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

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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 \textit{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. \textit{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

\textit{Anoxybacillus} sp.; detergents additive; lipase; oil-cake; response surface methodology; solid state fermentation

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.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[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, etc.\textsuperscript{[5]} Use of lipase as detergents additive has been reported several species of \textit{Bacillus} and \textit{Bulkhoderia}.\textsuperscript{[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.\textsuperscript{[4]} Further methodological up-gradation through solid substrate fermentation (SSF) is preferred over traditional submerged fermentation (SMF) for its economic benefits.\textsuperscript{[9]}

Waste biomass from oilseed 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.\textsuperscript{[10]} Bacterial lipase has been successfully produced using oil industry waste following solid-state fermentation.\textsuperscript{[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 meals has been 42.1 and 19.0 million metric tons (MMT) annually of which mustard contributes 9.75 and 3.2 MMT, respectively.\textsuperscript{[15]}

Various physical parameters like temperature, pH, moisture content and surfactants play an important role in interacting among themselves and influencing the lipase production when such solid wastes are used in fermentation process.\textsuperscript{[16–19]} In order to dispense with the influence and interaction of more than one physical parameters during lipase fermentation, central composite design (CCD), and response surface methodology (RSM) statistical models were employed.\textsuperscript{[20,21]} Optimization of lipase production in vitro using mathematical prediction models before scaleup is a justified method employed here.
Therefore, in the present study, we investigated and characterized the lipase produced by an alkali tolerant thermophilic isolate *Anoxybacillus* sp. ARS1 from a tropical hot spring Taptapani, Odisha, India using mustard oil cake by solid substrate fermentation. We further assayed the compatibility of the lipase produced to different surfactants, few oxidizing agents as well as in the presence of other commercially available laundry detergents.

### Materials and methods

#### Materials

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

#### Development of lipase producing consortia

Soil sample was collected from Taptapani hotspring and inoculated in Bushnell Haas Broth (BHB) supplemented with 1% (v/v) olive oil and incubated for 72 h at 50°C. After 72 h, 5 ml from the flask was transferred into fresh BHB olive oil medium to develop aerobic consortia. The process was repeated for another two weeks at interval of 72 h to acclimatize the microbes for oil utilization.

#### Isolation and screening of lipase producing bacteria

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

#### Molecular identification of lipase producing bacteria

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

#### Process optimization for lipase production using CCD

CCD was followed in order to find the most suitable conditions for synthesis of lipase enzyme through solid substrate fermentation as well as for the identification of variables controlling it. The parameters were optimized using the mathematical model following RSM to quantify their substantial influence on lipase production and to evaluate the existing interaction among them. Production of lipase (IU/g) was considered as a dependent response variable whereas A (Temperature°C), B (pH), C (Moisture %) and D (biosurfactant in mg) were taken as independent variables. Table S1 represents the range and levels for different variables.

#### Validation of prediction model

In order to find the goodness of fit of the employed model, the experimental lipase production was compared with the predicted lipase quantity under model predicted parameters such as temperature 57.5°C, pH 8.31, moisture 50%, biosurfactant 1.2 mg using mustard cake and *Anoxybacillus* sp. following SSF.

#### Solid state fermentation (SSF) experiment

The SSF experiment was carried out in 250 ml of conical flasks containing 10 g of Mustard oil cakes and *Anoxybacillus* sp. (10⁸ CFU/ml). Biomass without culture served as control. All the flasks were incubated 120 h and measured lipase activity every 24 h. After regular interval, 0.1 gm of biomass was resuspended with 1 ml sterile water and centrifuged at 10,000 rpm for 10 min. The cell free extract was used as crude lipase. Lipase activity was measured as described by Sahoo et al.[22]

#### Effect of chemicals and commercial detergents

The compatibility and stability study of crude lipase with chemical detergents (Tween 20, Tween 80, Triton X 100, SDS and CTAB) and commercial detergents (Surf Excel, Ghadi, Wheel, Ariel, and Ezee) were determined by preincubating the mixture of crude enzyme and detergents (each at 1% w/v) for 1 h.

#### Statistical analysis

The analysis of variance (ANOVA) was used for statistical analysis. The associated probability p(F) and the degree of fit to the regression model were determined by Fisher’s test (F-test) and coefficients (R and R²) to establish the
significance of the model. Graphical examination of influence of independent process variables on the experimental results were studied from two-dimensional contour plots and three-dimensional response surface curves using Design-Expert® Software Version 6.0.7 (Stat-Ease Inc, Minneapolis, USA).

**Result and discussion**

**Consortium analysis**

In this study, the aerobic consortium was developed by augmentation technique using olive oil as the sole source of carbon. After 72 h of incubation, the bacterial consortium produced 22.7 U/ml of lipase. Twelve isolates (ARS-1 to ARS-12) were obtained from the consortium by spreading on BHA\(\text{+}\)olive oil\(\text{+}\)Rhodamine B Plate produced orange halo zones under UV light. After 72 h of incubation in BHB medium supplemented with olive oil, 12 isolates (ARS-1 to ARS-12) showed lipase activity ranging from 3.89 U/ml to 19.26 IU/ml (Figure 1) and the highest activity was exhibited by ARS-1.

16S rRNA gene sequencing of the most efficient lipase producer (ARS-1) showed 99% similarity with *Anoxybacillus* species and was named as *Anoxybacillus* sp. ARS-1. Phylogenetic tree of *Anoxybacillus* sp. ARS-1 was constructed based on the 16S rRNA gene with reported lipase producing *Anoxybacillus* species. The tree showed *Anoxybacillus* sp. ARS-1 was phylogenetically distinct from other lipase-producing species of *Anoxybacillus* (Figure 2).

Olusesan et al. (2009)\[25\] reported the range of lipase production to be 0.56 to 2.62 U/ml and the highest being by a species of *Anoxybacillus*. However, another *Anoxybacillus* species isolated from thermophilic environment (Bakir and Metin 2016)\[26\] produced a higher amount of lipase (51 U/mg).

**Effect of parameters on lipase production**

As designed by CCD, 30 different batch runs under various parametric conditions were performed of which 16, 8, and 6 points were respectively assigned to factorial, axial and center points. The experimental and predicted production of lipase enzyme (IU/g) was given in Table S2. The principal effect of variable parameters like; moisture, biosurfactant, and temperature were found to be remarkable and that of pH was quadratic as found from the Fisher F-test. The quadratic model of response surface was analyzed by using ANOVA (Table S3). The \(p\)-value is <0.0001, which indicate that model is significant and confirmed by \(F\) value (3251.927). From the ANOVA analysis, the pH with high \(F\) value (142.522) has indicated the crucial factor for lipase production. Similarly, effect of pH with high \(F\) value (15939.05) on lipase production was observed in *Ochrobactrum intermedium* MZV101 lipase.\[27\]

Among all the variables, pH was found to be highly interactive with others significantly and thus considered for the model equation at higher order (Equation 1). \(F\) value at 99.99% confidence level establishes the significance of the model. The regression analysis having the value of \(R^2\) to be 0.955 indicates the quadratic model as best fitting with the experimental data and the value of the coefficient of variation at 6.9 establishes that the experiments are reliable.

\[
Y = -348.9 + 0.24A + 59.6B + 4.8C + 3.18D + 1.3A^2 - 3.636B^2 - 0.047C^2 - 8.68D^2 - 029AB + 0.020AD + 2.052B.................(1)
\]

The lipase activity (\(Y\)) has been predicted from the above Equation (1) and presented in Table S1. 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 on lipase activity was presented in Figure S2. (Graph not shown)
and biosurfactant on lipase production are shown in Figure 3A,B, respectively. The biosurfactant at 1.25 mg concentration and moisture at 50% was contributing significantly toward 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 4A,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 (Figure 5A,B). The highest lipase produced was 29.4 IU at temperature 47°C and pH 8.3. Similar, effects of temperature (35°C) and pH (8.5) on lipase production were observed using Pseudomonas aeruginosa employing Plackett–Burman design (PBD) for parameter optimization. In contrast to our result, Khoramnia et al. (2011) found that pH had no significant effect in process optimization for lipase production from Acinetobacter sp, while temperature plays important factor.

Validation of the predicted model

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

**Effect of chemical and commercial detergents**

The compatibility and stability study of crude lipase was depicted in Table 1. It was found that the crude lipase showed maximum activity in presence of nonionic detergents like Tween 80 (107.6%), Triton X 100 (99%) and Tween 20 (83.6%) but activity was reduced to 48.3% in presence of ionic detergent SDS. Sajna et al. (2013)\cite{31} also reported that the activity of lipase from *Pseudomonas* sp.
was increased in presence nonionic detergents when compared with ionic detergents. Longer acyl ester chains of Tween 80 have favored lipase enzyme activity when compared to Tween 20[32]. Recently, Sahoo et al. (2019)[22] reported that nonionic detergent has low hydrophilic balance which might be enhancing the lipase activity. Lipase activity was found to be enhanced in presence of commercial detergents Ezee (104.3%) and Ariel (103%) but the activity was slightly decreased to 95.6% and 84.3% in presence of Surf excel and Ghadi detergents respectively. Saraswat et al. (2017)[33] also reported similar influence of chemical as well as commercial detergents in his experiments using thermo-tolerant, alkalophilic lipase producing Bacillus subtilis isolate. In our findings, 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.

Conclusion

The industrially suitable thermostable (57.50°C) and alkaline stable (pH 8.31) isolate Anoxybacillus sp. ARS-1 obtained from Taptapani hot spring. The statistical model employed in the present work was found to be the best fitting and was validated by the experiment and thus can be utilized for pilot scale production of lipase from oil cake at an economic cost. 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.

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Disclosure statement

The authors declare that they have no conflict of interest.

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