ACETOCHLOR DEGRADATION BY A MIXED CULTURE OF *P. fluorescens* KT3 AND *B. subtilis* 2M6E IMMOBILIZED IN ALGINATE

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Abstract

In this study, the acetochlor degradation by two microbial isolates, P. fluorescens KT3 and B. subtilis 2M6E, was determined. The immobilization of the bacterial mixture in alginate beads resulted in higher degradation rates compared to their free cells. The addition of glycerol as a cryoprotectant reducing adverse effects in long-term storage. After storing at 4°C for three months, the cell survivals of free cell with and without the cryoprotectant were 43.0 ± 6.1% and 57.3 ± 5.9%, while data for immobilized bacteria were 64.0 ± 5.3% and 77.6 ± 4.0%, respectively. These results prove that the immobilization of bacteria in alginate and the addition of glycerol can be applied for storing bacteria in a long-term period.

Keywords: *Acetochlor, degradation, P. fluorescens KT3, B. subtilis 2M6E, immobilization.*

PHÂN HỦY ACETOCHLOR BỞI HỖN HỢP VI KHUẨN *P. fluorescens* KT3 VÀ *B. subtilis* 2M6E ĐƯỢC CỐ ĐỊNH TRONG ALGINATE

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Tóm tắt

Trong nghiên cứu này, sự phân hủy acetochlor của hai chủng vi khuẩn P. fluorescens KT3 và B. subtilis 2M6E được khảo sát. Sự cố định hỗn hợp vi khuẩn này trong hạt alginate giúp tăng cường tốc độ phân hủy acetochlor của chúng. Việc bổ sung glycerol như một chất phụ gia làm giảm ảnh hưởng bất lợi của vi khuẩn trong thời gian dài lưu trữ. Sau ba tháng lưu trữ ở 4°C, tỷ lệ sống sót của vi khuẩn không cố định và không có chất phụ gia là 43,0 ± 6,1%, còn không cố định nhưng có chất phụ gia là 57,3 ± 5,9%, trong khi tỷ lệ này đối với vi khuẩn được cố định tương ứng là 64,0 ± 5,3% và 77,6 ± 4,0%. Những kết quả này chứng tỏ việc cố định vi khuẩn trong alginate và bổ sung glycerol có thể được ứng dụng để lưu trữ vi khuẩn trong một thời gian dài.

Từ khóa: *Acetochlor, phân hủy, P. fluorescens KT3, B. subtilis 2M6E, cố định.*

1. Introduction

Acetochlor is one of the herbicides frequently used for controlling annual grasses and broad-leaved weeds. The chemical is relatively high water-solubility but low soil sorption (Lengye and Földényi, 2003). Thus, it is easy to transfer to other media after initial application. The wide use of acetochlor resulted in seriously environmental pollution, especially in aquatic bodies. For example, both the parent and the degraded acetochlor metabolites have been detected in surface and groundwater (de Guzman *et al.*, 2005).

The water pollution by pesticides is a seriously concerned problem because pesticides pose immediate and long-term risks for ecosystems and also for humans. Acetochlor and some other chloroacetanilides are now considered to be endocrine disruptors, and they have been classified as carcinogenic effect classified by the U.S. Environmental Protection Agency (EPA) in 1994, and caused other human problems (Garcia, 2003), and highly toxic to freshwater algae (Junghans *et al.*, 2003). The presence of acetochlor and other chloroacetanilide herbicides in natural waters may represent a risk for the aquatic biota.

Acetochlor is slowly dissimilated from the natural environment. It is found that only 33% of acetochlor in soil was degraded after one month application with 10 mg of acetochlor/kg soil (Jablonkai, 2000). Even though acetochlor can be degraded by chemical and physical methods, the herbicide is principally dissipated by biodegradation process (Souissi *et al.*, 2013). Some acetochlor-degrading bacteria were isolated such as *Catellibacterium caeni* (Zheng *et al.*, 2012), *Pseudomonas oleovorans* LCa2 (Xu *et al.*, 2013), and *Pseudomonas aeruginosa* DJ115 (Luo *et al.*, 2015). However, most publications showed the degradation by freely suspended bacteria, and no report on cell immobilization in alginate matrix for acetochlor degradation has been published.

The immobilized cells have shown advantages in biodegradation rather than free suspended ones. Alginate is a natural and cheap material, and non-toxic to bacteria, which is preferred being used for immobilizing bacteria. Moreover, freeze-dried immobilization of bacteria is conveniently stored and transported, so a frozen-dried formulation should be developed. In this study, the mixed culture of *P. fluorescens* KT3 and *B. subtilis* 2M6E showing effective degradability towards acetochlor Duc and Oanh (2019) was investigated for its degradation after a long-term storage at different conditions.

2. Materials and methods

The mineral medium (MM medium) was used for chemical degradation with the components of 1.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.5 g/L NaCl, 0.2 g/L $MgSO_4$, 0.5 g/L $CaSO_4$, 1.0 g/L ammonium sulfate, 1.0 g/L succinate, and 1.0 mL of trace elements solution (39.9 mg $MnSO_4 \cdot H_2O$, 42.8 mg $ZnSO_4 \cdot H_2O$, 3.8 mg $CuSO_4 \cdot 5H_2O$, 11.6 mg H_3BO_4, and 27.8 mg $FeSO_4 \cdot 7H_2O$ per litter). The pH was adjusted to 7.0 ± 0.1 using HCl (12%) and NaOH solution (5.0 M). Solid medium was obtained by adding 2% (w/v) agar. All media were sterilized at 121°C for 15 min. All chemicals were purchased from Sigma-Aldrich (Singapore) or Merck (Germany).

2.1. Immobilization method

For the preparation for immobilizing, each bacterial strain was cultured in MM medium for 12 h. Bacteria were collected by centrifugation at 8,000 rpm for 15 min. Cell pellets of each strain were washed twice with the sterile MM medium and mixed together. The cell pellets were used for immobilization, degradation and storage. The mixture was then re-suspended in 2×MM medium. The immobilization process was carried out according to the previous report (Bai *et al.*, 2010) with modifications. The concentrated bacterial solution was mixed with the sterilized solution of alginate and glycerol to give final cell numbers of approximately 10^9 CFUs/mL,

3% alginate, and 10% glycerol. Other beads without glycerol were also used for acetochlor degradation. The cell numbers of each strain were the same (0.5×10^9 CFUs/mL). The solution was blended carefully and dripped into a solution containing 3% $CaCl_2$ (w/v) using a syringe. The beads formed in the solution were stirred for one hour using a magnetic bar, and then stored for 24 hours at 4°C in this solution. The beads were collected and washed twice with the MM medium before being used in experiments.

2.2. Acetochlor degradation by freely suspended and immobilized bacteria

The acetochlor degradation performances by freely suspended and immobilized bacteria were carried out in the MM medium with 10^9 CFUs/mL. Acetochlor was added at 150 mg/L. The incubation processes were conducted at room temperature (from 28.0 to 31.0°C) with a shaking speed of 150 rpm for 24 hours.

2.3. Viable cell enumeration of free-suspended and entrapped cells

For enumeration of non-immobilized cells, the solution was serially diluted and spread on the MM agar plates. The number of bacteria was determined based on colonies emerging after being incubated for 24 hours at 30°C.

For the immobilized bacteria, the number of viable bacterial cells in an alginate bead was determined as described by Schoebitz *et al.* (2012) with some modification. 1.0 g of alginate beads was transferred to 10 mL of sterile sodium citrate (6%, w/v). The beads were dissolved at 30°C on a rotary shaker for 30 min. Then, the solution was serially diluted with sterile sodium citrate and spread on MM agar plate. The number of bacteria in a bead was determined based on colonies emerging on the agar plate.

Each bacterial strain in the mixture was identified based on their characteristics of morphological colonies forming on agar plates. *P. fluorescens* KT3 formed circular colonies, while *B. subtilis* 2M6E formed irregular colonies on agar plates (Duc and Oanh, 2019).

2.4. Long-term storage condition

For a long-term storage of entrapped bacteria, the beads were stored in a polyethylene bag for one and three months in the dark at the room temperature and 4°C. The non-immobilized bacteria were also stored in plastic tubes in the same condition. After the storage time, the acetochlor degradation and bacteria survival of immobilized and non-immobilized bacteria were determined.

2.5. Bacteria

Two bacterial strains *P. fluorescens* KT3 (MG966445.1) and *B. subtilis* 2M6E (MG966466.1) isolated from soil were used in this study (Duc and Oanh, 2019). *P. fluorescens* KT3 transformed acetochlor to 2-methyl-6-ethylaniline. *B. subtilis* 2M6E could not degrade acetochlor, but it degraded 2-methyl-6-ethylaniline at a high rate. Two strains co-operated in acetochlor degradation resulted in a higher degradation rate of acetochlor compared to the rates of each individual isolate (Duc and Oanh, 2019).

2.6. Statistical analysis

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

3. Results and discussion

3.1. Acetochlor degradation by freely suspended bacteria and bacteria immobilized in fresh beads

After immobilizing in the alginate beads (Figure 1), the degradation of acetochlor by immobilized bacteria was compared to those of freely suspended ones. As seen from Figure 2, the acetochlor degradation rate of bacteria immobilized in the alginate beads without glycerol was highest, while the degradation by freely suspended cells in the medium with glycerol was slow. The degradation rates of the

immobilized bacteria were higher than those of freely suspended counterparts in all experiments, which was probably because the alginate layer protected bacteria from toxicity of the herbicide.

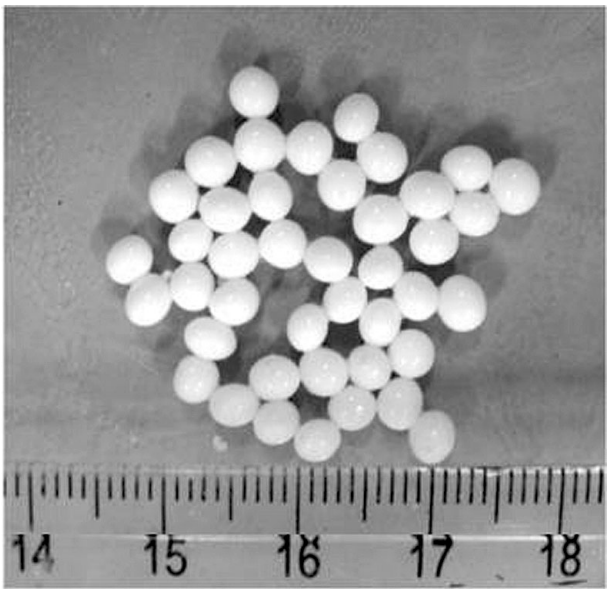

Figure 1. Alginate beads with bacteria used in acetochlor degradation

The supplementation with glycerol in the medium reduced the degradation rates. With the presence of glycerol in the medium, bacteria might use this substrate as a nutrient source instead of acetochlor, resulting in lower degradation rates. The abiotic control without beads showed no degradation, while about nearly 20% acetochlor was reduced in the medium with the abiotic beads (Figure 2). The results indicated that a small amount of acetochlor was absorbed into the beads.

3.2. Acetochlor degradation by the mixture of *P. fluorescens* KT3 and *B. subtilis* 2M6E after one month storage

After one month storage, the acetochlor degradation rates by free cells and immobilized cells, with and without glycerol, were compared. The degradation rates of most treatments were decreased after one month. The degradation performance and cell survival of freely suspended bacteria without glycerol was most reduced, while these reductions did not statistically occur for bacteria immobilized in alginate with glycerol (Table 1). For example, the degradation by free bacteria without glycerol decreased by 67%, and by free bacteria with glycerol reduced by 49% when they were stored at room temperature.

The reduction of the bacteria survival and biodegradation after one month storage at 4°C also occurred. However, the adverse effects were significantly lower at this temperature compared to the storage at room temperature in most treatments. The low activities of bacteria at the low temperature resulted in the reduction of adverse effects. Moreover, the alginate layer protects bacteria from environmental stresses. Immobilized bacteria could survive after the storage time better than free cells.

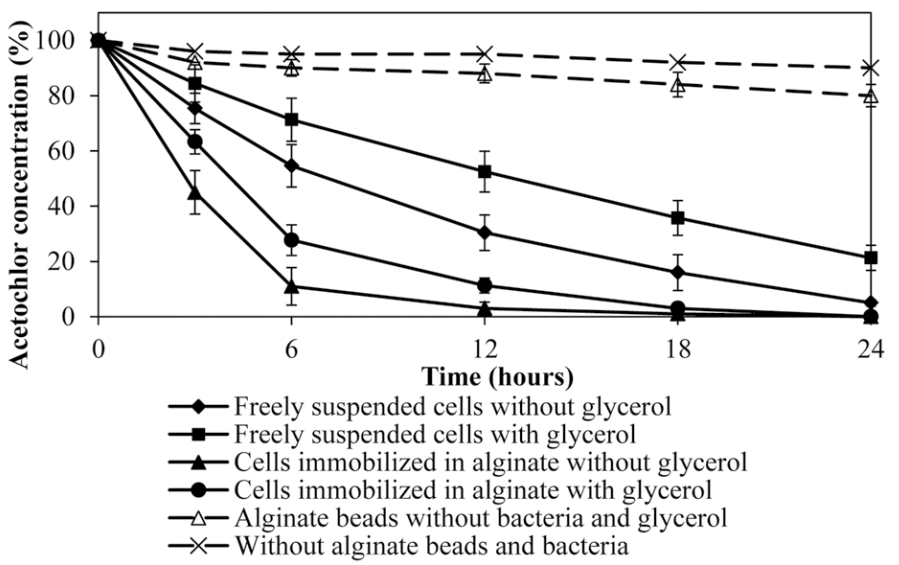

Figure 2. Acetochlor degradation by the mixed culture of *P. fluorescens* KT3 and *B. subtilis* 2M6E. The degradation processes were conducted by freely suspended and immobilized *P. fluorescens* KT and *B. subtilis* 2M6E

Table 1. Acetochlor degradation by the mixture of *P. fluorescens* KT3 and *B. subtilis* 2M6E after one month storage. The degradation processes were carried out for 24 hours

Storage of bacteria	Acetochlor degradation (%)(*)	Bacteria survival (%)(*)	
		P. fluorescens KT	*B. subtilis* 2M6E
Bacteria stored at room temperature			
Free bacteria without glycerol	33.3 ± 6.1^{a}	35.7 ± 4.9^{a}	48.0 ± 7.5^{a}
Free bacteria with glycerol	50.3 ± 6.7^{b}	55.7 ± 6.7^{b}	63.7 ± 5.5^{b}
Bacteria immobilized in alginate without glycerol	54.0 ± 5.3^{b}	54.0 ± 8.2^{b}	64.0 ± 6.9^{b}
Bacteria immobilized in alginate with glycerol	67.3 ± 4.9^{cd}	69.7 ± 5.5^{c}	79.3 ± 5.9^{c}
Bacteria stored at 4^{o}C			
Free bacteria without glycerol	58.7 ± 7.2^{bc}	58.0 ± 7.5^{b}	67.3 ± 4.6^{b}
Free bacteria with glycerol	64.7 ± 8.1^{cd}	64.0 ± 5.3^{c}	70.3 ± 7.8^{bc}
Bacteria immobilized in alginate without glycerol	71.7 ± 11.1^{d}	75.3 ± 7.6^{cd}	81.7 ± 8.1^{c}
Bacteria immobilized in alginate with glycerol	88.3 ± 5.5^{e}	86.7 ± 6.4^{d}	95.3 ± 2.5^{d}

Note: (*)Data are shown as means ± SD, in which different superscript letters (a, b, c, d and e) denote a significant difference ($p < 0.05$) among treatments in a column based on Duncan's test, whereas the same letter indicates no significant difference

3.3. Acetochlor degradation by the mixture of *P. fluorescens* KT3 and *B. subtilis* 2M6E after three month storage

Table 2. Acetochlor degradation by the mixture of *P. fluorescens* KT3 and *B. subtilis* 2M6E after three months storage. The degradation processes were carried out for 24 hours

Storage of bacteria	Acetochlor degradation (%)(*)	Bacteria survival (%)(*)	
		P. fluorescens KT	*B. subtilis* 2M6E
Bacteria stored at room temperature			
Free bacteria without glycerol	19.7 ± 3.2^{a}	17.7 ± 3.8^{a}	30.7 ± 4.0^{a}
Free bacteria with glycerol	31.3 ± 4.7^{b}	33.0 ± 5.6^{b}	44.7 ± 4.2^{b}
Bacteria immobilized in alginate with glycerol	30.6 ± 3.1^{b}	31.0 ± 3.6^{b}	42.7 ± 6.4^{b}
Bacteria immobilized in alginate without glycerol	41.7 ± 4.7^{c}	42.0 ± 5.3^{c}	59.3 ± 5.1^{c}
Bacteria stored at 4^{o}C			
Free bacteria without glycerol	43.0 ± 6.1^{c}	52.3 ± 5.9^{d}	60.7 ± 6.7^{c}
Free bacteria with glycerol	57.3 ± 5.9^{d}	64.0 ± 5.3^{e}	74.7 ± 4.7^{d}
Bacteria immobilized in alginate with glycerol	64.0 ± 5.3^{d}	63.7 ± 6.4^{e}	74.3 ± 5.9^{d}
Bacteria immobilized in alginate without glycerol	77.6 ± 4.0^{e}	78.3 ± 5.1^{f}	87.3 ± 4.2^{e}

Note: (*)Data are shown as means ± SD, in which different superscript letters (a, b, c, d and e) denote a significant difference ($p < 0.05$) among treatments in a column based on Duncan's test, whereas the same letter indicates no significant difference.

A number of alive bacteria were reduced after three months, especially free bacteria and immobilized cells without glycerol. The degradation percentages of bacteria were reduced from 21.4 to 75.3% compared to the fresh ones, and from 9.2 to 23.3% compared to those after one month-storage. The cell numbers in alginate beads also reduced, but significantly lower compared to free cells in the same storage conditions. The storage at 4oC reduced the death rate of bacteria. The low survival of bacteria in some treatments resulted in the reduction of degradation. The survival of *B. subtilis* 2M6E was better than *P. fluorescens* KT3 in all treatments (Table 2).

The addition of glycerol reduced the adverse effects of bacteria. Previous reports showed that the survival of entrapped microorganisms was enhanced with the addition of glycerol (Kearney *et al.*, 1990; Zohar-Perez *et al.*, 2002) . Glycerol is used as a cryoprotectant which could prevent ice-crystal formation after penetration into the cells (Madigan and Martinko, 1997). Another report showed that the addition of glycerol protected the microorganism, increased pore size in beads, and controls the structure of the dried macrocapsules (Zohar-Perez *et al.*, 2002).

4. Conclusion

P. fluorescens KT3 and *B. subtili*s 2M6E which were immobilized in alginate beads increased acetochlor degradation. Moreover, the addition of glycerol as the cryoprotectant reduced adverse effects in a long-term storage. The cell survival was increased, and degradation rates were reduced when bacteria were immobilized in alginate beads. These results indicate that the immobilization with the supplementation with glycerol in the alginate matrix can be applied in biodegradation and storing bacteria for a long time.

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