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Synthesis of geranyl acetate in non-aqueous media using immobilized *Pseudomonas cepacia* lipase on biodegradable polymer film: Kinetic modelling and chain length effect study

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ABSTRACT

Pseudomonas cepacia lipase (PCL) was immobilized on ternary blend biodegradable polymer made up of polylactic acid (PLA), chitosan (CH), and polyvinyl alcohol (PVA). Immobilized biocatalyst was characterized using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), % water content, protein and lipase activity assay. The lipase activity assay showed enhanced activity of immobilized lipase than crude lipase. Higher half life time ($t_{1/2}$) and lower deactivation rate constant (K_d) was found for the *n*-hexane among various tested solvent. Influence of various reaction parameters on enzyme activity were studied in detail. When geraniol (1 mmol) and vinyl acetate (4 mmol) in toluene (3 mL) were reacted with 50 mg immobilized lipase at 55 °C; then 99% geraniol was converted to geranyl acetate after 3 h. Various kinetic parameters such as r_{max}, $K_{i(A)}$, $K_{m(A)}$, $K_{m(B)}$ were determined using non-linear regression analysis for ternary-complex and Bi–Bi ping-pong mechanism. The kinetic study showed that reaction followed ternary-complex mechanism with inhibition by geraniol. Activation energy (*Ea*) was found to be lower for immobilized lipase (13.76 kCal/mol) than crude lipase (19.9 kCal/mol) indicating better catalytic efficiency of immobilized lipase. Immobilized biocatalyst demonstrated 4 fold increased catalytic activity than crude lipase and recycled five times.

1. Introduction

Esters of the terpinyl alcohols are considered to be natural flavours because of their organoleptic properties and wider applications in edible flavours, foods, fragrances, additives, cosmetics, pharma and home care products [1–5]. These esters can be obtained by conventional chemical synthesis or via extraction from the plant sources [1,2]. The extraction methods have major disadvantages like lower yield and high solvent costs while conventional chemical methodologies have several drawbacks such as harsh reaction conditions, use of hazardous chemicals, less selectivity, high activation energy and side product formation [1–4,6–11]. These techniques are inadequate for synthesis valuable fine chemicals present in various edible and home care products which invite safer and greener biocatalytic route with so give them as "Green Label" [8–10].

Biocatalysis is considered to be a greener catalytic system; as various enzymes are engaged for synthesis of fine chemicals at mild conditions [1–4]. The biocatalytic route involving lipase (triacylglycerol hydrolases, E.C. 3.1.1.3) has gained special importance because of the wide substrate array and the ability to carry out variety of organic reactions [4,6]. However, the use of crude enzyme in non-aqueous media has considerable drawbacks such as lower activity, stability and recyclability [8,9]. Hence, to overcome these disadvantages various immobilization techniques were employed [11–14]. Among these immobilization techniques; the use of beads or polymer films as a carrier has special importance because of their easy separation from reaction media to reuse [13].

Recently, Sheldon and Pelt [14] and Cantone et al. [15] stated that immobilization on the polymeric material has special rising significance in the enzyme immobilization and practical biocatalytic applications. Such type of the polymeric materials has great importance for the membrane reactor or bioreactor coating [14,15]. Immobilization of enzymes on biodegradable polymers or biopolymers represents an attractive choice for immobilization because of eco-friendly nature of biocatalyst which makes a greener immobilization protocol [14,15]. Hence, various biomaterials such as cellulose, polyvinyl alcohol, chitosan, starch, dextran, agarose, β-glucan, carboxy-methyl cellulose and gelatine were widely used for enzyme immobilization [1–4,7,11–17].

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In the present study lipase PCL was immobilized on biodegradable ternary blend of CH, PVA, PLA. CH is attractively used for immobilization because of its characteristic feature such as the lack of toxicity, inertness to chemical reactivity and ecofriendly nature [16]. PVA endows outstanding properties for immobilization on film such as high flexibility, biocompatibility, non-toxicity, adhesiveness and better resistivity to organic solvent [1,2]. PLA is a biodegradable and ecofriendly polymer obtained from the natural resources such as starch which maintains the biocompatibility in ternary blend [16]. Preparing ternary blend of CH, PVA and PLA is attractive as it offers all these individual advantages in single film [1,2,16]. This ternary blend immobilized lipase (biocatalyst) was then applied to synthesis of geranyl acetate which is recognized as a safe compound by U.S. Food and Drug Administration.

Geranyl acetate is a colourless liquid having pleasant floral-fruity smell; it is important component of 60 natural essential oils such as *Alpinia galangal*, *Ocimum basilicum*, *Citrus sanguinello*, *Cymbopogon nardus* etc. Furthermore it can be used as a flavouring agent for lavender, lime grass, peach, berry, fig, rose and citrus etc. Synthesis of food-flavour and fragrance compounds by the enzymatic route labelled them as a "green-natural" products [1,2]. In 2010, sales of immobilized enzymes for biocatalytic purpose were valued almost around $160 million [18]; and nearly 42% of the overall world enzyme market is covered for the food-beverage, pharma and home-care products [18]. Thus, considering the wide scope and importance of terpinyl esters, we make an attempt to explore kinetic modelling study for geranyl acetate synthesis, which has not been yet reported using biodegradable immobilized PCL as a biocatalyst and hence finds a great scope.

In the present study, we have characterized the ternary blend film (by SEM, FTIR, % water content, protein content, lipase activity) and then studied kinetics of geranyl acetate synthesis which involves study of the influence of various reaction parameters, energy of activation (Ea), half life time ($t_{1/2}$), deactivation rate constant (K_d) for various solvents and kinetic parameters (V_{max}, $K_{i(A)}$, $K_{m(A)}$, $K_{m(B)}$). Furthermore alkyl ester and alcohol chain length effect was studied to understand the influence of the chain length on immobilized lipase catalytic activity.

2. Materials and methods

2.1. Enzymes and chemicals

Candida rugosa (CRL, Lipase AYS, ≥30,000 U/g) and *Pseudomonas cepacia* lipase (PCL, lipase PS, ≥23,000 U/g) was gifted by Amano Enzymes (Nagoya, Japan) while *Rhizopus oryzae* (ROL, lipase ROL, ≥30,000 U/g), PVA (Mw – 9000–10,000), CH (Brookfield viscosity > 200.0 cps), *p*-NPP were purchased from Sigma–Aldrich Pvt. Ltd., India. Bovine serum albumin (BSA) and Folin-Ciocalteu reagent to measure protein content was purchased from Hi Media Pvt. Ltd., India. Other all chemicals were purchased from S.D. Fine Chemicals Ltd. with their highest purity available.

2.2. Immobilization of lipase

Preparation of support matrix ternary blend film of PVA, CH, and PLA was carried out with a marginal modification in the method described by the Grande et al. [16] and subsequently lipase PCL was immobilized. The PVA (300 mg) was dissolved in distilled water (2%, w/w solution) while CH (50 mg) was dissolved in distilled water (1%, w/w acetic acid solution) and PLA (50 mg) was dissolved in chloroform (2%, w/w solution) separately. Each solution was stirred for the 40 min at 1000–1200 rpm. Initially PVA and CH were mixed together vigorously for 30 min at speed of 2000 rpm, later on PLA solution was added in it and stirred vigorously for 2 h. On completion of 2 h, native lipase (100 mg dissolved in 1–2 mL of deionised water) was added to the ternary blend and moderately stirred at 120 rpm for 50 min. The mixture was carefully poured in a Teflon dish and allowed to dry at 40 °C for 40 h. A thin film of immobilized lipase PCL was formed which was then cut into small pieces of 2–4 mm^2 size and stored at 2–6 °C. Thus, theoretically 400 mg (0.4 g) support was loaded by 100 mg of crude lipase.

2.3. Characterization of the immobilized lipase

2.3.1. Scanning electron microscopy analysis (SEM)

Scanning electron microscope (SEM) analysis was performed to observe the change in surface morphology for control PLA:PVA:CH (1:6:1) and immobilized PLA:PVA:CH:PCL (1:6:1:2) lipase polymer film by the FEI-Quanta 200, instrument. The film sample was kept on carbon stub and images were recorded at 20 kV using LFD detector under low vacuum.

2.3.2. FTIR analysis

FTIR analysis was studied to confirm the presence of amide functionality after immobilization of lipase PCL on the ternary blend polymer film. The FTIR analysis was carried out by Perkin-Elmer, Spectrum 100.

2.3.3. Water content analysis for various crude and immobilized lipase

The % water content of various crude, immobilized lipases (PCL, ROL, CRL) and control support was measured using the Karl Fischer titration method (784 KFP Titrino).

2.3.4. Physical appearance and film thickness determination

The film thickness was determined by using a manual micrometre. The final thickness was determined by ten random various places of films.

2.3.5. Determination of lipase activity assay

Lipase activity of crude and ternary blend immobilized lipase was studied spectrophotometrically at 410 nm in triplicate by hydrolysis of *p*-nitrophenyl palmitate ester (*p*-NPP) with the minor modification in reported procedure by Yea et al. [19]. In standard condition, reaction mixture consists of the 1.5 mg of crude lipase (or equivalent quantity of the ternary blend immobilized lipase) was taken into the 1.5 mL of the cyclohexane. The reaction was started by addition of 0.5 mL of 25 mM, *p*-NPP substrate at 37 °C. After 3 min the clear supernatant was withdrawn and 1.5 mL of 2 mM, NaOH was added in it so that *p*-nitro phenol (*p*-NP) was extracted in the aqueous alkaline phase to give pale yellow colour [19]. It was then quickly used to measure the optical density at 410 nm. Lipase activity was defined as mM of *p*-NP released per min by per mg of lipase under given standard assay condition [19].

2.3.6. Determination of protein content

The amount of immobilized enzyme on the ternary blend was determined by following Folin-Ciocalteu method at 660 nm [2]. The initial amount of protein used to load on support was determined. Finally, after removal of ternary blend immobilized lipase polymer; the Teflon petri dish was rinsed and subjected to determine un-immobilized amount of protein. Thus, amount of protein immobilized is the difference between the initial amounts of protein used to load on support and un-immobilized amount of protein found after washing petri dishes. BSA was used as an internal standard to construct the calibration curve. % Protein loading, specific activity and activity yield was determined by following equations:

$$\%\,\text{protein loading} = \text{amount of protein immobilized} \div \text{initial amount of protein loaded}$$

$$\%\,\text{activity yield} = \text{immobilized lipase activity} \div \text{crude lipase activity}$$

$$\text{specific activity} = \text{lipase activity} \div \text{lipase protein content}$$

2.3.7. Effect of the organic solvent on activity and stability of immobilized lipase

The effect of various five organic solvents on the activity and stability of CH:PVA:PLA:PCL (1:6:1:2) immobilized lipase was determined. The immobilized lipase was placed in various five organic solvents at 140 rpm for incubation. After incubation, the solvent was removed by filtration and lipase activity was determined for immobilized lipase PCL as above indicated procedure.

2.3.8. Determination of the half-life time ($t_{1/2}$) and deactivation rate constant (K_d)

In another set of experiments, half-life time ($t_{1/2}$) and deactivation rate constant (K_d) was determined for immobilized lipase in different organic solvents using lipase activity assay.

2.4. Experimental setup and analysis

Geranyl acetate synthesis involves the addition of geraniol (1 mmol) and vinyl acetate (4 mmol) in a 10 mL glass reaction vessel of 1.6 cm i.d. with a glass lid. The reaction mixture was diluted by toluene to make volume of 3 mL. Later on, 50 mg of immobilized lipase PCL was added to initiate the reaction and was placed at 55 °C in orbital shaker with an agitation speed of 140 rpm (Scheme 1). Reaction mass sample 10 μl was withdrawn periodically and analyzed by using the Perkin Elmer, Clarus-400 gas chromatograph equipped with flame ionizing detector and capillary column. The oven temperature was kept at 80 °C for 3 min with a rise of 10 °C min^{-1} up to 240 °C for 30 min. The temperature of the detector and injector was maintained 260 and 60 °C respectively.

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