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Reusability of Surfactant-coated *Candida rugosa* Lipase Immobilized in Gelatin Microemulsion-based Organogels for Ethyl Isovalerate Synthesis

Dandavate, Vrushali and Datta Madamwar*

BRD School of Biosciences, Sardar Patel Maidan, Vadtal Road P.O. Box - 39, Satellite Campus, Sardar Patel University, Vallabh Vidyanagar-388120 Gujarat, India

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In our previous study, a surfactant-coated *Candida rugosa* lipase immobilized in microemulsion-based organogels was exploited for the synthesis of ethyl isovalerate. In the present study, we are focusing on the effective reuse of lipase immobilized in microemulsion-based organogels (MBGs) in terms of retainment of the catalytic activity. As water is one of the co-products in esterification reactions, the removal of water becomes a priority to allow the reaction to work in the forward direction and to prevent back hydrolysis. Taking this fact into consideration, the lipase-containing microemulsion-based organogels were given pretreatment and/or several intermittent treatments with dry reverse micellar solution of AOT in organic solvent during repeated cycles of ester synthesis. The pretreated MBGs with dry reverse micellar solution exhibited lower water content and higher initial rates of esterification in comparison with untreated freshly prepared MBGs. The esterification efficiency of untreated MBGs started decreasing after 5 cycles of reuse and was almost completely lost by the end of the 8th cycle. In contrast, pretreated MBGs exhibited a gradual decrease in esterification efficiency after 5 cycles and retained about 80% of the initial activity at the end of the 8th cycle. The intermittent treatment of MBGs after every 3 cycles resulted in enhanced reusability of immobilized lipase for up to 9 cycles without significant loss in esterification activity, after which it resulted in a slow decrease in activity with about 27% lower activity at the end of the 12th cycle. Furthermore, the treatment conditions such as concentration of AOT in liquid dessicant and time of treatment were optimized with respect to our system. The granulated MBGs proved to be better in terms of initial esterification rates (1.2-fold) as compared with the pelleted MBGs.

*Corresponding author
Phone: 91-2692-229380; Fax: 91-2692-231042/236475;
E-mail: datta_madamwar@yahoo.com, vdandavate@gmail.com

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Lipases (acylglycerol acylhydrolases, E.C. 3.1.1.3) are efficient catalysts for lipolytic reactions, initiating the catabolism of fats and oils by hydrolyzing the fatty acyl ester bonds of acylglycerols. The use of lipases in organic solvents is now well established and there are several advantages of conducting enzymatic reactions in water-poor media such as increased solubility of hydrophobic substrates, shifting of thermodynamic equilibrium in favor of synthesis over hydrolysis, and increased thermostability of the enzyme [26]. Many researchers have successfully designed a number of immobilization techniques, taking into account its processibility and feasibility, such as the immobilization of the catalyst onto a porous support by adsorption or deposition, entrapment in a gel matrix, or covalently attaching the enzyme to an immobilization carrier [11, 13, 20, 22], which have proved to be useful techniques for improving enzymatic activity. It is believed that the immobilization leads to dispersion of enzyme over a large surface area, thus preventing its aggregation and enhancing mass transfer [17].

Amongst the various methodologies studied for effective biotransformation in organic solvent media, the use of reverse micelles or water-in-oil microemulsion is an attractive approach [21]. The enzymes in a nearly anhydrous environment can exhibit exciting features, such as increased conformational rigidity, enhanced stability, altered substrate specificity, and more favorable thermodynamic equilibria. The enzyme molecules are very sensitive to the organic solvent involved, which results in low catalytic activity. To overcome this problem, embedding of enzymes in reverse micelles is in use [8]. The advantage of using this type of system, apart from dispersibility of the enzyme at the molecular level, is that it is capable of solubilizing a wide range of polar and

apolar substrates [23]. However, the recovery of product and regeneration of enzyme is challenging. To make enzyme recovery more easy, the use of gelled microemulsion systems, especially that of gelatin and Aerosol-OT w/o microemulsion systems, has been employed. The formation of microemulsion-based organogels was first demonstrated by Haering and Luisi *et al.* [14] in 1986. However, Jenta *et al.* [16] were the first to report the use of microemulsion based organogels as a matrix for lipase immobilization.

Water is recognized as playing a dual role in lipase catalyzed esterification reactions [16]. It is required in very small amounts to preserve the catalytic conformation of the enzyme in organic solvents, probably by adhering to the enzyme surface in form of a thin layer and acting as a protective sheath [1, 4]. Alternately, the presence of excess water can adversely affect the thermodynamic equilibrium of the ester synthesis by favoring the hydrolytic reaction [16]. Moreover, it should be noted that the optimum thermodynamic water activity varies from 0.3 to 1.0 for lipases from different microbial sources and it also depends on the type of immobilization matrix used in a given solvent [12, 18, 27]. This phenomenon poses the major limiting factor in repeated use of lipase immobilized in MBGs for ester synthesis, as the water produced during the esterification reaction accumulates in the gel. With an increasing amount of water in the gel, the rate of the esterification reaction decreases with time. Thus, if the water formed during the esterification reaction can be selectively removed in a continuous manner as it is produced, or at regular intervals, it should be possible to use the enzyme for more cycles of esterification. There are very meagre reports on the reusability of microemulsion-based organogel systems employing lipases for condensation reactions [16, 23]. The selective removal of water using molecular sieves has been demonstrated by several workers, in order to maintain the equilibrium toward synthesis; however, it is extremely difficult to remove water continuously using a molecular sieve to scale up the process [2, 5]. Jenta *et al.* [16] successfully demonstrated the use of a liquid dessicant for selective extraction of water from organogels at regular intervals during repeated cycles of ester synthesis [16].

In our laboratory, much work has been carried out in the synthesis of esters by immobilizing lipase in microemulsion-based organogels [9, 23]. In our previous study, we reported the enhanced catalytic activity of CTAB-coated *Candida rugosa* lipase immobilized in AOT-microemulsion-based organogels for synthesis of ethyl isovalerate [9]. In the present study, we have focused on the operational reusability of surfactant-coated *Candida rugosa* lipase immobilized in microemulsion-based organogels by providing pretreatments and intermittent treatments to the gels for the removal of the accumulated water and monitoring them for the production of ethyl isovalerate.

Materials and Methods

Materials

Sodium bis-2-(ethylhexyl) sulfosuccinate (AOT) was obtained from Fluka (Steinheim, Germany). Cetyl trimethyl ammonium bromide (CTAB) was obtained from Sisco Chem Industries (India). Ethyl isovalerate was purchased from Aldrich. Gelatin from porcine skin (Bloom 300) and *Candida rugosa* lipase (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) with a total activity of 1,140 U/mg of solid were supplied by Sigma (Japan). All organic solvents and chemicals used were of analytical reagent grade.

Immobilization of Surfactant-coated *Candida rugosa* Lipase in AOT-based Organogels

The method for preparation of the anionic MBGs is similar to the method described in our previous paper [9]. Thermodynamically stable reverse micellar solution consisting of AOT/buffer/isooctane was prepared by mixing each component in a suitable ratio. Surfactant-coated lipase containing MBGs were prepared by introducing a concentrated (60 mg/ml) aqueous enzyme solution (at 25°C) to the above prepared reverse micellar solution. After a brief mixing, this solution was then immediately added to a second solution of gelatin in buffer at 55°C. Gelatin obtained from porcine skin was dissolved in phosphate buffer (pH 7.2) at 50°C. The mixture was vigorously shaken and stirred until homogeneous and allowed to cool to room temperature to yield MBG. The Wo value, defined as the mole ratio of water to surfactant, was found to be 60 in the above preparation. The Wo value is a key parameter in the description of microemulsions because it relates both to the size of the microemulsion droplets and to the activity of water (a_w) present in the core. The gel was then poured into the plastic petriplates and kept overnight for air drying. On the next day, the dried gel was cut into small equal-sized pellets and used for esterification reaction. Alternatively, we used a directly pelleted form of MBGs; the pelleted form was subjected to mechanical grinding in the presence of liquid N_2, using a mortar and pestle to obtain granulated MBGs. Both pelleted and granulated forms of MBGs were used for further studies.

Treatments Given to Microemulsion-based Organogels

In the case of pretreated MBGs, the prepared granulated and pelleted MBGs were soaked in 1 M AOT dry reverse micellar solution for 24 h, and these MBGs were washed several times with hexane and used for the repeated cycles of esterification. The dry reverse micellar solution of AOT was prepared in isooctane. The pretreated MBGs were then used for several cycles without giving any intermittent treatment and monitored for the esterification activity at the end of each cycle.

In the case of intermittent treatment, the MBGs were treated with dry AOT reverse micellar solution for 24 h after every 3 consecutive cycles. This experiment was carried out until a significant decrease in the esterification efficiency was observed. This type of treatment was given to both the pelleted and granulated MBGs. For optimization of treatment conditions, MBGs were treated with dry reverse micellar solution of isooctane containing varying concentrations of AOT from 0.1 M to 2 M in isooctane. The time period for treatment also was varied from 3 h to 24 h, using 0.2 M AOT in isooctane.

Reaction Condition

The reaction mixture was comprised of 20 ml of hexane containing equimolar concentration (100 mM) of isovaleric acid and ethanol.

The reaction was initiated by adding enzyme entrapped in microemulsion-based organogels into the reaction mixture in a 250-ml glass-stoppered flask and kept on an orbital shaker at 37°C at 150 rpm for ethyl isovalerate production. The samples were withdrawn at regular interval of 72 h and analyzed for accumulation of ethyl isovalerate. Total of 3 samples were withdrawn from each cycle at 72 h, 144 h, and 216 h, respectively. After the completion of each cycle, the solvent containing product and unused substrates was completely removed and the MBGs were washed twice with hexane. The washed MBGs were then incubated with fresh reaction mixture for a subsequent cycle of ester synthesis.

Analytical Procedures

After initiation of esterification, 500 µl of sample from the reaction mixture was withdrawn periodically, and was immediately analyzed by a gas chromatograph (Perkin Elmer, Model Clarus 500, Germany) equipped with a flame ionization detector and 30 meter Rtx-R-20 (Cross bond 80% dimethyl-20% diphenyl polysiloxane) capillary column. Nitrogen served as a carrier gas at a split flow rate of 90 ml/min and the column temperature range from 40 to 280°C. The temperature was programmed to increase from 40 to 210°C at the rate of 6°/min and from 210 to 280°C at the rate of 15°/min. The temperature of the injector was 250°C and that of the detector was 280°C. Ester identification was done by comparing the retention time and peak area of the sample with a standard.

Scanning Electron Microscope (SEM) Analysis

Freeze-dried samples before and after esterification reaction were analyzed for the changes occurring in the gel, using Philips XL 30 ESEM (Environmental Scaning Electron Microscope) having a gaseous secondary state detector. Samples were mounted on aluminum stubs and placed in the chamber.

Determination of Water Content in Microemulsion-based Organogels

This was done by two different methods, *viz*., solid-state ^{1}H-NMR and Karl-Fischer titration. For solid-state NMR, the samples were mounted into a zirconium rotor and analyzed using a 300 MHz Bruker spectrophotometer at 25°C. Mobile H_2O is assumed to be contributing to the sharp resonance over a broad gel background. The relative ratios of resonance of mobile water protons to that of bound protons of gel were obtained by relative integrations of peaks after deconvolutions.

The water extracted from the MBGs in AOT/isooctane solution was determined by the Karl-Fischer method using Hydranal-E reagent by volumetric titration.

RESULTS AND DISCUSSION

The primary aim of immobilization technology is to reuse the enzyme, apart from easy downstream processing of the product. In the case of lipase-mediated esterification reactions, an equimolar amount of water is produced along with the ester. In our previous studies, we observed that lipase immobilized in MBGs could be successfully used for 5 cycles without significant reduction in the esterification efficiency. However, after the 5th cycle, the esterification efficiency was reduced profoundly by 50%, which was thought to be due to accumulation of water, a by-product of esterification reaction, leading to a shift in reaction equilibrium towards hydrolysis [9, 10, 12]. The treatment of MBGs with 1 M AOT/isooctane for 24 h, meant for extraction of excess water from the gel, resulted in partial recovery of lost esterification activity [9]. This observation encouraged us to investigate the reusability of surfactant-coated lipase immobilized in MBGs by removal of excess water using intermittent treatment with a dry reverse micellar solution of 1 M AOT/isooctane.

Repeated Use of Lipase Immobilized in MBGs With and Without Pretreatment

It has been reported earlier that lipase immobilized in MBGs with the lowest R value exhibited maximum esterification activity, which is commonly observed for synthetic activities of enzyme in microemulsion media [6, 25]. However, it is not possible to synthesize stable MBGs with a gelatin

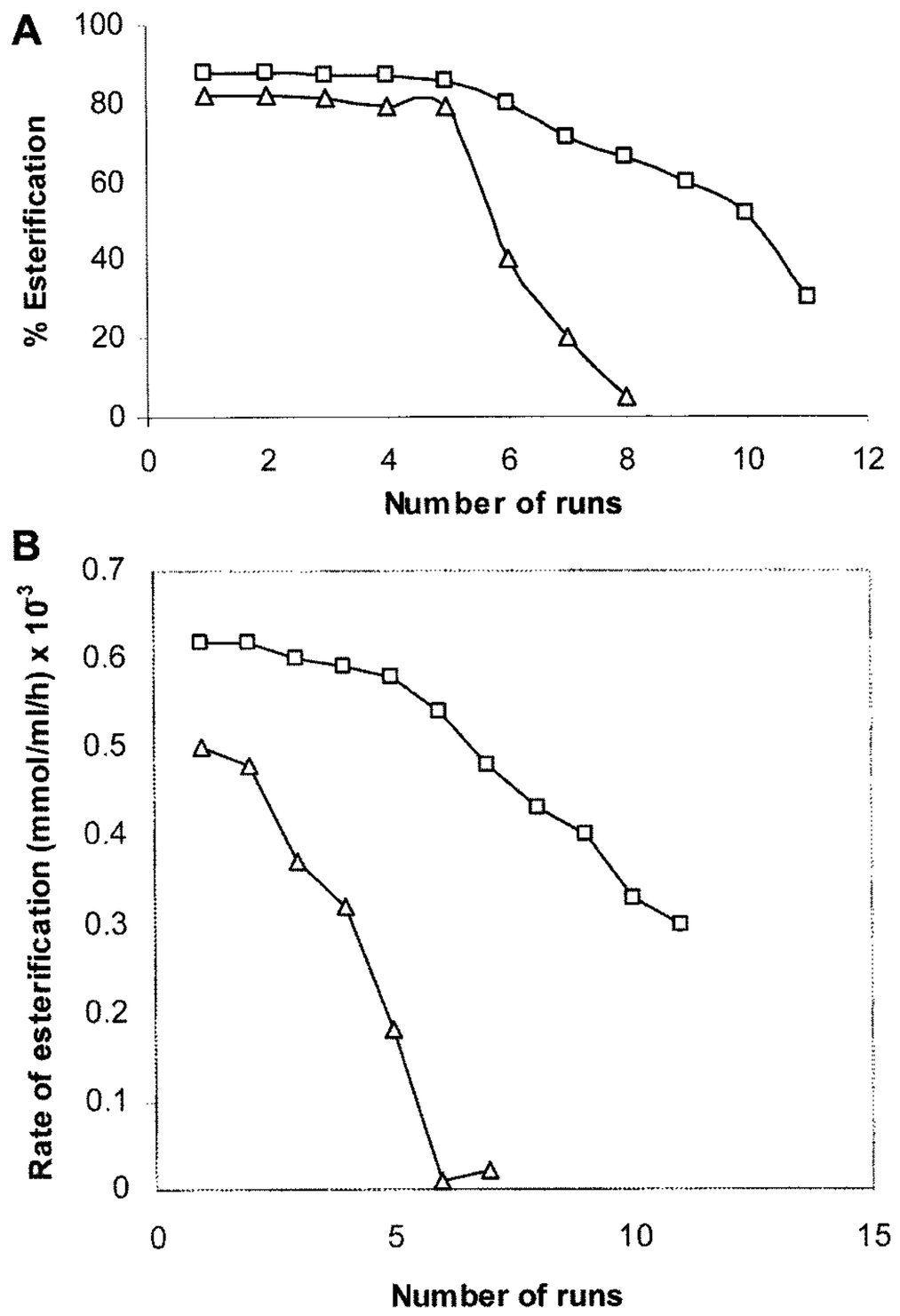

Fig. 1. A. Repeated cycles of ethyl isovalerate synthesis using surfactant-coated lipase immobilized in AOT-based organogels; △, Untreated MBGs; □, Pretreated MBGs. **B**. Initial rate of esterification during repeated cycles of ethyl isovalerate synthesis using surfactant-coated lipase immobilized in AOT-based organogels; △, Untreated MBGs; □, Pretreated MBGs.

concentration of 14% (w/v) having a R value lower than 60, but it has been shown earlier that the R value can be reduced below 60 by extraction of water from preformed MBGs using a dry reverse micellar solution as a liquid dessicant [16]. Thus, the freshly prepared MBGs (pellet form) were pretreated with 1 M AOT/isooctane solution for 24 h and then monitored for the repeated runs of esterification reaction. Figs. 1A and 1B show that pretreated MBGs exhibited slightly higher esterification activity, with enhanced reaction rate, up to 5 cycles in comparison with untreated MBGs. The initial rate of ethyl isovalerate synthesis using pretreated MBGs was found to be *ca.* 1.15-fold higher than for untreated MBGs. The esterification efficiency of untreated MBGs started decreasing after five cycles and was completely lost up to 8 cycles. However, in contrast to untreated MBGs, the esterification efficiency of pretreated MBGs declined or decreased slowly after 5 cycles, with about 50% reduction in esterification activity at the end of the 12th cycle. The amount of water extracted during pretreatment of MBGs with dry reverse micellar solution was monitored by Karl-Fischer titration and the R values of MBGs were calculated. The R value of freshly prepared MBGs was reduced from *ca.* 60 to 47.5 after pretreatment with 1 M AOT/isooctane solution for 24 h. The lower R value of pretreated MBGs could be accounted for by the 1.15-fold higher initial rate of ethyl isovalerate synthesis during the first cycle of esterification over that exhibited by untreated MBGs. Moreover, the lower initial R value enhanced the reusability of pretreated MBGs, with 40% loss of initial esterification activity after 11 cycles at which the R value was found to be *ca.* 89.7. The free water content of MBGs was also monitored *in situ* by solid-state ^{1}H-NMR by determining the ratio for protons of free water to bound protons of the gel. This ratio was found to increase from 0.27 for pretreated gel to 0.53 for MBGs after 11 cycles of reuse.

We monitored the changes in physical appearance of the gel (initial and after 10 cycles) using SEM. The SEM (Figs. 2A and 2B) images demonstrate that there was considerable swelling in the gel after 10 cycles, as can be seen by the increase in pore size in comparison with the initial gel. This swelling of the gel could be attributed to the accumulated water produced during ester synthesis. It has been reported earlier that with an increase in water concentration, coexisting w/o microemulsion droplets will be expected to increase in size [3, 15]. The decrease in esterification activity with increasing water concentration in MBGs may be due to several reasons. One of the reasons may be that increased water may alter the hydrophobicity of MBGs, thereby altering the partitioning of substrate and products and thus affecting the condensation activity. Another reason may be that the water produced in the esterification reaction hydrates the enzyme excessively, which can lead to lower reaction rates and/or unfavorable

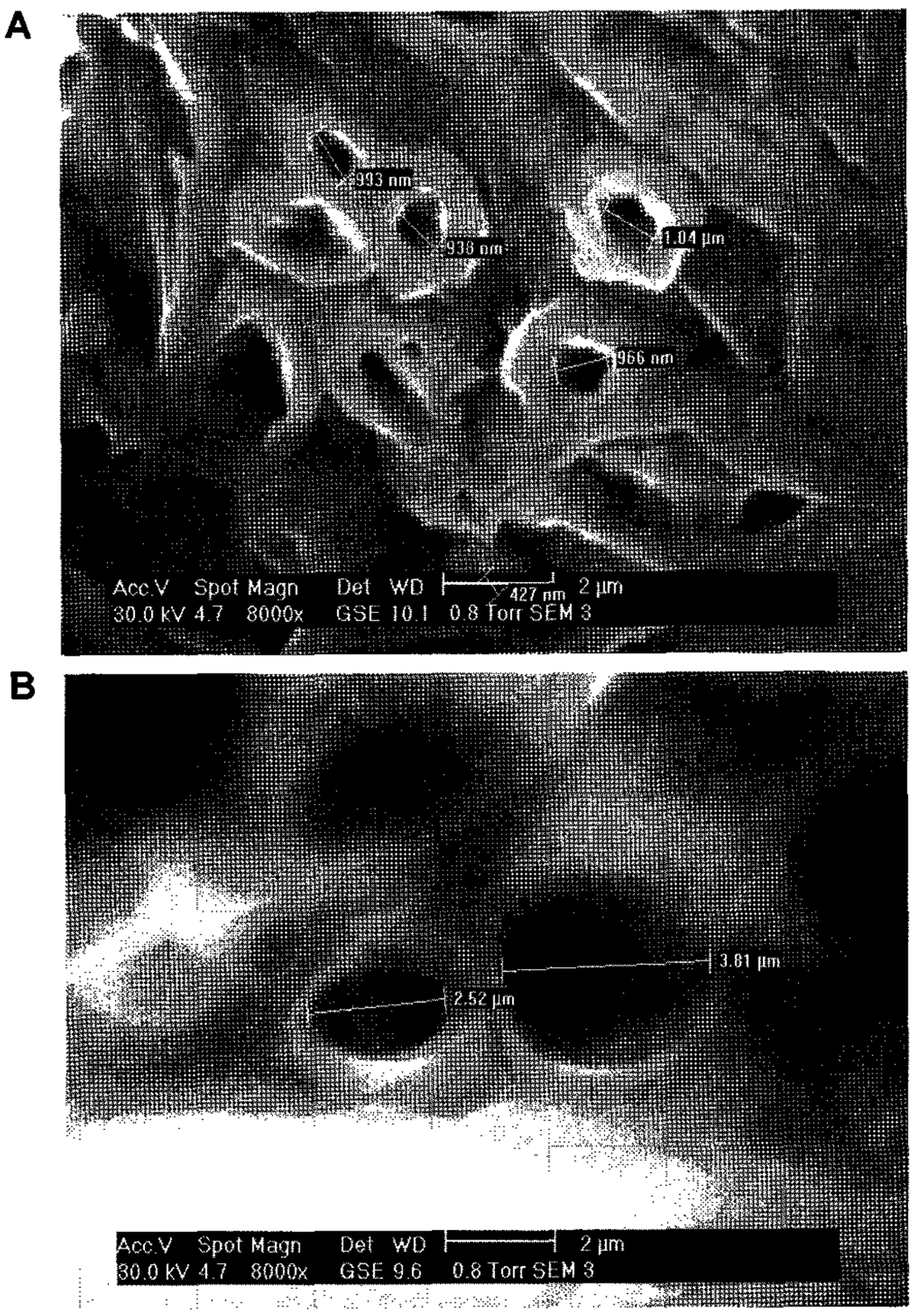

Fig. 2. A. SEM image of pretreated gel. **B**. SEM image of gel after 10 cycles.

equilibrium [10, 24]. The increasing water content is also expected to progressively favor the hydrolysis reaction of synthesized ester [12].

Thus, a decrease in activity upon repeated use of MBGs could be co-related to the increased concentration of free water, making it essential to remove excess water in order to enhance reusability [6, 19, 25].

Intermittent Treatment

It is essential to remove the water produced during esterification reactions in order to prevent its adverse effect on condensation activity. This was done by intermittent treatment of pelleted MBGs with AOT/isooctane dry reverse micellar solution after every 3 cycles. Fig. 3 shows that, upon intermittent treatment, the esterification activity could be retained (80%) up to 8 cycles over a period of 72 days, after which it reduced to 60% during the next 3 cycles. Thus, a considerable improvement in reusability could be achieved with intermittent treatment. The removal of excess water from MBGs upon treatment with dry reverse micellar solution was demonstrated by solid-state

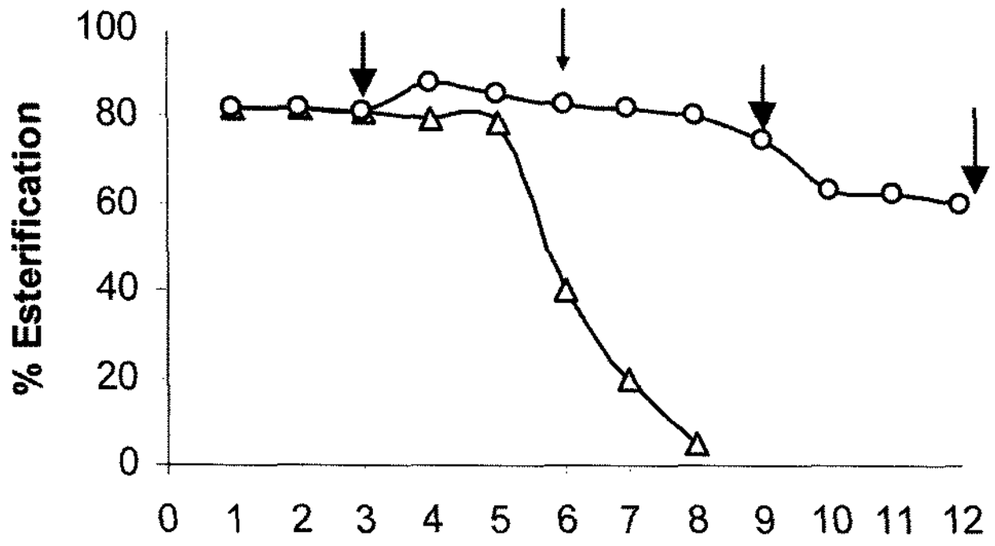

Fig. 3. Repeated cycles of ethyl isovalerate synthesis using surfactant coated lipase immobilized in AOT based organogels; Δ Untreated MBGs, Intermittently treated MBGs.
Arrows indicate the time of treatments.

^{1}H-NMR as well as Karl-Fisher titration for gels obtained after 3 cycles of esterification. The ratio of free water protons to bound protons of gel decreased from 0.47 to 0.39 after treatment of the gel with 1 M AOT/isooctane reverse micellar solution, which indicates removal of free water from the gel. The amount of water extracted from the gel in dry reverse micellar solution was monitored using Karl-Fischer titration and used to calculate R values of MBGs. The R value of MBGs increased from 60 (initial) to 82.6 after three cycles of ester synthesis, which upon treatment with dry reverse micellar solution was reduced to 68.6. Thus, by giving intermittent treatment to MBGs with dry reverse micellar solution after every three cycles, accumulation of water to inhibitory concentration was prevented. Jenta *et al.* [16] found the use of liquid dessicant to be most effective amongst various methods for removal of water from MBGs. They proposed that regeneration of biocatalytic activity upon treatment of gels with dry reverse micellar solution occurs predominantly because of the selective extraction of water from the gel matrix, rather than from the influx of surfactant into the MBG from the reservoir of dry micelles.

Optimization of Treatment Conditions for MBGs

The intermittent treatment of MBGs after every 3 cycles enabled the use of immobilized lipase for up to 9 cycles without significant loss in esterification activity. Thus, further studies were done to optimize the treatment conditions.

In all of the previous reports, dry reverse micellar treatment has been done using 1 M AOT in organic solvent [16, 23]. However, since the higher concentration of AOT may have deleterious effects on enzyme activity, we investigated treatments of MBGs with dry reverse micellar solutions at varying concentrations of AOT/isooctane. It was found that 0.2 M AOT in isooctane was found to be as efficient in the regeneration of esterification activity as 1 M AOT in isooctane. Thus, in further experiments, MBGs

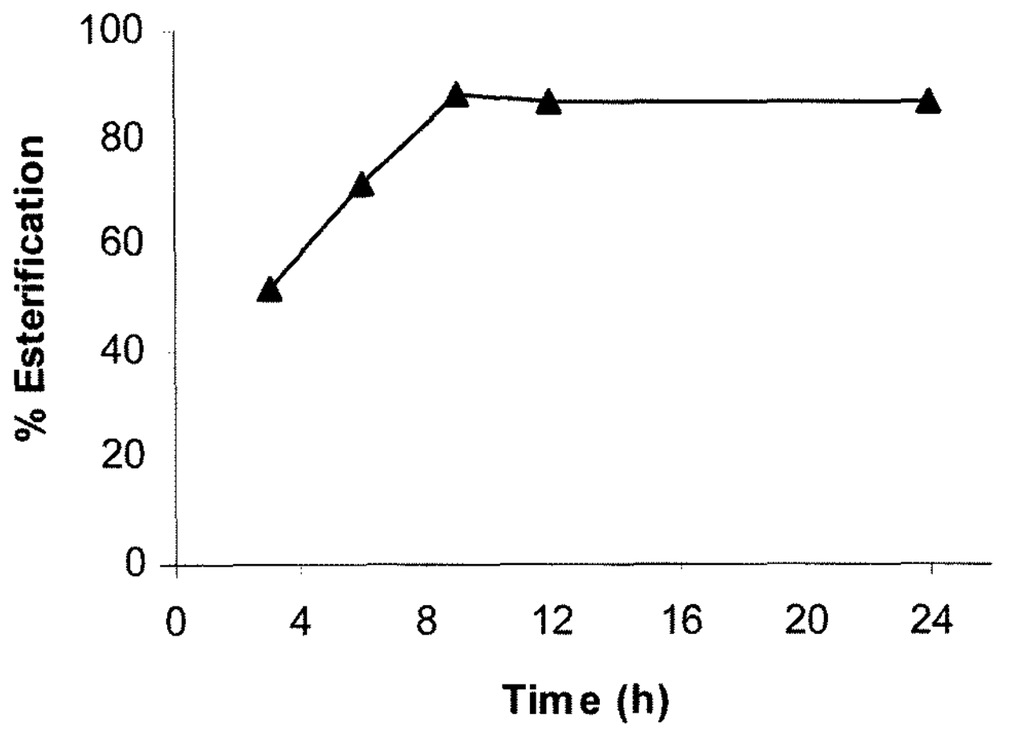

Fig. 4. Treatment of MBGs with 0.2 M AOT in isooctane for varying time periods.

were treated with reverse micelles containing 0.2 M AOT concentration. Then, in an attempt to optimize the treatment time with dry reverse micellar solution, MBGs were treated with 0.2 M AOT in isooctane solution for varying time periods. As per earlier reports, the dry reverse micellar treatment was given for 24 h. However, in our studies, we found that a 9-h treatment was enough for effective recovery of enzyme activity (Fig. 4).

Comparison Between Pelleted and Granulated MBGs

The rate of ester synthesis in MBGs also depends on enzyme loading and surface area per unit volume of immobilizate [15]. It has been very meticulously demonstrated earlier that in the case of pelleted MBGs at higher enzyme loading, because of the diffusional limitation of substrates/ products, the surface catalysis would predominate resulting in inefficient use of the enzyme [16]. In order to study the same phenomenon, we compared the esterification

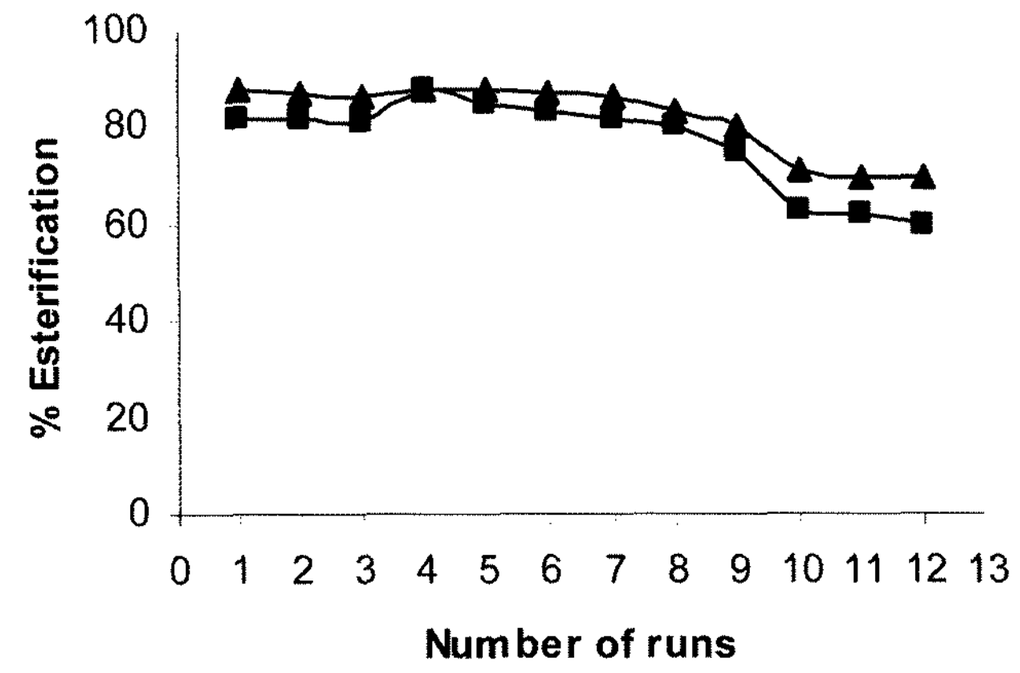

Fig. 5. Comparison of esterification efficiency of pelleted MBGs and granulated MBGs during repeated cycles of ethyl isovalerate synthesis; ■, Pelleted MBGs; ▲, Granulated MBGs.

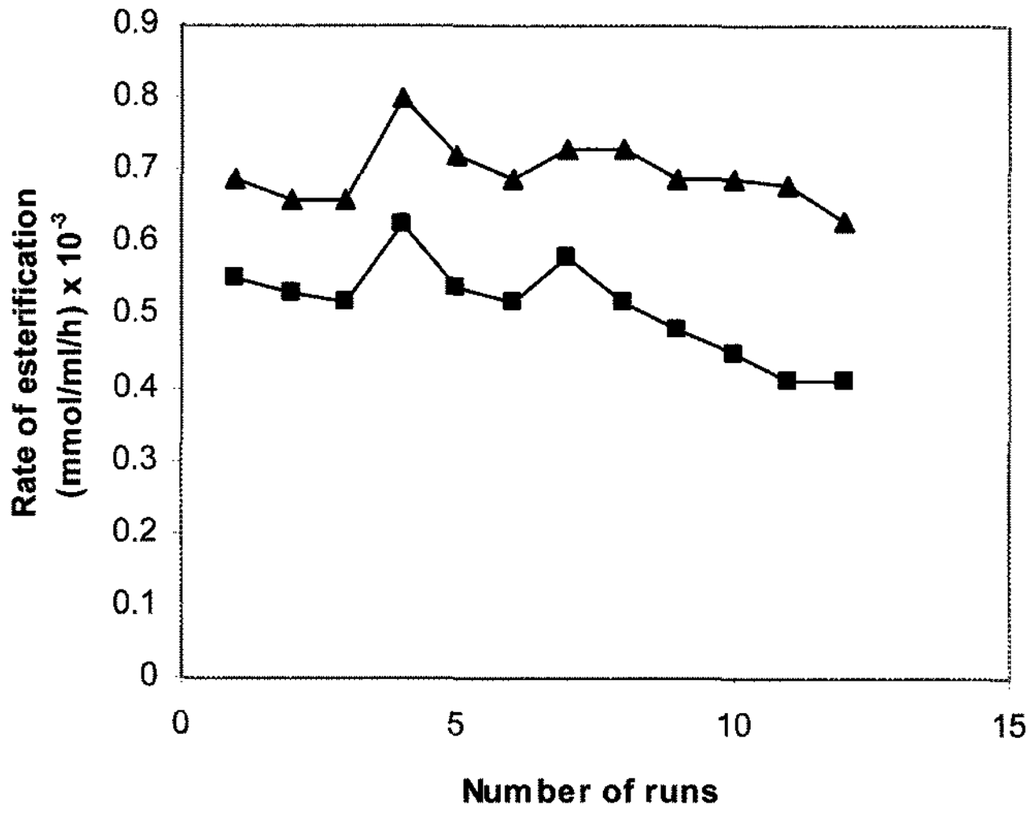

Fig. 6. Rate of ethyl isovalerate synthesis during repeated cycles using granulated and pelleted forms of surfactant-coated lipase immobilized in MBGs; ■, Pelleted MBGs; ▲, Granulated MBGs.

efficiency and reusability of untreated pelleted and granulated MBGs at equal enzyme loading, where MBGs were intermittently treated with dry reverse micellar solution of 0.2 M AOT in isooctane after every 3 cycles. The total surface area of pelleted and granulated MBGs was found to be 2.8×10^4 mm^2/cm^3 and 2×10^5 mm^2/cm^3 of the gel, respectively. It was observed that the yield of ethyl isovalerate at equilibrium using granulated MBGs was slightly higher in comparison with pelleted MBGs (Fig. 5); however, granulated MBGs exhibited significantly higher (1.2-fold) initial reaction rates (Fig. 6) than pelleted MBGs. Granulation of MBGs results in reduced transport distances in organogels, and a higher surface area to volume ratio, resulting in more efficient use of immobilized enzyme and thus higher initial rates of esterification in comparison with pelleted MBGs [7]. Furthermore, it would lead to more effective extraction of water during treatment with dry reverse micellar solution and, thus, more efficient reusability of enzyme in comparison with pelleted MBGs.

The treatment of MBGs with a dry reverse micellar solution of AOT in isooctane leads to selective extraction of free water from the gel, and this approach can be employed for removal of excess water from gels containing immobilized lipase, resulting in an enhanced rate and reusability of enzyme for condensation reactions. The reusability of lipase immobilized in MBGs can be improved by obtaining MBGs with lower R values upon pretreatment and/or intermittent treatment with liquid desiccant at regular intervals during repeated cycles of esterification. The granulated form of MBGs provides a higher surface area and thus exhibits a higher rate of esterification in comparison with the pelleted MBGs.

Acknowledgments

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