Jurnal
Ilmu Alam dan Lingkungan

Jurnal Ilmu Alam dan Lingkungan 12 (2), (2021). 32 - 39

Open Acces https://journal.unhas.ac.id/index.php/jai2

Isolation of Indigenous Bacteria from Paddy Field for Methomyl Degradation

W Wartono[1*], RA Suwignyo[2], A Napoleon[2], Suheryanto[3]

[1]*Department of Agrotechnology, Agriculture Faculty, Musi Rawas University, Lubuklinggau, Indonesia*
[2]*Department of Agronomy, Faculty of Agriculture, Sriwijaya University, Ogan Ilir, Palembang-Indonesia*
[3]*Department of Chemistry, Faculty of Mathematical and Natural Science, Sriwijaya University, Ogan Ilir, Palembang- Indonesia*
E-mail: wartono@unmura.ac.id

Abstract

Methomyl is an active ingredient of carbamate group pesticide. The uncontrolled application of methomyl may contaminate the water and soil. The objective of this research was to find indigenous bacteria that could degrade the methomyl. The soil samples were taken from the soil of the rice field located in Musi Rawas District, South Sumatera, Indonesia. The soil bacteria that were found to degrade methomyl were isolated by using a medium containing methomyl. There were 2 of 16 isolates that could grow in a high concentration of methomyl and they were Acinetobacter baumannii and Bacillus megaterium.

Keywords*: bacteria, isolate, methomyl*

INTRODUCTION

The use of pesticides is a powerful way to control pests and diseases. One of the pesticides that were mostly applied by Indonesian rice farmers was methomyl. Uncontrolled this pesticide application may contaminate the soil and water (Ntow, *et al.*, 2006; Ngowi, *et al.*, 2007). Methomyl with chemical formula $C_5H_{10}N_2O_2S$ (EPA US, 1998), was grouped in insecticide oxime carbamate form of white crystals with a melting point of 78-79°C, slightly soluble in water (5.8 g/100 ml) and toxicity acute oral for rats, 17mg/kg. Dermal rather low toxicity (rabbits 5,000 kg/mg). Methomyl was applied to control pests with a wide spectrum such as spiders, ticks, moths, flies, beetles, aphids, leafhoppers, and mites (Mourato & Huxtley, 2000; Chen, *et al.*, 2015).

Methomyl is a highly toxic pesticide that may kill other non-target organisms (Malhat, *et al.*, 2015; Xu, *et al.*, 2009). Methomyl in the soil that is not degraded will be a residue and danger for the environment (Kronmann, *et al.*, 2011; Parven & Nakgoshi, 2001). Degradation can take place through oxidation to form SO, SO_2, and hydrolysis (Tamimi, *et al.*, 2006; Barzman, *et al.*, 2011). Soil bacteria degradation of some pesticides had been isolated from soil. Some degrading bacteria were found in the contaminated soil of methomyl were *Bacillus cereus* and *Bacillus safensis* and *Pseudomonas*

P ISSN: 2086 - 4604
E ISSN: 2549 - 8819

aeruginosa (Tien, *et al.*, 2013; Amritha, *et al.*, 2014). Degradation of pesticides in the soil can be degraded by biotic and abiotic pathways (Kevin, *et al.*, 2012). Methomyl is the most widely used pesticide to control pests on rice cultivation of Musi Rawas District, South Sumatera Province, Indonesia, which reaches 19.20% (Wartono, *et al.*, 2018). This condition would danger for the environment because it made residue on soil and water. It is crucial to find the soil bacteria that may degrade the methomyl. The objective of this research was to find the indigenous soil bacteria which has the capability of methomyl degradation.

MATERIALS AND METHODS

Soil Sampling

Samples of soil were collected from a rice field in Musi Rawas District, South Sumatera, Indonesia where methomyl was applied uncontrolled. Soil samples were taken on the upper layer of soil (15 to 25 cm depth) into glass jars and stored at 30°C.

Media Preparation

Mineral salts medium (MSM) was prepared contained the following compositions gL^{-1}: 0.2 $MgSO_4.7H_2O$, 1 KH_2PO_4, 1 K_2HPO_4, 0.5 $(NH_4)_2SO_4$, 0.01 $CaCl_2$, 0,001 $FeSO_4$ dan 0.5 $NaNO_3$ (Fan *et al*. 2012) and added methomyl 1 ppm. The isolation of methomyl degrading bacteria was performed using an enrichment culture. Soil sample 10 g was suspended in distilled water 90 ml containing 1 ppm of methomyl and then shook using a rotary shaker at 120 rpm and 30°C for 5 days. 1 ml of each enrichment culture transferred to the tube test contains NaCl 0.85% with volume 9 m and make series dilution 10^{-6} at dilution of 10^{-4}, 10^{-5}, and 10^{-6} was inoculated on MSM at 37^{o}C for 2 days.

Purification was perfumed by streak plate method, the isolated bacteria in Nutrien Borth (NB) media enriched insecticide active ingredient then scratch in MSM and incubated for ± 24 hours, after growing these colonies were cultured in media MSM again to obtain pure bacterial culture. Pure isolates were coded TM (Soil Bacteria Methomyl), Gram's staining is done by taking one ose bacteria in aseptic, was transferred in a glass preparations contain physiological saline solution 0.85%, further stirred by vortex and left for 1-2 hours, then taken using a micropipette and then dropped on the glass slide and given a solution of crystal violet for 3 minutes and then washed with water, the next stage in the given Lugol for 1 minute and then washed with water, and then given alcohol 75 for 30 seconds and last wash with water. then given seframin for 1 minute and dry. it is observed using a digital microscope.

Isolates Growth Test

Pure isolates obtained were grown on MSM containing methomyl (Bestway, *et al*., 2013), and then incubated for 24 hours at room temperature. Isolates were grown in NB media plus methomyl of 0; 0.5; 1; 1.5; 2.3 and 6 ppm. Test growth was observed every three hours up to 30 hours, observations were made by using a spectrophotometer at a wavelength of 625 nm. Bacterial isolates were selected as a bioremediation agent is isolates grown in media containing the highest concentrations of pesticides. As a comparison was made of other growing medium without active ingredients of pesticides as control (George & Smotzer, 2007).

The standard curve was made by counting the number of colonies using Total Plate Count (TPC) and the observation of density using a spectrophotometer at a wavelength of 625 nm every 3 hours. The standard curve created by regressing the number of bacteria colonies and density values using a standard curve line equation y = ax + b, y = the number of colony-where, x = Optical Density (figure 1). The observation of the number of colonies and the optical density (OD) values shown in the following table 1.

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Table 1. Measurement of OD Value and Number of Colonies

Hour	OD	TPC
0	0:06	38
1	0:42	187
2	0716	277
3	0936	321
6	1,121	387
9	1,207	455

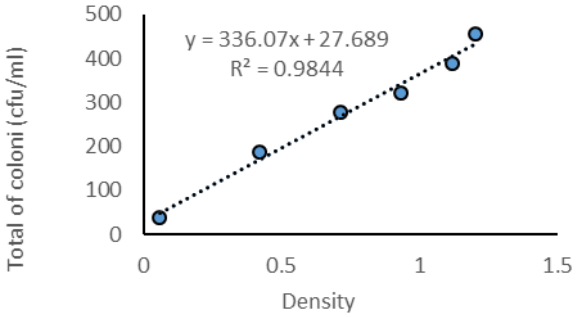

Figure 1. Standard curve.

Identification of the Isolates

Superior bacterial isolates that were found then performed morphological identification, identification of 16S RNA sequences phenotypic and molecular. Identification was done in the microbiology laboratory.

RESULTS AND DISCUSSION

Isolation of Bacteria Resistant

The results showed that most of the bacterial colonies look visible white with spread positions inside and on the surface of the media and flat edges. Numbers of colonies in each sample (table 2) showed that bacterial colonies in samples less survived than the control. 517.

Table 2. Numbers of Colonies in Each Sample

No.	Sample	Number of Colonies
1	1	$1{,}5 \times 10^5$
2	2	$1{,}9 \times 10^4$
3	3	$6{,}0 \times 10^6$
4	4	$5{,}6 \times 10^6$
5	5	$1{,}3 \times 10^6$
6	Control	$9{,}6 \times 10^6$

It means that there were bacteria that survive in polluted soils of methomyl 1 ppm. This bacteria is would be bacteria-tolerant of methomyl (Malhat, *et al*., 2015), these bacteria can eliminate toxic compounds and produce enzymes that degrade toxic compounds (Zhoua, *et al*., 2012). On the other hand, intolerant bacteria would die. The higher the concentration of methomyl, the slower the bacteria grow and capable of lowering the concentration of methomyl (Tien, *et al*., 2013).

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E ISSN: 2549 - 8819

Results Purification Isolates

The results of bacterial purification of all samples contained 16 isolates namely the code TM1 to TM16. It was methomyl-resistant bacteria that resistant to methomyl residues.

Selection using Growth Test

Bacteria that grow on media containing methomyl can not be ascertained that methomyl degraded by bacteria, but these bacteria showed the ability to adapt to the media contaminated methomyl and accumulated in cells or in combination with a compound found in nature (Pradhan, *et al*., 2008; Fan, *et al*., 2012). Bacteria are necessary to test the growth with an increase in the concentration of methomyl. Test 19 isolates growth at a concentration of 6 ppm is shown in (figure 2).

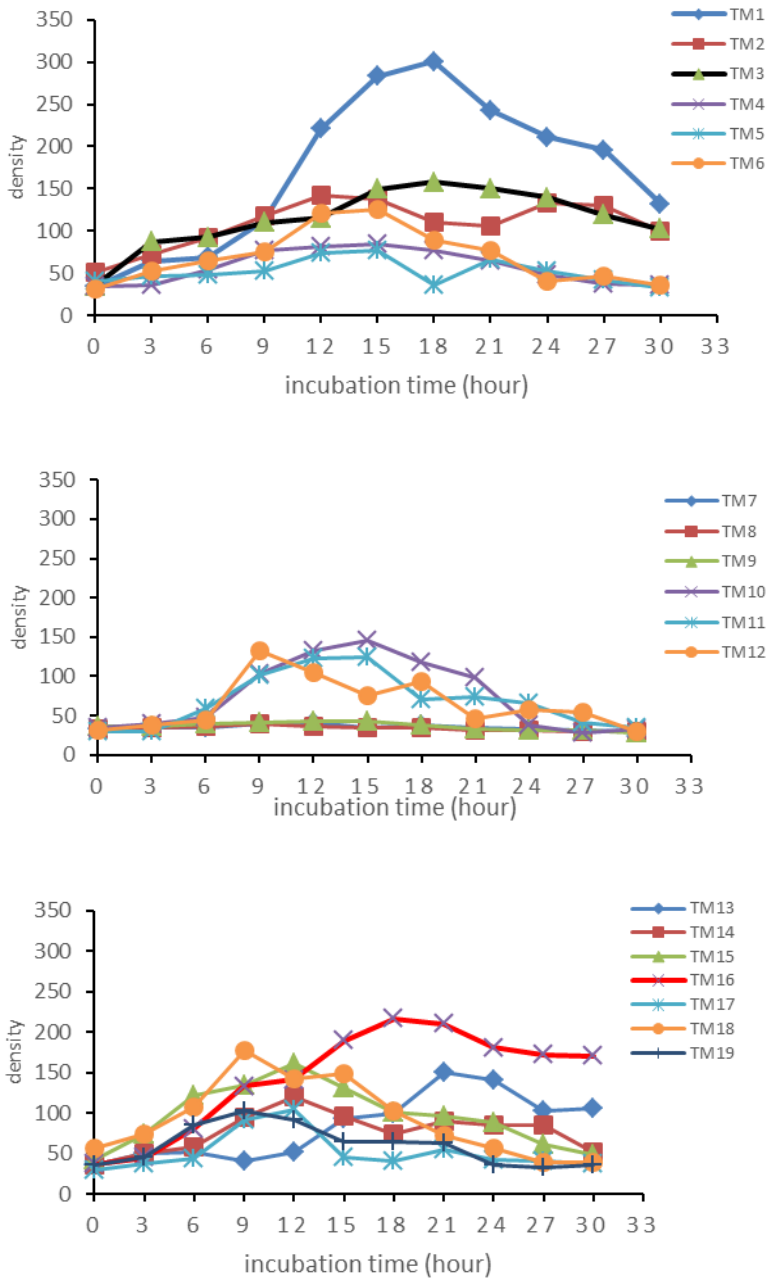

Figure 2. TM1-TM19 growth curve at 6 ppm methomyl concentration.

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E ISSN: 2549 - 8819

Bacterial growth generally grouped into four phases consisting of phase lag (adaptation), the exponential phase (propagation), stationary phase (static) and the death phase (Kharisma & Prazard, 2014). The results of this study indicate that each isolate was in the lag phase at the 0th hour until the 3rd hour, in that phase the bacterial isolate adapted to the new environment, namely media containing methomyl (Tamimi, *et al*., 2006; Kevin, *et al*., 2012). A lag phase transition to an exponential phase after the initial population has doubled (Matthew, *et al*., 2012), this phase lasts from a few minutes up to several hours (Yates & Smotzer, 2007).

The exponential growth phase is at the 6th hour until the 18th hour, in this phase of cell division isolates perform optimally because the bacteria utilize the enzyme to perform the hydrolysis of the chemical in the process of metabolism and cell division (Eric, 2012). The exponential growth phase is the phase of the fastest growth of bacteria, the rate of increase in the medium of cells proportional to the number of cells that exist at any given time (Yates & Smotzer, 2007), during the exponential growth of the number of cells increased two-fold. The exponential growth phase may occur within a short period of cell division occurs briefly in 20 minutes (Matthew, *et al*., 2012).

The growth of each isolate at the concentration of methomyl 3 and 6 ppm at the 21st hour has decreased, this shows that the isolate has entered a phase of death as indicated by a decreased density value (Yates & Smotzer, 2007), the phase of the death of bacteria due to the unavailability of nutrients and accumulated toxic waste products that cause bacterial cells are dead (Margesin**,** 2009; Kelly & Kenneth, 2008). The result of the growth of 16 isolates (figure 1) shows that all isolates were able to survive in media containing methomyl, both at low concentrations or high, but the ability to grow and the growth of cells of each isolate different (Xu, *et al*., 2009; Roy & Dast, 2017). Some isolates that are able to adapt well at a concentration of 6 ppm can be seen from the fluctuation of density values, isolates TM1, TM3, and TM16 with the time of growth in the exponential phase, which is longer that is the 9th hour to the 24th hour.

Isolates TM1 able to adapt more quickly to the hour-0 until the 3rd and entered a phase of exponential growth until the stationary phase at the 24th hour by the number of colonies of 354.349 cfu x 10^7, up to an hour to 30 races was still able to survive with the number of colonies that are still high at 131.871 x 10^7 cfu, 16S RNA sequence identification results in phenotypic and molecular TM1 resemblance 100% with *Acinetobacter baumannii* strain DSM 30 007 (figure 3). TM1 isolates were able to utilize methomyl as a food source for cell-division with 6 ppm (Singh, *et al*., 2012).

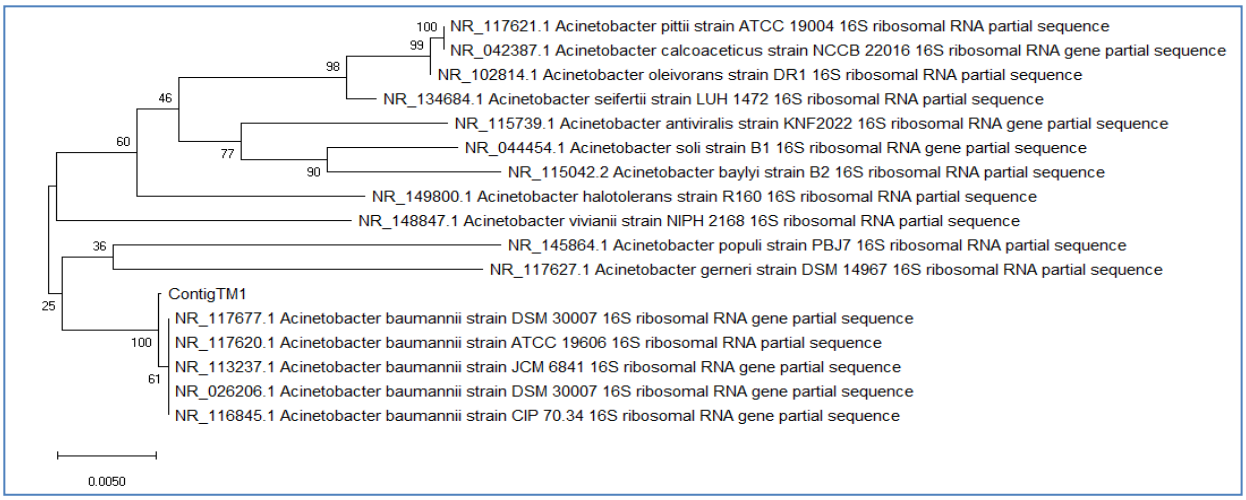

Figure 3. TM1 sequensing.

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E ISSN: 2549 - 8819

TM3 isolates were also able to grow and adapt to methomyl concentration of 6 ppm, has a phase of adaptation on hour-0-3, the exponential phase of the clock to the 6th until the 24th hour, and began to decline entered a phase of death in the 27 hours up to 30th hour. The bacteria that persist in the environment, making use of methomyl as an energy source. These bacteria are the best candidate for the bioremediation of pesticide methomyl on the ground (Roy & Dast, 2017). Based on the identification of 16S RNA sequences TM3 (Figure 4). Isolates were identified as *Bacillus megaterium* strain NBRC 15 308.

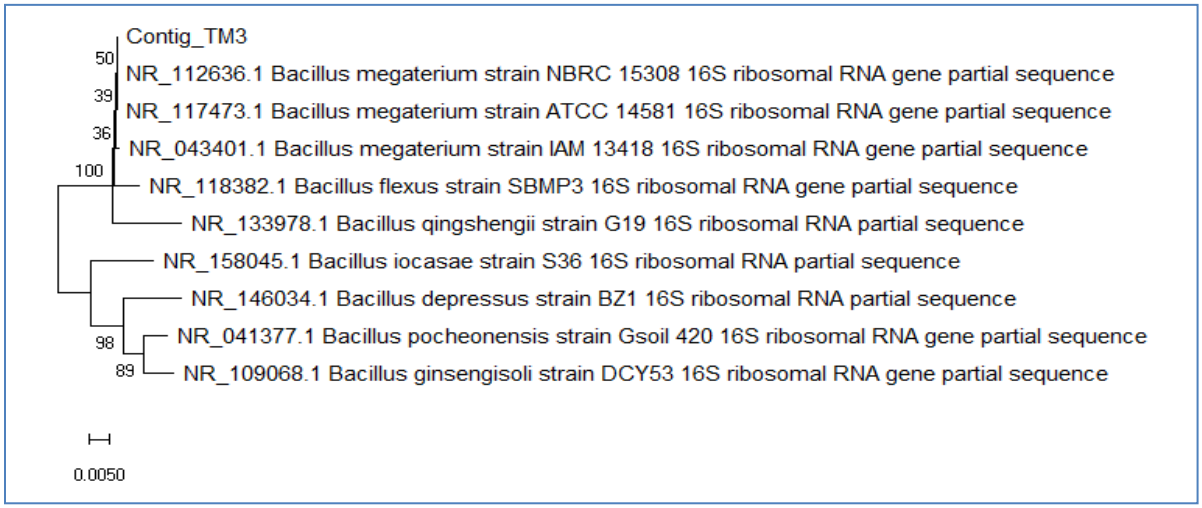

Figure 4. TM3 sequensing.

Isolate TM16 also isolates capable of utilizing methomyl as an energy source and is able to survive longer, these isolate have almost the same period exponential phase with TM1, TM16 isolate sequencing results are shown in Figure 5. The availability of essential energy sources such as carbon, nitrogen, phosphorus, and oxygen can also limit the ability of bacteria to degrade pesticide residues (Malik, 2006; Eric, 2012).

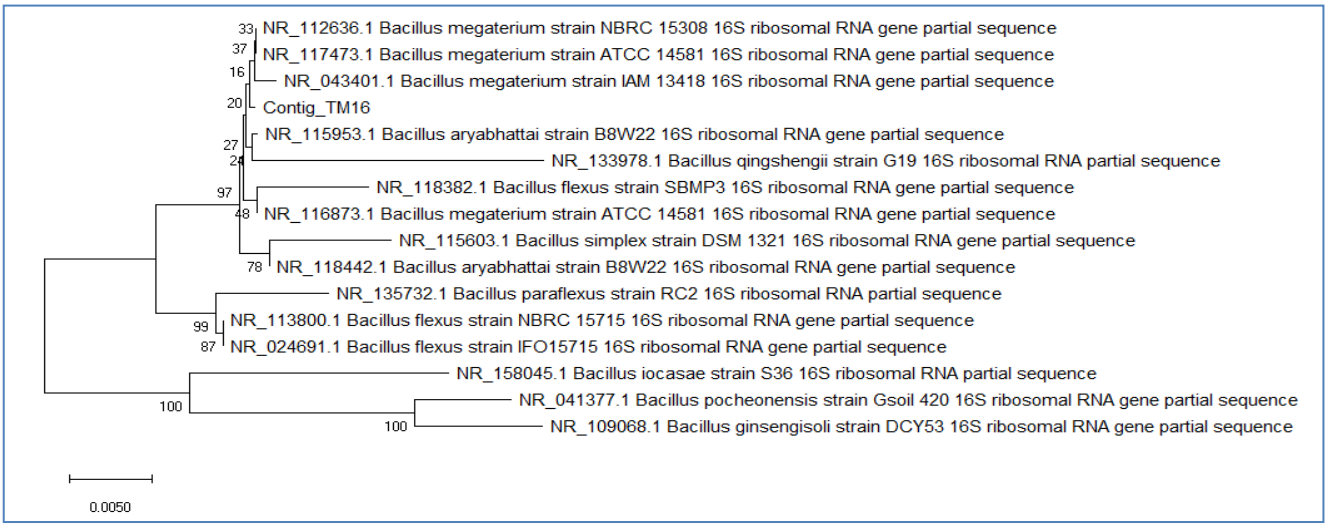

Figure 5. TM16 sequensing.

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E ISSN: 2549 - 8819

The concentration of highly toxic pesticide residues and are too low can cause biodegradation is not running due to lack of induction of degradative enzymes (Pradhan, *et al*., 2008; Kevin, *et al*., 2012), and appropriate uptake mechanisms. Failure to induce sufficient enzyme activity, or problems in providing sufficient energy for cell maintenance even (Jilani & Khan, 2004). Bacterial isolate have in common with TM3 isolates such as *Bacillus megaterium*. These bacteria included in the class of mesophilic bacteria because the bacteria are able to survive at a pH ranging from 5.5 to 8. In alkaline conditions relatively more stable growth than at acidic pH. In addition, Bacillus megaterium can grow at temperatures 30-37^{o}C (Yates & Smotzer, 2007; Patel, *et al*., 2016).

CONCLUSION

There were 2 of 19 isolates were superior and able to grow with a high concentration of methomyl in concentration 6 ppm, they were *Acinetobacter baumannii* and *Bacillus megaterium.* It is a prospect to use them for degrading the contaminated soil of methomyl.

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P ISSN: 2086 - 4604
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