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Application of lipase immobilized on the biocompatible ternary blend polymer matrix for synthesis of citronellyl acetate in non-aqueous media: Kinetic modelling study

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ABSTRACT

This work reports the use of new support for immobilization of lipase *Burkholderia cepacia* (BCL) matrix made up of polylactic acid (PLA), chitosan (CH), and polyvinyl alcohol (PVA). Initially lipase from various microbial sources and immobilization support composition was screened to obtain a robust biocatalyst. Among various biocatalysts preparation, the PLA:PVA:CH:BCL (1:6:1:2) was worked as a robust biocatalyst for the citronellyl acetate synthesis. Various reaction parameters were studied in detail to obtain the suitable reaction conditions for model citronellyl acetate synthesis reaction. Various kinetic parameters such as r_{max}, $K_{i(citronellol)}$, $K_{m(citronellol)}$, $K_{m(vinyl\ acetate)}$ were determined using non-linear regression analysis for the ternary complex as well as bi–bi ping-pong mechanism. The experimental results and kinetic study showed that citronellyl acetate synthesis catalyzed by immobilized lipase BCL followed the ternary complex mechanism with inhibition by alcohol (citronellol). The energy of activation for citronellyl acetate synthesis was found to be lower for immobilized lipase (8.9 kcal/mol) than aggregated lipase (14.8 kcal/mol) enzyme. The developed biocatalyst showed four to fivefold higher catalytic activity and excellent recyclability (up to six cycles) than the aggregated lipase.

1. Introduction

The terpinyl esters are nature's essence compounds of the great importance in the perfumery, cosmetics, pharmaceuticals, flavour and fragrance applications [1–3]. The synthesis of terpinyl esters can be achieved by the conventional chemical catalysis and natural extraction from the plant sources [4,5]. The conventional chemical catalysis has numerous disadvantages like harsh reaction conditions, use of unsafe chemicals, less selectivity and high energy of activation (Ea) [4]. Meanwhile natural extraction method has the major limitations such as lower yield, feasibility for large scale industrial production and higher capital expenditure [5]. These techniques are not adequate for the mercantile synthesis and hence encouraging to researcher for the production of these compounds enzymatically which can labelled them as "natural" esters [1,2].

Biocatalysis is labelled a greener catalysis as various natural enzymes were employed to synthesize of the fine chemicals at mild reaction conditions [3,6,7]. In biocatalysis lipase (E.C. 3.1.1.3) is ubiquitous enzymes with considerable catalytic significance and possessing wide industrial potential to carry many promiscuous reactions [2,3,7]. However, biocatalysis have limitations for the production efficiency as there are many practical difficulties arises to employ free or aggregated enzyme in organic media such as lower activity–stability, reusability and poor solvent tolerance capability [8,9]. To overcome these limitations several immobilization techniques were employed [10,11]. Immobilizations help for better dispersion of enzyme on support matrix and simplify the product recovery which is an important proficient parameter for enzymatic reactions [5–11]. Besides this; immobilization may also improve other enzyme properties such as pH, thermal, organic-solvent-operational stability and catalytic activity [8,12–21]. These improvements in enzyme properties may be the result of the change in enzyme conformation after immobilization [8,19–21]. When lipases are immobilized on (hydrophobic) support then it may act as a tool for the opening of the lid to make the active site available [7,8]. This mechanism is known as interfacial activation which leads to remarkable improvement in enzyme activity [7,8,19–21]. However, improper immobilization may also deactivate the enzyme activity and stability [7,8,19,20].

Amongst several immobilization techniques [7–11]; the use of the beads and polymer matrix films as a support has several advantages due to their stability, easy separation and recyclability [8–11,22]. Sheldon [11] stated that immobilization on various ecofriendly biodegradable carriers (support) have rising significance for immobilization and

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biocatalysis to accomplish greener aspect [10,11,23]. A range of biopolymers like hydroxypropylmethyl cellulose, β-glucan, cellulose, chitosan, poly(hydroxybutyrate), poly(3-hydroxybutyrate-co-hydroxyvalerate), carboxymethyl cellulose and starch are used as a support for immobilization in form of beads or polymer matrix [11,22–27].

In the present protocol we have carried out immobilization of lipase BCL on biocompatible ternary blend matrix made up of CH, PVA and PLA [26]. CH has excellent features for immobilization because of its distinct trait such as the lack of toxicity, inertness to chemicals reactivity and ecofriendly nature [25]. Additionally existence of free hydroxyl-amino-, groups in CH provided the enhanced immobilization binding sites [25,26]. PLA is a polymer obtained from starch-amylum like natural resources [26]. Use of PVA is important for immobilization as it has features like flexibility, non-toxicity, adhesiveness; better diffusion capability and biocompatibility [26,27]. Preparing ternary blend support has added advantage that it provides better immobilization capability, biodegradability and flexibility in physical forms. In this study, entrapment methodology was used to immobilize lipase BCL; as immobilization of the enzyme within the matrix is incorporated at the time of matrix formation [5,7–11]. Entrapment is an easy method of immobilization which is commonly free from the use of any cross linking chemical reagent [7–11,16,18]. Entrapment methodology has several advantages like decrease in enzyme leaching, improvement in thermal and hydrophobic solvent stability [7–11]. Thus it may leads to improve the enzyme activity and stability in organic media along with operational stability [7–10,16–18,26].

The worldwide market for the cosmetics and toiletries contributed almost 200 bn € while the global market for flavours and fragrances was estimated around 20.3 bn US $ in 2008 including food and beverages products [8]. Considering the grate scope and importance of these products we tried to apply an immobilization protocol for the synthesis of citronellyl acetate. Citronellyl acetate is a colourless liquid having pleasant smell of the fruits like orange, lemon, lychee, pear and plum and hence used as a flavouring agent [5,28]. Furthermore, it is essential component and has fragrance like *Pelargonium graveolens*, Lavandula and rose [28]. The European council categorized citronellyl acetate as an edible flavouring agent which can be used safely and hence, considered as commercially important compound in flavour and food industry [28,29].

To the best of our knowledge investigation of enzymatic synthesis of citronellyl acetate and its kinetic modelling study by using the lipase immobilized on biodegradable polymer matrix as a biocatalyst has not been reported. In present study, the effect of the various reaction parameters was studied in detail. Furthermore, Ea, kinetic parameters and possible mechanism were determined. In addition to this, recyclability and the applicability of developed protocol were tested for the various industrially important ester syntheses.

2. Materials and methods

2.1. Chemicals and enzymes

Lipase BCL (*Burkholderia cepacia* lipase, BCL, activity ≥ 23,000 U/g; white powder) and Lipase AYS (*Candida rugosa*, lipase, CRL, activity ≥ 30,000 U/g; white powder) was gifted by Amano Enzymes (Nagoya, Japan). Lipase HPL (Hog pancreas lipase, HPL, activity ≥ 30,000 U/g; brown powder) was purchased from the Fluka India Pvt. Ltd. CH (Brookfield viscosity > 200.0), PVA (M_w: 9000–10,000), *p*-NPP, and citronellol were purchased from Sigma-Aldrich Pvt. Ltd., India. Bovine serum albumin and all other chemicals were bought from Hi-Media Pvt. Ltd., India.

2.2. Immobilization of lipase

Lipase BCL was immobilized on a ternary blend matrix of CH, PLA and PVA prepared by Badgujar et al. method with minor modification [26]. CH (60 mg) was dissolved in 1% acetic acid solution while PVA (360 mg) was dissolved in 2% (w/w) solution in distilled water and PLA (60 mg) was dissolved in 2% (w/w) solution of chloroform separately. Each solution was then stirred (on magnetic stirrer) for nearly 1 h at 1200–1400 rpm and filtered through the Whatman filter paper to remove the undissolved particles. PVA and CH solutions were mixed together in a beaker and stirred for 40–50 min at 1000–1200 rpm; then PLA solution was added in it and allowed to stir vigorously for the 3 h at 1600 rpm. Then lipase (120 mg) dissolved in 1–2 ml of deionised water was added into the ternary blend and stirred at 140 rpm for 1 h. Finally, immobilized ternary blend solution was carefully transferred into a Teflon dish and dried at 40 °C for 35–40 h. A thin uniform immobilized lipase polymer matrix was formed and which was cut off into small pieces of 2–4 mm^2 size. Prepared ternary blend immobilized lipase biocatalyst was then stored in air tight plastic container at 5 °C. Thus, theoretically, 120 mg of lipase was present on 600 mg of immobilized polymer matrix.

2.3. Lipase activity and protein content assay

Lipases from various microbial sources were studied for the determination of protein content and lipase activity assay. Lipase activity was examined by Pencreac'h and Baratti method [30] with slight modification for the aggregated and immobilized lipase in triplicate to minimize the errors. In standard condition, reaction media consist of 1.5 mg of aggregated lipase (or equal amount of immobilized lipase) was taken into the 1.5 ml of the *n*-hexane. The reaction was started by addition of 0.5 ml; 20 mM of *p*-nitrophenyl palmitate (*p*-NPP) as a substrate followed by the incubation in water bath for 5 min at 40 °C. Immediately after incubation the reaction was terminated by addition of the 2 ml; 2 mM NaOH. Later on vortex it for a second, so that *p*-nitrophenol obtained was extracted in aqueous phase; which was then instantly used to measure the optical density at 405 nm. Lipase activity was described as mM of *p*-nitrophenol released min^{-1}/mg^{-1} of lipase under given standard assay condition.

Protein amount was determined in set of triplicate to minimize error by the Bradford method at 595 nm wavelength using bovine serum albumin as internal standard reference [24].

2.4. Experimental setup

For the synthesis of citronellyl acetate, reaction assembly consist of 8 ml glass reaction vial of 1.5 cm internal diameter with a glass stopper was used. In a typical experiment, citronellol (1 mmol) and vinyl acetate (4 mmol) was diluted to make up reaction volume up to 3 ml by solvent cyclohexane. The reaction mixture was stirred at 150 rpm for 5 min; later on reaction was initiated by adding 50 mg of immobilized lipase biocatalyst and kept at 55 °C in rotary orbital shaker with speed of 150 rpm.

2.5. Analysis

At specific time interval; 10 μl sample was withdrawn, diluted and analyzed by gas chromatography (Perkin Elmer Clarus-400-GC) having flame ionizing detector and a capillary column (Elite-1, 30 m × 0.32 mm × 0.25 μm). The temperature of the GC oven was set at 80 °C for 3 min with a rise of 10 °C min^{-1} up to 240 °C for 30 min while the temperature of the detector and injector was kept 260 °C and 80 °C, respectively. Conversion was determined based on the area under the curve of limiting reactant; for this purpose authentic sample of citronellyl acetate was used.

2.6. Recyclability of immobilized lipase

Various immobilized lipase support compositions were subjected to determine the repetitive use of citronellyl acetate synthesis. After finishing the reaction; reaction mass was filtered through the Whatman filter paper and rinsed three times with cyclohexane solution to remove traces of product/reactant. Later it was dried at 35–40 °C for 8–10 h and stored at 4–6 °C until used for next cycle.

3. Result and discussion

3.1. Lipase activity and protein content

Aggregated and immobilized lipases were tested for *p*-NPP activity assay and it showed following order of reactivity: lipase BCL > CRL > HPL (Table 1). Enzyme immobilized on polymer matrix showed lipase activity indicating that lipase is immobilized on the ternary blend polymer matrix [5,24]. Immobilized lipase showed some better activity than aggregated lipase (Table 1; columns A and B). This can be explained as immobilized enzymes dispersed on the support matrix and agglomeration will no longer possible, while aggregated enzyme may suffer from agglomeration and mass transfer diffusion in *n*-hexane, which showed lower enzyme activity [7]. Furthermore, improvement in catalytic activity may also be a cause of the interfacial activation [7,8]. The protein content