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Biodegradation of carbofuran in soils within Nzoia River Basin, Kenya

DANIEL O. ONUNGA[1], ISAAC O. KOWINO[1], ANASTASIAH N. NGIGI[2], AGGREY OSOGO[3], FRANCIS ORATA[1], ZACHARY M. GETENGA[1] and HASSAN WERE[3]

[1]*Department of Pure and Applied Chemistry, Masinde Muliro University of Science and Technology, Kakamega, Kenya*
[2]*Department of Physical Sciences, Multimedia University of Kenya, Nairobi, Kenya*
[3]*Department of Biological Sciences, Masinde Muliro University of Science and Technology, Kakamega, Kenya*

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) has been used within the Nzoia River Basin (NRB), especially in Bunyala Rice Irrigation Schemes, in Kenya for the control of pests. In this study, the capacity of native bacteria to degrade carbofuran in soils from NRB was investigated. A gram positive, rod-shaped bacteria capable of degrading carbofuran was isolated through liquid cultures with carbofuran as the only carbon and nitrogen source. The isolate degraded 98% of 100-μg mL^{-1} carbofuran within 10 days with the formation of carbofuran phenol as the only detectable metabolite. The degradation of carbofuran was followed by measuring its residues in liquid cultures using high performance liquid chromatography (HPLC). Physical and morphological characteristics as well as molecular characterization confirmed the bacterial isolate to be a member of *Bacillus* species. The results indicate that this strain of *Bacillus sp*. could be considered as *Bacillus cereus* or *Bacillus thuringiensis* with a bootstrap value of 100% similar to the 16S rRNA gene sequences. The biodegradation capability of the native strains in this study indicates that they have great potential for application in bioremediation of carbofuran-contaminated soil sites.

Keywords: Carbofuran, biodegradation, bioremediation, *Bacillus thurigiensis*, *Bacillus cereus*.

Introduction

Carbofuran (2, 3-dihydro-2, 2 dimethyl-7-benzofuranoyl N-methylcarbamate) is a broad-spectrum carbamate insecticide, acaricide and nematicide.[1–2] It belongs to the N-methylcarbamate class that is extensively used in pest control. It is widely used in the control of pests such as corn root worm, wire worms, boll weevils, mosquitoes, alfalfa weevils and white grubs.[3] It has been also used worldwide for the control of rice pests such as green leafhoppers, brown planthoppers, stem-borers and whorl maggots.[1] For example, in South Korea, this pesticide was used as early as 1975 to control brown hoppers, green rice leaf hoppers and rice stem borers in rice paddies.[4] It has been reported that Carbofuran has rapid action against both nymphs and adults, killing them within 20 min.[1] However, accidental exposure of carbofuran can result in acute toxicities and fatalities even to human beings, thereby making it highly hazardous.[1,3]

As in 2010, commercial carbofuran (Furadan®) with 10% active ingredient was allowed for restrictive use by informed users in Kenya.[1] Studies showed that carbofuran was still being imported mainly for use in seed dressing at the rate of 0.5–4 kg a.i. ha^{-1} (active ingredient per hectare) for control of soil-dwelling and foliar-feeding insects. It was used in Isiolo and Laikipia districts to control agricultural pests and its residues were found in the soils, plants and water sources in such areas.[2] Carbofuran has also been used in Nzoia River Drainage Basin, especially in Bunyala Rice Irrigation Schemes nuclear estates.[5] A study done by Kimosop[6] in the same area on the dissipation behavior of carbofuran in the soil established that residual levels of carbofuran ranged from 0.01–1.08 μg g^{-1} in the soils, indicating environmental contamination. Therefore, the detoxification of the contaminated sites is necessary.

Conventional (chemical) methods of carbofuran detoxification, such as oxidation with ozone, photodegradation, fenton degradation, ozonation, membrane filtration and adsorption,[7] have been used. However, these methods, including pyrolysis of carbofuran, are more cumbersome, less effective and more costly to set up and run than

Address correspondence to Isaac O. Kowino, Department of Pure and Applied Chemistry, Masinde Muliro University of Science and Technology, P.O. Box 190-50100, Kakamega, Kenya; E-mail: ikowino@gmail.com
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biological methods.[8,9] Also in some cases, particularly in pyrolysis, it leads to the introduction of other chemicals such as mono-aromatics and polycyclic aromatic hydrocarbons (PAHs) into the atmosphere, thereby contaminating it.[10]

Bacterial degradation offers an attractive alternative to chemical methods of carbofuran remediation, as it is less expensive and eco-friendly.[11,12] Isolation of indigenous soil bacteria capable of metabolizing carbofuran provides environment friendly means of detoxification.[12] Such microbes develop as a result of the repeated use of carbofuran, leading to the adaptation of native soil bacteria capable of degrading it.[13] Therefore, the most important route for its removal from the environment is through microbial degradation.[14] Several native bacteria capable of degrading carbofuran have been isolated and characterized in diverse geographical locations. These include bacterial strains, such as *Achromobacter sp*., *Sphingomonas sp*., *Paracoccus sp*., *Novosphingobium sp*. and *Alcaligenes faecalis*, among others.[15–21] Such isolations have given great insights in understanding the role of bacteria in the removal of carbofuran from the environment.

These studies have mostly been done in temperate regions; therefore, there are limited studies on microbial degradation of carbofuran in soils with repeated use of the chemical in tropical regions, especially in the Nzoia River Basin (NRB) in Kenya, where it has been used frequently.[22] Thus, the objective of this study was to isolate and characterize indigenous soil bacteria capable of degrading carbofuran from the soils within NRB in Kenya, evaluate the capability of bacterial isolates to degrade carbofuran in mineral salt medium, and identify them by analyzing the sequence of the gene encoding 16S rRNA.

Materials and methods

Sample collection

Soil samples were collected from the three sites of Bunyala Irrigation Scheme paddy fields (young rice field, flowering rice field and harvested rice fields), with a history of carbofuran use. Stratified random sampling method was used to collect soils from the three strata. From each stratum, plots (farms) were identified by simple randomization. Soil samples were collected 10–20-cm depth according to Anderson and Ingram,[23] aseptically put into labelled 250-mL sterile culture bottles and taken in ice-box to laboratory for use in degradation studies and isolation of carbofuran-degrading bacteria.

Reagents and medium

Commercial carbofuran was purchased from Cabrando Enterprises (Kakamega, Kenya). Nutrient Agar and nutrient broth were purchased from Fluka Biochemica and Himedia Laboratories Pvt. Ltd through Kobian Company, Kenya. Carbofuran (purity 98.5%) and carbofuran phenol (purity 99.0%) pesticide standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Both high-performance liquid chromatography (HPLC) grade methanol and analytical grade chloroform were purchased from Sigma Aldrich (Augsburg, Germany through Kobian, Kenya). Nylon 66 Microfilter (0.45 μm) was purchased from Kobian Company (Nairobi, Kenya). The universal primer pair 616F, 5′-AGAGTTTGA TYMTGGCTCAG-3′ and 630R, 5′-CAKAAAG GAGGTGATCC-3' was provided by Helmholtz Zentrum München, German Research Center for Environmental Health. All other chemicals were of analytical grade unless otherwise stated.

For HPLC calibration, individual standard stock solutions of 100 $\mu g\ mL^{-1}$ for carbofuran and 50 $\mu g\ mL^{-1}$ for carbofuran phenol were prepared in LC grade methanol. The working standard solutions were prepared by appropriately diluting the stock solution with LC grade methanol. Both the stock solutions and the standard dilutions were stored at 4°C for use.

Mineral salt medium (MSM) was constituted in distilled water as follows (in grams per liter, pH 7.2): K_2HPO_4, 3.75; KH_2PO_4, 1.0; NaCl, 0.25; $MgSO_4.7H_2O$, 0.1; $CaCl_2.H_2O$, 0.01; 10 mL of trace elements containing 10 mg of Na_2–$MoO_4.2H_2O$, 25 mg of H_2BO_3, 15 mg of $ZnCl_2$, 5 mg of $CuCl_2$ and 10 mg of $FeCl_3$ per liter. The pH was then adjusted to 7.2 using sodium hydroxide (NaOH) solution. The Luria–Bertoli medium (LB) was also prepared with the following components: 10-g tryptone, 5-g yeast extract, 5-g NaCl and 15-g mineral agar in 1-L distilled water.

Enrichment culturing and bacteria isolation

Soil bacteria were extracted by the use of serial 10-fold dilution technique as described in Collins and Lyne[24] with modifications. Briefly, 1 g of each soil sample was suspended in 10 mL of sterile MSM containing 0.475 mM of commercial carbofuran as the sole carbon source.[25] Subsequent dilutions were prepared from the dilution until a dilution of 10^{-10} was achieved. The dilutions were thoroughly agitated using a mechanical shaker to ensure maximum extraction of the bacteria.

For liquid culture experiments, 200-μL aliquots of appropriate 10-fold serial dilutions of the soil samples were inoculated into 100-mL MSM supplemented with 0.475 mM in 250-mL flask in triplicate under aseptic conditions. The cultures were incubated at 32°C with shaking in a Wisecube rotational shaker incubator at 150 revolutions per minute (rpm) for 14 days. Control experiments contained the same inoculum without carbofuran. Using the pour plate method[24] and spread plates method,[26]

200-μL aliquot of the incubated culture was inoculated in agar (Oxoid CM – 314) plates containing 10–15-mL MSM. The MSM was prepared by autoclaving at 121°C for 15 min, supplemented with 0.475 mM of commercial carbofuran and then incubated at 35°C for 48 h in an oven. The cultures in the above plates were then streaked into agar plates containing MSM supplemented with 0.475 mM of commercial carbofuran using a sterile wire loop. Inoculated plates were then incubated at 35°C for 48 h. Control experiments contained the same inoculum without carbofuran. Single colonies obtained were re-suspended in MSM containing 0.475 mM of commercial carbofuran for seven days to confirm the ability of isolates to utilize the pesticides. Using wire loop, cultures were then streaked into plates containing the Luria–Bertoli medium. Sub-culturing was done periodically on pesticide-supplemented medium (agar) until pure colonies were obtained.[25] The pure isolates with ability to degrade carbofuran (labeled as isolates 2, 3, 4, and 8) were then divided into two portions: one portion was kept at –80°C in MSM containing 50% glycerol awaiting molecular characterization. The other isolates were used for physical and morphological characterization and degradation studies.

Degradation studies

Growth of isolates in carbofuran-supplemented medium

The ability of isolates to utilize commercial carbofuran as the only C and N source was tested through liquid culture experiments. One milliliter of the bacterial culture that had been stored in glycerol was centrifuged at 10,000 g rpm for 10 min. The cultures were then resuscitated by inoculating, using a wire loop, into 15 mL of sterile nutrient broth media in a boiling tube. The MSM was autoclaved at 121°C for 15 min and supplemented with 0.475 mM of commercial carbofuran. The inoculated culture in the tube was then incubated at 32°C in Wisecube rotational shaker incubator at 150 rpm for 24 h. A 1-mL aliquot of isolates from the nutrient broth was centrifuged at 10,000 g rpm for 10 min and repeatedly washed for three times with 1 mL of sterile MSM. Washed isolates in MSM, 200 μL, were then incubated in 100-mL MSM supplemented with 0.475-mM commercial carbofuran as the only source of carbon and nitrogen at 32°C in a Wisecube rotational shaker incubator at 150 rpm. Two sets of experiments were conducted; one set was spiked with 0.475-mM commercial carbofuran once at the beginning, and the other set was spiked at the beginning and at intervals of 10 days for 90 days. The control experiment contained only carbofuran without isolates. The growth of isolates was monitored through optical density measurements using Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) at 600 nm, and the growth curves were obtained by plotting the optical density (OD_{600}) against time.

Growth of isolate 2 in standard cabofuran

The method by Kim et al.[4] was used with a few modifications. Briefly, time-course carbofuran degradation and bacterial cell growth were determined concurrently in the same culture flasks. An aliquot of 200 μl of isolate 2 (grown for 90 days in MSM supplemented with commercial carbofuran at an optical density of approximately 0.36 at OD600) were inoculated in MSM supplemented with 100 μg mL^{-1} standard carbofuran (in triplicate) as the sole source of carbon and nitrogen. The inoculums were then incubated at 32°C in a Wisecube rotational shaker incubator at 150 rpm for 10 days. Aliquots of the inoculum containing cells and the medium, 10 mL, were taken periodically on day 0, 2, 6, 8 and 10 respectively. Carbofuran and its metabolites were then extracted for three times from the aliquots with 3 mL of chloroform by vigorously shaking for 5 min. The organic extracts were combined, dried over anhydrous sodium sulfate and evaporated at 40°C by a vacuum rotary evaporator. The residues were re-dissolved in 1 mL of HPLC grade methanol and filtrated through nylon membrane filters (0.45 μm) for HPLC analysis of residual carbofuran and its metabolites.

HPLC analysis of carbofuran and metabolites

The HPLC analysis was performed under the following chromatographic conditions: Shimadzu LC-20AT prominence liquid chromatograph equipped with SPD-20A Shimadzu prominence UV/visible detector and phenomenex 00G-4420-E0 (250 × 4.60 mm, 5 micron, HyperClone 5u BDS C-18 130A) column. The mobile phase was methanol–water (1:1) at a flow of 0.7 mL min^{-1} at a detector wavelength of 280 nm and an injection volume of 20 μL. The experiments were done in triplicate. The mobile phase was prepared by first washing 500-mL bottles and drying in an oven for an hour before filling them with 500 mL of mixture of HPLC grade methanol and double distilled water in the ratio of 1:1. The mixture was then de-gassed for 15 min before connecting to a pump (LC-10AT VP-Shimadzu A). The retention time of carbofuran and its metabolite, carbofuran phenol, was identified using the reference standards.

Characterization of isolates

Morphological and physical characterization of isolates

The bacterial isolates were examined physically by noting the color, texture, consistency, elevation and margin of the colony. Cell morphology was determined by performing simple staining using methylene blue stain (0.05%) on each isolate, which was further examined under light microscope (Carl Zeiss, Germany) with × 1,000 magnification.

Molecular characterization of isolates

The isolates were further subjected to molecular characterization by extracting ribosomal DNA from isolates using a standard protocol.[27] The DNA was then subjected to polymerase chain reaction (PCR) amplification and partial sequence analysis of the 16S rRNA gene. The universal primer pair 616F, 5'-AGAGTTTGATYMTGGCTCAG-3' and 630R, 5'-CAKAAAGGAGGTGATCC-3',[28] corresponding to nucleotide positions 8–27 and 1528–1544 in 16S rRNA from *Escherichia coli*,[29] was used for amplification of a 1.4-kb region of 16S rRNA coding genes.

Amplification was performed using Primus 96 plus thermal cycler. The amplification mixture comprised 5 μL of 10 × TopTaq PCR buffer, 1 μL of dNTP mix, 5 μL of 10 × CoralLoad, 0.2 μL of 630R forward primer, 0.2 μL of 616F reverse primer, 1 μL of template DNA, 0.25 μL of TopTaq DNA polymerase and 37.35 μL of RNase-free water, while the control contained all the above except DNA template. Reaction mixtures were subjected to 30 cycles: Initial denaturation of the DNA was at 94°C for 5 min, denaturation at 94°C for 60 s, primer annealing at 54°C for 45 s, chain extension at 72°C for 45 s and a final extension at 72°C for 10 min.

The amplified 16S-rRNA gene fragments were further gel-purified in a 1% agarose gel in 1X TAE buffer, visualized by ethidium bromide staining[30] and subsequently cloned with the TOPO TA cloning-kit containing pCR_2.1 vector (Invitrogen, Paisley, UK). The amplified 16S rRNA gene fragments of the isolates were sequenced by a commercial service provider. The 16S rRNA gene sequences were compared with the sequences of public database using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity to sequences in the gene bank database.[31] The 16S rRNA gene sequences with high similarities to those determined in the study were retrieved and added to the alignment based on BLAST results. Phylogenetic tree was constructed by maximum likelihood method.

Results and discussion

Enrichment culturing and isolation of carbofuran degrading bacterial strains

Twelve isolates were obtained from the soils sampled from selected Nzoia River Drainage Basin, Kenya using independent enrichment liquid culture procedures. Isolates 1, 5, 6, 7, 9, 10, 11 and 12 did not show good growth in liquid enrichment cultures. Therefore, out of the 12 isolates, only four isolates (2, 3, 4 and 8) were selected, characterized and subjected to further experiments because of their efficiency in carbofuran utilization in liquid enrichment cultures. The four isolates showed similar physical–morphological and molecular characteristics, and isolate 2 was taken as a representative sample for degradation studies.

Growth of isolated degraders on commercial carbofuran

The growth of isolates expressed as optical density against time in MSM supplemented with 0.475 mM of commercial carbofuran was obtained (Fig. 1) through liquid culture experiments. From graph A, the growth of isolate 2 increased from the optical density of 0.077 on 0th day to a maximum of 0.419 on 90th day of incubation when carbofuran was spiked for several times at 10-day intervals. The isolate showed a lag phase of 10 days, possibly because the isolated microorganisms were adapting to carbofuran in the laboratory environment.[32] The isolate grew steadily in the presence of carbofuran that was added after every 10-day interval. The carbofuran was in constant supply, hence supporting the continued bacterial growth as shown by a steady increase in optical density.

The increase in optical density over a period of time indicated increase in the growth of isolate. There was a general increase in growth of microorganisms with few fluctuations. A slight decrease in growth observed at 10-day intervals before the spike may be attributed to decreases in concentration of carbofuran being utilized as an energy source by the bacteria. Observed subsequent increases in growth are due to acclimation of carbofuran degraders in MSM after its addition. No growth was observed in the control spiked with carbofuran severally at 10-day intervals without inoculation with isolated cultures.

The observed trend was a result of continual supply of carbofuran spiked after every 10 days which the isolate was utilizing for its growth. This means that the isolate had developed enzymes capable of degrading carbofuran,[25] thereby using it as a carbon and/or nitrogen and energy source[33] for growth. Hence, the increase in optical density was continually observed after every spiking.

The graph in Figure 1b where carbofuran was spiked once at the beginning of the experiment showed that isolate 2 declined in growth in the first eight days, grew exponentially to a maximum on the 21st day, and then started declining up to the 56th day. The decline in growth for the first eight days was attributed to the acclimatization of bacterial cells to start exploiting new carbofuran environmental conditions.[34] The bacteria had adapted to carbofuran by developing enzyme capable of utilizing it[25] for growth, which explains increase in optical density to a maximum of 0.063 on the 21st day. The decline after 21st day indicated that the spiked carbofuran was exhausted by the bacteria during the growth and cell death was being experienced as a result of the depletion of carbon or nitrogen source from carbofuran.[21] Similar trends were observed with isolates 3, 4 and 8.

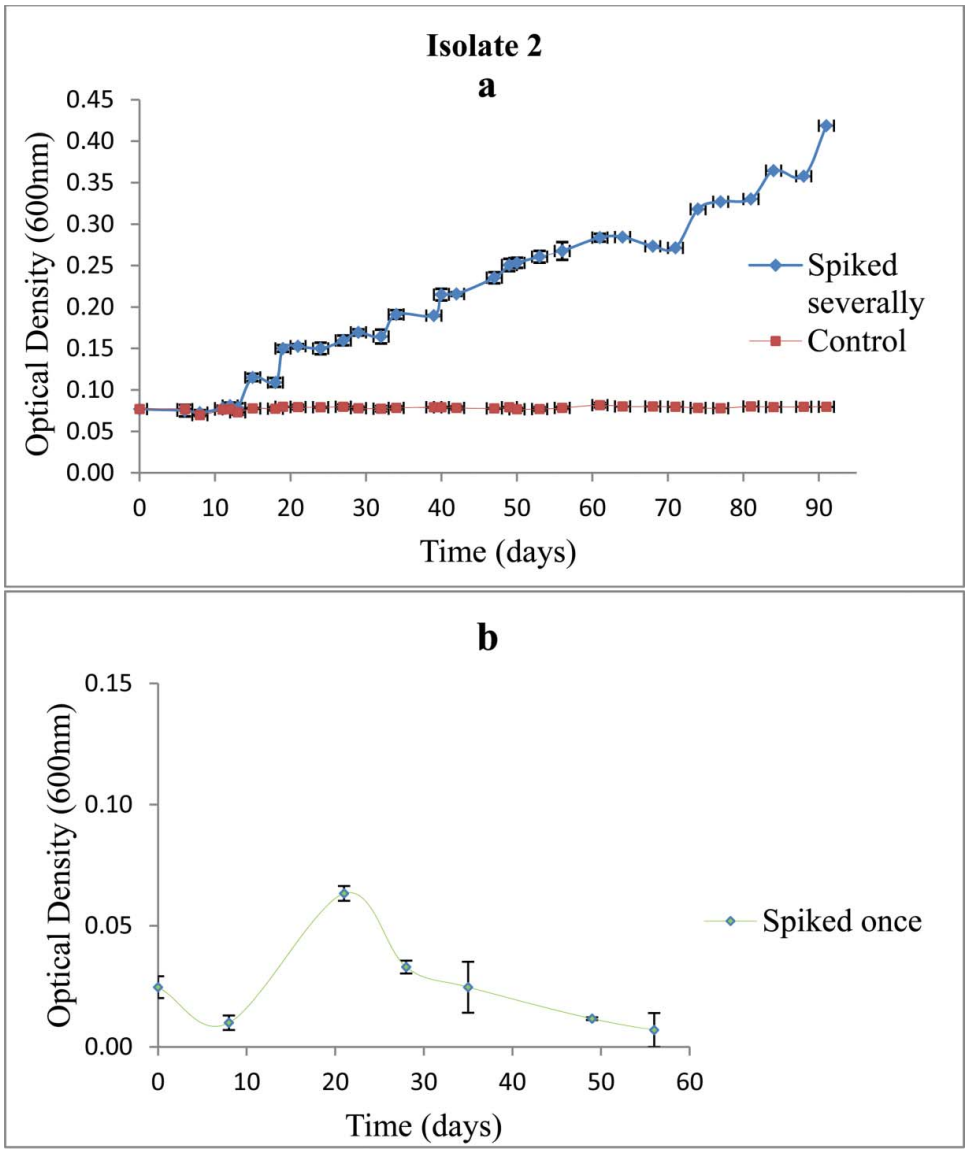

Fig. 1. Growth curves of isolate 2 incubated at 32°C in MSM supplemented with 0.475 mM of commercial carbofuran: (a) For 90 days and spiked severally at 10-day intervals and un-inoculated control under the same conditions, and (b) for 56 days and spiked once. Each point represents the mean value from the triplicate data. Error bars on the data represent ±SD of the three replicates.

These results show that the isolates degraded carbofuran. Carbofuran degradation was associated with an increase in the optical density (OD_{600}) of cultures, which indicated that the isolates used carbofuran as the sole carbon and nitrogen source for growth.

Degradation and HPLC analysis of carbofuran and degradation products

Carbofuran and carbofuran phenol standard peaks

Figure 2 shows the HPLC chromatogram of carbofuran and carbofuran phenol standards. The peaks at 12.5 and 15.3 min represented carbofuran and carbofuran phenol peaks respectively. The peak at 3.9 min was attributed to the solvent. These standard peaks were used to identify the peaks of the compounds in the liquid culture extracts.

The calibration curves for both carbofuran and carbofuran phenol were drawn using reference standard solutions of varied concentrations in methanol. For carbofuran, the concentrations ranged from 0 to 100 μg mL^{-1}, while that of carbofuran phenol ranged from 0 to 50 μg mL^{-1}. Peak areas for every injection of the standards were plotted against the concentrations of the standards. The graphs gave a correlation coefficient (R^2) of 0.9918, and the equations of the lines obtained for carbofuran and carbofuran phenol, respectively, were used for

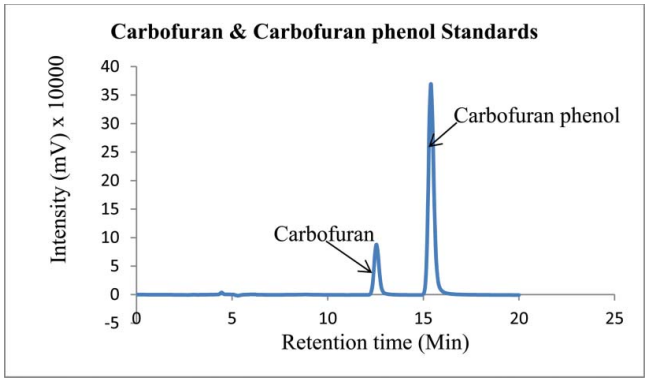

Fig. 2. HPLC chromatogram of a standard mixture of carbofuran ($R_t = 12.5$ min) and carbofuran phenol ($R_t = 15.3$ min).

the quantification of compounds in the samples. A correlation coefficient above 0.99 showed a good linearity of the HPLC detector response within specified ranges, making the calibration curve useful for quantification.[35]

Carbofuran degradation by isolate 2 in liquid cultures

The residual carbofuran and metabolites from liquid culture extracts of isolate 2 incubated for 0 to 10 days was determined by HPLC (Fig. 3). Carbofuran was detected at a retention time of 12.5 min and carbofuran phenol at 15.3 min. From Figure 2, the HPLC chromatogram peaks that matched the respective retention time periods of carbofuran and carbofuran phenol standard peaks from liquid culture extracts were attributed to carbofuran and carbofuran phenol respectively.[36] On day 0 of incubation, the liquid culture extract contained only carbofuran peak (Fig. 3a). This means that no degradation had taken place in the liquid culture, hence no metabolite peak was observed. On day 2, the isolate had started degrading carbofuran and a small peak of the metabolite, carbofuran phenol, was observed as well as a reduction in carbofuran peak (Fig. 3b). Reduction in carbofuran peaks with subsequent increase in carbofuran phenol peaks was observed on 6th, 8th and 10th day of incubation, with the 10th day having a much smaller carbofuran peak than carbofuran phenol peak (Figs. 3 c, d and e).

Growth of isolate 2 on carbofuran

Time course utilization of carbofuran as a sole carbon and nitrogen source for growth by isolate 2 was quantified. The growth of the isolate was monitored against change in the concentration of residual carbofuran and carbofuran phenol (Fig. 4). The concentration of residual carbofuran and the metabolite, carbofuran phenol, was determined in liquid cultures against their standard calibration curves.

The optical density measurements and the residual carbofuran analysis were done on the 0th, 2nd, 6th, 8th and 10th days. The isolate showed a lag phase of two days, and then grew exponentially from the optical density (OD_{600}) of 0.054 to a maximum of 0.728 by the 10th day. This could be explained by the fact that by the 10th day the residual carbofuran was very low (1.96 μg mL^{-1}), almost near depletion. Thus, 98% of carbofuran had been degraded in liquid cultures by the 10th day.

On the other hand, the metabolite, carbofuran phenol, was formed by the 2nd day and its concentration increased with time in the liquid culture throughout the 10-day incubation period, while the concentration of residual carbofuran decreased over time. The decrease in residual carbofuran is attributed to the utilization of carbofuran by isolate 2 to form carbofuran phenol as a degradation product.

Characterization of the isolates

Physical and morphological characterization of isolates

The isolates had similar physical and morphological characteristics (Table 1). The colony color was white-cream, the margin was wavy and the texture was smooth and flat elevation for all isolates. Gram staining reaction showed blue color on the cells, indicating that the isolates were gram positive bacteria and rod-shaped when observed under light microscope. Studies indicate that such physical characteristics, as observed in the isolates, are associated with gram positive rod-shaped bacteria.[37–43]

Molecular characterization

The taxonomic classification of the isolated strain was performed using 16S rRNA sequences of their genomic DNA since it is the most conserved region[44] for the identification of bacteria. Phylogenetic analysis of carbofuran degrading bacteria was done, and a phylogenetic tree was obtained (Fig. 5).

Partial 16S rRNA sequencing showed that isolates 2, 3, 4 and 8 were similar strains of *Bacillus sp*. as shown by the phylogenetic analysis. Linking the 16S rRNA sequences of isolates 2, 3, 4 and 8 with those in the GenBank databases showed close relationship with *Bacillus cereus* and *Bacillus thuringiensis*. The close relationship was indicated by high bootstrap values ranging from 99% to 100% (Fig. 6). Together with the

Table 1. Physical and morphological characteristics of isolates.

	Physical Characteristics				*Morphological*	
Isolate	*Color*	*Margin*	*Texture*	*Elevation*	*Shape*	*Gram stain*
2	White-cream	Wavy	Smooth	Flat	Rod	+
3	White-cream	Wavy	Smooth	Flat	Rod	+
4	White-cream	Wavy	Smooth	Flat	Rod	+
8	White-cream	Wavy	Smooth	Flat	Rod	+

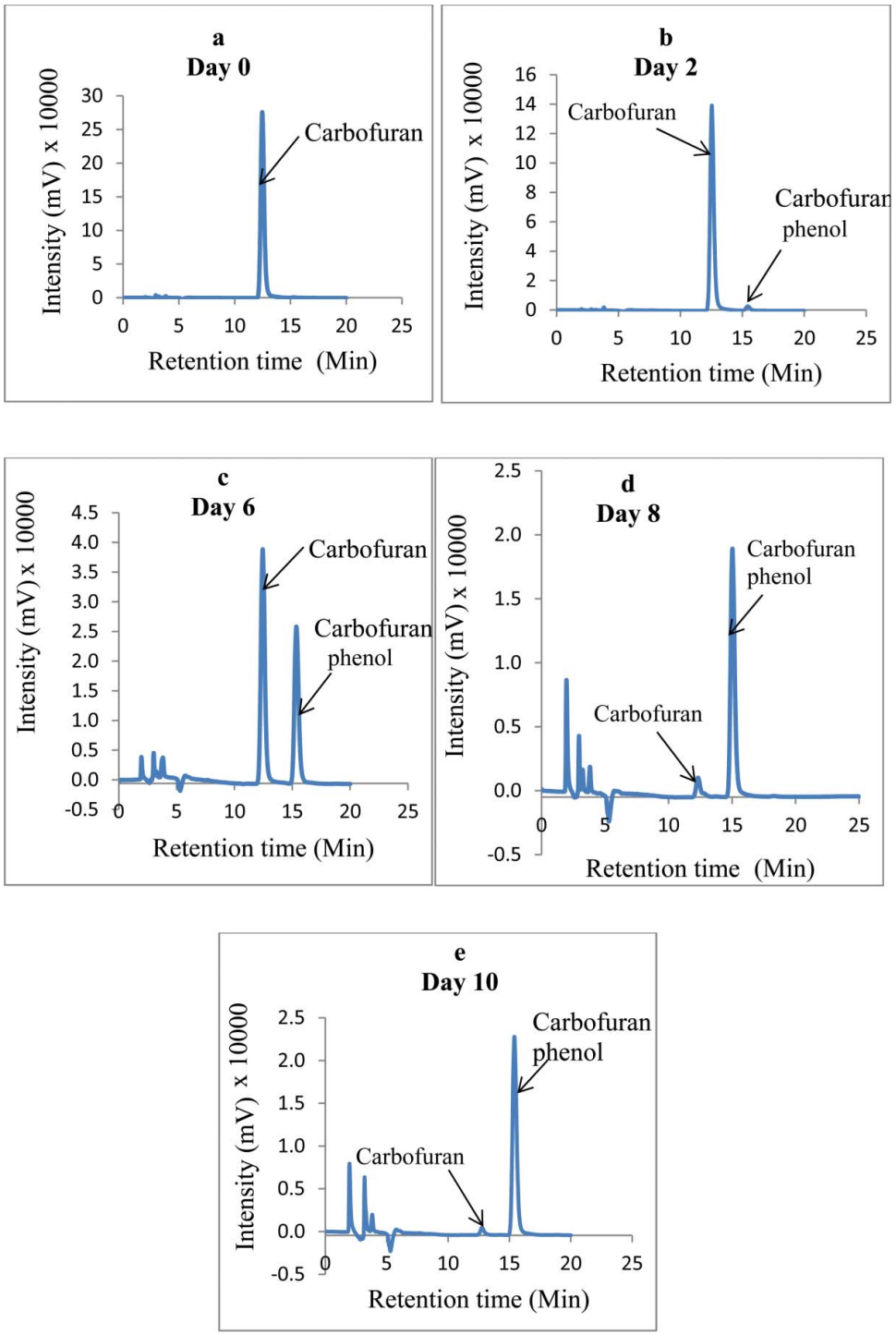

Fig. 3. Chromatograms showing carbofuran and its metabolite, carbofuran phenol, peaks at various days (0, 2, 6, 8 and 10) of incubation of isolate 2 at 32°C in MSM supplemented with 100-μg mL^{-1} carbofuran.

physical–morphological characteristics supporting the similarity of isolates (Table 1), they were assigned as *Bacillus cereus* or *Bacillus thuringiensis* strains. Further, given the very close percentage similarities, it was very difficult to know exactly whether the isolates were either *Bacillus cereus* or *Bacillus thuringiensis*, hence the above assignment.

Bacillus cereus is very closely related to *Bacillus thuringiensis* and appears as a single species; some literature suggests that they be treated as a single species on conceptual grounds[45,46] because of the difficulty of discriminating the strains.[47,48] Although there is no consensus as to whether they should be considered as specialized variants of one species or distinct species.[49] Also, their genomic

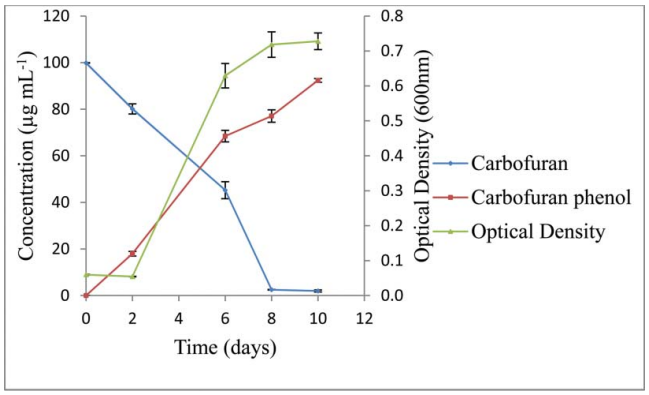

Fig. 4. Growth and carbofuran degradation curves for isolate 2 incubated at 32°C in MSM supplemented with 100 μg mL^{-1} of carbofuran. Each point represents the mean value from triplicate data. Error bars on the data represent ±SD (standard deviation) of three replicates. When the error bar is not visible, it is within the data point.

data sequence are very closely related[50] to their 16S rRNA gene sequence, sharing more than 99% similarity,[51] hence good correlations with the results of this study.

Characterization of *Bacillus cereus* and *Bacillus thuringiensis* capable of degrading diesel fuel and other xenobiotic compounds has been reported by other authors. For example, Kebria et al.[39] isolated a strain from the soil of oil refinery field with more than 98% similarity to *Bacillus cereus* or *Bacillus thuringiensis*. Reddy et al.[52] characterized from soil an isolate which was 100% similar to *Bacillus cereus* 149 and very closely related to *Bacillus thuringiensis* and other *Bacillus* species. This strain showed ability to efficiently metabolize and degrade 2-picoline. Similarly, Shukor et al.[53] characterized an isolate from soil which was 100% similar to *Bacillus cereus* and capable of degrading acrylamide. Liu et al.[54] also isolated a chlorpyrifos-degrading strain having 98% similarities to *Bacillus cereus*.

In addition, Singh et al.[55] isolated from soil a malathion-degrading strain which showed 99% similarities to *Bacillus cereus* and *Bacillus thuringiensis*. Kamal et al.[56] and Kumari et al.[57] reported isolated strains of *Bacillus thuringiensis* MOS-5 and *Bacillus sp*., respectively, capable of degrading malathion. Other characterization of soil microbes include *Bacillus thuringiensis*, capable of degrading polychlorinated biphenyls (PCBs),[58] and *Bacillus sp*. strain PHN 1, capable of degrading phenolic compound p-cresol.[59]

In spite of several reports of characterization of *Bacillus thuringiensis* and *Bacillus cereus* degradation activity on other xenobiotic substances as discussed above, there are no reports on the degradation of carbofuran by these microbes to date. Therefore, this study presents for the first time the characterization of *Bacillus cereus* or *Bacillus thuringiensis* as novel carbofuran degraders.

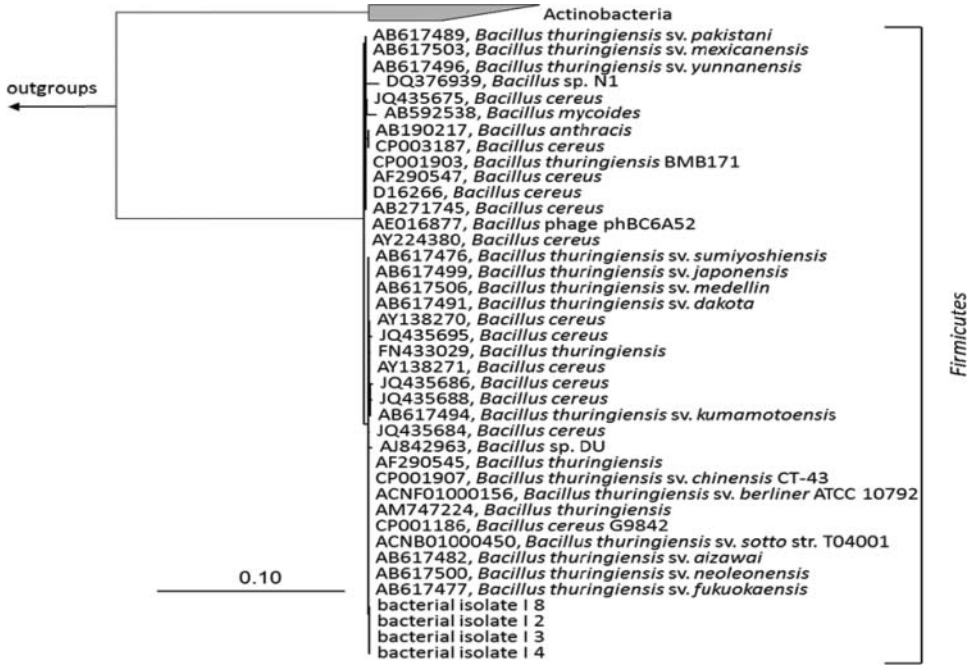

Fig. 5. Phylogenetic tree showing the position of carbofuran-degrading isolates 2, 3, 4 and 8 with reference organisms from the genus *Bacillus*. The bar indicates the estimated substitution per nucleotide position.

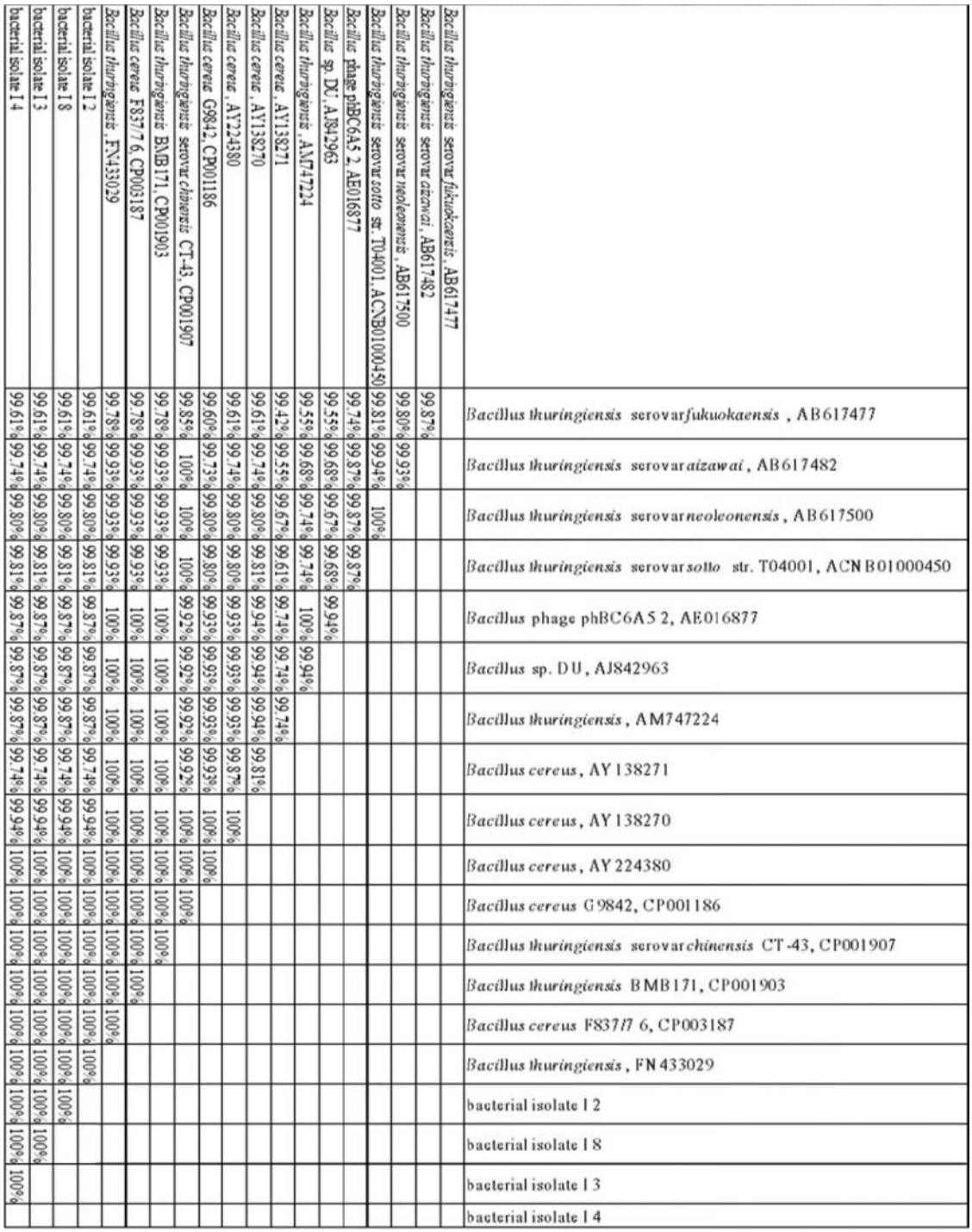

Fig. 6. Phylogenetic matrix showing percentage relatedness of *Bacillus cereus* and *Bacillus thuringiensis* species and the isolates.

Conclusions

This study identified *Bacillus cereus* or *Bacillus thuringiensis* as carbofuran degraders. The isolation of carbofuran-degrading bacteria from the soils of Nzoia River Basin indicates the presence of pesticide-degrading bacteria in rice fields with a history of carbofuran application. Carbofuran phenol was identified as the only metabolite formed. However, in order to fully understand carbofuran degradation in these soils, a complete elucidation of degradation mechanisms involved should be carried out. The identified native bacterial strain can be developed and used for bioremediation of carbofuran-contaminated environment.

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