VOLUME 13 NOMOR 1 JUNE 2026 ISSN 2548 – 611X

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejournal.brin.go.id/JBBI/index

THERMOHALOPHILIC BACTERIA PRODUCING THERMOSTABLE LIPASE FROM WAWOLESEA HOT SPRING, SOUTHEAST SULAWESI

JUDUL BAHASA INDONESIA

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ABSTRACT

This study aimed to isolate and characterize thermohalophilic bacteria producing lipase and to purify thermostable lipase from the Wawolesea hot spring in Southeast Sulawesi. A total of 24 isolates were obtained, all of which demonstrated lipase-producing capability. Three selected isolates (BT2.M3, CT2.M4, and DT1.M2) were identified as Gram-negative bacilli and showed lipase activities ranging from 0.17 to 21 U/mL, with the highest activity recorded for BT2.M3 at 50°C and pH 6. Purification through ammonium sulfate fractionation yielded the highest activity in the 60–80% saturation fraction (43 U/mL) at pH 6–7 and 50–60°C. Protein analysis showed the highest concentration in the 80% dialysis fraction (0.954 mg/mL). These findings highlight the potential of Wawolesea thermohalophilic bacteria as a valuable source of thermostable lipase for biotechnological applications.

Keywords: *Ammonium sulfate fractionation, Hotspring, Lipase, Thermohalophilic bacteria, Thermostable enzyme*

ABSTRAK

Penelitian ini bertujuan untuk mengisolasi dan mengkarakterisasi bakteri termohalofilik penghasil lipase serta memurnikan lipase termostabil yang berasal dari sumber air panas Wawolesea, Sulawesi Tenggara. Sebanyak 24 isolat berhasil diperoleh, dan seluruhnya menunjukkan kemampuan menghasilkan enzim lipase. Tiga isolat terpilih (BT2.M3, CT2.M4, dan DT1.M2) teridentifikasi sebagai bakteri berbentuk basil Gram-negatif dan menunjukkan aktivitas lipase berkisar antara 0,17 hingga 21 U/mL, dengan aktivitas tertinggi diperoleh pada isolat BT2.M3 pada suhu 50°C dan pH 6. Pemurnian melalui fraksinasi amonium sulfat menghasilkan aktivitas lipase tertinggi pada fraksi kejenuhan 60–80% (43 U/mL) pada pH 6–7 dan suhu 50–60°C. Analisis protein menunjukkan konsentrasi protein tertinggi pada fraksi hasil dialisis dengan kejenuhan amonium sulfat 80% (0,954 mg/mL). Temuan ini menunjukkan bahwa bakteri termohalofilik dari sumber air panas Wawolesea memiliki potensi sebagai sumber lipase termostabil yang bernilai untuk berbagai aplikasi bioteknologi.

Kata kunci: *fraksinasi amonium sulfat, sumber air panas, lipase, bakteri termohalofilik, enzim termostabil*

***Received**: 18 January 2026* ***Accepted**: 05 May 2026* ***Published**: 26 July 2026*

INTRODUCTION

Indonesia is one of the countries with the largest geothermal energy potential in the world, accounting for approximately 40% of the global total, or 29,000–29,544 MW, distributed across 252 locations in 26 provinces (White & Brannock, 1950; Schimel, 2007). However, the utilization of geothermal potential remains limited despite its abundance, particularly in volcanic regions with high-temperature ecosystems (Stetter, 2013; Cavicchioli, 2016). Among regions with geothermal activity, Southeast Sulawesi is known to have several hot springs, including Wawolesea.

The Wawolesea hot spring is located approximately 84 km from Kendari, the capital city of Southeast Sulawesi. It has temperatures ranging from 30.2 °C to 65.5 °C, a neutral pH, and relatively high salinity. Based on its geochemical characteristics, Wawolesea can be classified as mature water, reflecting extensive interaction and mixing with geological components, which in turn influences microbial colonization in geothermal systems (White & Brannock, 1950; Schimel, 2007). These environmental conditions make the hot spring an ideal habitat for extremophilic microorganisms, particularly thermohalophilic bacteria.

Thermohalophilic bacteria are microorganisms capable of growing optimally at high temperatures (≥45 °C) and salinity levels of 2%–30% (Jamaluddin et al., 2018; Sholeha & Agustini, 2021). These bacteria are known to produce various secondary metabolites with potential applications as antimicrobial, antitumor, antiparasitic agents, and raw materials for industrial chemicals (Gurumurthy et al., 2020). One of the most important extracellular enzymes produced by thermohalophilic bacteria is lipase, which catalyzes the hydrolysis of lipids into fatty acids and glycerol (Sharma et al., 2001; Jaeger & Eggert, 2002).

Lipase plays a crucial role in multiple industrial sectors, including food processing, pharmaceuticals, biodiesel production, and waste treatment. Its advantages include stability under diverse environmental conditions and the ability to catalyze various biochemical reactions such as esterification, interesterification, and transesterification (Fox et al., 2004). For industrial applications, however, lipases must possess high activity and strong thermal stability. Therefore, purification of the enzyme is essential to increase its specific activity and to obtain lipase with a higher degree of purity.

Previous research has reported the presence of thermohalophilic bacterial activity in the Wawolesea hot spring (Jamaluddin et al., 2018; Muzuni et al., 2024). However, comprehensive studies on the isolation, characterization, and purification of thermostable lipase from these bacteria are still lacking. Therefore, this study aims to isolate and characterize thermohalophilic bacteria, produce and purify thermostable lipase, and determine the optimum conditions for its enzymatic activity.

MATERIALS AND METHODS

Equipment

The equipment used in this study included: Petri dishes (Pyrex®), laminar flow cabinet (NuAire/BioBase), sterile inoculating loops, test tubes (Pyrex®), centrifuge, freezer, Erlenmeyer flasks (Pyrex®), hot plate, spatulas, glass funnels, glass rods, magnetic stirrer, graduated cylinders (Pyrex®), refractometer (Brix 0–20%), beakers (Pyrex®), pH meter and pH indicator paper, thermos bottles, analytical balance (Explorer Ohaus®), digital thermometer (−50–300°C), vortex mixer, micropipettes and tips, microtubes, UV transilluminator, incubator, Bunsen burner, aluminum foil, parafilm/plastic wrap, burettes (Pyrex®), ring stands, light microscope (Leica DM750), double-beam UV–Vis spectrophotometer (HITACHI U-2900 or equivalent), shaker incubator/water bath, ultrafiltration cartridges (Amicon, when needed), dialysis tubing (cellophane), and autoclave (WiseClave®).

Reagents and Materials

Samples and reagents included: hot spring water from Wawolesea (Southeast Sulawesi), distilled water (ddH_2O/aquadest), acetone, ethanol, cotton, gauze, rhodamine B, NaCl, tryptone, yeast extract, Nutrient Agar (NA, HiMedia/Oxoid), Luria–Bertani (LB) broth, polyvinyl alcohol (PVA, Merck), olive oil, beef tallow (for lipid substrates), phosphate buffer

components (KH_2PO_4/NaOH), NaOH for titration, phenolphthalein indicator, ammonium sulfate [$(NH_4)_2SO_4$], Folin–Ciocalteu reagent, $CuSO_4 \cdot 5H_2O$, bovine serum albumin (BSA) for protein standard, and other analytical reagents.

Sample collection and environmental measurements

Hot spring water samples (1,000 mL each) were collected from Wawolesea at approximately 50 cm depth into sterile bottles. Temperature, pH, and salinity were measured in situ prior to sampling. Samples were transported on ice to the laboratory and processed immediately or stored at 4 °C for short periods.

Media preparation

- **LB–Rhodamine B–Olive Oil agar (modified):** per liter — NaCl 14 g, tryptone 10 g, yeast extract 5 g; add 30 mL olive oil emulsified with 100 mL of 2% PVA; add rhodamine B as described. Pour into Petri dishes after sterilization.
- **Nutrient Agar (NA):** 3 g NA per 150 mL distilled water; autoclave at 121 °C, 15 psi, 15 min.
- **Nutrient Broth (NB):** 1.3 g NB per 100 mL distilled water; autoclave at 121 °C, 15 psi, 15 min.
- **Production medium:** NB supplemented with 10% (v/v) olive oil and mineral salts ($MgSO_4 \cdot 7H_2O$, K_2HPO_4, Na_2CO_3, NaCl as indicated in the protocol).

Isolation and purification of bacterial isolates

Filtered or unfiltered water samples were serially diluted, and 100 µL aliquots were spread onto LB–Rhodamine B–olive oil agar. Plates were incubated at temperatures corresponding to the sample source for 48 h. Lipase-producing colonies were identified by orange fluorescence under UV light and sub-cultured by streaking to obtain pure colonies (three successive streaks). Pure isolates were stored as NA slants for further study.

Microscopic characterization and Gram staining

Pure cultures were Gram-stained (crystal violet → Lugol's iodine → ethanol decolorizer → safranin counterstain) and observed under oil immersion (100×, 1.25 NA) to determine Gram reaction and cell morphology.

Production of crude lipase

Starter cultures (2–3 sterile loops) were inoculated into 90–500 mL NB containing 10% olive oil and incubated on a shaker at an appropriate temperature (e.g., 50 °C) at 200 rpm. Samples were taken periodically to construct production curves; the incubation optimum was determined by sampling every 3 h for 48 h. After incubation, cultures were centrifuged at 5,000 rpm for 15 min, and the supernatant was collected as crude enzyme extract.

Enzyme purification: ammonium sulfate fractionation and dialysis

Crude supernatant was fractionally precipitated by stepwise addition of solid ammonium sulfate to obtain saturation fractions: 0–20%, 20–40%, 40–60%, 60–80% and 80–100%. Ammonium sulfate was added slowly with stirring (~20 min) to avoid foaming. Precipitates were collected by centrifugation (5,000 rpm, 15 min), resuspended in 0.1 M phosphate buffer, pH 6.0, and dialyzed against the same buffer at 4 °C with several buffer changes until no sulfate remained (tested by addition of $BaCl_2$). Dialyzed fractions were assayed for lipase activity and protein concentration.

Enzyme activity assay (titrimetric method)

Lipase activity was measured by titration of free fatty acids released from olive oil: the reaction mixture contained 2 mL olive oil, 1 mL enzyme solution, and 4 mL phosphate buffer (pH 6–8 as required). Incubate for 30 min at specified temperatures (e.g., 50, 60, 70 °C). Stop the reaction with 10 mL acetone:ethanol (1:1) and titrate with 0.05 M NaOH using phenolphthalein until a persistent pink endpoint. A blank control containing all reaction components except the

enzyme solution was prepared to correct for non-enzymatic hydrolysis of the substrate. Additionally, negative controls consisting of heat-denatured enzyme were included to ensure that fatty acid release originated from enzymatic activity. Activity is calculated as:

$$Lipase\ Activity\ (U\ mL^{-1}) = \frac{(A-B) \times N \times 1000}{V_E \times t}$$

Where:
A = volume of NaOH for sample (mL)
B = volume of NaOH for blank (mL)
N = normality of NaOH
VE = enzyme volume (mL)
t = reaction time (min)

Protein determination (Lowry method)

Protein concentration was determined by the Lowry method using reagents prepared as follows: Reagent A (0.1 N NaOH with Na_2CO_3), Reagent B ($CuSO_4 \cdot 5H_2O$ with Na–K tartrate), Reagent C (mix of A and B), and diluted Folin–Ciocalteu reagent. Aliquots (0.1 mL) of the sample were reacted with the reagents, and the absorbance was measured at 540 nm after 30 min. BSA was used to construct the standard curve (Y = A·X + B) for concentration calculation. Specific activity (U mG^{-1} protein) was calculated for purified fractions.

Characterization of purified lipase

Optimum pH and temperature were determined by assaying activity across pH 6–8 and temperatures 50–70 °C. Thermal stability was assessed by pre-incubating the enzyme at selected temperatures and measuring residual activity.

The overall experimental design of this study consisted of several sequential steps, including sampling, bacterial isolation, screening of lipase-producing isolates, enzyme production, purification, and biochemical characterization. The general workflow of the experimental procedures performed in this study is illustrated in Figure 1.

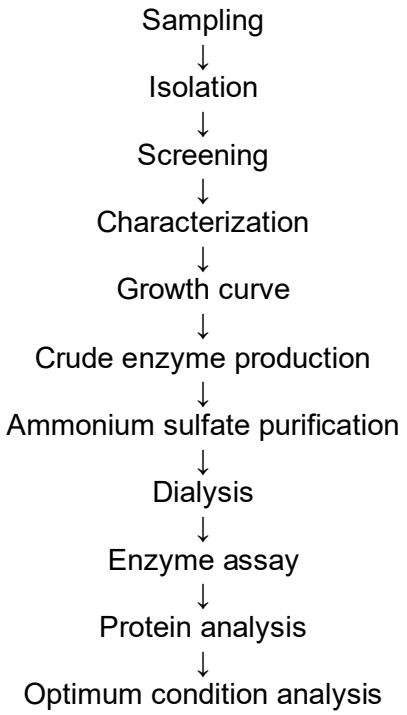

Figure 1. Experimental workflow of the isolation, screening, purification, and characterization of thermostable lipase-producing thermohalophilic bacteria from the Wawolesea hot spring

Statistical Analysis

All experiments were performed in triplicate and the results are expressed as mean ± standard deviation. Statistical significance between treatments was evaluated using one-way analysis of variance (ANOVA) at a confidence level of $p < 0.05$.

RESULTS AND DISCUSSION

Environmental Conditions of Wawolesea Hot Spring

The Wawolesea hot spring exhibited environmental variations conducive to the growth of thermohalophilic bacteria. Water temperature ranged from 41–53 °C, salinity from 15–16‰, pH from 6.40–6.77, and air humidity from 70–74%. These variations form ecological niches that allow thermophilic and halophilic microorganisms to thrive (Schimel, 2007). Temperatures above 45 °C support thermophilic growth due to the ability of their membranes and proteins to maintain structural stability under heat stress (Feller et al., 2012; Stetter, 2013).

A salinity level of 15–16‰ (1.5–1.6% NaCl) indicates selection toward slight halophiles (Oren, 2015). Halophiles maintain osmotic pressure by accumulating potassium ions, glycerol, or other compatible solutes, allowing them to survive in hypertonic environments (Ventosa et al., 2015). The pH of 6.4–6.7 corresponds to conditions favorable for neutrophilic bacteria, the dominant group in geothermal aquatic ecosystems (Madigan et al., 2018).

Table 1. Environmental parameters (water temperature, salinity, and pH) at sampling zones

Zone	Point	Water Temp (°C)	Salinity (‰)	pH
A	1	53	16	6.72
	2	52	16	6.77
B	1	50	15	6.51
	2	49	15	6.48
C	1	41	15	6.40
	2	50	15	6.45
D	1	50	15	6.48
	2	48	15	6.49

Overall, the combination of high temperature + moderate salinity + neutral pH indicates that bacteria inhabiting this area likely belong to thermohalophilic groups, consistent with other geothermal systems (Cavicchioli, 2016).

Isolation of Thermohalophilic Bacteria

A total of 32 isolates were obtained from four sampling zones (A–D). The relatively consistent number of isolates in each zone suggests that all hot spring pools provide nutrients and physical conditions capable of supporting microbial colonization. High concentrations of Ca^{2+}, Mg^{2+}, and silicates in geothermal water also support lithotrophic and heterotrophic microorganisms (White & Brannock, 1950).

Zone A produced no lipase-producing isolates, likely due to its higher salinity (16‰) and more open conditions with limited organic matter. Lipid availability is crucial for inducing lipase gene expression (Jaeger & Eggert, 2002), and the scarcity of lipid substrates in Zone A may limit the growth of lipolytic bacteria. BT2.M3, CT2.M4, and DT1.M2 showed the strongest, bright orange fluorescence radiating symmetrically around the colonies.

Table 2. Selected thermo-halophilic isolates showing strong lipolytic activity

Isolate	Zone	Sampling Point	Lipolytic Activity
BT1.M3	B	1	++
BT1.M4	B	1	++
BT2.M3	B	2	+++
CT2.M4	C	2	+++
DT1.M2	D	1	+++

Note:

- (+++) = strong lipolytic activity
- (++) = moderate lipolytic activity

Screening of Thermostable Lipase-Producing Isolates

Screening on LB Rhodamine B–Olive Oil agar identified 24 isolates with varying lipolytic activity. Orange fluorescence under UV light indicates the formation of a Rhodamine B–free fatty acid complex after lipase-mediated hydrolysis of triglycerides (Kouker & Jaeger, 1987; Gupta et al., 2004). Three isolates displayed the highest activity: BT2.M3, CT2.M4, DT1.M2.

Fluorescence intensity corresponded to lipase productivity, reflecting differences in enzymatic expression, catalytic efficiency, and tolerance to thermal–salinity stress (Sharma et al., 2017).

Microscopic Characterization

Gram staining revealed that all three isolates were Gram-negative bacilli, characterized by thin peptidoglycan layers and an outer lipopolysaccharide membrane (Radji, 2010). BT2.M3 and CT2.M4 appeared as long bacilli, whereas DT1.M2 appeared as short bacilli. These differences could be influenced by microhabitat variability, growth phase, or genetic factors (Madigan et al., 2018). Notably, many industrially relevant lipases originate from Gram-negative bacteria, especially Pseudomonas and Burkholderia (Salwoom et al., 2019).

Growth Curve Analysis

The growth curve analysis showed: BT2.M3 – exponential phase at 18 hours, CT2.M4 – exponential phase at 21 hours, DT1.M2 – exponential phase at 24 hours.

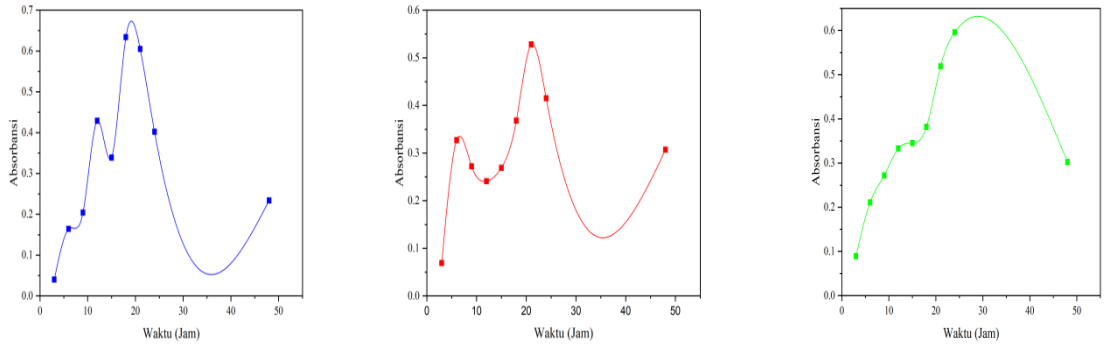

Figure 1. Growth curve analysis of thermohalophilic bacterial isolates.

This variation reflects differences in metabolic efficiency, nutrient utilization, and adaptation to thermal–salinity stress (Purkan et al., 2020). The exponential phase is the most productive period for enzyme synthesis, including lipase.

Lipase Activity and Optimum Conditions

All isolates showed maximum lipase activity at:50 °C, pH 6. Maximum activities were recorded as:BT2.M3: 21 U mL^{-1}, CT2.M4: 19.5 U mL^{-1}, DT1.M2: 17 U mL^{-1}.

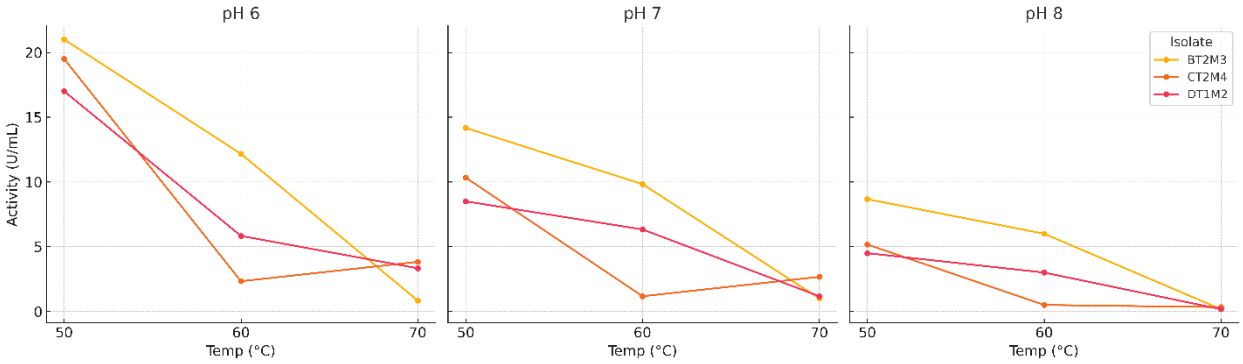

Figure 2. Lipase activity profiles of bacterial isolates at different temperatures

Activity decreased at 60 °C and dropped sharply at 70 °C. This trend reflects typical thermostable lipase behavior, which generally peaks at 45–60 °C (Gaur et al., 2016; Contesini et al., 2020). The decline at higher temperatures is associated with partial denaturation, disruption of active-site geometry, and loss of tertiary structure integrity (Bhardwaj et al., 2020).

Purification by Ammonium Sulfate Fractionation and Dialysis

Stepwise ammonium sulfate precipitation (0–100%) demonstrated highest activity in the fraction:60–80% saturation, yielding CT2.M4: 43 U mL^{-1}, BT2.M3: 29 U mL^{-1}, DT1.M2: 35 U mL^{-1}

Table 4. Summary. Maximum lipase activity of thermo-halophilic isolates at different ammonium sulfate fractions

Fraction (%)	CT2.M4 (U mL^{-1})	BT2.M3 (U mL^{-1})	DT1.M2 (U mL^{-1})
0–20%	**26.3**	**13.3**	**17.5**
20–40%	**35.0**	**20.0**	**30.0**
40–60%	**30.0**	**18.0**	**24.3**
60–80%	**43.0**	**29.0**	**35.0**
80–100%	**27.3**	**15.2**	**18.5**

This suggests that the lipase is hydrophilic, requiring higher ionic strength to precipitate (Neale et al., 1987). Lipases from thermophilic bacteria commonly precipitate at 50–80% saturation (Chatelet et al., 2022). Dialysis removed residual salts, indicated by the absence of $BaSO_4$ precipitate after $BaCl_2$ addition. Removing ammonium sulfate is essential as excess ions can inhibit substrate binding and catalytic efficiency.

Protein Content and Specific Activity

Highest protein concentrations were detected in the 80% fraction (0.954 mg mL^{-1}), while the lowest occurred in the 0–20% fraction.

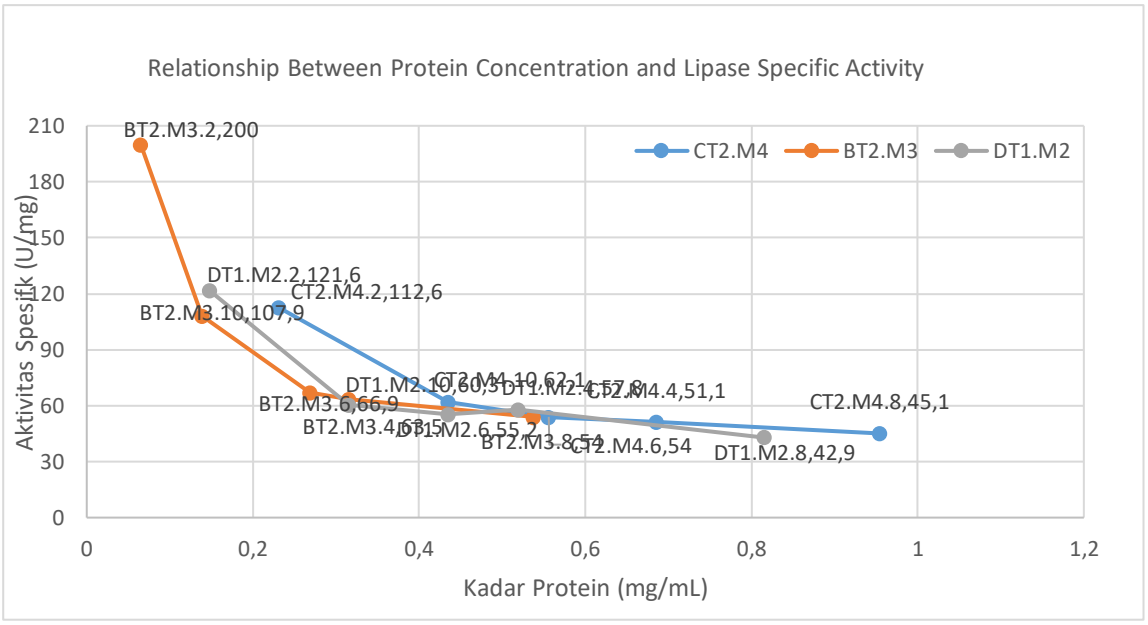

Figure 3. Protein content and specific activity of partially purified lipase fractions

Specific activity was highest in: BT2.M3: 200 U mG^{-1}, CT2.M4: 112.6 U mG^{-1}, DT1.M2: 121.6 U mG^{-1}. The decrease in specific activity at higher total protein levels suggests substantial contamination by non-enzymatic proteins within the fraction. Since specific activity reflects enzyme purity, it serves as a primary indicator of successful enzyme purification rather than total protein concentration (Neale et al., 1987).

Consequently, high catalytic activity cannot be directly inferred from high protein content alone, and further purification such as gel filtration may improve lipase purity and catalytic efficiency.

CONCLUSION

1. The Wawolesea hot spring environment supports thermohalophilic bacterial communities.
2. Three isolates (BT2.M3, CT2.M4, DT1.M2) displayed the strongest lipolytic activity and are Gram-negative bacilli.
3. Lipase activity was optimal at 50°C and pH 6, with BT2.M3 being the best performer.
4. Purification using ammonium sulfate revealed 60–80% saturation as the optimum fraction, indicating hydrophilic lipase characteristics.
5. Specific activities increased at lower protein concentrations, highlighting successful preliminary purification.

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