ISOLATION AND IDENTIFICATION OF CHLORPYRIFOS-DEGRADING BACTERIA FROM RICE-UPLAND CROP SOIL IN THE MEKONG DELTA

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ABSTRACT

The aim of this study is to isolate the bacterial strains which degrade Chlorpyrifos from rice-upland crop soils in the Mekong Delta. Twelve soil samples were taken at Cai Lay - Tien Giang, Cho Moi - An Giang and Binh Tan-Vinh Long. Soil bacteria were enriched in mineral salt medium solution containing 20 mg/L of Chlorpyrifos as the only carbon source for bacterial growth. The results showed that one bacterial community was enriched and degraded Chlorpyrifos. One bacterial strain (coded as BT_C8.9) that was isolated from this community degraded 41.07 % of Chlorpyrifos after 30 culture days. According to the sequencing of 16S rRNA gene, this bacterial strains was identified as Microbacterium sp. C8.9.

***Keyword**: Bacteria, Chlorpyrifos, degradation, isolate*

1. INTRODUCTION

Application of the pesticide on agricultural crop is now a common practice and is an important factor of integrated pest management (IPM) strategies. It adversely affects the properties of the soil as well as it alters the pH of the soil required for microbial activities of beneficial bacteria to act upon [1], [2]. Some of these pesticides persist in the soil to form pollutants which may occasionally lead to surface and groundwater contamination. One of such pesticides is Chlorpyrifos. It is a broad-spectrum organophosphate insecticide, which is widely used to control insect pests on grain, cotton, fruit, nut, and vegetable crops, as well as lawns and ornamental plants in Viet Nam. The environmental fate of Chlorpyrifos has been studied extensively, and the reported half-life in soil varies from 10 to 120 days, with 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product [3]. The manufacture and formulation process of Chlorpyrifos also generate waste that contains the compound, and this has to be treated by physicochemical or biological means [4]. If Chlorpyrifos is not degraded or detoxified rapidly enough, the risk of their off-site migration may pose a health risk to human [ref]. In soil, microorganisms play a very important role to promote the breakdown of Chlorpyrifos. For example, *Enterobacter strain* B-14 could degrade Chlorpyrifos [5]. *Alkaligenes faecalis* DSP3 was isolated, which was capable of degrading Chlorpyrifos and 3, 5, 6-trichloro-2-pyridinol (TCP) [6]. Six Chlorpyrifos-degrading bacteria were isolated using Chlorpyrifos as the sole carbon source by an enrichment procedure [7]. The main objective of the present study involved the isolation and identification of Chlorpyrifos-degrading bacteria from rice-upland crop soils in the Mekong Delta.

2. MATERIALS AND METHODS

2.1 Materials

Soil samples: 12 soil samples were collected from 12 fields in the Mekong Delta. Among them, 8 soil samples were collected from 8 fields in Vinh Long Province, 3 soil samples were collected from 3 fields in An Giang Province, 1 soil samples were collected from 1 fields in Tien Giang Province. Phosphate buffer used contain the following (in gram per liter): 23.99 g of NaH_2PO_4 and 15.59 g of Na_2HPO_4. Mineral salt midium: 870 mL of Q-water, 25 mL of buffer solution (35 g of $Na_2HPO_4.2H_2O$, 4 g of KH_2PO_4 were mixed in 1 L of milli-Q water), 100 mL of mineral salt solution (10 g of $(NH_4)_2SO_4$, 2 g of $MgCl_2.6H_2O$, 1 g of $Ca(NO_3)_2.4H_2O$ were mixed in 1 L of milli-Q water) and 5 mL of trace metal solution. Tryptic Soybean Agar (TSA): 30 g Tryptone Soya Broth and 15 g of agar were mixed in 1 L of milli-Q water. Chlorpyrifos (Dr.Ehrenstorfer, 99.5%) of Accustandard.

2.2 Methods

2.2.1 Collection of soil

Collection of soil: 12 soil samples from the fields was collected as described aboved . Soil was collected randomly from 0 - 20 cm depth. The samples were pooled together, brought to the laboratory in polyethylene bags and kept in refrigerator at 4 ^oC to maintain the biological activity of the soil microbes..

2.2.2 Isolation of Chlorpyrifos-degrading Bacteria

Chlorpyrifos-degrading bacteria were isolated from the soil samples by the enrichment culture technique on mineral salt medium, using Chlorpyrifos as the sole source of carbon [8]. The enrichment preparation comprised of 90 mL phosphate buffer in 250 mL Erlenmeyer's flask. They were autoclaved at 121^oC for 20 minutes before adding 10 g of the soil sample. The flasks were incubated in a rotary shaker at 130 revolutions per minute (rpm) and 30 ^oC for 1 hour. After shaking, 1 mL of the supernatant was inoculated in 24 mL of mineral salt mideum containing 20 mg/L of Chlopyrifos, and the flasks were incubated in a rotary shaker at 90 revolutions per minute (rpm) and 30 ^oC for 10 days.. This was repeated from 4 to 5 times. After that, the experiment was set up in tube containing 4 mL of mineral salt minimum and 20 mg/L of Chlorpyrifos to select bacterial community which could degrade Chlorpyrifos.

Using bacterial communities could degrade Chlorpyrifos to isolate bacteria strain. 50 µL of the bacterial commnity was inoculated on TSA, and the incubated at 30 ^oC for 3 days. Isolated Colonies were inoculated on tube containing 4 mL of mineral salt minimum and 20 mg/L of Chlorpyrifos. After 7 days, colony could grow which selected to set up in tube containing 4 mL of mineral salt minimum and 20 mg/L of Chlorpyrifos to select bacterial strain which could degrade Chlorpyrifos.

2.2.3 Chlorpyrifos Extraction and HPLC Analysis

After 30 days of incubation, 4 mL of the culture was taken from each flask and was placed in centrifuge tubes. Extraction of Chlorpyrifos was done by using a solution of Toluen (distilled) and Acetone (distilled) at a ratio of 2:1 and centrifuged at 3,000 rpm for 3 minutes. Concentration of Chlorpyrifos was determined on HPLC (High Performance

Liquid Chromatography) at 17.5 mins (Figure 1) . The C18 column (25 cm x 4.6 mm, 5 □m) was used as the stationay phase. The mobile phase is the mixture of methanol: water at ratio 80:20. The flow rate was set at 1 ml/min and the UV detector was set at 20 nm.

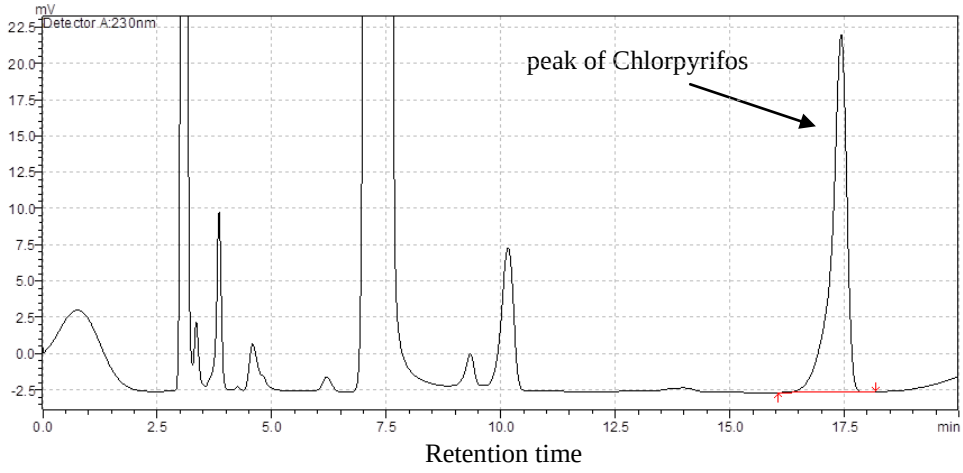


Figure 1: *Chromatogram of Chlorpyrifos*

2.2.4 Identification of the Isolates

The one bacterial strain isolate was grown on TSA to extract DNA by CTAB 3 % [9]. After that, DNA product was amplified by PCR with 27F/1492R primers [10]. The forward primer is, 5' AGA GTT TGA TCC TGG CTC AG 3' and the reverse primer id 5' TACGGT TACCTTGTTACGACT 3'. Pre denaturation 95°C (5 minutes), 30 cycles: Denaturation (95°C, 1 minute), Annealing (53°C, 30 seconds), Extension (72°C, 90 seconds), Final extension (72°C, 5 minutes) [10] and then use the sequence in a BLAST search limited to a bacterial data base. Identify their unknown bacteria by examining the top-scoring sequences from the BLAST search results.

2.2.5 Methods of processing, analysis and statistics data

Microsoft Excel (version) was used to calculate the percentage of decomposition and graphed. Minitab 16 software was used to compare the differences between pairs of data together.

3. RESULTS AND DISCUSSION

3.1. Enriched culture and identify Chlorpyrifos degradability of the bacterial communities

The results of this study selected one bacterial community which degraded Chlorpyrifos. After 30 days incubation, this bacterial community degraded 18.85 % of Chlorpyrifos (Figure 2).

Thus, bacterial community (was coded as 8) was selected from rice - upland crop soil in Vinh Long province. It could degrade Chlorpyrifos. So this community was used to isolate bacteria strains.

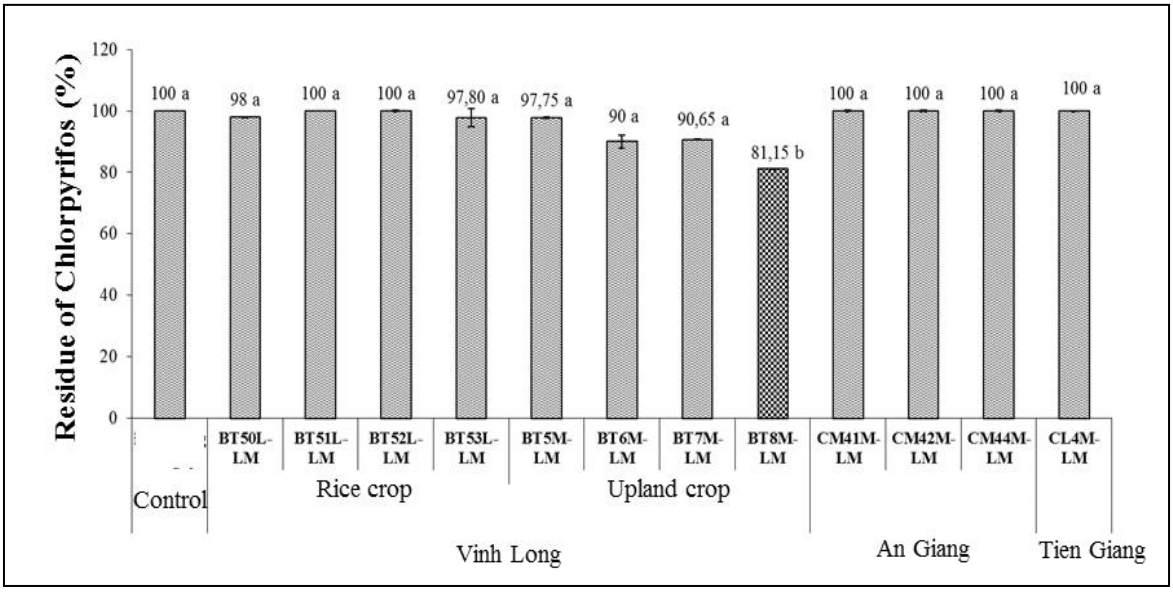


Figure 2. *Residue Chlorpyrifos after 30 days incubation with bacterial communities (n = 3, standard error)*

3.2. Identification of the Isolates

Bacterial strain was isolated from one bacterial community which was coded as 8. After 30 days incubation, this bacterial strain could degrade 41.07 % of Chlorpyrifos (Figure 3). Colony of this bacteria is circular, convex, entire, moderate and yellow (Figure 4a). This bacterial strain is Gram possitive (Figure 4b). It was sequenced and compared 16S rRNA gene sequence segments with the NCBI database. The results showed that the sequence of this bacterial strain sequences homologous about 98 % with 16S rRNA gene segments of *Microbacterium* sp. (Accession number: JQKT01000007.1). So, It was identified *Microbacterium* sp. C8.9.

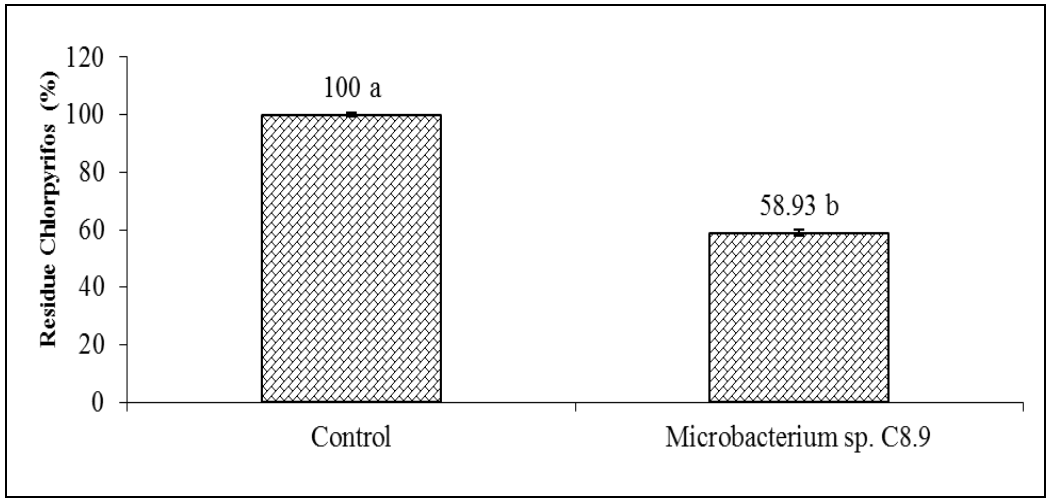
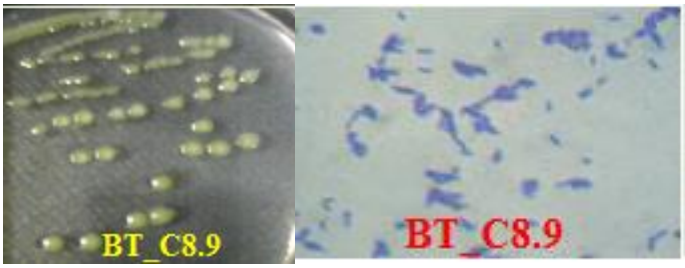


Figure 3. *Residue Chlorpyrifos after 30 days incubation with Microbacterium sp. C8.9 (n = 4, standard error)*



(a) *(b)*

Figure 4. *(a) Colony morphology ; (b) Gram staining of Microbacterium sp. C8.9*

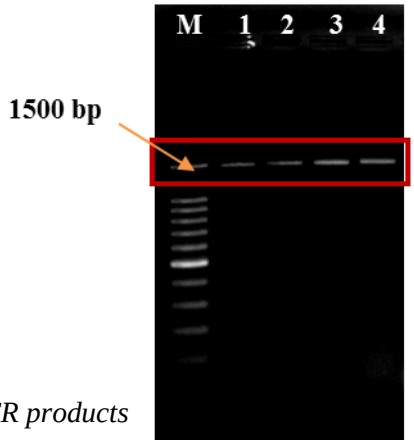


Figure 5. *Agarose gel electrophoresis of PCR products*

Notes: Lane M: 1kb DNA leadder; Lane 3: PCR products of *Microbacterium* sp. C8.9

Sequence nucleotide of *Microbacterium* sp. C8.9

2 GGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTA 61
62 GCGACTCCGACTTCATGAGGTCGAGTTGCAGACCTCAATCCGAACTGGGACCGGCTTTTT 121
122 GGGATTCGCTCCACCTCACGGTATTGCAGCCCTTTGTACCGGCCATTGTAGCATGCGTGA 181
182 AGCCCAAGACATAAGGGGCATGATGATTTGACGTCATCCCCACCTT-CCTCCGAGTTGAC 240
241 CCCGGCAGTATCCCATGAGTTCCCACCATTACGTGCTGGCAACATAGAACGAGGGTTGCG 300
301 CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC 360
361 TGTATACGAGTGTCCAAAGAGTTCTACATTTCTGCAGCGTTCTCGTATATGTCAAGCCTT 420
421 GGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGTCCC 480
481 CGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGAACTTAATGCG 540
541 TTAGCTGCGTCACGGAAACCGTGGAATGGTCCCCACAACTAGTTCCCAACGTTTACGGGG 600
601 TGGACTACCAGGGTATCTAAGCCTGTTTGCTCCCCACCCTTTCGCTCCTCAGCGTCAGTT 660
661 ACGGCCCAGAGATCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTGCGCATTCCACCGC 720
721 TACACCAGGAATTCCAATCTCCCCTACCGCACTCTAGTCTGCCCGTACCCACTGCAGGCT 780
781 GGAGGTTGAGCCTCCCGTTTTCACAGCAGACGCGACAAACCGCCTACAAGCTCTTTACGC 840
841 CCAATAATTCCGGATAACGCTTGCGCCCTACGTATTACCACGACTGCAGGCACGTAGTTC 900
901 ACCCGGCGCTTTTTCTGCAAGTACCGTCACTTTCGCTTCTTCCTTGCTAAAA-GAGGTAT 959
960 ACAACCCAGA-GGGCCGTCATCCCTCACGCGGGCGTGGCTGCATCAAG-CATGTAGCCCA 1017
1018 TTGAGG-AGTAGTCACCCACTGCTAACCTCCCGTATGAAATCTAGGACCG 1066

Thus, *Microbacterium* sp. C8.9 had Chlorpyrifos-degrading higher than its bacterial community. This result was explained by active of the bacteria in their community which had inhibited the activity of *Microbacterium* sp. C8.9.

4. CONCLUSIONS

This study was selected one bacterial community and *Microbacterium* sp. C8.9 was isolated from rice - upland crop soil in Vinh Long province. Bacterial community and *Microbacterium* sp. C8.9 was degraded Chlorpyrifos about 18.85 % and 41.07 %, respectively.

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