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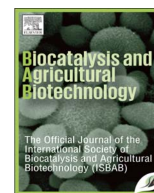

# Increasing esterification efficiency by double immobilization of lipase-ZnO bioconjugate into sodium bis (2-ethylhexyl) sulfosuccinate (AOT)- reverse micelles and microemulsion based organogels

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ABSTRACT

In the present study, we developed double immobilization system for entrapment of lipase to increase overall esterification efficiency. Lipase has been attached on functionalized ZnO (zinc oxide) particles. Fabricated lipase-ZnO conjugates were entrapped into sodium bis (2-ethylhexyl) sulfosuccinate (AOT)-reverse micelles. AOT-reverse micelles containing lipase-ZnO conjugate were further entrapped into microemulsion based organogels (MBGs), prepared from polyvinyl alcohol. These immobilization systems were characterized by fourier transform infrared spectroscopy (FTIR) and thermal gravimetric analysis (TGA). Double immobilized lipase (i.e. organogels) showed highest stability and esterification activity. Organogel immobilized lipase was therefore further applied for the synthesis of pentyl valerate and ethyl valerate esters in n-hexane. Successfully, 90% of ethyl valerate and 86% of pentyl valerate were synthesized using prepared double immobilization enzyme. Furthermore, the immobilized biocatalyst shows reusability for 10 cycles with meager loss of activity. The present study revealed a promising perspective to overcome the well-known drawbacks of the chemical-catalyzed route.

## 1. Introduction

Lipases (EC 3.1.1.3) are the enzymes responsible for the hydrolysis of triglycerides to fatty acids and glycerol (Faber, 2011; Patel and Madamwar, 2015; Lozano, 2010) and are attractive in organic chemistry because of their enantioselectivity, regioselectivity and stereoselectivity. The industrial applications of lipase include fine chemical synthesis, pharmaceutical chemistry, food and dairy industries, also biodiesel production (Faber, 2011; Patel and Madamwar, 2015; Ribeiro et al., 2010; Verma et al., 2013). Despite of all the benefits, the main drawback in carrying out the reaction in apolar organic solvents having tendency to strip away water molecules from the enzyme surface especially from the active site leaving the enzyme inactive (Raghavendra et al., 2014).

To improve lipase activity and stability, many approaches have been explored such as genetic engineering, protein engineering, medium engineering, immobilization and/or process alterations (Hara et al., 2009; Shaotao et al., 2010). "One of the approaches is to use nanostructured materials for enzymatic immobilization processes, although nanoparticles may have some drawbacks, including enzyme exposure to external interfaces, which implies sensitivity to proteolysis etc." ZnO nanoparticles have been investigated mostly for antibacterial and UV blocking function and in cosmetic and pharmaceutical industries (Wang et al., 2005; Selvarajan et al., 2015). Zn compounds have also been currently listed as GRAS, i.e. generally regarded as safe by the US Food and Drug Administration (21CFRI82.8991). However, ZnO nanoparticles do require some additional functional groups for attachment. There are many reports on the protective effect of polyethyleneimine (PEI), a water soluble cationic polymer with large number of primary amino groups, on the activity of enzymes such as lipases especially in organic media (Khoobi et al., 2014; Garcia-Galann et al., 2011; Patel et al., 2016). PEI grafting onto inert, inorganic supports and then cross-linking with bi-functional agents such as glutaraldehyde (GLU), hexamethylene diisocyanate (HMDI) is an extensively used approach for immobilization of various enzymes, such as lipase (Khoobi et al., 2014; Garcia-Galann et al., 2011; Patel et al., 2016), β-galactosidase (Selvarajan et al., 2015,) and tyrosinase (Arica and Bayramoglu, 2004).

Recently the enzyme-catalyzed biotransformation in non/micro-aqueous solvents has become the exciting field of enzymology (Hara et al., 2009; Klibanov, 2001). Among low water media, water-in-oil (w/o) microemulsions have been widely reviewed as tool for achieving

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various enzymatic reactions in hydrophobic environment (Biasutti et al., 2008; Pavlidis et al., 2009; Zanetteb et al., 2014). Reverse micelles form thermodynamically stable and optically transparent liquid medium with large interfacial area that provides an aqueous domain where hydrophilic enzymes can be located, an interface where the active site of enzymes can be anchored, and a non-polar organic phase where the hydrophobic substrates or products may be dissolved (Malik and Wani, 2012; Itabaiana et al., 2014; Zoumpanioti et al., 2010a, 2010b). However, practical applications of w/o microemulsions may be limited by the necessity of separating the surfactant from the reaction products. Nevertheless, some microemulsions can be transformed to gels by adding a gelling agent, usually a biopolymer such as gelatin, agar, or a cellulose derivative (Raghavendra et al., 2010; Dandavate and Madamwar, 2007; Wu et al., 2011) to form so-called microemulsion-based organogels (MBGs). MBGs are rigid and stable in various non-polar or relatively polar organic solvents and therefore can be used for several biotransformation in organic media, such as hydrolysis, esterification, and other syntheses (Dandavate and Madamwar, 2007; Wu et al., 2011). The network of the gel is considered to contain a bi-continuous phase that may coexist with conventional w/o microemulsion droplets containing the encapsulated enzyme.

The entrapment of lipases into a film proves to be a simple and efficient method for immobilization as they provide a high surface area for interaction of enzyme with substrate followed by ease of separation and greater enzyme stability with fewer chances of leaching (Wu et al., 2011; Vlierberghe et al., 2011). Eco-friendly polymers have gained a special importance for enzyme immobilization, drug delivery systems and tissue engineering due to their biodegradable nature (Badgujar et al., 2013; Dhake et al., 2011; Grande and Carvalho, 2011). Polyvinyl Alcohol (PVA) is widely known for its biodegradable, biocompatible and eco-friendly biopolymer properties (Grande and Carvalho, 2011). PVA has impressive features like excellent film formation, high interfacial adhesion, flexibility, and emulsification, high tensile strength, non-toxic nature, biodegradable, stabilization of blend and essentially resistant to organic solvent which makes them more ideal for lipase immobilization (Mandal et al., 2014; Hasan-Beikdashti et al., 2012). Dhake et al., 2011 reported immobilization of commercially *Rhizopus oryzae* lipase on a film using a blend of hydroxypropyl methyl cellulose (HPMC) and PVA. Ivaldo developed an efficient catalytic system for immobilizing *Candida antarctica* lipase B (CaLB) on an MBG matrix ($MBG_{CaLB}$) formed with (hydroxypropyl)methyl cellulose as a gelling agent for synthesis of monoacylglyceride (MAG) under both batch and continuous-flow conditions (Zoumpanioti et al., 2010b). Mandal et al., 2014 reported the development of cationic water-in-oil (w/o) microemulsion doped with newly designed nanocomposite comprising of gold nanoparticle (GNP) decorated single walled carbon nanotube (SWNT).

The present study focuses on the double immobilization of lipase-ZnO conjugate into AOT-reverse micelles, followed by entrapment into gelling matrix of PVA. ZnO nanoparticles were functionalized using PEI and cross linker GLU in order to produce functional groups for enzyme immobilization. Prepared lipase-ZnO bioconjugates were immobilized into AOT-reverse micelles, and these micelles were entrapped into gelling matrix of PVA. Thus in all three different immobilization systems viz: ZnO-E, ZnO-E@RM and ZnO-E-RM@PVA were prepared. Various characterization experiments were carried out which showed the improvement in pH, thermal and storage stability of enzyme after entrapment. In addition, the developed immobilization method for PVA films was further used for biocatalytic transformation of industrially important esters in n-hexane.

## 2. Materials and methods

### 2.1. Chemicals and the enzyme

Zinc oxide (ZnO) nanoparticles were synthesized according to the protocol described by Soni et al. (2011). *Candida rugosa* lipase with activity of 875U/g, (3-aminopropyl) triethoxysilane (APTES), polyethyleneimine (PEI), glutraldehyde (GLU) and 4-nitrophenyl palmitate (*p*-NPP) were obtained from Sigma-Aldrich, Germany. Pentyl valerate (≥98%), ethyl valerate (≥98%), 1-pentanol (≥97%) and valeric acid (≥97%) were purchased from Fluka-Chemica (Germany). Ethanol and n-hexane were procured from Spectrochem, India. All organic solvents used were of GC/HPLC grade.

### 2.2. Fabrication of lipase on ZnO functionalized with PEI

Fabrication of lipase on functionalized ZnO nanoparticles were carried out in two steps. Firstly, Branched polyethyleneimine (1.5 mL) was suspended in a solution containing 10% APTES in ethanol. To this mixture 10 mg of ZnO nanoparticles were added and stirred vigorously to react for 24 h. The obtained composite was purified by centrifugation (10000 × *g* for 15 min) and repeatedly washed with ethanol and milliQ in order to remove excess silane and PEI. Secondly, 10 mg of ZnO-PEI was incubated in 10 mL phosphate buffer solution (50 mM, pH 7.0) containing 1% (w/v) GLU and allowed to react for 10 min. Then, 1 mL of lipase solution (50 mg/mL) was incorporated into the mixture and stirred for overnight. The mixture was centrifuged and washed with phosphate buffer (50 mM) in order to eliminate unbound lipase. The resultant bioconjugant was dried under vacuum (37 °C for 1 h) and characterized as detailed in Section 2.4. The amount of immobilized lipase adsorbed on nanoparticles was determined by measuring the initial concentration and its final concentration in supernatant after immobilization using lipase activity.

### 2.3. Entrapment of lipase loaded ZnO into AOT/Iso-octane microemulsion and PVA gels

(i) Preparing microemulsions: In 10 mL screw cap vials, 0.1 M sodium bis (2-ethylhexyl) sulfosuccinate (AOT) with $W_o = 60$, 2 mL isooctane and 50 mM phosphate buffer (pH-8.0) were taken to attain the corresponding z([co-surfactant]/[surfactant]) and $W_o$([water]/[surfactant]) value, respectively. The mixture was vigorously vortexed for 5 min to obtain a clear homogeneous solution of 0.1 M AOT/iso-octane/buffer reverse micelle. Following the similar protocol, suspensions of previously prepared ZnO-E nanocomposite (108 µL) was added to AOT reverse micellar mixture instead of phosphate buffer to obtain the corresponding $W_o$ value. The enzyme entrapped into reverse micelles was designated as ZnO-E@RM. (ii) Preparing gelling matrix: The MBGs were prepared by introducing AOT microemulsion containing ZnO-E to a second solution of 2% PVA dissolved in water. Typically 2 mL of prepared microemulsions was introduced into 2% polymer solution and then moderately stirred to obtain homogenous mixture. Finally, immobilized matrix with entrapped lipase was carefully poured in a Teflon dish and allowed to dry at 40–45 °C for 40 h. A thin film of immobilized lipase was formed, which was then cut into small pieces (1 × 1 cm with 110 ± 10 µm thickness) and stored at 4 °C. Microemulsions prepared with AOT-reverse micelles were designated as ZnO-E-RM@PVA and further characterized as detailed in Section 2.4.

### 2.4. Lipase activity, Protein content and Immobilization Yield (%) determination

The catalytic activities of free as well as immobilized lipases were assayed using *p*-nitrophenyl palmitate (*p*-NPP) as substrate (Wrinkler and Stuckmaan). The reaction solution was prepared by addition of free or immobilized lipase to p-NPP solution (0.4 mM in 100 mM phosphate

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