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Isolation of chlorpyrifos-degrading bacteria from pesticide-polluted agricultural soil in Amansea, Anambra State, Nigeria

***Emeribe, Chiemeka Elochi and Onuorah, Samuel Chinedu**

Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University Awka Anambra State, Nigeria.

***Corresponding Author: ce.emeribe@unizik.edu.ng ; +2348163620586**

ABSTRACT

The extensive use of chlorpyrifos has been one of the major causes of pollution of soil and ground water. Chlorpyrifos-degrading bacteria from pesticide-polluted agricultural soil in Amansea, Anambra State, Nigeria was isolated. In this study, two chlorpyrifos degrading bacteria were isolated from Agricultural soil using mineral salts medium and characterized based on their physiological, biochemical, morphological and 16S rRNA sequencing as strains of *Bacillus cereus* ST06 and *Chryseobacterium* sp. 6024. In conclusion, two isolates (*Bacillus cereus and Chryseobacterium* sp.) capable of utilizing Chlorpyrifos as the only source of carbon and energy were identified. The bacteria consortium degraded 79% and 78% of 20mg/l and 60mg/l chlorpyrifos respectively better than the individual isolates. The bacteria consortium showed better results and possess potential to be used in biodegradation of 20mg/l and 60mg/l Chlorpyrifos than the individual isolates.

Keywords: Isolation, chlorpyrifos-degrading, bacteria, pesticide-polluted, agricultural, soil, Amansea, Anambra State, Nigeria

INTRODUCTION

Chlorpyrifos (O, O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate) is a broad spectrum moderately toxic organophosphorous insecticide [1,2] and acaricide, and is widely used for pest control on grain, cotton, fruits and vegetable crops, as well as lawns and ornamental plants [3] against rice leaf moth, plant hoppers, gall midge, wheat army worm, cotton boll worm, aphid and red spider [4]. Chlorpyrifos (CP) was first commercialized in the USA by Dow Chemical Co. (Midland, MI, USA) in 1965 [5]. The widespread and continuous application of chlorpyrifos has caused several toxicological, environmental contamination and residue problems, which seriously threaten human health, ecological and environment security [6]. The manufacturing and formulation process of chloropyrifos also generate waste that contain the compound [7]. Previous studies have shown that chlorpyrifos not only has acute and chronic toxicity to mammals, aquatic organisms and other non-target organisms, but also has neurotoxicity, genotoxicity and other multiple toxic influences [8]. A systematic review of data published between 2006 and 2018, supplemented by mortality data from WHO, found that there were approximately 740,000 annual cases of unintentional, acute pesticide poisoning (UAPP), with 7,446 fatalities and 733,921 non-fatal cases. On this basis, it is estimated that 385 million cases of UAPP occur annually world-wide including 11,000 fatalities [9]. According to the world health organization (WHO), about 1000,000

human being are affected by acute poisoning by contact with pesticide. Over 150,000 people die each year from pesticide poisoning. Most deaths result from self-poisoning by ingestion, rather than occupational or accidental exposures, which are typically topical or inhalation. Severe pesticide poisoning is more common in rural lower- and middle-income countries where pesticides are widely used in small holder agricultural practice and therefore freely available [10]. Work related contact with pesticides could be behind 70% of these mortalities. Furthermore, constant contact to lower pesticides dosages was associated with a group of syndromes in the medium and long term, involving numerous tumors and nervous system disorders [11]. WHO warns that every year, as many as 2.5 million people worldwide suffer from acute poisoning with pesticides and 0.2 million people die. (Atabila et al., 2022; George et al., 2014. Chlorpyrifos has also become a potential public health concern as it considered to be genotoxic, damage DNA and affect neurodevelopment in children [12]. Due to these problems, development of technologies that guarantee their elimination in a safe, efficient and economical way is paramount. A lot of significant studies related to the decontamination of soil and water pollution by chlorpyrifos were reported, including photochemical degradation using physical and chemical methods, nanometal materials or UV/H2O2 catalytic degradation [13]. Compared with these methods, biodegradation is an efficient and environmental friendly method to decontaminate soil and water contaminated by chlorpyrifos [14]. Biodegradation is a natural process by which organic chemicals in the environment are converted to simpler compounds, mineralised and redistributed through elemental cycles such as the carbon, nitrogen and sulphur cycles [15]. The biod egradation of organophosphate pesticide by soil microorganisms has been reported by many researchers and its biodegradation has helped to solve the problem of recalcitrance in water and soil.

[16] isolated *Enterobacter* B-14, a strain which could degrade chloropyrifos. [17], isolated *Bacillus cereus* MCAS02 and [18], isolated *Stenotrophomonas* sp and *Sphingomonas* sp respectively, which could utilize chloropyrifos as the source of carbon and phosphorous for their growth. Bacterial enzymes has also been implicated in chlorpyrifos degradation and one of the most important enzymes Phosphotriesterase (PTE) was first found in *Pseudomonas diminuta* MG and *Flavobacterium* sp which was able to hydrolyze organophosphatepesticide [19]. In the environment, chlorpyrifos has been reported as having from 10 to 120 days of half-life in various soil and major degraded non-toxic end product is 3,5,6-trichloro-2-pyridinol [20]. 3,5,6-Trichloro-2-pyridinol (TCP) is the main end metabolite of chlorpyrifos obtained by biodegradation [21]. 3,5,6-trichloro-2-pyridinol has been listed as a persistent and mobile organic pollutant by the U.S. Environmental Protection Agency (EPA) due to its higher solubility in water and longer half-life (65–360 days) than its parent compound chlorpyrifos [22]. It has reported that a combination of chlorpyrifos and TCP could cause greater toxic effects than either alone [23]. However, most bacteria can only degrade Chlorpyrifos but not TCP. Only a few bacteria are capable of simultaneously degrading both chlorpyrifos and TCP [24]. Considering that Chlorpyrifos is one of the most commonly applied insecticides for control of pests and insects, it is therefore of great significance to select bacteria strains that can efficiently degrade chlorpyrifos. So the aim of this study was to evaluate the biodegradation of Chlorpyrifos insecticide by bacteria isolated from agricultural soil in Amansea, Anambra State, Nigeria.

Aim of the Study

The aim of this study was to isolate chlorpyrifos-degrading bacteria from pesticide-polluted agricultural soil in Amansea, Anambra State, Nigeria.

MATERIALS AND METHODS

Study Area

The study was carried out in Ukukwa Amansea, Awka North Local Government Area of Anambra State, Nigeria. Amansea is in the tropical rainforest region and is located between latitude 6°16' 30" N and longitude 7° 07'30"E. Awka North has an estimated population of 112,192 in 2006 and 159,900 projected by Nigeria statistic of bureau 2022 (Nigeria Bureau of Statistic, 2022). The land mass is 460.2/km^2. Awka north consists of low-lying plains of agricultural land. It has derived savanna vegetation resulting from human activities. The people are mainly farmers and itinerant traders. Agricultural crops include yam, cocoyam, cassava, maize, fruits and vegetables.

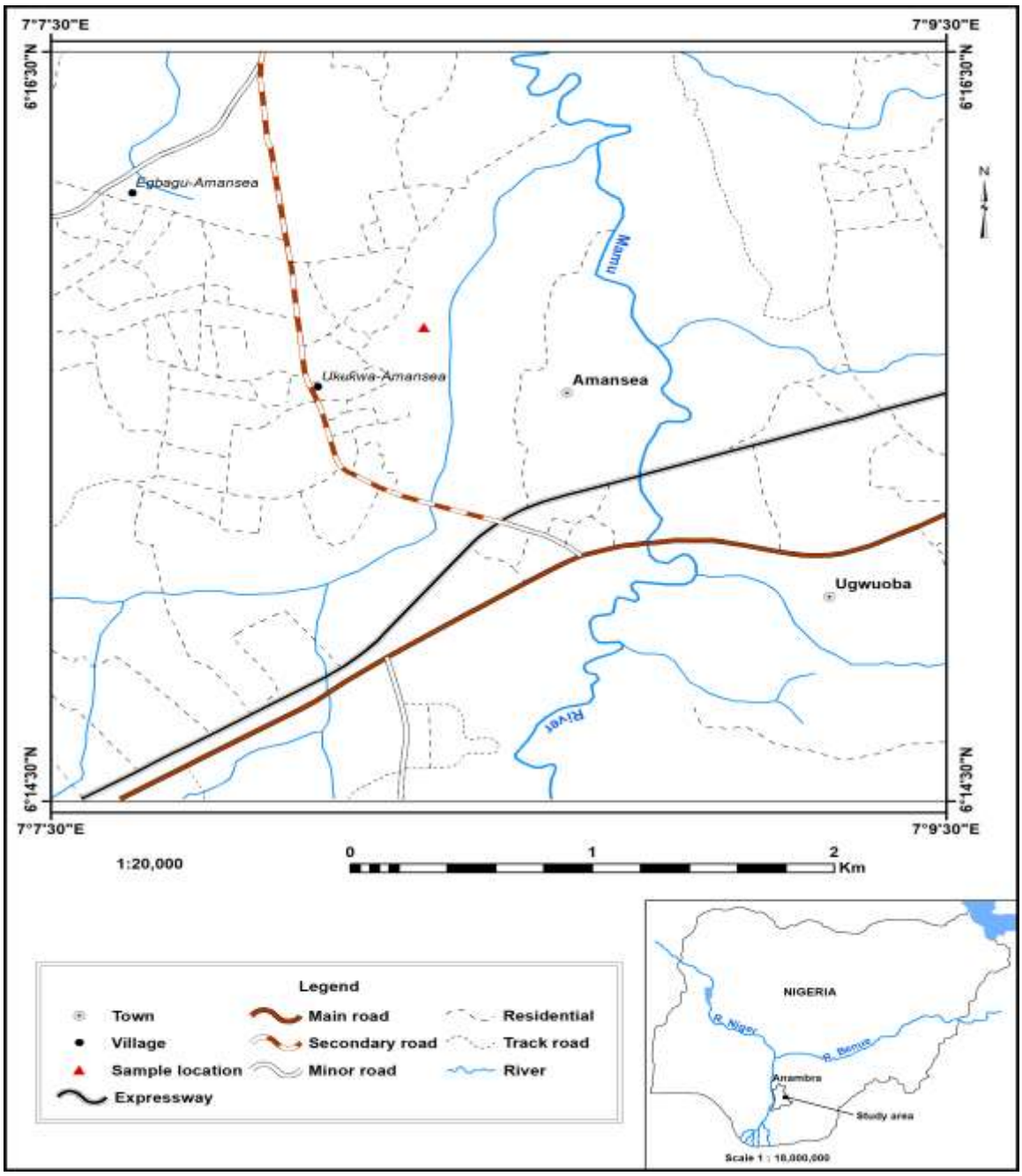

Figure 1: Geographic map Anambra state showing Awka North

Source: GIS Lab, Department of Geography and meterology Nnamdi Azikiwe University Awka Anambra State, Nigeria (2023).

Study Design and Sampling Procedures

The study was a community based cross-sectional survey conducted between January and March 2022 in one of the five villages. Sample selection involved the use of in person interview of the farmers that uses the insecticide for pest control. Within the selected farmers who pledged to have used the Chlorpyrifos insecticide constantly for at least a year were selected into the final sample. One farm was eligible for the study.

Insecticide

Commercial grade of chlorpyrifos commonly known as Perfect Killer® (containing 20g active ingredient/L, Emulsifiable concentrate 20%) and manufactured by Nantong Jinling Agrochemical co, LTD China was purchased from Eke-Awka Market Anambra State, Nigeria.

Media

The mineral salts medium (MSM) described by Benslama and Brulahruf (2013) containing(g/l) 1.5g of KH_2PO_4, 0.5g of NaCl, 0.6g of Na_2HPO_4, 2g NH_4SO_4, 0.2g $MgSO_47H_2O$, 0.01 $Cacl_2$ and 0.001g $FeSO_4.7H_2O$ was used for the isolation of bacteria. Agar agar, peptone water, nutrient agar and Simmon's citrate agar were also used.

Sample collection

Soil sample (1Kg) from a contaminated agricultural soil in ukukwa Amansea was collected. The properties of the soil were pH 6.5, 3.0% moisture content, 4.4% organic carbon. From the in person interview gotten from the farmer, it has been more than 2 years the farmer started using chlorpyrifos insect to control pest. The farmer confirmed obtaining a bountiful harvest during every harvesting period. Therefore, the soil has been contaminate with chlorpyrifos insecticide for more than 2 years. Four soil sample was randomly collected from all side of the Rhizosphere at a depth of 15cm. The soil sample was sorted, mixed and put into a sterile polyethylene bag and then conveyed immediately to the Laboratory of the Department of Applied Microbiology and Brewing Nnamdi Azikiwe University Awka for further analysis.

Isolation of Chlorpyrifos-degrading Bacteria

The soil sample was air-dried and sieved using a 2mm mesh. 5g of the soil sample was added into a 250ml Erlenmeyer flask containing an autoclaved mixture of 100ml of mineral salts medium and 0.05ml of Perfect killer ® (10mg/l of chlorpyrifos) at 121^OC for 15 minutes. This set up was done in triplicate. The flasks were incubated on a rotary shaker at 150 revolution per minute for 7 days at 30^OC. Isolation was done by inoculating 0.1ml of the broth culture on mineral salts medium agar supplemented with 10mg/l chlorpyrifos using the pour plate method. The plates were incubated at 30^OC for 72 hours [25].

Maintenance of Chlorpyrifos-degrading Isolates

Morphologically distinct colonies on the plate were selected and sub cultured on nutrient agar to obtain pure cultures at 72 hours of incubation. The pure cultures were stored on nutrient agar slant at 4^OC for further studies.

Identification of Isolates

Culture of the isolates inoculated on nutrient agar was observed morphologically after 24hours incubation. To identify the bacterial isolates, biochemical tests like gram staining test, motility test, catalase test, oxidase test, methyl red test, indole test, citrate utilization test, sugar fermentation test, urease production test, voges proskaeur test, gelatin hydrolysis test, spore test and nitrate reduction test were conducted as described by [26]

Gram staining

Thin smears of the 24 hours old bacteria culture were fixed on clean grease-free microscopic slides by applying heat for 5 seconds. The slides were flooded with 2% crystal violet stain for 1 minute and then washed off with tap water. The slides were then flooded with 1% Lugol's iodine solution for 1 minute to help fix the primary dye to Gram positive bacterium. The smears on the slides were then decolourized with 95% alcohol for 5 seconds and washed off immediately, then counterstained with 1% safranin solution for 30 seconds before washing off and blotted to dry. One drop of immersion oil was added to slides and viewed under X100 Objective lens to enhance resolution and avoid loss of light when light passes through the glass. Bacteria that retained the crystal violet were designated Gram positive. The ones that retained safranin stain were designated Gram negative.

Motility test

A loopful of the culture suspension from a 24 hours old culture was placed on a clean grease-free glass slide and a cover slip placed over it and sealed with plastecine. The slides were inverted (hanging drop method) and observed under the high power lens (X40) for motility.

Catalase test

Three drops of 3% hydrogen peroxide was placed on test colonies on grease-free glass slide and observed for gas bubbles. The appearance of prompt gas bubbles within 10seconds indicated a positive catalase test.

Oxidase test

With a sterile wire loop two drops of freshly prepared oxidase reagent was placed on a piece of whatman No 2 filter paper. Then using a sterile wire loop, a 24 hours culture was picked from a nutrient agar medium and smeared on the paper. A purple colour within 10 seconds indicated a positive test.

Methyl red test

Pure cultures of the organisms were inoculated into glucose phosphate peptone broth and incubated at 37^{O}C for a minimum 48 hours. 2 drops of methyl red reagent was added and pink colour change observed indicated a positive reaction.

Voges-proskaeur test

One (1) ml of 40% potassium hydroxide and 3ml of 5% alcohol∝- naphtol was added to a 5 days old culture of the negative methyl red test organism in glucose-phosphate peptone medium. A bright pink colour indicated a positive result.

Citrate utilization test

The test organisms were inoculated on Simmon's citrate agar slopes for 24 hours at 25^{O}C (this medium contains bromothymol blue as indicator). Colour change from green to blue indicated a positive result.

Sugar fermentation test

Peptone water containing 1% solution of fructose, lactose, sucrose, mannitol, glucose, maltose and galactose was prepared. Phenol red was added as indicator for acid production. Then Durham tubes were inserted inversely into the tubes and sterilized at 115^{O}C for 10 minutes. The test organisms were inoculated into the tubes and incubated at 37^{O}C for 24 hours. A red or pink colour indicated acid production while bubbles or space in the Durham's tubes indicated gas production.

Urease production test

Urea agar slopes were inoculated with the test organisms and then incubated at 37^{O}C for 24 hours. A pink colour indicated urease production.

Indole test

Test organisms were inoculated into peptone water and incubated at 37^{O}C for 96 hours. 3 drops of Kovac's reagent were added with gentle shaking and the development of a pink colour in the alcohol layer indicated a positive result.

Gelatine hydrolysis

Test organisms were inoculated onto nutrient gelatin tubes for 7 days. The liquefaction of gelatin after chilling the gelatin tubes in the freezer indicated a positive result while solidification of the gelatin indicated a negative result.

Nitrate reduction test

Test organisms were inoculated into 5ml of sterilized indole nitrate medium (nitrate broth) and incubated for 96 hours at 37^{O}C. 1ml of test reagents (sulphanilic acid and ∝-naphthylamine) was added. The development of a red colour within 2 minutes indicated the presence of nitrate.

Spore test

The test organisms were smeared on clean grease free slide; air dried and then was heat fixed for 8 seconds. The slides were flooded with primary stain malachite green and steam for 5 minutes. It was Allow to cool then rinsed with tap water. The slides were counter-stained with safranin for 30 seconds. The slides were rinsed with tap water and allowed to dry. The slides were viewed under X100 objective lens using immersion oil to enhance resolution and avoid loss of light when light passes through the glass. The isolate that retained malachite green indicated positive result.

Molecular Identification of Bacteria Isolates

Molecular identification was done for proper confirmation of the bacterial isolates in three stages namely DNA Extraction, PCR and 16S rRNA sequencing. Bacterial DNA was extracted by the method of Quick-DNATM Miniprep Plus Kit [27]. The 16S rRNA gene of the strains was amplified using two universal primers 27F:5'-AGAGTTTGATCGTGGCTCAG-3' and 5'- 1492R TACGGTTACCTTGTTACGACTT -3'. The PCR was performed with Eppendorf nexus gradient Mastercycler (Germany). The amplification conditions for the PCR was as follows: Initial denaturation for 30 seconds at 94^{O}C, followed by 35 cycles of denaturation at 94^{O}C for 20 seconds, primer annealing at 56^{O}C for 45 seconds and strand extension at 68^{O}C for 1 minute. Final extension at 68^{O}C for 5 minutes. The PCR fragments were purified on 2% agarose gel electrophoresis and visualized under Bio Doc-It imaging system (Ingeniuss UK). The genes were sequenced by the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit. The genes were compared with most similar sequences available in the Genbank nucleotide database using the NCBI BLAST Program.

Biodegradation Experiment

The inocula used for all the experiments were prepared using viable count method [28] by growing loopful of the bacterial isolates in separate 250ml Erlenmeyer flasks containing 100ml of mineral salt medium (MSM) conttaining 10mg/l chlorpyrifos at 120 revolutions per minute (rpm) and 30^{O}C on a

rotary shaker for 24 hours.1ml of the 24 hours culture containing $1.3X10^4$ CFU/ml (determined by viable count method) was used as inoculum for the biodegradation process.

Data Analysis

Statistical analysis of the data generated was carried out to indicate Arithmetic mean significant differences between the treatments using Statistical Package for Social Sciences (SPSS) version 23.0. Two ways analysis of variance (ANOVA) was used to determine if 20mg/l and 60mgl of chlorpyrifos have an effect on biodegradation potential of chlorpyrifos-degrading bacteria. Graphs and tables were also used for data presentation.

RESULTS

Isolation and Identification of Bacterial Isolates

The result showed that two morphologically distinct bacterial colonies were observed and characterised on the mineral salt agar supplemented with chlorpyrifos are shown in Table 1. They were identified as strains of *Bacillus cereus* ST06 and *Chryseobacterium* sp. 6024. The colonial morphology of both isolates are different. *Bacillus cereus* colony was circular, creamy, entire and opaque with rough edge. *Chryseobacterium* sp. colony was Rhizoid, yellow and smooth. *B. cereus* was characterized as gram positive and catalase positve bacterium, while *Chryseobacterium* sp. was characterized as gram negative and catalase positive bacterium. Both organisms are non lactose, non galactose and non mannitol fermenters. The result of the spore test showed that *Bacillus cereus* produce spore, the implication is that the spore could be invovled in expressing important enzymes for biodegradation purpose. The two bacterial isolates were identified by 16S rRNA gene amplification using thermocycler and were sequenced. The Partial 16Sr RNA gene sequences were compared with that of referred strains gene sequences in the Genbank. The FASTA Sequence of the 16S rRNA sequences (Figures 1 and 2.) of the bacterial strains showed that the two isolates are similar with 70.06% identity and 90% similarity according to Clustal omega sequence alignment.

Table 1: Morphological, Cultural and biochemical characteristics of isolated bacteria

Test	Isolate A1	Isolate A2
Colony morphology	Circular,creamy, entire,opaque and rough edge	Rhizoid,light yellow, smooth colonies
Grams reaction	Gram +ve rod	Gram –ve rod
Methyl red	-ve	-ve
Voges proskauer	+ve	-ve
Indole	-ve	+ve
Motility	+ve	-ve
Citrate	+ve	+ve
Oxidase	-ve	+ve
Gelatin hydrolysis	+ve	-ve
Urease test	-ve	-ve
Spore test	+ve	-ve
Nitrate reduction	+ve	-ve
Catalase	+ve	+ve
Lactose	-ve	-ve
Glucose	+ve	-ve
Sucrose	+ve	-ve
Maltose	+ve	-ve
Galactose	-ve	-ve
Fructose	+ve	-ve
Mannitol	-ve	-ve
Growth on MacConkey	-ve NLF	-ve NLF
Identity	*Bacillus cereus*	*Chryseobacterium* sp.

Key: NLF= Non Lactose fermenter

TGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTC

TAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATC

CTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAA

GTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTA

CACATGGAATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAA

TGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCA

CCTGCGCGCGCTTTACGCCCAATTATTCCGGATAACGCTTGCCACCTACG

TATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTA

CCGTCAAGGTGCCAGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACA

GAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTCCGTCA

GACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTC

TGGGCCGTGTCTCACCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTAC

GGATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGACGC

GGCTCCATCCATAAGTGACAGCCGAAGCCGCCTTTCAATTTCGAACCATG

CGCCTCAAAACGTTATCCGGTATTAGCCCCGGCTTCCCGGAGTTATCCCC

ACTCTTATGGGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGTCACT

TCATAAGAGCAACTCTTAATCCATTCCCTCGACTTGATGTATCAGGACSC

CGCCAGCGCTCATCTTGAACCATGATCAAACTTTAGGC

Figure 1: Partial sequence of *Bacillus cereus* ST06

TAACTTATCACTTTCGCTTAGTCTCTGAATCCGAAAACCCAAAAACGAGT

TAGCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCC

CCACGCTTTCGTCCATCAGCGTCAGTTGTTGCTTAGTAACCTGCCTTCGC

AATTGGTGTTCTAASTAATATCTATGCATTTCACCGCTACACTACTTATT

CCAGCTACTTCAACAACACTCAAGACCTGCAGTATCAATGGCAGTTTCAC

AGTTGAGCTGTGAGATTTCACCACTGACTTACAGATCCGCCTACGGACCC

TTTAAACCCAATAAATCCGGATAACGCTTGCACCCTCCGTATTACCGCGG

CTGCTGGCACGGAGTTAGCCGGTGCTTATTCTTATAGTACCTTCAGCTAC

CCTCACGAGAGTAGGTTTATCCCTATACAAAAGAAGTTTACAACCCATAG

GGCCGTCGTCCTTCACGCGGGATGGCTGGATCAGGCTCTCACCCATTGTC

CAATATTCCTCACTGCTGCCTCC

Figure 2: Partial sequence of *Chryseobacterium* sp. Strain *6024*

DISCUSSION

In this study, two chlorpyrifos degrading bacteria were isolated from Agricultural soil using mineral salts medium and characterized based on their physiological, biochemical, morphological and 16S rRNA sequencing as strains of *Bacillus cereus* ST06 and *Chryseobacterium* sp. 6024. The result showed that *Bacillus cereus* ST06 and *Chryseobacterium* sp. 6024 reached maximum growth on 20mg/l chlorpyrifos with an arithmetic mean difference of 0.23±0.20 and 0.42±0.02 respectively on day 16 than 60mg/l chlorpyrifos with arithmetic mean difference of 0.47±0.02 and 0.81±0.02 respectively, on day 20. The bacterial consortium also reached maximum growth on 20mg/l and 60mg/l of chlorpyrifos with an arithmetic mean difference of 0.21±0.31 and 0.29±0.02 on day 20 respectively. There were also significant (<0.05) difference in concentration of residual chlorpyrifos degraded by the isolates obtained from the GC-ECD curves. The bacteria consortium degraded 79% and 78% of 20mg/l and 60mg/l chlorpyrifos respectively better than the individual isolates. The bacteria consortium showed better results and possess potential to be used in biodegradation of 20mg/l and 60mg/l Chlorpyrifos than the individual isolates.

CONCLUSION

Two isolates (*Bacillus cereus and Chryseobacterium* sp.*)* capable of utilizing Chlorpyrifos as the only source of carbon and energy were identified. The bacteria consortium degraded 79% and 78% of 20mg/l and 60mg/l chlorpyrifos respectively better than the individual isolates. The bacteria consortium showed better results and possess potential to be used in biodegradation of 20mg/l and 60mg/l Chlorpyrifos than the individual isolates.

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