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UNVEILING A THERMO-ALKALIPHILIC BACTERIAL ISOLATE AS POTENTIAL LIPASE PRODUCER FROM LOCAL WATERBODY, KOCHARVA, VAPI, INDIA: MEDIA OPTIMIZATION STUDIES

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ABSTRACT

Our study reports thermo-alkaliphilic bacterial isolate C-1, with lipolytic potential, obtained from a local water body, located in Kocahrva village, Vapi, Gujarat, India, loaded with industrial effluents, identified as *Bacillus subtilis*, based on biochemical and molecular characterization (GenBank Accession no. MN068818). The present study is directed towards media parameters optimization for *Bacillus subtilis* C-1 lipase productio, using Plackett-Burman Design and response surface methodology. Various media variables viz., glucose, olive oil, $MgSO_4$, ammonium chloride, KH_2PO_4, peptone, tryptone, and $CaCl_2$ were tested using 16 runs design. In accordance with the Pareto chart, glucose, peptone, $MgSO_4$ and $CaCl_2$ were significant factors. The maximum lipase activity obtained was 21.48 Enzyme Units (EU)/ml. These significant media variables were further optimized for concentration, using randomized Central Composite Design, prepared using Design Expert (v.12.0.1). The lipolytic activity increased to 32.1 EU/ml, which was 1.49 times greater, with 3 gm% glucose, 3.5 gm/L peptone and 0.5 gm/L $MgSO_4$. Biochemical characterization of partially purified C-1 lipase, demonstrated optimum pH 10.0 and optimum temperature for 55°C. Hence, it was accomplished that *Bacillus* subtilis C-1 strain can be a prospective lipase source for large scale production.

Keywords: Thermo-alkaliphilic; *Bacillus subtilis*; Plackett-Burman Design; Central Composite Design; Response Surface method

INTRODUCTION

Microbial enzymes have always been more promising then plant or animal origin enzymes due to their vigorous nature. Lipases (EC 3.1.1.3), from hydrolases class of enzymes with a broad array of biological functions, have been the thrust area for research **(Thompson et al., 1999)**. Microbial lipases are unique as they catalyze hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis reactions in aqueous and non-aqueous media **(Vaisee et al., 2016; Javed et al., 2018; Tailor and Gadhvi, 2023; Tailor et al., 2024)**. Looking at the plethora of applications of bacterial lipase, especially those with thermo-, alkali- or halophilic nature, in detergent formulations and biodiesel synthesis **(Saraswat et al., 2017; Javed et al., 2018)**, potential new sources of such lipases are in demand.

Many bacterial and fungal species are known production of high activity lipase, but *Bacillus* species have gained enormous attention due to its extracellular lipase production. Furthermore, *Bacillus* lipases display high activity over a wide range which extends to highly alkaline pH upto 10 **(Saensenga et al., 2016) & 11.5 (Dhumri and Bayomi, 2019)** and temperature upto 60^{0}C **(Kumar et al., 2012) & 70^{0}C (Dhumri and Bayomi, 2019; Janssen et al., 1994)** under submerged conditions (SmF) **(Lee et al., 2015)**. Among vast number of *Bacillus* species which are reported to produce extracellular lipase, includes *Bacillus subtilis* **(Lesuisse et al., 1993; Eggert et al., 2003; Ahmed et al., 2010)**, *Bacillus aryabhattai* **(Adetunji and Olaniran, 2018)**, *Bacillus pumilus* **(Sangeetha et al., 2008)**, *Bacillus cereus* **(Vaisee et al., 2016)**, *Bacillus megaterium* **(Rani and Kaur, 2015)**, *Bacillus licheniformis* **(Sahoo et al., 2018)** and *Bacillus stratosphaericus* **(Gricajeva et al., 2016)**. Production of bacterial metabolites involves an imperative industrial bioprocess of submerged fermentation (SmF). Additionally, the technique is advantageous due to completely homogenous media composition. Therefore, approximately 90% of all industrial enzymes are produced by SmF **(Colla et al., 2010)**.

Diverse media parameters, including physico-chemical parameters such as temperature, pH, inoculum size, agitation; and carbon and nitrogen source, inducer substances and their concentrations influence production of extracellular lipase by bacterial strains **(Adetunji and Olaniran, 2018)**. It is decisive to have optimized fermentation conditions to obtain significant yields maintaining the process economics. Due to laborious, time-consuming and expensive working out of one-factor-at-a-time (OFAT) approach, statistical experimental designs of Plackett Burman design (PBD) and Response Surface Methodology (RSM) are preferred **(Hasan et al., 2006; Gururaj et al., 2016; Tailor et al., 2024)**.

In the present study, a native *Bacillus subtilis* strain C-1, obtained by screening and isolation procedures from a local water body of Vapi, Gujarat, was used for testing its potential for production of extremophilic lipase. In continuation with the objective, media parameter optimization was worked out using the statistical methods of PBD and RSM, applied for production of an extracellular lipase production. Eight media constituents from previous literature were first subjected to PBD optimization to find out the significant factors. The variables with significant contribution were further subjected to RSM optimization to determine their optimum concentrations. The data was analyzed, model validation was established and the potential of the *Bacillus subtilis*, as an extracellular lipase producer was evaluated.

MATERIALS AND METHODS

Chemicals used

All analytical grade chemicals were used in various experiments. Hi-media Laboratories Pvt. Ltd., Mumbai, India was the source for all media ingredients. Para-nitrophenyl palmitate and para-nitrophenol were purchased from Sigma-Aldrich, US.

Isolation and Screening of thermos-alkliphilic lipolytic bacteria

Various soil and water samples across regions of Silvassa, Dadra & Nagar Haveli, (UT of DNH & DD), India; and Vapi, Gujarat State, India, were collected at different time intervals. The collected samples were further analyzed for cultivable lipolytic bacterial strains diversity. The media used for screening lipase producers included Rhodamine B agar with composition as given by Kouker and Jaeger: Rhodamine B, 0.001%(w/v); nutrient broth, 0.8%(w/v), NaCl, 0.4%(w/v), agar, 1%(w/v) and olive oil 3%(v/v), in distilled water, with pH of 6.5-7.0 **(Kouker and Jaeger, 1987)**. Those bacterial isolates producing fluorescent orange halos around the colonies, indicating lipolytic activity were further studied. Selected isolates were then subjected to quantitative screening by carrying out lipase assays.

Quantitative screening for the most potential isolate

The positive lipolytic isolates were inoculated in production media with composition (gm/L): Yeast extract, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.38; KNO_3, 3.54; $FeSO_4.7H_2O$, 0.01; Olive oil, 10 ml; with final pH adjusted to 9±0.5 **(Bisht, 2011)**. The production media was incubated at 55^0C for 24-48 hours and then lipase activity was determined for each of the selected isolate.

Lipase assay

Crude enzyme, i.e. culture supernatant was used to assess the activity of bacterial lipase. The assay involved use of para-nitrophenyl palmitate (pNPP). The method for lipase assay was as developed by Winkler and Stuckman, with slight modifications as per Bussamara **(Winkler and Stuckmann, 1979; Bussamara et al., 2010)**. A stock solution (50 mM) of pNPP was prepared in isopropyl alcohol. Reaction mixture further consisted of 200 µl stock solution, added with 20 µl crude enzyme and 1.8 ml 0.1 M Tris buffer (pH 8.0). It was then incubated at 35^0C for 30 minutes. Amount of para-nitrophenol released was measured at 405 nm. One unit of lipase activity was defined as micromoles of pNP released $mL^{-1}min^{-1}$ under standard assay conditions.

Identification of the most potential isolate

Morphological, cultural and biochemical characteristics, based on Bergey's Manual of Determinative Bacteriology, VIII edition, were further used to identify the most potent isolate C-1. Even molecular characterization methods, involving 16S rDNA homology, was used to identify C-1 isolate.

16S rDNA homology characterization

Genomic DNA isolation kit was utilized for extraction of C-1 genomic DNA. It was further amplified using Polymerase Chain Reaction (PCR) with the help of 16S rDNA Universal Primers **(Turner et al., 2007)**.
Primer 8F (5'-→3') AGAGTTTGATCCTGGTCAG.
The PCR product quality was assessed by agarose gel electrophoresis (1% agarose). It was further subjected to sequence analysis using ABI 3730Xl (Applied Biosystems) Genetic Analyzer and BigDye Terminator (v3.1) cycle sequencing kit, with 11 nucleotide sequences. NCBI Basic Local Alignment Search Tools (BLASTn) program was used for sequence alignment. Weighbor software was used for phylogenetic tree construction, with 4 alphabet size and 1000 length size. Sequence deposition at GenBank, NCBI was done and an accession number was assigned to the submission.

Optimization of production media

Statistical approach was used for optimizing media parameters to maximize lipase production. Initial significant factors screening was done using Plackett Burman design (PBD) (Plackett and Burman, 1946). The optimum concentration of individual factors was further determined by subjecting to Response Surface Methodology (RSM).

Plackett Burman Design (PBD)

A set of 16 experiments was constructed using Design Expert (v.12.0.1) software (Stat-Ease Corporation, USA), for eight components: carbon sources including glucose and olive oil; nitrogen sources including ammonium chloride, peptone and tryptone; and inorganic salts including $MgSO_4$ and $CaCl_2$ and KH_2PO_4. Each component was tested at two concentration levels: low (-1) and high (+1). The concentration for each of the component tested was: glucose (1 & 3 gm%); olive oil (1 & 3 %v/v); ammonium chloride (0.5 & 2.5 gm/L); peptone (0.5 & 2.5 gm/L); tryptone (0.5 & 2.5 gm/L); $MgSO_4$ (0 & 0.5 gm/L); $CaCl_2$ (0 & 0.001 gm/L) and KH_2PO_4 (0 & 1 gm/L). Concentration levels were decided on the basis of experimental evaluation of each parameter at different concentrations as per previous literature. The shake flask studies were carried out in Erlenmeyer flasks (150 ml) with 50 ml media, incubated at 50^0C. Lipase activity was measured as response in periodically withdrawn samples (Ruchi et al., 2008) and analyzed for their effects based on the formula:

E = (Total response at high level) – (Total response at low level)/ N,

Where N = Total number of experiments (16 in this case) **(Mehta et al., 2019)**.

Response Surface Methodology (RSM)

A Central Composite Design (CCD) (2n factorial) was developed using software Design Expert (v.12.0.1), for optimization of concentrations of glucose, peptone and $MgSO_4$, compliant for a 20 experiments set. Low concentration values for the remaining factors were maintained throughout. Ammonium chloride and tryptone were omitted from the medium as these were showing negative regression coefficient. The shake flask studies were carried out in Erlenmeyer flasks (150 ml) with 50 ml media (pH 10.0), prepared as per the design. Inoculum size of 6% (v/v) was used for all sets, followed by incubation at 50^0C, recording lipase activity at the end of 24 hours. Response data was analyzed in the software to generate 2D contour plots and 3D response surface graphs.

Validation of the model

Validity of the quadratic model was evaluated by conducting experiments as per the Design Expert software (v.12.0.1), point prediction feature. Lipase activity was assessed by comparing predicted value and obtained value, to statistically validate the results. All the experiments were done in triplicate and variation was within ±5%.

RESULTS AND DISCUSSION

Isolation and Screening of thermos-alkliphilic lipolytic bacteria

Out of 5 soil samples and 7 water samples, a total of 5 potential lipolytic bacterial isolates were isolated, as indicated by orange halos on Rhodamine B agar plates, at 350 nm, utilized for further studies. Secondary screening demonstrated that highest lipolytic activity (6.87 EU/ml) was given by C-1 isolate, and hence was selected for further studies.

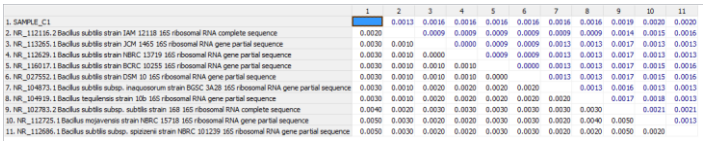


Figure 1 Evolutionary relationship of C-1 isolate

Figure 2 Phylogenetic tree for *Bacillus subtilis* C-1 isolate

Table 1 Characterization of the most efficient isolate C-1

Isolates	**C-1**
Morphological Characteristics	
Size	Long
Shape	Rods
Arrangement	Single/pairs
Gram reactivity	Positive
Cultural Characteristics	
Size	Large
Margin	Irregular
Elevation	Flat
Opacity	Opaque
Pigmentation	Nil
Consistency	Soft
Biochemical Characteristics	
Sugar fermentation	
Glucose	-
Galactose	-
Fructose	-
Lactose	-
Ribose	-
Sucrose	-
Mannose	-
Mannitol	-
Nitrate reduction	+
Oxidative sugar utilization	
Oxid	+
Ferm	-
Indole test	-
Oxidase test	+
Citrate utilization	+
Growth at	
4°C	-
25°C	+
40°C	++
55°C	++
Catalase	+++

Key: + = Positive reaction, - = Negative reaction, d = variable reaction, (+) = acid and gas production, +++ = strong positive reaction

Characterization of the most potential isolate

C-1 isolate was tentatively identified as *Bacillus* species, based on biochemical characteristics (Table 1). C-1was then confirmed to be *Bacillus subtilis* strain, based on 16S rDNA characterization. Evolutionary relationship showed that C-1 was having more than 99% sequence identity with *Bacillus subtilis* strain IAM 16118 (Fig 1). Neighbor joining method was used to construct Phylogenetic tree, which is displayed in Fig 2. The sequence information is available with NCBI gene database, provided with gene accession number MN068818 (https://www.ncbi.nlm.nih.gov/nuccore/MN068818.1/).

C-1, the most potent lipolytic bacterial isolate, was identified as *Bacillus subtilis*. Many species of *Bacillus* have been reported to produce extracellular lipase enzyme previously **(Sangeetha et al., 2008; Vaisee et al., 2016; Adetunji and Olaniran, 2018; Sahoo et al., 2018)**, including thermophilic lipases from *Bacillus* species and other bacterial isolates **(Castro-Ochoa et al., 2005; Saraswat et al., 2017; Tailor and Gadhvi, 2023)**. Many researchers have reported highly active extracellular lipase production by *Bacillus subtilis* **(Lesuisse et al., 1993; Eggert et al., 2003; Ahmed et al., 2010)**. Thus, *Bacillus subtilis* C-1 strain is likely to be a promising candidate for large scale production and application of lipase.

Optimization using Plackett Burman Design (PBD)

The PBD for 16 trials with two levels of concentration for eight different variables was carried out according to the experimental matrix as per Table No. 2. Maximum lipase activity (21.48 EU/ml) was observed in Run no. 3 and minimum lipase activity (1.58 EU/ml) was obtained with Run no. 7. The effect of media components on lipase production was reputed by means of E value and Pareto chart. Table no. 3 lists the E-values and ranks for the eight parameters tested. Although ammonium chloride was ranked 2, due to the negative value it was not significant. Hence, from the analysis, it was clear that glucose, peptone, $MgSO_4$ and $CaCl_2$ were significant factors. The significance of the variables is confirmed in Pareto Chart as shown in Figure 3. Both the observations led to common significant parameters including glucose, peptone, $MgSO_4$ and $CaCl_2$ were found to have significant effect on lipase production. Thus, the high (+1) concentration of each significant variable, i.e. 3 gm% glucose, 3.5 gm/L peptone and 0.5 gm/L $MgSO_4$, and hence selected for response surface optimization.

PBD optimization showed glucose as a significant factor in contrast to olive oil. This finding is in contradiction with many of the previous reports, stating olive oil and other vegetable oils induce lipases **(Shirazi et l., 1998; Kulkarni and Gadre, 1999)**. Glucose turned out as a significant factor, which was concurrent with previous studies which demonstrated maximum lipase production with carbohydrates **(Gupta et al., 2004)**. It was even reported that oils can be used in combination with saccharides for efficient lipase production **(Verma et al., 2012)**. Also, enzyme activity of *Bacillus* lipases has been shown to increase with glucose as carbon source **(Guncheva and Zhirakoya, 2011; Vasiee et al., 2016)**. However, some other agro-based products should be analyzed from the economic standpoint for industrial production.

For nitrogen source, being the second important constituent of media for bacterial cultivation, peptone and tryptone were tested as organic nitrogen sources, while ammonium chloride was tested among inorganic nitrogen source. Peptone gave maximum lipase production at higher value of 3.5 gm/L. These results are in contradiction with previous reports which shows yeast extract giving maximum lipase production and peptone had a negative effect on lipase production **(Verma et al., 2012)**. Peptone, being a complex nitrogenous source, containing co-factors and amino acids is well documented to promote lipase production **(Eltaweel et al., 2005; Shariff et al., 2007; Dutta and Ray, 2009; Dhumri and Bayoumi, 2019)**. Tryptone also being a complex organic nitrogenous compound, has been reported to have negative effects on lipase production **(Guncheva and Zhirakoya, 2011)**.

Investigation of effect of metal ions has always been useful in media optimization, as metal ions are shown to be involved in bacterial growth as well as lipase production **(Vasiee et al., 2016)**. Current study showed prominent effect of $MgSO_4$ and $CaCl_2$ on lipase production, in both PBD optimization, and then concentration optimization gave value of 3.5 gm/L $MgSO_4$ during RSM optimization. Similar findings were reported earlier, where Mg^{2+}, Ca^{2+} and Na^{+} were influencing lipase production to a higher extent.

Table 2 PBD for eight variables and response generated

Run	Factor 1 A: Glucose gm%	Factor 2 B: Olive oil % v/v	Factor 3 C: NH_4Cl gm/L	Factor 4 D: Tryptone gm/L	Factor 5 E: Peptone gm/L	Factor 6 F: $MgSO_4$ gm/L	Factor 7 G: $CaCl_2$ gm/L	Factor 8 H: KH_2PO_4 gm/L	Response 1 Lipase Activity EU/mL
1	3	2	1	2.5	0.5	0	0	1	6.82
2	0.5	0.5	1	0.5	0.5	0	0	0	1.54
3	**3**	**0.5**	**1**	**2.5**	**3.5**	**0.5**	**0**	**0**	**21.48**
4	3	0.5	3.5	0.5	0.5	0.5	0	1	9.85
5	3	0.5	1	0.5	3.5	0	0.001	1	19.85
6	0.5	2	3.5	2.5	0.5	0.5	0	0	5.85
7	0.5	2	3.5	0.5	0.5	0	0.001	1	1.53
8	3	0.5	3.5	2.5	0.5	0	0.001	0	3.76
9	0.5	0.5	3.5	2.5	3.5	0	0	1	3.65
10	0.5	2	1	0.5	3.5	0.5	0	1	2.64
11	3	2	3.5	0.5	3.5	0	0	0	12.43
12	0.5	0.5	3.5	0.5	3.5	0.5	0.001	0	8.45
13	3	2	3.5	2.5	3.5	0.5	0.001	1	15.64
14	3	2	1	0.5	0.5	0.5	0.001	0	18.27
15	0.5	0.5	1	2.5	0.5	0.5	0.001	1	6.56
16	0.5	2	1	2.5	3.5	0	0.001	0	6.89

Table 3 Variable Screening and significance ranks based on PBD performance

Sample No.	Parameter	Lipase Production	
		E value	Rank
1	**Glucose**	**4.437**	**1**
2	Olive oil	-0.317	8
3	Ammonium chloride	-2.512	2
4	Tryptone	-0.61	7
5	Peptone	2.295	3
6	$MgSO_4$	2.017	4
7	$CaCl_2$	1.043	5
8	KH_2PO_4	-0.758	6

The studies reported possible reason for influence of Mg can be traced to formation of ionized fatty acid complex, thereby altering the properties of cell membrane **(Vasiee et al., 2016)**. Influence of Mg^{2+} on lipase production has been documented as early as 1994 by **Janssen et al. (1994)** and more recently by **Vasiee et al. (2016) and Mazhar et al. (2017) (Janessen et al., 1994; Vasiee et al., 2016; Mazhar et al., 2017)**. Hence influence of $MgSO_4$ on lipase production by *Bacillus subtilis* C-1 is justifiable.

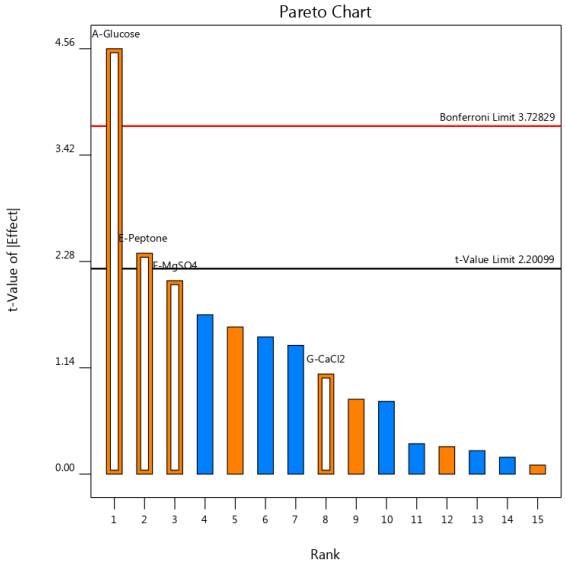


Figure 3 Pareto chart prepared in Design Expert software showing significant variables

RSM Optimization

As per the Pareto chart, significant factors, glucose, peptone and $MgsO_4$ were further subjected to concentration optimization using CCD. Table 4 shows CCD details for 20 run design and the response generated in the form of lipase activity. ANOVA analysis (Table 5) established the significance of the model, at 99% confidence level, as it had a p-value of <0.001. Final equation in terms of coded factors was obtained, which indicates that all the factors tested had a positive effect on LA.

R1 (Lipase Activity) = 13.88 + 2.15A + 3.84B + 3.36 C + 2.07AB + 1.90AC + 3.62 BC + 0.5227A² + 0.06 B² + 0.5737C²

Where, A = glucose, B = peptone and C = $MgSO_4$

RSM optimization of media parameters was done for determining optimal conditions for lipase production, due to laborious and time-consuming feature of one-factor-at-a-time (OFAT) method **(Hasan et al., 2006; Gururaj et al., 2016)**. As shown in the ANOVA analysis table (Table 5), the generated model was statistically significant with a p-value of <0.001, indicated that the model can very well describe lipase activity. The lack of fit value is used to measure the model unfitness for representing the data within experimental region. Hence, the non-significant lack-of-fit (p= 0.0696) indicates that the model is fit and significant **(Sifour et al., 2010)**, and that a suitable equation was established for lipase production simulation with any possible combination of these three variables. Detailing the observation, positive interaction was found amongst all the three factors selected. From the above equation it is evident that all the three factors have positive effect with peptone is highly influential (3.84), followed by MgSO4 (3.36). Also, the interaction between peptone and $MgSO_4$ has the most significant effect (3.62), as compared with the other two combinations of glucose with peptone or glucose with $MgSO_4$.

The 2D contour plots and 3D response surface graphically represent the regression equation. Model regression equation forms the basis for these plots, which helps in evaluation of interaction among the tested variables for the determination of optimum level of each of the glucose, peptone and MgSO4 for maximum lipase production. In contour plots and 3-D response surface generated by the experimental results are depicted in Figure 4a- 4c. These plots are constructed considering two factors at a time, while keeping the third one at fixed level. Fig 4a shows interaction graphs for glucose and peptone. It is observed that an increase in peptone concentration, keeping glucose concentration at high level, results in increase in LA. The same is not observed with low concentration of glucose. Similar pattern of interaction was evident with glucose and MgSO4, as shown in Fig 3b.

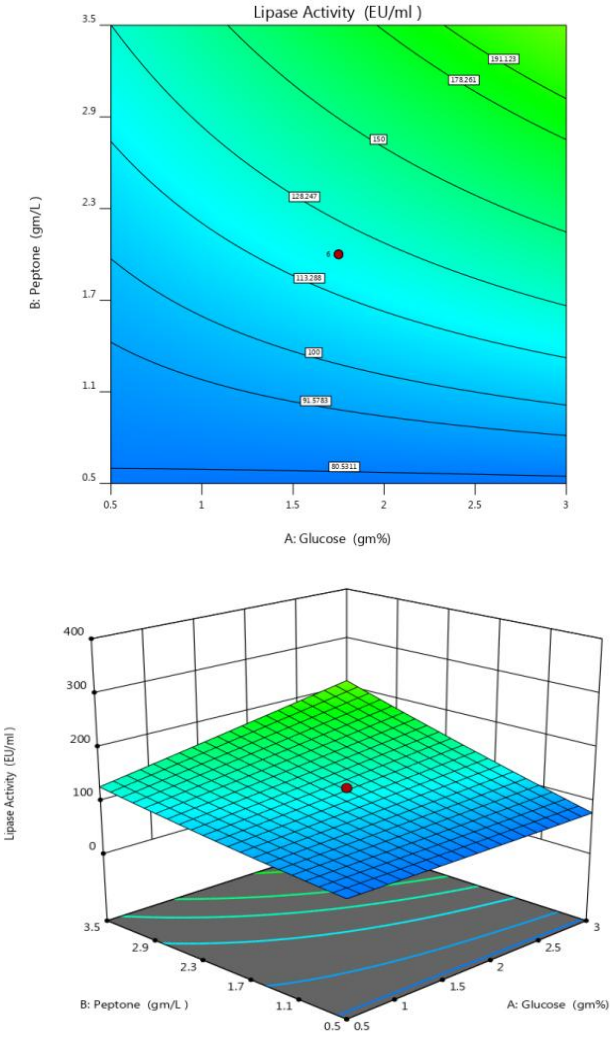
Table 4 CCD for RSM optimization with response generated

Run	Factor 1	Factor 2	Factor 3	Response 1: Lipase Activity	
	A: Glucose gm%	B: Peptone gm/L	C: MgSO4 gm/L	Experimental EU/ml	Predicted EU/ml
1	1.75	2	-0.170448	10	9.86
2	3	0.5	0	9.6	9.63
3	-0.352241	2	0.25	11.2	11.75
4	1.75	2	0.25	14.5	13.88
5	1.75	2	0.25	14.2	13.88
6	**1.75**	**4.52269**	**0.5**	**30.5**	**30.53**
7	1.75	2	0.25	13.8	13.88
8	3.85224	2	0.25	19.5	18.98
9	1.75	2	0.25	12.6	13.88
10	0.5	0.5	0	13.2	13.28
11	3	3.5	0	13.7	14.23
12	1.75	2	0.670448	21	21.16
13	1.75	2	0.25	14	13.88
14	3	0.5	0.5	12.5	12.91
15	1.75	2	0.25	14.2	13.88
16	0.5	3.5	0.5	19.8	19.73
17	0.5	0.5	0.5	9.5	8.96
18	0.5	3.5	0	10	9.58
19	3	3.5	0.5	32.1	31.98
20	1.75	-0.522689	0.25	7.6	7.59

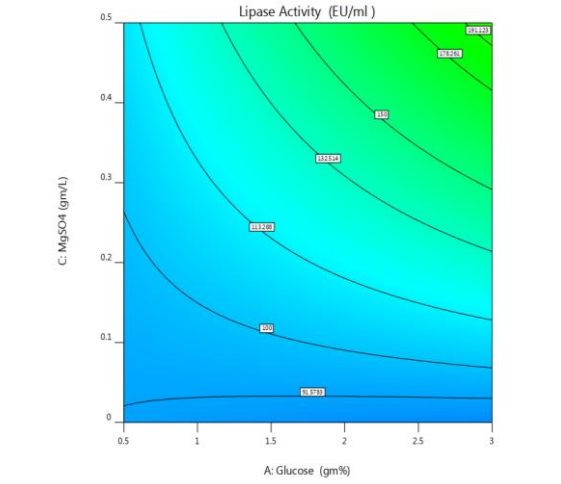
However, in Fig 4c, which depicts the interaction between peptone and MgSO4, keeping glucose constant. Surprisingly, a drastic increase in LA was observed by increasing either of the variable, keeping the other at high level concentration. This establishes a strong relationship between peptone and MgSO4 at their high concentrations. The optimum concentrations of the three factors for maximum lipase production were found out by studying responses in detail for all possible combinations using point prediction feature of the software. It was found that optimum concentration of the variables were, glucose, 3 gm%; peptone, 3.5 gm/L and $MgSO_4$, 0.5 gm/L. Thus, RSM optimization of media parameters for *Bacillus subtilis* C-1, gave 1.49 fold increase in lipase production.

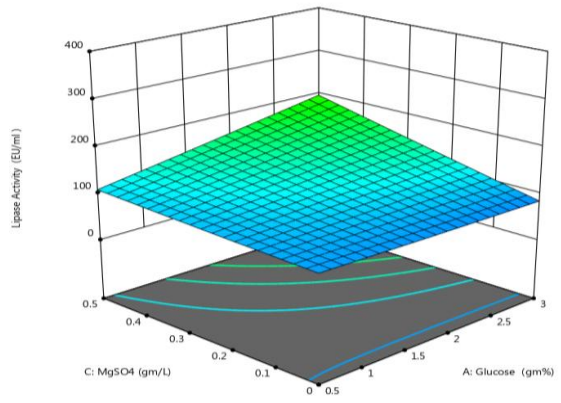
Table 5 ANOVA for quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	807.05	9	89.67	235.58	< 0.0001	significant
A-Glucose	63.11	1	63.11	165.81	< 0.0001	
B-Peptone	184.64	1	184.64	485.09	< 0.0001	
C-MgSO4	158.10	1	158.10	415.35	< 0.0001	
AB	34.44	1	34.44	90.49	< 0.0001	
AC	28.88	1	28.88	75.87	< 0.0001	
BC	119.52	1	119.52	314.00	< 0.0001	
A^2	3.88	1	3.88	10.19	0.0096	
B^2	0.0472	1	0.0472	0.1240	0.7321	
C^2	4.52	1	4.52	11.88	0.0063	
Residual	3.81	10	0.3806			
Lack of Fit	1.56	5	0.3116	0.6930	0.6514	not significant
Pure Error	2.25	5	0.4497			
Cor Total	810.86	19				

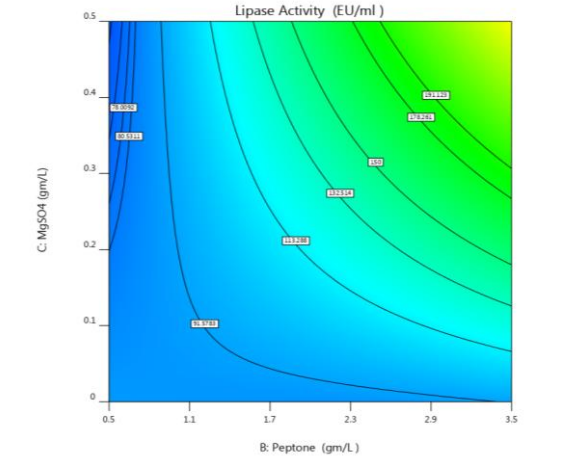


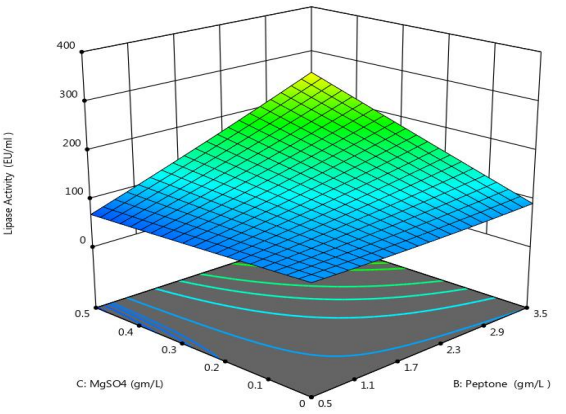
(a)





(b)





(c)

Figure 4 Contour plots and 3-D graphs showing effect of variable combinations 4a: Effect of glucose and Peptone; 4b: Effect of Glucose and MgSO4 and 4c: Effect of Glucose and Peptone

Table 6 Fit statistics

Std. Dev.	**0.6170**	R^2	**0.9953**
Mean	15.18	**Adjusted R^2**	0.9911
C.V. %	4.07	**Predicted R^2**	0.9772
		Adeq Precision	55.9091

The value of adjusted R^2 (0.9911) and predicted R^2 (0.9772), as evaluated in fit statistics (Table 6) were in accordance with each other, further supporting use of this regression model to describe the response trends **(Lo et al., 2012)**. The ratio of signal-to-noise was measured as adequate precision was 55.9091 (higher than 4), to affirm the sufficiency of the model **(Shabbiri and Adnan, 2011; Lo et al., 2012)**. The regression analysis showed R^2 value of 0.9953, during concentration optimization of significant factors using RSM, with central composite design. This was very close to predicted R^2 value of 0.9772, in reasonable agreement with adjusted R^2 of 0.9911, thus validating the model for media optimization.

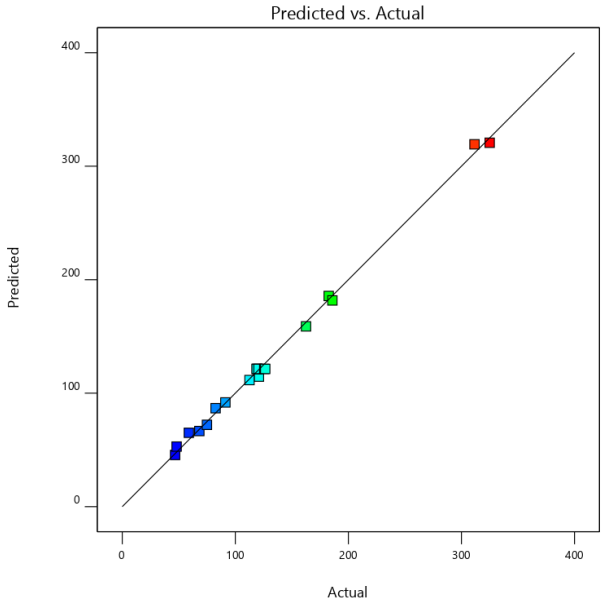


Figure 5 Predicted vs. Actual plot for RSM model

Predicted versus Actual values indicate that there is not much deviation from the fitted line indicating the satisfactoriness of the plot. The model F-value of 235.58 implies that model is significant. There is only 0.01% chance that an F-value this large could occur due to noise. Further, experimental value of highest lipase activity, 32.1 EU/ml is very close to predicted value of 31.96 EU/ml, indicating the competence of the model. This indicates satisfactoriness of the model to navigate design space.

The optimum concentrations of significant factors, glucose, peptone and MgSO4, were determined to be 3 gm%, 3.5 gm/L and 0.5gm/L. Thus, the studies proved the satisfactoriness of the RSM model for navigation of design space. **Mehta et al. (2019)** reported 1.8 gm% peptone as optimum concentration for maximum lipase production based on RSM optimization, using *Aspergillus fumigates* **(Mehta et al., 2019)**. In contrast, present RSM optimization results for *Bacillus subtilis* C-1, reveals much lower concentration (3.5 gm/L) as optimum for maximum lipase production. Also, **Suci et al. (2018)** reported 4.6 EU/ml lipase by *Bacillus subtilis*, whereas this strain is giving much higher activity of 32.1 EU/ml after RSM optimization **(Suci et al., 2018; Prabha et al., 2019)**. This establishes the promising production at cost-effective prices, owing to less amount of raw material required, to attain maximum lipase production.

Validation of the model

On comparing experimental data and predicted values (Table 4), the satisfactoriness of the RSM model was established, which was additionally confirmed by a fitted line plot. Fig 5 shows predicted versus actual response plot for lipase production by *Bacillus subtilis* C-1. It confirms an acceptable correlation between experimental and predicted values. The point clustering around the diagonal line indicates a good fit of the model. Fit statistics (Table 6) indicates that model is fir for navigation of design space. Maximum LA obtained was 32.1 EU/ml, higher than the predicted value of 31.96 U/ml, thus validating the model on statistical grounds.

CONCLUSION

In conclusion, this study has culminated in the isolation of potential thermos-alkaliphilic bacterial strain, *Bacillus subtilis* strain C-1, from local water body in Kocharva village, Vapi, Gujarat, India, which exhibited prominent lipolytic activity. To achieve media optimization with minimum number of experiments and

low chemical usage, statistical designs, such as PBD and RSM were applied. The results of PBD demonstrated glucose, peptone, $MgSO_4$ and $CaCl_2$ as significant factors, giving 3.19 fold increase in lipase activity. The R^2 value of 0.9 showed a good model of experimental data. The maximum lipase activity of 32.1 U/ml was obtained under optimum conditions of 3 gm% glucose, 3.5 gm/L peptone and 0.5 gm/L MgSO4, with incubation at 50^0C for 24 hours. This resulted in 1.46 fold increase in lipase production, giving 32.1 EU/ml. Thus, the present work identified optimum culture conditions for maximum LA using statistical methods and validation of models used. Also, candidature of *Bacillus subtilis* strain C1 was recognized as a prospective source of extracellular lipase.

Conflict of interest: Authors declare that there is no conflict of interest.

Author's contribution

Aparna J. Tailor: Conceptualization, Designing of Experimentation, Data submission and Analysis, Manuscript writing
Rekha R. Gadhvi: Data analysis; Reviewing Manuscript and Editing
Nipun K. Bariya: Designing and conducting Optimization protocols and Manuscript writing

REFERENCES

Adetunji AI and Olaniran AO. (2018). Optimization of culture conditions for enhanced lipase production by an indigenous *Bacillus aryabhattai* SE3-PB using response surface methodology. *Biotechnology and Biotechnology Equipment*. 32(6): 1514-1526. https://doi.org/10.1080/13102818.2018.1514985

Ahmed EH, Raghavendra T, Madamwar D. (2010) An alkaline lipase from organic solvent tolerant *Acinetobacter* sp. EH28: application for ethyl caprylate synthesis. *Bioresource and Biotechnology*, 101: 3628-3634. https://doi.org/10.1016/j.biortech.2009.12.107

Al-Dhumri SA and Bayoumi RA. (2019). Bacterial hyperthermostable alkaline lipase production by *B. stearothermophilus* isolated from oil polluted soil. *International Journal of Advanced Research in Biological Sciences*. 6(2): 166-184 http://dx.doi.org/10.22192/ijarbs.2019.06.02.019

Bussamara R, Fuentefria AM, Oliveira ES, Broetto L, Simcikova M, Valente P, et al. (2010). Isolation of a lipase-secreting yeast for enzyme production in pilot plant scale batch fermentation. *Bioresource Technology*, 101(1): 268-275 https://doi.org/10.1016/j.biortech.2008.10.063

Bisht D. (2011) *Studies on alkaline lipase production by an improved bacterial strain* (Doctoral dissertation). Retrieved from Shodhganga thesis. http://hdl.handle.net/10603/247076

Castro-Ochoa LD, Rodriguez-Gomez C, Valerio-Alfaro G, Ros RO. (2005). Screening, Purification and characterization of the thermoalkaliphilic lipase produced by *Bacillus thermoleovorans* CCR11. Enz. and Micro. Tech. 37:648-654 http://dx.doi.org/10.1016/j.enzmictec.2005.06.003

Colla LM, Rizzardi J, Pinto MH, Reinehr CO, Bertolin TE, Costa JA. (2010). Simultaneous production of lipases and biosurfactants by submerged and solid-state bioprocesses. *Bioresource Technology*, 101(21): 8308-8314. https://doi.org/10.1016/j.biortech.2010.05.086

Dutta S and Ray L. (2009). Production and characterization of an alkaline thermostable crude lipase from an isolated strain of *Bacillus cereus* C7. *Applied Biochemistry and Biotechnology*, 159: 142-154. https://doi.org/10.1007/s12010-009-8543-x

Eggert T, Brockmeier U, Droge MJ, Quax WJ, Jaeger KE. (2003). Extracellular lipases from *Bacillus subtilis*: regulation of gene expression and enzyme activity by amino acid supply and external pH. *FEMS Microbiology Letters*, 225(2): 319-324 https://doi.org/10.1016/s0378-1097(03)00536-6

Eltaweel MA, Rahman RNZRA, Salleh AB, Basri M. (2005). An organic solvent-stable lipase from *Bacillus* sp. strain 42. *Annals of Microbiology*, 55: 187-192

Gricajeva A, Bendikiene V, Kalediene L. (2016). Lipase from *Bacillus stratosphaericus* L1: cloning, expression and characterization. *International Journal of Biological Macromolecules*, 92: 96-104. https://doi.org/10.1016/j.ijbiomac.2016.07.015

Guncheva M, Zhiryakova D (2011). Catalytic properties and potential applications of Bacillus lipases. *Journal of Molecular Catalysis B: Enzymatic*, 68(1):1–21. https://doi.org/10.1016/j.molcatb.2010.09.002

Gupta R, Gupta N, Rathi P. (2004). Bacterial lipases: An overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology*, 64: 763-781. https://doi.org/10.1007/s00253-004-1568-8

Gururaj P, Ramalingam S, Nandhini Devi G, Gautam P. (2016). Process optimization for production and purification of a thermostable, organic solvent tolerant lipase from *Acinetobacter* sp. AU07. *Brazilian Journal of Microbiology*, 47(3): 647-657. https://doi.org/10.1016/j.bjm.2015.04.002

Hasan F, Shah A. A., and Hameed A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Biotechnology*, 39: 235-251. https://doi.org/10.1016/j.enzmictec.2005.10.016

Janssen P. H., Monk C. R., Morgan H. W. (1994). A thermophilic, lipolytic *Bacillus* sp. and a continuous assay of its p-nitrophenyl palmitate esterase activity. *FEMS Microbiology Letters*, 120: 195-200. https://doi.org/10.1111/j.1574-6968.1994.tb07030.x

Kaur, G., Singh, A., Sharma, R. *et al.* (2016). Cloning, expression, purification and characterization of lipase from *Bacillus licheniformis*, isolated from hot spring of Himachal Pradesh, India. *3 Biotech* **6:** 49 https://doi.org/10.1007/s13205-016-0369-y

Kouker G and Jaeger KE. (1987). Specific and sensitive plate assay for bacterial lipases. Applied and Environmental Biotechnology. 53(1): 211-213

Kulkarni N and Gadre RV. (1999) A novel thermostable, protease-free lipase from *Pseudomonas* sp. *Biotechnology letters*, 21: 897-899. https://doi.org/10.1023/A:1005591009596

Kumar R, Sharma A, Kumar A, Singh DK. (2012). Lipase from *Bacillus pumilus* RK31: Production, purification and some properties. *World Applied Sciences Journal*, 16(7): 940-948. CORPUS ID: 18508347

Lee LP, Karbul HM, Citartan M, Gopinath SCB, Lakshmipriya T, Tang TH. (2015). Lipase secreting *Bacillus* species in an oil-contaminated habitat: promising strains to alleviate oil pollution. *BioMed Research International*. http://dx.doi.org/10.1155/2015/820575

Lesuisse E., Schanck K. and Colson C. (1993). Purification and preliminary characterization of extraceullular lipase of *Bacillus subtilis* 168, an extremely basic pH tolerant enzyme. *European Journal of Biochemistry*. 216: 155-160. https://doi.org/10.1111/j.1432-1033.1993.tb18127.x

Lo C. F., Yu C. Y., Kuan I. C., Lee S. L. (2012). Optimization of lipase production by *Burkholderia* sp. using response surface methodology. *International Journal of Molecular Sciences*. 13: 14889-14897. https://doi.org/10.3390/ijms131114889

Massadeh MI, Sabra FM. (2011). Production and characterization of lipase from *Bacillus stearothermophilus*. *African Journal of Biotechnology*, 10(61): 13139-13146. http://doi.org/10.5897/AJB11.1481

Mazhar H, Abbas N, Ali S, Sohail A, Hussain Z, Ali SS (2017) Optimized production of lipase from *Bacillus subtilis* PCSIRNL-39. *African Journal of Biotechnology*, 16(19): 1106-1115 https://doi.org/10.5897/AJB2017.15924

Mehta A, Sharma R, Gupta R. (2019). Statistical optimization by response surface methodology to enhance lipase production by *Aspergillus fumigates*. *The Open Microbiology Journal*. 13: 86-93 http://dx.doi.org/10.2174/1874285801913010086

Plackett RL, Burman JP. (1946). The design of optimum multi-factorial experiments. *Biometrika*. 33:305–325. http://dx.doi.org/10.1093/biomet/33.4.305

Prabha S, Verma G, Pandey S, Singh BB, Dwivedi V. (2019). Utilization of agro-industrial by-products for production of lipase using mix culture batch process. *Bioscience Biotechnology Research Communications*. 12(3): 748-756. http://dx.doi.org/10.21786/bbrc/12.3/30

Rani S. and Kaur M. (2015). Parameter optimization for lipase production by *Bacillus megaterium* using response surface methodology. *Indian Journal of Biochemistry and Biophysics*. 52: 311-315 CORPUS ID: 55301290

Ruchi G., Anshu G., Khare S. K. (2008). Lipase from solvent tolerant *Pseudomonas aeruginosa* strain: Production optimization by response surface methodology and application. *Bioresource Technology*. 99: 4796-4802 https://doi.org/10.1016/j.biortech.2007.09.053

Saengsanga T, Siripornadulsil W, Sirpornadulsil S. (2016). Molecular and enzymatic characterization of alkaline lipase from *Bacillus amyloliquefaciens* E1PA isolated from lipid rich food waste. *Enzyme and Microbial Technology*, 82: 23-33. https://doi.org/10.1016/j.enzmictec.2015.08.005

Sahoo R. K., Kumar M., Mohanty S., Sawyer M., Rahman P. K. S. M., Sukla L. B., Subudhi E. (2018). Statistical optimization for lipase production from solid waste of vegetable oil industry. *Preparative Biochemistry and Biotechnology*, 48(4): 321-326. https://doi.org/10.1080/10826068.2018.1431785

Sangeetha R, Geetha A, Arulpandi I. (2008). Optimization of protease and lipase production by *Bacillus pumilus* SG2 isolated from an industrial effluent. *The Internet Journal of Microbiology*. 5(2).

Saraswat R, Verma V, Sistla S, Bhushan I. (2017). Evaluation of alkali and thermotolerant lipase from an indigenous isolated *Bacillus* strain for detergent formulation. Elec. J. Biotechnol. 30: 33-38. https://doi.org/10.1016/j.ejbt.2017.08.007

Shabbiri K and Adnan A. (2011). Bio-statistically optimized production of lipases by *Brevibacterium linens* DSM 20158. *World Applied Science Journal*. 13: 1059-1066.

Shariff FM, Leow TC, Mukred AD, Salleh AB, Basri M, Rahman RN. (2007). Production of L2 lipase by *Bacillus* sp. strain L2: nutritional and physical factors. *Journal of Basic Microbiology*. 47(5): 406-412. https://doi.org/10.1002/jobm.200610275

Shirazi SH, Rahman SR, Rahman MM. (1998). Production of Extra cellular lipases by *Saccharomyces cerevisiae*. *World Journal of Microbiology and Biotechnology*. 14:595–597. https://doi.org/10.1023/A%3A1008868905587

Sifour M, Saeed HM, Zaghloul TI, Berekaa MM, Abdel-Fattah YR. (2010). Purification and properties of a lipase from Thermophilic *Geobacillus stearothermophilus* strain 5. International Journal of Biological Chemistry. 4(4): 203-212 http://dx.doi.org/10.3923/ijbc.2010.203.212

Suci M, Arbianti R, Hermansyah h. (2018). IOP Conference series: Earth and Environmental Science. Lipase production from *Bacillus subtilis* with submerged

fermentation using waste cooking oil. 105: 12126 https://doi.org/10.1088/1755-1315/105/1/012126

Tailor A. J., and Gadhvi R. R. (2023) Thermo-alkaliphilic lipases and their applications, *In: Recent Advances in Microbiology*, Kripamoy Chakraborthy (ed.), First Edition, pg 79-104. (ISBN: 978-81-966651-1-1)

Tailor A. J., Bariya N. K. and Gadhvi R. R. (2024) Harnessing a thermos-active and alkaline lipase from novel strain of *Brevibacillus borstelensis* Gp-1, for application in biodiesel synthesis, *Biocatalysis and Biotransformation*, 1-15. https://doi.org/10.1080/10242422.2024.2391873

Thompson C. A., Delaquis P. J. and Mazza G. (1999) Detection and measurement of microbial lipase activity: A review. *Crit. Rev. Food Sci. Nutr*. 39(2): 165-187. https://doi.org/10.1080/10408399908500492

Turner S, Pryer KM, Miao VPW, Palmer JD. (2007). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *J. Eukaryot. Microbiol*. 46(4): 327-338. https://doi.org/10.111/j.1550-7408.1999.tb04612.x

Vaisee A, Behbahani BA, Yazdi FT, Moradi S. (2016). Optimization of the production conditions of the lipase produced by *Bacillus cereus* from rice flour through Plackett-Burman Design (PBD) and response surface methodology (RSM). Microbial Pathogen. 101: 36-43 http://dx/doi.org/10.1016/j.micpath.2016.10.020

Chandra P., Enepsa, Singh R., and Arora P. K. (2020) Microbial lipases and their industrial applications: A comprehensive review, *Microbial Cell Factories*, 19(1): 169. https://doi.org/10.1186/s12934-020-01428-8

Winkler UK, Stuckmann M. (1979) Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol. 138: 663-670. https://doi.org/10.1128/jb.138.3.663.670.1979