DIURON DEGRADATION BY A MIXED CULTURE OF BACTERIA IMMOBILIZED IN RICE STRAW

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Abstract

Diuron has been widely applied to control weeds causing serious environmental pollution. In the current study, the mixed culture of three bacterial isolates, *Bacillus subtilis* DU1, *Acinetobacter baumannii* DU, and *Pseudomonas* sp. DUK, was investigated for diuron degradation in a packed reactor and soil. The immobilization of the bacteria mixture in rice straw increased the degradation. The specific degradation rates of diuron by bacteria immobilized in rice straw in batch cultures increased from 0.38 ± 0.03 mg/L at the first cycle to 0.98 ± 0.10 mg/L at the fourth cycle. The degradation using the reactor was also carried out in a continuous operation. Moreover, the introduction of bacteria into soil increased diuron degradation. This study shows the potential of diuron degradation in the reactor and in soil using the mixed bacterial culture immobilized in rice straw.

Keywords: Degradation, diuron, immobilized bacteria, packed reactor.

ĐÁNH GIÁ SỰ PHÂN HỦY HOẠT CHẤT DIURON CỦA HỖN HỢP VI KHUẨN CỐ ĐỊNH TRONG RƠM CÂY LÚA

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Tóm tắt

Diuron là một hoạt chất của thuốc diệt cỏ được sử dụng rộng rãi và gây ô nhiễm môi trường nghiêm trọng. Trong bài báo này, hỗn hợp ba chủng vi khuẩn bao gồm *Bacillus subtilis* DU1, *Acinetobacter baumannii* DU và *Pseudomonas* sp. DUK đã được khảo sát về khả năng phân hủy diuron trong hệ thống xử lý (reactor) và trong đất ở điều kiện thí nghiệm. Hỗn hợp vi khuẩn được cố định trong rơm rạ giúp tăng cường sự phân hủy. Tốc độ phân hủy diuron khi thực hiện từng mẻ do hỗn hợp vi khuẩn này trong reactor tăng từ 0,38 ± 0,03 mg/L ở chu kỳ đầu tiên lên 0,98 ± 0,10 mg/L ở chu kỳ thứ tư. Sự phân huỷ sinh học này cũng được khảo sát khi reactor hoạt động liên tục trong. Ngoài ra, việc bổ sung vi khuẩn vào đất làm tăng sự phân hủy diuron. Nghiên cứu này cho thấy tiềm năng phân huỷ diuron nhờ hỗn hợp vi khuẩn cố định trong rơm rạ.

Từ khoá: Phân huỷ, diuron, vi khuẩn cố định, reactor.

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1. Introduction

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a phenylurea herbicide applied to control weeds in various crops such as cotton, coffee, sugarcane, citrus, etc. For its extensive application, the compound has been found in freshwater (Green & Young, 2006; Lapworth & Gooddy, 2006; Eriksson et al., 2007), Marine & Sediments (Thomas et al., 2002), and soil (Tucker, 1978). This herbicide is moderately toxic to mammals, birds, and aquatic invertebrates (Giacomazzi & Cochet, 2004). For humans, diuron causes damages to the liver, heart or kidney, and disturb the life at low concentrations (El-Nahhal & Lubbad, 2018).

The herbicide is quite persistent, with half-lives ranging from weeks to years in soil and aquatic environments (Tixier et al., 2001; El-Nahhal & Lubbad, 2018). Microbial degradation is the predominate method of natural removal of diuron from the environment (El-Nahhal & Lubbad, 2018). In some previous studies, diuron-degrading bacteria and fungi have been isolated, such as *Stenotrophomonas acidophila* TD4.7 and *Bacillus cereus* TD4.31 (Egea et al., 2017), *Arthrobacter sulfonivorans*, *Variovorax soli*, and *Advenella* sp. JRO (Villaverde et al., 2017), *Ganoderma lucidum* (Coelho-Moreira et al., 2018), and *Bacillus* spp. (Muendo et al., 2021).

In a previous report, a mixed culture of three bacterial strains, i.e., *Bacillus subtilis* DU1, *Acinetobacter baumannii* DU, and *Pseudomonas* sp. DUK, isolated from sugarcane soil, completely degraded diuron in liquid media at 20 mg/L within 48 hours (Duc & Oanh, 2019). These bacterial strains do not have any potential of negative health effect on either human or animals. For toxic substrates biodegradation, the immobilization of microorganisms is a preferred method due to high bacteria density and reduced reactor volume. Therefore, this study determined diuron degradation by the mixed bacterial culture immobilized in rice straw, using a pilot-packed reactor.

2. Materials and methods

2.1. Culture media

The mixed culture of three isolated bacteria (*Bacillus subtilis* DU1, *Acinetobacter baumannii* DU, and *Pseudomonas* sp. DUK) was cultured in mineral medium (MM). The MM components were described by Duc (2017), consisted of (g/L) Na_2HPO_4, 2.79; KH_2PO_4, 1.00; $(NH_4)_2SO_4$, 1.00; $MgSO_4 \cdot H_2O$, 0.20, and 1.00 mL/L trace mineral solution. Trade mineral solution contained (g/L) H_3BO_3, 0.30; $CoCl_2 \cdot 6H_2O$, 0.20; $ZnSO_4 \cdot 7H_2O$, 0.10; $Na_2MoO_4 \cdot 2H_2O$, 0.03; $MnCl_2 \cdot 4H_2O$, 0.03; $NiCl_2 \cdot 6H_2O$, 0.02; $CuCl_2 \cdot 2H_2O$, 0.01. The pH was adjusted to 7.0 ± 0.1. The medium was autoclaved at 121°C for 15 min.

2.2. Diuron degradation in a packed reactor

A cylindrical, pilot packed reactor was fabricated using a glass column connected to an air pump and a feed pump (Figure 1) as described by Duc & Oanh (2019). The feed was pumped into the reactor top, while filter sterile air was introduced from the bottom through a ceramic diffuser with 0.75 mL air/mL liquid medium/min. The height and inside diameter were 15.0 and 5.5 cm, respectively. The work volume was 130 cm^3. The packed reactor was operated at room temperature (~30°C) in batch and continuous processes.

The degradation in the packed reactor was conducted using freely suspended and immobilized bacteria. Each bacterial strain was separately cultured and then mixed before being added to new media for degradation experiments as described in a previous report (Duc *el al.*, 2021). The MM medium was inoculated with the mixed culture at an initial bacterial number of 10^6 CFU/mL (OD_{600} ~0.03). Each strain in the mixture had the same cell number. For degradation by freely suspended bacteria, the incubation was conducted for 48 hours. For degradation by immobilized cells, the reactor was operated in batch and continuous degradation. After each cycle (48 hours) in the batch operation, the spent medium was removed, and a new sterile MM medium was added for the next cycle. After four batch cycles, the degradation was carried out in continuous operation with a retention time of 12 hours. Liquid samples were collected to determine diuron concentrations.

The degradation was also carried out using bacteria immobilized in rice straw. Rice straw was collected from a rice field immediately after harvesting. The rice straw was first washed with

distilled water, and then cut into pieces (<1.0 mm in size). The fresh straw was dried in an oven (Ecocell-LIS-B2V/EC55, Germany) at 70°C for 2 days, and then sterilized at 121°C for 15 minutes. It was then transferred into the reactor to 70 cm^3. The volume of rice straw was determined based on the volume of the MM medium added to the reactor to a specific scale on the wall. Rice straw components were analyzed in a previous report (Duc, 2022).

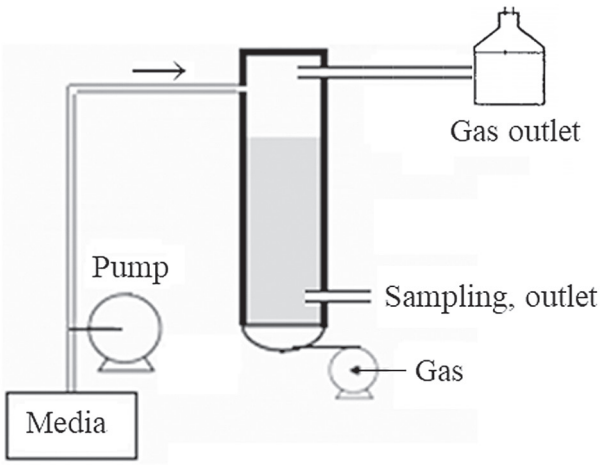

Figure 1. Schematic of a pilot packed reactor (Duc & Oanh, 2019)

2.3. Diuron degradation in soil

The soil sample was collected from a depth of 0-20 cm in a sugarcane field (9°40'06.1"N, 106°19'46.1"E) at which soil was collected to isolate the diuron-degrading bacteria before (Duc et al., 2021). Soil was also collected from a rice field (10°33'55.2" N, 105°51'2.4" E) where had been exposed to diuron. Moreover, a soil sample was taken from the campus of Dong Thap University, where the overall land has never been contaminated with pesticides. All soil samples were transferred to a lab for experiments. The soil was air-dried at room temperature, sieved through a mesh with a diameter of 2 mm. The soil components were determined against the soil texture triangle (Soil Science Division Staff, 2017). Other physicochemical properties were analyzed using the APHA method (APHA, 2005). The components of soil from the sugarcane field and rice field were not much different. Soil from the campus had higher fine sand and P_2O_5 than those of other soils, while silt, clay, total N and K_2O contents were lower. Therefore, the nutrient values for microorganisms in the sugarcane field and rice field were somewhat higher than those of soil from the campus. Diuron was not detected in all soils (Table 1).

Table 1. Physico-chemical characteristics dry soil samples

Soil properties	Soil collected from the sugarcane field	Soil collected from the rice field	Soil collected from the campus
Granulometric properties (%)			
Fine sand (0.02-0.2 mm)	48.5 ± 4.4	39.5 ± 5.5	62.8 ± 5.2
Silt (0.02-0.002 mm)	31.4 ± 2.7	35.8 ± 4.0	22.4 ± 2.8
Clay (< 0.002 mm)	20.1 ± 2.3	24.7 ± 2.2	14.8 ± 2.0
Agrochemical properties			
pH	6.3 ± 0.2	6.1 ± 0.3	6.6 ± 0.5
Total C (%)	3.6 ± 0.4	4.1 ± 0.4	2.2 ± 0.03
Total N (%)	0.15 ± 0.04	0.17 ± 0.03	0.07 ± 0.0
P_2O_5 (ppm)	36.7 ± 3.3	30.1 ± 3.1	48.8 ± 4.3
K_2O (ppm)	6.6 ± 0.4	5.3 ± 0.4	2.4 ± 3.0
Diuron (units)	0	0	0

Format diuron degradation in soil, 500 g soil was transferred into a plastic container (length×width×depth of 15×25×20 cm), and diuron was added to a final concentration of 20 mg/kg dry soil (w/w). The containers were closed with a plastic cover. The container was incubated for 30 days. Sterilized deionized water was sprayed during the incubation to maintain the soil moisture of 40%. The soil moisture was measured by the comparison between the wet and dried soil (soil dried at 80°C for 2 days).

Bacteria were cultured in the MM medium supplemented with 20 mg/L diuron. The medium was incubated for 24 hours with a shaking speed of 150 rpm and at room temperature (~ 30°C). Bacteria in the culture medium were collected by centrifuging it at a speed of 8,000 rpm for 5 min, washed twice with sterilized water and re-suspended in the water with approximately 5×10^7 CFUs/mL. Bacteria were added to the soil to give a final concentration of 10^6 cells/g dry soil for both free and immobilized cell treatments. Water was also added to approximately 40% soil moisture.

For chemical degradation by immobilized cells, 50 g dry straw was transferred into a sterile beaker which was then added with 500 mL of cell suspension (~ 10^9 CFU/mL sterile water) and stored for 12 hours. The difference between the cell densities in the liquid medium before and after the storage was determined by the bacteria numbers immobilized in the supporting material.

2.4. Determination of diuron concentration and bacteria number

The diuron extraction from soil was conducted as described in a previous study (Duc et al., 2022). Soil sample (3.0 g) was added into 7 ml of acetone/ethyl acetate (1/1, v/v), shaken at 500 rpm for 5 min. The extracts were filtered using 0.22 µm filters and concentrated. Concentrations of diuron and its degradation metabolites were measured using an HPLC system. The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of LC 20AD pumps, SIL-20A autosampler, and an SPDM20A photodiode array (PDA) detector. The mobile phase (acetonitrile and water (30:70, v/v)) was pumped isocratically at a rate of 0.5 mL/L, and a 5 µl sample was injected into the HPLC system. The column oven temperature was maintained at 40°C, and the detection was performed at a wavelength of 250 nm.

Bacteria density in liquid media was enumerated by counting CFUs emerging on agar plates containing the MM medium. The bacterial growth was also determined using a DU800 spectrophotometer (Beckman Coulter, USA) at 600 nm (OD_{600}).

2.5. Statistical analysis

Data obtained from at least three replicates are shown as the mean ± standard deviation. Significant differences among means were statistically analyzed using one-way Duncan's test ($p < 0.05$) in SPSS program version 22.0.

3. Results and discussion

3.1. Growth and degradation of diuron by freely suspended bacteria

Bacteria grew and degraded diuron in the packed reactor during the incubation (Figure 2). OD_{600} showing bacteria density increased from 0.03 ± 0.0 at the beginning to 0.93 ± 0.1 after 48 hours. The degradation occurred in the lag phase over 48 hours (Figure 2), in which diuron was completely removed, and bacteria were approximately (1.0 ± 0.1)×10^9 CFU/mL at the end of the incubation process. Meanwhile, the abiotic control showed no degradation.

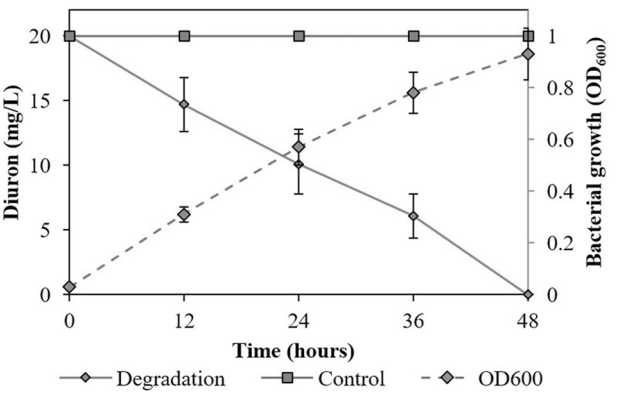

Figure 2. Diuron degradation by free bacteria of mixed culture of *Bacillus subtilis* DU1, *Acinetobacter baumannii* DU, and *Pseudomonas* sp. DUK

3.2. Diuron degradation by bacteria immobilized in rice straw

The degradation by bacteria immobilized in rice straw was conducted in the pilot-packed reactor. The degradation was first carried out in

batch culture experiments. At the first cycle, rice straw absorbed 38.1 ± 7.1% diuron, and degraded 61.9% ± 7.2% diuron after 36 hours (Figure 3.1). At the second one, corresponding data were 10.2 ± 4.1% and 89.8 ± 4.1% (Figure 3.2). The absorption by rice straw in the third and fourth cycles were not observed, while bacteria completely degraded the herbicide within 36 hours (Figure 3.3 and 3.4). The specific degradation rate at the first cycle was 0.38 ± 0.03 mg/L after 12 hours, which was not statistically different compared to the degradation in the lag phase of suspended bacteria (shown in Figure 1). The corresponding data for the second, third and fourth cycles were 0.58 ± 0.05, 0.88 ± 0.10, and 0.98 ± 0.10 mg/L, respectively. Diuron was not dissipated in the abiotic control in the two last cycles due to the saturation of diuron absorption. Meanwhile, the biodegradation increased due to a higher number of bacteria immobilized in the rice straw. The components of rice straw supported the bacterial growth and degradation of a pesticide was reported in a previous study (Duc, 2022).

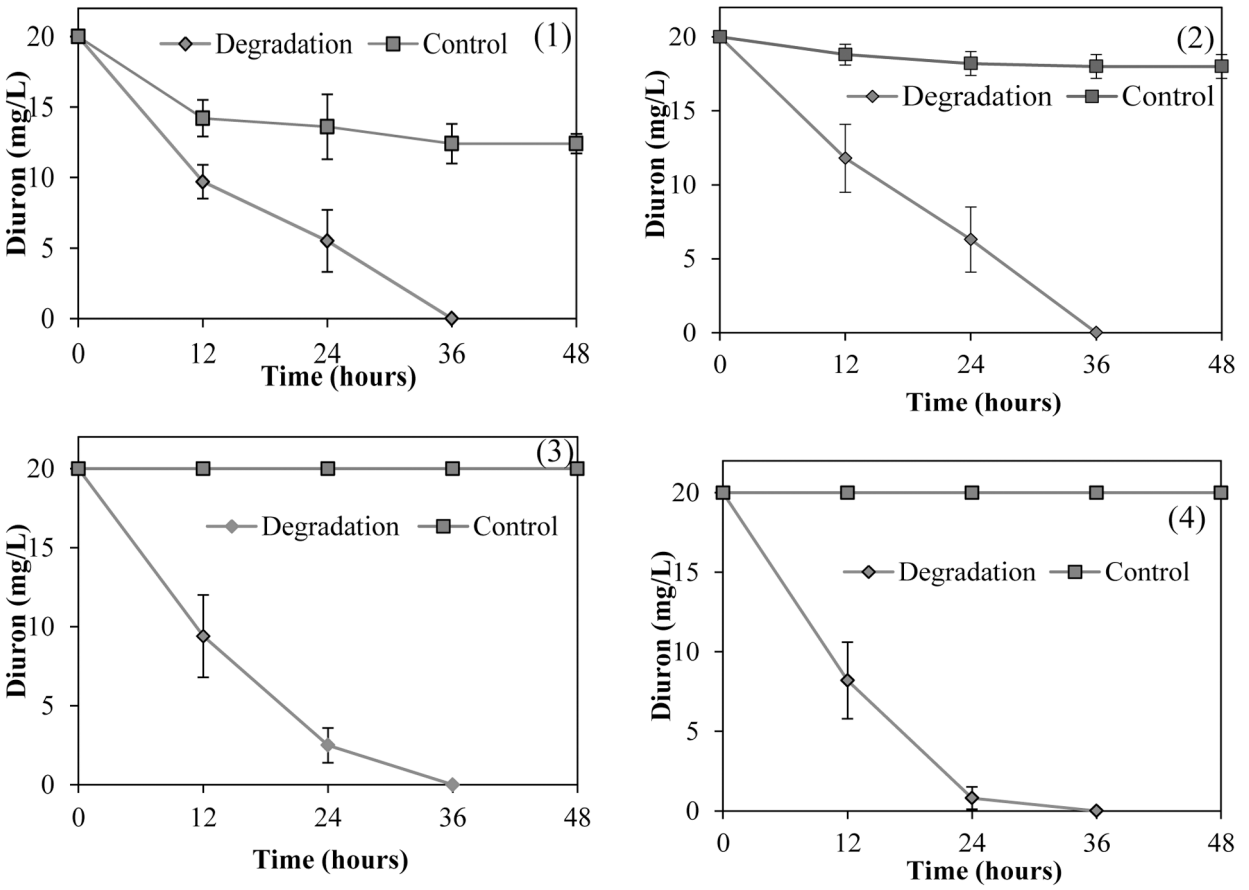

Figure 3. Diuron degradation by the mixed bacteria immobilized in rice straw. The degradation was conducted in batch culture in the packed reactor at the (1) first, (2) second, (3) third and (4) fourth cycles

After four cycles, the degradation was operated in continuous process with the retention time of 12 hours. Diuron was nearly completely removed at 10 mg/L. At the concentration of 20 mg/L, the degradation was 69.4 ± 9.5% at the first 12 hours, which was higher than the degradation at the fourth cycle in the batch culture by 13.2% on average. However, the degradation was quite stable at following cycles (Figure 4) probably because bacteria immobilized in the rice straw were saturated. The degradation at 40 mg/L was 25.0 ± 11.1% at the first 12 hours, increased to 52.5 ± 10.3% at the end of incubation process. The degradation percentages at 40 mg/L were slow at the beginning probably bacteria required more time to adapt to a high diuron concentration. The specific degradation rates at 10, 20

and 40 mg/L were 0.76 ± 0.04, 1.21 ± 0.08 and 1.27 ± 0.11 on average of four determined times (Figure 4), respectively. The results recorded no statistical difference in specific degradation rates at 20 and 40 mg/L, but their concentration rates were higher than that at 10 mg/L.

In a previous study, the absorption of carbofuran in rice straw was saturated after two days (Duc, 2022). Rice husk was used as a carbon source and a biofilm carrier for denitrification of wastewater (Shao et al., 2009), was also served as a lignocellulosic material to increase the growth and activity of fungus *Trametes versicolor* for carbofuran remediation (Ruiz-Hidalgo et al., 2014). The pilot packed reactor with bacteria immobilized in ceramic rings and polyurethane foam showed effective degradation of chlorobenzenes and chlorotoluenes (Duc & Oanh, 2019).

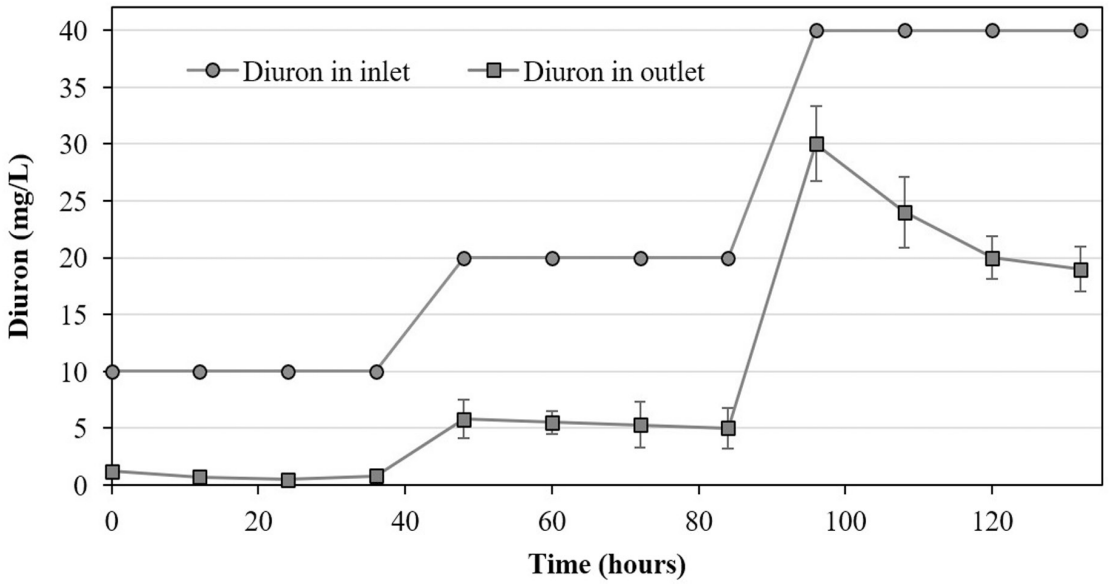

Figure 4. Diuron degradation by the mixed bacteria immobilized in rice straw. The degradation was conducted in the continuous process using the packed reactor

3.3. Diuron degradation in soil

Diuron degradation performances in soil transferred from the sugarcane field, rice field and campus are shown in table 2. The supplementation of bacteria in soils enhanced the diuron degradation in all treatments. The degradation rates in the soils without augmentation (from 23.5 ± 4.5 to 56.8 ± 7.7) were significantly lower than those of augmentation ones (from 82.6 ± 6.8% to 90.2 ± 4.4%). The diuron loss in the soils without augmentation was probably due to the degradation by indigenous microorganisms and chemical adsorption by soil components. The degradation rates in soil collected from the campus without augmentation and augmentation with free bacteria were significantly slower than those of other soil types. The soil collected from the sugarcane field and rice field had been contaminated by diuron; therefore, microorganisms in these soils had adapted to the herbicide, resulting in higher degradation rates than the soil collected from the campus by from 31.7% to 59.1% on average. Moreover, the structure and chemical components of the campus soil might be accounted for the lower degradation. The degradation performances in augmented soil with free and immobilized bacteria were not statistically different except for the campus soil (Table 2). The degradation percentage in the campus soil augmentation with immobilized bacteria was higher than the soil inoculated with free bacteria, probably rice straw supported bacteria. The rice straw probably provided a nutrient source for bacteria (Duc, 2022).

Our previous study showed that the inoculation of mixed bacteria boosted the degradation in soil collected from Nui Cam Mountain (An Giang) (Duc, 2022). These results signified that the mixed culture of

B. subtilis DU1, *A. baumannii* DU, and *Pseudomonas* sp. DUK could degrade diuron not only in the familiar soil (the sugarcane soil), but also in new environments (the soil from rice field and campus). The degradation in the soils in the current study is in line with previous reports. For example, Sørensen et al., (2008) showed that 78.8 ± 0.9% of diuron (2.0 mg/kg soil) remained in soil after 26 days, while the inoculation with *Arthrobacter globiformis* D47 and *Variovorax* sp. SRS16 resulted in almost the herbicide disappearing.

Table 2. Degradation of diuron (20 mg/kg dry soil) in soils

Treatments	Diuron degradation (%)		
	Soil from a sugarcane field	Soil from a rice field	Soil from a campus
Control	53.3 ± 6.8^{Ab}	56.8 ± 7.7^{Ab}	23.5 ± 4.5^{Aa}
Free bacteria	83.4 ± 7.4^{Bb}	81.8 ± 8.2^{Bb}	68.4 ± 7.4^{Ba}
Immobilized bacteria	90.2 ± 4.4^{Ba}	88.5 ± 7.0^{Ba}	82.6 ± 6.8^{Ca}

Different capital letters (A, B and C) and small superscript letters (a and b) indicate statistically significant differences ($p < 0.05$) among treatments within a column and a line, respectively.

4. Conclusion

The degradation by a mixed culture of *Bacillus subtilis* DU1, *Acinetobacter baumannii* DU, and *Pseudomonas* sp. DUK immobilized in rice straw was conducted. The degradation rates in the reactor in batch culture increased over the process, from the first to the fourth cycles, with corresponding cycles of 0.38±0.03, 0.58±0.05, 0.88±0.10 and 0.98±0.10 mg/L. Moreover, the inoculation of bacteria augmented the herbicide degradation in soils. Rice straw also stimulated the degradation in the soil with low C and N contents./.

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