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Biodegradation of Alachlor by a Newly Isolated Bacterium: Degradation Pathway and Product Analysis

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Abstract: Alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl]acetamide] is a chloroacetanilide herbicide and has been widely used as a selective pre-emergent and post-emergent herbicide to control weeds and grass. Due to its wide usage, direct application on the ground, high solubility in water, and moderate persistence, alachlor and its metabolites have been detected in various environments. Therefore, there is an increasing concern about the environmental fate of alachlor and its metabolites. Microbial biodegradation is a main method of removal of alachlor in the natural environment. In this study, we isolated new alachlor degrading bacterium and proposed a novel alachlor-degrading pathway. The alachlor-degrading bacterial strain, GC-A6, was identified as *Acinetobacter* sp. using 16S rRNA gene sequence analysis. *Acinetobacter* sp. GC-A6 utilized alachlor as its sole carbon source and degraded 100 mg L^{-1} of alachlor within 48 h, which was the highest alachlor degradation efficiency. The degradation pathway of alachlor was studied using GC-MS analysis. Alachlor was initially degraded to 2-chloro-*N*-(2,6-diethylphenyl) acetamide, which was further degraded to 2,6-diethylaniline and 7-ethylindoline, respectively. 2,6-Diethylaniline was transformed into *N*-(2,6-diethylphenyl) formamide. *N*-(2,6-diethylphenyl) formamide was a first-reported intermediate during the degrading pathway of alachlor by single isolate.

Keywords: biodegradation; alachlor; *Acinetobacter* sp. GC-A6; *N*-(2,6-diethylphenyl) formamide

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1. Introduction

Alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl]acetamide] is a chloroacetanilide herbicide and has been widely used as a selective pre-emergent and post-emergent herbicide to control weeds and grass in soybeans, peanuts, and corn crops. Since alachlor was first commercially registered in 1969, alachlor has been the most widely used herbicide. Alachlor has a low molecular weight (269.769 g mol^{-1}) and a high solubility in water (240 mg L^{-1} at 25 °C). Alachlor also shows moderate mobility in sand and silt [1] and is known to be persistent, with a half-life of over 70 days in soil and 808 to 1518 days in water [2]. Alachlor is mobile, with low adsorption to organic matters, and highly soluble in water. In addition, alachlor is stable to abiotic processes. Because of its wide usage, direct application on the ground, high solubility in water, and moderate persistence, alachlor and its metabolites have been detected in various environments, including soils, ground water, surface water, drinking water, and plants [3–5]. Elevated persistence in water could also amplify the exposure and risks for human and aquatic ecosystems.

Alachlor has been classified as a human carcinogen (Class B2) by the US Environmental Protection Agency (EPA) (EPA 1998). Degradation products of alachlor, such as alachlor ethanesulfonic acid (ESA) and alachlor oxanilic acid (OA), have been included in the US Environmental Protection Agency's Contaminant Candidate List (CCL4; USEPA, 2016). Alachlor has been reported as a probable carcinogen in nasal tumors [6] and olfactory mucosal tumors [7] in rats. Due to its toxicity and mutagenicity, the use of alachlor was controlled in the USA (1998) and forbidden in the Canada (1986) and the European

Union (2006) [8]. Moreover, alachlor has been reported as the endocrine-disrupting compound (EDC) that mimics the 17 β-estradiol hormone [9] and is included in the group of compounds disrupting the endocrine systems of humans and animals (ECD's) [10].

Alachlor is present in groundwaters at levels ranging from 0.1–16.6 μg L^{-1} [11]. This value exceeds the U.S. Environmental Protection Agency drinking-water maximum contaminant level criteria of 2 μg L^{-1} for alachlor [12]. Additionally, some reports suggest that even decades after legislative control, concentrations of the pollutant may still rise. Therefore, it is necessary to study the environmental fate of this herbicide to limit the spread of alachlor in environments.

There is a great concern about the environmental fate of this herbicide and its byproducts.

Alachlor is degraded by both abiotic and biotic processes, including chemical oxidation, gamma radiolysis, photo-oxidation, ultrasonic treatment, and biodegradation. Microbial degradation is the most important factor for the dissipation of alachlor from the environment. Several microbial consortium and microorganisms in pure culture, such as *Candida xestobii*, *Chaetomium globosum*, *Paracoccus* sp. FLY-8, *Streptomyces* sp. LS182, and fungus *Paecilomyces marquandii* have been reported to degrade alachlor [13–17]. In microbial degradation, alachlor is mainly degraded to 2-chloro-*N*-(2,6-diethylphenyl) acetamide by *N*-dealkylation, which is then converted to 2,6-diethylaniline, and then further degraded via aniline and catechol. Recently, Chen et al. [18] identified the three-component Rieske non-heme iron oxygenase (RHO) system catalyzing the *N*-dealkylation of chloroacetamide herbicide.

The main objectives of this study are (1) to isolate alachlor-degrading bacteria from agricultural soil using an enrichment process; (2) to investigate its ability to degrade alachlor in a mineral medium containing alachlor as the sole carbon source; and (3) to identify metabolic intermediates using gas chromatography–mass spectrometry and propose a novel biodegradation pathway of alachlor.

2. Materials and Methods

2.1. Chemicals and Media

Alachlor (99.8% purity), 2,6-diethylaniline (98.6% purity), aniline, and catechol were obtained from Sigma-Aldrich (Saint Louis, MO, USA). 2-chloro-*N*-(2,6-diethylphenyl) acetamide was purchased from Santa Cruz (USA). Mineral medium (MM) containing alachlor as the sole carbon source was used for the enrichment procedures. The mineral medium contained 1.42 g of Na_2HPO_4, 1.36 g of KH_2PO_4, 0.30 g of $(NH_4)_2SO_4$, 0.05 g of $MgSO_4 \cdot 7H_2O$, 5.75 mg of $CaCl_2 \cdot H_2O$, 2.75 mg of $FeSO_4 \cdot 7H_2O$, 1.15 mg $ZnSO_4 \cdot 7H_2O$, 1.70 mg $MnSO_4 \cdot 7H_2O$, 0.38 mg of $Co(NO_3)_2 \cdot 6H_2O$, 0.24 mg of $CuSO_4 \cdot 5H_2O$, and 0.13 of mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per liter. Peptone–tryptone–yeast extract–glucose (PTYG) medium [19] was used for the isolation of alachlor-degrading bacteria. PTYG medium contained 0.25 g of peptone (Difco), 0.25 g of tryptone (Difco), 0.5 g of yeast extracts (Difco), 0.5 g of glucose, 0.03 g of $MgSO_4$, and 0.003 g of $CaCl_2$ per liter.

2.2. Enrichment and Isolation of Alachlor-Degrading Bacteria

One hundred sixty-eight agricultural soil samples were collected from soybean fields in South Korea. Soils were taken from the top 15 cm of soil, sieved (2.0 mm mesh), and preserved at 4 °C prior to use. To isolate alachlor-degrading bacteria, an enrichment procedure was used. Alachlor was added to 20 g of each soil sample (the final alachlor concentration was 100 mg kg^{-1} of soil) and completely mixed. The mixture was incubated at room temperature and blended with a sterilized spatula every 3 days. After 4 weeks, 1 g of soil mixture was transferred to 9 mL of mineral medium containing 100 mg L^{-1} of alachlor, serially diluted in 3 mL of mineral medium containing 100 mg L^{-1} of alachlor, and incubated for 4 weeks in a shaking incubator (150 rpm) at 28 °C. After 4 weeks, the degradation of alachlor was examined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). When complete degradation of alachlor was observed, the culture medium was transferred to a fresh mineral medium containing

alachlor (final concentration was 100 mg L^{-1}), and the same process was repeated 25 times. Finally, the enriched culture medium was streaked on PTYG agar and incubated at 28 °C for 7 days. Morphologically different colonies were pure cultured using PTYG agar. A single colony of each isolate was transferred to 3 mL of mineral medium, supplemented with 100 mg L^{-1} of alachlor and incubated at 28 °C to examine its ability to degrade alachlor.

2.3. Identification and Characterization of Alachlor-Degrading Strain

The isolated alachlor-degrading bacterium, designated strain GC-A6, was identified by the 16S rRNA gene sequence, as previously described [19]. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using primers 27mf (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-GGYTACCTTGTTACGACTT-3′). The PCR was performed using the following protocol: denaturation of DNA at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and then a final extension at 72 °C for 10 min. The amplified PCR products were verified with an agarose gel electrophoresis and purified using a QIAquick PCR purification kit (QIAGEN). The amplified PCR product was sequenced with the sequencing primers 515r, 926f [20], and 1055r [21] and assembled using SEQMAN software (DNASTAR). The 16S rRNA gene sequence's similarity with bacterial type strains was determined using EzBioCloud server [22]. The 16S rRNA gene sequences of the isolate and related type strains were aligned according to the SILVA seed alignment [23]. Phylogenetic trees were constructed by neighbor-joining [24] and maximum-parsimony methods [25] using the MEGA6 program [26].

Morphological and physiological characteristics of the alachlor-degrading strain were identified. Bacterial growth at various temperatures (4, 10, 15, 20, 25, 28, 37, 40, 45, 50, and 55 °C) was examined after 7 days of incubation in PTYG broth. The growth at various pH was determined after 7 days of incubation at 28 °C in the PTYG broth (adjusted to pH 5.0–10.0 at intervals of 0.5 pH units). Tolerance to NaCl was assessed in the PTYG broth supplemented with 0–10.0% NaCl (w/v) (at intervals of 1%) after 7 days of incubation at 28 °C. Growth of the alachlor-degrading strain on a different medium was tested on R2A agar (BD), Luria–Bertani agar (LB; BD), nutrient agar (NA; BD), trypticase soy agar (TSA; BD), and MacConkey agar (BD) at 28 °C for 7 days of incubation. Growth under anaerobic condition was assessed by culturing the strain on PTYG agar in an anaerobic jar at 28 °C for 7 days. Gram staining was carried out using a Color Gram 2 kit (bioMérieux). Cellular morphology was determined using an inverted light microscope (AXIO; Zeiss) and transmission electron microscopy (80 kV, JEM1010), using cells grown on PTYG agar for 2 days at 28 °C. Catalase and oxidase activities were determined using an Oxidase Reagent and ID Color Catalase Reagent, respectively (bioMérieux). The abilities of hydrolysis of casein, cellulose, chitin, DNA, and starch were assessed according to the methods of Smibert and Krieg [27] and Ten et al. [28]. Other biochemical characteristics were examined using API ZYM systems (bioMérieux) according to the manufacturer's instruction. Cells of the alachlor-degrading strain grown on the PTYG agar medium at 28 °C for 2 days were used for API test. DNA G + C content (mol%) was examined using the fluorometric method [29].

2.4. Biodegradation of Alachlor

The alachlor-degrading strain was cultured in a mineral medium containing 100 mg L^{-1} of alachlor until the logarithmic phase. Exponentially growing cells of the isolate were collected by centrifugation, washed twice with mineral medium, and then adjusted to an optical density of 0.08 at 600 nm. A total of 1% (v/v) of the diluted cells were inoculated to the mineral medium containing 100 mg L^{-1} of alachlor as the sole carbon source. The inoculated medium was incubated at 28 °C in a rotary shaker at 150 rpm. At 12 h intervals, 3 mL of sample was collected from the culture medium. Bacterial growth was determined periodically by measuring the optical density at 600 nm. The concentration of alachlor was estimated by high-pressure liquid chromatography (HPLC). An amount of 3 mL of sample was mixed with an equal volume of acetonitrile and filtered (0.22 μm Millipore filter). The

filtered samples were used for HPLC analysis. The mobile phase was acetonitrile/water (70:30, *v*/*v*), and the flow rate was 1 mL min^{-1}. The injection volume was 20 µL, and the column elution was monitored at 220 nm. The concentration of alachlor was calculated from a peak area from the calibration curve. The medium with only alachlor in the absence of inoculation was used as a control to estimate the alachlor degradation caused by the abiotic process. All experiments were performed in triplicate.

2.5. Identification of Metabolic Intermediates

The metabolic intermediates of alachlor were identified using gas chromatography–mass spectrometry (GC-MS). Incubation conditions were the same as above. Culture samples were centrifuged with a swing rotor at 340 g for 10 min and filtered. The filtered samples were extracted and concentrated by solid-phase extraction. Solid-phase extraction was conducted with Sep-Pak C18 cartridges (Waters, Milford, MA, USA) using a Visiprep 24™ SPC vacuum manifold (Supelco, Bellefonte, PA, USA). The Sep-Pak C18 cartridges were conditioned with methanol, followed by distilled water. The samples were loaded onto the cartridges and eluted with methanol. The GC-MS analyses were performed in electron ionization (EI) mode (70 eV) using a Perkin Elmer Clarus 680 GC/600T with a TG-5MS column (length: 30 m, ID: 0.25 mm, FT: 0.25 µm). The initial oven temperature was 100 °C (2 min hold) to 280 °C at 10 °C min^{-1} and held for 20 min, then finally increased to 320 °C at 20 °C min^{-1} for 8 min. The helium was used as the carrier gas at a constant flow of 1 mL min^{-1}. The sample was analyzed in split mode (1:20) at an injection temperature of 280 °C. Chromatographic peaks were identified according to its mass spectra and NIST library identification program.

2.6. Deposition of 16S rRNA Gene Sequences and Bacterial Strains

The 16S rRNA gene sequence of the strain GC-A6 was deposited in the NCBI GenBank under accession number KT949414. The bacterial strain was deposited in the Korean Agricultural Culture Collection (KACC) under KACC number 18615.

3. Results

3.1. Isolation and Characterization of Alachlor-Degrading Strain

After twenty-five rounds of transfers, a single bacterial strain showing alachlor degradation was isolated from the soil sample collected from Gochang, Korea and designated as GC-A6. The strain GC-A6 was selected on the basis of its ability to degrade and utilize alachlor for its growth. The size of the 16S rRNA gene sequence of the isolate GC-A6 was 1483 bp. The 16S rRNA gene sequence analysis showed that the isolate GC-A6 had 99.9–95.9% similarities with the type strains of the genus *Acinetobacter*. The isolate GC-A6 showed especially high similarities with *Acinetobacter radioresistens* DSM 6976^T (99.9%) and *Acinetobacter venetianus* RAG-1^T (97.4%). The strain GC-A6 had 1 nucleotide difference with *Acinetobacter radioresistens* DSM 6976^T and 38 nucleotide differences with *Acinetobacter venetianus* RAG-1^T. The phylogenetic tree generated by the neighbor-joining method showed that strain GC-A6 grouped with members of the genus *Acinetobacter* (Figure 1).

Morphological, physiological, and biochemical characteristics were determined to identify strain GC-A6. The strain GC-A6 was Gram-stain-negative, an aerobe, a non-spore-forming coccobacilli, and 0.6–0.8 µm in length and 0.3–0.5 µm in width (Figure 2). The cells occurred singly or in pairs. Colonies were circular, convex, and opaque on the PTYG agar. The isolate grew on LB, TSA, NA, R2A agar, and MacConkey agar. The colonies were yellowish-white on PTYG and R2A agar and pale-yellow on LB, TSA, and NA. The temperature range for growth was 10–40 °C, with optimum growth at 25–40 °C. The pH range for growth was pH 5.5–9.5, with optimum growth at pH 6.0–8.5. The NaCl tolerance range was 0–7% (*w*/*v*). The isolate was positive for catalase but negative for oxidase. The isolate was not able to hydrolyze casein, cellulose, chitin, starch, and DNA. The G + C content was 47.4 mol%. The morphological, physiological, and biochemical characteristics

of the strain are listed in Table 1. These results support the idea that the isolate GC-A6 belongs to the *Acinetobacter* sp.

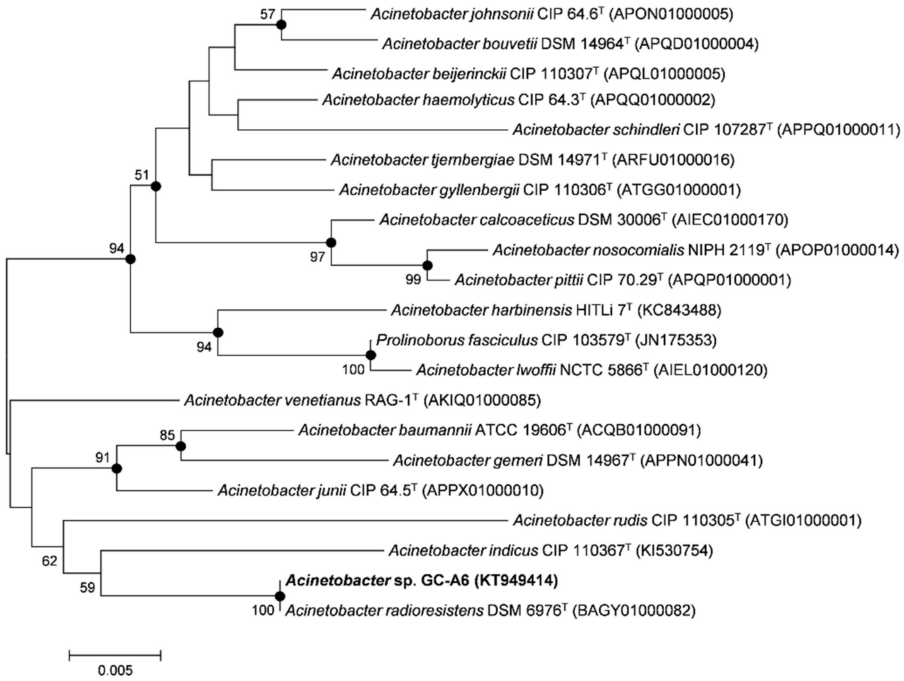

Figure 1. Neighbor-joining phylogenetic tree of *Acinetobacter* sp. strain GC-A6 and related species, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at branch points. Filled circles indicate nodes that were also recovered using maximum-parsimony algorithms. Bar, 0.005 nucleotide substitutions per position.

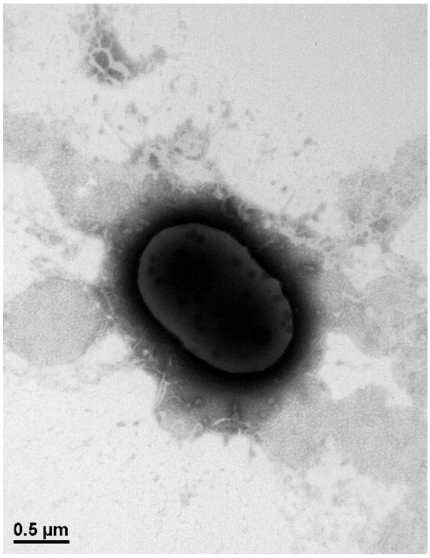

Figure 2. Transmission electron microscopy of cells of the strain GC-A6.

3.2. Degradation of Alachlor by Strain GC-A6

The growth and degradation patterns of the isolate GC-A6 are shown in Figure 3. The strain GC-A6 completely degraded 100 mg L^{-1} of alachlor within 48 h, and the bacterial cell density gradually increased from 0.006 to 0.115 at OD_{600}. There was an initial lag period within 12 h. Alachlor degradation was observed predominantly during the log phase. The results indicated that strain GC-A6 could efficiently degrade and utilize alachlor as the sole source of carbon and energy for growth.

Table 1. Morphological, physiological, and biochemical characteristics of the alachlor-degrading bacterial strain GC-A6.

Characteristics	GC-A6
Colony morphology	Circular, convex, and opaque (on PTYG agar)
Colony pigmentation	Yellowish white (on PTYG and LB agar)
Cell shape	Coccobacillus
Cell size (μm)	0.6–0.8 × 0.3–0.5
Gram staining	Negative
NaCl range for growth (*w*/*v*) (%)	0–7
pH range for growth	5.5–9.5
Temperature range for growth (°C)	10–40
Catalase	+
Oxidase	–
Alkaline phosphatase	+
Esterase (C4)	w
Esterase lipase (C8)	+
Lipase (C14)	–
Leucine arylamidase	+
Valine arylamidase	w
Cystine arylamidase	+
Trypsin	–
α-chymotrypsin	–
Acid phosphatase	w
Naphtol-AS-BI-phosphohydrolase	w
α-Galactosidase	–
β-Galactosidase	–
β-Glucuronidase	–
α-Glucosidase	–
β-Glucosidase	–
N-acetyl-β-glucosaminidase	–
α-Mannosidase	–
α-Fucosidase	–

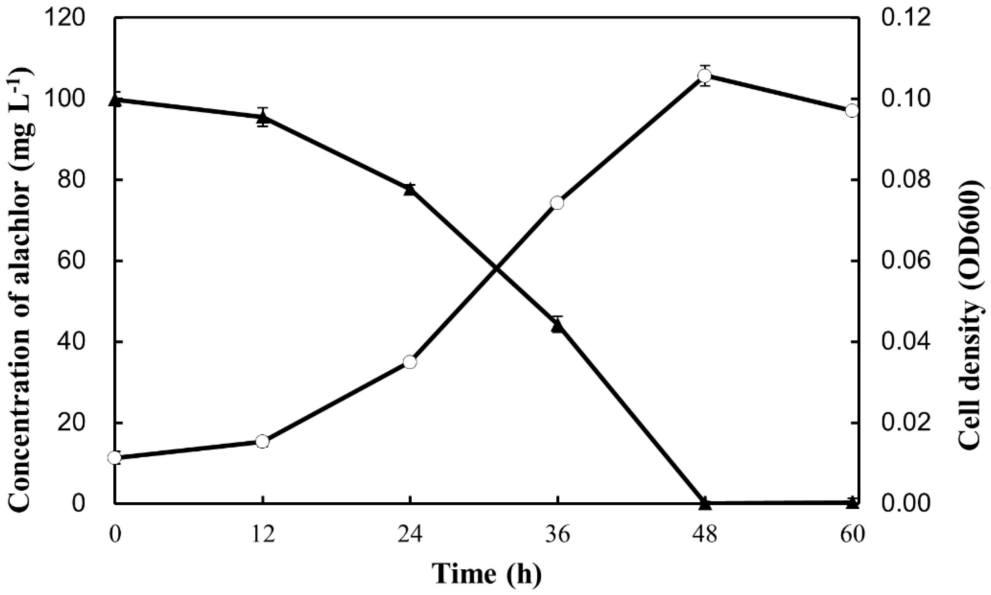

Figure 3. Utilization and degradation of alachlor by *Acinetobacter* sp. GC-A6 in alachlor mineral medium. (○) OD_{600} of strain GC-A6 cultures; (▲) residual concentration of alachlor in strain GC-A6 cultures. Error bars indicate the standard deviation of values measured in triplicate at each point.

HPLC spectrums obtained from the cultures at 0 h and 48 h are shown in Figure 4. HPLC spectra obtained from the culture at 0 h showed two large peaks. The peak

(Rt: 8.752 min) was alachlor originally added to the mineral medium. The other peak (Rt: 1.907 min) was also detected in the mineral medium without alachlor. Therefore, the peak at the retention time of 1.907 was the components of the mineral medium. HPLC spectra obtained from the culture at 48 h showed one large peak and two small peaks. The peak (Rt: 8.752 min) detected in the culture at 0 h was not observed at 48 h. This result suggests alachlor was completely degraded by the strain GC-A6.

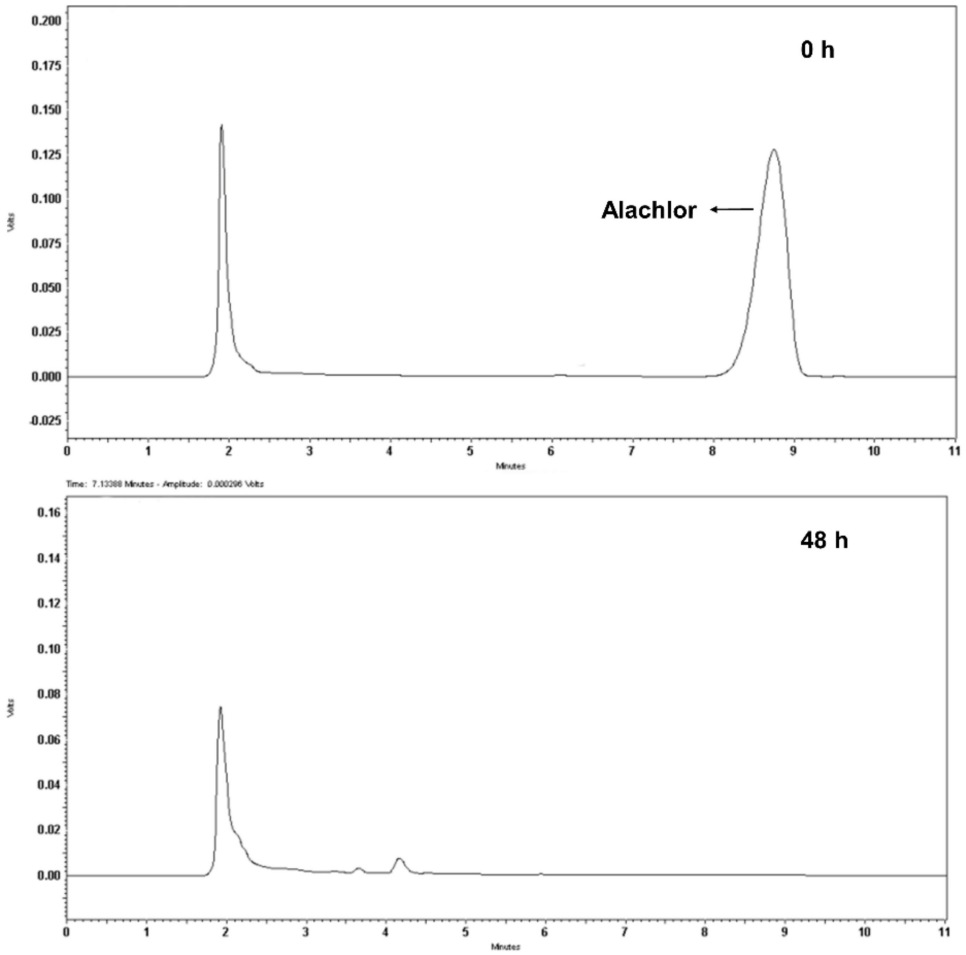

Figure 4. HPLC spectrum obtained from the culture at 0 h and 48 h.

3.3. Identification of Metabolites

To investigate the alachlor-degradation pathway, the metabolites of alachlor degradation by strain GC-A6 was identified by GC-MS. The gas chromatogram spectrum obtained from the culture extracts at 36 h and 60 h of incubation are shown in Figure 5A. The peaks were identified according to its mass spectra and NIST library identification program. The mass spectra of the compounds are shown in Figure 5B. GC-MS analysis of the culture at 36 h of incubation gave four peaks at retention times of 8.27, 11.58, 13.01, and 15.19 min, representing metabolites (3), (4), (2), and (1), respectively. Metabolite (1) was identified as alachlor, which was originally added to the mineral medium. Metabolite (2) was identified as 2-chloro-*N*-(2,6-diethylphenyl) acetamide (CDEPA), which was produced by *N*-dealkylation of alachlor. Metabolite (3) was identified as 2,6-diethylaniline (DEA). Metabolite (4) was identified as *N*-(2,6-diethylphenyl) formamide. GC-MS analysis of the culture at 60 h of incubation gave two peaks at retention times of 8.52 and 11.62 min, representing metabolites (5) and (4). Metabolite (5) was identified as 7-ethylindoline.

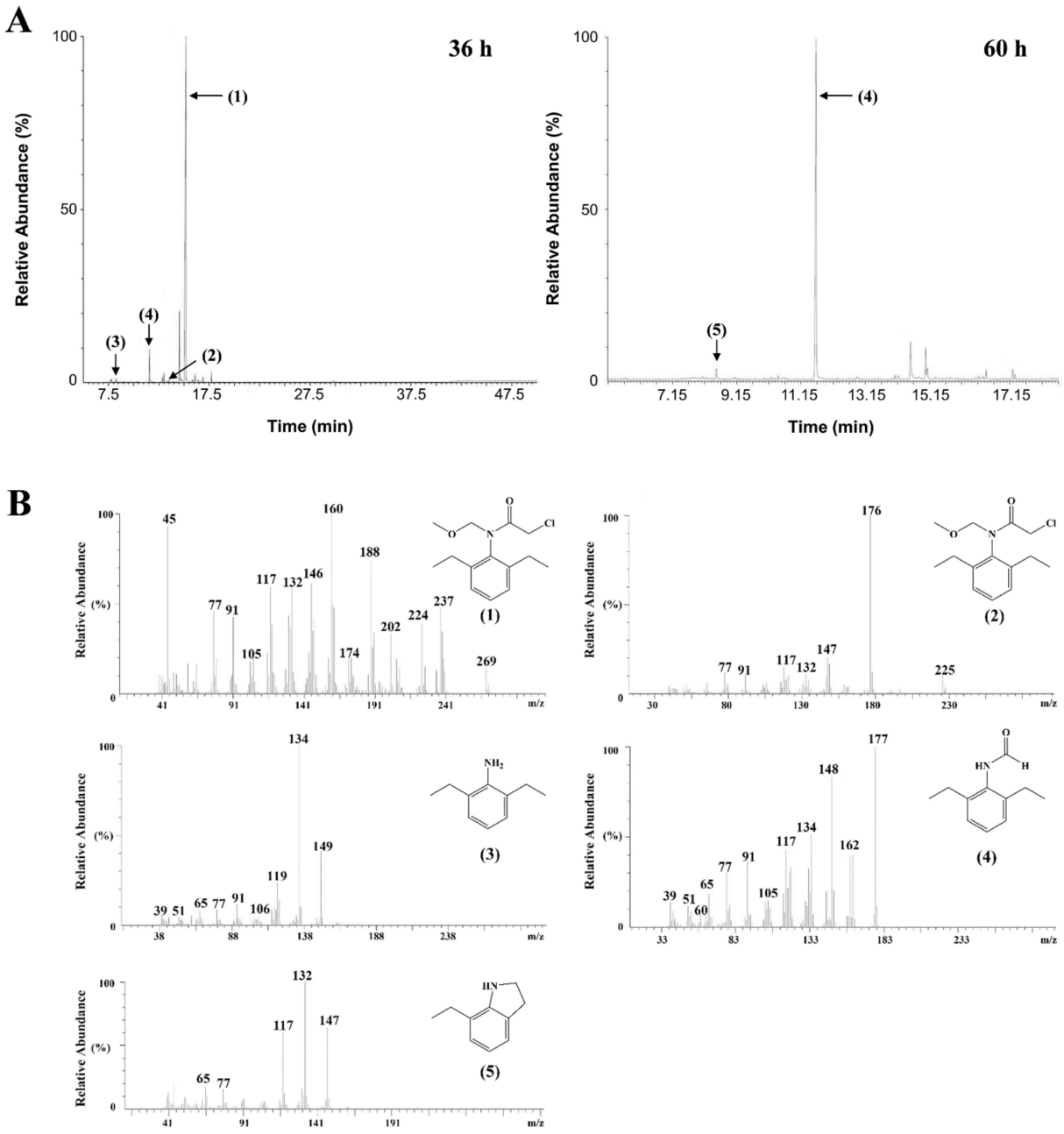

Figure 5. (**A**) Gas chromatogram spectrum of the extract from 36 h and 60 h of cultures. (**B**) Identification of metabolites in the degradation of alachlor by GC-MS. (1) alachlor (*m*/*z* 269); (2) 2-chloro-*N*-(2,6-diethylphenyl) acetamide (*m*/*z* 225); (3) 2,6-diethylaniline (*m*/*z* 149); (4) *N*-(2,6-diethylphenyl) formamide (*m*/*z* 177); and (5) 7-ethylindoline (*m*/*z* 147).

4. Discussion

In this study, a novel alachlor-degrading bacterial strain, *Acinetobacter* sp. GC-A6, was isolated from agricultural soil. The genus *Acinetobacter* is known to be involved in the biodegradation of various environmental contaminants. *Acinetobacter* sp. USTB-04 degraded methyl parathion, a widely used organophosphorus pesticide [30]. *Acinetobacter* sp. S13 was capable of transforming phenol and benzoate [31], and *Acinetobacter* sp. A6 degraded 250 mg L^{-1} of atrazine [32]. *Acinetobacter* sp. DL-2 was able to degrade fenoxaprop-*P*-ethyl, one of the 2-(4-aryloxyphenoxy) propionate acids herbicide [33]. As

far as we know, no information on the biodegradation of alachlor by *Acinetobacter* sp. has been reported.

To date, several studies on the biodegradation of alachlor have been reported. *Candida xestobii* transformed 81.9% of 50 mg L^{-1} of alachlor after 64 h [15]. *Chaetomium globosum* was able to degrade about 95% of 100 mg L^{-1} of alachlor after 60 h [13]. *Paracoccus* sp. strain FLY-8 degraded 98.7% of 100 mg L^{-1} of alachlor after 5 days of incubation [16]. *Streptomyces* sp. LS182 was able to degrade about 75% of 144 mg L^{-1} of alachlor after 14 days of incubation [14]. The fungus *Paecilomyces marquandii* transformed about 80% of 100 mg L^{-1} of alachlor after 7 days of incubation [17]. In this study, the strain GC-A6 completely degraded 100 mg L^{-1} of alachlor within 48 h, indicating that *Acinetobacter* sp. GC-A6 had greater alachlor-degrading ability than previously reported alachlor-degrading microorganisms.

On the basis of the results of the metabolites identification, a novel biodegradation pathway of alachlor was proposed in this study. As shown in Figure 6, alachlor is degraded with two pathways. In pathway 1, alachlor is transformed to 2-chloro-*N*-(2,6-diethylphenyl) acetamide (CDEPA), 2,6-diethylaniline, and *N*-(2,6-diethylphenyl) formamide. In pathway 2, alachlor is converted to CDEPA, and which is subsequently transformed to 7-ethylindoline.

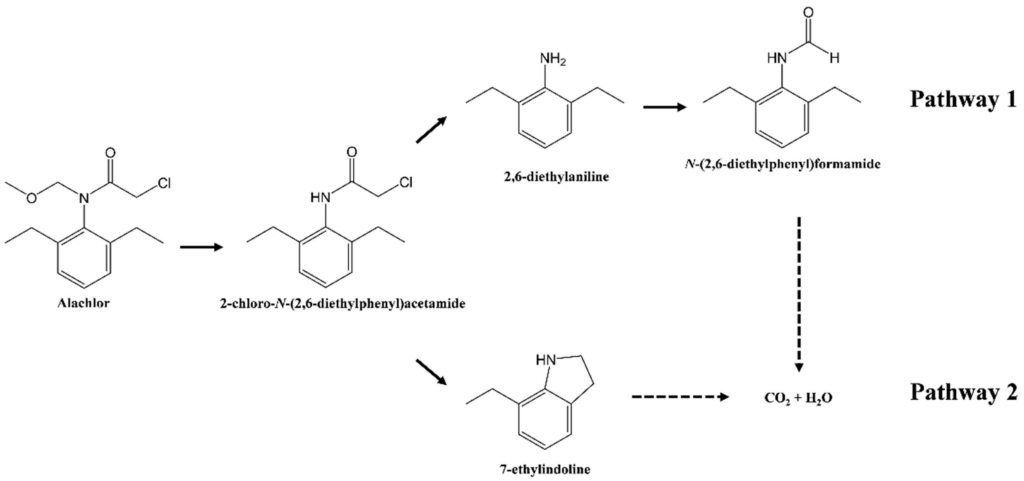

Figure 6. Proposed biodegradation pathway of alachlor by *Acinetobacter* sp. GC-A6.

In pathway 1, alachlor is *N*-dealkylated to CDEPA, which is converted to 2,6-diethylaniline. 2,6-diethylaniline is subsequently converted to *N*-(2,6-diethylphenyl) formamide. It is reported that *Paracoccus* sp. FLY-8 could transform butachlor through alachlor, 2-chloro-*N*-(2,6-diethylphenyl) acetamide (CDEPA), 2,6-diethylaniline, aniline, and catechol [14]. We analyzed the degradation of aniline and catechol by GC-A6. The strain GC-A6 could not utilize aniline and catechol for its sole source of carbon. Aniline and catechol were also not detected in the metabolites analysis by GC-MS. These results support the idea that the strain GC-A6 transforms 2,6-diethylaniline to other metabolites. Badriyha et al. [34] reported that 2,6-diethylaniline was degraded to *N*-(2,6-diethylphenyl) formamide, and this metabolite was subsequently transformed into 3-dihydro-1-formyl-7-ethylindole by the mixed cultures of fungal and bacterial strains. In this study, the presence of *N*-(2,6-diethylphenyl) formamide was confirmed by a comparison with the mass spectrum reported by Mangiapan et al. [35]. These results support the idea that the isolate GC-A6 transforms 2,6-diethylaniline to *N*-(2,6-diethylphenyl) formamide. However, in contrast to the previous study, the conversion of *N*-(2,6-diethylphenyl) formamide to 3-dihydro-1-formyl-7-ethylindole was not observed in this study. This is the first report on the biodegradation of alachlor to *N*-(2,6-diethylphenyl) formamide by single bacterial strain.

In pathway 2, alachlor is *N*-dealkylated to CDEPA, which is subsequently converted to 7-ethylindoline. 7-ethylindoline has been reported as a metabolite of alachlor by *Streptomyces* sp. LS182 [14]. Streptomyces sp. LS182 converted CDEPA to 1-chloroacetyl-2,3-dihydro-7-ethylindole, which was subsequently transformed into 7-ethylindoline. In contrast to the previous study, the intermediate product, 1-chloroacetyl-2,3-dihydro-7-ethylindole, was not observed in this study.

It is also reported that the carcinogenic product 2,6-diethylaniline is present in groundwater at twice the concentration of alachlor [36]. In this study, this carcinogenic product was detected with very low concentration, and its downstream metabolites were detected with relatively high concentrations. These results indicate that carcinogenic 2,6-diethylaniline is not accumulated in the degradation process of alachlor by strain GC-A6.

In conclusion, a novel alachlor-degrading bacterial strain, *Acinetobacter* sp. GC-A6, was isolated from agricultural soil. Strain GC-A6 degraded alachlor with a novel biodegradation pathway. The isolate GC-A6 degraded alachlor with two pathways. In pathway 1, alachlor was transformed into CDEPA, 2,6-diethylaniline, and *N*-(2,6-diethylphenyl) formamide. In pathway 2, alachlor was degraded to CDEPA, which was subsequently transformed into 7-ethylindoline.

The biodegradation of alachlor with a novel pathway supports the idea that this newly isolated strain may be useful for the removal of alachlor from contaminated environments.

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