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Bioprospecting of *Bacillus* species from East Kalimantan, Indonesia for developing sustainable mosquito larvicides

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Abstract. *Hariani N, Budiman, Patang F, Yuliatin E. 2025. Bioprospecting of* Bacillus *species from East Kalimantan, Indonesia for developing sustainable mosquito larvicides. Biodiversitas 26: 1360-1366.* Climate change, unplanned urbanization, environmental pollution, and the development of insecticide-resistant mosquito populations have increased Vector-Borne Disease (VBD) incidents in tropical and subtropical countries. They require an innovative and sustainable action strategy. As the endemic area of VBDs, East Kalimantan stores unexplored biocontrol agents such as *Bacillus* species. Therefore, this study tested the toxicity of nine single and three mixed isolates of *Bacillus* from East Kalimantan against mosquito larvae of both *Aedes aegypti* and non-*Ae. aegypti* species; and identified the selected *Bacillus* isolates using a 16S rDNA sequence approach. Five single isolates of *Bacillus* from East Kalimantan (BP1, BP2, BP3, BF2, and BF3) and their consortia (BPM, BFM, and BFP) decreased the larvae number over time. They demonstrated high effectiveness in reducing *Ae. aegypti* and non-*Ae. aegypti* larvae population with more than 50% mortality at 48 hours of exposure. However, utilizing a single isolate of *Bacillus* as a biocontrol agent against mosquitoes was preferred over using mixed isolates for efficiency in larvacide production, offering a sustainable alternative to chemical insecticides. Moreover, *Bacillus*-BP3 and *Bacillus*-BF2 isolates from East Kalimantan, identified as *Bacillus subtilis*, were strong and safe candidates for developing sustainable larvicides to control mosquito populations with larvae mortality percentage up to 100% in 24 hours, mainly *Ae. aegypti*. Other isolates were identified as *Bacillus cereus* (BP1) and *Bacillus tropicus* (BP2), which also showed high and consistent larvacide activity from the beginning of the exposure.

Keywords: 16s rDNA sequence, *Aedes aegypti*, *Bacillus subtilis*, larvae mortality, non-*Ae. aegypti* species

INTRODUCTION

Vector-Borne Diseases (VBDs) such as malaria, Japanese encephalitis, chikungunya, Zika, and dengue fever remain a significant threat to public health, especially in tropical areas (Golding et al. 2015; Wilson et al. 2020; Manikandan et al. 2023) such as East Kalimantan, Indonesia. These VBDs have caused more than 700,000 deaths each year (WHO 2020). Mosquitoes, the primary vectors of VBDs in humans, carry microorganisms or viruses that can cause diseases with high morbidity and mortality (Devi and Raju 2018; Gachelin et al. 2018). For example, dengue fever is transmitted to humans through the bite of a female *Aedes aegypti* infected with the dengue virus (Christofferson et al. 2022). Conditions like uncontrolled human population growth, unplanned urbanization, unhealthy housing, polluted environments, and climate change can create ideal habitats for mosquito reproduction and increase the population of infected mosquitoes, but with your expertise and interventions, these can be mitigated, reducing the risk of VBD transmission (Chala and Hamde 2021; Biswas 2022).

Integrated vector control is crucial for breaking the mosquito life cycle and preventing virus transmission concerning controlling VBDs (Wilson et al. 2020). In Indonesia, vector control typically involves fogging and spraying insecticides to eliminate mosquitoes. However, this approach is only effective for adult mosquitoes (Ridha et al. 2023). Additionally, fogging is ineffective against mosquito larvae in stagnant water (Usuga et al. 2019). Synthetic insecticides, especially those from the organophosphate and pyrethroid groups, during fogging, have also been linked to the development of mosquito resistance over time (Abeyasuriya et al. 2017; Usuga et al. 2019). Consequently, vector control strategies that rely on fogging require biennial assessments of mosquito insecticide resistance, making this method less effective in curbing the spread of VBDs (Richards et al. 2020). The current global context, characterized by rising socio-economic and environmental challenges coupled with the emergence of insecticide-resistant mosquito populations, highlights the pressing need for innovative and sustainable mosquito control methods. Developing these new strategies is vital for effectively reducing the incidence of VBDs (Moyes et al. 2021). This area of research is critical for advancing vector control and public health initiatives.

Microorganisms are being used to develop larvicides as a sustainable way to target mosquitoes in both the adult and larval life stages (De Melo Katak et al. 2023). *Bacillus*, Gram-positive bacteria species, have been extensively studied as biocontrol agents that protect soil and plants in

agroecosystems (Balakrishnan et al. 2015). The most well-known and used species is *Bacillus thuringiensis* (Bt), which produces toxic protein crystals for a specific range of insects and microbes. The proteins are known as cry toxins, classified as pore-forming toxins. They are initially water-soluble proteins that undergo a series of changes until they enter the host cell and act like a classical insect anti-growth hormone (Adang et al. 2014; Wu et al. 2021). It occurs naturally and is harmless to plants, humans, and non-target organisms, so it is an environmentally safe choice for developing a sustainable mosquito control agent (Rubio-Infante and Moreno-Fierros 2015).

Numerous studies have demonstrated the efficacy of *B. thuringiensis* in killing mosquito larvae, particularly *Ae. aegypti* (Land et al. 2023). However, more information must be provided regarding bioprospecting other *Bacillus* species, mainly from East Kalimantan, for larvacides against mosquitoes. As one of the endemic areas of dengue fever, East Kalimantan reserves numerous uncharacterized *Bacillus* species that may have similar larvacidal toxicity as *B. thuringiensis* in controlling not only *Ae. aegypti* but also other mosquito species. For example, metagenomic analysis showed that the rhizosphere of oil palm plantations and secondary forests in East Kalimantan reserved more than 2500 individuals of *B. thuringiensis* and some other *Bacillus* species (Yuliatin et al. 2025). Therefore, the study on exploring novel *Bacillus* species from East Kalimantan or other VBDs endemic areas and their prospects as larvicides are required for developing new, effective, and eco-friendly *Bacillus*-based larvacides to control the population of either *Ae. aegypti* or other mosquito species. As part of developing novel sustainable larvicides, this study aimed (i) to test the performance of single and mixed isolates of *Bacillus* from East Kalimantan against mosquito larvae and (ii) to identify the selected *Bacillus* isolates using a 16S rDNA sequence approach.

MATERIALS AND METHODS

Starter cultures and consortium preparation

Nine *Bacillus* isolates (BP1, BP2, BP3, BP4, BP5, BF2, BF3, BF4, and BF5) were isolated from the rhizosphere in the palm oil plantation (BP) and secondary forest (BF) in Samarinda, East Kalimantan, referring to the method of Patel et al. (2013) with modifications. One loop of each isolate was inoculated into every 50 mL of Luria Bertani (LB) medium. All starter cultures were agitated for 24 hours at 120 rpm at 28°C. The starter cultures' optical density and age were measured by spectrophotometer (λ=600 nm; Falqueto et al. 2021). The targeted OD for all bacterial cultures was approximately 1-1.2, with similar bacterial ages. Afterward, we formulated three different consortium bacteria, namely BPM (mixed BP1, BP2, and BP3), BFM (mixed BF2 and BF3), and BFP (mixed BP1, BP2, BP3, BF2, and BF3). Those consortia have been tested for synergistic and antagonistic. For consortia prep, the starter culture of 20% (w/v) of those selected isolates was inoculated into a new medium of Luria Bertani Broth 80% (w/v); hence, the total volume, including bacterial culture, was 100 mL. The bacterial culture was shaken again at 120 rpm for 24 hours at room temperature. This procedure was followed by Suharjono and Yuliatin (2022) with modification. In the end, this study tested 12 starter cultures: nine single isolates (BP1, BP2, BP3, BP4, BP5, BF2, BF3, BF4, and BF5) and three mixed isolates (BPM, BFM, and BFP).

Mosquito larvae preparation

Mosquito larvae samples were collected from three locations in Samarinda, East Kalimantan, Indonesia: Samarinda Utara, Sungai Pinang, and Sungai Kunjang sub-districts. Those locations were recorded as the highest cases of dengue fever in the last five years in Samarinda. Mosquito larvae samples were collected from water reservoirs and open containers (rainwater) found around residents' houses. Larva samples (mixed species of mosquitoes) were separated into two groups: *Ae. aegypti* larvae and non-*Ae. aegypti* larvae. *Ae. aegypti* larvae were identified based on several distinguishing features, namely having a siphon for the respiratory process, which is large, fat, and short in size, dark in color, with no fine hairs, and located in the last abdominal segment. On the eighth segment is a row of 8-12 comb teeth shaped like a crown. When resting or breathing, the position in the water is perpendicular to the surface. Each segment has a pair of fine hairs. For rearing purposes, each larvae group was placed in a new, clean container filled with rainwater. Using rainwater to rear mosquito larvae was preferred because it closely mimics their natural breeding habitats and avoids the potential effects of chlorine or other chemicals in tap water (Gerberg et al. 1994). During the rearing, the room temperature was maintained at around 28°C. They were fed groundfish pellets to support larval development without overfeeding. Rearing containers were regularly cleaned to prevent contamination, and airflow was controlled to maintain stable conditions. These carefully controlled parameters mimic natural habitats, promoting healthy larval development and reliable experimental outcomes. For the bioassay test, the larvae were third instar larvae (length ±5 mm, spines on the thorax, starting to become apparent). After the larvae were homogeneous in size and have been re-acclimatized, they were ready to be used for the bioassay test.

Toxicity test of *Bacillus* isolates against mosquito larvae

The procedure was set up to toxicity-test 12 different *Bacillus* isolates, namely BP1, BP2, BP3, BP4, BP5, BF2, BF3, BF4, BF5, BPM, BFM, and BFP on *Ae. aegypti* and non-*Ae. aegypti* larvae. There were three replicates of each bioassay, one each of 10-third instar larvae suspended in 100 mL of rainwater in disposable cups. Toxicity tests were performed with the dipping method, whereby 0.5 mL of 100% concentration starter cultures were dipped in each cup. Dead larvae were recorded at 4, 8, 24, and 48 hours intervals.

Identification of the selected *Bacillus* isolates based on 16S rDNA sequence

The chromosomal DNA of the selected *Bacillus* isolates was extracted using the Quick-DNA Magbed Plus Kit (Zymo Research, D4082) https://zymoresearch.eu/ products/ quick-dna-magbead-plus-kit. Bacterial DNA was amplified

using the MyTaq HS Red Mix kit (Bioline, BIO-25048) (https://www.bioline.com/mytaq-hs-red-mix.html) with universal primers 27f (5'-GAG AGT TTG CTG GCT CAG-3') and 1429r (5'-CTA CGG CTA TGT TAC GA-3'). The master mix bacterial suspension was 25 µL, with the composition: Nuclease-Free Water (9.5 µL), MyTaq HS Red Mix, 2x (12.5 µL), Primer 27f 10 µM (1.0 µL), Primer 1492r 10 µM (1.0 µL), and bacterial DNA template 15 µM (1.0 µL). Subsequently, the bacterial DNA was run with the PCR (cycle program: initial denaturation (95°C, 1 minute); 35 cycles: denaturation (95°C, 15 seconds), annealing (55°C, 15 seconds), extension (72°C, 10 seconds); final extension (72°C, 7 minutes)). The 16S rDNA amplicon was verified using electrophoresis. Bidirectional sequencing was conducted using the Sanger DNA sequencing with the Capillary Electrophoresis method (Eren et al. 2022).

Data analysis

The mortality percentage of *Ae. aegypti* and non-*Ae. aegypti* larvae at 0, 4, 8, 24, and 48 hours following inoculation with different *Bacillus* isolates were analyzed using descriptive statistics to determine their larvacidal toxicity. Moreover, the bacterial DNA sequences of the selected *Bacillus* isolates were analyzed bioinformatically using Sanger Sequencing, and the construction of bacterial phylogenetic trees was aligned with the ClustalW Multiple Alignment program MEGA V.11, constructed and inferred using the Neighbor-Joining (NJ) algorithm and analyzed the evolutionary distance matrix using the Tamura-Nei model using 1000 times bootstrap (Tamura et al. 2021).

RESULTS AND DISCUSSION

Performance of *Bacillus* isolates against mosquito larvae

Based on the toxicity test, five isolates of *Bacillus* from East Kalimantan reduced more than half of the initial population of *Ae. aegypti* and non-*Ae. aegypti* larvae within 48 hours. Those *Bacillus* isolates were BP1, BP2, BP3, BF2, and BF3. The consortium from those isolates (BFM, BPM, and BFP) also showed larvicidal activity as a single isolate. Meanwhile, isolates *Bacillus*-BP4, *Bacillus*-BP5, *Bacillus*-BF4, and *Bacillus*-BF5 were less toxic for those tested mosquito larvae (Figure 1). Moreover, the mortality of non-*Ae. aegypti* larvae were higher than *Ae. aegypti* larvae as a response to inoculating those *Bacillus* isolates (Table 1).

Based on Table 1, there were no *Bacillus* isolates that were able to kill more than 50% of the *Ae. aegypti* larvae at the 4th and 8th hours. However, at the 24th hour, six isolates (BF2, BF3, BP1, BP3, BFM, and BPM) were able to kill more than 50% of the *Ae. aegypti* larvae. Of the seven isolates, BP3 could kill all (100%) of the *Ae. aegypti* larvae. Both of the other isolates (BP2 and BFP) were able to kill 50% of the initial population of *Ae. aegypti* larvae at 48 hours after exposure. Meanwhile, in the toxicity test on the non-*Ae. aegypti* larvae, three *Bacillus* isolates (BF2, BF3, and BFM) could kill more than 50% of the larvae within 4 hours. Furthermore, there were four additional isolates at 8 hours (BP1, BP2, BPM, and BFP) and one isolate (BP3) at 24 hours that were able to kill half (50%) of the non-*Ae. aegypti* larvae population. Among the nine isolates, BP3 killed the entire (100%) population of the mosquito larvae within 24 hours.

Molecular identification of five superior *Bacillus* isolates from East Kalimantan for larvacide candidates against mosquitoes

Figure 2 and Table 2 showed the similarity of five selected *Bacillus* isolates that are potential larvacide candidates against mosquitoes to other *Bacillus* species in the GeneBank database: BP1, identified as *Bacillus cereus* ATCC 14579 (100%); BP2, identified as *Bacillus tropicus* MCCC 1A01406 (99,49%); and BP3 and BF2, identified as *Bacillus subtilis* DSM 10 (99.85% and 99.56%, respectively). However, isolate *Bacillus*-BF3 showed un-similarity with all reference isolates from the GeneBank database.

Discussion

Five single isolates of *Bacillus* from East Kalimantan (BP1, BP2, BP3, BF2, and BF3) and their consortium (BPM, BFM, and BFP) decreased the larvae number over time and demonstrated high effectiveness in reducing *Ae. aegypti* and non-*Ae. aegypti* larvae population with more than 70% mortality at 48 hours of exposure. These findings suggested that *Bacillus's* single and mixed isolates can be utilized as biocontrol agents against mosquito larvae, offering a reassuring and sustainable alternative to chemical insecticides. The mechanism of action of these *Bacillus* isolates in killing mosquito larvae may be similar to *B. thuringiensis*, which can produce crystal proteins (cry toxins) during sporulation, which are toxic to mosquito larvae. When mosquito larvae ingest these toxins, the proteins are activated in the alkaline environment of the larval gut. This transformation is essential for their toxic effect. The activated toxins bind to specific receptors on the cells lining the gut of the larvae. This binding creates pore formation in the midgut cells, causing the cells to lyse (break apart). The integrity of the gut wall is compromised. The larvae's digestive process is disrupted due to the lysis of gut cells, leading to a cessation of feeding. Eventually, the larvae become paralyzed and die due to the combined effects of gut cell destruction, starvation, and septicemia. The larvae's immune system may also react to the presence of the toxins, further causing physiological damage (Silva-Filha et al. 2021).

Larvicide-based single or consortium bacteria provide eco-friendly and new solutions for increasing mosquito larvae mortality. However, both bacteria treatment types have different strengths and limitations. Generally, single bacteria are effective for specific mosquito larvae (Ingabire et al. 2017) and simple to produce (Vitta et al. 2018). They also have expected action mechanisms and are direct for regulatory acceptance (Suryawanshi et al. 2015). Conversely, their efficacy is limited by abiotic factors, rapid onset of resistance, and temporary endurance. For instance, mosquito larvae mortality was lower when the single-strain culture was applied to the field than in the laboratory experiment. This highlights that single bacteria require specific treatment to improve their environmental adaptability, increasing

production costs (Derua et al. 2022). Meanwhile, consortium bacteria are an assembly of single bacteria that live, grow, and work together in a symbiotic relationship with synergistic activity (da Silva et al. 2017), enhancing resilience, long-lasting effectiveness, and resistance control (Derua et al. 2018). However, the bacteria consortium production needs a complex procedure, balancing precise strain, high cost, and regulatory challenges (Kajla 2020). As the effectiveness of mixed isolates of *Bacillus* is the same as that of single isolates, using *Bacillus* consortia (BPM, BFM, and BFP) is less efficient in larvicide production.

Bacillus-BP3 was highly effective in reducing *Ae. aegypti* larvae compared to other isolates. These isolates were rapid and complete larvacidal action with 100% mortality at 24 hours. This highlighted the ability of *Bacillus*-BP3 isolates from East Kalimantan to reduce *Ae. aegypti* larvae population was the same as the Bt performance in previous work (Salamun et al. 2021; Land et al. 2023). This isolate, in some cases, killed *Ae. aegypti* larvae more successfully than Bt (Pratiwi et al. 2013). *Bacillus*-BF2 isolates, on the other hand, had robust, predictable larvacidal activity from the start. These two isolates, *Bacillus*-BP3 and *Bacillus*-BF2, were both ideal candidates for generating viable larvicides for mosquito management in East Kalimantan.

Table 2. The similarity of five selected *Bacillus* isolates to other *Bacillus* species in the GenBank database

Isolates	Species	Accesion number	Similarity
BP1	*Bacillus cereus* ATCC 14579	NR 114582.1	100%
BP2	*Bacillus tropicus* MCCC 1A01406	KJ 812435.1	99,49%
BP3	*Bacillus subtilis* DSM 10	MN077147.1	99,85%
BF2	*Bacillus subtilis* DSM 10	MN077147.1	99,56%
BF3	*Bacillus*-BF3 (out of group)		

Table 1. Mortality (%) of *Ae. aegypti* and non-*Ae. aegypti* larvae at 0, 4, 8, 24, and 48 hours after exposure to different *Bacillus* isolates

Bacillus isolates	Percentage of mortality (mean±standard deviation)							
	Ae. aegypti larvae				non-*Ae. aegypti* larvae			
	4 hour	8 hour	24 hour	48 hour	4 hour	8 hour	24 hour	48 hour
Control	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
BP1	17±12	23±15	57±21	83±15	40±0	50±10	73±15	90±10
BP2	17±15	20±17	43±35	70±30	33±6	37±6	60±26	80±20
BP3	0±0	7±6	100±0	100±0	0±0	0±0	67±31	87±23
BP4	3±6	3±6	3±6	10±10	0±0	0±0	0±0	0±0
BP5	3±6	3±6	3±6	7±12	0±0	0±0	0±0	20±20
BF2	40±36	47±29	60±30	83±6	63±15	67±12	77±15	90±0
BF3	30±26	43±15	60±20	73±31	53±25	60±20	77±6	90±10
BF4	7±6	7±6	7±6	10±10	0±0	0±0	0±0	7±12
BF5	0±0	3±6	3±6	10±12	0±0	0±0	13±0	33±20
BPM	13±6	37±15	67±15	77±6	37±6	57±12	80±10	87±6
BFM	13±15	37±32	67±58	77±40	50±10	63±21	80±10	87±23
BFP	7±8	32±38	45±30	82±22	32±15	47±35	58±29	85±20

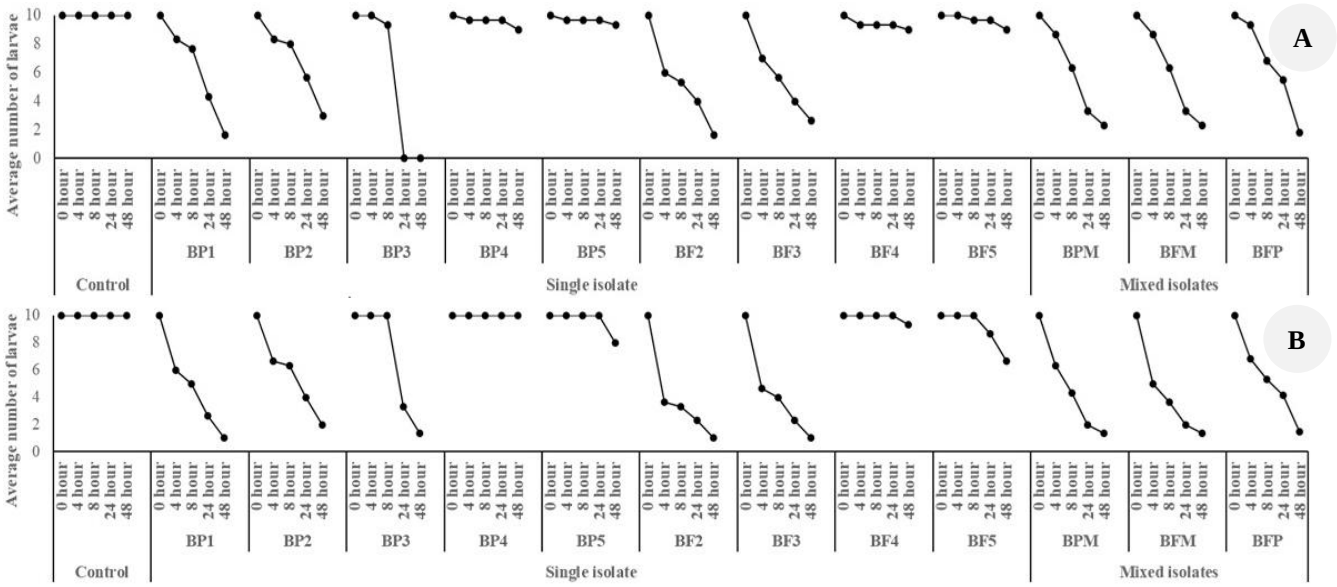

Figure 1. The average number of: A. *Ae. aegypti* larvae; and B. Non-*Ae. aegypti* larvae at 0, 4, 8, 24, and 48 hours after exposure to different *Bacillus* isolates

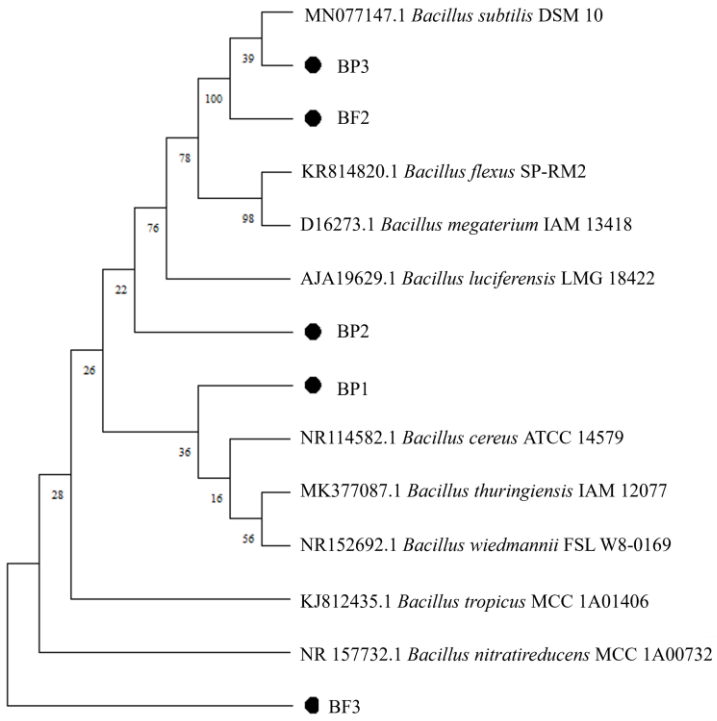

Figure 2. Phylogenetic tree of five selected *Bacillus* isolates (BP1, BP2, BP3, BF2, and BF3) from East Kalimantan

Based on the 16S rDNA sequence, the superior isolates (BP3 and BF2) were identified as *B. subtilis*. A previous study indicated that *B. subtilis* was present in larvae of *Ae. aegypti* collected from East Java, Indonesia, demonstrating a strong larvacidal effect with 96.7% mortality after 48 hours (Salamun et al. 2023). The effectiveness of *B. subtilis* in killing *Ae. aegypti* larvae are likely attributed to the cry toxin and secondary metabolites it produces (Salamun et al. 2024). In addition, *B. subtilis* can produce amphipathic surface active molecules (biosurfactants), including surfactin, iturin, and bacillomycin, which are reported to have high mosquito larvacidal and adulticidal activity even under extreme pH, temperature, and UV radiation (Ghribi et al. 2012; Parthipan at al. 2018; Parthasarathi et al. 2023). However, its moderate efficacy requires optimization for maximum and sustainable results (De Melo Katak et al. 2023; Parthasarathi et al. 2023). Furthermore, *B. subtilis* has been extensively utilized in the agricultural and health sectors to produce chemicals, enzymes, and antibiotics (Su et al. 2020), highlighting the safety of this species as an environmentally friendly larvicide. Furthermore, molecular identification of other *Bacillus* isolates from East Kalimantan showed that *Bacillus*-BP1 identified as *B. cereus* and *Bacillus*-BP2 similar to *B. tropicus*. Like *B. subtilis*, *B. cereus* is known as a biocontrol agent for suppressing mosquito larvae population (Mani et al. 2018). *B. cereus* can produce cry toxin similar to *B. thuringiensis*, which can directly damage the digestive tract of larvae, form long-lasting spores, and provide stable protection in the larval habitat (Ehling-Schulz et al. 2019). Although this *Bacillus* species can potentially be a candidate larvicidal agent against mosquito larvae, this *Bacillus* isolate requires comprehensive safety testing for application purposes. This is related to a previous study that found that *B. cereus* is an anthrax-like disease agent and resistant to antimicrobial substances (Milase et al. 2024). *B. cereus* was also reported as a highly resistant food poisoning bacterium important for human safety (Rahnama et al. 2022). Unlike the two previous *Bacillus* species, research on the application of *B. tropicus* for controlling mosquito populations is still emerging, and there is less knowledge about its long-term effects and potential risks. The logical reason for the high efficacy of *B. tropicus* in killing mosquito larvae is that this species has recently been identified as a subspecies of *B. cereus* (Milase et al. 2024). *B. tropicus* can also produce lipopeptides, such as the enzyme chitinase, which weakens the exoskeleton and larvae membrane and excels at adapting in tropical environments. Hence, the effectiveness level and environmental impact still require further research. However, *B. tropicus* is known as a biocontrol agent for plant pathogens (Bernal et al. 2024). In addition, *B. tropicus* is also broadly used for biodegradation and bioremediation pollution in various environments (Samanta et al. 2020; Malik et al. 2024). Testing is needed to apply this species as mosquito larvicide agents regarding safety for human health because it has been reported as the cause of anthrax-like disease (Milase et al. 2024; Santana de Cecco et al. 2024).

Meanwhile, *Bacillus*-BF3, which is not similar to all species from the GeneBank database, may be a discovery of a new *Bacillus* species from East Kalimantan, which has the potential as an environmentally friendly mosquito larvicidal agent. The BF3 isolate, a candidate of the new species of *Bacillus*, can be confirmed using physiological and biochemical characterization and molecular analysis. The physiological and biochemical characterization such as

morphology test (Gram staining, colony forming, mobility), environmental tolerance (pH, temperature, salinity), carbon substrate metabolism test, enzyme production test (amylase, protease, lipase), identification of secondary metabolites, fatty acid profile (FAME; Logan and De Vos 2015). Meanwhile, the molecular analysis of *Bacillus* performed by Whole Genome Sequencing analysis (WGS), digital DNA-DNA Hybridization (dDDH) analysis, percentage of GC content analysis, and identification of unique genes of the species such as *gyrB, rpoB, or recA* (Zhao and Kuipers 2016). In Whole Genome Sequencing analysis (WGS), the bacteria genome with other genomes in the database can be compared based on the Average Nucleotide Identity (ANI) calculation. An isolate can be a new species whose ANI value is <95% and <70% for dDDH analysis (Grubbs et al. 2017).

In conclusion, from nine tested single *Bacillus* isolates indigenous to East Kalimantan, BP1, BP2, BP3, BF2, and BF3 showed good performance in controlling the mosquito larvae population. Utilizing those single isolates of *Bacillus* as a biocontrol agent against mosquitoes was preferred over using mixed isolates (BPM, BFM, and BFP) for efficiency in larvacide production. *Bacillus*-BP3 and *Bacillus*-BF2 isolates from East Kalimantan, identified as *B. subtilis*, were strong and safe candidates for developing sustainable larvicides to control mosquito populations, mainly *Ae. aegypti*. However, using *Bacillus* isolates from East Kalimantan as a larvacide against mosquitoes, especially BP1 and BP2, similar to *B. cereus* and *B. tropicus*, respectively, requires profound research to assess their safety for humans, other non-target organisms, and the environment. As an initial stage of the development of *Bacillus*-based larvicides, this study requires further tests to determine the lethal dose of the five selected *Bacillus* species and is accompanied by a sub-lethal test. In addition, profiling the secondary metabolites produced by the five selected *Bacillus* species is required for further study to develop sustainable larvicides.

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