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Bacillus amyloliquefaciens PS35 ISOLATED FROM FAT AND OIL CONTAMINATED SITES: AUGMENTATION OF LIPASE PRODUCTION USING STATISTICAL EXPERIMENTAL DESIGN

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ABSTRACT

Lipase is an industrially important enzyme and is hence being extensively researched. Novel lipolytic bacterial strains capable of producing lipases with industrially useful properties are the need of the hour. In the present investigation, 52 bacterial strains were isolated from soil and wastewater samples high in fat and oil contents, out of which 12 showed significant lipolytic activity. The best lipase producing strain PS35 was identified to be *Bacillus amyloliquefaciens* by 16S rRNA gene sequencing. A temperature of 40°C and pH 8.0 were found to be the optimum conditions for lipase production by the strain. Dextrose, sunflower oil and casein were the chemical components in the culture medium that were conducive for lipase production. Subsequently, Box Behnken Design of Response Surface Methodology was applied and the model resulted in 1.44-fold increase in enzyme production. Analysis of variance indicated significance of the proposed model ($p < 0.0001$) and a good correlation between the experimental and predicted values. This study opens up promising avenues for further research on the properties of this enzyme, based on which appropriate applications could be devised.

KEY WORDS: Lipase, *Bacillus amyloliquefaciens*, 16S rRNA gene sequencing, response surface methodology

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INTRODUCTION

Hydrolases are the most widely applied class of industrial enzymes and lipases (triacyl glycerol acylhydrolases EC 3.1.1.3) are quite prominent among them. They belong to the superfamily of serine hydrolases and share certain common structural features such as the α/β hydrolase fold.[1-2] They catalyze the hydrolysis of esters formed from glycerol and long-chain fatty acids and the products released include di-acyl glycerol, mono-acyl glycerol, fatty acids and glycerol. The reverse reactions of esterification, interesterification and transesterification are catalyzed in non-aqueous environments. Lipases are chemo, regio and enantio selective in nature. They are applied widely in a variety of industrial sectors such as food, detergent, textile, leather, paper and pharmaceuticals.[3] The search for new lipases with improved properties continues, owing to the fact that only a miniscule fraction of the enormous microbial diversity has so far been exploited. After the initial screening step, optimizing lipase production by the isolate becomes pertinent. The traditional route to optimization is to vary one-factor-at-a-time (OFAT), while keeping the others at a constant level. This method becomes laborious when a large number of variables influence the process. Another drawback is its failure to incorporate the interaction effects of variables. Statistical tools such as Plackett-Burman and Response Surface Methodology (RSM) enable effective medium optimization using a limited number of trials and have been successfully applied in lipase production.[4] In this article, we report the isolation and screening of lipolytic bacterial strains from fat and oil containing wastewaters and their identification using 16S rRNA gene sequencing. Optimization of production medium for the best isolate was carried out through conventional single factorial method and RSM.

MATERIALS AND METHODS

Isolation, screening, identification and characterization

Samples were collected from five different sites in Coimbatore District of Tamil Nadu, India. They were obtained from the dairy industry, groundnut oil mill, palm oil mill, fast food restaurant, poultry slaughterhouse and coconut oil mill. At each site, the discharged wastewater, as well as soil sample exposed to the discharge was collected. They were serially diluted and the higher dilutions (10^{-5} - 10^{-8}) were plated on nutrient agar supplemented with 2% (v/v) olive oil and incubated overnight at 35°C. Discrete colonies were sub-cultured and the pure cultures were streaked on tributyrin agar plates to screen for lipase activity. Isolates showing positive results were further cultivated in glucose – yeast extract – peptone (GYP) broth supplemented with trace-elements and olive oil. The cells were harvested by centrifuging at 10,000 rpm and the cell-free supernatant was used as a crude enzyme extract. It was subjected to a quantitative assay using *p*-nitrophenyl palmitate (*p*-NPP) as the substrate.[5] The absorbance was measured (Shimadzu, UV-1800) at 410 nm, against an enzyme-free blank. Molar extinction coefficient of 0.0146 μM^{-1} cm^{-1} was used. One unit of lipase activity was defined as µM of *p*-nitrophenol released per minute under the assay conditions. Protein content was estimated by Lowry's method[6] and specific activity was calculated. The cellular, cultural and biochemical characteristics of the isolates were investigated.[7] The best lipase producer was identified using 16S rRNA gene sequencing. 16s rRNA gene primers 27F (5'-AGAGTTTGATC(A/C)TGGCTCAG-3') and 1492R(5'-ACGG(C/T)TACCTTGTTACGACTT-3') were used to amplify ~1.4 kb gene from the isolated genomic DNA.[8] The amplified PCR product was gel purified, quantified and sequenced using an ABI prism 3100 Genetic Analyzer (Applied Biosystem). Consensus sequence was generated from forward and reverse sequences using 'Aligner' software. It was subjected to BLAST with NCBI GenBank Database.[9] Phylogenetic tree was constructed using the neighbor joining method.

Conventional optimization of the production medium

The preserved stock culture of the best lipase producer was inoculated in GYP broth, cultured overnight and subsequently 2% (v/v) of this inoculum (43 x 10^6 CFU/ml) was transferred to the production medium. The physicochemical parameters were varied one-factor-at-a-time and their effects on enzyme production were studied. The glucose present in the medium was replaced with fructose, maltose, sucrose and lactose. They were added at a concentration of 20 g/L. Peptone present in the medium was replaced with other organic nitrogen sources such as tryptone, casein and inorganic sources such as $(NH_4)_2SO_4$, $NaNO_3$ and NH_4Cl. These components were added at a concentration of 10 g/L. Olive oil was replaced with sesame, palm, sunflower, groundnut and coconut oils, which were added at a concentration of 1% (v/v) in order to check which oil best induced lipase production. Next, the GYP medium was supplemented with different trace elements such as $CaCl_2$, $MgSO_4$, $MnSO_4$, KCl, $FeSO_4$ and $ZnCl_2$, which were added at a concentration of 0.50 g/L. The initial pH of the production medium was varied from 5.0 to 9.0, incubation temperature from 20 to 70°C and incubation time from 12 to 72 h. These experiments were carried out in triplicates and the data given represent the mean.

Response surface methodology

A Box - Behnken Design developed by the Design Expert Software, version 8.0.7.1 (Stat-Ease Inc. Minneapolis, USA, trial version) was used for this purpose. Sunflower oil (A), dextrose (B) and peptone (C) were the three parameters whose levels were optimized. Each one of these independent variables was studied at three different levels (-1, 0, +1). A matrix of 17 experiments was generated by the software. Accordingly, production was carried out in 250 ml Erlenmeyer flasks containing 100 ml culture medium of pH 8.0. The flasks were sterilized, inoculated with the culture under aseptic conditions and incubated at 40°C for 48 h, in an Orbital Shaker set at 120 rpm. The pH, time and temperature were selected based on results from OFAT studies. At the end of the incubation period, lipase assay was performed using the cell-free supernatant and activity was recorded as the response. All experiments were carried out in triplicates and the

data given represent the mean. The response data were analyzed by feeding into the software, which generated contour plots and standard analysis of variance (ANOVA). A quadratic polynomial regression model was assumed for the predicted response. For a three-factor system, the model equation was as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad \text{----------(1)}$$

Where, Y is the predicted response, β_0 is the intercept, β_1, β_2, β_3, are the linear coefficients, β_{11}, β_{22}, β_{33}, are the squared coefficients, and β_{12}, β_{13}, β_{23}, are the interaction coefficients.

RESULTS AND DISCUSSION

Isolation, screening, identification and characterization

Serial dilution and plating of the samples in olive oil supplemented nutrient agar led to the isolation of 52 bacterial strains. When they were inoculated in tributyrin agar, visible clearance zones, ranging from slight to well pronounced, were observed for 23 strains. They were:

DI2, DI4, DI5 and DI6 – Dairy Industry isolates
GOM7, GOM8, GOM10, GOM11 and GOM13 – Groundnut Oil Mill isolates
POM14, POM17 and POM20 – Palm Oil Mill isolates
FR22, FR26, FR27 and FR30 – Fast food Restaurant isolates
PS35, PS39, PS42, PS51 and PS52 – Poultry Slaughterhouse isolates
COM4A and COM6B – Coconut Oil Mill isolates

Upon performing *p*-NPP assay to confirm and quantify their lipase activity, 13 strains showed good color development and appreciable enzyme activity (Fig. 1). PS35 possessed the highest activity of 22.52 U/ml. This was followed by COM4A showing lipase activity of 18.23 U/ml and very closely by DI2 and GOM8.

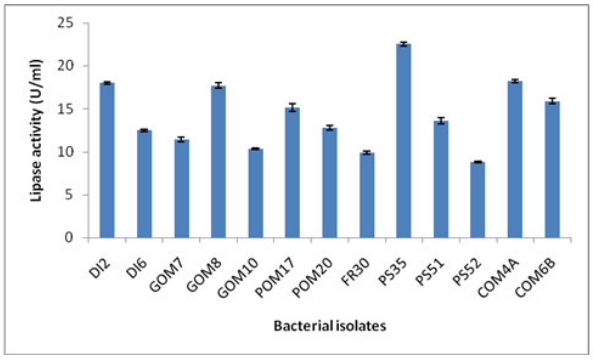

Figure 1
Quantification of lipase activity by p-NPP assay

The cultural characteristics of the bacterial isolates are listed in Table 1.Their cellular characteristics, as inferred through appropriate staining techniques, are given in Table 2. They were mostly seen to be Gram positive rods, motile and spore forming, with only a few exceptions. Biochemical characteristics are summarized in Table 3. It could be observed that all the isolates were uniformly capable of glucose fermentation, sucrose fermentation and catalase production, while they gave mixed results for the other biochemical tests.

Table 1
Cultural characteristics of the bacterial isolates

S. No.	Isolate	Colony morphology				
		Size	Color	Form	Margin	Elevation
1	DI2	Medium	Creamy	Irregular	Undulate	Flat
2	DI6	Medium	Creamy	Circular	Undulate	Raised
3	GOM7	Large	Creamy	Irregular	Lobate	Raised
4	GOM8	Large	White	Irregular	Serrate	Raised
5	GOM10	Large	White	Irregular	Serrate	Flat
6	POM17	Large	Creamy	Irregular	Undulate	Flat
7	POM20	Medium	Creamy	Irregular	Lobate	Flat
8	FR30	Large	White	Circular	Undulate	Raised
9	PS35	Large	White	Irregular	Undulate	Flat
10	PS51	Large	Creamy	Irregular	Lobate	Raised
11	PS52	Large	White	Circular	Undulate	Flat
12	COM4A	Small	Yellow	Circular	Entire	Raised
13	COM6B	Large	White	Circular	Lobate	Flat

Table 2
Cellular characteristics of the bacterial isolates

S. No.	Isolate	Cell morphology	Gram reaction	Endospore formation	Motility
1	DI2	Small rods	+	+	+
2	DI6	Small rods	+	+	+
3	GOM7	Small rods	+	+	+
4	GOM8	Rods	+	+	+
5	GOM10	Rods	+	+	+
6	POM17	Rods	+	+	+
7	POM20	Rods	+	+	+
8	FR30	Small rods	+	+	+
9	PS35	Rods	+	+	+
10	PS51	Small rods	+	+	+
11	PS52	Small rods	+	+	+
12	COM4A	Cocci	+	-	-
13	COM6B	Rods	+	+	+

Table 3
Biochemical characteristics of the bacterial isolates

Biochemical test	Isolate												
	DI2	D16	COM4A	COM6B	GOM7	GOM8	GOM10	POM17	POM20	FR30	PS35	PS51	PS52
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	-	-	-	+	-	-	-	+	-	-	-	-	-
Mannitol	+	-	+/-	-	+	-	+	-	+	+	+	+	-
Trehalose	+	+	+	+	-	-	+	+	-	+	+	+	-
Malonate	+	+/-	+	+	+/-	-	-	-	-	-	-	-	+/-
Voges Proskauer	-	-	-	-	+/-	-	-	-	+/-	+	-	-	-
Citrate	-	+	-	-	+	+	-	+	+	+	+	+	+
Arginine	-	-	+	+	-	-	-	-	-	-	-	-	+
ONPG	+	+	-	+	+	+	+/-	-	+	+	+	-	+
Nitrate reduction	+	+	+/-	+	-	+/-	-	+	+	+	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+

+ Positive result,- Negative result,+/- Variable result

16S rDNA sequencing and BLAST analysis revealed that the strain PS35 belonged to the genus *Bacillus*. Its sequence showed 99% identity to the 16S ribosomal RNA gene sequences of *Bacillus amyloliquefaciens* strains deposited earlier. Unlike *B. subtilis* that has been widely explored for lipase activity,[10-13] *B. amyloliquefaciens* is a novel species as far as its lipase activity is concerned. *Per se*, there are only scanty reports on lipase production from this species and hence a thorough investigation of its lipase encoding gene and enzymatic properties are warranted. Its phylogenetic tree is presented in Fig. 2. The sequence was submitted to NCBI GenBank Database and was allotted the Accession Number KJ000043.

Figure 2
Phylogenetic tree of Bacillus amyloliquefaciens PS35

Conventional optimization of the production medium

When different monosaccharides and disaccharides were tested for their effects on lipase production, dextrose was found to be the best carbon source, resulting in maximum enzyme production. Fairly good production was achieved with fructose as well, while lactose was least favored (Fig.3). In certain other studies, the carbon source did not have a significant effect on lipase production. For example, *Bacillus* sp. had shown no significant increase in lipase production when varying the carbon source at different pH values.[14] The presence of a lipid source in the production medium has been shown to stimulate enzyme production in most lipolytic strains. In our case, sunflower oil had optimal inducing effect (Fig.3). In another study, it has been shown that lipase production by *Aspergillus niger* was increased by 10% in the presence of palm oil.[15] Of the various nitrogen sources tested, peptone appeared to be the best organic nitrogen source that brought about highest enzyme production. Casein, a complex substrate was not preferred and so was the inorganic nitrogen source $NaNO_3$ (Fig. 3). Other authors have also established that organic nitrogen sources are preferred by *Bacillus* sp.[16] In one other study, lipolytic titers in the culture medium of *Bacillus* sp. were found to increase in the combination of inorganic (NH_4Cl) and organic (casein) nitrogen sources.[17]

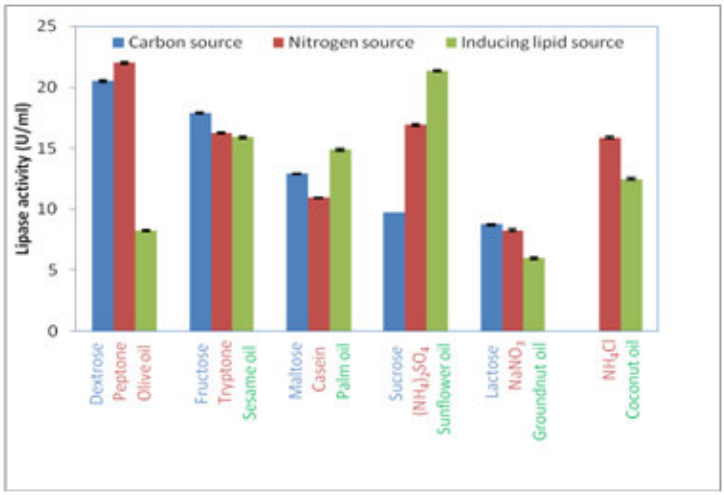

Figure 3
Effect of carbon, nitrogen and inducing lipid sources on lipase production

Certain metal ions when present in trace amounts in the production medium could induce lipase production. Peak enzyme production by PS35 was observed in the presence of Ca^{2+}, while Fe^{2+} resulted in least production (Fig. 4). Lipase production by *Burkholderia multivorans* has also been positively influenced by the presence of Ca^{2+} ions in the production medium.[4]

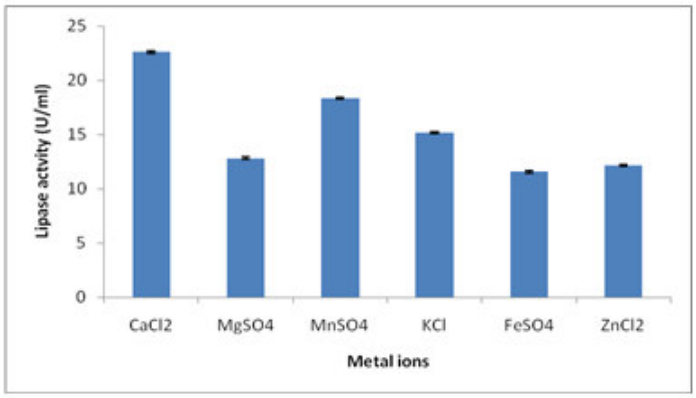

Figure 4
Effect of metal ions on lipase production

From incubation time, temperature and pH studies, it was observed that maximum lipase production was obtained in a culture medium of initial pH 8.0 (Fig. 5), after 48 h of incubation (Fig. 6) at a temperature of 40°C (Fig. 6). In the case of *B. coagulans*, maximum lipase production has been reported after 48 h of incubation at 55°C and pH 8.5.[12]

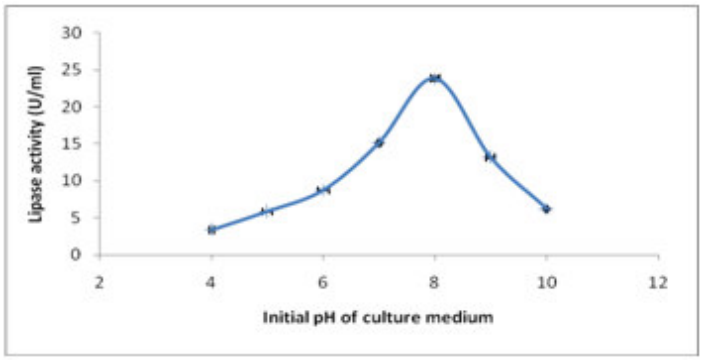

Figure 5
Effect of pH on lipase production

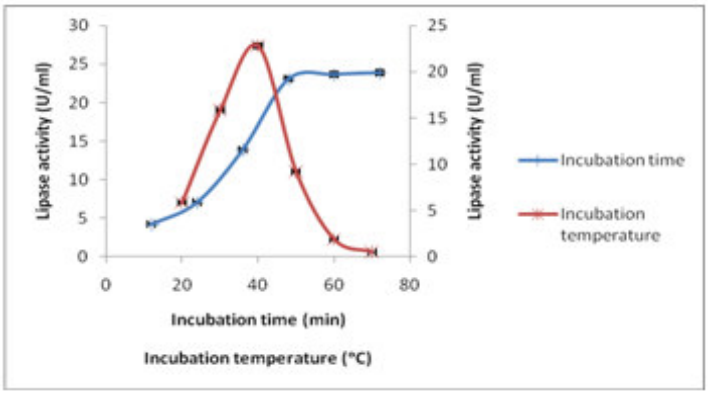

Figure 6
Effect of incubation time and temperature on lipase production

Response surface methodology

The experimental design and responses obtained are given in Table 4.The following second order polynomial equation illustrates the empirical relationship between the independent variables and the response:

$$Y = +15.40 + 7.81 \times A + 4.03 \times B + 1.56 \times C + 2.85 \times AB + 0.73 \times AC - 1.50 \times BC + 1.46 \times A^2 + 0.39 \times B^2 + 0.41 \times C^2 \quad \text{---------- (2)}$$

It can be inferred from the above equation that except for the interactive effects of dextrose and peptone, all other variables had positive impacts on lipase production.

Table 4
RSM experimental design and responses obtained for strain PS35

Run	Factor A: Oil (% v/v)	Factor B: Dextrose (g/L)	Factor C: Peptone (g/L)	Response: Lipase activity (U/ml)
1	1.75 (0)	17.5 (0)	10 (0)	15.1
2	1.75 (0)	17.5 (0)	10 (0)	15.1
3	0.5 (-1)	30 (+1)	10 (0)	10.2
4	1.75 (0)	30 (+1)	5 (-1)	20.6
5	3 (+1)	30 (+1)	10 (0)	32.4
6	1.75 (0)	17.5 (0)	10 (0)	15.1
7	0.5 (-1)	17.5 (0)	5 (-1)	8.6
8	0.5 (-1)	17.5 (0)	15 (+1)	11.2
9	1.75 (0)	17.5 (0)	10 (0)	15.1
10	3 (+1)	5 (-1)	10 (0)	18.6
11	3 (+1)	17.5 (0)	5 (-1)	21.9
12	1.75 (0)	17.5 (0)	10 (0)	16.6
13	0.5 (-1)	5 (-1)	10 (0)	7.8
14	1.75 (0)	30 (+1)	15 (+1)	19.8
15	1.75 (0)	5 (-1)	15 (+1)	14.8
16	1.75 (0)	5 (-1)	5 (-1)	9.6
17	3 (+1)	17.5 (0)	15 (+1)	27.4

ANOVA was used to check the adequacy of the model and F test was employed for this purpose (Table 5). Model F value of 106.64 and p-value < 0.0001 indicate that the model is highly significant.The p value serves as a tool for checking the significance of each of the coefficients and it also indicates the interaction strength of each independent variable.[18] In general, larger magnitudes of t, F values and smaller p values indicate that the corresponding coefficient terms are significant. All three linear coefficients (A,B,C), interaction coefficients AB, BC and quadratic coefficient A^2 were found to be significant.

Table 5
ANOVA for response surface quadratic model

Source	Sum of squares	Degrees of freedom	Mean square	F value	p-value Prob> F
odel	692.0513	9	76.89459	106.6394	< 0.0001
A-Oil	488.2813	1	488.2813	677.1607	< 0.0001
B-Dextrose	129.605	1	129.605	179.7395	< 0.0001
C-Peptone	19.53125	1	19.53125	27.08643	0.0012
AB	32.49	1	32.49	45.05795	0.0003
AC	2.1025	1	2.1025	2.9158	0.1315
BC	9	1	9	12.48143	0.0096
A^2	9.005921	1	9.005921	12.48964	0.0095
B^2	0.632237	1	0.632237	0.876802	0.3803
C^2	0.716447	1	0.716447	0.993587	0.3521
Residual	5.0475	7	0.721071	-	-
Lack of Fit	3.2475	3	1.0825	2.405556	0.2079
Pure Error	1.8	4	0.45	-	-
Cor Total	697.0988	16	-	-	-

The R^2 value gives a measure of how much variability in the observed response could be explained by the experimental parameters and their interactions. The closer the value of R^2 is to 1, the better the correlation between the experimental and predicted values and the better the model predicts the response.[19] The R^2 value for the quadratic model is 0.9927 and the adjusted R^2 value is 0.9834. Response surface methodology also attempts to detect which experimental parameters generate signals that are large in comparison to the noise. An adequate presision of 36.35 for lipase activity indicates adequate signal. The contour plot is a way of expressing the regression equation graphically (Fig. 7). It depicts the interactions among the variables and is used to determine the optimum concentration of each factor for good response.

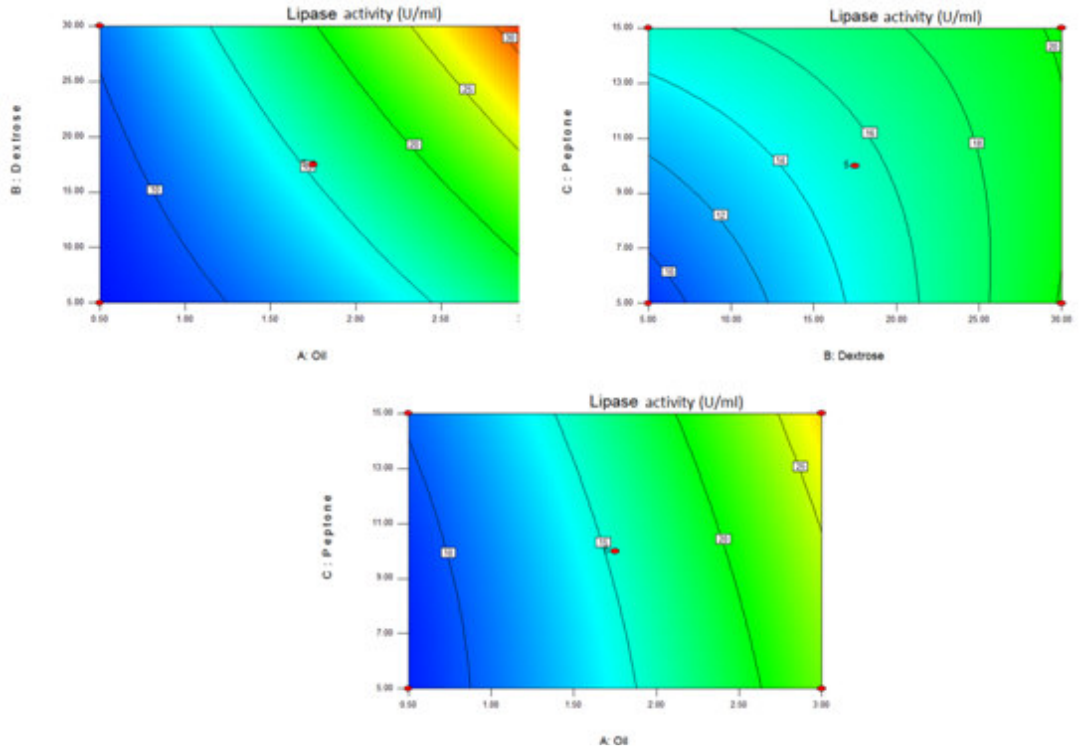

Figure 4.13
Contour plots depicting interactions between variables

Maximum lipase activity obtained after employing RSM was 32.40 U/ml, from run 5, with 3% (v/v) oil, 30 g/L dextrose and 10 g/L peptone. This is a 1.44-fold increase in comparison with the pre-optimization maximum of 22.52 U/ml. Thus, the proposed model has resulted in enhanced lipase production from *B. amyloliquefaciens*, thereby making the process all the more useful. The production of extracellular lipase from *Stenotrophomonas maltophilia* CCR11 has been similarly optimized via statistical design method.[20] Alkaline lipase production from *Burkholderia multivorans* has also been optimized by statistical methods and produced in a bioreactor.[4] Another work has utilized statistical experimental design methodology to improve lipase production from *Burkholderia* sp. C20.[21] In order to determine the correctness of the model, validation experiments were performed using the optimum values obtained for the parameters. Maximum lipase production achieved experimentally was 32.40 U/ml, while the predicted value from the model was 31.94 U/ml, thus indicating a strong agreement between the experimental and predicted values. This confirms the validity and precision of the model.

CONCLUSION

Our work has culminated in the isolation of several bacterial strains with significant lipase activity, from environmental samples. The best lipase producing strain PS35 has been identified as *B. amyloliquefaciens*, which is a novel species that has not been reported much in literature for its lipase activity. The effects of various physicochemical parameters on enzyme production by this isolate have been studied by varying one-factor-at-a-time. The work has also employed statistics-based experimental design tools to simultaneously screen multiple variables and optimize fermentation conditions for increasing the lipase yield. Box Behnken design of RSM has indicated sunflower oil 1% (v/v), dextrose 30 g/L and peptone10 g/L to be the optimum medium composition facilitating maximum lipase production. The model has been shown to be statistically significant as reflected by a very low p value (< 0.0001) and high F value (106.64). Cheap substrates derived from waste materials and large scale production in a bioreactor are to be explored with this isolate in future studies, so as to analyze scale-up feasibility, offset production costs and enhance the economic viability of the process.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest involved in this manuscript.

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