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MUTATION OF LIPASE-PRODUCING BACTERIAL ISOLATE FROM PALM OIL EFFLUENT FOR FAT HYDROLYSIS ON POME

Mutasi Isolat Bakteri yang Menghasilkan Lipase dari Limbah Minyak Kelapa Sawit untuk Hidrolisis Lemak pada POME

Dadang Suhendar*, Ika Rahmatul Layly, Gabriela Christy Sabbathini, Deden Rosid Waltam, Edi Wahjono, Catur Sriherwanto, Haniyya
Research Centre for Applied Microbiology, National Research and Innovation Agency, BRIN, Jln. Raya Jakarta-Bogor KM. 46, Cibinong, Bogor, West Java 16911, Indonesia
*Email: dadang.suhendar@brin.go.id

ABSTRACT
*Lipase digunakan untuk menghidrolisis minyak atau lemak yang tersisa dalam limbah cair pabrik kelapa sawit (POME), yang digunakan sebagai bahan baku dalam pembentukan biogas. Isolat bakteri (*Bacillus velezensis*) yang menunjukkan aktivitas lipase, sebelumnya diperoleh dari Malimping, Pandeglang, Banten, Indonesia, mengalami perlakuan berurutan yaitu iradiasi gamma (1, 2, dan 3 kGy), mutagenesis EMS (ethyl methane sulfonate) (0,5% v/v), dan NTG (N-methyl N-nitrosoguanidine) (1 mg mL^{-1}). Setelah setiap mutasi, aktivitas lipase dari koloni mutan diukur dan dibandingkan dengan strain tipe liar. Hasil penelitian menunjukkan bahwa semua koloni* B. velezensis *yang bermutasi menunjukkan nilai aktivitas lipase (7,78 ± 0,80 – 9,05 ± 0,23 U mL^{-1}) yang tidak berbeda signifikan dengan isolat tipe liar (8,31 ± 0,01 U mL^{-1}), menunjukkan ketahanan bakteri yang kuat terhadap perlakuan mutagenik. Preparasi lipase kasar secara efektif menghidrolisis POME, mengakibatkan penurunan nilai COD (dari 131.450 ppm menjadi 88.450 ppm) dan kandungan O&G (dari 41.400 mg L^{-1} menjadi 5.770 mg L^{-1}) dalam jangka waktu 72 jam.*

Keywords: *biogas, EMS, irradiasi gamma, lipase, NTG, POME*

ABSTRAK
Lipase is applied to hydrolyze the residual oil or fat in palm oil mill effluent (POME), which serves as a feedstock for biogas production. A bacterial isolate (*Bacillus velezensis*) exhibiting lipase activity, previously obtained from Malimping, Pandeglang, Banten, Indonesia, underwent sequential treatments of gamma irradiation (1, 2, and 3 kGy), EMS (ethyl methane sulfonate) mutagenesis (0.5% v/v), and NTG (N-methyl N-nitrosoguanidine) (1 mg mL^{-1}). Following each mutation, lipase activities of the mutant colonies were measured and compared to the wild-type strain. The results revealed that all mutated *B. velezensis* colonies exhibited lipase activity values (7.78 ± 0.80 – 9.05 ± 0.23 U mL^{-1}) that were not significantly different from the wild-type isolate (8.31 ± 0.01 U mL^{-1}), indicating strong bacterial resistance against the mutagenic treatments. The crude lipase preparation effectively hydrolyzed POME, resulting in a reduction of the COD value (from 131,450 ppm to 88,450 ppm) and O&G content (from 41,400 mg L^{-1} to 5,770 mg L^{-1}) within a 72-hour timeframe.

Kata Kunci: biogas, EMS, gamma irradiation, lipase, NTG, POME

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INTRODUCTION

Palm oil mill effluent (POME) is a liquid waste predominantly composed of water (94.2–95.3%) (Zubairi et al. 2016) generated during sterilization, extraction, and clarification processes in palm oil mills. POME poses significant environmental risks due to its elevated nutrient and phosphorous content (142 ± 19 mg L^{-1}) (Zubairi et al. 2016), as well as high levels of BOD and COD (approximately 100,000 mg L^{-1}) (Low et al. 2021). With an acidic pH range of 4.3–4.75, POME contains various constituents (mean values in mg L^{-1}), including mixed liquor suspended solids (38.070), mixed liquor volatile suspended solids (32.465), oil and grease (O&G) (16.050), total nitrogen (780), ammonia nitrogen (67.5), palmitic acid (7.460), acetic acid (2.005), propanoic acid (54.6), butanoic acid (31), and iso-butanoic acid (4.3). Additionally, it contains certain elements (mean values in %) such as carbon (47.7), hydrogen (7.2), nitrogen (1.8), sulfur (0.9), and oxygen (38) (Wong et al. 2014).

Currently, POME is commonly treated through open-lagoon systems, leading to the significant production of methane, which contributes approximately half of the total greenhouse gas emissions in conventional palm oil production (Krishnan et al. 2017). The capital and operational costs required for constructing methane-based energy generation facilities discourage many palm oil mills. As a result, methane is often flared, converting it into less harmful carbon dioxide (Krishnan et al. 2017). Therefore, further research and innovation are necessary to explore the utilization of POME as a valuable feedstock for the production of value-added products such as biogas (Aziz et al. 2020), offering potential as a renewable energy source (Khadaroo et al. 2019).

The upstream to downstream process of POME-based biogas production has been the subject of extensive research, including investigations into the most appropriate and efficient pre-treatment method (Aziz et al. 2020). Pre-treatment is essential to reduce the burden on the anaerobic digestion process, ensure sustainable biogas production, and improve the treated effluent to comply with strict environmental protocols (Khadaroo et al. 2019). However, the effective removal of residual oil in POME pre-treatment to prevent foam and other inhibitory issues during the anaerobic biogas production process remains challenging (Khadaroo et al. 2019). Consequently, there is a growing interest in the use of lipases for the hydrolytic removal of fats, oils, and grease (O&G) from the effluent due to their mild operating conditions (Baena et al. 2022). Lipases have also received considerable attention as a biological pre-treatment method for POME due to their pollution-free approach and extensive research in this area (Khadaroo et al. 2019).

Lipase enzymes for POME hydrolysis pre-treatment can be derived from microorganisms isolated from palm oil industrial waste. In a previous study, a lipase-producing bacterial isolate was obtained from the sewage sludge of a palm oil plant in Malimping, Banten, Indonesia (Layli et al. 2021). Through morphological observation, biochemical tests, and molecular biology characterization, the bacterium was identified as non-pathogenic *Bacillus velezensis*. However, the ability of this microorganism to hydrolyze fats and oils in POME has not yet been investigated. Therefore, the objective of this study is to characterize the crude lipase enzyme produced by the physically and chemically mutated *B. velezensis* isolate and evaluate its capacity for fat and oil hydrolysis in POME.

MATERIALS AND METHODS

Location and time

This study was conducted in 2018 at LAPTIAB, the National Research and Innovation Agency (BRIN), Science and Technology Park, South Tangerang, Banten, Indonesia. The gamma radiation mutation was performed with the support of the National Nuclear Energy Agency (BATAN).

Bacterial strain

In this study, *B. velezensis*, previously identified by Layli et al. (2021) as a non-pathogenic bacterium capable of lipase production, was used. The strain

was screened and isolated from palm oil processing sewage sludge in Malimping Sub-district, Lebak District, Banten Province, Indonesia (105º 1' 11" - 106º 7' 12" E; 5º 7' 50" - 7º 1' 1" S). The bacterium was subjected to stepwise mutation through gamma radiation, EMS (ethyl methane sulfonate) (Sigma, USA), and NTG (N-methyl N-nitrosoguanidine) (Sigma, USA).

Gamma radiation mutation

B. velezensis underwent mutation using gamma irradiation at the National Nuclear Energy Agency (BATAN) following the method described by Diep et al. (2016). Prior to irradiation, a cell suspension of *B. velezensis* was prepared by culturing the bacterium in LB broth medium (pH 9) at 37°C, 150 rpm, until it reached an optical density of 0.8 (logarithmic phase). A 0.1 mL portion of the bacterial suspension was spread onto LB agar media, immediately irradiated at doses of 0 (control, no irradiation), 1, 2, and 3 kGy (with a dose rate of 2.325 kGy/h), and then incubated at 37°C for 18-24 hours to allow viable irradiated bacterial cells to form colonies.

EMS-induced mutation

Chemical mutation using ethyl methane sulfonate (EMS) was conducted as described by Ifadah et al. (2011) on *B. velezensis* cells that exhibited the highest lipase activity after gamma irradiation treatment. The bacterial cells were cultured in 10 mL of LB broth media at 37 °C and 150 rpm for 18-24 hours, or until the cell suspension reached 10^8 CFU mL^{-1}. One milliliter of the cell suspension was diluted 10^4 times with reverse osmosis water, and EMS was added to achieve a final concentration of 0.5% (v/v). The mixture was incubated at 37 °C and 150 rpm for varied periods of 1, 2, 3, 4, and 5 hours. At the end of the incubation period, 5% of 0.4 M $Na_2S_2O_3$ (Merck, USA) was added to terminate the reaction. The cell suspension was then plated onto LB agar media and incubated overnight at 37 °C. The resulting colonies were counted to calculate the mortality ratio based on the number of colonies formed on control plates (without EMS treatment). The different incubation periods aimed to obtain colonies with a 90% mortality ratio, indicating successful mutant formation.

NTG-induced mutation

The previously EMS-induced colonies of *B. velezensis* with a mortality rate of ≥ 90% were subjected to further mutation using N-methyl N-nitrosoguanidine (NTG), following the methods described by Ifadah et al. (2011) and Ohinisi et al. (2008). The procedure was identical to the EMS-induced mutation, with the addition of NTG at a concentration of 1 mg mL^{-1} in a 0.2 M phosphate buffer (pH 7), and the incubation period was 4 hours.

Crude lipase production

Lipase activity of *B. velezensis* was measured after each mutation. The mutated cells were grown in a specific medium designed to induce lipase production, namely LB broth supplemented with 1% olive oil. The production process began by preparing a starter culture, where *B. velezensis* was grown in 10 mL of LB broth medium and incubated at 37 °C and 150 rpm until the optical density reached 0.8. Subsequently, 5% of the starter culture was transferred into the production medium and incubated at 37 °C and 150 rpm for 18 hours or overnight. The resulting cell suspension was centrifuged at 6000 rpm, 4 °C, for 10 minutes to obtain the supernatant (crude lipase enzyme) for lipase activity and POME hydrolysis assays.

Lipase activity test

Lipase activity was determined following the method of Kojima and Shimizu (2003) with slight modifications. A substrate mixture comprising 1.5% polyvinyl alcohol (PVA) (Sigma, USA), 25% olive oil (Le Riche), and distilled water (5 mL) was combined with 4 mL of 0.05 M Tris-HCl buffer, pH 8 (Merck), and 1 mL of the lipase enzyme. The mixture was then incubated at 37 °C, 150 rpm for 20 minutes. Subsequently, 5 mL of methanol (Merck, USA) and 2 drops of PP indicator (Merck) were added, followed by titration against 0.05 M NaOH (Merck, USA). Lipase activity (U mL^{-1}) was calculated using the formula:

$$(\mathrm{U}) = \frac{V_2 - V_1 \times \mathrm{n\ NaOH} \times 1000}{\mathrm{t}}$$

Where:
V1 = NaOH volume used in the blank titration (mL)
V2 = NaOH volume used in the sample titration (mL)
n = NaOH concentration (N)
t = incubation period (minutes)

Enzymatic hydrolysis of POME

Enzymatic hydrolysis of POME was conducted according to a previously described method (Muanruksa et al. 2021). A volume of 250 mL of POME was mixed with crude lipase at a concentration of 4% (v/v) in a cotton-plugged 500-mL Erlenmeyer flask. The flask was then incubated at room temperature (approximately 28 °C), 150 rpm for 96 hours, and samples were collected at 0, 24, 48, 72, and 96-hour time points. The resulting hydrolyzed POME samples were subjected to measurements of Chemical Oxygen Demand (COD) and oil and grease (O&G).

COD analysis

COD measurement was performed following the Indonesian National Standard method SNI 06-6989.2-2004 (BSN, 2004). Approximately 625 µL of the hydrolyzed POME sample was transferred to a vial bottle, and 375 µL of digestive solution (BSN, 2004) and 875 µL of sulfuric acid reactant (BSN, 2004) (Merck, USA) were added. The mixture was heated at 150 °C for 2 hours, cooled to room temperature, and then analyzed using a UV-Vis spectrophotometer at a wavelength of 620 nm. The absorbance values were obtained and plotted on a standard curve to determine the COD concentration.

O&G analysis (Oil and Grease)

Oil and grease determination was conducted according to the Indonesian National Standard method SNI 6989.10:2011 (BSN, 2011). Approximately 15 mL of the hydrolyzed POME sample in a separating funnel was mixed drop-wise with HCl (Merck, USA) until the pH was below 2. Then, 15 mL of n-hexane (Merck, USA) was added and mixed for 2 minutes to achieve homogeneity. The solvent layer was separated through a funnel with a filter paper (Whatman, USA). The remaining aqueous layer was extracted twice by adding 15 mL of n-hexane successively. The extracts were then distilled at 60 °C until no more water droplets were observed. Finally, the evaporator tube containing the sample was cooled in a desiccator and weighed.

Data analysis

Data analysis was performed using analysis of variance (ANOVA) at a significance level of 5%.

RESULTS AND DISCUSSION

Effect of gamma irradiation, EMS, and NTG-induced mutation

Gamma irradiation at doses of 1, 2, and 3 kGy did not result in any morphological changes in the *B. velezensis* isolate obtained from palm oil processing sewage sludge. The colonies remained round, yellow, and slimy, exhibiting high density on LB agar petri dishes for all radiation treatments (Figure 1). The viability of *B. velezensis* appeared unaffected by gamma radiation.

Following gamma irradiation, the average lipase activity of *B. velezensis* increased, with higher doses correlating to higher lipase activities (Table 1). The highest lipase activity recorded was 8.75 U mL^{-1} (5.3% higher than the non-irradiated sample), observed when the bacteria were exposed to 3 kGy gamma irradiation. However, when considering the standard deviation and the results of ANOVA statistical analysis, the lipase activities exhibited insignificantly different values among all radiation doses. Subsequently, the *B. velezensis* isolates irradiated at 3 kGy were designated as mutant strains and subjected to chemical mutagenesis using EMS.

Table 1. Lipase activity of *B. velezensis* after gamma irradiation treatment ($\bar{x} \pm \sigma$, n = 2)

Gamma Irradiation Dose (kGy)	Lipase Activity (U mL^{-1})
0 (wild-type)	8.31 ± 0.01[a]
1	8.44 ± 0.58[a]
2	8.64 ± 0.45[a]
3	8.75 ± 0.31[a]

Note: Values with the same superscript alphabets are not significantly different (P > 0.05).

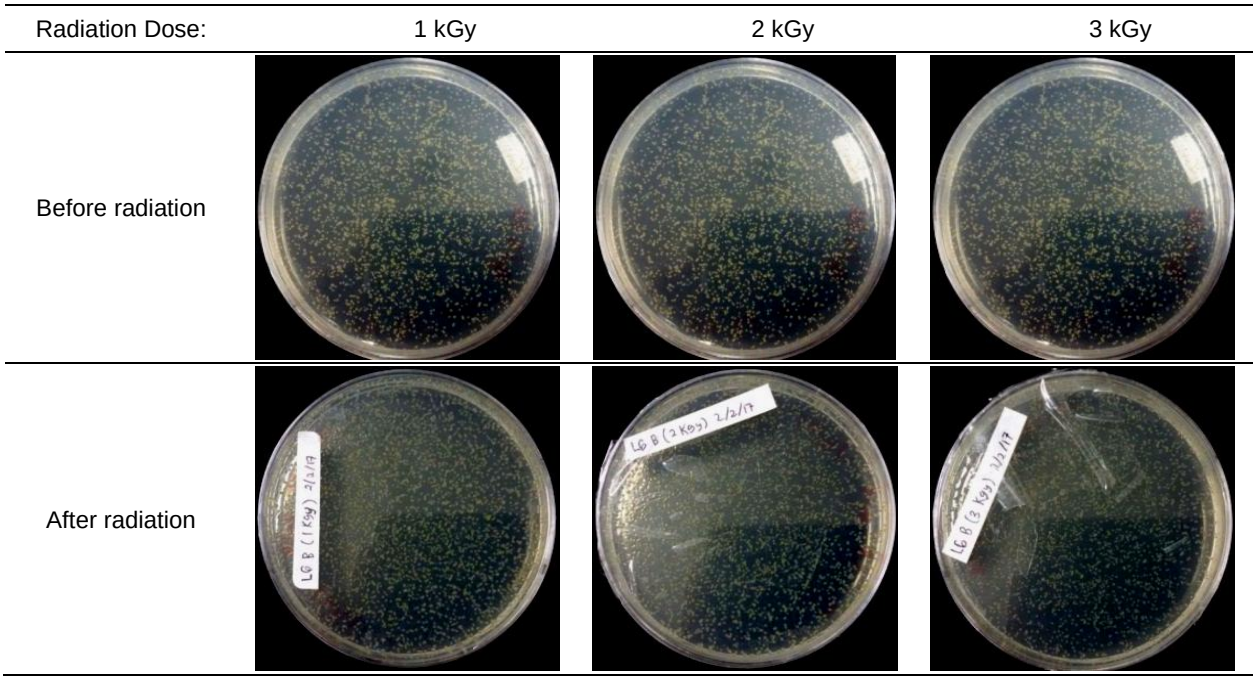

Figure 1. Colony density of *B. velezensis* before and after gamma irradiation

The *B. velezensis* mutant treated with 0.5% EMS exhibited significantly lower colony formation (viability) compared to the untreated sample across all tested incubation periods. Prolonged exposure to EMS appeared to increase the mortality rate (Figure 2 and Table 2). Mortality ratios exceeding 90% were observed after 4 and 5 hours of treatment with the chemical mutagen.

Thirteen surviving mutated colonies were obtained after 0.5% EMS mutation treatment for 4 hours (colonies 1-6) and 5 hours (colonies 7-13). These colonies were tested for lipase activity to assess the impact of chemical mutagenesis. Lipase activity values ranged from 8.73 to 9.05 U mL^{-1} across all colonies, with no significant differences observed between them or between the 4-hour and 5-hour treatments (Table 3). These mean values were also not significantly different from those of gamma-irradiated *B. velezensis* mutants or the wild-type strain. Further efforts to enhance lipase activity involved subjecting the *B. velezensis* mutant, which experienced more than 90% mortality rates with EMS, to chemical mutation using NTG.

The 13 colonies of 0.5% EMS-screened *B. velezensis* mutant were further subjected to 1 mg mL^{-1} NTG mutagen for 1, 2, 3, and 4 h. The treatment for 3-4 h resulted in the highest mortality ratio of above 90%, as demonstrated by colony 3 (Table 4). Thus, colony 3, which underwent 3-h NTG treatment, was considered mutated, and thus selected for lipase activity assay.

Five randomly selected colonies (labelled as colony A-E), obtained from the

Table 2. The mortality ratios of *B. velezensis* mutant colonies after 0.5% EMS treatment at different incubation periods ($\bar{x} \pm \sigma$, n = 2)

Incubation Period (h) of EMS Treatment	Number of colony[#]		EMS-Induced Mortality[##] Ratio (%)
	Control	Treatment	
1	52.5 ± 2.1^{a}	11.0 ± 0.0^{b}	79.03 ± 0.85^{p}
2	37.5 ± 4.9^{c}	13.0 ± 1.4^{d}	65.28 ± 0.81^{p}
3	86.5 ± 2.1^{e}	15.5 ± 0.7^{f}	82.09 ± 0.38^{q}
4	74.0 ± 8.5^{g}	6.5 ± 0.7^{h}	91.10 ± 1.98^{r}
5	103.5 ± 2.1^{i}	7.5 ± 0.7^{j}	92.76 ± 0.53^{r}

Note: Values compared within each row ([#]) or each column ([##]) with the same superscript alphabets are not significantly different (P > 0.05).

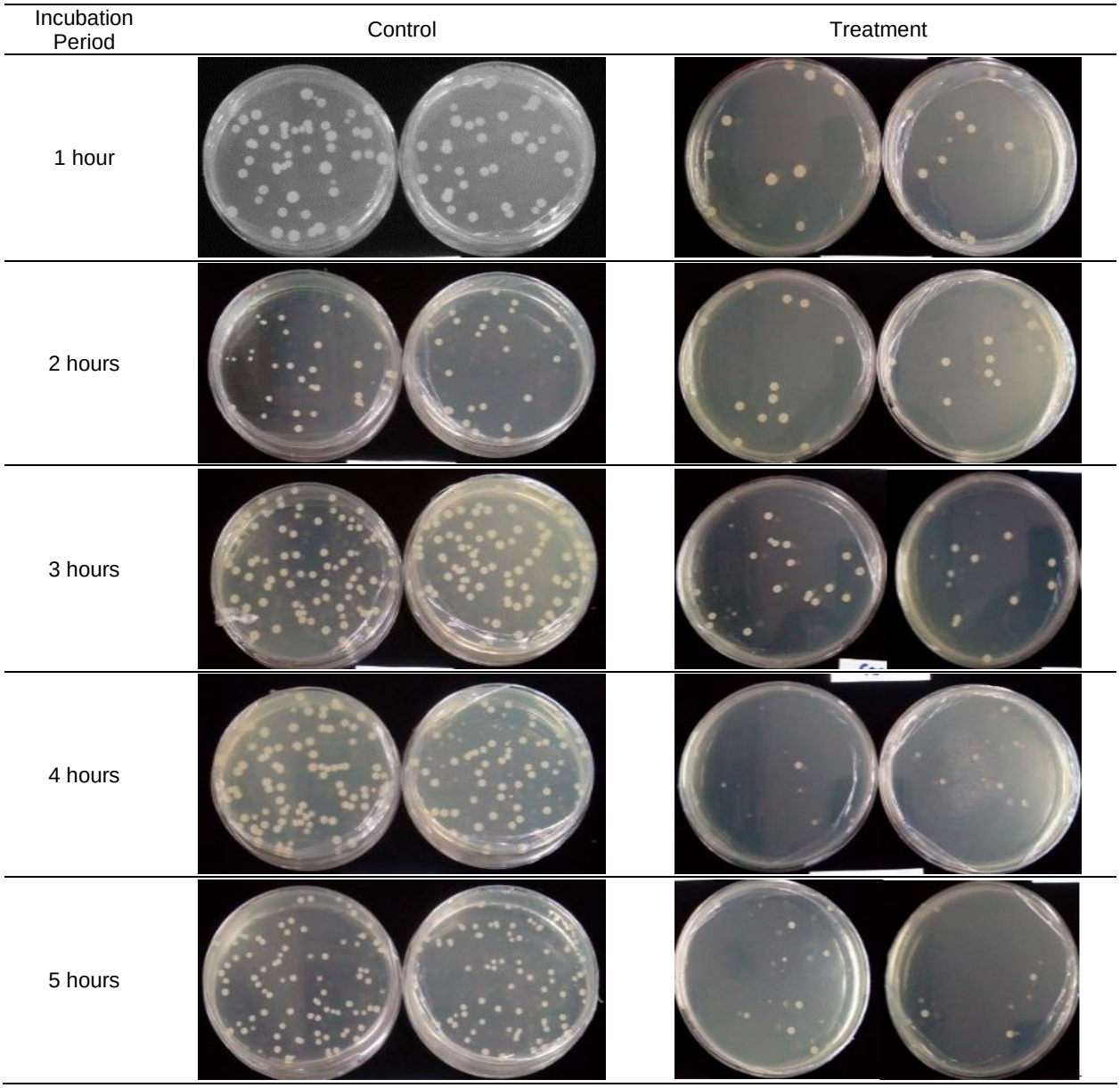

Figure 2. Colony density of *B. velezensis* mutant after treatment with 0.5% EMS mutagen at different incubation periods.

subculture of the NTG-mutated *B. velezensis* (colony 3), were tested for improvement in lipase enzyme activity (Table 5).

Although NTG-mutated colony B exhibited the highest lipase activity of 9 U mL^{-1}, statistical analysis revealed no significant difference among the tested colonies in terms of lipase activity, including when compared to the *B. velezensis* wild-type (Table 1). Therefore, colony B was arbitrarily chosen for application in the hydrolysis of POME.

Although several studies on mutation in *B. velezensis* have been reported (Cai et al. 2017; Kim et al. 2020), to the best of our knowledge, this study represents the first attempt to subject *B. velezensis* to sequential mutations using gamma irradiation, EMS, and NTG with the objective of enhancing bacterial lipase activity. However, gamma irradiation did not induce any mutations, while both EMS and NTG-induced mutations had no significant impact on the enzyme activity of the mutated *B. velezensis* compared to the parental strain. This finding contrasts with a previous report that obtained fungal lipase with nearly two-fold higher activity than the wild-type strain following 0.14-kGy gamma irradiation of an *Aspergillus niger* isolate (El-Batal et al. 2015). Similarly, enhanced lipase productivity was observed when UV and NTG were employed as mutagenic agents on *Rhizopus* sp. BTS-24

Table 3. Lipase activity of *B. velezensis* mutant after 0.5% EMS treatment for 4-h and 5-h incubation periods ($\bar{x} \pm \sigma$, n = 2)

EMS-Mutated Colony (4-h Incubation)	Lipase Activity (U mL^{-1})
Colony 1	8.83 ± 0.08[a]
Colony 2	8.91 ± 0.83[a]
Colony 3	8.92 ± 0.84[a]
Colony 4	8.71 ± 0.26[a]
Colony 5	8.89 ± 0.17[a]
Colony 6	9.03 ± 0.77[a]
Means:	8.88 ± 0.45[a]

EMS-Mutated Colony (5-h Incubation)	Lipase Activity (U mL^{-1})
Colony 7	9.05 ± 0.23a
Colony 8	8.87 ± 0.35a
Colony 9	8.73 ± 0.31a
Colony 10	9.03 ± 0.59a
Colony 11	8.81 ± 0.41a
Colony 12	9.02 ± 1.13a
Colony 13	9.02 ± 0.82a
Means:	8.93 ± 0.47a

Note: Values within all columns and rows with the same superscript alphabets are not significantly different (P > 0.05).

Table 4. The mortality ratio of *B. velezensis* mutant colonies after treatment with 1 mg mL^{-1} NTG mutagen at 3-4 h incubation periods ($\bar{x} \pm \sigma$, n = 2)

EMS-screened *B. velezensis* mutant	NTG Induced Mortality Ratio (%) at 3-4 h
Colony 1	65.00 ± 7.07[ac]
Colony 2	52.50 ± 3.54[a]
Colony 3	91.00 ± 5.66[bd]
Colony 4	83.50 ± 4.95[cd]
Colony 5	55.00 ± 7.07[a]
Colony 6	57.50 ± 3.54[a]

EMS-screened *B. velezensis* mutant	NTG Induced Mortality Ratio (%) at 3-4 h
Colony 7	72.50 ± 3.54[a]
Colony 8	62.00 ± 9.90[ab]
Colony 9	81.00 ± 5.66[ac]
Colony 10	62.50 ± 3.54[ab]
Colony 11	82.00 ± 2.83[ab]
Colony 12	60.00 ± 7.07[ab]
Colony 13	39.00 ± 8.49[bd]

Note: Values within all columns and rows with the same superscript alphabets are not significantly different (P > 0.05).

(Bapiraju et al. 2005). Additionally, a *Yarrowia lipolytica* mutant with 10.5-fold higher lipase productivity than the wild-type strain was generated using EMS and UV-light (Darvishi et al. 2011).

The resistance of *B. velezensis* against gamma irradiation, as demonstrated in this study, is a trait shared by other microorganisms previously reported (Kim et al. 2018; Lee et al. 2021; Lee et al. 2013; Sathiyaraj et al. 2018; Shirsalimian et al. 2018). Gamma irradiation eliminates bacteria by damaging their DNA and halting bacterial division (Khandpur, 2019). If such molecular and subsequent biological damage did not occur to the irradiated microorganisms, several factors could explain the resistance phenomenon, such as the irradiation dosage being too low or the protective sporulation ability of the microbes. It has been demonstrated that gamma irradiation at 5 kGy or higher rendered fungi and coliform bacteria undetectable as microbial contaminants in bee pollen, whereas yeasts

Table 5. Lipase activity of EMS-screened *B. velezensis* mutant after treatment with 1 mg mL^{-1} NTG mutagen at 3-h incubation period ($\bar{x} \pm \sigma$, n = 2)

NTG-Mutated Colony	Lipase Activity (U mL^{-1})
Colony A	7.78 ± 0.80[a]
Colony B	8.99 ± 0.17[a]
Colony C	8.69 ± 0.35[a]
Colony D	8.03 ± 0.29[a]
Colony E	7.90 ± 0.64[a]

Note: Values within each column with the same superscript alphabets are not significantly different (P > 0.05).

and aerobic bacteria were found to be more resistant and were only no longer detected at 7 kGy or higher (Álvarez Hidalgo et al. 2020). The study also identified spore-forming bacterial species as the most resistant to gamma irradiation. Hence, the combination of *B. velezensis'* sporulation ability (Xu et al. 2022) and low-dosage gamma irradiation (1-3 kGy) might elucidate the resistance exhibited by the Bacillus strain utilized in this investigation.

Hydrolysis of POME

The effectiveness of enzymatic hydrolysis of POME was evaluated by measuring the reduction in COD and O&G values. The application of crude enzyme preparation from selected mutants (B colonies) with enzymatic activity of 9 U mL^{-1} resulted in a 33% reduction in COD, decreasing the initial concentration of 131,450 ppm to 88,450 ppm within 72 hours (Table 6). Simultaneously, the lipase reduced the O&G values by 86%, from 41,400 mg L^{-1} to 5,770 mg L^{-1} (Table 7).

Compared to previous studies, the crude lipase preparation in this study achieved a lower COD removal rate (33%) but a higher O&G reduction rate (86%). For instance, a co-culture of yeast *Magnusiomyces spicifer* AW2 and bacterium *Staphylococcus hominis* exhibited higher COD removal (75.9 ± 2.8%) but lower O&G reduction (80.1 ± 1.3%) (Fibriana et al. 2021). Similarly, the indigenous yeast *M. guilliermondii* achieved higher O&G removal (92.4%) through its lipase activity (Ganapathy et al. 2019). Furthermore, the use of indigenous mixed microbial consortium comprising fungi and bacteria from the genera *Micrococcus, Stenotrophomonas, Bacillus, Providencia, Klebsiella, Aspergillus, and Meyerozyma* resulted in a 91.06% COD reduction when whole microbial cells were utilized instead of lipase (Bala et al. 2018). These findings indicate that microbial consortium and whole microbial cells are more effective than crude lipase preparation in reducing COD and O&G in POME.

Table 6. COD value during POME hydrolysis

Sampling Period (h)	Concentration (ppm)	COD Reduction Rate (%)
0	131,450	-
24	126,450	3.8
48	92,950	26.5
72	88,450	4.8

Table 7. O&G value (oil & grease)

Sampling Period (h)	Oil content (mg L^{-1})	O&G Reduction Rate (%)
0	41.400	-
24	19.100	54
48	13.440	30
72	5.770	57

The lipase produced by *B. velezensis* treated with EMS, a mutagen known for inducing random mutations through nucleotide substitution (guanine alkylation) in DNA and RNA (Yan et al. 2021), did not exhibit higher enzyme activity. This finding contradicts previous studies where EMS mutagenesis successfully yielded fungal lipases with superior activities compared to wild types. For instance, EMS-treated *Aspergillus eucalypticola* generated a mutated fungal strain with approximately 1.3-fold higher lipase activity than the untreated strain (Shreya et al. 2022). *Aspergillus tamarii* and *Aspergillus flavus* mutants obtained through EMS treatment displayed enhanced waste frying oil transesterification abilities (Elhussiny et al. 2020a). Similarly, EMS-mutated *Rhizopus americanus* and *Rhizopus stolonifer* also exhibited improved transesterification compared to their respective wild types (Elhussiny et al. 2020b). While exclusive use of EMS without additional mutagenic treatment worked well on fungi, it may not have the same effect on bacteria, as demonstrated in this study, in terms of generating mutants with improved lipase activities.

This study showed that NTG treatment resulted in mutated *B. velezensis* strains without a significant enhancement in lipase activity, contrary to previously published findings. For example, a *Streptomyces* sp. mutant was obtained following NTG-UV mutagenesis, exhibiting a lipase activity 12.6-fold higher than the unmutated strain (Mo et al. 2013). The different NTG treatment and microorganism used in this study compared to the previous report may account for the observed results. Instead of UV, gamma irradiation was employed, which might be less effective in facilitating

subsequent chemical mutation using NTG to generate lipases with improved activity.

CONCLUSION

All of the mutated *B. velezensis* colonies exhibited lipase activity values (7.78 ± 0.80 – 9.05 ± 0.23 U mL^{-1}) that were not significantly different from those of the wild-type isolate (8.31 ± 0.01 U mL^{-1}), indicating the strong resistance of the bacteria against the mutagenic treatments. The crude lipase preparation successfully hydrolyzed POME, resulting in a reduction of the COD value from 131,450 ppm to 88,450 ppm and the O&G content from 41,400 mg L^{-1} to 5,770 mg L^{-1} within 72 hours.

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