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Effective Immobilization of Lipase onto a Porous Gelatin-*co*-Poly (vinyl alcohol) Copolymer and Evaluation of Its Hydrolytic Properties

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ABSTRACT: Crosslinked copolymers of gelatin and poly(vinyl alcohol) (PVA) with excellent water absorption and water retention abilities were successfully synthesized using ^{60}Co γ radiation. Ammonium persulfate (APS), as a water-soluble initiator and sodium bicarbonate ($NaHCO_3$) as a foaming agent were used. The best synthesis conditions were evaluated with regard to the maximum percentage of swelling as a function of the APS concentration, $NaHCO_3$ concentration, amount of water, and reaction time. The maximum swelling percentage (1694.59%) of the copolymer gelatin-*co*-PVA, was obtained at the optimum parameters [APS] = 2.92×10^{-1} mol/L, [$NaHCO_3$] = 7.94×10^{-2} mol/L, and 1.5 mL of water with 31.104 kGy of the γ radiation dose. The copolymer was characterized by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) methods. The SEM analysis showed a highly nanoporous and cellular structure of the copolymer. The copolymer was used as a support for lipase immobilization. The optimization of the reaction conditions, including the pH and temperature for immobilization, on the basis of the hydrolysis of *p*-nitrophenyl palmitate, was carried out. An excellent efficiency for protein loading (70%) at pH 8.5 by the copolymer was observed. The results observed during the evaluation of the hydrolytic properties showed excellent activity of the bound lipase. The porous gelatin-*co*-PVA bound lipase was found to be stable at 75°C and pH 8.5; it displayed 2.326 ± 0.005 U/g of lipase activity. The stability and activity of the copolymer-bound lipase were also studied as a function of the time at 75°C, and the biocatalyst was found to be stable and active up to 1 h, beyond which the activity decreased. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 39622.

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INTRODUCTION

Copolymers are one of the most promising types of three-dimensional crosslinked hydrophilic polymeric networks, and they are used for various applications, including biomedical, immobilization, and pharmaceutical applications. These polymeric materials do not dissolve in water at physiological temperature and pH but swell considerably in an aqueous medium. In this study, the copolymer was prepared with a natural polymer, gelatin, and a synthetic polymer, poly(vinyl alcohol) (PVA). The selection of gelatin rested upon two reasons. First, gelatin is well known for its nontoxic, nonirritating, hydrophilic, and biodegradable properties and good living body compatibility, and second, because of the large number of functional side groups, it readily undergoes chemical crosslinking. The synthetic polymer PVA was selected because it endowed mechanical stability and flexibility to the polymer matrix that could not be provided well by the natural component. In this study, therefore, we fabricated a three-dimensional gelatin–PVA hybrid that had the capacity of absorbing a large quantity of water with improved properties over both polymers, and we used it as a support for enzyme immobilization.

Immobilization often hyperactivates and stabilizes the structure of the enzyme, thereby enhancing its stability and easy recovery and allowing its use, even under harsh environmental conditions of pH, temperature, and organic solvents.[1–3] Immobilization on a copolymer is advantageous in many ways for living cells and enzymes and has many merits compared to other matrices such as calcium alginate in which the chelators (e.g., phosphate buffer) may deteriorate the enzyme active site. These supports have certain advantages, such as a low cost, ease of enzyme accessibility, increased productivity, hydrophilic character, easy recovery, high stability, and the presence of hydroxyl groups on the surface capable of interaction with proteins, over other materials and have, therefore, received considerable attention in recent years. Enzyme immobilization on these supports is quick, apparently irreversible; this enhances the chances for

reuse and provides a nontoxic and biocompatible microenvironment conducive to the catalytic activity and stability of the enzyme.

Lipases (EC 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of long-chain triglycerides. They have vast importance for our health, not just in regard to commonly recognized diseases of fat metabolism, such as overweight and underweight conditions; cardiovascular diseases; diabetes; strokes; and degenerative muscle diseases but also for skin problems, autoimmune diseases of the brain and nervous system, and rejuvenation and regeneration, in general. In addition, lipases also constitute a most important group of biocatalysts for biotechnological applications. They are one of the most extensively investigated enzymes because of their abilities in fat splitting, esterification, transesterification, and other reactions of industrial importance; therefore, many attempts have been made to immobilize them on suitable supports. Available methods proposed for lipase immobilization, such as adsorption, covalent attachments to solid supports, gel entrapment,[4] and entrapments within polymer matrices[5,6] and on membranes[7] or resins,[8] have provided new possibilities in the field of material science.[9,10] Lipase obtained from the porcine pancreas was immobilized on polyacrylamide beads and partially hydrolyzed to active carboxylic groups.[11] Lipase from a lipase-producing strain, *Arthrobacter* spleen (ABL), was isolated and immobilized in calcium alginate beads by entrapment to increase its reusability and overall enzyme stability.[12]

Lipase hydrolyzes triglycerides and other carboxylic esters in aqueous media[13] but also shows good activity in hydrophobic organic solvents with limited water content,[14] where the chemical equilibrium is shifted toward ester formation. Hydrolysis predominates in a water-rich environment, whereas esterification predominates at low water contents. Reaction mediums composed of organic solvents having low water activities as biphasic solvent systems consisting of water and water-immiscible solvents, reverse micelle, or microaqueous systems shift the thermodynamic equilibrium toward ester synthesis.[15,16] In biphasic systems and in reverse micelles, the enzyme is solubilized in water, whereas in microaqueous systems, an enzyme as a suspension in organic solvent is used. The nature of the organic media in which the reaction is performed also influences the lipase activity from both a kinetic and a thermodynamic point of view.[17,18]

There are various reports in the literature that show the immobilization of lipase onto a copolymer and its use in synthetic reactions. In previous studies, poly(methacrylic acid-*co*-dodecyl methacrylate-cl-*N*,*N*-methylene bisacrylamide) [poly(MAAc-*co*-DMA-*cl*-MBAAm)] bound lipase showed optimal hydrolytic activity toward *p*-nitrophenyl palmitate (*p*-NPP) at 55°C and 8.5 pH.[19] Kumar and Kanwar[20] synthesized isopropyl ferulate using silica-immobilized lipase in an organic medium and concluded that the enzyme retained good activity and stability after repetitive use and a remarkable stability toward the organic solvent dimethyl sulfoxide (DMSO). A purified thermotolerant alkalophilic extracellular lipase of *Bacillus coagulans* MTCC-6375 was efficiently immobilized onto poly(MAAc-*co*-DMA-*cl*-MBAAm), a synthetic copolymer, by surface absorption, and the bound lipase was used to perform short-chain fatty acid ester synthesis in *n*-alkane(s).[21] The productivity of *Clostridium butyricum*, used for the production of 1,3-propanediol, can be enhanced by entrapment in a copolymer under nonsterile conditions over several weeks without any microbial contamination, and the copolymer-bound lipase also shows a high thermal stability.[22] Betigeri and Neau,[23] in their study, found that the immobilization of lipase with a copolymer in the form of beads showed the maximum entrapment efficiency with minimum leaching. In another study, lipase was immobilized onto a nitrocellulose membrane through physical adsorption; this also proved to be an efficient biocatalyst in organic media.[24] Lipolase T20 was immobilized onto natural fibers of dried coconut fruit and used for the synthesis of 2-octyl ferulate in an organic medium.[25] Kumar and Kanwar[26] carried out the synthesis of ethyl ferulate catalyzed by a commercial lipase (Steapsin) immobilized onto celite-545.

To investigate the effects of the structural aspects of the supports and those of the environmental conditions on the enzyme activity, we studied the immobilization of lipase onto a gelatin-*co*-PVA copolymer. The enzyme activity was studied as a function of the temperature, pH, incubation time and amount and nature of the solvent. In this study, gelatin-*co*-PVA enhanced the thermal stability up to 10°C. The immobilized lipase was used as a hydrolase, and the reproducibility of the enzyme activity was evaluated. This study has good potential to help in the development of model bioreactors for the synthesis and hydrolysis of useful esters.

EXPERIMENTAL

Materials and Methods

PVA (molecular weight = 14,000, E. Merck, Mumbai, India), gelatin (S. D. Fine Chemicals, Boisar, India), sodium bicarbonate (Loba Chemie, Mumbai, India), a foaming agent, and ammonium persulfate (APS; S. D. Fine Chemicals, Mumbai), a free-radical initiator, were used as received. Distilled water was used throughout the study as the reaction and swelling medium. Trishydroxymethylaminomethane (Tris) buffer (Sigma Aldrich), gum acacia (Merck Schurdt Germany), *p*-NPP (Lancaster Synthesis, Eastgate White Lund, Morecambe, England), and bovine serum albumin (Sisco Research Laboratory, Mumbai, India) were used as received. Toluene, *n*-hexane, acetone, *n*-ethanol, xylene, dimethyl sulfoxide, heptanes, diethylphthalate, *n*-butanol, and *n*-propanol (Thermo Fischer Scientific India) were analytical grade and were used as received. The commercial lipase (Lipolase 100T) was obtained from Novozyme Pvt., Ltd. (Bangalore, India).

Synthesis of the Gelatin-*co*-PVA Copolymer

The synthesis of the copolymer of gelatin and PVA was carried out with a γ-radiation-induced mutual method. Radiation-induced methods can be carried out at room temperature and prevent the use of a crosslinker and hence its removal. The process is easy to control. All of these factors made irradiation the method of choice for the synthesis of the copolymer.

PVA (0.250 g) was dissolved in 1.5 mL of distilled water at 90°C, and gelatin (0.250 g) was added to it. The mixture was

mixed by vigorous shaking for about 15 min to prepare a thick dispersion, and to it, the APS initiator (2.92×10^{-1} mol/L) was added. The resulting dispersion was stirred for 3–4 min at 100°C. In the meanwhile, $NaHCO_3$ (7.94×10^{-2} mol/L) was also added during stirring. The thick dispersion thus obtained was irradiated with ^{60}Co γ rays in air for 24 h at a constant dose rate of 1.296 kGy/h. The product obtained (weighing ca. 0.450 g) was washed with water and dried in oven at 50°C until a constant weight was achieved.

Characterization

The gelatin-*co*-PVA copolymer and gelatin-*co*-PVA copolymer with immobilized lipase were characterized by Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy (SEM). FTIR spectra were obtained with a 5700 Thermas IR spectrophotometer. The surface topology and homogeneity of the gelatin-*co*-PVA copolymer was studied by a LEO model 1430 VP SEM instrument.

Immobilization of Lipase onto Gelatin-*co*-PVA through Physical Adsorption

Lipase was immobilized onto the gelatin-*co*-PVA copolymer support through physical adsorption. About 5 g of powdered copolymer was submerged in 10 mL of lipase solution [1.914 ± 0.005 U/mL in tris buffer 0.02*M* (9 mL), pH 8.5] and was incubated at 4°C for 24 h. After incubation of the copolymer with lipase, the supernatant was decanted to record its volume and unbound protein concentration. The copolymer was taken out and thoroughly rinsed with tris buffer (0.02*M,* pH 8.5). The protein loading onto the copolymer was determined by subtraction of the protein content of the unbound enzyme (lipase) from the total lipase used for immobilization.

The copolymer-bound enzyme was assayed for its protein content and enzyme activity by a colorimetric method in which lipase catalyzed the hydrolysis of *p*-NPP.[27] Indirectly, the protein content was determined with bovine serum albumin as a reference protein.[28] The copolymer-bound lipase, the gelatin-*co*-PVA biocatalyst, was used to study its hydrolytic properties.

Enzyme Activity Assay

The activities of the free and immobilized lipase were assayed with 0.5% w/v *p*-NPP in 2-propanol as a substrate. The reaction mixture consisted of 2.9 mL of tris buffer (0.02*M,* pH 8.5) containing 0.095 mL of *p*-NPP solution. All of the additives, including buffer, were preincubated at 55°C for a short period (10 min) to initiate the reaction. To this was added, 0.005 g of immobilized polymer per 0.005 mL of the unbound lipase. The reaction was terminated by the placement of the reaction mixture at −80°C for 2 min. The activities of the free and immobilized enzyme were analyzed spectrophotometrically with a Shimadzu UV-160A spectrophotometer (Japan) by measurement of the increment in the absorption at 410 nm promoted by the hydrolysis of *p*-NPP.[27]

Evaluation of the Hydrolytic Properties of the Copolymer-Bound Lipase

The evaluation of the hydrolytic properties of the copolymer-bound lipase as a hydrolase was done, and the effects of the reaction parameters, such as the temperature, pH, time of reaction and organic solvents on the hydrolytic properties of the copolymer-bound lipase were evaluated.

Effect of the Temperature. The effect of the reaction temperature on the activity of the free and immobilized lipase for the hydrolysis of *p*-NPP was studied at pH 8.5 in the temperature range 55–75°C under shaking (150 rpm) for the maximal hydrolytic activity of the bound lipase.

Effect of the pH. The catalytic activity of the copolymer-bound lipase in the hydrolysis of *p*-NPP at 75°C under shaking was investigated at different pHs (6.5–10), and the residual lipase activity was measured.

Effect of the Organic Solvents. Because many of the reactions in organic synthesis are carried out in nonaqueous media, the stability and efficiency of the copolymer-bound lipase biocatalyst was studied in different organic solvents, including toluene, *n*-hexane, acetone, *n*-ethanol, xylene, dimethyl sulfoxide, heptanes, diethylphthalate, *n*-butanol, and *n*-propanol. The copolymer-bound lipase (0.005 g) was incubated with 200 μL of different organic solvents for 1 h. After the specified time, the mixture of the solvent and the biocatalyst was separated by centrifugation (10,000 rpm), and the supernatant was decanted. The biocatalyst was used immediately to check its activity against the hydrolysis of *p*-NPP in tris buffer (pH 8.5).

Effect of Salt Ions on the Copolymer-Bound Lipase. The effect of ions on hydrolytic activity of the copolymer-bound lipase was studied and compared to the initial lipase activity, which was determined in the absence of salt ions. The copolymer-bound lipase was incubated for 1 h at room temperature in aqueous solutions of different concentrations (1, 3, and 5 m*M* aqueous solutions of $FeCl_2$, $CoCl_2$, $MnCl_2$, $HgCl_2$, $MgCl_2$, NaCl, NH_4Cl, $CaCl_2$, and KCl). After 1 h, the bound lipase was separated from the salt ions by centrifugation, and was checked for lipase activity at 75°C.

Effect of Detergents on the Copolymer-Bound Lipase. Different concentrations of detergents (1, 5, 10, and 20% v/v) were used to study the effects of detergents on the activity of the bound lipase. The bound lipase was added separately to the mixture containing 2.95 mL of tris buffer and detergents at different concentrations. The lipase activity and the absorbance were measured at 75°C and 410 nm[27] respectively.

Stability of the Copolymer-Bound Lipase. The copolymer-bound lipase was incubated at 75°C in a water bath under shaking, and its activity was checked against the hydrolysis of *p*-NPP in tris buffer (pH 8.5) after regular time intervals of 1 h and compared with the activity of free lipase under the same conditions.

RESULTS AND DISCUSSION

Synthesis of the Porous Gelatin-*co*-PVA Copolymer

The porous gelatin-*co*-PVA copolymer was synthesized through a radiation-induced mutual method. The addition of APS and sodium bicarbonate to the aqueous mixture of PVA and gelatin caused foaming and led to spatial pores. Upon irradiation of the mixture, the crosslinking of the two polymers with a porous structure (gelatin-*co*-PVA) copolymer was obtained.

The synthesis of the copolymer was studied as functions of different reaction parameters and their effects on the swelling behavior of the copolymer.

Effect of the Gelatin–PVA Ratio. The formation of the gelatin-*co*-PVA copolymer was studied as a function of the ratio of gelatin to PVA, and the results are presented in Table I. When the total amount of the two polymeric substrates was fixed at 0.500 g, the ratio was varied between 0.100 and 0.400 g. It is shown in Table I that the stable copolymer, holding its structure at equilibrium swelling (1534.00%), was formed when a 1:1 w/w ratio of the two polymers was used. An increased amount of gelatin (0.400 g) and PVA (0.300 g) in the mixture gave very high swelling (1846.15 and 1677.14%, respectively), but the copolymer started disintegrating. The increased amount of either polymer increased the volume fraction of the polymer in the copolymer and led to gelling in the case of gelatin, whereas in the case of PVA, an enhanced degree of interaction with water did not help hold the structure.

Effect of the Amount of Water. The effect of water as a reaction medium on the swelling percentage of the copolymer was also studied. We observed that maximum percentage of swelling (1534.00%) was obtained with 1.5 mL of water. An increase or decrease in the amount of water beyond the optimum led to a decrease in the swelling percentage. The low swelling in case of 1 mL of water may have been due to the insufficient amount of water for the homogenization of the reaction, which led to a decrease in the formation of pores all through the network. However, amounts higher than the optimum were found to be excessive for holding the copolymer structure.

Effect of the APS Concentration. The relationship between the APS concentration and the water absorbency values was studied as a function of the APS concentration, and the results are presented in Table I. The maximum swelling (1534.00%) was obtained at $[APS] = 2.92 \times 10^{-1}$ mol/L, beyond which it decreased. The reduction of swelling beyond the optimum APS concentration may have been due attributed to an increase in the chain termination reactions via bimolecular collision; this led to the self-crosslinking of the polymeric chains. In addition, the free-radical degradation of the gelatin-*co*-PVA backbone by sulfate radical anions led to a decrease in the swelling percentage at higher APS concentrations.[29]

In another set of experiments, the copolymer was synthesized with only γ radiation in the absence of APS. The copolymer thus obtained showed a good swelling percentage (1600%), but in the process, it lost its strength and became hollow within, losing about 30% of the weight. This indicated that the APS acted as an initiator and helped bind the two polymers through the formation of crosslinks.

Table I. Effects of the Reaction Parameters on the Swelling of Gelatin-*co*-PVA

PVA (g)	Gelatin (g)	$[APS] \times 10^{-1}$ (mol/L)	$[NaHCO_3] \times 10^{-2}$ (mol/L)	Water (mL)	Dose (kGy)	Swelling (%) attained in the time (h)
0.10	0.40	2.92	15.87	1.5	31.104	1846.15 (24)
0.20	0.30	2.92	15.87	1.5	31.104	1214.29 (24)
0.25	0.25	2.92	15.87	1.5	31.104	1534.00 (24)
0.30	0.20	2.92	15.87	1.5	31.104	1677.14 (24)
0.40	0.10	2.92	15.87	1.5	31.104	1305.56 (24)
0.25	0.25	2.92	15.87	1.0	31.104	1397.29 (24)
0.25	0.25	2.92	15.87	1.5	31.104	1534.00 (24)
0.25	0.25	2.92	15.87	2.0	31.104	1413.89 (24)
0.25	0.25	2.92	15.87	2.5	31.104	1258.62 (24)
0.25	0.25	2.92	15.87	3.0	31.104	1295.83 (24)
0.25	0.25	1.46	15.87	1.5	31.104	847.37 (47)
0.25	0.25	2.92	15.87	1.5	31.104	1534.00 (24)
0.25	0.25	4.38	15.87	1.5	31.104	1039.47 (7)
0.25	0.25	5.84	15.87	1.5	31.104	1200.00 (7)
0.25	0.25	7.30	15.87	1.5	31.104	1215.38 (7)
0.25	0.25	2.92	7.94	1.5	31.104	1694.59 (25)
0.25	0.25	2.92	15.87	1.5	31.104	1534.00 (24)
0.25	0.25	2.92	23.81	1.5	31.104	1369.44 (72)
0.25	0.25	2.92	31.75	1.5	31.104	1607.89 (72)
0.25	0.25	2.92	39.68	1.5	31.104	850.00 (72)
0.25	0.25	2.92	7.94	1.5	31.104	1694.59 (25)
0.25	0.25	2.92	7.94	1.5	62.210	762.79 (96)
0.25	0.25	2.92	7.94	1.5	93.315	646.81 (96)

Effect of $NaHCO_3$ Concentration. Sodium bicarbonate was used as a foaming agent to increase the porosity of the copolymer. The effect of the $NaHCO_3$ concentration on the water absorbency of the synthesized copolymer is presented in Table I. It was observed that the maximum swelling (1694.59%) was obtained at the minimum $[NaHCO_3] = 7.92 \times 10^{-2}$ mol/L; beyond this value, it decreased continuously. The decrease may have been due to the fact that the excess sodium ions blocked the functional groups of gelatin/PVA for interaction with water; this led to a decrease in the swelling percentage.

Effect of the Irradiation Dose. The swelling percentage of the gelatin-*co*-PVA copolymer was studied as a function of the total dose, and the results are presented in Table I. We observed that swelling decreased continuously with increasing dose from 31.104 to 93.312 kGy. This was attributed to the fact that at higher total doses, an enhancement of the crosslinking process took place. This resulted in a dense network of crosslinks that lowered the free volume space for water molecules to diffuse and thereby affected swelling. Similar behavior has been reported by Dergunov et al.[30] and Singh et al.[31]

Characterization of the Porous Gelatin-*co*-PVA Copolymer

FTIR Spectroscopy. The IR spectrum of gelatin-*co*-PVA [Figure 1(a)] showed peaks at 3422.25 and 3199.02 cm^{-1} due to $\nu_{(O-H)}$ stretching of PVA and $\nu_{(N-H)}$ stretching of the secondary amide of gelatin, respectively. We also observed peaks at 2926.58 and 2854.61 cm^{-1} due to $\nu_{(C-H)}$ stretching (asymmetric) and peaks at 1636.23 and 1559.66 cm^{-1} due to the $\nu_{(N-H)}$ bending of amide I and the carbonyl function, amide II, respectively. A sharp peak at 1401.36 cm^{-1} due to $\nu_{(C-O-H)}$ bending vibrations, a peak at 1116.26 cm^{-1} due to the $\nu_{(C-C)}$ and $\nu_{(C-O)}$ stretching of PVA, a peak at 990.64 cm^{-1} due to $\nu_{(C-N)}$ stretching, and a peak at 618.68 cm^{-1} due to $\nu_{(O-C-N)}$ deformation due to gelatin were also observed. The presence of these peaks indicated that both gelatin and PVA were successfully reacted to give the gelatin-*co*-PVA copolymer.

In the IR spectrum of the gelatin-*co*-PVA copolymer with immobilized lipase [Figure 1(b)], the peaks at 3422.25 and 3199.02 cm^{-1} due to the $\nu_{(O-H)}$ stretching of PVA and $\nu_{(N-H)}$ stretching of the secondary amide of gelatin, respectively, disappeared, and a wide, sharper peak at 3286 cm^{-1} appeared and was associated with $\nu_{(N-H)}$ stretching; this indicated that both the OH and NH_2 of PVA and gelatin interacted with the polar functionalities of lipase. The peaks at 1636.23 and 1559.66 cm^{-1} due to the $\nu_{(N-H)}$ bending of amide I and the carbonyl function, amide II, shifted and appeared as sharp peaks at 1643 and 1543 cm^{-1}. In addition to these, a number of peaks appeared between 1041 and 1450 cm^{-1}; these were attributed to the $\nu_{(C-O)}$ and $\nu_{(C-O-H)}$ coupled vibrations of the reactive moieties, such as serine, histidine, and aspartic acid of the enzyme. The appearance of new peaks and the shifting of some peaks showed that the enzyme was successfully immobilized onto the copolymer.

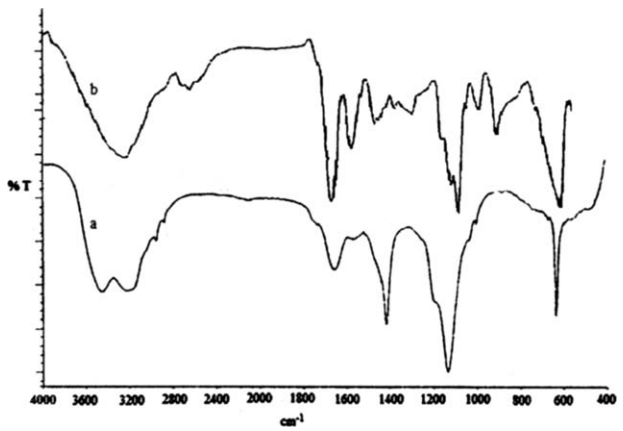

Figure 1. FTIR spectra of (a) gelatin-*co*-PVA and (b) gelatin-*co*-PVA-bound lipase.

SEM. Figure 2 depicts the surface topology and homogeneity of gelatin-*co*-PVA at different magnifications. At a magnification of 622×, the presence of cavities distributed on the entire surface in the copolymer matrix was distinctly observed. The porous structure depicting large cavities was further corroborated by scanning carried out at 1260× magnification. Still at higher magnification, that is, 2000×, the large cavities were visualized as independent pores spread within the polymer matrix; this influenced the extensive capillary channels, and hence, swelling was attained at near equilibrium in a matter of few hours.

Immobilization of Lipase onto the Gelatin-*co*-PVA Copolymer

The mode of binding of the protein and the ability of the gelatin-*co*-PVA copolymer to retain the enzyme in its active form are important characteristics that are responsible for lipase's usefulness in organic media. The immobilization of lipase from its suspension in tris buffer (0.02*M*, pH 8.5, 1.914 ± 0.005 U/mL activity) onto the copolymer through physical adsorption with a maximum of 70% protein binding efficiency at pH 8.5 was observed. The specific activity was also checked and was found 0.926 ± 0.005 U/g for the immobilized enzyme and 0.717 ± 0.005 U/mL for the decanted supernatant.

Evaluation of the Hydrolytic Properties of the Gelatin-*co*-PVA-Bound Lipase

The hydrolysis of *p*-NPP both by free lipase and by the copolymer-bound lipase was studied as a function of reaction variables such as the temperature and pH, which affected the activity of the enzyme and the effects of different salts and detergents on the activity of the bound enzyme.

Effect of the Temperature on Copolymer-Bound Lipase Activity. The effect of the temperature on the activity of the immobilized lipase and free lipase for the hydrolysis of *p*-NPP at pH 8.5 was studied, and the results are presented in Figure 3. As shown in the figure, in case of free lipase, the activity increased with increasing temperature and gave a maximum (1.54 ± 0.005 U/mL) at 65°C; beyond this, it decreased to as low as 0.543 ± 0.005 U/mL. In case of the immobilized lipase, the activity was found to increase continuously with increasing temperature, giving a maximum (2.326 ± 0.005 U/g) at 75°C. Kanwar et al.,[32] during their study on the use of the synthetic poly (Methacrylic acid-*co*-dodecyl methacrylate-*cl*-N,N-methylene bis acrylamide) poly(MAc-*co*-DMA-*cl*-MBAm) copolymer-immobilized lipase of *B. coagulans* MTCC-6375 for the synthesis of ethyl oleate, observed that the optimal synthesis of the ester

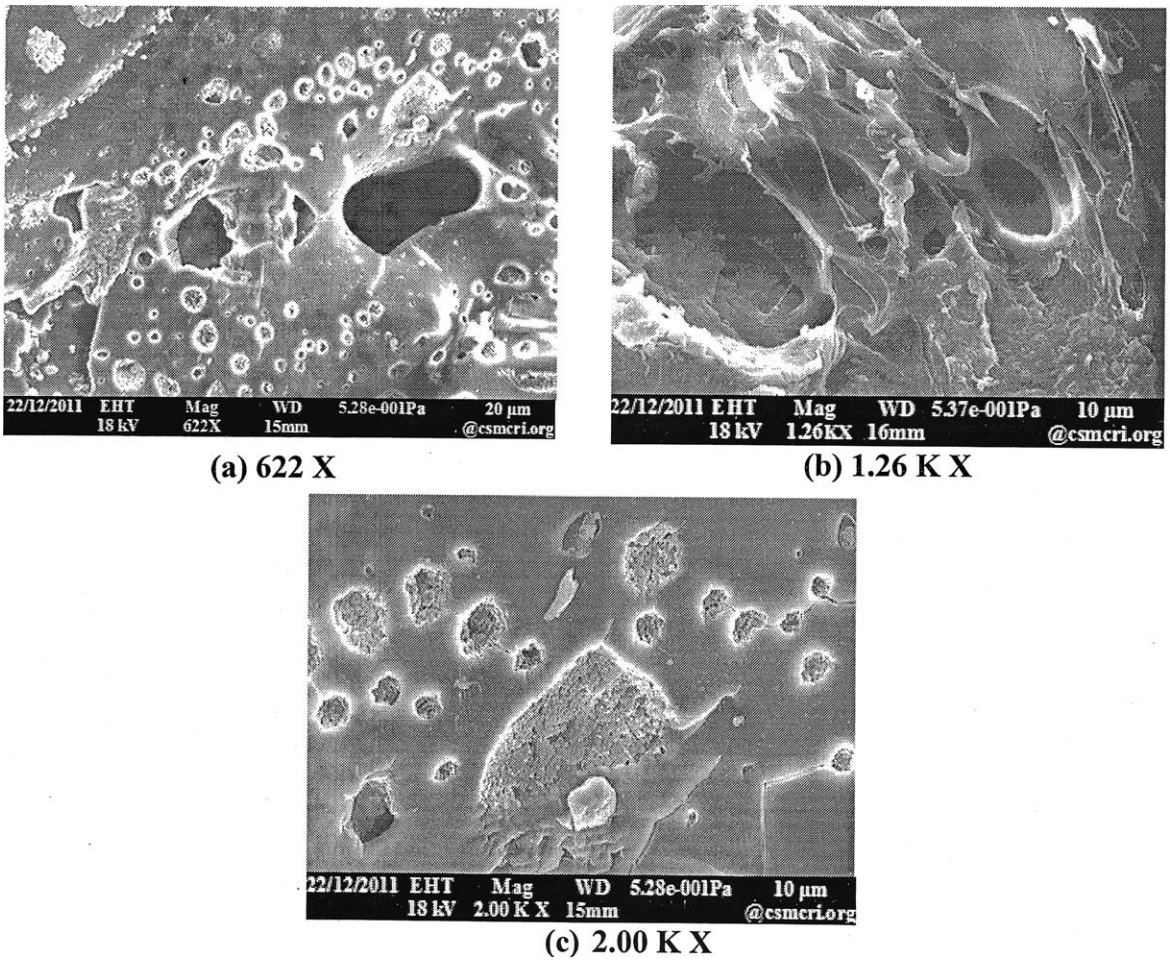

Figure 2. SEM micrographs of the gelatin-*co*-PVA copolymer.

(ca. 53% conversion) occurred in 24 h at 55 and 65°C. A decrease or an increase in the reaction temperature resulted in a significant decline in the amount of ethyl oleate produced. Thus, the gelatin-*co*-PVA copolymer bound biocatalyst was active at a higher temperature and had a higher activity compared to the free enzyme. The increase in the activity of the bound biocatalyst may have been due to the stabilization of the lipase molecule by its immobilization, which was due to the conformational limitation on the enzyme movement as a result of covalent bond formation between the enzyme and also the easy diffusion of the substrate at high temperatures.[33] This behavior was corroborated by Villeneuve et al.[34] when they considered a rigid structure of the encapsulated lipase, which prevented the split breaking of the interactions responsible for the proper globular, catalytically active structure of the lipase.

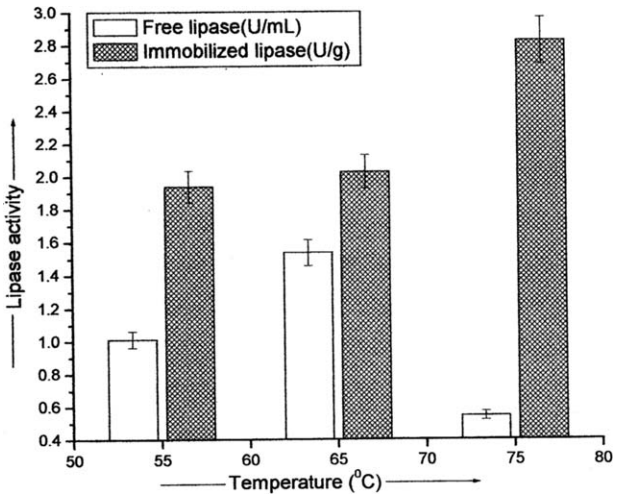

Figure 3. Effect of the temperature on the activity of the free and hydrogel-bound lipase: pH 8.5 and [Buffer] = 0.02*M*.

Effect of the pH on the Copolymer-Bound Lipase Activity. pH variation in the reaction medium is known to affect the stability of the enzyme and, consequently, its activity; therefore, in this study, the effects of the pH on the activity of the free lipase and immobilized lipase during the hydrolysis of *p*-NPP ester were studied, and the results are presented in Figure 4. As shown in the figure, the copolymer-bound lipase was more stable and showed a higher activity (2.802 ± 0.005 U/g) at pH 8.5 compared to the free lipase (1.826 ± 0.005 U/mL at pH 8.5). The pH effects could have manifested because of the partitioning of protons between the solution and the support surface, which thus affected the reaction under study. Kumar and Kanwar[24] in a recent study reported that the NC-membrane-bound lipase was more stable at high pH (pH 8–9.5) and displayed better hydrolytic activity than

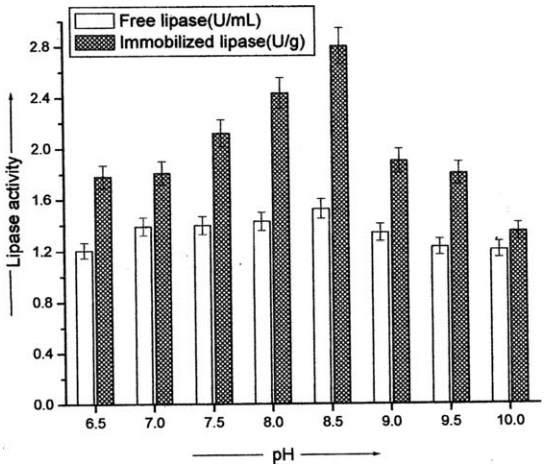

Figure 4. Effect of the pH on the activity of the free and hydrogel-bound lipases: temperatures = 65 and 75 for the free and hydrogel-bound lipase, respectively and [Buffer] = 0.02M.

the free lipase, which showed a sharp decline in its activity at pH > 8.5. Similarly, a purified alkaline thermoalkalophilic extracellular lipase of *Pseudomonas aeruginosa* MTCC-4713, efficiently immobilized onto a synthetic poly(acrylic acid-*co*-hydroxy propylmethacrylate-*cl*-ethylene glycol dimethacrylate) poly(AAc-*co*-HPMA-*cl*-EGDMA) copolymer, also showed optimal hydrolytic activity toward *p*-NPP at pH 8.5.[35]

Effect of Organic Solvents on the Copolymer-Bound Lipase. The effect of organic solvents on the hydrolytic activity of the copolymer-bound lipase incubated in different organic solvents, including toluene, octanol, heptane, hexane, xylene, ethanol, propanol, butanol, diethylphathalate (DEPH), and DMSO, was studied and compared to the initial lipase activity, determined in the absence of organic solvents. All of the evaluated organic solvents were found to decrease the activity of the immobilized lipase after 1 h of incubation (Figure 5); this might have been due to the suppression of the active sites on the biocatalyst.

Effect of Salt Ions on the Copolymer-Bound Lipase. The effect of salt ions on the hydrolytic activity of lipase incubated in salt ions (1, 3, and 5 m*M* aqueous solutions of $FeCl_2$, $CoCl_2$, $MnCl_2$, $HgCl_2$, $MgCl_2$, NaCl, NH_4Cl, $CaCl_2$, and KCl) was determined and compared to the initial lipase activity, which was determined in the absence of salt ions. As shown in Figure 6, all of the evaluated salt ions (in 3% solution) and $MgCl_2$ (in 1% solution) showed higher hydrolytic activities than that carried out in the absence of the salts. Among the various salts used, $MgCl_2$ increased the activity (2.037 ± 0.005 U/g) compared to the control (1.627 ± 0.005 U/g). The increase in the activity might have been due to the activation of active sites on the biocatalyst.

Effect of Detergents on the Copolymer-Bound Lipase. Detergents in general have a close resemblance to lipase substrates and form micelles with increasing solubility concentrations. In this study, Triton X-100, Tween-60, and Tween-80 were used at different concentration (1, 5, 10, and 20%) to determine their effects on the lipase activity. All of the evaluated detergents were found to decrease the lipase activity (Figure 7). Triton X-100 [critical micelle concentration (cmc) = 0.0155%] and Tween-80 (cmc = 0.0016%), when used at a 1% concentration, showed activities greater than 1 U/g although lower than the control (1.648 ± 0.005 U/g) whereas at higher concentrations, the activity was much lower. At higher concentrations, micelle formation took place; this favored an increase in activity but also resulted in the denaturation of protein, which might not have supported the conformational changes in the active site of the enzyme. Also, at higher concentrations, the detergents formed complexes with lipophilic proteins; this resulted in an inhibition of the activity. These factors ultimately led to a decrease in the lipase activity. This was also supported by the findings of Salameh and Wiegel[36] in that the activity profiles of the nonionic detergents and sodium dodecyl sulfate were similar on the basis of the fact that the activation started at low concentration below their cmc values and, therefore, did not involve micelles, but maximal activation and inhibition occurred in the presence of micelles.

Stability of the Copolymer-Bound Lipase. The hydrolytic activities of the free lipase and copolymer-bound lipase were

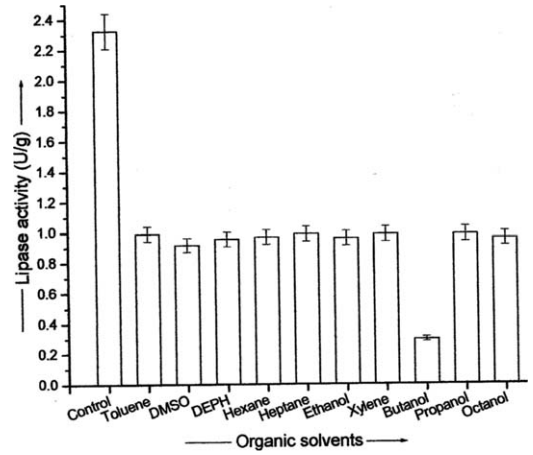

Figure 5. Effect of the organic solvents on the activity of the hydrogel-bound lipase: pH 8.5, temperature = 75, [Buffer] = 0.02*M*, and centrifugation = 5000 rpm for 5 min at 4°C.

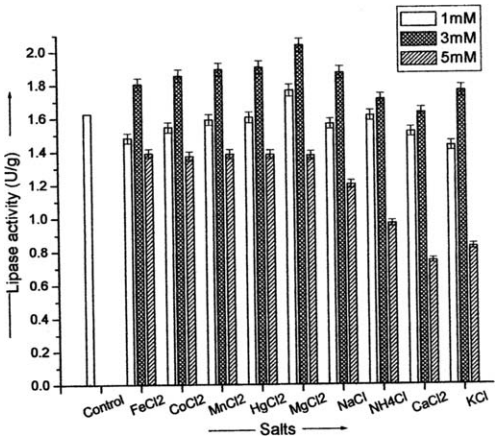

Figure 6. Effect of the salts on the activity of the hydrogel-bound lipase: pH 8.5, temperature = 75, [Buffer] = 0.02*M*, and centrifugation = 5000 rpm for 5 min at 4°C.

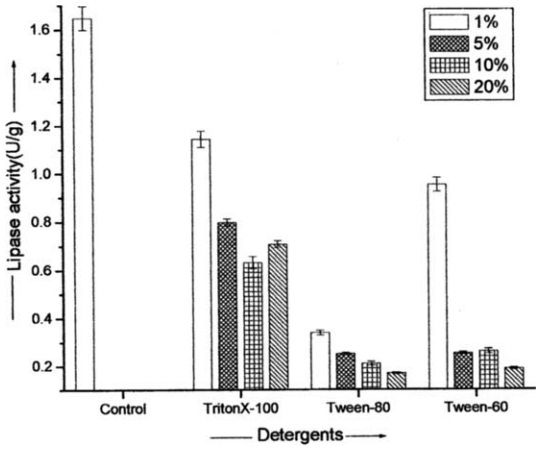

Figure 7. Effect of the detergents on the activity of the hydrogel-bound lipase: pH 8.5, temperature = 75, [Buffer] = 0.02*M*, and centrifugation = 5000 rpm for 5 min at 4°C.

measured and compared, and the results are presented in Figure 8. It is shown in the figure that the activity of free lipase decreased with increasing time of incubation from the maximum (1.535 ± 0.005 U/mL) at room temperature to (0.306 ± 0.005 U/mL) after 3 h of incubation, whereas the copolymer-bound lipase almost retained its hydrolytic activity after 1 h of incubation (2.319 ± 0.005 U/g) to that observed at room temperature (2.368 ± 0.005 U/g), which decreased to 1.588 ± 0.005 U/g after 2 h of incubation and was little higher than that of the free lipase at room temperature. After 3 h of incubation, the activity of both the free lipase and the immobilized lipase showed almost similar activity (0.306 ± 0.005 and 0.326 ± 0.005 U/g, respectively). The drastic decrease in stability after 3 h of incubation was attributed to the fact that the exposure of the active site to high temperature for a long time affected the activity of the enzyme.

CONCLUSIONS

The successful synthesis of the gelatin-*co*-PVA copolymer by a mutual irradiation method and the successful immobilization of the lipase enzyme was achieved. The hydrolytic activity of the bound enzyme toward the hydrolysis of *p*-nitrophenyl ester was successfully carried out and compared with that of the free enzyme. The bound enzyme was more stable at different temperatures and pH values and also retained a higher hydrolytic activity.

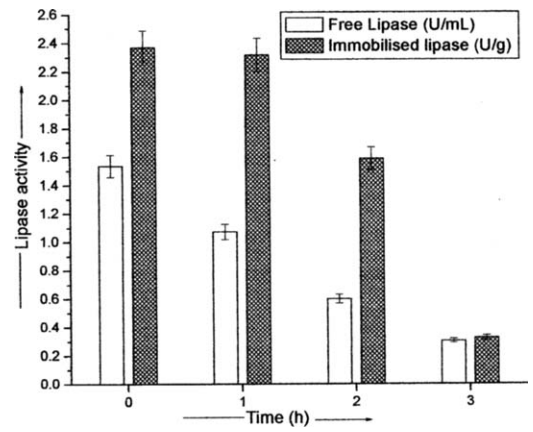

Figure 8. Stability of the free and hydrogel-bound lipase: pH 8.5, temperature = 65 and 75 for the free and hydrogel-bound lipase, respectively, and [Buffer] = 0.02*M*.

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