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Synthesis of the 'green apple ester' ethyl valerate in organic solvents by *Candida rugosa* lipase immobilized in MBGs in organic solvents: Effects of immobilization and reaction parameters

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ABSTRACT

Ethyl valerate, also known as the green apple flavor is well known for its wide applications in the areas of food, pharmaceuticals and cosmetics industries. *Candida rugosa* lipase was immobilized in microemulsion based organogels (MBGs) and used for ethyl valerate synthesis in organic solvents. Various immobilization and reaction parameters were scrutinized for enhancement of ester production. Among the immobilization parameters, sodium bis-2-(ethylhexyl) sulfosuccinate (AOT), n-heptane and gelatin were found to be the highest yielding combination. Cyclohexane was found to be the solvent of choice as the reaction medium while pH 7, 40 °C and 1:1.6 ratio of valeric acid to ethanol were the reaction parameters exhibiting highest ester formation. The organogels were highly stable in the solvents and were reused for nine cycles with meager loss of activity. Also, immobilized enzyme was thermostable at 50–70 °C for ten hours. Hence, MBGs verify to be a promising enzyme immobilization system for ester synthesis in organic solvents.

1. Introduction

From the time enzymes were discovered, they have been a major subject of intense research owing to their spectacular properties. Enzymes, being highly specific and extremely enantio- and regioselective catalysts, are being used extensively in the industrial production of bulk chemicals, pharmaceutical and agrochemical intermediates and food ingredients [1,2]. Though water is the natural milieu for enzymes, non-aqueous enzymology emerged as the cutting edge for a variety of reactions which are otherwise virtually impossible in the aqueous counterpart, along with elimination of interfering side reactions [3,4] and thereafter, this mode of biocatalysis is being widely used in various processes [5,6].

Among the enzymes used in organic syntheses, the most frequently used enzyme is lipase [7,8]. Biocatalysis involving lipases has been well studied and understood in the past few decades [9,10]. Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes that are activated when adsorbed to an oil–water interface [11,12]. Due to their widely diversified enzymatic properties, in particular, the ability to perform enantioselective hydrolytic reactions and catalyze the formation of a wide range of ester and amide bonds, microbial lipases have become very attractive for industrial applications [13–15]. One of the numerous applications of lipases is production of flavor esters. In recent times, flavors represent over a quarter of the world market for food additives and it has been shown that consumers prefer foodstuff that can be labeled as "natural" [16]. The tag "natural" and value added features of these biochemically produced esters excelling their chemical counterparts by having better odor and flavor [17] have intensified research activities in this field [18].

However, high costs and tough purification procedures render the enzymes economically unattractive. Moreover, reactions involving fats and oils require high temperatures for long durations meant for their (fats) solubility in the medium, which may result in enzyme denaturation. Hence, an enzyme has to fulfil important requisites such as high turnover, solvent tolerance, reusability and thermostability if it has to be used for bulk production processes. A possible solution to this problem is its recovery and reuse by immobilizing in/on a solid support which makes it cost effective [19]. Various modes of immobilization of the enzymes have been used till date including adsorption, ionic binding, covalent attachment, entrapment and encapsulation [20–22]. One of the well-explored methods in non-aqueous biocatalysis is immobilization of enzymes in microemulsion based organogels (MBGs). Structure of MBGs has been very well established as an extensive, rigid, interconnected network of gelatin/water rods stabilized by a monolayer of surfactants, in co-existence with a population of conventional w/o microemulsion droplets [23,24]. Though MBGs have been used for production of a variety of flavor esters [25–27], very scanty infor-

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mation is available on production of ethyl valerate, especially by immobilized enzyme [28–30]. Ethyl valerate, also known as green apple flavor is an important ester used as a constituent in many fruity flavors such as plum, apricot, peach, strawberry and pear aromas [31].

In this study, ethyl valerate has been synthesized by condensation of ethanol and valeric acid catalyzed by MBGs containing *Candida rugosa* lipase (CRL) in small-scale model studies. The immobilization parameters i.e. surfactants, organic solvents and gelling agents were assessed for preparation of MBGs. Effect of reaction parameters such as water/surfactant mole ratio (also known as Wo), pH of buffer, substrate concentration, organic solvents and temperature on the esterification reaction was investigated. Further, the MBGs were subjected to reusability and thermostability studies.

2. Materials and methods

2.1. Materials

Sodium bis-2-(ethylhexyl) sulfosuccinate (AOT), Tween 80 and n-hexanol were obtained from Fluka (Switzerland). Cetyl trimethyl ammonium bromide (CTAB) and Triton X-100 were procured from Hi-Media (India). Ethyl valerate and valeric acid were purchased from Fluka (Switzerland). *C. rugosa* lipase (triacylglycerol acyl hydrolase, E.C. 3.1.1.3, type VII) was supplied by Sigma (Germany), with the total activity of 965 U/mg of solid. Agarose was from Bangalore Genei (India), Carboxyl methyl cellulose (CMC) of high viscosity and agar-agar were acquired from Hi-media (India). All other organic solvents used were of HPLC/GC grade.

2.1.1. Preparation of reverse micelles using different surfactants

Thermostable reverse micellar solutions were prepared according to the procedure of Dandavate and Madamwar [32]. Anionic, cationic and non-ionic reverse micellar solutions were prepared using AOT, CTAB, Triton X-100 and Tween 80 respectively. The organic solvents used were isooctane, n-hexane and n-heptane and the aqueous phase consisted of lipase (CRL) dissolved in buffer of pH 7.2 (except for pH studies). Water/surfactant mole ratio (Wo) and ratio of surfactant to co-surfactant, abbreviated as Po (in case of cationic and non-ionic surfactants) were determined for each reverse micellar solution.

2.1.2. Preparation of w/o based microemulsion gels using various gelling agents

Microemulsion based organogels were prepared following the procedure used by Dandavate and Madamwar [32] with slight modifications, as follows. Gelatin obtained from porcine skin was dissolved in double distilled water, autoclaved and cooled down to 55 °C. To 1 mL of thermodynamically stable reverse micellar solution, 1.5 mL gelatin (of concentrations ranging from 10 to 20%) maintained at 55 °C in a hot water bath was added and the mixture was vortexed vigorously for 2–5 min. The resulting mixture was cooled to 25 °C, poured into plastic petriplates and kept for air drying overnight. The dried gels were cut into small pieces and used for esterification reaction. In a similar fashion, AOT based MBGs of agarose, CMC and agar-agar (1–10% concentration each) were prepared using various concentrations of the gelling agents.

2.1.3. Esterification reaction conditions

The reaction was carried out in 250 mL glass stoppered flasks. The reaction mixture consisted of 20 mL of solvent and equimolar concentration (0.1 M) of valeric acid and ethanol (except for substrate effect studies). To initiate the reaction, the MBG pieces were added to the reaction mixture and kept on orbital shaker at 37 °C (except for temperature effect studies) and 150 rpm. 500 μL of the reaction mixture was withdrawn every 24 h and immediately analyzed by gas chromatography for accumulation of ethyl valerate.

2.1.4. Effect of solvents on esterification

To study the interactive effect of the two solvents, one used for MBG preparation and another as reaction medium on esterification, AOT based organogels containing lipase (5790 U/mg of solid) were used to carry out esterification in various organic solvents. Solvents used for MBG preparation were isooctane, n-hexane and n-heptane while those used as reaction medium were isooctane, n-hexane, n-heptane, cyclohexane, acetone, acetonitrile and DMSO.

2.1.5. Effect of Wo on esterification

Reverse micellar solutions of Wo ranging from 10 to 100 were prepared in AOT/Buffered lipase/isooctane and gelatin system while cyclohexane was used as reaction medium. The reaction was performed under standard conditions as mentioned in Section 2.1.3.

2.1.6. Effect of buffer pH on esterification

It has been shown that the water present within reverse micelles exhibits different properties as compared to bulk water [33]. This may result in a different pH of the buffer inside the water pool of reverse micelles when compared to pH of the same solution measured in bulk. Hence, to determine the optimum pH required for esterification using MBGs, lipase (5790 U/mg of solid) was dissolved in five buffers of different pH: acetate buffer—pH 5, sodium phosphate buffer—pH 6, 7 and 8, Tris–HCl buffer—pH 8.8. All the buffers were of 0.1 M strength. The buffers containing lipase were immobilized in AOT based organogels and monitored for esterification reaction.

2.1.7. Effect of temperature on esterification

Effect of temperature was studied by carrying out the reaction at five different temperatures (25, 30, 37, 40 and 45 °C). The reaction system consisted of MBGs of AOT/buffered lipase (5790 U/mg of solid)/isooctane in cyclohexane. A control was kept at each temperature with free lipase as the catalyst.

2.1.8. Effect of substrate on esterification

For determination of effect of substrates on esterification, the concentration of one substrate was kept constant while the other was varied. The concentration of the varied substrate corresponding to complete utilization of both substrates was then kept constant and the other (kept constant in first set) was varied. In first set of reactions, ethanol concentration was kept constant at 0.1 M while varying valeric acid concentration from 0.02 to 0.09 M, and in second set of reactions, ethanol concentration was varied from 0.04 to 0.2 M while keeping valeric acid concentration constant at 0.06 M. The reaction system comprised AOT/buffered lipase (5790 U/mg of solid)/isooctane in cylcohexane.

2.1.9. Reusability of the MBG preparation

After completion of a reaction cycle, the MBGs prepared using three different solvents viz. isooctane, n-hexane and n-heptane were washed with solvent 2–3 times for complete removal of substrates and product. They were air dried and reused for ester production. After 3–4 runs, when a sharp decline in percentage esterification was observed, the MBGs were treated with dry reverse micellar solution of 1 M AOT overnight for removal of excess water (byproduct of esterification reaction); given 2–3 solvent washes for complete removal of AOT and used for esterification.

2.1.10. Thermostability studies

Thermostability studies were carried out for free as well as immobilized lipase in order to evaluate the innate stability of the enzyme and the additional stability offered by immobilization against thermal denaturation. The analysis was done by preincubating free and immobilized enzyme (5790 U/mg of solid each) at 50, 60 and 70 °C for 1–10 h in cyclohexane. After incubation, substrates were added to the flasks and esterification was carried out under standard conditions.

2.2. Analytical procedure

After initiation of reaction, 500 μL sample of the reaction mixture was withdrawn periodically every 24 h and immediately analyzed by gas chromatography (PerkinElmer, Model Clarus 500, USA) equipped with a flame ionization detector and 30 m TRX-R-20 (Crossband 80% dimethyl-20% diphenyl polysiloxane) capillary column. The carrier gas was nitrogen at a split flow rate of 90 mL/min. The injector and detector temperatures were 250 and 280 °C respectively and oven temperature was programmed to increase from 100 to 160 °C at the rate of 20 °C/min, from 160 to 280 °C at the rate of 2 °C/min and from 165 to 175 °C at the rate of 1 °C/min. Ester identification and quantification were done by comparing the retention time and peak area of the sample with standard. Pure ethyl valerate was used as external standard.

3. Results and discussion

Ethyl valerate, which is well known for its fragrance, has been synthesized biocatalytically by many investigators. However, many of them have reported catalysis using whole cells [29,34] whilst a few used immobilized enzyme [28] and others have tried both ways [30].

The total yields obtained by immobilized enzyme [28,30] and those from whole cells [30] have however not been very appreciable. Hence, this study was focused mainly on immobilization of purified enzyme for the same purpose of obtaining higher percentage conversions.

3.1. Preparation of stable reverse micelles using different surfactants

Due to amphipathic nature, surfactants behave as the interface between the organic phase and the enzyme harboring buffer providing first level of protection to the enzyme entrapped within the micelle. Thus, it is important to use a surfactant which is stable in such conditions. All the four surfactants i.e. AOT, CTAB, Triton X-100 and Tween 80 formed thermostable reverse micellar solutions with the solvents. However, optimum value of Wo and Po (in case of cationic and non-ionic surfactants) for stable micelle formation using different surfactants in the three solvents was dissimilar (Table 1). Addition of buffer above this optimum Wo value resulted in a turbid solution indicating dissociation of reverse micelles. The stable reverse micelles thus produced were immobilized in gelling agents for further studies.

Table 1
Optimum values of Wo (ratio of water to surfactant) and corresponding Po (ratio of co-surfactant to surfactant) of reverse micellar solutions prepared using the surfactants (0.1 M)-AOT, CTAB, Triton X-100 and Tween 80; solvents—isooctane, n-hexane and n-heptane; co-surfactant-n-hexanol and 0.1 M sodium phosphate buffer.

Surfactant	Organic solvents					
	Isooctane		n-Hexane		n-Heptane	
	Wo	Po	Wo	Po	Wo	Po
AOT	60	–	50	–	70	–
CTAB	30	7.84	60	5.49	90	10.98
Triton X-100	50	11.76	30	25.88	50	15.68
Tween 80	40	15.68	40	14.11	50	18.04

3.2. Esterification using MBGs prepared by various gelling agents

Enzymes are relatively expensive, and therefore discarding them after a single use is not economical. Another problem is their general instability towards heat, organic solvents, acids or bases, etc. One method to circumvent these issues is to immobilize the enzyme on a suitable supporting medium. Not only does this facilitate easier recovery and reuse of the enzyme, but also in many cases, immobilized enzymes show a higher stability than free species [35]. For this reason, various gelling agents have been used to immobilize them making them easily recoverable [36,37]. Four gelling agents viz. gelatin, carboxy methyl cellulose (CMC), agarose and agar-agar were used in different concentrations for preparation of AOT based organogels. MBGs prepared using gelatin (14%) were uniformly porous and thick. For agar-agar, agarose and CMC, 5% gelling agent was sufficient for organogel preparation though the MBGs were thin, translucent and non-porous. Also, concentrations above 5% gelling agent either resulted in gels with phase separation or highly viscous gels that could not be poured into the petriplates at all. The esterification yield of gelatin MBG (~99%) was many folds higher than the yield obtained using CMC (25.55%) and agarose/agar-agar (negligible).

Thus, different concentrations of gelatin were used for preparation of MBGs using CTAB, Triton X-100 and Tween 80. The physical characteristics of the same are displayed in Table 2a. The finest organogels thus obtained were further used for esterification. As seen from Table 2b, AOT based organogels catalyzed the reaction more efficiently than the others. Highest esterification was seen in AOT/n-heptane organogels in n-heptane medium (97.82%) which can be attributed to high Wo value for the same (Wo = 70). Being anionic in nature, AOT does not require any co-surfactant for stabilization, and therefore, it has been the surfactant of choice for many investigators [38–40]. Thus, AOT based organogels using gelatin were used for further experimentation. Gelatin has been the gelling agent of choice for this purpose for many years [41–43].

3.3. Effect of organic solvents

It is crucial to introduce organic solvents for bioconversions of lipophilic compounds for improving the poor solubility of the substrates in water. Organic solvents produce various physicochemical effects on enzyme molecules, and the effects differ depending on the kinds of organic solvents and enzymes used. It has been reported that suspension of enzymes in organic solvents results in conformational changes and thereby the specificity of substrates [44,45]. Among the various organic solvents used, there was no esterification observed in acetonitrile which can be attributed to its highly polar nature. It has been hypothesized that polar solvents strip away the essential water bound to the enzyme by participating in non-covalent solvent protein interaction resulting in their active conformational distortion. This is observed in the form of lowered or complete absence of catalytic properties of the enzyme. DMSO dissolved the organogels completely which may have been due to loss of surfactant in this medium. Similar effects with DMSO have been observed by Aguiar et al. [46] and Dave and Madamwar [47]. Deleterious effects of other solvents such as ethanoic acid and butanoic acid have also been reported [36,48]. It can be seen from Fig. 1a–c that all the organogels showed high ester production in isooctane, n-heptane, n-hexane and cyclohexane. They showed exceptionally higher yield at faster rates when cyclohexane (Fig. 1a–c) was used as the medium. Highest yields with all three MBGs were seen in shortest time period in this reac-

Table 2a
Physical characteristics of organogels prepared using reverse micellar solutions containing surfactants (0.1 M) AOT, CTAB, Triton X-100, Tween 80 and solvents n-hexane, n-heptane and isooctane with various concentration of gelatin.

Surfactant	Organic solvent	Gelatin (%)	Physical characteristics of organogel
AOT	Isooctane	<14	Thin, fragile, transparent
		14	Porous, translucent, strong
		>14	Thick, opaque, non-porous
	n-Hexane	<14	Thin, fragile, transparent
		14	Porous, translucent
		>14	Thick, opaque, non-porous
	n-Heptane	<14	Thin, fragile, transparent
		14	Porous, translucent
		>14	Thick, opaque, non-porous
CTAB	Isooctane	<16	Thin, fragile, chalky
		16	Porous, white, brittle
		>16	Thick, non-porous
	n-Hexane	<20	Thin, chalky
		20	Porous, white, brittle
		>20	Very thick, chalky, non-porous
	n-Heptane	<20	Thin, chalky
		20	Porous, white
		>20	Very thick, chalky, non-porous
Triton X-100	Isooctane	<18	Thin, transparent, brittle
		18	Thin, translucent, elastic
		>18	Thick, translucent
	n-Hexane	<20	Thin, transparent, brittle
		20	Translucent, elastic
		>20	Thick, opaque
	n-Heptane	<18	Thin, transparent
		18	Thin, translucent, elastic
		>18	Thick, translucent
Tween 80	Isooctane	<16	Thin, very frail, transparent
		16	Translucent, elastic
		>16	Thin, hard, non elastic
	n-Hexane	<16	Thin, fragile, transparent
		16	Translucent, elastic
		>16	Thin, hard
	n-Heptane	<16	Thin, fragile, transparent
		16	Translucent, elastic
		>16	Thin, hard, non elastic

tion medium (Fig. 1a–c). This can be attributed to the extreme hydrophobic nature of the solvent stabilizing the hydrated enzyme structure. However, the free enzyme showed poor product formation under similar conditions (Fig. 1a–c). Furthermore, it was observed that better results were obtained when two different solvents were used for the two purposes (one for MBG preparation and the other as reaction medium) as compared to the reactions using the same solvent for both purposes. Similar effects of these organic solvents have been observed by Yesiloglu and Kilic [49], Gogoi et al. [50] and Jenta et al. [42].

As Wo of MBG prepared in isooctane was lower (60) than n-heptane (70), it follows that the AOT/isooctane system used less lipase than the AOT/n-heptane system and contributed to equivalent production (Higher the Wo value, more is the enzyme used). Hence, AOT/isooctane MBG was chosen for further experimentation in combination with cyclohexane as reaction medium.

3.4. Effect of Wo on esterification

The water/surfactant mole ratio, given the symbol Wo is directly proportional to the size (radius) of the dispersed water droplets. Fig. 2 shows that ester formation increased with increase in Wo from 10 to 60. Reverse micellar solutions of Wo higher than 60 were turbid and showed a slight decrease in esterification (Fig. 2). Thus, optimum value of Wo for AOT/isooctane was 60 for maximum esterification. Turbidity above a certain Wo value indicated that the reverse micelles which could not hold more water started breaking and hence showed lowered values of esterification. Similar observations have been made by Jenta et al. [42] and Soni and Madamwar [48].

3.5. Effect of pH on esterification

Water inside reverse micelles displays unusual properties when compared to those of bulk water [33]. This had led many investigators to analyze properties of reverse micellar water [51]. Thus, in this study, the optimum pH required for esterification reaction using lipase entrapped in MBGs was investigated for the same purpose. High esterification values were observed at low pH values of pH 5 (85.7%), pH 6 (97.3%) and pH 7 (98.91%). Further increase in pH resulted in decrease in ester production to a mere 22.34% at pH 8.8. As is evident from Fig. 3, the immobilized lipase was stable and showed highest esterification rates in acidic to neutral pH than in alkaline pH. Similar results of esterification maxima at low pH have been observed by others [51,52].

It has also been shown that at alkaline pH, there is a negative potential that appears in the active site in *C. rugosa* lipase resulting in higher activity towards ester hydrolysis [53] and hence, the vice-versa activity at low/acidic pH resulting in reverse reaction (condensation to form ester) can be expected.

3.6. Effect of temperature on esterification

The activity of CRL (free and immobilized in MBGs) towards the esterification reaction was monitored as a function of incubation temperature. Increase in temperature from 25 to 45 °C showed increase in lipase activity (Fig. 4).

It was observed that at higher temperatures (>37 °C), increase in incubation temperature led to significant increase of the reaction's initial rate resulting in high product formation at 37, 40 °C as well as 45 °C whereas at lower temperatures (25 and 30 °C) very low conversions were observed (Fig. 4). This suggests that at higher temperatures, the conversion rate is controlled by reaction steps and hence strongly increases with increase in temperature. However, at lower temperatures, the reaction rate is limited by mass transport phenomena and not by the reaction steps which can be attributed to the low increase in ester production with increase in temperature [40]. Similar results have been observed by Dave

Table 2b
Ester synthesis using AOT, CTAB, Triton X-100 and Tween 80 based organogels using buffer of pH 7.2 containing lipase at 37 °C and 150 rpm. The reactions reached completion on 9th day.

Surfactant	Solvent used for organogel preparation as well as for reaction medium					
	Isooctane		n-Heptane		n-Hexane	
	Gelatin (%)	Esterification (%)	Gelatin (%)	Esterification (%)	Gelatin (%)	Esterification (%)
AOT	14	78.13 ± 0.8	14	97.82 ± 0.9	14	75.38 ± 1.6
CTAB	16	19.21 ± 0.9	20	74.74 ± 1.1	20	32.20 ± 0.8
Triton X-100	18	11.01 ± 0.7	16	12.46 ± 0.8	20	4.27 ± 0.7
Tween 80	16	10.84 ± 0.7	16	27.13 ± 0.9	16	12.50 ± 0.7

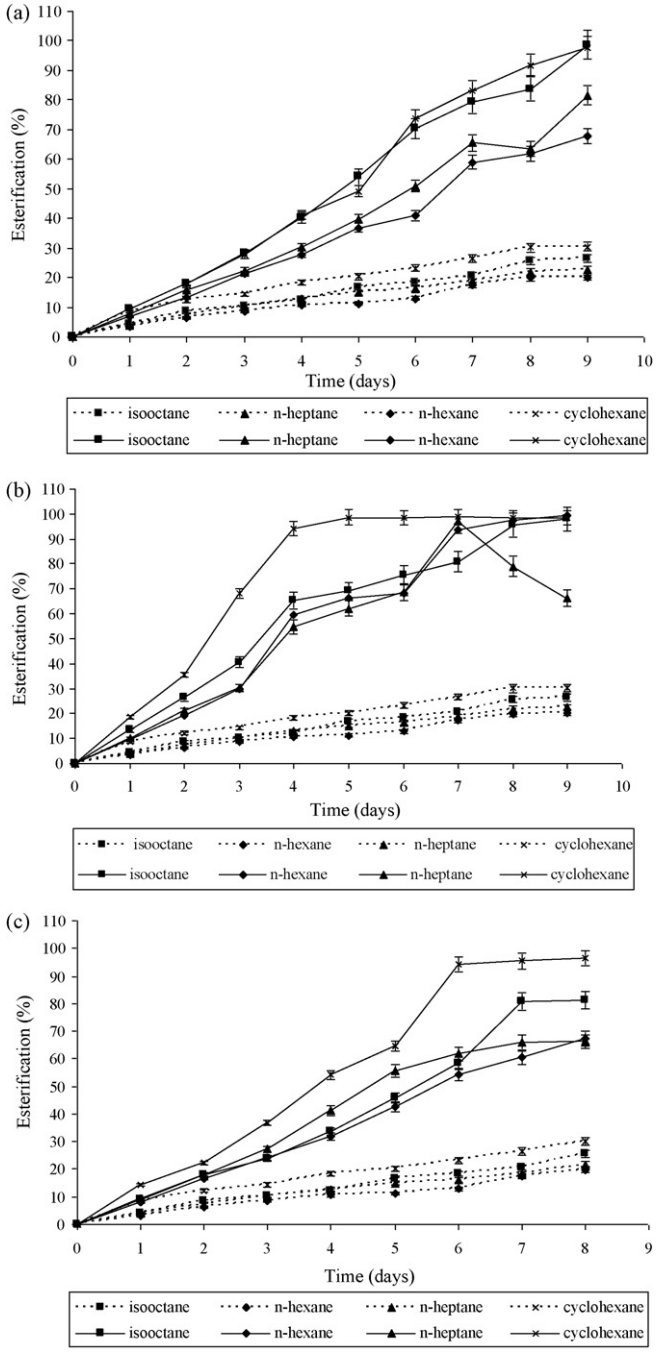

Fig. 1. (a) Synthesis of ethyl valerate using AOT/isooctane organogels in isooctane, n-heptane, n-hexane and cyclohexane at 37 °C and 150 rpm for 9 days. The solid lines represent lipase entrapped in MBGs and the dotted lines represent free lipase. (b) Synthesis of ethyl valerate using AOT/n-heptane organogels in isooctane, n-hexane, n-heptane and cyclohexane at 37 °C and 150 rpm for 9 days. The solid lines represent lipase entrapped in MBGs and the dotted lines represent free lipase. (c) Synthesis of ethyl valerate using AOT/n-hexane organogels in isooctane, n-hexane, n-heptane and cyclohexane at 37 °C and 150 rpm for 8 days. The solid lines represent lipase entrapped in MBGs and the dotted lines represent free lipase.

and Madamwar [54] and Schlatmann et al. [39]. Optimum temperature was found to be 40 °C, showing 99.87% product formation on 6th day whereas the reactions proceeded till days 8–9 at other temperatures. Also, free lipase used as control showed extremely meager ester production at higher temperatures when compared to the immobilized lipase (Fig. 4). However, the productivities of free as well as immobilized lipase were similar at 25 and 30 °C which showed that the immobilized lipase preparation needed higher

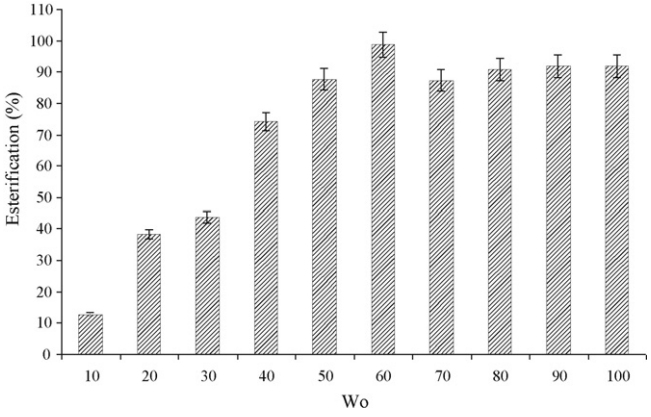

Fig. 2. Effect of Wo on esterification using AOT based organogels at 37 °C and 150 rpm.

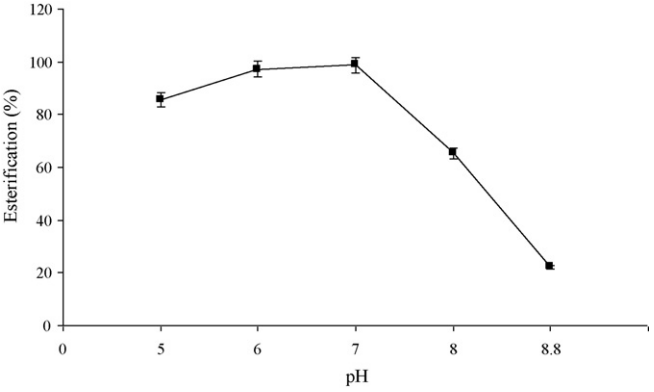

Fig. 3. Effect of pH on esterification using AOT based organogels at 37 °C and 150 rpm.

temperature for optimum activity. It also conveyed the effective protection to lipase offered by immobilization process against thermal denaturation.

3.7. *Effect of substrate concentration on esterification*

As substrate concentration is a very significant parameter in any enzyme catalyzed reaction, it is important to determine their effects on the enzyme and reaction kinetics. It is also very essential to set up reactions in such a way that result in maximum pro-

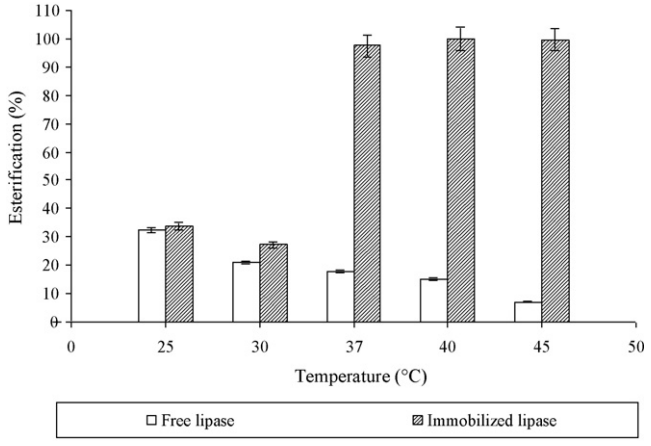

Fig. 4. Effect of different temperatures on ethyl valerate synthesis by free and AOT based organogels 150 rpm.

Table 3a
Effect of valeric acid concentration on ester production using AOT based organogel using buffer of pH 7.2 containing lipase at 37 °C and 150 rpm. Ethanol concentration was kept constant at 0.1 M. The reactions were carried out for 8 days.

Valeric acid concentration (M)	Esterification (%)	Residual substrate (%)
0.02	41.57 ± 1.2	0.11 ± 0.7
0.03	52.90 ± 1.1	0.37 ± 0.9
0.04	63.21 ± 0.8	0.58 ± 0.8
0.05	90.23 ± 0.7	2.66 ± 0.7
0.06	98.89 ± 0.9	5.50 ± 1.2
0.07	96.67 ± 0.7	17.41 ± 1.1
0.08	85.73 ± 1.2	18.44 ± 0.9
0.09	86.66 ± 0.8	21.27 ± 0.8

duction with minimal wastage/usage of substrates. To determine the optimum ratio of the substrates, the concentrations of ethanol and valeric acid were varied one at a time keeping the other constant and carrying out esterification reaction. In case of variation of valeric acid concentration (ethanol 0.1 M), 98.89% esterification was observed with 0.06 M valeric acid on 8th day and both the substrates were nearly completely utilized (Table 3a). In reactions with less than 0.06 M valeric acid, esterification increased till a maximum of 90% with complete utilization of the acid but some residual presence of the alcohol and in those with more than 0.06 M valeric acid, the vice-versa was observed i.e. considerable quantities of unutilized valeric acid was present. When the same was repeated keeping valeric acid at a constant concentration of 0.06 M (obtained from the previous study) and varying ethanol concentration from 0.04 to 2.0 M, highest yield was obtained at 0.08–0.12 M concentrations (Table 3b). The reactions proceeded very quickly at low alcohol content (0.05–0.08 M) and reached a maximum in 5–6 days while higher concentrations of alcohol exhibited an inhibitory effect on reaction slowing it down drastically showing minimum esterification of 45% at 0.16 M ethanol concentration (Table 3b). Similar phenomena have been observed by Gogoi et al. [50] and Somashekar and Divakar [52]. Thus, the molar ratio of valeric acid to alcohol was found to be 1:1.6 corresponding to maximum esterification with complete utilization of both substrates.

3.8. Reusability of the immobilized enzyme preparation

The MBGs were subjected to reusability examination for determining the efficiency of immobilization. The enzyme preparation was given solvent washes after every cycle and reused in fresh media supplemented with substrates. Three organogels viz. AOT/isooctane, AOT/n-hexane and AOT/n-heptane were reused 9 times with intermittent dry AOT/isooctane reverse micellar treatment after declination in ester production. It was observed that the MBGs catalyzed the reaction appreciably for first three cycles after which their activity started declining slowly (Fig. 5). A sharp decline in ester production was observed in 4th and 8th run with respect to all the three organogels. This may be due to accumulation

Table 3b
Effect of ethanol concentration on ester production using AOT based organogel using buffer of pH 7.2 containing lipase at 37 °C and 150 rpm. Valeric acid concentration was kept constant at 0.06 M. The reactions were carried out for 8 days.

Ethanol concentration (M)	Esterification (%)	Residual ethanol (%)
0.04	81.45 ± 1.2	0.60 ± 1.1
0.06	88.67 ± 0.9	1.23 ± 1.2
0.08	97.67 ± 0.7	4.54 ± 0.9
0.1	98.68 ± 0.9	14.65 ± 0.9
0.12	98.25 ± 1.1	19.38 ± 1.2
0.14	73.61 ± 1.1	21.33 ± 1.2
0.16	45.22 ± 0.7	25.76 ± 0.9
0.2	59.82 ± 0.9	29.76 ± 0.7

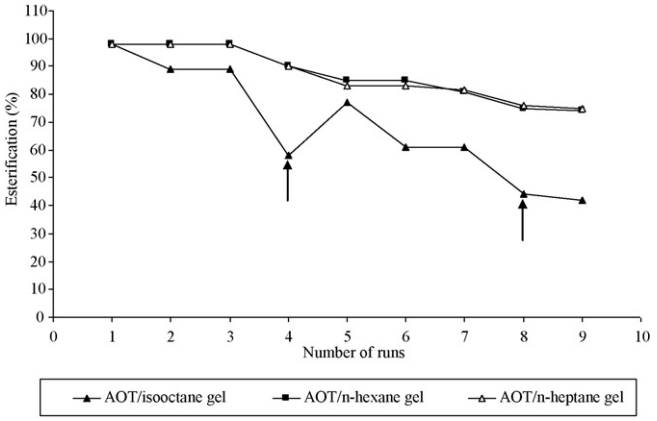

Fig. 5. Reusability of AOT/isooctane, AOT/n-hexane and AOT/n-heptane organogels. The up arrows indicate intermittent treatment of the organogels with 1 M AOT/isooctane dry reverse micellar solution for removal of excess water. The reactions were carried out at 37 °C and 150 rpm for 9 cycles.

of excess water formed as a byproduct of esterification reaction. As water may result in hydrolysis reaction behaving as a substrate, it is important to remove this water. Thus, 1 M dry AOT/isooctane reverse micellar treatment was given to the MBGs for 24 h. Significant increase or maintenance in esterification (no further decrease) was observed in all three MBGs demonstrating partial recovery of the lost activity (Fig. 5). The AOT/isooctane, AOT/n-hexane and AOT/n-heptane organogels exhibited 44%, 75% and 76% residual esterification respectively in 9th run. These results indicate better immobilization efficiency and reaction systems depicted by the high reaction rates even after 8–9 runs as compared to others [40,47,55].

3.9. Thermostability studies

It is well known that proteins, including enzymes are sensitive biological molecules which tend to get denatured under harsh physical and chemical environments (except for a few belonging to extremophiles). High temperatures may result in alteration of spatial structure of many proteins rendering denaturation and consequently loss of their activity. On the other hand, high temperature may also induce minor structural changes resulting in superactivity of the enzyme [56]. In this study, a similar phenomenon was observed at 50 and 60 °C wherein incubation of the organogels for prolonged periods at these temperatures led to increase in ester synthesis providing evidence of some structural change resulting in high activities (Fig. 6). This may also be attributed to slow adaptations to high temperature as incubation for 1–2 h led to loss in activity while in longer incubation this loss was restored, perhaps by adaptation to the temperature. Also, all the organogels were physically stable at all three temperatures. More than 80% esterification was observed even after 10 h incubation at 50 and 60 °C but incubation at 70 °C for more than 6 h resulted in drastic reduction in ester production (Fig. 6). However, in case of free lipase, it was observed that though the enzyme activity was low compared to its immobilized counterpart, but the performance in terms of esterification was more or less constant at all three temperatures even after 10 h of incubation (Fig. 6). These observations point towards an inherent stability of the enzyme against high temperatures which was further enhanced by immobilization. Nevertheless, the product yields were lower than unincubated free enzyme (Figs. 1a–c and 6). However, many reports have shown total or high loss of activities of enzymes (free or immobilized) at such high temperatures and shorter incubation periods [47,57,58]. Hence, the results obtained in this study may be a combined consequence of an

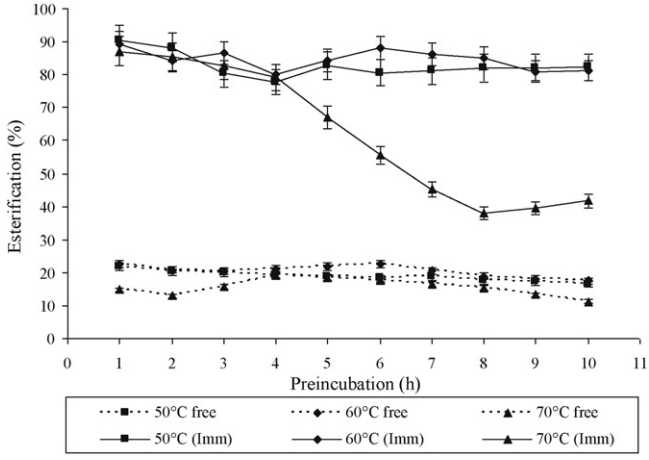

Fig. 6. Thermostability of free and immobilized lipase at 50, 60 and 70 °C after preincubation for 1–10 h. Esterification was carried out at 37 °C and 150 rpm for 8 days. The solid lines represent immobilized lipase and the dotted lines represent free lipase.

innate stability of the enzyme along with the fortification offered by immobilization.

This also signifies that in organogels, the enzyme molecules are not only physically entrapped but also form additional bonds with the water and gelatin networking molecules providing it strong protection from harsh temperatures. This paves way for a variety of processes requiring high temperatures and also reduces/eliminates chances of contamination (if any) and viscosity.

4. Conclusion

Production of ethyl valerate by condensation of ethyl alcohol and valeric acid was performed using CRL immobilized in MBGs under nearly non-aqueous conditions. AOT proved to be the ideal surfactant for organogels among other surfactants yielding as high as 98–99% product under optimum conditions. Gelatin was the gelling agent of choice due to the stronger, stable organogels and higher esterification rates. The combination of the two organic solvents viz. isooctane as reverse micellar constituent and cyclohexane as reaction medium exhibited highest yield. Polar solvents such as acetonitrile and DMSO showed their non-applicability in this area of catalysis by their deleterious effects on MBGs and the reaction. The optimum temperature for this reaction was found to be 40 °C for MBGs. The enzyme showed high activities at acidic to neutral pH (5–7), pH 7 being the optimum while a steep decline was seen in basic pH (8–8.8). The ratio of acid:alcohol for maximum ester production and complete substrate utilization corresponded to 1:1.6 while higher ethanol concentrations displayed substrate inhibition. The organogels could be reused for 9 cycles with excellent activity retention. Also, they were highly stable at 50, 60 and 70 °C for 1–10 h of incubation prior to reaction. This confirms the staturation of microemulsion based organogels in non-aqueous enzymology, especially in production of flavor esters.

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