Mapana Journal of Sciences
2022, Vol. 21, No. 4, 21 - 45
ISSN 0975-3303|https://doi.org/10.12723/mjs.63.2

Statistical Optimization of Lipase Production From Mutagenic Strain of Newly Isolated *Bacillus licheniformis* MLP

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ABSTRACT

In the present study, seven separate bacterial strains were isolated from oil contaminated soil and screened using 1%(v/v) olive oil based on high lipase productivty. Maximum lipase produced bacterium was identified as *Bacillus licheniformis* based on 16s ribotyping. Then this selected wild strain was subjected to UV irradiation and produced a mutant as *B. licheniformis* MLP. The nutritional parameters statistically optimized using fractional factorial Central Composite Design - Response Surface Methodology were sunflower oil 5.5%, glucose 5.5%, peptone 5.5% and Zn 55µML^{-1} with maximum lipase activity of 37.21±0.12 UmL^{-1}. Further, the maximum lipase production of 52.22±0.21 UmL^{-1}was obtained by *B. licheniformis* MLP when grown in an optimized medium at 48th h under the optimized pH 7 and temperature 40^{o}C. The results of the present study specify the possibility of utilization of the mutant *B. licheniformis* MLP for extracellular lipase production employing submerged fermentation. The lipase production by *B. licheniformis* MLP using sunflower oil, glucose, peptone and Zn

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employs statistical modeling towards industrial production.

Keywords: *Bacillus licheniformis,* Lipase, Mutation, Ribotyping, statistical modeling, Responce Surface Methodology

1. Introduction

Lipase enzymes (Triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous and hydrolysis the triacyglycerol into glycerol and free fatty acids in the oil water interface (Kamini et al., 2000). They belong to serine hydrolases, hence do not require any cofactor for hydrolysis. In future lipases will dominate the global enzyme market because they comprise the most significant group of biocatalysts for novel applications in the field of biotechnology (Chandra et al., 2020). The chemo, regio, and enantio- specific behavior of these enzymes have caused remarkable interest among the industrialists and scientists (Saxena et al., 2003). Currently, the lipase enzymes are using to clean septic tanks, grease traps and other systems (Al Mohaini et al., 2022).

Lipase enzymes are universally produced by various microorganisms, plants and animals. Microbial lipases on the other hand, are the most widely used enzymes because of ease of genetic manipulation, low-cost production, high productivity, stereospecificity, regiospecificity, broad substrate specificity, their selectivity, and their ability to catalyze heterogeneous reaction at both the interface of water soluble and insoluble systems (Gupta et al., 2004; Isiaka Adetunji and Olufolahan Olaniran, 2018). Microorganisms, such as bacteria and fungi, have the capability to produce intracellular and extracellular lipases. Eijkmannin isolated the first microbial lipases from *Bacillus prodigiosus, B. fluorescens,* and *B. pyocyaneus*, now they are known as *Serratia marcescens*, *Pseudomonas aeruginosa* and *P. fluorescens* respectively (Szymczak et al., 2021).

Bacterial enzymes are preferred over fungal enzymes because of their higher activities and neutral or alkaline pH conditions. Relatively very few bacterial lipases have been well studied when compared to plant and fungal lipases. Most of the bacterial lipases reported ti ll date are constitutive and nonspecific in their substrate specificity, and a few bacterial lipases are thermostable. Extracellular lipases production by bacteria influenced via fermentation

conditions includes temperature, pH, inoculum concentration, culture shaking speed, inducer sources and its concentrations (Kumari et al., 2009; Treichel et al., 2010). Explore for best conditions/concentration of the aforementioned parameters are essential for achieving high lipase production with low cost by fermentation. Classical and conventional method of achieving this is by changing one parameter- at- a time, while keeping other parameters at constant levels. This approach is very time-consuming, laborious and highly expensive for simultaneous consideration of several parameters at a time. In addition to this, illustrating the interactive effect among the parameters and finding the optimum conditions is very difficult. The statistical experimental design central composite design (CCD) in response surface methodology (RSM) has been accepted as a successful method to overcome these drawbacks in numerous biotechnology processes (Oliveira et al., 2017; Olufolahan Olaniran, 2018).

RSM is one of the statistical techniques that are used for designing and modeling experiments to search the optimum conditions of effective parameters influencing the responses. Besides, it is extensively used to study interactions between parameters and quantify the relationships (Gupta et al., 2004; Acikel et al., 2011). Furthermore, this technique has been successfully applied to find the optimum conditions for process parameters for lipase production. A CCD is the most frequently used design while the experimental design is defined with lower and upper limits of each variable. Many fermentation parameters had been optimized for high lipase production using CCD (Colla et al., 2016).

The constant demand for exploring the indigenous and robust bacterial strains for production of high lipases with potential biotechnological applications are necessitated. A focus should be made on getting the right microbial strain through screening and modification, selection of appropriate medium components, identification of physical parameters and intended applications of the produced lipases (Salihu and Alam, 2012). The aim of the present study is to optimize the lipase production by using response surface methodology with newly isolated mutant bacteria. In the present study, optimization of culture parameters for enhanced extracellular lipase production by native, newly isolated mutant bacterium was investigated using RSM.

MATERIALS AND METHODS

Materials

High purity or analytical grade chemicals used in the experiment were purchased from Hi-media and SRL (Mumbai, India) for preparing various media and reagents.

Sample collection and isolation of lipase producing bacterial strains

Soil samples collected aseptically from five different sites, viz. site1, site2, site3, site4 and site5 of oil spilled areas of the small-scale oil extraction industry, located in Kancheepuram District, Tamil Nadu state, India, for isolation of potent lipase producing bacterial strains under laboratory conditions. The obtained soil samples pooled into a single sample and used 1g of mixed soil sample for soil extracts by dissolving in 100mL distilled water. Then the sample was serially diluted by added about 1mL of sample into 9mL sterilized distilled water. This is the first dilution, and it gives a 10^{-1} dilution. Like this, up to 10^{-9} dilutions were made. Finally, the dilutions of 10^{-3} to 10^{-5} were chosen and spread 100 µL of diluted sample on nutrient agar plates supplemented with olive oil (1%, v/v). Then plates were incubated at 30°C until the strains with lipolytic activity.

Maintenance of isolated bacterial strains

Isolated bacterial strains were stored at 4 °C in a nutrient agar slant (Peptone-5.0 g, Beef extract-3.0 g, Sodium Chloride -5.00 g, Agar-15.0 g, Distilled water -1000.0 mL, pH - 7.1± 0.2) for further uses. The cultures were revived every month. An inoculum was prepared by transferring a loopful of seed culture to the nutrient medium. Then incubated at 35 °C until an optical density value of 0.6 was reached at 600 nm.

Screening of maximum lipase producing bacterial strains

Erlenmeyer flasks (250mL) containing 100 mL of basal medium (Yeast extract-3g, K_2HPO_4 -1.5g, KH_2PO_4 -1.5g, CaCl - 0.3g, $MgSO_4.7H_2O$ -0.4g, KCl-0.1g, $FeSO_4$. $7H_2O$-0.3 g, Distilled water-1000mL, pH-7.0) was supplemented with 1.0 %(v/v) of olive oil and inoculated 1% (v/v) of newly isolated bacterial strains. Then the Erlenmeyer flasks were incubated at 30 °C for 3 days. End of the experiment, culture broth was withdrawn and centrifuged at 14000

rpm, at 4 ^{o}C for 15 min. The collected supernatant was used as a crude lipase for lipase activity estimation under the standard assay conditions. Then the maximum lipase producing bacterial strain was selected and used for further studies.Each experiment was carried out in triplicate for each bacterial strain and presented the data values as mean± standard deviation.

Lipase assay

Lipase assay was done by spectrophotometrically using the method of Krieger et al., (1999). About 2.4 mL of substrate solution p-nitrophenyl palmitate (p-NPP) was added to 0.1 mL of enzyme solution and then incubated at 37 oC for 10 min. End of the experiment the reaction was terminated by adding 2 mL of 0.2 M Na_2CO_3 solution. Immediately the p-nitrophenol (p-NP) concentration was measured at 410 nm using the UV-Visible spectrophotometer (PC-Based Double Beam UV-VIS Spectrophotometer 2206, Systronics India Ltd, Gujarat). The obtained OD value was interpolated in the standard curve of the p-NP and found the lipase activity. One unit of enzyme activity is defined as the amount of enzyme that released 1 µmole p - nitrophenol per minute at 37 °C and pH 8.

Identification of selected bacterial strain using 16S rRNA based molecular techniques

Genomic DNA was extracted from the selected isolate using the Biogeno Genomic DNA isolation kit (BGKT16). The amplification of 16S rRNA gene was carried out by polymerase chain reaction (PCR) technique using primer (F) 5'-AGAGTTTGATCCTGGCTCAG-3' as the forward primer and (R) 5' -TACGGTTACCTTGTTACGACTT-3' as the reverse primer. PCR technique and sequencing were performed according to the procedure of Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Then the sequence of 16S rRNA was aligned by using the BLASTn programme (Altschul et al., 1997), and a Neighbour - Joining phylogenetic tree was constructed using the Clustal W algorithm with the help of MEGA (Molecular Evolutionary Genetics Analysis) software version 4.1 (Tamura et al., 2013).

Strain development by ultraviolet -mutation

The selected wild parent strain such as LLP4 was exposed to ultraviolet (UV) irradiation at different time intervals of 0, 3, 6, 9, 12,

15 and 18 min in a "Laminar flowhood – Cabinet" fitted with a TUP 40W germicidal lamp that has about 90% of its radiation at 2540-2550A at an affixed distance of 35 cm away from the lamp. The mutation frequency was mentioned to be high when the survival rates were between 10 and 0.1% (Hopwood *et al*., 1985). Plates having less than a 1% survival rate were selected for the isolation of mutants. Eight isolates of LLP4 were selected and screened for maximum lipase production. The suffix MLP was designated for each selected mutant strain of their respective parent strains. The selected mutant strain was further used for lipase production. Every month, the mutant strain was revived in a nutrient agar slant.

Screening the best nutritional factors for lipase production

The best nutritional factors were screened to achieve the maximum lipase production using a selected mutant strain by one parameter-at-a-time (classical method). The experiments were carried out in a 250 mL Erlenmeyer flask using 100 mL of each sources free basal medium, which contained . The medium was autoclaved at 1.1 bar pressure for 15 min at 121°C. The fermentation was then performed at 30 °C for 72 h at 120 rpm using 1% inoculum. At the end of the experiment, 10 mL of culture broth was withdrawn and centrifuged at 14,000 rpm at 4 °C for 15 min. The cell free supernatant was used as the crude lipase for determination of lipase activity under standard assay conditions. The essential nutritional factors such as oil sources (1%v/v of coconut oil, cottonseed oil, mustard oil, sunflower oil, soybean oil and wheat bran oil), carbon source (1 % w/v, glucose, sucrose, and glycerol), nitrogen source (1% w/v of yeast extract, peptone and ammonium sulphate) and metal ions (10 μML^{-1} of Copper (Cu), Selenium (Se), and Zinc (Zn)) were optimized. In the present investigation, each experiment was carried out in triplicate and presented the data values as mean± standard deviation.

Optimization of media components for lipase production using statistical approach

The optimization of lipase production medium components were carried out by the screened variables namely sunflower oil, glucose, peptone, and Zn and their interactions were studied. Other medium components kept constant. Each factor in the CCD was studied at

three different levels (Table 1). All the variables were taken at a central coded value considered as zero. A matrix of 31 experiments with four factors was generated, using a design expert software MINITAB 12. The fermentation was carried out separately for each run with replicates. The average maximum lipase activity was taken as the response (Y). A regression analysis was done for the obtained data and this resulted in an empirical model that related the response measured to the independent variables of the experiment. For any system, the model equation is represented as

$$Y = \beta o + \Sigma \beta i Xi + \Sigma \beta i Xi^2 + \Sigma \beta ij XiXj \quad (1)$$

Where, Y is the predicted response, βo the intercept, βi the linear coefficient, and βij is the interaction coefficient. The statistical significance of the regression coefficients was determined by student's t-test. The second order model equation was determined by Fischer's test, and the proportion of variance explained. An analysis of variance (ANOVA) was performed, and three dimensional response surface curves were plotted to study the interaction among these factors.

Table 1 Experimental range of the independent variables used in CCD

Independent variables	Code	Levels		
		-1	0	+1
Sunflower oil (%, v/v)	X_1	1	5.5	10
Glucose (%, w/v)	X_2	1	5.5	10
Peptone (%, w/v)	X_3	1	5.5	10
Zn (μML^{-1})	X_4	10	55	100

Optimization of physical parameters

The essential physical parameters such as pH (4-9) and temperature (25-50 ^oC) were also optimized using classical methods of optimization. The experiments were carried out in Erlenmeyer flasks (250 mL) containing 100 mL of screened basal medium with an optimum concentration of sunflower oil, glucose, peptone and Zn. Each experiment was carried out in triplicate and presented the data values as mean ± standard deviation.

RESULTS AND DISCUSSION

Isolation and screening of lipase producing bacterial strains

There is a vast need to explore the natural habitats to isolate potential lipase producing bacteria, since microbial isolation and screening is a significant stage for evaluating potential lipase producing bacteria from diverse natural habitats. In the present investigation, seven different bacterial strains were isolated from the soil samples under aerobic conditions. Then each strain was individually screened by cultivated in the basal medium supplemented with 1% olive oil for 72 hrs. It was observed that all the seven bacterial strains were grown well and produced lipase in 1% olive oil supplemented basal medium. The lipase production was 12.01±0.31, 13.34±0.11, 11.14±0.14, 17.21±0.21, 12.11±0.22, 11.03±0.21 and 11.21±0.32 UmL^{-1} for the isolates, LLP1, LLP2, LLP3, LLP4, LLP5, LLP6 and LLP7 respectively. Among the seven strains, the strain LLP4 produced the maximum lipase of 17.21±0.21UmL^{-1}. Therefore, the strain LLP4 was selected for further study. This result supports the information that lipase production between the bacterial species could be notably dissimilar (Hasan *et al*., 2006).

Molecular level identification of isolate LLP4

A 16S rRNA sequence analysis was done for molecular level identification of the selected isolate LLP4. The amplification of 16S rRNA gene was carried out by a PCR technique and it was found to be ~1181 bp. The selected bacterial strain sequence was aligned with 26 most similar sequences in the Genbank database and then a phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). In the phylogenetic tree (Fig. 1) the marked isolate LLP4 was 100% similar to the *Bacillus licheniformis* ATCC14580 DSM13. Data from phylogenetic analysis studies unveiled and confirmed the selected bacterial strain LLP4 was *Bacillus licheniformis*. Then it was used for further investigation. *Bacillus* spp is a most common bacterial genera present in soil, and different species have been found in a variety of environmental niches (Saxena et al., 2020; Al Mohaini et al., 2022).

Selection of mutants with high lipase production

The strain improvement programmes are required for commercial application. The remarkable increases in fermentation productivity

and the resulting decreases in production costs have come about essentially by using mutagenesis. In the present study, the suspension of a selected wild strain such as *B. licheniformis*-LLP4 was separately exposed to UV radiation for 3, 6, 9, 12, 15 and 18 min. Then The UV survival percent was tabulated for each exposure time (Table 2). Plates having a survival rate of between 10 to 0.1% of the parent strain were selected. Eight colonies of *B. licheniformis*-LLP4 were obtained and screened for high lipase production. They were designated as 1 LLP4 to 8 LLP4. The maximum lipase production was obtained from the isolate number 5 LLP4. Then it was chosen as a potential mutant bacterium and further designated as *B. licheniformis* MLP. The selected mutant bacterial strain was further used for the following studies.

Figure 1 The constructed phylogenetic tree of *Bacillus licheniformis*-LLP4 by Neighbor-Joining method

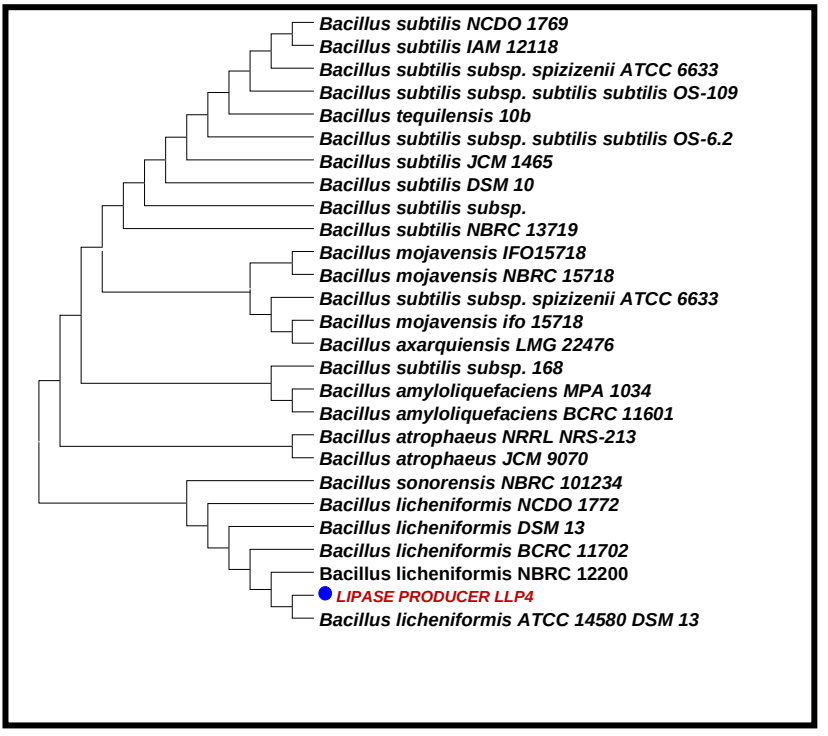


Table 2 Effect of UV irradiation on selected strain 5 LLP4

UV Irradiation time(min.)	**Lipase Activity (UmL-1)**		
	No. of cells/mL after irradiation	**Survival percent**	**Percentage kill**
0	2.3X107	100	0
3	5.6X106	24.34	75.63
6	5.1X106	22.17	77.83
9	8.9X105	3.86	96.14
12	7.3X105	3.17	96.83
15	8.3X104	0.36	99.64
18	6.1X104	0.26	99.74

All values (No. of cells/mL after irradiation) are represented as mean ± SD of three replications

Screening best nutritional factor for lipase production by *B. licheniformis* MLP

The present study reported on the favorable condition of the lipase production by mutant *B. licheniformis* MLP using media component optimization. The following important factors were tested for their effects on lipase yield with the aim of reducing production costs. The best nutritional factor for lipase production by *B. licheniformis* MLP was screened by the one parameter-at-a-time (classical method). In this study, each nutritional factor [oil sources (coconut oil, cottonseed oil, mustard oil, sunflower oil, soybean oil, and wheat bran oil), carbon source (glucose, sucrose, and glycerol), nitrogen source (yeast extract, peptone and ammonium sulphate) and metal ions (Cu, Se, and Zn)] was screened individually by studied their effect on maximum lipase production by *B. licheniformis* MLP.

The result indicates that the supplementation of various natural oil sources are induced lipase production (Table 3). However, among the various oil sources tested, sunflower oil was found as a best inducer for lipase production because the medium containing sunflower oil showed the highest lipase production of 25.12±0.11UmL-1 by *B. licheniformis* MLP at 72 h. This finding is on

par with the reports of Al Mohaini et al., (2022). It was reported that the addition of sunflower oil in the fermentation medium as a carbon source resulted in a significant increase in lipase production by *Bacillus salmalaya*. The lipid which contains the highest C18:n will serve as a best inducer for lipase production by bacteria. There were significant differences between the lipase production in different oil sources ($p<0.05$). Hence, it was used as the best inducer for further media optimization.

As shown in the Figure 2, among the three different carbon sources used, the mutant strain *B. licheniformis* MLP utilized glucose as a carbon source for the maximum lipase yield of 27.21±0.13UmL^{-1}, in the presence of 1% sunflower oil. This study was in accordance with the report of Rathi *et al.,* (2001) and Soleymani et al., (2017). There were significant differences between the lipase production in carbon sources ($p<0.05$). Hence, glucose was used as the best carbon source for further media optimization. On the other hand, the presence of nitrogen source in the fermentation medium also influences maximum lipase production by different microorganisms (Tembhurkar *et al.,* 2012). As illustrated in Fig. 3, the maximum lipase production of 28.99±0.41 UmL^{-1} was observed in the 1% (w/v) peptone supplemented medium. In the present investigation, the best nitrogen source was peptone, similar to the results shown by Haniya et al., (2017). There were significant differences in the lipase production from nitrogen sources ($p<0.05$). Hence, peptone was used as the best nitrogen source for further media optimization.

This study indicates that the tested metal ions are required for the growth as well as lipase production of mutant bacterial strain. The lipase production by the mutant bacterium *B. licheniformis* MLP was found to be 32.21±0.05 UmL^{-1} in 10 µML^{-1} Zn supplemented medium (Figure 4). When the metal ions were utilized for the lipase production, Zn^{2+} was observed to stimulate lipase production by *Hypocrea patella* (Christianah *et al.,* 2012). However, Haniya et al., (2017) who showed the inhibitory effects of Zn on lipase production by *B. subtilis* strain PSCIRNL-39. There were significant differences in the lipase production from metal ion sources ($p<0.05$).

In this study, the influence of various oil sources, carbon sources, nitrogen sources, and metal ions on lipase production in submerged fermentation was studied and found that 1% (v/v) of sunflower oil,

1% (w/v) of glouse, 1% (w/v) of peptone, and 10 μML^{-1} of Zn were the best nutritional factors for maximum lipase production by the mutant bacterium *B. licheniformis* MLP. The influence of these nutritional factors on lipase production was found to be statistically significant ($p<0.05$). Besides, these nutritional factors were used for further medium optimization by RSM.

Table 3 Effect of different oil sources on lipase production by *B. licheniformis* MLP

Name of the oil sources (1%, v/v)	**Lipase Activity (UmL^{-1})**
Control (Basal medium)	5.01±0.14
Coconut oil	18.24±0.11
Cottonseed oil	23.14±0.23
Mustard oil	19.32±0.12
Sunflower oil	25.12±0.11
Soybean oil	19.33±0.21
Wheat bran oil	21.21±0.13

Figure 2 Effect of different carbon source on lipase production by *B. licheniformis* MLP

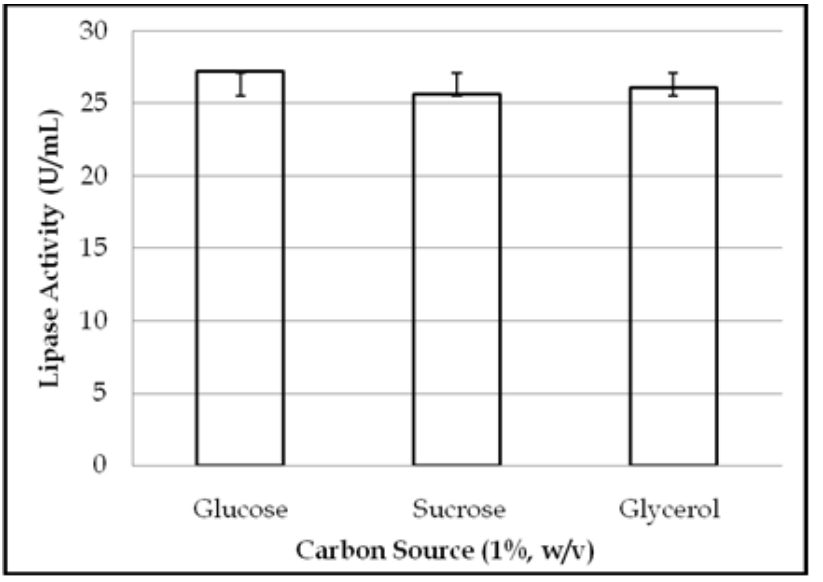


Figure 3 Effect of different nitrogen source on lipase production by *B. licheniformis* MLP

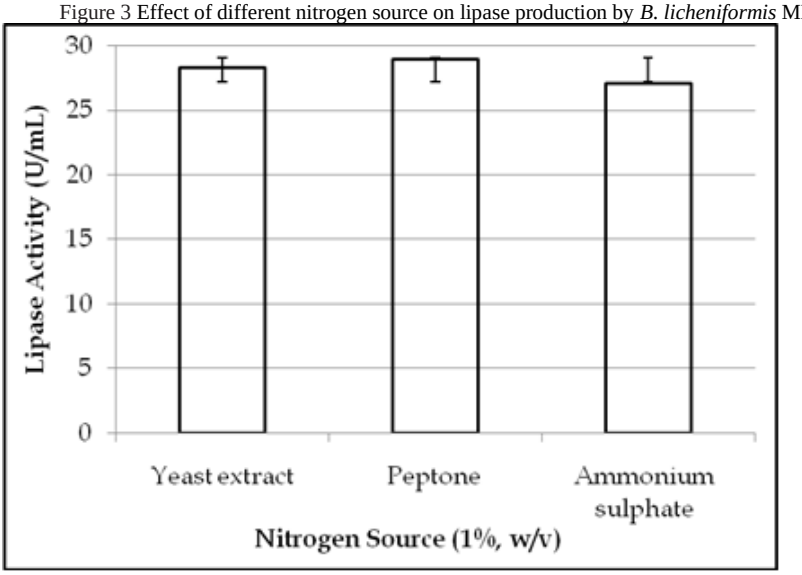
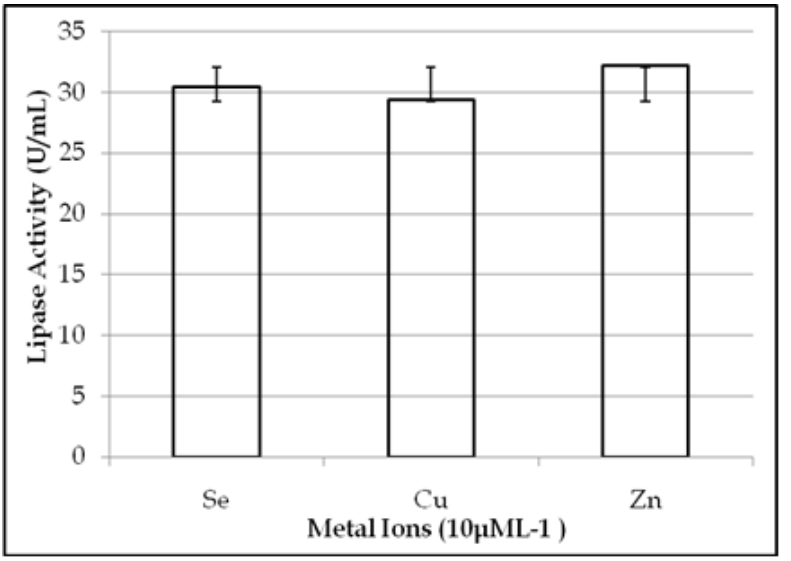


Figure 4 Effect of different metal ions on lipase production by *B. licheniformis* MLP



Media optimization using CCD method

To improve the maximum lipase production by *B. licheniformis* MLP, the screened best nutritional factors sunflower oil (1-10 %v/v), glucose (1-10 %w/v), Peptone (1-10% w/v) and Zn (10-100 μML^{-1}) were statistically optimized and their interactions studied using

RSM. Thus, in order to determine optimum concentration of these components for maximum yield of lipase, analysis using CCD, a set of 31 experiments was done. All the experiments were carried out in duplicate. The design matrix of the variables in coded units along with the predicted and experimental values of response (lipase production) of *B. licheniformis* MLP. The obtained average lipase yield was present in Table 4.

The obtained t, and the p values for the linear, quadratic and combined effects of all the four independent variables on lipase yield by *B. licheniformis* MLP are given in Table 5. The p-value and Fisher's test value of this model was <0.001. It demonstrates statistically significant results of this model at >95% confidence level (p-<0.05). From the Table 5, it was observed that the three individual (X_2, X_3, and X_3), four quadratic coefficients (X_1^2, X_2^2, X_3^2, and X_4^2) and two combined effects (X_2X_3, X_2X_4) were statistically significant at $p< 0.05$, while the interaction between factors was also significant. Among them, quadratic coefficients X_2^2 (glucose), X_3^2 (peptone) and X_4^2 (Zn) were the most significant, which confirmed the fact that they are very important for the lipase production by *B. licheniformis* MLP. The equation second order polynomial regression for lipase production was interrelated with an empirical correlation and the process variable in coded formats is specified in Eq. 2.

$$\textbf{Lipase Yield } (UmL^{-1}) = 37.007 - 0.106*X_1 - 0.718*X_2 - 2.526*X_3 - 3.020*X_4 - 6.830*X_1^2 - 4.175*X_2^2 - 4.830*X_3^2 + 1.790*X_4^2 + 0.539*X_1X_2 + 0.666*X_1X_3 - 2.479*X_1X_4 + 0.9337*X_2X_3 - 2.238*X_2X_4 - 0.148*X_3X_4 \quad (2)$$

The coefficient of determination (R^2) states that the developed model is fit to be used. The value of R^2 was measured to be 0.981 for lipase production which indicates that 98.10% of the responses confidence level would be clarified by the model. (Pramitasari and Ilmi, 2021). In this study, the obtained experimental values are comparatively similar to predicted values. This supports the correctness of the data obtained in various experimental combinations. It is apparent from the ANOVA (Table 6) that the model thus developed is fitting and significant for lipase production since the value of R^2 for lipase production recorded was >96% confidence level.

Std Order	Media components, (%,(v/v),%,(w/v), μML^{-1})				Lipase Yields (UmL^{-1})	
	X1	X2	X3	X4	Experimental	Predicted
1	1.0	1.0	1.0	10	29.21±0.11	28.93
2	10.0	1.0	1.0	10	31.02±0.13	31.39
3	1.0	10.0	1.0	10	30.21±0.14	29.36
4	10.0	10.0	1.0	10	34.12±0.11	33.98
5	1.0	1.0	10.0	10	22.31±0.21	21.91
6	10.0	1.0	10.0	10	27.04±0.14	27.04
7	1.0	10.0	10.0	10	26.07±0.11	26.07
8	10.0	10.0	10.0	10	33.41±0.13	33.36
9	1.0	1.0	1.0	100	33.55±0.12	33.54
10	10.0	1.0	1.0	100	26.09±0.15	26.09
11	1.0	10.0	1.0	100	25.01±0.13	25.01
12	10.0	10.0	1.0	100	20.01±0.14	19.72
13	1.0	1.0	10.0	100	26.14±0.11	25.93
14	10.0	1.0	10.0	100	21.14±0.12	21.11
15	1.0	10.0	10.0	100	21.13±0.17	21.79
16	10.0	10.0	10.0	100	18.51±0.12	18.71
17	1.0	5.5	5.5	55	23.21±0.13	23.33
18	10.0	5.5	5.5	55	23.04±0.11	23.67
19	5.5	1.0	5.5	55	27.12±0.11	26.00
20	5.5	10.0	5.5	55	25.12±0.15	24.00
21	5.5	5.5	1.0	55	29.24±0.16	29.67
22	5.5	5.5	10.0	55	21.01±0.14	21.33
23	5.5	5.5	5.5	10	37.41±0.13	36.33
24	5.5	5.5	5.5	100	27.21±0.11	26.67
25	5.5	5.5	5.5	55	37.15±0.11	37.08
26	5.5	5.5	5.5	55	37.21±0.21	37.08

27	5.5	5.5	5.5	55	37.18±0.21	37.08
28	5.5	5.5	5.5	55	37.11±0.12	37.08
29	5.5	5.5	5.5	55	37.12±0.21	37.08
30	5.5	5.5	5.5	55	37.13±0.20	37.08
31	5.5	5.5	5.5	55	37.11±0.21	37.08

Table 4 CCD matrix of independent variables used in RSM for lipase production by *B. licheniformis* MLP

Table 5 Estimated regression coefficients of second order polynomial model for optimization of lipase production by *B. licheniformis* MLP

Variables	**Coefficient**	**Estimated Coefficients**	**t- value**	**p-value**
Constant	β_0	37.007	85.409	<0.001[a]
X_1	β_1	-0.106	-0.307	0.753[b]
X_2	β_2	-0.718	-2.087	<0.001[a]
X_3	β_3	-2.526	-7.336	<0.001[a]
X_4	β_4	-3.020	-8.772	<0.001[a]
X_1^2	β_{11}	-6.830	-7.533	<0.001[a]
X_2^2	β_{22}	-4.175	-4.605	<0.001[a]
X_3^2	β_{33}	-4.830	-5.327	<0.001[a]
X_4^2	β_{44}	1.790	1.974	<0.001[a]
X_1X_2	β_{12}	0.539	1.475	0.066 [b]
X_1X_3	β_{13}	0.666	1.825	0.160[b]
X_1X_4	β_{14}	-2.479	-6.788	0.087[b]
X_2X_3	β_{23}	0.9337	2.557	<0.001[a]
X_2X_4	β_{24}	-2.238	-6.131	<0.001[a]
X_3X_4	β_{34}	-0.148	-0.407	0.689[b]

Parameter: X1 - Sunflower oil, X2-Glucose, X3- Peptone, X4- Zn.

[a]Significant at p< 0.05 level

[b]Not Significant at p< 0.05 level

Table 6 ANOVA of second order polynomial model for optimization of lipase production by *B. licheniformis* MLP

Source	**Degree of freedom**	**Sum of squares**	**Mean square**	**f-value**	**p-value**
Regression	14	1768.78	126.342	59.22	<0.001[a]
Linear	4	288.47	72.117	33.80	<0.001[a]
Square	4	1275.77	318.941	149.50	<0.001[a]
Interaction	6	204.55	34.092	15.98	<0.001[a]
Residual Error	16	34.13	2.133		
Lack of fit	10	34.13	3.413		
Pure Error	6	0.000	0.000		
Total	30	1802.92			

R2=0.981%, aSignificant at p< 0.05 level

3D plots of the influence of sunflower oil (X1), glucose (X2), peptone (X3) and Zn (X4) on the lipase production determined using CCD in RSM are given in Figure 5 (a - f). The 3D figure shows the effect of two selected independent variables on lipase production while other two factors kept constant. It was discovered that increasing the levels of four factors increased lipase production up to its middle level, after which it decreased.

The predicted optimum value of lipase production was sunflower oil 5.5 % (v/v), glucose 5.5% (w/v), peptone 5.5% (w/v) and Zn 55 µML-1. The predicted maximum lipase yield was 37.08 UmL-1 in the optimized medium. These values were experimentally validated by conducting each experiment in triplicate at optimum process conditions, and the comparable maximum lipase production of 37.21±0.21 UmL-1 was observed.

Optimization of environmental factors for lipase production by the classical method of optimization

The initial pH of the production medium was maintained from 4 to 9 with 1 increment. As shown in Figure 6, the maximum lipase production of 41.02±0.32 UL^{-1} was obtained at pH 7.0 by *B. licheniformis* MLP. The low lipase activity was obtained for all other initial pH values. Most of the bacteria reported for lipase production were grown at neutral initial pH for optimal growth and lipase production (Sumanjelin *et al.*, 2013; Sathyavrathan and Jaya, 2013). In the present study, production media with an initial pH 7.0 was found to have maximum lipase production by *B. licheniformis* MLP. Hence, the pH 7.0 was used for further process optimization. The effect of culture temperature on lipase production was studied by varying the temperature range from 25 to 50 ^{0}C using the optimized medium. As shown in Figure 7, when increasing the culture temperature from 25 to 40 0C resulted in a noteworthy increase in lipase production from 15.12 to 42.22±0.14UmL^{-1}. In the temperature range from 30 - 50 ^{0}C, decrease in lipase production was recorded. This finding is on par with the reports of Isiaka Adetunji and Olufolahan Olaniran, (2018). Therefore, the maximum lipase production was obtained at 40 ^{0}C and is considered the optimum.

The *B. licheniformis* MLP produced 8.4- fold increase in lipase yield when grown in the optimized culture condition than grown in an unoptimized medium as control. The observed experimental lipase production values are comparatively similar to the predicted lipase values. Hence, the generated model proves to be valid and accurate. Therefore, this statistical experimental design and analysis were found to be very efficient for maximum lipase production

Figure 5 (a-f) 3D Surface plot of combined effect of screened nutritional factors on production by *B. licheniformis* MLP

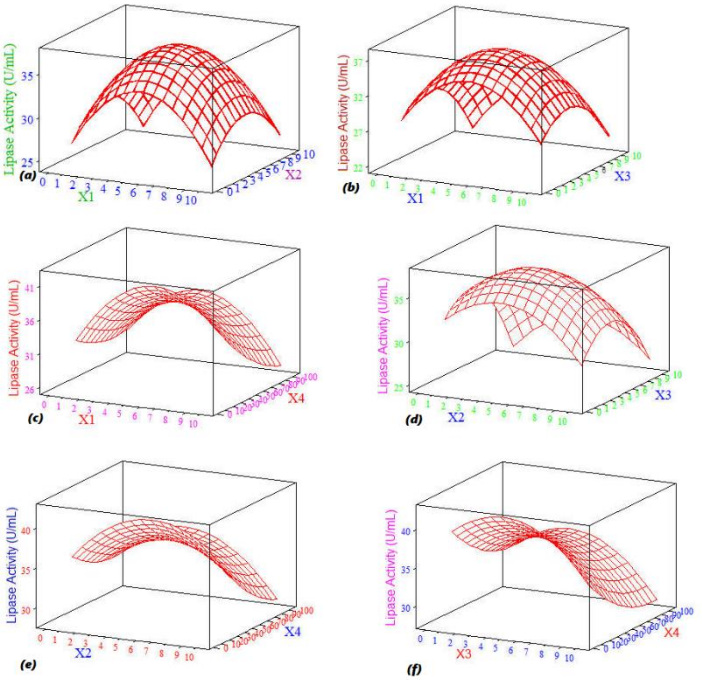


Figure 6 Effect of different initial medium pH on lipase production by *B. licheniformis* MLP

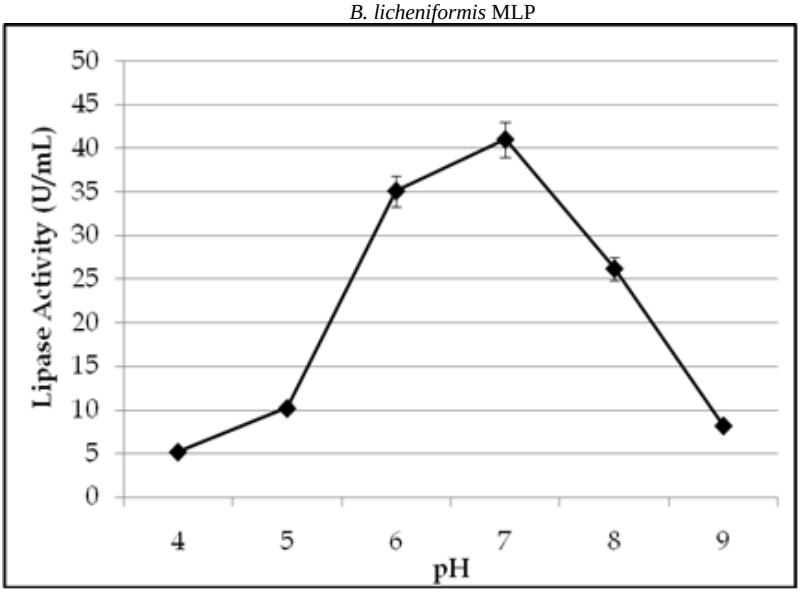
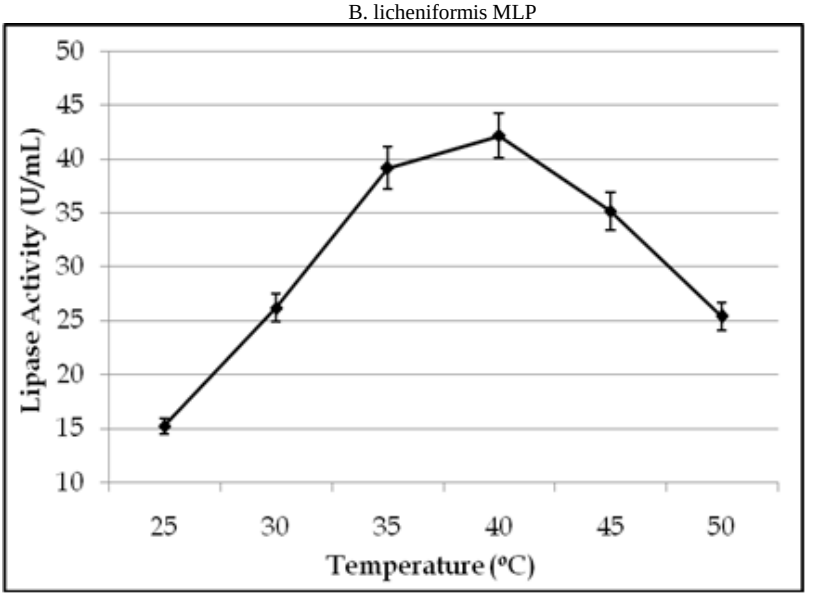


Figure 7 Effect of different temperature on lipase production by B. licheniformis MLP



CONCLUSION

Seven separate bacterial strains were isolated from oil-contaminated soil and screened using 1% (v/v) olive oil based on high lipase productivity. A maximum lipase produced bacterium strain was identified as *B. licheniformis* based on 16S ribotyping. Then this selected wild strain was subjected to UV irradiation and produced a mutant as *B. licheniformis* MLP. The nutritional parameters statistically optimized using CCD-RSM were sunflower oil 5.5%, glucose 5.5%, peptone 5.5%, and Zn 55 μML^{-1} with maximum lipase activity of 37.21±0.12 UmL^{-1}. Further, the maximum lipase production of 42.22±0.14 UmL^{-1} was obtained by *B. licheniformis* MLP when grown in the optimized medium at 48 h under the optimized pH of 7, and temperature of 40 ^0C. The observed experimental lipase production values are comparatively similar to the predicted lipase values. Hence, the generated model proves to be valid and accurate. As a result of the experiment it was recorded that the lipase production increased to 8.4 fold after the optimization of culture parameters by RSM. The findings of the new investigation suggest that the optimum conditions of the parameters studied by the statistical method of optimization will enable the production of lipase in a large-scale with minimized expenditure by a newly

isolated mutant *B. licheniformis* MLP, over the one variable at a time method.

Disclosure and Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding information

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Credit author statement

D.Lakshmi: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **K. Dhandayuthapani:** Data curation, Conceptualization, Supervision, Writing - original draft, Formal analysis, Investigation.

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