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DEGRADATION OF CARBOFURAN USING MALAYSIAN ISOLATE *Bacillus* sp.

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Abstract

Pesticides are beneficial for the agricultural sector. Carbofuran is one of the most common pesticides used in the agricultural industry. Acting as a neurotransmitter inhibitor through Acetyl Cholinesterase activity, carbofuran can become a harmful substance to animals and humans in uncontrolled situations. Another concern is the persistence of carbofuran in the environment, especially at soil surfaces and in surface water. Thus, remediation of this contaminant is essential to prevent or minimise toxic effects towards animals and humans. Green technology is the optimum method to achieve this objective with minimum side effects. Using potential bacteria to remediate the contaminant is proven to reduce the carbofuran toxicity. Local bacteria from a carbofuran contaminated area were isolated and identified as *Bacillus* sp. isolates S2B, S3C, S4B and S5B with accession numbers MN689686, MN689687, MN689688 and MN689689, respectively. These four bacteria showed significant potential in degrading carbofuran. Isolate S2B showed the fastest degradation rate with 91% degradation within 7 days. Isolates S4B and S5B showed 99% degradation after 14 days of incubation. The results of this study are vital, since current knowledge and data on potential bacterial isolates that can degrade carbofuran in Malaysia are still deficient. Our findings will be beneficial in the future for treating similar contaminants.

INTRODUCTION

Pesticides are used in the agricultural industry to control the pest distribution, which can significantly affect agricultural production. These pesticides are considered harmful due to their persistence in the environment[1]. Carbofuran is a carbamate insecticide that is commonly used in agriculture to control pest infection towards crops[2, 3]. Carbofuran is mainly used to control soil-dwelling and foliar-feeding insects[4]. This insecticide inhibits an Acetyl Cholinesterase (AChE) enzyme in the insect nervous system that interrupts its behaviour[5]. Due to this mechanism, the European Union have now banned the use of carbofuran in the agricultural sector[6].

Carbofuran can be degraded through a photolysis and hydrolysis process in the environment. This process takes 67 d to reduce the amount of carbofuran by up to 65% in ground water[7]. Even though the half-life of carbofuran is considered short, it can still have a tremendous effect on human and animal populations. This has been proven by a recent review claiming that the worldwide vulture population is decreasing due to their unintentional exposure to various types of pesticides, including carbofuran[8]. Carbofuran is also used illegally in several European states, as claimed by research conducted in the Canary Islands, Spain, where domestic animals, such as cats and dogs, and also endangered animals, such as hedgehogs and owls, are accidently exposed to carbofuran[9].

The prolonged existence of this pesticide in the environment may also lead to soil surface contamination that affects the surface water supply to human and agricultural products. If not prevented, this situation will have an effect on humans as the final consumer[10–12]. In Malaysia, the application of carbofuran is common among farmers, especially in the paddy cultivation industry[13]. Several toxicity reports related to carbofuran contamination have been reported in Malaysia with a fatal incident occurring in 2004[14]. Carbofuran has also been claimed to cause DNA damage towards "orang asli" children in Selangor Malaysia[15].

The existence of carbofuran in soil surfaces needs to be treated. There are several techniques for degrading carbofuran contamination, with one of them being through biodegradation by bacteria. Numerous researchers have utilised bacteria for carbofuran degradation, with some using bacterial consortia and some using single bacterial colonies[16–18]. In the last decade, numerous bacterial species have been identified for their ability to detoxify carbofuran from the environment. Some of the bacteria identified were *Paracoccus* sp. YM3, *Burkholderia cepacia* PCL3, *Agrobacterium* sp. strain Yw12, *Klebsiella pneumoniae* ATCC13883T, *Enterobacter* sp. G12, *Bacillus* sp., *Pseudomonas putida*, *Sphingomonas* sp. strain CDS-1 and *Cupriavidus* sp. ISTL7[16, 18–26]. Most of these bacteria are grouped under rod-shaped gram-negative bacteria, except for *Bacillus* sp., which is classified as a rod-shaped Gram-positive bacterium. Based on the literature, it is most common that gram-negative bacteria are more favourable in degrading carbofuran in soil compared to gram-positive bacteria.

Even though numerous carbofuran degrading bacteria have been identified, information on Malaysian soil bacteria that can degrade carbofuran from the environment is still lacking. In this report, bacteria isolated from a Malaysian agriculture landfill are evaluated for their ability to degrade carbofuran through high-performance liquid chromatography (HPLC) analysis.

MATERIALS AND METHODS

Materials

All chemicals used in this research were purchased from Merck (M) Sdn. Bhd., Fisher Scientific (M) Sdn. Bhd. The DNeasy Blood and Tissue Kit used for total genomic extraction was purchased from Qiagen Biotechnology Malaysia Sdn. Bhd. The purification of genomic DNA was based on a Wizard®Genomic DNA purification kit from Promega. The polymerase chain reaction (PCR) analysis was conducted using a GoTaq® Green Master Mix from Promega.

Bacterial Isolation

Soil Sampling

Soil samples were taken from a paddy cultivation landfill at Tanjong Karang, Selangor. This area was exposed to a certain amount of carbofuran from the paddy cultivation activity [27]. Five different sampling spots were selected. A five centimetre depth of soil was taken and transferred to a sterilised container and then brought to the lab for further analysis. Soil parameters (pH and temperature) were recorded for soil characterisation.

Bacterial Colony Screening

The preliminary bacterial colony screening was conducted using a nutrient agar (NA). Each soil from different spots was transferred into a 250 mL conical flask containing a minimal salt medium. In total, five conical flasks were incubated at room temperature and shaken at 100 rpm for 24 h. After 24 h of incubation, 100 µL of the aqueous part of the media were aspirated out using a micropipette and transferred onto the NA plate. Each flask was inoculated onto three NA plates. NA plates were later incubated at room temperature for 24 hours.

Isolation of Carbofuran Resistant Bacteria

Screening for carbofuran resistant strains was conducted using an M3 carbofuran specific agar. This specific composition was described in [28] and was used with a slight modification. This medium was composed of 2.1 gL^{-1} Na_2HPO_4, 0.01 gL^{-1} $MgSO_4$, 0.1 gL^{-1} NaCl, 0.001 gL^{-1} $FeSO_4$, 0.04 gL^{-1} $CuSO_4$, 0.002 gL^{-1} Na_2MoO_4 and 0.1 gL^{-1} Furadan 3G. Each colony isolated from the preliminary screening was transferred onto the M3 agar and incubated at room temperature for 24 hours.

Identification of Bacteria

16s rDNA Analysis

Genomic DNA was extracted from bacterial single colonies using the DNeasy Blood and Tissue Kit purchased from QIAGEN. PCR amplification was performed using a thermal cycler. The PCR mixture contained 0.5 µL of each primer, 0.5 µL of each deoxynucleotide triphosphate, 1× reaction buffer and 0.5 µL of Taq DNA polymerase (Promega) to achieve a final volume of 50 µL. The 16s rDNA gene from the genomic DNA was amplified by the PCR using the following primers: 5'-AGA GTT TGA TCA TGG CTC AG-3' and 5'-ACG GTT ACC TTG TTA CGA CTT-3' corresponding to the forward and reverse primers of 16s rDNA, respectively[29]. The PCR protocol consisted of initial denaturation at 94 °C for 3 min, 25 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. Observations using an agarose electrophoresis gel were conducted to obtain a single band of DNA. The single band PCR product was cleaned up with the PCR purification kit purchased from PROMEGA. The sequence results were compared with the GenBank database using the Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic Analysis

Twenty sequences of the 16s rDNA gene, which are closely related to the local isolate, were initially obtained from GenBank and aligned using the program Muscle from MEGA X. The evolutionary history was inferred using the neighbour-joining method[30]. The bootstrap consensus tree inferred from 100 replicates[31] is taken to represent the evolutionary history of the taxa analysed[31]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches[31]. The evolutionary distances were computed using the p-distance method[32] and are in units of the number of base differences per site. The analysis involved 24 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1523 positions in the final dataset. Evolutionary analyses were conducted in MEGA X[33].

Carbofuran Degradation Rate

The decline in carbofuran concentration upon degradation was monitored for 21 d with an interval on the seventh day using HPLC on a reverse phase C-18 column, 25 cm × 4.6 mm, fitted with a C-18 silica reverse phase guard column and equipped with an ultraviolet detector and retention time identified using carbofuran and its metabolite (phenol carbofuran) as reference standards. The injection volume was set at 5 µL. The HPLC pumps were set at a flow rate of 1.0 mL min^{-1}.

Statistical Analysis

All data were subjected to one-way analysis of variance (ANOVA). Treatment means were compared using Tukey's multiple comparison tests. Statistical software Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA) was used for all statistical analyses.

RESULTS AND DISCUSSION

Isolation of Bacteria

Twenty bacterial colonies were isolated from five different sampling points (Table 1). These 20 colonies were screened for their carbofuran resistance ability using the modified M3 medium. Only four colonies showed a positive response towards the medium containing carbofuran. These four colonies were labelled as S2B, S3C, S4B and S5B accordingly. Table 1 shows a resistance bacteria strain isolated in weak acidic conditions. This is shown by the soil pH condition where the sample taken. Only sample site S1 showed a low pH value (4.35), with the others showing a higher pH range (5.4–6.4). Only four locations (S2, S3, S4 and S5) from five showed an existence of carbofuran resistance bacteria.

These four locations (S2, S3, S4 and S5) showed a different soil pH condition from the S1 location. Soil pH condition is one of the significant factors influencing the presence of pesticide degrading bacteria[34]. A recent report claims that low pH levels affect bacterial community existence in soil areas in general[35]. It is known that the optimum condition for carbofuran degrading bacteria in soil is around pH 7[24, 36]. Due to this fact, it is clear that none of the bacterial colonies isolated from acidic soil in this study show resistance towards carbofuran.

Table 1. Soil characteristics and bacterial colonies isolated from five different sampling points.

No	Sampling Point	Coordinates	pH	Temperature (°C)	Isolate Name	Carbofuran Resistance
1	S1	3.4443716751020896 N	4.35	28.4	S1A	-ve
2		101.21409729123116 E			S1B	-ve
3					S1C	-ve
4					S1D	-ve
5					S1E	-ve
6	S2	3.444583186824491 N	6.45	30.1	S2B	+ve
7		101.21396854519844 E			S2A	-ve
8	S3	3.4449232118999547 N	5.97	29.0	S3A	-ve
9		101.21418580412865 E			S3B	-ve
10					S3C	+ve
11	S4	3.445166851839955 N	5.45	28.4	S4A	-ve
12		101.21436014771461 E			S4B	+ve
13					S4C	-ve
14					S4D	-ve
15	S5	3.4443020636388693 N	6.36	28.2	S5A	-ve
16		101.21379151940345 E			S5B	+ve
17					S5C	-ve
18					S5D	-ve
19					S5E	-ve
20					S5F	-ve

Identification of Bacteria

All four colonies appeared as a white coloured colony form with bacilli or rod shapes. Gram staining analysis indicates that all four bacterial isolates were gram-positive bacteria. Further analysis using 16s rDNA was conducted. With multiple alignment analysis using Muscle from MEGA X, 20 closely matched sequences were chosen from the NCBI GenBank and compared to the four isolate sequences. The evolutionary distance is shown adjacent with the taxa (Figure 1). All isolates significantly showed a high evolutionary distance value (>50%) to *Bacillus* sp. group. Thus, these isolates can be grouped under the Bacillus genus. All four isolates, S2B, S3C, S4B and S5B, were submitted to the GenBank and obtained GenBank accession numbers MN689686, MN689687, MN689688 and MN689689, respectively.

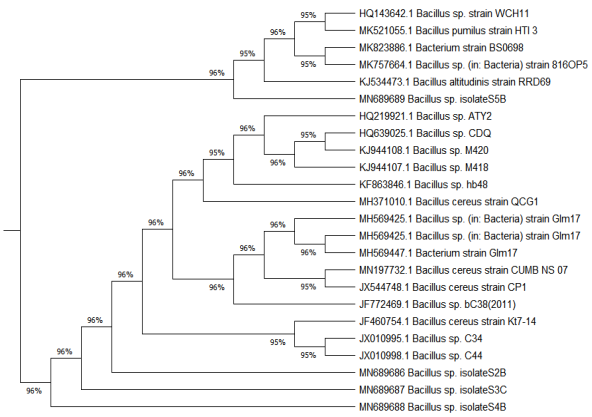

Figure 1. Phylogenetic tree of isolated bacteria. Four isolate gene sequence were compared with others sequence and give percentage value of similarity with compared sequence. All four isolates were group under Bacillus group with high similarity.

Based on the literature, numerous bacterial strains were isolated related to carbofuran degradation. Gram-negative bacteria are considered as a vulgar strain that possesses this characteristic. Only a few gram-positive bacteria possess the ability to degrade carbofuran and they usually belong to the Bacillus genus[25, 34, 37, 38]. *Bacillus* sp. is one of the common gram-positive bacteria found in soil[39]. As a result, it is well understood that the Bacillus genus possesses a high probability of exposure to various types of soil contaminants, including carbofuran, with continuous exposure building the capability of Bacillus in utilising carbofuran as its carbon source.

Carbofuran Degradation Rate

Figure 2 represents the correlation between the data on the carbofuran degradation rate and the population size of the *Bacillus* sp. isolate raziS2B. The carbofuran degradation rate is determined by the percentage of degradation over time, whereas the population size is determined by the colony absorbance at an optical density of 600 nm (OD 600) over time. Overall, the degradation rate of carbofuran by isolate S2B increases over time with 91% degradation by day 7. The degradation rate then slowly increases until day 21 with 99% degradation. The degradation rate is proportional to the population size of the bacteria. This result shows that there is an increment in bacterial colony over time. The initial absorbance of bacteria increases from 0.2 to 0.8 by day 7. Later, the trend shows a descending pattern until day 21. The increment in colony number indicates that the bacteria can utilise carbofuran as their carbon source.

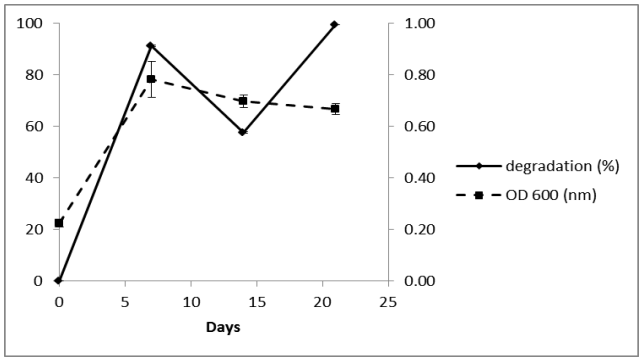

Figure 2. Isolate S2B showed that the amount of bacteria was increased over time porpotion with the carbofuran degradation rate. Graph showed that 91% of carbofuran were degraded at day 7 and reached maximum rate (99%) after 21 days of incubation. Error bar represent standard deviation value of three replicates.

Figure 3 shows almost the same degradation pattern between isolates S3C and S2B. The degradation rate for S3C showed a 90% degradation rate by day 7. No significant difference in the degradation rate was observed for day 7 and day 14 incubation, but the degradation rate increased to 99% for day 21. The same pattern was observed for the OD 600 data for isolate S3C. There is an increase in the OD 600 value for isolate S3C from day 0 to day 7, but the colony formation showed no significant different from day 7 to day 21 accordingly.

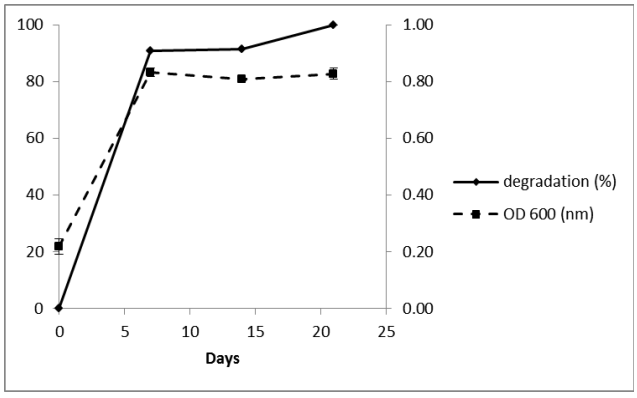

Figure 3. Isolate S3C showed that the amount of bacteria cells is propotion with the carbofuran degradation rate. The carbofuran degradtion rate reached 90% after 7 days incubation and reached maximum percentage (99%) after 21 days incubation. Error bar represent standard deviation value of three replicates.

Figure 4 indicates that the isolate S4B also can degrade carbofuran from the environment. Slightly different from the previous two isolates, isolate S4B shows a lower degradation rate at day 7 with only 88% of the degradation rate. Whilst the degradation rate is low compared to S2B and S3C, isolate S4B achieve its maximum degradation rate (99%) at day 14. The same pattern observed for isolate S5B, the degradation rate is at 89% for day 7 (Figure 5) but the degradation rate reached its optimum level with no significant difference at day 14. The bacterial population size showed almost similar pattern for all isolates. The graphs show an increment in the bacterial population size over time. The bacterial population either decreases slowly or maintains a plateau after day 7 to day 21, this situation is mainly due to the limitation of carbon source that is used by the bacteria to support their growth.

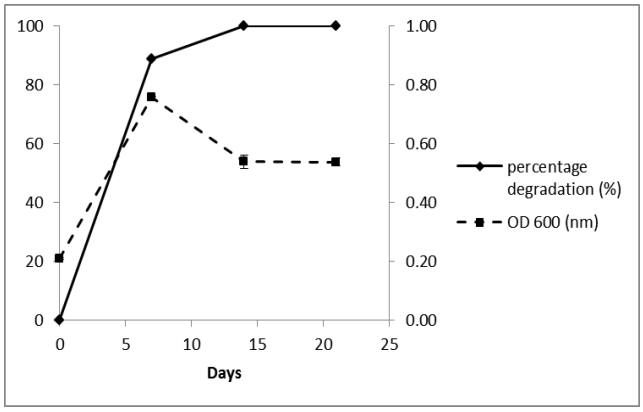

Figure 4. Isolate S4B showed an increament in colony numbers at day 7th. After day 7, the colony number showed significant low at day 14 onwards. The degradation rate showed about 88% of carbofuran degradation at day 7th. The degradation rate increase over time and reached 99% at day 14. Error bar represent standard deviation value of three replicates.

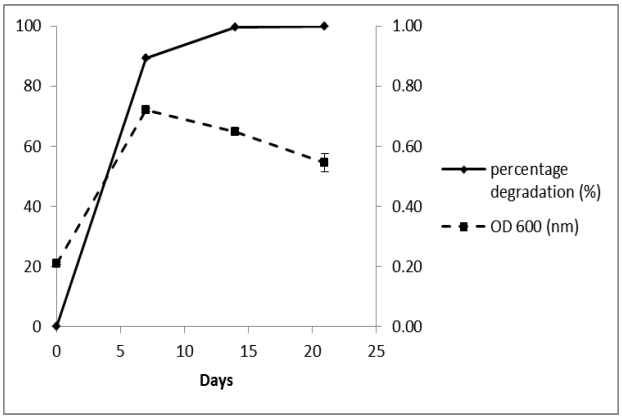

Figure 5. Isolate S5B showed the colony numbers increase untill day 7. The amount decreased after day 7 onwards. The carbofuran degradation rate showed and increament at 89% after 7 days incubation. The degradation rate reached 99% degradation at day 14 of incubation. Error bar represent standard deviation value of three replicates.

Compared with the same genus, the current isolates show a slightly low overall performance. *Bacillus* sp. isolated from Kenyan soil showed a faster degradation rate where their isolate can degrade 100% of carbofuran from the environment after 10 days of incubation[25]. Another report regarding carbofuran degradation by *Bacillus* sp. stated that the degradation by this bacteria can be up to 90% within 7 days of incubation[40]. Based on these two reports, we can see that the degradation pattern by *Bacillus* sp. varies. This situation can be influenced by several factors, such as initial carbofuran concentration, environment condition and the types of bacteria itself.

CONCLUSION

Bacteria are known for their ability to remediate environmental pollution, particularly carbofuran. Four bacterial isolates from Malaysian soil are classified as gram-positive bacteria with phylogenetic analysis giving similarity to *Bacillus* sp. for all four isolates and genetic information for these bacteria deposited in GenBank with accession numbers MN689686, MN689687, MN689688 and MN689689. Degradation analysis for the four isolates indicates that all four can degrade carbofuran with different kinetic properties. Isolate S2B showed the fastest degradation with 91% degradation within 7 days, while isolates S4B and S5B show 99% degradation within 14 days incubation. Even though all isolates were grouped under *Bacillus* sp., the degradation rate for each isolate is different, indicating that all isolates are from different species. Isolated bacterial strains can be beneficial in the future for the bioremediation of carbofuran contaminated sites.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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