



Analysis of 2,2-DCP degrading bacteria isolated from a paddy field at a rural area in Malang, Indonesia

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ABSTRACT

Aims: The use of herbicide effectively controls weeds in agricultural practice. However, its release to the surrounding surface water bodies may lead to environmental issues. The aim of this study was to isolate the bacteria that were able to remove 2,2-dichloropropionic acid (2,2-DCP) from a paddy field located in Malang.

Methodology and results: The 2,2-DCP degrading bacteria were isolated and their ability to grow on higher 2,2-DCP concentrations (50 and 80 mM) was tested. Bacterial degradation of 2,2-DCP was examined through measurement of released chloride ions. The potential isolates were identified according to their 16S rDNA sequences. Two potential isolates, BB9.2 and BC14.3 were observed for their growth on 20, 50, and 80 mM 2,2-DCP. Isolate BC14.3 had the shortest cell doubling time of approximately 4.1 h with 100% 2,2-DCP (20 mM) utilization, whereas BB9.2 was only able to degrade 80% of 2,2-DCP at the same concentration. The 16S rDNA gene sequences suggested that BB9.2 and BC14.3 belong to *Acinetobacter calcoaceticus* and *Pseudomonas plecoglossicida*, respectively.

Conclusion, significance and impact of study: Bacterial strains with 2,2-DCP degrading potentials were successfully isolated from long-term exposed agricultural soil. They demonstrated notable utilization of the organic halide. This is the first time that strains of *A. calcoaceticus* and *P. plecoglossicida* were reported to utilize 2,2-DCP.

Keywords: Paddy field, 2,2-DCP utilizing bacteria, *Acinetobacter*, *Pseudomonas*

INTRODUCTION

Rice is a very important commodity in Indonesia and the stable yield is maintained to meet the domestic demand. A substantial increase had been recorded in rice production from 50 Tg to 75 Tg within the last twenty years (Anonymous, 2016). Nevertheless, plant pests and weeds are still a major concern prior to harvest time. The productivity of rice paddy fields was negatively influenced by the successive growth of accompanying weeds. Uncontrolled growth of weeds significantly delayed the harvest time and even reduced both the quantity and quality of the crop's yield (Abouziena and Haggag, 2016).

For decades, traditional farmers in Indonesia intensively use herbicides as the absolute solution for controlling weed growth in the fields. Moreover, during the rainy season, the application of herbicides was more frequent, and the effective dose was raised to compensate for dilution by rain water (Hudayya and

Jayanti, 2013). Such intensive application of herbicides is undoubtedly important for the agricultural activity. However, their excess is potentially harmful to animals and humans when they enter the environment through irrigation runoff.

One of the most common herbicides used in Indonesia is 2,2-dichloropropionic acid (2,2-DCP; the commercial name is Dalapon). As an active compound, it prevents growth of targeted weeds by inhibiting lipid and protein biosynthesis. However, the organic halogen compound is water soluble and known to be toxic for human (Wong and Huyop, 2012). It has been reported that human exposure to 2,2-DCP leads to several issues, such as skin irritation, corneal damage, diarrhea, slowing of pulse, and irritation of the respiratory tract (Malaie and Malaie, 2013). These health risks have encouraged a

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series of experiments on the effective removal of the toxic halogen compound through bacterial degradation.

The 2,2-DCP herbicide is initially biodegraded by removal of the halogen functional groups using bacterial dehalogenase enzyme. The reaction releases two chloride ions and one pyruvate. Pyruvate is subsequently oxidized by the cell to obtain energy and assimilated as a carbon source (Sinha *et al.*, 2009; Abel *et al.*, 2012). Bacteria are capable of evolving new enzymes, pathways, and regulatory mechanisms for the degradation of almost all xenobiotic compounds, due to their short life cycle (Timmis and Pieper, 1999). The ability to degrade the xenobiotic 2,2-DCP is possibly due to bacterial long-term adaptation in the contaminated area (Schroll *et al.*, 2004).

Paddy fields in a rural area in Malang rely on a continuous application of 2,2-DCP for years. It is effective at controlling the growth of weeds such as *Echinochloa crusgalli* that is commonly found in the paddy fields (Kadir, 2007). However, the unabsorbed 2,2-DCP settled in the soil and may be dissolved in the irrigation water. It is hypothesized that 2,2-DCP degrading bacteria are readily present in the paddy field. Therefore, this study was aimed to isolate bacteria for potential agents of 2,2-DCP biodegradation.

MATERIALS AND METHODS

Collection of soil samples

The area of interest was a paddy field that received intensive application of 2,2-DCP for a decade as told by several local farmers at Sumber Ngepoh, Malang, East Java, Indonesia (Figure 1). Three specific locations were chosen based on their gradual proximity to the primary water source of irrigation. Location A was 0-10 m to the water source (S 07° 49' 36.3" and E 112° 43' 46.9", 368 meter amsl), while location B (S 07° 49' 36.3" and E 112° 43' 47.2", 362 meter amsl) and C (07° 56' 44.5" and E 112° 37' 45.7", 360 meter amsl) were 20-30 m and 30-40 m to the water source, respectively. Multiple locations were used to increase the possibility in obtaining potential 2,2-DCP degrading bacteria. Soil samples were collected from the ground surface to 10 cm depth using a soil borer. Composite samples obtained from each location were placed in sterile plastic bags and kept cold in an ice box until the next treatment in the laboratory.

Isolation of 2,2-DCP degrading bacteria

Bacterial isolation was achieved by making three suspensions of 25 g composite samples in 0.85% NaCl solutions (1:9 w/v) and subjected to subsequent serial dilutions (Panda *et al.*, 2012). Aliquots of 0.1 mL were spread onto agar plates of minimal salt medium (MSM) containing 20 mM 2,2-DCP and incubated at 30 °C to obtain single colonies. The colonies were purified by dilution and spreading on MSM agar plates. Pure colonies were confirmed by gram staining. The MSM medium composed of basal salts K₂HPO₄ (4.25 g/L), NaH₂PO₄·2H₂O (1.0 g/L), (NH₄)₂SO₄ (2.5 g/L) and trace

metal salts MgSO₄·7H₂O (1.0 g/L), FeSO₄·7H₂O (60 mg/L), MnSO₄·4H₂O (15 mg/L), and ZnSO₄·H₂O (15 mg/L) (Merck) in distilled water (Hareland *et al.*, 1975). The medium was autoclaved at 121 °C for 15 min. The sole carbon source, 2,2-DCP (Merck) was sterilized separately using membrane filtration and mixed aseptically with the autoclaved media achieving final concentration of 20 mM. In order to prepare solid medium, 1.5% bacteriological agar (Oxoid) was added prior to autoclaving.



Figure 1: Satellite map of the paddy fields. Pinpoints indicate the sampling locations where soil samples were obtained. Scale bar represents 60 ft.

Growth potential on 2,2-DCP

Five milliliter cultures of purified isolates with equivalent cell numbers were inoculated in 45 mL MSM containing 20 mM 2,2-DCP. The cultures were incubated for 48 h and the cell concentrations were estimated by measuring the turbidity of the suspension at Abs_{500nm}. A medium without bacterial inoculum was used as a control. This step was done in triplicates. The optical density (OD) values were analyzed using one-way analysis of variance (ANOVA) and Games-Howell test ($\alpha = 0.05$) using a licensed software of SPSS for Windows V16.0. Isolates with high growth yield as indicated by high OD values were chosen for further investigation.

Bacterial growth on higher 2,2-DCP concentration

To assess growth ability of the bacterial isolates on higher 2,2-DCP concentration, their growth curves were generated in triplicates. Ten milliliter cultures with equivalent cell numbers were inoculated in 90 mL MSM containing 20, 50, and 80 mM 2,2-DCP and incubated for 72 h. The cell concentrations were estimated by measuring the turbidity of the suspension at Abs_{500nm} at 6-h interval. Doubling time of each isolate was calculated according to a period of exponential growth phase.

Chloride ion release from 2,2-DCP

Dehalogenase activity was measured by determining the release of chloride ion during bacterial growth on 2,2-DCP

using colorimetric method (Bergman and Sanik, 1957). Five milliliter bacterial cultures with equivalent cell numbers were inoculated in 45 mL MSM containing 20 mM 2,2-DCP and incubated for 48 h. The concentration was used since it was the best concentration used to determine the dehalogenase enzyme activity from the previous study (Hussein and Huyop, 2014). Chloride ion concentration was measured at before and after the incubation. The maximum concentration of released chloride ion per stoichiometric calculation was 40 $\mu\text{mol Cl}^-/\text{mL}$ for the 20 mM 2,2-DCP in the medium. Negative control was used without bacterial inocula. A chloride test kit HI 3815 (Hanna Instruments) was used to measure free chloride ion following the manufacturer's protocols. The chloride ion standard curve was constructed within range of 0-0.2 $\mu\text{mol NaCl}$. All measurement were done in triplicates. Data of chloride ion released were analyzed using ANOVA and Games-Howell test ($\alpha = 0.05$) using a licensed software of SPSS for Windows V16.0.

Bacterial 16S rDNA identification

Bacterial isolates were grown in Nutrient Agar (NA) medium and the colonies were subjected to DNA extraction using i-genomic soil DNA extraction mini kit (iNtRON Biotechnology, Inc). The polymerase chain reaction (PCR) was carried out by using primers 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1495r (5'-CTACGGCTACCTTGTACGA-3') to amplify 16S rDNA (Varma and Oelmüller, 2007). Amplification of the 16S rDNA by PCR was performed for 35 cycles and was set initial denaturation of 94 °C for 5 min, then denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 1.5 min and final extension at 72 °C for 7 min. Amplicons were purified and sequenced by 1st BASE Malaysia. The obtained 16S rDNA sequences were aligned according GenBank database using BLAST nucleotides to define the genetic similarities (Altschul *et al.*, 1997). The phylogenetic tree was constructed based on the Maximum Likelihood method using CLUSTAL W alignment in MEGA 6 software (Tamura and Nei, 1993; Tamura *et al.*, 2013).

RESULTS

Growth potential on 2,2-DCP

A total of 20 distinctive colonies from three locations were obtained and cultured in the liquid medium containing 20 mM 2,2-DCP. They showed visible turbidity after 48 h incubation in the medium while no growth was observed in control. Two isolates, coded as BB9.2 (from location B) and BC14.3 (from location C), were selected due to their high growth yields since 2,2-DCP was the sole carbon source to support growth. Their OD values were twofold, as high as the average of the other isolates (data not shown). Isolate BB9.2 was confirmed as a gram-negative cocco-bacillus while BC14.3 was a Gram-negative rod.

Growth on higher 2,2-DCP concentration and chloride ion release

The cell doubling time of both isolates was measured for all growth in the presence of 20, 50, and 80 mM of 2,2-DCP concentrations.

Table 1: Doubling time of isolate BB9.2 and BC14.3 growth on various 2,2-DCP concentrations.

Isolate	2,2-DCP concentration (mM)	Doubling times (h)
BB9.2	20	9.9 ± 0.8
	50	7.1 ± 1.6
	80	5.4 ± 0.5
BC14.3	20	9.2 ± 0.5
	50	4.1 ± 0.1
	80	8.6 ± 0.3

The doubling time for isolate BB9.2 was 9.9 h, 7.1 h and 5.4 h, respectively. On the other hand, the isolate BC14.3 showed shorter doubling times on 20 and 50 mM 2,2-DCP which were 9.2 h and 4.1 h, respectively (Table 1). The total amount of chloride ion released in the growth medium was measured following 48 h growth on 20 mM 2,2-DCP. Total chloride ion released by BB9.2 was 34 $\mu\text{mol Cl}^-/\text{mL}$ while BC14.3 was 46 $\mu\text{mol Cl}^-/\text{mL}$ (Figure 2). The results showed that there were significant differences for total chloride ion released in the medium of both isolates (p -value $\alpha > 0.05$).

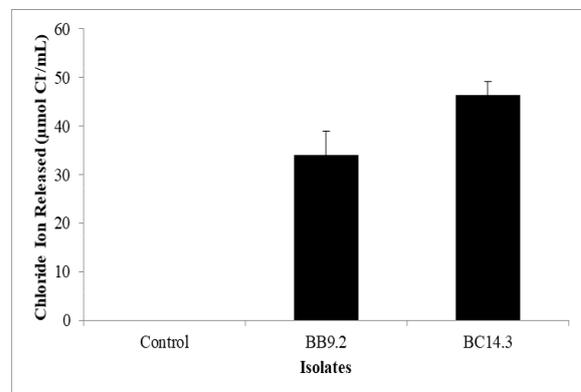


Figure 2: Total chloride ion released by isolates in MSM containing 20 mM 2,2-DCP after 48 h incubation. Negative control used was MSM without inocula.

Bacterial 16S rDNA identification

The bacterial isolates were identified on the basis of 16S rDNA gene sequences. The sequences were analyzed and aligned to the sequences in GenBank using the BLASTn analysis tool. The result showed 99% identity to *Acinetobacter calcoaceticus* strain H3 and *Pseudomonas plecoglossicida* strain S8 (Figures 3 and 4). Therefore, both isolates were designated as *A. calcoaceticus* strain BB9.2 and *P. plecoglossicida* strain BC14.3.

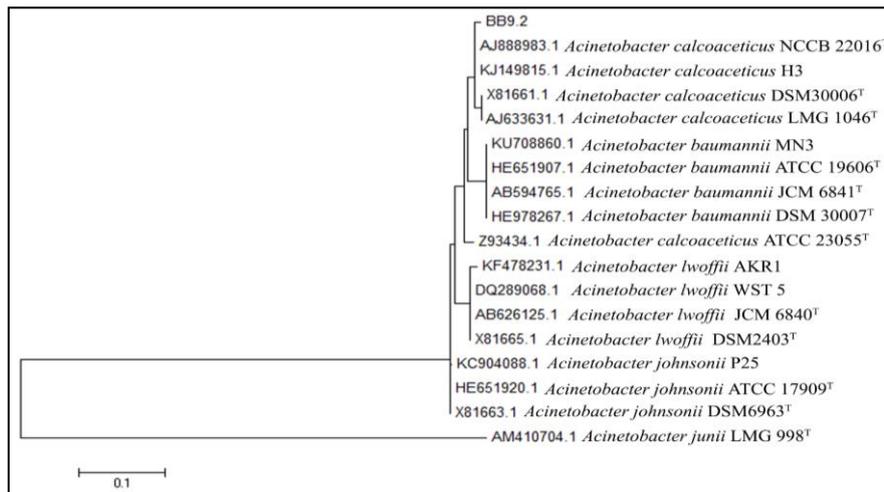


Figure 3: Phylogenetic tree constructed by Maximum Likelihood method (1000 bootstraps) for 16S rDNA sequences. The scale bar indicates the proportion of sites changing along each branch. BB9.2 is the isolate investigated in this study.

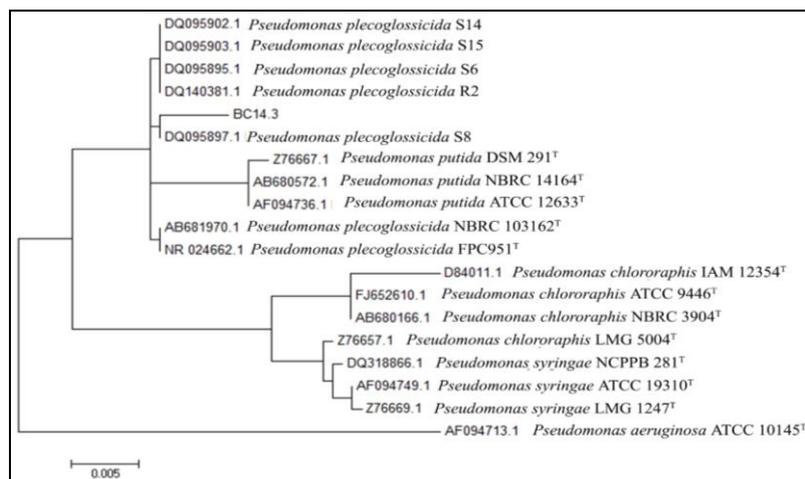


Figure 4: Phylogenetic tree constructed by Maximum Likelihood method (1000 bootstraps) for 16S rDNA sequences. The scale bar indicates the proportion of sites changing along each branch. BC14.3 is the isolate investigated in this study.

DISCUSSION

In this study, two potential 2,2-DCP degrading bacteria were isolated from soil of contaminated paddy field, *A. calcoaceticus* strain BB9.2 and *P. plecoglossicida* strain BC14.3. Those species have not previously been reported according to their nature in degradation of 2,2-DCP. However, several strains of the same species were reported for their roles in degradation of other organic halides. *A. calcoaceticus*, as reported by Zeitsev and Baskunov (1985), was known to degrade 3-chlorobenzoic acid (3-CBA) to chloride and muconic acid. A strain of *P. plecoglossicida* isolated from wastewater could degrade 2-chloropropionic acid (2-CPA) and 2-monochloroacetic acid (2-MCA) (Song *et al.*, 2003). Moreover, within the same genus, *A. baumannii* was reported for its capability

in hydrolyzing halogenated propionic acid with a halide group (Abel *et al.*, 2012b). *P. aeruginosa* from a coast was reported for its ability in using α -haloalkanoic acid and 2,2-DCP as a sole carbon source (Edbeib *et al.*, 2016).

During growth on 20 mM 2,2-DCP, the growth rates of *P. plecoglossicida* strain BC14.3 was faster than *A. calcoaceticus* strain BB9.2. The doubling time of *A. calcoaceticus* strain BB9.2 was 9.9 h, while that of *P. plecoglossicida* strain BC14.3 was 9.2 h. The cells doubling time may have varied between species as reported in earlier investigations (Hussein and Huyop, 2014). Bacteria that have the ability to degrade 2,2-DCP will have different characteristics, based on the level of organic-halide contamination in the location used for bacterial isolation. In a previous study, bacteria isolated

from the hot spring in Thailand and wastewater from Tioman island have doubling times of more than 20 h in medium containing 20 mM 2,2-DCP (Hussein and Huyop, 2014; Niknam *et al.*, 2014). Meanwhile, 2,2-DCP degrading bacteria isolated from contaminated soil at Johor has doubling times of less than 5 h in medium containing 20 mM 2,2-DCP (Abel *et al.*, 2012a). *Burkholderia* sp. isolated from a rubber estate area in Malacca has a doubling time of 6 h in medium containing 20 mM 2,2-DCP (Wong and Huyop, 2012). *Bacillus cereus*, isolated from feces of ruminant animal that consumed contaminated grass has a doubling time of less than 3 h in medium containing 20 mM 2,2-DCP (Ismail *et al.*, 2016).

In comparison to the exposure of higher 2,2-DCP concentrations, 50 and 80 mM, the growth rate of *A. calcoaceticus* strain BB9.2 was getting faster as the 2,2-DCP concentration in the medium increased. The shortest doubling time of the isolate (5.4 h) was observed in medium containing 80 mM 2,2-DCP. Meanwhile, *P. plecoglossicida* strain BC14.3 peaked the doubling time in medium containing 50 mM 2,2-DCP. Those isolates responded differently to the same 2,2-DCP concentration. Each species may differ in the regulation of substrate uptake and tolerance mechanism to toxic compounds (Ghoreishi *et al.*, 2017). Nevertheless, both strains have a considerable ability to utilize 2,2-DCP at high concentration. As previously reported, the growth of 2,2-DCP degrading bacterium was significantly inhibited while exposed to the concentration of higher than 20 mM due to its toxicity effect (Wong and Huyop, 2012). Another study demonstrated that *Pseudomonas* sp. strain R1 has the best growth in medium containing 40 mM monochloroacetic acid (Ismail *et al.*, 2008). Similarly, as reported by Edbeib *et al.* (2016), *Pseudomonas aeruginosa* MX1 showed the best growth in medium containing 20 mM 2,2-DCP and the growth rate decreased at 30 mM concentration of 2,2-DCP. *Enterobacter cloacae* strain D9 and *Burkholderia* sp. strain Dw, isolated from a rubber estate agricultural area, could degrade 2,2-DCP in up to 40 mM concentrations, while at 80 mM both of the strains showed no growth (Wong and Huyop, 2012). The tolerance to high concentration of 2,2-DCP may be related to frequent exposure of the organic halide on the sampling location. Bacterial species survived in a polluted soil would lead to a more efficient pollutant biodegrader (Ghoreishi *et al.*, 2017).

The ability of growth on organic halide as a sole carbon source was presented by their growth rate, as described in the previous paragraph, and the amount of released chloride ion. In this study, the chloride ions released by both isolates were assumed proportional to their doubling time during growth on 20 mM 2,2-DCP. The *P. plecoglossicida* strain BC14.3 with doubling time of 9.2 h released chloride ion of 40 $\mu\text{mol Cl}^-/\text{mL}$ which is 100% degradation of 20 mM 2,2-DCP. Meanwhile, the isolate *A. calcoaceticus* strain BB9.2 (9.9 h) released 34 $\mu\text{mol Cl}^-/\text{mL}$ (80% 2,2-DCP degradation). Chloride ions were released into the medium following enzymatic hydrolysis

of 2,2-DCP inside the cells (Abel *et al.*, 2012a). The accompanying product, pyruvate, was essential for cells. It is catabolized to supply carbon and to generate energy required by cells to grow. Therefore, the high concentration of released chloride ion may indicate the high demand of pyruvate as required for a rapid grow.

CONCLUSION

The 2,2-DCP degrading bacteria isolated from paddy agricultural fields were identified as *A. calcoaceticus* and *P. plecoglossicida*. They grew well on 2,2-DCP as the sole carbon source and one of them showed chloride ion release up to 40 $\mu\text{mol Cl}^-/\text{mL}$ on 20 mM 2,2-DCP. The isolates also exhibited tolerance to higher concentrations of the organic halide. A study in employing the isolates within a microcosm is needed to observe the feasibility as potential bioremediation agents.

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