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Characterisation of novel acidic lipase of *Corynebacterium nuruki* PM2 from palm oil mill effluent for palm oil derivatisation

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Abstract

The palm oil industry generates significant amounts of palm oil mill effluent (POME), which harbors diverse microbial communities with potential biotechnological applications. Yet, only a limited number of lipolytic bacteria have been isolated and studied. Limited nutrient medium selection was previously demonstrated for isolating unique indigenous bacteria. This study aimed to isolate novel lipolytic bacteria from POME and characterize the lipase for potential palm oil derivatization. *Corynebacterium nuruki* PM2 was successfully isolated and identified. Distinct colony morphologies were observed on different agar media. Lipase from *C. nuruki* PM2 (LipCN) exhibited optimum activity at 40-60°C, pH 5.0-8.0, and with vegetable oil as substrate. The enzyme also showed the highest stability at a temperature between 30-40°C and pH 7.0-8.0 after 30 min of incubation. The enzyme remained stable in methanol, isopropanol, and *n*-hexane. Metal ions such as Mn^{2+} and surfactants (e.g., Triton X-100, Tween-80, and Tween-20 significantly inhibit LipCN activity. Thin-layer chromatography identified 2-palmitoylglycerol in the hydrolyzed product of LipCN, suggesting *sn*-1,3 specific activity of the enzyme.

[Keywords: biocatalysis, enzymatic, microbial lipase, transesterification, palm oil residue]

Introduction

The palm oil industry has seen a significant trend in valorizing its side-products for high-value downstream applications, particularly in the derivatization of palm-based compounds (Chandra et al., 2020). Among these efforts, there has been growing interest in isolating enzymes from palm oil by-products to support biotransformation and industrial processing. Among various enzymes explored, lipase has emerged as one of the most promising due to its broad applicability, including biodiesel production, human milk fat substitute synthesis, as well as margarine and shortening production (Hasibuan et al., 2021; Salas et al., 2024; Suwanno et al., 2017; Zhang et al., 2024).

This wide applicability arises from the unique characteristics of lipase. The degumming process requires lipases capable of hydrolyzing phospholipids specifically under pH 6–8 and temperature 20–40°C (Bajpai et al., 2023). In contrast, fractionation requires lipases that perform well at low temperatures to prevent crystallization and degradation while also exhibiting selectivity for specific fatty acid chains (Salas et al., 2024). Similarly, biodiesel production from POME benefits from lipases with broad substrate specificity, high thermal stability, metal-inhibitor tolerance, and optimal activity at neutral to basic pH (Chandra et al., 2020; Suwanno et al., 2017). Meanwhile, for margarine and shortening production, lipases with high thermal and oxidative stability and regioselectivity to avoid trans-fat formation are crucial (Zhang et al., 2024). Finally, human milk fat substitute production relies on *sn*-2 regioselective lipases to optimise palmitic acid content in the *sn*-2 position (Hasibuan et al., 2021; Li et al., 2021)

A key source of microbial lipase with untapped potential is POME—a nutrient-rich waste stream generated in large volumes during palm oil processing. POME is known to harbor a highly diverse microbial community, particularly bacteria, which makes it an ideal reservoir for novel enzyme discovery (Karno et al., 2024; Periadnadi et al., 2024). However, despite high microbial diversity in POME, only a limited number of lipolytic bacteria have been successfully isolated and studied to date (Ibiyemi & Julius, 2022; Nwuche & Ogbonna, 2011; Okwute et al., 2015). This limited exploration, therefore, poses a challenge for the discovery of novel lipases with unique characteristics and broader industrial relevance. To overcome this limitation, recent studies have demonstrated that nutrient-limited medium selection can enhance the

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isolation of functionally diverse and rare bacterial strains by mimicking competitive environmental conditions (Kamagata, 2015; Narihiro et al., 2014; Putra et al., 2019; Shi et al., 2024). This approach has been proven effective in recovering novel microbial species, as shown by previous successful isolations using such strategies.

In light of this, the objective of this research was to isolate and characterize the lipase enzyme from indigenous microbial populations in POME by employing a nutrient-limited medium as a selective pressure. This focus on bacterial lipases is relevant because they offer advantages over those derived from fungi or plants, including broader substrate specificity, higher thermal and pH stability, and more straightforward genetic modifications (Fatima et al., 2019; Santos et al., 2022). Furthermore, the scale-up of bacterial lipase production is easier with genetic engineering, as bacteria have faster and more flexible growth cycles and simpler prokaryotic genetic systems than eukaryotic systems (Ali et al., 2023; Contesini et al., 2020). From an economic perspective, bacterial cultivation is less demanding, requiring simpler growth media compared to fungi or plants, which depend on complex media and seasonal factors (Ali et al., 2023).

Materials and Methods

Bacterial sources

POME was collected from the oil palm Cikasungka Plantation (PTPN 8), Bogor Regency, West Java. *Escherichia coli* BL21(DE3) was provided by the Indonesian Oil Palm Research Institute (IOPRI), Bogor, Indonesia.

Lipolytic bacteria isolation

A nutrient-limited medium was used for screening indigenous lipolytic bacteria (Ma et al., 2010). One milliliter of well-mixed POME was inoculated onto nutrient-limited rhodamine agar plates, comprising 0.4% peptone, 0.1% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 12% olive oil emulsion with 2% polyvinyl alcohol (PVA), 0.1% rhodamine B, and 2% agar. Plates were incubated at 37°C for 48 h. Unique lipolytic bacteria, indicated by a clear zone generated around the colonies, were chosen and re-inoculated on rhodamine agar containing 8% Luria Bertani (LB) powder (HiMedia Laboratories), 12% olive oil (Filippo Berio) emulsion with 2% PVA, 0.1% rhodamine B, and 2% agar.

Lipolytic Index (LI)

Pre-screening of lipase activity was performed on tributyrin agar by calculating the lipolytic index (LI) defined as the ratio between the diameter of the clear zone and that of the bacterial colony (Willerding et al., 2011). Selected lipolytic isolates were cultured on tributyrin agar comprising 8% LB agar and 1% tributyrin emulsion with 10% gum arabic, then incubated at 37°C for 72 h. Strains with the highest LI were chosen for bacteria identification and enzyme characterization.

Bacteria identification

Bacteria identification was carried out using morphological characterization and the 16S rRNA identification method. Morphological characterization included colony observation on rhodamine agar and LB agar (Ma et al., 2010). The 16S rRNA identification was conducted based on Marchesi et al. (1998). Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit, and sequencing was outsourced to 1st BASE, Apical Scientific Sdn. Bhd., Malaysia. Full-length 16S rRNA sequences were reconstructed using pairwise alignment in BioEdit, and nucleotide BLAST (BLASTn) was conducted to determine the similarity of the isolate's 16S rRNA gene to sequences in the NCBI database.

Bacterial growth curve

The growth curve of the selected isolate was observed with *Bacillus cereus* PM3 (hereafter referred to as isolate PM3) and *E. coli* BL21(DE3) used as a comparison. A single colony of each bacterium is inoculated into 50 mL LB broth and incubated at 37°C until its OD_{600} reaches 0.6-0.8. One milliliter of the culture is transferred into a new 100 mL LB broth containing 5% olive oil and incubated at 37°C. 100 µL culture is taken at 1, 3, 6, 9, 24, 48, and 72 h and transferred into LB agar through serial dilution (up to 10^9). Colony-forming unit (CFU) is defined by conducting a total plate count on the grown colony.

Crude protein extraction

Crude protein extracts were prepared from the selected isolate. Seed culture was initiated by inoculating a single colony into 10 mL of LB medium and incubating at 37°C for 16 h. A main culture was then prepared by transferring 1% of the seed culture into 50 mL of liquid LB medium containing 5% olive oil, followed by incubation at 37°C for 24 h. The culture was centrifuged at a high speed at 4°C for 15 min. The pellet was collected and ground into a powder using liquid nitrogen. The powder was resuspended in lysis buffer containing 35 mL Tris-HCl (pH 8.5), 100 mM NaCl, 1 mL of 250 mM PMSF, and 4 mL of β-mercaptoethanol. The mixture was vortexed for 5 min at 4°C and centrifuged at 11,000 rpm at 4°C for 30 min. The

supernatant was collected and stored at a temperature of 4°C or -20°C.

Lipase assay

Lipase activity was measured using a modified titration method (Xing et al., 2021). A reaction mixture of 9 mL containing 4 mL substrate (1% olive oil), 4.5 mL Tris-HCl buffer (pH 8) and 0.5 mL $CaCl_2$, pre-incubated at 37°C for 5 min. Subsequently, 1 mL of the crude protein extract was added, and the reaction was incubated for 15 min. The reaction was terminated by adding 1 mL of absolute ethanol. Phenolphthalein indicator (1%) was added (2–3 drops), and the mixture was titrated with 0.5 N NaOH until a subtle pink color was observed. One unit of lipase activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per min under the assay conditions.

Lipase characterisation

Lipase activities in varying temperatures, pH, substrates, organic solvents, metal ions, and surfactants were evaluated using the lipase assay described above. Optimal temperature was determined within the range of 30-80°C. Thermostability was determined by pre-incubating the protein within the same temperature range for 30 min at pH 8.0, before conducting the assay at the optimal temperature.

Optimal pH was determined within the range of 3.0 to 9.0. The enzyme stability at different pH levels was determined by pre-incubating the protein within the same pH range for 30 min before conducting the assay at the optimal pH. Buffer systems included 0.1 M citrate buffer (pH 3.0–5.0), phosphate buffer (pH 5.0–7.0), Tris-HCl buffer (pH 7.0–9.0), and glycine-NaOH buffer (pH 9.0–10.0). Substrate specificity was evaluated with olive oil, vegetable oil, and tributyrin (emulsified with 1% gum arabic).

The effect of organic solvents, metal ions, and surfactants was evaluated by incubating the protein at the optimal temperature and pH for 30 min in the presence of the following substances: 10% organic solvent (methanol, ethanol, butanol, isopropanol, acetonitrile, or *n*-hexane); 1 mM metal ion ($CaCl_2$, $MgCl_2$, $ZnCl_2$, $MnSO_4 \cdot H_2O$, $FeCl_3 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, or $CoCl_2 \cdot 6H_2O$); and 1% surfactant (dimethyl sulfoxide (DMSO), Triton X-100, Tween-80, Tween-20, or sodium dodecylsulfonate (SDS)). As a control, the protein was incubated at the same temperature and pH for 30 min without the presence of any substances mentioned above.

Relative activity (%) was calculated by normalizing the activity under each condition to the highest activity observed within that parameter, which was defined as 100%. Residual activity (%) was calculated by comparing the activity after pre-incubation at each condition to the activity measured at the optimum condition (set as 100%).

Synthesis of 2-palmitoylglycerol

Synthesis of 2-palmitoylglycerol was conducted to determine the *sn*-1,3 regioselectivity of LipCN. The measurement was determined using thin-layer chromatography (TLC) (Duan et al., 2019). The mixture of CPO (0.3%) and lipase (0.1mL) was dissolved in 4 mL Tris-HCl buffer (20 mM, pH 7) and incubated at 30°C and 150 rpm for 1 hour. The reaction was extracted using 2 mL of petroleum benzene. The upper phase was collected and spotted onto a prepared TLC plate along with the standards (palm oil and 2-palmitoylglycerol). The plate was placed into a pre-saturated eluent chamber and allowed to develop until it reached the predetermined solvent front. The plate was dried and placed in an iodine chamber for spot visualization. The retention factor (Rf) for each spot is measured by the ratio of the distance traveled by the compound to the distance traveled by the solvent.

Statistical analysis

Measurements of the lipolytic index, bacterial growth rate, protein concentration, specific enzyme activity, and enzymatic characterisations were performed in triplicate. Statistical difference of each enzyme characteristic was determined using one-way ANOVA with $\alpha = 0.05$. Optimum temperature, pH, and substrate were determined based on the statistical difference between the characteristic group with the highest activity and those below using Tukey's post hoc test. Enzyme stability in different temperatures, pH, organic solvents, metal ions, and surfactants was determined based on the statistical difference between control and characteristic groups using Tukey's post hoc test.

Results and Discussion

Lipolytic bacteria isolation and identification

Four distinct lipolytic bacterial isolates were successfully obtained from POME and designated as PM1, PM2, PM3, and PM4. Among these isolates, PM2 exhibited the highest lipolytic index of 1.83 ± 0.18, followed by PM1 (1.68 ± 0.21), PM3 (1.49 ± 0.04), and PM4 (1.27 ± 0.14), respectively. Based on 16S rRNA gene analysis, isolate PM2 showed 98.72% sequence similarity to *C. nuruki* S6-4 (Accession: CP042429.1).

Notably, in this study, *C. nuruki* was isolated from POME for the first time, thereby demonstrating the effectiveness of the nutrient-limited medium. Previously, this species had only been isolated from nuruk (an alcohol fermentation

starter) and human skin. (Fernandez-Palacios et al., 2025; Shin et al., 2010). The colonies of *C. nuruki* PM2 appeared smooth, opaque, convex, slightly viscous, and cream-coloured after 48 h of incubation, which is consistent with the characteristics described by Shin et al. (2010). Furthermore, the lipolytic activity of *C. nuruki* PM2 was also observed for the first time, as evidenced by the formation of light red colonies on rhodamine agar and white colonies on LB agar. In addition, a clear zone was formed on tributyrin agar after 72 h of incubation (Figure 1).

Growth curve

The growth of *C. nuruki* PM2 was further investigated and compared with that of isolate PM3 and *E. coli* BL21(DE3) (Figure 2). Isolate PM3 was further included because it exhibited the highest growth rate among PM1 and PM4. Meanwhile, the *E. coli* BL21(DE3), a standard industrial strain for enzyme production, was used as the benchmark for industrial comparison (Marisch et al., 2013).

All strains followed a similar trend with an exponential phase up to 24 h, a stationary phase between 24-48 h, and a death phase past 48 h. However, *C. nuruki* PM2 exhibited a slower growth rate at the first 6 h, yet maintained a more sustained population after 48 h of growth compared to isolate PM3. The benchmark *E. coli* BL21(DE3) showed significantly higher growth than the other species. Overall, the stable population of *C. nuruki* PM2 after 48 h, comparable to the *E. coli* BL21(DE3), suggests its potential for continuous whole-cell production.

Figure 1. *C. nuruki* PM2 grown in different nutrient-limited media: LB (left), rhodamine agar (middle), and tributyrin agar (right). Clear zone can be observed on the tributyrin agar.

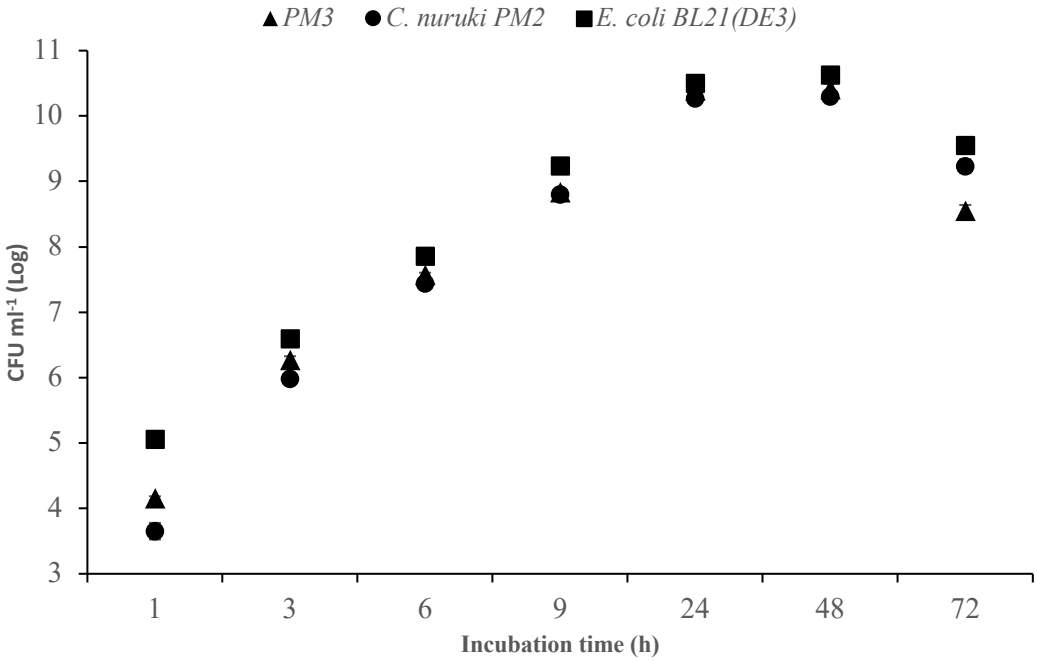

Figure 2. Growth curve of *C. nuruki* PM2, isolate PM3, and *E. coli* BL21(DE3)

Effect of temperature on LipCN activity

Lipase from *C. nuruki* PM2 (LipCN) was extracted and characterised. The optimum temperature and thermostability of LipCN were observed within the range of 30-80°C (Figure 3). ANOVA was conducted with $p < 0.05$, revealing a significant difference in enzyme activity at various temperatures. Furthermore, Tukey's post hoc test suggested thermophilic characteristics of LipCN with an optimal temperature between 40-60°C. In terms of thermostability, LipCN was stable at 30°C, with no significant difference from the control. Interestingly, lipase from *Corynebacterium acnes* showed similar characteristics with optimal temperature between 30-50°C (Pablo et al., 1974).

Effect of pH on LipCN activity

The optimum pH and pH stability of LipCN were observed within the range of pH 3.0-9.0 (Figure 4). ANOVA was conducted with $p < 0.05$, suggesting a significant difference in enzyme activity at various pH levels. According to Tukey's post hoc test, LipCN exhibited acidophilic characteristics with an optimal pH between 5.0-8.0. Regarding pH stability, the enzyme was stable between 7.0-8.0 with no significant difference between those ranges and the control. Similarly, lipase from *C. acnes* also showed similar characteristics with optimum pH between 7.5-8.0. Although the protein analysis is not covered in this study, thermostability and pH stability have been associated with the presence of disulfide bonds, where a higher number of such bonds enhances protein stability (Liu et al., 2016).

Effect of substrates on LipCN activity

Substrate specificity was observed towards different edible oils/fats, including olive oil, vegetable oil, and tributyrin (Figure 5). ANOVA was conducted with $p < 0.05$, showing a significant difference in enzyme activity at various pH levels. According to Tukey's post hoc test, there was no significant difference in enzyme activity between olive oil and palm oil as substrates, whereas both showed significantly higher activity than tributyrin. This indicates that LipCN has a stronger preference towards long-chain triglycerides (C_{16}-C_{18}) than short-chain triglycerides like tributyrin (C_4). Notably, POME contains edible oils as a natural environment of *C. nuruki* PM2, which may partly explain the substrate specificity of its enzyme (Suwanno et al., 2017). Additionally, LipCN may prefer a hydrophobic environment for its activity, thereby showing higher activity on edible oils than emulsified tributyrin.

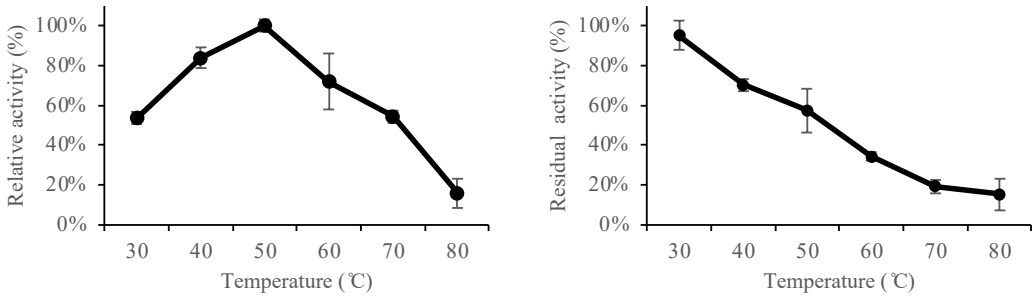

Figure 3. Effect of temperature on the activity and stability of LipCN. (Left) The optimum temperature was determined by assaying enzyme activity at different temperatures (30–80°C). Relative activity (%) was calculated by setting the maximum activity (0.89 U mg^{-1} at 50 °C) to 100 %. (Right) Enzyme residual activity (%) was normalized to the activity measured after incubation at 50°C (0.89 U mg^{-1}).

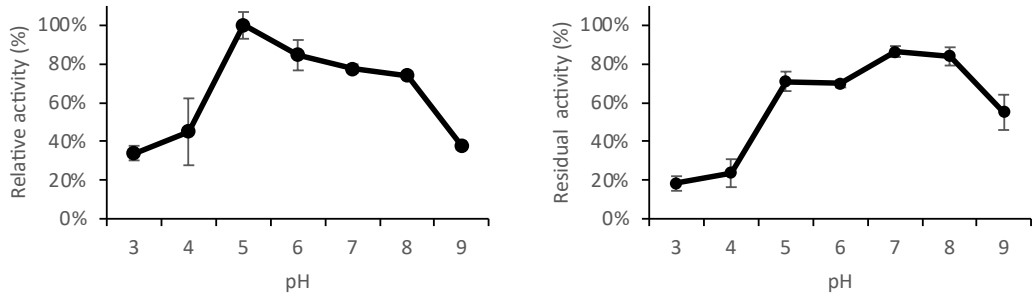

Figure 4. Effect of pH on the activity and stability of LipCN. (Left) The optimum pH was determined by measuring enzyme activity in different buffer systems (pH 3–9). Relative activity (%) was calculated by setting the highest observed activity (1.21 U mg^{-1} at pH 7.0) to 100%. (Right) Enzyme residual activity (%) was normalized to the activity measured after incubation at pH 7.0 (1.21 U mg^{-1}).

Effect of organic solvent on LipCN activity

The stability of LipCN was observed in the presence of organic solvents, including methanol, ethanol, 2-propanol, and *n*-hexane (Table 1). Statistical analysis indicated a significant difference in enzyme activity at various organic solvents. Overall, LipCN shows high stability towards methanol, 2-propanol, and *n*-hexane, while being less stable in the presence of ethanol. The trend can be explained by the polarity of organic solvents, which influences the lid region of the lipase catalytic site and thereby affects the enzyme activation (Kamat et al., 2013; Skjold-Jørgensen et al., 2015). In this report, LipCN showed a preference towards non-polar solvents such as *n*-hexane, which provides a more hydrophobic environment. Although methanol, ethanol, and 2-propanol are polar solvents, the molecular size and concentration of each solvent may also affect the enzyme stability (Wang et al., 2016). For instance, methanol's small size and 2-propanol's longer alkyl chain may reduce disruptive interaction with the enzyme structure. On the other hand, ethanol's intermediate size may be significant enough to disrupt hydrogen bonding, which leads to denaturation. While the current study does not cover the effect of solvent concentration, Kotogán et al. (2018) showed a higher denaturation effect of ethanol than other polar solvents at higher concentrations (>10%).

Effect of metal ions on LipCN activity

The stability of LipCN was observed in the presence of various metal ions (Table 2). Statistical analysis again revealed a significant difference in enzyme activity at various metal ions. According to Tukey's post hoc test, LipCN was inhibited in the presence of Mn^{2+}, while there was no significant effect from the other metal ions. Generally, metal ions can affect lipase stability by attaching to the active site (Pham et al., 2021). In this case, Mn^{2+} is likely to interact with a sensitive region of the catalytic residue, leading to either protein misfolding or denaturation.

Effect of surfactants on LipCN activity

The stability of LipCN was observed in the presence of surfactants, such as Triton X-100, Tween-20, Tween-80, and SDS (Table 3). The analysis revealed a significant difference in enzyme activity at various organic solvents. LipCN was more inhibited by Triton X-100, Tween-20, and Tween-80, while it is relatively stable in the presence of SDS. These findings suggest that the inhibiting surfactants may have a compatible structure with the active site of LipCN, thereby causing competitive inhibition with the actual substrate.

Synthesis of 2-palmitoylglycerol

The hydrolysis product of LipCN was further observed using TLC (Figure 6). Both extracellular and intracellular hydrolysates generated three spots with Rf = 0.11, 0.30, and 0.54, while palm oil (TAG standard) generated two spots at Rf = 0.13 and 0.19. The 2-palmitoylglycerol standard generated a single spot at Rf = 0.56. The presence of a band near this Rf value in the LipCN samples indicates that LipCN can synthesize *sn*-1,3 product, 2-palmitoylglycerol. However, further investigation is needed to determine whether the lipase is strictly *sn*-1,3 specific or non-specific.

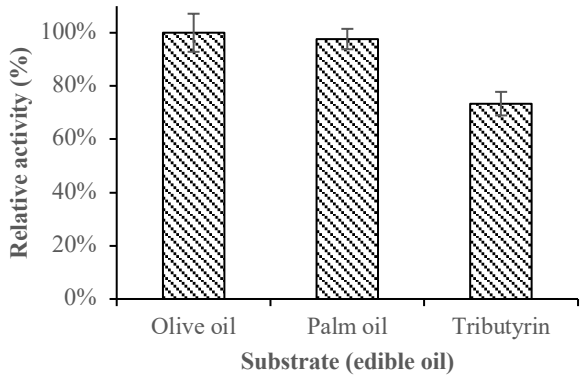

Figure 5. Effect of substrate types on LipCN activity. Enzyme activity was measured using olive oil, vegetable oil, and emulsified tributyrin as substrates. Relative activity (%) was calculated by setting the highest activity (1.21 U mg^{-1}) to 100%. Error bars indicate standard deviations (N = 3).

Table 1. Effect of different organic solvents on LipCN stability after 30 min of incubation at 30°C and pH 7.0. Residual activity (%) was calculated by setting the activity measured in the control (1.04 U mg^{-1}) to 100%. Values represent means ± standard deviations (N = 3).

Organic solvent	Residual activity (%)
Control	100 ± 3
Methanol	106 ± 8
Ethanol	25 ± 9
2-propanol	104 ± 6
n-hexane	126 ±11

Table 2. Effect of metal ions on LipCN stability after 30 min of incubation at 30°C and pH 7.0. Residual activity (%) was calculated by setting the activity measured in the control (1.04 U mg^{-1}) to 100%. Values represent means ± standard deviations (N = 3).

Metal ion	Residual activity (%)
Control	100 ± 3
$CaCl_2$	103 ± 8
$MgCl_2$	77 ±15
$ZnCl_2$	64 ±11
$MnSO_4 \cdot H_2O$	32 ± 9
$FeCl_3 \cdot 6H_2O$	73 ±17
$CuSO_4 \cdot 5H_2O$	80 ±10
$CoCl_2 \cdot 6H_2O$	85 ±10

Table 3. Effect of surfactants on LipCN stability after 30 min of incubation at 30°C and pH 7.0. Residual activity (%) was calculated by setting the activity measured in the control (1.04 U mg^{-1}) to 100%. Values represent means ± standard deviations (N = 3).

Surfactant	Residual activity (%)
Control	100 ± 3
Triton X-100	58 ± 4
Tween-20	26 ± 7
Tween-80	51 ±13
SDS	77 ± 6

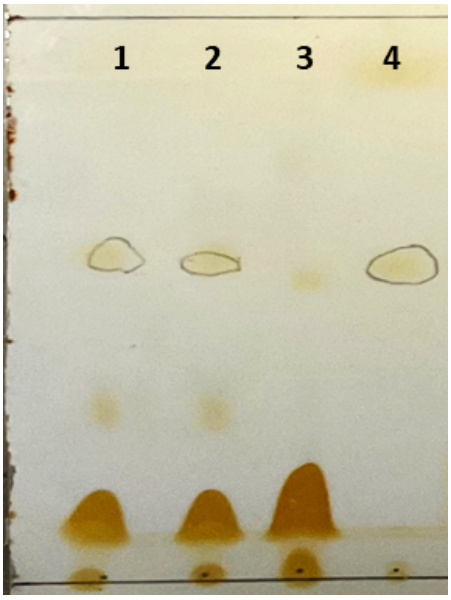

Figure 6. TLC analysis for the synthesis of 2-palmitoylglycerol by LipCN. Reaction mixtures containing CPO and (1) extracellular LipCN or (2) intracellular LipCN were incubated and analyzed by TLC together with (3) palm oil and (4) 2-palmitoylglycerol standards.

Conclusion

In this study, *C. nuruki* PM2 has been successfully isolated from POME for the first time, thereby demonstrating the potential of nutrient-limited medium as an effective method for novel lipase exploration. The findings indicate that the characteristics of LipCN suggest strong potential as a catalyst for biodiesel production from palm fatty acid distillate, with its high thermostability, acidic profile, and high stability in the presence of methanol. For future improvements, its specific activity and stability towards metal ions could be enhanced by immobilisation. In this regard, ionic liquid modification could be used as an immobilisation strategy, as it's proven effective to enhance not only enzyme stability and reusability, but also affinity towards metal ions (Xing et al., 2024). Additionally, heterologous expression of LipCN in *E.coli* BL21(DE3) could also be an alternative for higher enzyme activity or whole-cell production.

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