

Isolation and Characterization of Atrazine-Degrading *Xanthomonas* sp. ARB2 and Its Use in Bioremediation of Contaminated Soils

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Abstract—Thai degrading-bacteria, strain ARB2, was isolated from sugarcane field soils in Ratchaburi province of Thailand. Strain ARB2 was gram-negative bacteria, rod shape in pair and a single cell. The genomic DNA was extracted following the standard protocol for bacterial genomic DNA preparations. The partial 16S rDNA genes were amplified by polymerase chain reaction (PCR) using the universal primers of 16S rDNA gene. Sequence analysis of the PCR product indicated that the 16S rRNA gene in strain ARB2 was ranging from 89-91% identical to the same region in *Xanthomonas* sp. and were named *Xanthomonas* sp. ARB2. *Xanthomonas* sp. ARB2 was capable of degrading 300 mg L⁻¹ and 100 mg kg⁻¹ of atrazine in mineral salts liquid medium and soil at 81% and 62% in 7 days, respectively. GC-MS analysis detected the formation of two metabolites, deethylatrazine (DEA) and deisopropylatrazine (DIA) during the process of degradation of atrazine.

Index Terms—Atrazine, biodegradation, bioremediation, contaminated soil.

I. INTRODUCTION

Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino) -1, 3, 5-triazine) is a selective, systemic triazine herbicide, widely used for the control of annual broadleaf and grassy weeds in corn, maize and sugarcane. Although banned for use in many European countries, atrazine is still a commonly used herbicide in many countries like China, India and USA [1], [2]. In states with high atrazine use, surface and ground water commonly have detectable levels of atrazine [3], [4]. Atrazine persists in soil and ground water and has the potential to leach into drinking water supplies [5]. One result of this concern is that research has increased on microorganisms found in atrazine-impacted areas, as atrazine dissipation is often accelerated after continued exposure to the herbicide [6]-[8].

Microbial degradation has been regarded as the primary mechanism of atrazine degradation in contaminated sites [9]. Bioremediation, the use of microorganisms to clean up contaminated site, is an environmentally safe solution for atrazine removal [10], [11]. Up to date, a number of atrazine-degrading strains from different bacterial genera have been isolated. Atrazine can be degraded by *Arthrobacter*

sp. [12], *Chelatobacter heintzii* [13], *Rhodococcus* *sp.*, *Acinetobacter* *sp.*, *Streptomyces* *sp.*, *Pseudomonas aeruginosa*, *Clavibacter michiganense* [14], *Enterobacter cloacae* [15], *Bacillus megaterium*, *Alcaligenes faecalis*, *Klebsiella ornithinolytica*, and *Agrobacterium tumefaciens* [16]. Under aerobic conditions, *Rhodococcus* strain TE1 can metabolize the atrazine into deethylatrazine (DEA) and deisopropylatrazine (DIA) [17]. *Pseudomonas* strain ADP metabolizes atrazine into cyanuric acid via three enzymatic steps, encoded by the genes *atzABC* [18] and cyanuric acid acts as a nitrogen source for many bacteria.

In Thailand, a number of atrazine-degrading bacterial strains, belonging to diverse genera, have been isolated and characterized for research and bioremediation purposes. These include strains of *Klebsiella*, *Rhizobium* and *Stenotrophomonas* [19], [20].

This study aims to isolate and investigate atrazine-degrading bacteria from agricultural soil samples in Ratchaburi province. Once atrazine-degrading bacteria were isolated, we sequenced their 16S rRNA genes and compared with previously reported bacteria. Experiments were conducted to determine the bioremediation capacity of atrazine contaminated in culture and in soils.

II. MATERIALS AND METHODS

A. Chemicals and Media

Atrazine (98.9% purity) was purchased from Chem Service Inc. (West Chester, PA, USA). Enrichment medium consisted of a mineral salts medium (MSM) and 100 mg L⁻¹ atrazine as the sole nitrogen source, plus sucrose 3 g L⁻¹ as carbon source, and was autoclaved at 121 °C for 30 minutes. The mineral salts medium (MSM) contained (per liter) 1.6 g of K₂HPO₄, 0.40 g of KH₂PO₄, 0.20 g of MgSO₄·7H₂O and 0.10 g of NaCl [21]. Trace element solution contained (per liter): EDTA, 2.5 g; FeSO₄·7H₂O, 1.0 g; ZnSO₄·7H₂O, 5.0 g; MnSO₄·H₂O, 1.0 g; CuSO₄·5H₂O, 0.40 g; Na₂B₄O₇·10H₂O, 0.20 g; Na₂MoO₄·2H₂O, 0.25 g. The pH of the medium was adjusted to 7.0 with 1 M NaOH solution. For solid medium, 2% (w/v) agar was added to the same atrazine containing liquid mineral salts medium.

B. Enrichment and Isolation

The soil samples were collected from an agricultural field cultivated with sugarcane in Ratchaburi province, Thailand. The soil samples were collected from 0 to 15 cm of soil depth with a hand-driven soil auger and stored at 4 °C until they were used. Soil samples (10 g) were suspended in 30 ml of 0.1

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M phosphate buffer (pH = 7.5) and centrifuged at 6000×g for 15 min at 4 °C and the supernatants were discarded to remove the quantity of residual nitrogen sources in soil [22].

Enrichment of the atrazine-degrading microorganism was performed in 100 ml MSM supplemented with 2.5 mg L⁻¹ of atrazine [23] as the sole carbon and nitrogen source from the 10 g soil sample and incubated aerobically by shaking at 150 rpm at 37 °C for 7 days. All enrichment cultures were subcultured on the same medium at 7 day interval for a total of three passes. The final culture was diluted and plated on MSM agar plates containing atrazine (300 mg L⁻¹). Developed colonies were repeatedly streaked on atrazine agar plates for isolation of a pure culture. Colonies which developed cleared zones in the atrazine-containing MSM agar were purified and routinely maintained on this medium. A bacterial isolate, designated strain ARB2, was selected for Gram staining and further studies.

C. Identification of Strain ARB2 by 16S rDNA Sequence

The genomic DNA of the strains was extracted and precipitated following the standard protocol for bacterial genomic DNA preparations [24]. The primer pairs of 27F (5'-AGAGTTTGATCMTGGCTCAG -3') and 1492R (5'-TACGGHTACCTTGTTACGACTT -3') were used for amplification of 16S rDNA of ARB2, and the product was approximately 1400 bp. Reactions were carried out in 25 µL volumes containing 5 µL of template DNA, 2.5 µL of 10X PCR buffer (0.5 M KCl, 0.1 M Tris-HCl, pH 9.0 and 1% Triton X-100), 2.0 µL of 25 mM MgCl₂, 0.5 µL of 10 mM deoxyribonucleoside triphosphate, 1.0 µL of 10.0 µM of each primer, and 0.1 µL of 5 U *Taq* polymerase. The cycles used were as follows: 1 cycle at 94 °C for 5 min; 35 cycles at 94 °C for 60 s, 55 °C for 60 s, 72 °C for 120 s; and 1 cycle of 72 °C for 15 min. Sequencing of the 16S rDNA was performed by First BASE Laboratories Sdn Bhd., Malaysia. The DNA sequences were analyzed using the BLAST program of the National Centre for Biotechnology. Sequences were analyzed and concatenated by using DNASTAR (DNASTAR, Inc., Madison, Wis.) A multiple-sequence alignment was prepared by using CLUSTAL W [25], and phylogenetic trees were constructed by using the neighbor-joining method.

D. Degradation of Atrazine by Isolate ARB2

Strain ARB2 was incubated in MSM with 100 mg L⁻¹ of atrazine as carbon and nitrogen source. ARB2 fresh cell suspension (OD₆₀₀ = 0.8) was incubated aerobically with shaking (150 rpm) at 37 °C in sterile flask (250 mL) containing 100 mL of the liquid medium supplemented with 300 mg L⁻¹ of atrazine. The medium without inoculation of isolated strain was used to confirm the biodegradation mechanism. All these tests were performed in triplicate. The changes of atrazine concentrations in the liquids were monitored by high performance liquid chromatography (HPLC) analysis [26]. The samples were collected at the incubation time of 0, 1, 3, 5 and 7-day and analyzed to determine the remained atrazine concentration at each time interval.

E. Bioremediation of Atrazine-Contaminated Soil

Soil samples (1 kg) were sterilized by autoclave. Subsamples (25 g) of autoclaved, 2.5 mg of atrazine (100

ppm), 1 mL of mineral salts liquid medium, and 1 mL of ARB2 liquid culture were mixed in sterile Petri dishes and incubated at 37°C. Negative control contained no live cells. The soil was kept damp by spraying with sterilized water daily. Three dishes from each treatment were removed on day 0, 1, 2, 3, 5 and 7, and analyzed to determine the remained atrazine concentration at each time interval.

III. RESULTS AND DISCUSSIONS

A. Isolation of Atrazine-Degrading Bacteria by Enrichment

An atrazine-degrading bacterium, designated strain ARB2, was isolated from an agricultural soil. The pure cultures were gram-stained and a phylogenetic analysis of their 16 S rRNA gene sequences was performed. Morphological characterization indicated that ARB2 was a Gram-negative bacteria, rod shape in pair and a single cell. The 16S rRNA sequence of strain ARB2 was compared to those of the bacterial sequences in GenBank. Nucleotide sequences of 16S rRNA gene of ARB2 were closely related to those of *Xanthomonas* sp. FSBSY16, *Xanthomonas* sp. QSt2, *Xanthomonas* sp. FSGSD7 and *Xanthomonas* sp. CU12 with nucleotide sequence identity ranging from 89% to 91%. Fig. 1 shows that strain ARB2 formed a closely related group with many *Xanthomonas* sp. strain such as FSBSY16, QSt2, FSGSD7 and CU12 and showed a greater sequence homology among them than with other *Xanthomonas theicola*, *Xanthomonas melonis* and *Xanthomonas albilineans*, whereas genus *Pseudoxanthomonas*, *Lysobacter*, *Bacillus* and *Arthrobacter* were clearly separated to the other clades. Based on morphological characterization, sequence comparisons and phylogenetic analyses, strain ARB2 was identified as new strain of *Xanthomonas* and named *Xanthomonas* sp. ARB2. Further characterization of this strain may lead to a better affiliation in the future.

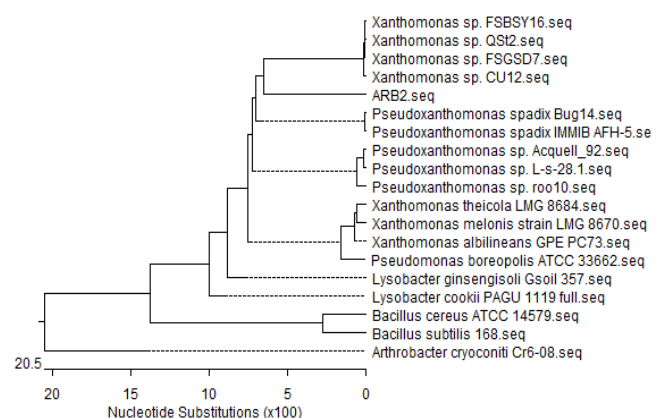


Fig. 1. Neighbor-joining tree based on the 16S rDNA sequences of the atrazine-degrading isolates (ARB2) with related 16S rDNA sequences found in GenBank database.

B. Degrading Ability of Xanthomonas Strain ARB2

Atrazine degradation by strain ARB2 was performed thrice in bacterial medium containing atrazine of 300 mg L⁻¹. At 37°C, atrazine degradation in media containing atrazine as sole carbon and nitrogen source showed the maximum degradation of 81.7 % in 7 days of incubation (Fig. 2),

whereas the loss of atrazine in uninoculated, sterile controls was not evident. Atrazine was rapidly degraded by ARB2 and it produced the metabolites. The Gas chromatography–mass spectrometry (GC-MS) analysis revealed that the metabolites produced by the isolate during atrazine degradation were deethylatrazine (DEA) and deisopropylatrazine (DIA) (Fig. 3).

C. Bioremediation of Atrazine-Contaminated Soil

The reduction in atrazine concentration during the biodegradation assay is shown in Fig. 4. Atrazine biodegradation rates were significantly enhanced in the inoculated soils as compared to uninoculated control soils. Degradation of atrazine in control autoclaved soils (without inoculation) was minimal where less than 2.2% of the applied concentration was degraded in 7-day incubation studies. On day 0, the degradation rates of atrazine in soil inoculated with ARB2 was low (below 0.1 %). After 1, 3, 5 and 7 days, ARB2 removed atrazine 13%, 25%, 41% and 62%, respectively. The metabolites produced by ARB2 during atrazine degradation were DEA and DIA (Fig. 5). Whereas the compounds DIA and DIA were not detected in uninoculated soil.

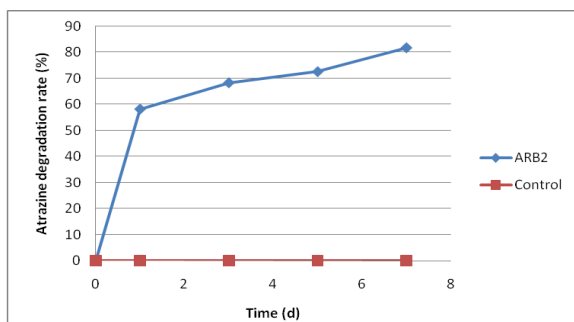


Fig. 2. Biodegradation rate of atrazine by strain ARB2 in liquid medium.

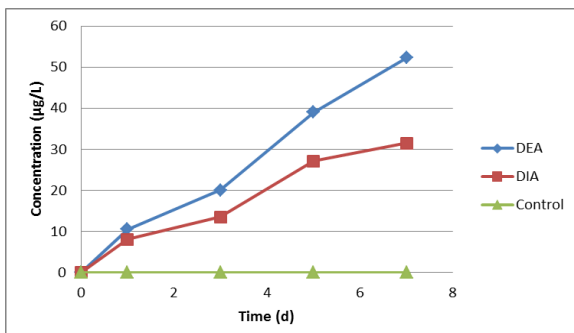


Fig. 3. Degradation metabolites deethylatrazine (DEA) and deisopropyl atrazine (DIA) in the spent atrazine mineral salts medium produced by the isolates ARB2.

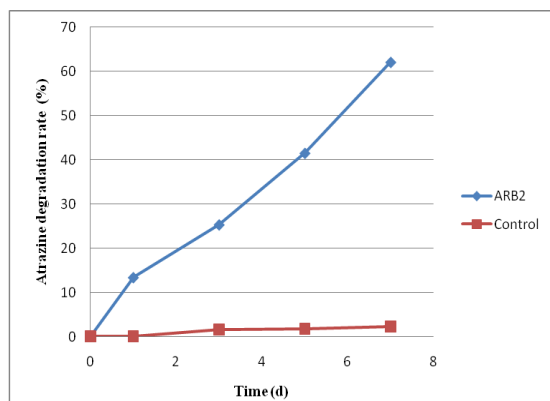


Fig. 4. Biodegradation rate of atrazine by strain ARB2 in soil.

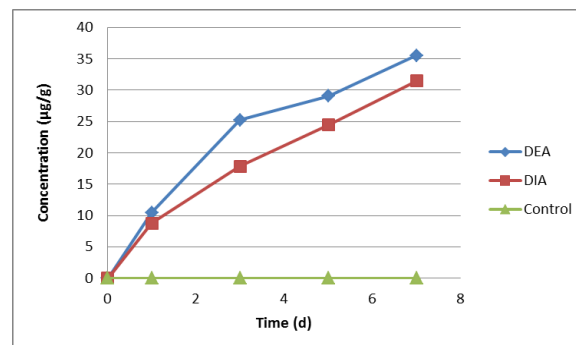


Fig. 5. Degradation metabolites deethylatrazine (DEA) and deisopropyl atrazine (DIA) in soil produced by the isolates ARB2.

IV. CONCLUSIONS

From the presented results, we were successful in isolating new strain of atrazine-degrading microorganism from a Central Thai agricultural soil, ARB2, gram-negative and short rod which were able to use this herbicide as a sole source of nitrogen. The 16S rDNA nucleotide sequence of strain ARB2 exhibited a 89-91% nucleotide identity with *Xanthomonas* sp. This isolates studied were grouped with microorganisms in the genera *Xanthomonas* and named *Xanthomonas* sp. ARB2.

Strain ARB2 was shown to be capable of removing 81% and 62% atrazine in a mineral salts liquid medium and contaminated soil, respectively. The *Xanthomonas* sp. ARB2 generated deethylatrazine (DEA) and deisopropylatrazine (DIA) by N-dealkylation in the upper degradation pathway. The present knowledge concerning the degradation of atrazine using bacteria species may be relevant for the development of effective bioremediation strategies for contaminated agricultural water and soil.

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