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Enzymatic synthesis of fruit flavor esters by immobilized lipase from *Rhizopus oligosporus* optimized with response surface methodology

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ABSTRACT

Short chain aliphatic esters have immense applications as flavors in fruit juices, cheeses, baked goods, candies, beverages and ice creams. Two such flavor esters (*n*-butyl acetate and *n*-propyl acetate) were synthesized enzymatically by transesterification of vinyl acetate with alcohols namely *n*-butanol and *n*-propanol, respectively, in solvent-free systems. These two synthesized esters occur naturally in various fruits like apple, strawberry, pear, etc. Lipase from *Rhizopus oligosporus* NRRL 5905 immobilized onto cross-linked silica gel was used for synthesis of the esters. The process parameters (reaction time, enzyme amount, additional water and shaking speed) were studied to achieve the highest yield of the esters. Maximum conversion of *n*-butyl acetate (50%) and *n*-propyl acetate (56%) were achieved after 24 h of reaction at 30 °C, at an enzyme concentration of 25% (w/v) of reaction mixture. This process was further optimized using response surface methodology (RSM) based on a three-level, four-variable central composite design (CCD). The optimum molar conversion of 54.6% using 27.5% enzyme concentration at 215 rpm and 26.5 °C for 28 h and 56.5% molar conversion using 29.8% enzyme concentration at 101 rpm and 28.2 °C for 28 h were achieved for *n*-butyl and *n*-propyl acetates, respectively. Immobilized lipase could be used for three cycles for synthesis of *n*-butyl acetate and *n*-propyl acetate, respectively, with almost 100% retention of molar conversion. The K_m and V_{max} values were determined to be 227 mM and 322 μmol/(g-h), respectively, for *n*-butyl acetate while for *n*-propyl acetate the respective values were 222 mM and 385 μmol/(g-h).

1. Introduction

Short chain aliphatic esters are small enough to be volatile and are therefore capable of producing pleasant fruity notes. Many aliphatic acetate esters are components of natural flavors. These esters are versatile components of flavors and fragrances which are widely used in the food, beverage, cosmetic, and pharmaceutical industries [1]. Traditional methods of synthesizing these flavor compounds include extraction from natural sources and chemical syntheses, which nowadays are being replaced by enzymatic syntheses as products obtained from enzyme mediated reactions are considered natural [2]. Since lipases (EC 3.1.1. 3) are capable of hydrolyzing ester bonds, catalyzing the reverse reaction of esterification/transesterification, in the present study lipase from a generally regarded as safe (GRAS) organism has been selected as the catalyst for synthesis of the flavor esters. The enzyme was made suitable for repetitive use by immobilization on a solid support. Use of immobilized biocatalyst enabled easy recovery from the reaction mixture. Although synthesis of flavor esters in presence of various organic solvents are well documented [3,4], in order to avoid organic solvent toxicity and to facilitate downstream processing, attempts have been made for synthesis of these esters in absence of added solvents. Based on previous reports [5–7] that direct esterification with acetic acid leads to inhibition of the biocatalyst, in the present study transesterification was attempted using vinyl acetate as the acyl donor. Vinyl alcohol formed after transesterification of vinyl acetate tautomerises to acetaldehyde, thus making the process irreversible [8].

In the present study, enzymatic synthesis of two short chain flavor esters (*n*-butyl acetate and *n*-propyl acetate) with fruity aroma have been attempted using a lipase produced by *Rhizopus oligosporus* NRRL 5905, a new strain, immobilized onto cross linked

Abbreviations: SIL, silica immobilized lipase; GRAS, generally regarded as safe; RSM, response surface methodology; CCD, central composite design; NMR, nuclear magnetic resonance; IR, infra-red; BA, *n*-butyl acetate; PA, n-propyl acetate; MC, molar conversion; K_{mA}, Michaelis constant for respective alcohols; K_{mB}, Michaelis constant for vinyl acetate; V, initial rate; V_{max}, maximum velocity; [A], concentration of respective alcohols; [B], concentration of vinyl acetate.

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silica gel 60. Both the esters are naturally present in apple, strawberry and pear [9–13].

Although only a few reports [14–16] are available for lipase-mediated synthesis of *n*-butyl acetate, this is the first report for enzymatic synthesis of *n*-propyl acetate. The work focuses on the optimization of the parameters for the synthesis of *n*-butyl acetate and *n*-propyl acetate by transesterification of vinyl acetate with *n*-butanol and *n*-propanol, respectively, using immobilized lipase from *R. oligosporus* employing RSM. The acylating agent (vinyl acetate) was used in large excess compared to alcohol and this reduced the most accepted "*Ping-Pong Bi-Bi*" mechanism to simpler Michaelis–Menten model. Efficiency of the silica immobilized lipase (SIL) for synthesis of the aforesaid esters were estimated from the respective K_m and V_{max} values which were evaluated by using a pseudo-first-order kinetic model.

2. Materials and methods

2.1. Microorganism

The strain *R. oligosporus* NRRL 5905 was maintained on potato dextrose agar slants.

2.2. Chemicals

Vinyl acetate was purchased from HiMedia. All other solvents and reagents were either of HPLC grade or AR grade and were obtained from Merck.

2.3. Lipase production from R. oligosporus

For production of extracellular lipase from *R. oligosporus* NRRL 5905, fermentation was carried out in a Erlenmeyer flask (250 ml) containing wheat bran (8 g) supplemented with modified Czapekdox medium (12 ml) (KH_2PO_4 1.0 g/l, $MgSO_4{\cdot}7H_2O$ 0.5 g/l, KCl 0.5 g/l, $NaNO_3$ 2.5 g/l, glucose 50 g/l and mahua (*Madhuka indica*) oil 100 ml/l, pH 5). The medium was sterilized by autoclaving at 15 psi for 20 min and was inoculated with spore suspension (1 ml, 10^8 spores/ml) followed by incubation at 35 °C and 85% relative humidity. After 5 days, the fermented biomass was soaked in water (32 ml) followed by extraction through wet-cheese cloth. The supernatant was collected by centrifugation at 10,000 rpm for 10 min and was partially purified by ammonium sulphate (60% saturation) at 4 °C. The precipitate was dissolved in phosphate buffer (50 Mm, pH 7) and the concentrated enzyme was lyophilized and used for subsequent experiments.

2.4. Preparation of activated silica support for immobilization

Support for immobilization of the enzyme was made by amination of silica gel 60 followed by cross-linking with glutaraldehyde. For the preparation of the aminated support silica gel 60 (5 g) was refluxed with ethylenediamine (EDA) (50 ml) for 4 h. After the reaction, the aminated silica was filtered, washed several times with ethanol and de-ionized water. The aminated silica was further washed with phosphate buffer (50 mM, pH 8) and was stirred for 2 h at 4 °C with 2.5% glutaraldehyde (100 ml) in the same buffer. After the completion of the reaction, the cross-linked silica (activated silica) was washed with phosphate buffer (50 mM, pH 8) followed by phosphate buffer (10 mM, pH 6.5) to make the silica support completely free from glutaraldehyde.

2.5. Immobilization of lipase on activated silica

Lyophilized lipase (1 g, 1.13 U/mg of protein) dissolved in phosphate buffer (pH 7, 10 mM, 20 mL) was mixed with activated silica gel (4 g) support. Cold acetone (80 ml, −20 °C) was added to it and the mixture was stirred at 4 °C with a magnetic stirrer in an ice bath for 30 min. SIL was filtered, washed with acetone (5 ml, −10 °C) and dried in air. The immobilized catalyst was stored at −4 °C. The activity of the immobilized enzyme was 60 U/g.

2.6. Determination of enzymatic activity

Lipase assay was performed spectrophotometrically using *p*-nitrophenyl palmitate as the substrate [17]. *p*-Nitrophenol was liberated from *p*-nitrophenyl palmitate by lipase-mediated hydrolysis. One unit (U) of lipase was defined as the amount of enzyme that liberates 1 μM of *p*-nitrophenol per min under the assay conditions. Enzyme activity was expressed as U/g of silica.

2.7. Transesterification reaction

The reactions were carried out in screw-capped vials (5 ml) containing solution of the alcohols (500 mM, *n*-butanol and *n*-propanol) in vinyl acetate. The reaction was initiated by addition of SIL. The vials were placed in an orbital shaker (200 rpm) at 30 °C, along with the respective controls (reaction mixture without SIL). Samples were collected at regular intervals, dried over 4 Å molecular sieves and analyzed by gas chromatography. All the experiments were carried out in triplicate and the values are represented as mean of the three different replicates.

2.8. Experimental setup for kinetic study

For determining the kinetics of solvent-free synthesis of flavor esters, reaction mixtures were prepared with varying alcohol (*n*-butanol or *n*-propanol) concentrations (25–1000 mM) in vinyl acetate. Reaction was initiated by addition of 5 mg of SIL in each of the reaction mixtures. The reaction temperature was maintained at 30 °C. The maximum initial rate (V_{max}) of consumption of the substrates and Michaelis–Menten constants (K_m) were determined by linear regression using the Lineweaver–Burk approach. The apparent K_m and V_{max} values for synthesis of flavor esters by the immobilized lipase preparation were determined from the slope (K_m/V_{max}) and the intercept ($1/V_{max}$) of Lineweaver–Burk plot.

2.9. Analysis procedure

GC analysis was performed with (Agilent GC-6820) equipped with HP-5 capillary column (0.25 μm × 0.25 mm × 30 m) and flame ionization detection (FID) detector. For *n*-butyl acetate, injector and detector temperatures were set at 200 and 280 °C, respectively. The temperature of the oven was initially maintained at 40 °C for 3 min, then was raised to 50 °C at the rate of 5 °C per min and then maintained at 50 °C for 1 min. For *n*-propyl acetate, the oven temperature was initially maintained at 40 °C for 3 min, thereafter raised to 100 °C at a rate of 5 °C per min. The injector and detector temperatures were set to 150 and 280 °C, respectively. Nitrogen was used as the carrier gas with a flow rate of 1 ml/min. The retention times of *n*-butyl acetate and *n*-propyl acetate were 5.2 and 3.14 min, respectively.

2.10. Optimization of flavor esters synthesis using RSM

A three-level, four-variable central composite design (CCD) was adopted for optimizing the syntheses of flavor esters (*n*-butyl acetate and *n*-propyl acetate) using MINITAB 14 software. The different variables considered for the syntheses of the flavor esters (*n*-butyl acetate and *n*-propyl acetate) include the reaction time

Table 1
Transesterification variables and levels for central composite design.

Sl. no.	Variable descriptions	Notation		Levels		
		Coded	Uncoded	High (+1)	Middle (0)	Low (−1)
1	Reaction time (h)	X_1	*A*	30	25	20
2	Temperature (°C)	X_2	*B*	35	30	25
3	Agitation (rpm)	X_3	*C*	300	200	100
4	Enzyme concentration (%)	X_4	*D*	30	25	20

Table 2
Experimental results based on central composite design.

Sl. no.	Input parameters			EC (%)	Response molar conversion (%)	
	RT (h)	Temperature (°C)	Agitation (rpm)		BA	PA
1	20	25	100	20	18.3	22.3
2	30	25	100	20	32.6	37.7
3	20	35	100	20	26.6	30.0
4	30	35	100	20	34.3	38.3
5	20	25	300	20	21.6	26.3
6	30	25	300	20	45.2	48.7
7	20	35	300	20	21.8	24.7
8	30	35	300	20	42.2	46.6
9	20	25	100	30	23.0	30.0
10	30	25	100	30	47.2	49.94
11	20	35	100	30	29.4	33.45
12	30	35	100	30	52.7	56.62
13	20	25	300	30	22.3	25.32
14	30	25	300	30	49.9	53.92
15	20	35	300	30	28.2	29.25
16	30	35	300	30	52.5	56.45
17	20	30	200	25	39.7	42.65
18	30	30	200	25	55.6	59.75
19	25	25	200	25	46.6	47.76
20	25	35	200	25	45.3	48.67
21	25	30	100	25	46.2	50.73
22	25	30	300	25	47.6	50.63
23	25	30	200	20	44.6	47.62
24	25	30	200	30	55.6	58.63
25	25	30	200	25	51.0	56.00
26	25	30	200	25	51.0	56.00
27	25	30	200	25	51.0	56.00

(20–28 h), temperature (25–35 °C), agitation speed (100–300 rpm) and enzyme concentration (20–30%). The independent variables, their levels and real values are presented in Table 1. *n*-butyl acetate and *n*-propyl acetate syntheses were carried out in screw-capped vials at different reaction times, temperatures, agitation speeds and varying lipase concentrations according to CCD experimental design presented in Table 2. The collected samples were analyzed by gas chromatography.

3. Results and discussion

3.1. Shaking speed

In immobilized enzyme catalyzed reactions, a bi-phasic catalytic system exists due to the insolubility of enzyme in organic reaction mixture resulting in external mass transfer limitations in such reaction systems. To observe the effect of mass transfer limitations, experiments were carried out at different agitation speeds varying from 50 to 300 rpm. It was found (Fig. 1) that the substrate conversion increased progressively to reach a maximum at 200 rpm, beyond which the conversion remained almost unchanged. With the increase in shaking speed, the film thickness around the solid SIL particles decreased and subsequently the mass transfer resistance decreased. Minimum mass transfer resistance was noticed at 200 rpm which did not further decrease upon increasing the shaking speed beyond 200 rpm implying that the reaction rate was not limited by the mass transfer limitation above this shaking speed.

3.2. Effect of initial addition of water

Water has immense importance in lipase-mediated reactions both for the maintenance of three-dimensional structural integrity and also for optimal catalytic activity of the enzyme [18]. The effect of initial water on enzymatic activity was examined by adding water ranging from 2 to 20% (v/v) to the reaction mixture and the molar conversion was measured after 6 h. For both the esters, molar conversion decreased with the increase in added water. Maximum molar conversion was obtained in absence of water (10% for *n*-butyl acetate and 13% for *n*-propyl acetate), while

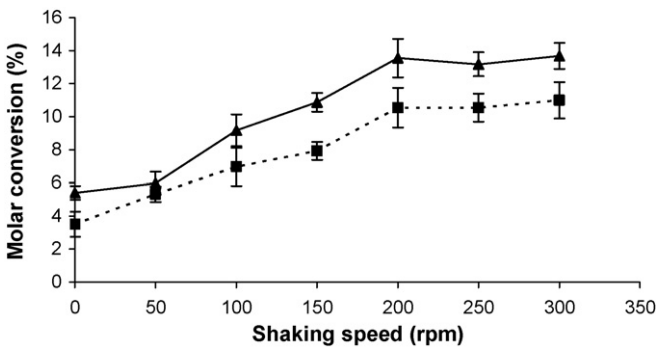

Fig. 1. Effect of shaking speed on synthesis of *n*-butyl acetate (■) and *n*-propyl acetate (▲). Reaction conditions: temperature: 30 °C, SIL concentration: 10% (w/v) and reaction time: 6 h.

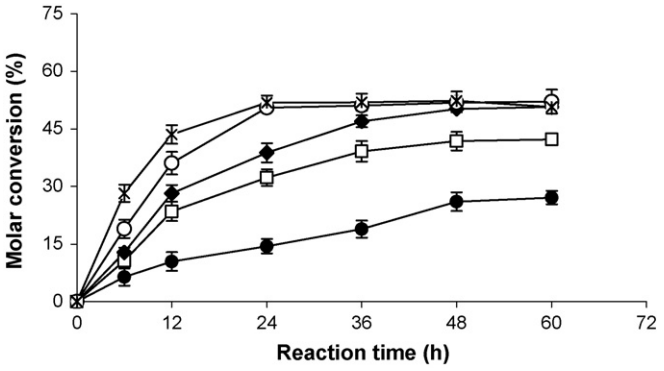

Fig. 2. Effect of enzyme amount on enzymatic synthesis of *n*-butyl acetate (●) 5% (w/v) SIL, (□) 10% (w/v) SIL, (♦) 20% (w/v) SIL, (○) 25% (w/v) SIL and (x) 30% SIL. Reaction conditions: temperature: 30 °C, shaking speed: 200 rpm and additional water: 0%.

the conversion dropped to about 5% for both the esters at 20% water in the reaction mixture. This implies that the amount of water initially adsorbed on the cross-linked silica is sufficient for the enzyme to exhibit highest activity. With addition of water beyond a critical point the cross-linked silica support gets strongly hydrated and the thickness of water layer around the enzyme molecule increases. This results in problem in diffusion of the substrates to the active site of the enzyme disfavoring the esterification reaction. Similar results were reported by Ghamgui et al. [19] for the enzymatic synthesis of butyl oleate. Mukherjee [20] suggested that low water content favors ester synthesis over hydrolysis.

3.3. Effect of enzyme concentration

The effect of enzyme amount on synthesis of flavor esters was studied by varying the SIL concentration from 2.5% (1.5 U/ml) to 30% (18 U/ml) (w/v) of the reaction mixture keeping all other parameters constant. From Figs. 2 and 3, it was observed that 50 and 56% molar conversion for *n*-butyl acetate and *n*-propyl acetate synthesis could be achieved after 24 h of reaction at an enzyme concentration of 25% (15 U/ml). Upon increasing the enzyme amount further, the reaction rate did not increase significantly which may be due to the lack of substrate to access the active site of enzyme, and/or difficulty in maintaining uniform suspension of the biocatalysts at higher enzyme concentration [21].

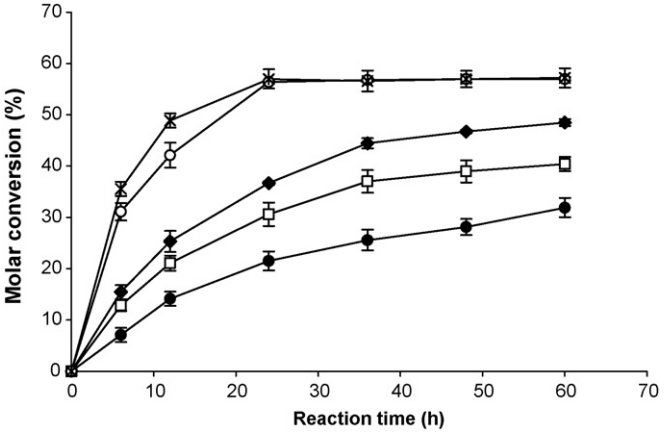

Fig. 3. Effect of enzyme amount on enzymatic synthesis of *n*-propyl acetate (●) 5% (w/v) SIL, (□) 10% (w/v) SIL, (♦) 20% (w/v) SIL, (○) 25% (w/v) SIL, (x) 30% (w/v) SIL. Reaction conditions: temperature: 30 °C, shaking speed: 200 rpm, additional water: 0%.

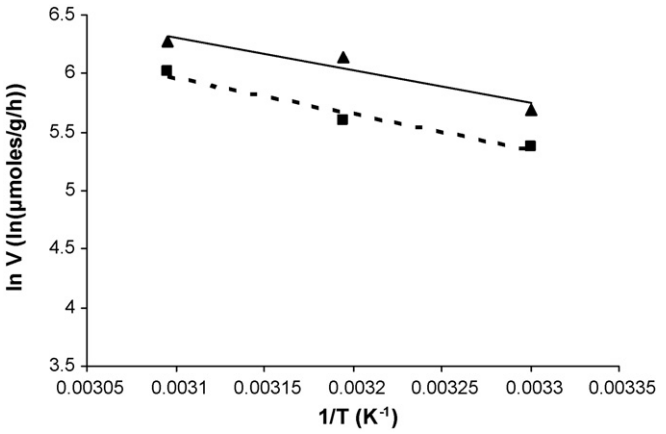

Fig. 4. Arrhenius plot for determination of activation energies for *n*-butyl acetate (■) and *n*-propyl acetate (▲) synthesis.

3.4. Effect of temperature

In lipase-catalyzed reactions, temperature significantly influences both the initial rate of the reaction and stability of the enzyme. In most cases, rate of reaction increases with temperature, while the stability of enzymes declines [22,23]. Increase of enzymatic reaction rate with temperature is explained by the transition state theory [24]. Considering the boiling point of vinyl acetate (73 °C), reaction rate was explored at various temperatures in the range of 30–70 °C. With increase in temperature from 30 to 50 °C, the rate of reaction increased following Arrhenius model and beyond 50 °C, the reaction rate dropped. For *n*-butyl acetate, the rate increased from 217 to 410 μmol/(g-h) while for *n*-propyl acetate, the rate increased from 298 to 529 μmol/(g-h). Hence activation energies were determined in this temperature range from the Arrhenius plot (Fig. 4) based on initial reaction rate. The apparent activation energies for synthesis of *n*-butyl acetate and *n*-propyl acetate by SIL were calculated to be 26 and 23 kJ/mol, respectively. This indicated that the enzyme tertiary structure might have been disrupted causing it to denature at higher temperatures [7]. The result of the present study is in confirmation with the earlier reports where the optimum temperatures for lipase action lie between 30 and 62 °C [25]. Yong and Al-Duri [26] suggested that almost all enzymes suffer from thermal denaturation at temperatures above 45 °C.

3.5. Stability study of SIL

The stability of SIL in reaction mixture was studied from the residual activity of the biocatalyst at different time intervals at 30 °C. The stability of the biocatalyst was not affected by the reaction mixture (500 mM alcohol in vinyl acetate). Moreover, it was observed that even on increasing the concentration of the alcohols in vinyl acetate up to 1000 mM, the activity of SIL remained unaltered (data not shown). For estimating thermal stability of the biocatalyst, reaction mixtures were pre-equilibrated at three different temperatures (30, 40 and 50 °C) prior to addition of SIL. Enzyme samples were collected from the reaction mixture at definite time intervals for activity test. Fig. 5 shows that although SIL lost 25% of its activity at 50 °C after 24 h of reaction, this deactivation was not observed at lower temperatures which were reflected in the reusability of the enzyme at 30 °C.

3.6. Optimization of flavor esters synthesis using RSM

The experimental design (Table 2) was carried out in order to study the effect of different variables on the synthesis of *n*-butyl

Table 3
Results of significance test for BA and PA molar conversion (coded form).

Sl. no.	Terms	Coefficient		SE coefficient		*T*		*P*	
		BA	PA	BA	PA	BA	PA	BA	PA
1	Constant	51.7	55.4	0.8	0.66	64.7	84.6	0.00	0.00
2	X_1	10.1	10.2	0.51	0.42	19.7	24.4	0.00	0.00
3	X_2	1.46	1.22	0.51	0.42	2.86	2.93	0.01	0.01
4	X_3	1.18	0.71	0.51	0.42	2.31	1.71	0.04	0.11
5	X_4	4.09	3.97	0.51	0.42	8.01	9.49	0.00	0.00
6	X_1^2	−4.37	−3.95	1.35	1.11	−3.23	−3.56	0.01	0.00
7	X_2^2	−6.09	−6.94	1.35	1.11	−4.50	−6.26	0.00	0.00
8	X_3^2	−5.08	−4.48	1.35	1.11	−3.76	−4.03	0.00	0.00
9	X_4^2	−1.86	−2.03	1.35	1.11	−1.38	−1.83	0.19	0.09
10	X_1X_2	−0.91	−0.36	0.54	0.44	−1.67	−0.81	0.12	0.43
11	X_1X_3	1.65	2.08	0.54	0.44	3.03	4.69	0.01	0.00
12	X_1X_4	2.09	1.92	0.54	0.44	3.84	4.32	0.00	0.00
13	X_2X_3	−1.01	−0.97	0.54	0.44	−1.87	−2.18	0.09	0.05
14	X_2X_4	0.82	0.75	0.54	0.44	1.51	1.69	0.16	0.12
15	X_3X_4	−1.16	−1.45	0.54	0.44	−2.14	−3.26	0.06	0.01

	SS	*R*-sq	*R*-sq (adj)
For BA	2.165	98.5%	96.8%
For PA	1.778	99.0%	97.8%

Table 4
Results of ANOVA—BA and PA molar conversion (%).

Source	DF		Seq SS		Adj SS		Adj MS		*F*		*P*	
	BA	PA	BA	PA	BA	PA	BA	PA	BA	PA	BA	PA
Regression	14	14	3742	3776	3742	3776	267	270	57.0	85.3	0.00	0.00
Linear	4	4	2189	2205	2189	2205	547	551	116	174	0.00	0.00
Square	4	4	1378	1384	1378	1384	345	346	73.5	109	0.00	0.00
Interaction	6	6	174	188	174	188	29	31	6.19	10	0.00	0.00
Residual error	12	12	56.3	38	56.3	38	4.69	3.17				
Lack-of-fit	10	10	56.3	38	56.3	38	5.63	3.8				
Pure error	2	2	0.00	0.00	0.00	0.00	0.00	0.00				
Total	26	26	3798	3814								

and *n*-propyl acetates. The effect of each variable, as well as the interaction between them, is reported in Table 3 for *n*-butyl acetate and *n*-propyl acetate synthesis, respectively. The Analysis of variance (ANOVA) is presented for *n*-butyl acetate and *n*-propyl acetate syntheses in Table 4. The pure errors were very low indicating the good reproducibility of the obtained data. The coefficient of regression was found to be 96.8 and 97.8% for *n*-butyl acetate and *n*-propyl acetate synthesis and the results of *F*-test were good indicators for the models (coded equations) representative of the actual relationship between the percent molar conversion as the response and the variables.

The molar conversion (MC) (%) of *n*-butyl acetate was expressed as a non-linear function of the input process parameters in coded form as follows:

$$\mathrm{MC}(\%) = 51.7 + 10.1X_1 + 1.46X_2 + 1.18X_3 + 4.09X_4 - 4.36X_1^2 - 6.08X_2^2 - 5.08X_3^2 - 1.86X_4^2 - 0.90X_1X_2 + 1.64X_1X_3 + 2.08X_1X_4 - 1.01X_2X_3 + 0.82X_2X_4 - 1.16X_3X_4 \quad (1)$$

The molar conversion (MC) (%) of *n*-propyl acetate was expressed as a non-linear function of the input process parameters in coded form as follows:

$$\mathrm{MC}(\%) = 55.43 + 10.2X_1 + 1.23X_2 + 0.72X_3 + 3.97X_4 - 3.95X_1^2 - 6.94X_2^2 - 4.47X_3^2 - 2.03X_4^2 - 0.36X_1X_2 + 2.09X_1X_3 + 1.92X_1X_4 - 0.97X_2X_3 + 0.75X_2X_4 - 1.44X_3X_4 \quad (2)$$

Regression Eqs. (1) and (2) were optimized by response optimizer using MINITAB 14 (MINITAB Inc., Pennsylvania, USA). The optimum molar conversion of 54.6% was obtained using 27.5% enzyme concentration at 215 rpm and 26.5 °C for 28 h in case of *n*-butyl acetate. Whereas for *n*-propyl acetate the optimal molar conversion of 56.5% was obtained using 29.8% enzyme concentration at 102 rpm and 28.2 °C for 28 h.

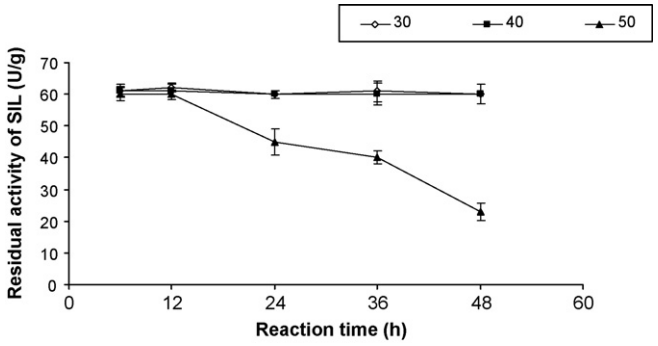

Fig. 5. Stability study of silica immobilized lipase (SIL).

3.7. Reusability of immobilized lipase

The major advantage of immobilized lipase lies in its reusability, which makes the process cost effective. To evaluate the reusability of the immobilized biocatalyst after completion of reaction, the enzyme was collected by centrifugation at 5000 rpm for

10 min, washed with hexane to remove the reactants and products adsorbed on the matrix, dried at room temperature and resuspended in the reaction mixture for the next run under same conditions. It was observed that the molar conversion of the substrates remained unchanged for three successive cycles for synthesis of either of the esters. Thereafter, the substrate conversion dropped by almost 30 and 37% for *n*-butyl acetate and *n*-propyl acetate, respectively, in the subsequent runs. This could be attributed to the enzyme leaking or denaturation of the enzyme on repetitive use.

3.8. Characterization of purified product

3.8.1. IR spectroscopic study

FTIR spectrum of the isolated products was analyzed for *n*-butyl acetate and *n*-propyl acetate, transmittance bands were observed around 1200 cm^{-1} (corresponding to C–O stretch of the ester group), 1680 cm^{-1} (corresponding to the C=O stretch of the ester group), 3000 cm^{-1} (corresponding to aliphatic C–H stretch).

3.8.2. NMR study

The identities of the synthesized flavor esters (*n*-butyl acetate and *n*-propyl acetate) were confirmed by: ^{1}H NMR (400 MHz, $CDCl_3$) spectroscopy. Five signals in the NMR spectrum of *n*-butyl acetate correspond to the five different protons present in the molecule. The signals and the corresponding protons are underlined below:

δ4(2H, t, $CH_3CH_2CH_2\underline{CH_2}OC(=O)CH_3$), δ2(3H, s, $-OC(=O)\underline{CH_3}$),

δ1.6(2H, m, $CH_3CH_2\underline{CH_2}CH_2OC(=O)CH_3$),

δ1.3(2H, m$CH_3\underline{CH_2}CH_2CH_2OC(=O)CH_3$),

δ0.9(3H, t$\underline{CH_3}CH_2CH_2CH_2OC(=O)CH_3$).

For *n*-propyl acetate the four signals correspond to four different kinds of protons as are underlined below:

δ4(2H, t, $CH_3CH_2\underline{CH_2}OC(=O)CH_3$), δ2(3H, s, $-OC(=O)\underline{CH_3}$),

δ1.6(2H, m, $CH_3\underline{CH_2}CH_2OC(=O)CH_3$),

δ0.9(3H, t, $\underline{CH_3}CH_2CH_2CH_2OC(=O)CH_3$).

n-Butyl acetate and *n*-propyl acetate have five and four different kind of protons, respectively. The C-1 protons of both the molecules appear most downfield (δ4) and the three acetyl protons appear as a singlet around δ2. The rest of the protons on C-2, C-3 and C-4 atoms of *n*-butyl acetate depending on their environment had chemical shifts at δ1.6, δ1.3 and δ0.9, respectively. Theoretically, with the decrease in electronegativity the signals appear more upfield. The C-2 and C-3 protons of *n*-propyl acetate appeared at δ1.6 and δ0.9, respectively.

3.9. Kinetics study

To evaluate the kinetic parameters of SIL mediated synthesis of *n*-butyl acetate and *n*-propyl acetate, pseudo-first-order model was adopted as was reported by Luhong et al. [27]. The most general and accepted description of the catalytic action of lipases is *Ping-Pong Bi-Bi* mechanism [28]. Previous reports [29–31] are available where kinetic study of acetylation of various alcohols in organic solvents obeyed "*Ping-Pong Bi-Bi*" mechanism.

The rate equation for such type of reactions was given by Romero et al. [32] as

$$V = \frac{V_{\max}[A][B]}{K_{mB}[A] + K_{mA}[B] + [A][B]} \quad (3)$$

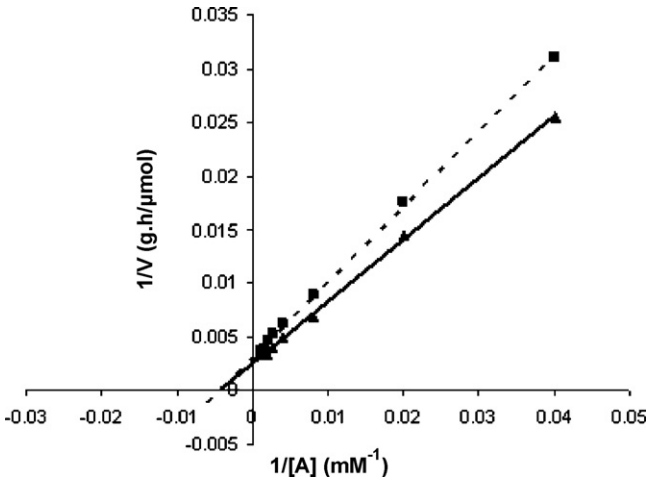

Fig. 6. Double-reciprocal plot (Lineweaver–Burk) of initial velocity (V) against initial substrate (A: *n*-butyl alcohol (■)/*n*-propyl alcohol (▲)) concentration Reaction conditions: temperature: 30 °C, shaking speed: 200 rpm and initial water: 0%.

where V is the initial velocity, V_{max} is the maximum velocity and K_{mA} is the Michaelis constant for the respective alcohols, K_{mB} is the Michaelis constant for vinyl acetate, [A] and [B] are the initial concentrations of alcohol and vinyl acetate, respectively.

Although for synthesis of each of the two short chain acetate esters, two substrates (alcohol (A) and vinyl acetate (B)) were used, the reaction mixture was saturated with vinyl acetate so that the concentration of vinyl acetate remained constant and the reaction rate depended only on alcohol concentration. Thus although the reactions followed *Ping-Pong Bi-Bi* mechanism, the reactions obeyed Michaelis–Menten kinetics for single substrate as observed from the relationship between initial rate and alcohol concentration.

This fact can be supported further from the following derivation. Eq. (3) can be rearranged as

$$V = \frac{V_{\max}[A]}{K_{mB}[A]/[B] + K_{mA} + [A]} \quad (4)$$

In presence of large excess of substrate B (vinyl acetate), the first term in the denominator of Eq. (4) tends to be zero. Thus Eq. (4) takes the following form:

$$V = \frac{V_{\max}[A]}{K_{mA} + [A]} \quad (5)$$

Thus for all initial concentrations of the respective alcohols [A], the reaction rate became independent of the concentration of vinyl acetate [B] and the reaction followed pseudo-first-order reaction kinetics.

The double-reciprocal plot of initial velocity and alcohol concentration was linear from which the kinetic parameters, K_m and V_{max} were evaluated to be 227.4 mM and 322.6 μmol/(g-h) for *n*-butyl acetate, respectively, while for *n*-propyl acetate the respective values were 221.6 mM and 384.6 μmol/(g-h) (Fig. 6).

4. Conclusion

The present work is a comprehensive study on enzymatic synthesis of *n*-butyl acetate and *n*-propyl acetate through transesterification reaction using lipase immobilized onto cross linked silica gel 60. The immobilized biocatalyst worked efficiently for the synthesis of two new flavor esters yielding 50 and 56% of *n*-butyl acetate and *n*-propyl acetate, respectively. The optimum molar conversion of 54.6% using 27.5% enzyme concentration at 215 rpm and 26.5 °C for 28 h and 56.5% using 29.8% enzyme concen-

tration at 102 rpm and 28.2 °C for 28 h were achieved for *n*-butyl and *n*-propyl acetates, respectively, using RSM approach. So this immobilized biocatalyst can be commercially exploited for the synthesis of other short chain flavor esters. The reaction conditions to carry out the synthesis of the esters are mild and use of immobilized lipase enabled repetitive use of the biocatalyst. The article focuses on effective application of pseudo-first-order reaction mechanism that followed Michaelis–Menten rate law for single substrate on systems where one of the substrates were present in large excess as compared to the other, particularly in solvent-free condition.

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