Journal of Molecular Catalysis B: Enzymatic 75 (2012) 50–59

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Covalently immobilized lipase catalyzing high-yielding optimized geranyl butyrate synthesis in a batch and fluidized bed reactor

Jasmina J. Damnjanović[a,b,*], Milena G. Žuža[a], Jova K. Savanović[a], Dejan I. Bezbradica[a], Dušan Ž. Mijin[c], Nevenka Bošković-Vragolović[d], Zorica D. Knežević-Jugović[a]

[a] Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, Karnegijeva 4, University of Belgrade, Belgrade, Serbia
[b] Laboratory of Molecular Biotechnology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan
[c] Department of Organic Chemistry, Faculty of Technology and Metallurgy, Karnegijeva 4, University of Belgrade, Belgrade, Serbia
[d] Department of Chemical Engineering, Faculty of Technology and Metallurgy, Karnegijeva 4, University of Belgrade, 11000 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 4 November 2011
Accepted 8 November 2011
Available online 17 November 2011

Keywords:
Geranyl butyrate
Covalent immobilization
Enzymatic esterification
Fluidized bed reactor
Optimization study

ABSTRACT

Three commercially available polymers (Sepabeads® EC-EP, Sepabeads® EC-HA and Purolite® A-109) were tested for potential application as supports for covalent immobilization of lipase from *Candida rugosa* by analyzing some critical properties of immobilized enzymes such as enzyme loading, activity and activity immobilization yield. Among them, lipase covalently immobilized on Sepabeads® EC-EP via epoxy groups appeared to show the best performance in a standard hydrolytic reaction. Therefore, it was selected and assayed in the esterification of butyric acid and geraniol to produce geranyl butyrate, first in a batch system followed by continuous geranyl butyrate synthesis in a fluidized bed reactor, as one being potentially applicable for large-scale production.

Based on statistical analysis, optimal conditions for the production of geranyl butyrate by selected, immobilized lipase in the batch system are recommended as: temperature at 25–30 °C, water concentration at 3.6% (v/v) and acid/alcohol molar ratio at 2.5. A set of optimal conditions for the ester synthesis in a fluidized bed reactor system has also been determined, specifically, flow rate at 10 mL min^{-1}, temperature at 35 °C, water concentration at 2% (v/v), substrate concentration at 0.1 M and acid/alcohol ratio at 2.0. Implementation of the optimized parameters in a batch system and in a fluidized bed reactor enabled production of target ester with high molar conversion, at > 99.9% for 48 h in the batch process, and 78.9% for 10 h in fluidized bed reactor. Although when assayed at their optimal conditions, lower molar conversion was achieved in the fluidized bed reactor system compared to the batch system, the volumetric productivity in fluidized bed reactor was more than five fold higher than that obtained in the batch system.

1. Introduction

Candida rugosa lipase (CRL) is a versatile, robust, frequently used lipase since it can be easily produced in lyophilized form and efficiently prepared in large amounts. Reports have been made describing successful CRL catalyzed synthesis of terpene esters [1–3]. In industrial scale, aromatic terpene esters are currently synthesized in a nonspecific chemical process obtaining low yields and poor quality of the product [4,5]. Also, chemical processes demand high costs for additional separation and purification steps [6]. Besides higher product yields, mild operating conditions, synthesis of products that do not need further purification, enzymatic reaction delivers "natural product", in terms of its origin, which is of a high importance, especially in the food industry [7].

However, industrial-scale synthesis using soluble enzymes is economically unacceptable, since these enzymes lack reusability as well as possibility of continuous type synthesis due to their low stability and complex separation techniques [8]. One way to address these issues is enzyme immobilization.

Covalent immobilization provides formation of a very stable catalyst via multipoint covalent attachment by maintaining enzyme's active conformation and reducing denaturing effects of environmental factors [9]. Among materials used for covalent immobilization, Sepabeds® EC series show physical and chemical stability, high protein binding capacity, low swelling tendency in

Abbreviations: CRL, *Candida rugosa* lipase; FBR, fluidized bed reactor; CCRD, central composite rotatable design.

* Corresponding author at: Laboratory of Molecular Biotechnology, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, 464-8601 Nagoya, Japan. Tel.: +81 90 9180 1389.
E-mail address: damnjanovic.jasmina@b.mbox.nagoya-u.ac.jp (J.J. Damnjanović).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.11.009

high molar solutions and resistance to the microbial degradation, making them suitable for application in industrial bioprocesses [10–13]. Specifically, Sepabeads® EC-EP particles are shown to efficiently stabilize penicillin G acylase providing hundreds-fold more stable catalyst compared to the one attached to Eupergit® C [14]. Hilterhaus et al. successfully immobilized three industrially important enzymes, endoglucanase, benzoylformate decarboxylase from *Pseudomonas putida* and lipase from *Candida antarctica* on Sepabeads® EC-EP, EC-EA and EC-BU, producing stable and active enzymes suitable for industrial application [15].

The selection of a proper reactor configuration is another important aspect in designing industrial enzymatic synthesis. Fluidized bed reactors (FBRs) are widely and successfully used in many industrial processes, like aerobic fermentation processes, catalytic reactions and biological waste-water treatment, due to continuous operational mode as well as for improved heat and mass transfer [16]. However, information on their application for ester synthesis is currently rather limited in the literature. The majority of studies concerning ester synthesis focused on batch systems and packed bed reactors [17], while ester synthesis in FBRs remains yet unexplored.

In studies conducted so far, geranyl butyrate was produced in yields between 85 and 99.9% using free or immobilized lipases [5,18–20]. Recently, it was shown that CRL immobilized on Sepabeads® EC-EP can be employed as a robust biocatalyst in esterification of geraniol with butyric acid in a low aqueous system [21]. However, these studies have been generally performed in the flasks with magnetic stirring or in the vials immersed in an orbital shaker. Analysis of the effect of reactor configuration and hydrodynamic conditions on the reaction rate and enzyme stability was not included in these contributions. To the best of our knowledge, this report presents the first study of the parameters affecting the synthesis of geranyl butyrate with the immobilized lipase in FBR system.

Previous research of Saponjic et al. regarded application of CRL immobilized on Sepabeads® EC-EP for amyl caprylate synthesis in FBR [22]. The study revealed that both batch and continuous synthesis of amyl caprylate can be accomplished in a high yield. Continuous esterification had slightly improved kinetics since 90.2% yield was achieved within 14 h compared to the batch synthesis where almost complete conversion was observed within 24 h [22]. As a further research, we aimed to prove robustness and versatility of immobilized CRL and efficiency of the reaction optimization pathway by employing them for geranyl butyrate synthesis. Using a different reaction model which includes acid substrate of three fold higher polarity and synthesis of a higher polarity ester would pose additional limitations to the ongoing esterification, since lipase activity is known to be affected by changes in substrate partitioning between organic phase and water layer surrounding the enzyme [23,24].

Focus was set on designing an economical fluidized-bed-immobilized-enzyme system for geranyl butyrate synthesis employing an efficient and inexpensive commercial support and immobilization method. In the first part of the study, characteristics of CRL covalently immobilized on Sepabeads® EC-EP have been compared to those obtained with other two commercial supports. Some critical properties of immobilized enzymes such as protein loading, activity, specific activity and immobilization yield were considered. Selected highly active immobilized lipase was used for statistical assessment of relevant process conditions in the batch system and in further study as a biocatalyst and constituent of the FBR. The bioreactor hydrodynamic characteristics and the reaction conditions have been investigated in order to improve the process performance.

2. Experimental

2.1. Materials

Nonspecific lipase AY, Type VII, L 1754, in powdered form from *C. rugosa*, lipase substrate (stabilized olive oil emulsion), Triton X-100, bovine serum albumin (BSA) and terpene alcohol, geraniol (98.0%) were purchased from Sigma–Aldrich Inc. St. Louis, MO, USA. Butyric acid from Merck, Darmstadt, Germany was used as acid substrate while isooctane (HPLC grade) from Arcos Organics, New Jersey, USA, was used as organic solvent. Sepabeads® EC-EP and Sepabeads® EC-HA were donated by Resindion S.R.L., Mitsubishi Chemical Corporation, Milan, Italy. Purolite® A-109 was purchased from Purolite International Ltd. (Llantrisant, United Kingdom). All other chemicals were purchased from Merck, Darmstadt, Germany.

2.2. Immobilization method

Immobilization of CRL on epoxy-Sepabeads® (EC-EP): Immobilization of CRL on epoxy-activated support, Sepabeads® EC-EP, was achieved by direct lipase coupling to the polymer via epoxy groups in the presence of very high salt concentration (standard protocol recommended by Resindion S.R.L., Mitsubishi Chemical Corporation). Immobilization was performed in 70 mL of 1.25 M potassium phosphate buffer, pH = 8.0 at 22.5 °C with orbital mixing for 48 h. Immobilized enzyme was then washed with water and buffer and kept at 4 °C followed by drying *in vacuo* for 48 h, prior to the reaction.

Immobilization of CRL on Sepabeads® EC-HA and Purolite® A-109: The immobilization procedure on amino-supports consisted of two steps: (1) oxidation of the lipase by sodium periodate; and (2) coupling of the oxidized enzyme to the amino-supports.

Therefore, first step was the lipase oxidation by sodium periodate following the methodology previously described [25]. According to this protocol, 1 mg mL^{-1} of crude enzyme solutions, corresponding to 0.25 mg mL^{-1} of pure protein determined by the Bradford method [26] were incubated in 5 mM solution of sodium periodate in sodium acetate buffer, pH = 5.0, for 6 h in the dark at 4 °C. The reaction mixture was stirred occasionally and the reaction was quenched with 10 mM ethylene glycol for 30 min. To remove by-products, the oxidized lipase solution was then dialyzed against 50 mM sodium acetate buffer, pH = 5.0, for 18 h.

Polymers with amino groups (1.0 g) were incubated with 35 mL of oxidized lipase solution in sodium acetate buffer at pH = 5.0 and 4 °C for 48 h. Afterwards, obtained biocatalysts were washed with water and sodium phosphate buffer, pH = 7.0 and stored in the same buffer at 4 °C until use. In previous research, it was reported that Schiff's bases formed between oxidized enzyme and aminated supports proved as very stable, therefore, additional reduction step was not applied to the immobilized preparations [25,27]. The lipase oxidation was checked using FT-IR spectrometry as described bellow.

2.3. Immobilization parameters

Protein loading, P_g defined as the amount of the pure protein coupled to the supports (mg of protein/g of the supports) is calculated as a difference between protein amount (mg) added in the immobilization process and the protein amount (mg) found in the filtrate and wash-through after immobilization. The protein loading efficiency, Y_P (%) was calculated according to Eq. (1):

$$Y_P\,(\%) = \frac{C_0V_0 - (C_fV_f + C_wV_w)}{C_0V_0} \quad (1)$$

where C_0 is the protein concentration of the initial immobilization solution (mg mL^{-1}); V_0 its volume (mL); C_f the protein concentration in the filtrate (mg mL^{-1}); V_f the filtrate volume (mL); C_w the

protein concentration in washing solution (mg mL^{-1}) and V_w its volume (mL).

Specific activity of the immobilized enzyme, SA_{IE} was defined as the hydrolytic activity of the immobilized protein, A (IU g^{-1}) divided by the protein loading and expressed as IU mg^{-1} protein. The activity immobilization yield, Y_A (%) was calculated by dividing the specific activity of the immobilized lipase by specific activity of the free lipase.

2.4. Desorption study

100 mg of the immobilized enzyme samples were suspended in 10 mL of 10 mM sodium phosphate buffer at pH = 7.0 containing 0.5% (v/v) Triton X-100 or 0.50–1.25 M NaCl and incubated at room temperature in orbital shaker (150 rpm). Samples were taken from the supernatant after 60 min and the amount of lipase was determined as described above. Desorption study from different supports was performed for two values of protein loading in each support. Desorption yield, Y_D (%) was defined as the amount of desorbed protein (calculated per 1 g of biocatalysts) divided by the protein loading expressed in percentage [25].

2.5. Geranyl butyrate synthesis in a batch system

Syntheses were done in a screw capped flasks (100 mL) containing precalculated proportions and concentrations of geraniol, butyric acid and water in isooctane. 500 mg of immobilized lipase was added to the preheated reaction mixture. Flasks were incubated at variable temperatures in orbital shaker (150 rpm) for 48 h. The reaction time was selected based on preliminary results of the batch runs showing that the reaction reached the equilibrium after 48 h. Substrate concentration was fixed at 0.25 M. Other reaction parameters varied according to experimental design (Supplemental Table 1). Blank experiments were done under identical conditions.

Five-level-five-factor central composite rotatable design (CCRD) was used which included 32 experiments consisting of 16 factorial, 10 axial, and 6 central points [28]. Experiments were run in random order to avoid bias. Obtained data were fitted to a second-order polynomial equation:

$$Y = \beta_{k0} + \sum_{i=1}^{5}\beta_{ki}X_i + \sum_{i=1}^{5}\beta_{kii}X_i^2 + \sum_{i=1}^{4}\sum_{j=i+1}^{5}\beta_{kij}X_iX_j \quad (2)$$

where Y is the response (molar conversion in %), β_{k0}, β_{ki}, β_{kii} and β_{kij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables. The values and statistical significances of the response function coefficients were calculated using the method of least squares in the MATLAB software (version 6.5, Release 13, The MathWorks, Juc, Matick, MA, USA). Contour plots were obtained by using fitted model. Ester synthesis was optimized and obtained response equation enabled prediction of molar conversion from known values of the five main factors.

2.6. Analysis

2.6.1. Enzyme concentration assay

Enzyme concentration in samples was determined spectrophotometrically at 215 nm and 225 nm using CRL as a standard [29]. Calibration plots for enzyme were generated for both wave lengths by plotting averaged absorbance against enzyme concentration and presented by their linear regression formulas. Line slopes (l_s) are used in the calculation of enzyme mass (m_e) in samples. Calculation is based on the following equation:

$$m_e = \frac{A}{l_s}V_s \quad (3)$$

where V_s stands for sample volume, and A stands for the absorbance. The final value of enzyme concentration is the average of two values obtained on two wave lengths. Modified standard curves in the presence of Triton X-100 or NaCl were used for the desorption experiments with the detergent (0.5%, v/v), or NaCl (0.50–1.25 M).

2.6.2. Fourier transform infrared spectroscopy (FT-IR) analysis

Samples of free lipase and oxidized CRL were subjected to FT-IR analysis and the spectra were obtained using a Bomem MB 100 FT-IR Spectrophotometer. The amount of 10 wt% of the sample was mixed and ground with 100 wt% of potassium bromide and then compressed into a pellet under a pressure of 11 t, for about a minute, using Graseby Specac Model: 15.011. The Spectra were recorded in the 400–4000 cm^{-1} wave number range.

2.6.3. Ester analysis

Ester synthesis was monitored by determination of the residual acid content by titration against sodium hydroxide using phenolphthalein as an indicator and methanol as a quenching agent. After 48 h reaction time, 0.5% phenolphthalein in methanol was added in the reaction mixture followed by titration with 0.1 M NaOH solution to determine the amount of residual butyric acid [30]. The reported molar conversion was calculated as

$$Y\,(\%) = \frac{B_0 - A_0}{B_0} \times 100 \quad (4)$$

where Y stands for molar conversion (%), B_0 (mL) and A_0 (mL) stand for volume of 0.1 M NaOH spent to titrate reaction mixture before and after synthesis, respectively.

The accuracy of this method was tested by determination of ester concentration on Varian 3400 gas chromatograph as described previously [22]. The ester yield (%) was defined as the amount of geranyl butyrate produced from initial substrate in defect (mol ester/mol initial substrate in defect × 100). The ester yield (%) and molar conversion (%) were found to be in good agreement.

2.7. Lipase activity assay

Activities of immobilized enzymes were determined in the reaction of olive oil hydrolysis by Sigma method as described previously [25] and expressed in IU. This activity assay was carried out with reaction mixtures containing 3 mL of Sigma lipase substrate, 1 mL of Trizma buffer, and 3.5 mL of distilled water. 1 IU is defined as the amount of enzyme required to produce 1 μmol of free fatty acid per minute under the assay conditions (37 °C, pH 7.7).

2.8. Geranyl butyrate synthesis in a fluidized bed reactor (FBR)

2.8.1. Hydrodynamic characteristic of the FBR system

Tracer response analysis was used to characterize and model the flow through the reactor, according to previously described methodology [31]. The system was disturbed by introducing a step input change of the tracer (black ink) concentration and system response was then monitored at the reactor output. Experimental setup is given in Fig. 1a. Effluent of the reactor was sampled at timed intervals, and tracer concentration was determined spectrophotometrically at 400 nm. The experiments were performed in duplicates. Obtained tracer concentration profiles were normalized

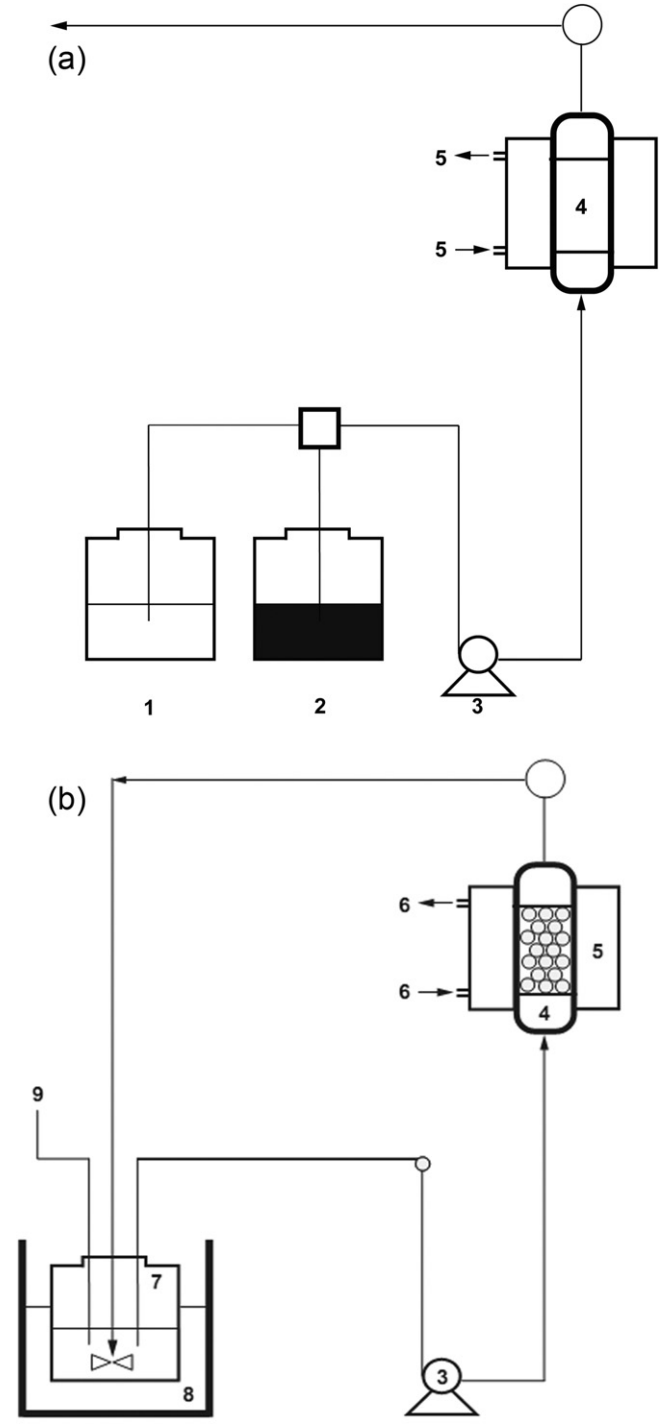

Fig. 1. Experimental setup for flow pattern analysis (a) and for lipase catalyzed esterification (b): (1) distilled water reservoir; (2) solution of labeled substance (black ink); (3) peristaltic pump; (4) fluidized bed reactor; (5) reactor water jacket; (6) cooling/heating water; (7) substrate reservoir; (8) reactor temperature control; (9) sampling.

with respect to the inlet tracer concentration, and resulting normalized C/C_0 functions were plotted against time to give experimental F curve. Experimental E curves (exit age distribution functions) were then obtained by derivation, as follows:

$$E = \frac{dF}{dt} \tag{5}$$

In this study, Tanks-in-series model, presented by Eqs. (6) and (7) was used to characterize non-ideal flow within the reactor:

$$F = 1 - e^{(-N(t/\bar{t}))}\left[1 + N\frac{t}{\bar{t}} + \frac{(N(t/\bar{t}))^2}{2!} + \cdots + \frac{(N(t/\bar{t}))^{N-1}}{(N-1)!}\right] \tag{6}$$

$$E = \frac{N^N}{\bar{t}^{N-1}}\frac{t^{N-1}}{(N-1)!}e^{-(Nt/\bar{t})} \tag{7}$$

where N is the number of stirred tanks, $\bar{t}$ is mean residence time in the system, and t is time of sampling.

2.8.2. Enzymatic reactor setup

Fluidized bed reactor (FBR) was a cylindrical glass column (10 mm i.d. × 136 mm length) packed with 2.0 g of immobilized enzyme (3.75 cm g^{-1}, density 1.13 g mL^{-1}) and equipped with a water jacket for temperature control. The reaction mixture (80 mL, containing 2% (v/v) water) was fed upwards through the column using a peristaltic pump (Behrotest® Labor-Schlauchpumpe PLP 66, Dusseldorf) at different flow rates. Prior to entry into the reactor zone, the substrate mixture was preheated to a predetermined temperature by thermostatic bath, while the identical temperature in the reactor zone was maintained constant by circulating preheated water through the reactor jacket. Reaction was carried out by recirculation of the reaction mixture through the FBR. Sampling was conducted at specified times in the substrate reservoir. Experimental set-up is shown in Fig. 1b.

For estimation of optimal flow rate and substrate concentration, parameter P or volumetric productivity, was used. It describes efficiency and feasibility of the process regarding the concentration of the product formed and a reaction time. Calculation is presented by the following equation:

$$P = \frac{[E]}{t} \tag{8}$$

where [E] stands for ester concentration and t stands for the reaction time needed for production of [E].

3. Results and discussion

3.1. Enzyme immobilization study

As reported in our previous studies, CRL was successfully immobilized on epoxy activated supports [22,25]. This time, CRL was covalently immobilized on epoxy-activated Sepabeads® EC-EP, and two amino-supports, Sepabeads® EC-HA and Purolite® A-109, in order to compare the immobilization performances of obtained biocatalysts. The selected supports used in this work are relatively inexpensive and readily available commercially. The coupling of enzyme to epoxy-activated carrier, Sepabeads® EC-EP, involved reactions between enzyme's available amino or sulfhydryl groups and active epoxy groups of the polymer in the presence of high salt concentrations. In fact, the reactivity of epoxy groups towards nucleophilic groups of the enzyme is very low under mild experimental conditions (neutral pH, low ionic strength) [32]. On the other hand, successful immobilization could be achieved by using hydrophobic epoxy supports such as Sepabeads® EC-EP and performing the enzyme coupling in the presence of high salt concentrations (e.g. in 1.25 M potassium phosphate buffer). The immobilization is reported to follow a very interesting two step mechanism: first, a rapid mild physical adsorption between the enzyme and the support occurs followed by covalent reaction between adsorbed protein and epoxy groups of the support [32,33]. Immobilization of CRL on amino-supports, Sepabeads® EC-HA and Purolite® A-109, could be achieved via lipase carbohydrate moiety previously modified by periodate oxidation. The exact way of enzyme attachment is not well known, but covalent coupling

between supports' amino groups and enzyme carbohydrate moiety was expected to be the most probable mechanism. The idea behind the method was the formation of a Schiff's base linkage between carbonyl groups of the modified lipase and free amino groups on the supports [34].

Immobilization data for three tested supports and covalent techniques are summarized in Table 1, along with desorption data for each immobilization system. Although the enzyme binding capacity of supports often largely depends on the surface density of functional groups and the nature of the coupling reactions, the protein loading for all three tested supports was similar and quite satisfactory. Protein loading increased with increasing protein concentration in the coupling solution reaching maximum values of 19.2, 16.1 and 20.0 mg g^{-1} dry supports for Sepabeads® EC-EP, Sepabeads® EC-HA and Purolite® A-109, respectively. The slight decrease in protein loading on Sepabeads® EC-EP at higher lipase concentration than 0.75 mg mL^{-1} may be due to the fact that this support has a finite number of binding sites and the amount of lipase for immobilization was overloading in this instance. Reasons affecting protein loading to decrease at higher protein concentrations could also include effects of steric hindrances and diffusional limitations, typical for concentrated enzyme solutions. Specific activity of immobilized enzyme appeared to follow the opposite trend, with maximal value reached at 1.98 IU mg^{-1} for the Sepabeads® EC-EP sample having the lowest amount of protein loading (6.9 mg g^{-1}). Influences of diffusional limitations and steric effects are notable in values of specific activities of CRL immobilized on all three supports. As protein loading increased, these effects were more pronounced and likely limited the amount of substrate reaching active site, thus causing decrease in specific activity.

Successful immobilization on Sepabeads® EC-EP is probably the consequence of high affinity of epoxy groups present on the polymer surface towards nucleophilic groups of the enzyme, as well as of simple, mild and effective immobilization procedure [13].

Immobilization outcome can also be influenced by type and origin of the enzyme. In previous studies, Kunamneni et al. immobilized recombinant laccase expressed in *Aspergillus* on Sepabeads® EC-EP3 using enzyme/support ratio 100 mg g^{-1} and reported enzyme loading efficiency to be 32.6% [11]. However, Hilterhaus et al. immobilized *C. antarctica* lipase on Sepabeads® EC-EP using enzyme/support ratio of 138.7 mg g^{-1} and immobilization process succeeded with enzyme loading efficiency of 85% [15]. Differences in enzyme loading efficiencies might be affected by the content and availability of the groups reacting with the support in different enzymes. Also, different degree of enzyme glycosilation may influence enzyme-support interactions [11].

In order to obtain more information concerning the nature of the lipase attachment to amino-supports, immobilized preparations were incubated in 10 mM sodium phosphate buffer, pH 7 containing 0.5% (v/v) Triton X-100 or 0.5–1.25 M NaCl. The oxidation of CRL was also confirmed by FT-IR analysis (supplemental Fig. 1). The FT-IR spectrum of the lipase exhibited characteristic bands of 1657.74 cm^{-1} (amide I), 1538.01 cm^{-1} (amide II) while the broad absorption bands at 3400 cm^{-1} and 2934.52 cm^{-1} were apparently caused by the symmetrical amine N–H vibration. The absorption bands at 1410.92 cm^{-1} (–CH symmetric bending vibration in –CHOH–), 1122.38 cm^{-1} (–CO stretching vibration in –CH–O–CH) and 889.442 cm^{-1} (–CN stretching vibration) could be characteristic for enzyme's carbohydrate moiety. It is evident that some major changes occurred due to CRL oxidation by sodium periodate. The band 1122.38 cm^{-1} shifted to 1127.59 cm^{-1} showing decreased intensity, revealing major changes in primary hydroxyl group. In addition, bands 1685.39 cm^{-1} (C=O stretch vibration) appeared indicating the appearance of free carbonyl groups. Two bands in the range 2900–3010 cm^{-1} could be also related to free carbonyl group (=C–H stretch) but they were moved to higher values since other functional groups covered oxidized lipase carbonyl bands detectable in that range. However, it is clear that CRL and oxidized CRL showed differences in the FT-IR spectra, more specifically in the position and intensities of the primary hydroxyl group and carbonyl group related bands, characterizing differences in the oxidation states.

The nature of the linkage between enzyme and amino-supports was further highlighted using desorption study and the results are presented in Table 1. It appeared that the lipase was tightly bound to Sepabeads® EC-EP and was not removed by treatment with Triton X-100 or NaCl (desorption yield was lower than 5%). This provided evidence that most of the enzyme has been bound covalently on this support rather than by hydrophobic or ionic interactions.

Lower than 5 and 10% of bound lipase was desorbed from Sepabeads® EC-EP, and from other two types of amino-supports, respectively, after incubation with 0.5% Triton X-100 for 60 min. However, non-covalently bound lipase was easily lost after treatment with salt solutions of high ionic strength, revealing that the non-covalent binding was primary ionic. Overall, the results emphasized that non-covalent binding of CRL occurred in addition to covalent coupling of enzyme to amino-supports indicating the importance of the removal of this non-covalently bound lipase after covalent coupling.

In conclusion, among three supports and coupling chemistry tested, lipase immobilized on Sepabeads® EC-EP is the immobilization process of choice. This support is a unique material for enzyme immobilization and the process of immobilization is simple, effective and very economical. The stable binding achieved in this case is a useful characteristic for practical applications of biocatalysts that would allow enzyme recycling with minimal leaching.

Since immobilized enzyme obtained using protein concentration 0.75 mg mL^{-1} showed highest activity and protein loading, still retaining high specific activity, it was selected for geranyl butyrate synthesis optimization.

3.2. Optimization of geranyl butyrate synthesis in the batch system by statistical approach

Reaction parameters were optimized using response surface methodology based on 5-level-5-factor central composite rotatable design (CCRD) requiring 32 experiments. The variables studied in the batch process of geranyl butyrate synthesis were: water concentration (1.0–5.0%, v/v), temperature (25–45 °C), enzyme concentration during immobilization, indicating enzyme loading (2.0–4.0 g L^{-1}), initial substrate molar ratio (acid/alcohol: from 1:2 to 5:2) and time of molecular sieves introduction for removal of excess water (0–32 h). The range and levels of the variables were chosen based on our previous study [21] where temperature was found to have a significant negative influence on ester yield, masking the effects of all other tested variables. Thus, in the new experimental design used in this paper, the range for temperature was decreased (25–45 °C) while for other variables remained the same. A higher temperature would not increase molar conversion, but could cause denaturation of the immobilized lipase.

The experimental data obtained for 32 experiments of the statistical design are presented in supplemental Table 2. Significances of the factors were estimated based on the *t*-test and *p*-value statistical parameters. The effects of temperature, water concentration and temperature-substrate molar ratio interaction seem to be significant ($p < 0.05$), as well as three quadratic coefficients ($p < 0.05$). The fit of the model is verified by Fisher test (F) for 5% level of significance. Since the experimental F value (−3.31) was lower compared to theoretical (4.58), it was concluded that the model is adequate for description of this reaction system. After the introduction of

Table 1
Comparison of the methods applied for covalent immobilization of CRL on three different supports using two methods.

Support	C_0 (mg mL^{-1})	P_g (mg g^{-1})	Y_P (%)	A (IU g^{-1})	SA_{IE} (IU mg^{-1})	Y_A (%)	Y_D (%), for 0.5% Triton X-100	Y_D (%), for 0.50 M NaCl	Y_D (%), for 1.00 M NaCl	Y_D (%), for 1.25 M NaCl
Sepabeads® EC-EP	0.25	6.9	39.3	13.7	1.98	90.6	3.2	Minor	Minor	Minor
	0.50	11.4	32.6	19.4	1.70	77.35				
	0.75	19.2	36.5	26.2	1.36	62.1				
	0.90	17.5	28.6	17.2	0.98	44.6	4.3	Minor	Minor	Minor
Sepabeads® EC-HA	0.25	1.5	17.4	4.94	3.29	–				
	0.50	7.1	40.4	4.78	0.67	76.8	9.3	12.4	13.5	24.2
	0.75	9.4	36.0	5.76	0.61	69.3				
	1.25	16.0	36.6	5.76	0.36	40.8	8.7	12.0	13.0	24.0
	1.40	16.1	33.4	5.58	0.35	39.4				
Purolite® A-109	0.25	2.6	29.7	3.9	1.50	–				
	0.50	6.0	34.4	3.0	0.50	56.6	9.9	10.5	15.4	33.7
	0.75	10.1	38.4	1.7	0.17	19.2				
	1.25	16.2	37.0	1.3	0.08	9.1	9.3	12.5	17.8	30.2
	1.50	20.0	38.0	1.1	0.05	6.3				

C_0, the protein concentration of the initial immobilization solution (mg mL^{-1}); P_g, the protein loading (mg protein/g of the support); Y_P (%), the protein loading efficiency; A, the hydrolytic activity of the immobilized lipase (IU g^{-1}); SA_{IE}, the specific activity of the immobilized enzyme lipase (IU mg^{-1}); Y_A (%), the activity immobilization yield; Y_D (%), the desorption yield.

calculated regression coefficients into second-order polynomial equation, following model was obtained:

$$Y = 86.28 + 6.05X_1 - 15.8X_2 - 4.64X_1^2 - 4.84X_2^2 - 10.6X_2X_4 + 4.34X_3^2 \quad (9)$$

Contour plots obtained using the model are given in Fig. 2.

Temperature appears to have the most significant effect on ester synthesis, although there are other factors, such as water concentration, substrate molar ratio, enzyme content, and temperature–substrate molar ratio interaction which should also be considered.

Significant negative interactive effect of temperature and substrate molar ratio, indicate that high molar conversion could be expected at low temperatures and high acid/alcohol molar ratios (Fig. 2a). Molar conversion increased as reaction temperature decreased, reaching maximum at 25–30 °C. Several previous researches also indicated lower reaction temperatures as favorable for production of butyric acid esters catalyzed by lipases. Shieh et al. reported optimal temperature of 35 °C for geranyl butyrate synthesis in transesterification reaction catalyzed with CRL [20]. Also, Hari Krishna et al. reported optimal temperature of 30 °C for isoamyl butyrate synthesis in esterification catalyzed by Lipozyme IM-20 [35]. Further on, it is apparent form Fig. 2a that the increase in acid/alcohol molar ratio had a positive influence on ester synthesis. Similar conclusions were made by Pereira et al., in the study of butyl butyrate synthesis catalyzed by CRL immobilized on porous chitosan grains. Optimal initial molar ratio of butanol/butyric acid was 1:1.5 [36]. In esterification where butyric acid is present as acyl donor, usually moderate acid excess favors synthesis from the aspect of reaction equilibrium, thus having a beneficial effect on molar conversion. Furthermore, excess acid in the esterification is often necessary to prevent competition of two acyl donors, acid and ester formed during the reaction [37]. Also, CRL shows affinity towards small chain fatty acids, but high acid concentrations eventually inhibit lipase activity. Polar butyric acid molecules tend to concentrate in the water layer around the enzyme changing its pH, and thus causing enzyme inactivation. Excess geraniol proved to have an adverse effect on molar conversion, as also found by Chatterjee et al. in the research regarding production of geranyl capronate, geranyl caprylate, geranyl caprate and geranyl laurate catalyzed by lipase from *R. miehei* [38].

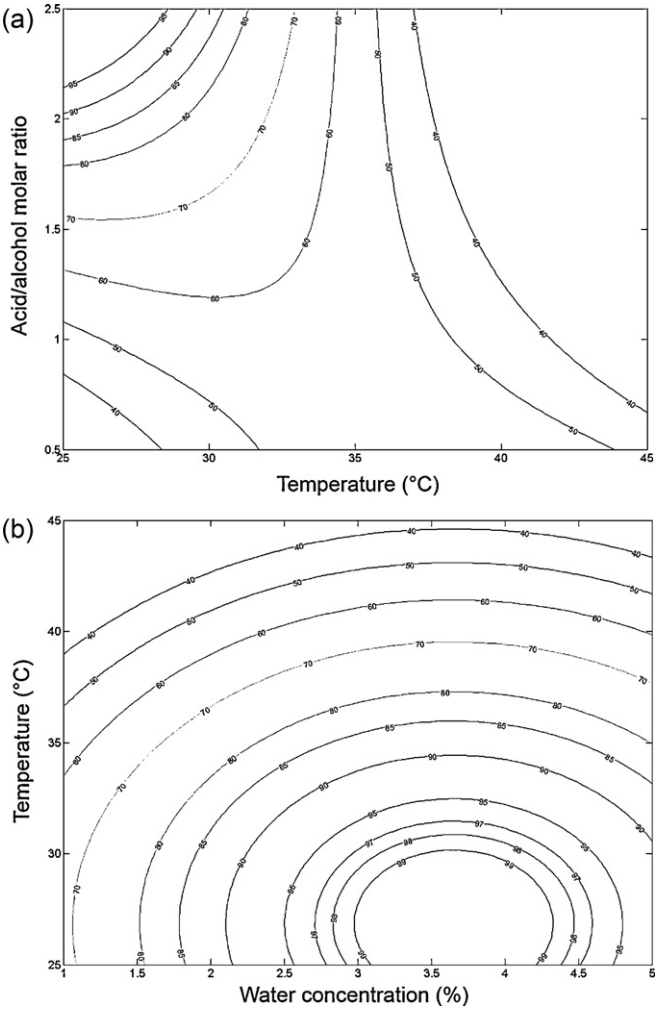

Fig. 2. Contour plots for molar conversion as a function of temperature and initial substrate molar ratio (a) and for the molar conversion as a function of temperature and water concentration (b). Reaction conditions: reaction time 48 h, limiting substrate concentration 0.25 M.

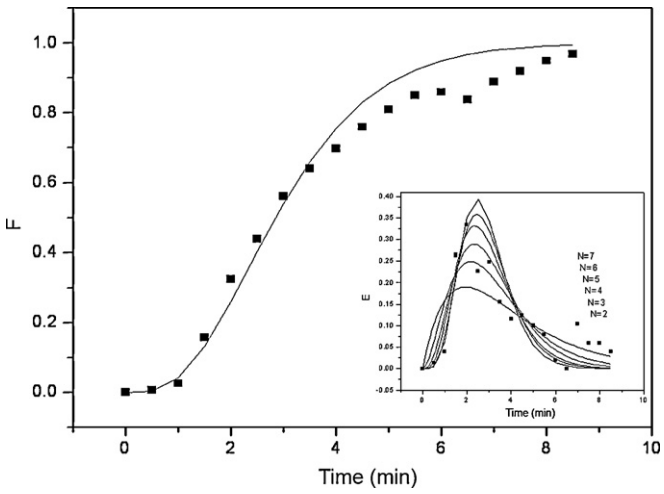

Fig. 3. Application of Tanks-in-series model on experimental *F* and *E* curves. Flow rate was set to 3.70 mL min^{-1}. Simbols stand for experimental data while lines stand for model predictions. In case of *E* curve, best fitting between experimental and predicted data stand for $N = 4$ (according to criteria of lowest STD).

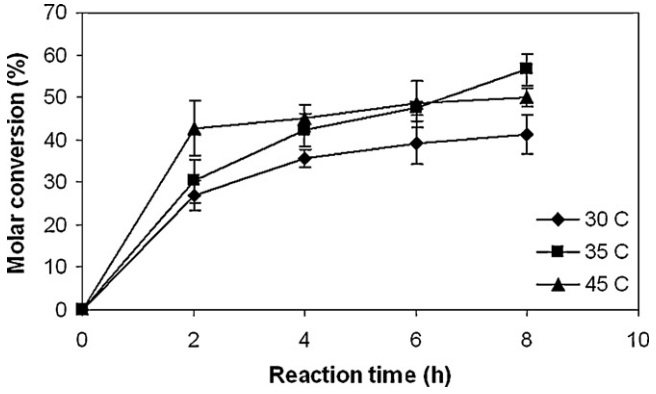

Fig. 4. Effect of temperature on geranyl butyrate synthesis in a FBR. Reaction conditions: substrate concentration 0.1 M, substrate molar ratio 1:1, flow rate 3.70 mL min^{-1}, water concentration 2% (v/v) and enzyme loading 76.7 mg g^{-1}.

The effect of temperature and water concentration is presented in Fig. 2b.

Ester production is represented by a contour plot with a maximum at a low temperature of around 25–30 °C for a water concentration of 3.6% (v/v). Low water content is necessary to retain enzyme catalytic activity by keeping the integrity of the molecule in active conformation. However, exceeding the critical amount of water, the thickness of water layer around the enzyme is increasing causing increase in enzyme flexibility and eventually denaturation [39]. Excess water would also favor the reverse hydrolytic reaction.

Data obtained by surface response analysis were used as reference to further optimize geranyl butyrate synthesis in a FBR.

3.3. Esterification in a FBR

The majority of studies concerning ester synthesis focused on stirred tank and packed bed reactors. Fluidized-bed reactors could present a number of advantages such as improved heat and mass transfer, optimal liquid mixing and absence of plugging. Also, pressure drop across the bed appears to be much lower than that in the corresponding packed bed reactor. Thus, this study aimed to further test fluidization properties of biocatalyst beads (CRL-Sepabeads® EC-EP system, 150–300 μm) and their ability to catalyze geranyl butyrate synthesis in a FBR.

3.3.1. Hydrodynamic characteristic of the FBR system

For continuous reactors, residence time distribution is an essential tool to assess fluid velocity profile, which can be used to describe flow regime in the reactor. Since flow regime affects reactor performance, its description may enable better process control. Flow pattern studies conducted previously by Saponjic et al. revealed nearly plug flow for the FBR studies of amyl caprylate synthesis, using immobilized CRL in the similar FBR system [22]. Due to differences in scale-up strategy, size of particle layer used to fill reactor vessel for geranyl butyrate synthesis was around 40% higher compared to the amount used for amyl caprylate synthesis. Thus, flow pattern analysis was conducted once more.

Using Tanks-in-series model (Eqs. (6) and (7)), flow pattern in this system can be described by the cascade of four ideal continuous flow stirred tanks (Fig. 3). This number of tanks describes the flow through tubular fluidized bed reactor as nearly plug flow with moderately low deviations, due to a certain degree of axial dispersion. Mean residence time, determined by the model, for $N = 4$, t_{mod}, is identical with residence time calculated using reactor volume and flow rate, t_{emp}, and was 3.1 min, indicating absence of dead regions within the reactor volume.

3.3.2. Optimization of esterification conditions in FBR

3.3.2.1. Effects of water content and temperature. Preliminary study revealed increase in molar conversion as the initial water concentration in the reaction mixture rose from 0 to 2% (v/v) (data not shown). However, a sharp decrease in molar conversion was observed if water content further increased from 2 to 6% (v/v). Also, the effect of temperature (30, 35 and 45 °C) on geranyl butyrate production in FBR was investigated using fixed initial acid/alcohol molar ratio at 1:1, substrate concentration at 0.1 M, and flow rate at 3.70 mL min^{-1}. Time course of molar conversion at different temperatures is shown in Fig. 4. Although the highest conversion was achieved at 35 °C, initial reaction rate was higher at 45 °C, revealing that the biocatalyst was inactivated when subjected to higher temperature for longer period under non-aqueous conditions. Therefore, control of water and temperature at optimal values (2%, v/v, 35 °C) in the system is important to optimize esterification in FBR.

3.3.2.2. Effect of substrate concentration. Enzymatic reactions, especially esterifications, are often challenged by high substrate concentrations due to the inhibitory effects of acid, alcohol or both of the substrates. This influence was not part of the initial study in the batch system, but may have a strong impact on the reaction course. Therefore, we compared the influence of different substrate concentrations, namely, 0.05 M, 0.1 M and 0.25 M on molar conversion for stoichiometric amounts of reactants. Results are given in Fig. 5a.

Increase in substrate concentration caused a decrease of molar conversion. As the substrate concentration increases, inhibitory effects of the substrates might become more pronounced. Observed effect could also be partially attributed to intensified water formation in the presence of higher substrate concentrations. However, efficient water removal in FBR still remains to be established. The highest molar conversion (67.3%) was obtained for the lowest substrate concentration 0.05 M, but the volumetric productivity (P defined by Eq. (8)) was 38% lower than the corresponding value obtained for the highest substrate concentration 0.25 M. Thus, to make a correct comparison, the volumetric productivity has been calculated for each substrate concentration. The highest P value (7.1 mmol L^{-1} h^{-1}) is obtained for the system with substrate concentration 0.1 M, which was selected as optimal. Lowest P value (4.2 mmol L^{-1} h^{-1}) is calculated for initial substrate concentration 0.05 M, since in this case substrate concentration is unnecessarily

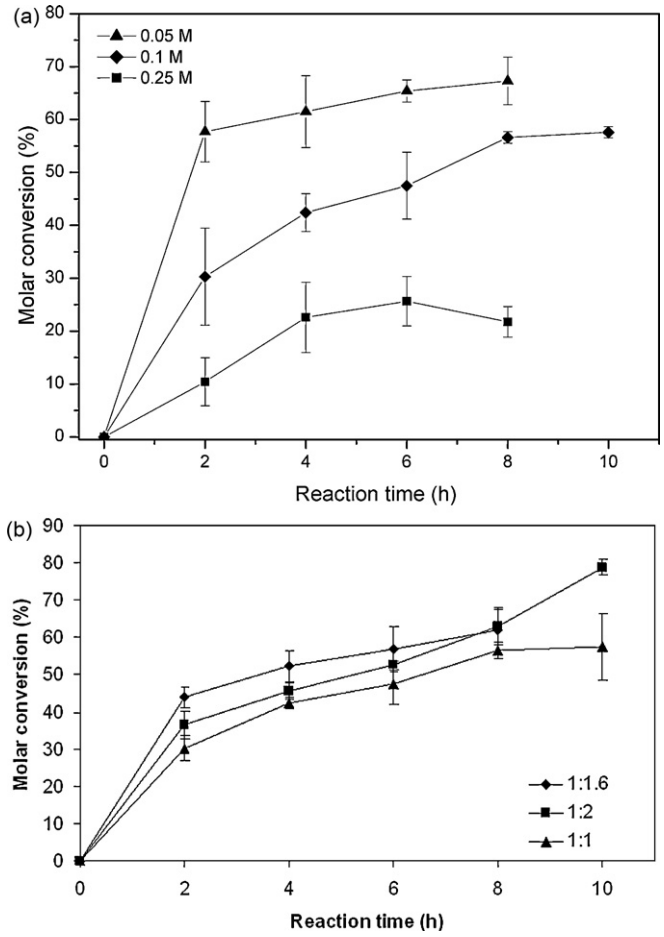

Fig. 5. Effect of substrate concentration (a) and initial substrate molar ratio (b) on geranyl butyrate synthesis in a FBR. Reaction conditions: substrate molar ratio 1:1, flow rate 3.70 mL min^{-1}, water concentration 2% (v/v), enzyme loading 76.7 mg g^{-1}, temperature 35 °C (a) and, limiting substrate concentration 0.1 M, flow rate 3.70 mL min^{-1}, water concentration 2% (v/v), enzyme loading 76.7 mg g^{-1} and temperature 35 °C (b).

low, enzyme/substrate ratio very high, indicating that amount of enzyme in the reactor was not yet used with its full catalytic capacity. Therefore, this system was characterized as economically unfeasible even though, from Fig. 5a, it produced highest molar conversion (%).

Effect of substrate molar ratio on esterification in FBR (Fig. 5b) followed the same trend as determined by statistical analysis for batch conditions, providing maximal molar conversion of 78.9% (P = 7.9 mmol L^{-1} h^{-1}) obtained within 10 h by introducing excess butyric acid.

3.3.2.3. Effect of flow rate. In continuous type reactors, flow rate proved to be significant for reactions catalyzed by immobilized enzymes, since it has reciprocal relationship with residence time. Usually, longer residence time enhances the reaction by enabling longer enzyme–substrate contact, which can enhance conversion.

In this work, we used three different flow rates (residence times): 2.67 mL min^{-1} (4.3 min), 3.70 mL min^{-1} (3.1 min) and 10.0 mL min^{-1} (1.1 min). Flow rate 2.67 mL min^{-1} is a minimal flow rate that fluidized layer of immobilized enzyme particles, while 10.0 mL min^{-1} is a maximal flow rate which caused maximal layer expansion without particle leakage. It was shown that biocatalyst beads can be used to create stable expanded beds at all flow rates. Determined relation between molar conversion and flow rates is given in Fig. 6.

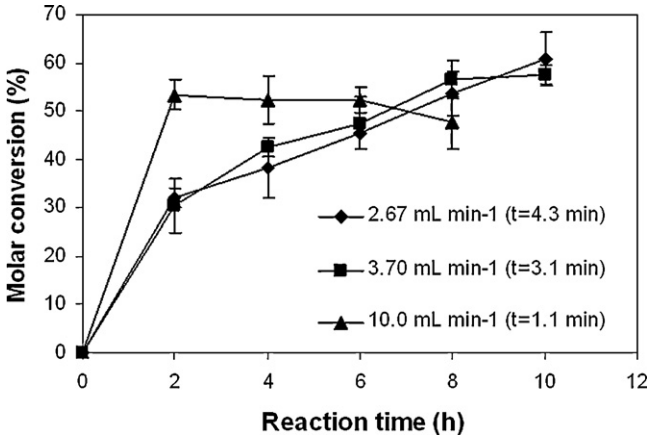

Fig. 6. Effect of flow rate on geranyl butyrate synthesis in a FBR. Reaction conditions: substrate molar ratio 1:1, substrate concentration 0.1 M, water concentration 2% (v/v), enzyme loading 76.7 mg g^{-1} and temperature 35 °C.

It seems that the flow rate strongly influenced ester production by immobilized CRL in FBR. According to P value, which was highest at flow rate 10.0 mL min^{-1} reaching the value of 26.7 mmol L^{-1} h^{-1}, this flow rate is selected as optimal. It appears that the esterification reaction in FBR was mass transfer limited and thus can be improved by increasing flow rate. At high flow rates, mass transfer is enhanced and diffusional limitations reduced. These reasons affect molar conversion and could be used to explain obtained results. However, one adverse effect was also observed, specifically, increased flow rate led to a reduction of contact time between the enzyme surface and substrates; the reaction was incomplete causing reduction in maximal molar conversion [22,40]. This effect seems to be predominant at lower flow rates. Thus, if flow rate is set to 2.67 mL min^{-1}, P value reaches only 6.1 mmol L^{-1} h^{-1}, while at flow rate 3.70 mL min^{-1}, P value is even less and reaches 5.8 mmol L^{-1} h^{-1}. Results obtained by other authors suggest lower flow rates and longer residence times as more productive due to prolonged contact between enzyme and substrates [40,41]

3.3.3. Operational stability of the immobilized CRL

Operational stability was analyzed in a semi-continuous manner, with the biocatalyst regeneration after each esterification cycle, as previously done in this research group [22]. Trend in relative molar conversions for eight successive cycles conducted in a batch system is given in Fig. 7.

During seven complete reaction cycles, immobilized lipase retained high level of stability reflected in high relative molar

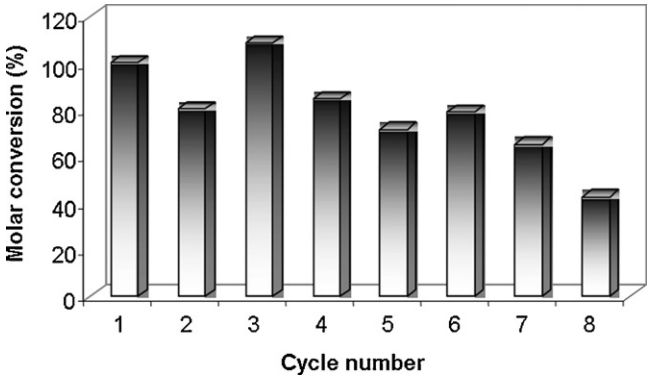

Fig. 7. Relative operational stability of lipase immobilized on Sepabeads® EC-EP. Molar conversion is presented as a relative value referred to initial reaction cycle. Reaction conditions: substrate concentration 0.25 M, substrate molar ratio acid/alcohol 2:1, enzyme loading 76.7 mg g^{-1} and temperature 30 °C.

conversion. By the end of the eighth cycle, approximately 50% decrease in molar conversion was observed indicating loss of operational stability (the operational enzyme half-life was 368 h). Operational stability achieved in the batch system corresponds to the results published previously using lipases in flavor ester synthesis. Claon et al. reported five and 10 cycles operational stability of the commercial enzyme preparations SP382 and SP435 (industrially immobilized lipase from *C. antarctica*) used in a batch geranyl acetate synthesis [5]. In a similar way, Yee et al. investigated operational stability of lipase from *Pseudomonas* sp. immobilized on Duolite and PVP in the reaction of citronellyl butyrate and geranyl capronate synthesis. The sudden drop in enzyme activity was registered after the fifth cycle of synthesis [42].

Generally, stability might be affected by the method of catalyst regeneration, immobilization mechanism or nature of substrates. Immobilization mechanism would influence the strength of the enzyme-support bond. In this sense, covalent immobilization provides strong enzyme-support bond preventing enzyme leakage from the reactor vessel [16]. Immobilized enzyme regeneration was done by thorough rinsing with the solvent followed by overnight drying at room temperature in a dessicator containing silica gel. This regeneration could be insufficient to completely remove adsorbed polar substrate resulting in accumulation of acid molecules on the carrier surface causing diffusional limitations to the substrates migrating from the reaction medium towards enzyme, as proposed by Marty et al. [43] and verified by Saponjic et al. [22]. Therefore, highly effective catalyst regeneration remains yet to be established.

3.3.4. Comparison of the batch and FBR systems for geranyl butyrate synthesis

To compare batch system with FBR system, when assayed at their optimal conditions, the volumetric productivity was calculated for each one. It was shown that in the batch system, a rather high molar conversion >99.9% can be obtained. However, a drawback of this system was low reaction rate thus the conversion was achieved after 48 h, corresponding to the volumetric productivity of 5.2 mmol L^{-1} h^{-1}. The kinetics in the FBR system seems to have a better profile compared to batch system since the highest conversion of 78.9% was achieved in 10 h, corresponding to the volumetric productivity of 7.9 mmol L^{-1} h^{-1}. At flow rate of 10 mL min^{-1}, the molar conversion of 53.3% may have been reached after 2 h reaction (volumetric productivity 26.7 mmol L^{-1} h^{-1}), indicating, as expected, improved reaction kinetics compared to the batch system. Observed molar conversion is possibly still susceptible to further increase by implementation of effective control of water concentration, and by better understanding of transfer phenomena inside the reactor zone.

4. Conclusions

A process to obtain geranyl butyrate has been developed using batch and FBR systems with CRL immobilized on Sepabeads® EC-EP by covalent binding. Batch geranyl butyrate synthesis catalyzed by immobilized CRL was efficiently optimized using response surface methodology. Derived optimal conditions were temperature of 25–30 °C, water concentration of 3.6% (v/v) and substrate molar ratio acid/alcohol 2.5. Enzyme concentration and time of molecular sieves addition did not significantly influence molar conversion. Preliminary results in repeated batch esterification showed an acceptable stability of the immobilized enzyme.

Geranyl butyrate synthesis in the FBR was optimized at temperature 35 °C, substrate concentration 0.1 M, acid/alcohol ratio 2.0 and flow rate 10.0 mL min^{-1}. When assayed at their optimal conditions, higher molar conversion was achieved in the batch system, but the productivity in FBR was more than 5-fold higher compared to that obtained in the batch system.

Generally, reaction conditions in a batch reactor and FBR followed the same trend with minor differences originating from different reactor set-ups and consequently different transfer phenomena. Both, however, enabled highly productive synthesis of geranyl butyrate. Covalently immobilized CRL applied in FBR synthesis proved to be robust and versatile way for aroma ester production even in the medium posing additional limitations due to substrate polarity and unfavorable substrate partitioning between organic phase and microaqueous layer.

Results of the conducted study imply that this system has high potential for further improvement and scale-up as well as for application in continuous enzymatic synthesis of other esters or products of enzymatic conversions. Besides of enzyme stabilization achieved by immobilization, conditions in the reactor provide improved mass transfer necessary for the reaction advancement.

Acknowledgements

The authors are grateful to Resindion S.R.L. (Mitsubishi Chemical Corporation, Milan, Italy) for donation of Sepabeads® EC-EP and Sepabeads® EC-HA carriers. Also, the authors are grateful to the Ministry of Education and Science of the Republic of Serbia (Project No. III 46010) for the financial support during this research which was conducted at the Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, from 2006 to 2009.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.11.009.

References

[1] C. Akoh, G.-C. Lee, J.-F. Shaw, Lipids 39 (2004) 513–526.
[2] N. Gandhi, Journal of the American Oil Chemists Society 74 (1997) 621–634.
[3] D. Bezbradica, D. Mijin, S. Siler-Marinkovic, Z. Knezevic, Journal of Molecular Catalysis B: Enzymatic 45 (2007) 97–101.
[4] M. Karra-Chaabouni, S. Pulvin, D. Touraud, D. Thomas, Biotechnology Letters 18 (1996) 1083–1088.
[5] P. Claon, C. Akoh, Journal of the American Oil Chemists Society 71 (1994) 575–578.
[6] Y. Ikeda, Y. Kurokawa, Journal of the American Oil Chemists Society 78 (2001) 1099–1103.
[7] B. Gillies, H. Yamazaki, D.W. Armstrong, Biotechnology Letters 9 (1987) 709–714.
[8] M.G. Carneiro-da-Cunha, J.M.S. Rocha, F.A.P. Garcia, M.H. Gil, Biotechnology Techniques 13 (1999) 403–409.
[9] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Enzyme and Microbial Technology 40 (2007) 1451–1463.
[10] S.R.L. Resindion, Sepabeads® EC series-enzyme carriers (Instruction manual), M.C. Corporation, Milan, Italy, 2006.
[11] A. Kunamneni, I. Ghazi, S. Camarero, A. Ballesteros, F.J. Plou, M. Alcalde, Process Biochemistry 43 (2008) 169–178.
[12] C. Mateo, V. Grazu, B.C. Pessela, T. Montes, J.M. Palomo, R. Torres, F. Lopez-Gallego, R. Fernandez-Lafuente, J.M. Guisan, Biochemical Society Transactions 35 (2007) 1593–1601.
[13] C. Mateo, V. Grazu, J.M. Palomo, F. Lopez-Gallego, R. Fernandez-Lafuente, J.M. Guisan, Nature Protocols 2 (2007) 1022–1033.
[14] C. Mateo, O. Abian, G. Fernández-Lorente, J. Pedroche, R. Fernández-Lafuente, J.M. Guisan, Biotechnology Progress 18 (2002) 629–634.
[15] L. Hilterhaus, B. Minow, J. Müller, M. Berheide, H. Quitmann, M. Katzer, O. Thum, G. Antranikian, A. Zeng, A. Liese, Bioprocess and Biosystems Engineering 31 (2008) 163–171.
[16] M. Chaplin, Enzyme Technology, Faculty of Engineering, Science and the Built Environment, South Bank University, London, UK, 2004.
[17] S.-W. Chang, J.-F. Shaw, C.-K. Yang, C.-J. Shieh, Process Biochemistry 42 (2007) 1362–1366.
[18] F. Molinari, G. Marianelli, F. Aragozzini, Applied Microbiology and Biotechnology 43 (1995) 967–973.
[19] S.S. Kanwar, S. Gehlot, M.L. Verma, R. Gupta, Y. Kumar, G.S. Chauhan, Journal of Applied Polymer Science 110 (2008) 2681–2692.

[20] C.-J. Shieh, C.C. Akoh, L.N. Yee, Biotechnology and Bioengineering 51 (1996) 371–374.
[21] Z.D. Knežević-Jugović, J.J. Damnjanović, D.I. Bezbradica, Ž.D. Mijin, Chemical Industry and Chemical Engineering Quarterly 14 (2008) 245–249.
[22] S. Saponjic, Z.D. Knezevic-Jugovic, D.I. Bezbradica, M.G. Zuza, O.A. Saied, N. Boskovic-Vragolovic, D.Z. Mijin, Electronic Journal of Biotechnology 13 (2010).
[23] P.J. Halling, Enzyme and Microbial Technology 16 (1994) 178–206.
[24] R.H. Valivety, P.J. Halling, A.R. Macrae, Biochimica et Biophysica Acta (BBA): Protein Structure and Molecular Enzymology 1118 (1992) 218–222.
[25] Z. Knezevic, N. Milosavic, D. Bezbradica, Z. Jakovljevic, R. Prodanovic, Biochemical Engineering Journal 30 (2006) 269–278.
[26] B.M. Marion, Analytical Biochemistry 72 (1976) 248–254.
[27] R. Prodanović, S. Jovanović, Z. Vujčić, Biotechnology Letters 23 (2001) 1171–1174.
[28] W.G. Hunter, G.E.P. Box, J.S. Hunter, Statistics for experimenters: An introduction to design, data analysis and model building, Wiley, New York, 1978.
[29] J.M. Walker, Protein Protocols Handbook, Humana Press Inc, New York, 1996.
[30] J. Nelson, Chemistry: The Central Science, Prentice-Hall, 1985.
[31] Z. Knezevic, G. Kukic, M. Vukovic, B. Bugarski, B. Obradovic, Process Biochemistry 39 (2004) 1377–1385.
[32] J.M. Guisan, C. Mateo, G. Fernandez-Lorente, O. Abian, R. Fernandez-Lafuente, Biomacromolecules 1 (2000) 739–745.
[33] J.B. Wheatley, D.E. Schmidt Jr., Journal of Chromatography A 644 (1993) 11–16.
[34] Z.D. Knežević-Jugović, D.I. Bezbradica, D.Ž. Mijin, M.G. Antov, in: S.D. Minteer (Ed.), Enzyme stabilization and immobilization: Methods and protocols, Humana Press, New York, 2011, pp. 99–111.
[35] S. Krishna, B. Manohar, S. Divakar, N. Karanth, Journal of the American Oil Chemists Society 76 (1999) 1483–1488.
[36] G.M.Z.E.B. Pereira, H.F. de Castro, Brazilian Journal of Chemical Engineering 20 (2003) 343–355.
[37] E.B.P.H.F. de Castro, W.A. Anderson, Journal of the Brazilian Chemical Society 7 (1996) 219–224.
[38] T. Chatterjee, D.K. Bhattacharyya, Biotechnology Letters 20 (1998) 865–868.
[39] G.D. Yadav, P.S. Lathi, Journal of Molecular Catalysis B: Enzymatic 27 (2004) 113–119.
[40] L.M. Esteban, d.M. Muñío, A. Robles, E. Hita, M.J. Jiménez, P.A. González, B. Camacho, E. Molina, Biochemical Engineering Journal 44 (2009) 271–279.
[41] A. Sánchez, F. Valero, J. Lafuente, C. Solà, Enzyme and Microbial Technology 27 (2000) 157–166.
[42] L. Yee, C. Akoh, R. Phillips, Journal of the American Oil Chemists Society 74 (1997) 255–260.
[43] A. Marty, V. Dossat, J.-S. Condoret, Biotechnology and Bioengineering 56 (1997) 232–237.