

Dissipation of butachlor by a new strain of *Pseudomonas* sp. isolated from paddy soils

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ABSTRACT: Butachlor (BUT) is a chloroacetanilide herbicide widely applied to rice paddies to control annual grass and broad-leaf weeds. A BUT-degrading bacterial strain (PK) was isolated from paddy soils. Biochemical and 16S rRNA sequencing characteristics confirmed the strain as *Pseudomonas aeruginosa* (99% resemblance). The isolate dissipated BUT (100 µg/mL) in an M9 liquid medium with a rate of $0.5 \pm 0.03 \text{ day}^{-1}$ and DT₅₀ and DT₉₀ of 1.38 ± 0.10 days and 4.58 ± 0.32 days, respectively. Soil dissipation of BUT was investigated under flooded conditions. In sterile soils, the isolate increased the dissipation of BUT (200 µg/g) (DT₅₀ = 12.38 ± 1.83 days, DT₉₀ = 41.12 ± 6.09 days, $k = 0.06 \pm 0.01 \text{ day}^{-1}$) compared to sterile non-inoculated samples (DT₅₀ = 26.87 ± 2.82 days, DT₉₀ = 89.25 ± 9.36 days, $k = 0.03 \pm 0.00 \text{ day}^{-1}$). In non-inoculated non-sterile soil experiments, the dissipation of BUT was faster (DT₅₀ = 15.17 ± 2.11 days, DT₉₀ = 50.38 ± 7.02 days, $k = 0.05 \pm 0.00 \text{ day}^{-1}$) compared to non-inoculated sterile ones, and inoculating the isolate accelerated the removal of BUT in non-sterile soils significantly (DT₅₀ = 8.03 ± 1.20 days, DT₉₀ = 26.68 ± 3.97 days, $k = 0.09 \pm 0.01 \text{ day}^{-1}$). BUT inhibited soil respiration (SR) initially for 5 days, followed by an increase until day 20. The increase in SR was more pronounced in the co-presence of BUT and the isolate. The results of this research suggest *P. aeruginosa* PK as a suitable candidate for BUT bioremediation.

Keywords: Bacterial isolate, Bioremediation, *Pseudomonas aeruginosa*, Paddy, Soil respiration.

INTRODUCTION

Butachlor (BUT) (N-butoxymethyl-2-chloro-2,6-diethylacetanilide) is a pre-emergence chloroacetanilide herbicide widely applied to rice paddies in Asian countries to control a wide range of annual grass and broad-leaf weeds (Iwafune et al., 2010). In Iran, this herbicide is applied at a rate of 3-4 kg/ha in

the Northern provinces (Armanpour & Bing, 2015). Rice paddies are mainly located alongside rivers, and consequently, pesticide runoff linked with poor water management and heavy rainfalls is reported to contaminate aquatic ecosystems (Ateeq et al., 2006; Watanabe et al., 2007; Geng et al., 2010; Armanpour & Bing, 2015). BUT can affect soil microbial communities and enzymatic activities. Also, initial inhibition

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of soil respiration (SR) flowing BUT treatment has been observed (Min et al., 2001). The toxicity of BUT to earthworms (Singh & Singh, 2016; Wang et al., 2016) and some aquatic organisms (Vajargah & Hedayati, 2017; Akan et al., 2019), and its potential carcinogenic effects on animals and humans (Geng et al., 2005; Dwivedi et al., 2012) has been reported. Therefore, exploring through bioremediation strategies to reduce BUT residues in paddy soils seems crucial.

Degradation of BUT in soil occurs through both abiotic and biotic processes, and microbial biodegradation has been reported as the principal procedure responsible for the dissipation of this herbicide (Lin et al., 2000; Pal et al., 2006; Mohanty & Jena, 2019; Wu et al., 2020). Hence, bioremediation by employing soil microorganisms seems a promising tool to reduce the residues of this herbicide in the environment. A few researchers have so far addressed the isolation of BUT-degrading microorganisms including bacterial strains of *Stenotrophomonas acidaminiphila*, *Paracoccus* sp., *Catellibacterium caeni*, *Rhodococcus* sp., *Mycobacterium* sp., *Sphingobium* sp., *Rhodopseudomonas marshes*, and *Pseudomonas putida*, and have reported their efficiency in removing various concentrations of the herbicide in soils and liquid media (Dwivedi et al., 2010; Zhang et al., 2011; Zheng et al., 2012; Liu et al., 2012; Kim et al., 2013; Mohanty & Jena, 2019; Wu et al., 2020). However, due to the extensive application and hazards of BUT, this topic requires more attention to explore new BUT-degrading strains and evaluate their efficacies for removing and bioremediation of this agrochemical.

In this research, a BUT-degrading new strain of *Pseudomonas* sp. was isolated from paddy soil. In addition, the ability of the isolate to dissipate BUT liquid media and flooded soil samples was assessed. Finally, the effect of BUT on SR was

evaluated in the presence or absence of the isolate.

MATERIAL AND METHODS

BUT standard (97.7%) was purchased from Sigma-Aldrich, Germany (Supplementary information (SI) section A). All solvents and salts were of analytical-grade purity and obtained from either from Sigma-Aldrich or Merck, Germany. Primary-secondary amine (PSA) was supplied by Supelco, USA. A stock solution of BUT (1 g/L) was prepared in acetone (ACE) and used for liquid medium and soil spiking tests. For chromatographic analyses, BUT was dissolved in methanol (MeOH) (1 g/L). All solutions were kept at -20 °C.

Soil samples were collected from the topsoil (0–15 cm) of a paddy field located in the Rice Research Institute of Iran (Rasht City, Guilan Province). 20 randomly-selected locations were sampled, and a final amount of 20 kg was transferred to the laboratory and kept at 4 °C (Cycoń et al., 2009). Before experiments, samples were homogenized, sieved (2 mm mesh), and a portion was used to measure physical and chemical properties based on ISO standard procedures (<http://www.iso.org/>) (Table 1).

Table 1. Physico-chemical properties of the experimental soil

Soil texture classification (FAO and USDA system)	Clay
Sand (%)	12 (1.2) ^a
Silt (%)	32 (1.5)
Clay (%)	56 (2.7)
pH (in water)	8.2 (0.8)
FC (%)	46 (4.2)
EC (dS/m)	1.63 (0.1)
CEC (cmol _c /kg)	23.41 (3.2)
C _{org} (%)	0.05 (0.0)
N _{tot} (%)	1.2 (0.2)
Na ⁺ (meq/L)	3.5 (0.5)
Ca ⁺ (meq/L)	11.8 (1.1)
Mg ⁺ (meq/L)	1.8 (0.3)

^a means of triplicate measurements with standard deviations in parentheses.

FC: field capacity, EC: electrical conductivity, CEC: cation exchange capacity, C_{org}: organic carbon content, N_{tot}: total nitrogen content

Enrichment process was conducted in Erlenmeyer flasks containing 100 mL Nutrient Broth (NB) plus filter-sterilized BUT ACE solution (20 µg/mL). After solvent evaporation, flasks were inoculated with 2 g soil in triplicates. For controls, sterile soils (Berns et al., 2008) were used. After incubation (7 days, 28 °C, 120 rpm), 5 mL of each suspension was transferred to new flasks containing the M9 medium (See SI section B for details) plus BUT (100 µg/mL), and incubation was done at the same conditions mentioned above for 7 days. Six additional cycles of enrichment were performed in M9 + BUT, increasing BUT concentration up to 300 µg/mL. 0.1 mL tenfold dilution series of each liquid culture from the last enrichment cycle was spread on M9 agar (Liofilchem, Italy) + BUT (300 µg/mL). Plates were incubated at 28 °C for 72 h. At this concentration, only one morphologically distinct bacterial colony was separated and purified. The isolate was named PK, and an inoculum of 3×10^8 cells/mL was prepared in M9 + BUT (50 µg/mL). The PK isolate was characterization biochemically based on the Bergey's manual of systematic bacteriology, and its optimal growth conditions were evaluated in either NB or M9 medium (SI section C). Finally, the PK isolate was identified 16S rRNA gene sequencing (SI section D). The sequence of the PK isolate was submitted in GenBank database with the accession number of MN918259.

The dissipation of in liquid media was evaluated by adding a bacterial suspension of the isolate (3×10^6 cells/mL) to Erlenmeyer flasks containing 100 mL of M9 + BUT (100 µg/mL) + 0.2% yeast extract in triplicates (pH 7). Triplicate flasks without the isolate were also set as controls. All samples were incubated in the dark (30 °C, 120 rpm) and sampled periodically up to 5 days to measure BUT concentration. In soil, the dissipation of BUT by the PK isolate (3×10^6 cells/mL) and SR variations were evaluated with six

sets of experiments in triplicates including (i) sterile soil + PK isolate + BUT (SS + PK + BUT), (ii) sterile soil + PK isolate without BUT (SS + PK), (iii) sterile soil + BUT without the isolate (SS + BUT), (iv) non-sterile soil + BUT (nSS + BUT), (v) non-sterile soil + PK isolate without BUT (nSS + PK), and (vi) non-sterile soil + PK isolate + BUT (nSS + PK + BUT). In all experiments, BUT (200 µg/g) was added as a filter-sterilized ACE solution to 200 g of soil, and the solvent was evaporated. To simulate flooded conditions, filter-sterilized deionized water was added up to 2 cm above the soil surface. Well-mixed samples were kept in the dark at 30 °C, and the dissipation of BUT along with variations in SR was monitored at up to 20 days following incubation.

SR was measured based on Froment (1972) (See SI section E for details). BUT was extracted according to Xue et al. (2014) using *n*-Hexane. The extracts were purified with PSA (Anastassiades et al., 2003), evaporated with nitrogen, and re-diluted in MeOH. A high-performance liquid chromatography (HPLC) apparatus equipped with a UV detector at 248 nm and a reverse-phase C₁₈ column (250 mm×4mm ID; Knauer®, Germany) was used for BUT quantification. The mobile phase was acetonitrile:water (72:28, v/v) pumped at 1 mL/min. Method validation was performed based on standard guidelines of the European Commission (2006) (See SI section F). Dissipation kinetics for BUT were estimated using a simple first-order kinetic (SFOK) model according to the instructions of the North American Free Trade Agreement (NAFTA) guidance (US-EPA; <https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/guidance-evaluating-and-calculating-degradation>). SRs were compared in soils samples using the least significant difference (LSD) method ($P = 0.05$) (Williams & Abdi, 2010).

RESULTS AND DISCUSSION

16S rRNA gene sequencing and homology searches within the GenBank database showed a 99% resemblance of the PK isolate to *P. aeruginosa* strains (SI section D). These results were consistent with the biochemical tests revealing that the isolate was a gram-negative and rod-shaped bacterium with the ability to produce blue-

green pigment and showing positive catalase and oxidase activities (Table 2).

PK isolate was able to dissipate BUT (100 µg/mL) in the M9 medium with a rate of $0.5 \pm 0.03 \text{ day}^{-1}$ and DT₅₀ and DT₉₀ of 1.38 ± 0.10 and 4.58 ± 0.32 days, respectively. PK isolate dissipated 80 and 95% of 100 µg/mL BUT after 3 and 5 days, respectively (Table 3).

Table 2. Biochemical characteristics BUT-degrading PK isolate

Characteristics	PK isolate
Cell shape	Bacilli (rod-shape)
Gram reaction	-
Catalase	+
Oxidase	+
Oxidative/fermentative (O/F)	+/-
Triple Sugar Iron (TSI)	Alk/Alk
Pigment production	+
Citrate	+
Indole production	-

Table 3. Dissipation (%) ± SD of BUT in liquid medium and soil

Time (day)	M9 + PK + BUT	SS + BUT	nSS + BUT	SS + PK + BUT	nSS + PK + BUT
0	0 ± 4	0 ± 5	0 ± 3	0 ± 4	0 ± 5
1	33 ± 2	-	-	-	-
3	80 ± 3	-	-	-	-
5	95 ± 4	19 ± 2	36 ± 2	41 ± 2	48 ± 6
7	-	-	-	-	-
10	-	30 ± 7	46 ± 5	53 ± 4	60 ± 3
15	-	35 ± 3	50 ± 1	56 ± 2	67 ± 8
20	-	38 ± 2	51 ± 3	59 ± 1	73 ± 9

M9 + PK + BUT: M9 liquid medium + PK isolate + BUT (100 µg/mL), **SS + BUT:** sterile soil + BUT (200 µg/g), **nSS + BUT:** non-sterile soil + BUT (200 µg/g), **SS + PK + BUT:** sterile soil + PK isolate + BUT (200 µg/g), **nSS + PK + BUT:** non-sterile soil + PK isolate + BUT (200 µg/g).

Studies using other isolates showed *Paracoccus* sp. FLY-8 degraded 64.98% of 100 µg/mL BUT within 5 days and *Catelibacterium caeni* sp. DCA-1^T degraded 80.02% of 50 µg/mL BUT after 4 days of inoculation (Zhang et al., 2011; Zheng et al., 2012). Liu et al. (2012) showed that *Rhodococcus* sp. strain B1 utilized BUT as a sole carbon and energy source and completely degraded 100 µg/mL of the herbicide within 5 days. Syntrophic degradation of BUT by *Mycobacterium* sp. J7A and *Sphingobium* sp. J7B dissipated 100 µg/mL of the herbicide at 28 °C within 24 h (Kim et al., 2013). The ability of *P. aeruginosa* in

degrading various insecticides and herbicides has been proved (Krishna & Philip, 2011; Thabit & El-Naggar, 2013; Ramu & Seetharaman, 2014; Egea et al., 2017). *P. alcaligenes*, which has been placed in the *P. aeruginosa* group based on 16S rRNA analysis (Anzai et al., 2000), has been shown to biodegrade BUT (Abd-Alrahman & Salem-Bekhit, 2013). Furthermore, *Pseudomonas* sp. has been demonstrated to be capable of degrading sulfonylurea herbicides (Li-Feng et al., 2007; Ma et al., 2009) and other xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs) (O'Mahony et al., 2006).

Our results revealed that the inoculation of sterile soils with the PK isolate (SS + PK + BUT) accelerated the dissipation of BUT significantly compared to the SS + BUT experiments (Tables 3 and 4). This shows that the isolate was able to well-stabilize in the soil and utilize the herbicide as a source of energy. Inoculation of *Catellibacterium caeni* sp. nov DCA-1 cells into soils significantly accelerated the degradation of BUT in both sterile and non-sterile soils, with 57.2% – 90.4% of 50 µg/g of the herbicide dissipated within 5 days compared to 5.4% – 36% in non-inoculated controls (Zheng et al., 2012). Furthermore, BUT declined faster in nSS + PK + BUT experiments compared to nSS + BUT (Tables 3 and 4), which reveals that the isolate was able to sustain oneself in the presence of competition between other

soil microorganisms at the inoculated concentration of 3×10^6 cells/mL. It has been shown previously that sufficient initial concentration of an isolate inoculate is necessary to maintain its degradation efficiency in non-sterile soils (Rousseaux et al., 2003; Singh et al., 2006; Cycoń et al., 2009; Pourbabaee et al., 2018). The cooperation between the PK isolate and the microbial consortia already present in the soil may have resulted in the faster dissipation of BUT in nSS + PK + BUT compared to SS + PK + BUT experiments (Tables 3 and 4). It has been shown that the interactions between several components of a mixed microbial population can result in faster and more efficient biodegradation of pesticides compared to a single strain (El-Fantroussi, 2000; Sutherland et al., 2000; Kim et al., 2013; Torabi et al., 2017).

Table 4. Dissipation kinetics of BUT in liquid medium and soil derived from the SFOK model

Treatment	SFOK model parameters			Dissipation Times	
	r ²	C ₀ (µg/mL or µg/g) ± SE	k (day ⁻¹) ± SE	DT ₅₀ (day) ± SE	DT ₉₀ (day) ± SE
M9 + PK + BUT	0.97	104.40 ± 3.23	0.50 ± 0.03	1.38 ± 0.10	4.58 ± 0.32
SS + BUT	0.88	193.97 ± 3.28	0.03 ± 0.00	26.87 ± 2.82	89.25 ± 9.36
nSS + BUT	0.83	194.98 ± 6.23	0.05 ± 0.00	15.17 ± 2.11	50.38 ± 7.02
SS + PK + BUT	0.82	183.69 ± 6.86	0.06 ± 0.01	12.38 ± 1.83	41.12 ± 6.09
nSS + PK + BUT	0.91	181.07 ± 5.90	0.09 ± 0.01	8.03 ± 1.20	26.68 ± 3.97

C₀: Initial concentration of BUT at time 0, **k**: dissipation rate of BUT, **M9 + PK + BUT**: M9 liquid medium + PK isolate + BUT (100 µg/mL), **SS + BUT**: sterile soil + BUT (200 µg/g), **nSS + BUT**: non-sterile soil + BUT (200 µg/g), **SS + PK + BUT**: sterile soil + PK isolate + BUT (200 µg/g), **nSS + PK + BUT**: non-sterile soil + PK isolate + BUT (200 µg/g), **SE**: standard error obtained from the regression analysis of the SFOK model.

Exposure to pesticides can alter soil microbial activities transiently and in an inhibitory or a stimulatory manner (Zabaloy et al., 2008; Cycoń & Piotrowska-Seget, 2009; Gomez et al., 2009; Crouzet et al., 2010; Wang et al., 2010; Radivojević et al., 2012). These effects are mainly driven by parameters such as pesticide concentration and physicochemical properties, i.e., water solubility and K_{oc}, as well as soil properties, especially water and organic matter content, which determine the bioavailable fraction of these chemicals in soil (Dungan et al., 2003; Gomez et al.,

2009; Filimon et al., 2015; Šantrić et al., 2016). SR represents the overall soil microbial activity and can be used as an indicator of pesticide exposure and toxicity to soil microorganisms (Heinonen-Tansk et al., 1984; Radivojević et al., 2012).

Our results showed inhibition of SR in non-sterile soils (nSS + BUT and nSS + PK + BUT) after application of BUT at days 0 (4 h after application) and five when compared to nSS + PK experiments (Fig. 1). This initial inhibition can be the result of high BUT dose applied to soil samples under flooded conditions, resulting in BUT initial high bioavailability and probable

toxicity to soil microbes. It has been shown previously that the application of BUT at a high concentration to paddy soils results in an 8-day inhibition of SR (Min et al., 2001). However, this inhibition was transient and was followed by a significant recovery of SR following day 5 (Fig. 1). This, along with the pronounced dissipation of BUT in the non-sterile soils (Tables 3 and 4), indicate that during the time, the population of microorganisms

adapted for degrading BUT might have become more metabolically active (Crouzet et al., 2010; Vandana et al., 2012). For both sterile and non-sterile experiments, the increase of SR was more obvious in the co-presence of BUT and the PK isolate, which evidences the ability of the isolate to colonize successfully, utilize BUT as a source of energy, and boost the metabolic activity in the soil (Fig. 1).

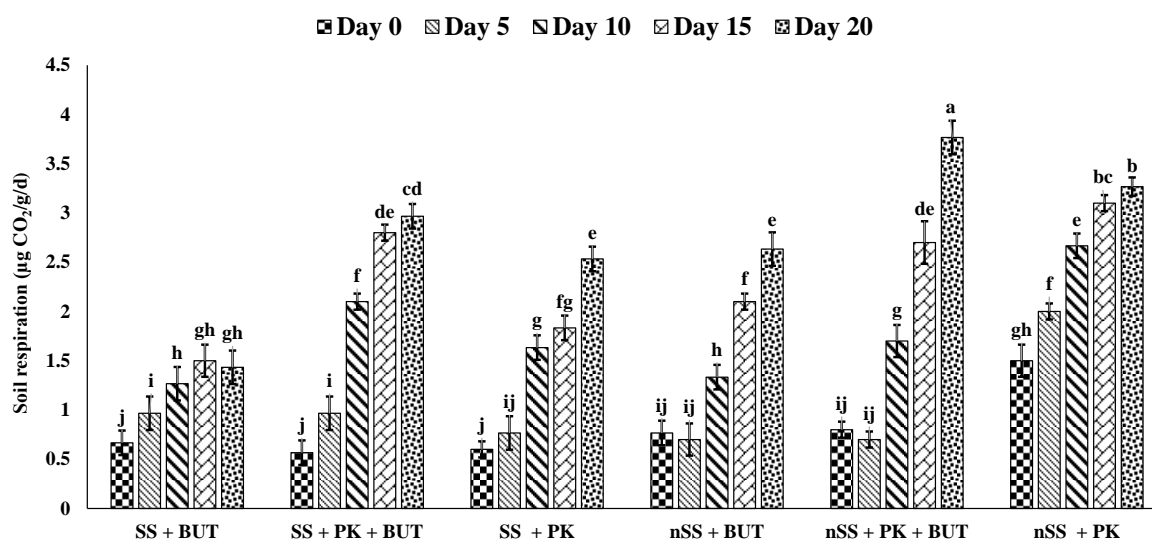


Fig. 1. Soil respiration in various soil experiments. SS + BUT: sterile soil + BUT (200 µg/g), SS + PK + BUT: sterile soil + PK isolate + BUT (200 µg/g), SS + PK: sterile soil + PK isolate (without BUT), nSS + BUT: non-sterile soil + BUT (200 µg/g), nSS + PK + BUT: non-sterile soil + PK isolate + BUT (200 µg/g), nSS + PK: non-sterile soil + PK isolate (without BUT). Error bars represent ± standard deviations (SD) of triplicates. Columns without similar letters differ significantly (LSD, $P = 0.05$)

CONCLUSION

In this research, a BUT-degrading bacterial strain (named PK) was isolated from rice paddies. Based on biochemical tests and 16S rRNA sequencing data, the isolate was identified as *P. aeruginosa*. The isolate was capable of dissipating 100 µg/mL of BUT in an M9 liquid medium with a decline rate of $0.50 \pm 0.03 \text{ day}^{-1}$. In sterile and non-sterile paddy soil experiments under flooded conditions, the isolate was able to well-colonize in soil, accelerate the decline of BUT (200 µg/g), and increase the rate of SR compared to non-inoculated experiments. The highest rate of BUT degradation and SR was observed in the co-presence of BUT and the PK isolate, in

non-sterile soil experiments. Overall, these results introduce *P. aeruginosa* PK as a potential BUT-degrader in flooded paddy soils.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double

publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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