Production and purification of an alkaline lipase from *Bacillus* sp. for enantioselective resolution of ($\pm$)-Ketoprofen butyl ester

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Abstract

The present study was conducted to purify lipase from indigenous *Bacillus subtilis* strain Kakrayal_1 (BSK-L) for enantioselective resolution of racemic-ketoprofen. The production of lipase (BSK-L) was optimized using Plackett–Burman and central composite design of response surface methodology (RSM). The optimized media containing olive oil (3.5%), MnSO_4 (8 mM), CaCl_2 (5 mM), peptone (20 g/l), pH (8), agitation (180 rpm) and temperature (37 °C) resulted in maximum lipase production of 7500 U/g of cell biomass. The lipase was purified using sequential method to an overall purification fold of 13% with 20% recovery, 882 U/mg specific activity and a molecular weight of 45 kDa. Optimal pH and temperature of purified lipase were found to be 8 and 37 °C, respectively. Furthermore, BSK-L displayed good stability with various organic solvents, surfactants and metal ions. K_m and V_{max} values of lipase were observed to be 2.2 mM and 6.67 mmol of product formed/min/mg, respectively. The racemic ketoprofen butyl ester was hydrolyzed using lipase with 49% conversion efficiency and 69% enantiomeric excess (ee) which was superior to the commercially procured lipase (*Candida antarctica* lipase). Thus, this enzyme could be considered as a promising candidate for the pharmaceutical industry.

Keywords *Bacillus* sp. · Lipase · Purification · Enantioselective resolution

Introduction

Lipases are triacylglycerol acyl-hydrolases (E.C. 3.1.1.3) that catalyze the hydrolysis of carboxyl esters of triacylglycerols (Casas-Godoy et al. 2012), and esterification and transesterification reactions in non-aqueous conditions (Kumar et al. 2013). Importance of lipase is deduced from their applications in various industries such as food, detergent, cosmetics, leather, textile, pharmaceutical, diagnosis, biodiesel and biotransformation (Bhushan et al. 2008;

Bento et al. 2017; Cubides-Roman et al. 2017; Shi et al. 2017). The microbial lipase market in 2018 is envisaged to be \$425 million and it is anticipated that by 2023 lipase market can encompass \$590 million (MarketsandMarkets 2018). Although lipases have a wide range of industrial applications, their uses are still hindered due to high cost, poor stability and requirement for specific characteristics of the enzyme in various industrial processes such as in ester synthesis, organic solvent-stable lipases are required, while in the detergent formulation, alkaline stable lipases are usually preferred.

Owing to the industrial requirement, the optimization of production process is crucial to facilitate economical lipase production (Rigo et al. 2010; Kumar et al. 2014; Bhosale et al. 2015). The use of statistical tools, such as RSM, has emerged as a promising alternative to conventional methods for optimization of physio-chemical factors for enzyme production processes. Although RSM has been studied exhaustively, rare studies on intracellular lipase from *Bacillus subtilis* have been reported which is optimized using RSM.

Synthesis of enantiopure compounds is one of the significant applications of lipase to produce life-saving drugs. Enantiopure drugs are highly demanded pharmaceutically to

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avoid the side effects caused by unwanted enantiomer present in the chiral drugs and, therefore, enantiopure drugs are recommended by FDA and European legislation (Qin et al. 2013). A bioactive drug such as ketoprofen is chemically synthesized as a racemic drug having both R and S enantiomers which display significantly different pharmacological activities. The S-Ketoprofen is used to treat rheumatoid arthritis and osteoarthritis while R-Ketoprofen causes gastrointestinal side effects (Kantor 1986). Hydrolysis of ketoprofen vinyl ester for enantioselective resolution has been catalyzed by lipase from *Aspergillus terreus*, *Rhizomucor miehei*, *Mucor javanicus*, *Candida rugosa* and *Pseudomonas cepacia* and continuously proposed for better efficiency (Caroline et al. 2015). In the present study, optimization of lipase production, followed by purification, characterization and enantioselective resolution of ketoprofen using lipase was carried out.

Materials and methods

Materials

Lipase-producing indigenous *Bacillus subtilis* strain Kakrayal_1 was isolated from Katra region of Jammu and Kashmir, India. Identification of this strain was performed by sequencing 16S rRNA gene and the sequence was submitted to NCBI and obtained accession no.: KT985358 (Saraswat et al. 2017). Chemicals and biochemicals were acquired from GE Healthcare, Sigma, Hi-Media, Merck, Loba-Chemie, and S.D Fine Chemicals Ltd.

Methods

Cultivation strategy and lipase production

The *B. subtilis* strain Kakrayal_1 was cultivated in the medium consisting of 0.5% (w/v) yeast extract, 0.5% (w/v) beef extract, 1% (w/v) peptone and 0.2% (w/v) NaCl at pH 7 for 24 h at 37 °C. The cultivated broth was withdrawn at different time intervals, centrifuged and analyzed for lipase activity both in the supernatant as well as in the cell biomass.

Statistical optimization of lipase production

Plackett–Burman design Plackett–Burman is a two-level factorial design used for the identification of media components that contribute significantly for lipase production. High (+1) and low (−1) level of each factor such as peptone, yeast extract, olive oil, CaCl₂, MnSO₄, pH, temperature and agitation were investigated. The level of each factor was selected on basis of literature. Design Expert 10 software

(State-Ease Inc., Minneapolis, U.S.A.) was used to generate and analyze the experimental design of Plackett–Burman.

Response surface methodology (RSM) Based on the results of the Plackett–Burman design, three significant independent factors, i.e. olive oil, MnSO₄ and temperature were applied to central composite design (CCD) in process optimization tool, i.e. RSM. Three different levels, high (+1), medium (0) and low (−1), were used to study the independent factors. High and low levels of different factors are given in Table 1. Twenty experiments designed by Design Expert 10 software were conducted. The results obtained were fitted in the quadratic polynomial regression equation given below:

$$\begin{aligned} \text{Lipase activity} = & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 \\ & + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \\ & + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2, \end{aligned}$$

where X_1 , X_2 , and X_3 represent independent variables; β_0 represents intercept; β_1 , β_2 and β_3 represent linear coefficient; β_{12} , β_{13} and β_{23} represent interaction coefficient; β_{11} , β_{22} and β_{33} represent quadratic coefficients. The significance of effects was evaluated using analysis of variance (ANOVA). Values of “Prob > F” less than 0.05 indicated the significance of the factor. All experimental runs were conducted thrice.

Enzyme assay and protein determination

Titrimetric method Lipase activity was assayed by titrimetric method using olive oil as substrate at 37 °C in a reaction mixture (15 ml) consisting of 1% (v/v) olive oil, 1% (w/v) gum acacia, 2% (w/v) CaCl₂ and 1M NaCl. The initial pH of the reaction mixture was adjusted at pH 7 and the reaction was initiated by adding lipase. On hydrolysis of olive oil, fatty acid obtained was neutralized with 10 mM NaOH by simultaneous maintenance of pH at 7 for a standard time. The amount of triglycerides hydrolyzed by enzyme was calculated by the volume of 10 mM NaOH used for the neutralizing fatty acid produced by hydrolysis of olive oil (Bakir and Metin 2016). A unit of lipase activity is defined as the amount of lipase required to liberate 1 μmol of fatty acid from triglycerides per min under standard conditions.

Table 1 Levels of different factors used in central composite experimental design

Factors	Low level	High level
Olive oil	2%	5%
MnSO ₄	4 mM	8 mM
Temperature	35 °C	40 °C

Lipase activity was determined by titrimetric method using cell biomass and cell-free extract of lipase/crude lipase. The units of lipase activity were determined in U/g of cell biomass or U/ml of cell-free extract/crude lipase added to the reaction mixture.

$$\text{Lipase activity (U/mg)} = \frac{\text{Volume of NaOH consumed} \times \text{Molarity of NaOH}}{\text{Wt. of cell pellet} \times \text{Reaction time}}$$

or

$$\text{Lipase activity (U/ml)} = \frac{\text{Volume of NaOH consumed} \times \text{Molarity of NaOH}}{\text{Volume of lipase} \times \text{Reaction time}}.$$

Spectrophotometric method The lipase was assayed spectrophotometrically using p-nitrophenyl palmitate as substrate (Kaur et al. 2016). The reaction mixture of 1 ml contained 0.8 ml of 50 mM Tris–HCl buffer (pH 8), 100 µl lipase and 100 µl of 1 mM p-nitrophenyl palmitate. The reaction was performed at 37 °C for 30 min and deactivated by addition of 0.25 ml sodium carbonate (100 mM). The lipase activity was determined at 420 nm in LABINDIA UV 3000⁺ UV/Vis spectrophotometer against appropriate blank. One unit of lipase activity was defined as the amount of lipase required to liberate 1 µmol of p-nitrophenol per min from p-nitrophenyl palmitate under standard conditions.

Determination of protein concentration The protein concentration was determined by the Bradford method using bovine serum albumin as standard protein (Bradford 1976).

Preparation of cell-free extract (CFE) by sonication Since the enzyme is intracellular, 10 g of cell biomass was washed with 50 mM Tris–HCl buffer (pH 8) and resuspended in 50 ml of same buffer, followed by cell disruption by ultrasonication of cell biomass at 4 °C (five cycles consisting of 1-min pulse and 4-min rest) in MSE Manor Roy Crawley RH 10 2QQ cell disrupter at 18 kHz. The disrupted suspension was centrifuged at 10,000 rpm for 15 min at 4 °C (Sigma-3K3, Germany) and the supernatant (cell-free extract) obtained was analyzed for lipase activity.

Purification of lipase

The cell-free extract obtained was precipitated with ammonium sulphate precipitation by taking ten saturation fractions of ammonium sulphate, i.e. 0–10%, 10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, 70–80%, 80–90% and 90–100% and were precipitated at 4 °C (Duong-Ly and Gabelli 2014). Each fraction was analyzed for lipase activity and protein content. The fractions with lipase activity were pooled and applied on the pre-equilibrated phenyl-Sepharose

CL-4B column for binding with support. The bounded enzyme was eluted with a negative linear gradient of ammonium sulphate in 50 mM Tris–HCl buffer (pH 8) at a flow rate of 1 ml per min and fractions were collected. Lipase activity of all collected fractions was determined and active

fractions with lipase activity were pooled and again applied on pre-equilibrated DEAE Sepharose column. A linear gradient of 0.1–1M NaCl prepared in 50 mM Tris–HCl buffer (pH 8) was used to elute the bounded proteins. The active fractions with lipase activity were pooled, desalted and once more applied to Sephadex G-100 column. The bounded lipase was eluted with 50 mM Tris–HCl buffer (pH 8) from Sephadex G-100 column. Active fractions collected from Sephadex G-100 column were pooled and purification of the lipase was confirmed by SDS-PAGE.

Poly acrylamide gel electrophoresis (PAGE)

SDS-PAGE: the relative molecular weight and protein purity of lipase were determined by SDS-PAGE (Laemmli 1970) using 5% stacking gel and 15% resolving gel. The protein bands were stained by Coomassie Brilliant Blue R-250.

Specific staining: native (non-denaturing) PAGE was performed using discontinuous gel consisting of 5% stacking gel and 15% resolving gel. Specific staining was performed with 50 mg/ml β-naphthylacetate and 20 mg/ml Fast Blue RR dye.

Characterization of lipase

Effect of pH The optimum pH of purified lipase was determined by incubating the enzyme in different buffers of 50 mM, i.e. glycine–HCl buffer (pH 2), acetate–acetic acid buffer (pH 3–5), phosphate buffer (pH 6 and 7), Tris–HCl buffer (pH 8 and 9), glycine–NaOH buffer (pH 10), phosphate–NaOH buffer (pH 11) and KCl–NaOH buffer (pH 12). The lipase activity was plotted as 100% of initial activity.

Effect of temperature The optimum temperature of lipase was estimated by incubating aliquots of the purified enzyme at various temperatures ranging from 20 to 90 °C in 50 mM Tris–HCl buffer at pH 8 with 200 rpm for 24 h. At the begin-

ning of incubation, the maximum activity was considered 100%.

Effect of organic solvents The effect of organic solvent on purified lipase was studied by incubating the lipase in various organic solvents at 50% (v/v) concentration for 6 h at 37 °C with constant agitation at 200 rpm. The enzyme activity of the control (without an organic solvent) was plotted as 100% activity.

Effect of metal ions The effect of metal ions at 1 mM concentration was investigated by incubating lipase with different metal ions at 37 °C with 200 rpm for 6 h. No metal ion was used in the control sample solution. The relative activity of the lipase was evaluated as the change in the activity of the lipase in the presence of metal ions when compared to the activity of the control.

Effect of surfactant The stability of the purified lipase was determined in the presence of various surfactants at 1% (v/v) concentration for 6 h at 37 °C and 200 rpm. The relative activity without surfactant was used as a control. The relative activity of the lipase was evaluated as the change in the activity on treatment with surfactants when compared to the activity of the control.

Kinetic parameters of lipase

The influence of substrate concentration on the reaction velocity of lipase was examined using p-nitrophenyl

palmitate as a substrate, at various substrate concentrations (0.1 to 10 mM) in 50 mM Tris–HCl buffer (pH 8). The K_m (Michaelis constant) and V_{max} (maximum velocity) were determined from the Lineweaver–Burk plot.

Synthesis of (±) ketoprofen butyl ester

1.5 g of racemic ketoprofen was added to 25 ml butanol with five to six drops of sulphuric acid. The reaction mixture was refluxed for 4 h at 80 °C. The product was purified using silica gel column chromatography to obtain (±) ketoprofen butyl ester.

Enantioselective hydrolysis of (±) ketoprofen butyl ester

The reaction mixture was prepared using 30 mg (±) ketoprofen butyl ester, 7 ml of 50 mM Tris–HCl buffer (pH 7.5), 50 μ L Tween 80 and 50 U/ml lipase (Scheme 1) at 30 °C and 200 rpm. The conversion (C) and enantiomeric excess (ee) of the hydrolyzed products were measured by HPLC (Shimadzu Corp.), using an AD-H chiral column with a flow rate of 0.5 ml/min (hexane:isopropanol:acetic acid in 90:9.9:0.1). Retention time periods of ketoprofen butyl ester, (R)-ketoprofen and (S)-ketoprofen were 12.6, 30.2 and 38.8 min, respectively. All the experiments were conducted in triplicates.

Scheme 1 Chemical synthesis and enzymatic enantioselective resolution of ketoprofen butyl ester

Results and discussion

Lipase production

Lipase can be produced from microorganisms, plants and animals (Treichel et al. 2010). However, to meet the large demands of industries, lipases are produced from microorganisms. The advantages of microbial lipases include the availability of high catalytic activities, high production yields, ease of genetic manipulation, less influence of seasonal fluctuations and high growth rate of microorganisms on inexpensive media. Moreover, microbial lipases are more stable than plant and animal lipases. Lipase from *B. subtilis* is suitable for large-scale industrial production because it is identified as 'Generally Recognised as Safe' (GRAS) microorganism, easy to handle, have shorter processing time, produces endospore for survival in harsh reaction conditions, has ability to adapt to environmental changes and capable of being genetically manipulated (van Dijk and Hecker 2013; Ma et al. 2015). The lipase activity of 128 U/g was obtained from cell biomass of *B. subtilis* strain, whereas no lipase activity was observed in supernatant. Therefore, it can be inferred from the results that the lipase was intracellular. Initially, lipase production media was optimized by one variable at a time, a classical method, in which carbon and nitrogen sources were studied (data not shown) and resulted in enhancement of lipase activity from 128 U/g to 628 U/g cell biomass.

Statistical optimization of lipase production

Lipases produced from microorganisms are influenced by various physio-chemical factors. Lipids (oil) are critical carbon sources that impact on lipase by enhancing the production remarkably. However, nitrogen and other micronutrients should also be considered as important parameters to optimize lipase production (Treichel et al. 2010).

Plackett–Burman Design

Plackett–Burman design is based on a first-order model. The effect of peptone, yeast extract, olive oil, CaCl_2 , MnSO_4 , pH, temperature and agitation was examined by the Plackett–Burman design experiment using a set of 13 runs. The maximum lipase activity of *B. subtilis* strain Kakrayal_1 was observed to be 4800 U/g cell biomass. The analysis of regression coefficient values depicted that olive oil, temperature, MnSO_4 , CaCl_2 and agitation had a positive impact on lipase production whereas peptone, yeast extract and pH affected enzyme production negatively (Supplementary Fig. 1). Analysis of variance (ANOVA) depicted the significance of the model (Supplementary Table 1). Olive oil

(A), MnSO_4 (B) and temperature (C) were found to be the most significant factors as their probability value was below <0.05 and were selected for further analysis using RSM.

Response surface methodology (RSM) It is an exploratory and investigative approach to understand the relationship between factors that influence lipase production. RSM allows establishing the relationship between multiple factors proficiently than conventional design. Central composite design (CCD) was used to determine the linear, quadratic and interactive effects. Three factors, olive oil (A), MnSO_4 (B) and temperature (C) were optimized by CCD for maximum lipase production (Table 2). The obtained data were fitted in the quadratic polynomial regression equation. The model and regression equation was generated by design expert software. The regression equation obtained in terms of coded factors is given below:

$$Y = +63.40 + 6.60 (A) + 8.69 (B) - 5.99 (C) \\ - 11.20 (AB) - 5.07 (AC) + 0.000 (BC) \\ - 8.76 (A^2) + 5.81 (B^2) - 8.76 (C^2),$$

where Y represents the predicted response of lipase production and A , B and C represent olive oil, MnSO_4 and temperature, respectively. The errors of the coefficients are given in Table 3.

Table 2 Central composite experimental design

Run	A: olive oil (%)	B: MnSO_4 (M)	C: temp. (°C)	Lipase activity (U/g)
1	0	0	0	4800
2	−1	−1	−1	600
3	0	0	0	4800
4	1	−1	1	2400
5	0	0	0	4800
6	−1	1	−1	4800
7	−1.7	0	0	600
8	0	0	1.7	600
9	0	1.7	0	7500
10	−1	1	1	4800
11	−1	−1	1	600
12	1.7	0	0	2400
13	0	0	0	4800
14	1	1	1	2400
15	1	−1	−1	4800
16	0	0	0	4800
17	0	0	0	1200
18	1	1	−1	4800
19	0	0	−1.7	2400
20	0	−1.7	0	4800

Table 3 Errors of the coefficients of the regression equation

Factors	Coefficient	Standard error
Intercept	63.4	2.42
A–A	6.6	0.93
B–B	8.96	0.93
C–C	– 5.98	0.93
AB	– 11.19	1.83
AC	– 5.07	1.83
BC	0	1.83
A ²	– 8.76	0.85
B ²	5.8	0.85
C ²	– 8.76	0.85

The quadratic regression model analysis of variance (ANOVA) is given in Table 4. The *F* value of 5.79 implied that the model is significant with low probability for the regression model. The values of *p* < 0.05 indicated that model terms were significant. In this model, the linear effect of olive oil and MnSO₄ was significant while the linear effect of temperature was insignificant. The quadratic effect of olive oil and temperature was significant but quadratic effect of MnSO₄ was insignificant. Similarly, the interactive effect of olive oil and MnSO₄ was statistically significant. The “Lack of Fit *F*-value” of 0.18 suggests that Lack of fit is not significant relative to the pure error. The model’s goodness of fit was examined by the determination coefficient, i.e. R square. The R square of 0.90 was obtained wherein closer the value is to 1.0, better is the correlation between the observed and predicted response. “AdEq Precision” measures the signal to noise ratio and a ratio greater than 4 is desirable. This model designated a ratio of 9.1, indicating

an adequate signal and hence, the model is significant to explain the lipase production process.

The three-dimensional response surface graphs and contour plots (Fig. 1) are based on the interaction between the independent factors and the dependent response. Figure 1a depicts the interaction of olive oil and temperature (AC) with lipase activity where the 3D-plot is dome shaped indicating that the maximum lipase activity near the mid value of both the factors. Lipase activity decreased with an increase in temperature above 37 °C. Decrease in lipase activity, due to rise in temperature could be because of thermal damage to bacterial cells or lipase (Esmaeili et al. 2015). Lipase production was enhanced drastically in the presence of olive oil which acts as an inducer (Mobarak-Qamsari et al. 2011), suggesting that lipase in our study is lipid inducible. Sharma et al. (2014) reported the enhancement in production of extracellular lipase by RSM on supplementing with olive oil. The interactive effects of factor BC and BA with lipase activity are shown in Fig. 1b, c. Non-elliptical nature of contour plots in Fig. 1b, c showed the interaction between the two factors. The regression analysis equation was used to acquire the optimal values for each factor and thus optimized media composition is given as olive oil (3.5%), MnSO₄ (8 mM), temperature (37 °C), CaCl₂ (5 mM), peptone (20 g/L), agitation (180 rpm) and pH (8). Optimized media components were validated and obtained a satisfactory result of 7500 U/g cell biomass, which was much higher than classical optimization method (627 U/g cell biomass), i.e. about 12-fold enhancement in lipase activity was obtained. The optimization of intracellular *Bacillus subtilis* lipase using Plackett–Burman design and RSM was reported for the first time. Generally, the yield of lipase activity is a crucial factor for industrial application of enzymes. The optimized

Table 4 ANOVA for response surface quadratic model (A: olive oil, B: MnSO₄ and C: temperature)

Source	Sum of squares	Degree of freedom (Df)	Mean square	<i>F</i> value	<i>P</i> value [Probability > <i>F</i>]	
Model	6139.35	9	682.15	5.79	0.005	Significant
A–A	595.55	1	595.55	5.06	0.048	
B–B	1031.76	1	1031.76	8.76	0.014	
C–C	489.71	1	489.71	4.16	0.068	
AB	1002.94	1	1002.94	8.52	0.015	
AC	205.89	1	205.89	1.75	0.215	
BC	0	1	0	0	1	
A ²	1106.18	1	1106.18	9.4	0.011	
B ²	485.67	1	485.67	4.13	0.069	
C ²	1106.18	1	1106.18	9.4	0.011	
Residual	1177.16	10	117.72			
Lack of Fit	177.16	5	35.43	0.18	0.959	Not significant
Pure error	1000	5	200			
Cor total	7316.5	19

Fig. 1 Response surface 3D graphs of centre composite design depicting interactive effects. **a** AC: olive oil and temperature, **b** BC: MnSO₄ and temperature, **c** AB: olive oil and MnSO₄

media of *B. subtilis* PTCC 1720 yielded 3.06 U/mL extracellular lipase (Esmaeili et al. 2015). Extracellular lipase production from *Candida sp.* was optimized using RSM and obtained activity as 6,230 U/mL (Treichel et al. 2010). *Bacillus safensis* DVL-43 fermentation medium was optimized using Plackett–Burman design and response surface methodology which resulted in 11.4-fold increase in extracellular lipase production (Kumar et al. 2014). Plackett–Burman and Box–Behnken designs were used for optimizing the culture conditions to improve lipase production from *P. aeruginosa* strain BUP2; and the optimized significant factors were determined to be temperature (28 °C), pH (6) and incubation time (24 h) that increased lipase production by 11% (Unni et al. 2016).

Purification of lipase

Purification of an enzyme is an important step to isolate a protein of interest and to remove unwanted proteins. The intracellular BSK-L was sequentially purified using sequential techniques. The ammonium sulphate fractions, 70–80% and 80–90%, showing lipase activity were pooled, and the specific activity of 116 U/mg protein was determined. The partially purified BSK-L was then subjected to hydrophobic interaction chromatography using Phenyl Sepharose CL-4B column. The hydrophobic regions of lipase interact with hydrophobic support and thus bind with it (Saxena et al. 2003). The eluted fractions which displayed lipase activity were pooled and specific activity was determined

to be 248 U/mg protein. Further purification was achieved by ion-exchange chromatography wherein the relative negative charge on lipase aids in binding to the positively charged DEAE-cellulose. The eluted lipase from ion-exchange chromatography (DEAE-cellulose) has shown a specific activity of 677 U/mg protein. Additional purification step was conducted using Sephadex G-100 resin by gel filtration chromatography. The purification process resulted in 13-fold purification factor and a final recovery of 20.3%, with 882U/mg specific activity (Table 5). The purity of lipase was confirmed by SDS-PAGE which revealed the appearance of a single protein band (Fig. 2). The molecular weight of intracellular BSK-L was found to be 45 kDa, which was similar to *Acinetobacter* sp. AU07 lipase (Gururaj et al. 2016). Most of the *Bacillus* lipases have molecular weight in the range of 11–70 kDa, such as 11 kDa lipase was obtained from *B. thermoleovorans* (Castro-Ochoa et al. 2005), 19 kDa lipase from *B. subtilis* 168 (Lesuisse and Schanck 1993) and 31 kDa lipase from *B. coagulans* BTS3 (Kumar et al. 2005). The *Bacillus* sp. isolated from furnace secreted lipase which was purified by ammonium sulphate precipitation and ion-exchange chromatography leading to 8.6-fold purification with 13% recovery. Molecular weight of lipase confirmed from SDS-PAGE was obtained to be

45 kDa (Sivaramakrishnan and Muthukumar 2012). Shah and Bhatt (2011) reported purification of extracellular lipase from *B. subtilis* Pa2 by acetone precipitation and ion-exchange chromatographic methods. A 29-kDa extracellular lipase of *Pseudomonas aeruginosa* SRT 9 was purified to apparent homogeneity to 98-fold using ammonium sulphate precipitation tailed by phenyl Sepharose CL-4B and Mono Q HR 5/5 column chromatographic techniques. The specific activity of lipase was determined to be 12307.8 U/mg (Borkar et al. 2009).

Characterization of lipase

Effect of pH

The optimum pH of purified lipase (BSK-L) was found to be 8 and showed stability at pH range from 4 to 10 which retained more than 60% of the relative activity on incubation at pH 10 for 24 h, whereas only 11% and 34% of relative activity were retained at pH 4 and pH 5, respectively (Supplementary Fig. 2). The alkaline lipase can find application in the detergent formulation, leather industry and sewage treatment (Joseph and Ramteke 2013). The pH optima of *Bacillus* sp. lipase was found to be at pH 7

Table 5 Purification of BSK-L

	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	29,530 ± 8.45	436 ± 1.61	67.7 ± 0.07	1	100
Ammonium sulphate precipitation	24,410 ± 7.21	210 ± 1.77	116 ± 0.61	1.71	83
Hydrophobic interaction chromatography	19,390 ± 5.99	78 ± 0.14	248.5 ± 1.56	3.67	66
Ion-exchange chromatography	9480 ± 6.34	14 ± 0.07	677 ± 1.83	10	32
Gel filtration chromatography	5997 ± 5.81	6.8 ± 0.01	882 ± 2.01	13.01	20

Fig. 2 SDS-PAGE of lipase obtained from *Bacillus subtilis* (BSK-L). Lane 1: molecular marker, lane 2: cell-free extract, lane 3: ammonium sulphate precipitation, lane 4: hydrophobic interaction chromatography, lane 5: ion-exchange chromatography, lane 6: gel filtration chromatography and lane 7: specific staining of lipase

(Sivaramakrishnan and Muthukumar 2012). The extracellular lipase from *B. subtilis* Pa2 was active in the pH range of 7–9 with pH optima of 8 (Shah and Bhatt 2011). *P. aeruginosa* SRT 9 lipase was observed to have maximum lipase activity at pH 6.9 (Borkar et al. 2009). *Cohnella* sp. A01 lipase exhibited optimal activity at pH 8.5 and was highly stable at the pH range of 8.5–10 for 180 min (Golaki et al. 2015).

Effect of temperature

The optimum temperature of purified BSK-L lipase was 37 °C, which is comparable as reported for *B. subtilis* 168 lipase (Lesuisse and Schanck 1993). Lipase (BSK-L) was active at the temperature range from 20 °C to 60 °C. Relative activity observed at 40 °C (91%), 45 °C (68%), 50 °C (32%), 55 °C (12%) and 60 °C (7%) depicted that the BSK-L is thermotolerant lipase (Supplementary Fig. 3). On further increase in temperature, lipase activity diminished indicating thermal disruption of the tertiary structure. The *B. subtilis* Pa2 lipase was most active in temperature range 30–50 °C (Shah and Bhatt 2011). The optimum activity of the intracellular *A. flavithermus* lipase was at 50 °C and was observed to be stable at 25 °C, 40 °C, and 50 °C for 24 h (Bakir and Metin 2016).

Effect of organic solvents

Purified lipase (BSK-L) was treated with organic solvents for 6 h at 50% (v/v) concentration and observed remarkable tolerance with diethyl ether (76.8%), dimethyl sulfoxide (77.18%), ethanol (82.2%), hexane (89.4%), dimethylformamide (92.8%) and isopropanol (93.2%), while moderately tolerant to methanol (42.2%) and chloroform (42%). Lipase activity was almost completely inhibited with acetonitrile (9.3%) and dichloromethane (0.8%) which expressed that this lipase exhibited good stability with most of the organic solvents. Likewise, about 40% relative activity was demonstrated with lipase from *Bacillus stearothermophilus* after incubation for 30 min in 50% concentration of ethanol, isopropanol, butanol, acetone, chloroform, diethyl ether and hexane (Kambourova et al. 2003). Organic solvent-tolerant lipases are indispensable for the synthesis of fine chemicals, biodiesel production and biopolymeric material production (Cadirci and Yasa 2010).

Effect of metal ions

The purified lipase was treated with 1 mM metal ions for 6 h and observed stimulation in relative activity of lipase on treatment with Mn^{2+} (46.3%), Ca^{2+} (38.3%), K^+ (34.6%), Zn^{2+} (26.7%) and Fe^{2+} (21.9%), while good tolerance was observed in the presence of Mg^{2+} (85.2%), Na^+ (83.3%),

Cu^{2+} (69.2%) and Co^{2+} (66.4%). Similarly, enhancement in lipase activity in the presence of Ca^{2+} and K^+ ions was expressed (Wang et al. 2012). Golaki et al. (2015) stated the reduction in lipase activity on addition of metal ion chelator (EDTA) with Mn^{2+} (62.8%) and Ca^{2+} (71.5%), because metal ions aid in function and structural maintenance of enzyme. It is noteworthy that lipase stabilized in the metal ions can be useful in detergent formulations. Lipase activity of *Bacillus* sp. was stimulated by divalent metal ions Ca^{2+} , Mg^{2+} and Mn^{2+} (Sivaramakrishnan and Incharoen-sakdi 2016). The Cu^{2+} and Zn^{2+} ions strongly inhibited the enzyme activity of *B. cereus* C71 lipase (Chen et al. 2007). Enhancement in *Bacillus* sp. RSJ-1 lipase activity was observed in the presence of Ca^{2+} , Na^+ , Mg^{2+} and Ba^{2+} but was strongly inhibited by Cs^+ , K^+ , Co^{2+} and Zn^{2+} , whereas EDTA had no influence on enzyme activity (Sharma et al. 2002).

Effect of surfactants

The effect of various surfactants on purified lipase was investigated by incubating lipase with different surfactants for 6 h. The relative activity was improved by the addition of Tween 20 (27%) and Tween 80 (15%) while lipase was observed to be tolerant of Triton X-100 (95.4%). On the other hand, biocatalytic activity was significantly reduced to 16.2% by SDS (Castro-Ochoa et al. 2005; Golaki et al. 2015). Non-ionic detergents cause disaggregation by reducing the hydrophobic interaction within the enzyme and thus stabilizing its activity, whereas SDS causes denaturation of the enzyme by breaking disulphide linkages (Liebeton et al. 2001; Sajna et al. 2013).

Kinetic parameters of lipase

The kinetic parameters (K_m and V_{max}) of lipase were determined by the Lineweaver–Burk plot. The K_m and V_{max} values of lipase using p-nitrophenyl palmitate (pNPP) as substrate were found to be 2.2 mM and 6.67 mmoles of product formed/min/mg, respectively (Fig. 3). A low K_m value signifies a high affinity of enzyme towards its substrate, while a high K_m signifies a low affinity. The K_m and V_{max} values of 3.2 mM and 0.005 mmoles/min/mg for *Microbacterium* sp. lipase (Tripathi et al. 2014), 0.03 mM and 188.6 mmoles/min/mg for *Pseudomonas* sp. lipase (Borkar et al. 2009) were reported, which showed the attraction of enzymes toward its substrates.

Synthesis of (±) ketoprofen butyl ester

Ketoprofen (2-3-benzoyl phenyl-propionic acid) is a non-steroidal anti-inflammatory drug (NSAID) of the 2-aryl propionic acid derivative family which is used for the treatment

Fig. 3 Line weaver–Burk plot (double reciprocal plot) for determination of K_m and V_{max} values of BSK-L lipase using p-nitrophenyl palmitate as substrate

Table 6 Enantioselective resolution of ketoprofen butyl esters

Lipases	Conversion	Reaction Time (h)	Enantiomeric excess (%)	Enantio-preference
Purified BSK-L	49.3	24	69.37	R
<i>C. antarctica</i> lipase-B	48	24	62.9	R

of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (Kantor 1986). The racemic Ketoprofen butyl ester was prepared using ($\pm$) ketoprofen and butanol by chemical esterification and the purified product was confirmed by NMR (nuclear magnetic resonance).

$C_{20}H_{22}O_3$: $C_{20}H_{22}O_3$: 1H NMR (400 MHz, $CDCl_3$) δ 7.83–7.77 (m, 2H), 7.75 (t, $J=1.7$ Hz, 1H), 7.70–7.65 (m, 1H), 7.63–7.52 (m, 2H), 7.47 (ddd, $J=18.9, 11.6, 4.6$ Hz, 3H), 4.13–4.03 (m, 2H), 3.79 (q, $J=7.2$ Hz, 1H), 1.63–1.51 (m, 6H), 1.29 (dq, $J=14.6, 7.4$ Hz, 2H), 0.88 (t, $J=7.4$ Hz, 3H).

Enantioselective hydrolysis of ($\pm$) ketoprofen butyl ester

The chemically prepared ($\pm$) ketoprofen butyl ester was hydrolyzed using purified BSK-L and commercially purchased *Candida antarctica* lipase-B to yield (S/+) or (R/–) enantiomers. The absolute configurations, i.e. S(+) or R(–) enantiomer, of optically active profens, were assigned based on a comparison of their sign (+/–) of rotation reported in the literature (Abdel-Aziz et al. 2012). BSK-L displayed

49.3% conversion efficiency (C) and 69.37% of enantiomeric excess (ee) compared to *Candida antarctica* lipase (48% C, 62.95% ee) on hydrolysis of racemic ketoprofen butyl ester (Table 6). Both purified lipase (BSK-L) and *Candida antarctica* lipase showed enantioselectivity towards R-ketoprofen. Thus, purified BSK-L lipase in our study presented better conversion efficiency and enantiomeric excess, suggesting a potential candidate for the chemical industry. Lipase from *Arthrobacter* sp. has been used for the kinetic resolution of chiral drug intermediates (Bhushan et al. 2008). Since lipases possess significant properties regio-, chemo-, and enantioselectivity, so they are most frequently used in the manufacture of chiral drugs and their intermediates by racemic resolution (Köse et al. 2002; Dlugy and Wolfson 2007; Chang et al. 2008; Zhang et al. 2015; Seddigi et al. 2017).

Conclusion

Production of lipase (BSK-L) from *Bacillus subtilis* strain was enhanced by optimizing the physio-chemical factors using response surface methodology. The lipase was sequentially purified using ammonium sulphate precipitation, hydrophobic interaction, and ion-exchange and gel filtration chromatography, obtaining molecular weight of 45 kDa with 13-fold purification and 882U/mg specific activity. It exhibited higher conversion efficiency and enantiomeric excess compared to commercially procured lipase for enantioselective resolution of racemic ketoprofen butyl ester. Therefore, BSK-L proved to be a potential candidate for the synthesis of pharmaceutical intermediates and high

value-added compounds. Moreover, because of its pronounced biochemical characteristics such as tolerance to organic solvents, metal ions and surfactants, this enzyme could contribute to organo-synthesis, detergent formulation and sewage treatment.

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Compliance with ethical standards

Conflict of interest The author declares that they have no conflict of interest and the article also complies with the ethical standard.

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