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Metagenomic Study and Biodegrading Capability of Bacterial Community in Monocrotophos Treated Tea Soil

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Authors' contributions

This work was carried out in collaboration between all authors. Author SB designed the study, performed the experiments and statistical analysis. Author MG performed the bioinformatics analysis, author RP carried out the HPLC analysis. Authors PC, AZ and HS managed the analyses of the study. Author TB provided critical feedback and reviewed the manuscript. Author AKB was the PI and supervised the project. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study was undertaken to reveal the diversity of bacteria in organophosphate (monocrotophos) pesticide-treated tea soil to provide new insights on monocrotophos degrading bacterial community.
Study Design: A metagenomic study of monocrotophos treated and untreated soil to isolate and identify pesticide-degrading microflora.

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Place and Duration of Study: Tea soil was collected from Borbhetta Tea Estate of Tocklai Tea Research Institute, Jorhat, Assam and the experiments were carried out from December 2015 to December 2017.

Methodology: Tea soil was enriched up to 300 ppm of monocrotophos for four weeks (TREATED sample) and 16S rRNA V3 region gene amplicon metagenomic sequencing was carried out on untreated (CONTROL) and spiked (TREATED) soil. The treated soil was cultured in mineral salt medium up to 600 ppm of monocrotophos and bacterial growth, and degrading capacity was studied for three isolated bacterial species at three different pH and identified by sequencing the 16s rRNA region. The bacterial species in the metagenome were also compared and grouped with the bacterial species in NCBI database based on the presence and absence of organophosphate hydrolase (OPH) gene.

Results: Metagenomic sequencing revealed the presence of 20 bacterial phyla distributed across 119 families and 433 genera. 147559 sequences remained taxonomically unclassified suggesting the presence of unique undescribed bacteria. Pesticide-contaminated tea soil was mostly dominant with *Acidobacteria*, *Actinobacteria*, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Nitrospirae* and *Verrucomicrobia* phyla. The number of species observed in the control and treated soil was 1036 and 910 respectively. Three species were isolated and characterised from the TREATED soil in mineral salt medium with pH 5 to 7 and their monocrotophos degradation was determined by UV-Vis microplate reader and HPLC at 2-time points.

Conclusion: Organophosphate-degrading bacteria namely, *Pseudomonas pseudoalcaligenes, Pseudomonas fluorescence, Pseudomonas putida, Serratia species, Cupriavidis metallidurans, Burkholderia species, Achromobacter xylosoxidans, Achromobacter species, Sphingomonas species, Ochrobactrum gallinifaecis, and Brucella species* were present in increased numbers in the treated sample. 52.2% monocrotophos degradation was observed in *Serratia fonticola* in 48 hrs in acidic pH of 5.

Keywords: Tea soil; metagenomics; bacterial diversity; organophosphate hydrolase; monocrotophos degradation.

1. INTRODUCTION

Microbes, essential for tea ecosystem, play an active role in maintaining biogeochemical cycles [1], plant growth promotion [2] and inhibition of destructive tea pest and pathogens [3]. Tea production is facing challenges due to pest and pathogen attack. Wide ranges of pesticides are used in tea gardens to combat pest attack. Many published reports indicated that the tea industry has been facing problems of pesticide residues and organophosphate (OP) class is one of them [4]. This class of pesticide enters the tea ecosystem and presenece of residues is reported in tea leaves as well as soil leading to health concerns amongst the tea consumers. To reduce the pesticide load in tea soil, identification of the organophosphate pesticide-degrading bacterial species is important. Monocrotophos, one of the most toxic OP pesticide, is not recommended in tea cultivation as it is highly neurotoxic, but it is widely used by some tea growers for its low price and broad spectrum activity. In a recent study, monocrotophos was widely detected in tea samples [5]. Majority of the registered bacterial products for OP pesticide degradation are based on species of *Bacillus* and *Pseudomonas,* which are conducive to degradation in alkaline soils [6] and may not work in acidic soils of tea garden. There is a need for characterisation of organophosphate pesticide-degrading bacteria from acidic tea soil (pH-4.5- 5.5). Culture-independent methods are required to probe the true diversity of OP degrading bacteria in the tea soil as only a small fraction of environmental bacteria can be isolated into pure cultures. Multiple species of microorganisms occupy one niche and there are scope to discover the dominant species that can adapt and degrade the OP pesticide. Metagenomics has aided to close the gaps and provide insights into the *in situ* microbial community including information on the identity and potential metabolic capabilities of community members [7]. Phylogenetic, genomic and biochemical characterisation of micro organisms from the environment is crucial to develop bioremediation strategies. The present study was conducted to reveal the diversity of soil bacterial community in the pesticide contaminated tea soil with the help of metagenomics and provide new insights into the monocrotophos degrading microbial community. Three species with monocrotophos degrading capacity were functionally characterised.

2. MATERIALS AND METHODS

2.1 Soil Sampling and Enrichment Culture

Tea soil was collected from Borbhetta Tea Estate of Tocklai Tea Research Institute, Jorhat, Assam, India. Soil within 0-15 cm layer was collected using sterile spatulas considered as CONTROL sample. Soil moisture content was 10.23% and temperature of 30°C. An amount of 50 g soil was further spiked with monocrotophos (Sigma) from 10 ppm to final concentration of 300 ppm at 3 days interval for four weeks at an incubation temperature of 30°C and was considered as TREATED sample.

2.2 Screening and Identification of Monocrotophos Degrading Bacteria

An amount of 5 g treated soil was used for isolation of monocrotophos degrading bacteria in Mineral Salt Medium [8] containing 100 to 600 ppm of monocrotophos. Morphologically 6 different bacteria were isolated and pure cultures were established after repeated subcultures in MSM medium at pH 5 to 7. The pH of the medium was adjusted with 1N HCl. Bacteria S1, S2 and S3 were isolated from 600 ppm of monocrotophos enriched MSM medium while S4, S5 and S6 were isolated from 300 ppm monocrotophos concentration. Pure stock cultures of the isolated strains were maintained in Nutrient Agar. Colony forming units (CFU) of the bacterial strains were observed in 10^{-7} dilution and morphology of the bacterial colonies was studied by Gram staining and microscopic analysis. Based on the bacterial optical density (OD) at 600 nm and degrading capacity of the six strains in 3ml of MSM media with 300 ppm of monocrotophos at pH 7, three bacterial strains were selected for further degradation studies at pH 5 and molecular identification. DNA was extracted using the Bacterial DNA extraction kit (Himedia) and PCR amplified to identify the bacterial strains using 16S rRNA primers (Forward 5´TGGTAGTCCACGCCCTAAAC, Reverse 5´CTGGAAAGTTCCGTGGATGT). PCR reactions were carried out in 10 µl volume containing 50 ng of total DNA, 1X Taq Buffer B (Genei), 1.25 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 mM primer and 0.1 U Taq DNA polymerase in a programmable Veriti (Applied Biosystems, USA) thermocycler. The amplification protocol included initial denaturation at 94°C for 5 min followed by 35 successive cycles of denaturation at 94°C for 30 sec. Then primer annealing was done at 60°C for 30 sec and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 7 min. The amplicons were then separated in 1% agarose gels, visualised in a Protein Simple AlphaImager MINI gel documentation system (Cell Biosciences Inc., USA).The PCR ampicon was purified using Gel extraction kit (Sigma). Forward and reverse DNA sequencing reaction was carried out using Big Dye® Terminator v3.1 Cycle sequencing kit following manufacturer's instructions on ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

2.3 Biodegradation Activity of Monocrotophos

The bacterial growth and degradation kinetics of the best three strains were studied in 5 ml of MSM broth with 300 ppm of monocrotophos in three different pH (pH 5 to 7) in a UV-Vis Microplate Reader (SkanIt Software 4.1 for Microplate Readers RE, ver. 4.1.0.43, Thermo Scientific) after 24 hrs. After 48 hrs, only the culture with pH 5 were centrifuged, and supernatant filter sterilised and percent degradation of monocrotophos was quantified using high-performance liquid chromatography (Water's HPLC equipped with Photodiode Array detector). This was separated on Sunfire column 250 x 4.6 mm, 5 µm C18 using 100% methanol as mobile phase at a flow rate of 1 mL-min-1 and column temperature at 40°C. Quantification was performed against monocrotophos standard at a wavelength of 215 nm. Under this condition, the retention time of monocrotophos was 2.8 min, the limit of detection was 0.1 ppm and the average recovery was 92.0–94.0%.

2.4 Metagenomic and Bioinformatics Analysis

Total DNA was extracted from the CONTROL and TREATED samples using bacterial DNA extraction kit (Himedia) and metagenome was sequenced from the 16S V3 region paired-end library using Illumina MiSeq. Usually, a paired-end sequence from V3 metagenomics contains some portion of the conserved region, spacer and V3 region. The spacer and conserved region from paired- end reads were removed. After trimming, a consensus V3 region sequence was constructed using Clustal O program. Multiple filters such as, conserved region filter, spacer filter and mismatch filter were performed to take only the high quality V3 region sequences for various downstream analyses. While making consensus V3 sequence, the passed reads

aligned to each other with 0 mismatches with an average contig length of ~130 to ~160 bp. The quality trimmed reads were converted to FASTQ and uploaded in NCBI Bioproject. FASTQ files were concatenated, the quality was filtered and uploaded to the MG-RAST server [9] for further analysis using annotation source M5NR. Determination of alpha diversity and rarefactionwere performed within MG-RAST by applying the ''Best Hit Classification'' option using the M5NR database as a reference with the following settings: a maximum e-value cutoff of 1*1025, the minimum identity of 80%. The distribution of taxonomic categories at different levels of the resolution was projected against the NCBI taxonomic tree and determined by the lowest common ancestor (LCA) with the same cutoff mentioned above using ribosomal RNA similarities to entries in the RefSeq protein database. The bacterial species identified from the metagenomic analysis were further grouped based on the presence of OPH gene in those bacterial species. For this grouping, the names of the bacterial species having OPH genes were retrieved from the NCBI database and compared with the name of the species identified in the metagenome data.

3. RESULTS AND DISCUSSION

3.1 Soil Metagenome Analysis

The metagenomes of the monocrotophos untreated (CONTROL) and monocrotophos spiked (TREATED) tea soil was analysed using MG-RAST automated processing pipeline. The dataset CONTROL contained 680,994 sequences totalling 74,404,814 base pairs with an average length of 109± 13 bps. Post QC sequence count of CONTROL was 60746 with a mean GC content of 55±4% and base pair count of 5,176,681. The TREATED dataset contains 608,758 sequences with a total of 66500632 base pairs, and 49,548 sequences passed QC with GC content of 56±4% with a total number of 5,176,681 base pairs. The bacterial diversity in the CONTROL and TREATED tea soil showed a species count of 1036 (Supplementary Table 1) and 910 (Supplementary Table 2), respectively. The principal component analysis showed both the CONTROL and TREATED samples as two different groups (Supplementary Fig. 1). Rarefaction curve (the total number of distinct species annotations as a function of the number of sequences sampled) showed that the CONTROL sample had the highest species richness and evenness (Fig. 1). The steep slope of the rarefaction curve on the left indicates that a large fraction of the tea soil species diversity remained to be discovered. The alpha diversity (calculated by MG-RAST server as an antilog of the Shannon index) was higher in CONTROL (14.75) than TREATED (12.16) sample. The rank abundance plot, where the data showed an abundance of bacterial phyla (Fig. 2), was normalised to 0 and 1 values and the calculated p values are given in Supplementary Table 3. The abundance of *Bacteriodetes*, *Synergisetetes*, *Thermodesulfobacteria*, *Chloroflexi*, *Verrucomicrobia* decreased when monocrotophos was treated (Fig. 2). Phylum *Planctomycetes*, *Chlorobi*, *Aquificae* was not observed in the TREATED sample (Fig. 2).

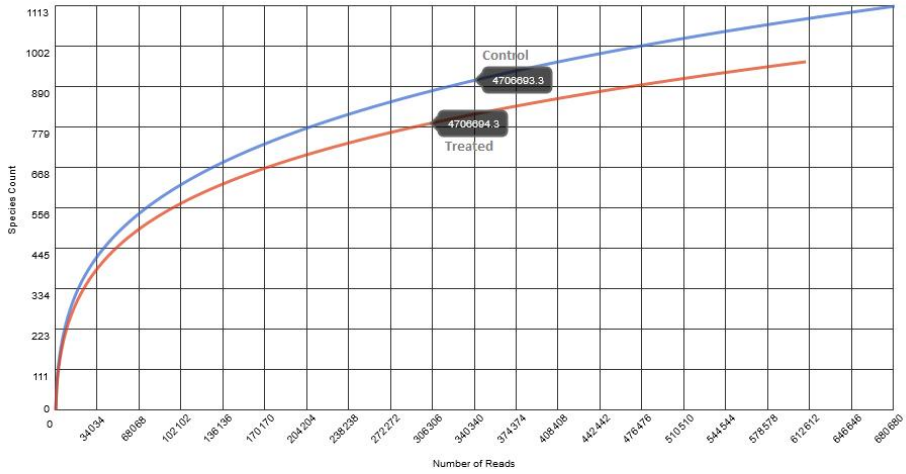

Fig. 1. Rarefaction curve showing species richness in the CONTROL and TREATED samples

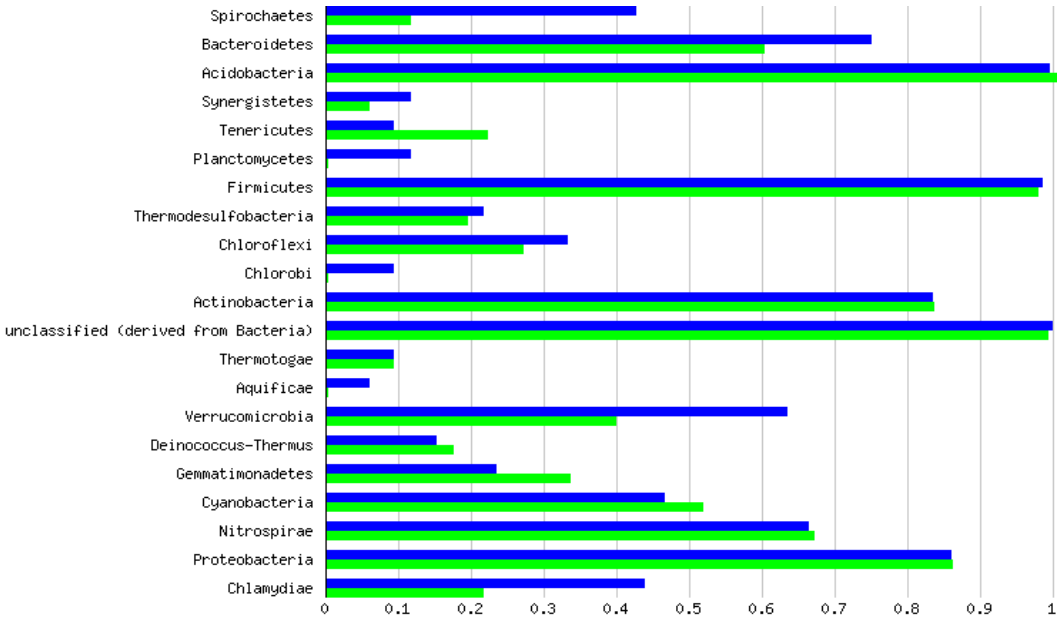

Fig. 2. Bar chart comparing normalized abundance of bacterial phyla in CONTROL (blue) and monocrotophos TREATED (green)

The metagenome of CONTROL represented 98.94% bacteria, 0.96% eukaryote and 0.10% other soil organisms, while the TREATED showed 99.34% bacteria, 0.5% eukaryotes and 0.16% others soil organisms. Unclassified phyla derived from bacteria remained the largest group with 34.50%, while *Firmicutes* (29.37%), *Acidobacteria* (21.57%), *Proteobacteria* (5.98%) and *Actinobacteria* (4.72%) were the most abundant bacterial phyla in the control sample (Fig. 3a). The same phyla were also found to be dominant in the treated sample (Fig. 3b), but increase in abundances was observed in case of *Acidobacteria* (25.87%) and *Proteobacteria* (6.22%). Percent abundance of *Firmicutes* was lesser (28.47%) in the TREATED sample than the CONTROL. Culture-independent 16S-23S intergenic spacer regions were utilised to amplify the surface and subsurface soil bacteria of Brahmaputra Valley, Assam, North-east India and reported that the tea garden soil clustered tightly in its phylogeny with b-Proteobacterium [10]. *Acidobacteria, Actinobacteria, Proteobacteria, Bacteroidetes* and *Firmicutes* were reported as the five major dominant bacterial groups across a range of soil habitats with high and low pH [11]. Soil pH was identified as an important factor in shaping the bacterial community composition with lower diversity in acidic soil pH [12]. Tea grows best in the acidic pH with the range of 4.5-5.5. Results of Metastats runs comparing bacterial classes between CONTROL and TREATED population indicated that eleven taxonomic classes including unclassified groups are differentially abundant in the two samples. The most abundant classes were the unknown group followed by *Acidobacteria* (Fig. 3c). Soil pH strongly regulates the abundance of *Acidobacteria* in the soil [13]. As tea grows best in the acidic soil, a large portion of the bacterial community was represented by *Acidobacteria*. At the class level, Solibacters showed a significant increase (24.62%) with *Candidatus solibacter* representing 24.62% at the genus level in the TREATED sample (Fig. 3d). Heat map comparison showed that *Actinobacteria*, *Acidobacteria*, *Proteobacteria* and Solibacters dominated irrespective of the presence or absence of pesticides (Fig. 4). Epsilon *proteobacteria* and Gamma *proteobacteria* showed positive response with increasing numbers on monocrotophos spiked soil. The normalized scale for heat map construction at the class level is given in the Supplementary Fig. 2. The order level was represented by Burkholderiales, Bacillales, Rhizobiales, Myxococcales, Legionales and Spingobacteriales (Figs. 3e and 3f). Both CONTROl and TREATED samples were variable for the high abundance of some genera (Fig. 3g and 3h). The abundant genus in the TREATED sample includes *Candidatus solibacter* (24.62%), *Alicyclobacillus* (24.43%), *Desulfotomaculum* (2.12%), genus derived from

Alpha *proteobacteria* (1.59%), *Candidatus koribacter* (1.24%) and *Burkholderia* (1.04%) (Fig. 3h). Thus, it can be concluded from the data that a distinct difference occurred in the numbers of bacterial species between the phyla types when monocrotophos was applied. The abundant species in the TREATED sample with above 1000 reads were *Acidobacterium capsulatum, Candidatus solibacter usitatus, Candidatus koribacter versatilis, Rhodococcus opacus, Thermomonaspora curvata, Alicyclobacillus hesperidum, Alicyclo bacillus tolerans, Staphylococcus pasteuri, Desulfotomaculum acetooxidans, Candidatus nitrospira defluvii, Bradyrhizobium elkanii, Burkholderia cepacia, Burkholderia Sp. Pseudomonas pseudoalcaligenes,* uncultured gamma proteobacterium, uncultured bacteria, uncultured forest soil bacterium, uncultured *Alphaproteobacterium*, uncultured *Epsilonproteobacterium* and unidentified sulphate reducing bacterium (Supplementary Table 2). The DNA sequences from this metagenomic project were deposited in the Sequence Read Archive under the Bio Project ID: PRJNA339673, Accession SAMN05687777 and SAMN05687776. Most of the metagenomic studies in tea sector were done only for the identification of bacterial communities present during the tea leaves fermentation stage [14]. *Saccharopolyspora, Bacillus, Brevibacterium, Brachybacterium, Kocuria, Streptococcus* and *Staphylococcus* were found to be the dominant genera. These genera were also observed in the bacterial community of monocrotophos treated and untreated tea soil exhibiting diverse interactions of the tea plant with soil-dwelling microbes. Bacteria like *Enterobacter sp., Flavobacterium sp., Pseudomonas stutzeri, Pseudomonas putida, Micrococcus* sp., *Arthrobacter sp., Bacillus sp., Bacillus megaterium, Xanthomonas sp., Alcaligene sp., Agrobacterium sp., Geobacillus sp., Clavibacter michiganense and Burkholderia* species, responsible for the detoxification of organophosphate [15] like Chloropyriphos, Parathion, Methyl parathion, Glyphosate, Coumaphos, Monocrotophos, Fenitrothion, Diazinon, were present in the monocrotophos treated tea soil and has the potentiality or may contribute for xenobiotic degradation in the plant or in the soil. Several bacterial strains like *Bacillus* and *Pseudomonas* identified in the current study were also reported in tea soils [16]. Potentiality of microbes, such as *Bacillus subtilis, Pseudomonas corrugata, Rhizobium* sp., and *Streptomyces nojiriensis* in enhanced growth of tea along with pest and disease suppression was reported [17]. Genera like *Pseudomonas, Bacillus, Enterobacter, Rhizobium, Mesorhizobium, Burkholderia, Azotobacter, Serratia, Azospirillum* and *Erwinia* identified in the metagenome were reported as phosphate solubilising bacteria [18]. Rhizosphere bacteria like *Azotobacter, Serratia* etc. also play an important role in biological nitrogen fixation [19].

3.2 Comparison of the Bacterial Species in Metagenome with OPH Degrading Bacteria in the NCBI Database

From the NCBI database, 34 hits of bacterial species containing OPH gene were identified based on the presence of OPH gene. Out of these bacterial species, 16 species were observed in the CONTROL and 13 in the TREATED sample. *Pseudomonas putida, Pseudomonas sp., Achromobacter sp, Brucella sp., Serratia sp., Serratia marcescens, Flavobacterium sp., Burkholderia cepacia, Burkholderia multivorans, Burkholderia sp., Arthobacter sp. Achromobacter sp., Sphingomonas sp., Ochrobactrum sp., Streptomyces sp.* were observed in the treated sample (Supplementary Table 4). Research over the past decade provided considerable knowledge of different bacteria capable of degrading organophosphate pesticides [20-24]. The bacteria capable of degrading organophosphates possess OPH enzymes which are encoded by the OPD (organophosphate degradation) gene and members of amidohydrolase superfamily. These enzymes are reported to have evolved in the soil bacteria to counter the toxic effects of OP insecticide residues released into agricultural soils [25]. However, the adaptability of the microorganisms for the bioremediation is important for microbial action to be able to release certain enzymes to metabolise or degrade pesticides. The identified bacterial species with OPH gene from the tea soil metagenome may have the potential to degrade organophosphate pesticides.

3.3 Screening of Monocrotophos Degrading Bacteria

In this study, the isolation of bacterial colonies from 300ppm monocrotophos spiked soil (Fig. 5a-b) and the screening in liquid mineral salts media reduced the number of bacterial colonies from 47 to 4 colonies per plate with an increase in sole carbon source, monocrotophos concentration from 100 to 600 ppm (Fig. 5d). The same bacterial species were observed in pH 5, 6

and 7. High CFU/ml was observed in 300 ppm of monocrotophos culture than 600 ppm at pH 5 (Supplementary Table 5). Six morphologically different bacterial colonies were isolated from the media containing 300 ppm (Fig. 5b) and 600 ppm (Fig. 5c) monocrotophos. The colony characteristics of the six selected bacterial strains are given in Supplementary Table 6. They were found to form round, opaque colonies and most colonies were formed by Gram-negative strains. In most of the metagenomic studies, a disparity was observed between the number of

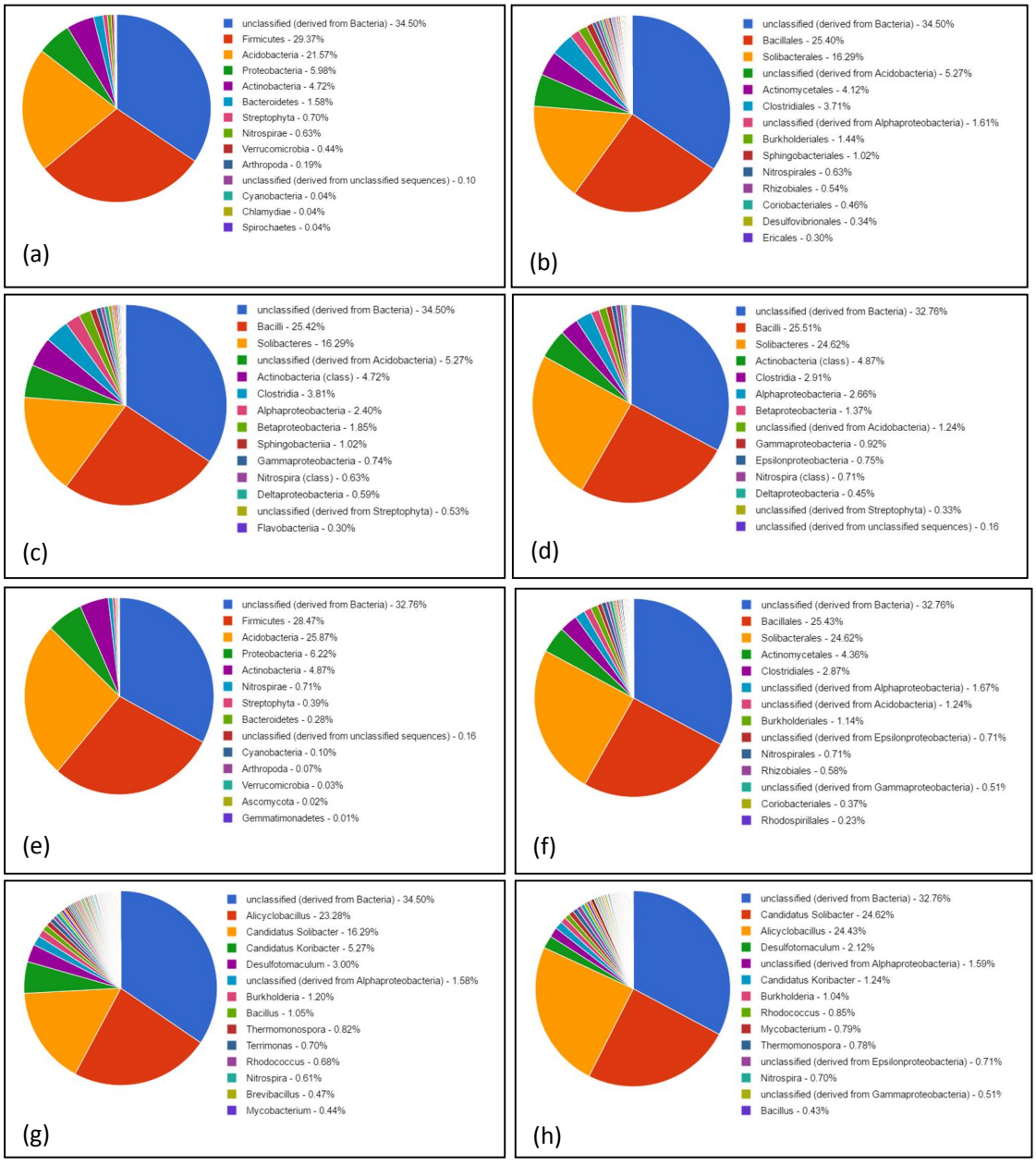

Fig. 3. Pie chart of the relative abundance of MG-RAST taxonomic hits at the phylum level in (a) control and (b) treated sample; Class level in (c) control and (d) treated sample; Order level in (e) control and (f) treated sample; genus level in (g) control and (h)treated sample

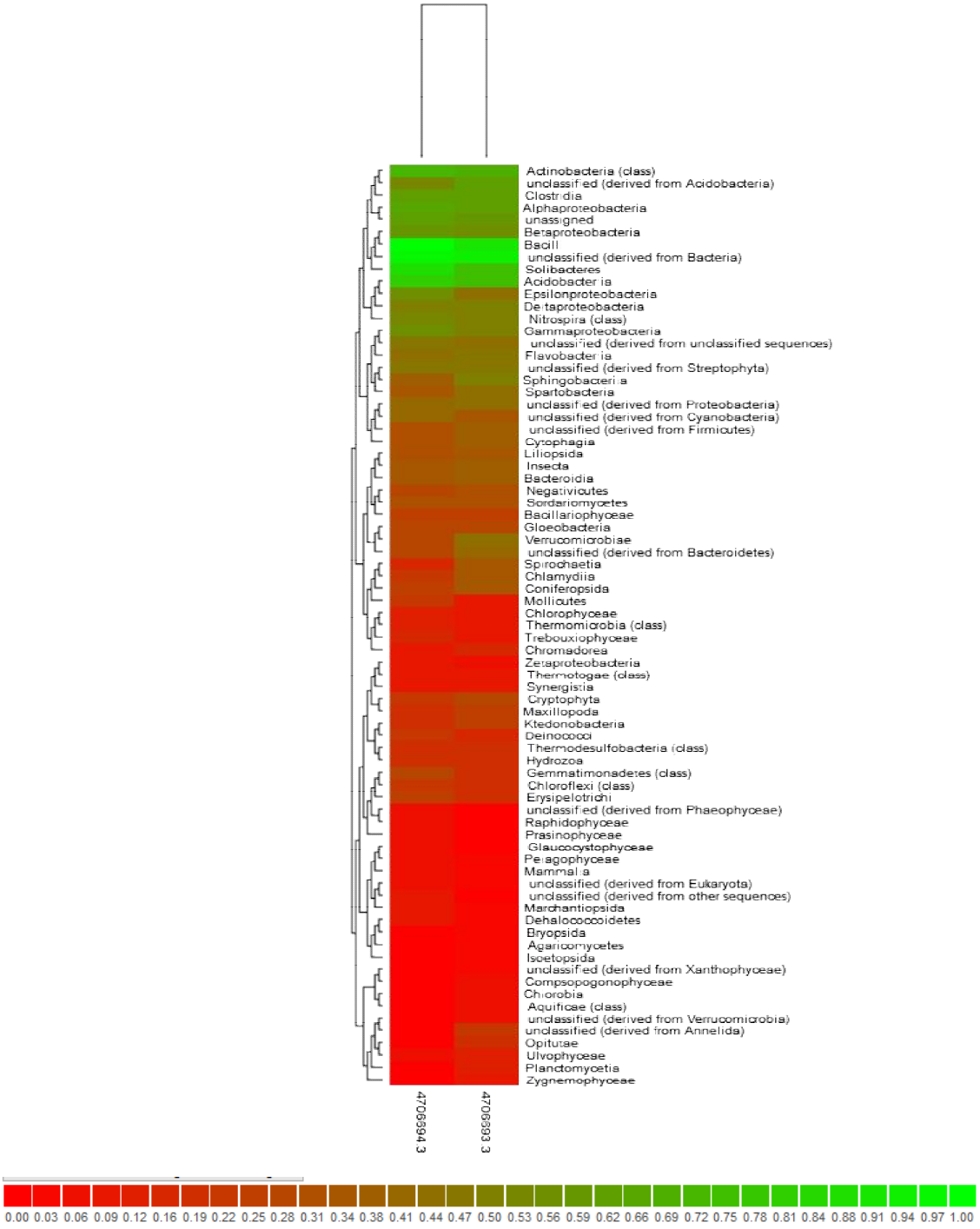

Fig. 4. Heat map for the comparison of bacteria at class level

bacterial species present in the metagenome and the culturable bacterial species (about 1%) [26]. Failure of the bacterial colonies to grow is a complex ecological response which depends on the cell's environment [27]. However, changing the environment of the culture media using high or low nutrient, different pH or culturing in a soil environment can increase the percentage of culturable bacterial species. In order to screen the best bacterium for monocrotophos

degradation studies at pH 5, the present study first determined the optical density of culturable bacteria in 3 ml of MSM media with 300ppm monocrotophos at pH 7 (Fig. 5d) and selected three strains. The efficacy of a bacterial strain to effectively degrade monocrotophos can be determined from the growth of the bacterial culture in a medium supplemented with the pesticide. S2, S3 and S6 strains were found to show a noticeable increase in growth within 48 hours (Fig. 5d). The strain S3 showed the highest growth at pH 7 with OD 1.807 and 2.776 after 24 and 48 hrs respectively (Supplementary Table 7).

3.4 Monocrotophos Pesticide Degradation of the Best Three Bacterial Strains

The bacterial growth and monocrotophos degradation kinetics of the best three strains S3, S6 and S2 were studied in three different pH (pH 5, 6 and 7) and data recorded in a UV-Vis Microplate Reader after 24 hrs (Table 1, Fig. 6). Degradation by the three strains was found to be modulated when the pH of the medium was changed. It was observed that pH 6 showed the maximum degradation by the three strains after 24 hrs. There are reports which show an increase of bacterial degradation activity with increasing pH [28]. The effect of soil pH on the bacterial degradation capability in bioremediation studies revealed that low soil pH (about 5) may lead to low microbial activity but it does not lead to total inhibition of activity [29], although bacterial growth and degradation prefers neutral pH. At pH 5, the highest optical density at 600 nm was shown by the strain S3 (0.681) followed by S6 (0.640) and S2 (0.542) (Supplementary Table 8). Strain S3 showed the highest degradation of 21.6%, followed by 18.6% (S6) and 16.7% (S2) (Table 1, Fig. 6). Again, hydrolysis of monocrotophos is pH dependent and quicker hydrolysis occurs at higher pH and the half-lives of monocrotophos in pH 3 and 9 at 25°C are 131 and 26 d respectively Bhadbhade et al. [30]. So, very high rates of monocrotophos degradation were not observed in 24 hrs as it is dependent on its hydrolysis capacity as well as other factors like pH, bacteria, temperature, etc. HPLC analysis against monocrotophos standard with spiking level 300 µg/mL (Fig. 7a) revealed that the S3 strain was able to degrade 52.2% of 300ppm monocrotophos within 48 hours (Fig. 7b) while S2 and S6 showed a moderate degradation of 32.5% (Fig. 7c) and 39.1% (Fig. 7d), respectively at pH 5. The short time required by these three strains at pH 5 to degrade monocrotophos was first reported from tea soil and a promising source for bioremediation of monocrotophos contaminated soil. Several microorganisms were isolated from soil sources capable of degrading monocrotophos and thereby minimising the toxic effect of this compound Singh and Singh [31], Bhadbhade et al. [32]. Studies revealed that many microorganisms have the higher degradation potential in bioremediation of organophosphate pesticides in alkaline soil [33]. Environmental factors such as temperature, pH and inoculum size play an important role in the microbial biodegradation of xenobiotics. Bacterial strains capable of degrading pesticides like cypermethrin in a variable range of pH 5-10 was reported [34]. As tea grows best in acidic soil, it is very important to identify the microbial community associated with tea to develop measures for bioremediation of tea soil at this particular pH. The findings indicated that the three strains capable of degrading monocrotophos was able to grow in a pH range of 5 to 7.

Table 1. Bacterial growth and monocrotophos degradation shown by S3, S6 and S2 in different pH in UV-Vis microplate reader

Name	Composition of culture (Controls)	Absorbance at 600 nm (Initial reading)	Absorbance at 215 nm (Initial reading)	Monocrotophos degradation % (Initial reading)
Blank1 B08	MSM	0.033	0.484	0
Std0011 B09	MSM + Monocrotophos 300 ppm	0.040	3.003	0
Std0012 B10	MSM + Monocrotophos 300 ppm +S3 , 1 hr	0.044	3.013	0
Std0013 B11	MSM + Monocrotophos 300 ppm +S6 , 1 hr	0.041	3.052	0
Std0014 B12	MSM + Monocrotophos 300 ppm +S2 , 1 hr	0.043	3.062	0

Name	Composition of culture (Test samples)	Absorbance at 600 nm (After 24 hrs)	Absorbance at 215nm (After 24 hrs)	Monocrotophos degradation % (After 24 hrs)
Un0009 C08	MSM + Monocrotophos 300 ppm + S3, pH 5	0.681	2.361	21.6
Un0010 C09	MSM + Monocrotophos 300 ppm + S3, pH 6	0.746	2.242	25.5
Un0011 C10	MSM + Monocrotophos 300 ppm + S3, pH 7	0.722	2.536	15.8
Un0012 C11	MSM + Monocrotophos 300 ppm + S6, pH 5	0.640	2.483	18.6
Un0013 C12	MSM + Monocrotophos 300 ppm + S6, pH 6	0.538	2.416	20.8
Un0014 D01	MSM + Monocrotophos 300 ppm + S6, pH 7	0.556	2.566	15.9
Un0015 D02	MSM + Monocrotophos 300 ppm + S2, pH 5	0.542	2.549	16.7
Un0016 D03	MSM + Monocrotophos 300 ppm + S2, pH 6	0.501	1.607	47.5
Un0017 D04	MSM + Monocrotophos 300 ppm + S2, pH 7	0.843	2.272	25.8

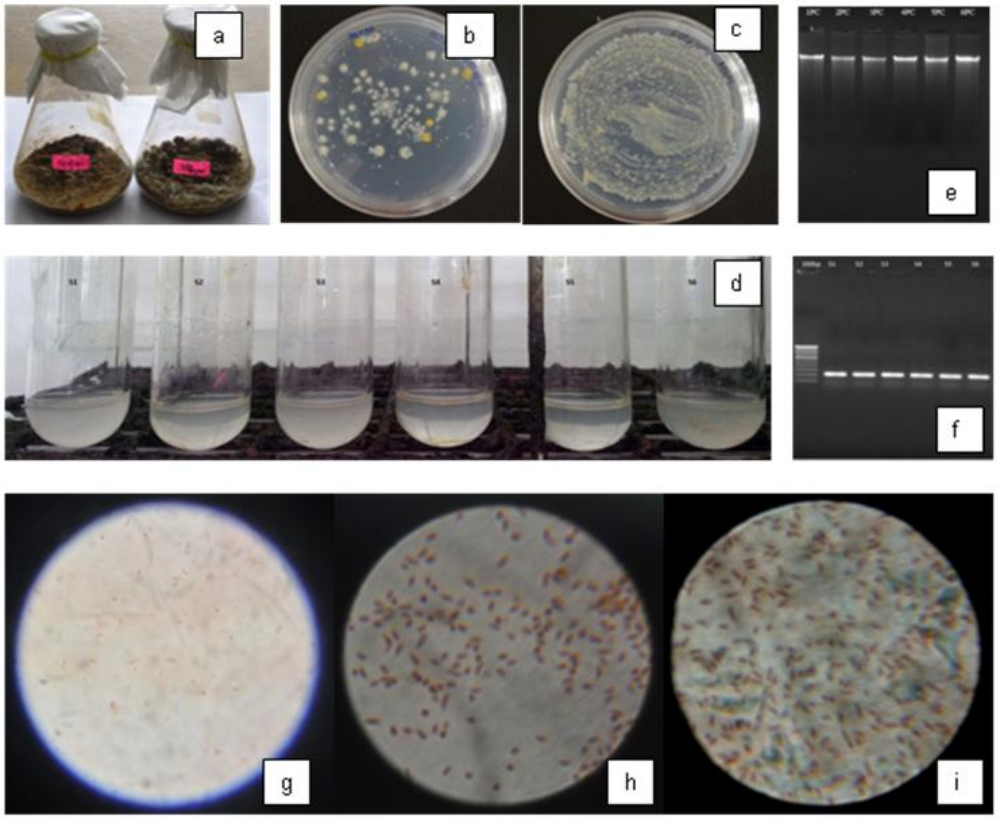

Fig. 5 a) Spiking of soil with increasing concentrations of pesticide Monocrotophos to a final concentration of 300 ppm. b) Bacterial colonies in MSM media with 300 of Monocrotophos. c) Bacterial colonies in MSM media with 600 ppm of Monocrotophos. d) Six bacterial cultures for pesticide degradation study in 300 ppm of Monocrotophos at pH 7. e)Genomic DNA extraction from the 6 colonies. f) 16SrRNA PCR amplification of bacterial colonies. g) Gram staining of strain S2. h) Gram staining of strain S3. i) Gram staining of strain S6

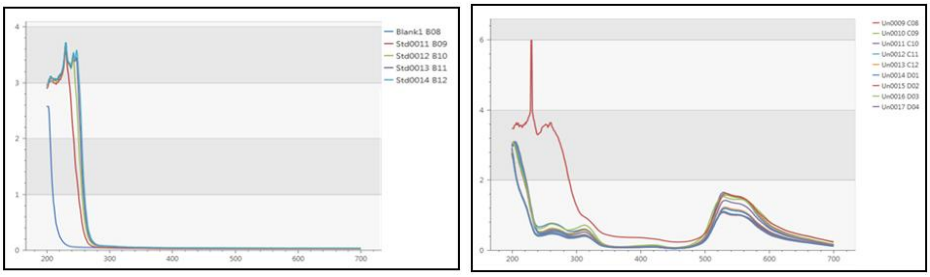

Fig. 6. Bacterial growth and monocrotophos degradation shown by S3, S6 and S2 strains in pH 5 to 7 in UV-VIS microplate reader (Labels are defined in Table 1)

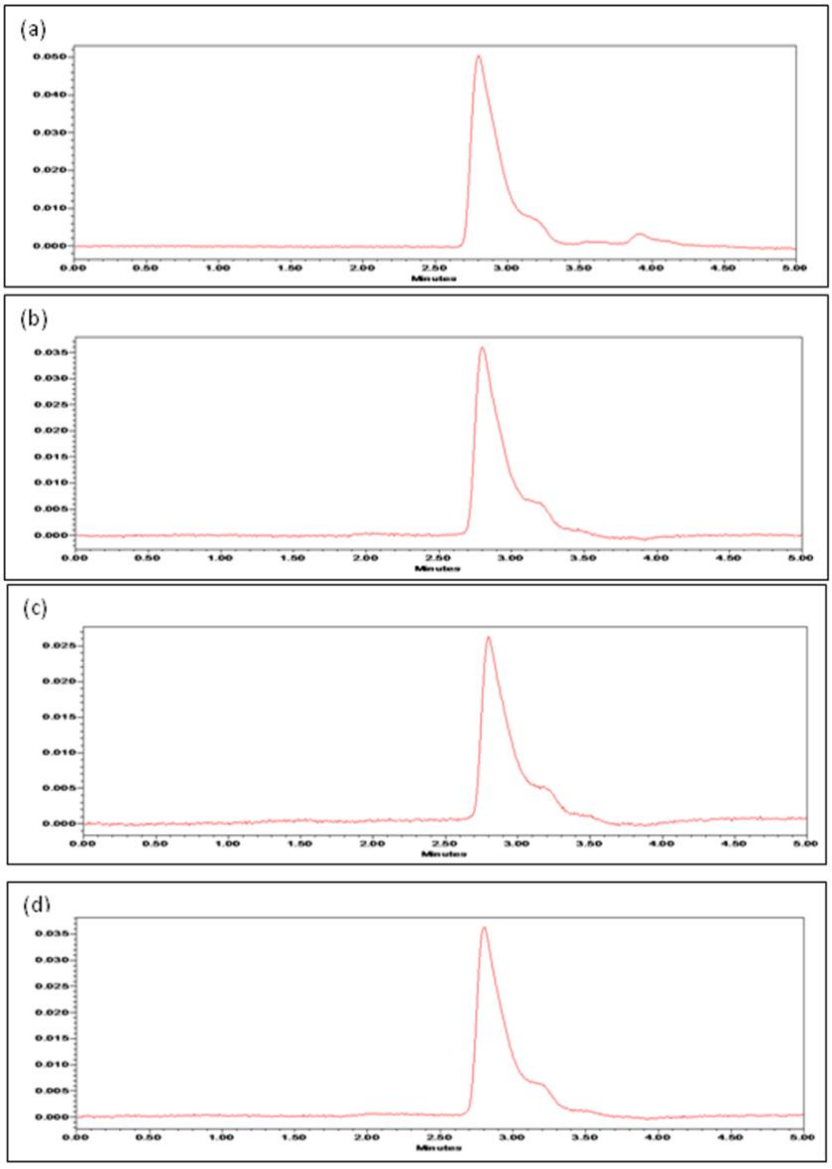

Fig. 7. HPLC peaks for monocrotophos at 215 nm for standard and three bacterial samples spiked with monocrotophos (a) Monocrotophos standard (b) (S3) *Paraburkholderia kururiensis* (c) (S2) *Serratia fonticola* (d) (S6) *Pseudomonas putida*

3.5 Molecular Identification of the Bacterial Strains Capable of Degrading Monocrotophos

Three species (GenBank: KX197245.1, GenBank: KX197246.1 and GenBank: KP637150.1) of tea OP pesticide (monocrotophos) degrading bacteria were identified from the tea soil. Strain S2 is identified as *Paraburkholderia kururiensis* of *proteobacteria* family. The genus *Paraburkholderia* is an ecologically diverse group of Gram-negative bacteria and inhabits a wide range of ecological niches, ranging from soil, plant, animals to the human respiratory tract [35-37]. They are reported to degrade a wide range of recalcitrant xenobiotics [38]. Strain S3 is identified as *Serratia fonticola,* a Gram-negative, facultatively anaerobic, endospore-forming, rod-shaped bacteria of Enterobacteriaceae family. The strain could utilise monocrotophos as the sole source of carbon for its growth and exhibited positive chemotactic response towards it. *Serratia* species are capable of degrading organophosphates, tetrachlorovinphos [39] and is an ideal species for waste degradation as well as bioremediation of soil and water [40]. Strain S6 is identified as *Pseudomonas putida*. This species has shown high potentiality in degrading OP pesticide residues [41]. The enzyme systems involved in biodegradation of OP compounds were extensively studied [15]. The organophosphorus hydrolase (OPH) are members of amidohydrolase superfamily and it is encoded by the highly conserved opd gene capable of hydrolysing organophosphorus compounds with P-O, P-F and P-S bonds. Monocrotophos belongs to the vinyl phosphate group and its hydrolytic cleavage occurs at the vinyl phosphate bond. Thirteen OP degrading bacteria were also found in this metagenomic study with the help of bioinformatics in the TREATED sample.

4. CONCLUSION

This study characterised three bacterial strains capable of degrading monocrotophos pesticide in an acidic environment. The study also confirmed the presence of taxa previously found to inhabit the tea soil and identified many bacterial species not yet reported along with the metagenomic survey of the bacterial community. The identified strains from the metagenome were reported to have plant growth promoting characteristics as well as the capability to survive in the presence of monocrotophos. The 13 bacterial species found in the monocrotophos treated tea soil metagenome are reported with OPH gene in the NCBI database may be important for organophosphate pesticide bioremediation in tea. The unknown microbial resources of tea soil deserve immediate attention in terms of documentation and bioprospection. Knowledge on microbial populations, their relationship and function are required to develop bioremediation strategy which has still not been attempted in tea soil. This study will aid to design a pesticide degrading bacterial formulation which can be useful in organophosphate contaminated tea soil to build soil health for tea replantations or in conversions to organic tea plantations.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rousk J, Bengtson P. Microbial regulation of global biogeochemical cycles. Front Microbiol. 2014;5:103.
2. Phukan I, Madhab M, Bordoloi M, Sarmah SR, Dutta P, et al. Exploitation of PGP microbes of tea for improvement of plant growth and pest suppression: A novel approach. Two Bud. 2012;59(1):69-74.
3. Balamurugan A, Jayanthi R, Nepolean P, Vidhya Pallavi R, Premkumar R. Studies on cellulose degrading bacteria in tea garden Soils. Afr J Plant Sci. 2011;5(1): 22-27.
4. Barooah AK. Present status of use of agrochemicals in tea industry of eastern India and future directions. Science and Culture. 2011;77(9-10):385-390.
5. Barooah AK, Kanrar B, Kalita JN, Pal S, Sharma N, Barman T. Competency of the laboratories in analyzing pesticide residue in tea and the probable challenges to meet the export criteria – case study. In Proceedings of the International Conference on Emerging Issues on Health Effects of Pesticide Residues in Food and Environment, Food and Drug Toxicology

Research Center, Hyderabad. 2013;105-116.
6. Singh BK. Organophosphorus degrading bacteria: Ecology and industrial applications. Nature. 2009;7:156-164.
7. Maphosa F, Lieten SH, Dinkla I, Stams AJ, Smidt H, Fennell DE. Ecogenomics of microbial communities in bioremediation of chlorinated contaminated sites. Front Microbiol. 2012;3:351.
8. Weissenfels WD, Beyer M, Klein J. Degradation of phenanthrene, fluorene and fluoranthene by pure bacterial cultures. Appl Microbiol Biotechnol. 1990;32(4): 479-484.
9. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, et al. The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 2008;9:386.
10. Bhattacharyya P, Tanti B, Barman P, Jha D. Culture-independent metagenomic approach to characterize the surface and subsurface soil bacterial community in the Brahmaputra valley, Assam, North-East India, an Indo-Burma mega-biodiversity hotspot. World J Microbiol Biotechnol. 2013;30(2):519-528.
11. Lauber CL, Hamady M, Knight R, Fierer N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community composition at the continental scale. Appl Environ Microbiol. 2009;75: 5111–5120.
12. Wu Y, Zeng J, Zhu Q, Zhang Z, Lin X. pH is the primary determinant of the bacterial community structure in agricultural soils impacted by polycyclic aromatic hydrocarbon pollution. Sci. Rep. 2017;7:40093. DOI: 10.1038/srep40093
13. Jones RT, Robeson MS, Lauber CL, Hamady M, Knight R, Fierer N. A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. The ISME Journal. 2009;3(4):442–453.
14. Fu J, Lv H, Chen F. Diversity and variation of bacterial community revealed by MiSeq sequencing in Chinese dark teas. Plos One. 2016;11(9):e0162719. DOI: 10.1371/journal. pone.0162719
15. Singh BK, Walker A. Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev. 2006;30:428–471.
16. Bhattacharyya P, Sarmah S, Dutta P, Tanti A. Emergence in mapping microbial diversity in tea (*Camellia sinensis* (L.) *O. Kuntze*) soil of Assam, North-East India: A novel approach. Eur J Biotech Biosci. 2015;3:20-25.
17. Phukan I, Madhab M, Bordoloi M, Sarmah SR, Dutta P, Begum R, et al. Exploitation of PGP microbes of tea for improvement of plant growth and pest suppression: A novel approach. Two Bud. 2012;59(1):69-74.
18. Haas D, Defago G. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Rev Microbiol. 2005;3:307-319.
19. Egamberdiyeva D. Plant-growth-promoting Rhizobacteria isolated from a Calcisol in a semi-arid region of Uzbekistan: Biochemical characterization and effectiveness. J Soil Sci Plant Natr. 2005;168(1): 94–99.
20. Chang TC, Chang HC, Yu FW, Leni D, Mario V, Chung TT. Species level identification of isolates of the *Acinetobacter calcoaceticus*, *Acinetobacter baumanni* complex by sequence analysis of the 165-23S rRNA gene spacer region. J. Clin. Microbiol. 2005;43(4):1632-1639.
21. Horne I, Harcourt RL, Sutherland TD, Russel RJ, Oakeshott JG. Isolation of a *Pseudomonas monteili* strain with a novel phosphotriesterase. FEMS Microbiol Lett. 2002;206:51-55.
22. Zhang RF, Cui ZL, Jiang JD, He J, Gu XY, Li, SP. Diversity of organophosphorus pesticide-degrading bacteria in a polluted soil and conservation of their organophosphorus hydrolase genes. Can J Microbiol. 2005;51:337–343.
23. Yang H, Carr PD, McLoughlin SY, Liu LW, Horne I, Qui X, Jeffries CM, Russell RJ, Oakeshott JG, Ollis DL. Evolution of an organophosphate-degrading enzyme: A comparison of natural and directed evolution. Protein Eng. 2003;16:135–145.
24. Chino-Flores C, Dantan-Gonzalez E, Vazquez Ramos A, Tinoco-Valencia R, Diaz Mendez R, Sanchez-Salinas E, et al. Isolation of the opdE gene that encodes for a new hydrolase of *Enterobacter sp.* capable of degrading organophosphorus pesticides. Biodegradation. 2012;23(3): 387-97.
25. Chakka D, Gudla R, Madikonda AK, Pandeeti AVP, Parthasarathy S, Nandavaram A, et al. The organophosphate degradation (opd) island-borne esterase-induced metabolic diversion in *Escherichia coli* and its

influence on p-nitrophenol degradation. J Biol Chem. 2015;290(50):29920–29930.
26. Torsvik V, Ovreas L. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. 2002;5: 240–245.
27. Ernebjerg M, Kishony R. Distinct growth strategies of soil bacteria as revealed by large-scale colony tracking. Appl Environ Microbiol. 2012;78:1345–1352.
28. Vidali M. Bioremediation: An overview. J. Appl. Chem. 2001;73(7):1163-1172.
29. Pawar RM. The effect of soil PH on bioremediation of polycyclic aromatic hydrocarbons (PAHS). J Bioremed Biodeg. 2015;6:291.
30. Bhadbhade BJ, Sarnaik SS, Kanekar PP. Biomineralization of an organophosphorus pesticide, monocrotophos, by soil bacteria. J Appl Microbiol. 2002;93(2):224-234.
31. Singh S, Singh DK. Utilization of monocrotophos as phosphorus source by *Pseudomonas aeruginosa* F10B and *Clavibacter michiganense* subsp. insidiosum SBL 11. Can J Microbiol. 2003; 49:101–109.
32. Bhadbhade BJ, Dhakephalkar PK, Sarnik SS, Kanekar PP. Plasmid-associated biodegradation of an organophosphorus pesticide, monocrotophos, by *Pseudomonas mendocina*. Biotechol Lett. 2002;24:647–650.
33. Singh BK, Walker A, Morgan JA, Wright DJ. Effects of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. Appl Environ Microbiol. 2003;69(9):5198-5206.
34. Jabeen F, Ahmed M, Ahmed F, Sarwar MB, Akhtar S, Shahid AA. Characterization of cypermethrin degrading bacteria: A hidden micro flora for biogeochemical cycling of xenobiotics. Adv. Life Sci. 2017; 4(3):97-107.
35. Xu Y, Buss E, Boucias DG. Culturing and characterization of the gut symbiont *Burkholderia* from the Southern chinch bug, *Blissus insularis* (*Hemiptera: Blissidae*). Appl Environ Microbiol. 2016; 82:3319–3330.
36. Verstraete B, Janssens S, Smets E, Dessein S. Symbiotic ß-*Proteobacteria* beyond Legumes: *Burkholderia* in *Rubiaceae*. Plos One. 2013;8:e55260.
37. Compant S, Nowak J, Coenye T, Clement C, Ait Barka E. Diversity and occurrence of *Burkholderia* spp. in the natural environment. FEMS Microbiol Rev. 2008;32:607–626.
38. Coenye T, Vandamme P. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ Microbiol. 2003;5:719–729.
39. Ortiz-Hernandez ML, Sanchez-Salinas E. Biodegradation of the organophosphate pesticide tetrachlorvinphos by bacteria isolated from agricultural soils in Mexico. Rev Int Contam Ambient. 2010;26(1):27-38.
40. Abo-Amer A. Biodegradation of diazinon by *Serratia marcescens* DI101 and its use in bioremediation of contaminated environment. J. Microbiol. Biotechnol. 2011;21(1): 71-80.
41. Rani NL, Lalitha-kumari D. Degradation of methyl parathion by *Pseudomonas putida*. Can J Microbiol. 1994;4:1000–1004.

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