International Journal of Psychosocial Rehabilitation, Vol. 24, Issue 03, 2020
ISSN: 1475-7192

Characterization and Growth Evaluation of Carbofuran-degrading Local Bacteria Isolated from Brinchang Cameron Highlands Malaysia

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Abstract--- *The insecticide carbofuran is a broad spectrum carbamate pesticide often used to control pests in agricultural sector. However, despite its ability to effectively kill the insects in the farms, health associated problems are still been reported due to its higher level of toxicity. The biodegradation is an effective method used for the removal of these compound from the environment since previous methods using chemical process of degradation prove to be ineffective due to the presence of highly stable bonds. Bacterial strain BRC05 isolated from vegetable plantation area of Cameron highlands was found to have carbofuran-degrading ability. The morphology and growth at different concentration of carbofuran was studied. The growth of the isolate was evaluated in Carbofuran medium under stable and shaking conditions. The gram negative motile and rod-shaped BRC05 show good growth on Carbofuran medium after 12 hours of incubation. The optical densities of the isolate was more under shaking condition and differed significantly than under static conditions. There is no significant difference ($p>0.05$) between growth at 25 and 50 mg/l under static conditions. At 25 mg/l under shaking condition the insecticide has less effect on the growth of the isolate. It was found that BRC05 could grow well and reach the largest biomass in the medium containing 25 mg/l of carbofuran and could keep active growth even in medium with high concentration of carbofuran 100 mg/l. These showed that the bacteria could grow and remove carbofuran in soils effectively and safely. The present study may provide a basis for bio treatment and bioremediation of carbofuran-contaminated soils.*

Keywords--- *Bacteria, Carbofuran, Degrading, Biochemical Methods, Growth.*

I. INTRODUCTION

Pesticides generally become an unavoidable part of the modern societies as they are extensively used in general aspect of life such as agriculture, domestic and public health division. Pesticide fate in the environment is affected by microbial activity. Some pesticides are readily degraded by microorganisms, others have proven to be recalcitrant (Li *et al.*, 2016). Depending on their fate, carbamate pesticides may become bioavailable for microbial degradation. One of the methyl carbamate pesticide is Carbofuran (2, 3-dihydro-2, 2 dimethylbenzofuran-7-yl methyl-carbamate) a broad-spectrum insecticide extensively used to prevents crops from insects and nematodes (Jayasumana *et al.*, 2018). Carbofuran has molecular weight of 221.256 g/mol an odorless white crystalline solid. Contact with skin may

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DOI: 10.37200/IJPR/V24I3/PR200951
Received: 20 Jan 2020 | Revised: 06 Feb 2020 | Accepted: 12 Feb 2020

burn skin and eyes. When exposed to heat or flames it may emit toxic oxides of nitrogen. It is toxic by inhalation, skin contact, and/ or ingestion. It is used as a pesticide (Fenyves *et al.*, 2006). Carbofuran, granule form, is banned in the U.S. A U.S. EPA restricted Use Pesticide (RUP). The insecticide is not approved for use in EU countries. According to the Ecological Incident Investigation System, carbofuran has been responsible for more avian deaths than any other pesticide (EPA, 2013).

Carbofuran is one of the pesticides which is banned or strictly restricted in many countries, but however, it is still used in many part of the world (Chiari *et al.*, 2017). Carbofuran insecticide is of ecological concern due to its higher mobility in soil and solubility in water causing high risk for groundwater contamination (Mabury *et al.*, 1996). It causes acute toxicity to mammals by inhibiting cholinesterase. It has been reported that carbofuran has relatively high mammalian toxicity with oral LD_{50} of 11 mg/kg in rats and can also be toxic to invertebrates, fish and birds (Evert, 2002).

The insecticide mode of action in target organisms is mostly by inhibition of the acetylcholine esterase (AChE) causing accumulation of acetylcholine at the junction of the nerve cell and the receptor areas and ultimately causes the nerve to fire uninterruptedly leading to convulsions and death (Mansano *et al.*, 2018). Therefore, to avoid environmental pollution and incidental exposure the use of carbofuran insecticide has to be handled very carefully (Pan *et al*., 2006; Pohanka, 2012; Jayasumana *et al.*, 2018).

Although there is no sufficient information with regard to environmental concern of carbofuran metabolites such as hydroxylated carbofuran or carbofuran-7-phenol. The ecological concern of carbofuran or its metabolites, has over the years prompted researches on biodegradation of the compound. Therefore, it has become increasingly possible to isolate microorganisms that are capable of degrading such xenobiotic compounds as well as recalcitrant compounds from environments contaminated with such toxic compounds (Ishag *et al.*, 2016).

Soil bacteria that constantly encounter this toxic compounds may develop new adaptive abilities to degrade such chemicals. Many of these chemicals are known to be carcinogenic or endocrine disrupting chemicals (Cheung *et al.*, 2007; Otieno, Lalah and Schramm, 2010) as such, they affects the diverse gene pool that is present in soil, influencing gene expression and genetic recombination among the microbial population in soil (Nguyen *et al.*, 2014).

Traditional approaches of carbofuran decontamination, using chemicals bond methods such as membrane filtration, oxidation with ozone, fenton degradation, adsorption and ozonation, have been less effective and more costly to operate than biological methods using microbes (Tien *et al*., 2013; Zhang and Bennett, 2005). Many bacterial isolates were reported to have degrade carbofuran insecticides, in an effort to fully understand the role of bacteria in degrading carbofuran in the environment strains such as *Proteus, Pseudomonas, Sphingomonas, Flavobacterium, Achromobacterium, and Arthrobacter*, etc (Bano and Musarrat, 2004; Chaudhry and Ali, 1988; Onunga *et al.*, 2015; Yang *et al.*, 2011). But, only few of the studies reported bacteria that hydrolyze Carbofuran insecticide or completely degrade the aromatic ring structure. In the present study, bacterial cultures were enriched and isolated from carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methyl carbamate) polluted areas. The growth and pesticide degradation potentials of these cultures both under static and shaking conditions were

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evaluated, using carbofuran as supplementary source of carbon.

II. MATERIALS AND METHODS

Pesticides and Chemicals

High purity Carbofuran, 98 % PESTANAL® analytical standard carbofuran was purchased from Sigma–Aldrich. Acentonitrile used was of high pressure liquid chromatography (HPLC) grade, purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Medium

The enrichment medium contained the following constituents (in grams per liter): The mineral salt medium (MSM) contained the following constituents (in grams per liter): $NaHPO_4$, 5.57; KH_2PO_4, 2.44; NH_4Cl, 2.00; $MgCl_2.6H_2O$, 0.20; $MnCl_2.4H_2O$, 0.0004; $FeCl_3.6H_2O$, 0.001; $CaCl_2$, 0.001. The MSM medium pH was adjusted to 7 then autoclaved at 121 °C for 15 minutes (Nguyen *et al.*, 2014).

Nutrient Agar

The nutrient agar contained; Agar, 15.0 g; Peptone, 5.0 g; Beef Extract, 3.0 g; Distilled Water, 1000 ml; Final pH 7 then autoclaved at 121 °C for 15 min.

Sample Collection

Soil samples were collected from a vegetable farm with a history of pesticide application in Brinchang Cameron Highlands Malaysia. Soil samples were collected 10–20-cm depth and aseptically put into labelled and sterile culture polyethene and taken in ice-box to laboratory for isolation of carbofuran-degrading bacteria and degradation studies (Yang *et al*., 2011).

Enrichment and Isolation

Enrichment was performed by serial 10-fold dilution method, 1 g of each soil sample was suspended in 10 mL of sterile Mineral salts medium (MSM) containing initial 5 mg/l of carbofuran as the sole carbon source. Subsequent dilutions were made from the dilution until a dilution of 10^{-10}. The dilutions were thoroughly agitated using a shaker, 0.2 ml aliquots of appropriate 10-fold serial dilutions of the soil samples were inoculated into 100-mL MSM added with 50 mg/l carbofuran in 250-mL flask the experiment was done under aseptic conditions in triplicate. The cultures were incubated at 32°C agitated for 14 days in a shaker incubator at 150 rpm. Flask having the same inoculum without addition of carbofuran serves as control experiment. For isolation pure culture was obtained by plating the enrichment culture on MSM medium petri plate supplemented with 50 mg/L carbofuran, then incubated at 32°C (Chaudhry and Ali, 1988).

Morphological and Biochemical Characterization of Isolates

The bacterial isolate was examined physically and morphologically which was determined by performing simple gram staining and colony morphology for the isolate, and further examined under light microscope with × 100 magnification (Omolo *et al.*, 2012).

Growth Evaluation of the Isolate

The microbial growth can be affected by many variables, main among these being temperature, pH and aeration.

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Bacterial cell growth were determined in 250ml flasks. An aliquot of 250 µl of isolate BRC05 grown in MSM supplemented with carbofuran at an optical density of approximately 0.45 at OD600 were inoculated in MSM supplemented with 25, 50, 75 and 100 mg/l standard carbofuran as the sole source of carbon and nitrogen. The inoculums were then incubated at 32°C in a rotational shaker incubator at 150 rpm (shaking) for culture aeration and 0 rpm (non-shaking) conditions for 96 hours so as to see the effect of shaking on the growth of bacteria in carbofuran medium. Aliquots of the inoculum containing cells and the medium, 1 mL, were taken periodically on every 12 hour the absorbance was measured at 600 nm using spectrophotometer (Jiang *et al*., 2007).

III. RESULTS AND DISCUSSION

Soil samples were collected from vegetable farms in Brinchang Cameron Highland and enriched with initial 5 mg/L of initial carbofuran insecticides as sol carbon and energy sources. Carbofuran degrading strains were then isolated from these soil samples by serially diluting these enriched soil samples in MSM supplemented with 50mg/L of carbofuran. The isolated bacterial colonies that shows resistant to carbofuran were further purified by streaking them separately on Nutrient Agar plate. Data shows that when initial 5 mg/L was used many bacterial isolates grow but after subsequent transfer to new medium and increase in carbofuran concentration to 50 mg/L. It was found that carbofuran has an adverse effect on soil bacterial population as indicated by a lower count of soil bacteria upon treatment of the soil with high carbofuran concentrations. One bacterial strains capable of growing in carbofuran medium BRC05 isolates was selected, characterized and are subjected to further experiments as a result of its effectiveness in carbofuran utilization in liquid enrichment cultures and the results of microscopic analysis of bacterial strain and its colony characteristics were presented in Table 1, respectively.

Table 1: Colony Characteristics of Carbofuran-degrading BRC05 Strain

Isolate	BRC05
Location	Brinchang Cameron Highlands
Size	Small (0.5 µm).
Gram stain	Negative (-)
Length	6 µm
Form	Circular
Pigmentation	Creamy-white
Margin	Entire
Opacity	Opaque
Shape	Rod
Surface	Smooth
Elevation	Raised

The colony color was white-cream, the margin was entire and the texture was smooth as well as raised elevation for the carbofuran degrading isolate. Gram staining reaction revealed a pinkish red color on the cells, signifying that the isolates was gram negative motile bacteria and rod-shaped when observed under light microscope. Studies indicate that such physical characteristics, as observed in the isolates, are associated with gram negative rod-shaped bacteria. BRC05 was negative for citrate utilization test and found to be positive to starch hydrolysis test. Carbofuran degrading isolate BRC05 was also positive for H_2S production test. The gram-negative, rod-shaped strain was found to possess cytochrome oxidase enzyme as it was test positive with N'N'N'N tetramethyl-p-phenylenediamine dihydrochloride which act as an artificial electron acceptor for the enzyme oxidase. The strains

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also possessed catalase enzyme which was able to form hydrogen peroxide as an oxidative end product of the aerobic breakdown of sugars. MacConkey test was also conducted to differentiate the isolates as the bile salt content in this differential and selective medium inhibits the growth of mostly gram positive bacteria. The growth of bacteria isolate BRC05 was able to grow on MacConkey agar confirming further that the strain is gram negative isolate as shown in Table 2.

Li *et al.* (2007), isolated carbofuran degrading bacteria and identified as *Sphingomonas sp*. It was also reported that carbofuran degrading bacteria isolated from agricultural soil was able to remediate carbofuran insecticides (Yan *et al.*, 2007). However, it has been reported that pesticides that are newly introduced into soils are typically resistant to biodegradation by indigenous soil microbes (Nguyen *et al.*, 2015). Yet, studies have shown that repeated applications of the same pesticide or its equivalents over time may result in buildup of microbial populations capable of utilizing the compound as their source of carbon, nitrogen as well as energy source (Plangklang and Reungsang, 2011; Yan *et al.*, 2007).

The growth of the carbofuran degrading bacteria and pesticides degrading ability were dependent on pH and temperature. Optimal pH for growth of carbofuran resistant bacteria was reported at 6.5 (Head *et al.*, 1992). It was also reported that the optimum temperature for the growth of most bacteria is between 30 and 37°C (Singh, 2008). Previous literatures reported isolated local carbofuran degrading bacterial species from different geographical locations. These includes strains, such as *Achromobacter sp., Sphingomonas sp., Flavobacterium, Paracoccus sp., Novosphingobium sp. and Alcaligenes faecalis,* among others (Kim *et al.*, 2004; Plangklang and Reungsang, 2011; Yan *et al.*, 2007).

Table 2: Biochemical Tests for the Isolated Bacterial Strain

Test	BRC05
Catalase test	+
Oxidase test	+
Citrate utilization test	-
Starch hydrolysis	+
Indole production test	-
Gelatin	+
Spore	+
Spore position	Terminal
Carbohydrate fermentation	+
H_2S production test	+
Methyl red test	-
VP test	-
Growth in Nutrient agar	+
Growth in Nutrient Broth	Uniform with fine turbidity
Growth in LB agar	+
Growth in MSM	+
Growth in Mackonkey agar	-
Growth at 5	-
Growth at 10	-
Growth at 15	+
Growth at 25	+
Growth at 35	+
Growth at 45	-

(+), Positive Result; (-), Negative Result

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Growth Evaluation of Carbofuran-degrading Bacteria

The widespread usage of insecticides in agricultural sector caused persistence of most of these substances in the environment. Several studies shows the involvement of soil microbes in the enhanced degradation of pesticides (Jiang *et al.,* 2007; Omolo *et al.*, 2012). Bacterial growth analysis was carried out based on increase bacterial populations through turbidity measurement of colony growth on agar medium determined by taking the (OD_{600}) using spectrophotometer at static and shaking conditions. It was found that isolates BRC05 was able to show good growth on 25 and 50 mg/L carbofuran medium after 12 hours of incubation. There is no significant difference ($p>0.05$) between growth at 25 and 50 mg/l under static conditions as shown in Figure 1(A) and (B). The optical density of the isolate was more under shaking condition which might be due to aeration and oxygen in addition to nutrient availability and differed significantly than under static conditions. There was significant differences in growth with the increase concentrations of carbofuran between 50, 75 and 100 mg/L under shaking condition ($p<0.05$). Thus, It has been observed that the bacterial isolates had been utilizing the carbofuran as source of carbon to aid their growth. Bacterial growth in carbofuran containing medium, as shown by an increase in OD_{600}, is an indication of adaptation to carbofuran concentrations by the growing isolates. From the study it is clear that static and shaking conditions do not have much effect on the initial adaptation of bacteria against pesticide, but shaking condition hastens the bacterial growth.

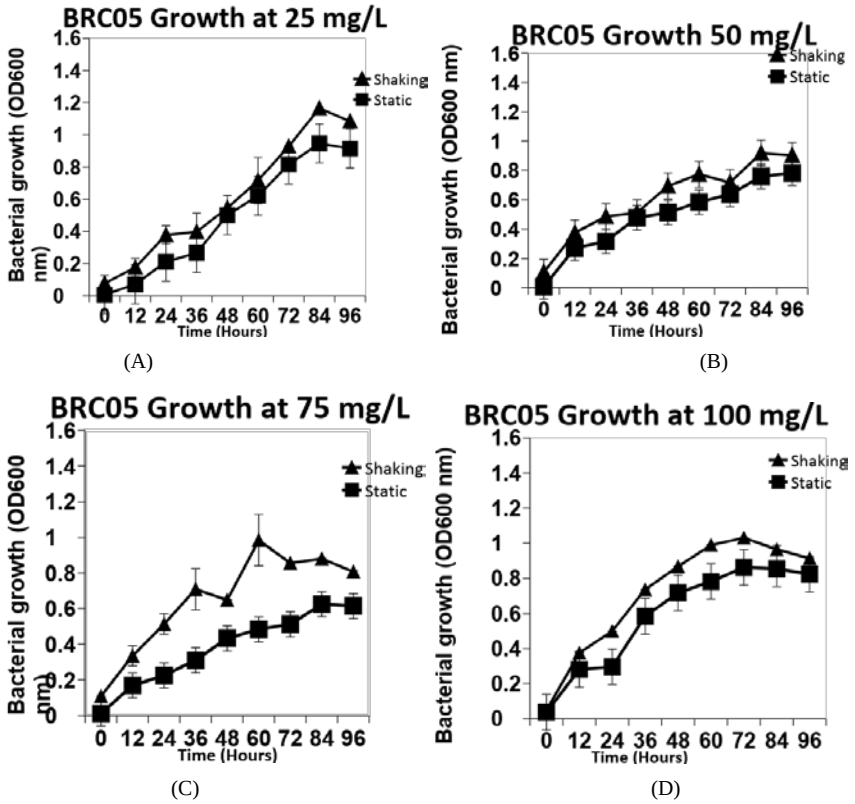

Figure 1: Growth and Adaptation of Isolate BRC05 under Static and Shaking Conditions on Medium Supplemented with (A) 25 mg/L (B) 50 mg/L (C) 75 mg/L (D) 100 mg/L of Carbofuran Concentration. Data Represent Mean ± STDEV, n=3

The decrease in the growth rate under static condition with an increase in initial carbofuran concentrations indicates that the insecticide act as an inhibitor to the bacteria. The fact that the bacterial growth starts slowly with

DOI: 10.37200/IJPR/V24I3/PR200951
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longer lag phase at higher concentrations means it requires an acclimation period and needed longer time to initiate and achieve enhanced growth.

Many scientists have reported the growth and biodegradation of various pesticides by several isolated bacterial species under different conditions (Gupta *et al*., 2014; Doddamani *et al*., 2001; Gonzalez *et al*., 2012; Plangklang and Reungsang, 2011). Arshad *et al*. (2013), reported that the growth and biodegradation under shaking conditions was more pronounced than the static conditions. According to Cycon *et al.* (2017), microbial adaptability during growth and biodegradation releases certain enzymes, which metabolizes wide-range of toxic organic compounds. A gram-negative isolate *Novosphingobium* sp. strain FND-3 capable of growing and degrading carbofuran was isolated and characterized. The strain FND-3 was investigated under various culture conditions (Yan *et al.*, 2007). Arshad (2007) reported that with aeration, bacterial growth was maximum for biodegradation than under static conditions, respectively. Du *et al.* (2011) also reported an increase growth under shaking than static conditions. Guan (2013), observed significant difference between growth and biodegradation of fenvalerate by the bacterium under static versus shaking conditions, and reported maximum growth and degradation under shaking conditions (30°C, 160 rpm, $OD_{initial}$= 0.4, 72 h) as compared to static conditions. No significant difference was found between shaking and non-shaking condition during growth and degradation of endosulfan and chlorpyrifos (Kumar *et al*., 2011).

IV. CONCLUSION

In this study, we isolated a carbofuran-degrading bacterium BRC05 strain that can use carbofuran as source of carbon, the isolate show good growth in carbofuran medium. Therefore, the strain could degrade carbofuran in soils, even in the presence of indigenous microbial competition. The newly isolated bacteria will be potentially useful in decontamination of pesticides polluted soils.

ACKNOWLEDGMENTS

This work was supported and funded by Research Grant Scheme (IPS), Universiti Putra Malaysia (IPM9636200) and the Faculty of Environmental studies, Universiti Putra Malaysia.

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Received: 20 Jan 2020 | Revised: 06 Feb 2020 | Accepted: 12 Feb 2020