

## **Parameter optimization for thermostable lipase production and performance evaluation as prospective detergent additive**

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Keywords: Response surface methodology, solid state fermentation, lipase, oil-cake, *Anoxybacillus* sp, detergents additive

## Parameter optimization for thermostable lipase production and performance evaluation as prospective detergent additive

### Abstract

Lipase based formulations has been a rising interest to laundry detergent industry for their eco-friendly property over phosphate-based counterparts and compatibility with chemical detergents ingredients. A thermo-stable *Anoxybacillus sp.* ARS-1 isolated from Taptapani Hotspring, India was characterized for optimum lipase production employing statistical model central composite design (CCD) under four independent variables (temperature, pH, % moisture and bio-surfactant) by solid substrate fermentation (SSF) using mustard cake. The output was utilized to find the effect of parameters and their interaction employing response surface methodology (RSM). A quadratic regression with  $R^2=0.955$  established the model to be statically best fitting and a predicted highest lipase production of 29.4 IU/g at an optimum temperature of 57.5 °C, pH 8.31, moisture 50% and 1.2 mg of bio-surfactant. Experimental production of 30.3 IU/g lipase at above conditions validated the fitness of model. *Anoxybacillus sp.* ARS-1 produced lipase was found to resist almost all chemical detergents as well as common laundry detergent, proving it to be a prospective additive for incorporation.

**Keywords:** Response surface methodology; solid state fermentation; lipase; oil-cake; *Anoxybacillus sp.*; detergents additive

## Introduction

Lipase is the second largest commercially produced enzyme used in various industries for the production of fine chemicals, cosmetics, pharmaceuticals and biodiesel including detergents. [1] More than thousands of tons of lipase has been used for the production of laundry detergents as an additive or to replace the chemical detergents because of its advantage of being eco-friendly and better ability to remove oil stains with not harming the texture of the cloth. [2,3] The value of lipase producing industry was estimated to reach 1.8 billion dollars by 2018 and expected to be much more by now. [4] Commercially used lipase is found to be stable under high pH and temperature and should be unaffected in the presence of other detergents, oxidizing agents and surfactants. [5] As detergent additive has been reported several species of *Bacillus* and *Bulkhoderia*. [6-8] However, reduction of the cost of biotechnologically produced lipases has always been a challenge, despite its extensive reports on production and use. High cost incurring media components are the common constraints which compels to search for low-cost solid substrates. [4] Further methodological up-gradation through solid substrate fermentation (SSF) is preferred over traditional submerged fermentation (SMF) for its economic benefits. [9] Waste biomass from oil seed industries are left with rich residual nutrients even after the extraction of oil, which has been popularly used as solid substrate among all other agro-industrial waste. It acts as a very good resource for growth of microbes to produce enzymes. [10] Bacterial lipase has been successfully produced using oil industry waste following solid state fermentation. [11-14] According to NMOOP's (National Mission on Oilseeds and Palm) mission statement, current Indian fiscal year term targets (IFY 2017/18 to IFY 2021/22) for the production of oilseeds and oil

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3 meals has been 42.1 and 19.0 million metric tons (MMT) annually of which mustard contributes  
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5 9.75 and 3.2 MMT respectively. [15]  
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8 Various physical parameters like temperature, pH, moisture content and surfactants play an  
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10 important role in interacting among themselves and influencing the lipase production when such  
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12 solid wastes are used in fermentation process. [16-19] In order to dispense with the influence and  
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14 interaction of more than one physical parameters during lipase fermentation, central composite  
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16 design (CCD), and response surface methodology (RSM) statistical models were employed. [20,21]  
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18 Optimization of lipase production in vitro using mathematical prediction models before scaleup is  
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20 a justified method employed here.  
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24 Therefore, in the present study, we investigated and characterized the lipase produced by an alkali  
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26 tolerant thermophilic isolate *Anoxybacillus sp* ARS1 from a tropical hot spring Taptapani, Odisha,  
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28 India using mustard oil cake by solid substrate fermentation. We further assayed the compatibility  
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30 of the lipase produced to different surfactants, few oxidizing agents as well as in the presence of  
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32 other commercially available laundry detergents.  
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## 35 **Materials and methods**

### 36 ***Materials***

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38 The oil cakes used in the present work were collected from oil producing company of the locality.  
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40 Specific reagents like; p-nitrophenyl laureate was supplied by Sigma-Aldrich Chemicals, India and  
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42 other routine reagents were from HIMEDIA (Mumbai, India).  
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### 45 ***Development of lipase producing consortia***

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47 Soil sample was collected from Taptapani hot spring and inoculated in Bushnell Haas Broth (BHB)  
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49 supplemented with 1% (v/v) olive oil and incubated for 72 h at 50 °C. After 72 h, 5 ml from the  
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51 flask was transferred into fresh BHB olive oil medium to develop aerobic consortia. The process  
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3 was repeated for another two weeks at interval of 72 h to acclimatize the microbes for oil  
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5 utilization.  
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### 7 ***Isolation and screening of lipase producing bacteria***

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10 The developed consortium was plated on Bushnell Haas agar medium supplemented with olive oil  
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12 (0.5% v/v) and Rhodamine B (0.01% w/v) and incubated at 50 °C for 72 h. After 72 h, the colonies  
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14 showing halo zone under UV light were picked and inoculated in BHB+ olive oil (0.5% v/v) to  
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16 determine their ability to produce lipase. After 72 h incubation, cells were separated by  
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18 centrifugation and supernatant was used as crude lipase and it was assayed by spectrophotometric  
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20 method by using 4-nitrophenyl laurate as a substrate described by Sahoo et al [22]. One unit (IU) of  
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22 lipase was defined as the amount of enzyme that liberate one micro-mole of 4-nitrophenol per  
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24 minute under assay condition.  
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### 28 ***Molecular identification of lipase producing bacteria***

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30 Genomic DNA of selected bacteria was extracted by using DNeasy ultraclean microbial kit  
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32 (Qiagen, India). 16S rRNA gene was amplified by using universal primers 8F (5'-  
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34 AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACGACTT-3'). 25 µl of  
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36 PCR reaction mixture contained 12.5 µl of PCR master mix (2X) (Thermo Fisher Scientific, India)  
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38 and 1 µl of each forward and reverse primer (10 µM/µl). Temperature profiles for PCR reaction  
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40 was followed: 35 cycles of 94 °C (60s), 55 °C (45s) and 72 °C (60s) with initial denaturation at  
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42 94°C (3 min) and final extension at 72°C (10 min). PCR product was sequenced by AgriGenome  
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44 Labs Pvt Ltd, India and analyzed through NCBI's BLAST tool. [23] The 16S rRNA gene sequence  
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46 of bacterial strain was submitted in the GeneBank database (Gene bank accession number  
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48 KR606390). The phylogenetic tree was constructed by retrieving 16S rRNA gene of already  
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3 reported lipase producing *Anoxybacillus* species using MEGA v6.0 [24] with 1000 bootstrap  
4 replicates.  
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### 7 ***Process optimization for lipase production using CCD***

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10 CCD was followed in order to find the most suitable conditions for synthesis of lipase enzyme  
11 through solid substrate fermentation as well as for the identification of variables controlling it. The  
12 parameters were optimized using the mathematical model following RSM to quantify their  
13 substantial influence on lipase production and to evaluate the existing interaction among them.  
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15 Production of lipase (IU/g) was considered as a dependent response variable whereas A  
16 (Temperature °C), B (pH), C (Moisture %) and D (biosurfactant in mg) were taken as independent  
17 variables. Table 1 represents the range and levels for different variables.  
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### 26 ***Validation of prediction model***

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28 In order to find the goodness of fit of the employed model, the experimental lipase production was  
29 compared with the predicted lipase quantity under model predicted parameters such as,  
30 temperature 57.5 °C, pH 8.31, moisture 50%, bio-surfactant 1.2 mg using mustard cake and  
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33 *Anoxybacillus* sp. following SSF.  
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### 37 ***Solid state fermentation (SSF) Experiment***

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40 The SSF experiment was carried out in 250ml of conical flasks containing 10g of Mustard oil  
41 cakes and *Anoxybacillus* sp. ( $10^8$  CFU/ml). Biomass without culture served as control. All the  
42 flasks were incubated 120h and measured lipase activity every 24h. After regular interval, 0.1gm  
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45 of biomass was resuspended with 1 ml sterile water and centrifuged at 10,000 rpm for 10 min. The  
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48 cell free extract was used as crude lipase. Lipase activity was measured as described by [22] ***Effect***  
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51 ***of chemicals and commercial detergents***  
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3 The compatibility and stability study of crude lipase with chemical detergents (Tween 20, Tween  
4 80, Triton X 100, SDS and CTAB) and commercial detergents (Surf excel, Ghadi, Wheel, Ariel  
5 and Ezee) were determined by preincubating the mixture of crude enzyme and detergents (each at  
6 1% w/v) for 1 h.  
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### 10 **Statistical analysis**

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12 The analysis of variance (ANOVA) was used for statistical analysis. The associated probability  
13 p(F) and the degree of fit to the regression model were determined by Fisher's test (F-test) and  
14 coefficients (R and R<sup>2</sup>) to establish the significance of the model. Graphical examination of  
15 influence of independent process variables on the experimental results were studied from two-  
16 dimensional contour plots and three-dimensional response surface curves using Design-Expert®  
17 Software Version 6.0.7 (Stat-Ease Inc, Minneapolis, USA).  
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### 28 **Result and discussion**

#### 29 **Consortium analysis**

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31 In this study, the aerobic consortium was developed by augmentation technique using olive oil as  
32 the sole source of carbon. After 72 h of incubation, the bacterial consortium produced 22.7 U/ml  
33 of lipase. Twelve isolates (ARS-1 to ARS-12) were obtained from the consortium by spreading on  
34 BHA + olive oil + Rhodamine B Plate produced orange halo zones under UV light. After 72 h of  
35 incubation in BHB medium supplemented with olive oil, 12 isolates (ARS-1 to ARS-12) showed  
36 lipase activity ranging from 3.89 U/ml to 19.26 IU/ml (Fig. 1) and the highest activity was  
37 exhibited by ARS-1.  
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49 16S rRNA gene sequencing of the most efficient lipase producer (ARS-1) showed 99% similarity  
50 with *Anoxybacillus* species and was named as *Anoxybacillus* sp. ARS-1. Phylogenetic tree of  
51 *Anoxybacillus* sp. ARS-1 was constructed based on the 16S rRNA gene with reported lipase  
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3 producing *Anoxybacillus* species. The tree showed *Anoxybacillus* sp. ARS-1 was phylogenetically  
4 distinct from other lipase-producing species of *Anoxybacillus* (Fig. 2). Olusesan et al. (2009) [25]  
5 reported the range of lipase production to be 0.56 to 2.62 U/ml and the highest being by a species  
6 of *Anoxybacillus*. However, another *Anoxybacillus* species isolated from thermophilic  
7 environment (Bakir and Metin 2016) [26] produced a higher amount of lipase (51 U/mg).  
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### 14 ***Effect of parameters on lipase production***

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17 As designed by CCD, 30 different batch runs under various parametric conditions were performed  
18 of which 16, 8 and 6 points were respectively assigned to factorial, axial and center points. The  
19 experimental and predicted production of lipase enzyme (IU/g) was given in Table 2. The principal  
20 effect of variable parameters like; moisture, biosurfactant and temperature were found to be  
21 remarkable and that of pH was quadratic as found from the Fisher F-test. The quadratic model of  
22 response surface was analyzed by using ANOVA (Table S3). The p value is <0.0001, which  
23 indicate that model is significant and confirmed by F value (3251.927). From the ANOVA  
24 analysis, the pH with high F value (142.522) has indicated the crucial factor for lipase production.  
25 Similarly, effect of pH with high F value (15939.05) on lipase production was observed in  
26 *Ochrobactrum intermedium* MZV101 lipase [27].  
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40 Among all the variables, pH was found to be highly interactive with others significantly and thus  
41 considered for the model equation at higher order (Eq. 1). F value at 99.99% confidence level  
42 establishes the significance of the model. The regression analysis having the value of  $R^2$  to be  
43 0.955 indicates the quadratic model as best fitting with the experimental data and the value of the  
44 coefficient of variation at 6.9 establishes that the experiments are reliable.  
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50 Y representing the lipase enzyme activity for MOC as shown by the model equation (Eq. 1) where  
51 temperature, pH, moisture and biosurfactant are represented as A, B, C and D respectively.  
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$$Y = -348.9 + 0.24A + 59.6B + 4.8C + 3.18D + 1.3A^2 - 3.636B^2 - 0.047C^2 - 8.68D^2 - 0.29AB + 0.020AD + 2.052B \dots \dots \dots Eq. 1$$

The lipase activity (Y) has been predicted from the above equation (1) and presented in Table 1.

The predicted average percentage absolute error was found to be 5.8% (Data not given) which was within the acceptable limit and thus chosen model could be employed for optimization of parameter for the highest lipase production.

Statistical software Design-Expert v6.0 (State-Ease Inc., Minneapolis, USA) was used to generate graphs; the contour plots and three-dimensional surface response plots based on the equation taking two parameters at a time and keeping the other parameters at optimum. The effect of moisture and biosurfactant on lipase production are shown in figure 3A and 3B respectively. The biosurfactant at 1.25 mg concentration and moisture at 50% was contributing significantly towards production of 29.4 IU/g lipase from mustard cake. Nevertheless, the lipase production was affected at low moisture condition even if the biosurfactant was present at higher quantity, which establishes that the interaction between biosurfactant and moisture content was significant. In another experiment, by keeping moisture content and temperature at optimum, the influence and the possible interaction of pH and biosurfactant on lipase production was observed and presented in figure 4(A and B). A strong influence of pH at different values was observed irrespective of the biosurfactant content on the highest lipase production showing the interaction of pH with biosurfactant. The most favorable conditions for pH and biosurfactant concentration were 8.3 and 1.2 mg respectively. Under optimal conditions for biosurfactant and moisture content, the effect of other two process parameters (temperature, pH) was also found out. Change in temperature had no effect on lipase production whereas pH was found to influence irrespective of variation in temperature (Fig. 5A and 5B). The highest lipase produced was 29.4 IU at temperature 47 °C and

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3 pH 8.3. Similar, effects of temperature (35 °C) and pH (8.5) on lipase production were observed  
4 using *Pseudomonas aeruginosa* employing Plackett–Burman design (PBD) for parameter  
5 optimization [28]. In contrast to our result, Khoramnia et al (2011) [29] found that pH had not  
6 significant effect in process optimization for lipase production from *Acinetobacter* sp, while  
7 temperature plays important factor.  
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### 14 ***Validation of the predicted model***

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17 Central composite design model was employed to find out the best conditions of process  
18 parameters in order to maximize the production of lipase from mustard oil cake. The highest  
19 predicted lipase activity was found to be 29.4 IU/g at optimized conditions of pH, temperature,  
20 moisture and biosurfactant respectively at 8.31, 57.50 °C, 50 % and 1.2 mg. Under the above  
21 conditions solid substrate lipase fermentation was carried out using mustard cake employing the  
22 thermophilic isolate *Anoxybacillus* sp. ARS-1 in order to experimentally validate the predicted  
23 model. The experimental lipase production was found to be at a near higher value (30.3 IU/g) as  
24 compared to the predicted value (29.4 IU/g) proving the goodness of fit of the model in predicting  
25 optimum enzyme production. It is further confirmed by Pearson's Chi-square test. The p value is  
26 >0.05, which indicates there is no significant difference between experimental and predicted lipase  
27 activity (Supplementary figure). Kai and Peisheng (2016) [30] reported similar fitness of the  
28 prediction model for lipase production employing RSM using novel strain *Thalassospira*  
29 *permensis*.  
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### 46 ***Effect of chemical and commercial detergents***

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49 The compatibility and stability study of crude lipase was depicted in table. It was found that the  
50 crude lipase showed maximum activity in presence of non-ionic detergents like Tween 80  
51 (107.6%), Triton-X 100 (99%) and Tween 20 (83.6%) but activity was reduced to 48.3% in  
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3 presence of ionic detergent SDS. Sajna et al. (2013) [31] also reported that the activity of lipase  
4 from *Pseudomonas* sp. was increased in presence non-ionic detergents when compared with ionic  
5 detergents. Longer acyl ester chains of Tween 80 have favored lipase enzyme activity when  
6 compared to Tween 20 [32]. Recently, Sahoo et al. (2019) [22] reported that non-ionic detergent has  
7 low hydrophilic balance which might be enhancing the lipase activity. Lipase activity was found  
8 to be enhanced in presence of commercial detergents Ezee (104.3%) and Ariel (103%) but the  
9 activity was slightly decreased to 95.6% and 84.3% in presence of Surf excel and Ghadi detergents  
10 respectively. Saraswat et al. (2017) [33] also reported similar influence of chemical as well as  
11 commercial detergents in his experiments using thermotolerant, alkalophilic lipase producing  
12 *Bacillus subtilis* isolate. In our findings, *Anoxybacillus* sp ARS-1 produced lipase was found to  
13 resist almost all chemical detergents as well as common laundry detergent, proving it to be a  
14 prospective additive for incorporation.  
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### 30 **Conclusion**

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32 The industrially suitable thermostable (57.50 °C) and alkaline stable (pH 8.31) isolate  
33 *Anoxybacillus* sp. ARS-1 obtained from Taptapani hot spring, The statistical model employed in  
34 the present work was found to be the best fitting and was validated by the experiment and thus can  
35 be utilized for pilot scale production of lipase from oil cake at an economic cost. *Anoxybacillus*  
36 sp. ARS-1 produced lipase was found to resist almost all chemical detergents as well as common  
37 laundry detergent, proving it to be a prospective additive for incorporation.  
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### 47 **Conflict of Interest**

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49 The authors declare that they have no conflict of interest.  
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### Figure Captions

Figure 1: Lipase production of bacterial strains isolated from the consortium.

Figure 2: Phylogenetic tree of 16S rRNA genes of lipase-producing bacteria was constructed by the neighbour-joining method using the MEGA6 software. One thousand bootstrap analyses were conducted. Scale bar = 0.01 change per nucleotide position.

Figure 3: (A) Effect of biosurfactant and moisture on lipase production, (B) response surface graph showing interaction among them

Figure 4: (A) Effect of bio-surfactant and pH on lipase production, (B) response surface graph showing interaction among them.

Figure 5: (A) Effect of pH and temperature on lipase production, (B) response surface graph showing interaction among them.

Supplementary figure: (A) Comparison of experimental and predicted lipase activity. (B) Scatter plot of experimental vs predicted lipase activity.

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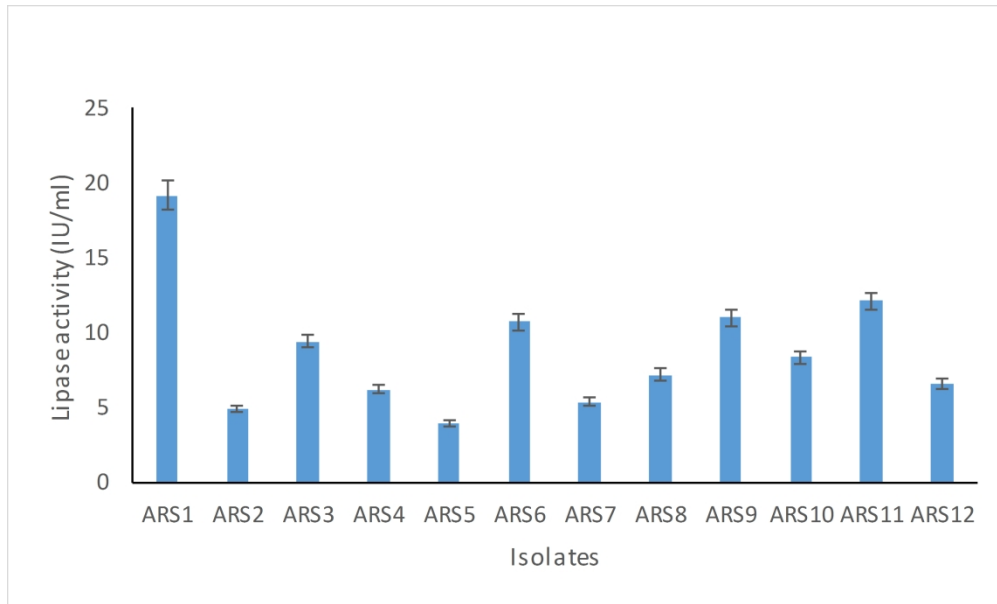


Figure 1: Lipase production of bacterial strains isolated from the consortium.

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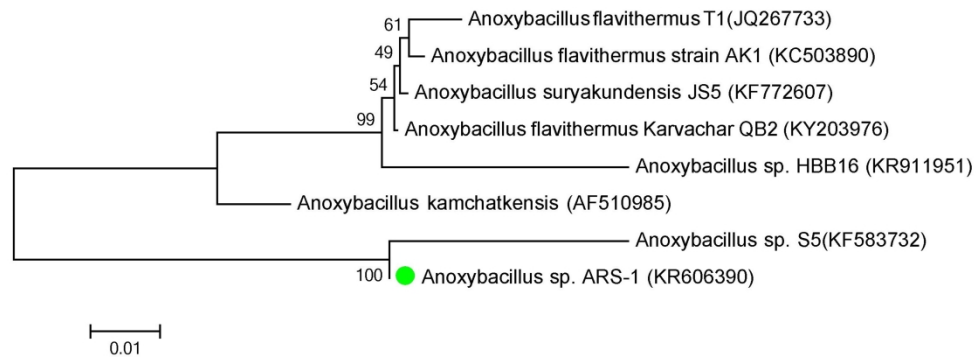


Figure 2: Phylogenetic tree of 16S rRNA genes of lipase-producing bacteria was constructed by the neighbour-joining method using the MEGA6 software. One thousand bootstrap analyses were conducted. Scale bar = 0.01 change per nucleotide position.

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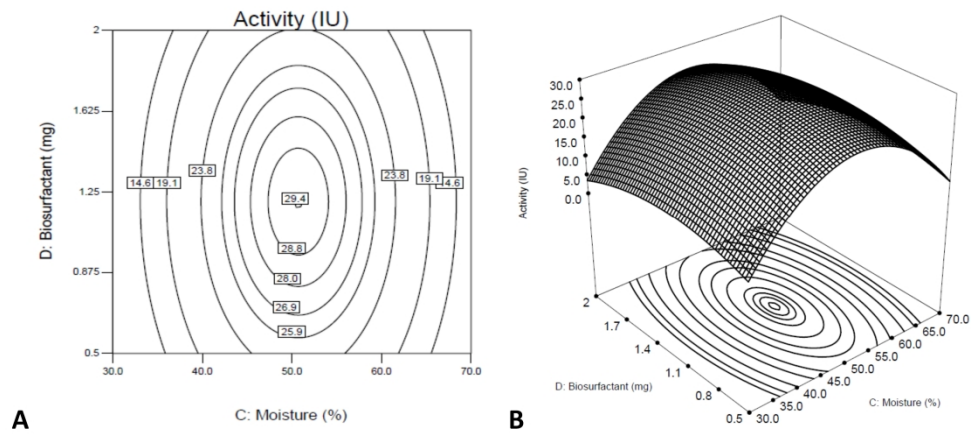


Figure 3: (A) Effect of biosurfactant and moisture on lipase production, (B) response surface graph showing interaction among them

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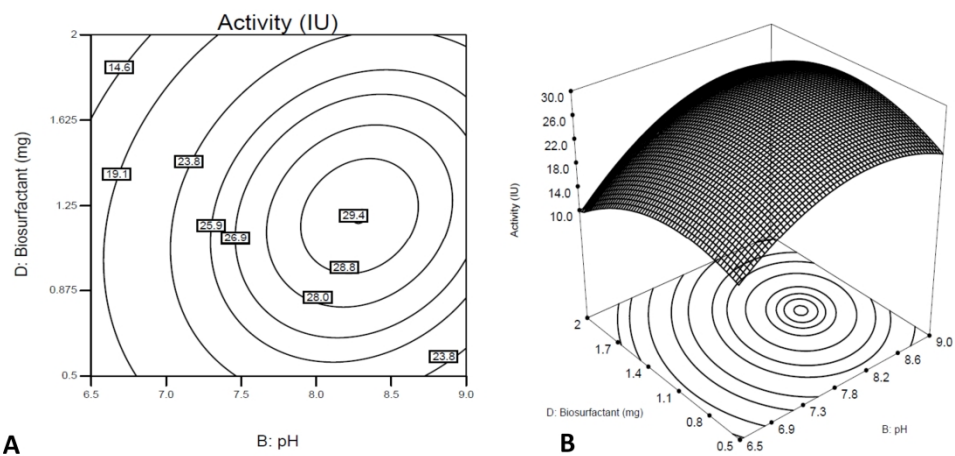


Figure 4: (A) Effect of bio-surfactant and pH on lipase production, (B) response surface graph showing interaction among them

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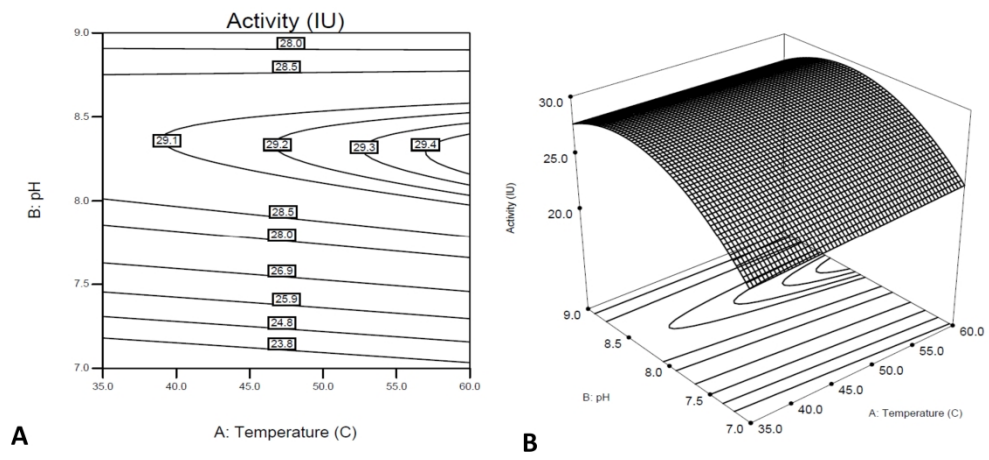


Figure 5: (A) Effect of pH and temperature on lipase production, (B) response surface graph showing interaction among them

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**Table S1: Variable parameters (independent variables) range and codes for their level**

Variables of the Process		Codes employed for Range and Level		
		Low (-1)	Medium (0)	High (+1)
A	Temperature (°C)	35	50	65
B	pH	7	8	9
C	Moisture (%)	30	50	70
D	Biosurfactant (mg)	0.5	1	1.5

**Table S2: Design of experiment using CCD for optimization of process parameters**

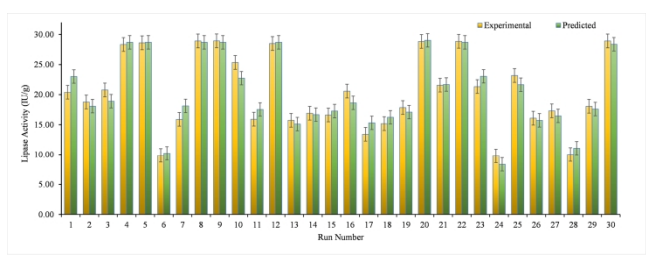
Run order	Run Type	Variables				Response (Y)	
		biosurfactant	moisture	temperature	pH	Lipase yield (IU/g)	
		mg	%	°C		Observed	Predicted
1	Fact	1.5125	60	42.5	9	20.78	23.01
2	Axial	1.025	50	50	10	19.01	18.08
3	Fact	0.5375	60	42.5	9	20.78	18.90
4	Center	1.025	50	50	8	28.64	28.69
5	Center	1.025	50	50	8	28.45	28.69
6	Axial	1.025	50	50	6	10.34	10.20
7	Axial	0.05	50	50	8	15.67	18.12
8	Center	1.025	50	50	8	28.98	28.69
9	Center	1.025	50	50	8	29.56	28.69
10	Axial	2	50	50	8	24.8	22.74
11	Fact	0.5375	40	42.5	9	15.76	17.54
12	Center	1.025	50	50	8	27.9	28.69
13	Fact	0.5375	40	42.5	7	15.45	15.10
14	Fact	1.5125	60	42.5	7	16.63	16.67
15	Fact	0.5375	40	57.5	9	16.43	17.27
16	Fact	0.5375	60	57.5	9	20.52	18.63
17	Fact	1.5125	40	42.5	7	13.56	15.31
18	Fact	1.5125	40	57.5	7	14.56	16.23
19	Fact	0.5375	60	57.5	7	18.23	17.09
20	Axial	1.025	50	65	8	28.67	29.02
21	Fact	1.5125	40	57.5	9	21.87	21.68
22	Center	1.025	50	50	8	28.5	28.69
23	Fact	1.5125	60	57.5	9	21.68	23.04
24	Axial	1.025	30	50	8	9.59	8.35
25	Fact	1.5125	40	42.5	9	22	21.65
26	Fact	0.5375	40	57.5	7	16.27	15.73
27	Fact	0.5375	60	42.5	7	17.89	16.47
28	Axial	1.025	70	50	8	9.45	11.08
29	Fact	1.5125	60	57.5	7	18.5	17.60
30	Axial	1.025	50	35	8	28.94	28.37



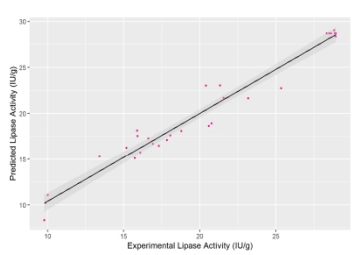
**Table S3:** ANOVA for Response Surface Quadratic Model [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	3251.927	11	295.6298	150.8089	< 0.0001
A	1.924068	1	1.924068	0.98152	0.3249
B	279.3854	1	279.3854	142.5222	< 0.0001
C	33.5517	1	33.5517	17.11565	< 0.0001
D	95.88817	1	95.88817	48.9152	< 0.0001
A2	0.000043	1	0.000043	0.000022	0.9962
B2	1088.319	1	1088.319	555.1817	< 0.0001
C2	1852.421	1	1852.421	944.971	< 0.0001
D2	350.9202	1	350.9202	179.0141	< 0.0001
AB	2.407552	1	2.407552	1.228159	0.2712
AD	0.280602	1	0.280602	0.143143	0.7062
BD	45.72755	1	45.72755	23.32688	< 0.0001
Residual	152.9029	78	1.960294		
Lack of Fit	132.2704	13	10.17465	32.05391	< 0.0001
Pure Error	20.63249	65	0.317423		
Cor Total	3404.83	89			

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**Table: Effect of chemicals and commercial detergents on stability of crude lipase from *Anoxybacillus* sp. ARS1**

<b>Detergents (1% w/v)</b>	<b>Relative activity</b>
Control	100.0
SDS	48.3 ± 3.05
Tween 20	83.6 ± 4.72
Tween 80	107.6 ± 2.51
Triton X 100	99 ± 4.58
Ezee	104.3 ± 2.51
Surf Excel	95.6 ± 3.51
Ghadi	84.3 ± 4.5
Ariel	103 ± 6.24