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Characterization of multi-degrading bacterium associated with corals on pesticide compounds isolated from Karimunjawa Island, Indonesia

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Abstract

The contamination of marine ecosystems with organic compounds from agricultural and industrial sources has fuelled research into these frequently toxic compounds. The purpose of this study was to characterize the bacteria associated with corals, which are competent at degrading phenylurea diuron, diazinon, and s-triazine pesticide compounds. The study of pesticide degradation on bacteria associated with corals was conducted by isolating the bacteria from coral tissues, purifying it and implementing a sensitivity test, degradation test and microbiological and molecular characterization. The result showed that among 392 bacterial isolates, only 111 isolates were able to degrade phenylurea diuron herbicide compounds on EMBA indicator media. KA5 isolate was selected based on the best results of the sensitivity and degradable test. This strain is facultative anaerobic with that pesticide serving as the only known energy sources. Microscopy of isolate revealed that strain KA5 is gram-positive, catalase-positive, rod, spore-forming bacterium, non-motile, and unpigmented colonies. The bacterium could be identified on the basis of its carbon-source-utilization pattern as a genus *Bacillus* sp. A partial sequencing of the 16S rDNA analysis suggest that this strain is closely related to *Bacillus haldenitrificans*.

Introduction

The coral reef is an ecosystem in the tropical ocean floor which has been built primarily by scleractinian corals and coralline algae. About 85.707 km² or 14 percent of total corals in the world extends round the islands of Indonesia. Sea pollution has increased recently because of industry waste disposal and agriculture chemical residues. Pesticides are the existing pollutant that endangers Indonesian coral conservation. Glynn *et al* (1984) stated that 2,4-D and 2,4,5-T herbicide kills corals at low concentrations in brief exposure.

Microbes are ubiquitous in the marine environment and, not surprisingly, mucus-covered coral surfaces are often colonized by bacteria and other microorganisms (Paul *et al.*, 1986; Coffroth, 1990). Several bacteria with the ability to degrade a wide range of compounds have been isolated, including chlorinated aromatic compounds such as chlorinated benzoic acids, chlorinated phenol, chlorinated benzenes and chlorinated biphenyls. Recent reviews present a detailed and up-to-date account of the degradation of halogenated aromatic compounds by various microorganisms (Reineke and Knackmuss, 1988; Chaudry and Chapalamadugu, 1991).

The association of biologically active microorganisms with corals of the Karimunjawa Archipelago was examined. These studies included a cultivation of the microorganisms, a determination of the identity by 16S rDNA analysis and different biological tests.

Materials and Methods

Bacterial isolation

Corals representing different life forms (massive, sub-massive, branching and foliose) were collected from Karimunjawa Archipelago, North Java Sea (Fig. 1) by scuba diving, and were put in a small sterile plastic bag (Whirl-Pak, USA), stored in ice and immediately brought to the laboratory at the Marine Station of Diponegoro University. Coral mucus was collected by scraping the coral surface and putting the sample into a sterile glass beaker containing 90 ml sterile seawater. One ml of this dilution was transferred into a test tube containing 9 ml sterile seawater and shaken for homogeneity. Dilution series were then prepared and 100 μ l of diluted sample spread onto Zobell 2216 E medium and incubated for 48 hours in room temperature. Based on the morphological features, each colony was streaked on to Zobell 2216E solid medium and transferred several times until a pure isolate was obtained.

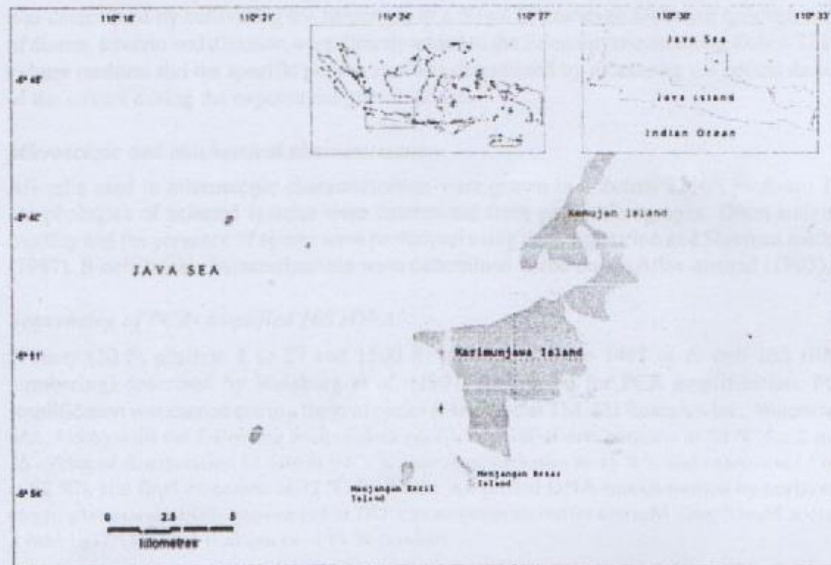


Fig. 1. Karimunjawa Archipelago.

Screening test of diuron, ametrin and diazinon degradation

The coral associated-bacterial isolates were tested for their ability to qualitatively degrade pesticides using an EMBA media indicator (Loos, 1975). Isolates were streaked on the surface of a ZoBell medium containing 100 ppm diuron, ametrin and diazinon and incubated for 24 hours and observed by the color change of colonies into red. In order to assess quantitatively the effects of the diuron, ametrin and diazinon, positive isolates were grown in a liquid Zobell 2216E that was amended with 0.02 % yeast extract, after which the pH was adjusted to 7.6. The sterilized stock solutions of diuron, ametrin and diazinon were added to the desired concentration (80 ppm) after autoclaving. Liquid media was inoculated with bacteria and was incubated at room temperature while shaking at 700 rpm. Degradation of the diuron, ametrin and diazinon in representative acid-producing cultures were confirmed by diluting culture samples after centrifugation (1:2) with distilled water and determining their ultraviolet absorption spectra on a Beckman DB recording spectrophotometer over the wavelength range 200 to 310 nm. Acid production was invariably associated with partial or complete disappearance of the diuron, ametrin and diazinon absorption peak. Growth rate was measured with UV-vis spectrophotometer OD₆₀₀. The best isolate was selected and used for further study on kinetic growth and pesticide degradation.

Kinetic constants regarding the growth of microorganisms on diuron, ametrin and diazinon was determined by cultivating the bacterium in a 50 ml Erlenmeyer. Different concentrations of diuron, ametrin and diazinon were directly added to the Erlenmeyer containing Zobell 2216E culture medium and the specific growth rate was determined by measuring the optical density of the culture during the exponential growth phase.

Microscopic and biochemical characterization

All cells used in microscopic characterization were grown in a Zobell 2216E medium. The morphologies of selected isolates were determined from photomicrographs. Gram staining, motility and the presence of spores were performed using the Cappuccino and Sherman method (1987). Biochemical characterizations were determined based on the Atlas method (1993).

Sequencing of PCR-amplified 16S rDNA

Primers (20 F; position 8 to 27 and 1500 R; position 1510 to 1492 of *E. coli* 16S rRNA numbering) described by Weisburg *et al.* (1991) were used for PCR amplification. PCR amplification was carried out in a thermal cycler (Mini Cycler TM; MJ Research Inc., Watertown, MA, USA) with the following temperature profile: an initial denaturation at 94 °C for 2 min; 25 cycles of denaturation (2 min at 94 °C), annealing (1.5 min at 45 °C), and extension (2 min at 72 °C), and final extension at 72 °C for 3 min. Amplified DNA was examined by horizontal electrophoresis on 1.0% agarose gel in TAE electrophoresis buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA) with 1 μ l aliquots of PCR product.

Sequencing was conducted as previously described by Urakawa *et al.* (1999). The PCR product was purified and concentrated with Microcon 100 microconcentrator (Amicon, Beverly, MA,

USA) according to the manufacturer's instructions. Sequencing was carried out with a SequiTherm Long-Read Cycle Sequencing Kit Epicentre Technologies, Madison, WI, USA) and an automated sequencer (the ALF DNA sequences: Pharmacia LKB Biotech, Uppsala, Sweden).

Results and Discussion

Isolation and screening of pesticide-degrading strains

111 bacterial isolates which utilize pesticide as their sole source of carbon and energy were obtained from 392 enrichment cultures. Screening of pesticide-degrading bacteria was carried out by selecting the red coloured colonies. Colonies which produced relatively little or no acid failed to mobilize the dyes and remained colorless. Loos (1975) stated that many pesticide molecules contain chlorine which may be released as chloride ions by the metabolic action of microorganisms. Chlorinated pesticide breakdown is shown by the reaction of indicator dyes. However, the indicator action depends on the mobilization of the dyes at low pH and their movement into acid-producing colonies. All isolates were capable of growth at the expense of pesticide (80 mg/l) as evidenced by the decrease of UV-absorbing compounds (200 to 310 nm) and by an increase in biomass produced during passages through Zobell 2216E containing only pesticide as a carbon source. The 111 isolates were sorted into two groups based on the biomass product and the percentage of pesticide degradation. Strain KA5 which has the highest capability of degrading pesticide compounds and biomass production was selected.

Diuron degradation

Diuron degradation of the five concentrations showed distinct patterns (Fig. 2). Strain KA5 degraded 75 ppm diuron rapidly. In this concentration approximately 50% of applied diuron was degraded after 18 hours, following which there was a period of rapid loss, with almost constant degradation after 48 hours. Similarly, approximately 50% of the 100 ppm diuron concentration had been degraded in medium after 24 hours. There was a period of slow degradation from three further concentrations, so that complete degradation did not occur. So far, strain KA5 is the first strain of coral bacterium reported to be able to degrade the aromatic ring and to remove the chloride ions of diuron. It is assumed that this strain converts this phenylurea herbicide at least to a 3,4-dichloroaniline compound. It was not surprising that this bacterium could not degrade diuron completely. It seems that cooperative metabolic activities in bacteria of different cultures are needed to degrade diuron herbicide. Dejonghe *et al.* (2003) reported that among five strains, only *Variovorax* sp. strain WDL1 was able to use linuron as the sole source of C, N, and energy. WDL1 first converted linuron to 3,4-dichloroaniline (3,4-DCA). Two strains, *D. acidovorans* WDL34 and *C. testosteroni* WDL7 were found to be responsible for degradation of the intermediate 3,4-DCA. In another study, De Souza *et al.* (1998) determined that in a four-member atrazine-degrading consortium, *Clavibacter michiganense* ATZ1 initiates the degradation of this s-triazine herbicide by removing the side chain while *Pseudomonas* sp. strain CN1 subsequently cleaves the ring.

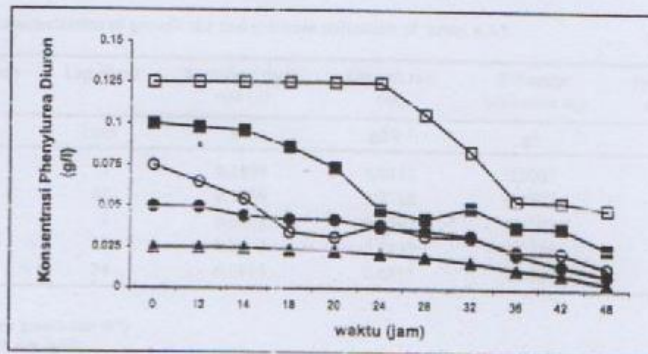


Fig. 2. Diuron degradation of the five different concentrations. (Symbols: ▲: 25 ppm ●: 50 ppm ○: 75 ppm ■: 100 ppm □: 125 ppm diuron)

Cell Growth and Substrate Utilization

The relationship of the concentration of the diuron to growth rate strain KA5 was determined by using batch culture methods. Values of specific growth rate (μ) obtained from turbidity measurements ranged from 0.1599 h^{-1} to 0.2935 h^{-1} and the rate of substrate utilization (δ) ranged from 0.00001 h^{-1} to 0.01950 h^{-1} (Table 1). Furthermore, the highest growth rate of 0.6501 h^{-1} occurred at 75 mg/liter diuron. Above this level, growth was strongly inhibited. The observation of threshold concentration at 75 ppm diuron and subsequent linear decline in growth rate with increasing concentration was probably more consistent with such general toxicity than with any single-enzyme model. Tyler and Finn (1974) observed linear inhibition curves occurred at 500 ppm 2,4-D, above this concentration, growth was inhibited. The solid line in Fig. 2 is best described by the empirical equation: $Y = 0.1076 X + 2.1722$

As shown in Fig. 2, the estimate of half-saturation growth constant (C_s) was 49.5 ppm diuron and the maximum growth rates (μ_{max}) was 0.46 h^{-1} . Tyler and Finn (1974) demonstrated that the value of μ_{max} was dependent on the initial inoculum density or initial substrate/biomass ratio. Greer *et al.* (1992) found that the value of μ_{max} was from 0.32 h^{-1} and C_s value was 33.8 ppm.

Phenotypic properties of strain KA5

Microscopy bacterium revealed that strain KA5 is rod, motile and gram-positive. On solid pesticide-Zobell 2216E agar the strain forms hair-like outgrowths, filamentous, colorless and opaque colonies. Liquification of gelatine and degradation of starch could be detected positively. Catalase reaction was positive. Nitrate was reduced under aerobic and anaerobic conditions. Good growth was observed on the following carbon sources: glucose, maltose and fructose.

Table 1. Characterization of growth rate and substrate utilization of strain KA5

Concentration Diuron	Lag phase	Specific growth rate (μ)	Growth rate (rx)	Substrate utilization (r_s)	Specific substrate utilization (ϕ)
mg/l	hour	h^{-1}	g/l h^{-1}	g/l	h^{-1}
25	12	0.1599	4.0151	0.0003	0.00001
50	12	0.1739	3.2788	0.0023	0.00010
75	8	0.6501	6.4507	0.0107	0.00110
100	12	0.2677	5.5899	0.0136	0.00070
125	24	0.2935	0.6853	0.0456	0.01950

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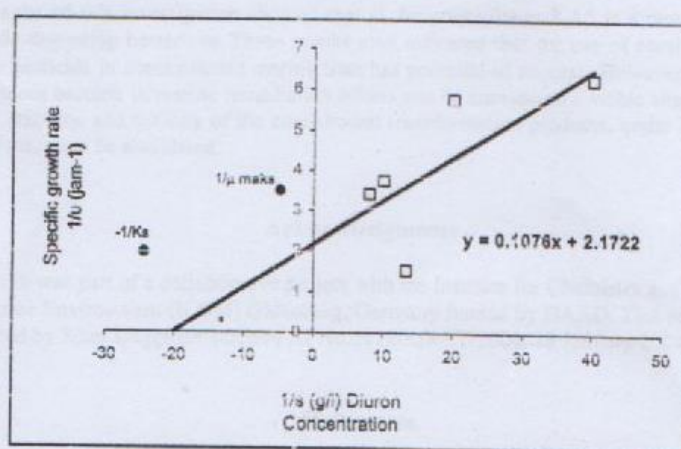
 μ : specific growth rate (h^{-1}) r_s : growth rate (g/l/h) r_s : the rate of substrate utilization (g/l/h) ϕ : the specific rate of substrate utilization (h^{-1})

Fig. 3. Specific growth rate of coral bacterium Strain KA5 at various concentration of diuron

On the other hand, the following features were negative on lactose, sucrose, mannitol, inositol, xylose and galactose.

Previously most research focused on the selection and isolation of microbial communities which grow on halogenated compounds under aerobic conditions. Some Gram-negative bacteria, belonging to the Protobacteria, have been described in publications that utilize chlorinated phenoxyalkanoic acids and their chlorophenol derivatives, i.e. *Alcaligenes eutrophus* JMP 134

(Don and Pemberton, 1985), *Pseudomonas* sp. (Bhat et al., 1995), *Vibrio natriegens* PP202 (Sabdono et al., 2000), *Flavobacterium* sp. (Chaudry and Huang, 1988) and *Ochrobactrum anthropi* strain LMG3331 (Lechner et al., 1995). However, unlike other bacterial degraders mentioned above, strain KA5 is gram positive. In addition, this strain is facultative anaerobic. These observations suggest that a wide variety of bacterial species have the potential to survive under chlorinated conditions.

The comparison of 16S rDNA with known 16S rDNA sequences from the FASTA Database showed that the closest sequence similarity (98%) of strain KA5 was found to *Bacillus haldenitrificans*. It is interesting to note that *B. haldenitrificans* is the new species of pesticide-degrading coral bacterium.

Conclusion

The results of this investigation showed that *B. haldenitrificans* KA5 is a new species of pesticide-degrading bacterium. These results also indicated that the use of coral bacteria to deplete pesticide in contaminated marine sites has potential of success. However, before the use of these bacteria in marine remediation efforts can be considered a viable alternative, the nature, stability, and toxicity of the coral-bound transformation products, under a variety of conditions, must be elucidated.

Acknowledgments

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References

- Bending, G.D., S. D. Lincoln, S. R. Sørensen, J. A. W. Morgan, J. Aamand, and A. Walker. 2003. In-Field Spatial Variability in the Degradation of the Phenyl-Urea Herbicide Isoproturon Is the Result of Interactions between Degradative *Sphingomonas* spp. and Soil pH. *Appl. Environ. Microbiol.* 69: 827-834.
- Cullington, J. E., and A. Walker. 1999. Rapid biodegradation of diuron and other phenylurea herbicides by a soil bacterium. *Soil Biol. Biochem.* 31: 677-686.
- Daprah, S. Y. and G.A. Hill 1992. Biodegradation of chlorophenol mixtures by *Pseudomonas putida*. *Biotechnol. Bioeng.* 40: 1353-1358.
- De Souza, M. L., D. Newcombe, S. Alvey, D. E. Crowley, A. Hay, M. J. Sadowsky, and L. P. Wackett. 1998. Molecular basis of a bacterial conjugative interspecies catabolism of atrazine. *Appl. Environ. Microbiol.* 64: 178-184.

- Esener, A.A., T. Veerman, J.A. Roels, and N.W.F. Kossen 1982. Modelling of bacterial growth: formulation and evaluation of a structured model. *Biotechnol. Bioeng.* 24: 1749-1764.
- Freifeider, D.M. 1987. *Microbial genetics*. Jones and Bartlett Publisher, Inc.
- Greer, L.E., J.A. Robinson and D.R. Shelton 1992. Kinetic comparison of seven strains of 2,4-Dichlorophenoxyacetic acid-degrading bacteria. *Appl. Environ. Microbiol.* 58: 1027-1030.
- Sabdon, A., O.K. Radjasa and J. Soedarsono 2003. Characterization and Identification of Strain KM221, A Novel MCPA Herbicide-Degrading Bacterium Isolated from Coral Surface, Menjangan Kecil Island, Karimunjawa. *J. Coastal Dev.* 6: 127-133.
- Sabdon, A., J. Soedarsono, H. Hartiko and W.T. Artama 2000. Identification and classification of 2,4-D-degrading coral bacteria based on PCR and 16S rRNA gene. *Ilmu Kelautan* 19: 181-186.
- Sørensen, S. R., Z. Ronen, and J. Aamand. 2001. Isolation from agricultural soil and characterization of a *Sphingomonas* sp. able to mineralize the phenylurea herbicide isoproturon. *Appl. Environ. Microbiol.* 67: 5403-5409.
- Tixier, C., M. Sancelme, S. Ait-Aissa, P. Widehem, F. Bonnemoy, A. Cuer, N. Truffaut, and H. Veschambre. 2002. Biotransformation of phenylurea herbicides by a soil bacterial strain, *Arthrobacter* sp. N2: structure, ecotoxicity and fate of diuron metabolite with soil fungi. *Chemosphere* 46: 519-526.
- Tixier, C., M. Sancelme, F. Bonnemoy, A. Cuer, and H. Veschambre. 2001. Degradation products of a phenylurea herbicide, diuron: synthesis, ecotoxicity, and biotransformation. *Environ. Toxicol. Chem.* 20: 1381-1389.
- Turnbull, G.A., J. E. Cullington, A. Walker, and J. A. W. Morgan. 2001. Identification and characterisation of a diuron-degrading bacterium. *Biol. Fertil. Soils* 33:472-476.
- Tyler, J.E. and R.K. Finn 1974. Growth rates of a *Pseudomonad* on 2,4-Dichlorophenoxy-acetic acid and 2,4-Dichlorophenol. *Appl. Microbiol.* 28 (2): 181-184.