Malaysian Journal of Microbiology, Vol 17(4) 2021, pp. 345-351
DOI: http://dx.doi.org/10.21161/mjm.200997

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Bacillus thuringiensis isolated from soil in oil palm plantation with high toxicity against *Oryctes rhinoceros* larvae

Flatya Indah Anggraini[1], Maria Sugiharti[2], Antonius Suwanto[1]*, Nisa Rachmania[1] and Bibiana Widiawati Lay[3]

[1]Department of Biology, Faculty of Mathematics and Natural Science, IPB University, Bogor 16680, Indonesia.
[2]R&D Biotechnology PT Wilmar Benih Indonesia, Bekasi 17530, Indonesia.
[3]Faculty of Biotechnology, Atma Jaya Catholic University, Tangerang 15345, Indonesia.
Email: antoniussuwanto@gmail.com

Received 11 September 2020; Received in revised form 7 December 2020; Accepted 28 April 2021

ABSTRACT

Aims: *Oryctes rhinoceros* beetle is one of the most damaging pests of oil palm and cause high oil palm mortality. The empty fruit bunch mulch and rotten old trunk of oil palm in the field provide the organic matter for the breeding sites and increases the number of *O. rhinoceros* larvae. *Bacillus thuringiensis* as bioinsecticide can synthesize crystal proteins toxic to the larvae. The present study was aimed to find effective *B. thuringiensis* isolates as biopesticide against *O. rhinoceros* larvae.
Methodology and results: Screening process was carried out through heating of soil sample suspension at 80 °C to eliminate the non-spore formers and plated onto T3 medium. Colony morphology was observed, followed by Gram and endospore staining. The crystal protein was observed by Coomassie Brilliant Blue (CBB) staining. Bioassay test was conducted by force-feed method followed by food contamination method. The results showed isolates SBB33 and SBB35 were able to infect and caused high mortality to the *O. rhinoceros* larvae. Isolates SBB33 and SBB35 showed the highest mortality against 1st instar larvae (94.44% and 75% respectively) and 3rd instar larvae (64.8% and 60% respectively) compared to control treatments. The 16S rRNA gene sequencing showed SBB33 has high similarity with *B. thuringiensis* strain 3S2-3, while SBB35 has high similarity with *B. thuringiensis* strain GCU_BTi10. Protein separation of the spore-crystal mixture by SDS-PAGE showed the prominence of 66 kDa protein band that was predicted to be Cry toxins which is specific to coleopterans insect.
Conclusion, significance and impact of study: *Bacillus thuringiensis* isolates SBB33 and SBB35 have high potential as biopesticides against *O. rhinoceros* larvae and could be used to control major pests in oil palm plantation.

Keywords: Bioinsecticides, Coleoptera larvae, sustainable palm

INTRODUCTION

Oryctes rhinoceros (Coleoptera: Scarabaeidae) or commonly known as the rhinoceros beetle, is widely distributed throughout the tropical regions of the world. Adult beetles are known as the main pests of oil palm which can cause severe damage and reduce the economic value of oil palm plantations. It is reported that oil palm tree which infected by these adult beetles can decrease 20% to 25% of oil palm yields (Abidin *et al*., 2014). Adult beetles fly onto the crown of oil palm and attack the plant by perforating the inner cluster of leaf spear of oil palm tree and chewing the soft young tissue in the unopened frond causing a V shape-cut (Gopal *et al.*, 2001). This pest could reduce the economic value in oil palm plantation, it is start from weakening petiole followed by reduction of photosynthesis activity and consequently resulting in plant death (Manjeri *et al.*, 2014).

The *O. rhinoceros* adult beetles and larval stages have different feeding preferences, and in this case, the damage to oil palms is caused by the adult stages. Meanwhile, the larval stage feed and live on layered or stacked decomposed matter or soil in oil palm plantation (Ooi *et al*., 2004). However, the oil palm trunk, empty fruit bunches, and rotted mesocarp fruit waste can be a good breeding sites for *O. rhinoceros* beetles and can protect the larval stage, resulting in an increased population (Ponnamma *et al.*, 2001). To control *O. rhinoceros*, it seems an effective way to control at larval stage due to less mobility of *O. rhinoceros* during this immature stage.

Bacillus thuringiensis has good potential as a pesticide because *B. thuringiensis* has the ability to produce parasporal proteinaceous crystalline inclusion (Cry toxin) that are toxic to insect which ingest it (Heckel,

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

2020). The Cry toxin have a great potential to control the targeted insect which belong to Lepidopteran, Dipteran and Coleopteran (Vidyarthi *et al*., 2002), however currently there is no report on effective way to suppress *O. rhinoceros* in oil palm plantation. As such, this study aimed to determine the toxicity of *B. thuringiensis* isolates from soil in oil palm plantation as the breeding site of *O. rhinoceros* larvae, as most adapted isolate to control this pest in oil palm plantation. Moreover, the identification of the isolates by 16S rRNA gene sequencing and molecular weight of the *B. thuringiensis* isolates also have been performed.

MATERIALS AND METHODS

Samples

Soil samples were collected from the Wilmar Group oil palm plantation in West Sumatra, Indonesia. *Bacillus thuringiensis galleriae* (Bt-Gal) was obtained from Institute Pertanian Bogor Culture Collection (IPBCC), while *Bacillus thuringienis* strain D20 Bt-M4 isolate was obtained from the Wilmar Benih Indonesia (WBE), Research and Development Laboratory. Bt-Gal and Bt-M4 were served as positive controls in this study.

Isolation of *Bacillus thuringiensis*

A total of 5 g of soil sample was dissolved in 50 mL of sterilize physiological saline 0.85% solution. The mixture was homogenized by shaking and allowed to sediment into two distinct phase – the soil phase and the liquid phase. The liquid phase (10 mL) was boiled at 80 °C for 10 min. Serial dilution (10^{-6}) was done and aliquots were plated on the T3 agar medium (Travers *et al*., 1987). The plate then incubated at 30 °C overnight to allow bacterial growth. The colony morphology observed was determined by Gram and endospore staining. Colonies positive for Gram and endospore staining were subsequently stained with Commassie Brilliant Blue (CBB) [0.25% (w/v)] in 60% ethanol and 7% (v/v) acetic acid (Ammons *et al*., 2002) to determine the presence of crystal proteins. Isolates containing crystal proteins were cultured in the Casitone Yeast Glucose (CYS) broth medium and standardized to 10^8 spore/mL spore-crystal mix suspension for use in bioassay test (Yamamoto, 1990).

Bioassay test

Oryctes rhinoceros larvae were collected from oil palm plantations in West Sumatra, Indonesia and acclimatized for one week for physiological adjustment and adaptation to the greenhouse environment. All bioassays were performed in the glass jar (diameter 6 cm, height 9 cm) in the greenhouse under 75% humidity at 25-27 °C. The bioassay was conducted with a completely randomized research design with three replications. Negative controls were sterile water and *Escherichia coli*, while positive controls were Bt-Gal and Bt-M4. The 1st and 3rd instar larvae were used in this bioassay. Bioassay on 3rd instar larvae was done by the modified force-feeding method (Jackson and Savile, 2000) by inoculating 25 µL of 10^8 spores/mL of spore-crystal mix suspensions into the larva's mouthpart with 200 µL micropipette tip. The treated larvae were placed on 50 g of sterilized compost and observed every week for 4-weeks observation period.

Highly toxic isolates were further tested using a food contamination-based method (Theunis and Aloalii, 1999) with completely randomized design of 3 replicates in the 1st and 3rd instar larvae. Non-sterilized compost was placed in the jar and mixed with 5 mL of 10^8 spores/ mL of spore-crystal mix suspension. The larvae were starved for 24 h, and subsequently placed individually in jar. Observations were carried out every week for 4 and 8 weeks for 1st and 3rd instar larvae respectively. Lethal time 50 (LT_{50}) is used to calculate the time needed to kill 50% of the test insect population and the data were analyzed using probit analysis.

16S rRNA gene sequencing

The 16S rRNA gene were isolated from 24 h cultured isolates and amplification was done using forward 63F primer (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*., 1998). PCR cycles were carried out in 24 cycles using a thermal cycler under the following conditions: denaturation 96 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 60 °C for 90 sec, and final extension at 72 °C for 90 sec to yield 1300 bp PCR product. The amplified product was then purified using Qiaquick DNA purification kit, followed by ExoSAP-IT™. The purified amplicon was sequenced by dideoxy method using BigDye X-terminator™ Purification Kit, followed by analysis using 3310 genetic analyzers. The 16S rRNA sequence obtained was analyzed with BLASTN in the NCBI database.

Protein profiling by SDS-PAGE analysis

The spore protein crystals from selected isolates were analyzed by SDS-PAGE. A loopful of colony of each isolates were inoculated into CYS broth medium (Yamamoto, 1990) and incubated at 30 °C with agitation at 200 rpm for 72 h. Subsequently, the bacterial culture was centrifugated and the pellet was suspended in 1 M Tris-HCl at pH 8. About 20 µL of the pellet suspension was mixed with 20 µL of loading buffer (60 mM Tris-HCl (pH 6.8) 25% (v/v) of glycerol, 10% (w/v) SDS, 14 mM β-mercaptoethanol and 0.1% (w/v) bromophenol blue) and heated in the boiling water (100 °C) for 10 min. About 20 µL (10 mg/mL) of the sample-loading buffer mixture was loaded into each well and the gel was run for 135 min at 90 V. The molecular weight of the proteins was determined by comparison with standard protein ladder (Biorad, USA).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(4) 2021, pp. 345-351
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RESULTS

Isolation and identification of *Bacillus thuringiensis*

The isolates were screened based on their colonies phenotypic characteristics. Thirty-seven bacterial isolates with characteristics identifying them with *Bacillus* sp., such as white to milky white color, irregular to rough wavy edge, smooth-looking surface texture, were selected for Gram staining. All 37 isolates also presented a purple coloration on Gram staining and demonstrated the presence of endospore. However, on staining for the presence of crystal protein, only 10 isolates demonstrated a positive result. The crystal protein observed under the phase contrast microscope which characterized with circular to cuboidal (Figure 1).

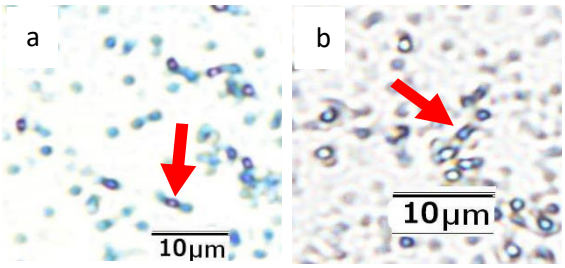


Figure 1: Microscopic analysis of cells with crystal protein of *B. thuringiensis* strain (1000× magnification with CBB staining). Arrows showed the crystal protein for (a) SBB33 and (b) Bt-Gal as positive control.

Bioassay

Larval mortality for each treatment was analyzed by one-way ANOVA with Tukey's test (α <0.05). The bioassay with the force-feeding method showed that positive control (Bt-M4) resulted in the highest mortality (76.65%) on the 3rd instar larvae (Table 1) with lethal time 50% of population (LT_{50}) of 2.89 weeks (Table 2). Meanwhile, among the 10 isolates, only SBB33 and SBB35 demonstrated high mortality, about 56.68% and 50%, on the 3rd instar larvae (Table 1), with LT_{50} of 3.77 weeks and 3.78 weeks respectively (Table 2). Isolates SBB33 and SBB35 were then tested on the 1st and 3rd instar larvae by the food contamination method.

Bioassay with food contamination method also showed that SBB33 and SBB35 are effectively caused high mortality to *O. rhinoceros*, about 94.44% and 75%, respectively, on 1st instar larva (Table 1). Based on one-way ANOVA analysis, followed by Tukey's test (α <0.05), it is confirmed that both SBB33 and SBB35 produced mortality rate against 1st instar larvae at a level comparable (no significant difference at α <0.05) to positive control strains, Bt-Gal and Bt-M4 which has established toxicity against coleopteran larvae. Meanwhile, both the positive control and the new isolates demonstrated a significantly higher mortality against 1st instar larvae compared to larvae that received water (negative control) and *E. coli* treatment. The LT_{50} of 1st instar larvae were 2.50 weeks and 2.87 weeks for SBB33 and SBB35 compared to 2.91 weeks of Bt-Gal and 2.94 weeks of Bt-M4 respectively (Table 2).

Furthermore, in bioassay with food contamination method using 3rd instar larvae, SBB33 and SBB35 isolates show caused 64.08% and 60% mortality respectively. This mortality level is not significantly from the positive controls, Bt-M4 (71.48%) and Bt-Gal (43.70%). However, LT_{50} of SBB33 (5.89 weeks) and SBB35 (6.15 weeks) are faster compared to positive control Bt-Gal (8.22 weeks), but slower compared to Bt-M4 (5.67 weeks) (Table 2).

Table 1: Toxicity of spore-crystal mixture of *B. thuringiensis* isolates against 1st and 3rd instar larvae of *O. rhinoceros*.

Sample	Average mortality (%) 3rd instar (force-feeding method)	Average mortality (%) (food contamination method)	
		1st instar	3rd instar
Water (negative control)	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
E. coli (negative control)	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
Bt-Gal (positive control)	53.35 ± 11.52^{a,b,c}	83.33 ± 16.67^{a}	43.70 ± 20.50^{a}
Bt-M4 (positive control)	76.65 ± 15.30^{a}	77.84 ± 19.20^{a}	71.48 ± 5.70^{a}
SBA3A	0.00 ± 0.00^{c}	not tested	not tested
SBA3B	13.33 ± 23.09^{b,c}	not tested	not tested
SB15B5	20.00 ± 20.00^{b,c}	not tested	not tested
SBA3E	13.33 ± 23.09^{b,c}	not tested	not tested
SBB3F	6.67 ± 11.55^{b,c}	not tested	not tested
SBB33 *	56.68 ± 15.25^{a,b}	94.44 ± 9.62^{a}	64.08 ± 7.56^{a}
SBB34	0.00 ± 0.00^{c}	not tested	not tested
SBB35 *	50.00 ± 45.80^{a,b,c}	75.00 ± 8.33^{a}	60.00 ± 20.00^{a}
SBA316	10.00 ± 10.00^{b,c}	not tested	not tested
SBA321	20.00 ± 20.00^{b,c}	not tested	not tested

Values in the same column bearing the same letters are significantly different at α 5% confidence level interval (Tukey test).
*Potential isolates

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(4) 2021, pp. 345-351
DOI: http://dx.doi.org/10.21161/mjm.200997

Table 2: LT_{50} of *B. thuringiensis* isolates against *O. rhinnoceros* larvae with force feeding and food contamination methods.

Isolates	LT_{50} (weeks)		
	Force feeding method	Food contamination method	
	3rd instar	1st instar	3rd instar
Bt-M4	2.89	2.94	5.67
Bt-Gal	3.59	2.91	8.22
SBB33	3.77	2.50	5.89
SBB35	3.78	2.87	6.15

16S rRNA gene sequencing

The 16S rRNA PCR amplification of SBB33 and SBB35 produced DNA bands with the size of 1300 bp (Figure 2). The sequencing analysis result of PCR product showed that SBB33 and SBB35 has a 100% similarity level to *B. thuringiensis* strain 3S2-3 (MG738340.1) which was isolated from animal farm soil and *B. thuringiensis* strain GCU_BTi10 (KR048279.1) isolated from leaf litter respectively. The sequences obtain in this study were uploaded to the NCBI GenBank with accession number MW269691 for SBB33 and MW269692 for SBB35.

Protein profiling by SDS-PAGE

Protein separation by 12% SDS PAGE analysis showed differences and similarities in band patterns among the isolates and the positive control (Figure 3). The pellet suspension samples of the positive control strain Bt-Gal produce two prominent protein bands of 130 kDa and 66 kDa, while SBB33 and SBB35 sample produced several bands, with the most prominent band being the 66 kDa band. Since SBB33 and SBB35 showed similar band size to positive control Bt-Gal at 66 kDa band size, it is predicted that the 66 kDa is the Cry toxin which is responsible for the toxic action of all three strains. However, further research is needed to validate the hypothesis.

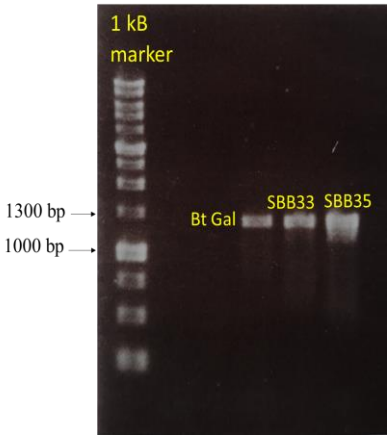


Figure 2: Electrophoretic analysis of amplified 16S rRNA gene of Bt-Gal (as positive control), SBB33 and SBB35. The 16S rRNA amplicons have size of 1300 bp.

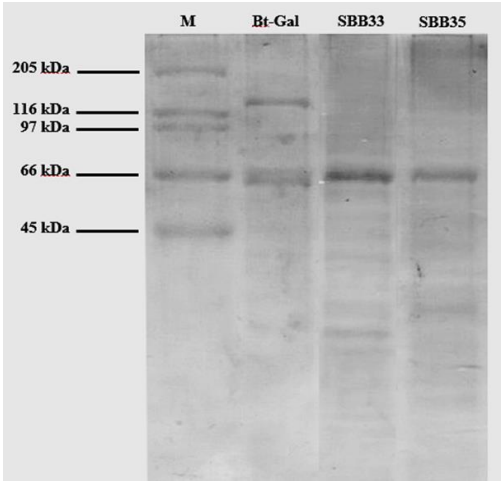


Figure 3: Molecular weight determination of pellet of isolates SBB33 and SBB35 compared with high molecular weight marker (HMW) by SDS-PAGE. The label indicates prominent bands.

DISCUSSION

Bacillus thuringiensis is known to exists in various habitats; however, they are relatively more abundant in soil environment (Schnepf *et al*., 2005). Soil is the main reservoir of *B. thuringiensis* spores and is widely used as the source for the isolation of *Bacillus* species, such as *B. subtilis* and *B. cereus* (Soares-da-Silva *et al*., 2015; El-Kersh *et al*., 2016). In this study, *B. thuringiensis* was isolated from the soil in oil palm plantation which hypotheses that it will be more adaptable to use as biological control of *O. rhinoceros* pest in the oil palm. During isolation, suspension heating is used to eliminate non-spore forming bacteria and thus facilitating preliminary screening.

Since *B. thuringiensis* is a closely related species with abundant similarities in morphology, the crystal protein is the only feature that can differentiate *B. thuringiensis* from other *Bacillus* species such *B. subtilis* and *B. cereus* (Mukhija and Khanna, 2018). Biotoxicity assay of *B. thuringiensis* against larvae and adult insects is one of the ways to screen the isolates for their potential as bioinsecticides and is preferably carried out either by force-feeding or food contamination method. The

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(4) 2021, pp. 345-351
DOI: http://dx.doi.org/10.21161/mjm.200997

bioassay of isolates SBB33 and SBB35, against the 3rd instar larvae revealed the ability of these isolates to cause high mortality, at level comparable to positive controls, in both force feeding and food contamination method. This indicated that SBB33 and SBB35 have the ability to infect *O. rhinoceros* larvae and cause death. SBB33 and SBB35 might be more adaptable to applicated in oil palm plantation as indigenous isolate from oil palm plantation.

The finding of our study is akin to previous reports on the isolation of *B. thuringiensis.* For instance, Sihombing *et al.* (2014), obtained mortality percentage of *O. rhinoceros* 3rd instar larvae of 56.26% at a 25 g/ L dose of *B. thuringiensis*. Mortality caused by *B. thuringiensis* infection in Coleoptera larvae also reported in the study of Gonzales *et al*. (2002) on *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae) adults with an LT_{95} of 24 days. Oppert (2010) also reported the mortality of *Tenebrio molitor* neonate larvae up to 90% after 35 days of treatment with pure protoxin of Cry3A. Another report by Asano *et al.* (2003) described how the residual activity of *B. thuringiensis* caused the mortality of *Anomala cuprea* larvae, reaching 100% with an observation time of up to 106 days. The difference in the level of toxicity in native isolates of *B. thuringiensis* could be caused by differences in the strain, weight of crystal protein and type of cry protein produced in each isolate (Khaeruni and Ningrum, 2012). Nevertheless, the agreement of the results of the force-feeding and food contamination methods in this study proves that both SBB33 and SBB35 isolates have the potential as a bioinsecticide for controlling *O. rhinoceros* larvae.

Surprisingly, the results of *B. thuringiensis* bioassay on the 1st instar larvae showed an even higher mortality compared to the 3rd instar at the same dose using food contamination method. Based on the results, it shows that SBB33 can cause more than 90% death in the 1st instar larvae in lesser time compared to the 3rd instar. This can be explained based on the report of Jackson and Saville (2000) which stated that feeding treatment on scarab larvae usually has greater mortality when using the early-stage larvae compared to using late-instar larvae, as they will be quiescent to the molting process.

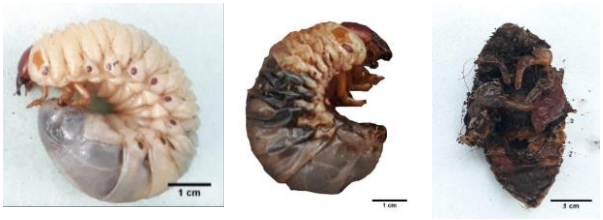
The force-feeding method gave shortest LT_{50} with Bt-M4 and cause faster mortality of larvae when compared to the food contamination method on all treated isolates. Besides, Jackson and Saville (2000) previously demonstrated that the force-feeding method is able to administrate a known dose of bacterial suspension directly into the insect mouthpart.

On the other hand, the food contamination method has similarities to the application principle in the field by mixing the bacterial suspension in the compost medium. In the oil palm plantation to control this pest, pesticide will be spraying into soil or organic matter heap to target the larvae which live in there. This process allows the larvae to ingest the mixture of compost that has been naturally contaminated with the suspension. Besides, the food contamination method consistently exposes the bacteria to their host, by mixing food and bacterial suspensions to promote germination of spores (Theunis and Aloalii, 1999).

Bacillus thuringiensis-infected larvae show the symptom in the form of low movement activity and subsequently death. The death is characterized by a soft body texture (Fig 4b), emission of a foul odor and discoloration in the larva's body in form of blackening. It was previously reported by Federici *et al.* (2010) that larval infection causes the body to soften. Pujiastuti *et al*. (2020) explained that *B. thuringiensis* isolates against *O. rhinoceros* 1st instar larvae was indicated by body color changes, texture, and shape.

Another mechanism reported by Zhang *et al.* (2006) describes that Cry toxin binds to the receptor and stimulates G protein and adenylyl cyclase which can increase the level of cyclic adenosine monophosphate(cAMP); therefore, it activates kinase A protein. This process can affect the ion flux, leading to cell lysis. Infection by *B. thuringiensis* in this study caused rot at the pre-pupal and pupal stages therefore the pupae fail to become adult beetles and died (Figure 4c). The failure of the pupa to become a beetle can reduce the population of beetles that grows, thereby reducing attacks on oil-palm plantations.

Bacillus thuringiensis is unique in synthesizing protein crystals during the stationary phase. The production of protein crystals varies in number and type. Each type of protein crystal can exhibit toxicity against specific insect larvae (Schnepf *et al.,* 2005). The Cry-3 group was classified as a *B. thuringiensis* toxin which able to cause mortality in the Coleoptera group (Crickmore *et al*., 1998). While Cry3Aa and Cry3Bb proteins are highly toxic to Colorado Potato Beetle (CPB) (Ochoa-Campuzano *et al.*, 2007). In this study, a 66 kDa protein found in SBB33, SBB35 and Bt-Gal is predicted to be the Cry protein. Bt-Gal also have a 130 kDa compared to SBB33 and SBB35 which has lower protein molecular weight. In addition, Cry3 does not have a large C-terminal region that is protoxin at a molecular weight of 130 kDa like Cry1 (Loseva *et al.*, 2002). Meanwhile, Oppert (2010) showed that Cry3Aa has a high molecular weight of 67-73 kDa. However, further research involving protein purification, direct protein administration to larvae/cell culture, or antibody detection is required to validate this claim.



(a) (b) (c)

Figure 4: Third instar larvae of *O. rhinoceros* with the force feeding and food contamination methods caused death which characterized by a soft body texture and the larva's body discoloration into blackening. (a) healthy larvae (b) dead treated larvae (c) dead treated pupa.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(4) 2021, pp. 345-351
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CONCLUSION

In conclusion, *B. thuringiensis* isolates SBB33 and SBB35 obtained in this study could significantly cause mortality to *O. rhinoceros* larvae and could be potential as an environmentally-friendly biopesticides against *O. rhinoceros* in oil palm plantation. Further investigation on bacterial mass production, as well as field trials should be conducted to evaluate their efficacy and economic feasibility.

ACKNOWLEDGEMENTS

We wish to acknowledge PT. Wilmar Benih Indonesia for funding this research.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538