Chemoselective Hydrolysis of Methyl 2-Acetoxybenzoate Using Free and Entrapped Esterase in K-Carrageenan Beads

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ABSTRACT: Porcine liver esterase was entrapped in natural polysaccharides K-carrageenan and retention of its activity was determined using *p*-nitrophenyl acetate as the substrate. The optimum pH for esterase activity of entrapped enzyme showed a little shift towards acidic side. Immobilized enzyme showed improved thermal and storage stability. The entrapped esterase retained 50% of its activity after eight repetitive cycles. Michaelis constant K_m for the free and entrapped enzymes was almost same indicting no conformational change during immobilization. Maximum velocity V_{max} was observed to decrease on immobilization. The free and entrapped esterase was used for selective hydrolysis of methyl 2-acetoxybenzoate to methyl 2-hydroxybenzoate in batch process as well as in a fixed bed reactor. The hydrolysis was observed to be 99% within 2 h for free as well as immobilized enzyme in batch process. The rate of hydrolysis was found to depend on pH. The turn over number of selective hydrolysis in batch and fixed bed reactor was 3.08×10^6 and 1.19×10^7, respectively. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 108: 2617–2622, 2008

Key words: porcine liver esterase; K-carrageenan; immobilization; fixed bed reactor; selective hydrolysis

INTRODUCTION

Porcine liver esterase (PLE) (EC 3.1.1.1) a serine type of esterase has several advantages such as low cost, prolonged stability at low temperature, purity and ability to catalyze stereoselective hydrolyses of a large range of esters without any cofactor or metal ions. It can be conveniently assayed spectrophotometrically by examining hydrolysis of *p*-nitro phenyl esters and carbonates.[1,2]

PLE has been also used to catalyze enatioselective transesterification of chiral alcohols in a biphasic organic-aqueous medium[3] as well as for the enantioselective hydrolysis of racemic esters to separate enantiomers have also been reported.[4–7] It has been widely used in the preparation of chiral building blocks useful in the synthesis of biologically important molecules.[8]

K-carrageenan belongs to a family of linear sulphated food grade polysaccharide obtained from red seaweeds. Shukla et al.[9] have reported the immobilization of horseradish peroxidase in k-carrageenan by entrapment. Esterase has been successfully immobilized on different supports like crosslinked polymer of 2-vinyl-4,4-dimethylazalactone and trimethylol propane trimethacrylate,[10] polysulphone membrane,[11] agarose gel,[12] methoxypolyethylene glycol[13] microporous nylon membrane[14] etc. PLE recognizes the enantiotopic ligands, and is also useful in discriminating electronically anisotropic functional groups. However, PLE has not been much used for chemoselective transformations. Some examples of use of PLE for chemoselective transformations such as hydrolysis of saturated methyl ester preferentially over its unsaturated counter part,[15] aliphatic methyl ester in the presence of aromatic ester[16] and phenolic acetate over an aromatic methyl acetate where both groups present in the same moiety[17] are reported. To the best of our knowledge these chemoselective transformations with PLE have been reported with the soluble enzyme. Hence attempts are made to study chemoselective transformation using esterase immobilized in K-carrageenan beads. Optimization of the conditions for the entrapment of PLE in K-carrageenan and its use in the selective hydrolysis of methyl 2-acetoxy benzoate to methyl 2-hydroxy benzoate through batch and fixed bed reactor is carried out.

EXPERIMENTAL

Materials

PLE and *p*-nitrophenyl acetate were purchased from Sigma Chemicals Co., USA. K-carrageenan was

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TABLE I
Effect of Enzyme Concentration on Immobilization of Esterase

Enzyme taken (μg)	Protein (μg/g) of support	Protein entrapped (%)	Active protein (μg/g) of support	Active protein (%)	Retention of enzyme activity in beads (%)
50	13.6	27	6.3	13	46.3
100	32.0	32	14.7	15	45.9
200	87.9	44	42.4	21	48.2
300	96.9	32	45.7	15	47.2

dry wt. of K-carrageenan: 60 mg, 2% PEI in 0.1 *M* potassium hydrogen phosphate buffer, curing time: 2 h).

obtained as a gift from Central Salt Marine Research Institute, Bhavnagar, India. All other chemicals were of analytical grade.

Assay of esterase activity

The esterase activity of free and immobilized enzyme was determined spectrophotometrically as per the method reported earlier,[18] using 1.85 mL of 0.05 *M* phosphate buffer of pH 6.5 and 0.15 mL *p*-nitrophenyl acetate solution (50 m*M*) in acetonitrile. After addition of 1.0 mL of enzyme solution of different concentration, the increase in absorbance was measured after 10 min at 400 nm. The activity of esterase was determined from the calibration plot considering one unit of esterase activity as the amount of esterase that hydrolyzes 1 μmol of *p*-nitrophenyl acetate to produce *p*-nitrophenol per minute at 30°C. The amount of protein was determined by Lowry method.[19]

Preparation of immobilized esterase

Entrapment of esterase in K-carrageenan was done as reported earlier.[20] The effect of enzyme loading on entrapment of enzyme in beads was studied by using 50–300 μg esterase and 3.0 mL of 3% K-carrageenan solution for the preparation of beads, which were further cured in polyethyleneimine and phosphate buffer of pH 7. The retention of enzyme activity is the ratio of percentage of active protein to the protein entrapped in the bead.

Effect of pH and temperature on the stability of free and immobilized esterase

The effect of pH on the activity of free and immobilized enzyme was examined by incubating enzymes at 35°C for 10 min in 100 m*M* phosphate buffer of pH 5–8 using *p*-nitro phenyl acetate as a substrate. Absorbance due to formation of *p*-nitro phenol was measured at 400 nm and activity was determined from calibration plot.

The influence of temperature on the activity of free and entrapped esterase was determined by incubating them in the buffer solution of the optimum pH at various temperatures (30–60°C) for different time intervals. The thermodeactivation constant (K_d) was calculated as described earlier.[20]

Storage stability

The residual activities of the free and immobilized enzymes stored at room temperature (~ 35°C) were determined and were expressed as the percentage retention of the activities at various time intervals.

Reusability of immobilized enzyme

To evaluate the reusability of the immobilized esterase, the beads containing esterase were washed with water and buffer after use and then suspended again in a fresh reaction mixture for 10 min. The enzymatic activity was measured at 35°C and pH 6.5. This procedure was repeated till 50% of its original activity is retained.

Determination of kinetic constants

The kinetic parameters such as Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were

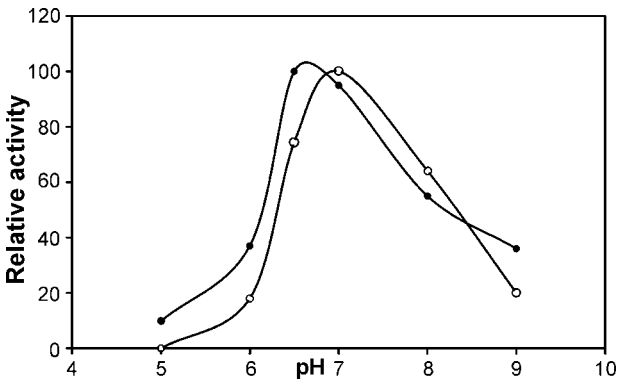

Figure 1 Effect of pH on the activity of free and immobilized esterase at 35°C, free enzyme (○) and immobilized enzyme (●).

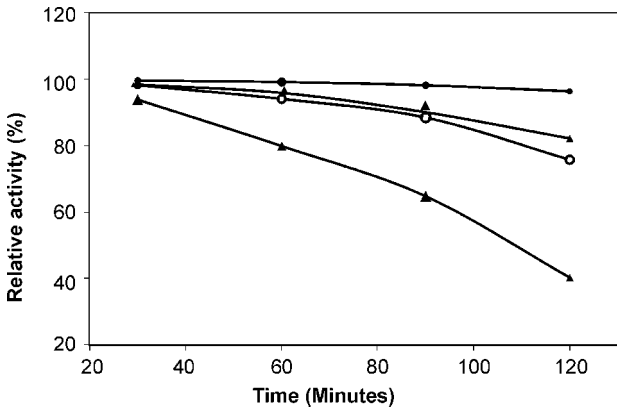

Figure 2 Effect of temperature on the activity of free and immobilized esterase at pH 6.5, free enzyme 50°C (○), 60°C (△), immobilized enzyme 50°C (●), 60°C (▲).

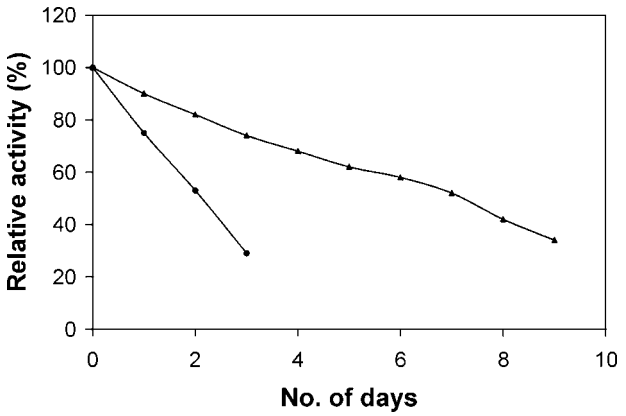

Figure 3 Storage stability of free and immobilized enzyme at 35°C, free enzyme (●) and immobilized enzyme (▲).

measured by varying the substrate concentration at constant enzyme concentration and vice-versa following the Lineweaver-Burk of $1/S$ versus $1/V$ where S is substrate or enzyme concentration and V is reaction velocity.[20]

Selective hydrolysis of methyl 2-acetoxybenzoate

Generally, hydrolysis of phenolic acetate is easier than the aromatic ester because of higher conjugation in the aromatic ester carbonyl. However, hot alkali hydrolysis both the functional groups of methyl 2-acetoxy benzoate, whereas PLE catalyzes the hydrolysis of only phenolic acetate keeping the methyl ester unhydrolyzed as shown below.

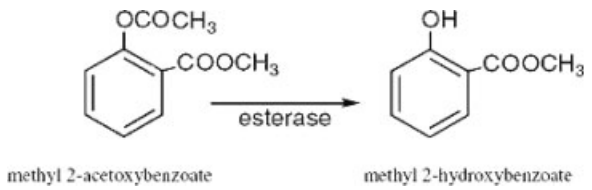

TABLE II
Thermodeactivation Constant for Free and Entrapped Enzyme

Time (min)	Temperature (°C)	Deactivation rate constant $K_d \times 10^3$ Free	ENT-esterase
30	50	0.63	0.13
	60	2.13	0.57
60	50	1.03	0.15
	60	3.75	0.72
90	50	1.37	0.21
	60	4.83	1.17
120	50	2.32	0.32
	60	7.59	1.64

Batch hydrolysis of methyl 2-acetoxybenzoate (0.125 g) was carried out using different amounts of free and immobilized esterase in 14 mL of 90 : 10 v/v acetone and phosphate buffer of pH 5–8. The reaction mixture was stirred at 30°C.

A fixed bed glass reactor of 20 cm length and 1.2 cm diameter was packed with 5 g immobilized enzyme system. Hydrolysis of 1–4% methyl 2-acetoxybenzoate in 90 : 10 v/v phosphate buffer of pH 5–8 and acetone was carried out by passing it through reactor. The operational conditions of reactor such as flow rate, substrate concentration, pH of buffer were varied and their respective effects on conversion were examined. After completion of each cycle, a sample was withdrawn for the analysis of reaction mixture and extracted with ethylacetate and analyzed by HPLC.

Analytical methods

Analysis was carried out using Waters (LC-4000) HPLC system with UV detector and Millenium 2010

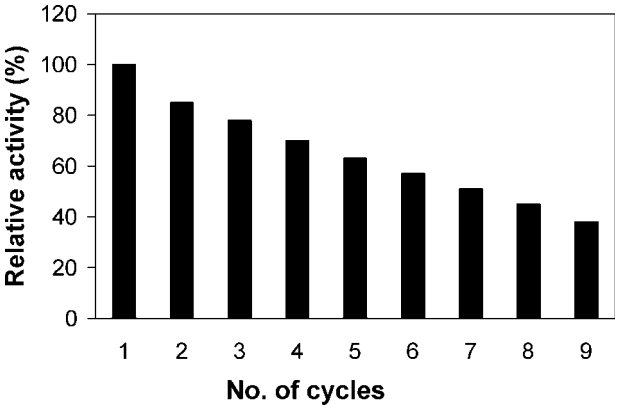

Figure 4 Reusability of immobilized enzyme at 35°C and pH 6.5.

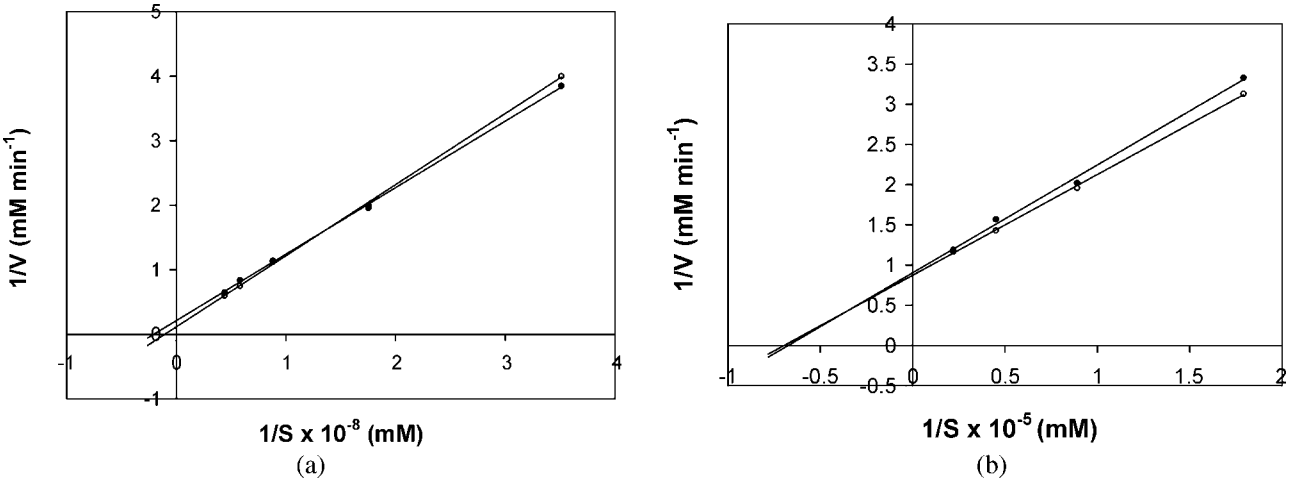

Figure 5 Lineweaver burk plots for *p*-nitrophenyl acetate hydrolysis temperature 35°C, pH 6.5, time 10 min, free enzyme (○) and immobilized enzyme (●). (a) When enzyme concentration kept constant, (b) When substrate concentration kept constant.

software. The concentration of methyl 2-acetoxybenzoate and methyl 2-hydroxybenzoate was determined at 234 nm using a reversed phase column C_8 μ Bondapak (150 × 4 mm) and methanol and water (60 : 40% v/v) as mobile phase at 1 mL min^{-1} flow rate.

RESULT AND DISCUSSION

Entrapment of esterase

Entrapment process for immobilization has been observed to be simple and reproducible one. From the results given in Table I it is observed that with increasing enzyme concentration, the amount of protein and active protein in beads decreases. The percentage protein entrapped and retention of enzyme activity is observed to be optimal at the enzyme concentration of 200 μg.

Effect of pH

Immobilization of enzyme is likely to result in conformational changes of enzyme resulting into a variation in optimum pH for enzyme activity. Results obtained in relative activity as a function of pH are given in Figure 1. It was observed that maximum enzyme activity is exhibited at pH 7.0 and 6.5 by free and entrapped esterase, respectively, indicating a little effect of polycationic character of support on the pH activity profile of immobilized enzyme.

Thermal stability

The catalytic activity of enzymes depends on temperature and is lost at temperatures above a certain limit because of the denaturation of enzyme protein. Relative activity as a function of time and temperature are illustrated in Figure 2. It is observed that entrapped enzyme shows better thermal stability at all temperatures and times. Thermodeactivation constants calculated are given in Table II. The thermodeactivation constants for immobilized esterase are lower than those for free ones at all temperatures, indicating thereby that the immobilized esterase is less prone to denature due to temperature.

Storage stability

The enhancement of stability is advantageous in the industrial applications of immobilized enzymes and

TABLE III
Kinetic Parameters for Free and Immobilized Enzyme

Conditions	System	Parameters	
		K_m (mM)	V_{max} (mM/min)
Enzyme concentration constant			
Substrate conc 0.22 to 1.79 $\times 10^{-2} M$	Free enzyme	1.39×10^{-5}	1.11
Enzyme conc $1.32 \times 10^{-11} M$	Entrapped enzyme	1.40×10^{-5}	1.10
Substrate concentration constant			
Enzyme conc 0.44 to $3.51 \times 10^{-11} M$	Free enzyme	4.30×10^{-8}	10.2
Substrate conc $0.45 \times 10^{-2} M$	Entrapped enzyme	9.32×10^{-8}	4.71

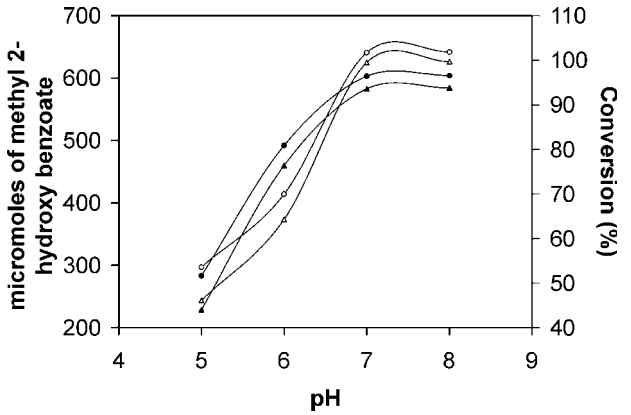

Figure 6 Effect of pH on selective hydrolysis. Conversion for free enzyme (○) and immobilized enzyme (●). Micro moles of methyl 2-hydroxybenzoate for free enzyme (○) and immobilized enzyme (△).

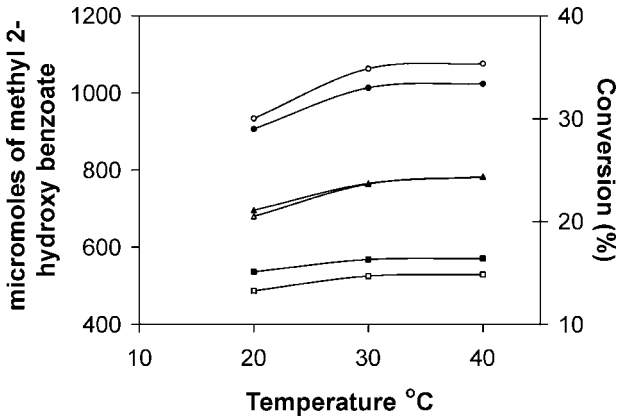

Figure 8 Effect of flow rate on hydrolysis. Substrate conc: 1%, flow rate: 1 cm^3 min^{-1} (●), 2 cm^3 min^{-1}(▲), 3 cm^3 min^{-1} (■).

it is thus important in determining the feasibility of immobilized enzymes for a particular reaction. The free enzyme and immobilized enzyme were stored in dry state for various time periods at 35°C. The residual activity of the enzyme was determined as a function of time using *p*-nitrophenyl acetate as substrate and results are given in Figure 3. It is observed that the free enzyme loses its 50% activity in 2 days, whereas the immobilized one in dry state could retain its 50% activity for 7 days.

Reusability of esterase

The reusability of enzyme is an important factor and directly affects the costs of the process. It is observed from Figure 4 that the 50% of enzyme activity is retained after seven repeated cycles, improving cost to performance ratio. The turn over number was calculated considering moles of product produced per mole of enzyme per unit time. The turn over number for the hydrolysis of *p*-nitro phenyl acetate is 3.22×10^6.

Determination of kinetic constants

The effect of the substrate concentration and enzyme concentration on the kinetics of the reaction catalyzed by free and immobilized esterase was studied using *p*-nitrophenyl acetate as substrate. From Lineweaver Burk plot of $1/V$ versus $1/S$. [Fig. 5(a,b)] Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the free and immobilized enzymes were calculated and results are given in Table III. No significant change in K_m values for the free and immobilized enzyme is observed at constant substrate and enzyme concentrations, which indicates that there is no possibility of conformational change taking place during immobilization. Though no significant change in V_{max} was observed when enzyme concentration was kept constant, but V_{max} for immobilized enzyme was twofold smaller than that for the free esterase when substrate concentration was kept

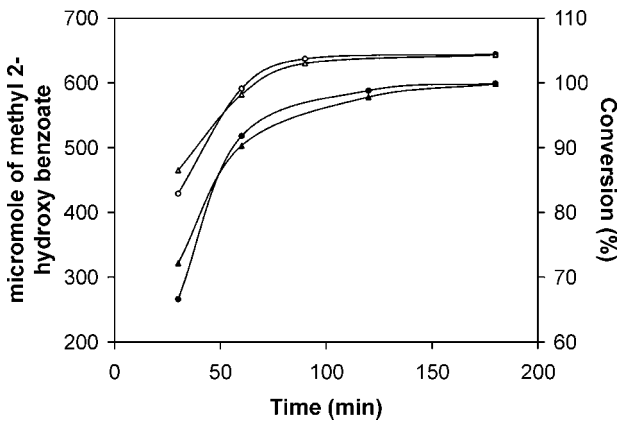

Figure 7 Progress of selective hydrolysis reaction with time. Free enzyme: 500 μg, immobilized enzyme: 5 g, pH 8, Conversion for free enzyme (●) and immobilized enzyme (▲). Micro moles of methyl 2-hydroxybenzoate for free enzyme (○) and immobilized enzyme (△).

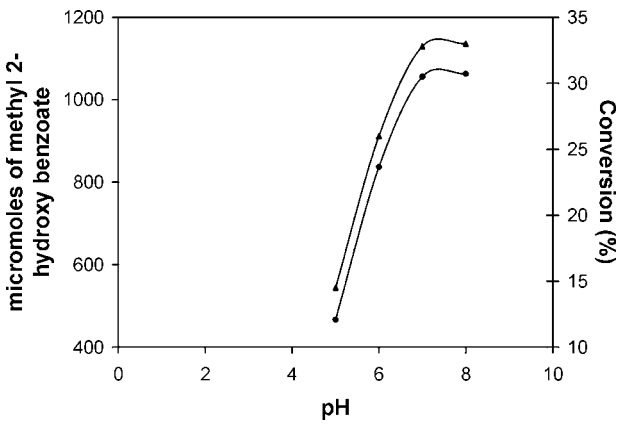

Figure 9 Effect of pH on hydrolysis. Flow rate: 1 cm^3 min^{-1}, substrate conc.: 1%.

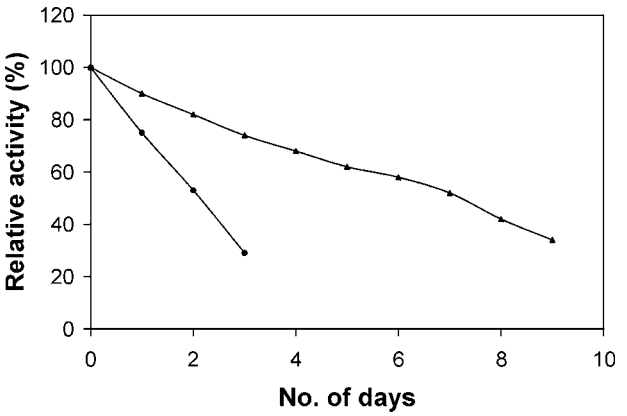

Figure 10 Reactor stability for the hydrolysis. Batch reactor (●), fixed bed reactor (■).

constant, which may be due to increased resistance for the substrate diffusion in immobilized enzyme system.

Chemoselective hydrolysis of methyl 2-acetoxybenzoate

Basik et al.[17] have reported 92% conversion of methyl 2-acetoxybenzoate to methyl 2-hydroxybenzoate within 15 min when 500 mg of free PLE per gram of reactant was used. In the present study, free as well as immobilized esterase was used and also the different conditions were optimized.

Batch reactor

In the present study, it is observed from the Figure 6 that the pH factor makes significant effect on the conversion. The minimum and maximum conversion is observed at pH 5 and 8, respectively. The reaction rate of chemoselective hydrolysis as a function of time is observed in Figure 7. The reaction progresses with time, and 65–70% conversion is completed within half an hour. Thereafter rate of reaction is slow. This may be probably due to enzyme denature, product inhibition and/or limitation of chemical equilibrium.

Fixed bed reactor

It is observed in our study that with increasing flow rate the conversion decreases because of lesser time of contact between substrate and immobilized enzyme. It is observed that the temperature did not make significant effect on conversion (Fig. 8). The maximum conversion observed in one cycle is 33.4% (1.078 m*M*) at 40°C and 1.0 cm^3 min^{-1} flow rate and 1% substrate concentration in one cycle. We have observed similar results for fixed bed reactor of those observed for batch reactor i.e. minimum and maximum conversion at pH 5 and 8, respectively, in batch and fixed bed reactors (Fig. 9).

The most important and realistic assessment of the utility of an immobilized enzyme is in determining its operational stability over the number of cycles. This was studied for batch and fix bed reactor and it is observed (Fig. 10) that 50% residual activity is retained for 4 and 9 cycles, respectively, in batch and fixed bed reactors.

CONCLUSION

The entrapment of esterase in K-carrageenan is an operationally convenient method. The immobilized catalyst exhibited an appreciable catalytic along with improved stability to different parameters, including pH, temperature, and storage stability. The immobilized esterase also exhibited good operational stability, maintaining 50% of its residual activity after 10 cycles of hydrolysis of a model substrate *p*-nitro phenylacetate. The selective hydrolysis of methyl 2-acetoxy benzoate to methyl 2-hydroxy benzoate is performed in batch as well as fixed bed reactor and it is observed that the fixed bed reactor retained residual activity for more repeated cycles than the batch reactor.

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