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Lipase Production By The *Bacillus Sp*. MSF4 Isolate and Its Compatibility with Surfactants for Potential Detergent Applications

Aulia Narjisatus Syiva[1], Suharti Suharti[1*], Eli Hendrik Sanjaya[1], Intan Kusuma Dewi[1]

[1]Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Jl. Semarang 5, Malang, Indonesia

**Corresponding Author :suharti.fmipa@um.ac.id*

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Abstract: *The development of environmentally friendly detergents requires enzymes that remain active and stable under alkaline and surfactant-rich conditions. This study focused on the production and characterization of a lipase enzyme from the indigenous isolate MSF4 obtained from poultry waste. Morphological observation revealed a rod-shaped bacterium, while Gram staining produced an atypical Gram-negative appearance. However, molecular identification based on 16S rRNA gene sequencing showed 100% similarity to Bacillus cereus strain DS-2, indicating that the isolate belongs to the Bacillus cereus group and is most closely related to B. cereus. The atypical Gram-staining result was interpreted as a phenotypic variation rather than evidence contradicting the molecular identification. Lipase production reached its optimum after 24 h of submerged fermentation at pH 10 and 57 °C. Subsequent enzyme characterization showed maximum lipase activity of 75.85 ± 0.85 U/mL at 57 °C and 150.76 ± 0.36 U/mL at pH 9.5, indicating moderately thermostable and alkaliphilic properties. The enzyme maintained moderate activity in the presence of nonionic surfactants, retaining 32.34% activity with Triton X-100 and 28.99% with Tween 20. These findings highlight the potential of Bacillus cereus MSF4 lipase as a detergent-compatible biocatalyst for sustainable, eco-friendly formulations aligned with Sustainable Development Goal 9 (Industry, Innovation, and Infrastructure).*

Keywords: *Bacillus cereus MSF4, Lipase, Optimization, Surfactant Stability, Sustainable Biotechnology*

INTRODUCTION

The industrial enzyme market primarily comprises amylase, protease, and lipase, which are extensively utilized in the food, health, textile, biofuel, and detergent industries. Enzyme-based detergents demonstrate superior washing performance compared to conventional formulations (Korkmaz, 2022). Detergent-compatible enzymes account for approximately 25–30% of the global enzyme market (Bektas et al., 2023), valued for their eco-friendly nature, effectiveness at low temperatures, and ability to reduce the use of non-biodegradable surfactants (Aehle, et.al., 2007; Bhange et al., 2016). Protease, lipase, and amylase are responsible for degrading protein, fat, and carbohydrate stains, respectively, with protease representing about 60–65% of the market share (Kumar et al., 2008). To be effective in detergent formulations, these enzymes must remain stable under alkaline and surfactant-rich conditions (Rosalin et al., 2022). Microbial sources are preferred over plant or animal origins due to their lower production costs, faster growth rates, and greater stability (Fatimah, 2021; Niyonzima & More, 2014; Bektas et al., 2023)

Several Bacillus species, including *Bacillus cereus*, *B. subtilis*, *B. flexus*, and *B. licheniformis*, have been reported to produce lipase and other hydrolytic enzymes with potential industrial applications (Oni et al., 2022; Niyonzima & More, 2014; Baltaci et al., 2020). However, enzyme production and functional properties are highly strain-dependent,

and the lipase-producing capability of the indigenous isolate MSF4 remains unknown. Previous work by Azizah (2024) identified MSF4 as a protease-producing isolate recovered from poultry-waste environments, but its potential to produce lipase has not been investigated. Furthermore, no information is available regarding the compatibility of lipase produced by this isolate with surfactants, which is a critical requirement for detergent applications.

Therefore, a significant knowledge gap exists regarding whether MSF4 can serve as a source of detergent-compatible lipase. Addressing this gap is important because poultry-waste environments may harbor microorganisms adapted to lipid-rich and alkaline conditions, potentially yielding enzymes with desirable industrial characteristics. The novelty of this study lies in the first investigation of lipase production, optimization of fermentation and enzyme activity conditions, and evaluation of surfactant compatibility of *Bacillus cereus* MSF4. The findings are expected to provide scientific insight into the potential application of this indigenous isolate as a source of environmentally friendly biocatalysts for detergent formulations.

This study aims to evaluate the capability of *Bacillus* MSF4 to produce a stable crude lipase preparation under alkaline and surfactant-rich conditions, supporting the development of eco-friendly detergents in alignment with Sustainable Development Goal (SDG) 9.

RESEARCH METHODS

Materials and Tools

The primary equipment utilized in this study included Petri dishes, Erlenmeyer flasks, wire loops, spatulas, an autoclave (Tomy), laminar air flow and biological safety cabinets (Kewaunee), a water bath shaker (Thermo Scientific), vortex mixer (Dragon Lab MX-5), centrifuge (Precisa XT 120 A), VIS spectrophotometer (Genesys 30), pH meter (Toledo), and analytical balance (Shimadzu). The chemicals employed were of pro-analytical grade, including sodium chloride (NaCl) (Merck, Germany), bacto-peptone (Difco, USA), yeast extract (Difco, USA), Tris(hydroxymethyl)aminomethane (Merck, Germany), casein (Sigma-Aldrich, USA), hydrochloric acid (HCl) (Merck, Germany), p-nitrophenol palmitate (pNPP) (Sigma-Aldrich, USA), Tween 20 (Merck, Germany), Triton X-100 (Merck, Germany), and distilled water. Technical-grade materials included skim milk, olive oil, and Rhodamine B.

Methods

Bacterial Regeneration

The regeneration step was conducted to recover the physiological state of the stored isolate and to obtain a fresh, metabolically active culture for inoculum preparation. Since enzyme production is strongly influenced by inoculum quality, regeneration helped ensure consistent cell viability, growth kinetics, and preservation of the characteristic keratinase-producing phenotype previously reported for the MSF4 isolate. This step also minimized the risk of culture deterioration or contamination prior to subsequent lipase production experiments. The *Bacillus* MSF4 isolate was revived from a glycerol stock stored at –80 °C using solid Luria Bertani (LB) medium composed of 1 g bacto agar, 0.25 g yeast extract, 0.5 g NaCl, and 0.5 g peptone in 50 mL of distilled water. The medium was autoclaved and poured into Petri dishes. A single colony was then inoculated onto the fresh medium and incubated in an inverted position at 37 °C for 24 hours.

Screening of MSF Isolates Producing Lipase

The enzymatic activity of the isolates was assessed using a modified medium based on Niyonzima et al. (2015), containing 5% skim milk, 2% olive oil, 0.001% Rhodamine B, 0.01% $MgSO_4$, and 1.5% agar. Lipase production was screened using Rhodamine B–olive oil agar plates. After incubation at 37 °C for 48 h, the plates were exposed to UV light at 350 nm and examined visually for the presence of orange fluorescent halos surrounding bacterial colonies. The appearance of fluorescence was used as a qualitative indicator of lipase activity. No positive or negative controls were included because the assay was intended as a preliminary screening of the MSF isolates. Furthermore, halo diameter and fluorescence intensity were not quantitatively measured; therefore, the screening results were used only to identify potential lipase-producing isolates for further characterization.

Gram Staining

Bacterial smears were heat-fixed, then sequentially stained with crystal violet, iodine, 95% alcohol, and safranin. The preparations were examined under a light microscope at 1000× magnification using immersion oil.

Genetic Analysis of MSF4 Isolate Species

Genetic identification was carried out by amplifying and sequencing the 16S rRNA gene using primers 785F and 907R (Macrogen Inc., South Korea). The obtained sequences were analyzed with BioEdit, compared to reference sequences in the GenBank database using BLASTN, and a phylogenetic tree was constructed with MEGA X to determine species similarity and evolutionary relationships.

Production of Crude Enzyme Extract

Bacillus MSF4 grown on solid LB medium was inoculated into 25 mL of liquid LB and incubated at 37 °C with shaking at 100 rpm for 5 hours. A 1% (v/v) inoculum was transferred into 100 mL of fermentation medium containing 0.5% casein, 0.5% olive oil, and 0.2% NaCl at pH 10. Casein was included because the MSF4 isolate had previously been reported as a protease-producing bacterium and was expected to support the production of extracellular hydrolytic enzymes, whereas olive oil served as the primary substrate and inducer for lipase production.

Optimization of Lipase Production

Optimization of lipase production was performed using a One-Factor-at-a-Time (OFAT) approach by varying fermentation temperature (37–62 °C) and initial medium pH (7–11) while maintaining all other cultivation parameters constant. Lipase production was evaluated based on the activity of the crude enzyme extract obtained after fermentation. The temperature and pH resulting in the highest lipase yield were considered the optimum conditions for enzyme production

Optimization of Lipase Activity

The effects of temperature and pH on lipase activity were evaluated using the crude enzyme extract obtained under the optimum fermentation conditions. Enzyme activity was determined using p-nitrophenyl palmitate (pNPP) as substrate. For temperature optimization, enzyme assays were performed at various temperatures (37–65 °C) under constant pH conditions. For pH optimization, enzyme assays were conducted using appropriate buffer

systems covering pH 7–11 while maintaining the optimum assay temperature. The temperature and pH yielding the highest catalytic activity were considered the optimum operating conditions of the enzyme.

Enzyme Stability in Surfactant Environment

Surfactant compatibility was evaluated by incubating the crude lipase extract with Tween 20 and Triton X-100 at concentrations of 1–5% (v/v). The enzyme solution without surfactant was used as the control treatment and was considered to have 100% relative activity. After 20 min of incubation, residual lipase activity was measured using p-nitrophenyl palmitate (pNPP) as the substrate under the optimum assay conditions. All treatments were performed in triplicate, and the results were expressed as mean ± standard deviation. No inferential statistical test was applied; therefore, the surfactant compatibility results were interpreted descriptively based on relative residual activity compared with the control.

RESULTS AND DISCUSSION

Regeneration of MSF Isolates on Solid LB Medium

The regeneration step was performed to restore the physiological state of the stored isolate and obtain a fresh, metabolically active culture for inoculum preparation. Since enzyme production is strongly influenced by inoculum quality, regeneration helped ensure consistent cell viability, growth kinetics, and keratinase biosynthesis during subsequent fermentation experiments. Bacterial regeneration was carried out to obtain active cultures, as glycerol stocks stored at −80 °C exhibited no growth activity. The nutrient-rich solid Luria Bertani (LB) medium, containing peptone, nitrogen sources, amino acids, vitamins, NaCl, and essential growth factors, effectively supported bacterial proliferation (Trisiswanti et al., 2022). After 24 hours of incubation at 37 °C, all MSF1–MSF11 isolates demonstrated robust growth on solid LB medium.

Screening of Lipase Producing MSF Isolates

The formation of hydrolysis and/or fluorescent zones was used as a qualitative indicator of extracellular enzyme production during the initial screening stage. These plate-based assays were intended only to confirm the presence of enzymatic activity and were not used for quantitative comparison among isolates. Quantitative evaluation of enzyme production was subsequently conducted using enzyme activity assays under submerged fermentation conditions. Lipase activity was screened using an olive oil–Rhodamine B medium, a preferred substrate system that allows direct visualization of enzyme activity through fluorescence under UV light (Azizah, 2024; Oni et al., 2022; Kim et al., 2001; Hube et al., 2000). The lipase screening assay was done qualitatively, and future studies should include appropriate controls and quantitative measurements of halo diameter or fluorescence intensity to improve the reliability of isolate selection. Figure 1a shows the selective medium before being colonized by isolates MSF1, MSF2, MSF3, MSF4, MSF5, MSF6, MSF7, MSF9, MSF10, and MSF11, while

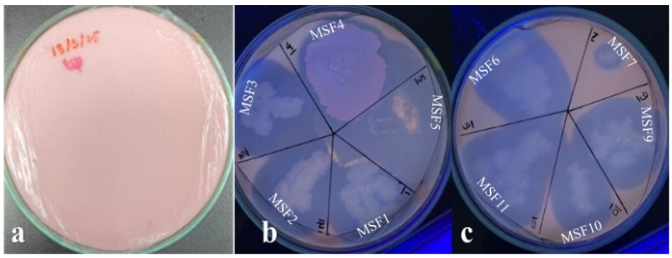


Figure 1. (a) Selective lipase medium before inoculation with isolates, (b) and (c) Selective lipase medium after inoculation with isolates

Figures 1b and 1c show the medium after inoculation and colonization by the same isolates. After 48 hours of incubation at 37 °C followed by UV illumination, only the MSF4 isolate produced an orange fluorescent halo, indicating lipase-mediated fatty acid release, whereas the other isolates showed no detectable activity. Therefore, MSF4 was selected for further enzyme production studies.

Purity Analysis of MSF4 Isolates

Cell purity analysis (Figure 2) revealed that the MSF4 isolate exhibited a bacillus-shaped morphology and was Gram-negative. The cells lost the crystal violet stain during decolorization and appeared red after counterstaining with safranin, resulting in a Gram-negative staining reaction (Velu et al., 2012). The consistent cell morphology and Gram reaction confirmed the purity of the isolate for subsequent species identification.

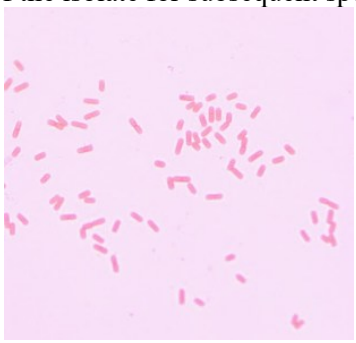


Figure 2. Gram staining results for MSF4

Although species of the genus *Bacillus* are generally regarded as Gram-positive bacteria, the MSF4 isolate consistently exhibited a Gram-negative staining reaction in repeated Gram-staining assays. This atypical result may be associated with Gram variability, a phenomenon reported in several *Bacillus* species, especially during sporulation or in older cultures (Logan & De Vos, 2009). Changes in cell wall structure related to physiological condition, culture age, nutrient availability, or sporulation can reduce the retention of the crystal violet–iodine complex, causing cells to appear Gram-negative despite belonging to a Gram-positive lineage. Therefore, the staining result was interpreted with caution and deemed insufficient for definitive taxonomic identification. Given the limited taxonomic resolution of morphological and staining characteristics, the isolate was further characterized using 16S rRNA gene sequence analysis. The obtained sequence was compared with reference sequences available in the GenBank database using BLASTN, and phylogenetic analysis was subsequently performed to determine the genetic affiliation of the isolate. Molecular

identification based on 16S rRNA gene homology provides a more robust and reliable approach for bacterial classification than phenotypic characteristics alone, particularly when atypical staining reactions are observed.

Genetic Determination of MSF4 Isolate Species

Sequencing was conducted using the Sanger method, in which fluorescently labeled ddNTPs terminate DNA strand elongation, enabling precise nucleotide identification. The amplified PCR products were analyzed through electrophoresis followed by fluorescence detection. Sequences obtained with primers 785F and 907R were assembled using BioEdit, resulting in a 1501 bp contig, of which 1379 bp showed homology to the 16S rRNA gene; non-homologous regions were excluded from further analysis.

BLASTN analysis (NCBI) conducted on August 7, 2025, produced 102 sequence hits—86 identified as *Bacillus cereus* and 16 as unclassified *Bacillus* species—showing close similarity to *B. thuringiensis*, *B. paramycoides*, *B. tropicus*, and *B. anthracis*. The ten top matches, each exhibiting 100% query coverage and identity, were selected to construct a phylogenetic tree comprising *B. paranthracis*, *B. cereus*, *B. tropicus*, and *Bacillus* sp. PM5. The tree was generated using the Neighbor-Joining method in MEGA11 (Figure 3).

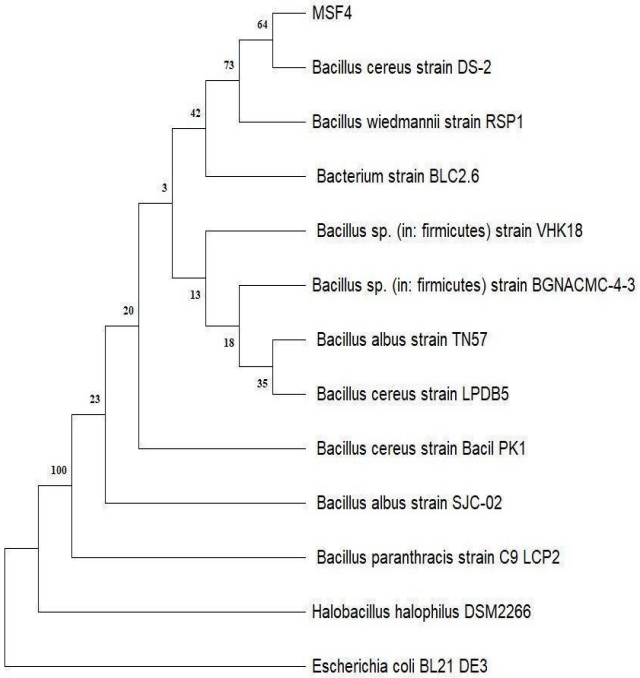


Figure 3. MSF4 phylogenetic tree

The phylogenetic tree distinctly separated the *Bacillus* cluster from the outer clade consisting of *Halobacillus halophilus* and *Escherichia coli* BL21 (DE3), supported by a bootstrap value of 100. The MSF4 isolate clustered closely with *Bacillus cereus*, together with *B. wiedmannii*, *Bacterium* strain BLC2.6, *Bacillus* sp., and *B. albus*. The closest genetic relationship was observed with *B. cereus* strain DS-2, supported by a bootstrap value of 64, indicating high genetic similarity. Although Gram staining showed that MSF4 appeared as a Gram-negative, rod-shaped bacterium, molecular identification provided stronger taxonomic evidence. BLASTN analysis of the 16S rRNA gene sequence revealed 100% similarity to

Bacillus cereus strain DS-2, and phylogenetic analysis clustered MSF4 closely with *B. cereus* and other members of the *Bacillus cereus* group. However, species-level identification within the *Bacillus cereus* group should be interpreted cautiously because 16S rRNA gene sequences are highly conserved among closely related taxa, including *B. cereus*, *B. thuringiensis*, *B. anthracis*, and related species.

The apparent discrepancy between the Gram-staining result and molecular identification was also interpreted with caution. Although *Bacillus cereus* is traditionally recognized as a Gram-positive rod-shaped bacterium, Gram-variable or Gram-negative staining responses have occasionally been reported in members of the genus *Bacillus*. Such variability may be associated with culture age, physiological state, sporulation, or alterations in cell wall integrity that affect retention of the crystal violet–iodine complex. Therefore, the Gram-negative appearance was considered an atypical phenotypic observation rather than definitive taxonomic evidence

Based on the available molecular data, the isolate was considered a member of the *Bacillus cereus* group and was provisionally designated as *Bacillus cereus* MSF4 according to its closest sequence match. Nevertheless, additional analyses, such as multilocus sequence analysis (MLSA) or whole-genome sequencing, would be required to confirm species-level identification.

Growth Curve of MSF4 Isolate in LB Liquid Medium

The growth patterns observed at different agitation speeds are presented in Figure 4. Increased turbidity in the LB medium indicated active bacterial growth. At an agitation speed of 150 rpm, cells exhibited rapid growth (OD_{600} > 0.6) followed by a sharp decline, likely due to mechanical stress. In contrast, incubation at 100 rpm maintained stable growth (OD_{600} 0.4–0.6), representing the optimal logarithmic phase for culture development.

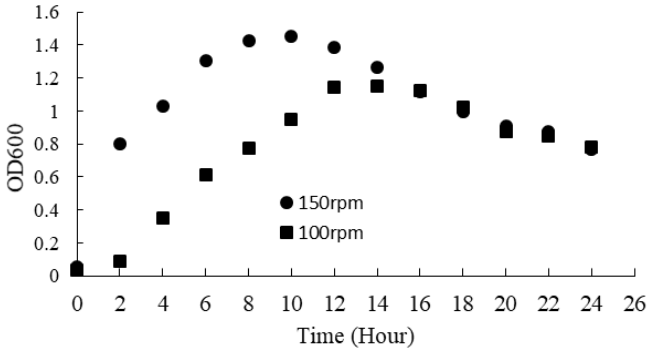


Figure 4. Growth curve of MSF4 isolate in liquid LB medium

Therefore, 100 rpm was chosen to maintain active bacterial growth, promote enzyme production, and minimize both cell damage and energy consumption. An OD_{600} value of approximately 0.5 was considered to represent the exponential growth phase of the MSF4 isolate under the cultivation conditions used in this study, whereas lower and higher OD_{600} values corresponded to the lag and stationary phases, respectively.

Standard Curve of p-Nitrophenol

Lipase activity was determined using *p*-nitrophenyl palmitate (pNPP) as the substrate, which undergoes hydrolysis to yield palmitic acid and *p*-nitrophenol (pNP), producing a yellow coloration under alkaline conditions. The absorbance of *p*-nitrophenol

was measured at 410 nm, and its concentration was calculated using the standard curve equation y = 0.0058x + 0.0111 (R^2 = 0.9955), as presented in Figure 5, demonstrating high analytical precision and reliability.

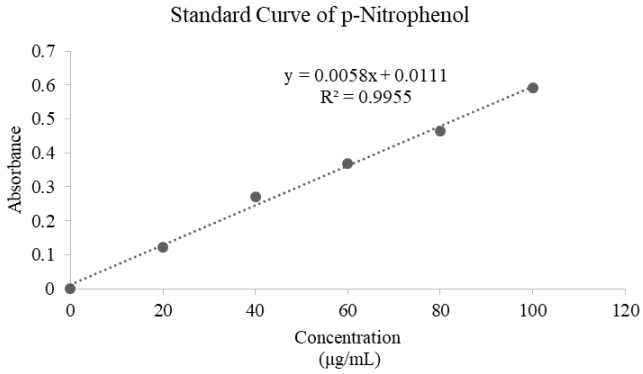


Figure 5. Standard curve for p-Nitrophenol

One unit (U) of lipase activity is defined as the enzyme amount producing 1 µg pNP per minute per mL under assay conditions. Activity was calculated using Equation (1) (Kai & Peisheng, 2016):

$$\text{Enzyme activity (U/mL)} = \frac{C \times Ves}{t \times Ve}$$

Where,
C = pNP concentration (µg/mL)
Ves = total reaction volume (mL)
t = incubation time (min)
Ve = enzyme volume (mL)

Production Curve of Lipase by MSF4 Isolate

Production of extracellular lipase by isolate MSF4 occurred rapidly during the early stages of submerged fermentation. As illustrated in Figure 6, enzyme activity reached 48.40 ± 0.06 U/mL after 24 h and remained nearly unchanged at 48 h (48.61 ± 0.24 U/mL). The minimal difference between these values suggests that lipase production had already approached its maximum level within the first day of cultivation. Beyond 48 h, enzyme activity gradually decreased, which may be attributed to nutrient limitation, accumulation of metabolic byproducts, or reduced enzyme stability during prolonged incubation. Although the highest activity was recorded at 48 h, the difference relative to 24 h was negligible. Therefore, 24 h was selected as the optimal harvest time because it provided nearly maximal lipase yield while reducing fermentation time and associated production costs. A similar production profile has been reported for several Bacillus species, in which maximum lipase production was achieved within the first 24–48 h of cultivation (Alkan et al., 2007; Oni et al., 2022).

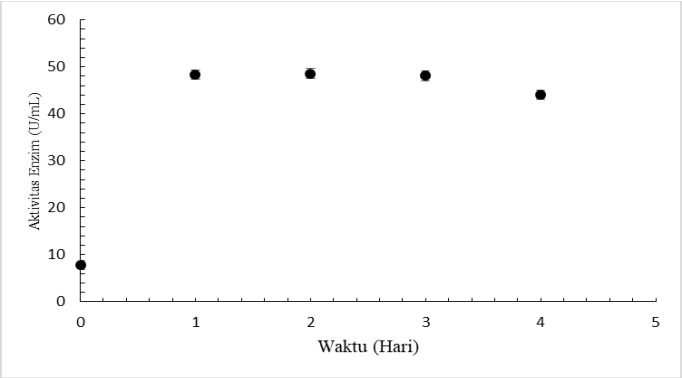


Figure 6. Changes in Lipase Activity as a Function of Incubation Time

Optimization of Lipase Fermentation by MSF4 Isolate Using the One Factor at a Time (OFAT) Method

Fermentation Temperature Optimization

Temperature plays a crucial role in bacterial growth by regulating enzymatic activity—low temperatures reduce metabolic rates, while excessively high temperatures induce thermal stress, protein denaturation, and ultimately cell death (Oni et al., 2022; Baltaci et al., 2020)

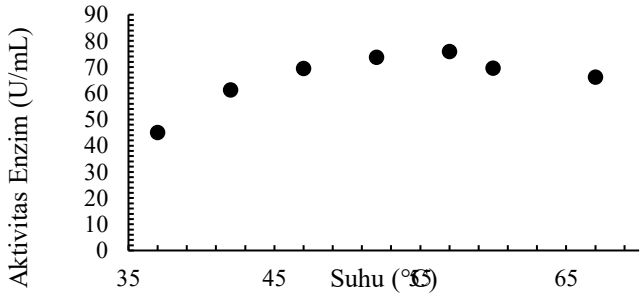


Figure 7. Effect of incubation temperature on lipase enzyme production

As shown in Figure 7, lipase activity reached its maximum at approximately 75 U/mL at 57 °C, then declined at higher temperatures (60–65 °C) due to enzyme denaturation. This observation aligns with previous reports indicating that lipase activity typically peaks within the thermophilic temperature range, confirming 57 °C as the optimal fermentation temperature for MSF4 lipase (H. K. Kim et al., 1998).

The Effect of Fermentation pH

Medium pH influences both enzyme activity and microbial growth rate (Oni et al., 2022; Kim et al., 1998). The results presented in Figure 8 indicate that lipase production increased progressively with increasing medium pH and reached its highest value at pH 10. This result indicates that alkaline conditions favor lipase biosynthesis by the MSF4 isolate. The enhanced production under alkaline conditions may be associated with improved cellular metabolism and more efficient secretion

of extracellular enzymes. Similar observations have been reported for alkaline lipase-producing *Bacillus* species (H. K. Kim et al., 1998).

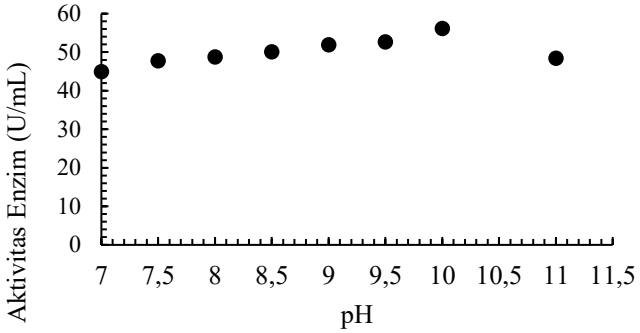


Figure 8. Effect of pH medium on enzyme production

Optimization of Lipase Working Conditions by MSF4 Isolate Using the One Factor at a Time (OFAT) Method

The Effect of Temperature on Enzyme Activity

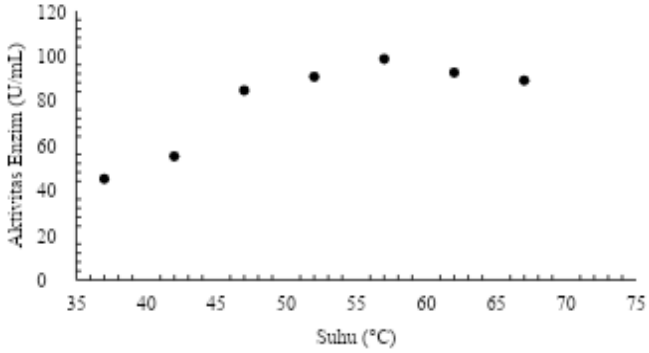


Figure 9. Effect of temperature on lipase working conditions

Figure 9 shows the effect of assay temperature on lipase activity**.** Enzyme activity increased progressively with increasing temperature and reached a maximum of 75.85 ± 0.85 U/mL at 57 °C. Above this temperature, activity decreased, suggesting reduced enzyme stability at elevated temperatures. The optimum temperature observed in this study is comparable to the temperature range reported for several thermostable Bacillus lipases, indicating that MSF4 lipase possesses moderate thermostability and potential for operation at elevated temperatures (Kim et al., 1998).

Effect of pH on Lipase Activity

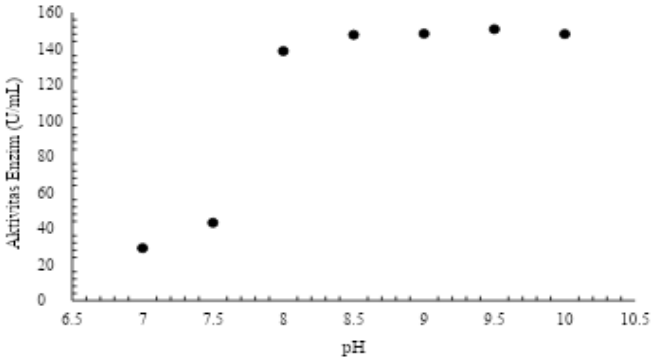


Figure 10. Effect of temperature on pH on lipase activitydarant

The enzyme exhibited maximum activity at pH 9.5 (150.76 ± 0.36 U/mL), as presented in Figure 10. Activity increased progressively from neutral to alkaline conditions but declined above pH 10, suggesting that excessive alkalinity may affect the structural integrity of the catalytic site. These results indicate that MSF4 lipase is moderately alkalophilic and remains highly active under basic conditions. The alkaline optimum observed for MSF4 lipase indicates that the enzyme is moderately alkaliphilic. Similar alkaline activity profiles have been reported for thermostable Bacillus lipases, which retain high catalytic activity under alkaline conditions (Kim et al., 1998). These characteristics support the potential application of MSF4 lipase in detergent formulations and other alkaline industrial processes.

Stability Testing of Lipase Enzymes in a Surfactant Environment

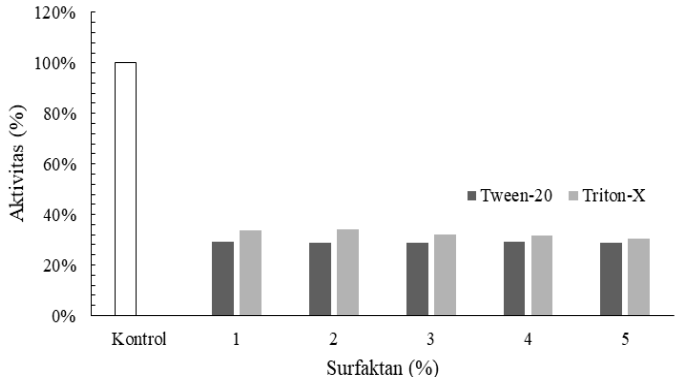


Figure 11. Activity test in a surfactant environment

Figure 11 illustrates that Tween 20 and Triton X-100 reduced lipase activity to 28.99% and 32.34% of the control (2,861 U/mL), respectively. This reduction is attributed to surfactant interactions with the enzyme's hydrophobic regions, which disrupt its tertiary structure and alter the active site conformation, thereby diminishing catalytic efficiency (Ozyilmaz & Eski, 2020; Goswami, 2020; Wang et al., 2012).

CONCLUSION

The isolate *Bacillus sp.* MSF4 exhibited an atypical Gram-negative staining reaction and showed maximum lipase production after 24 h of fermentation. The crude lipase

displayed optimum activity at 57 °C and pH 9.5–10 and retained activity in the presence of nonionic surfactants, indicating its potential for detergent-related applications.

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