BUTACHLOR DEGRADATION BY *Pseudomonas* sp. But1 AND *Pseudomonas* sp. But2 IMMOBILIZED IN POLYURETHANE FOAM (PUF)

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Abstract

Butachlor has been extensively applied to control weeds, increasing food production and reducing labor. In this study, butachlor degradation by Pseudomonas sp. But1 and Pseudomonas sp. But2 immobilized in polyurethane foam (PUF) was determined to compare with degradation by free cells. The degradation percentages of the pure compound by Pseudomonas sp. But1 and Pseudomonas sp. But2 were 100% and 96% within 24 hours at a concentration of 50 mg/L, respectively. Meanwhile, butachlor degradation in an Cantanil 550EC herbicide was completely after 30 hours. The determination of butachlor degradation by bacteria immobilized in PUF showed that degradation rates of immobilized Pseudomonas sp. But1 were more effective than those of Pseudomonas sp. But1. Even though degradation rates by immobilized bacteria were decreased after long-term storage of 90 days, Pseudomonas sp. But2 immobilized in PUF could be used to degrade butachlor in liquid media.

Keywords: *Butachlor, herbicide, immobilized bacteria, long-term storage, polyurethane foam.*

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PHÂN HỦY BUTACHLOR BỞI *Pseudomonas* sp. But1 VÀ *Pseudomonas* sp. But2 CỐ ĐỊNH TRONG POLYURETHANE (PUF)

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Tóm tắt

Butachlor đã được ứng dụng rộng rãi để kiểm soát cỏ dại, giúp tăng sản lượng lương thực và giảm công lao động. Trong bài báo này, sự phân huỷ butachlor do Pseudomonas sp. But1 và Pseudomonas sp. But2 cố định trong polyurethane (PUF) được xác định để so sánh với sự phân hủy được thực hiện bởi các tế bào tự do. Kết quả cho thấy sự phân hủy hợp chất butachlor tinh khiết do Pseudomonas sp. But1 và Pseudomonas sp. But2 tương ứng là 100% và 96% ở nồng độ tương ứng là 50 mg/L trong vòng 24 giờ. Trong khi đó, sự phân hủy 100% butachlor trong thuốc trừ cỏ Cantanil 550EC cần 30 giờ. Sự phân huỷ butachlor của vi khuẩn cố định trong PUF thì Pseudomonas sp. But2 hiệu quả hơn so với Pseudomonas sp. But1. Mặc dù tốc độ phân hủy của vi khuẩn cố định bị giảm sau khi bảo quản trong thời gian 90 ngày, Pseudomonas sp. But2 cố định trong PUF có thể được sử dụng để phân hủy butachlor trong môi trường lỏng.

Từ khóa: *Bảo quản, butachlor, polyurethane, thuốc trừ cỏ, vi khuẩn cố định.*

1. Introduction

Herbicides have been extensively used to control weeds in the agricultural sector in order to increase food production and reduce the need for labor. Among various herbicides, butachlor has been worldwide used. The herbicide is mostly used for post-emergent treatment in crop fields, especially in rice *(Oryza sativa)* cultivation (Yang et al., 2011). Butachlor is used to control various annual grasses and some broadleaf weeds (Dwivedi et al., 2010). In Asia alone, about 4.5×10^7 kg butachlor is used each year (Singh et al., 2018; Kaur & Goyal, 2020). Since propanil is usually mixed with butachlor, both compounds can co-contaminate the environment. Commercial herbicides contain adjuvants and the principle active ingredients. The adjuvants are used to enhance herbicide performance (Mesnage & Antoniou, 2018).

The indiscriminate use of herbicides generally leads to environmental contamination and causes severe problems for non-target animals, microorganisms, and humans. Butachlor has genotoxic effects on amphibians and various freshwater fishes (Geng et al., 2005; Hsu et al., 2005), adversely affects earthworms (Muthukaruppan et al., 2005). Moreover, this herbicide is suspected to be carcinogenic; it produces mitochondrial dysfunction and chromosomal and DNA damage (Dwivedi et al., 2012).

Given their persistence and toxicity, propanil and butachlor residues must be removed from contaminated sites. The microbial degradation of these herbicides may be inhibited by other xenobiotic compounds. For example, propanil degradation by Acinetobacter baumannii DT was influenced by the presence of butachlor (Nguyen et al., 2020). The substrate inhibition of butachlor degradation occurred at high concentrations (Mohanty et al., 2019). However, little is known about the effects of adjuvants and other herbicides on butachlor remediation.

Cell immobilization is a preferred method used to degrade toxic organic compounds because it may enhance degradation rates, reduce cell leakage and is convenient for transportation. Some common immobilization methods such as using alginate and PUF have been widely applied. In this study, *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 isolated from soil (Ha et al., 2020) were determined for their degradation by free-resting cells and immobilized counterpart.

2. Materials and methods

2.1. Media for bacterial growth and degradation

The mineral medium (MM medium) was prepared by adding the following salts to double-distilled water (mg/L): Na_2HPO_4, 2.79; KH_2PO_4, 1.00; $(NH_4)_2SO_4$, 1.00; $MgSO_4\bullet H_2O$, 0.20 and 1.00 mL trace mineral solution. The trace mineral solution consisted (in grams per liter) of H_3BO_3, 0.30; $CoCl_2\bullet6H_2O$, 0.20; $ZnSO_4\bullet7H_2O$, 0.10; $Na_2MoO_4\bullet2H_2O$, 0.03; $MnCl_2\bullet4H_2O$, 0.03; $NiCl_2\bullet6H_2O$, 0.02; $CuCl_2\bullet2H_2O$, 0.01. After adjusting to pH 7.0±0.1, the medium was sterilized at 121°C for 15 minutes. Chemicals were purchased from Sigma-Aldrich or Merck.

2.2. Immobilization method

For the immobilizing preparation, each bacterial strain was cultured in MM medium for 12 h. Bacteria were collected by centrifugation at 8,000 rpm for 15 minutes. Cell pellets of each strain were washed twice with the sterile MM medium and mixed together. The solution of resting cells with about 10^9 CFU/mL of each strain *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 was used for immobilization, degradation, and storage. The PUF immobilization was carried out by the method described by (Ha & Bui, 2017) with modification. The PUF was cut into 2.5 cm cubes, placed in 250-mL Erlenmeyer flasks, autoclaved and the dried at 80°C in a Memmert cabinet (Germany). 100 mL of resting cell culture and five dry PUF cubes were added to each flash. The flash was kept stable allowing bacteria to immobilize in PUF cubes. After two hours, liquid media were removed, and the PUF cubes were rinsed with sterile saline (0.85% NaCl) twice.

2.3. Experiments on butachlor degradation

The degradation process was carried out at room temperature (~30°C) with a shaking speed of 150 rpm. Pure butachlor was supplemented into the mineral medium at 50 mg/L. For butachlor degradation in herbicide Cantanil 550EC, the herbicide was added into the liquid mineral medium to give a butachlor concentration of 50 mg/L. Herbicide Cantanil 550EC containing 275 mg/L propanil, 275 mg/L butachlor and adjuvants was produced by Thanhson Agrochem Company, Vietnam.

The degradation by resting cells was conducted with 10^9 CFU/mL in liquid MM medium. After degradation, the medium was centrifuged to collect bacteria. Bacteria were rinsed twice with the sterile MM, placed in 1.5-mL eppendorfs and stored at 4°C. For chemical degradation by immobilized bacteria, the degradation was conducted for five cycles, each cycle was 12 hours. After each cycle, the PUF cubes were rinsed twice with sterile saline. The cubes used in the last cycle were divided into two groups and stored at 4°C in a dark condition. One group was stored for 30 days, and another was 90 days. Abiotic control was also run in parallel.

2.4. Statistical analysis

All obtained data from at least three experiment 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. Butachlor degradation by free cells

The degradation rates of pure butachlor and butachlor in herbicide Cantanil 550EC by condensed free bacteria were compared. The results showed that the reductions of the pure compound in media were significantly higher than those of the compound in the herbicide (Figure 1). This result was similar to the previous study (Nguyen et al., 2020). The degradation rates by free cells of *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 were not significantly different. The degradation of pure compound by But1 was complete (100%), and by But2 was nearly complete (96%) within 24 hours. Meanwhile, the butachlor degradation in herbicide Cantanil 550EC by both bacterial strains was complete after 30 hours.

The butachlor degradation in herbicide was always slower than that of pure compound (Figure 1), which agreed with a previous report by Nguyen et al. (2020). Indeed, propanil in the herbicide is an active gradient which inhibited the butachlor degradation process (Nguyen et al., 2020). Moreover, the presence of adjuvants in the herbicide was also a factor to limit this process. The degradation performances by condensed bacteria as well as the degradation rates in this study were higher than those by growth cells in exponential phase described in a previous report due to higher cell numbers at the outset (Nguyen et al., 2020).

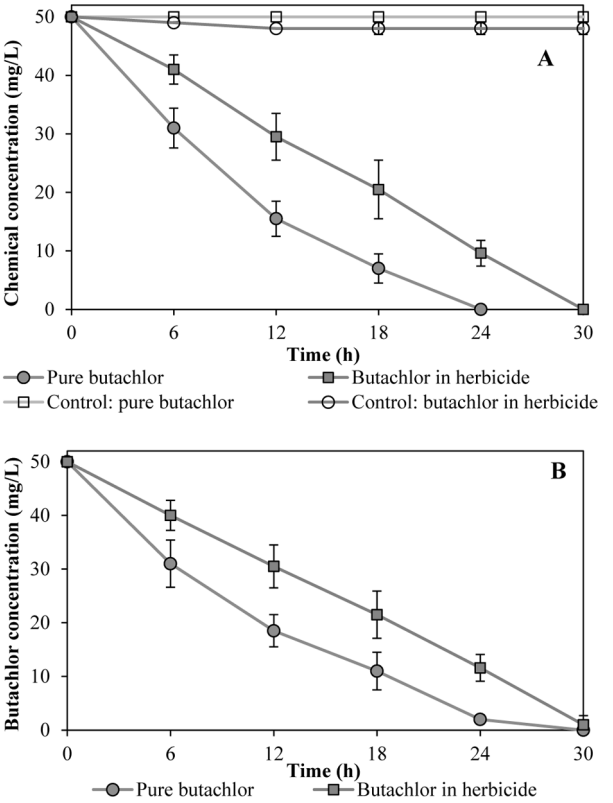

Figure 1. Butachlor degradation by (A) *Pseudomonas* sp. But1 and (B) *Pseudomonas* sp. But2 in mineral medium. The controls without bacteria showed insignificant degradation

3.2. Butachlor degradation by bacteria immobilized in PUF

After immobilization of *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 in PUF, the degradation of pure butachlor and butachlor in herbicide Cantanil 550EC by both bacterial strains was determined. Figure 2 indicated the degrading capacity of *Pseudomonas* sp. But1 on pure butachlor and butachlor in herbicide Cantanil 550EC.

Immobilized *Pseudomonas* sp. But1 strain could degrade pure butachlor about 61.0% at the first cycle, over 79.2% from the following cycles (Figure 2A). Butachlor concentration in Cantanil 550EC was removed by strain But1 is lower than pure butachlor one, which was 33.0% at the first cycle and by 47.0% at the 5th cycle (Figure 2B).

For degradation by immobilized *Pseudomonas* sp. But2, the concentrations of pure butachlor and butachlor in Cantanil 550EC were reduced by 59.0% and 39.0% on average at the end of the first cycle

(Figure 3), respectively. These results were not statistically different from the degradation by free cells. The degradation of pure substrate and butachlor in herbicide Cantanil 550EC was complete at the 4th and 5th cycle, respectively.

Even though the degradation rates at the first cycle by free cells of But1 and But2 were similar, the degradation by But2 was increased at following cycles. The previous report showed that But2 formed biofilm with a higher level than that of But1 (Ha et al., 2020. But2 probably formed biofilm inside PUF, and the bacteria numbers in biofilm increased at following cycles. The higher biofilm formation resulted in higher bacteria numbers, which was probably the cause of a complete degradation by But2 after some cycles (Figure 3).

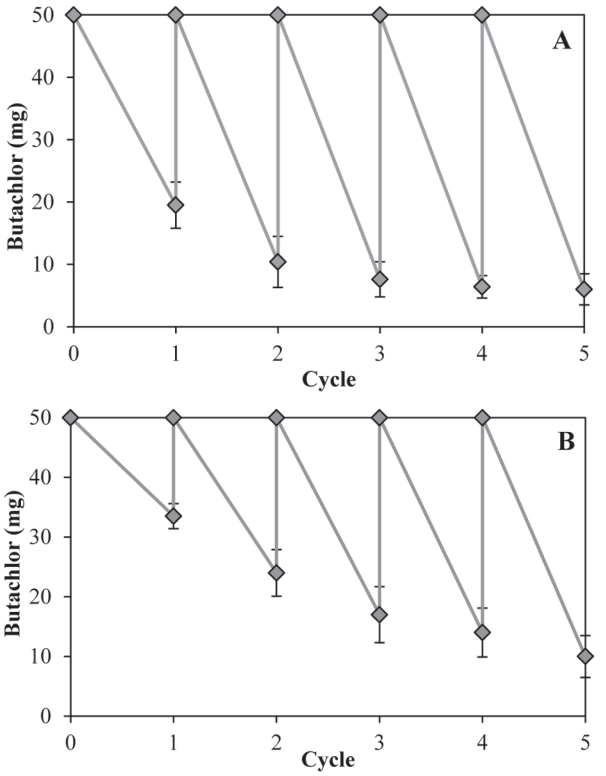

Figure 2. Degradation of pure butachlor (A) and butachlor in herbicide Cantanil 550EC (B) by *Pseudomonas* sp. But1 immobilized in PUF. Each cycle was carried out for 12 hours

The immobilization of bacteria used to degrade herbicides has been reported by our research. For example, alginate used to immobilize *Pseudomonas fluorescens* HH for degrading 2,4-dichlorophenoxyacetic acid (Nguyen et al., 2018, *Pseudomonas fluorescens* KT3 and Bacillus subtilis 2M6E for degrading acetochlor Ha et al., 2020) were reported. The immobilization of *Comamonas testosterone* and *Bacillus subtilis* DKT in PUF for degrading chlorobenzenes and chlorotoluenes was also reported (Ha & Nguyen, 2019). These studies showed that immobilized bacteria enhanced the degradation and cell survival after long-term storage.

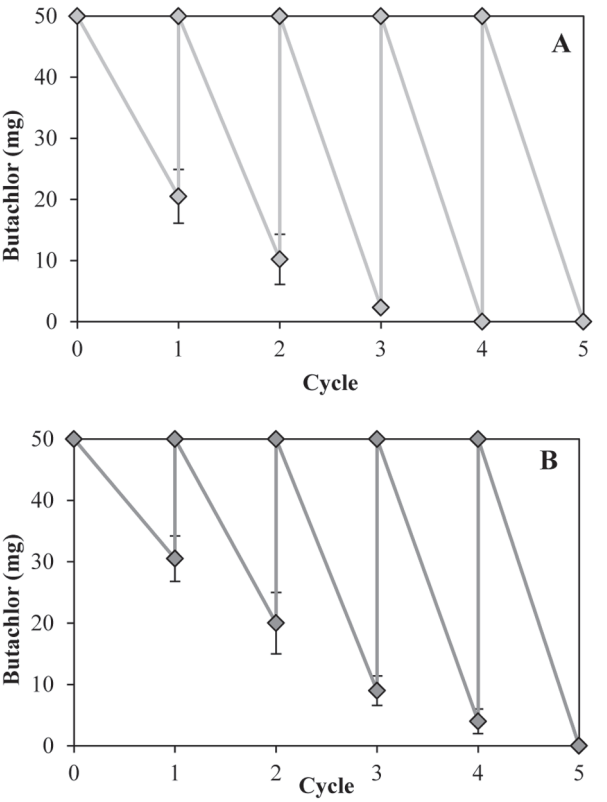

Figure 3. Degradation of pure butachlor (A) and butachlor in herbicide Cantanil 550EC (B) by *Pseudomonas* sp. But2 immobilized in PUF. Each cycle was carried out for 12 hours

3.3. Butachlor degradation after long-term storage

The degradation percentages by free and immobilized bacteria after storing for one and three months were presented in Table 1. The degradation rates were mildly reduced after one month storage, and significantly decreased after three months. However, the degradation by free and immobilized bacteria after long-term storage was incomparable due to different bacteria numbers.

The degradation percentages of pure butachlor by *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2

after one month storage were reduced by 7.1% and 10.1% on average, respectively. The corresponding data for the compound in herbicide Cantanil 550EC were 13.0% and 16.9%. After three months, the degradation rates of pure compound by immobilized cells of *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 were decreased by 24.6% and 29.2% on average, respectively. The corresponding data for the compound in Cantanil 550EC were 32.2% and 33.1% after three months. The decrease in degradation occurred after long-term storage probably because bacteria were dead during the storage time.

For degradation by free bacteria, the degradation percentages of pure butachlor by *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 after one month storage were reduced by 8.2% and 8.3% on average, respectively, compared to the degradation shown in Figure 1. After three months, the degradation of pure compound by immobilized cells of *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 was reduced by 22.8% and 29.1% on average, respectively. Corresponding data for the compound in herbicide Cantanil 550EC was 18.8% and 20.6%.

The decrease in degradation by immobilized bacteria after long time storage have been reported, and higher cell survival in immobilized matrixes than free cells was confirmed (Nguyen et al., 2018; Ha & Nguyen, 2019; Ha et al., 2020). Bacteria immobilized in PUF effectively degraded chlorobenzenes and chlorotoluenes (Ha & Nguyen, 2019). Moreover, the immobilization of bacteria in PUF showed lower adverse effects than those of non-immobilized cells for long-term storage (Ha & Nguyen, 2019). In another report, PUF-immobilized cells were more stable in aniline biodegradation and could be stored for three months at 4°C with little reduction in degradation capacity (Ha Danh Duc and Bui Minh Triet, 2017). However, the differences in reduction of degradation by free and immobilized bacteria after storing were not apparent in this study.

Table 1. Comparison of degrading effectiveness between stored free and immobilized bacteria in pure butachlor and butachlor in herbicide. The degradation was carried out in liquid mineral media for 12 hours

		Degradation (%) by free bacteria		Degradation (%) immobilized bacteria	
Bacteria	Storing time	Pure butachlor	Butachlor in Cantanil 550EC	Pure butachlor	Butachlor in Cantanil 550EC
Pseudomonas sp. But1	One month	61.5 ± 6.1^{Bbc}	37.7 ± 4.6^{Ab}	78.2 ± 6.5^{BCbc}	67.0 ± 7.1^{Bbc}
	Three months	40.6 ± 5.5^{Ba}	21.7 ± 4.6^{Aa}	57.3 ± 6.2^{Ca}	43.1 ± 5.0^{Ba}
Pseudomonas sp. But2	One month	55.5 ± 7.4^{Bb}	33.5 ± 5.2^{Ab}	93.0 ± 3.3^{CDd}	83.1 ± 6.6^{Cd}
	Three months	40.6 ± 5.5^{Ba}	20.6 ± 4.4^{Aa}	71.8 ± 7.7^{CDc}	61.8 ± 5.2^{Cb}

Different superscript letters indicate statistically significant differences ($p < 0.05$) among treatments within a column. Data are means of the results from at least three individual experiments, and mean values and standard deviations are shown.

4. Conclusion

Two bacterial strains including *Pseudomonas* sp. But1 and *Pseudomonas* sp. But2 showed similar degradation rates in degrading butachlor when they were used with free cells. These bacteria could remove pure butachlor approximately 100% and 96% within 24 hours at a concentration of 50 mg/L, respectively as well as eliminate butachlor in an herbicide completely after 30 hours. However, strain But2 immobilized in PUF showed higher efficiency in degrading butachlor than that of But1. The higher biofilm formation of But2 might be the cause of higher degradation. Even though the immobilized bacteria of both bacterial isolates reduced the degradation after long-term storing of 90 days, immobilization is a suitable applying way for degrading butachlor in liquid media.

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