Process Biochemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

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

Kirtikumar C. Badgujar, Bhalchandra M. Bhanage*

Department of Chemistry, Institute of Chemical Technology, Matunga, Mumbai 400 019, India

ARTICLE INFO

Article history:
Received 22 December 2014
Received in revised form 15 April 2015
Accepted 29 April 2015
Available online xxx

Keywords:
Laurate compounds
Biocompatible co-polymer
Supercritical carbon dioxide
Biocatalysis
Response surface methodology
Immobilization

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

* Corresponding author. Tel.: +91 22 3361 2601/2222; fax: +91 22 2414 5614.
E-mail addresses: bm.bhanage@ictmumbai.edu.in, bm.bhanage@gmail.com, bhalchandra_bhanage@yahoo.com (B.M. Bhanage).

http://dx.doi.org/10.1016/j.procbio.2015.04.019
1359-5113/

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.

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

2.3.3. Lipase activity and protein content determination

The lipase activity of free and immobilized PVA/CHI lipase was studied in triplicate through spectrophotometrically at 405 nm by hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) with minor modification in reported procedure by Pencreach and Baratti [25]. In a standard assay condition consists of 1 mg of free lipase (or equivalent quantity of the PVA/CHI immobilized lipase) in 0.5 mL of *n*-hexane solvent. The reaction was commencing by addition of 0.5 mL of 12 mM, *p*-NPB dissolved in isopropyl alcohol (IPA) as a substrate and incubated at 45 °C for 10 min. After 10 min, 100 μL of reaction mixture was taken out and added to 600 μL of deionized water to extract *p*-nitro phenol in an aqueous phase. Finally, 600 μL of the potassium phosphate buffer solution (50 mM) of pH 7.8–8.0 was added to above solution in order to extract, *p*-nitro phenol (*p*-NP) in the aqueous phase which provided pale yellow colour which was then used to measure absorbance. One unit (U) lipase activity was defined as micromoles of *p*-NP released per minute by hydrolysis of *p*-nitro phenyl butyrate (*p*-PNB) by 1 mg of lipase under the given standard assay conditions at 45 °C. Furthermore, % residual activity was determined by equation:

(1) % protein binding yield = (amount of protein bound after immobilization)/(initial protein used for immobilization).
(2) % lipase activity = (immobilized lipase activity)/(free lipase activity).
(3) Specific activity of free lipase = (free lipase activity)/(free or initial amount of protein content).
(4) Specific activity of immobilized lipase = (immobilized lipase activity)/(amount of protein bound after immobilization).
(5) % activity yield = (specific activity of immobilized lipase)/(specific activity of free lipase).
(6) % residual activity = (activity of incubated or treated sample)/(activity of non-incubated or fresh sample).

2.3.4. Determination kinetic parameters

The kinetic parameters Km and Vmax were determined by Lineweaver–Burk plot in the range of 3–18 mM concentration of substrate (*p*-NPB). For this, we have used method indicated in Section 2.3.3.

2.4. Synthesis of laurate esters in SC-CO_2

The enzymatic synthesis of citronellyl laurate esters was carried out in 50 mL high pressure reactor vessel. The instrument was equipped with the pressure reading controller and regulator model JASCO-PU-2080-CO_2 plus. At first, given amount of vinyl laurate was added followed by addition of given amount of citronellol in the 50 mL reaction vessel. Finally, the reaction was started by addition of immobilized PVA/CHI lipase and the reactor vessel was closed appropriately and assembled to SC-CO_2 high pressure reactor. The liquid SC-CO_2 was pumped inside the reactor vessel with a flow rate of 3.5 mL/min. The reaction was conducted at given pressure (MPa) and temperature (°C) designed by RSM software for given period. After, the completion of reaction, the SC-CO_2 was slowly depressurized through a thermostat restrictor having temperature 50 °C which leaves back the residual reaction mass inside the reactor by leaving CO_2. The reaction mass was then analyzed using the Perkin-Elmer, Clarus-400 Gas Chromatography (GC) equipped with a flame ionizing detector (FID) and capillary column. The oven temperature of GC was kept at 90 °C for 4 min and then rises at 10 °C/min up to 240 °C. The product formed was also confirmed by the gas-chromatography–mass spectroscopy analysis (GC–MS) by Shimadzu QP-2010 instrument.

2.5. Experimental design and statistical analysis

The RSM was used to optimize the enzymatic synthesis of the citronellyl laurate ester in SC-CO_2 to study the effect of various process variables along with the interaction in between them. The combination of RSM and five-factor-three level Box–Behnken employed in present study to optimize the enzymatic citronellyl laurate synthesis. The independent variables (reaction parameters) were citronellol (mmol), vinyl laurate (mmol), PVA/CHI/lipase biocatalyst amount (mg), reaction temperature (°C) and pressure (MPa) whereas the responses (dependent variables) chosen was the % yield. The coded values for the various five independent variables were provided in Table 1, while the five-factor-three-levels Box–Behnken design was consisted of 46 experiments as presented in Table 2. The responses were then examined through the numerical tools provided by RSM Design Expert Software (version 7.0.10). The experiments were analyzed by means of a response surface regression procedure to fit the following second-order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{i=1}^{k} \beta_{ij} X_i X_j$$

Herein, Y is response % yield, β_0 is a constant, β_i is a linear coefficient, β_{ii} is the squared coefficient, β_{ij} is the cross product coefficient, and k is the number of factors. The second order polynomial coefficient and analysis of variance (ANOVA) was determined using analytical tools of the Design Expert Software (version 7.0.10). The 3D contour and response surface plots were obtained for each response, furthermore multiple response optimizations were studied using numerical tools provided within the Design Expert software. Various separate experiments at the optimized process parameters were carried out to evaluate the response model which is designed by the Design Expert software (Scheme 1).

3. Results and discussion

3.1. Characterization of immobilized lipase and control support

3.1.1. Selection of the biocatalyst

Selection of the biocatalyst was performed by determining the 'protein binding yield (%)' and hydrolytic 'lipase activity (%)' assay. Both of these are combinely expressed in terms of the 'specific activity' and 'activity yield (%)' to determine the best suitable biocatalyst. Hydrolytic activity assay of free as well as immobilized lipase with various support compositions was determined for *p*-PNB assay (Table 3). The % protein binding yield was found to be >88% for various immobilized support composition, which assured that support matrix is compatible for immobilization of enzyme. Interestingly it was observed that, immobilized lipase activity was found to be higher after immobilization (Table 3). The basic reason for increment of lipase activity is interfacial activation of the lipase after immobilization on hydrophobic PVA/CHI matrix [3,14]. Moreover, free lipase aggregation was avoided after immobilization since, lipases are well scattered into PVA/CHI matrix after immobilization which made easy mass transfer phenomenon for the substrate reactivity [11–13] These better results of the 'lipase activity (%)' and 'protein bound (%)' provided higher 'specific activity' and 'activity yield (%)' of immobilized lipase as compared to free lipases [11–14]. In present study highest specific activity and activity yield was observed for various support composition PVA:CHI:BCL (6:4:2.5). Hence among all support compositions, PVA:CHI:BCL (6:4:2.5) was chosen as a robust biocatalyst and used further to carry out the organic transformation (Table 3; entry 13). Similar type of improvement in catalytic activity was observed by

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

Table 1
Independent variable and their levels.

Name	Coded symbol	−1	0	+1
Alcohol (mmol)	A	6	8	10
Laurate (mmol)	B	12	16	20
Catalyst loading (mg)	C	50	125	200
Pressure (MPa)	D	7.2	8.2	9.2
Temperature (°C)	E	35	45	55

Table 2
Box–Behnken design matrix of independent variables with their corresponding response (% yield).

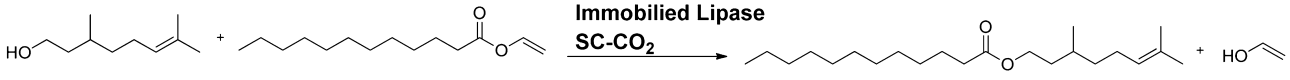
Run (no)	Citronellol (mmol)	Laurate (mmol)	Biocatalyst (mg)	Pressure (MPa)	Temperature (°C)	Yield (%)
1	8	16	125	9.2	55	72
2	6	20	125	8.2	45	78
3	8	20	125	9.2	45	94
4	10	16	50	8.2	45	40
5	10	12	125	8.2	45	32
6	8	12	50	8.2	45	38
7	8	16	125	8.2	45	82
8	8	20	125	8.2	35	58
9	8	12	200	8.2	45	88
10	10	16	125	8.2	35	33
11	8	12	125	8.2	35	30
12	6	16	125	8.2	55	68
13	8	12	125	7.2	45	66
14	6	16	125	9.2	45	66
15	8	16	125	8.2	45	82
16	10	16	125	9.2	45	50
17	8	12	125	9.2	45	76
18	8	16	50	9.2	45	36
19	10	20	125	8.2	45	72
20	8	16	200	9.2	45	94
21	10	16	200	8.2	45	70
22	8	16	50	8.2	55	58
23	8	16	50	7.2	45	39
24	8	16	125	8.2	45	84
25	8	20	50	8.2	45	66
26	8	16	200	8.2	55	88
27	8	20	125	7.2	45	81
28	10	16	125	7.2	45	46
29	8	16	125	7.2	35	44
30	8	16	125	9.2	35	60
31	8	16	200	7.2	45	89
32	8	16	125	8.2	45	83
33	6	16	200	8.2	45	93
34	6	16	50	8.2	45	44
35	6	12	125	8.2	45	58
36	8	16	125	8.2	45	76
37	8	16	50	8.2	35	42
38	8	20	200	8.2	45	93
39	6	16	125	8.2	35	49
40	8	16	125	7.2	55	88
41	10	16	125	8.2	55	60
42	8	16	200	8.2	35	44
43	8	16	125	8.2	45	82
44	8	20	125	8.2	55	86
45	8	12	125	8.2	55	68
46	6	16	125	7.2	45	60

Time: 3.5 h.

Fernandez-Lorente et al. [12] due to interfacial activation of lipase after immobilization of enzyme on the hydrophobic support.

3.1.2. Mechanism of enzyme immobilization on PVA:CHI support

It is essential to know the possible support–lipase interaction and immobilization mechanism to achieve higher activity–stability of immobilized lipase and to avoid random denaturation of enzyme after immobilization [26–33]. In the present study, an entrapment methodology was used for immobilization of lipase BCL since, immobilization of lipase within polymer matrix was carried out at the time of PVA:CHI matrix formation [26,27]. Also, it is expected that, lipases not only entrapped within the PVA:CHI polymer matrix, but also forms an extra multi-point interaction within the matrix via H-bonding, covalent, and hydrophobic interactions, which produces an efficient stable and robust biocatalyst [28,29]. Basically free —OH of PVA and CHI leads to form H-bonding,



Scheme 1. Synthesis of citronellyl laurate.

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

Table 3
Screening of various support composition (PVA:CHI:BCL).

No	PVA:CHI:BCL	% protein binding yield	% lipase activity	Specific activity of immobilized lipase (U/mg)	% activity yield
1	0:10:2	94.362	129.396	1.815	137.127
2	2:8:2	94.016	133.173	1.875	141.650
3	3:7:2	93.064	135.474	1.927	145.571
4	4:6:2	92.934	138.152	1.968	148.657
5	5:5:2	92.012	140.728	2.024	152.945
6	6:4:2	91.619	142.103	2.045	155.103
7	7:3:2	91.376	140.941	2.042	154.242
8	8:2:2	91.333	140.350	2.034	153.669
9	10:0:2	91.420	139.320	2.017	152.396
10	6:4:1.5	93.806	134.864	1.903	143.769
11	6:4:2	91.619	142.103	2.045	155.103
12	6:4:2.25	91.309	144.778	2.068	158.557
13	6:4:2.5	91.620	152.284	2.196	166.214
14	6:4:2.75	89.899	148.622	1.939	165.320
15	6:4:3	91.336	125.408	1.932	137.304

Table 4
Kinetic parameter study.

Kinetic parameter	Immobilized lipase (PVA:CHI:BCL)	Free BCL
Vmax (μmol/mg/min)	50.0	36.36
Km (mM)	2.60	3.00
Catalytic efficiency	19.19	12.07
% catalytic efficiency	158.6	100

while free $-NH_2$ group of the CHI leads to form hydrogen bonding as well as covalent bonding (covalent bonding causes Schiff's reaction/bond formation) [28,29]. Furthermore, the hydrophobic nature of PVA:CHI support matrix lead to improve catalytic activity [30]. More recently, Kosaka et al. proposed that, immobilization of lipase onto polysaccharide base matrix favours the lipase activation and stabilization via open state confirmation [30]. The Leucine 73, Alanine 76, Leucine 80, Valine 86, and Valine 90 amino acid residues are actively involved in hydrophobic bonding which causes the activation of lipase molecules [30,31]. Thus hydrophobic support enhances the phenomenon of lipase immobilization [26,27,29,31]. In addition to this, entrapment methodology has several advantages such as (i) it is simple, (ii) it is usually free from the use of cross-linking chemical agent, (iii) it does not involve multistep immobilization process and (iv) moreover, entrapment methodology shields enzyme molecules by direct exposure of outer reaction environment, which ultimately enhances the stability and activity of biocatalyst in reaction media. Besides this, immobilization via entrapment avoids direct leaching of enzyme from support matrix, which leads to improve operational stability of the biocatalyst [26–29].

3.1.3. Determination of kinetic parameters and catalytic efficiency

To investigate the catalytic efficiency and activity, we have performed kinetic study via hydrolytic activity assay. Interestingly, kinetic study demonstrated higher activity of lipase BCL after immobilization on hydrophobic support. The Vmax value for immobilized lipase BCL was found to be higher (50 μmol/mg/min) than that of crude/free lipase BCL (36.36 μmol/mg/min) (Table 4). This increment of Vmax values of the PVA:CHI immobilized lipases could be explained by mechanism of interfacial activation which could benefit the "open state" conformation of lipase [26–28,30]. The Km value for immobilized lipase BCL was found to be lower (2.6 mM) than that of free lipase (3 mM), which indicating higher affinity of immobilized lipase towards substrate (Table 4). This higher affinity of PVA:CHI immobilized lipase was attributed to more substrate concentration around well dispersed/scattered lipase which could make easy contact with substrate molecules [28].

This PVA:CHI:BCL immobilized lipase demonstrated higher % catalytic efficiency (158%) which indicating the hyperactivation of lipase BCL after immobilization on PVA:CHI. This hyperactivation was attributed to dynamic interfacial activation of lipase on hydrophobic support [31,32]. The hydrophobic support–lipase interactions promote stabilization of active (open) state conformation of lipase molecules [32]. This open state conformation of lipases causes easy mass transfer of substrate molecules to active catalytic sites and improves the bio-catalytic activity of immobilized lipase dramatically [28–30,32].

3.1.4. Surface texture analysis

The visual observation showed uniform continuous texture of the control PVA:CHI and PVA:CHI:BCL immobilized film matrix. The surface of control support PVA:CHI and immobilized PVA:CHI:BCL represented fine structural integrity, without any fracture or crack. The control PVA:CHI was slightly transparent with the off-white colouration while, after immobilization, polymer matrix lost somewhat transparency and showed slight pale yellow colouration. The control PVA:CHI support represented thickness value in between 64 and 68 μm; while immobilized PVA:CHI:BCL (6:4:2.5) film represented thickness values in between 78 and 82 μm. The SEM analysis was performed to observe the surface morphology of support PVA:CHI and immobilized PVA:CHI:BCL (Fig. 1). The SEM analysis showed change in the surface morphology after immobilization of lipase into polymer, as surface of the PVA/CHI immobilized lipase indicated distribution of lipase molecules into the polymer matrix while control film showed plane uniform surface.

3.1.5. TGA analysis

Thermo-gravimetric analysis (TGA) was used to investigate the change in mass of the sample with respect to temperature which provides an idea of thermal stability [33]. In general, physically adsorbed water molecules are eliminated at 100–120 °C while closely associated (tightly bound) water eliminated at 220–250 °C temperature from proteins. This closely associated water plays an essential role to maintain the conformational flexibility and catalytic activity of enzyme [33]. Similar type of observations was also made in the present study for free as well as PVA/CHI immobilized lipase (Fig. 2). Physically adsorbed water molecules eliminated at 100–120 °C while closely associated water eliminated at 220–250 °C. Free lipases showed 21 and 52% weight loss at 200 °C and 300 °C respectively. In contrast to this, immobilized PVA:CHI:BCL showed 10 and 23% weight loss at 200 °C and 300 °C respectively [33]. The higher stability of immobilized biocatalyst was attributed due to entrapment of lipase within the PVA:CHI polymer matrix. This PVA:CHI support matrix provided suitable

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

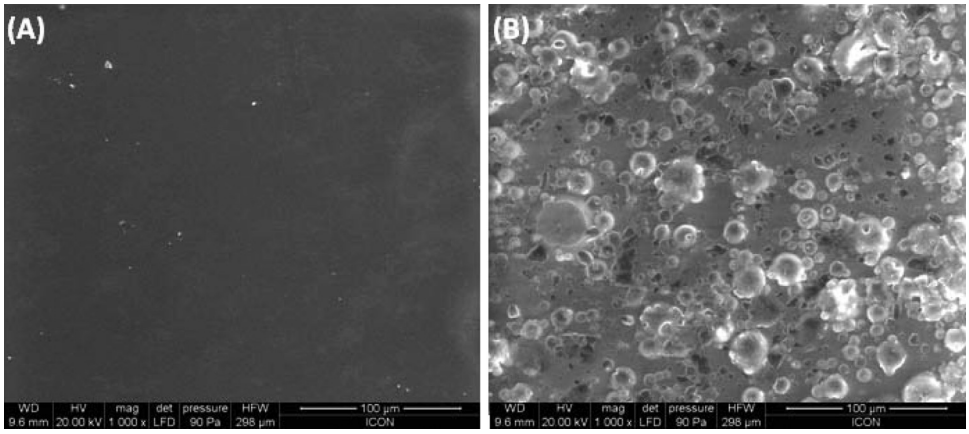


Fig. 1. SEM anlysis of the (A) plane PVA:CHI co-polymer and (B) lipase immobilized on PVA:CHI co-polymer.

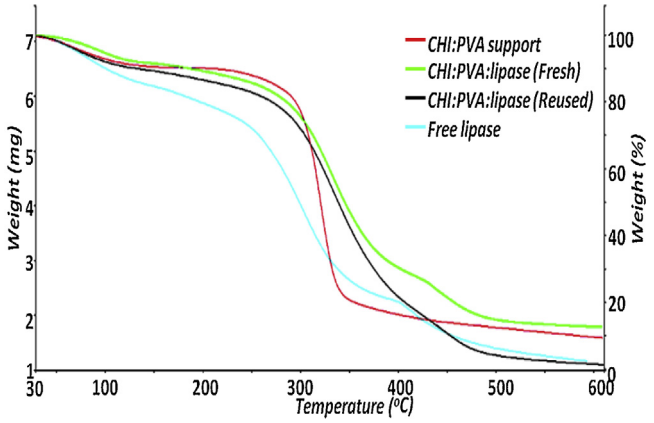


Fig. 2. TGA analysis.

surface for immobilization which can shelter the lipase molecules from outside environment and avoids the breaking of various stabilizing forces [28,29]. Conversely, the case is different for free BCL which openly exposed to outer higher temperature and may causes damage to tertiary structure of lipases via breaking of important stabilizing forces such as H-bonding/Vander-waals forces. Thus, immobilization protect lipase molecules from direct exposure of higher temperature, while free lipases expose directly to higher temperature and causes denaturation quickly [28,29,33]. Moreover, reused biocatalyst sample also showed sufficient stability as like fresh immobilized lipase sample. Thus, present study indicated better thermal stability of the PVA/CHI immobilized lipase as compared to free lipases.

3.2. Model fitting and process optimization

The main intention of the present paper is to develop and evaluation of a statistical approach for better understanding of the co-relation between the various variables of a lipase-catalyzed synthesis of citronellyl laurate reaction. As compared to the one-factor-at-a-time design, the RSM with five-factor-three-level employed in this study was more efficient in reducing the experimental runs, time and energy to investigate the optimized synthesis of citronellyl laurate with an affordable cost. One-factor-at-a-time design for optimization purpose has been adopted most often in the literature, which suffers from the drawback such as more experimental time, run, and energy. Among the various treatments, the lowest % yield was 30% obtained (when alcohol:vinyl laurate is 8:12; biocatalyst 125 mg; pressure 8.2 MPa and temperature 35 °C) and highest % yield obtained was 94% (Table 2). The second-order polynomial equation for % yield for citronellyl laurate synthesis was as follows.

$$Y = \%\,\text{yield} = 81.5 - 7.06A + 10.75B + 18.5C + 2.18D + 14.25E + 5AB - 4.75AC - 0.5AD + 2AE - 5.75BC + 0.75BD - 2.5BE + 2CD + 7CE - 8DE - 16.60A^2 - 2.85B^2 - 8.02C^2 - 4.77D^2 - 14.18E^2$$

Analysis of variance (ANOVA) was showing that second order polynomial model was extremely significant and ideal to present the actual relationship between the response Y (% yield) and significant variables (citronellol and vinyl laurate quantity, biocatalyst amount, pressure and temperature) with very small p-value and satisfactory coefficient of determination (R^2 = 0.909) (Table 5). Moreover, the overall effect of various five variables which affecting the synthesis of citronellyl laurate % yield was further analyzed by the joint test. The results showed that, amount of the laurate ester (B), biocatalyst amount (C) and temperature (E) are the most important factors which exerting a statistically significant p-value (<0.0001) for the response % yield of citronellyl laurate synthesis (Table 5).

3.3. Mutual effects of various reaction parameters

In present study, we have investigated the influence of mutual reaction parameters for citronellyl laurate synthesis. It was observed that, as moles of vinyl laurate increased from 12 to 20 mmol then % yield also increased linearly but slowly. In contrast to this it was observed that, as moles of citronellol increases from 6 to 8 mmol then % yield also increases however, further increase in mole quantity from 8 to 10 mmol showed decrease in the % yield which may be attributed due to enzyme inhibition phenomenon (Fig. 3). Most of the literature study revealed that alcohol acts as potential inhibitor for the enzyme activity because of the polar nature of the alcohol [34]; similar type of the citronellol alcohol inhibition was attributed in the present study also. The % yield of citronellyl laurate was increased steeply with amount of the immobilized PVA/CHI biocatalyst, as more catalytic sites are available with more amount of biocatalyst loading [34–36]. The sufficient enzyme loading and activated acyl donors (vinyl laurate) accelerate the reaction rate by formation of lauryl–lipase (acyl–enzyme) complex which consequently break-down into the product after attack of the alcohol (citronellol) and thus increases the reaction progress and % yield [3].

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

Table 5
ANOVA table for response surface model.

Source	Sum of squares	df	Mean square	*F* value	*p*-value	Remark
Model	15,774.77	20	788.73	12.62	<0.0001	Significant
β_A	798.06	1	798.06	12.77	00015	–
β_B	1849	1	1849	29.59	<0.0001	–
β_C	5476	1	5476	87.66	<0.0001	–
β_D	76.56	1	76.56	1.22	0.2788	–
β_E	3249	1	3249	52.01	<0.0001	–
β_{AB}	100	1	100	1.60	0.2174	–
β_{AC}	90.25	1	90.25	1.44	0.2406	–
β_{AD}	1	1	1	0.01	0.9003	–
β_{AE}	16	1	16	0.25	0.6172	–
β_{BC}	132.25	1	132.25	2.11	0.1581	–
β_{BD}	2.25	1	2.25	0.03	0.8510	–
β_{BE}	25	1	25	0.40	0.5327	–
β_{CD}	16	1	16	0.25	0.6172	–
β_{CE}	196	1	196	3.13	0.0887	–
β_{DE}	256	1	256	4.09	0.0537	–
β_A^2	2406.09	1	2406.09	38.51	<0.0001	–
β_B^2	71.09	1	71.09	1.13	0.2962	–
β_C^2	561.45	1	561.45	8.98	0.0061	–
β_D^2	198.64	1	198.64	3.17	0.0867	–
β_E^2	1756.67	1	1756.67	28.12	<0.0001	–
Residual	1561.66	25	62.46	–	–	–
Lack of fit	1522.16	20	76.10	9.63	0.0098	Significant
Pure error	39.5	5	7.9	–	–	–
Corrected total	17,336.43	45	–	–	–	–
R^2	–	–	–	–	–	0.909
R^2 adjusted	–	–	–	–	–	0.878

The temperature and pressure of SC-CO_2 system has mutual effect on the rheological properties, including density and viscosity of reaction media, mass-transfer and substrate diffusivity at the active catalytic sites of enzyme [20,21,36–38]. In present study it was observed that, % yield increases slowly as the SC-CO_2 pressure increases from 7.2 to 8.8 MPa, however further increase in the pressure had insignificant effect on yield and reaction rate (Fig. 4) [37,38]. Increase in the temperature causes increase of reaction rate and subsequent % yield due to increase in kinetic energy of reactant molecules. However, precaution must be taken that enzymes may lose their catalytic activity at higher temperatures due to thermal denaturation of protein structure [37–42]. In the present study, temperature was varied from 32 to 55 °C (Fig. 4). Increase in the temperature showed increase of catalytic activity of biocatalyst and % yield of reaction. The significant yield (>85%) was obtained when temperature was ≥45 °C. In addition to this, temperature effect is related to the enzyme activity, stability, density and solvating power of SC-CO_2 [37–41]. Thus, enzymes should have optimal effective temperature with suitable SC-CO_2 density where they can offer utmost catalytic activity with proper mass-transfer at given density for the corresponding reaction system [37–39].

3.4. Attaining the optimal reaction parameters and model verification

Consequently, the optimal points provided by Design Expert were used to find out the optimal reaction conditions, which were mole ratio of citronellol (8.5 mmol), vinyl laurate (19.87 mmol), immobilized PVA/CHI biocatalyst amount (175.6 mg), pressure of SC-CO_2 (8.81 MPa) and temperature of system (46.02 °C), leads to provide 94.42% (predicted) yield of the desired product (Table 6). The validity of software proposed model was scrutinized by performing additional independent experiments at the suggested optimal reaction parameters. Table 6 showed software predicted

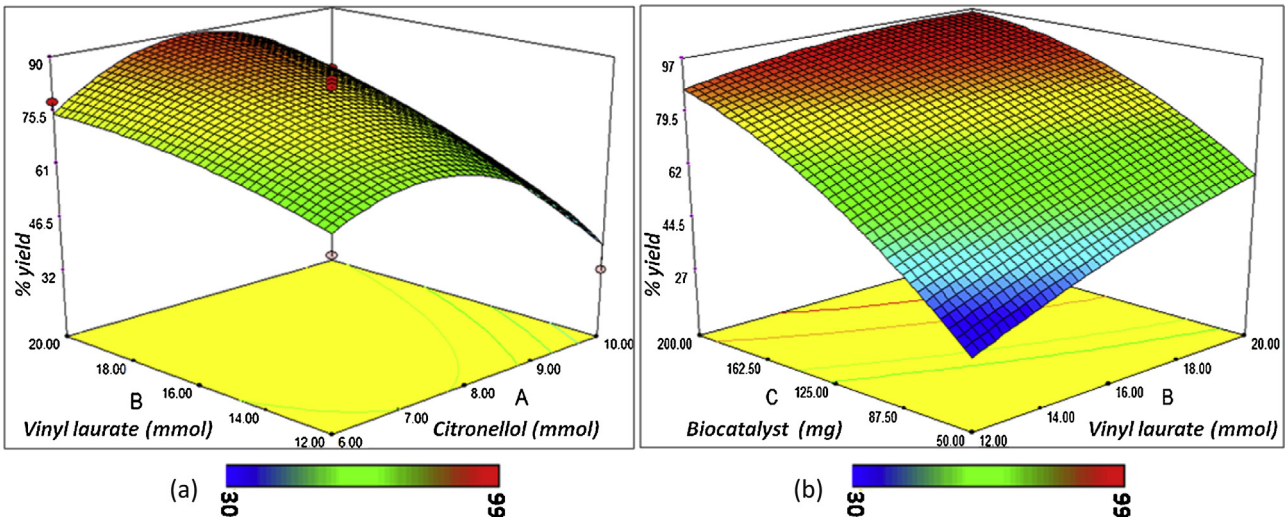


Fig. 3. (a) Plot showing the effect of moles of vinyl laurate and citronellol on % yield and (b) plot showing the effect of vinyl laurate moles and biocatalyst amount on % yield.

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

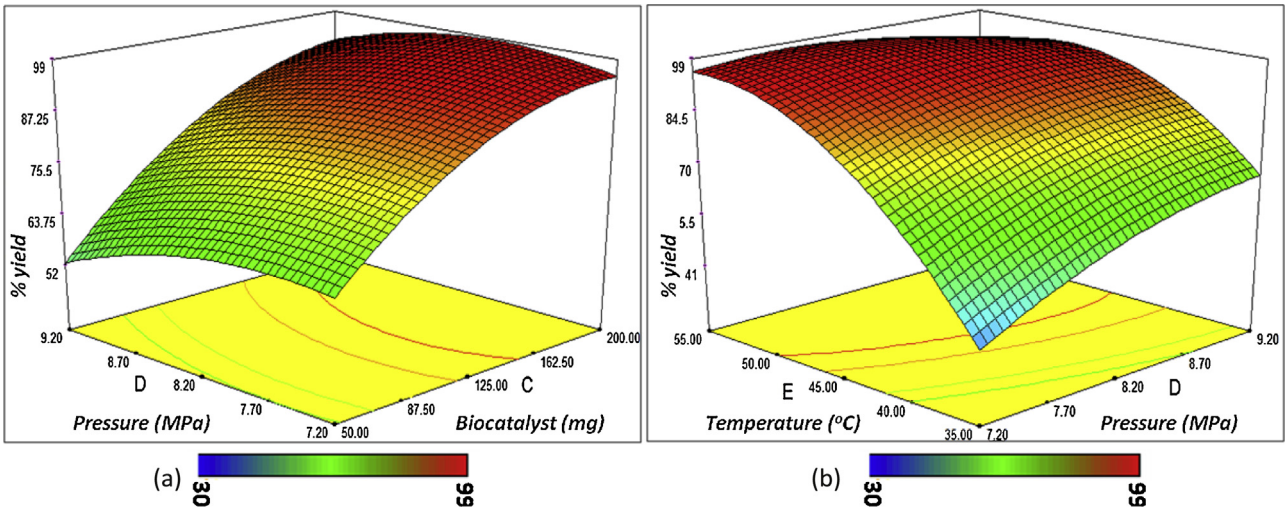


Fig. 4. (a) Plot showing the effect of pressure and biocatalyst amount on % yield and (b) plot showing the effect of pressure and temperature on % yield.

Table 6
Model solution and verification.

Run (no)	Citronellol (mmol)	Laurate (mmol)	Biocatalyst (mg)	Pressure (MPa)	Temperature (°C)	Predicted yield (%)	Experimental yield (%)
1	8.50	19.87	175.60	8.81	46.02	94.42	94.00 ± 1.52
2	6.84	16.13	193.88	8.74	43.91	92.00	91.66 ± 1.15
3	6.50	18.62	178.34	7.76	52.38	93.63	94.33 ± 0.57
4	8.24	19.81	139.21	7.61	51.90	92.89	92.33 ± 2.08
5	8.08	18.50	172.73	7.98	46.15	93.90	95.00 ± 1.00
6	8.59	18.80	160.21	8.28	51.82	93.89	94.33 ± 1.52

Time: 3.5 h.

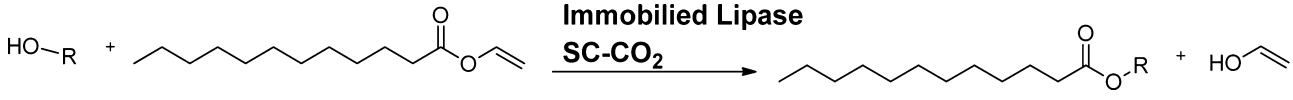
and experimentally observed values for given responses at optimal reaction conditions for synthesis of citronellol laurate. The experimentally provided values were average of three values and very close to the RSM software predicted values which indicating that model generated was acceptable (Table 6). Accordingly, this present model may be acts as an important reference model for further industrial application to synthesize the citronellyl laurate. Thus, optimization of PVA/CHI immobilized lipase-catalyzed synthesis of citronellyl laurate was successfully performed by RSM.

3.5. Scope of the developed protocol

To extend the application and scope of developed protocol, we performed various substrate studies at optimal reaction condition (Scheme 2; Table 7). Herein, it was observed that, various alcohols reacted with vinyl laurate to provide excellent yield of desired laurate ester at a given reaction condition. Interestingly, the shorter chain alcohols (such as ethanol, propanol and butanol) reacted fast as compared to the longer chain alcohol to provide slightly better yield (Table 7; entries 1–6). This might be attributed to mass-transfer limitations of long chain alcohols at the active sites of enzyme [4,34]. Moreover, branched alcohols also suffered from mass transfer limitations and provided slightly lower yield (Table 7; entries 7–9). The aromatic benzyl alcohol derivatives provided excellent yield as compared to long chain alcohol because of the higher nucleophilicity (Table 7; entries 10 and 11). These all fatty acid esters (laurate) are widely used in/as lubricant, emulsifiers or oiling agents for foods, spin finishes (fibre lubricant) in textiles, lubricants for plastics, paint or ink additives, and surfactants [4,34,37–39]. Moreover, these are also used as solvents or co-solvents and base materials in pharma, food-flavour and perfumery industries [34,37–39]. Considering scope and importance, the present protocol provided an important optimized synthesis of various fatty laurate esters (Table 7).

3.6. Operational stability study

In view of economic aspect, we studied recyclability of developed protocol for various laurate ester syntheses at the given optimal condition (Table 7). After each recycle, the immobilized lipase was filtered, washed with the solvent toluene 3 times, dried at 40 °C for 12 h and reused as such when needed. It was observed that, there was an insignificant decrease in % yield for first two cycles, whereas after five recycles the % yield was reduced upto 65–76% depending on the nature of substrate for laurate ester synthesis. The reduction in % yield (activity) with higher number of recycle may be endorsed due to continuous exposure of enzymes to alcoholic substrates at higher pressure [4,34,37,39,43,44]. Similar type of decrease in yield/activity after subsequent reuse was observed to the Friedrich et al. [5]. Moreover, recyclability study demonstrated that biocatalyst was sufficiently stable in SC-CO_2 media upto studied five recycles.



Scheme 2. Synthesis of various valuable laurate compounds.

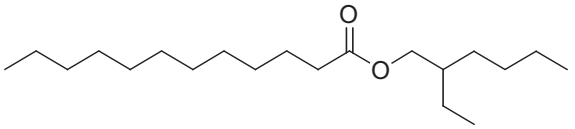
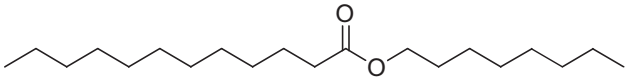
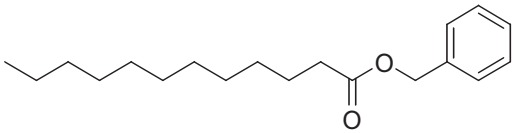
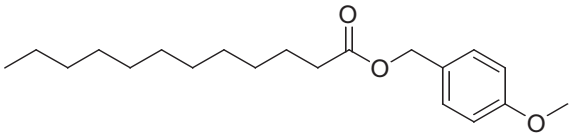
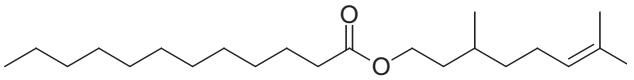
Please cite this article in press as: Badgujar KC, Bhanage BM. Immobilization of lipase on biocompatible co-polymer of polyvinyl alcohol and chitosan for synthesis of laurate compounds in supercritical carbon dioxide using response surface methodology. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

Table 7
Scope and applications of developed protocol for synthesis of various valuable laurate compounds and their recyclability study.[a]

No.	Substrate	Fresh and recycle run yield (%)[b]				Free lipase[c]	GCMS analysis (product confirmation)
		Fresh	I	III	V		
1		99	96	88	74	31	41, 43, 53, 57, 73, 88 (base peak), 101, 157, 181, 183, 199, 201, 214, 228 (M+), 229 (M+1)
2		99	94	86	73	30	41, 43, 53, 57, 61 (base peak), 73, 102, 115, 129, 143, 157, 171, 183, 201, 213, 243(M+)
3		98	95	87	71	31	41, 43, 56 (base peak), 73, 85, 101, 116, 129, 143, 157, 171, 183, 201, 213, 227, 241, 256 (M+)
4		98	94	86	73	33	41, 43, 56 (base peak), 57, 73, 85, 98, 101, 116, 129, 143, 157, 171, 183, 201, 256 (M+), 256 (M+1)
5		98	96	87	74	34	41, 43, 56, 57, 70 (base peak), 85, 98, 115, 129, 143, 157, 171, 183, 201, 255, 271 (M+)
6		99	95	85	75	32	41, 43, 56, 57, 68 (base peak), 71, 85, 98, 109, 115, 123, 141, 163, 171, 183, 201, 268 (M+)
7		97	93	87	70	29	41, 43, 56, 57, 77, 84 (base peak), 85, 98, 115, 129, 143, 157, 171, 183, 201, 213, 284 (M+), 284 (M+1)

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

Table 7 (*Continued*)

No.	Substrate	Fresh and recycle run yield (%)[b]				Free lipase[c]	GCMS analysis (product confirmation)
		Fresh	I	III	V		
8		95	90	79	69	28	41, 43, 56, 57 (base peak), 70, 71, 84, 112, 129, 143, 157, 171, 183, 201, 213, 283, 312 (M+), 313 (M+1)
9		93	89	76	65	23	41, 43, 56, 57 (base peak), 70, 71, 84, 85, 112, 113, 129, 143, 157, 183, 200, 201, 312 (M+), 313 (M+1)
10		96	92	84	71	27	41, 43, 57 83, 91 (base peak), 108, 125, 139, 163, 181, 199, 217, 272, 290 (M+)
11		95	90	81	69	29	40, 57, 78, 91, 109, 121 (base peak), 138, 320 (M+), 321 (M+1)
12		94	91	80	70	23	41, 55, 57, 81, 97, 109, 124, 138 (base peak), 163, 180, 196, 221, 244, 265, 338 (M+), 339 (M+1), 340 (M+2)

[a] Reaction condition: alcohol: 8.5 mmol; laurate: 19.87 mmol; immobilized biocatalyst: 175.6 mg; pressure: 8.81 MPa; temperature: 46.02 °C; time: 3.5 h.
[b] Yield obtained by immobilized lipase.
[c] Yield obtained by free lipase.

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

Table 8
Determination of activity of lipase incubated in the SC-CO_2.

No.	Temperature (°C)	Pressure (MPa)	Time (h)	% residual activity of PVA:CHI:BCL	% residual activity of free lipase
Fresh sample non-incubated					
1	25 (Fresh)	0	0	100.00	100.00
Influence of incubation time in SC-CO_2 system					
2	45	8.5	2	99.927	99.214
3	45	8.5	4	99.768	98.643
4	45	8.5	6	99.476	97.786
5	45	8.5	8	99.317	97.443
6	45	8.5	10	99.012	96.657
7	45	8.5	12	98.829	93.071
Influence of the pressure of SC-CO_2 system					
8	45	7.6	10	99.707	99.443
9	45	8	10	99.451	97.629
10	45	8.5	10	99.012	96.657
11	45	9	10	99.805	95.329
12	45	9.5	10	98.646	93.229
13	45	10	10	98.151	91.186
Influence of the temperature of SC-CO_2 system					
14	36	8.5	10	99.854	98.557
15	40	8.5	10	99.976	97.943
16	44	8.5	10	99.463	97.057
17	48	8.5	10	99.134	96.286
18	52	8.5	10	98.902	95.143
19	56	8.5	10	98.746	92.114

Lipase activity assay performed by procedure indicated in Section 2.3.3. Actual activity of the fresh (non-incubated) PVA:CHI:BCL immobilized lipase: 41.23 U/mg. Actual activity of the fresh (non-incubated) free lipase: 29.11 U/mg.

3.7. Determination of the residual activity of immobilized lipase

It is noteworthy to study the influence of the temperature, pressure and time in SC-CO_2 phase on the enzyme activity since; enzymes are highly sensitive to high temperature and pressure [30,41]. In the present study, it was observed that higher pressure treatment with compressed SC-CO_2 could maintain or marginally decreases the immobilized lipase activity; while free lipase activity was slowly decreased at the prescribed condition (Table 8). This might be due to reason that, free lipases was directly exposed to outer compressed SC-CO_2 environment while, immobilized lipases were well protected by the immobilization support matrix [17,40,41]. This decrease in the activity was associated to removal of essential trace amount of water at high pressure [40,41]. Since, enzymes need a specific amount of bound water, while this trace amount of bound water is removed in high pressure and during the depressurization of SC-CO_2 which tends to reduces the enzyme activity [40]. Moreover, Knez et al. [22] reported that high pressure in supercritical phases causes change in *Rhizomucor miehei* lipase' lid region, which results into the loss of the lipase activity. Similar type of the results was observed to Chen et al. [40] who showed decreases of the enzyme activity due to removal of the essential water in pressurized supercritical system [40]. In contrast to this, there are some reports which reported that, high pressure treatment could improve the lipase activity may be due to changes in the secondary and tertiary structure at high-pressure carbon dioxide treatment [41]. Thus in present study, the increase of incubation time (2–12 h), pressure (7.6–10 MPa) and temperature (36–56 °C) has insignificant effect on the PVA:CHI immobilized enzyme activity as compared to that free lipase.

3.8. Comparison of immobilized enzyme to free enzyme and time study

At the given optimal parameters, we have catalyzed similar reaction with the equivalent quantity of free lipase. It was observed that, reaction yield was greatly enhanced and providing 94.00 ± 1.51% yield when reaction was catalyzed by PVA/CHI immobilized matrix. However, free lipase provided only 23% yield (Fig. 5). The decrement in activity of the free lipase was observed, as free lipases were directly expose to outer compressed SC-CO_2 environment while, immobilized lipases were well protected by immobilization support matrix [17,40,41]. Moreover after immobilization, lipases were well scattered into the surface of matrix and promoted mass transfer diffusion of substrate to active sites of lipase [4,12,17]. Thus in comparison, immobilized lipase showed almost 4-fold better catalytic activity in SC-CO_2 as compared to free lipase. In all substrate cases it was observed that, yield obtained by immobilized lipase is 3–5-folds higher than the free lipases (Table 7, last column). Moreover, no yield was found when reaction was carried without the biocatalyst.

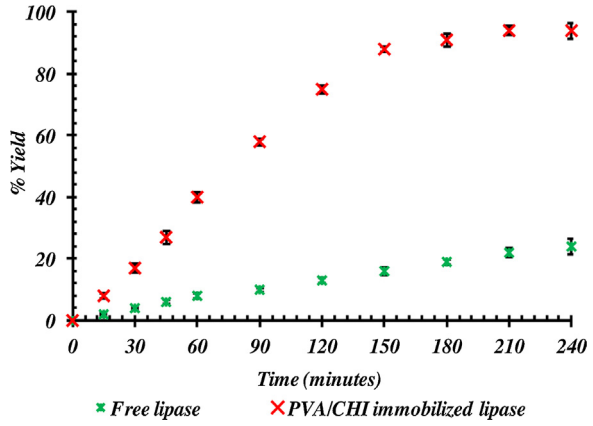


Fig. 5. Comparision of the free liapse with immobilized biocatalyst and time study to synthsize the citronellyl laurate.

3.9. Merits and comparison of the present protocol with literature protocols

The present protocol was compared with the previously reported various literature protocols for the chosen model reaction of citronellyl laurate synthesis (Table 9) [17,34,37–39,42]. It is observed that, present protocol is one of the best biocatalytic protocol among all reported potential protocol which providing better yield (94 ± 1.52) and good catalyst reusability. Moreover,

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019

Table 9
Literature survey for the citronellyl laurate synthesis.

No	Biocatalyst	Reaction condition	Time (h)	Conversion/yield (%)	Reuse no.	Reference no.
1	Novozym 435; 3% loading	Citronellol:lauric acid (0.005 M:0.005 M), solvent heptane up to 15 mL; temperature: 30 °C	2	63	3	[34]
2	Novozym 435;12 wt%	Citronellol:lauric acid (0.005 M:0.005 M), co-solvent: 15 mL (87 wt%) EMK, temperature: 60 °C; pressure: 10 MPa	1	(3.95 mmol/g substrates)	–	[37]
3	Novozym 435; 12 wt%	Citronellol:lauric acid (5 mmol:5 mmol), 85 wt% EMK; temperature: 60 °C; pressure: 10 MPa	1.5	81	–	[38]
4	*Candida rugosa* immobilized on Amberlite MB-1; 60 g/L;	Citronellol:lauric acid (0.03 M:0.02 M); temperature; 37 °C; 10 mL iso-octane	24	89	–	[42]
5	*Candida rugosa* immobilized on SBA-15; 0.6 gm	Citronellol:vinyl lauarte (30 mM:20 mM), non-aqueous media; temperature: 37 °C	24	93	4	[39]
6	*Candida rugosa* immobilized on functionalized SBA-15; 0.6 gm	Citronellol:vinyl lauarte (30 mM:20 mM), non-aqueous media; temperature: 37 °C	24	95	4	[39]
7	Immobilized *Burkholderia cepacia* matrix of polymer 175.6 mg	Citronellol:vinyl laurate (8.5 mmol:19.87 mmol), temperature: 45 °C; pressure: 8.81 MPa	3.5	94 ± 1.51	5–6	Present study

as compared to other reported protocols; the time required for present protocol is sufficiently lesser. Thus in present protocol, we use SC-CO_2 and lipase immobilized into biocompatible support as a greener reaction medium and greener biocatalyst which accomplishing the greener enzymatic synthetic protocol.

4. Conclusion

In the present study, the process of synthesis of citronellyl laurate was optimized successfully by applying a RSM with Box–Behnken design. Initially free lipase biocatalyst was immobilized on a PVA/CHI biocompatible polymer which was characterized by the SEM, TGA, % lipase activity assay and % protein binding yield. Various support compositions were screened systematically to obtain the robust biocatalyst. The robust biocatalyst was then subjected to synthesize citronellyl laurate via response surface methodology. The RSM study provided optimal condition to produce 94 ± 1.51% yield of the citronellyl laurate. Moreover, the optimized reaction conditions were applied for the synthesis of various industrially important laurate esters. Interestingly, protocol provided excellent yield of various laurate esters upto five recycles. Moreover, we have investigated the activity and stability of the developed immobilized biocatalyst in SC-CO_2 media, which showed that immobilized biocatalyst, is robust and sufficiently stable in SC-CO_2 media. Thus in present study, we have performed (i) synthesis of immobilized biocatalyst, (ii) characterization of immobilized biocatalyst, (iii) activity assessment in SC-CO_2 media, (iv) optimized synthesis of citronellyl laurate in SC-CO_2 and (v) extended application of optimized parameters to synthesize various laurate esters with excellent recyclability.

Acknowledgements

Author (Kirtikumar) is grateful to Council of Scientific & Industrial Research (CSIR), India (File No. 09/991(0015)/2011-EMR-I) and Department of Biotechnology (DBT), India (File No. BT/PR4192/PID/6/623/2011) for providing research fellowship and funds. Also author (Kirtikumar) is thankful to Prof. C. S. Mathpati for the helpful discussion.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2015.04.019

References

[1] Ansorge-Schumacher MB, Thum O. Immobilised lipases in the cosmetics industry. Chem Soc Rev 2013;42:6475–90.
[2] Carla CCR, Carvalho D, Manuela MR, Fonseca D. Biotransformation of terpene. Biotechnol Adv 2006;24:134–42.
[3] Badgujar KC, Bhanage BM. Application of lipase immobilized on the biocompatible ternary blend polymer matrix for synthesis of citronellyl acetate in non-aqueous media: kinetic modelling study. Enzyme Microb Technol 2014;57:16–25.
[4] Badgujar KC, Bhanage BM. Synthesis of geranyl acetate in non-aqueous media using immobilized *Pseudomonas cepacia* lipase on biodegradable polymer film: kinetic modelling and chain length effect study. Process Biochem 2014;49:1304–13.
[5] Friedrich JLR, Pena FP, Garcia-Galan C, Fernandez-Lafuente R, Ayub MAZ, Rodrigues RC. Effect of immobilization protocol on optimal conditions of ethyl butyrate synthesis catalyzed by lipase B from *Candida antarctica*. J Chem Technol Biotechnol 2013;88:1089–95.
[6] Dhake KP, Thakare D, Bhanage BM. Lipase A potential biocatalyst for the synthesis of valuable flavour and fragrance ester compounds. Flavour Fragr J 2013;28:71–83.
[7] Mendes AA, Castro HF, Andrade GSS, Tardioli PW, Giordano RLC. Preparation and application of epoxy–chitosan/alginate support in the immobilization of microbial lipases by covalent attachment. React Funct Polym 2013;73:160–7.
[8] Rós PCM, Guilherme AM, Silva Mendes AA, Santos JC, Castro HF. Evaluation of the catalytic properties of *Burkholderia cepacia* lipase immobilized on non-commercial matrices to be used in biodiesel synthesis from different feedstocks. Bioresour Technol 2010;101:5508–16.
[9] Martinsa AB, Graebin NG, Lorenzoni ASG, Fernandez-Lafuente R, Ayub MAZ, Rodriguesa RC. Rapid and high yields of synthesis of butyl acetate catalyzed by Novozym 435: reaction optimization by response surface methodology. Process Biochem 2011;46:2311–6.
[10] Badgujar KC, Bhanage BM. Factors governing dissolution process of lignocellulosic biomass in ionic liquid: current status, overview and challenges. Bioresour Technol 2015;178:2–18, http://dx.doi.org/10.1016/j.biortech.2014.09.138.
[11] Adlercreutz P. Immobilisation and application of lipases in organic media. Chem Soc Rev 2013;42:6406–36.
[12] Fernandez-Lorente G, Cabrera Z, Godoy C, Fernandez-Lafuente R, Palomo JM, Guisan JM. Interfacially activated lipases against hydrophobic supports:

effect of the support nature on the biocatalytic properties. Process Biochem 2008;43:1061–7.
[13] Mateo C, Palomo JM, Fernandez-Lorente G, Fernandez-Lafuente R, Guisan JM. Improvement of enzyme activity, stability and selectivity via immobilization technique. Enzyme Microb Technol 2007;40:1451–63.
[14] Giorno L, Drioli E. Biocatalytic membrane reactors: applications and perspectives. Trends Biotechnol 2000;18:339–49.
[15] Cantone S, Ferrario V, Corici L, Ebert C, Fattor D, Spizzo P, et al. Efficient immobilisation of industrial biocatalysts: criteria and constraints for the selection of organic polymeric carriers and immobilisation methods. Chem Soc Rev 2013;42:6262–76.
[16] Mendes AA, Castro HF, Rodrigues DS, Adriano WS, Tardioli PW, Mammarella EJ, et al. Multipoint covalent immobilization of lipase on chitosan hybrid hydrogels: influence of the polyelectrolyte complex type and chemical modification on the catalytic properties of the biocatalysts. J Ind Microbiol Biotechnol 2011;38:1055–66.
[17] Dhake KP, Deshmukh KM, Patil YP, Singhal RS, Bhanage BM. Improved activity and stability of *Rhizopus oryzae* lipase via immobilization for citronellol ester synthesis in supercritical carbon dioxide. J Biotechnol 2011;156:46–51.
[18] Wang Y, Chen H, Wang J, Xing L. Preparation of active corn peptides from zein through double enzymes immobilized with calcium alginate–chitosan beads. Process Biochem 2014;49:1682–90.
[19] European Commission: commission directive 96/77/EC-European Commission.
[20] Lozano P. Enzymes in neoteric solvents: from one-phase to multiphase systems. Green Chem 2010;12:555–69.
[21] Lozano P, García-Verdugo E, Piamtongkam R, Karbass N, Diego T, Burguete Luis S, et al. Bioreactors based on monolith-supported ionic liquid phase for enzyme catalysis in supercritical carbon dioxide. Adv Synth Catal 2007;349:1077–84.
[22] Knez Z, Laudani CG, Habulin M, Reverchon E. Exploiting the pressure effect on lipase-catalyzed wax ester synthesis in dense medium. Biotechnol Bioeng 2007;97:1366–75.
[23] Leffingwell & Associates Survey for the Flavor & Fragrance Industry Leaders.
[24] Bezerraa MA, Santellia RE, Oliveira EP, Villara LS, Escaleira LA. Response surface methodology as a tool for optimization in analytical chemistry. Talanta 2008;76:965–77.
[25] Pencreac'h G, Baratti JC. Comparison of hydrolytic activity in water and heptane for thirty-two commercial lipase preparations. Enzyme Microb Technol 2001;28:473–9.
[26] Rodrigues RC, Ortiz C, Berenguer-Murcia A, Torres R, Fernández-Lafuente R. Modifying enzyme activity and selectivity by immobilization. Chem Soc Rev 2013;42:6290–307.
[27] Jochems P, Satyawali Y, Diels L, Dejonghe W. Enzyme immobilization on/in polymeric membranes: status, challenges and perspectives in biocatalytic membrane reactors (BMRs). Green Chem 2011;13:1609–23.
[28] Reetz MT. Entrapment of biocatalysts in hydrophobic sol–gel materials for use in organic chemistry. Adv Mater 1997;9:943–54.
[29] Dave R, Madamwar D. Esterification in organic solvents by lipase immobilized in polymer of PVA-alginate–boric acid. Process Biochem 2006;41:951–6.
[30] Kosaka PM, Kawano Y, El Seoud OA, Petri DFS. Catalytic activity of lipase immobilized onto ultrathin films of cellulose esters. Langmuir 2007;23:12167–73.
[31] Kuo C, Chen GJ, Twu Y, Liu Y, Shieh C. Optimum lipase immobilized on diamine-grafted PVDF membrane and its characterization. Ind Eng Chem Res 2012;51:5141–7.
[32] Fernandez-Lafuente R, Armisen P, Sabuquillo P, Fernandez-Lorente G, Guisan JM. Immobilization of lipases by selective adsorption on hydrophobic supports. Chem Phys Lipids 1998;93:185–97.
[33] Tuner NA, Vulfson EN. At what temperature can enzymes maintain their catalytic activity? Enzyme Microb Technol 2000;27:108–13.
[34] Yadav GD, Lathi PS. Synthesis of citronellol laurate in organic media catalyzed by immobilized lipases: kinetic studies. J Mol Catal B: Enzym 2004;27:113–9.
[35] Salis A, Svensson I, Monduzzi M, Solinas V, Adlercreutz P. The atypical lipase B from *Candida antarctica* is better adapted for organic media than the typical lipase from thermomyces lanuginose. Biochim Biophys Acta 2003;1646:145–51.
[36] Chen D, Zhanga H, Xub J, Yana Y. Effect of sub- and supercritical CO_2 treatment on the properties of *Pseudomonas cepacia* lipase. Enzyme Microbiol Technol 2013;53:110–7.
[37] Habulin M, Sabeder S, Paljevac M, Primozi M, Knez Z. Lipase-catalyzed esterification of citronellol with lauric acid in supercritical carbon dioxide/co-solvent media. J Supercrit Fluids 2007;43:199–203.
[38] Habulin M, Sabeder S, Sampedro MA, Knez Z. Enzymatic synthesis of citronellol laurate in organic media and supercritical carbon dioxide. Biochem Eng J 2008;42:6–12.
[39] Abdullah AZ, Sulaiman NS, Kamaruddin AH. Biocatalytic esterification of citronellol with lauric acid by immobilized lipase on aminopropyl-grafted mesoporous SBA-15. Biochem Eng J 2009;44:263–70.
[40] Chen D, Peng C, Zhanga H, Xub J, Yana Y. Assessment of activities and conformation of lipases treated with sub- and supercritical carbon dioxide. Appl Biochem Biotechnol 2013;169:2189–201.
[41] Liu Y, Chen D, Xud X, Yand Y. Evaluation of structure and hydrolysis activity of *Candida rugosa* Lip7 in presence of sub-/super-critical CO_2. Enzyme Microb Technol 2012;51:354–8.
[42] Serri NA, Kamaruddin AH. Studies of reaction parameters on synthesis of citronellyl laurate ester via immobilized *Candida rugosa* lipase in organic media. Bioprocess Biosyst Eng 2006;29:253–60.
[43] Badgujar KC, Dhake KP, Bhanage BM. Immobilization of *Candida cylindracea* lipase on poly lactic acid, polyvinyl alcohol and chitosan based polymer film: characterization, activity, stability and its application for N-acylation reactions. Process Biochem 2013;48:1335–47.
[44] Badgujar KC, Bhanage BM. Solvent stability study with thermodynamic analysis and superior biocatalytic activity of *Burkholderia cepacia* lipase immobilized on biocompatible hybrid matrix of poly(vinyl alcohol) and hypromellose. J Phys Chem B 2014;118:14808–19.

Please cite this article in press as: Badgujar KC, Bhanage BM. 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. Process Biochem (2015), http://dx.doi.org/10.1016/j.procbio.2015.04.019