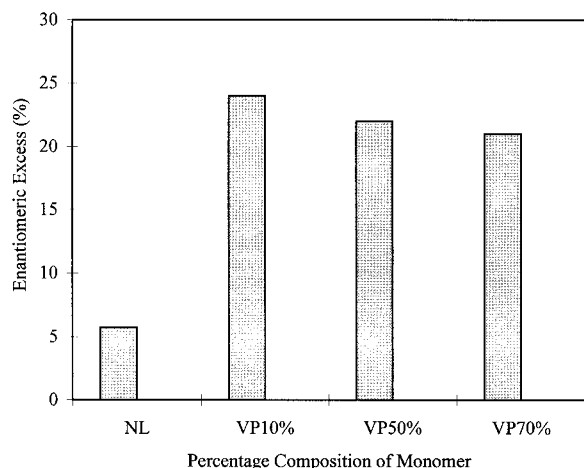

Figure 2 Enantioselectivity of (VP-*co*-ST) hydrogel-immobilized lipase reaction at 30°C for 24 h in CCl_4. NL, native lipase; VP10%, VP50%, VP70%, hydrogels with compositions VP(%)/ST(%), 10:90, 50:50, and 70:30 [% = % of monomer in total weight of (VP + ST)].

the conversion of 2-(4- chlorophenoxy)-propanoate. In the case of the hydrogel-immobilized lipases, the (VP-*co*-ST) hydrogel with composition of monomers (VP)%: (ST)%; 10:90, exhibited the highest percentage of ester conversion at the optimum reaction time.

Generally, immobilization of lipase onto hydrogel with different compositions of VP and ST showed that, as the composition of VP, which corresponded to the high hydrophobicity, decreased the percentage conversion of ester increased. The (VP-*co*-ST) hydrogel-immobilized lipase with higher composition of ST may absorb more solvent, which helps the diffusion of organic solvents in the hydrogel and therefore the enzyme has more chance to attack the substrate that is soluble in the solvents. In contrast, the lower percentage of ester conversion when hydrophilic (VP-*co*-ST) hydrogel-immobilized lipase was used may be due to the water layer around the enzyme being stripped off by the hydrophilic hydrogel. The lack of the surrounding water will prevent the mantainance of the three-dimensional structure of the enzyme, which may reduce the catalytic activity of the enzyme. Hence, it may also reduce the conversion of ester. A similar result was reported by Basri et al.[2] The decrease in the percentage conversion of ester after 24 h may be due to the reversible reaction that has occurred since the reaction is a reversible reaction.

Effect of different composition of monomers

Poly(VP-*co*-ST) hydrogel-immobilized lipases showed a higher value of enantiomeric excess (% ee) compared to native lipase (Fig. 2). The immobilized lipases were more stereoselective toward (*R*)-2-(4-chlorophenoxy)-propanoic acid to form (*R*)-2-(4-chlorophenoxy)-propanoate compared with native lipase. The % ee value played an important role in recognizing the optical purity of the product formed, whereby it indicated how much of the (*S*)-acid was left in the reaction medium. That is why the enzyme with enantiomeric preference toward the (*R*)-acid would form more (*R*)-ester, thus leaving a higher % ee of (*S*)-acid after the reaction.

The highest percentage of enantiomeric excess was obtained from the poly(VP-*co*-ST) hydrogel-immobilized lipase (VP)%(ST)%; 10:90, which corresponds to higher hydrophobicity of the hydrogel. Increasing the hydrophobicity of the respective hydrogel caused the rigidity properties to be increased and the flexibility to be decreased; hence, one substrate was recognized better than others. Therefore, that substrate may display the right conformation for higher selectivity compared to the others. As the hydrophobicity of the hydrogel-immobilized lipases decreased, it seemed that the % ee also decreased, that is 22% for hydrogel-immobilized lipase (VP)%(ST)%; 50:50, and 21% for (VP)%(ST)%; 70:30, respectively. This result might be because the more hydrophilic hydrogel-immobilized lipase tends to restrain the recognition of one substrate and therefore it may prevent the right conformation for higher selectivity. This result is in agreement with the result reported by Bastida *et. al.*[5], whereby the enantioselectivity was strongly improved when using the lipase from *Pseudomonas fluorescens* immobilized on the actyl agarose (hydrophobic gel) to catalyze the hydrolysis of ethyl α-hydroxyphenyl butyrate. The results also indicated that the hydrophilic hydrogel, which was more flexible, would make the dimensional mesh and porous network unstable and therefore unable to hold the enzyme in its place[6]; therefore, it may provoke the enzymes not to identify the right conformation to select the target substrate.

Effect of temperature

As shown in Figure 3, the optimum temperature for high enantioselectivity seemed to be 40°C, whereby the poly(VP-*co*-ST) hydrogel-immobilized lipases exhibited higher % ee compared to native lipase. However, the % ee decreased gradually until the reaction temperature reached 70°C. The poly(VP-*co*-ST) hydrogel-immobilized lipase with the composition of monomer (VP)%:(ST)%; 10:90, exhibited the highest value of % ee at optimum reaction time.

As the temperature was increased, the enantioselectivity drastically decreased. This result is due to the higher temperature, which may interfere with the controlled porosity of the gel, thus, to prevent the rigidity of the enzyme. This particular process may decrease the high enantiomeric preference to the target substrate. Moreover, higher temperature may also denature the enzyme, although ultimately, it might change

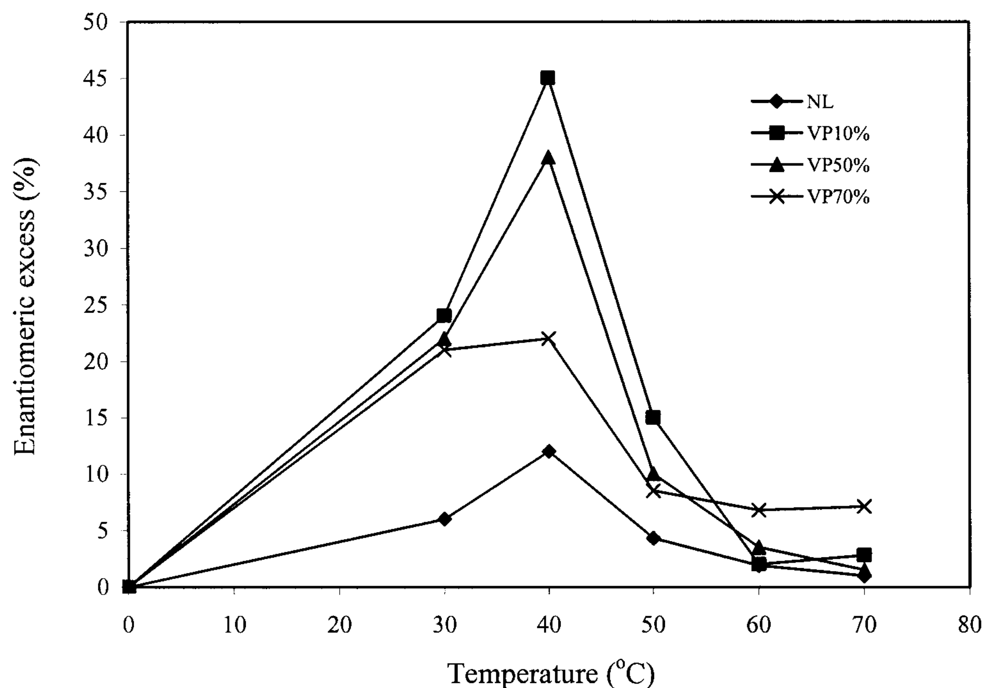

Figure 3 Enantioselectivity of (VP-*co*-ST) hydrogel-immobilized lipase reaction at 30°C, 24 h in CCl_4 at different reaction temperatures. NL, native lipase; VP10%, VP50%, VP70%, hydrogels with compositions VP(%)/ST(%), 10:90, 50:50, and 70:30 [% = % of monomer in total weight of (VP + ST)].

the enzyme conformation, thus resisting the enantiomeric preference on the enzyme to the target substrate. Besides, Wu et al.[7] reported that the percentage of % ee of menthyl propionate decreased when temperature increased. Philips[6] also reported that the higher temperature would lead to lower enantiomeric purity in the product and, that lower temperature would yield higher enantiomeric purity.

The effect of organic solvents

In general, the correlation of the % ee on the effect of log *P* of different organic solvents for native lipase and hydrogel-immobilized lipases is not very well defined (Fig. 4). The percentage of % ee of all hydrogel-immobilized lipases seemed to be higher than that of native lipase. The enantioselectivity was relatively higher in carbon tetrachloride for all immobilized lipases. The highest enantioselectivity was exhibited by hydrogel-immobilized lipase (VP)%:(ST%); 10:90, at 46%, % ee. In chloroform, hydrogel-immobilized lipase (VP)%:(ST%); 50:50, showed the highest % ee at 50%. It seemed that enantioselectivity could be enhanced by using certain solvents and/or immobilizing the enzyme on polymers made up of different composition of monomers. On the other hand, the % ee was lower in hexane, heptane, and isooctane (log *P*, 3.5 to 6.0). The lower enantioselectivity in benzene and toluene could not be explained.

The effect of initial water activity (a_w)

Generally, all lipases showed an increase in % ee as a_w increased until an optimum a_w, whereby hereafter the % ee decreased as the a_W further increased (Fig. 5). The optimum a_w for NL, VP(%):ST(%), 10:90 and 50:50. was 0.328 while that for VP(%):ST(%), 70:30, was

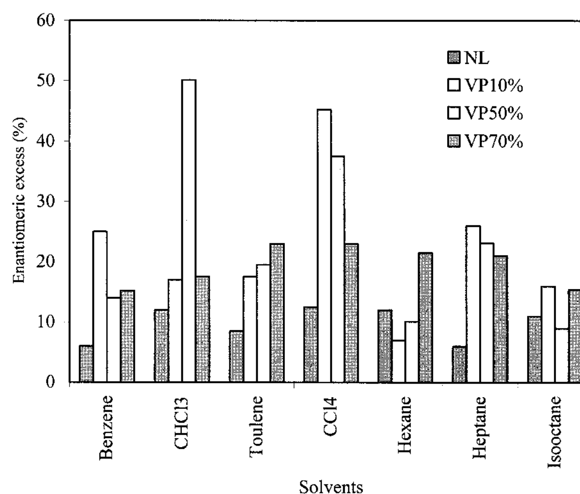

Figure 4 Enantioselectivity of (VP-*co*-ST) hydrogel-immobilized lipase reaction at 30°C, 24 h in different organic solvents. NL, native lipase; VP10%, VP50%, VP70%, hydrogels with compositions VP(%)/ST(%), 10:90, 50:50, and 70:30 [% = % of monomer in total weight of (VP + ST)].

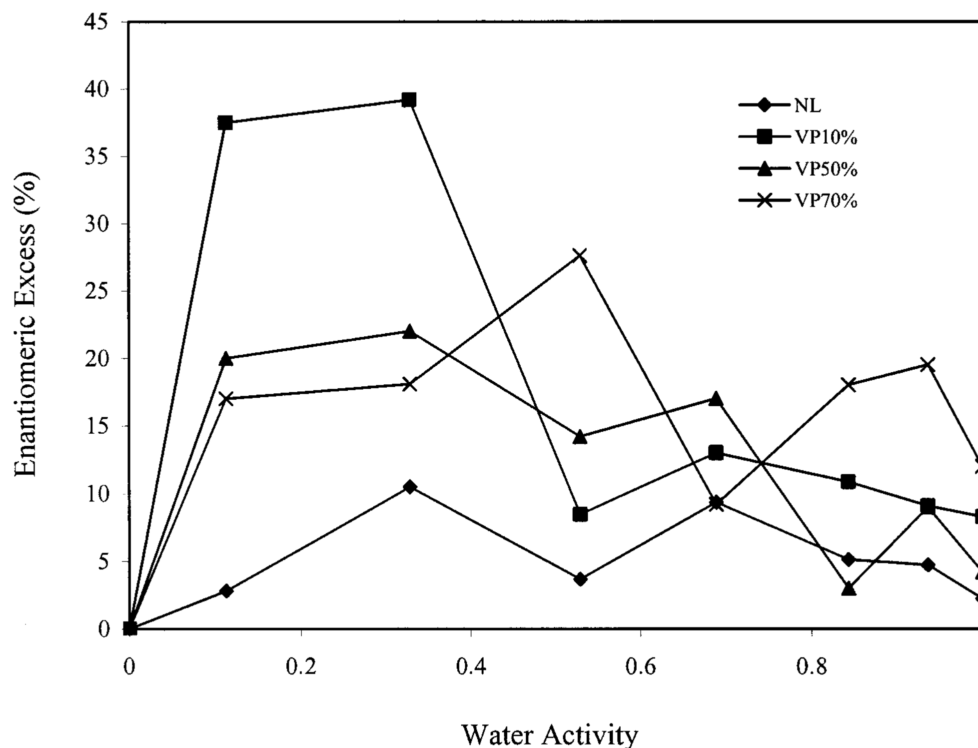

Figure 5 Enantioselectivity of (VP-*co*-ST) hydrogel-immobilized lipase reaction at 30°C, 24 h in different water activity. NL, native lipase; VP10%, VP50%, VP70%, hydrogels with compositions VP(%)/ST(%), 10:90, 50:50, and 70:30 [% = % of monomer in total weight of (VP + ST)].

0.55. At optimum a_w, all poly(VP-*co*-ST) hydrogel-immobilized lipases showed higher enantioselectivity compared to native lipase whereby (VP: ST)%, 10:90, exhibited the highest percentage of enantioselectivity. The results suggested that immobilization might improve the enantioselectivity of the enzymes under all different a_w conditions.

The increment of the percentage of % ee in the a_w ranging from 0.12 to 0.328 may be due to the increasing of a_w, which led to the right hydration level of the enzymes; thus, the enzymes would have the right conformation to recognize one substrate over the other. The result reported is in agreement with the result reported by Jonsson et al.[8] On the other hand, a_w ranging from 0.69 to 1.0, which corresponds to an even higher a_w level, suggested that at this position the enzymes were fully hydrated (too much water) and thus rendered the enzyme to have less rigidity and therefore diminution of enantioselectivity.

CONCLUSION

Our results affirmed that immobilization of lipase on (VP-*co*-ST) hydrogels enhanced its enantioselectivity at moderate temperature and controlled initial water activity in certain organic solvents. These immobilized lipases may thus have potential for use as a niche biocatalyst for the preparation of many chiral compounds.

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