IMMOBILIZATION OF *CANDIDA RUGOSA* LIPASE ONTO N-VINYL-2-PYYROLIDONE – CO-STYRENE FOR USE IN ENANTIOSELECTIVE ESTERIFICATION

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Abstract: Lipase from *Candida rugosa* was immobilized onto N-vinyl-2-pyyrolidone –co-styrene (VP-co-ST) hydrogel by an entrapment method. The percentage composition of monomer used to synthesize the VP-co-ST hydrogel is (VP:ST)% ; 10:90 . The VP-co-ST hydrogel immobilized lipase was used to catalyze the enantioselective esterification of *(R,S)*-2-(4-chlorophenoxy) propanoic acid with n-tetradecanol to produce *(R)*- 2- (4-chlorophenoxy) propanoate and *(S)*- 2- (4-chlorophenoxy) propanoic acid. The VP-co-ST hydrogel immobilized lipase with the percentage of monomers 10:90 had beed chosen since it exhibited higher enantioselectivity compared with free lipase at optimum time 24 hours. The optimum temperature for the VP-co-ST hydrogel immobilized lipase and free lipase to catalyze the enantioselective esterification is at 40°C whereby the VP-co-ST hydrogel immobilized lipase exhibited higher enantioselectivity (enantiomeric excess) at 45%. The best solvent media for both lipases to catalyze the same reaction when using carbon tetrachloride, in which VP-co-ST hydrogel immobilized lipase showed higher percentage of enantioselectivity that is almost 50% compared to free lipase.

Keywords: Immobilized lipase, Hydrogel, Entrapment

INTRODUCTION

Enzymes are biological catalysts that allow chemical reactions to occur in living organism at ambient condition. The ability of enzymes compared to any catalysts is that enzymes catalyze the reaction under mild condition at normal temperature, pressure and with high substrate specificity. Chibata [1] reported that most enzymes have been applied in microbiology and genetic engineering.

Because of enzymes are generally unstable in organic solvent or at elevated temperature, immobilization is one of the best method to produce a modified enzyme which finally can withstand in organic solvents or high temperature. Moreover, based on Bailey and Ollis [2] using immobilized enzymes are more effectives such as an ease of separation from the product, improved stability, continuous operation and possibility of obtaining superacivity.

Since immobilized enzymes are now very important catalysts in industries, the immobilization technique especially entrapment can be used because enzyme entrapped in the polymer possess no stearic problems or obstructed the active site of enzyme which is important for catalytic activities. Trevan [3] reported that by using three-dimensional gel the enzymes could be protected from environmental stress such as pH, temperature, solvents, inhibitors and poison.

The most attractive characteristic that makes enzyme far superior to conventional catalyst is their high enantioselectivity. Enzymes often exhibited a high selectivity for a target substrate, thus it can be used as a biocatalyst for the resolution of chiral compound such as *(S)*-ibuprofens and anti-cancer drugs. Santoyo [4] reported that with recent development of enzyme technology, the immobilized enzyme could be utilized to produce the optically active materials by the enantioselective esterification. The gel was chosen as a support material because the uniform size can be formed by a mild and simple immobilization procedure.

In this work, the immobilized lipase was prepared by entrapped the *Candida rugosa* lipase in the VP-co-ST hydrogel which is used to catalyze the production of *(S)*-2-(4-chlorophenoxy) propanoic acid (chiral compound) form its racemic mixture via enantioselective esterification of racemic acid *(R,S)*-2-(4-chlorophenoxy) propanoic acid with n-tetradecanol. The effect of temperature and organic solvents were investigated.

MATERIALS AND METHODS

Purification of Monomers and Water Extraction of Lipase

Before the polymerization was carried out, the monomer Styrene and VP were purified by filtration via a glass column, which was prepared before. The filtration was repeated until no colour was detected.

For water extraction lipase, 0.5g of commercial lipase powder was dissolved in 1 mL of distilled water and stirred for 5 minutes. After that the lipase solution was centrifuged at 13000 rpm for 5 minutes. After centrifugation, the solid suspension was discarded and the supernatant was collected and stored at –4°C prior to use.

Polymerization and Immobilization of Lipase

A total weight of 10g monomers was mixed in a test tube. Into the polymer mixture, a crosslinker EDMA was added and followed by AIBN to initiate the polymerization process. The polymer mixture was degassed with nitrogen for 20 minutes. After degassing, the mixture was incubated in water bath at temperature 50°C - 60°C until the polymer mixture become viscous. As well as the viscosity of the polymer solution was satisfied, the lipase solution that was previously degassed with nitrogen was added into the polymer solution. Again, the mixture was incubated at temperature below 50°C until the solid hydrogel immobilized lipase formed. The hydrogel was cut into pieces or disk shape ant stored at 0°C before used.

Enantioselective Esterification Reaction

The reaction system consisted of 0.5g VP-co-ST hydrogel immobilized lipase, 200mM (*R,S*)-2-(4-chlorophenoxy) propanoic acid, 200mM 1-tetradecanol in 10mL organic solvents. The mixture was immediately shaken at 120 rpm at 30°C. When the shaking was over, filtrating out the hydrogel from the solution mixture to terminate the reaction. The solvent in the filtrate was removed by evaporation with rotary evaporator until the yellowish viscous oil was obtained. This oily solution was actually consisted of ester, alcohol and remaining acid. The remaining acid was separated through chromatographic separation.

Chromatographic Separation

The yellowish viscous oil was placed on top of the glass column, which was prepared before. In order to separate the ester and alcohol from the mixture, an eluent solvent of n-hexane:ethyl acetate (8:2, v/v) was used and for acid the eluent was n-hexane: ethyl acetate: acetic acid (10:3:1,v/v/v). Because of ester was less polar, it will be eluted first, followed by alcohol and lastly the acid. The Thin Layer Chromatography (TLC) was used to detect the fraction of the remaining acid. For acid, the developing solvent, n-hexane: ethyl acetate: acetic acid (10:3:1, v/v/v) was used. The solvent in acid fractions was discarded by using rotary evaporator until white acid crystal remained in the flask.

Measurement of Optical Rotation

The optical rotation, α of the remaining acid was measured in absolute ethanol with polarimeter. In this method, the acid crystal was dissolved in 15 mL absolute ethanol and was poured into the cell and placed in the polarimeter. The rotational angle of the remaining acid solution was detected by the polarimeter reading. From the value of the rotational angle, the enantiomeric excess (ee) can be calculated by using the following equation:

$$\%ee = [\alpha] / -40.1^{\circ} \times 100$$

$$[\alpha] = \alpha / c \times l$$

$[\alpha]$ = specific rotational angle
α = optical rotation from polarimeter reading
c = concentration of acid solution
l = the length of the cell

Characteristic of Lipase

- Time Course Study
  The seal vials which were consisted of esterification mixture were shaken conat 120 rpm for 1,2,3,4,5,10,24,48,72,96,120 hours to determine the optimum reaction time for immobilized lipase and free lipase.

- Effect of Temperature
  The reaction mixtures in the seal vials were shaken at 120 rpm at various temperatures (30°C, 40°C, 50°C, 60°C, 70°C) for 24 hours separately. For each of the temperature, the enantioselectivity (enantiomeric excess) was determined, as it had been discussed before.

- Effect of Organic Solvents
  The reaction mixtures along with organic solvent carbon tetrachloride were shaking continuously at 120 rpm for 24 hours. The enantioselectivity was determined as discussed before. The experiment was repeated with other organic solvents such as chloroform, benzene, toluene, hexane, heptane and isooctane.

RESULTS AND DISCUSSIONS

Effect of Reaction Time On the Enantioselective Esterification

The enantioselective esterification of *(R,S)*-2-(4-chlorophenoxy) propanoic acid with 1-tetradecanol catalyzed by VP-co-ST hydrogel immobilized lipase and free lipase were carried out at different reaction time. From the result (Figure 1), the conversion of ester 2-(4-chlorophenoxy) propanoate increases as the time of reaction increases. After 24 hours, the conversion decreases slowly until it reaches 120 hours. Hence, the reaction time 24 hours has become the optimum reaction time for the enantioselective esterification.

As shown in Figure 1, the VP-co-ST hydrogel immobilized lipase catalyzed the conversion of ester 2-(4-chlorophenoxy) propanoate at 24 hours more effective rather than free lipase. The VP-co-ST hydrogel used for immobilizing the lipase was a rigid support which might held the enzyme in one position, thus provided stability for the enzyme structure. The higher the stability of VP-co-ST hydrogel immobilized lipase over a free lipase, the more effective it catalyzed the reaction. The result was quite similar to those reported by Pan et al [5] which stated that by using immobilized lipase from celite-absorbed lipase, the percentage of ester formed was much higher, more than 50% after 72 hours.

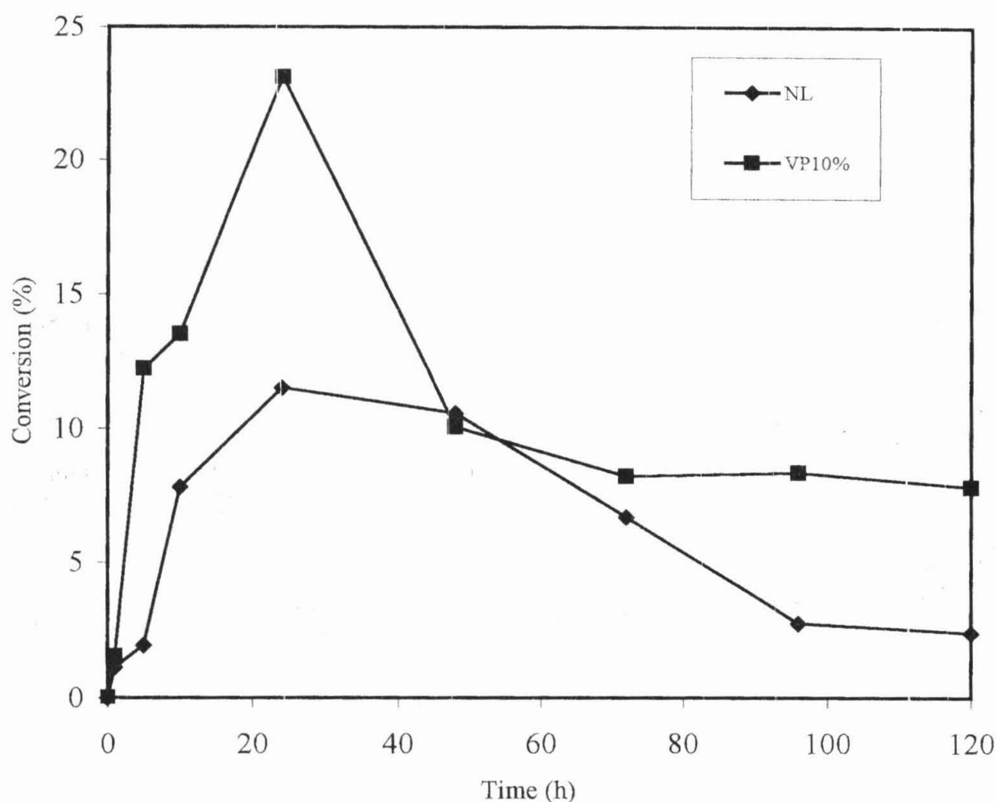

Figure 1: Effect of Reaction Time on Enantioselective Esterification

The Effect of Temperature on The Enantioselective Esterification

The VP-co-ST hydrogel immobilized lipase and free lipase catalyzed the enantioselective esterification of (R,S)-2-(4-chlorophenoxy) propanoic acid with 1-tetradecanol were carried out at different temperatures. It was observed (Figure 2) that temperature could affect the enantioselectivity. For both lipases, the percentage of enantiomeric excess increases up to 40°C and after that its percentage decreases gradually until the reaction temperature reached 70°C. It was also discovered that VP-co-ST hydrogel immobilized lipase has higher percentage of enantiomeric excess than free lipase. The results also proved that VP-co-ST hydrogel immobilized lipase has an effort to enhance the enantioselectivity. Moreover, the trend of the results was quite similar with Wu et al [6] who's reported that the percentage of enantiomeric excess of menthyl propionate decreased when temperature was increased. The higher temperatures would lead to lower enantiomeric purity in the product and conversely, the lower temperatures would yield higher enantiomeric purity. Philips [7].

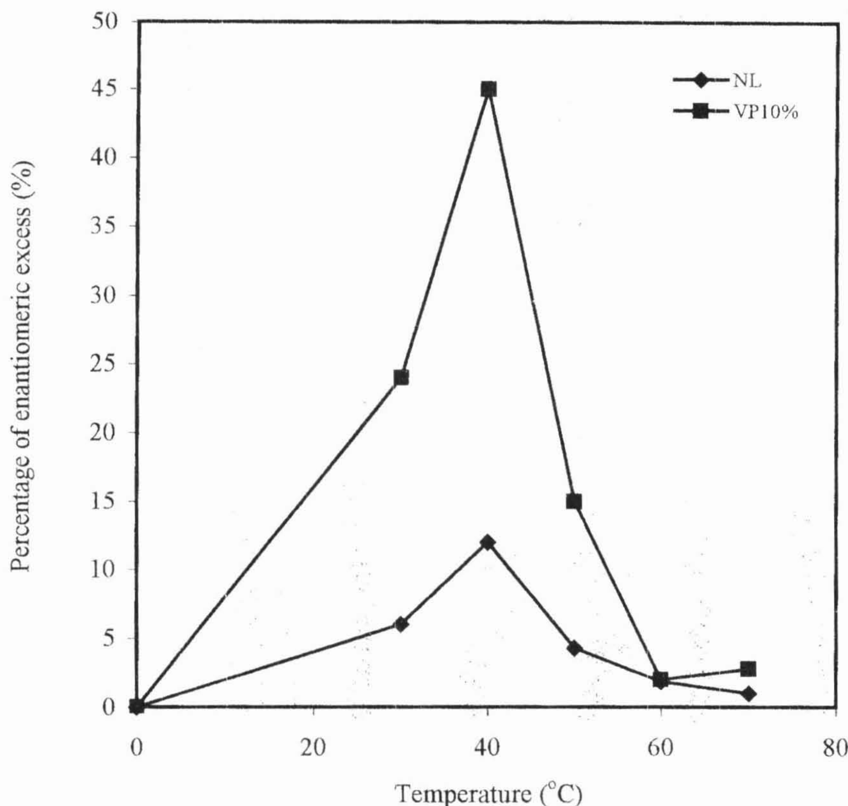

Figure 2: Effect of Reaction Temperature on Percentage of Enantiomeric Excess at 40°C for 24 hours

The Effect of Organic Solvents

The enantioselective esterification of (R,S)-2-(4-chlorophenoxy) propanoic acid with 1-tetradecanol was carried out at 40°C for 24 hours by using a series of different organic solvents. According to Figure 3, generally the correlation of percentage of enantiomeric excess in different organic solvents for VP-co-ST hydrogel immobilized lipase and free lipase were not very clear. However, it seems that the percentage of enantiomeric excess was relatively higher when using VP-co-ST hydrogel immobilized lipase compared when using free lipase in all cases of organic solvents. It was observed that with the presence of VP-co-ST hydrogel immobilized lipase; the percentage of enantiomeric excess was enhanced at approximately 45% in carbon tetrachloride compared with free lipase at approximately 10%. The results obtained concluded that the organic solvents might enhance the enantioselectivity of VP-co-ST hydrogel immobilized lipase. Since carbon tetrachloride (CCl_4) is hydrophobic organic solvent, the results obtained were quite similar to those reported reported by Holmberg and Hunt [8] where the enantioselectivity of enzyme was observed higher in the more hydrophobic solvent. The higher enantioselectivity in solvent with relatively higher hydrophobicity indicated that the enzyme molecule in hydrophobic solvent was fully hydrated and become more rigid and less flexible thus recognized one substrate better than other.

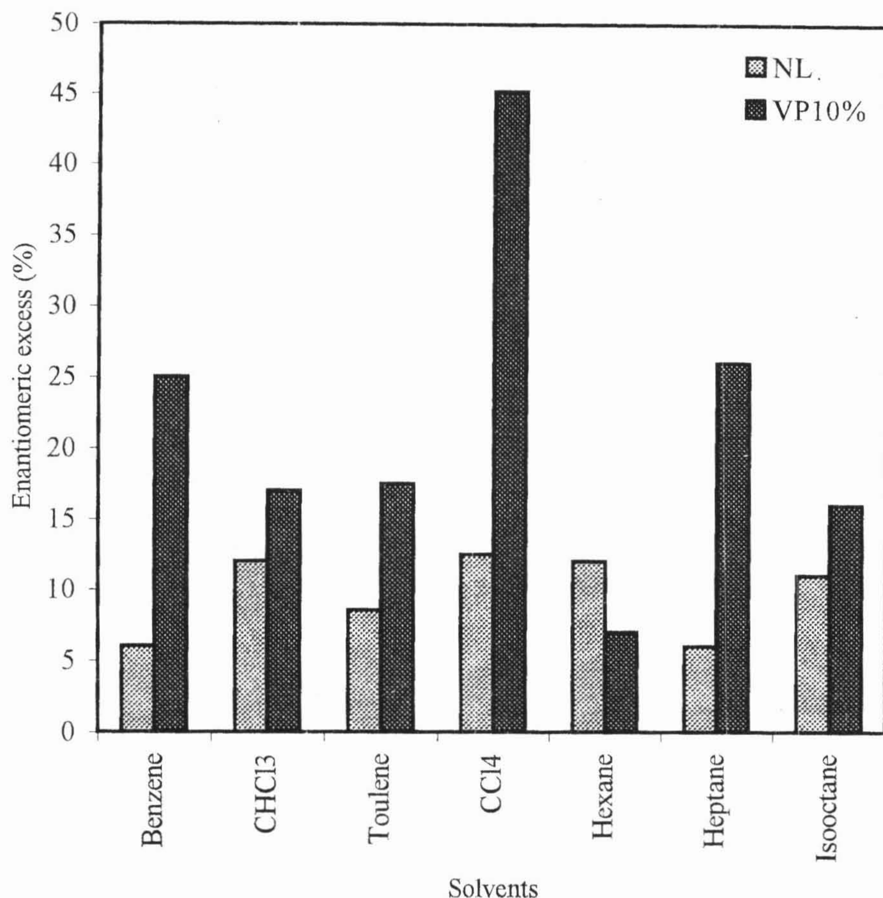

Figure 3: Effect of Organic Solvents on Percentage of Enantiomeric Excess at 40°C for 24 hours

CONCLUSION

From these studies, the immobilization of enzyme enhanced the properties of enzyme such as increase stability and reusability, thus making the use of immobilized enzyme in industrial scale more attractive than free lipase.

Generally, we can conclude that the immobilized lipase was relatively more enantioselective compared with free lipase. In the case of reaction temperature, the optimum temperature for VP-co-ST hydrogel immobilized lipase to catalyze the enantioselective esterification is at 40°C whereby it exhibited higher percentage of enantiomeric excess at approximately 45% than free lipase.

On the effect of organic solvents, the higher higher percentage of enantiomeric excess was obtained when using CCl_4, compared with other organic solvents whereby the use of VP-co-St hydrogel immobilized lipase enhanced the percentage of enantiomeric excess at approximately 45%. Hence the result concluded that with the presence of suitable organic solvent, the VP-co-ST hydrogel immobilized lipase still could enhance the enantioselectivity.

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