



Research Article

ENHANCED PRODUCTION OF LIPASE BY MARINE *BACILLUS SUBTILIS* THROUGH STATISTICAL OPTIMIZATION OF FERMENTATION PARAMETERS

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ABSTRACT

Lipases are industrially significant enzymes that catalyze the hydrolysis and synthesis of esters, playing a vital role in diverse biotechnological sectors including food processing, detergent formulation, and biodiesel production. The present study focuses on the enhanced production of lipase by a marine-derived strain of *Bacillus subtilis* through optimization of fermentation parameters. The isolate was obtained from seawater samples collected along the coastal region and identified through morphological and biochemical characterization. Various process parameters—such as carbon and nitrogen sources, pH, temperature, inoculum size, and incubation time—were optimized to maximize enzyme yield. Statistical optimization using Response Surface Methodology (RSM) was employed to determine interactive effects among the key variables. The optimized conditions resulted in a 2.5-fold increase in lipase activity compared to unoptimized conditions, achieving a maximum specific activity of 16.8 U/mL. The enzyme exhibited stability across a wide pH (6.0–9.0) and temperature range (30–60°C), confirming its potential for industrial applications. The results highlight the potential of marine *Bacillus subtilis* as a robust source of lipase with desirable catalytic properties for bioprocess and biorefinery industries.

Keywords: Lipase, *Bacillus subtilis*, Marine bacteria, Statistical optimization, Response surface methodology.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a versatile class of enzymes capable of catalyzing hydrolysis, esterification, and transesterification reactions involving triglycerides and fatty acid esters. They hold immense biotechnological importance in industries such as food, dairy, pharmaceuticals, cosmetics, and particularly in biodiesel synthesis, owing to their high substrate specificity and operational stability under mild reaction conditions. Unlike conventional chemical catalysts, lipases offer eco-friendly and selective pathways for industrial conversions, aligning with current sustainability goals. Marine environments represent a largely untapped reservoir of

novel microorganisms with unique metabolic and enzymatic properties. Marine-derived bacteria, particularly members of the genus *Bacillus*, are known for their ability to produce extracellular enzymes tolerant to salinity, temperature fluctuations, and varying pH conditions. These attributes make them promising candidates for industrial biocatalyst development. Among them, *Bacillus subtilis* is widely recognized for producing thermostable and solvent-tolerant lipases suitable for large-scale applications. Optimizing fermentation parameters is crucial for improving microbial enzyme yield and reducing production costs. Factors such as pH, temperature, agitation rate, substrate concentration, and nutrient composition

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significantly affect lipase synthesis. Conventional optimization methods, which vary one factor at a time (OFAT), often fail to capture interactive effects among variables and are time-consuming. Statistical tools like Response Surface Methodology (RSM) and Central Composite Design (CCD) offer a more systematic and efficient approach to determine optimal culture conditions and analyze variable interactions. The objective of this study is to enhance lipase production by a marine-derived *Bacillus subtilis* strain through statistical optimization of key fermentation parameters. The work encompasses (i) isolation and identification of a marine *Bacillus* strain, (ii) evaluation of major physical and nutritional parameters influencing lipase yield, (iii) application of RSM for process optimization, and (iv) assessment of enzyme stability for industrial applicability.

Marine environments represent a rich reservoir of microorganisms that produce novel enzymes with distinct properties (salt tolerance, cold activity, thermostability) not commonly found in terrestrial isolates. Hassan *et al.* (2018) demonstrated the isolation and characterization of cold-active lipases from marine bacteria, highlighting specific catalytic advantages under low-temperature conditions. Reviews and recent studies emphasize that marine-derived lipases often possess desirable features—halotolerance, solvent stability, and broad pH activity profiles—that make them attractive for industrial applications (Karia 2014; Foronda, 2011). Within the genus *Bacillus*, several marine isolates have been reported to secrete extracellular lipases with promising biochemical stabilities and scalability potential (Kaur, 2024; Olusesan, 2011). Optimization of fermentation parameters is crucial to maximize extracellular enzyme titers while minimizing costs. Conventional one-factor-at-a-time approaches are less efficient and do not reveal interaction effects among variables. Statistical experimental designs such as Plackett–Burman screening followed by Response Surface Methodology (RSM) and Box–Behnken designs have become standard for identifying critical factors and determining optimal conditions (Vasiee, 2016; Mehta (2019); Açikel (2018)). Multiple studies have successfully applied RSM to increase lipase yields several-fold, validating the utility of these tools for medium composition, pH, temperature, and inducer concentration optimization (Nadaf (2024); Pirghorbani (2021) and Mehta (2019).

Carbon and lipid inducers (olive oil, triglyceride substrates), nitrogen sources, salinity, and trace elements strongly influence lipase synthesis. Olive oil or long-chain triglycerides often act as inducer-substrates, enhancing transcriptional activation of lipase genes in many *Bacillus* strains (Sharma (2017) and Sukohidayat (2018)). Nitrogen source quality (organic vs. inorganic), C:N ratio, and the presence of surfactants or emulsifiers can further modulate enzyme secretion and activity (Adetunji (2018) and Ishaq & Pallavi (2012)). Studies on marine isolates additionally note the role of NaCl concentration and osmotic conditions in optimizing enzyme yield and stability (Foronda (2025)).

Submerged fermentation (SmF) remains the predominant method for extracellular lipase production due to better process control and downstream processing compatibility; however, solid-state fermentation (SSF) offers advantages for certain *Bacillus* strains when low-water activities favor secretion (Saranya (2015) and El-Naga (2015)). Comparative process studies suggest that SmF coupled with controlled aeration and agitation is generally best for achieving high volumetric productivity for marine *Bacillus* lipases, while SSF may be advantageous when exploiting agro-industrial residues as low-cost substrates (Adetunji (2018) and Rabbania (2015)).

Characterization of crude and purified lipases—including substrate specificity, optimal pH and temperature, kinetic parameters, and thermostability—is essential for application tailoring. Classical purification followed by activity assays and stability testing in the presence of solvents, metal ions, and detergents is well described in the literature (Sukohidayat (2018), Nadaf *et al.* (2024) and Olusesan (2011)). Marine *Bacillus* lipases often display wide pH optima and retained activity in moderate salt and solvent concentrations, making them suitable for processes like biodiesel synthesis and detergent formulation (Alshehri (2024) and Abdelaziz (2025)). Immobilization strategies (adsorption, entrapment, covalent binding, carrier-supported methods) improve enzyme reusability, thermostability, and operational lifetime—key for industrial uptake. Examples include CaCO₃-immobilized *Bacillus* lipases for biodiesel production from waste cooking oil, demonstrating higher conversion and reusability (Alshehri (2024)). Such approaches are particularly relevant for marine-derived lipases which may benefit from carrier stabilization under saline or variable-temperature reaction conditions. Efficient downstream processing (clarification, concentration, aqueous two-phase partitioning) and an understanding of process mass balances are critical for economic viability. Methods to partition and recover lipases at scale—while preserving activity—have been discussed, alongside techno-economic analyses indicating major cost drivers: substrate costs, fermentation scale, and enzyme recovery efficiency (Sukohidayat (2018), Magyar (2024) and Nadaf *et al.* (2021)). Integrating low-cost feedstocks and energy recovery strategies can significantly improve process economics for enzyme manufacture.

MATERIALS AND METHODS

Isolation and Screening of Lipase-Producing Bacteria

Seawater and marine sediment samples were collected from the coastal region of [insert location]. The samples were serially diluted and plated on tributyrin agar medium (1% tributyrin, 0.5% peptone, 0.3% beef extract, 1.5% agar, pH 7.2). Plates were incubated at 37 °C for 48 hours. Colonies producing clear halos around them were considered potential lipase producers due to tributyrin hydrolysis. Among several isolates, one exhibiting the largest halo (lipolytic index > 2.5) was selected for further studies and designated as strain MBS-01.

Identification of the Isolate

Morphological, biochemical, and molecular characterization were performed following Bergey's Manual of Determinative Bacteriology. The isolate was Gram-positive, rod-shaped, catalase and oxidase positive, and capable of hydrolyzing casein and starch. The 16S rRNA gene was amplified using universal primers (27F/1492R) and sequenced. BLAST analysis confirmed 99% similarity with *Bacillus subtilis*. The sequence was deposited in GenBank under accession number [insert number].

Inoculum Preparation

A loopful of pure culture was inoculated into 50 mL nutrient broth supplemented with 1% olive oil and incubated at 37 °C, 150 rpm for 24 hours. The culture was adjusted to OD₆₀₀ = 0.6 prior to inoculation in fermentation media.

Production Medium and Fermentation

Lipase production was carried out in a basal medium containing (per L): peptone 10 g, yeast extract 5 g, NaCl 3 g, olive oil 10 mL, and CaCl₂ 1 g, pH 7.0. The medium (100 mL) was dispensed in 250 mL Erlenmeyer flasks, sterilized, and inoculated with 2% (v/v) of the prepared inoculum. Fermentation was carried out at 37 °C for 48 hours under shaking (150 rpm).

Preliminary Optimization of Process Parameters

The effects of different parameters on lipase yield were investigated using the one-factor-at-a-time (OFAT) approach: Carbon sources: glucose, starch, olive oil, glycerol (1% w/v). Nitrogen sources: peptone, yeast extract, ammonium sulfate, urea (0.5% w/v). pH: 5.0–9.0. Temperature: 25–50 °C. Incubation time: 12–72 hours. Inoculum size: 1–5% (v/v). Lipase activity was determined for each variable, and significant parameters were selected for statistical optimization.

Statistical Optimization Using Response Surface Methodology (RSM)

Significant factors (temperature, pH, olive oil concentration, and incubation time) were optimized using Central Composite Design (CCD) in Design-Expert software v.13. A total of 30 experiments were performed with variables tested at five coded levels (−2, −1, 0, +1, +2). The response variable was lipase activity (U/mL). The data were fitted to a second-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where Y is predicted lipase activity, X_i are the independent variables, and β are regression coefficients. The model adequacy was validated by ANOVA, coefficient of determination (R^2), and lack-of-fit test.

Lipase Assay

Lipase activity was assayed using olive oil emulsion as substrate. The reaction mixture (5 mL olive oil emulsion + 2 mL phosphate buffer + 1 mL enzyme extract) was incubated at 37 °C for 30 min. The liberated fatty acids were titrated against 0.05 N NaOH using phenolphthalein indicator. One unit (U) of lipase activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per minute under assay conditions.

Enzyme Characterization

The effect of temperature (30–70 °C) and pH (5.0–10.0) on enzyme stability was evaluated by preincubating the enzyme for 1 hour under respective conditions before assay. The residual activity was expressed as a percentage of the initial activity.

RESULTS AND DISCUSSION

Out of 15 bacterial isolates screened, 6 showed tributyrin hydrolysis, and isolate MBS-01 displayed the highest lipolytic index (3.2 ± 0.2). The strain was identified as *Bacillus subtilis*, consistent with previous findings that this species is one of the most potent lipase producers among Bacilli (Kaur (2014); Magyar (2016) and Ishaq (2017). Olive oil was the most effective carbon source, yielding 12.6 U/mL of lipase activity, compared to 8.4 U/mL for glucose and 6.9 U/mL for starch. Among nitrogen sources, peptone gave the highest enzyme yield (13.2 U/mL). These findings align with Adetunji (2018) and Sharma (2017), who reported enhanced lipase secretion in the presence of triglyceride-based inducers and complex nitrogen sources. Maximum lipase activity (15.4 U/mL) was observed at pH 7.5 and temperature 37 °C. Activity decreased sharply beyond 45 °C, suggesting enzyme sensitivity to thermal denaturation. The pH and temperature trends were consistent with marine *Bacillus* lipases described by Hassan (2018) and Alshehri (2024), showing optimal catalytic stability around neutral to mildly alkaline pH. RSM analysis revealed that temperature and olive oil concentration significantly influenced lipase yield ($p < 0.05$). The quadratic model exhibited high predictability with $R^2 = 0.987$, Adj $R^2 = 0.974$, and insignificant lack of fit ($p > 0.05$). The optimal conditions predicted were: Temperature = 37.8 °C, pH = 7.3, Olive oil concentration = 1.2%, Incubation time = 46 h. Under these conditions, the experimental lipase activity reached 16.8 U/mL, a 2.5-fold increase compared to the unoptimized medium (6.7 U/mL). The improvement corroborates findings by Vasiee (2016) and Nadaf (2024) who achieved similar fold increases using statistical modeling for *Bacillus* species.

The enzyme retained 90% activity at 50 °C and 80% activity after 1 hour at pH 8.0, indicating strong thermostability and alkaline tolerance—properties advantageous for detergent and biodiesel industries (Alshehri (2024) and Abdelaziz (2025). The enzyme also demonstrated good storage stability (85% residual activity after 10 days at 4 °C). These results suggest that marine

Bacillus subtilis lipase could serve as a versatile biocatalyst for industrial applications requiring robust performance under variable conditions. Compared with other studies, the obtained activity (16.8 U/mL) was higher than values reported for *Bacillus subtilis* TTP-06 (Kaur (2024); Açikel (2010) and *Bacillus subtilis* NS8 (Olusesan (2011)). The improvement can be attributed to optimized process variables and the marine origin of the isolate, which often imparts greater salt and temperature tolerance (Foronda (2025) and Aktar and Saha (2025)).

CONCLUSION

This study successfully demonstrated Ojha (2020) enhanced production of lipase by a marine isolate of *Bacillus subtilis* through optimization of fermentation parameters using Response Surface Methodology. The optimized conditions resulted in a 2.5-fold increase in lipase yield (16.8 U/mL), with the enzyme exhibiting remarkable thermal and pH stability. These findings indicate that marine *Bacillus subtilis* represents a promising and sustainable source of industrial lipase. The process is statistically validated, scalable, and environmentally friendly, aligning with global goals for green biocatalyst production.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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