Malaysian Journal of Microbiology, Vol 17(6) 2021, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.211186

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

Preliminary study on thiamethoxam degrading bacteria isolated from corn plantation

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Received 29 May 2021; Received in revised form 9 August 2021; Accepted 18 October 2021

ABSTRACT

Aims: Thiamethoxam (THIA) is a pesticide that has been widely used for its effectiveness in controlling and preventing insect pests. However, the use of THIA diffused in soil, surface and groundwater pose severe toxicity to the ecosystem. The hazardous pollution caused by the toxicity of THIA demands for remediation to ensure degradation of THIA into its safe constituent elements. Thus, the aim of this study is to isolate and identify potential THIA degraders for future bioremediation.
Methodology and results: Bacteria were isolated from soil sample collected at a corn plantation which utilizes THIA as a source of pesticide. Overall, two bacterial isolates were isolated from the soil sample. The bacterial isolates were screened and identified for their ability to degrade pesticide by culturing in minimal salt media (MSM) supplemented with 50 mg/L THIA. The growth of isolates was observed and analyzed through spectrophotometry analysis, standard plate count method and pH value of culture medium. As a result, isolate THIA 1 had been found to possess the ability to degrade pesticide as it showed a high rate in growth of bacteria compared to its controls. Meanwhile, isolate THIA 2 showed no degrading activities while under treatment as it showed similar rate of growth towards its control. Isolate THIA 1 was identified as *Acinetobacter* sp. UMTFA THIA 1.
Conclusion, significance and impact of study: The isolation and identification of the pesticide degrading bacteria will provide promising source of pesticide degrading enzyme that can be further developed for enzymatic pesticide biodegradation. This will pave the way forward in bioremediation where new effective degradation tools can be developed for pesticide residue which otherwise lead to serious ecological problem.

Keywords: Thiamethoxam, bioremediation, isolation, identification, pesticide degradation

INTRODUCTION

Pesticides are synthetic organic chemicals used to control various pests that feed on crops. It is undeniable that extensive use of pesticides has improved the agricultural productivity by many folds. The ever-increasing global demand for food and productive crops has escalated the demand of pesticides for various crops from damaging effects of insects, reducing losses from the weeds and diseases. However, excessive and continued use of these toxic and relatively non-biodegradable chemicals leads to a substantial health hazard resulting from active uptake and accumulation of these compounds in the food chain and drinking water (Alavanja *et al*., 2013; Rahman *et al*., 2018). Pesticides plays a vital role in increasing economic potential in term of increased production of food (Aktar *et al*., 2009; Hussain *et al*., 2016). One of the examples of widely used pesticides is thiamethoxam (THIA), a pesticidal active substance in the neonicotinoid class of pesticides (Tosi and Nieh, 2017; Rana and Gupta, 2019). Neonicotinoid insecticides are amongst the most effective pesticides used worldwide in the prevention and control of insect pests such as aphids, whiteflies, leafhoppers and plant hoppers, thrips, some microlepidoptera and several coleopteran pests (Zhou *et al*., 2013; Oliveira *et al*., 2014). This tremendous success is based on their unique chemical and biological properties, such as broad-spectrum insecticidal activity, low application rates, excellent systemic characteristics, favorable safety profile and a new mode of action (Liqing *et al*., 2006; Rana and Gupta, 2019). Although the uses of THIA give beneficial outputs, it has contributed to the pollution of chemicals in soil, surface and groundwater (Monard *et al*., 2013). Once it was introduced to a field, THIA can leach into groundwater or collect in runoff, affecting local drinking water and wildlife (Aktar *et al*., 2009; Briceño *et al*., 2020). Other than killing insects or weeds, THIA can be toxic to a host of other organisms including birds, fish, beneficial

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

DOI: http://dx.doi.org/10.21161/mjm.211186

insects and non-target organisms. In this respect, it is crucial for THIA to degrade into its constituent elements which is less or non-toxic to prevent them from contaminating the environment (Gupta *et al*., 2008; Pang *et al*., 2020).

Generally, pesticides can be degraded by either chemical or microbiological processes (Huang *et al*., 2018; Barba *et al*., 2019). However, the use of microbes with degradative ability (microbiological process) is considered the most efficient and cost-effective option to clean pesticide-contaminated sites (Massiha *et al*., 2011). This process makes use of various microorganisms in soil to break up the pesticide and use as nutrient source. The genetic adaptation in microorganisms induces mechanisms of degradation due to extensive use of these compounds in agricultural soils. The extensive use of THIA often led to the use of THIA by these microorganisms as the source of carbon, nitrogen, sulphur or phosphorus thus, facilitates the elimination of the compound's toxicity (Wang *et al*., 2013; Rana *et al*., 2019; Briceño *et al*., 2020) as illustrated in Figure 1.

Biodegradation of THIA by microbial species is a biological way to reduce the contaminant as it offers eco-friendly and cost-effective remediation approach. This study focuses on the isolation and characterization of bacterial strains of the THIA degrading microbes from fortified soil cultures from a corn plantation in Tanjung Karang, Selangor. These isolates will enable future bioremediation strategy to dissipate THIA commonly used in agriculture.

MATERIALS AND METHODS

Collection of soil sample

Soil sample was collected at a corn plantation that is located at Jalan Masjid Baruh, Kg. Sungai Tengi Kanan, 45500, Tanjung Karang, Selangor (N3°25'49" E101°11'6"). This site was chosen based on their prolonged use of pesticide THIA in controlling aphid insects from infecting corn plants, seed and fruit. Approximately 100 g of the total weight of soil sample was taken from a surface layer of soil at depth 10 cm, from five different areas within the corn plantation. Soil samples were collected in sterile condition before it was transported back to the laboratory for further analysis.

Enrichment and isolation of soil bacteria

Serial dilution and plating techniques were carried out to enrich and isolate the THIA degrading bacteria from the soil as previously described (Hedge *et al*., 2017). Individual colonies of bacteria which varied in macroscopic characteristics such as shape and color was picked and purified by streaking on nutrient agar (NA) plate. As for the stock culture preparation, the bacteria isolate of each pure colony was cultured on NA slant and incubated at 37 °C for 24 h. It was then stored at 4 °C.

Morphological and biochemical characterization

The morphology of THIA degrading bacterial isolates was observed under microscope for basic cellular forms and aggregation of cell. Gram staining was performed to identify the Gram profile of bacteria: crystal violet for Gram-positive and safranin for Gram negative. Bacterial isolates were also observed and identified based on their reaction towards certain chemicals or selective medium. Motility of the bacteria was examined using Sulfide Indole Motility (SIM) medium (BBL, Thomas Scientific, USA) where the isolates are inoculated by stabbing through center of media and incubated at 37 °C, 24 h. Indole test was done by using Kovacs reagent into the bacterial isolates grown in motility medium (Hemraj *et al.*, 2013).

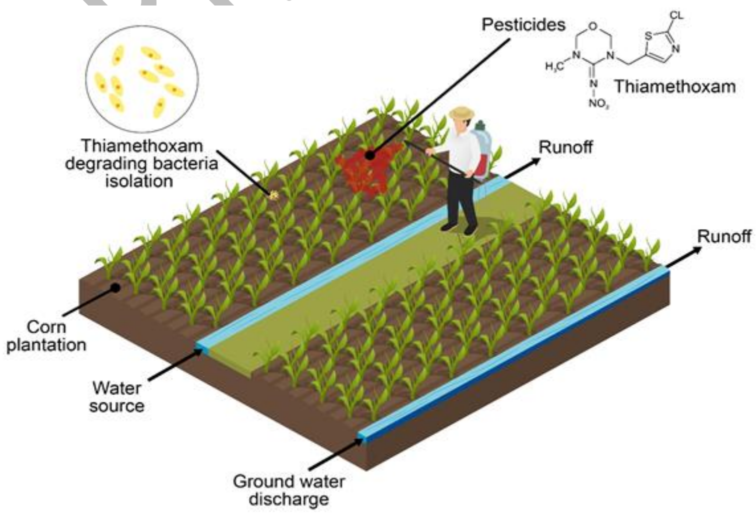

Figure 1: Scheme shows that excessive and continued use of THIA pesticides leads to a substantial environmental hazard from accumulation of these compounds in the food chain and drinking water. The use of microbes with THIA degradative ability will enable bioremediation to dissipate THIA commonly used in agriculture.

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

Any change of colour was recorded within seconds after added. Oxidase test was carried out using moistened FLUKA oxidase strip (FLUKA 70439, Thomas Scientific, USA). Change of colour was observed within 2 min and the result was analyzed and recorded.

Identification of thiamethoxam degraders

The identification of THIA degraders were carried out by performing the 16S rRNA sequencing. Direct sequencing of 16S rRNA gene sequence was done by PCR-amplified 16S rDNA. The bacterial 16S rDNA, full-length of 1.5 kb was amplified using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR was performed as follow: 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 sec; 53 °C for 30 sec; 68 °C for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified by standard method and directly sequenced with primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018b).

Screening of bacteria degrading thiamethoxam

The mineral salts medium (MSM) containing THIA as a sole nutrient source was used to screen pesticide degrading bacteria. MSM was prepared in conical flask consisting of as follows: 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.05 g $CaCl_2$, 2.44 g Na_2HPO_4 and 1.52 g KH_2PO_4 dissolved in 1 L of distilled water, pH at 6.8. It was then sterilized at 121 °C for 15–20 min. THIA (Actara® 25WG, Syngenta Corporation Sdn. Bhd., USA) was used at concentration of 50 mg/L based on previous research carried out (Hedge *et al*., 2017). Pure bacterial isolate was inoculated in nutrient broth and incubated at room temperature for 24 h at 120 rpm. The screening process was conducted in three replicates and control for each of the sample was MSM medium with bacteria but without THIA. All the treatments were incubated at 27 °C for 30 days at 120 rpm. The growth of the bacteria was determined based on three quantitative measurements, which were spectrophotometry analysis and standard plate count method. The pH value of each treatment was also determined (Akbar and Sultan, 2016).

Spectrophotometry analysis

The growth of bacteria was determined based on the optical density (OD) value of the MSM enriched with THIA. An amount of 3 mL of each sample was transferred into a glass cuvette and scanned at the wavelength of 600 nm by using UV spectrophotometer (Shimadzu UV spectrophotometer UK-1800, Japan). The blank control used was MSM medium and the analysis was done from day 0 to day 30 at 3 days interval and the data was recorded (Rahman *et al*., 2018).

Standard plate count method

The growth of bacteria was also measured based on the number of colony forming unit per mL of bacteria (CFU/mL). Using aseptic technique, 0.1 mL of bacteria in culture solution was taken from flasks and spread into NA plate by using glass spreader. It was incubated at 37 °C for 24 h before counted using a Stuart Scientific Colony Counter, UK. The enumeration of bacteria was performed at 3 days interval starting from day 0 to day 30.

Determination of pH value

The pH of MSM media enriched with THIA was measured for detection of the growth of bacteria. It was conducted by transferring 10 mL of enrichment media from each flask into a centrifuge tube and measured using pH meter. It was measured from day 0 to day 30 at 3 days interval. The pH value was recorded (Chaussonnerie *et al*., 2016).

RESULTS AND DISCUSSION

Identification of thiamethoxam degraders

A total of two strains of isolated bacteria with THIA degrading ability were obtained based on serial dilution and plating techniques for further study. Initial studies based on the morphological characteristics and the biochemical analysis are summarized in Table 1. Based on the initial test carried out, it has been deduced that the isolate THIA 1 is a Gram-positive coccus which shows negative for indole, motility and oxidase test. As for the isolate THIA 2, it is a Gram-negative rod. However, in Table 2, based on the identification of the THIA-degraders carried out using molecular analysis of 16S rDNA, the 16S rDNA sequence of strain THIA 1 exhibited 99.9% similarity to *Acinetobacter* sp. Meanwhile, the isolate THIA 2 exhibited 99.8% similarity to *Enteroccocus* sp. The phylogenetic tree comparison of these strains within their genus were shown in Figure 2 (isolate THIA 1) and Figure 3 (isolate THIA 2). Therefore, isolate THIA 1 was denoted as *Acinetobacter* sp. USMFA THIA 1 and isolate THIA 2 was denoted as *Enteroccocus* sp. THIA 2. Generally, microbial population in an ecosystem is influenced by the adaptation to utilize the most abundant material in the surroundings environment (Kumar *et al*., 2018a). Previous studies have shown *Acinetobacter radioresistens*, *Pseudomonas frederiksbergensis*, *Bacillus* sp. (Sundaram *et al.*, 2013), *Serratia liquefaciens*, *Serratia marcescens*, *Burkholderia gladioli* (Iqbal and Bartakke, 2014; Hussain *et al*., 2016), *Achromobacter* spp. and *Diaphorobacter* sp. (Ahn *et al.*, 2018; Rahman *et al*., 2018) are commonly isolated bacterial strains from pesticide-applied farmland soil.

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

Malays. J. Microbiol. Vol 17(6) 2021, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.211186

Table 1: Morphological and biochemical characteristics THIA degrading bacteria isolates.

Characteristics		THIA 1	THIA 2
A.	Morphology		
	Gram staining	+	-
	Shape	Cocci	Rod
B.	Biochemical Test		
	Motility test	-	-
	Indole test	-	-
	Oxidase test	-	-

(+) positive response; (-) negative response.

Table 2: Similarity of genus identification of THIA-degrader.

Strains [a]	Genus [b]	Similarity index [b]
THIA 1	*Acinetobacter* sp.	99.9%
THIA 2	*Enteroccocus* sp.	99.8%

[a] Strains isolated from the soil sample collected at a corn plantation which utilizes THIA.
[b] Identification based on the 16s rDNA.

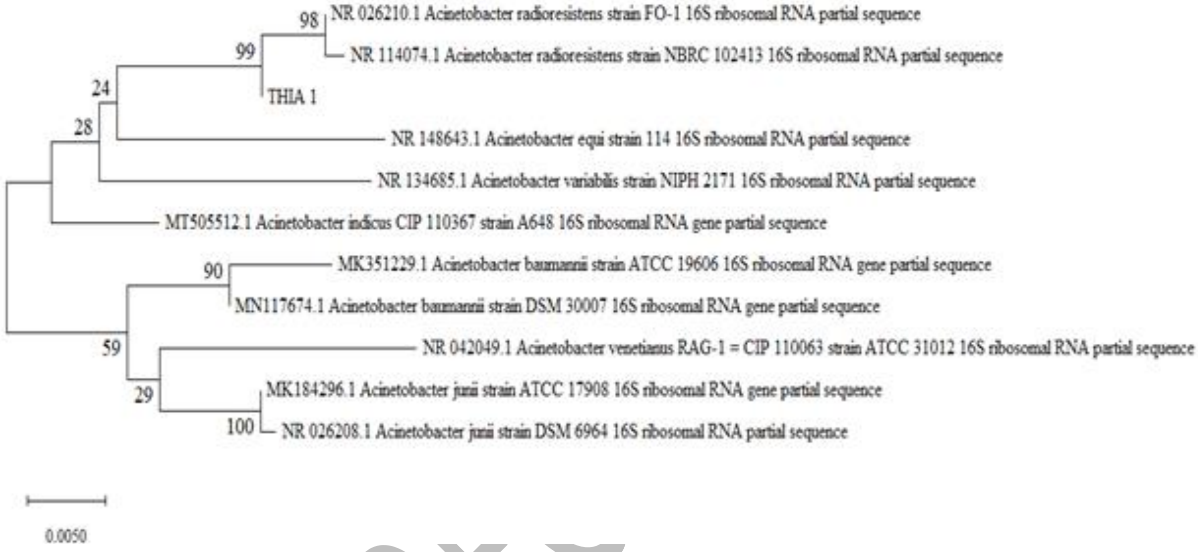

Figure 2: Phylogenetic tree of *Acinetobacter* sp. USMFA THIA 1 and related bacterial strains based on the 16S rRNA comparisons with accession numbers.

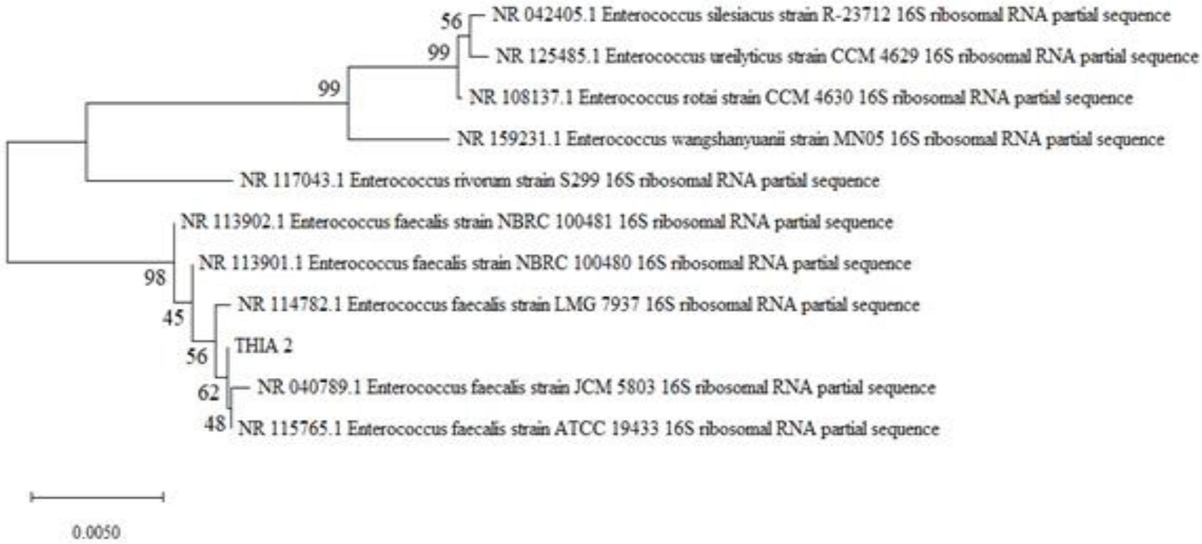

Figure 3: Phylogenetic tree of *Enterococcus* sp. THIA 2 and related bacterial strains based on the 16S rRNA comparisons with accession numbers.

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

Malays. J. Microbiol. Vol 17(6) 2021, pp. xxx-xxx
DOI: http://dx.doi.org/10.21161/mjm.211186

Screening for THIA degrading bacteria

The Figure 4 (a) shows the graph of absorbance at the wavelength of 600 nm against days of treatment for isolate THIA 1, THIA 2 and control. Based on the trend, isolate THIA 1 had a high increase in the absorbance from 0.02 (day 0) to 0.09 (day 30). This increase in absorbance indicates the growth of bacterial isolate in the culture media and a log phase in the growth curve. Degradation of pesticide is often related to the stimulation of microbial growth which leads to typical sigmoidal curves with significant delay, exponential increase and saturation phase (Wirsching *et al*., 2020). However, it was observed that growth curves of isolate THIA 2 were almost similar to the control. This suggested that THIA 2 was unable to degrade THIA as their source of nutrient.

Figure 4 (b) shows the colony forming unit per millilitre (CFU/mL) of bacterial isolate against days of treatment. The result indicated that the growth of bacteria increased by 83.3% from day 3 to day 15 for THIA 1. This indicated that THIA supported the growth of bacterial isolate as the growth was higher than the control (Mehta *et al*., 2021). Based on Figure 4 (c), the pH value of the enrichment media showed a gradual decline from 6.8 to 6.5 for isolate THIA 1 from day 0 to day 30. This decline could probably be attributed to the accumulation of waste product over time, causing an increase inhibition to the growth of bacterial isolate as time passes (days of treatment). Based on the Figure 4 (c), the pH of media with isolate THIA 2 showed there was only a small decline in the pH reading from 6.8 to 6.7, suggesting that the rate of waste accumulation that led to the inhibition of the growth of bacterial isolate was the same.

Based on the results (Figure 4), it could be concluded that there was no degrading activity of THIA occurred in the culture treatment of isolate THIA 2, as the bacteria could have only used MSM as the source of nitrogen supplied for growth. Hence, it can be deduced that isolate THIA 2 was not capable of degrading THIA. Basically, microbes with appropriate degradative enzymes catalyzes the degradation of THIA (Conde-Avila *et al*., 2020). In the present study, the purified bacterial isolates were screened for their efficiency to degrade THIA based on the bacterial log phase in the growth curve. The longer growth phase indicates the degradative ability of the isolated strains in the present study (Dangi *et al*., 2019).

Acinetobacter sp. USMFA THIA 1 showed evidence utilization of THIA for the bacterial growth which was observed in the MSM supplemented with THIA as the sole nutrient source. This can be attributed to the adaptation of the microbes to the pesticide whereby the growth rate of the pesticide degrading bacteria increased (Wang *et al*., 2011). The ability of this strain to utilize THIA for growth was determined by the prolonged log phase when cultured in MSM with THIA as compared to MSM without supplemented with any other carbon source. An increase in turbidity of culture medium was also recorded and this further supports the theory of isolate *Acinetobacter* sp. USM FA THIA 1 being able to degrade and utilize THIA.

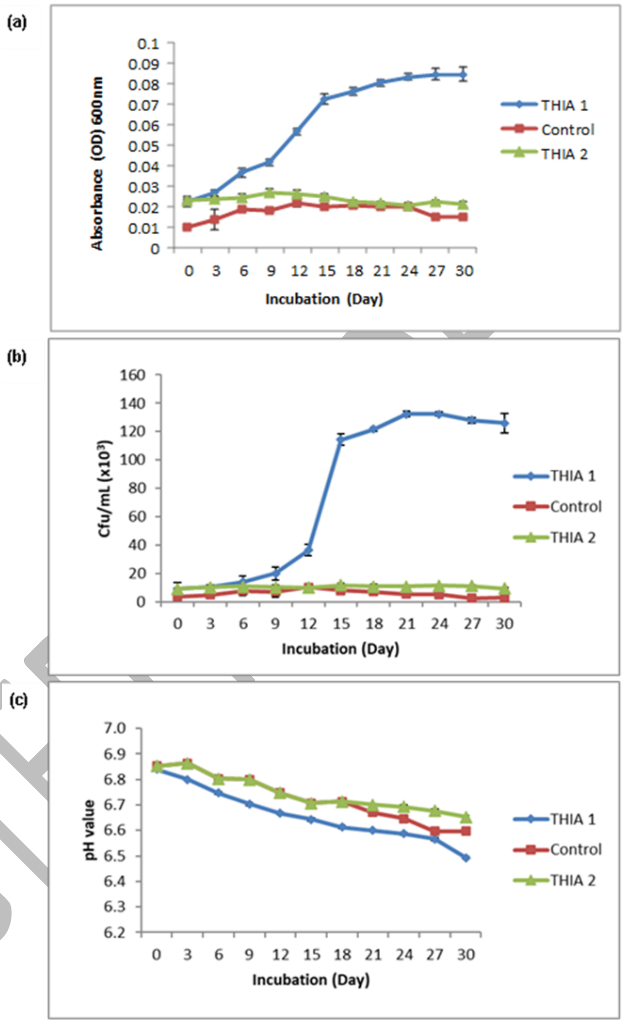

Figure 4: Growth profile of isolate THIA 1 and THIA 2. (a) absorbance, (b) plate count measurement (CFU/mL) and (c) pH of isolate THIA 1 and THIA 2 during 30 days of treatment.

In the present study, the utilization of MSM supplemented with THIA as the sole nutrient sources, enable the identification of bacteria that are able to degrade THIA and distinguish from the non-degraders of THIA. Hence, those bacteria that could grow in the enrichment medium in 30 days in this study are those strains that can degrade THIA (Hedge *et al*., 2017). These days, pesticides have become an integral part in the modern agriculture hence, widely distributed throughout the environment (Stamm *et al*., 2013; Rana *et al*., 2019). Therefore, rapid and robust remediation is essential. Over the past years, physical, chemical and biological degradation have been used for soil remediation. However, the accumulation of toxic intermediates further exhilarated environmental pollution (Kong *et al*., 2018; Sidhu *et al*., 2019). Thus, microbe-sustainable alternative approach to bioremediate the

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

contaminated soils with no or minimal toxic intermediates (Choussonnerie *et al*., 2016; Ortiz-Hernández *et al*., 2018).

The growth of bacterial isolates in a culture medium supplemented with THIA was directly compared with the control. The result showed that the bacterial isolated had higher increase in the reading of absorbance and plate count in the treatment than the control. It can be deduced that the isolate used THIA as its source of nutrient. Thus, the growth of bacterial isolate is promoted (Conde-Avila *et al*., 2020).

CONCLUSION

There were two strains of bacteria that had been isolated, which are Gram-positive *Acinetobacter* sp. USM FA THIA 1 and Gram-negative *Enteroccocus* sp. THIA 2. They were distinguished primarily based on their macroscopic characteristics before identified to molecular level. However, the results of this study indicated that *Acinetobacter* sp. USMFA THIA 1 is capable in degrading THIA. On the other hand, *Enteroccocus* sp. THIA 2 was not capable in degrading THIA as it did not show a significant increase in growth as compared to the control during the log phase with the MSM supplemented THIA. Thus, the present study indicates that the bacterial strain *Acinetobacter* sp. USMFA THIA 1 isolated from fortified soil cultures from a corn plantation in Tanjung Karang, Selangor can degrade THIA. Hence, this study signifies that this strain can be further improved to focus on the characterization of the THIA degradative-enzyme which will pave the way for the microbe-mediated bioremediation to be of great importance to treat THIA contaminated agricultural soil.

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