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Immobilization of lipase on biocompatible co-polymer of polyvinyl alcohol and chitosan for synthesis of laurate compounds in supercritical carbon dioxide using response surface methodology

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ABSTRACT

Biocompatible co-polymer matrix has great importance for enzyme immobilization and subsequent bio-catalytic applications to synthesize important organic moieties. Citronellyl laurate is a fatty-acid-ester having pleasant fruity aroma and widely used as/in emulsifier, lubricant in textile, paint or ink-additives, surfactants, perfumery and food-flavouring ingredient. In present study, *Burkholderia cepacia* lipase (BCL) was immobilized on biodegradable co-polymer of chitosan (CHI) and polyvinyl alcohol (PVA). The synthesized bio-catalyst {PVA:CHI:BCL (6:4:2.5)} was characterized by SEM, TGA, lipase assay and protein-content analysis. This biocatalyst was applied to synthesize citronellyl laurate in supercritical carbon-dioxide ($SC\text{-}CO_2$) using response surface methodology with five-factor-three-level Box–Behnken-design to optimize reaction parameters (citronellol: 8.5 mmol; vinyl laurate: 19.87 mmol; biocatalyst: 175.6 mg; temperature: 46.02 °C; pressure: 8.81 MPa) which provided 94 ± 1.52% yield. The protocol is extended to synthesize various important 12 laurate compounds with excellent yield (90–98%) and noteworthy recyclability (upto studied 5 recycles). Interestingly, immobilized PVA/CHI/lipase biocatalyst showed 4-fold higher bio-catalytic activity than free lipase in $SC\text{-}CO_2$. Moreover, the biocatalyst activity assessment study showed remarkable activity–stability of immobilized biocatalyst in $SC\text{-}CO_2$ media as compared to free enzyme. Thus, present protocol demonstrated potential biocatalytic applications for synthesis of important laurate compounds with excellent recyclability in $SC\text{-}CO_2$ as greener biocatalyst and reaction medium.

1. Introduction

The terpenyl esters of the fatty-acids finds several potential applications in cosmetics foods, beverages, flavour–fragrances, toiletries, surfactants, lubricants, pharmaceuticals and various house-hold products [1–3]. Among the acyclic class of the terpinyl alcohols, the geraniol and citronellol are commercially most important terpinyl alcohols [2–4]. Traditionally, these valuable fatty-acid esters are chemically synthesized by using strong mineral acid or base at very higher temperature ranging from 150 to 250 °C and suffers from numerous disadvantages like the use of hazardous chemicals, lower-yield, lower-selectivity, lower-reaction rate, need of higher activation energy, need of acid or corrosion resistant reactors, intense down streaming processes, by-product formation, waste minimization, and several environmental issues [3–7]. Moreover, traditional process involves extraction of these fatty-acid esters from various natural sources which having several drawbacks such as poor yield, large amount of solvent use and its distillation which make the process economically nonviable [2–8]. These traditional techniques are not sufficient and appeals to various research communities to search for the "safer, greener and ecofriendly" option for synthesis of these valuable long chain fatty-acid esters compounds [4–9].

Use of enzyme based technology in biocatalysis has attracted significant attention because of the cleaner, greener and eco-friendly aspect which make available a number of advantages like higher-selectivity, higher-yield, mild reaction parameters and environmental compatibility [6,10–13]. Besides these, valuable esters synthesized via bio-catalysis can be marked as "natural products" and used "safely" without "chemo-phobia-attitude". Basically, "lipase" from the "hydrolases" group fascinated more concern, since lipases possessing the wide substrate array to carry out different promiscuous organic reactions at gentle reaction conditions [6,10,13,14]. However, proteomic nature of enzymes is frequently hampered the practical use of free enzyme and

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industrial process economics because of poor solvent-, thermal- and operational stability [10–12]. To conquer above said limitations, various advanced immobilization methods were developed which can improve the catalytic performance and activity–stability of various enzymes [11–14]. Among above immobilization protocols, the use of natural polymer matrices for immobilization is especially of great interest since; it can be practically applied for the biocatalytic reactor and membrane reactors coating [2,3,11,14]. The preparation of such type of active matrices can be achieved by different immobilization techniques such as entrapment, adsorption, ionic-binding, covalent-linkage, etc. which leads to form a highly stable biocatalyst [1,11,14]. Till time, a variety of natural polymers such as cellulose, carrageenan, alginate, β-glucan, poly (hydroxybutyrate), hydroxypropyl methyl cellulose, polylactic acid etc. were employed in the form of spherical beads or films for immobilization of different enzymes [11–16].

In present study, biocompatible matrix of PVA and CHI was used as an attractive immobilization support. PVA was extensively used in bio-medical applications as a potential bio-polymer with excellent properties such as biocompatibility, high adhesivity, high flexibility, high tensile strength, non-toxicity, better resistivity to chemicals and organic solvent [17]. CHI is a linear β-1,4-linked polysaccharide matrix which possess various useful features such as high mechanical strength, inertness to chemical reactivity, good adhesion, lack of toxicity and biodegradability which can hold up enzyme immobilization ability [18]. Both of these polymers possessing the hydroxyl group while, free-amino groups of each unit of CHI offer a higher extent of immobilization [17,18]. This PVA:CHI:BCL biocatalyst was then applied to synthesize citronellyl laurate as a biocatalytic application. The Commission of European community has restricted the use of volatile organic solvents for synthesis of the food ingredients to maintain its purity, which appeals to researchers to look for alternative greener solvent for synthesis of valuable food ingredient additives/commodities [19].

Use of these volatile organic solvent is restricted as, these solvents are the major source of volatile organic compounds (VOCs) which severely affect the environment and human health [20]. The utilization of the volatile organic solvent can cause inhibition of enzyme catalytic activity, difficulty in use because of flammable nature, expensive down-streaming process and increment of E-factor (E-factor is defined as the ratio of mass of waste generated per unit of desired product; higher the E factor more is the waste and subsequently, having negative environmental impact) [17,20,21]. Hence, use of supercritical carbon dioxide (SC-CO_2) as a solvent is the best alternative for synthesis of drug and food-additives which can overcome the above said shortcomings [17,21]. Moreover, employment of the SC-CO_2 has been accepted as a 'Clean and Green' solvent with noteworthy potential for commercial purposes, as it is non-combustible, harmless, chemically inert solvent, reduces work-up procedure and provide final product by simple depressurization [20–22]. Additionally, this SC-CO_2 is available in large quantity in environment and SC-CO_2 is a low-viscous solvent which may endorse easy mass-transfer phenomenon between reaction mass and active sites of catalyst [17–22]. Thus, use of enzyme catalysis in SC-CO_2 is extremely attractive system and can be considered as an "Eco-friendly and Safe" technique [17–22].

Citronellyl laurate is a colourless liquid having pleasant fruity Citus (lemon type) aroma which is widely used in pharma, cosmetics, emulsification, perfumery and food-flavour ingredient. In 2009, the overall international estimated market for the essential food, flavour and fragrance was nearly 20 thousands million USD, which increases upto 24 thousands million USD by 2013 [23]. Hence, in view of the present extensive scope and importance of these valuable fatty-acids esters, we make an attempt to investigate the synthesis of citronellyl laurate using PVA:CHI:lipase as an immobilized biocatalyst in SC-CO_2 by response surface methodology. The response surface methodology (RSM) is a mathematical and multivariate statistical technique to optimize the process, which is aimed to reduce the cost of expensive analysis methods [24]. To the best of our knowledge, such type of membrane or polymer base biocatalyst (PVA:CHI:lipase) for their potential biocatalytic applications in SC-CO_2 was not explored in details, which inspire us to explore the influence of various reaction parameters on the enzyme activity in SC-CO_2 and corresponding synthetic applications of designed biocatalyst. In present study, we (i) synthesized biocatalyst, (ii) characterized it, (iii) used for citronellyl laurate synthesis using RSM, (iv) explored various substrate scope as well as recyclability in SC-CO_2, and (iv) finally studied effect of SC-CO_2 parameters on the immobilized biocatalyst activity.

2. Materials and methods

2.1. Enzymes and chemicals

The lipase BCL (*Burkholderia cepacia* lipase, BCL), CHI (Brookfield viscosity >200), PVA (Mw 9000–10,000), vinyl laurate (VL), and *p*-nitro phenyl butyrate (*p*-PNB) citronellyl alcohol, bovine serum albumin (BSA) and all other solvents or chemicals were purchased from Sigma–Aldrich Pvt. Ltd.

2.2. Immobilization of lipase

The lipase was immobilized onto the PVA/CHI biocompatible matrix simply in water as a greener solvent at room temperature (~30 °C). The PVA (600 mg) was dissolved in distilled water (40–50 mL) while CHI (400 mg) was dissolved in distilled water (1.5%, w/w, acetic acid solution) in a separate beaker and stirred at 1200–1400 rpm for 60 min. Both these solutions were then filtered to remove the undissolved particles. Finally, both solutions were mixed and stirred vigorously for 4–5 h at 1500–1600 rpm. After that, parent lipase BCL (250 mg) was dissolved in deionised water (6–8 mL) which was added to the PVA/CHI blend and stirred it gently at 160–180 rpm for 60 min. The PVA/CHI/BCL immobilized lipase blend was then carefully poured into a Teflon-dish and allowed it to dry at 40–46 °C for 45–48 h. A uniform plane thin film of PVA:CHI:BCL was formed, which was afterwards cut off into the small pieces of 2–3 mm^2 size and stored at 8–12 °C in freeze. Thus, the theoretically 1000 mg (1 g) support was loaded by 250 mg of native lipase, and this composition was denoted as PVA:CHI:BCL (6:4:2.5) means PVA:CHI:BCL (600:400:250).

2.3. Characterization of immobilized lipase

2.3.1. Surface texture analysis

Scanning electron microscope (SEM) analysis was performed to observe the change in surface texture of the control PVA:CHI and immobilized PVA:CHI:lipase by the FEI-Quanta 200, instrument. The film sample was placed on a carbon stub and images were captured at 15–20 kV using LFD detector under the lower vacuum. The film thickness of control support and immobilized lipase was determined by using a manual micrometre at 8–10 random places of films.

2.3.2. TGA analysis

The thermo gravimetric analysis (TGA) was performed using Q-series 600 analyzer; for this 7–8 mg of sample was kept in ceramic crucible and the analysis was examined from 30 to 600 °C with 10 °C/min rise in temperature, under the 99.99% pure nitrogen atmosphere with flow of 100 mL/min. The reference control run was performed with an empty sample crucible pan.