Process Biochemistry 48 (2013) 819–830

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

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

Immobilized *Pseudomonas* sp. lipase: A powerful biocatalyst for asymmetric acylation of (±)-2-amino-1-phenylethanols with vinyl acetate

Deniz Yildirim*, S. Seyhan Tükel

University of Cukurova, The Faculty of Sciences and Letters, Department of Chemistry, 01330 Adana, Turkey

ARTICLE INFO

Article history:
Received 30 October 2012
Received in revised form 25 April 2013
Accepted 26 April 2013
Available online 7 May 2013

Keywords:
Enantiopure 2-amino-1-phenylethanols
Asymmetric acylation
Response surface methodology
Box–Behnken design

ABSTRACT

Pseudomonas sp. lipase was immobilized onto glutaraldehyde-activated Florisil® support via Schiff base formation and stabilized by reducing Schiff base with sodium cyanoborohydride. The immobilization performance was evaluated in terms of bound protein per gram of support (%) and recovered activity (%). A 4-factor and 3-level Box–Behnken design was applied for the acylation of (±)-2-(propylamino)-1-phenylethanol, a model substrate, with vinyl acetate and the asymmetric acylations of other (±)-2-amino-1-phenylethanols with different alkyl substituents onto nitrogen atom such as (±)-2-(methylamino)-1-phenylethanol, (±)-2-(ethylamino)-1-phenylethanol, (±)-2-(butylamino)-1-phenylethanol and (±)-2-(hexylamino)-1-phenylethanol were performed under the optimized conditions. The optimal conditions were bulk water content of 1.8%, reaction temperature of 51.5 °C, initial molar ratio of vinyl acetate to amino alcohol of 1.92, and immobilized lipase loading of 47 mg mL^{-1}. (*R*)-enantiomers of tested amino alcohols were preferentially acylated and the reaction purely took place on the hydroxyl group of 2-amino-1-phenylethanols. The increase of alkyl chain length substituted onto nitrogen atom caused an increase in the acylation yield and ee values of (*S*)-enantiomers. Enantiomeric ratio values were >200 for all the reactions. Our results demonstrate that the immobilized lipase is a promising biocatalyst for the preparation of (*S*)-2-amino-1-phenylethanols and their corresponding (*R*)-esters via *O*-selective acylation of (±)-2-amino-1-phenylethanols with vinyl acetate.

1. Introduction

Beta-adrenergic receptor blocking agents (β-blockers) are important drugs consumed for the treatment of high blood pressure, heart failure, hypertension and myocardial ischemic diseases [1–5]. Many β-blockers available in markets are still used as racemates in therapy although it is well known that chirality is a key factor in the efficacy of β-blockers and distomers are possible to cause the adverse effects [6–8]. Therefore, the syntheses of single enantiomeric forms of β-blockers have found increasing demands in pharmaceutical industry due to supplying more effective and safer drugs [8]. Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) have been known as unsurpassed biocatalysts in organic syntheses because they catalyze a variety of reactions including esterification, interesterification, transesterification and aminolysis [9–12]. They do not require any cofactors for their activities and show good to excellent enantioselectivity on their substrates and are ubiquitous in nature [13]. Due to their aforementioned properties, lipases have been used in free or immobilized forms for the preparations of pharmaceutically important enantiopure alcohols, esters, amines and amides in non-aqueous media [14–17]. However, due to the denaturation, deactivation and recycling problems of lipases in free form, lipases have been immobilized onto various types of supports to prepare more active, cost-effective and more enantioselective biocatalysts toward their free forms in the preparation of aforementioned enantiopure compound groups in non-aqueous media [15,18–20]. Furthermore, immobilization of lipase occasionally helps to positively modulate catalytic properties of lipases such as improvement of stability and activity in drastic reaction conditions, enhancement of enantioselectivity toward non-natural substrates [21–23]. For example, Palomo et al. [24] reported that the enantioselectivity of *Rhizopus oryzae* lipase toward (±)-glycidyl butyrate was modulated depending on the used immobilization technique. Chaubey et al. [25] reported that the catalytic and enantioselectivity properties of *Arthrobacter* sp. was modulated by immobilizing onto *Arthrobacter* sp. with different techniques such as hydrophobic binding, covalent binding and sol–gel entrapment. Palomo et al. [26] immobilized a new lipase from porcine pancreas with two techniques. First, the lipase was immobilized onto polyethyleneimine-coated agarose support by adsorption and then the adsorbed lipase was treated with glutaraldehyde. The results showed that the enantioselectivity of lipase treated with glutaraldehyde was enhanced 10-fold toward (±)-glycidyl butyrate. Volpato et al. [27] reported that a

* Corresponding author. Tel.: +90 322 3386081/25; fax: +90 322 3386070.
E-mail addresses: dozyildirim@gmail.com, dyildirim@cu.edu.tr (D. Yildirim).

1359-5113/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2013.04.019

lipase from *Staphylococcus warneri* EX17 immobilized onto glyoxyl, cyanogen bromide or octyl agarose beads exhibited different enantioselectivities for the hydrolysis of chiral esters, such as (±)-methyl mandelate, (±)-2-*O*-butyryl-2-phenylacetic acid and (±)-2-hydroxy-4-phenyl-butyric acid ethyl ester depending on the immobilization protocol used.

Enantiopure 2-amino-1-phenylethanol derivatives are the group of sympathomimetic drugs. They generally interact with α- and β-adrenergic receptors, resulting in numerous physiological effects such as vascular constriction, reduction of blood flow or decrease in mucus secretion into nasal passages [28]. The structure–activity relationship for ligands interacting with adrenergic receptors showed that the essential structural features for direct activity on the receptors were the 2-amino-1-phenylethanol skeleton, various substituents on the phenyl ring and substituent onto amino group [29]. Lundell et al. reported that α-adrenoceptor agonist activity of (*R*)-noradrenaline decreased notably and its β-activity increased dramatically when (*R*)-noradrenaline was *N*-substituted and the substituent was larger than methyl group [28].

Response surface methodology (RSM) is, an effectual optimization process, combination of statistical and mathematical techniques based on the multivariate non-linear model and provides more advantageous compared to other methods due to its simplicity and reduction of time requirement. RSM includes three steps: (1) proposing a mathematical model to predict response; (2) calculating the coefficients of proposed mathematical model; and (3) testing model adequacy [30–32]. In the literature, RSM has been employed for optimization of lipase-catalyzed synthesis of various enzymatic reactions [33–37]. Box–Behnken designs (BBDs) are rotatable or nearly rotatable designs and require 3 levels of each factor. BBDs necessitate fewer experiments than full factorial designs (FFDs) or central composite designs (CCDs) with the same number of factors [38].

In this study, RSM was performed to optimize for the asymmetric acylation of (±)-2-amino-1-phenylethanols with vinyl acetate catalyzed by immobilized *Pseudomonas* sp. lipase, a facile approach for the preparation of enantiopure 2-amino-1-phenylethanols which may be used as potential new β-blockers. For this purpose, a 4-factor and 3-level BBD was applied for the optimization study. The independent parameters investigated were the bulk water content of reaction medium, reaction temperature, initial molar ratio of vinyl acetate to amino alcohol and immobilized lipase loading. The effects of alkyl chain length substituted onto amino group were investigated onto the product yield and enantioselectivity of the immobilized lipase. For this aim, (±)-2-amino-1-phenylethanols with different alkyl chain lengths substituted onto amino group were tested under the optimized conditions.

2. Materials and methods

2.1. Materials

Pseudomonas sp. lipase (Type XIII, lyophilized powder, ≥15 U/mg solid), (3-aminopropyl)triethoxysilane (3-APTES), glutaraldehyde solution (Grade I, 50% in H_2O), *para*-nitrophenol (*p*-NP), *para*-nitrophenol palmitate (*p*-NPP) and (*R*/*S*)-styrene oxide ((*R*/*S*)-SO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Florisil® (magnesium silicate, particle size 150–250 μm, pore size 6–8 nm and surface area 170–300 m^2/g), sodium cyanoborohydride ($NaCNBH_3$), methylamine hydrochloride (99%), ethylamine (70%, v/v), propylamine (99%, v/v), butylamine (99%, v/v), hexylamine (99%, v/v), acetonitrile, diisopropyl ether (DIPE), diethyl ether (DEE), dioxane (DIOX), tetrahydrofurane (THF), *tert*-butyl methyl ether (TBME), acetic acid and triethyl amine (99%, v/v) were supplied from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of Florisil® support for lipase immobilization

Florisil® support was silanized with 3-APTES and activated with glutaraldehyde before the lipase immobilization as follows. Ten grams of Florisil® supports were washed with 50 mL of HNO_3 solution (5% in water, v/v) at 80–90 °C for 60 min and

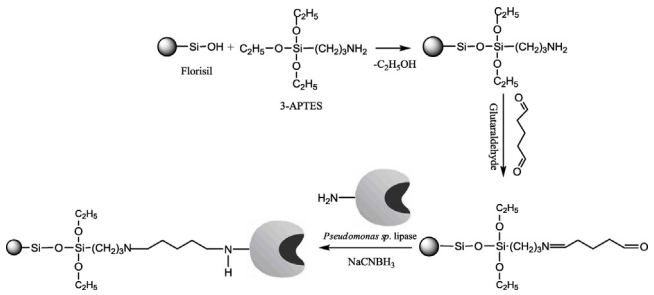


Fig. 1. The modification steps of Florisil® support and the immobilization of *Pseudomonas* sp. lipase onto the modified Florisil® support.

subsequently rinsed with distilled water. To 1 g of acid-washed Florisil® support 25 mL of 3-APTES solution (4% in acetone, v/v) was added and the mixture was kept at 45 °C until dryness [39]. The silanized-Florisil® support was extensively rinsed with distilled water and dried overnight at 120 °C. The glutaraldehyde activation of silanized-Florisil® support was performed according to Alptekin et al. [40]. To 1 g of the silanized-Florisil® support, 25 mL of glutaraldehyde solution (2.5%, w/v, in phosphate buffer (50 mM, pH 7.0)) was added and the mixture was agitated at room temperature for 2 h. The glutaraldehyde-activated Florisil® support was collected by the filtration on a Buchnel funnel, extensively washed with distilled water until the detection of glutaraldehyde was not observed in the filtrate. The detection of glutaraldehyde in the filtrate was determined according to Boratynski and Zal [41]. Then, the obtained support was dried overnight at 120 °C. The amounts of free primary amine ($-NH_2$) groups onto Florisil® support after 3-APTES treatment and glutaraldehyde activation were measured using ninhydrin reagent [40]. To 10 mg of Florisil® support obtained after 3-APTES treatment and glutaraldehyde activation, 100 μL H_2O and 200 μL ninhydrin reagent were added and the mixture was kept in a boiling water bath for 30 min. Subsequently, 5 mL of a 50/50 (v/v) mixture of ethanol/water was added onto the mixture and mixed well. The absorbance of the colored complex known as Ruhemann's purple was measured at 570 nm. A calibration curve was plotted using ethylenediamine as standard to determine the amounts of $—NH_2$ groups after 3-APTES treatment and glutaraldehyde activation. The amount of unreacted $—NH_2$ groups was calculated by subtracting the amount of $—NH_2$ groups remaining after glutaraldehyde activation from the amount of $—NH_2$ groups formed after 3-APTES treatment.

2.2.2. Immobilization of lipase

The immobilization procedure was carried out under predetermined optimal conditions. A total 2 g of glutaraldehyde-activated Florisil® support was suspended in 8 mL of lipase solution (1 mg mL^{-1}) prepared in a citrate buffer (50 mM, pH 6.0) at 5 °C. Then, 10 mL of $NaCNBH_3$ solution (0.25 M, pH 6.0) was slowly added onto it and the mixture was gently stirred for 2 h (Fig. 1). The immobilized lipase preparations were filtrated and rinsed with the same acetate buffer having different concentrations (50–200 mM) to remove the unbound proteins and dried under vacuum to eliminate inter-particle water. The protein amounts of filtrates were measured using the Lowry protein assay [42].

2.2.3. Optimization of immobilization conditions

The optimal conditions for the immobilization of lipase were investigated by changing individually the immobilization pH, immobilization temperature, immobilization time and initial loading protein concentration. The optimal immobilization pH of lipase was determined by performing the immobilization process at different pH values ranging from 5.0 to 8.0 (50 mM acetate buffers for pH 5.0–5.5; 50 mM citrate buffer for pH 6.0; 50 mM phosphate buffers for pH 6.5–8.0). The immobilization process was carried out at the temperature values of 5, 15 and 25 °C in order to determine optimal immobilization temperature. The immobilization of lipase was conducted for 1, 2, 3, 6, 12 and 24 h at the predetermined optimal immobilization pH and temperature. The effect of initial loaded protein concentration was investigated in the range of 0.25–2.0 mg mL^{-1}.

2.2.4. Measurement of hydrolytic activity

The hydrolytic activity of lipase was measured using *p*-NPP as substrate [43]. A defined amount of immobilized lipase (10 mg) was added onto 1 mL of phosphate buffer (50 mM, pH 7.0) and incubated for 5 min at 35 °C. The reaction was started by the addition 1 mL of *p*-NPP solution (0.2% in absolute ethanol, w/v) and the mixture was agitated at 100 rpm for 5 min. The reaction was stopped by adding 2 mL of Na_2CO_3 solution (0.25 M in water) and centrifuged at 5000 rpm for 5 min. One milliliter of supernatant was diluted to 5 mL with distilled water and the quantity of formed *p*-NP was detected at 410 nm by a spectrophotometer (Shimadzu UV-1800).

2.2.5. Chemical synthesis of racemic amino alcohols

(±)-2-Amino-1-phenylethanols were synthesized by reacting (*R*/*S*)-SO with different primary amines, such as methylamine, ethylamine, propylamine, butylamine and hexylamine at room temperature. To 20 mmol of (*R*/*S*)-SO, 25 mmol of each

Table 1
The coded values and levels of each independent variable.

Variables	Symbol	Variable level		
		Low −1	Center 0	High +1
Water content of reaction medium (%)	X_1	0	2	4
Reaction temperature (°C)	X_2	40	50	60
Molar ratio of VA/AA	X_3	1.0	1.5	2.0
Immobilized lipase loading (mg mL^{-1})	X_4	10	30	50

primary amine was separately added. The mixtures were stirred for 12 h and subsequently the unreacted amines were extracted with distilled water (3× 10 mL). The racemic amino alcohols formed were extracted with diethyl ether and the solvent was evaporated under vacuum then the racemic amino alcohols were dried on anhydrous Na_2SO_4. The characterizations of amino alcohols were achieved by high-performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR).

^{1}H NMR and ^{13}C NMR spectra in DMSO-d6 were recorded on a Bruker Ultrashield TM NMR 300 MHz spectrometer with tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) were expressed in parts per million (ppm), multiplicity (s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet) and coupling constants (J) were expressed as Hertz (Hz). A FTIR instrument (Perkin Elmer Spectrum RX/FTIR system) was used to determine the functional groups after the pellet was prepared in KBr. HPLC analyses were performed on a Shodex ORpak CDC-453 HQ chiral column at 220 nm. The mobile phase used was water/acetonitrile/acetic acid/triethylamine mixture (80/20/1.4/0.7, v/v/v/v) at a flow rate of 0.25 mL min^{-1}. The retention times of the amino alcohols and formed products were given in Section 2.2.11.

2.2.6. Experimental design and data analysis

A 4-factor and 3-level Box–Behnken design (BBD) was applied to assess optimal reaction conditions and to understand the relationships between reaction variables. (±)-2-(propylamino)-1-phenylethanol was selected as model substrate for the optimization studies. Several preliminary studies have been performed before the choosing of reaction parameters and the bulk water content of reaction medium (X_1), reaction temperature (X_2), initial molar ratio of vinyl acetate to amino alcohol (VA/AA) (X_3) and immobilized lipase loading (X_4) were determined as important parameters. The low, zero and high levels of parameters were symbolized as −1, 0 and +1, respectively, and are given in Table 1. The experimental studies were performed according to conditions demonstrated in Table 3. The results were analyzed by RSM to fit the following quadratic model:

$$Y = \beta_0 + \sum_{i=1}^{N} \beta_i \times X_i + \sum_{i=1}^{N} \beta_{ii} \times X_i^2 + \sum_{i=1}^{N-1} \sum_{j=i+1}^{N} \beta_{ij} \times X_{ij} \quad (1)$$

where Y is the predicted response (acylation yield), N, number of variables, X_i the independent variable, β_0, β_i, β_{ii} and β_{ij} are the intercept term, the linear effect, the squared effect and the interaction effect, respectively. The experimental design and data analysis were performed by using Design Expert statistical software (Design-Expert 8.0.7).

2.2.7. Lipase catalyzed asymmetric acylation reaction

The immobilized lipase catalyzed asymmetric acylation reaction was performed in a glass vial equipped with a screw stopcock. The reactions designed by RSM were carried out at different experimental conditions as given in Table 3. Briefly, to 800 μL of TBME containing desired amount of water (0–4%), 100 μL of desired concentration of vinyl acetate solution (1, 1.5 and 2 M in TBME) and 100 μL of ±-2-(propylamino)-1-phenylethanol solution (1 M in TBME) were added and then the acylation reaction was initiated by adding the defined amount of immobilized lipase preparation and allowed to continue by agitating at 100 rpm for 2 h (Fig. 2). Subsequently, 100 μL of sample was taken out, dried over sodium sulfate and then analyzed by HPLC equipped with a Shodex ORpak CDC-453 HQ chiral column (4.6 mm × 150 mm) at 220 nm after dilution to 500 μL with acetonitrile/water mixture (1/4, v/v). The calibration graphs were plotted via peak area of the each compound versus its concentration and the amount of the each compound was calculated from their peak areas. The mobile phase consisted of water/acetonitrile/acetic acid/triethylamine mixture (80/20/1.4/0.7, v/v/v/v) and its flow rate was adjusted to 0.25 mL min^{-1}. The column temperature was kept at 25 °C during analysis. The enantiomeric excess values (ee) of amino alcohols and esters were calculated from the equations given below:

$$ee_{alcohol} = \frac{[S-R]_{alcohol}}{[S+R]_{alcohol}} \quad \text{and} \quad ee_{ester} = \frac{[R-S]_{ester}}{[R+S]_{ester}}$$

The enantiomeric ratio values (E) of immobilized lipase were calculated from the ee values of related ester (ee_{ester}) and amino alcohol ($ee_{alcohol}$) and conversion degree (C) [44].

2.2.8. Asymmetric acylation of different N-substituted 2-amino-1-phenylethanols

The reactions were carried out under the optimized conditions estimated by RSM. Five reactions were made and only one aliquot of 100 μL was taken from each mixture for each time interval (15, 30, 60, 120 and 240 min). Aliquots were diluted to 500 μL with acetonitrile/water mixture (1/4, v/v) and analyzed by the chiral HPLC for the quantification of substrate and product concentrations, their ee values and E values of the immobilized lipase.

To purify the reaction products, the TBME layer was loaded onto a silica gel column after 240 min reaction time, and the products were eluted with hexane–ethyl acetate mixture (9/1, v/v). After the removal of solvent, the products were characterized by chiral HPLC, FTIR and NMR techniques.

2.2.9. Thermal stability

The thermal stability of immobilized lipase was investigated at 40, 50 and 60 °C under the optimal conditions. The first-order inactivation constant (k_i) and half-life ($t_{1/2}$) of the lipase were calculated from the equation:

$$\ln V_t = \ln V_0 - k_i t$$

Table 2
The results of immobilization of *Pseudomonas* sp. lipase onto Florisil® at different immobilization conditions.

Immobilization pH	Immobilization temperature	Immobilization time	Initial protein concentration (mg mL^{-1})	Bound protein (mg/g support)	Bound protein (%)	Specific activity (U/mg protein)	Recovered activity (%)
5.0		2 h		3.0 ± 0.08	75	1130 ± 48	33.5
5.5				3.0 ± 0.08	75	1130 ± 45	33.5
6.0				3.2 ± 0.10	80	1250 ± 40	37.0
6.5	5 °C		1.0	3.2 ± 0.10	80	1190 ± 56	35.2
7.0				2.8 ± 0.08	70	1060 ± 52	31.4
7.5				2.8 ± 0.07	70	1050 ± 40	31.1
8.0				2.4 ± 0.07	60	880 ± 42	26.1
	5 °C			3.2 ± 0.10	80	1250 ± 35	37.0
6.0	15 °C	2 h	1.0	3.2 ± 0.10	80	1250 ± 40	37.0
	25 °C			3.2 ± 0.10	80	1130 ± 55	33.5
		1 h		2.4 ± 0.08	60	1010 ± 40	29.9
		2 h		3.2 ± 0.09	80	1260 ± 40	37.3
		3 h		3.2 ± 0.09	80	1260 ± 50	37.3
6.0	15 °C	6 h	1.0	3.2 ± 0.09	80	1260 ± 45	37.3
		12 h		3.2 ± 0.10	80	1200 ± 30	35.5
		24 h		3.2 ± 0.10	80	1130 ± 50	33.5
			0.25	1.0 ± 0.02	100	390 ± 15	11.5
			0.5	2.0 ± 0.05	100	670 ± 25	19.8
6.0	15 °C	2 h	1.0	3.2 ± 0.06	80	1290 ± 60	38.2
			1.5	5.1 ± 0.15	85	1030 ± 50	30.5
			2.0	7.2 ± 0.18	90	950 ± 40	28.1

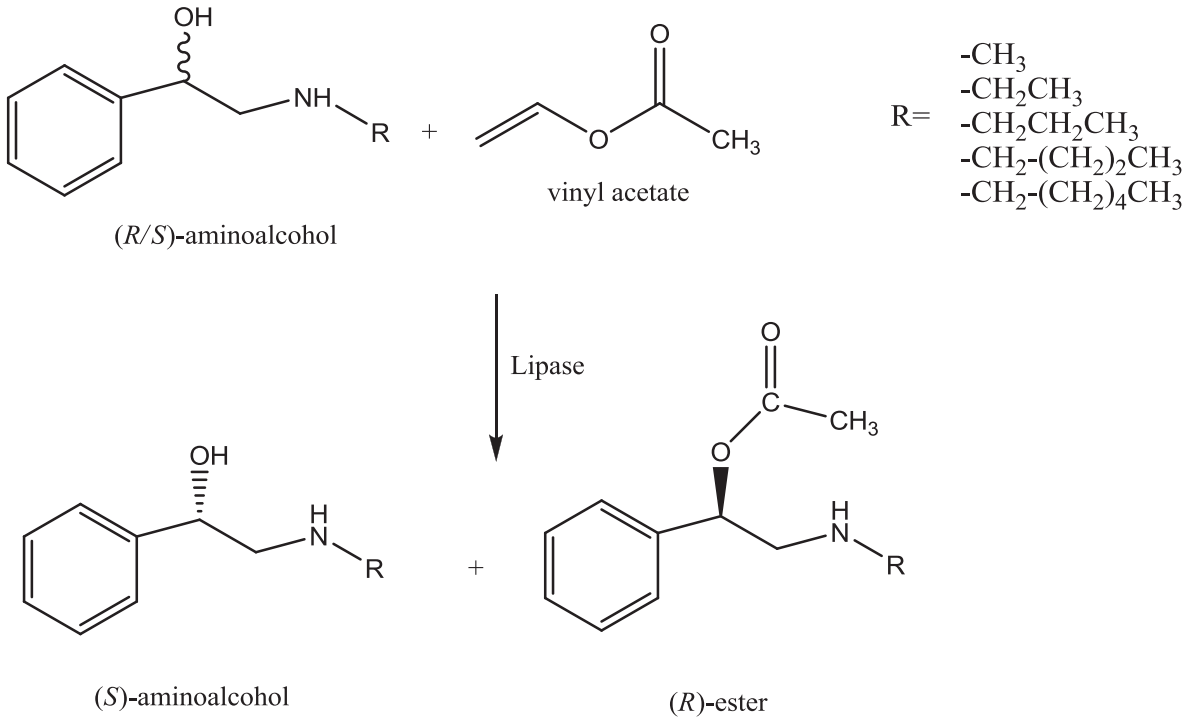


Fig. 2. The immobilized *Pseudomonas* sp. lipase catalyzed the asymmetric acylation reaction of ±-2-amino-1-phenylethanols.

where V_0 and V_t are the initial activity and the activity after t time incubation, respectively. $t_{1/2}$ values of the free and immobilized lipase preparations were estimated from the equation:

$$t_{1/2} = \frac{-\ln(0.5)}{k_i} = \frac{0.693}{k_i}$$

where ln(0.5) is residual activity of 50%.

2.2.10. Reusability of immobilized lipase

The operational stability of immobilized lipase was investigated in a batch type reactor. To 8 mL of TBME, 1 mL of ±-2-(hexylamino)-1-phenylethanol solution (5.0 M) and 1 mL of vinyl acetate (9.60 M) were added. The reaction was started by the addition 1 g of immobilized lipase at 50 °C. After 4 h reaction time, the separation of immobilized lipase preparations from the reaction mixture was achieved by filtration. After each experiment, the immobilized lipase preparations were washed with 5 mL of TBME and then dried under vacuum. For the next operation, the fresh reaction medium was loaded onto the reactor and these procedures were repeated 10 times.

2.2.11. Spectral studies of reaction products

2.2.11.1. 2-(Methylamino)-1-phenylethanol (1a). ($C_9H_{13}NO$): Yellow liquid, purity 99% (HPLC).

^{1}H NMR δ **(ppm)**: 2.17 (s, 1H, —N**H**), 2.26 (d, 1H, J = 5.70 Hz, —NHC**H**$_2$—), 3.44 (t, 1H, J = 12.09 Hz, —NHC**H**$_2$—), 3.46 (s, 3H, —NC**H**$_3$), 3.60 (s, 1H, —O**H**), 4.67 (dd, 1H, J = 2.32 Hz, J = 2.16 Hz, ArC**H**OH), 7.29–7.32 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 39.8 (—N**C**H$_3$), 56.5 (—N**C**H$_2$—), 70.2 (—H**C**OH), 127.7–138.7 (Ar**C**).
HPLC: Retention times of (*S*)-1a and (*R*)-1a are 5.27 and 9.10 min, respectively.
IR (film) cm^{-1}: 4056, 3244, 3086, 2881, 1952, 1885, 1758, 1659, 1555, 1493, 1338, 1268, 1201, 1091, 1026.

2.2.11.2. 2-(Ethylamino)-1-phenylethanol (2a). ($C_{10}H_{15}NO$): Pale yellow solid, purity 99% (HPLC).

^{1}H NMR δ **(ppm)**: 1.0 (t, 3H, J = 12.50 Hz, —CH$_2$C**H**$_3$), 2.58 (q, 2H, J = 12.09 Hz, —C**H**$_2$CH$_3$), 2.60 (s, 1H, —N**H**), 2.61 (d, 1H, J = 4.23 Hz, —NHC**H**$_2$—), 2.63 (t, 1H, J = 6.96 Hz, —NHC**H**$_2$—), 3.65 (s, 1H, —O**H**), 4.63 (dd, 1H, J = 2.35 Hz, J = 2.33 Hz, ArC**H**OH), 7.31–7.33 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 15.6 (—**C**H$_3$), 43.6 (—N**C**H$_2$—CH$_3$), 58 (—**C**H$_2$N), 70.6 (—H**C**OH), 127.2–142 (Ar**C**).
HPLC: Retention times of (*S*)-2a and (*R*)-2a are 5.3 and 9.14 min, respectively.
IR (film) cm^{-1}: 4046, 3224, 3086, 2881, 1955, 1885, 1758, 1659, 1555, 1416, 1268, 1201, 1065, 1026.

2.2.11.3. 2-(Propylamino)-1-phenylethanol (3a). ($C_{11}H_{17}NO$): Yellow liquid, purity 99% (HPLC).

^{1}H NMR δ **(ppm)**: 0.85 (t, 3H, J = 12.50 Hz, —CH$_2$C**H**$_3$), 1.39 (m, 2H, —C**H**$_2$CH$_3$), 2.48 (s, 1H, —N**H**), 2.59 (t, 2H, J = 5.81 Hz, —C**H**$_2$CH$_2$CH$_3$), 2.62 (d, 1H, J = 5.81 Hz, —NHC**H**$_2$—), 3.37 (t, 1H, J = 5.57 Hz, —NHC**H**$_2$—), 3.63 (s, 1H, —O**H**), 4.62 (dd, 1H, J = 4.55 Hz, J = 3.21 Hz, ArC**H**OH), 7.31–7.32 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 12.2 (—**C**H$_3$), 40.2 (—**C**H$_2$CH$_3$), 51.4 (—N**C**H$_2$—CH$_2$), 58.2 —H**C**NH—CH$_2$—), 71.5 (—H**C**OH), 127.2–142 (Ar**C**).
HPLC: Retention times of (*S*)-3a and (*R*)-3a are 5.33 and 8.85 min, respectively.
IR (film) cm^{-1}: 3416, 3032, 2532, 1994, 1729, 1618, 1500, 1394, 1324, 1296, 1185, 1048, 1026.

2.2.11.4. 2-(Butylamino)-1-phenylethanol (4a). ($C_{12}H_{19}NO$): Yellow liquid, purity 99% (HPLC).

^{1}H NMR δ **(ppm)**: 0.86 (t, 3H, J = 12.50 Hz, —CH$_2$C**H**$_3$), 1.31 (m, 2H, —C**H**$_2$CH$_3$), 1.36 (m, 2H, —C**H**$_2$CH$_2$CH$_3$), 2.49 (t, 2H, J = 6.00 Hz, —C**H**$_2$CH$_2$—), 2.60 (s, 1H, —N**H**), 2.99 (d, 1H, J = 6.95 Hz, —NHC**H**$_2$—), 3.31 (t, 1H, J = 9.57 Hz, —C**H**HNH—CH$_2$—), 3.65 (s, 1H, —O**H**), 4.63 (dd, 1H, J = 4.18 Hz, J = 3.21 Hz, ArC**H**OH), 7.31–7.33 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 14.4 (—**C**H$_3$), 20.4 (—**C**H$_2$CH$_3$), 39.9 (—CH$_2$—**C**H$_2$—CH$_2$—), 49.2 (—N**C**H$_2$—CH$_2$), 58.2 (—**C**H$_2$NH—CH$_2$—CH$_2$—), 70.6 (—H**C**OH), 127.2–142 (Ar**C**).
HPLC: Retention times of (*S*)-4a and (*R*)-5a are 5.27 and 8.84 min, respectively.
IR (film) cm^{-1}: 3422, 2975, 2958, 2738, 2491, 2365, 1621, 1475, 1433, 1397, 1171, 1036.

2.2.11.5. 2-(Hexylamino)-1-phenylethanol(5a). ($C_{14}H_{23}NO$): White solid, purity 99% (HPLC).

^{1}H NMR δ **(ppm)**: 0.86 (t, 3H, J = 11.90 Hz, —CH$_2$C**H**$_3$), 1.24 (m, 4H, J = 12.50 Hz, —C**H**$_2$C**H**$_2$CH$_2$CH$_3$), 1.28 (m, 2H, —CH$_2$C**H**$_2$CH$_3$), 1.38 (m, 2H, —C**H**$_2$CH$_2$CH$_2$—), 2.52 (t, 2H, J = 9.24 Hz, —NHC**H**$_2$CH$_2$—), 2.62 (s, 1H, —N**H**CH$_2$—), 2.64 (d, 1H, J = 4.50 Hz, —CH**H**NH—CH$_2$—), 3.41 (t, 1H, J = 9.60 Hz, —C**H**HNH—CH$_2$—), 3.65 (s, 1H, —CO**H**), 4.62 (dd, 1H, J = 3.73 Hz, J = 2.30 Hz, ArC**H**OH), 7.30–7.32 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 14.3 (—**C**H$_3$), 22.6 (—**C**H$_2$CH$_3$), 25.4 (—**C**H$_2$CH$_2$CH$_3$), 26.9 (—**C**H$_2$CH$_2$CH$_2$CH$_3$), 30.0 (—**C**H$_2$CH$_2$CH$_2$—), 49.5 (—N**C**H$_2$—CH$_2$—), 58.2 (—**C**H$_2$N), 71.8 (—**C**OH), 126.4–142 (Ar**C**).
HPLC: Retention times of (*S*)-5a and (*R*)-5a are 5.58 and 8.84 min, respectively.
IR (film) cm^{-1}: 3418, 2977, 2940, 2739, 2491, 2359, 1475, 1397, 1172, 1036.

2.2.11.6. 2-(Methylamino)-1-phenylethyl acetate (1b). ($C_{11}H_{15}NO_2$): Brown liquid, purity 96% (HPLC).

^{1}H NMR δ **(ppm)**: 2.36 (s, 3H, —O—C—**CH$_3$**), 2.72 (s, 1H, —N**H**—CH$_3$), 3.35 (t, 1H, *J* = 12.57 Hz, —C**H**HNH—CH$_3$), 3.37 (s, 3H, —NH—C**H$_3$**), 3.93 (d, 1H, *J* = 4.90 Hz, —CH**H**NH—CH$_3$), 5.90 (dd, 1H, *J* = 4.90 Hz, *J* = 4.90 Hz, ArC**H**O—), 7.35–7.37 (m, 5H, **H**Ar).
^{13}C NMR δ **(ppm)**: 21.4 (O—C—**C**H$_3$), 39.9 (—HN**C**H$_3$), 52.6 (—**C**H$_2$HNCH$_3$), 73.6 (—**C**OH), 127.1–149.8 (Ar**C**), 172.6 (—**C**—O).
HPLC: Retention time of (*R*)-1b is 6.90 min.
IR (film) cm^{-1}: 4068, 3400, 3033, 2601, 1970, 1715, 1597, 1490, 1371, 1264, 1025, 1005.

2.2.11.7. 2-(Ethylamino)-1-phenylethyl acetate (2b). ($C_{10}H_{15}NO$): Brown liquid, purity 96% (HPLC).

^{1}H NMR δ **(ppm)**: 1.90 (t, 3H, *J* = 12.50 Hz, —CH$_2$C**H$_3$**), 2.02 (s, 3H, —O—C—C**H$_3$**), 2.11 (q, 2H, *J* = 12.09 Hz, —C**H$_2$**CH$_3$), 2.58 (s, 1H, —N**H**CH$_2$—CH$_3$), 3.20 (d, 1H, *J* = 5.57 Hz, —CH**H**NH—CH$_2$), 3.22 (t, 1H, *J* = 6.0 Hz, —C**H**HNH—CH$_2$—), 5.37 (dd, 1H, dd, 1H, *J* = 4.60 Hz, *J* = 4.50 Hz, ArC**H**O—), 7.36–7.38 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 14.0 (—**C**H$_3$), 21.3 (O—C—**C**H$_3$), 44.4 (—HN**C**H$_2$—CH$_3$), 50.6 (—**C**H$_2$NHCH$_2$), 73.6 (—**C**OH), 128.2–149.7 (Ar**C**), 172.7 (—**C**—O).
HPLC: Retention time of (*R*)-2b is 6.93 min.
IR (film) cm^{-1}: 3401, 3034, 2604, 1969, 1719, 1624, 1490, 1375, 1269, 1006.

2.2.11.8. 2-(Propylamino)-1-phenylethyl acetate (3b). ($C_{11}H_{17}NO$): Brown liquid, purity 97% (HPLC).

^{1}H NMR δ **(ppm)**: 0.80 (t, 3H, *J* = 12.80 Hz, —CH$_2$C**H$_3$**), 1.47 (m, 2H, —C**H$_2$**CH$_3$), 2.19 (s, 3H, —O—C—C**H$_3$**), 2.55 (t, 2H, *J* = 11.40 Hz, —C**H$_2$**CH$_2$CH$_3$), 2.65 (s, 1H, —N**H**CH$_2$—), 3.07 (d, 1H, *J* = 4.80 Hz, —CH**H**NH—CH$_2$—), 3.61 (t, 1H, *J* = 12.57 Hz, —C**H**HNH—CH$_2$—), 5.90 (dd, 1H, *J* = 4.58 Hz, *J* = 4.50 Hz, ArC**H**O—), 7.35–7.37 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 11.5 (—**C**H$_3$), 21.4 (O—C—**C**H$_3$), 39.7 (—**C**H$_2$CH$_3$), 51.0 (—**C**H$_2$NHCH$_2$—), 56.8 (—HN**C**H$_2$—CH$_2$—), 73.6 (—**C**OH), 127.3–149.9 (Ar**C**), 172.6 (—**C**—O).
HPLC: Retention time of (*R*)-3b is 6.99 min.
IR (film) cm^{-1}: 3063, 3033, 2970, 2939, 2881, 2597, 1960, 1721, 1619, 1440, 1371, 1256, 1066, 1035, 1006.

2.2.11.9. 2-(Butylamino)-1-phenylethyl acetate (4b). ($C_{12}H_{19}NO$): Brown liquid, purity 97% (HPLC).

^{1}H NMR δ **(ppm)**: 0.87 (t, 3H, *J* = 6.90 Hz, —CH$_2$C**H$_3$**), 1.26 (m, 2H, —C**H$_2$**CH$_3$), 1.39 (m, 2H, —C**H$_2$**CH$_2$CH$_3$), 2.19 (s, 3H, —O—C—C**H$_3$**), 2.51 (t, 2H, *J* = 6.90 Hz, —NHC**H$_2$**CH$_2$—), 2.68 (s, 1H, —N**H**CH$_2$—), 3.19 (d, 1H, *J* = 3.90 Hz, —CH**H**NH—CH$_2$—), 3.55 (t, 1H, *J* = 12.90 Hz, —C**H**HNH—CH$_2$—), 5.90 (dd, 1H, *J* = 4.50 Hz, *J* = 4.60 Hz, ArC**H**O—), 7.37–7.39, (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 13.8 (—**C**H$_3$), 21.0 (O—C—**C**H$_3$), 21.4 (—**C**H$_2$CH$_3$), 39.5 (—CH$_2$—**C**H$_2$—CH$_2$—), 40.1 (—HN**C**H$_2$—CH$_2$), 50.4 (—**C**H$_2$NH—), 73.6 (—**C**OH), 126.8–149.9 (Ar**C**), 172.5 (—**C**—O).
HPLC: Retention time of (*R*)-4b is 7.17 min.
IR (film) cm^{-1}: 3033, 2961, 2939, 2875, 2593, 1959, 1723, 1623, 1439, 1372, 1237, 1065, 1027, 1005.

2.2.11.10. 2-(Hexylamino)-1-phenylethyl acetate (5b). ($C_{14}H_{23}NO$): Brown liquid, purity 97% (HPLC).

^{1}H NMR δ **(ppm)**: 0.82 (t, 3H, *J* = 11.90 Hz, —CH$_2$C**H$_3$**), 1.19 (m, 4H, —C**H$_2$**C**H$_2$**CH$_2$CH$_3$), 1.20 (m, 2H, —CH$_2$C**H$_2$**CH$_3$), 1.41 (m, 2H, —C**H$_2$**CH$_2$CH$_2$—), 1.90 (t, 2H, *J* = 12.50 Hz, —NHC**H$_2$**CH$_2$—), 2.0 (s, 1H, —N**H**CH$_2$—), 2.05 (s, 3H, —O—C—C**H$_3$**), 3.17 (d, 1H, *J* = 3.60 Hz, —CH**H**NH—CH$_2$—), 3.54 (t, 1H, *J* = 12.30 Hz, —C**H**HNH—CH$_2$—), 5.90 (dd, 1H, *J* = 4.50 Hz, *J* = 4.50 Hz, ArC**H**O—), 7.37–7.39 (m, 5H, **H**Ar—).
^{13}C NMR δ **(ppm)**: 14.2 (—**C**H$_3$), 21.3 (O—C—**C**H$_3$), 22.4 (—**C**H$_2$CH$_3$), 31.5 (—CH$_2$—CH$_2$—CH$_3$), 26.4 (—CH$_2$—**CH$_2$**—CH$_2$—), 31.3 (—NHCH$_2$—**C**H$_2$—), 49.9 (—HN**CH$_2$**—CH$_2$—), 53.1 (—**C**H$_2$NHCH$_2$—), 73.6 (—**C**OH), 126.8–149.8 (Ar**C**), 172.5 (—C—O).
HPLC: Retention time of (*R*)-5b is 7.21 min.
IR (film) cm^{-1}: 3572, 2933, 2598, 1958, 1720, 1597, 1490, 1440, 1370, 1263, 1005.

3. Results and discussion

Florisil® is mainly composed of MgO (15%) and SiO_2 (85%) and has been used as support material for the immobilizations of different enzymes since it exhibits an excellent mechanical character and resists against microbial attack and organic solvents [40,45,46]. Before covalent enzyme immobilization, the inert hydroxyl groups on the surface of Florisil® support should be modified and activated in order to bind enzymes. In this study, the surface of Florisil® was modified with 3-APTES to provide free primary amine groups followed by glutaraldehyde activation to provide free aldehyde groups that can bind the enzyme molecules. The amount of free $-NH_2$ groups introduced after 3-APTES treatment was measured as 250 μmol g support^{-1}. After glutaraldehyde activation, the amount of $-NH_2$ group was 45 μmol g support^{-1}. These results indicate that 86% of total $-NH_2$ groups introduced after 3-APTES treatment are activated with glutaraldehyde. These results also show that a small portion of the support may behave as anion exchanger due to protonation of free $-NH_2$ groups remaining after glutaraldehyde activation [47]. However, the immobilized lipase preparations were washed with the different concentrations of immobilization buffer (50–200 mM) to prevent ionic adsorption of the lipase. The covalent immobilization of *Pseudomonas* sp. lipase onto glutaraldehyde-activated Florisil® support was achieved via Schiff base formation reaction between the free carbonyl group of glutaraldehyde and the amino functional groups of the lipase. Due to instability of Schiff base, they were reduced using sodium cyanoborohydride and stabilized as highly stable alkylamine bonds. Sodium cyanoborohydride was preferred as reducing agent because it was reported that it was milder reductant than sodium borohydride and would rapidly reduce the Schiff base. However, it would not reduce aldehyde groups unlike sodium borohydride [48]. It was reported that the activities of immobilized enzymes, proteins and also some labile monoclonal antibodies preserved after the treatment of sodium cyanoborohydride [49–51].

Since success of an immobilization study (high activity and protein recovery yields) affects from various parameters such as immobilization pH, temperature and immobilization time and initial loaded enzyme amount, the conditions of lipase immobilization were optimized for the obtention of maximum performance from the immobilized lipase preparation. In this study, the immobilization of lipase onto glutaraldehyde-activated Florisil® support was investigated at different pH values and the results are given in Table 2. For the immobilized lipase, the same specific activity was observed as 1130 ± 45 U/mg protein at pH 5.0 and 5.5 and the maximum specific activity was determined at pH 6.0. At the immobilization pHs above 6.0, the specific activity of immobilized lipase decreased and observed as 880 ± 42 U/mg protein for the immobilization pH of 8.0. These results indicate that the activity of immobilized lipase was affected from immobilization pH. The nearly same behavior was observed for the amount of bound lipase and the highest amounts of bound protein were determined for the immobilization pH 6.0 and 6.5. The pH stability of free *Pseudomonas* sp. lipase was evaluated for various pH values and the results were given in Fig. 3. The three-dimensional structure of *Pseudomonas* sp. lipase nearly protected during 2 h incubation time and a 90% of relative activity of *Pseudomonas* sp. lipase was protected at the end of 6 h incubation time for all the investigated pHs. At the end of 24 h incubation time, the relative activities observed were 86, 90, 88, 87 and 82%, respectively, for pH values of 5.0, 6.0, 6.5, 7.0 and 8.0.

The effect of immobilization temperature on the relative activity of immobilized lipase and bound protein amount was studied for 5, 15 and 25 °C at the immobilization pH of 6.0, immobilization time of 2 h and initial loaded protein concentration of 1.0 mg mL^{-1}. The amount of bound protein did not affect from the change of immobilization temperature investigated in the range of 5–25 °C and found as 80% of initial loaded protein. The specific activities of immobilized lipase were determined as the same for the immobilization temperature of 5 and 15 °C whereas the relative activity decreased slightly for the immobilization temperature 25 °C (Table 2).

The percentage of bound protein increased from 60 to 80% when the immobilization time was changed from 1 to 2 h at the immobilization pH of 6.0, immobilization temperature of 5 °C and initial

Table 3
BBD results of asymmetric acylation of 2-(propylamino)-1-phenylethanol with vinyl acetate catalyzed by the immobilized *Pseudomonas* sp. lipase. The experimental results were average of three replicates. The predicted results were estimated using Eq. (3).

Run	X_1/water content (%)	X_2/reaction temperature (°C)	X_3/molar ratio of VA/AA	X_4/immobilized lipase loading (mg mL^{-1})	Y/yield (%) (experimental)	Y/yield (%) (predicted)
1	2	60	1.0	30	41.1	41.0
2	4	50	1.5	50	43.1	43.2
3	4	60	1.5	30	41.5	41.6
4	0	40	1.5	30	37.7	37.7
5	0	50	1.0	30	37.5	38.1
6	4	50	1.5	10	19.3	19.1
7	4	40	1.5	30	37.7	37.7
8	2	50	2.0	10	21.0	21.6
9	2	40	2.0	30	38.6	40.2
10	2	40	1.5	10	19.1	18.2
11	2	50	2.0	50	46.8	45.8
12	4	50	1.0	30	37.7	38.1
13	2	60	1.5	50	45.2	46.1
14	2	50	1.5	30	45.2	45.2
15	0	50	1.5	10	19.1	19.1
16	0	50	2.0	30	41.2	41.2
17	2	40	1.0	30	37.4	37.1
18	2	60	2.0	30	45.0	44.1
19	0	50	1.5	50	42.8	43.2
20	2	50	1.5	30	45.2	45.2
21	2	40	1.5	50	42.7	42.3
22	2	50	1.0	50	42.7	42.7
23	2	50	1.5	30	45.3	45.2
24	2	50	1.5	30	45.1	45.2
25	2	50	1.0	10	19.1	18.5
26	0	60	1.5	30	42.6	41.6
27	2	50	1.5	30	45.4	45.2
28	4	50	2.0	30	41.5	41.2
29	2	60	1.5	10	20.9	22.0

loaded lipase concentration of 1.0 mg mL^{-1}. After that point, the increase in immobilization time did not affect on relative percentage of bound protein under the same conditions. The specific activity of immobilized lipase was calculated as 1010 ± 40 U/mg protein after 1 h immobilization time. For 2–6 h immobilization times, the specific activities reached a maximum value (Table 2). Further increase in immobilization time resulted in decrease of relative activity of immobilized lipase. These findings may be explained that glutaraldehyde causes distortion effect in three dimensional structure of the lipase for the immobilization times of 12 and 24 h due to the high reactivity of glutaraldehyde with other nucleophilic groups of the lipase [52,53]. In view of these results, the optimal immobilization time was chosen as 2 h for the next investigation.

All the amount of loaded protein bound on the support for the initial protein concentrations of 0.25 and 0.5 mg mL^{-1}. When initial protein concentration was increased, the percentage of bound protein decreased. For 1.0, 1.5 and 2.5 mg mL^{-1} protein concentrations, the percentage of bound protein were determined as 80, 85 and 90%, respectively. However, the immobilized lipase activity was higher for the protein concentration of 1.0 mg mL^{-1} than those of 1.5 and 2.5 mg mL^{-1}. At the protein loadings of 1.5 and 2.5 mg mL^{-1}, the possibility of reaction between glutaraldehyde and other nucleophilic groups of the lipase increases and this situation cause distortion of the three-dimensional structure of the lipase, resulting in decrease of the immobilized lipase activity. Besides, the diffusion of substrate to active site of the lipase may be prevented at the protein loadings of 1.5 and 2.5 mg mL^{-1} [52,53].

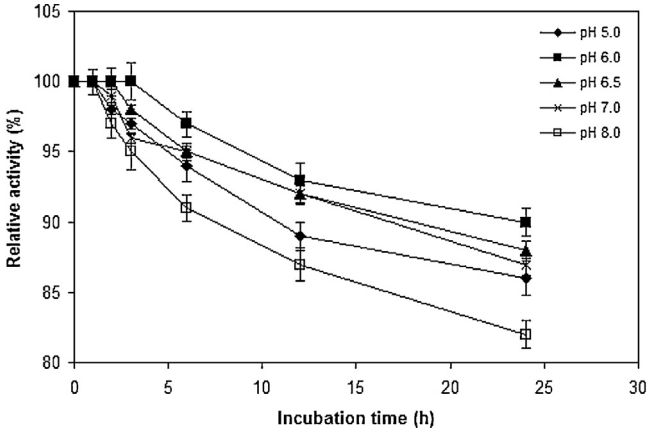


Fig. 3. The pH stability of free *Pseudomonas* sp. lipase at different pH values.

In view of these results, the optimal immobilization conditions were selected as pH value of 6.0, immobilization temperature of 5 or 15 °C, immobilization time of 2 h and initial protein concentration of 1.0 mg mL^{-1}. Under the optimized conditions, the amount of loaded protein on the support was determined as 3.2 mg protein g support^{-1} which corresponds to 80% of initial loaded protein and the lipase preserved 38.2% of its hydrolytic activity with the immobilization.

In this study, the immobilized lipase-catalyzed asymmetric acylation reaction of ±-2-(propylamino)-1-phenylethanol with vinyl acetate was optimized by using RSM. For this purpose, a 4-factor and 3-level BBD was applied as given in Table 3. The results of 29 experiments were analyzed by using Design-Expert software and the coefficients of quadratic model proposed (Eq. (2)) were estimated as given below:

$$Y = +45.24 - 8.333 \times 10^{-3}X_1 + 1.92X_2 + 1.55X_3 + 12.07X_4 - 0.28X_1 \times X_2 + 0.025X_1 \times X_3 + 0.025X_1 \times X_4 + 0.67X_2 \times X_3 + 0.18X_2 \times X_4 + 0.55X_3 \times X_4 - 3.29{X_1}^2 - 2.32{X_2}^2 - 2.31{X_3}^2 - 10.78{X_4}^2 \quad (2)$$

Table 4
ANOVA analysis of the proposed quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	*F* value	*F* tabulated	*p*-Value, Prob > *F*	
Model	2588.81	14	184.92	366.28	2.48	<0.0001	Significant
X_1	8.333×10^{-4}	1	8.333×10^{-4}	1.651×10^{-3}	4.60	0.9682	
X_2	44.47	1	44.47	88.08	4.60	<0.0001	Significant
X_3	28.83	1	28.83	57.11	4.60	<0.0001	Significant
X_4	1747.25	1	1747.25	3460.97	4.60	<0.0001	Significant
X_1X_2	0.30	1	0.30	0.60	4.60	0.4518	
X_1X_3	2.5×10^{-3}	1	2.5×10^{-3}	4.952×10^{-3}	4.60	0.9449	
X_1X_4	2.5×10^{-3}	1	2.5×10^{-3}	4.952×10^{-3}	4.60	0.9449	
X_2X_3	1.82	1	1.82	3.61	4.60	0.0782	
X_2X_4	0.12	1	0.12	0.24	4.60	0.6299	
X_3X_4	1.21	1	1.21	2.40	4.60	0.1439	
X_1^2	70.42	1	70.42	139.50	4.60	<0.0001	Significant
X_2^2	34.91	1	34.91	69.16	4.60	<0.0001	Significant
X_3^2	34.54	1	34.54	68.41	4.60	<0.0001	Significant
X_4^2	754.13	1	754.13	1493.79	4.60	<0.0001	Significant
Residual	7.07	14	0.50				
Lack of Fit	7.02	10	0.70	53.97	2.98	0.0008	Significant
Pure Error	0.052	4	0.013				
$R^2 = 0.9973$	Adj $R^2 = 0.9946$						

Eq. (2) was reduced by discarding the insignificant model terms and Eq. (3) was obtained.

$$Y = +45.24 + 1.92X_2 + 1.55X_3 + 12.07X_4 - 3.29{X_1}^2 - 2.32{X_2}^2 - 2.31{X_3}^2 - 10.78{X_4}^2 \quad (3)$$

where Y is the predicted response (% acylation yield), X_1 is the bulk water content of reaction medium, X_2 is the reaction temperature, X_3 is the initial molar ratio of vinyl acetate to amino alcohol (VA/AA) and X_4 is the immobilized lipase loading. A positive sign before a term shows a synergistic effect, while a negative sign shows an antagonistic effect [32]. The equation indicates that the immobilized lipase loading (X_4) with a coefficiency of 12.07 was the greatest positive linear effect while the bulk water content of reaction medium was the negative linear effect on the yield of asymmetric acylation reaction. The results of analysis of variance (ANOVA) test are given in Table 4. *F*-value of proposed quadratic model and *F*-tabulated value are 366.28 and 2.48, respectively, implying that the model is significant. The model terms with p level value lower than 0.05 (i.e. at a 95% confidence level) are accepted as significant term. Values between $0.05 < X < 0.1$ are considered marginally with significance. Then values higher than 0.1 are insignificant. In this case X_2, X_3, X_4, ${X_1}^2$, ${X_2}^2$, ${X_3}^2$ and ${X_4}^2$ are significant model terms. The coefficient of determination value (R^2) was estimated as 0.9973 showing that the model explains 99.73% of total variations. The adjusted coefficient of determination value (Adj $R^2 = 0.9946$) is in agreement with the R^2. As shown in Table 3, the predicted results fit very well with the experimental results within the range investigated indicating that the proposed quadratic model provides an adequate approximation for explaining the asymmetric acylation reaction of ±-2-(propylamino)-1-phenylethanol with vinyl acetate catalyzed by immobilized *Pseudomonas* sp. lipase.

Fig. 4a illustrates the contour graph of the influence of reaction temperature and initial water content of reaction medium on the asymmetric acylation yield of ±-2-(propylamino)-1-phenylethanol when initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol and immobilized lipase loading were 1.5 and 30 mg mL^{-1}, respectively. Water plays an important role for lipase activity in organic media and a small amount of water should be present in the reaction medium to acquire a proper lipase conformation. As shown in Fig. 4a, the acylation yield slightly increased with increasing the bulk water content of reaction medium from 0 to 2%. This increase in activity may be ascribed to the change of conformational flexibility of lipase in order to access substrate easily. Further increase in water content of reaction medium caused a slight decrease in the acylation yield. This may be occurrence of the hydrolysis reaction. Meanwhile, the increase in reaction temperature from 40 to 50 °C had a slight positive effect on the acylation yield and the further increase in reaction temperature was no effect on the acylation yield.

Fig. 4b demonstrates the contour graph of the influence of reaction temperature and initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol at bulk water content of 2% and immobilized lipase loading of 30 mg mL^{-1}. In the literature, enol esters, particularly vinyl acetate, were reported as more effective acylating reagents for lipase catalyzed acylation reaction due to their high affinity to serine residues in catalytic site of lipases. Furthermore, the acylation reaction is irreversible because of keto-enol tautomerization [50,54]. The increase of reaction temperature was no significant effect on the yield of reaction, however, the increase in initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol was significant effect on the yield. The acylation yield increased from 41.1 (run number 1) to 45.0% (run number 18) by increasing the initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol from 1.0 to 2.0, respectively, when the reaction temperature was 60 °C.

Enzyme loading is an important factor for application of an immobilized enzyme in industry. Fig. 4c shows the response surface graph of the effect of immobilized lipase loading and initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol at bulk water content of 2% and 50 °C. As shown in Fig. 4c, the acylation yield affected markedly from the immobilized lipase loading. The acylation yield increased linearly with the increase of immobilized lipase loading from 10 to 30 mg mL^{-1}. The acylation yield was observed as 19.1% when initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol was 1.0 and 10 mg mL^{-1} of immobilized lipase used as catalyst (run number 25). When the immobilized lipase loading was increased to 50 mg mL^{-1} and the other reaction conditions were kept constant, the acylation yield increased to 42.7% (run number 22). The increasing of initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol had a very small positive effect on the acylation yield. The acylation yield reached from 42.7% (run number 22) to a maximum point as 46.8% (run number 11) when the initial molar ratio of vinyl acetate to ±-2-(propylamino)-1-phenylethanol was increased from 1.0 to 2.0.

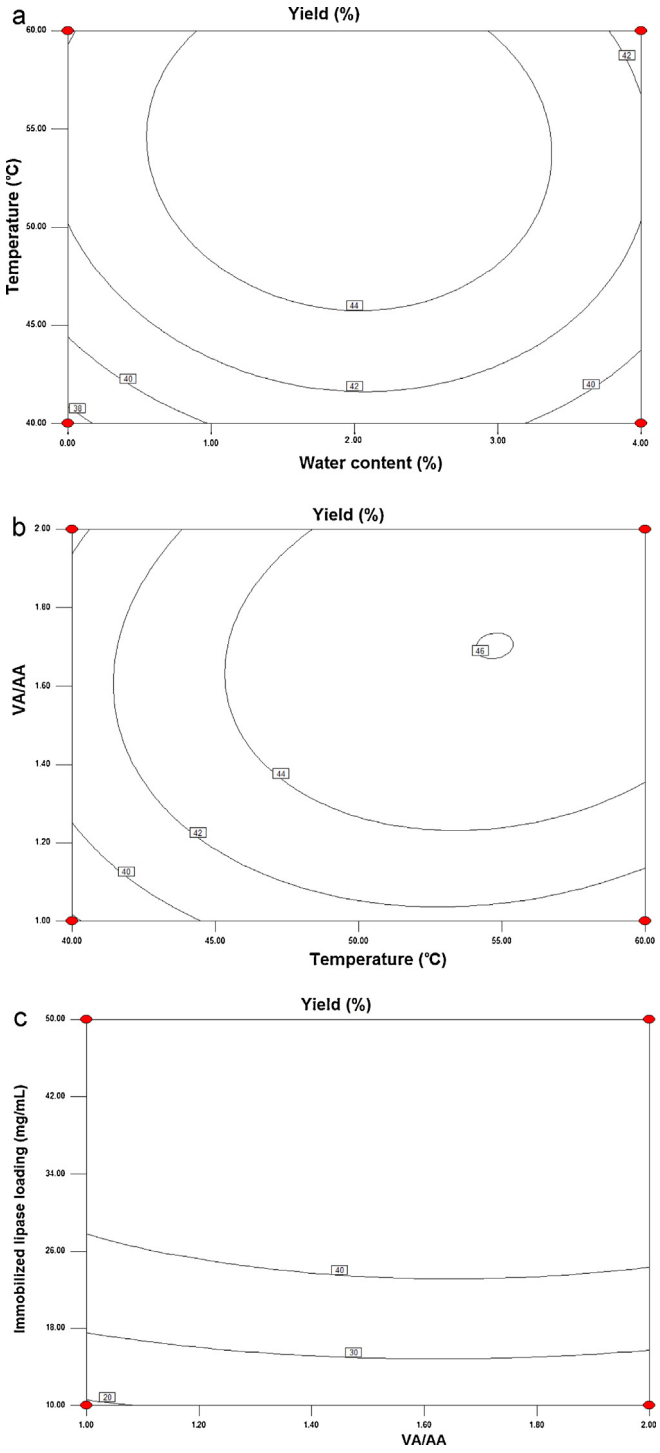


Fig. 4. (a) The contour graph for the asymmetric acylation of ±-2-(propylamino)-1-phenylethanol with vinyl acetate as a function of reaction temperature and water content of reaction medium at a constant lipase loading (30 mg mL^{-1}) and vinyl acetate/±-2-(propylamino)-1-phenylethanol molar ratio (1.5/1.0). (b) The contour graph for the asymmetric acylation of ±-2-(propylamino)-1-phenylethanol with vinylacetate as a function of reaction temperature and initial vinyl acetate/±-2-(propylamino)-1-phenylethanol molar ratio at a constant lipase loading (30 mg mL^{-1}) and bulk water content (2%) of reaction medium. (c) The contour graph for the asymmetric acylation of ±-2-(propylamino)-1-phenylethanol with vinylacetate as a function of immobilized lipase loading and initial vinyl acetate/±-2-(propylamino)-1-phenylethanol molar ratio at a constant reaction temperature (50 °C) and bulk water content (2%) of reaction medium.

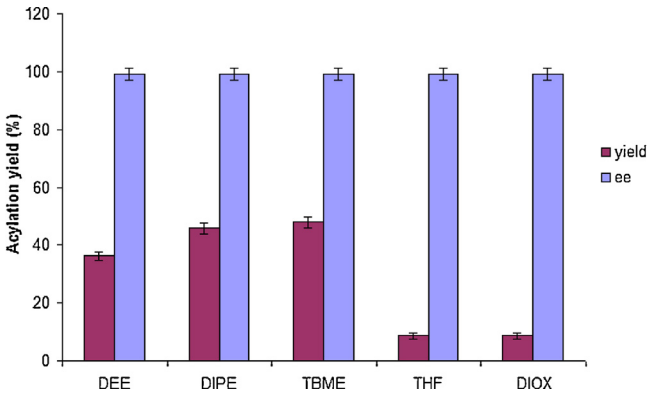


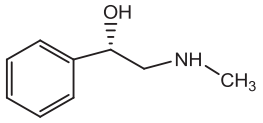
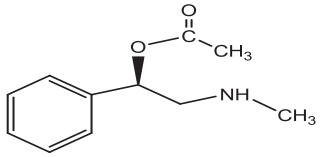
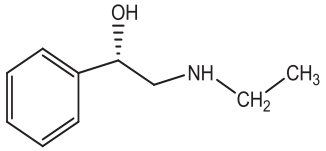
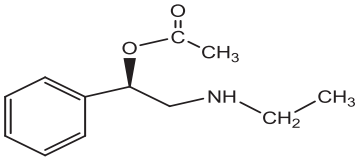
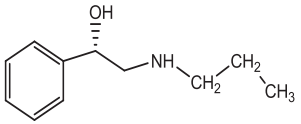
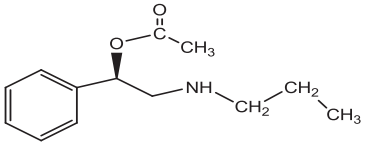
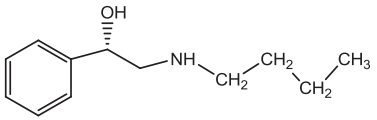
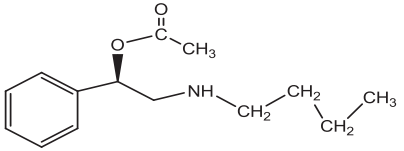
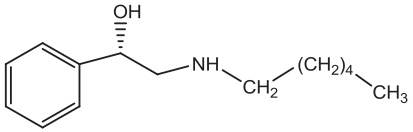
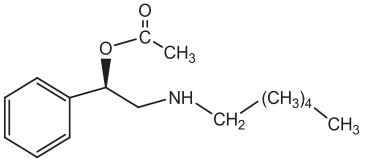
Fig. 5. The performance of the lipase catalyzed acylation reaction in different ether solutions.

To assess the optimum conditions for the acylation reaction, the desired acylation yield was chosen as "maximize" and the bulk water content of reaction medium, reaction temperature, initial molar ratio of vinyl acetate to amino alcohol and immobilized lipase loading were selected as "within the range" on the desirability section of software menu. For this way, the optimal conditions were bulk water content of 1.8%, reaction temperature of 51.5 °C, initial molar ratio of vinyl acetate to amino alcohol of 1.92, and immobilized lipase loading of 47 mg mL^{-1}. At these conditions, the acylation yields were obtained as 47.7 ± 1.2, 48.2 ± 0.8 and 48.1 ± 1.1% for 3 individual studies.

The performance of the immobilized *Pseudomonas* sp. lipase catalyzed acylation reaction was compared in different ether solvents such as DEE (log *P* 0.83), DIPE (log *P* 1.40), DIOX (log *P* −0.27), TBME (log *P* 0.94) and THF (log *P* 0.45) for ±-2-(propylamino)-1-phenylethanol because yield of a lipase catalyzed acylation reaction and enantioselectivity of lipase depend strictly on the nature of the solvent used as the reaction medium that different solvents may exhibit different behaviors to solvate the substrate and, thus, may influence enzyme activity. We selected ether solvents for the reason that the dissolution of amino alcohols used was higher in these solvents than the other commonly used reaction media for lipase catalyzed reaction such as hexane, heptane. The reaction was carried out under the determined optimum conditions. As shown in Fig. 5, the maximum acylation yield was obtained as 48% in TBME. The immobilized lipase catalyzed acylation of ±-2-(propylamino)-1-phenylethanol with vinyl acetate performed sluggishly in DIOX and THF and the acylation yields were determined as 8.7% for the both solvents. The lower lipase activity in DIOX and THF may be attributed to higher hydrophilic character of both solvents than other used ether solvents which cause to strip the essential water molecule around lipase molecule [55,56]. However, the ee values of formed ester did not affect from hydrophobicity or hydrophilicity of the solvents and were determined as >99% for all the reaction media.

Thermal stabilities of the free and immobilized lipase were studied at 40, 50 and 60 °C. The relative activities of free lipase almost decreased linearly for each investigated temperature. After 72 h incubation time, the relative activities of free lipase were measured as 44, 39 and 31%, respectively, for 40, 50 and 60 °C (Fig. 6a). k_i values of the free lipase were calculated as 10.5×10^{-3}, 12.3×10^{-3} and 15.5×10^{-3} h^{-1}, respectively, for 40, 50 and 60 °C and the corresponding $t_{1/2}$ values were estimated as 66.0, 56.3 and 44.7 h. After 4 h incubation time, the relative activities of the immobilized lipase were observed as 100, 95 and 92%, respectively, for 40, 50 and 60 °C. At the end of 72 h incubation time, the corresponding relative activities of immobilized lipase were measured as 86, 81 and 71% (Fig. 6b). For the immobilized lipase, k_i

Table 5
The yield and ee values of remaining aminoalcohols and formed esters and *E* values of the immobilized lipase.

Remaining aminoalcohol	Yield (%)	ee of remaining aminoalcohol (%)	Formed ester	Yield (%)	ee of formed ester (%)	*E* value
 **(*S*)-2-(methylamino)-1-phenylethanol** 1a	50	80	 **(*R*)-2-(methylamino)-1-phenylethyl acetate** 1b	44	>99	>200
 **(*S*)-2-(ethylamino)-1-phenylethanol** 2a	50	80	 **(*R*)-2-(ethylamino)-1-phenylethyl acetate** 2b	45	>99	>200
 **(*S*)-2-(propylamino)-1-phenylethanol** 3a	50	90	 **(*R*)-2-(propylamino)-1-phenylethyl acetate** 3b	48	>99	>200
 **(*S*)-2-(butylamino)-1-phenylethanol** 4a	50	93.2	 **(*R*)-2-(butylamino)-1-phenylethyl acetate** 4b	48.2	>99	>200
 **(*S*)-2-(hexylamino)-1-phenylethanol** 5a	50	>99	 **(*R*)-2-(hexylamino)-1-phenylethyl acetate** 5b	50	>99	>200

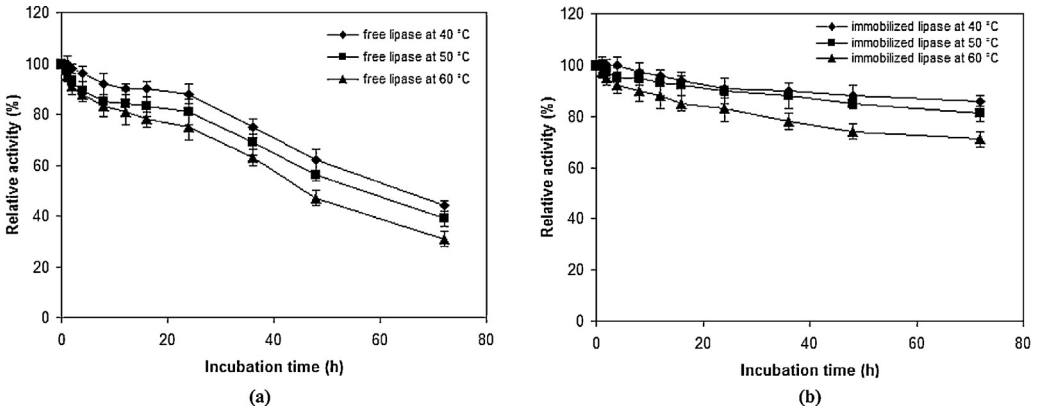


Fig. 6. The thermal stability curves of free and immobilized lipase preparations.

values were estimated as 2.5×10^{-3}, 3.4×10^{-3} and 5.9×10^{-3} h^{-1}, respectively, for 40, 50 and 60 °C and the corresponding $t_{1/2}$ values were determined as 277.2, 203.8 and 117.4 h. These results indicated that thermal stability of the free lipase considerably increased by the immobilization. Mendes et al. [53] showed that the $t_{1/2}$ value of *Thermomyces lanuginosus* lipase immobilized onto chitosan modified with 2,4,6-trinitrobenzene sulfonic acid followed by activation with glutaraldehyde was around 3.90 h at 70 °C. Rodrigues et al. [57] reported that *Candida antarctica* lipase B immobilized onto agarose and chitosan beads via glutaraldehyde retained around 15 and 30% of their initial activities, respectively, at 50 °C after 24 h incubation time whereas the initial activity of free lipase nearly depleted at the same conditions. The corresponding $t_{1/2}$ values were 6.3 and 5.4 h for the aforementioned conditions. Pahujani et al. [58] reported that an alkaline lipase from thermo tolerant *Bacillus coagulans* BTS-3 immobilized onto glutaraldehyde activated Nylon-6 protected 88% of its initial activity at 55 °C after 2 h incubation. Zhu and Sun [59] studied the thermal stability of free and immobilized *Candida rugosa* lipase onto glutaraldehyde-activated nanofibrous membrane and they reported that the free lipase retained around 20% of its initial activity within 75 min incubation at 55 °C, while the immobilized lipase retained 90% of its initial activity under the same conditions.

The immobilized lipase-catalyzed resolution studies were examined for a range of *N*-substituted ±-2-amino-1-phenylethanols under the optimized conditions. The results showed that the selectivity of immobilized lipase for these substrates obeyed the Kazlauskas rule [60]. This rule suggests that the enantiodiscrimination of secondary alcohols by a lipase is based on the size of the substituents [61]. As shown in Fig. 7a–e, (*R*)-enantiomers of tested amino alcohols reacted faster than their (*S*)-enantiomers and the acylation yields of the tested amino alcohols increased depending on the alkyl chain length substituted onto amino group. (*R*)-ester yields obtained were 44, 45, 48, 48.2 and 50%, respectively, for 1b—5b and their ee values were found to be >99% for all the formed esters (Table 5).

E value represents the ability of the enzyme to discriminate between enantiomers. It is generally accepted that a resolution reaction proceed with *E* value above 20 is useful for industrial applications [13]. *E* values of immobilized lipase were determined as >200 toward all the tested substrates, indicating that the immobilized lipase was promising biocatalyst for industrial production

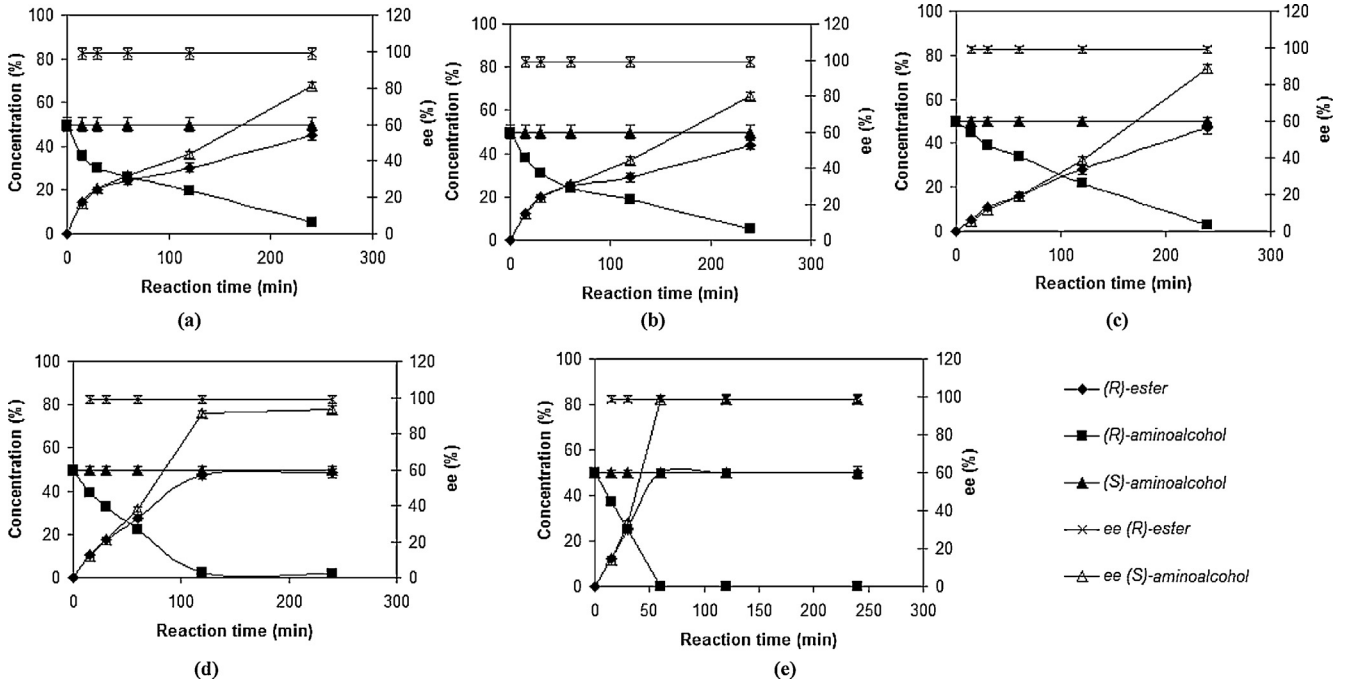


Fig. 7. Time courses of asymmetric acylation of tested ±-2-amino-1-phenylethanols.

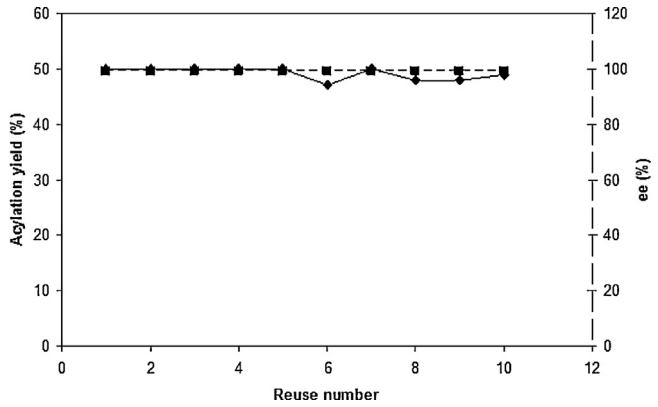


Fig. 8. The repeated-batch operation for the immobilized lipase. The acylation yield and ee values of formed ester are symbolized with (♦) and (■), respectively.

of enantiopure 2-amino-1-phenylethanols and their corresponding esters. The chiral HPLC analyses showed that only (*R*)-enantiomer of the related racemic amino alcohol was acylated for each tested amino alcohol by the immobilized lipase and no spontaneous conversion and byproducts were determined. The results of IR and NMR spectra indicated that the acylation reaction purely took place on the hydroxyl group of 2-amino-1-phenylethanols.

The reusability has a crucial importance for biocatalysts and an enhancement of operational stability utilizes more advantageous. The reusability studies were conducted for only ±-2-(hexylamino)-1-phenylethanol since the highest acylation yield was obtained when it was used as substrate in the experimental studies. To show the possibility of large scale industrial application, the reaction was carried out at the substrate concentration of 5 M. As shown in Fig. 8, the initial activity of immobilized lipase was protected after 5 reuses and 98% of its initial activity was retained at the end of 10 recycles. The ee value of formed ester was determined as >99% after each reuse. This result indicates that the lipase catalyzed asymmetric acylation of ±-2-(hexylamino)-1-phenylethanol may be attractive for large scale industrial application.

4. Conclusion

The results of RSM studies indicated that the proposed mathematical model provided a statistically significant accuracy in the prediction of the response. The results of acylation reactions showed that the immobilized lipase reacted preferentially with (*R*)-enantiomers of tested amino alcohols. The acylation yields increased with the increase of alkyl chain length substituted onto nitrogen atom and the highest acylation yield was obtained as 50% for (*R*)-2-(hexylamino)-1-phenylethyl acetate. *E* values of immobilized lipase were calculated as >200 for all the asymmetric acylation reaction. A retained activity of 98% was obtained for the immobilized lipase after 10 repeated-batch operations. The results of IR and NMR spectra analyses proved that the acylation reaction purely took place on the hydroxyl group of 2-amino-1-phenylethanols. As a result, the asymmetric acylations of (±)-2-amino-1-phenylethanol derivatives with vinyl acetate catalyzed by immobilized *Pseudomonas* sp. lipase can be a very promising tool for the production of (*S*)-2-amino-1-phenylethanol derivatives and their corresponding (*R*)-esters.

Acknowledgement

This study was financially supported by Cukurova University with the project number of FEF 2005D15.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2013.04.019.

References

[1] Agustian J, Kamaruddin AH, Bhatia S. Single enantiomeric β-blockers – the existing technologies. Process Biochem 2010;45:1587–604.
[2] Cullington D, Yassin A, Cleland J. Beta-blockers in the treatment of cardiovascular disease. Prescriber 2008;19:31–9.
[3] Frishman WH. Beta-adrenergic blockers. Circulation 2003;107:117–9.
[4] Kennedy HL. Current utilisation trends for beta blockers in cardiovascular disease. Am J Med 2001;110:2–6.
[5] Mehvar R, Brocks DR. Stereospecific pharmacokinetics and pharmacodynamics of beta-adrenergic blockers in humans. J Pharm Sci 2001;4:185–200.
[6] Barret AM. Cardio beta-adrenoceptor blockade: the guest for selectivity. J Pharm Sci 1985;2:95–108.
[7] Maciejewski M, Półtorak K, Kamińska JE. Lipase resolution of new (±)-3-aryloxy-1-halogenopropan-2-ols: versatile building blocks for β-adrenergic receptor antagonists. J Mol Catal B: Enzym 2010;62:248–56.
[8] Patel RN. Biocatalysis for synthesis for chiral pharmaceutical intermediates. In: Patel RN, editor. Biocatalysis in the Pharmaceutical and Biotechnology Industries. Boca Raton: CRC Press; 2006. p. 105–58.
[9] Baig MN, Santos RCD, Zetzl C, King J, Pioch D, Bowra S. Evaluation and modelling the utility of $SCCO_2$ to support efficient lipase mediated esterification. Enzyme Microb Technol 2011;49:420–6.
[10] Couturier L, Taupin D, Yvergnaux F. Lipase-catalyzed chemoselective aminolysis of various aminoalcohols with fatty acids. J Mol Catal B: Enzym 2009;56:29–33.
[11] Edwinoliver NG, Thirunavukarasu K, Naidu RB, Gowthaman MK, Kambe TN, Kamini NR. Scale up of a novel tri-substrate fermentation for enhanced production of *Aspergillus niger* lipase for tallow hydrolysis. Bioresour Technol 2010;101:6791–6.
[12] Majumder AB, Gupta MN. Stabilization of *Candida rugosa* lipase during transacetylation with vinyl acetate. Bioresour Technol 2010;101:2877–9.
[13] Bornscheuer UT, Kazlauskas RJ. Hydrolases in organic synthesis: regio- and stereoselective biotransformations. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2005. p. 61.
[14] Andrade LH, Silva AV, Pedrozo EC. First dynamic kinetic resolution of selenium-containing chiral amines catalyzed by palladium ($Pd/BaSO_4$) and *Candida antartica* lipase (CAL-B). Tetrahedron Lett 2009;30:4331–4.
[15] Chaubey A, Parshad R, Gupta P, Taneja SC, Qazi GN, Rajan CR, Ponrathnam S. *Arthrobacter* sp. lipase immobilization for preparation of enantiopure masked β-amino alcohols. Bioorg Med Chem 2009;17:29–34.
[16] Pilissão C, Nascimento MG. Effects of organic solvents and ionic liquids on the aminolysis of (*RS*)-methyl mandelate catalyzed by lipases. Tetrahedron: Asymmetry 2006;17:428–33.
[17] Yilmaz E, Can K, Sezgin M, Yilmaz M. Immobilization of *Candida rugosa* lipase on glass beads for enantioselective hydrolysis of racemic Naproxen methyl ester. Bioresour Technol 2011;102:499–506.
[18] Sanfilippo C, Nicolosi G. Enantiomeric resolution by lipase-catalysed esterification of a trans-5,6-dihydro-1,10-phenanthroline possessing helical and central chirality. Tetrahedron: Asymmetry 2007;18:1828–32.
[19] Gotor-Fernández V, Brieva R, Gotor V. Lipases: useful biocatalysts for the preparation of pharmaceuticals. J Mol Catal B: Enzym 2006;40:111–20.
[20] Kamal A, Malik MS, Shaik AA, Azeeza S. Lipase mediated resolution of γ-azidoalcohols in aqueous and organic media: synthesis of (*R*)- and (*S*)-fluoxetine and duloxetine. J Mol Catal B: Enzym 2009;58:132–7.
[21] Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb Technol 2007;40:1451–63.
[22] Hernandez K, Fernandez-Lafuente R. Control of protein immobilization: coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. Enzyme Microb Technol 2011;48:107–22.
[23] Garcia-Galan C, Berenguer-Murcia Á, Fernandez-Lafuente R, Rodrigues RC. Potential of different enzyme immobilization strategies to improve enzyme performance. Adv Synth Catal 2011;353:2885–904.
[24] Palomo JM, Segura RL, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R. Enzymatic resolution of (±)-glycidyl butyrate in aqueous media. Strong modulation of the properties of the lipase from *Rhizopus oryzae* via immobilization techniques. Tetrahedron: Asymmetry 2004;15:1157–61.
[25] Chaubey A, Parshad R, Koul S, Taneja SC, Qazi GN. Enantioselectivity modulation through immobilization of *Arthrobacter* sp. lipase: kinetic resolution of fluoxetine intermediate. J Mol Catal B: Enzym 2006;42:39–44.
[26] Palomo JM, Segura RL, Fernandez-Lorente G, Fernandez-Lafuente R, Guisan JM. Glutaraldehyde modification of lipases adsorbed on aminated supports: a simple way to improve their behaviour as enantioselective biocatalyst. Enzyme Microb Technol 2007;40:704–7.
[27] Volpato G, Filice M, Rodrigues RC, Heck JX, Guisan JM, Mateo C, Marco AAZ. Modulation of a lipase from *Staphylococcus warneri* EX17 using immobilization techniques. J Mol Catal B: Enzym 2009;60:125–32.

[28] Lundell K, Katainen E, Kiviniemi A, Kanerva LT. Enantiomers of adrenaline-type amino alcohols by *Burkholderia cepacia* lipase-catalyzed asymmetric acylation. Tetrahedron: Asymmetry 2004;15:3723–9.
[29] Lee TJF, Stitzel RE. Adrenomimetic drugs. In: Craig CR, Stitzel RE, editors. Modern pharmacology with clinical applications. Philadelphia: Lippincott Williams & Wilkins; 2004. p. 109–21.
[30] Box GEP, Draper NR. Empirical model building and response surfaces. New York: John Wiley & Sons; 1987. p. 477.
[31] Sarabia LA, Ortiz MC. Response surface methodology. In: Brown SD, Tauler R, Walczak B, editors. Comprehensive chemometrics chemical and biochemical data analysis. Amsterdam: Elsevier; 2009. p. 345–90.
[32] Myers RH, Montgomery DC, Anderson-Cook CM. Response surface methodology: process and product optimization using designed experiments. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2009. p. 219.
[33] Soo EL, Salleh AB, Basri M, Rahman RNZA, Kamaruddin K. Response surface methodological study on lipase-catalyzed synthesis of amino acid surfactants. Process Biochem 2004;39:1511–8.
[34] Sharma M, Rastogi NK, Lokesh BR. Synthesis of structured lipid with balanced omega-3:omega-6 ratio by lipase-catalyzed acidolysis reaction: optimization of reaction using response surface methodology. Process Biochem 2009;44:1284–8.
[35] Wu J, Wang J-L, Li M-H, Lin J-P, Wei D-Z. Optimization of immobilization for selective oxidation of benzyl alcohol by *Gluconobacter oxydans* using response surface methodology. Bioresour Technol 2010;101:8936–41.
[36] Martins AB, Graebin NG, Lorenzoni ASG, Fernandez-Lafuente R, Ayub MAZ, Rodrigues 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.
[37] Yücel Y. Optimization of immobilization conditions of *Thermomyces lanuginosus* lipase on olive pomace powder using response surface methodology. Biocatal Agric Biotechnol 2012;1:20–4.
[38] Ray S, Lalman JA, Biswas N. Using the Box–Benkhen technique to statistically model phenol photocatalytic degradation by titanium dioxide nanoparticles. Chem Eng J 2009;150:15–24.
[39] Weetall HH. Covalent coupling methods for inorganic support materials. In: Mosbach K, editor. Methods in enzymology. London: Academic Press; 1976. p. 134–48.
[40] Alptekin Ö, Tükel SS, Yildirim D, Alagöz D. Covalent immobilization of catalase onto spacer-arm attached modified Florisil: characterization and application to batch and plug-flow type reactor systems. Enzyme Microb Technol 2011;49:547–54.
[41] Boratynski J, Zal T. Colorimetric micromethods for glutaraldehyde determination by means of phenol and sulfuric acid or phenol and perchloric acid. Anal Biochem 1990;184:259–62.
[42] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagents. J Biol Chem 1951;193:265–75.
[43] Chiou S-H, Wu W-T. Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. Biomaterials 2004;25:197–204.
[44] Faber K, Hönig H. The computer program was obtained in the ftp-server at ftp://borg185.kfunigraz.ac.at/2001
[45] Tukel SS, Alptekin O. Immobilization and kinetics of catalase onto magnesium silicate. Process Biochem 2004;39:2149–50.
[46] Ozyilmaz G, Tukel SS, Alptekin O. Activity and storage stability of immobilized glucose oxidase onto magnesium silicate. J Mol Catal B: Enzym 2005;35:154–60.
[47] Barbosa O, Torres R, Ortiz C, Fernandez-Lafuente R. Versatility of glutaraldehyde to immobilize lipases: effect of the immobilization protocol on the properties of lipase B from *Candida antarctica*. Process Biochem 2012;47:1220–7.
[48] Borch RF, Bernstein MD, Durst HD. Cyanohydridoborate anion as a selective reducing agent. J Am Chem Soc 1971;93:2897–904.
[49] Hansen EH, Mikkelsen HS. Enzyme-immobilization by the glutardialdehyde procedure. An investigation of the effects of reducing the Schiff-bases generated, as based on studying the immobilization of glucose oxidase to silanized controlled pore glass. Anal Lett 1991;24:1419–1430.
[50] Park SW, Lee JW, Hong SI, Kim SW. Immobilization of glutaryl-7-aminocephalosporanic acid acylase on silica gel and enhancement of its stability. Appl Biochem Biotechnol 2003;104:185–98.
[51] Hermanson GT. Bioconjugate techniques. London: Academic Press; 2008. p. 231.
[52] Mendes AA, Giordano RC, Giordano RLC, de Castro HF. Immobilization and stabilization of microbial lipases by multipoint covalent attachment on aldehyde-resin affinity: application of the biocatalysts in biodiesel synthesis. J Mol Catal B: Enzym 2011;68:109–15.
[53] Mendes AA, de Castro HF, Rodrigues DS, Adriano WS, Tardioli PW, Mammarella EJ, Giordano RC, Giordano RLC. 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.
[54] Wielechowska M, Plenkiewicz J. Lipase-catalyzed separation of the enantiomers of 1-substituted-3-arylthio-2-propanols. Tetrahedron: Asymmetry 2005;16:1199–205.
[55] Hazarika S, Goswami P, Dutta NN, Hazarika AK. Ethyl oleate synthesis by *Porcine pancreatic* lipase in organic solvents. Chem Eng J 2002;85:61–8.
[56] Pilissão C, Carvalho PO, Nascimento MG. Enantioselective acylation of (*RS*)-phenylethylamine catalysed by lipases. Process Biochem 2009;44:1352–7.
[57] Rodrigues DS, Mendes AA, Adriano WS, Gonçalves LRB, Giordano RLC. Multipoint covalent immobilization of microbial lipase on chitosan and agarose activated by different methods. J Mol Catal B: Enzym 2008;51:100–9.
[58] Pahujani S, Kanwar SS, Chauhan G, Gupta R. Glutaraldehyde activation of polymer Nylon-6 for lipase immobilization: enzyme characteristics and stability. Bioresour Technol 2008;99:2566–70.
[59] Zhu J, Sun G. Lipase immobilization on glutaraldehyde-activated nanofibrous membranes for improved enzyme stabilities and activities. React Funct Polym 2012;72:839–45.
[60] Kazlauskas RJ, Weissfloch ANE, Rappaport AT, Cuccia LA. A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*. J Org Chem 1991;56:2656–65.
[61] Sorgedrager MJ, Malpique R, van Rantwijk F, Sheldon RA. Lipase catalysed resolution of nitro aldol adducts. Tetrahedron: Asymmetry 2004;15:1295–9.