Menara Perkebunan 2025, 93(2), 148-157
p-ISSN: 0125-9318/ e-ISSN: 1858-3768
http://dx.doi.org/10.22302/iribb.jur.mp.v93i2.673
Accreditation Number: 177/E/KPT/2024

Characterization of *sn*-1,3 extracellular lipases of *Aspergillus niger* and *Rhizopus oryzae* for the crude palm oil hydrolysis

Ludwinardo PUTRA[1], Eneng NURHASANAH[2], , Eti ROHAETI[2], & Irma KRESNAWATY[1*]

[1)] Indonesia Oil Palm Research Institute – Bogor Unit, Jl. Taman Kencana No. 1, Bogor 16128, Indonesia
[2)] Department of Chemistry, IPB University − Jl. Tanjung Kampus, Dramaga, Bogor 16680, Indonesia

Received 25 Sep 2025/ Revised 11 Nov 2025/ Accepted 13 Nov 2025

Abstract

Hydrolysis of triacylglycerol-rich oils is a crucial step in producing structured lipids such as *sn*-2 palmitate, which requires *sn*-1,3-specific lipases capable of selectively cleaving ester bonds at the outer positions of the glycerol backbone. This study characterized extracellular *sn*-1,3 lipases from *Aspergillus niger* and *Rhizopus oryzae* cultivated in crude palm oil (CPO)-enriched submerged fermentation (SmF) media. The resulting culture filtrates exhibited lipase activities of 2.76 and 2.10 U mL^{-1}, respectively. Partial purification through ammonium sulfate fractionation (0–40%) yielded lipases with the highest specific activities of 4.89 U mg^{-1} (*A. niger*) and 9.64 U mg^{-1} (*R. oryzae*), with estimated molecular masses of 35 and 29 kDa. Both enzymes showed *sn*-1,3 positional specificity toward CPO hydrolysis and exhibited optimal activity at pH 6–7 and 35 °C. Although thermal stability was moderate, both lipases maintained substantial activity in benzene, ethanol, and methanol, indicating tolerance to both non-polar and low-polarity solvents. These findings highlight the potential of indigenous fungal lipases as promising biocatalysts for CPO-based structured lipid synthesis under mild and sustainable conditions.

[Keywords: enzymatic hydrolysis, filamentous fungi, regioselectivity, *sn*-2 palmitate, vegetable oil]

Introduction

Crude palm oil (CPO), extracted from the mesocarp of oil palm fruit, is a major source of vegetable oil rich in triacylglycerols (TAGs) composed of 39–44% palmitic acid, 39–40% oleic acid, 10–11% linoleic acid, about 5% stearic acid, and smaller proportions of lauric, myristic, palmitoleic, linolenic, and arachidic acids (Man et al., 1999; Stavila et al., 2023; Bomfima et al., 2024). Previous enzymatic study has demonstrated that CPO can be effectively modified through glycerolysis reactions using microbial lipases, particularly those from *Rhizopus oryzae*, to generate monoacylglycerol (MAG) and diacylglycerol (DAG) with desirable structural characteristics (Tri-Panji et al., 2019). Extending from these findings, the rich palmitic acid content and balanced fatty acid composition of CPO highlight its potential as a cost-efficient and sustainable substrate for producing structured lipids such as *sn*-2 palmitate, a key component in human milk fat substitutes (HMFS). *Sn*-2 palmitate refers to palmitic acid esterified at the *sn*-2 position of the TAG molecule, commonly found in 1,3-dioleoyl-2-palmitoylglycerol (OPO) and 1-oleoyl-2-palmitoyl-3-linoleoylglycerol (OPL) (Wei et al., 2020). HMFS are structured lipids designed to mimic the fatty acid composition and positional distribution of human milk fat (Yuan et al., 2020). The enzymatic modification of CPO using *sn*-1,3-specific lipases offer a promising biocatalytic route to generate these high-value lipids under mild and environmentally friendly conditions.

Lipase are enzymes capable of catalyzing the hydrolysis of TAGs into free fatty acids (FFA) and glycerol (Köse et al., 2016). This biocatalyst acts on the carboxylate ester bond (Emmanuel et al., 2020). Based on their positional regioselectivity toward the carboxylate ester bond of TAG, lipases can be classified into *sn*-1,3-specific and non-specific lipases. *Sn*-1,3-specific lipases can direct the cleavage of ester bonds at the *sn*-1 (R_1) and sn-3 (R_3) positions (Figure 1), while non-specific lipases completely hydrolyze TAG at all three ester bond positions (Girelli & Chiappini,2023). The use of lipases with *sn*-1,3 regioselectivity can contribute to the processing of CPO as a source of *sn*-2 palmitate through hydrolysis.

[*)]Corresponding author: irmakresnawaty83@gmail.com

Menara Perkebunan is DOAJ indexed Journal and accredited as Sinta 2 Journal (https://sinta.kemdikbud.go.id/journals/profile/3125)
How to Cite: Putra, L., Nurhasanah, E., Rohaeti, E., & Kresnawaty, I. (2025). Characterization of sn-1,3, extracellular lipases of *Aspergillus niger* and *Rhizopus oryzae* for the crude palm oil hydrolysis. *Menara Perkebunan, 93*(2), 148-157. http://dx.doi.org/10.22302/iribb.jur.mp.v93i2.673

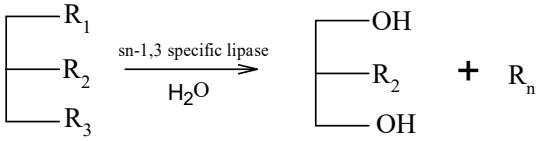


Figure 1. Enzymatic hydrolysis of TAG with *sn*-1,3-specific lipase

Lipases are enzymes capable of catalyzing the hydrolysis of TAGs into free fatty acids (FFA) and glycerol (Köse et al., 2016). This biocatalyst acts on the carboxylate ester bond (Emmanuel et al., 2020). Based on their positional regioselectivity toward the carboxylate ester bond of TAG, lipases can be classified into *sn*-1,3-specific and non-specific lipases. *Sn*-1,3-specific lipases can direct the cleavage of ester bonds at the *sn*-1 (R_1) and *sn*-3 (R_3) positions (Figure 1), while non-specific lipases completely hydrolyze TAG at all three ester bond positions (Girelli & Chiappini,2023). The use of lipases with *sn*-1,3 regioselectivity can contribute to the processing of CPO as a source of *sn*-2 palmitate through hydrolysis.

Commercial production of *sn*-2 palmitate commonly employs *sn*-1,3-specific lipases such as *Rhizomucor miehei* (Lipozyme RM IM), *Thermomyces lanuginosus* (Lipozyme TL IM), and *Candida antarctica* (Novozyme 435). These immobilized enzymes are widely available from manufacturers such as Novozymes and Sigma-Aldrich, typically priced between USD 100-900 per kilograms (Souza-Gonçalves, 2023). While these imported biocatalysts dominate industrial use, their high cost and limited local supply highlight an opportunity for indigenous enzyme production in Indonesia.

Fermentation using native Indonesian filamentous fungi offers a low-cost and sustainable approach to lipase production (Tri-Panji et al., 2019). Extracellular lipases secreted into the fermentation medium by fungi such as *Aspergillus niger* and *Rhizopus oryzae* have gained considerable attention for their biotechnological potential in oil and fat modification (Emmanuel et al., 2020; Vidal et al., 2023). These fungal lipases are advantageous because they function under mild reaction conditions, remain active in organic solvents and elevated temperatures, and operate across a broad pH range (Javed et al., 2018). Understanding their catalytic stability and physicochemical properties is therefore essential for evaluating their suitability in CPO hydrolysis toward *sn*-2 palmitate formation for human milk fat substitute (HMFS) development. Therefore, this study aims to isolate and characterize the extracellular lipases from both fungi to evaluate their functional suitability for CPO hydrolysis in HMFS production.

Materials and Methods

Materials

Aspergillus niger was obtained from the isolate collection of Indonesian Oil Palm Research Institute (IOPRI), Bogor. *Rhizopus oryzae* was isolated from tempeh purchased from a local market in Bogor. All other materials and reagents were supplied by IOPRI.

Culture filtrate production

Production was carried out in a jar containing 100 mL of media with a composition of 2 g CPO, 1.5 g peptone, 1.5 g yeast extract, 0.04 g $MgSO_4.7H_2O$, and an adjusted pH of 5 (Helal et al., 2021). The medium was sterilized in an autoclave at 121 °C for 20 minutes and cooled to room temperature. Spores of *A. niger* and *R. oryzae* were inoculated into the fermentation medium at a concentration of 1×10^6 spores mL^{-1} using 5 % (v/v) inoculum and incubated at 25 °C under static conditions for 5 days. The culture broth was filtered through Whatman No. 1 filter paper (11 µm pore size) to remove mycelial debris, and the resulting clear filtrate was used as the crude enzyme source.

Lipase activity assay

Lipase activity was measured by the titrimetric method (Tri-Panji et al., 2019). The hydrolysis reaction mixture (total volume 45 mL) consisted of 1 mL of enzyme isolate, 3 g of CPO, 1 g of poly(vinyl alcohol) (PVA), and 40 mL of 0.05 M phosphate buffer (pH 5). The mixture was homogenized using a laboratory blender at 10,000 rpm for 3 minutes to obtain a fine emulsion and then gently stirred for 5 minutes to stabilize the dispersion before incubation at 37 °C for 30 minutes. The reaction was stopped with 10 mL of an acetone : ethanol mixture to completely denature the lipase (1:1 v/v). 1% of phenolphtalein was added into the mixture, then titrated with 1N NaOH until a persistent faint pink color appeared. A blank sample underwent the same procedure without the addition of the enzyme isolate. Lipase activity was calculated using the following equation:

$$\text{Lipase activity} = \frac{(A - B) \times [NaOH] \times 1000}{v \times t}$$

A : Sample titration volume (mL)
B : Blank titration volume (mL)
[NaOH] : NaOH concentration (N)
t : Incubation time (minutes)
v : Total reaction volume (mL)

Protein concentration determination

Protein concentration was determined using the method of Goldring (2018) using Bovine Serum Albumin (BSA) as a standard. Through the mathematical equation of the protein standard curve, the total dissolved protein contained in the crude fraction and fractions I and II was obtained.

Partial purification of culture filtrate

The crude enzyme fraction was partially purified through $(NH_4)_2SO_4$ fractionation with two saturation fraction variations: fraction I (0-40%) and fraction II (40-80%). The $(NH_4)_2SO_4$ was added gradually at 4 °C using a magnetic stirrer. Then, the fractions were centrifuged at 8000 × g at 4 °C for 30 minutes. The precipitate was suspended in 0.1 M phosphate buffer, pH 7, and dialyzed using a semipermeable membrane (cut-off 10-14 kDa) against the same buffer overnight at 4°C (Barathi et al., 2019). The extracellular lipase fractions from *A. niger* and *R. oryzae* in this study consisted of three types: a crude fraction and two partially purified fractions (Fractions I and II). All fractions were analyzed for total protein content and lipase activity to determine which had the highest specific activity.

Determination of lipase molecular mass profile

The molecular masses of both lipases were measured using SDS-PAGE electrophoresis (Goldring, 2018). Non-induced *Escherichia coli* BL21(DE3) was used as a negative control for the non-lipase protein. A 10% polyacrylamide gel was used as a porous membrane for protein migration (10 kDa-250 kDa). The gel was stained with Coomassie Brilliant Blue R-250 and visualized under white light using BIO-RAD GeldocGo Imaging System.

Lipase specificity assay

The positional specificity of lipase was assessed following the method of Tri-Panji et al. (2019). The reaction mixture consisted of 0.3 g CPO and 0.1 mL of crude lipase suspended in 0.1 M phosphate buffer (pH 7) and was incubated at 35 °C for 60 minutes. The hydrolysis products were extracted using n-hexane, and the organic phase was analyzed on a Thin Layer Chromatography (TLC) Silica Gel 60 F254 plate developed in n-hexane : diethyl ether : acetic acid (95 : 5 : 1). Visualization was performed by exposing the developed plate to iodine vapor in a closed chamber until brown lipid spots appeared distinctly. The Rf value of each spot was calculated as the ratio between the migration distance of the compound and the solvent front. The apparent *sn*-1,3 activity was inferred from the appearance of spot corresponding to the Rf of 2-palmitoylglycerol (MAG standard) together with the glyceryl tripalmitate (TAG standard). The optimum pH and temperature, as well as the stability of each enzyme against pH, temperature, and organic solvents, were evaluated using the lipase activity assay described above.

Lipase characterization

The optimum pH and temperature, as well as the stability of each enzyme towards pH, temperature, and organic solvents were evaluated using the lipase activity assay described above. Each treatment was performed in triplicate, and the results are presented as the mean with error bars representing standard deviation. The optimum pH was evaluated using 0.05 M phosphate buffer at pH levels of 4, 5, 6, 7, and 8, with incubation at 35 °C for 30 minutes. The effect of temperature was evaluated at 25, 35, 45, 55, and 65 °C under the previously determined optimal pH condition for 30 minutes. Lipase activity at both optimum pH and temperature was expressed as relative activity, calculated by comparing the activity under each test condition to that of the enzyme under optimal assay conditions, which was defined as 100%.

The pH stability was evaluated by pre-incubating the enzyme at pH 4, 5, 6, 7, and 8 in 0.05 M phosphate buffer at 35 °C for 60 minutes. Temperature stability was evaluated by pre-incubating the enzyme at 25, 35, 45, 55 and 65 °C under the previously determined optimum pH stability for 60 minutes. After incubation, each sample was subjected to the lipase assay at the determined optimum pH and temperature. Lipase activity of both temperature and pH stability was expressed as residual activity, defined as the percentage of enzymatic activity retained after pre-incubation compared to the initial activity measured under optimal assay conditions.

The effect of organic solvents was evaluated by incubating the enzyme at room 25°C for 60 minutes in the presence of the 10% of the following solvents: acetone, ethanol, methanol, isopropanol, and benzene. As a control, the enzyme was also incubated in 0.05 M phosphate buffer. Lipase activity of organic solvent stability was expressed as residual activity defined as the percentage of lipase activity of each sample compared to the control measured under optimal assay conditions.

Results and Discussion

Extracellular lipase activity of culture filtrate

The culture filtrates of *A. niger* and *R. oryzae* in submerged fermentation (SmF) showed lipase activities of 2.76 and 2.10 U mL^{-1}, respectively (Table 1). Several studies have previously reported the enzyme activity of both isolates, ranging from 0.6 to 63 U mL^{-1} (Mukhtar et al., 2015; Hermansyah et al., 2019; Yassein et al., 2021). This suggests potential improvements to the current method, including optimizing culture conditions and nutrient availability. Innoculation of *A. niger*

Table 1. Activity and total protein of the extracellular lipase in ammonium sulfate fraction

Test sample	Lipase activity (U mL^{-1})	Total protein (mg mL^{-1})	Specific activities (U mg^{-1})	Recovery of enzyme activity (%)	Purification fold
Aspergillus niger					
Crude fraction	2.76	1.23	2.23	100	1.00
Fraction I (0-40%)	1.97	0.40	4.89	71.50	2.19
Fraction II (20-40%)	1.18	1.86	0.63	42.86	0.28
Rhizopus oryzae					
Crude fraction	2.10	0.87	2.42	100	1.00
Fraction I (0-40%)	1.42	0.15	9.64	67.57	3.99
Fraction II (20-40%)	1.05	1.56	0.67	50.00	0.28

and *R. oryzae* spores into SmF medium aimed to establish a fungal metabolic system that secretes lipase. Several bioprocess components are utilized by fungal species for lipase secretion, including CPO as an inducer and carbon source, peptone and yeast extract as nitrogen sources, and $MgSO_4.7H_2O$ as a source of metal ion compounds (Al-Dahlan et al., 2024). Both fungal species are responsive to the environmental conditions of the media containing these components, enabling them to secrete lipase into the culture filtrate.

Extracellular lipase fraction

The maximum specific activity was obtained in Fraction I (0–40% ammonium sulfate saturation), reaching 4.89 U mg^{-1} for *A. niger* and 9.64 U mg^{-1} for *R. oryzae*. The corresponding enzyme activity recoveries were 71.50% and 67.57%, with purification folds of 2.19 and 3.99, respectively (Table 1). These results indicate that the initial fraction retained most of the lipase activity during the partial purification process. Similar findings were reported by Putra et al. (2019), who observed that recombinant lipase fractions with low salt saturation (20–30%) exhibited higher specific activity than advanced fractions. Likewise, Fathi et al. (2021) found that initial low-salt fractions from *Lactobacillus fermentum* retained superior lipase activity compared to later fractions. Based on these results, Fraction I was selected as the enzyme source for subsequent characterization.

The precipitate of fractions I and II is formed due to the salting-out process during fractionation, by changing the solubility of the protein (Nargotra et al., 2022). $(NH_4)_2SO_4$ can increase the saturation of the fraction, so that components with low solubility will be precipitated (Du et al., 2022). Protein precipitates of fractions I and II from *A. niger* and *R. oryzae* were suspended in 0.1 M phosphate buffer, pH 7, and dialyzed in a semipermeable membrane. Small molecules in the sample move through the membrane into the dialysis buffer, while large macromolecules (including lipase) are retained within the membrane (Thomas et al., 2020).

Lipase molecular mass profile

SDS-PAGE electrophoresis of 10% protein fraction I of *A. niger* and *R. oryzae* produced bands with estimated molecular masses of 35 and 29 kDa, respectively (Figure 2). The determination of the molecular masses of lipase from these two species is relatively consistent with previous studies, with each of *A. niger* and *R. oryzae* being found to be 31.5 - 35 kDa and 29-31 kDa, respectively (Chattopadhyaya, 2000; López-Fernández et al., 2020; Xing et al., 2020; Namboodiri & Vidal et al., 2023).

Lipase specificity

The hydrolysis products of crude palm oil (CPO) by lipases from *A. niger* and *R. oryzae* produced three visible spots on TLC (Figure 4). Two of these spots matched those of identified as glyceryl tripalmitate and 2-palmitoylglycerol standards (Figure 3), which suggests that the lipases preferentially hydrolyze ester bonds at the *sn*-1 and *sn*-3 positions. However, this interpretation should be considered indicative rather than conclusive, as TLC alone cannot distinguish between positional isomers. Additional analyses, such as co-chromatography with authentic 1-MAG standards or instrumental techniques like gas chromatography (GC) or high-performance liquid chromatography (HPLC), would be required to confirm the regioisomeric specificity.

Effect of pH and temperature

The *A. niger* lipase was optimal at pH 6 with a relative activity of 92.59 ± 6.42%, while the *R. oryzae* lipase was optimal at pH 7 with a relative activity of 88.89 ± 11.11 % (Figure 4A). Another *sn*-1,3 lipase, *Rhizomucor miehei,* was also optimal at pH 7 (Tako et al., 2017). At optimum conditions, the enzyme conformation is in a highly electrostatically stable form, so the binding of substrate-enzyme is effective (Yao et al., 2021). Exposure of the enzyme to unfavorable pH causes changes in the ionization state of the enzyme's amino acid groups, leading to changes in the characteristic electrostatic interaction patterns in the enzyme structure (Nargotra et al., 2022).

The lipase from fraction I of both fungal species showed optimal activity at 35 °C (Figure 5B). The *sn*-1,3 extracellular lipase from *A. niger* in the study by Xing et al. (2020) also had an optimum temperature of 35 °C. Similarly, the non-specific lipase from *R. oryzae* in the study by Helal et al. (2021) showed an optimum temperature of 37 °C. Lipase activity at the optimum temperature indicates that the affinity of substrate for lipase is at optimum conditions for hydrolysis (Remonatto et al., 2021). The lowest activity of both fungal species was achieved when the hydrolysis reaction temperature exceeded 45 °C. However, it should be noted that enzymes are proteins subjected to protein denaturation at high temperatures, preventing them from maintaining their structure and function (Alabdalall et al., 2021).

pH and temperature Stability

The stability range of *A. niger* lipase was broader (pH 5–7) than that of *R. oryzae* lipase (pH 6–7) (Figure 5). However, the overall stability of both free lipases was lower compared to the immobilized lipases characterized by Nuraliyah et al. (2021) and Yang et al. (2021). This result indicates that free lipases lose more activity after exposure to extreme pH and temperature for 60 minutes, compared to immobilized ones. According to Kumar et al. (2021), enzyme immobilization enhances catalytic stability, and further improvements can also be achieved through lipase engineering (Ali et al., 2023).

Effects of organic solvents

Both *A. niger* and *R. oryzae* lipases followed a similar stability trend, retaining the highest residual activity in benzene, followed by methanol, ethanol, and isopropanol, while losing most activity in acetone (Figure 6). This pattern suggests a preference for non-polar environments that preserve the enzyme hydrophobic core, as found in solvents like benzene. Although industrial studies often preferred n-hexane and toluene as non-polar media, benzene's comparable polarity helps indicate the enzyme compatibility within such hydrophobic systems.

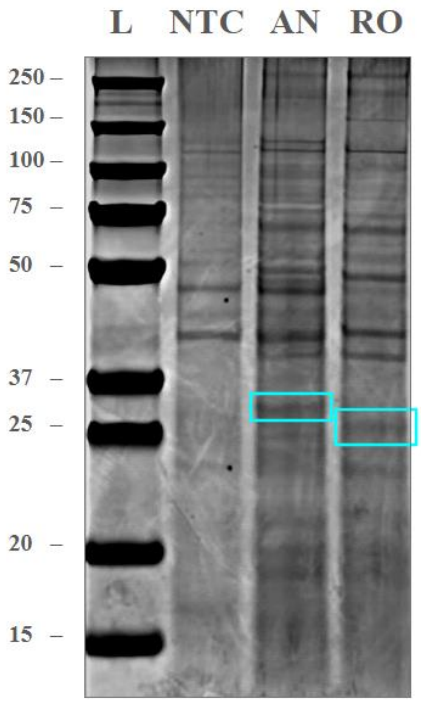
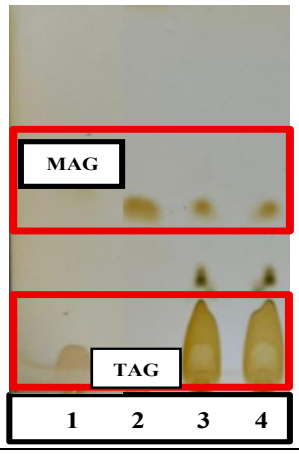


Figure 2. SDS-PAGE profile (10% resolving gel) of crude lipase fractions visualized using Coomassie Brilliant Blue R-250 staining. **L**: pre-stained Bio-Rad Precision Plus Protein™ Dual Color Standards (kDa indicated); **NTC**: negative control (non-induced *E. coli* BL21(DE3)); **AN**: *Aspergillus niger* lipase fraction; **RO**: *Rhizopus oryzae* lipase fraction. Light blue boxes indicate the putative lipase protein bands.



Spot	Rf TAG*)	Spot	Rf MAG**)
(1)	0.20	(2)	0.56
(3)	0.20	(3)	0.56
(4)	0.20	(4)	0.56

*)Retention factor triacylglycerol, **)retention factor 2-monoacylglycerol

Figure 3. TLC in the eluent system n-hexane: diethyl ether: acetic acid (95:5:1). (1) Glyceryl tripalmitate standard, (2) 2-palmytoylglycerol standard, (3) CPO hydrolysis product by lipase fractions I *A. niger*, (4) CPO hydrolysis product by lipase fractions I *R.oryzae*. The reaction was carried out at pH 7 and a temperature of 35 ℃

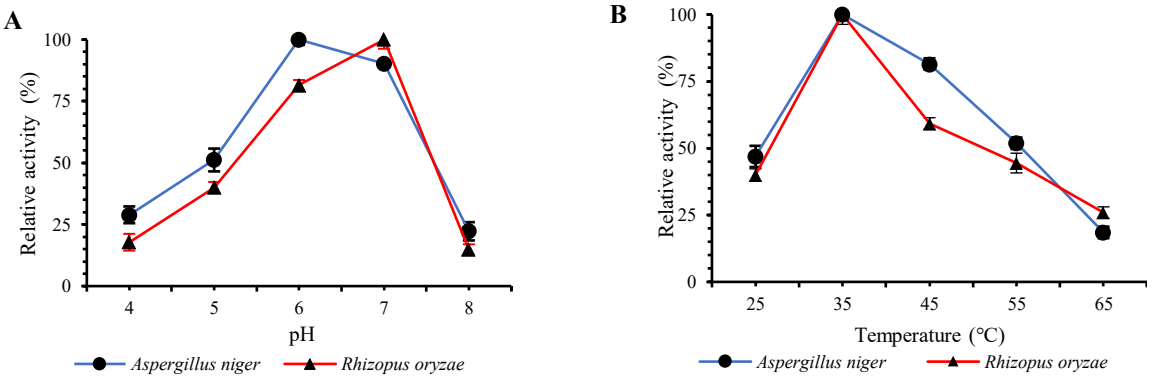


Figure 4. Characteristics of optimal conditions for lipase in CPO hydrolysis. (A) At pH 4-8 and 35 ℃, (B) at temperatures 25-65 ℃ and optimal pH

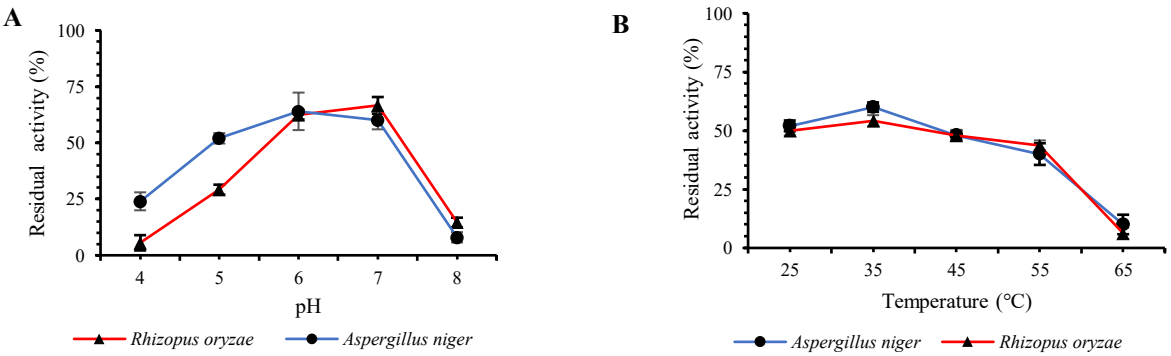


Figure 5. Characteristics of stability conditions for 60-minute lipase in CPO hydrolysis. (A) At pH 4-8 and 35 ℃, (B) at temperatures 25-65 ℃ and optimal pH

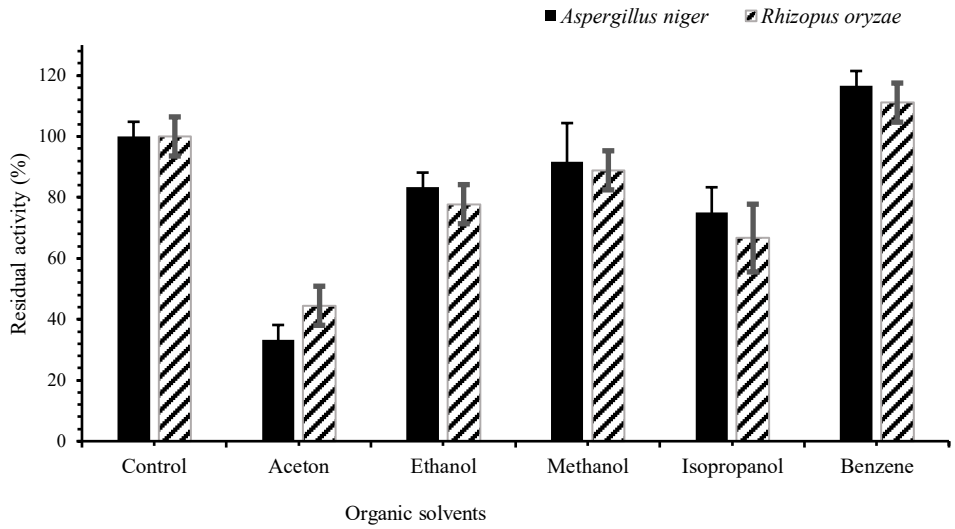


Figure 6. Effect of organic solvents on lipase stability for 60 minutes

Both lipases also showed good activity in water–methanol and water–ethanol systems, confirming their tolerance toward low-polarity alcohols. Similar findings were reported by Ayinla et al. (2021) and Xing et al. (2021), who demonstrated strong methanol compatibility in extracellular lipases. According to Tian et al. (2022), enhancing methanol tolerance and catalytic activity in water-soluble lipases is critical for improving biodiesel conversion efficiency.

High lipase activity at the water–polar solvent interface is attributed to interfacial activation (Albayati et al., 2020), though a minor decline in hydrolytic activity was noted. The lower FFA levels detected by NaOH titration likely resulted from a shift in reaction equilibrium toward ester formation through esterification (Dasetty et al., 2017). Despite moderate stability in polar alcohols (methanol, ethanol, isopropanol), the sharp decline in acetone activity indicates that moderately polar solvents are the most disruptive—being polar enough to disturb enzyme–water interactions but not hydrophobic enough to stabilize the enzyme's structure (Wang et al., 2016).

Conclusion

The submerged fermentation of *Aspergillus niger* and *Rhizopus oryzae* in CPO-enriched media successfully produced extracellular lipases with specific activities of 4.89 and 9.64 U mg^{-1}, respectively. The highest enzymatic performance was obtained from the 0–40% ammonium sulfate fraction, corresponding to molecular masses of approximately 35 kDa (*A. niger*) and 29 kDa (*R. oryzae*). Both enzymes exhibited *sn*-1,3 positional specificity during CPO hydrolysis, indicating potential application in structured lipid synthesis, although further analysis is needed to confirm regioisomer distribution. The optimal catalytic conditions were pH 6–7 and 35 °C, with notable stability in benzene, ethanol, and methanol, suggesting good compatibility in both non-polar and low-polarity environments.

Acknowledgements

We thank the Indonesian Oil Palm Research Institute - Bogor Unit – PT Research Perkebunan Nusantara for the research funding. We also thank the Bogor Agricultural Institute for supporting this research.

References

Alabdalall, H. A., Al-Anazi, N. A., Aldakheel, L. A., Amer, I. H. F., Aldakheel, A. F., Ababutain, M. I., Alghamdi, I. A., & Al-Khaldi, M. E. 2021. Application and characterization of crude fungal lipases used to degrade fat and oil wastes. *Scientific Reports, 11*(19670), 1-10. https://doi.org/10.1038/s41598-021-98927-4

Albayati, H. S., Masomia, M., Ishak, H. S., Ali, M. S. M., Thean, L. A., Shariff, Noor, M. D. N., & Rahman, A. R. J. N. 2020. Main structural targets for engineering lipase substrate specificity. *Catalysts, 10*(747), 1-34. https://doi.org/10.3390/catal10070747

Al-dahlan, Baghdadi, M. A., & Alkattan, O. M. 2024. Microbial lipases: Between production, purification, and their biotechnological applications. *Current Science International. 13*(1), 75-86. https://doi.org/10.36632/csi/2024.13.1.6

Ali, S., Khan, A. S., Hamayun, M., & Lee, I. 2023. The recent advances in the utility of microbial lipases, *11*(540), 1-26. https://doi.org/10.3390/microorganisms11020510

Ayinla, A. Z., Ademakinwa, N. A., & Agboola, K. F. 2017. Studies on the optimization of lipase production by *Rhizopus* sp. ZAC3 isolated from the contaminated soil of a palm oil processing shed. *Journal of Applied Biology amd Biotechnology*, *5*(2), 30-37. https://doi.org/10.7324/JABB.2017.50205

Barathi, D., Rajalakshmi, G., & Komathi, S. 2019. Optimization and production of lipase enzyme from bacterial strains isolated from petrol spilled soil. *Journal of King Saud University, 31,* 898-901. https://doi.org/10.1016/j.jksus.2017.12.018

Bomfima, S. R., Velasco, J., Cardoso, A. L., Ribeiro, F. D. C., Marinho, M. Q. L., Ribeiro, R, P., & Almeida, T. D. (2024). Processing practices and quality of crude palm oil produced on a small scale in Valença, Bahia, Brazil. *Grasas Y Aceites, 75*(2), 1-12, https://doi.org/10.3989/gya.1191231.2084

Dasetty, S., Blenner, A., M., & Sarupria, S. 2017. Engineering lipases: walking the fine line between activity and stability. *Materials Research Express,* 1-32. https://doi.org/10.1088/2053-1591/aa9946

Du, M., Hou, Z., Liu, L., Xuan, Y., Chen, X., Fan, L., Li, Z., & Xu, B. 2022. Progress, applications, challenges and prospects of protein purification technology. *Frontiers in Bioengineering and Biotechnology, 10*, 1-26. https://doi.org/10.3389/fbioe.2022.1028691

Emmanuel, B. M., Evans, C. D., Abubakar, A., Labaran, M. L., Ali, V. A., & Zabe, M. 2020. Production, partial purification and characterization of lipase enzyme expressed by *Klebsiella pnemoniae* of vegetable oil contaminated soil. *International Journal of Biochemistry and Biophysics, 8*(2), 30-39. https://doi.org/10.13189/ijbb.2020.080202

Fathi, F., Kasra-Kermanshahi, Moosavi-Nejad, Z., & Qamsari, M. E. 2021. Partial purification, characterization and immobilization of a novel lipase from a native isolate of *Lactobacillus fermentum*. *Iranian Journal of Microbiology, 13*(6), 871-877. https://doi.org/10.18502/ijm.v13i6.8093

Girelli, G. M., & Chiappini, V. 2023. Renewable, sustainable, and natural lignocellulosic carriers for lipase immobilization: A review. *Journal of Biotechnology, 365*, 29-47. https://doi.org/10.1016/j.jbiotec.2023.02.003

Goldring, D. P. J. 2018. Measuring protein concentration with absorbance, Lowry, Bradford Coomassie Blue, or the smith bicinchoninic acid assay before electrophoresis. *Electrophoretic Separation of Protein: Method and Protocols, 1855*, 31-39. https://doi.org/10.1007/978-1-4939-8793-1_3

Helal, E. S., Abdelhady, M. H., Abou-Taleb, A. K., Hassan, G. M., & Amer, M. M. 2021. Lipase from *Rhizopus oryzae* R1: in-depth characterization, immobilization, and evaluation in biodiesel production. *Journal of Genetic Engineering and Biotechnology, 19*(1), 1-13. https://doi.org/10.1186/s43141-020-00094-y

Hermansyah, H., Andikoputro, M. I., & Alatas, A. (2019). *Production of lipase enzyme from Rhizopus oryzae by solid state fermentation and submerged fermentation using wheat bran as substrate*. In T. Ariyanto, I. Prasetyo, Rochmadi, & N. R. E. Putri (Eds.), *11th Regional Conference on Chemical Engineering, RCChE 2018 (AIP Conference Proceedings, Vol. 2085, Article 020013)*. American Institute of Physics. https://doi.org/10.1063/1.5094991

Javed, S., Azeem, F., Hussain, S., Rasul, I., Siddique, M. H., Riaz, M., Afzal, M., Kouser, A., & Nadeem, H. 2018. Bacterial lipases: a review on purification and characterization. *Progress in Biophysic and Molecular Biology, 132*, 23-34. https://doi.org/10.1016/j.pbiomolbio.2017.07.014

Köse, K., Erol, K., Köse, A. D., Evci, E., & Uzun, L. 2016. Affinity purification lipase from wheat germ: comparison of hydrophobic and metal chelation effect. *Artificial Cells, Nanomedicine, and Biotechnology, 45*(3), 574-583. https://doi.org/10.3109/21691401.2016.1161642

Kumar, A., Mukhia, S., Kumar, N., Acharya, V., Kumar, S., & Kumar, R. 2021. A broad temperature active lipase purified from a psychrotrophic bacterium of sikkim himalaya with potential application in detergent formulation. *Frontier in Bioengineering and Biotechnology, 8*(642), 1-16. https://doi.org/10.3389/fbioe.2020.00642

López-Fernández, J., Benaiges, M. D., & Valero, F. (2020). *Rhizopus oryzae* lipase, a promising industrial enzyme: Biochemical characteristics, production and biocatalytic applications. *Catalysts, 10*(11), 1277. https://doi.org/10.3390/catal10111277

Man, C. B. Y., Haryati, T., Ghazali, M. H., & Asbi, A. B. 1999. Composition and thermal profile of crude palm oil and its products. *JAOCS*, *76*(2), 237-242. https://doi.org/10.1007/s11746-999-0224-y

Mukhtar, H., Hanif, M., Rehman, A., Nawaz, A., & Haq, I. (2015). *Studies on the lipase production by Aspergillus niger through solid state fermentation*. *Pakistan Journal of Botany*, 47(SI), 351-354. Retrieved from https://www.pakbs.org/pjbot/PDFs/47%28SI%29/48.pdf

Namboodiri, V.M., Chattopadhyaya. R. (2000). Purification and biochemical characterization of a novel thermostable lipase from Aspergillus niger. *Lipids*, 35(5): 495-502. https://doi.org/10.1007/s11745-000-549-3.

Nargotra, P., Sharma, V., Sharma, S., Bangotra, R., & Bajaj, K. B. 2022. Purification of an ionic liquid stable cellulase from *Aspergillus aculeatus* PN14 with potential for biomass refining. *Environmental Sustainability 5*, 313-323. https://doi.org/10.1007/s42398-022-00232-x

Nuraliyah, A., Perdani, Putri, N. D., Sahlan, M., & Hermansyah, H. 2021. Effect of additional amino group to improve the performance of immobilized lipase from *Aspergillus niger* by adsorption-crosslinking method. *Frontiers in Energy Research, 9*, 1-11. https://doi.org/10.3389/fenrg.2021.616945

Putra, L., Natadiputri, H. G., Meryandini, A., & Suwanto, A. 2019. Isolation, cloning and co-expression of lipase and foldase genes of *Burkholderia territorii* GP3 from Mount Papandayan Soil. *Journal Microbialogy biotechnology, 29*(6), 944-951. https://doi.org/10.4014/jmb.1812.12013

Remonatto, D., Ferrari, R. B., Bassan, C. J., Santos-Ebinuma, C. V., & Paula, V. A. 2021. Utilization of clay materials as support for *Aspergillus japonicus* lipase: an eco-friendly approach. *Catalysts, 11*(10), 1-17. https://doi.org/10.3390/catal11101173

Stavila, E., Yuliati, F., Adharis, A., Laksmono, J. A., & Iqbal, M. (2023). Recent advances in synthesis of polymers based on palm oil and its fatty acids. *RSC Advances, 13*(28), 14747–14775. https://doi.org/10.1039/D3RA01913F.

Souza-Gonçalves, J., Fialho, A., Soares, C. M. F., Osório, N. M., & Ferreira-Dias, S. (2023). Continuous production of dietetic structured lipids using crude acidic olive pomace oils. *Molecules, 28*(6), 2637. https://doi.org/10.3390/molecules28062637

Tako, M., Katogan, A., Papp, T., Kadaikunnan., Alharbi, N., & Vágvölgyi, C. 2017. Purification and properties of extracellular lipases with transesterification activity and 1,3-regioselectivity from *Rhizomucor miehei* and *Rhizopus oryzae*. *Journal Microbiology and Biotechnology, 27*(2), 277-288. https://doi.org/10.4014/jmb.1608.08005

Thomas, L. S., Thacker, B. J., Schung, A. K., & Maráková, K. 2020. Sample preparation and fractionation techniques for intact proteins for mass spectrometric analysis. *Journal of Separation Science, 44*(1), 211-246. https://doi.org/10.1002/jssc.202000936

Tian M, Yang L, Wang Z, Lv P, Fu J, Changlin M, Li Z, Li L, Liu T, Du W, Luo W. 2022. Improvement of methanol tolerances and catalytic activity of *Rhizomucor miehei* lipase for one-step synthesis of biodiesel by semi-rational design. *Bioresource Technology, 348,* 1-31. https://doi.org/10.1016/j.biortech.2022.126769

Tri-Panji, Kresnawaty, I., Dimawarnita, F., Saadah, S., Aminingsih, T., & Miranti, M. 2019. Gliserolisis enzimatik CPO dengan lipase amobil untuk produksi diasil dan monoasil gliserol. *Menara Perkebunan*, *87*(1), 11-19. https://doi.org/10.22302/iribb.jur.mp.v87i1.321

Wei, W., Sun, C., Wang, X., Jin, Q., Xu, X., Akoh, C.C., & Wang, X. 2020. Lipase-catalyzed synthesis of sn-2 palmitate. *Engineering, 6*(406), 1-14. https://doi.org/10.1016/j.eng.2020.02.008

Xing, S., Zhu, R., Li, C., He, L., Zeng, X., & Zhang, Q. (2020). Gene cloning, expression, purification and characterization of an sn-1,3 extracellular lipase from *Aspergillus niger* GZUF36. *Journal of Food Science and Technology, 57*(7), 2669–2680. https://doi.org/10.1007/s13197-020-04303-x.

Xing, S., Zhu, R., Cheng, K., Cai, Y., Yuedan, Z. X., Zhu, Q., & He, L. 2021. Gene expression, biochemical characterization of sn-1,3 extracellular lipase from *Aspergillus niger* GZUF36 and its mode-structure analysis. *Frontiers in Microbiology. 12*, 1-16. https://doi.org/10.3389/fmicb.2021.633489

Wang, S., Meng, X., Zhou, H., Liu, Y., Secundo, F., & Liu, Y. (2016). Enzyme Stability and Activity in Non-Aqueous Reaction Systems: A Mini Review. *Catalysts*, *6*(2), 32. https://doi.org/10.3390/catal6020032

Yang, X., Zhang, Y., Pang, H., Yuan, S., Wang, X., Hu, Z., Zhou, Q., He, Y., Yan, Y., & Xu, L. 2021. Codisplay of *Rhizopus oryzae* and *Candida rugosa* lipases for biodiesel production. *Catalysts, 11*(4), 1-13. https://doi.org/10.3390/catal11040421.

Yao, W., Liu, K., Liu, H., Wang, R., Wang, W., & Wang, T. 2021. A valuable product of microbioal cell factories: microbial lipase. *Frontiers in Microbiology, 12*, 1-16. https://doi.org/10.3389/fmicb.2021.743377

Yassein, A. S., Hassan, M. M., & Elamary, R. B. (2021). Prevalence of lipase producer *Aspergillus niger* in nuts and anti-biofilm efficacy of its crude lipase against some human pathogenic bacteria. *Scientific Reports, 11*(1), 7981. https://doi.org/10.1038/s41598-021-87079-0

Yuan, T., Wei, W., Wang, X., & Jin, Q. 2020. Biosynthesis of structured lipids enriched with medium and long-chain triacylglycerols of human milk fat substitute. *Food Science and Technology, 128*, 1-7. https://doi.org/ 10.1016/j.lwt.2020.109255