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Degradation of propanil by *Acinetobacter baumannii* DT immobilized in alginate

Danh Duc Ha*, Thi Oanh Nguyen

Dong Thap University

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Abstract:

The herbicide propanil has been widely applied in Vietnam and around the world to control weeds. In this study, *Acinetobacter baumannii* DT isolated from soil was used to determine propanil degradation. Degradation experiments were carried out with condensed cells at 10^9 CFU/ml with both free and immobilized forms of the bacteria. Propanil degradation rates by bacteria immobilized in alginate were higher than those of free cells at the same concentrations. The degradation curve as a function of concentration fit well to the degradation kinetics described by the Edwards model with a maximum degradation of 0.034±0.003 mM/h for free cells and 0.053±0.005 mM/h for immobilized cells. Moreover, the immobilized bacteria could tolerate higher propanil concentrations and degrade propanil in a well-known herbicide more effectively compared to the freely suspended bacteria. These results demonstrate that *A. baumannii* DT immobilized in alginate is suitable for degradation of propanil in herbicides.

Keywords: ***Acinetobacter baumannii* DT, degradation, immobilized cells, kinetics, propanil.**

Classification number: **2.2**

Introduction

The proper use of herbicides in agricultural production saves money, time and labor, especially considering they have become indispensable in today's agricultural sector. Propanil is a contact herbicide [1], which is used globally. This herbicide inhibits the photosynthesis of broadleaf weeds that leads to leaf chlorosis and the subsequent necrosis of leaves and other organs [2]. The herbicide is mainly applied to rice fields, which results in surface water, groundwater, and soil contamination [3-5]. Propanil has been detected at concentrations up to 3.6 mg/l in water [5], while the acceptable level of propanil in drinking water is 0.1 µg/l [6].

Propanil is usually mixed with butachlor to increase the efficiency of weed eradication [7]. The negative effects of butachlor on soil microorganisms have also been studied [8-10]. Therefore, the biodegradation of propanil may be influenced by the presence of butachlor. However, few reports describing the effects of butachlor on the degradation of propanil have been published [11].

Alginate is a natural polymer universally employed to immobilize cells in biodegradation experiments because alginate is relatively mild, chemically inert, inexpensive, and non-toxic [12]. Several bacteria and fungi such as *Fusarium oxysporum* [13], *Paracoccus* sp. FLN-7 [14], *Ochrobactrum* sp. PP-2 [15], and *Spirosoma sordidisoli* TY50^T [16] have been isolated. However, to our knowledge, no publication has yet described the degradation of propanil by microorganisms immobilized in alginate.

In our previous report, propanil degradation by *A. baumannii* DT during the exponential growth phase with low cell density was determined [11, 17]. In this study, propanil degradation by *A. baumannii* DT immobilized in alginate and condensed counterparts is compared.

Materials and methods

Cultivation media

The mineral medium (MM) used in this study was prepared according to Ha Danh Duc (2017) [18]. The MM contained (in grams per liter) 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 of trace mineral solution. The trace mineral solution consisted of (in grams per liter) 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; and $CuCl_2 \cdot 2H_2O$, 0.01. The pH of the MM was adjusted to 7.0±0.1. Ammonium sulfate (0.1%, w/v) and succinate (0.1%, w/v) were supplemented as a

*Corresponding author: Email: hadanhduc@gmail.com

nitrogen and carbon source, respectively. The medium was sterilized for 15 min at 121°C and stored at 4°C until further use. Pure propanil (99.6%) and herbicide with the trade name Cantanil 550EC (Thanhson Agrochem Company, Vietnam) were used. Cantanil 550EC contained 275 mg/l of propanil, 275 mg/l of butachlor, and adjuvant was used to determine the biodegradation of propanil.

Resting cells and immobilization method

To prepare for immobilization, *A. baumannii* DT was cultured in MM amended with ammonium sulfate and succinate medium for 12 h. Bacteria were collected by centrifugation at 10,000 rpm for 5 min. Cell pellets were washed twice with sterile MM. The mixture was then re-suspended in MM used for degradation by free cells (resting cells) while 2×MM was used for immobilization.

The immobilization process was carried out according to a previous report [19] with modifications. The concentrated bacterial solution was mixed with a sterilized solution of Ca-alginate and glycerol to give final cell numbers of approximately 10^9 CFUs/ml, 3% alginate, and 10% glycerol. The solution was carefully blended and dripped into a solution containing 3% $CaCl_2$ (w/v) using a syringe. The beads formed in the solution were stirred for 1 h using a magnetic bar and then stored for 24 h at 4°C in this solution. The beads were collected and washed twice with MM before being used in experiments.

Propanil degradation by resting cells and immobilized cells

The degradation processes of propanil were carried out in MM with 10^9 CFUs/ml of both freely suspended and immobilized bacteria. Propanil was dissolved in absolute ethanol at 0.1 M and used as a stock solution. The degradation was carried out at propanil concentrations ranging from 0.1 to 0.7 mM. The degradation rates at various propanil concentrations were determined and expressed in mM/h. Nonlinear regression analysis was used to fit the trends of the degradation process. The obtained data were best fit by kinetic models that incorporate the equation for logarithmic degradation such as the V.H. Edwards model (1970) [20] given by $V=V_{max}[e^{(-S/Ki)}-e^{(-S/Ki)}]$ or the Haldane equation [21] $V=(V_{max}S)/(K_s+S+S^2/K_i)$ where V is the degradation velocity, V_{max} is the maximum degradation rate, S is the propanil concentration, K_i is the inhibition coefficient, and K_s is the half-saturation coefficient. The kinetic parameters were then calculated based on linear regression fitting of the H. Lineweaver, D. Burk (1934) [22] plot or double reciprocal plot. The Dixon plot was used to determine the inhibition constant in which the reciprocal of the velocity (1/V) was plotted against the inhibitor concentrations [*i*].

For the degradation of propanil in herbicide, Cantanil 550EC was added to 0.1 mM of propanil. The effects of butachlor on propanil degradation were also carried out. Pure butachlor or butachlor in the herbicide Cantanil 550EC were used at the same quantity (weight) as propanil. The incubation processes were conducted at room temperature (from 28 to 31°C) with a shaking speed of 150 rpm.

Long-term storage condition

For long-term storage, resting and entrapped bacteria were stored in Corning™ polyethylene terephthalate centrifuge tubes in the dark at 4°C. After 2 months of storage, both free and immobilized bacteria were kept at room temperature for 2 h before determining cell survival and biodegradation. The results were compared with bacteria numbers and degradation rates of fresh ones.

The number of viable bacterial cells in an alginate bead has been described by M. Schoebitz, et al. (2012) [23] with some modification. The beads (1.0 g) were transferred to 10 ml of sterile sodium citrate (6%, w/v) to dissolve at 30°C on a rotary shaker for 30 min. Then, the solution was serially diluted with MM and spread on an agar plate containing MM supplemented with ammonium sulfate and succinate. For the enumeration of non-immobilized cells, the bacteria solution was serially diluted and also spread on the plates. The number of survival bacteria was determined based on colonies emerging after being incubated for 24 h at 30°C in an incubator.

Analytical method

Propanil concentrations were measured using reverse phase high performance liquid chromatography (HPLC) (LC-10AD, Shimadzu, Japan) with a C18 column (5 µm, 250×4.6 mm; Hyperclone, Phenomenex, USA) at an absorbance of 240 nm. A mixture of acetonitrile and ultrapure water (7:3, v/v) served as a mobile phase at a flow rate of 1 ml/min.

Statistical analysis

The obtained data are shown as the mean ± standard deviation (SD). Duncan's multiple range test in the SPSS program (version 22.0) were used to determine differences among the treatments ($p<0.05$).

Results and discussion

Propanil degradation by A. baumannii DT

A. baumannii DT isolated from soil can degrade propanil and 3,4-dichloroaniline [11]. *A. baumannii* DT was shown to utilize propanil as a sole carbon and nitrogen source in a previous report [11]. However, supplementation with succinate and ammonium sulfate increased the degradation

rates [11]. So, all experiments in this work were carried out with the amendment of these co-substrates.

The degradation by free bacteria and their immobilized counterparts were compared. The results showed that the degradation rates of propanil by immobilized cells were significantly higher than those of freely suspended cells at the same concentrations. At 0.5 mM, the complete degradation of propanil by immobilized bacteria was accomplished after 20 h while propanil degradation by free cells was not complete until after 24 h (Fig. 1). No propanil was lost in the control without alginate beads and bacteria, while the beads absorbed about 12% of the substrate after 24 h.

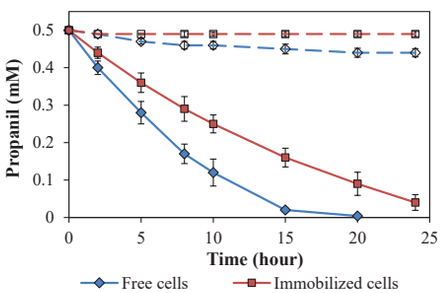

Fig. 1. Propanil degradation by free and immobilized cells (solid lines). The controls (dashed lines) were also run in parallel.

Degradation kinetics of freely suspended and immobilized cells.

The degradation rates at various concentrations showed that propanil degradation by freely suspended resting cells and immobilized cells in alginate beads both followed the Edwards model, in which degradation rates increase at low concentrations but are inhibited at high concentrations (Fig. 2). The degradation rates caused by the resting cells and their immobilized counterparts increased by 0.3 and 0.4 mM, respectively.

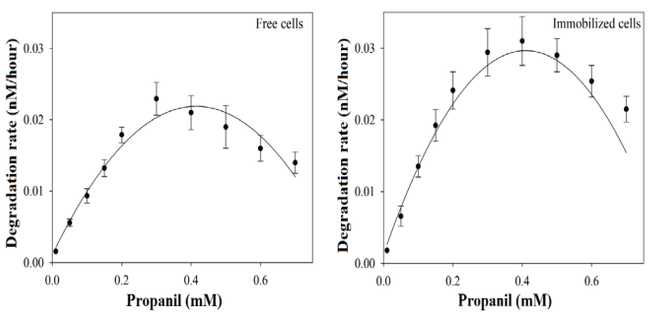

Fig. 2. Relation between propanil concentrations and degradation rates of free resting and immobilized cells.

The parameters of degradation kinetics were extracted from the Edwards equation and are shown in Table 1. The maximum degradation rate of immobilized bacteria was statistically higher than that of the free counterparts. The inhibition coefficient of degradation, which is the concentration required to produce half maximum inhibition, by free cells was also significantly lower compared to the immobilized cells. However, the half-saturation coefficients, which are the reaction rates at half-maximum, of free cells and immobilized bacteria were not statistically different. These results showed that immobilized bacteria were more tolerant to the toxicity of propanil. Similarly, the degradation of toluene and chlorotoluene by *Comamonas testosterone* KT5 immobilized in alginate was higher than that of freely suspended cells [24]. Propanil is a toxic compound, so the substrate inhibition occurred. The enhanced degradation of immobilized cells was because bacteria were protected by alginate matrix. In alginate beads, substrate has to diffuse through the immobilization barrier, and will then be available for the cells to utilize, which reduced the toxicity to bacteria.

Table 1. Degradation parameters of free suspended cells and immobilized cells.

Parameters	Free suspended cells	Immobilized cells
Maximum degradation rate (mM/hour)	0.034±0.003[a]	0.053±0.005[b]
Half-saturation coefficient (mM)	0.249±0.022[a]	0.296±0.030[a]
Inhibition coefficient (mM)	3.50±0.24[a]	4.32±0.41[b]

Data are shown as mean ±SD and different superscript letters (a and b) denote a significant difference ($p<0.05$) between treatments in a line based on Duncan's test, whereas the same letter indicates no significant difference.

In our previous report, V_{max}, K_s and K_i of propanil utilization by freely suspended bacteria in the exponential growth phase were 0.027±0.003 mM/h, 0.16±0.02 mM and 0.33±0.03 mM, respectively [11]. In this study, the V_{max} and K_s of concentrated bacteria were comparable higher than those of low bacteria density as described in the previous report [11]. The degradation and growth rates of bacteria in the exponential growth phase showed the activities of *A. baumannii* DT to propanil, while the degradation by concentrated bacteria provided a potential application to remediate the herbicide. Propanil detected in contaminated sites by E.G. Primel, et al. (2007) [5], of course, was mostly lower concentrations in this study. *A. baumannii* DT was investigated for degradation of various propanil concentrations, which showed the application ability to remediate in contaminated sites.

Effects of butachlor on degradation of pure propanil and degradation of propanil in herbicide Cantanil 550EC

Figure 3 shows that the percentage of propanil degradation for immobilized bacteria were significantly higher compared to those for free cells under the same conditions. The addition of pure butachlor and Cantanil 550EC decreased

the degradation rates. Similarly, the effects of butachlor on propanil degradation and growth of *A. baumannii* DT have been described in previous studies [11, 17]. A number of trace herbicides contain both butachlor and propanil, but both substrates inhibited each other in biodegradation [17]. The degradation was the lowest with the amendment of the herbicide Cantanil 550EC. These results can be explained by the negative effects from the adjuvants in the herbicide on the degradation propanil, which was also described by H.D. Duc, et al. (2020) [17].

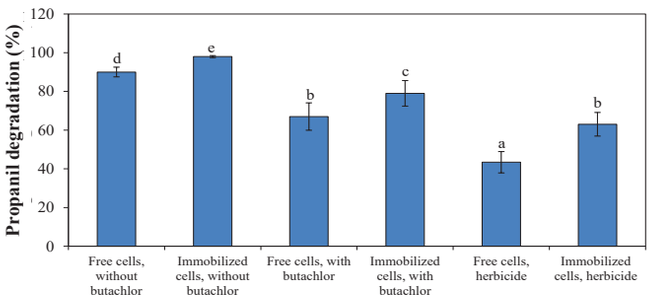

Fig. 3. Effects of butachlor on degradation of pure propanil and degradation of propanil in herbicide Cantanil 550EC. The degradation processes were carried out for 12 h at 0.1 mM propanil. Different letters (a, b, c, d and e) above the columns denote a significant difference ($p<0.05$) among treatments.

Bacteria survival and propanil degradation for long-term storage

The number of live bacteria decreased after two months of storage. The survival percentages of bacteria amended with glycerol were higher than those of treatments without the cryoprotectant (Fig. 4). These results indicated that using glycerol as a cryoprotectant agent reduced the adverse effects of the bacteria. The survival of free and entrapped bacteria was not statistically different under the same conditions.

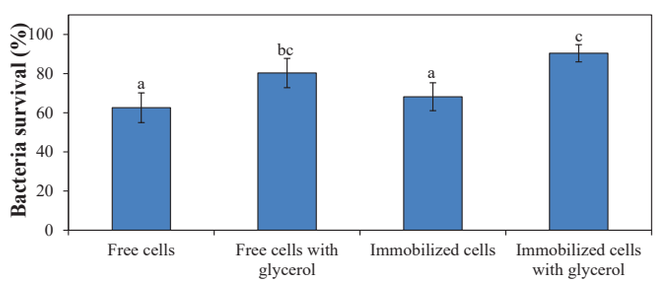

Fig. 4. Survival of *A. baumannii* DT after two months' storage at 4°C. Different letters (a, b, and c) above the columns denote a significant difference ($p<0.05$) among treatments.

Table 2 shows that the degradation by immobilized bacteria was significantly higher than that of the resting cells, which was similar to the experiments described above. The addition of glycerol reduced propanil degradation from about 8.2 to 15.6% at the beginning. However, the presence of glycerol resulted in a non-statistically significant reduction of degradation after two months of storage. The degradation rates of the treatments with glycerol decreased from only 6.1 to 12%, while data of treatments without glycerol were reduced from 27.9 to 36.4%.

Table 2. Propanil degradation by free cells and immobilized cells. The degradation processes were carried out for 10 h at 0.1 mM propanil.

		Propanil degradation (%)			
		Free suspended cells		*Immobilized cells*	
		At the beginning	*After two months*	*At the beginning*	*After two months*
Pure propanil	Without glycerol	92.1 ± 4.2^{Dc}	55.5 ± 8.1^{Ba}	98.4 ± 1.5^{Cc}	68.4 ± 7.9^{Bb}
	With glycerol	80.6 ± 6.3^{Cab}	72.8 ± 7.0^{Ca}	90.2 ± 4.2^{Cb}	78.2 ± 7.3^{Bab}
Herbicide	Without glycerol	43.4 ± 5.5^{Bc}	15.5 ± 4.3^{Aa}	63.4 ± 6.1^{Bd}	30.4 ± 5.2^{Ab}
	With glycerol	30.2 ± 5.6^{Aa}	22.6 ± 5.6^{Aa}	47.8 ± 6.5^{Ab}	41.7 ± 6.2^{Ab}

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

The results show that the addition of glycerol reduced the adverse effects of bacteria. Previous reports also presented that the survival of entrapped microorganisms is enhanced by the addition of glycerol [25, 26]. Glycerol is used as a cryoprotectant that can prevent ice crystal formation after penetration into the cells [27]. Another report shows that the addition of glycerol protects the microorganisms, increases pore size in beads, and controls the structure of dried macrocapsules [26].

Conclusions

A. baumannii DT immobilized in alginate showed more effective propanil degradation than free cells. It was confirmed that the chemical degradation over a wide range of concentrations by condensed free cells and immobilized cells followed the Edwards model with higher degradation rates and inhibition coefficients for immobilized bacteria. Besides, immobilized bacteria degraded propanil in an herbicide with higher rates than its free counterparts. Moreover, bacteria entrapped in alginate beads amended with glycerol reduced the adverse effects of long-term storage. These results indicate that *A. baumannii* DT immobilized in alginate can be applied to remediate propanil.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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