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ENHANCED DEGRADATION OF PRETILACHLOR IN SOIL AND SEDIMENT SLURRIES BY INOCULATION OF A MIXED BACTERIAL CULTURE UNDER ANAEROBIC CONDITION

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ABSTRACT

Pretilachlor is a main component of herbicides widely applied to control weeds, causing serious environmental problems. However, its degradation is so slow under anaerobic conditions. This study evaluated the enhancement of pretilachlor degradation using a mixed culture of *Pseudomonas* sp. Pr1, *Proteiniclasticum* sp. Pr2 and *Paracoccus denitrificans* Pr3. The result showed that the degradation using a horizontal-flow anaerobic immobilized biomass bioreactor gave a degradation rate of 3.11 ± 0.31 µM/day after six cycles. Moreover, the inoculation of these bacteria significantly augmented the degradation in slurries of soil collected from a paddy field and sediments from a river. In addition, the determination of pretilachlor degradation in water and soil collected from a paddy field showed that the degradation rates followed an order: a slurry of soil and water (20:80, w/w) > water ≈ soil and water (50:50, w/w). However, adjuvants in an herbicide significantly caused adverse effects on substrate degradation. This study showed the role of isolated bacteria in degradation augmentation in liquid media. It also provided information on the degradation differences in water soil slurry, and soil collected from a paddy field.

Keywords: Pretilachlor, degradation, enhancement, bioreactor, soil, sediment.

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INTRODUCTION

Pretilachlor (2-chloro-2', 6'-diethyl-N-(2-propoxyethyl) acetanilide) is an ingredient of chloroacetamide herbicides. This ingredient is commonly used to control annual weeds, sedges, and broadleaf weeds in rice fields (Shilpakar et al., 2020). The compound kills weeds by inhibiting the biosynthesis of fatty acids, lipids, proteins, and flavonoids (Shilpakar et al., 2020). Pretilachlor is a main ingredient in herbicides, and it is usually mixed with fenclorim and other adjuvants to protect rice and increase its effectiveness. Fenclorim is a safener that reduces lipid peroxidation and oxidative damage induced by pretilachlor and enhances the disappearance of pretilachlor in rice tissues (Hu et al., 2020). The ratio of pretilachlor to fenclorim in herbicides is usually 3/1.

Due to its vast application, pretilachlor has been detected in water, soil, and sediment. For example, it was found in the water system of a rice field in Malaysia (Sapari & Ismail, 2012), in surface water, and in drinking water in the Vietnamese Mekong Delta (Toan et al., 2013; Chau et al., 2015). Pretilachlor was also found in groundwater, harvested rainwater, flocculated canal water, and bottled water in Viet Nam (Chau et al., 2015). It was also accumulated in sediment (Vidotto et al., 2004; Wang et al., 2022), in soil and plant biomass (Kobayashi et al., 1999; Marin-Morales et al., 2013), rice straw components (Braschi et al., 2003), and milk (Jouyban et al., 2020). Pretilachlor negatively affects enzyme activity in rat liver (Hisato, 1998), zebrafish (Jiang et al., 2016), and soil bacteria under aerobic conditions (Murata et al., 2004; Saha et al., 2012; Bhowmick et al., 2014; Sahoo et al., 2016). It causes acute oral intoxication in humans similar to clinical manifestations of organophosphate toxicity (Shilpakar et al., 2020). Therefore, the elimination of the compound should be conducted.

In a previous study, two mixed cultures of pretilachlor-degrading and fenclorim-degrading bacteria isolated from soil from a paddy field were determined for their degradation in liquid media under anaerobic conditions (Duc et al., 2024). However, the degradation by these strains during the growth phase was quite slow, especially the degradation of the compounds in an herbicide. The mixed culture of pretilachlor- and fenclorim-degrading bacteria required 30 days to degrade 80.2 ± 5.4% of pretilachlor (100 µM) and 100% of fenclorim (46.2 µM) in the mixed pure compounds.

One method to enhance the degradation rate is to increase the bacterial biomass by immobilization. Horizontal-flow anaerobic reactors with high length-to-diameter ratios and using polyurethane foam (PUF) to immobilize biomass exhibited effective degradation of benzene, toluene, ethylbenzene and xylene (BTEX) (Ribeiro et al., 2013), sulfamethazine (Oliveira et al., 2017), limonene (Rodrigues et al., 2021), and thiobencarb (Oanh & Duc, 2022). In this study, degradation enhancement using a horizontal-flow anaerobic reactor filled with immobilized cells of mixed pretilachlor-degrading bacteria in PUF was carried out. Moreover, the augmentation of pretilachlor degradation in slurries of soil collected from a paddy field, sediments collected from a river, and mangrove by inoculation of mixed degrading bacteria was conducted.

MATERIALS AND METHODS

Bacteria and mineral medium (MM medium)

The mixed culture of pretilachlor-degrading bacteria, including *Pseudomonas* sp. Pr1 (PP002273.1), *Proteiniclasticum* sp. Pr2 (PP002283.1) and *P. denitrificans* Pr3 (PP002293.1) (Duc et al., 2024) were used in this study. Each bacterial strain was individually cultured in MM medium supplemented with 100 µM pretilachlor and incubated at 30 ^{o}C with a shaking speed of 150 rpm for 10 days. Bacteria were collected by centrifuging at 10,000 rpm for 5 min, rinsed twice with the MM, and re-suspended in the MM at about 10^{11} CFU/mL.

The MM medium was prepared as described in a previous study (Duc et al., 2024). The medium components consisted of 1,360 mg/L KH_2PO_4, 840 mg/L $NaHCO_3$,

1,000 mg/L $NaNO_3$, 1,000 g/L D-glucose, 60.2 mg/L $MgSO_4$, 55.5 mg/L $CaCl_2$, 81.0 mg/L $FeCl_3$, 16.1 mg/L $ZnSO_4$, 16.0 mg/L $CuSO_4$, 6.2 m/L H_3BO_4 and 1.3 mg/L $CoCl_2 \cdot 6H_2O$. The pH was adjusted to 7.0 ± 0.1. The medium was sterilized at 121 ^oC for 15 min.

Chemical degradation using a bench-scale horizontal anaerobic reactor

In this study, a bench-scale horizontal anaerobic fixed bed reactor (Fig. 1) was fabricated as described in previous studies (Oanh & Duc, 2022; Rodrigues et al., 2021). The reactor made from a polyvinyl chloride tube had a total volume of 1.991 mL, a length (L) of 100 cm, and a diameter (D) of 5 cm. Five sampling points were evenly distributed at L/D of 3.3, 6.6, 10, 13.3, and 16.7.

PUF serving as the support for immobilizing biomass with a density of 23 kg/m^3 and porosity of 40% was cut to a particle size of 0.3–0.4 cm. 20.8 g PUF on a dry basis was transformed into a 250 mL bottle containing 100 mL of the cell condensed solution, shaken at 50 rpm for 12 hours. After the immobilization of bacteria for two cycles, PUF was filled in the reactor. In the medium with condensed pretilachlor-degrading strains, each bacterial strain was 10^{10} CFU/mL. All reactors were run for six periods, and each lasted for 1 day. Liquid samples were collected to determine the remaining chemical concentrations and degradation intermediates. Each experiment was performed with at least three replicates.

In this study, the degradation of pretilachlor at 100 µM (31.2 mg) was studied using it as a pure compound and a trade herbicide named Prefit 300EC (Central Plant Protection Joint Stock Company 1, Vietnam). The herbicide contains 300 g/L pretilachlor, 100 g/L fenclorim, and other adjuvants. The degradation of pure pretilachlor with the effects of pure fenclorim and the effects of other components in the Prefit 300EC was analyzed. Pure fenclorim was added to the media at 10.4 mg/L.

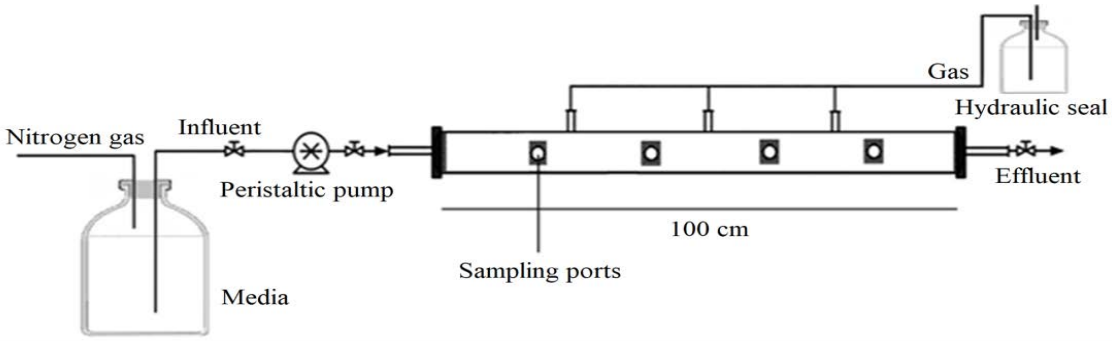

Figure 1. Schematic of pilot fed horizontal-flow anaerobic immobilized biomass bioreactor (Oanh & Duc, 2022)

Pretilachlor degradation in slurries of soil and sediments

The soil was collected from a paddy field, while sediment samples were collected from a river and a mangrove, as described in a previous study (Duc et al., 2024). In this study, the degradation of pure pretilachlor and Prefit 300EC in slurries of soil and sediments was conducted (Duc et al., 2024). The soil and sediments were individually mixed with site water to form slurries with 20% dry soil (w/w). The slurries were transferred into 50-mL glass vials (25 mL/vial). The mixed bacteria cultures were inoculated into the slurries with 0.33 × 10^6 CFU/mL of each strain. Vials were then flushed with nitrogen gas to create an anaerobic condition. Abiotic control without bacteria inoculation was run in parallel.

Effect of water content on pretilachlor degradation in samples collected from a paddy field

Water and soil samples were collected from the paddy field as described above. Pure pretilachlor or Prefit 300EC was added to media with 100% water, 80% water + 20% soil and 50% water + 50% soil. The degradation was carried out without and with augmentation with the mixed culture of *Pseudomonas* sp. Pr1, *Proteiniclasticum* sp. Pr2 and *P. denitrificans* Pr3 (10^6 CFU/mL in total). All vials were incubated at 30 ^oC with diffused light condition for 15 days. The vials were shaken at 150 rpm for 30 min a day.

Chemical analysis

Pretilachlor and its metabolites were extracted from liquid media and slurries of soil and sediment twice with dichloromethane, according to a previous report (Duc et al., 2024). The concentrations of all chemicals were determined using HPLC, and the identification of degradation metabolites was analyzed using the GC-MS technique, as described in a previous study (Duc et al., 2024).

Statistical analysis

Degradation percentages and degradation rates were calculated based on the chemical concentrations at the beginning (C_0) and after a specific time (C_t). Half-life ($t_{1/2}$) was calculated using the formula: $t_{1/2} = \ln 2/k$ where k is the degradation rate constant. The degradation rate constant was obtained from the formula: $C_t = C_0 e^{-kt}$. Data obtained from at least three replicates were presented as the mean ± one standard deviation. Duncan's test in the SPSS software program version 22.0 was used to analyze the significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Degradation of individual pure pretilachlor by a mixed culture using a horizontal-flow anaerobic immobilized biomass bioreactor

The mixed culture of *Pseudomonas* sp. Pr1, *Proteiniclasticum* sp. Pr2 and *P. denitrificans* Pr3 immobilized in PUF were determined for their degradation in the horizontal-flow reactor. The bacterial attachment at the beginning was 192.6 mg dry biomass, which increased to 287.5 mg dry biomass/g dry material after six cycles. The increase in attachment biomass of bacteria resulted in the enhancement of degradation. The degradation of pure pretilachlor (100 µM) increased during the operation, from the first to the sixth period (sixth cycle). The reduction of pure pretilachlor in the first period was 32.4 ± 4.3%, giving a degradation rate of 1.15 ± 0.18 µM/day. The degradation increased to 74.8 ± 7.5% (3.1 ± 0.31 µM/day) at the last period (Fig. 2). For the effect of fenclorim on pretilachlor degradation, fenclorim was added to the medium, and no significant change was observed during pretilachlor degradation in all periods.

Besides, abiotic controls were run in parallel. The result showed that the reduction of pretilachlor was not found, meaning that these compounds were not absorbed into the material in the reactor.

The biodegradation of pretilachlor and fenclorim occurred slowly during their growth phase (Duc et al., 2024), but the rates were enhanced in this study by using immobilized microorganisms. The degradation rates in this study were significantly higher than those in the previous report using suspended cells (Duc et al., 2024). Bacterial biomass immobilized in PUF in the reactor grew significantly higher bacteria compared to suspended cells, resulting in a higher degradation rate. Moreover, the survivability of immobilized microorganisms was probably better than freely suspended cells. Immobilized cells might decrease the toxic effects of chemicals on cells, resulting in higher activities of microorganisms (Mulla et al., 2013). For example, bacteria might form biofilms or be absorbed in PUF to enhance thiobencarb degradation in the horizontal-flow anaerobic bioreactor (Oanh & Duc, 2022). Microorganisms immobilized in PUF showed higher degradation than those immobilized in alginate and polyvinyl alcohol for degrading aniline (Oanh & Duc, 2022), and polyacrylamide, alginate and agar for

degrading carbofuran phenol (Kadakol et al., 2011). PUF is suitable for microorganism immobilization due to its high porosity, mechanical strength, stability and adsorbing capacity (Manohar et al., 2001; Kadakol et al., 2011).

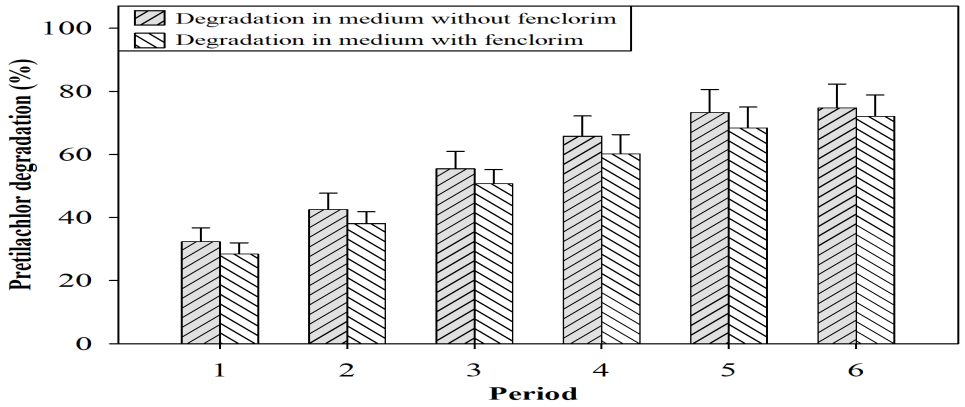

Figure 2. Degradation of pure pretilachlor by mixed culture of *Pseudomonas* sp. Pr1, *Proteiniclasticum* sp. Pr2 and *P. denitrificans* Pr3 in the bioreactor (a) without and (b) with supplementation with fenclorim in different periods

Degradation of pretilachlor in an herbicide by mixed culture using a horizontal-flow anaerobic immobilized biomass bioreactor

In this experiment, pretilachlor in Prefit 300EC herbicide added in the MM medium was degraded by a mixed culture of pretilachlor-degrading bacteria. The degradation rates of these compounds in Prefit 300EC herbicide were significantly slower than those of pure compounds. The degradation of pretilachlor at the end of the first period was 22.6 ± 3.4%. The corresponding data over the operation of the sixth period was 49.6 ± 5.1% (Fig. 3). The results showed that the degradation of the compounds in the herbicide was significantly slower than those of pure substrates, which was in line with the previous study that pure herbicide compounds were more easily degraded than those in a commercial herbicide (Duc et al., 2024). Adjuvants in Prefit 300EC herbicide probably caused negative effects on the activities of bacteria.

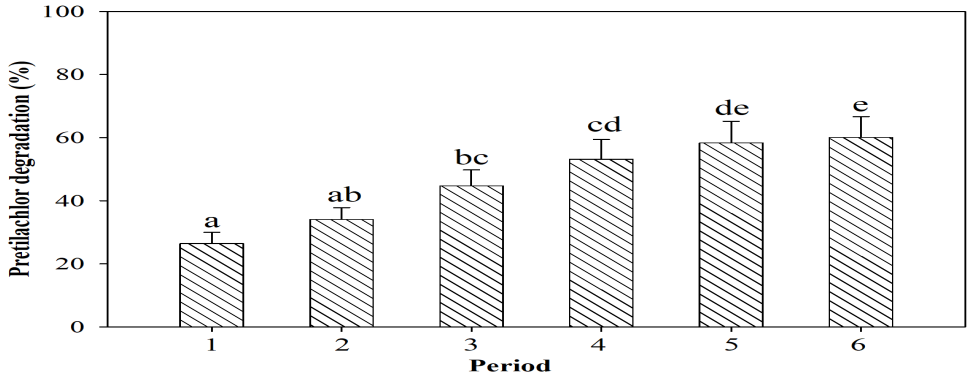

Figure 3. Degradation of pretilachlor in Prefit 300 EC herbicide by mixed culture in different periods of the bioreactor operation. Different letters (a, b, c, d and e) above each column indicate significantly different degradation ($p < 0.05$) among periods

The reactor presents an adequate performance and stability of the process due to long cell retention times and low hydraulic retention times. The fundamental studies are essential to suggest a potential application using a higher scale for the removal of pretilachlor. However, the removal of components in contaminated water, such as BOD and COD has not been determined. Moreover, a biogas collecting system, such as methane produced during the anaerobic condition, should be set up. In addition, the configuration for avoiding excessive introduction of O_2 from the atmosphere should be considered at a higher scale of the reactor. Therefore, more research should be conducted to overcome these issues.

The degradation at different sampling sites of the horizontal-flow reactor

The degradation at different sampling sites of the horizontal-flow anaerobic immobilized biomass bioreactor was determined and shown in Figure 4. The degradation percentages of pure pretilachlor by the mixed culture at the first sampling port were 17.0 ± 4.8% (Fig. 4a), while corresponding data for Prefit 300 EC herbicide were 8.0 ± 3.1% (Fig. 4b). The degradation increased at the following sampling ports with longer retention time. The increase in degradation at higher L/D was due to the higher retention time of the liquid media in the reactor.

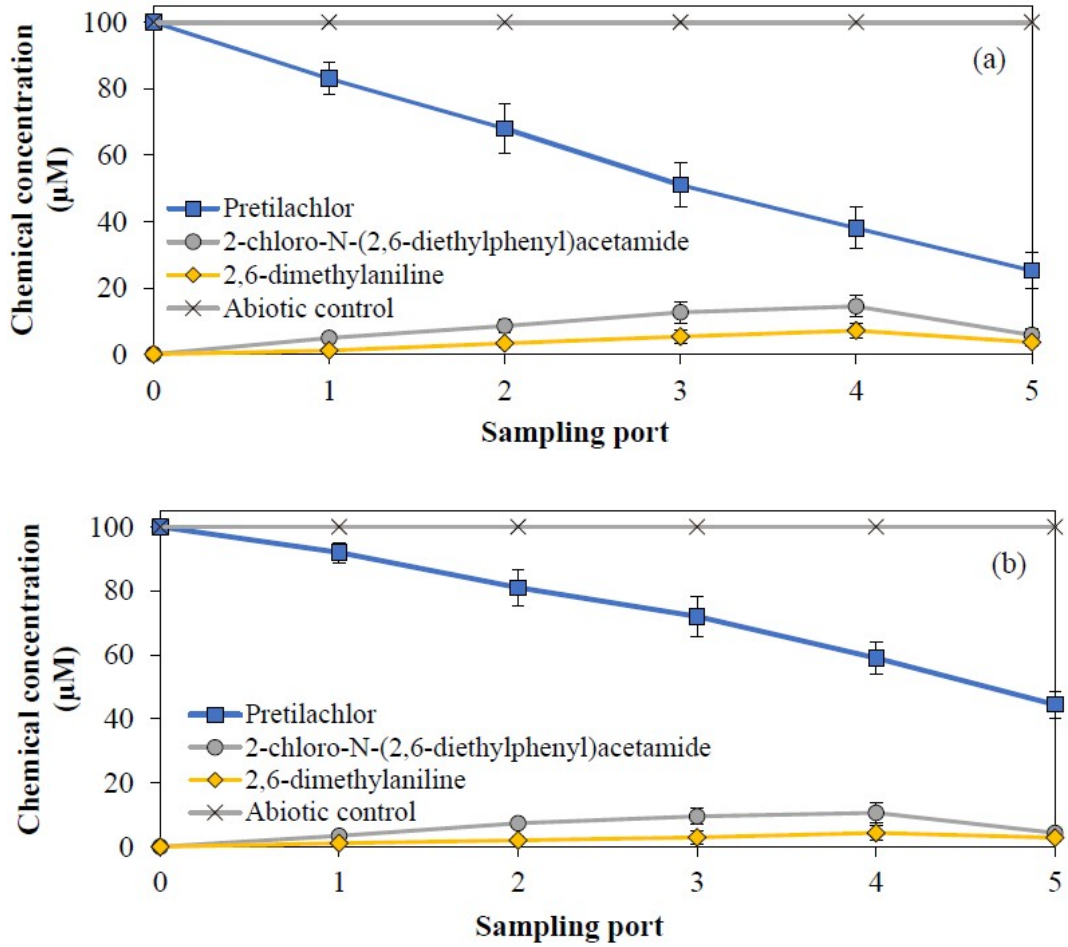

Figure 4. Degradation of pretilachlor using (a) pure substrates and (b) substrates in Prefit 300 EC herbicide at different sampling ports in the sixth period of the reactor

Some metabolites produced from pretilachlor and fenclorim transformation were found. GC-MS analysis showed that a metabolite with m/z 225, 176, 147, 77 was identified as 2-chloro-N-(2,6-diethylphenyl) acetamide. Another one with m/z 149, 134, 119 was considered to be 2,6-dimethylaniline. The concentrations of these metabolites increased from the first to the fourth sampling ports and degreased at the last port (Fig. 4). These metabolites were also produced during the degradation in the previous study (Duc et al., 2024). *Proteiniclasticum sediminis* BAD-10T was another strain showing pretilachlor degradation under anaerobic conditions, but its degradation pathway was not determined (Liu et al., 2022).

Pretilachlor degradation in soil and sediment slurries

The degradation by indigenous microorganisms and with augmentation in soil slurries from the paddy field, sediment from the river, and sediment from the mangrove was compared. The dissipation rates of pretilachlor in soil slurries from the rice field and sediment from the river were not statistically different and significantly higher than those in sediment from the mangrove. The dissipation percentages of the pure compounds were higher than those in the herbicide in most treatments (Table 1). The inoculation with degrading bacteria increased pretilachlor's degradation percentages into soil slurries from rice fields and sediment from a river from about 1.8 to two times. Meanwhile, the inoculation into sediment from the mangrove enhanced the degradation of pretilachlor by about 1.4 times. The natural dissipation and degradation with inoculation were in order: slurry from the paddy field ≈ sediment from the river > sediment from the mangrove (Table 1). The result could be explained that the mangrove is far from the sites where herbicides had been applied; therefore, the indigenous microorganisms in the mangrove sediment were not adapted well to pretilachlor. *Pseudomonas* sp., *Proteiniclasticum* sp. Pr2 and *P. denitrificans* Pr3 isolated from a paddy field could also not adapt to the herbicide well. Moreover, the physico-chemical characteristics of all media were different resulting in differences in the degradation rates.

Moreover, bacteria isolated from the paddy soil were well adapted to sediment from the river, but sediment from the mangrove. Similarly, half-life values of pure pretilachlor and fenclorim in the mixed compounds were significantly shorter than those of the compounds in the herbicide in most treatments. The inoculation of pretilachlor-degrading bacteria reduced the half-life values of both substrates (Table 1).

Table 1. Pretilachlor degradation in soil and sediment slurries by indigenous microorganisms and augmentation with inoculated bacteria for 15 days under anaerobic conditions

Treatment			Soil slurry from a paddy field	Sediment from a river	Sediment from a mangrove
Natural degradation	Pure pretilachlor	Degradation (%)	47.2 ± 5.5^{bBC}	41.8 ± 4.6^{bB}	30.1 ± 3.5^{bA}
		Half-life (day)	16.3 ± 1.9	19.2 ± 2.1	29.6 ± 3.4
	Herbicide	Degradation (%)	30.6 ± 4.0^{aB}	27.5 ± 3.2^{aAB}	20.5 ± 2.0^{aA}
		Half-life (day)	28.5 ± 3.7	32.3 ± 3.8	45.3 ± 4.4
Inoculation	Pure pretilachlor	Degradation (%)	84.6 ± 2.5^{dBC}	78.4 ± 4.4^{dB}	40.8 ± 4.5^{cA}
		Half-life (day)	5.6 ± 0.2	6.8 ± 0.4	19.8 ± 2.8
	Herbicide	Degradation (%)	62.4 ± 6.6^{cBC}	55.2 ± 6.5^{cB}	29.7 ± 4.4^{bA}
		Half-life (day)	10.6 ± 1.1	12.9 ± 1.5	29.5 ± 4.4

Note: The lowercase capitalized superscript letters show statistically significant differences in degradation among treatments within a column, while the capitalized superscript letters indicate statistically significant differences a line ($p < 0.05$).

In a previous report, most pretilachlor remained in the water phase and slowly percolated into the soil, in which the half-lives were 4.68–6.77 days in water and 15.01–28.76 days in soil (Vidotto et al., 2004). In this study, all vials were shaken, so the compound was probably distributed equally in both the water and soil phases. Duc et al. (2024) showed that the half-life values of pure pretilachlor individually added to the soil from the paddy rice, sediment from the river, and sediment from the mangrove were 16.1 ± 1.65, 16.7 ± 1.35, and 26.9 ± 2.73 days, respectively. These results were not statistically different from the results in this study, indicating that pure fenclorim did not significantly affect the degradation of pretilachlor. Under anaerobic condition, the half-life degradation of pretilachlor in soil was 2.78 days (Liu et al., 2020). Half-life values of the compound in the sediment of paddy fields were reported to be 15.01–28.76 days (Vidotto et al., 2004). In another report, half-life values of the compound under reductive conditions were 11.3 and 45.0 days in non-sterile soil and sterile soil, and 45.0 days, respectively, and significantly slower than those under oxidative conditions (Fajardo et al., 2000). For fenclorim, half-life was about 26 days in soil (Oloye et al., 2021). All results showed that the half-life values of pretilachlor depended on different conditions. However, previous studies determined the degradation of individual pure compounds. This study showed half-life values of pure compounds and the compounds in an herbicide with the presence of adjuvants.

Effects of water contain on pretilachlor degradation in soil from a paddy field

Pretilachlor is usually applied in paddy fields where irrigation is required. Therefore, this experiment determined the dissipation of the compound in soil collected from a paddy field with different water regimes. The result showed that the degradation performances in water were not statistically different compared to those in soil with 50% water and significantly slower than those in the slurry of 80% water (Table 2). Abundant microorganisms in the soil were probably the reason for the higher degradation performance in this medium. The degradation was slow in water, probably due to the shortage of available mineral ions and nutrients. At the same time, limited diffusion of pretilachlor in the media with 50% soil might also reduce contact with microorganisms.

The inoculation of pretilachlor-degrading bacteria increased the degradation of all water regimes, with around two times higher in both non-sterile treatments of 20% and 50% water. Moreover, a significant amount of pretilachlor was dissipated in treatments with the presence of sediment because it was absorbed in the solid component.

Table 2. Pretilachlor degradation (at 100 µM) in water and soil collected from a paddy field for 15 days

Pretilachlor		Soil contain (%)					
		0		20		50	
		Sterile	Non-sterile	Sterile	Non-sterile	Sterile	Non-sterile
Non-inoculation	Pure	0	0	7.1 ± 2.3	41.8 ± 4.6	10.3 ± 2.5	23.5 ± 3.3
	Herbicide	0	0	7.3 ± 2.4	30.6 ± 4.0	8.6 ± 2.8	19.4 ± 2.7
Inoculation	Pure	32.2 ± 4.1	35.6 ± 4.4	58.4 ± 6.8	84.6 ± 2.5	36.6 ± 5.1	55.8 ± 6.7
	Herbicide	24.3 ± 3.6	27.8 ± 3.2	46.1 ± 5.7	62.4 ± 6.6	28.1 ± 4.4	40.3 ± 5.2

A previous study showed that the sterile controls absorbed about 10% of pretilachlor and fenclorim into soil and sediment (Duc et al., 2024). In another report, 57.0% of pretilachlor remained in the surface water and 20.5% in the soil surface (0–2 cm) after 10 days, and no degradation occurred after 30 days (Flori et al., 2003). Fajardo et al. (2000) added pretilachlor in the paddy field and found that half-lives of the compound ranged from 7 to 10 days in soil and

3.5 days in water. Bhardwaj et al. (2024) showed that about 97% of 0.68 μg pretilachlor/g soil was degraded in soil within 45 days in a flooded rice field. The slow reduction of these compounds indicated that native microorganisms poorly degraded both compounds using pure substrates and an herbicide.

CONCLUSION

Three anaerobic pretilachlor-degrading isolates, i.e., *Pseudomonas* sp. Pr1, *Proteiniclasticum* sp. Pr2 and *P. denitrificans* Pr3 were immobilized in PUF, increasing from 32.4 ± 4.3% in the first cycle to 74.8 ± 7.5% in the sixth cycle in the MM medium. Moreover, the inoculation of the mixed bacteria significantly increased the degradation in slurries of soil collected from a paddy field, sediment from a river, and somewhat enhanced the degradation in sediment from a mangrove. The determination of water regimes in samples collected from a rice field showed that pretilachlor degradation was the highest in the slurry of soil (20%) and water (80%). Moreover, the role of pretilachlor-degrading isolates, inside degradation of pretilachlor by native microorganisms and bioaugmentation in soil and sediments, and the effects of water regimes on the degradation processes were elucidated. The horizontal-flow anaerobic immobilized biomass bioreactor shows an interesting alternative treatment of pretilachlor. However, a higher scale reactor should be developed for future application.

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