

Biosynthesis and characterization of lipase produced by *Bacillus subtilis* DB104 and its potential in biodiesel production

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Abstract

Lipases hydrolyse fats into glycerol and free fatty acids and extensively used in various industries, including food, pharmaceuticals, biodiesel production, and detergents. The present study aimed to optimize fermentation conditions for lipase production by *Bacillus subtilis* DB104 in shake flask cultivations using one factor at a time (OFAT) method. The optimal conditions identified included the supplementation of basal salt medium with 0.5% waste cooking oil (WCO) and 15 mM ammonium nitrate at an initial medium pH 7.0 and resulted in the highest lipase activities of 8.97 U/mL. In enzyme stability tests, the lipase exhibited optimal activity at pH 7.0 and 37°C. Metal ions, CaCl₂ and MnCl₂, enhanced lipase activity, as did non-ionic detergents such as Tween 80, and Tween 20. Lipase produced retained its stability (>70%) in the presence of 1% inhibitors like EDTA, and PMSF, showing its robustness. Also, it maintained full activity (100%) in 1% of isopropanol, DMSO, and hexane. SDS-PAGE and Zymogram analyses showed the molecular weight of lipase obtained was in the range from 33-35 kDA. Lipase catalysed- transesterification of WCO has resulted in FAME conversion of 68.8%. Further production and optimization in a bigger scale fermenter and lipase purification are ongoing to meet the mass biodiesel production demand in Indonesia.

Keywords; *Bacillus subtilis* DB104, biodiesel, fatty acid methyl ester, lipase production, optimization.

1. Introduction

Indonesia, a country rich in natural resources, has seen a significant rise in its biodiesel production over the past decade. This increase has been driven by government policies, the growing demand for renewable energy, and efforts to reduce greenhouse gas emissions (Wirawan *et al.* 2024). Since the implementation of the B30 program in 2020, which requires a 30% blend of biodiesel in diesel fuel, this program has encouraged domestic biodiesel use and decreasing the country's reliance on fossil fuel imports (Mokhtar *et al.* 2023). Indonesia has set renewable energy goals by aiming for 23% of its energy must come from renewable sources by 2025 (Budiarto *et al.* 2021; Erdiwansyah *et al.* 2021). As one of the world's leading producers of palm oil, Indonesia has steadily increased its biodiesel production capacity (Purwoko and Wibowo, 2020). The government's consideration of adopting even higher biodiesel blends, such as B40 and possibly B50, shows the commitment to further expanding the biodiesel sector.

Biodiesel production from lipase-catalyzed process has attracted attention due to the enzyme's effectiveness in facilitating the transesterification process, which converts fats and oils into biodiesel (Wancura *et al.* 2023). Various

feedstocks, such as non-edible oils (Zambare *et al.* 2021), waste cooking oils (Alshehri *et al.* 2024), and microalgae for biodiesel synthesis (Fattah *et al.* 2020) are gaining popularity suiting circular bioeconomy concepts. Lipase-catalyzed transesterification works well for feedstocks with high free fatty acid content, which can be challenging for chemical catalysts (Wancura *et al.* 2023; Alshehri *et al.* 2024; Akhter *et al.* 2022). The use of lipases was reported to be able to decrease the need for energy-intensive processes and harsh chemicals, which may lower production costs. Enzyme-catalyzed methods are also seen as more environmentally friendly, producing fewer by-products and waste compared to traditional chemical methods (Ali *et al.* 2023).

Lipase production from waste cooking oil (WCO) by *Bacillus* species has gained considerable research interest due to the enzyme's effectiveness in biocatalytic processes and the chance to use sustainable waste materials (Binhayeeding *et al.* 2020). Using WCO as a feedstock for lipase production may address environmental issues related to waste disposal and offers a cost-effective option for enzyme production (Akhter *et al.* 2022). Various pretreatment methods to remove impurities and enhance the suitability of waste cooking oil for microbial fermentation have been reported. Several *Bacillus* strains with exceptional lipase production have been isolated from

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different environments (Suci *et al.* 2018; Akhter *et al.* 2022; Khattab *et al.* 2022).

Improving the operational stability of *Bacillus* lipases under industrial settings remains a big challenge. Transitioning laboratory-scale achievements to large-scale production necessitates overcoming various technical and economic hurdles. Research on optimizing fermentation conditions such as pH, temperature, nutrient composition, and aeration, to maximize lipase yields are progressing (Suci *et al.* 2018; Akhter *et al.* 2022; Moschona *et al.* 2024). Current research is aiming to develop more robust enzymes through genetic modifications and immobilization techniques. Optimizing fermentation conditions and utilizing low-cost substrates are also among the initiatives to reduce production costs (Nawani *et al.* 2006).

Due to the problems or issues stated above, the present study aimed to optimize the lipase production using WCO under different cultivation conditions using one factor at a time (OFAT) by *B. subtilis* DB104. Partially purified lipase was characterized, and the stability of lipase was tested in terms of thermal stability; substrate specificity was carried out. Potential application of lipase produced by *B. subtilis* DB104 from WCO for biodiesel conversion was also reported in the present work.

2. Materials and Methods

2.1. Preparation of bacterial cell culture

B. subtilis DB104 was obtained from the Indonesian National Research and Innovation Agency (BRIN) Culture Collection and stored as a glycerol stock at -80 °C. This strain was then streaked onto a nutrient agar (NA) plate and incubated at 37 °C for 24 h. To prepare the seed culture, a single colony of *B. subtilis* DB104 was inoculated into a sterile test tube containing 5 mL of nutrient broth (NB) and incubated at 37 °C, 170 rpm for 15-16 h or until reaching OD 0.6-0.8 measured using UV spectrophotometer at 600 nm.

2.2. Medium and fermentation conditions for lipase production

Mineral salt media (MSM) containing following components per liter of distilled water: NH_4Cl (2.0 g/L), Na_2HPO_4 (6.0 g/L), MgSO_4 (0.1 g/L), NaCl (5.0 g/L), KH_2PO_4 (3.0 g/L) was prepared as proposed by Zhang *et al.* (2012). The MSM was autoclaved at 121°C for 20 min, separately from the carbon source prior to a 10% inoculum addition to media. For the optimization study, *B. subtilis* DB104 was incubated under different conditions, including temperature, initial medium pH, concentrations of carbon sources, nitrogen sources, and concentrations of the chosen nitrogen source, for 48 h with an agitation speed at 170 rpm.

2.3. Optimization conditions for lipase production

2.3.1. Type of carbon sources

Various carbon sources (glucose, glycerol, WCO, palm cooking oil, and soybean oil) at a 1% concentration were evaluated. *B. subtilis* DB104 was inoculated into MSM containing each carbon source, and the initial pH of the medium was adjusted to 7.0 using acid or base. The

cultures were incubated at 37 °C for 48 h with agitation at 170 rpm.

2.3.2. Concentrations of carbon sources

The optimal carbon source, WCO, identified in the experiment described in section 2.3.2 was further investigated for its concentrations on the growth and lipase production by *B. subtilis* DB104. The cultures were supplemented with 0.5-2.0% of WCO, while only MSM served as the control. The initial medium pH was adjusted to 7.0 using an acid or base. All cultures were incubated at 37 °C for 48 h with an agitation speed at 170 rpm.

2.3.3. Type of nitrogen sources

The cultures were supplemented with 0.5% WCO and different nitrogen sources (ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3), ammonium sulphate, peptone, yeast extract and urea) at a concentration of 15 mM. The initial medium pH was set at 7.0, and the cultures were incubated at 37 °C for 48 h with agitation speed at 170 rpm.

2.3.4. Concentrations of NH_4NO_3

The cultures were cultivated in MSM containing 0.5% WCO and NH_4NO_3 at varying concentrations from 15 to 100 mM. This experiment was performed to investigate the effects of different NH_4NO_3 concentrations on the growth and lipase production by *B. subtilis* DB104. The initial medium pH was adjusted to 7.0 using either 1M NaOH or 1M HCl, and the cultures were incubated at 37 °C for 48 h with agitation at 170 rpm.

2.3.5. Initial medium pH

B. subtilis DB104 was cultivated in an optimized MSM supplemented with 0.5% WCO, 15 mM of NH_4NO_3 and the initial medium pH was adjusted to the selected pH range of 6.5 to 8.5 using either 1M NaOH or 1M HCl. The culture was then incubated at 37°C for 48 h with agitation at 170 rpm.

2.4. Analyses

2.4.1. Extraction of crude enzyme

The crude lipase was extracted from the supernatant of the fermentation broth. The fermentation mixture was centrifuged at 12,000 rpm at 4 °C for 5 min to separate the enzyme-containing supernatant (Falony *et al.* 2006).

2.4.2. Partial purification using acetone precipitation

Protein precipitation using acetone was performed according to the method described by Ewelina *et al.* (2010) with slight modifications. The crude enzyme extract was gradually mixed with chilled acetone at a ratio of 2:1 (acetone:enzyme, v/v) and incubated at -20 °C overnight. The mixture was then centrifuged at 10,000 rpm for 20 min. The resulting precipitate was allowed to air-dry for 20 min to remove residual acetone and subsequently resuspended in phosphate buffer.

2.4.3. Lipase activity assay

A standard titrimetric method was employed to assay lipase activity. The substrate for the enzyme assay was prepared by emulsifying olive oil with Arabic gum in 5 mL of phosphate buffer at pH 7 and 2 mL of 0.6% CaCl_2 solution. During estimation, 500 μL of the crude enzyme extract was added, and the substrate-enzyme mixture was

incubated in a rotary shaker at 150 rpm and 30°C for 15 min. A solution containing 3 mL of ethanol and 3 mL of acetone was used to terminate the reaction and extract the fatty acids. Phenolphthalein was used as an indicator dye during the titration of the fatty acids with 0.1M NaOH at pH 10.5. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 µmol of fatty acids per minute from the triglyceride substrate (tributylin) (Kojima *et al.* 2003).

2.4.4. Lipase activity test

The lipase activity was measured using a spectrophotometric assay with *p*-nitrophenyl palmitate as the substrate (Silva *et al.* 2005). The substrate was prepared by mixing two solutions: Solution A containing 30 mg of *p*-nitrophenyl palmitate in 10 mL of isopropanol, and Solution B containing 100 mg of gum Arabic and 400 mg of Triton X-100 in 90 mL of 50 mM Tris-HCl buffer. The substrate was created by combining these two solutions evenly. Next, 100 µL of lipase was mixed with 900 µL of the substrate. Blank samples were prepared by replacing the lipase with 50 mM Tris-HCl buffer. The lipase-substrate and blank mixtures were incubated for 30 min at 37°C in a water bath shaker. Then, the absorbance was then measured using a UV-Vis spectrophotometer at a wavelength of 410 nm. The enzyme activity was calculated as the amount of *p*-nitrophenol released enzymatically from the substrate per minute. The absorption measurement results were converted into the standard curve equation of *p*-nitrophenol, which was used to determine the lipase activity.

2.4.5. Protein content

Protein content of *B. subtilis* DB 104 was determined using Bradford assay (Bradford, 1976) with slight modification. Protein concentrations were measured by adding a 1:50 dilution of the sample to the Bradford reagent, incubating for 30 min, and the absorbance was recorded at 595 nm. The specific activity was then calculated by dividing the enzyme activity by the measured lipase protein content.

$$\text{Specific activity} = \frac{\text{Lipase activity} \left(\frac{\mu}{\text{ml}} \right)}{\text{Protein content} \left(\frac{\text{mg}}{\text{ml}} \right)}$$

2.5. Lipase stability tests

2.5.1. pH and temperature

The experiment on the effect of pH and temperature on lipase stability was performed according to method proposed by Lu *et al.* (2010). The effect of pH on lipase activity was carried out in the pH range from pH 5 – pH 10 using following buffers; pH 5 (acetate); pH 6-8 (phosphate); and pH 9-10 (Glycine-NaOH).

Meanwhile, the effect of temperature on lipase stability was determined at 35 °C, 37 °C, 40 °C, 45 °C, and 50 °C.

2.5.2. Effect of ions

The effect of several ions on lipase stability was determined by measuring lipase activity after incubation for 30 min at optimum temperature and pH in a buffer containing 5 mM of CaCl₂, MgCl₂, ZnCl₂, MnCl₂, FeCl₃, KCl, and NaCl (Kotogan *et al.* 2018).

2.5.3. Effect of detergents

The effect of different detergents on lipase stability was determined by measuring lipase activity after incubation for 30 min at optimum temperature and pH in a buffer containing 1% (w/v) of SDS, Triton X, Tween 80 and Tween 20 (Guncheva *et al.* 2007).

2.5.4. Effect of inhibitors and activators

The influence of inhibitors and activators on lipase stability was determined by measuring lipase activity after incubation for 30 min at optimum temperature and pH in a buffer containing 1% of DTT (Dithiothreitol), PMSF (Phenyl Methyl Sulfonyl Fluoride), β Mercaptoethanol and EDTA (Ethylene Diamine Tetra Acetic Acid) (Kumar *et al.* 2020).

2.5.5. Effect of organic solvents

The effect of organic solvents on lipase stability was investigated by measuring the lipase's catalytic performance following a 30 min incubation at the optimal temperature and pH in the presence of 1% (v/v) of DMSO, methanol, chloroform, n-hexane, isopropanol, and acetone (Vahidi *et al.* 2020).

2.5.6. SDS Page and Zymogram

SDS-PAGE was conducted according to protocol suggested by Laemmli (1970) using 12% polyacrylamide gels. After electrophoresis, the gel was stained with Coomassie brilliant blue. Subsequently, the proteins were renatured by incubating the gel in a renaturation buffer (0.05 mM phosphate buffer (pH 7) and 2.5% Triton X-100) for 30 min. The gel was kept at 4°C for 15 min and then soaked in 0.1% Congo red for 30 min. Then, the gel was soaked in 1 M NaCl until the bands became visible, with the lipolytic activity shown as a red band (Bora and Bora, 2012).

2.5.7. Biodiesel production

Biodiesel production via enzymatic route was carried out by reacting WCO with methanol at a molar ratio of 1:5 and lipase amounting of 10% (v/v) relative to the WCO was added into the mixture. The reaction was conducted at 40°C with continuous stirring at 500 rpm for 24 h. The samples were centrifuged at 12,000 rpm for 5 min at 15°C to obtain the upper layer and solution from this layer was then used to determine the percentage and composition of biodiesel produced from the synthesis reaction (Husar *et al.* 2021).

2.5.8. Analysis of biodiesel content

The biodiesel content of fatty acid methyl ester (FAME) was determined using gas chromatography (GC-17A, Shimadzu, Japan) with a flame ionization detector (GC-FID). The GC unit was equipped with a Zb-5TH column (0.25 mm x 15 m) along with an auto sampler. The injector and detector temperatures were set at 140°C and 280°C, respectively, and helium gas was used as the carrier at a flow rate of 35 mL/min. The FAME composition for each reaction was determined by peak area normalization and calculating as a percentage through comparison with authentic FAME standards including methyl myristate, methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate. Quantification was further supported by the use of standards comprising methyl myristate, methyl

palmitate, methyl stearate, arachidate, behenate, tetracosanoate, hexacosanoate, octacosanoate, and triacontanoate.

3. Results and Discussion

3.1. Optimization for lipase production

3.1.1. Effect of carbon sources

Using waste cooking oil (WCO) as a substrate greatly influenced lipase production, which is an enzyme that helps break down fats. *B. subtilis* DB104, achieved the highest lipase activity at 4.49 U/mL when using WCO as a substrate. In comparison, soybean oil produced 3.37 U/mL and palm oil resulted in 3.17 U/mL (Table 1).

Table 1 Effect of carbon sources on the growth and lipase production by *B. subtilis* DB 104.

Carbon sources	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
Glucose	1.02 ± 0.07	0.03 ± 0.00	0.42 ± 0.02
Glycerol	0.13 ± 0.01	0.06 ± 0.00	1.42 ± 0.00
Waste cooking oil	1.34 ± 0.01	0.07 ± 0.00	4.49 ± 0.01
Palm cooking oil	0.75 ± 0.06	0.07 ± 0.00	3.17 ± 0.15
Soybean cooking oil	0.52 ± 0.03	0.14 ± 0.00	3.37 ± 0.06

Lower lipase activity was obtained when the culture media was supplemented with glucose and glycerol, with activity recorded at 0.42 U/mL and 1.42 U/mL, respectively. A substantial increase in lipase activity with lipid carbon sources (WCO, palm and soybean cooking oil) is likely due to the availability of fatty acids during growth on these compounds. Similarly, Carvalho *et al.* (2022) also found the lipase production from fatty acid esters is stronger than that observed with glucose or other non-lipid substrates. *Bacillus* species was reported to be efficiently utilized WCO, with high lipase yields (Fattah *et al.* 2020; Carvalho *et al.* 2022). The present work shows the triglycerides and free fatty acids found in WCO was an ideal substrate for lipase induction, promoting increased enzyme production.

3.1.2. Effect of WCO concentration

Mineral salt medium with supplementation of 0.5% WCO showed the best growth and lipase activity, measuring 1.67 OD and 5.35 U/mL, respectively (Table 2). Similar results were obtained from a study reported by Baltaci *et al.* (2019), which also found the highest lipase activity was obtained at 0.5% WCO as a substrate. WCO is a cost-effective substrate because it contains free fatty acids, glycerides, and various impurities that *B. subtilis* DB104 can use as a carbon source.

Table 2 Effect of WCO concentrations on the growth and lipase production by *B. subtilis* DB 104.

WCO concentration(%)	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
0	0.29 ± 0.08	0.02 ± 0.00	0.40 ± 0.01
0.5	1.67 ± 0.07	0.08 ± 0.02	5.35 ± 0.17
1.0	1.62 ± 0.08	0.13 ± 0.02	4.31 ± 0.27
1.5	0.65 ± 0.13	0.05 ± 0.00	1.19 ± 0.06
2	0.59 ± 0.04	0.04 ± 0.00	1.16 ± 0.06

Using WCO as a carbon source in fermentation process is a good choice as it helps in waste management and turning a waste product into useful biological products. The excess supply of WCO in the fermentation medium may result in reduction of microbial growth and lipase activity. The decrease in growth and lipase activity can be explained due to the buildup of toxic by-products, feedback inhibition caused by free fatty acids released during hydrolysis, and impurities that may inhibit microbial growth or lipase activity (Khodadai *et al.* 2020). Furthermore, high WCO concentrations can create a viscous environment that hinders oxygen transfer in aerobic fermentation processes (Ismail *et al.* 2018).

3.1.3. Effect of nitrogen sources

Types and concentration of nitrogen are necessary for microbial growth and lipase production, though its optimal concentration varies with the microbial strain and fermentation conditions (Al Mohaini *et al.* 2022). *B. subtilis* DB104 produced the highest lipase at 8.97 U/mL when using NH_4NO_3 as the nitrogen source and followed by other nitrogen sources ($\text{NH}_4)_2\text{SO}_4$ and NH_4Cl at 8.63 U/mL and 7.51 U/mL, respectively (Table 3). Inorganic nitrogen sources are preferable for lipase production than organic ones. Similar findings were reported by Akter *et al.* (2022) and Ali *et al.* (2023), which found that no growth was observed with yeast extract in *B. cereus* NC7401. NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$ are fully soluble in water and readily available for microbial growth, and high ionic strength of ammonium ions from these sources help protein synthesis by being easily incorporated into amino acids (Ismail *et al.* 2018). However, it can be inferred that the nitrogen sources significantly affect lipase production, and the optimal nitrogen concentrations vary by species.

Table 3 Effect of nitrogen sources on the growth and lipase production by *B. subtilis* DB104.

Nitrogen sources (15 mM)	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
Ammonium chloride	1.43 ± 0.11	0.32 ± 0.00	7.51 ± 0.25
Ammonium sulfate	5.34 ± 0.05	0.11 ± 0.01	8.63 ± 0.31
Ammonium nitrate	5.03 ± 0.14	0.13 ± 0.01	8.97 ± 0.42
Urea	1.89 ± 0.16	0.19 ± 0.14	6.10 ± 0.07
Peptone	1.33 ± 0.28	0.53 ± 0.03	6.81 ± 0.21
Yeast extract	5.35 ± 0.16	0.42 ± 0.02	5.59 ± 0.40

3.1.4. Effect of NH_4NO_3 concentrations

Table 4 Effect of NH_4NO_3 concentrations on the growth and lipase production by *B. subtilis* DB104 using WCO.

$(\text{NH}_4)_2\text{NO}_3$ (mM)	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
0	1.45 ± 0.11	0.21 ± 0.05	1.40 ± 0.11
15	4.96 ± 0.15	0.27 ± 0.02	8.94 ± 0.21
25	1.80 ± 0.14	0.23 ± 0.03	8.50 ± 0.70
35	0.99 ± 0.17	0.14 ± 0.03	7.88 ± 0.55
45	1.35 ± 0.06	0.13 ± 0.03	7.57 ± 0.11

MSM was supplemented with NH_4NO_3 at concentrations from 0 to 45 mM after identifying the optimal nitrogen source for growth and lipase production (Table 4). The best growth and lipase activity was obtained at 15 mM NH_4NO_3 , with values of 4.96 OD and 8.94 U/mL. Sufficient nitrogen is essential for synthesizing amino acids, nucleotides, and other cellular components, leading to strong microbial growth and metabolism, which can boost lipase production (Pham *et al.* 2021). However, increasing nitrogen levels further reduced growth and resulted in a plateau for lipase activity. Akhter *et al.* (2022) reported that high nitrogen levels may suppress the production of lipase, by shifting microbial metabolism to primary growth rather than secondary processes like lipase production. Thus, the present study suggests that finding the right nitrogen concentration is important for ensuring the growth of *B. subtilis* DB104 and balanced metabolism, ultimately promoting more lipase production. Obtaining the right nitrogen source, type of nitrogen used, carbon/nitrogen ratio, and overall nutrient balance are key to maximizing lipase production in fermentation.

3.1.5. Effect of initial medium pH

Table 5 Effect of initial medium pH on the growth and lipase production by *B. subtilis* DB 104 using WCO.

Initial medium pH	OD (660 nm)	Protein (mg/mL)	Lipase activity (U/mL)
6.5	1.27 ± 0.31	0.55 ± 0.18	3.28 ± 0.11
7.0	4.97 ± 0.14	1.12 ± 0.10	8.55 ± 0.22
7.5	3.09 ± 0.14	0.79 ± 0.12	8.55 ± 0.22
8.0	1.35 ± 0.34	0.63 ± 0.13	3.66 ± 0.22
8.5	1.00 ± 0.34	0.42 ± 0.02	2.99 ± 0.22

The highest lipase activity, 8.55 U/mL, was obtained when the culture grew at initial medium pH 7.0. Lipase activity varied significantly across different pH levels (pH 6.5 to 8.5) with lipase activity recorded from 2.99 U/mL to 8.55 U/mL (Table 5). *B. subtilis* PCSIRNL-39 was reported to produce the highest lipase at neutral pH (Mazhar *et al.*, 2017). In other reports, *Bacillus* sp. LBN2 achieved maximum lipase production within the alkaline range of 8.0 to 10.0 (Bora and Bora, 2012). Obtaining an optimal pH is vital for nutrient absorption, bacterial growth, and boosting enzyme production through signaling pathways, including the release of extracellular enzymes via proteolytic mechanisms (Ismail *et al.* 2018). Maintaining the right pH throughout cultivation process is essential for maximizing lipase yield and often needs strain-specific optimization and careful control during fermentation (Ebrahimipour *et al.* 2017).

3.1.6. Lipase stability under selected conditions pH

Lipase was very sensitive to pH changes. *B. subtilis* DB104 lipase work best at pH 7 and 8 with lipase activity of 9.03 U/mL and 8.36 U/mL, respectively (Fig. 1). Outside this range, the enzyme's activity dropped significantly from 9.02 U/mL to 7.39 U/mL and 7.29 U/mL at pH 5 and pH 10, respectively. This can be explained in that pH affects the ionization state of amino acid residues at the active site and the overall shape of the enzyme protein, impacting its ability to catalyze reactions (Di Russo *et al.* 2012). Maintaining high enzyme activity and stability all the time with suitable pH range is important, especially during processes like enzymatic transesterification for biodiesel production (Moschona *et al.* 2024).

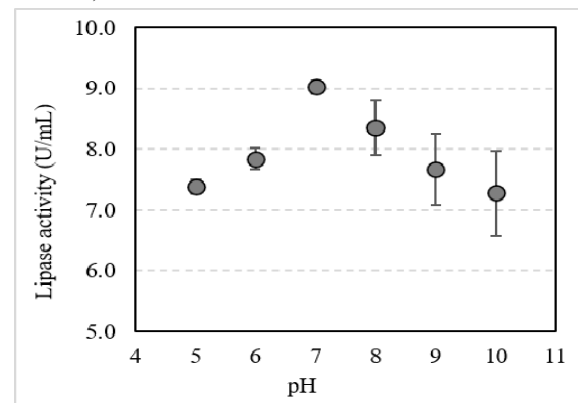


Figure 1 Comparison of Lipase activity of *B. subtilis* DB 104 under different pH

Temperature

B. subtilis DB104 strain had optimal lipase activity at a temperature of 37 °C (Fig. 2). Gradual decrease in lipase activity was observed when lipase was exposed to higher temperature (up to 50 °C). This can be explained in that when temperature increased, the molecules gained more kinetic energy, which resulted in more frequent collisions between the enzyme and substrate molecules (Ali *et al.* 2023), and eventually enzyme reached its optimal temperature. However, going beyond this temperature caused a sharp decrease in the enzyme's activity due to thermal inactivation. High temperatures could break essential hydrogen bonds, ionic bonds, and hydrophobic interactions that keep the enzyme's three-dimensional structure intact (Kumar *et al.* 2020).

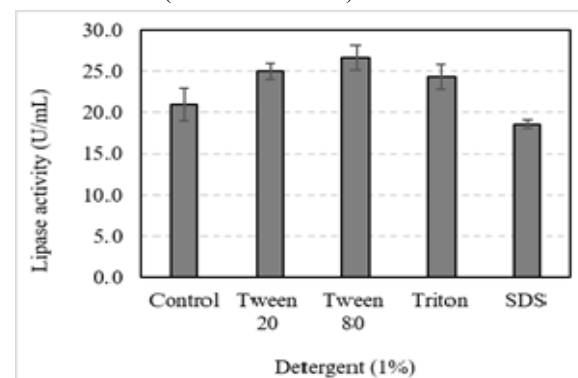


Figure 2 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different detergents

Effect of metal ions

Some metal ions acted as cofactors, enhancing enzyme activity, while others inhibited or destabilized the enzyme. This study showed that NaCl, KCl, FeCl₃, MnCl₂, ZnCl₂, MgSO₄, and CaCl₂, all at a concentration of 5 mM, increased lipase activity (Fig. 3). The ranking of lipase activity from highest to lowest was: CaCl₂ > MnCl₂ > Control > ZnCl₂ > NaCl > KCl > MgSO₄ > FeCl₃. Neha *et al.* (2023) reported that CaCl₂ boosted lipase activity, with activity increasing as CaCl₂ concentrations rose. Other metal ions like Ca²⁺ and Mg²⁺ are known to enhance lipase activity by stabilizing the enzyme structure or taking part in catalytic processes (Lu *et al.* 2018). These ions helped maintain the enzyme's shape, making it more effective in catalysis. On the other hand, heavy metal ions like Cu²⁺ and Zn²⁺ inhibited lipase activity by binding to the enzyme and disrupting its active site, which decreased its ability to catalyze reactions (Pham *et al.* 2021). However, the concentration of metal ions, microbial source of lipases, and the pH of the environment might influence the activity and stability of lipase.

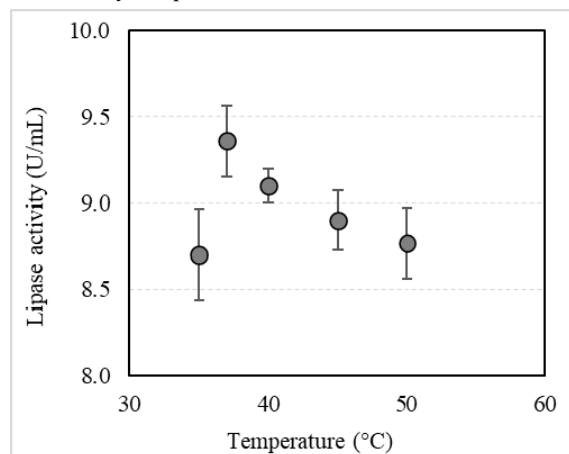


Figure 3 Comparison of Lipase activity of *B. subtilis* DB 104 under different temperature

Effect of detergents

Detergents can act either as inhibitors or activators, depending on their concentration and type. Non-ionic detergents like Tween 80, Tween 20, and Triton X-100 affected lipase activity, with values of 26.67 U/mL, 25 U/mL, and 24.33 U/mL, respectively (Fig. 4). Paz *et al.* (2023) reported that non-ionic detergents such as Triton X-100 and Tween 20 might boost lipase activity at low concentrations by improving substrate solubility or changing the enzyme's shape favorably. Also, the increase in lipase activity could result from higher availability of substrates (oil) or the detergent acting as an emulsifier to boost substrate uptake for lipase production (Al Mohaini *et al.* 2022). Lipase of *B. subtilis* DB104, when treated with SDS, showed lower activity but maintained at least 90% of its activity compared to the control. However, SDS was often reported as strong detergent and anionic surfactant to denature proteins that lead to a loss of enzyme activity (Ali *et al.* 2023).

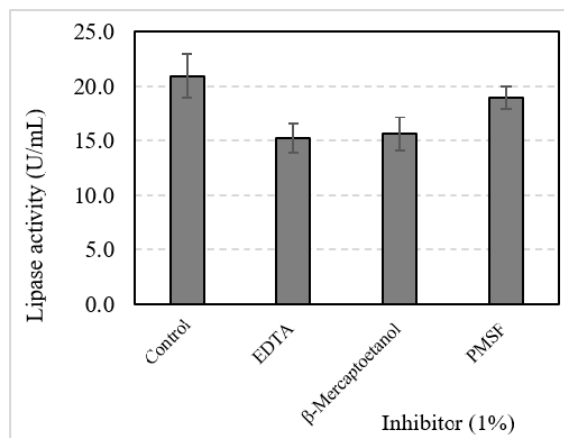


Figure 4 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different inhibitors

Effect of inhibitors

Various inhibitors were tested on lipase from *B. subtilis* DB104 with the aim to provide insight into enzyme regulation and applications. It was observed that the lipase treated with 1% concentrations of EDTA, β-mercaptoethanol, and PMSF retained at least 75-90% of its activity (Fig. 5). The highest inhibition on lipase activity was observed from EDTA (15 U/mL) since EDTA is a chelating agent and might sequester essential metal ions needed for the enzyme to function (Ali *et al.* 2023). PMSF, on the other hand, a serine protease inhibitor, might target serine residues at the active site and eventually reduce enzyme activity (Pham *et al.* 2021). Overall, the resistance of *B. subtilis* DB104 lipase shown in the present study towards these inhibitors suggests its potential use in various industrial applications.

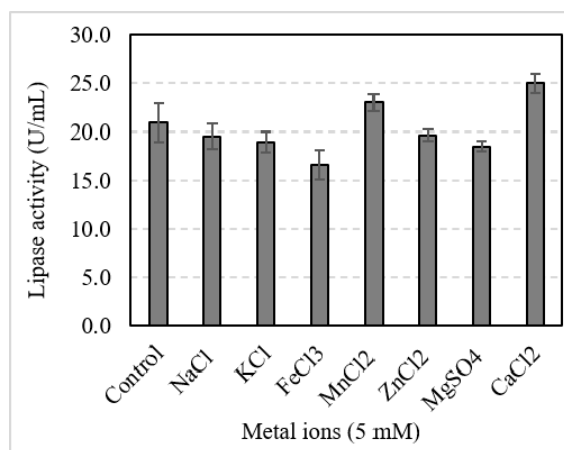


Figure 5 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different metal ions

Effect of organic solvents

Lipase from *B. subtilis* DB104 maintained 100% of its activity after treatment with 1% concentrations of isopropanol, DMSO, n-hexane, methanol, and DMSO (Fig. 6). However, its activity was reduced (18 U/mL and 19 U/mL) after exposure to acetone and chloroform, respectively might be due to the disruption of the lipase structure or unfavorable interactions with the active site. Organic solvents are often used in biocatalysis but can affect enzymatic activity and stability in various ways. Moschona *et al.* (2024) and Lotti *et al.* (2015) reported that

lipase activity can be boosted by low concentrations of methanol and ethanol since these solvents may increase substrate solubility or favorably altering the enzyme's shape. Ismail *et al.* (2018) reported that the presence of hydrophobic solvents like toluene and hexane at high concentrations might reduce enzyme activity due to the denaturation or partial unfolding of the enzyme. Water-immiscible solvents can hinder enzyme activity by limiting their shape, but adding water to such solvents can significantly enhance enzymatic activity in enzyme suspensions (Ali *et al.* 2023).

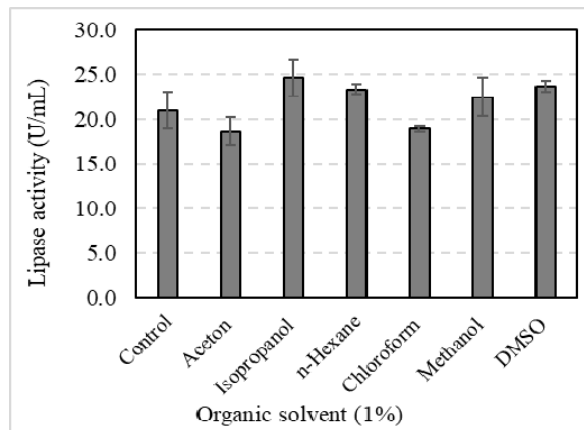


Figure 2 Comparison of lipase activity of *B. subtilis* DB104 in the presence of different organic solvent

3.2. SDS-PAGE and Zymogram

SDS-PAGE analysis of *B. subtilis* DB104 lipase revealed a protein band profile with molecular weights ranging from about 15 to 180 kDa and several thick protein bands appeared at 33 and 65 kDa (Fig. 2). Lipases from various *Bacillus* species were reported to have specific bands with high lipolytic activity around 35 kDa (Ali *et al.* 2023; Paz *et al.* 2023). Same molecular weight of 35 kDa was observed from *B. pumilus* V1 lipase, using fructose and yeast extract as carbon and nitrogen sources (Kandasamy *et al.* 2023). A slightly higher molecular weight lipase (38 kDa) was obtained from *B. halotolerans*

VSH 09 when grown on olive oil for 72 hours (Mahnashi *et al.* 2022). *B. cereus* ATA179 and *B. safensis* produced greater molecular weights of 47 kDa and 55 kDa respectively when grown on sucrose and olive oil (Demirkan *et al.* 2021; Patel *et al.* 2022). From this study, it can be concluded that the molecular weight of lipases produced from *Bacillus* sp. is species dependent and heavily depending on substrate and other cultivation factors.

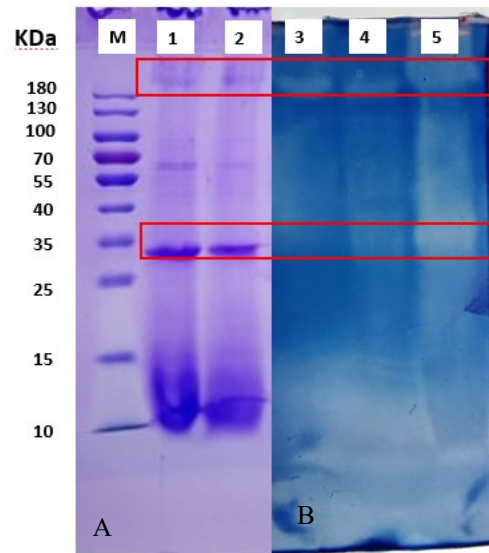


Figure. 7. SDS-PAGE protein profiling (A) and Zymogram lipolytic detection (B) of lipase produced by *B. subtilis* DB104 using WCO.

Table 6 summarizes recent lipase production from selected *Bacillus* species under specific fermentation conditions. It is worth noting that lipase production depended on the species and relied heavily on certain growth and nutrient needs. The growth requirements and lipase activity from this study were also compared with other *B. subtilis* species (Nadaf *et al.* 2020; Soeka *et al.* 2020; Kaur *et al.* 2024).

Table 6 Lipase-producing *Bacillus* species under optimal fermentation conditions.

No.	Microorganism	Fermentation conditions	Lipase activity	References
1.	<i>B. subtilis</i> DB104	Substrate: WCO Nitrogen: 15 mM (NH ₄) ₂ SO ₄ pH: 7.0 Temperature: 37 °C Fermentation: Submerge SDS-PAGE: 33-35 kDA Duration: 48 h	8.94 U/mL 21.0 U/mL (partial purified)	This study
2.	<i>B. subtilis</i> KUBT4	Substrate: 1% sucrose Nitrogen: 1% tryptone pH: 7.5 Temperature: 36 °C Fermentation: Submerge Duration: 48 h	46-49 U/mL	Nadaf and Thimmappa (2020)
3.	<i>B. subtilis</i> C2	Substrate: 0.1% olive oil Nitrogen: pH: 9.0 Temperature: 30 °C Fermentation: Submerge Duration: 120 h	1.76 umol/ mL	Soeka and Sulistiani (2020)
4.	<i>B. subtilis</i> TTP-06	Substrate: 2.75% glucose Nitrogen: 1.6% peptone pH: 7.5 Temperature: 30 °C Fermentation: Submerge Duration: 24 h	8.89 U/mL	Kaur and Gupta (2024)
5.	<i>B. subtilis</i> B-1-4	Substrate: WCO Nitrogen: 1.6% tryptone and 1.6% yeast extract pH: 8.0 Temperature: 45 °C Fermentation: Submerge Duration: 24 h	1.7 U/ mL	Al Shehri <i>et al.</i> (2024)
6.	<i>B. cereus</i> ATA179	Substrate: Sucrose Nitrogen: (NH ₄) ₂ HPO ₄ pH: 6.0 Temperature: 55 °C Fermentation: Submerge SDS-PAGE: 47 kDA Duration: 24 h		Demirkan <i>et al.</i> (2021)
7.	<i>B. cereus</i> NC7401	Substrate: Maltose Nitrogen: tryptone pH: 8.0 Temperature: 55 °C Fermentation: Submerge	40 U/mL	Akhter <i>et al.</i> (2022)
8.	<i>B. cereus</i> I ₁₃	Substrate: Glucose and olive oil Nitrogen: Yeast extract pH: 7.0 Temperature: 20 °C Fermentation: Submerge Duration: 96 h	11.42 U/mL	Kattathayil <i>et al.</i> (2024)
9.	<i>B. licheniformis</i> SMIA-3	Substrate: 0.5% orange flour and 0.5% WCO Nitrogen: - pH: 7.5 Temperature: 50 °C Fermentation: Submerge Duration: 42 h	0.35 U/mL	Carvalho <i>et al.</i> (2022)
10.	<i>Bacillus</i> sp. VITL8	Substrate: 6% olive oil Nitrogen: 0.7% peptone pH: 7.5 Temperature: 36 °C Fermentation: Submerge Duration: 25 h	325.0 U/ mL	Balaji <i>et al.</i> (2020)

11.	<i>B. halotolerans</i> VSH 09	Substrate: Olive oil pH: 7.0 Temperature: 55 °C Fermentation: Submerge SDS-PAGE: 38 kDA Duration: 72 h	41.66 IU/mL	Mahnashi <i>et al.</i> (2022)
12.	<i>B. safensis</i>	Substrate: 2% olive oil Nitrogen: 2g/L peptone pH: 7.0 Temperature: 37 °C Fermentation: Submerge SDS-PAGE: 55 kDA Duration: 48-72 h	13.71 U/mg	Patel and Parikh (2022)
13.	<i>B. salmalaya</i> 139SI	Substrate: Olive oil Nitrogen: - pH: 8.0 Temperature: 37 °C Fermentation: Submerge Duration: 48 h	11.0 U/ mL	Al Mohaini <i>et al.</i> (2022)
14.	<i>B. amyloliquefaciens</i>	Substrate: soybean extract Nitrogen: Yeast extract pH: 7.0 Temperature: 40 °C Fermentation: Solid state Duration: 72 h	41.20 U/mL	Mazhar <i>et al.</i> (2017)
15.	<i>B. aryabhatai</i> BA03	Substrate: 25% olive mill wastewater Nitrogen: Yeast extract pH: 8.0 Temperature: 27 °C Fermentation: Submerge	28.34 U/mL	Paz <i>et al.</i> (2023)
16.	<i>B. pumilus</i> V1	Substrate: Fructose Nitrogen: Yeast extract pH: 8.0 Temperature: 37 °C Fermentation: Submerge SDS-PAGE: 35 kDA	16.6-27.8 ug/mL	Kandasamy <i>et al.</i> (2023)
17.	<i>B. halotolerans</i> VSH 09	Substrate: 2.5% molasses Nitrogen: 2% peptone pH: 7.0 Temperature: 35 °C Fermentation: Submerge Duration: 72 h	26.04 IU/mL	Hombalimath and Gurumurthy (2024)

3.3. Lipase catalyzed-biodiesel production

Lipase-catalyzed biodiesel synthesis showed that the transesterification reaction successfully converted waste cooking oil (WCO) into FAME. The chromatogram profile at retention times 5, 6, 7, 8, 10, 16, and 23 matched the internal standard chromatogram profile. The enzymatic

synthesis of biodiesel using *B. subtilis* DB104 lipase yielded FAME compositions of 0.7% methyl myristate, 22% methyl palmitate, 17.9% methyl stearate, 23.7% methyl oleate, 4.1% methyl linoleate, and 0.4% methyl linolenate (Fig. 8b).

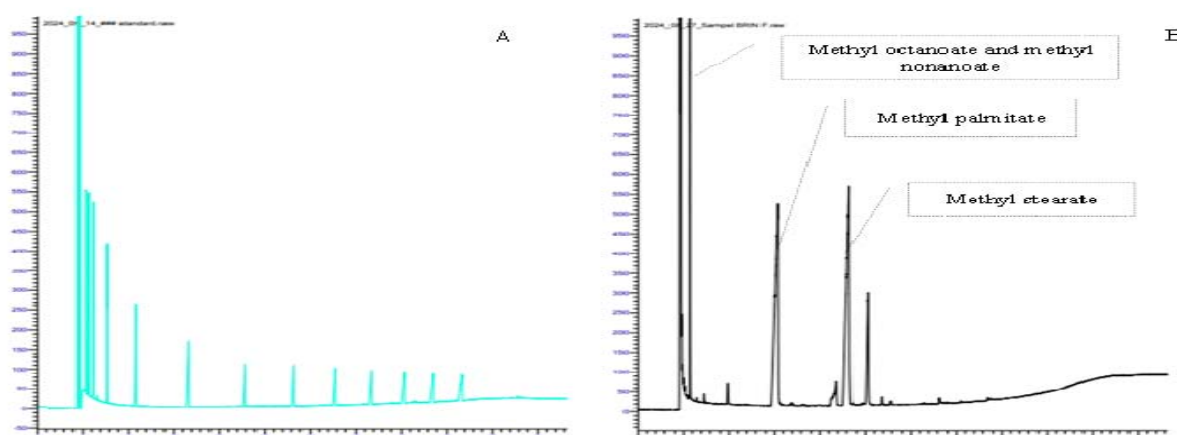


Figure 8. Chromatogram of FAME standard (A), Enzymatic biodiesel synthesis (B)

The total FAME conversion from WCO was 68.8%, which fell short of the required standards for FAME content. According to the EN 14103 (GC) testing method, a minimum FAME content from the transesterification reaction should be 96.5% (m/m) (Husar *et al.* 2021). *B. subtilis* DB104 lipase showed both hydrolysis and transesterification activities. Biodiesel production through lipase-catalyzed transesterification is affected by several key factors, such as the ratio of WCO to methanol, lipase purity, lipase volume and activity, and the resulting biodiesel conversion efficiency.

Theoretically, oil-to-alcohol molar ratio in the transesterification reaction is 1:3, although this ratio varies based on the type of oil and alcohol used (Susanty *et al.* 2013). Enzymatic transesterification using *Pseudomonas fluorescens* lipase was performed with oil and methanol molar ratios of 1:3, 1:6, and 1:9. The highest FAME percentage (4.55%) was achieved with a molar ratio of 1:6. Methanol was reduced during the reaction due to evaporation at a 1:3 ratio, while at a 1:9 ratio, lipase enzyme inhibition occurred. Enzymatic transesterification using lipase extracted from *Leonotis nepetifolia* (Christmas candlestick) seeds was successfully performed at a molar ratio of 1:3 for oil and methanol (Vazquez *et al.*, 2023).

Even though FAME conversion using lipase extracted from *B. subtilis* DB104 was below the minimum requirement for biodiesel production, there is a room for the improvement since this study is still in the optimization of cultivation stage. The mineral salt medium (MSM) used with WCO as sole carbon source showed that lipase can be produced at minimal and cost-effective conditions.

4. Conclusion

The production of lipase by *B. subtilis* DB104 reached its optimum of 8.97 U/mL activity when the initial medium had pH 7.0 and contained 0.5% of WCO and 15 mM of NH_4NO_3 as the most effective carbon and nitrogen sources, respectively. The optimal activity of partial purified lipase was observed at pH 7 – 9, and a temperature of 37 °C. An enhancement of enzyme activity was shown with the addition of CaCl_2 Tween 80, Tween 20, and Triton X-100. Meanwhile, treatment with EDTA, β -mercaptoethanol, and PMSF, and organic solvent such as isopropanol, DMSO, n-hexane, and methanol showed a stable activity, indicating high stability and robustness for industrial applications. The lipase from *B. subtilis* DB104 exhibited a molecular weight of 33 – 35 kDa and effectively catalyzed transesterification reaction, resulting in a 68.8% biodiesel yield.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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