Sains Malaysiana 54(12)(2025): 2837-2846
http://doi.org/10.17576/jsm-2025-5412-03

Enhanced Production of Lipase from 'NC Mutant' Moulds for Transesterification Reactions

(Peningkatan Penghasilan Lipase daripada Kulat Mutant NC untuk Tindak Balas Transesterifikasi)

NISA RACHMANIA MUBARIK[3], TRISMILAH SISWODARSONO[2,*] & GALIH CENDANA NABILASANI[1],

[1]Study Program of Biotechnology, Graduate School of IPB University, Bogor 16680, Indonesia
[2]Center for Bioindustrial Technology-BRIN (National Innovation Research Agency), Bld.610-614 PUSPIPTEK, South Tangerang, Banten, Indonesia
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia

Received: 25 February 2024/Accepted: 17 November 2025

ABSTRACT

Lipase can be used for biodiesel production, specifically in transesterification reactions. Nut C (NC) is a fungal isolate derived from palm kernels and palm kernel waste, capable of producing lipase. The objective of this study was to obtain a mutant mould with higher transesterification activity compared to its wild type counterpart (NC). The mutation process on NC mould was carried out using ultraviolet (UV) radiation followed by ethyl methane sulfonate (EMS). UV-induced mutation on NC produced four isolates, with m4.3NC1 exhibiting higher transesterification activity than the wild type, with an increment of 138.8% (from 0.121 U/mg to 0.168 U/mg). Subsequent EMS mutations of the mutant m4.3NC1 isolate, designated as m5.4NC, increased the transesterification activity from 0.168 U/mg to 2.048 U/mg (1119% increment as compared to the wild type). Molecular identification of the NC isolate showed 100% similarity with the *Aspergillus fumigatus* CMXY15837 strain. The highest specific enzyme activity of the NC mutant was observed at pH 6 and a temperature of 50 °C. This study showed that the mutation of NC mould using UV, followed by EMS, significantly enhanced its transesterification activity.

Keywords: Ethyl methane sulfonate; lipase; NC mutant mould; transesterification reaction; ultraviolet

ABSTRAK

Lipase boleh digunakan untuk pengeluaran biodiesel, khususnya dalam tindak balas transesterifikasi. Nut C (NC) ialah pencilan kulat yang diperoleh daripada isirung sawit dan sisa isirung sawit yang mampu menghasilkan lipase. Objektif kajian ini adalah untuk mendapatkan kulat mutan yang mempunyai aktiviti transesterifikasi yang lebih tinggi berbanding jenis liarnya (NC). Proses mutasi pada kulat NC dijalankan menggunakan sinaran ultraungu (UV) diikuti oleh etil metana sulfonat (EMS). Mutasi akibat UV pada NC menghasilkan empat pencilan dengan m4.3NC1 menunjukkan aktiviti transesterifikasi yang lebih tinggi daripada jenis liar, dengan kenaikan sebanyak 138.8% (daripada 0.121 U/mg kepada 0.168 U/mg). Mutasi EMS seterusnya terhadap pencilan m4.3NC1 mutan yang ditetapkan sebagai m5.4NC meningkatkan aktiviti transesterifikasi daripada 0.168 U/mg kepada 2.048 U/mg (kenaikan 1119% berbanding jenis liar). Pengenalpastian molekul bagi pencilan NC mendedahkan 100% persamaan dengan strain *Aspergillus fumigatus* CMXY15837. Aktiviti enzim khusus tertinggi mutan NC diperhatikan pada pH 6 dan suhu 50 °C. Kajian ini menunjukkan bahawa mutasi terhadap mutan NC menggunakan UV diikuti oleh EMS telah meningkatkan aktiviti transesterifikasi dengan ketara.

Kata kunci: Etil metana sulfonat; kulat mutan NC; lipase; tindak balas transesterifikasi; ultralembayung

INTRODUCTION

Lipase demonstrates its ability to catalyse various reactions, including hydrolysis, esterification, transesterification, and aminolysis. The multifaceted catalytic potential of lipases is attributed to their complex stereochemistry and the presence of flexible 'backbone' proteins (Ozturk 2001). As an enzyme, lipase exhibits catalytic prowess against fats and oils, and can be applied for biodiesel production, specifically in the transesterification reaction (Sundaramahalingam et al. 2021; Tran et al. 2012). Transesterification involves a reaction between triglycerides and alcohol facilitated by a catalyst, yielding methyl esters possessing akin physical properties to diesel fuel (Suryanto, Suprapto & Mahfud 2015). Methyl palmitate, methyl oleate, methyl elaidate, methyl stearate, and methyl linolenic are methyl ester compounds obtainable through transesterification (Aziz, Aisyah & Ilyas 2016). Commonly employed chemical catalysts in transesterification reactions include

NaOH, KOH, and $NaOCH_3$ (Wang et al. 2023). However, using chemical catalysts in transesterification presents drawbacks, such as toxicity, cost, and impurities in the resulting product, which necessitate a purification stage (Puspitaningati, Permatasari & Gunardi 2013). In contrast, employing enzymes as biocatalysts offers environmental friendliness, energy efficiency, and a purer final product (Chandra et al. 2020). Enzymes exhibit high efficiency and specificity, do not generate by-products, and yield purer final products, thereby potentially reducing production costs and environmental damage.

Lipase can be sourced from plants, animals, and microorganisms. Microorganisms stand as the primary source of current lipase production, displaying varied abilities depending on their lipase type, environmental conditions and media utilised for enzyme production. Despite its potential, the use of lipase as a catalyst in industrial fields remains limited due to its high cost. NaOH serves as a common basic catalyst in transesterification owing to its lower price compared to commercial lipases such as Novozym 435, with a price of USD 42.15 per g, which is higher than the price of NaOH (Merck) USD 0.09 per g (José et al. 2021).

Enhancing microorganism capabilities extends beyond genetic engineering and media optimisation; it can also be achieved through mutation using ultraviolet light and chemicals (Eregie et al. 2024). For instance, *Aspergillus* sp. with the code TL-12(3) exhibited increased lipase enzyme activity after mutation. Lipase activity in *Aspergillus* sp. TL-12(3) amplified by 175%, 340%, and 575% post-mutation with ultraviolet light followed by EMS (ethyl methane sulfonate), showcasing the potential of the resulting lipase in diverse industrial fields (Tagore & Narasum 2014).

The mould isolate NC, isolated from palm kernel and palm kernel waste in Malingping, Banten, West Java, Indonesia, is capable of producing the enzyme lipase. This isolate is part of the collection of the Bioindustry Technology Laboratory, LAPTIAB, located in the PUSPIPTEK area, Serpong, South Tangerang, Indonesia. The lipase enzyme exhibits low transesterification activity, measuring at 0.168 U/mg (data not shown), when compared to the commercial lipase AK Amano, which yields a transesterification activity of 1.210 U/mg. This study aims to induce mutations in the NC mould isolate using UV light and EMS chemicals to obtain mutant moulds with higher transesterification activity compared to their wild type (NC).

MATERIALS AND METHODS

The materials used in this study consisted of mould isolates coded Nut C (NC) from the Bioindustry Technology Laboratory collection in the PUSPIPTEK area, Serpong, South Tangerang. Additionally, the materials included soybean flour (from Bumiku), NaCl 0.85% (Merck), olive oil (Le Riche), p-nitrophenyl (pNP) (Sigma Aldrich), p-nitrophenylphosphate (pNPP) (Sigma Aldrich), and n-heptane (Sigma Aldrich).

NUT C (NC) MOULD REJUVENATION

NC mould rejuvenation involved aseptical inoculation one loop of the stock isolate onto sterile slanting PDA (Potato Dextrose Agar) media. NC isolates were incubated at 28 °C for 5 days.

PHYSICAL MUTATIONS

The physical mutations of mould isolates were performed according to Uyar and Uyar (2019) with slight modifications. Modifications included UV germicidal lamp irradiation (250 nm) for durations of 0, 60, 120, and 180 s in an open petri dish placed approximately 18 cm from the lamp. Spore suspensions (diluted to 10^{-5}) were irradiated and then incubated for 5 days at 28 °C. On the fifth day, growing colonies were enumerated. Tests yielding a lethal dose value of ≥ 99% were cultured on PDA slanted media for lipase production to determine enzyme and transesterification activity.

CHEMICAL MUTATIONS

Selected mould isolates, with the best outcomes from UV light mutations, underwent subsequent mutations using the chemical compound EMS. The mutation process was performed using the method modified from Rao et al. (2019), differing in spore suspension used, EMS concentration, and incubation time on PDA media. The spore suspension was diluted to 10^{-5}, and incubated with EMS concentrations ranging from 0% to 7% for 60 min at 750 rpm and 28 °C using a thermomixer. The resulting spore suspension from mutation was inoculated onto PDA media (0.1 mL) using the scatter method and incubated for 5 days at 28 °C. Counts of growing moulds were conducted on the fifth day of incubation. Tests showing lethal doses of ≥ 99% were chosen for further evaluation.

PREPARATION OF ENZYME PRODUCTION MEDIA

The production media consisted of a liquid culture medium comprising 3% commercial soybean flour boiled for approximately 60 min, followed by filtration using gauze. Subsequently, 0.1% technical olive oil (not pure olive oil) was added to the boiled soybean flour water and sterilised using an autoclave at 121 °C and 2 atm pressure for 15 min.

LIPASE ENZYME PRODUCTION

Lipase enzyme production involved a modification of the method from Karanam and Medicherla (2008), where the production medium and the filtration results were not saturated using ammonium sulphate. A 5-day-old mould spore suspension was inoculated at 10% (v/v) in a sterile enzyme production medium and incubated in a rotary shaker at 200 rpm for 5 days at 28 °C. The crude enzyme was produced by centrifugation for 30 min at 3800 rpm at 4 °C. The supernatant was tested for lipase enzyme activity.

LIPASE ACTIVITY ASSAY

The lipase activity assay was performed using the method of Linfield et al. (1984). A total of 1.5 g of polyvinyl alcohol (PVA) was put into a 250 mL Erlenmeyer flask and mixed with 25 mL of olive oil and 75 mL of reverse osmosis (RO) water. The mixture was homogenised using a homogeniser. Then, 5 mL of the substrate was transferred into a 50 mL flask before 4 mL of 0.05 M phosphate buffer (pH 6) and 1 mL of crude enzyme extract were added. The mixture was incubated at 50 °C for 20 min and shaken at 150 rpm. After incubation, 5 mL of methanol was added to stop the reaction. Two drops of phenolphthalein (PP) indicator were added, and the solution was titrated using 0.05 M NaOH until it turned pink. The blank solution was prepared in the same way, but methanol was added before incubation. Lipase activity was calculated using the following formula:

$$\text{Lipase activity (U/mL)} = \frac{(V2 - V1) \times n \times 1000}{t}$$

where V1 is the volume of NaOH required for blank titration (mL); V2 is the volume of NaOH required for sample titration (mL); n is the concentration of NaOH (N); and t is the incubation time (min).

PROTEIN CONTENT DETERMINATION

The concentration of the crude enzyme was determined by the Bradford assay, which used bovine serum albumin (BSA) as a standard to determine the protein concentration. Crude enzyme extract of 0.2 mL was mixed with 2 mL of Bradford reagent, vortexed for 30 s, and incubated for 20 min in the dark. The absorbance of the solution was measured at 595 nm. For the blank, 0.2 mL of water was used instead of the enzyme extract, and the solution was measured at the same wavelength. The specific activity of the enzyme was calculated based on the ration of lipase activity (U/mL) to protein content (mg/mL).

ENZYMATIC TRANSESTERIFICATION

The transesterification activity of the crude lipase extract was determined spectrophotometrically (Kotogan et al. 2014). The crude enzyme was added with 450 µL of n-heptane containing 10 mM pNPP and 50 mL of absolute ethanol. The mixture was homogenised and incubated for 30 min at 50 °C. The extracted pNP was monitored at 405 nm. One unit of lipase activity in transesterification is the amount of enzyme that releases 1 µmol of product (pNP) per min under the assay condition. A blank solution was prepared without the addition of crude enzyme extract. Transesterification activity was calculated using the equation:

$$\text{Transesterification activity (U/mg)} = \frac{S \times 1000}{m \times t \times y}$$

where S is the sample concentration; m is mr pNPP (417.3); t is the incubation time (min); and y is the amount of enzyme used in the reaction (mg).

MOLECULAR IDENTIFICATION OF ISOLATE NC

Molecular identification of NC was pursued due to the production of its mutant, which had the highest transesterification activity. The procedure commenced with DNA isolation of NC and PCR amplification with ITS. The nucleotide sequences were analysed using the sequence comparison tool on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). A phylogenetic tree analysis was executed using a 2-parameter Kimura Maximum Likelihood tree with a bootstrapping test of 1000 replications.

DETERMINATION OF OPTIMUM pH AND TEMPERATURE OF LIPASE ISOLATES NC MUTANTS

The study aimed to ascertain the effect of pH and temperature on lipase activity. Tests covered various pH variations (pH 5-9) and temperature ranges (30 °C-70 °C) (Banerjee et al. 2013).

RESULTS AND DISCUSSION

MUTATION WITH ULTRAVIOLET (UV) LIGHT

UV radiation was applied for 0 to 3 min to the NC mould. The selection of mutant NC mould isolates was determined based on the lethal dose values after UV radiation. The exposure to UV radiation resulted in 97.9 - 100% spore death of the NC isolate (Table 1). The lethal dose figures were obtained by observing mould growth on a PDA medium incubated for 5 days following UV radiation exposure. The UV mutation yielded four mutants from the NC isolate. The treatment resulting in a lethal dose value of ≥99% was chosen for further stages in lipase enzyme production. Consequently, the UV radiation treatment of the m4.3NC1 mutant isolate of the NC mould was selected to test its lipase activity and transesterification activity.

Lipase enzyme production was conducted by incubating 50 mL of production medium at pH 7 for 5 days in a rotary shaker at 200 rpm and 28 °C. Irradiation with UV light can cause mutations in microorganisms by disrupting the activity of enzymes and other cellular systems due to the formation of pyrimidine dimers. Pyrimidine nitrogenous bases consist of thymine (T) and cytosine (C). UV radiation can cause bonds between T-T or C-C located close together. Pyrimidine dimer bonds can obstruct DNA replication, disrupt DNA stability, and causing death in microorganisms exposed to UV radiation (Eregie et al. 2024; Goto et al. 2015). The m4.3NC1 mould yielded the highest transesterification activity compared to the other three mutants and the wild-type NC (Figure 1).

TABLE 1. Mutation results of NC isolates with UV light

Time (min)	Repeat (colony)			Number of colony	Lethal dose (%)
	1	2	3		
0	336	280	406	1022	
1	2	0	0	2	99.8
2	1	0	0	1	99.9
3	1	0	0	1	99.9

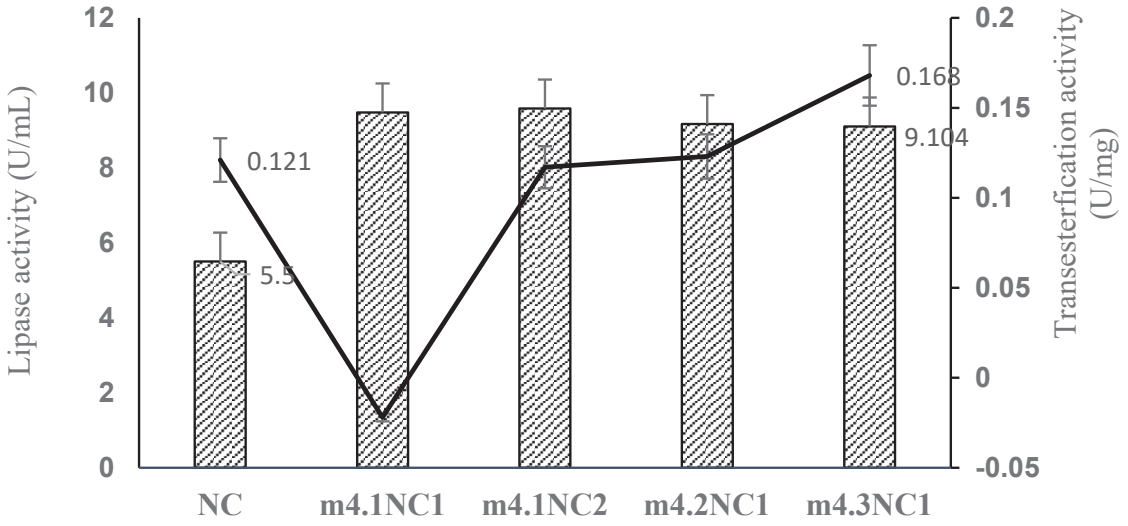

FIGURE 1. Lipase (▨) and transesterification (—) activities of NC (wild-type) and UV-mutated Nut C (NC) mould mutants, after fermentation in 50 mL Erlenmeyer working volume on 200 rpm agitation rotary shaker, t = 5 days, T = 28 °C, n=3

Previous research utilising UV radiation has shown potential to enhance lipase enzyme activity. *Rhizopus oligosporus* var. *microsporus* IIB-63-10 generated lipase enzyme activity 158.7% higher than its wild type, *R. oligosporus* var. *microsporus* IIB-63 (Iftikhar et al. 2010). *Aspergillus fumigatus* RA204, mutated using gamma radiation, exhibited a 15.85% increase in transesterification activity compared to its wild type. *Aspergillus fumigatus* RA204 exhibited a 42.11% enhancement in transesterification activity compared to its wild type after 4 h of UV radiation mutation (Indriawan 2018). UV mutation also led to a 127% increase in activity in *A. japonicus* MTCC 1975 (Karanam & Medicherla 2008).

Enhanced lipase hydrolysis activity in mutants may stem from the overexpression of genes involved in enzyme secretion. Isolate m4.3NC1 exhibited the highest lipase enzyme activity and transesterification activity, reaching 5.5 to 9.104 U/mL (65.5%) and 0.121 to 0.168 U/mg (38.8%), respectively (Figure 1). Previous studies using *R. miehei* NRRL 5282 reported a lipase activity of 735.80 U/mL, but only a transesterification activity of 4.80 U/mg (Kotogan et al. 2014). This discrepancy is attributed to the enzyme's response to the solvent used during testing. Each microorganism produces enzymes with distinct properties; thus, their response to organic solvents may hinder enzyme function. For example, when the enzyme is dispersed in an aqueous system and the substrate is dissolved in an organic system, the substrate partitions into the aqueous phase where catalysis occurs. Subsequently, the products of the reaction will move back into the organic system.

The phase separation between the organic system and the aqueous system can cause the enzyme denaturation process. This challenge can be overcome by creating microaqueous conditions, namely a reaction environment with a very limited amount or concentration of water (usually no more than 0.1% v/v) (Kumar et al. 2016). According to Kotogan et al. (2014), this phenomenon may occur due to temperature and organic compounds utilised. For example, high temperatures will reduce viscosity problems and increase the reaction rate. In selecting an organic solvent, it is necessary to consider the overall characteristics of the reaction system, especially the

polarity of the substrate and reaction products, and their possible interactions with the solvent used (Kumar et al. 2016). The transesterification activity produced by the m4.3NC1 mutant exhibited a 27.98% increase from its wild type (NC). This increased activity may be explained by the DNA changes caused by ultraviolet radiation. These changes can cause cells to undergo genetic modification to survive and may change the gene sequence coding for lipase. According to Indriawan (2018), *Aspergillus fumigatus* is more resistant to ultraviolet light than other moulds. Based on these findings, the m4.3NC1 isolate was selected for further mutation with EMS.

MUTATION OF THE m4.3NC1 ISOLATE WITH EMS

The EMS mutation was conducted by incubating the selected mutant mould resulting from the UV mutation, namely m4.3NC1, in various concentrations of EMS solutions (0%, 1%, 2%, 3%, 4%, 5%, 6%, and 7%). Treatment with varying concentrations of EMS was carried out to determine the mould's ability to survive in unsuitable environments. Microorganisms undergo genetic changes to defend themselves and survive, resulting in phenotypic differences from the original phenotype. The naming of mutant moulds was done using m5.4NC; m5 indicates the resulting mutant mould from the UV mutation followed by EMS. The number 4 after m5 signifies the EMS concentration producing a mutant with a lethal dose of 94.12% for NC, while the NC code denotes the wild type of the resulting mutant. In the EMS mutation test against m4.3NC1, one mutant isolate was produced with a mortality rate of 94.12% after incubation with EMS at a concentration of 4%. Subsequently, this isolate was coded as m5.4NC, detailed in Table 2 for further clarity.

The NC mutant mutated using EMS has a specific activity value of 24.04 U/mg (Figure 2). Meanwhile, the m5.4NC mutant experienced a decrease in specific lipase activity of up to 88% compared to the NC mutant.

TABLE 2. Results of the m4.3NC1 mutation with EMS

EMS concentration (%)	Repeat (colony)			Number of colony	The Lethal dose (%)
	1	2	3		
0	6	8	7	17	
1	7	4	3	14	17.65
2	7	2	5	14	17.65
3	1	6	4	11	35.29
4	0	0	1	1	94.12

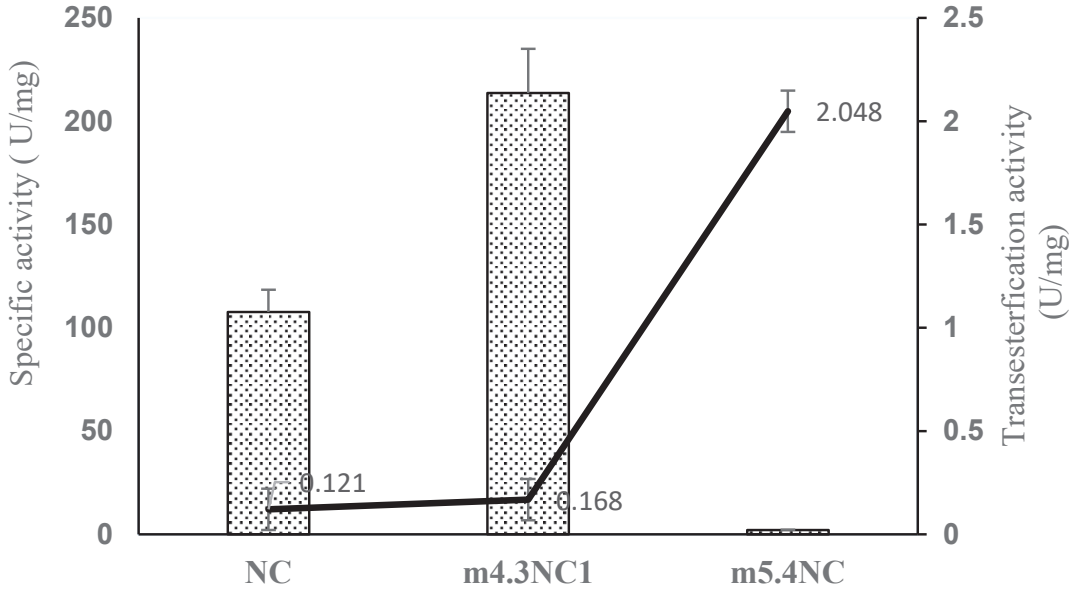

FIGURE 2. Lipase specific (▨) and transesterification (—) activities of the NC (wild-type), m4.3NC1 mutant (UV mutation only), and m5.4NC mutant (UV and EMS mutations), after fermentation in an Erlenmeyer working volume of 50 mL on a 200 rpm agitation rotary shaker, t = 5 days, T = 28 °C, n=3

The m5.4NC mutant, on the other hand, has a higher transesterification activity than the m4.3NC1 mutant. Its activity value goes from 0.168 U/mg to 2.048 U/mg, which is a 1119% or 11.2-fold increase compared to m4.3NC1. High lipase activity does not necessarily correlate with the transesterification activity produced. For instance, *Rhizopus miehei* NRRL 5282 demonstrates a lipase activity of 735.80 U/mL but performs transesterification at only 4.80 U/mg (Kotogan et al. 2014). This discrepancy is believed to stem from microorganisms producing enzymes with diverse properties, resulting in varied responses to reactions. Kotogan et al. (2014) suggest that the incongruity between lipase and transesterification activities could be due to temperature and the organic compounds used.

EMS is classified as an alkylating agent, which leads to the formation of O6-ethyl guanine by bonding its ethyl group to guanine at the 7-N and 6-O positions in DNA, resulting in replication errors. Alkylating agents induce point mutations, events that impact the phenotype of a living organism. This process occurs during point mutations, where DNA undergoes transitions, transversions, insertions, or deletions, resulting in changes in the nucleotide sequence's reading frame, subsequently altering the arrangement of formed amino acids (Sreeju et al. 2011). Living organisms possess the ability to repair damages within themselves, albeit with limitations. When DNA undergoes alkylation by EMS, there exists the MGMT enzyme, or O6-methylguanine methyltransferase, capable of directly repairing alkylated DNA. However, this enzyme is irreversible and can only repair a single altered base. Once repaired, the MGMT enzyme is promptly destroyed by the cell. Cells that fail to repair after mutation are presumed to hinder normal replication processes, leading to inherited mutations or cell death (Ifadah, Kusnadi & Wijayanti 2016).

MOLECULAR ANALYSIS OF MOULD ISOLATES NC

The molecular analysis of the NC mould isolate began with DNA isolation from NC and subsequent PCR amplification using ITS primers. The PCR amplification results were visualised by electrophoresis, yielding a DNA amplicon with a band size of approximately 600 bp (Figure 3(A)). Nucleotide bases were subjected to BLAST analysis on the NCBI, providing information regarding

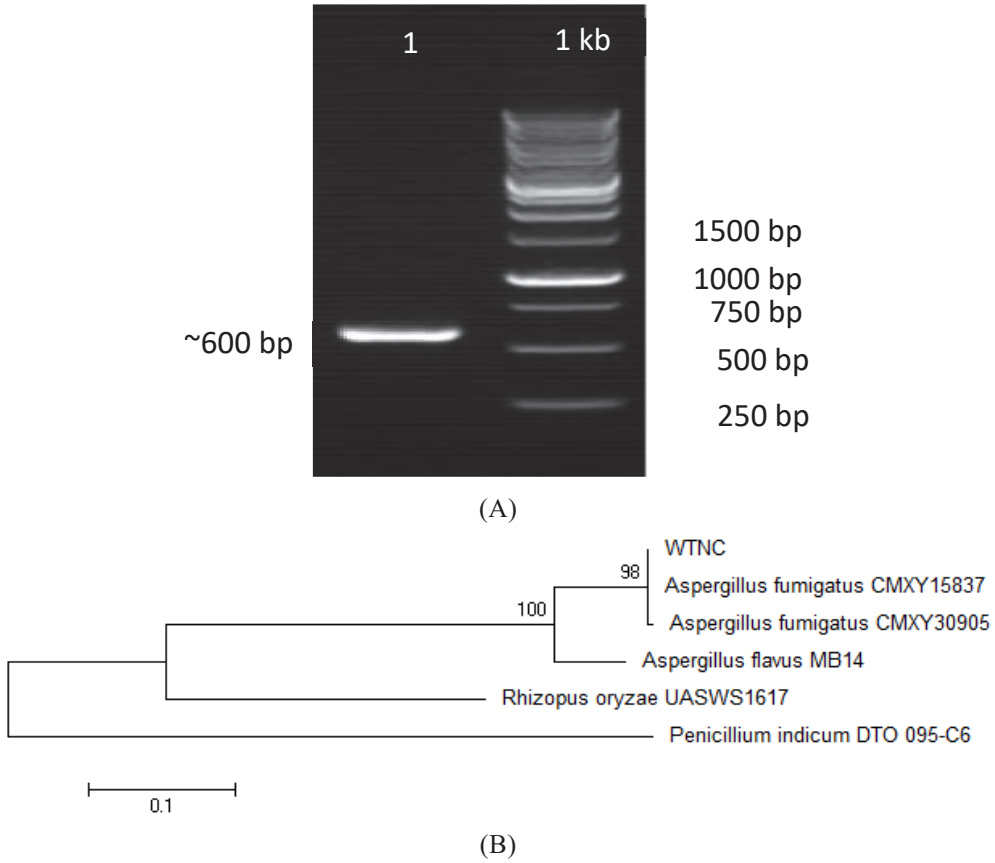

FIGURE 3. (A) Visualization of genomic DNA of NC mould isolates on 1% agarose gel electrophoresis (1 kb = Marker, well 1: NC isolate). (B) Phylogenetic tree of NC mould using Maximum Likelihood method with 1000 times bootstrap evaluation

the similarity between the tested nucleotide bases and the GenBank database. This information is outlined in Table 3. Parameters such as max score, query cover, e-value, and 'ident' were utilized to assess the BLAST results. The max score indicates the value of base pair similarity, while ident denotes the percentage of identification accuracy. The query cover displays the percentage of sample usage in the BLASTn analysis (Nugraha et al. 2014). NC displays 100% identity to *Aspergillus fumigatus* CMXY15837 with an E-value of 0.0 (Table 3), signifying close relatedness between NC and *A. fumigatus* CMXY15837.

A phylogenetic tree analysis was conducted, and the results indicated that NC comprises approximately 978 nt. The analysis indicates that NC falls within the *Aspergillus* sp. clade and is closely related to *A. fumigatus* CMXY15837 compared to *A. flavus* MB14 based on evolutionary lines within the clade. *Penicillium indicum* DTO 095-C6, exhibits a considerable genetic distance from the NC isolate (Figure 3(B)). *A. fumigatus* isolated from fruit waste holds potential in biodiesel production as a catalyst in transesterification reactions (Asci et al. 2020). Indriawan (2018) reported that *A. fumigatus* RA204 produces lipase with transesterification activity. Apart from *A. fumigatus*-produced lipase, lipase from *A. niger* and *A. niger* MYA 135 can also serve as catalysts in transesterification reactions (Collin, Baigori & Pera 2011; El-Batal et al. 2016).

DETERMINATION OF OPTIMUM pH AND TEMPERATURE OF LIPASE ACTIVITY OF THE NC MUTANT

The optimum temperature and pH for lipase activity of the wild-type NC isolate determined from Nabilasani et al. (2019) are 50 ºC and pH 6, respectively. This study aimed to examine the effects of pH and temperature on the lipase activity of the NC mutant, specifically the *A. fumigatus* m5.4NC isolate, which is 100% identical to the *A. fumigatus* strain CMXY1583. Various pH variations ranging from pH 5 to pH 9 and temperatures from 30 °C to 70 °C were employed in the assessment to determine the optimum pH and temperature for the lipase. The NC mutant lipase exhibited the highest specific lipase activity at pH 6, similar to the wild-type (Figure 4). Optimum enzyme activity at pH 6 was also observed in *Aeromonas sobria* LP004 (Lotrakul & Dharmsthiti 1997) and solid-state fermented *Aspergillus* sp. (Colla et al. 2015).

At pH levels of 7 to 9, the lipase activity experienced a continuous decline, likely due to ionisation changes affecting the substrate's bond with the active site. pH alterations can impact enzyme activity by influencing the ionisation changes within the protein structure, leading to modifications in the amino acid ionisation within the enzyme (Rajakumara et al. 2008). Amino acid residues in the enzyme structure act as substrate binders and maintain the enzyme's tertiary and quaternary structure (Sharma et al. 2002). The tertiary enzyme structure comprises hydrogen bonds, hydrophobic interactions, disulphide bridges, and ionic bonds. Ionisation changes can cause the hydrophobic segments of the tertiary structure to interact with water, reducing enzyme solubility. Reduced solubility leads to a gradual decrease in enzyme activity.

According to Shah et al. (2024), the catalytic mechanism of microbial lipase is based on a catalytic triad that is highly similar to that identified in serine hydrolases. The catalytic triad, consisting of serine, histidine, and aspartic acid residues, operates through a cyclic mechanism in which histidine activates serine to attack the substrate's carbonyl carbon. This process yields a tetrahedral intermediate and a transient acyl–enzyme complex, which is then hydrolysed by water to release the fatty acid and regenerate the active site.

Another factor that influences changes in enzyme structure and activity is temperature (Figure 5). The NC mutant lipase displayed enzyme activity at temperatures ranging from 30 °C and 70 °C, with the optimum activity observed at 50 °C, exhibiting a specific lipase activity of 128 Um/g, which is also comparable to that of wild-type. At temperatures of 60 °C and 70 °C, lipase activity decreased. Elevated temperatures enhance kinetic energy, intensifying substrate-enzyme collisions. However, excessively high temperatures can lead to enzyme denaturation (Chandra et al. 2020) and diminish enzyme activity due to conformational changes inhibiting the interaction between the active site and the substrate binding. Enzymes are categorised as proteins, and high temperatures can damage their secondary protein structure. This damage alters the protein's three-dimensional structure, making it challenging for substrates to bind to the enzyme's active site (Daniel et al. 2010).

TABLE 3. Bioinformatic analysis of ITS gene sequences isolate NC

Comparison strain (GenBank)	Max Score	Total Score	Query cover	E Value	Ident	Accession Number
Aspergillus fumigatus CMXY15837	1088	1088	100%	0.0	100%	MG991618.1
Aspergillus fumigatus CMXY30905	1083	1083	100%	0.0	99%	MG991641.1
Aspergillus fumigatus CMXY13495	1083	1083	100%	0.0	99%	MG991612.1
Aspergillus fumigatus CMXY13433	1083	1083	100%	0.0	99%	MG991611.1
Aspergillus fumigatus EMBT12	1081	1081	99%	0.0	99%	MG552677.1

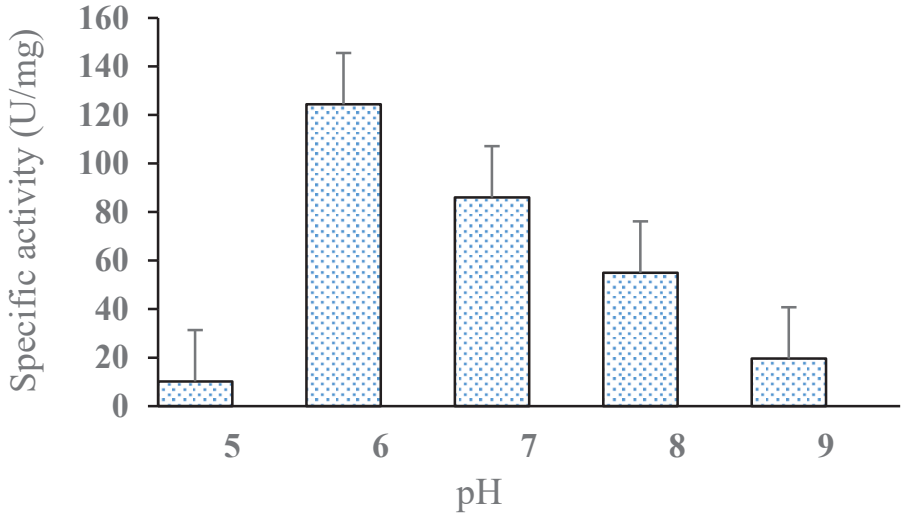

FIGURE 4. The effect of pH on the specific activity of the mutant NC *Aspergillus fumigatus* m5.4NC lipase (n=3)

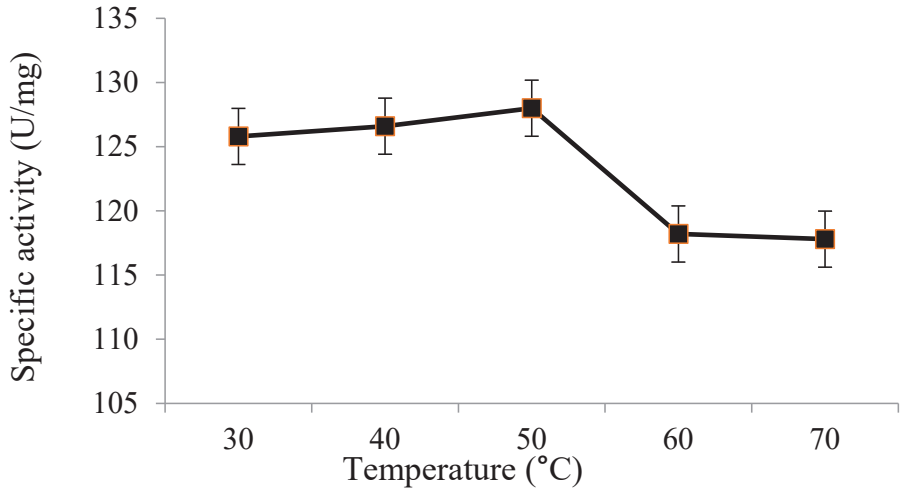

FIGURE 5. The effect of temperature on the specific activity of the mutant NC *Aspergillus fumigatus* m5.4NC lipase (n=3)

Acinetobacter calcoaceticus LP009 exhibits optimum lipase activity at pH 7 and 50 °C (Pratuangdejkul & Dharrnsthiti 2000). *A. niger* F044 produces lipase with optimal activity at pH 7 and 45 °C (Shu, Yang & Yan 2007). Meanwhile, *A. fumigatus* MTCC 9657 has the highest lipase activity at 30 °C and pH 8.5 (Rajan & Nair 2011). Another strain, *A. niger* NRLL3, has an optimum temperature of 60 °C and pH of 7.2 (Adham & Ahmed 2009), while *A. terreus* NCFT 4269.10 shows optimum lipase activity at pH 6 and 50 °C (Sethi, Nanda & Sahoo 2016). These microorganisms are capable of producing lipase, each with distinct characteristics, which can be used in various fields according to specific needs.

CONCLUSION

Mutation using UV followed by EMS on the NC mould isolated from palm kernel and palm kernel waste in Malingping, Banten, West Java, Indonesia, successfully increased its transesterification activity. The UV-induced mutant m4.3NC1 exhibited a 138.843% increase in transesterification activity compared to the wild-type NC mould, while the EMS-induced mutant m5.4NC showed a 1219% increase in transesterification activity compared to the m4.3NC1 mutant. The highest specific enzyme activity of the NC mutant was achieved at pH 6 and a temperature of 50 °C. Molecular identification of the NC isolate showed 100% similarity to the *Aspergillus fumigatus* CMXY15837 strain.

ACKNOWLEDGMENTS

This research was supported by the Center for Bioindustrial Technology (PTB), Agency for the Assessment and Application of Technology (BPPT), PUSPIPTEK Area, Serpong, South Tangerang, which provided laboratory facilities to conduct this study.

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*Corresponding author; email: tris001@brin.go.id