Environmental Science and Pollution Research (2023) 30:4555–4569
https://doi.org/10.1007/s11356-022-22098-6

RESEARCH ARTICLE

Multi-substrate sequential optimization, characterization and immobilization of lipase produced by *Pseudomonas plecoglossicida S7*

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Received: 12 February 2022 / Accepted: 14 July 2022 / Published online: 16 August 2022

Abstract
Lipases are important biocatalysts having the third largest global demand after amylases and proteases. In the present study, we have screened 56 potential lipolytic *Pseudomonas* strains for their lipolytic activity. *Pseudomonas plecoglossicida S7* showed highest lipase production with specific activity of 70 U/mg. Statistical optimizations using Plackett Burman design and response surface methodology evaluated fourteen different media supplements including various oilcakes, carbon sources, nitrogen sources, and metal ions which led to a 2.23-fold (156.23 U/mg) increase in lipase activity. Further, inoculum size optimization increased the overall lipase activity by 2.81-folds. The lipase was active over a range of 30–50° C with a pH range (7–10). The enzyme was tolerant to various solvents like chloroform, methanol, 1-butanol, acetonitrile, and dichloromethane and retained 60% of its activity in the presence of sodium dodecyl sulfate (0.5% w/v). The enzyme was immobilized onto Ca-alginate beads which increased thermal (20–60 °C) and pH stability (5–10). The purified enzyme could successfully remove sesame oil stains and degraded upto 25.2% of diesel contaminated soil. These properties of the lipase will help in its applicability in detergent formulations, wastewater treatments, and biodegradation of oil in the environment.

Keywords *Pseudomonas* · Lipase · Response surface methodology · Solvent stability · Immobilization

Introduction

Lipases are serine hydrolases that catalyze the conversion of triglycerides into glycerol and free fatty acids. It is due to this unique catalysis that lipases have applications in food, pharmaceutical, and biofuel production industries (Bayramoglu et al. 2015; Devi et al. 2020). Additionally, they take part in inter-esterification, esterification, aminolysis, and alcoholysis activities which makes the lipases one of the most sought after enzymes in the industries (Chandra et al. 2020). Microbial lipases are one of the most studied enzymes due to their ease of genetic manipulation, non-toxicity, no harmful residues, scale-up production in fermenters, and eco-friendly approach. In order to enhance the cost–benefit ratio and to meet the global demands, new sources of lipases are constantly being explored and reported (Ahmad et al. 2019; Coelho et al. 2020). With the aim to meet the energy demands of the ever growing population, countries have shifted their focus from the depleting non renewable to renewable energy sources (Ogino and Amoah 2019; Putri et al. 2020). Lipases have a major role in that context as they help in cost effective biofuel production and help in reduction of import duties of a country (Geetha et al. 2020). Biodiesel (fatty acid alkyl esters) is one such alternative liquid fuel which is almost similar to petro-based diesel and the role of lipases in processing glycerides and fatty acids from animal or plant fats/oil sources is indispensable and eco-friendly. The global markets of lipases were projected at $ 590.5 million by 2020 with Asia–Pacific region as the largest market. Increase in the Compound Annual Growth Rate (CAGR) of lipases at 6.5% from 2015

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to 2020 has being attributed to the rising awareness for animal health and numerous feeds for livestock.

Various microbial sources of lipases have been reported such as *Candida rugosa*, *Pseudomonas* sp., *Arthrobacter* sp., *Chromobacterium* sp., *Serratia* sp., and *Aspergillus niger* (Rios et al. 2019a; Phukon et al. 2020). *Pseudomonas* spp. such as *P. fluorescens*, *P. cepacia*, and *P. aeruginosa* have been known for their versatile nature and as source of industrially important enzymes (Paggiola et al. 2020; Verma et al. 2020). They are chemoorganotrophs and can catabolize a large number of organic compounds. In response to fluctuating external nutrients, they secrete a number of extracellular enzymes including lipases (Paggiola et al. 2020; Verma et al. 2020). *Pseudomonas* spp. have another advantage as they easily survive on low cost substrates like agro-industrial wastes, drainage spills, and sludge (Kezrane et al. 2020; Rocha e Silva et al. 2014).

Optimization of carbon and energy sources, media components, temperature, pH, and other factors are required to check the yield of an enzyme in proportion to the cost of production (Musa et al. 2019). Various statistical designs are used for the optimization experiments (Yele and Desai 2014; Balaji et al. 2020; Sahoo et al. 2020). Low productivity and stability issues of the enzyme presents a great challenge towards the applicability and availability on a large scale. Immobilization of *Pseudomonas* lipases have also been reported to increase the stability, storability, and efficiency of the enzyme (Pereira et al. 2019; Rios et al. 2019b). The choice of the carrier depends on the chemical and thermal stability, capability of easy rejuvenation, biocompatibility and reusability as well as cost effectiveness (Chandra et al. 2020).

The present study aims to optimize the production of lipase from *Pseudomonas plecoglossicida* S7 by using various substrates and increase its downstream applicability by immobilization.

Materials

Microbial cultures

Forty isolates of *Pseudomonas* spp. were obtained from National Agriculturally Important Microbial Culture Collection (NAIMCC), Maunath Bhanjan, India (Table 1). Sixteen *Pseudomonas* cultures were generously provided by Dr. Pandiyan Kuppusamy (ICAR-NBAIM, Mau). The cultures were revived and maintained on King's B media for further use.

Chemicals and reagents

All the media, buffer components, and p-nitrophenyl acetate were purchased from HiMedia Laboratories, India. The solvents used in the study were of analytical grade and procured from Sigma-Aldrich Co. (St. Louis, MO, USA). Supplementary Table 1 contains the details of all the chemicals used in the study.

Screening for lipolytic isolates and lipase assay

The cultures were streaked on media containing beef extract (3.0 g), peptone (5.0 g), sodium chloride (5.0 g), $CaCl_2$. $2H_2O$ (0.1 g), Tween 80 (1% v/v), and agar (2% w/v) per liter of distilled water (Sierra 1957). The lipolytic activity was indicated by the appearance of a visible precipitate, resulting from the deposition of crystals of the calcium salt formed by the fatty acid liberated by the enzyme (Mehta et al. 2018).

The lipase activity was determined using p-nitrophenyl acetate as substrate with slight modifications (Choo et al. 1998). 200 mM of p-nitrophenyl acetate solution was prepared using DMSO (Extrapure, analytical grade) as solvent and stored at 4 °C for further use. The assay mixture consisted of 0.25 mL of bacterial broth culture, 0.2 mL of toluene (Extrapure, analytical grade), 2 mL of MUB buffer (pH-7.0, 100 mM), and 1 mL of p-Nitrophenyl acetate (200 mM). The mixture was mixed and incubated at 37 °C for 1 h at 150 rpm. The mixture was then centrifuged at 10,000 rpm (4 °C) for 15 min and 0.1 mL of the supernatant (diluted with 1 mL of 0.5 M NaOH and volume made upto 10 mL using distilled water) was used for final assay. Absorbance was taken at 430 nm. A standard curve using p-nitrophenol (100–1000 µg/mL) was prepared after taking absorbance at 430 nm. One unit of lipase activity was defined as amount of enzyme required to release one micromole of p-nitrophenol from the substrate per minute under standard assay conditions. Total protein was estimated using Bradford's method (Bradford 1976).

Medium optimization for higher lipase production

Initial screening of multiple media components

A total of fourteen different media components were taken for the screening of best lipolytic activity (Table 2). Seven lipid rich sources including four oilcakes (groundnut, sesame, *Brassica nigra*, and *Sinapis alba*), three surfactants (Tween 20, Tween 40, Triton X-100 (2X) and Triton X-100), three carbon sources (glucose, sucrose, and maltose), and one metal ion source ($MgSO_4$. $7H_2O$) was used initially. The basal lipase medium reported by Sierra (1957) was amended for the experiments. The concentration for each media component used has been mentioned in Table 2. The culture (1% v/v) was inoculated in 20 mL to amend the lipase media at 37 °C for 24 h at 150 rpm and the lipase activity was recorded as described in the "Chemicals and reagents" section. The results were analyzed by Duncan's Multiple Range Test (DMRT) using the SPSS software package 16.0 using Microsoft excel 2007 (SPSS Inc. 2007).

Table 1 List of *Pseudomonas* cultures used in the study

Sl no	Isolate name	NAIMCC accession number	Sl no	Isolate name	NAIMCC accession number	Sl no	Isolate name	NAIMCC accession number
1	*Pseudomonas* sp. NBP1	B-00647	11	*P. aeruginosa* R13	B-00334	21	*P. fluorescens* PF11	B-00687
2	*P. koreensis* P3	B-01504	12	*P. fluorescens* 373	B-00367	22	*P. aeruginosa* DKH3	B-01390
3	*P. fluorescens* PF1	B-00680	13	*Pseudomonas* sp. NBP-14	B-00705	23	*Pseudomonas* sp. NBP-17	B-00708
4	*P. fluorescens* NCIM 2141	B-00340	14	*P. putida* NBP2	B-00692	24	*P. aeruginosa* FU2	B-01386
5	*P. koreensis* P2	B-01747	15	*P. taiwanensis* MPF2	B-01337	25	*P. aeruginosa* BBK3	B-01388
6	*Pseudomonas* sp. NCIM 5095	B-00374	16	*P. stutzeri* TS3	B-00901	26	*P. arsenicoxydans* P1	B-01746
7	*P. putida* GAP-P45	B-00923	17	*P. aeruginosa* PsaMB-TDI0909-101	B-00856	27	*P. morganii* NCIM 2040	B-00383
8	*Pseudomonas* sp. MB65	B-01025	18	*P. plecoglossicida* S7	B-00397	28	*P. fluorescens* 245	B-00363
9	*P. fluorescens* biovar III232	B-00762	19	*P. aeruginosa* MZP4	B-00881	29	*P. fluorescens* 136	B-00361
10	*P. putida* CPCRI4	B-01275	20	*P. fluorescens* ID6-PF3	B-00570	30	*P. fluorescens* 83	B-00358
Sl no	**Isolate name**	**NAIMCC accession number Code**	Sl no	**Isolate name**	**NAIMCC Code**	Sl no	**Isolate name**	**NAIMCC Code**
31	*Pseudomonas fluorescens* 59	B-00357	41	*Pseudomonas* sp. 28		51	*Pseudomonas* sp. 80	
32	*Pseudomonas fluorescens* 35	B-00356	42	*Pseudomonas* sp. 76		52	*Pseudomonas* sp. 12	
33	*Pseudomonas aeruginosa* NBRAJG 92	B-00331	43	*Pseudomonas* sp. 29		53	*Pseudomonas* sp. 44	
34	*Pseudomonas fluorescens* 101	B-00360	44	*Pseudomonas* sp. 26		54	*Pseudomonas* sp. 10	
35	*Pseudomonas fluorescens* 324	B-00364	45	*Pseudomonas* sp. 53		55	*Pseudomonas* sp. 81	
36	*Pseudomonas indica* CSR-GHB2	B-01978	46	*Pseudomonas* sp. 21		56	*Pseudomonas* sp. 16	
37	*Pseudomonas* sp. PS1	B-00352	47	*Pseudomonas* sp. 48				
38	*Pseudomonas* sp. NBP9	B-00699	48	*Pseudomonas* sp. 46				
39	*Pseudomonas putida* NCIM 2650	B-00325	49	*Pseudomonas* sp. 58				
40	*Pseudomonas* sp. SCL 1		50	*Pseudomonas* sp. 77				

Screening of factors using Plackett Burman experimental design

Four best substrate screened using initial screening were subjected to Plackett Burman experimental design as depicted in Table 3. Three nitrogen sources, viz., ammonium di-hydrogen orthophosphate (ADP), urea, and potassium nitrate (PN), were also added for evaluation as substrates for the experiment. The higher limit was denoted by + 1 and lower limit by − 1 indicating the amount of substrate used per run. Eight runs were performed in duplicates using a total of seven substrates. The results were analyzed using Design-Expert software package version 9.0.6.2 (serial number: 1619–5898-8149–2390). Factors were screened using normal probability plot (Fig. 2) from where it can be seen that four factors, namely ADP, Maltose, PN, and TX-100, were having positive effects on lipase

Table 2 Various substrates and salts used in the study along with their respective concentrations

S. no	Substrate	Conc
1	Groundnut oilcake (GOC)	1% (w/v)
2	Sesame oilcake (SOC)	1% (w/v)
3	*Brassica nigra* oilcake (BNOC)	1% (w/v)
4	*Sinapis alba* oilcake (SAOC)	1% (w/v)
5	Tween 20 (T20)	0.5% (v/v)
6	Tween 40 (T40)	0.5% (v/v)
7	Triton X-100 (TX100)	0.5% (v/v)
8	Maltose	1% (w/v)
9	Glucose	1% (w/v)
10	Sucrose	1% (w/v)
11	Tween 80 (T80) (2X)	1% (v/v)
12	Magnesium sulfate	1% (w/v)

activity. Therefore, out of these four factors, for the present investigation, three important factors, namely ADP, Maltose, and TX-100, were opted for further investigation.

Central composite design for optimization of parameters

For quadratic model

Central composite design (CCD) is a second-order rotatable design (SORD) which demands fitting of a second order or quadratic polynomial for getting more precision in analyzing the results. For the present investigation, a 20 run CCD was used with these three factors (Table 5). For three factors, the second-order polynomial model generally used is as follows:

$$y = \beta_0 + \beta_1 * A + \beta_1 * A + \beta_2 * B + \beta_3 * C + \beta_{11} * A^2 + \beta_{22} * +B^2 + \beta_{12} * AB + \beta_{13} * AC + \beta_{23} * BC \quad (1)$$

So, first as expected, a second-order quadratic model was fitted with the data. Based on second-order model fitting, it was observed that for the given dataset, the overall model was not significant even at 10% level of significance (p-value: 0.1774) although the quadratic effect of TX-100 (p-value: 0.0465) and the interaction effect of ADP and TX-100 (p-value: 0.0277) were significant at 5% level of significance. The non-significance of lack of fit error although desirable but the low p-value of 0.1938 against the lack of fit error indicated there was still scope for model improvement. The $R^2 = 0.6237$ for the model indicates the model was able to explain only 62.37% variability which was also in lower side. The adjusted $R^2 = 0.2851$ indicated the significant portions of variations explained by the model was only 28.51% which was low. It is to be noted here that the adjusted R^2 will increase if only significant variables included in the model. The adequate precision value of 5.161 although desirable as it is more than 4 but it was still on the lower side. Thus, all these criteria suggest that the model is not appropriately representing the given data and there is a need to improve the model by adding significant terms.

Modified model From the results obtained based on fitting of quadratic model as discussed above, it can be seen that

Table 3 Plackett Burman experimental setup to analyze the effect of multiple substrates

Runs	GOC (%)	SOC (%)	Ammonium dihydrogen phosphate (ADP) (%)	TX100 (%)	Maltose (%)	Urea (%)	Potassium nitrate (%)	Lipase (U)
1	1 (1.5)	1 (1.5)	−1 (0.5)	1 (0.75)	−1 (0.5)	−1 (0.5)	−1 (0.5)	43.20
1	1 (1.5)	1 (1.5)	−1 (0.5)	1 (0.75)	−1 (0.5)	−1 (0.5)	−1 (0.5)	43.82
2	1 (1.5)	1 (1.5)	1 (1.5)	1 (0.75)	1 (1.5)	1 (1.5)	1 (1.5)	75.30
2	1 (1.5)	1 (1.5)	1 (1.5)	1 (0.75)	1 (1.5)	1 (1.5)	1 (1.5)	76.47
3	1 (1.5)	−1 (0.5)	−1 (0.5)	−1 (0.25)	−1 (0.5)	1 (1.5)	1 (1.5)	44.95
3	1 (1.5)	−1 (0.5)	−1 (0.5)	−1 (0.25)	−1 (0.5)	1 (1.5)	1 (1.5)	46.54
4	−1 (0.5)	−1 (0.5)	1 (1.5)	1 (0.75)	−1 (0.5)	−1 (0.5)	1 (1.5)	78.58
4	−1 (0.5)	−1 (0.5)	1 (1.5)	1 (0.75)	−1 (0.5)	−1 (0.5)	1 (1.5)	77.95
5	−1 (0.5)	−1 (0.5)	−1 (0.5)	1 (0.75)	1 (1.5)	1 (1.5)	−1 (0.5)	51.46
5	−1 (0.5)	−1 (0.5)	−1 (0.5)	1 (0.75)	1 (1.5)	1 (1.5)	−1 (0.5)	53.44
6	1 (1.5)	−1 (0.5)	1 (1.5)	−1 (0.25)	1 (1.5)	−1 (0.5)	−1 (0.5)	70.88
6	1 (1.5)	−1 (0.5)	1 (1.5)	−1 (0.25)	1 (1.5)	−1 (0.5)	−1 (0.5)	72.64
7	−1 (0.5)	1 (1.5)	1 (1.5)	−1 (0.25)	−1 (0.5)	1 (1.5)	−1 (0.5)	61.27
7	−1 (0.5)	1 (1.5)	1 (1.5)	−1 (0.25)	−1 (0.5)	1 (1.5)	−1 (0.5)	58.90
8	−1 (0.5)	1 (1.5)	−1 (0.5)	−1 (0.25)	1 (1.5)	−1 (0.5)	1 (1.5)	54.91
8	−1 (0.5)	1 (1.5)	−1 (0.5)	−1 (0.25)	1 (1.5)	−1 (0.5)	1 (1.5)	54.06

Numbers in brackets indicate the original levels tried

there is still enough scope for improving the model. In case of the quadratic model although lack of fit test remains non-significant (which should remain non-significant for a goodness of fit test), the p value of 0.1938 is on the lower side. Thus, cubic model may be a good choice.

It should be note that the data under consideration were obtained based on a 20 run central composite designs with three factors which is although enough for estimating all the 10 parameters (including intercept) of a quadratic model, but the same resources were not enough to estimate all the 20 parameters (including intercept) of a cubic model. However, the existing resources can be used to estimate some additional parameters apart from all the 10 parameters of quadratic model. Therefore, in order to improve the performance of the model and keeping the resource constraint, based on hit and trial method, the following modified model with AB^2 was fitted again:

$$y = \beta_0 + \beta_1 * A + \beta_1 * A + \beta_2 * B + \beta_3 * C + \beta_{11} * A^2 + \beta_{22} * B^2 + \beta_{33} * C^2 + \beta_{12} * AB + \beta_{13} * AC + \beta_{23} * BC + \beta_{133} * AB^2 \quad (2)$$

All the above analysis and the optimization were carried out using Design-Expert Software package version 9.0.6.2 (serial number: 1619–5898-8149–2390). The final optimized media compositions were tested for lipase production in triplicates.

Optimization of inoculum size and effect of temperature, pH, organic solvents, and SDS on the activity and stability of lipase

Inoculum concentration for the assay was tested with a range from 1.0 to 5.0% (v/v) with an interval of 1%. The activity and stability were determined under a range of physiological and chemical factors like temperature, pH, organic solvents, and SDS. Temperature optimization was checked by incubating the substrate and enzyme at 20–60 °C (with an interval of 10° C) for 1 h and the enzyme activity was recorded. A range of pH from 5.0 to 10.0 (with an interval of 1) was taken by adjusting the pH of the MUB buffer (0.1 M) by citrate buffer (5.0–6.0, 0.1 M) and 1 M NaOH (8.0–10.0). The final concentration of all the buffers were 57.14 mM in all the experimental setups. Similarly, organic solvents like chloroform, methanol, 1-butanol, acetonitrile, and dichloromethane were tested with a concentration of 1 and 2% (v/v). Sodium dodecyl sulfate, being an important component of detergents was also used to test the enzyme activity (0.1–0.5% w/v). The above optimization experiments were checked for lipase activity after 24 and 48 h of the incubation at 150 rpm and repeated three times.

Purification of lipase and SDS-PAGE

After optimization, the selected isolate was grown in the optimized media at pH 7 for 2 days. The cells were harvested by centrifugation at 10,000 g for 20 min. The cell-free supernatant was subjected to precipitation by 50–70% ammonium sulfate at 37 °C with continuous stirring (150 rpm) followed by centrifugation at 10000 g for 30 min. Ammonium sulfate precipitate was dialyzed against 100 mM phosphate buffer in a dialysis membrane (HiMedia) with a molecular weight cut-off of 12–14 kDa (Priyanka et al. 2019). The dialyzed fraction was further purified by ion exchange chromatography using a DEAE-cellulose column (HiMedia, India) equilibrated with 100 mM phosphate buffer (with 0.002 M sodium azide) at pH 7. The enzyme was eluted at a flow rate of 0.5 ml/min with linear gradient of 0.1–1.0 M NaCl in the same buffer. Lipase activity and protein concentration of the eluted fraction were measured spectrophotometrically, and the active fractions were pooled together and stored for further analysis. The molecular weight of the active fraction was determined by SDS-PAGE, as mentioned by Laemmli (1970).

Entrapment in Ca-alginate beads using ionotropic gelation

The biopolymer solution was prepared using 3% (w/v) sodium alginate in 10 mL of the cell-free crude as well as purified enzyme extracts separately. Using an intravenous syringe (20 mL), the biopolymer solution was added drop wise to an aqueous solution of calcium chloride (0.14 M) while keeping the system on a magnetic stirrer at 500 rpm for 5 min (Pereira et al. 2019). After the encapsulation process, the beads were washed with 0.1 M phosphate buffer and dried at 4 °C. The dried microcapsules were used to check the enzyme activity retention after encapsulation along with its reusability. The beads were used three times subsequently to analyze the retention of their enzyme activity and efficiency. Beads were also prepared without containing crude/purified enzyme to check the effect of alginate and calcium chloride on the substrate. Also, the microcapsules were characterized at different temperature, pH, organic solvents, and SDS as performed for the free enzyme. A schematic representation of the immobilization process has been depicted in Fig. 1.

The effect of commercial detergent on lipase stability, laundry additive, and oil stain removal

Stability of lipase in the presence of detergents was investigated using five commercial detergent powders:

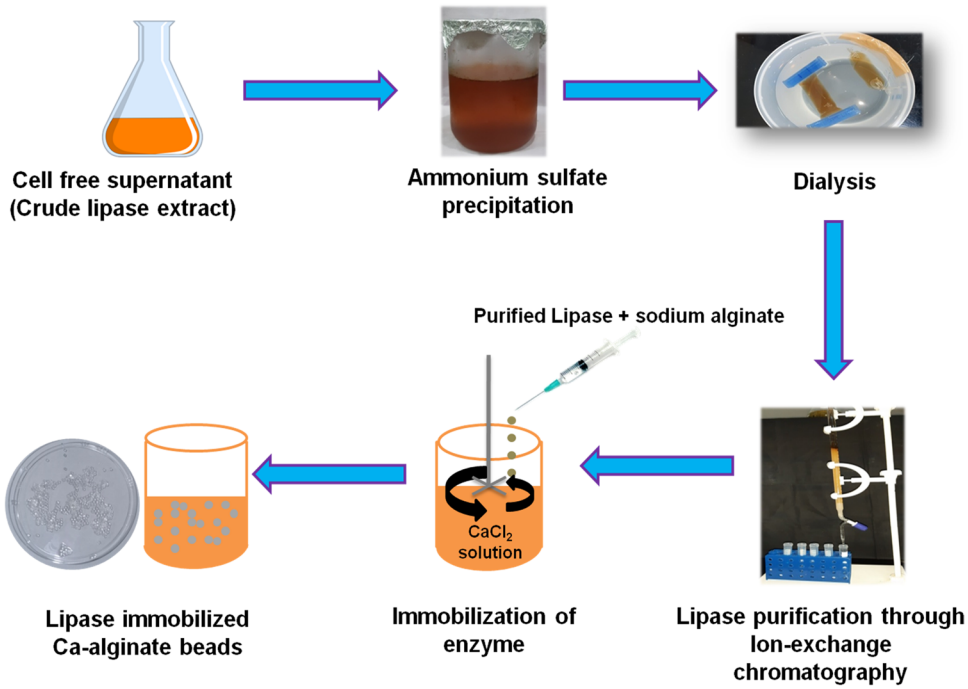

Fig. 1 A schematic of the production, extraction, and immobilization of *Pseudomonas plecoglossicida* S7 lipase

Tide® and Ariel® (Procter & Gamble home products Ltd., India), Vidhisa® (Vidhisa Group, Kanpur, India), Surf excel® (Hindustan Unilever Limited-Mumbai, India), and Active Wheel® (Hindustan Unilever Limited-Mumbai, India). Solution of 1% (w/v) commercial detergents was prepared by dissolving each detergent in distilled water and boiling for 15 min to inactivate the enzyme present in the detergent formulation (Sahay and Chouhan 2018). A positive control without detergent was run under similar conditions to calculate the residual lipase activity of each test sample. Lipase activity was analyzed as method described earlier and residual enzyme activity was calculated with respect to control (without detergent) as 100%.

The purified lipase was tested for its ability to remove oil stains from fabrics. Polycotton fabric was cut into pieces (3 cm × 3 cm) and each piece was stained with two drops of commercially available sesame oil (Organic India) used for deep frying. The fabric pieces were allowed to dry and subjected to various oil-destaining treatments such as distilled water, commercially available detergent 1% (w/v) and water + lipase (Das et al. 2016). The oil-stained fabric pieces were separately soaked in each treatment solution taken in a petri-plate and gently agitated. Post-incubation for 30 min, the fabric pieces were removed, dried, and observed for residual oil stains. The efficiency of lipolytic activity was evaluated by visualization of the oil stain.

Determination of diesel degradation through gravimetric analysis

Soil samples were collected from the fields of Indian Institute of Seed Science, Mau. It was sterilized thrice through tyndallization before proceeding with the experiment. Diesel was procured from a nearby fuel station and filter sterilized prior to use. *P. plecoglossicida* S7 was raised in broth media and the following treatments were set up: (1) T1 consisted of 25 g soil + 1 mL diesel, (2) T2: 25 g soil + 1 mL diesel + 100 mL *P. plecoglossicida* S7 bacterial suspension, and (3) MS medium (control) in sterile 250-mL flasks. The treatments were incubated at 37 °C for 10 days at 120 rpm. Residual diesel was extracted using n-hexane as described earlier by Zakaria et al. (2021). The degradation rate was determined using the following equation:

$$\% \text{ Biodegradation} = \frac{\left[\text{Mass of residual diesel abiotic control (g)} - \text{Mass of residual diesel treatment (g)}\right]}{\text{Mass of diesel in abiotic control (g)}} \times 100 \quad (3)$$

Results

Screening of isolates

Out of fifty six isolates used in the study, thirty nine formed a clear halo zone on the Tween 80 supplemented media (Fig. 2a). Upon quantitative screening of the 39 positive cultures, 27 showed lipase activity in a range between 1.083 and 28.89 µmole p-NP/min (Table 4). *Pseudomonas plecoglossicida* S7 (NAIMCC-B: 00,397) showed a maximum activity of 28.9 µmole p-NP min^{-1} and a specific activity of 70 U/mg protein. Hence, it was selected for further optimization and analysis.

Substrate optimization

Screening with multiple substrates

DMRT ranking showed four substrates, viz., groundnut oilcake, sesame oilcake, Triton-X 100, and maltose, which enhanced the production of lipase significantly as compared to control substrate Tween 80 (Fig. 2b). Hence, these substrates were selected for the Plackett Burman design along with three nitrogen sources, viz., ADP, potassium nitrate, and urea.

Figure 3 showed the substrates which contributed to the enhancement in lipase production. Results of Plackett Burman analyses revealed Triton X-100, ADP, maltose, and potassium nitrate to be the most dominant components. ADP was selected over potassium nitrate as it was more effective. Finally, Triton X-100, maltose, and ADP were selected for further optimization.

Table 4 Quantitative screening of twenty seven *Pseudomonas* cultures for lipase activity

Sl. no	Culture name	Lipase activity (µmole p-NP/min)
1	*P. putida* CPCRI4	26.06
2	*P. plecoglossicida* S7	28.88
3	*Pseudomonas* sp. NBP-14	22.14
4	*P. aeruginosa* FU2	13.63
5	*Pseudomonas* sp. NBP9	20.98
6	*P. putida* NCIM 2650	22.58
7	*P. aeruginosa* R13	18.01
8	*P. fluorescens* NCIM 2141	21.93
9	*P. aeruginosa* PsaMBTDI0909-101	23.77
10	*P. putida* GAP-P45	14.05
11	*P. aeruginosa* BBK3	18.41
12	*Pseudomonas* sp. MB65	22.45
13	*P. aeruginosa* DKH3	25.29
14	*P. koreensis* P2	1.33
15	*P. koreensis* P3	17.32
16	*Pseudomonas* sp. 12	10.96
17	*P. fluorescens* 101	2.97
18	*Pseudomonas* sp. 29	10.98
19	*Pseudomonas* sp. 26	1.50
20	*Pseudomonas* sp. PS1	5.17
21	*Pseudomonas* sp. 46	6.80
22	*P. fluorescens* 83	2.02
23	*Pseudomonas* sp. 44	12.50
24	*P. fluorescens* 245	1.08
25	*Pseudomonas* sp. 81	17.69
26	*Pseudomonas* sp. 55	1.133
27	*Pseudomonas* sp. 21	12.25

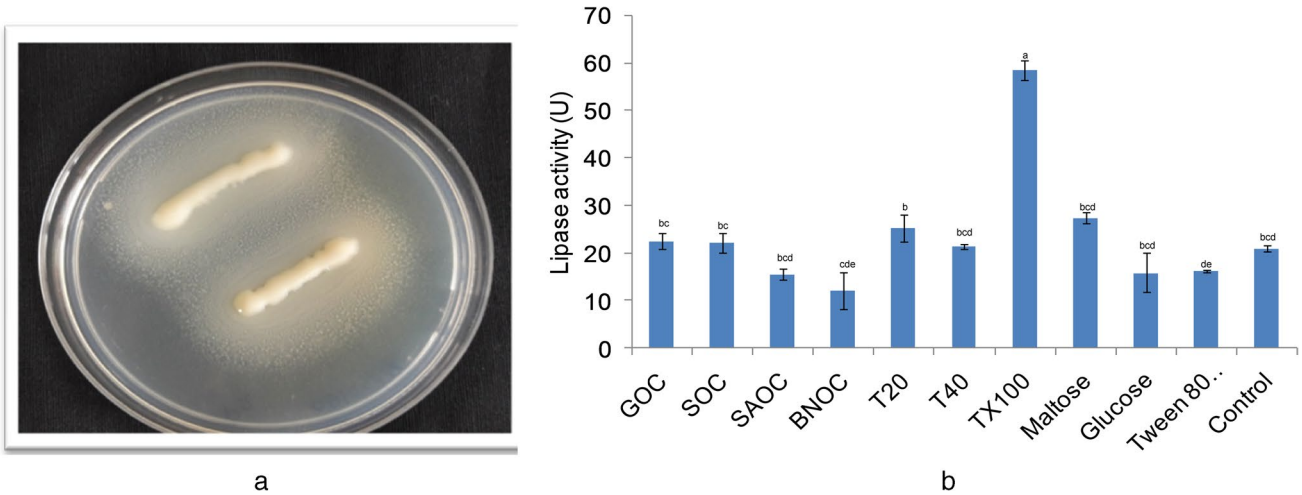

Fig. 2 **a** The isolate *Pseudomonas plecoglossicida* S7 formed a halo zone on Tween 80 supplemented media. **b** The effect of various substrates on the production of lipase. Magnesium sulfate and sucrose was excluded as it had a negative impact on lipase production. The letters placed above the bars are DMRT ranks. Different letters present significantly different mean values. GOC, groundnut oilcake; SOC, sesame oilcake; BNOC, *Brassica nigra* oilcake; SAOC, *Sinapis alba* oilcake

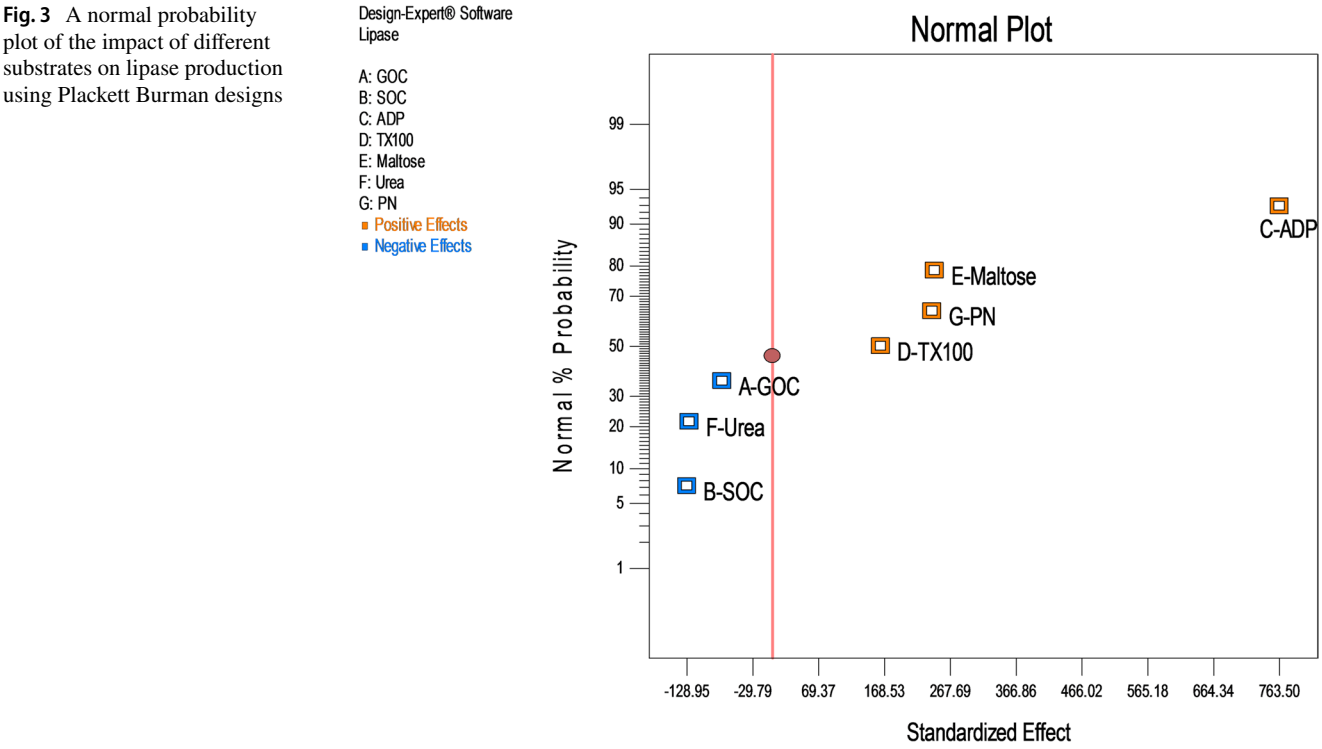

Fig. 3 A normal probability plot of the impact of different substrates on lipase production using Plackett Burman designs

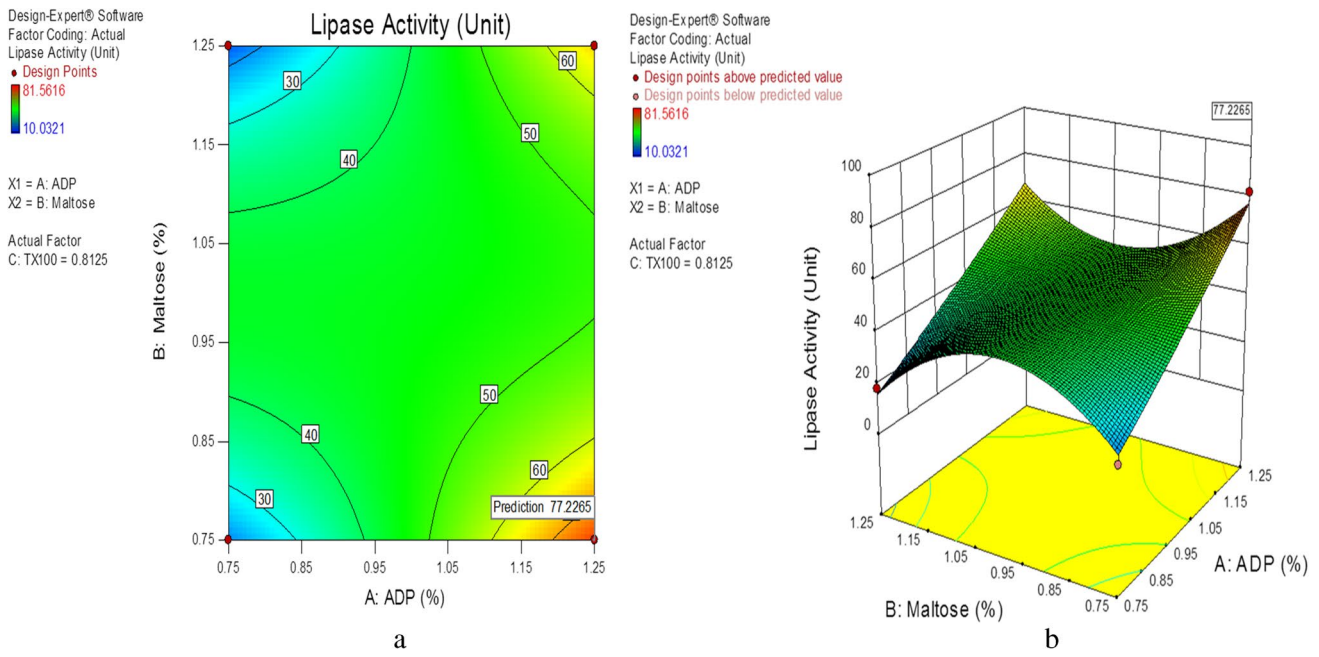

Fig. 4 **a**, **b** Keeping Triton X-100 (%) at its optimum level, maxima with high desirability lay towards the extremes which was towards higher value of ADP (%) and lower value of maltose (%)

Response surface optimization based on CCD using modified model

Figures 4, 5, and 6 showed that keeping ADP % at intermediate level, maxima with high desirability lied towards the extremes which was towards the lower concentration of maltose and higher concentration of Triton X-100. The results of projected composition of the selected substrates are presented in Table 5. The actual factor concentrations predicted by the RSM cubic model were: Maltose: 0.75%,

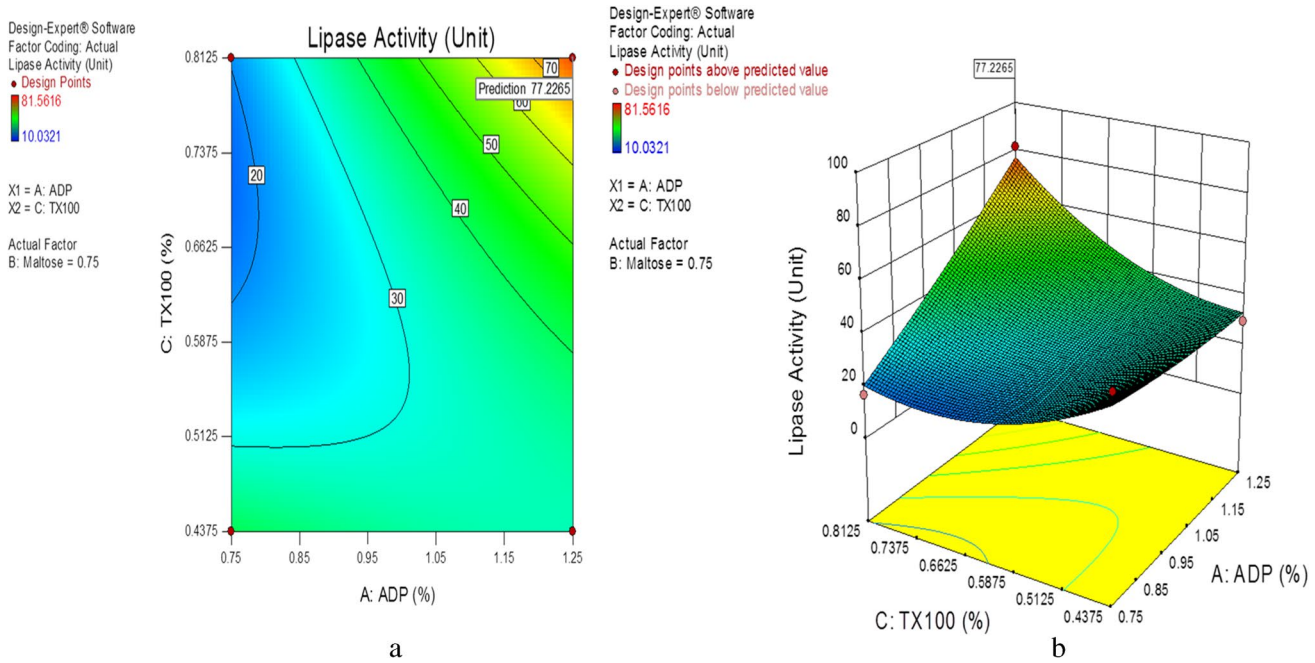

Fig. 5 **a**, **b** Keeping maltose % at its optimum level, maxima with high desirability lay towards the extremes which was towards higher value of both ADP % and TX100%

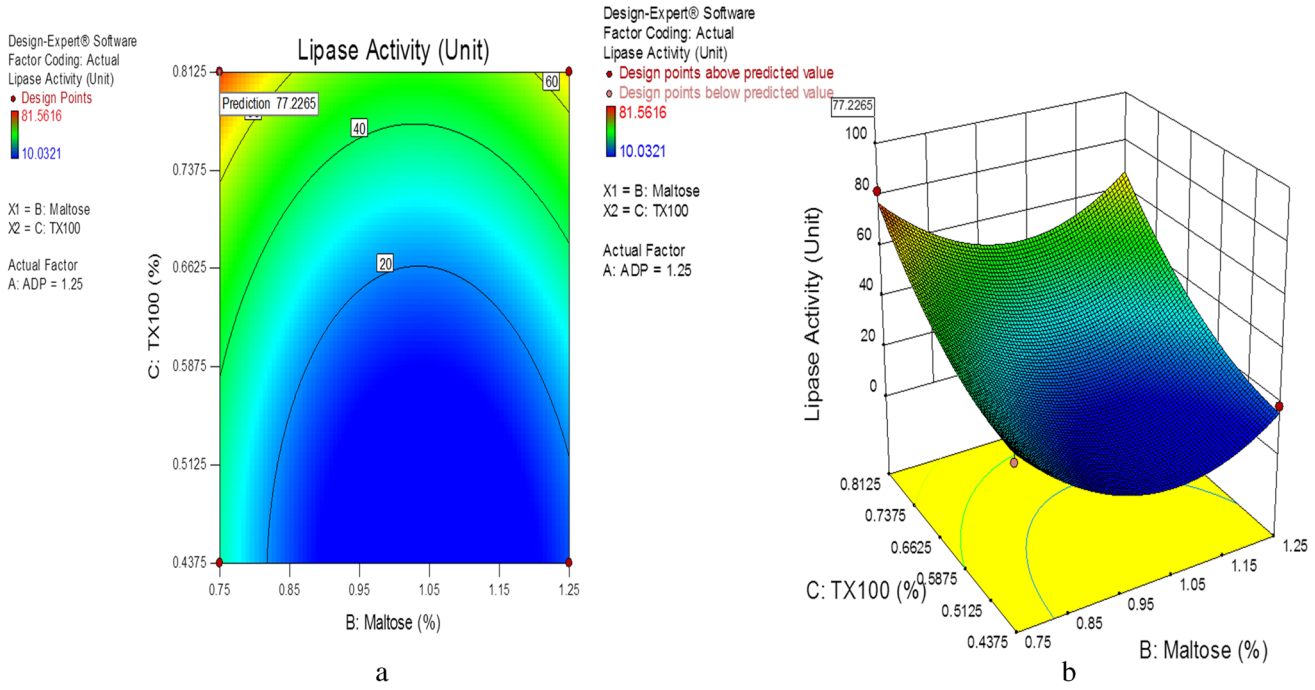

Fig. 6 Fig. 5(a) and Fig. 5(b) indicated that keeping ADP % at optimum level; maxima with high desirability lay towards the extremes which was towards lower value of maltose % and higher value of TX100%

Triton X-100: 0.81%, and ADP: 1.25% with a prediction maxima of 77.22 µmole p-NP min^{-1} of lipase and a desirability of 0.939. The final lipase activity obtained was 70.32 µmole p-NP min^{-1} with a specific activity of 156.23 U/mg protein. An increase by 38% in the lipase production was achieved after the optimization of substrate concentrations.

Table 5 Experimental run of the predicted substrate concentrations after response surface methodology

Runs	ADP (%)	Maltose (%)	TX-100 (%)	Lipase activity (U)
1	0.75	1.25	0.44	21.93
2	0.75	0.75	0.81	16.70
3	1.0	1.0	0.625	13.71
4	1.0	1.0	0.31	42.54
5	1.0	1.0	0.625	20.41
6	1.0	1.0	0.625	30.38
7	1.0	1.0	0.625	20.00
8	0.75	1.25	0.81	36.31
9	1.25	0.75	0.81	81.56
10	1.42	1.0	0.625	10.03
11	1.0	0.579	0.625	61.62
12	1.25	1.25	0.81	70.41
13	1.0	1.0	0.94	30.84
14	1.0	1.0	0.625	51.63
15	1.25	0.75	0.44	29.57
16	0.579	1.0	0.625	53.34
17	0.75	0.75	0.44	44.17
18	1.25	1.25	0.44	18.44
19	1.0	1.0	0.625	20.93
20	1.0	1.42	0.625	17.25

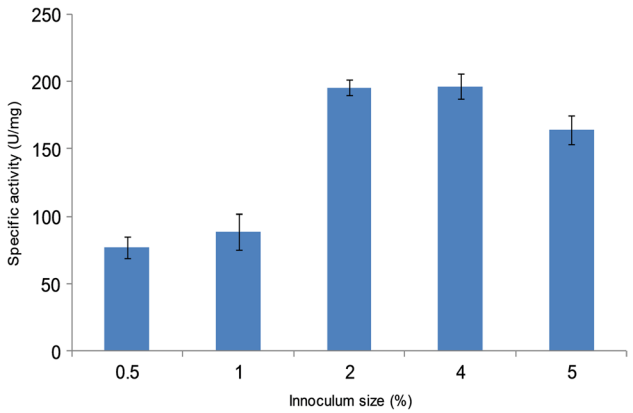

Fig. 7 The effect of varying inoculum sizes (1–5%) on the production of lipase

The effect of inoculum size, temperature, pH, organic solvents, and SDS of the optimal lipase

Maximum lipolytic activity (197 U/mg protein) was obtained with 4% (v/v) inoculum increasing the enzyme production by 1.26-folds (Fig. 7). There was no activity at acidic pH of 5 and 6 whereas it retained ~ 43% activity at pH 10 when compared to neutral pH (Fig. 8a). Likewise, the enzyme retained its activity in the temperature 30–50 °C with optimal activity at 37 °C (Fig. 8b). The lipase showed good organo-tolerance to a variety of organic solvents and could tolerate 0.5% of SDS retaining 60% of its activity (Fig. 8c, d).

Purification of lipase enzyme and determination of molecular weight through SDS-PAGE

An overall 9.2-fold purification was obtained after purification using ion exchange chromatography; however, the final yield was ~ 11% (Table 6). The purified enzyme was eluted at 0.9 M NaCl. The purified protein was ~ 67 KDa as determined by SDS-PAGE (Fig. 9a).

Determination of activity of lipase (crude/purified) in free and bound form under various growth parameters

The immobilized enzyme beads showed greater stability as compared to the unbound enzyme in both crude and purified form. Under immobilized condition, the enzyme retained 85% of its activity at pH of 5 and 6 whereas the unbound crude enzyme lost its activity in acidic pH (Fig. 8a). Notably, the purified free enzyme showed enhanced activity even at acidic pH as compared to the free crude enzyme. Similarly, the purified immobilized enzyme showed excellent activity at 60 °C and showed sustained activity over the range 20–60 °C (Fig. 8b). The activity of both bound and unbound crude enzymes was almost the same in the case of varying concentrations of SDS (Fig. 8c) showing enhanced tolerance in purified bound form. It was noted that the activity of the bound crude enzyme lowered (27–34%) in the presence of various organic solvents but increased significantly in its purified bound form (Fig. 8d). After third re-use, the immobilized beads could retain 66.2% of its initial specific activity as compared to the initial activity. Overall, the immobilized purified enzyme beads showed maximum retention of lipase activity when subjected to various external parameters.

The effect of commercial detergents, SDS-PAGE, and oil stain removal capacity of the purified lipase

Among the five detergents tested, the purified lipase showed best compatibility retaining 99% of its activity in the presence of Vidhisa detergent powder (Fig. 10). Ariel showed the least compatibility as the enzyme could retain only 29% of its activity in its immobilized form. The rest of the three detergents were moderately compatible with the purified enzyme. As compared to the detergent + water formulation, the purified lipase + water solution proved to be more effective against the sesame oil stain on the cloth fabric as shown in Fig. 9b.

Diesel degradation using P. plecoglossicida S7

After gravimetric analysis of the different samples, it was observed that the bacterial isolate was able to degrade 25.2%

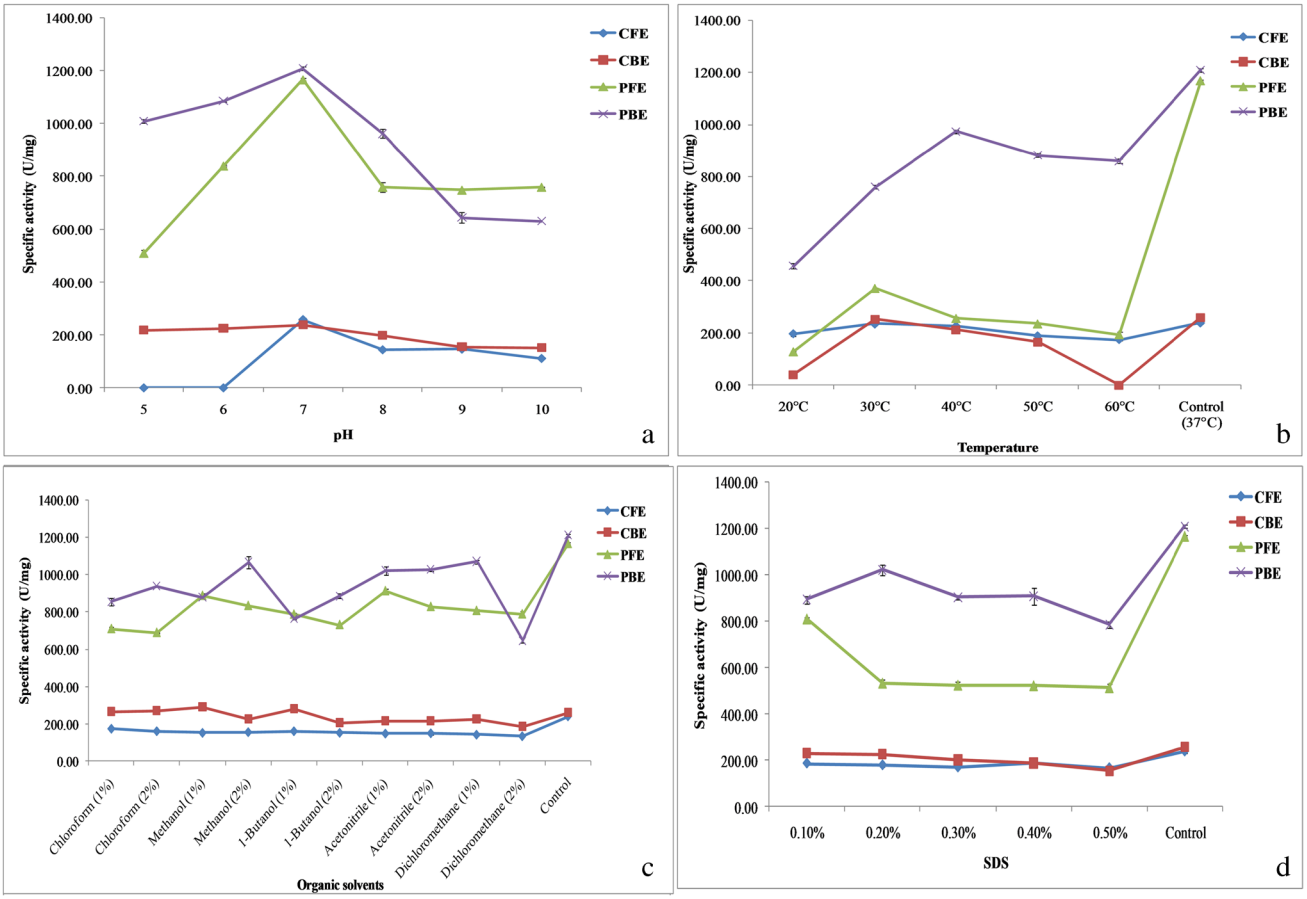

Fig. 8 **a–d** A comparative profile of the free and bound (Ca-alginate beads) of crude and purified lipase in the presence of organic solvents and SDS along with the varying lipase production over a range of pH and temperature. CFE, crude-free enzyme; CBE, crude bound, enzyme; PFE, purified free enzyme; PBE, purified bound enzyme

Table 6 Summary of step-wise purification of lipase from *P. plecoglossicida* S7

Purification step	Total Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Crude enzyme	500	18.9	0.15	128.4	1	100
Ammonium sulfate fractionation (70%)	249	33.73	0.183	183.74	1.43	69.9
Dialysis	10	11.4	.037	302.4	2.35	42.5
Ion exchange chromatography	20	107.5	.091	1181.3	9.2	10.8

of the diesel added to the soil as compared to the abiotic control.

Discussion

Pseudomonas spp. are known as potential source of diverse lipases. This is due to the versatile metabolic machinery present in *Pseudomonas* spp. Studies have reported application of *Pseudomonas* lipases in detergent, paper pulp, biodiesel production industries, etc. (Phukon et al. 2020; Nathan and Rani 2020). Optimization of the production of lipase from *Pseudomonas plecoglossicida* S7 was performed using sequential statistical designs with multiple substrates. Numerous studies have been undertaken to enhance the production of lipase using cost effective substrates (Jain et al. 2020; Ilesanmi et al. 2020). Also, the use of statistical models comes in quite handy while designing such experiments. Devi et al. (2020) used castor oil as lipase inducer for the optimization of lipase production (220 U/mL) from *P. guariconesis*. Phukon et al. (2020) applied response surface methodology using tryptone, yeast extract, and NaCl and

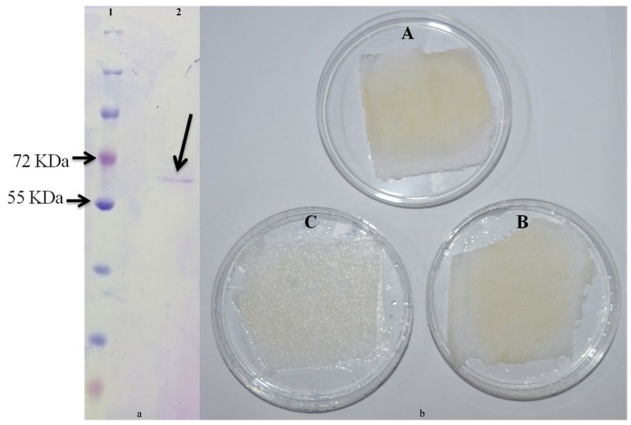

Fig. 9 **a** Lane 1: protein marker (10–250 kDa, pure gene); lane 2: Purified lipase. **b** oil stain removal using detergent + lipase and water + lipase solutions

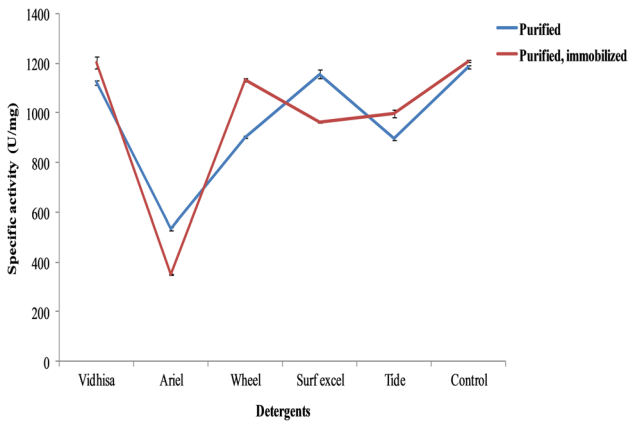

Fig. 10 The effect on lipase activity in the presence of five commercially available detergents

obtained a 2.69-fold increase in lipase activity (179.3 U/mg) from *P. helmanticensis* HS6. Azevedo et al (2020) used cacay butter and wheat bran as lipase inducers producing 308.14 U/g of lipase from *Aspergillus terrus* NRRL-255. Similarly, Nema et al. (2019) used a tri-substrate mixture of rice husk, cottonseed cake, and red gram husk in the ratio of 2:1:1 to maximize the lipase production from *Aspergillus niger* MTCC 872. In this study, optimization of the medium resulted in production of 197 U of lipase per mg protein. The focus on agro-industrial wastes and cost effective oils helps in reducing the cost of production of the enzyme significantly. Moreover, the recycling of agricultural and industrial waste has positive impact on the environment contributing to their valorization (Federici et al. 2009). This study included various oilcakes of groundnut, sesame, *Brassica nigra*, and *Synapis alba* although they were eliminated as the Plakket Burman design experiment showed that the oilcakes had a negative impact on the lipase production during their comparative analysis with other substrates. However, amendments of media components like maltose, Triton X 100 and ammonium di-hydrogen phosphate (ADP) increased the lipase activity by 2.23-folds. Al-Dhabi et al. (2020) used six carbon sources in which maltose was reported to enhance the production of lipase by 10%. Generally *Pseudomonas* spp. have been reported to use glucose more effectively (Salwoom et al. 2019; Sooch and Kauldhar 2013). Triton X-100, a nonionic surfactant, showed the highest effect on lipase activity in the screening experiment and is a well-known inducer of lipase production (Mesa et al. 2018; Perna et al. 2017). The surfactant has also been reported to contribute to the stability and catalytic property of the enzyme (Mesa et al. 2018). In the present study, further optimization of inoculum amount (4% v/v) resulted in increase in activity by 1.26-folds. Priyanka et al. (2020) reported 2.5% inoculum to be optimum for lipase activity in *P. brenneri*. 3% inoculum was found to be most favorable for lipase production in *P. yamanorum* LP2 (Komesli et al. 2020).

Lipases have a broad range of action acting on various substrates demanding different environmental conditions like temperature, pH, tolerance to organic solvents, and detergents. A wide number of thermophilic enzymes have been characterized such as amylases, xylanases, proteases, esterases, and lipases (Chakdar et al. 2016; Curci et al. 2019; Gazali and Suwastika 2018). Similarly, pH stability becomes an important factor during the mass production of an enzyme (Dumorné et al. 2017). Variations in pH change the conformation of the catalytic site, as well as extreme changes, may completely alter the enzyme structure. Lipase produced by *P. plecoglossicida* S7 showed an optimum activity at 37 °C at neutral pH. Molecular weight of the purified lipase was determined to be ~ 67 KDa. *Pseudomonas* lipases with high molecular weight have been reported previously (Ramani et al. 2010). The enzyme retained its activity over a range of 30-–50° C (Fig. 8) whereas it retained 43% of its activity at pH 10. Similar results have been reported earlier showing alkaline nature of *Pseudomonas* lipases and optimal activity at temperature ranging from 30 to 45 °C C (Devi et al. 2020; Azevedo et al. 2020). The catalytic activity of enzymes is altered or lost by the presence of ionic detergents like SDS and CTAB. *P. plecoglossicida* S7 lipase retained 60% of the activity at 0.5% concentration of SDS. Hu et al. (2018) reported that the lipase activity of *P. aeruginosa* HFE733 was lost at 10 mM/L concentration of SDS. Also, *P. plecoglossicida* S7 lipase activity increased by 4% in the presence of chloroform (2% v/v) as compared to control without organic solvents. In the presence of other organic solvents like dichloromethane, 1-butanol, methanol, and acetonitrile, the lipase activity was lost by 28.6, 21.3, 13, and 17% respectively (Fig. 8c). The bound enzyme showed relative stability (27–34% less activity) in the presence of organic solvents (1% & 2% v/v) (Bayramoglu et al. 2022;

Acıkgoz-Erkaya et al. 2021). Klibanov (2001) discussed the applicability and commercialization of enzyme-catalyzed reactions in organic solvents as compared to natural aqueous reaction media. Sonkar and Singh (2020) reported that the organic solvents showed inhibitory effect on enzyme activity of *P. punonensis* except in the case of acetone and 2-propanol. Bisht et al. (2014) attributed this loss of activity to the hydrophilic nature of some organic solvents. Purification of lipase led to 9.2-fold purification in specific activity of the protein. Previous studies have used column chromatographic techniques to enhance the purification of lipase enzyme (Priyanka et al. 2020; Hu et al. 2018). Hu et al. (2018) achieved 9.9-folds of alkaline lipase from *Pseudomonas aeruginosa* HFE733. Mehta et al. (2018) reported a 6.9-fold purification of lipase from *Aspergillus fumigatus* with a yield of 11.03% using Octyl Sepharose chromatography.

In order to have industrial applications of an enzyme, stability over broad range of temperature and pH is important for downstream processing. Since all natural enzymes are not suitable for large-scale production, they are immobilized/bound to inert substances to enhance their downstream processing including selectivity, stability, and kinetics (Velasco-Lozano 2020). Reusability of the bound enzyme also contributes to enhancing cost–benefit ratio. In this study, we carried out the ionotropic gelation of lipase onto Ca-alginate beads. As compared to the free enzyme, the stability of the bound enzyme improved in terms of both pH and temperature. The free crude enzyme lost its activity at acidic pH whereas the bound enzyme retained 84% of its activity at pH 5 as compared to the activity of free enzyme at neutral pH. The enzyme bound to Ca-alginate beads showed considerable thermal tolerance at 60 °C retaining 72% of its activity. Importantly, the purified bound form showed a more improved and sustained lipase stability when subjected to varying temperature ranges. Immobilization of lipases has been reported to improve the range of thermo stability as well as stability due to changes in pH. Pereira et al. (2019) showed that the bound enzyme in alginate-chitosan beads shifted lipase optimum pH (to 8.5 from 7) and temperature (to 40 °C from 35 °C). Notably, both the free and bound enzymes were stable in the presence of SDS which makes it a suitable candidate for usage in detergent industries. The bound enzyme could retain 66.2% of its activity which highlighted the fact that effective immobilization could save repeated production of the lipase for industrial applications. Marín-Suárez et al. (2019) analyzed the reusability of immobilized Novozyme 435 on environment-friendly biodiesel production. Further, the present study evaluated the compatibility of the purified lipase with commercially popular detergents and found that it worked extremely well in the presence of them. Earlier studies also point to the fact that enzymes compatible with commercial products prove more useful on an industrial scale (Das et al. 2016; Prazeres et al. 2006). Additionally, the action of lipase on stubborn oil stains proved the versatility of the purified enzyme (Fig. 10a). Also, the applicability of *P. plecoglossicida* S7 lipase to degrade diesel in soil can have major impact on environmental friendly removal of diesel spills. Sharma et al. (2014) reported that 66% of the diesel contaminated soil could be degraded by augmenting *P. aeruginosa*. Looking at the use of diesel by various industries and research stations, biodegradation of diesel spills becomes more relevant (Brooks et al. 2018; Zakaria et al. 2021). The use of enzymes is restricted to their natural, aqueous reaction media, and the scope of industrial bioconversions, especially for the production of speciality chemicals and polymers gets limited (Klibanov 2001). Clearly, the purified immobilized lipase in this study showed great promise to be utilized for industrial applications.

Conclusions

The present study has used sequential statistical optimization to increase lipase production in *Pseudomonas plecoglossicida* S7. Inoculation of 4% (v/v) *P. plecoglossicida* S7 culture in basal media supplemented with Maltose-0.75% (w/v), ADP-1.25% (w/v), and Triton X-100–0.81% (v/v) followed by growth at 37 °C with pH 7 resulting in 2.81-fold increase in lipase production. The free enzyme was considerably organo and detergent tolerant and active at alkaline pH. The purified enzyme when bound to Ca-alginate beads showed significant increase in the stability over a wide range of temperature (20–60 °C) and pH (5–10) with distinct efficiency and reusability. The results of this study will be very helpful to utilize *P. plecoglossicida* for mass production of lipase for industrial applications such as in dairy, pharmaceuticals, animal feed, detergent formulations, and environmental engineering like wastewater treatments, biodegradation of oil, and diesel spills in the environment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-022-22098-6.

Author contribution HC conceptualized the study, edited, and revised the manuscript. PC executed all experimental part and prepared the first draft of the manuscript. SV helped in screening of lipolytic Pseudomonads. PC and SV compiled all experimental results. AB designed the statistical set up for the experiments and analyzed the statistical data. HC, SS, and AKS finalized the manuscript in its final form.

Funding The authors received from to ICAR-NBAIM, Mau, financial assistance and infrastructural facilities.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare competing interests.

Disclaimer This is a part of the Ph.D. work of the first author under supervision of the corresponding author as co-supervisor.

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