Biological Forum – An International Journal **15(4): 584-589(2023)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Molecular profiling of a novel bacteria *Cronobacter sakazakii* (ON248143), degrading organophosphate (MCP) pesticide from contaminated soil of agricultural land

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(Received: 11 February 2023; Revised: 15 March 2023; Accepted: 19 March 2023; Published: 20 April 2023)

(Published by Research Trend)

ABSTRACT: The widespread and indiscriminate use of pesticides in agriculture for pest control has caused major harm and issues for both humans and biodiversity. It has been thought to be useful to cleanse areas that have been contaminated by pesticides via microbial biodegradation of pesticides in contaminated soils. A commonly used organophosphorus insecticide called monocrotophos has negative consequences, particularly those on the central nervous system. This study's objective was to use an enrichment culture approach to isolate bacteria from Monocrotophos (MCP)-contaminated soil. The isolates received a high dose (1000 ppm) of MCP as a carbon source supplement. Based on the 16S rRNA gene sequence, the isolate was determined to be *Cronobacter sakazakii* (ON248143), a microorganism that may degrade MCP. The outcomes were validated with an analytical tool, High-Performance Liquid Chromatography (HPLC).

Keywords: Pesticides, central nervous system, organophosphorus, enrichment culture technique, HPLC.

INTRODUCTION

The economy of India were largely dependent on the quality and quantity of its agricultural production. Better harvests require intensive cultivation, irrigation, fertilizers, and what is more important the use of chemicals to protect plants from pests and plant diseases. In India, 15–20% of all produce is destroyed by pests (Bhalerao and Puranik 2007). This emphasizes the paramount importance of pesticides in India in preventing agricultural loss and enhancing production. The enormous use of pesticides, however, has added to environmental pollution. A great proportion of acute poisoning cases were caused by exposure to pesticides, especially organophosphate (OP) compounds. The primary mechanism of action of OP pesticides was based on the inhibition of the acetylcholinesterase (ache) enzyme (Alarami, 2015). Once AChE has been inactivated, acetylcholine (ach) accumulates throughout the nervous system, resulting in overstimulation of muscarinic and nicotinic receptors. Organophosphate insecticides such as Monocrotophos, Parathion, Malathion, Methylpa-rathion are well known for their bioaccumulation and neurotoxic properties because they act on the enzyme acetylcholinesterase.

Monocrotophos was one of the widely used organophosphorus pesticides. It is a non-specific and systemic insecticide (Fox, 1995) belonging to the vinyl phosphate group. Monocrotophos is manufactured using monocloromonomethyl aetoacetamide and trimethyl phosphate. It was highly toxic in nature and affects many beneficial, non-target organisms like honey bees (Kasturi Bai and Reddy 1977; Qadri *et al.,* 1982) fishes (Thangnipon *et al.,* 1995), birds and mammals (Skripsky and Loosli 1994). But, it was still in use in some developing countries like India due to lake of awareness and alternative replacements (Banerjee *et al.*, 2000).

Owing to its highly toxic nature, degradation of MCP from the environment is very essential. Degradation of MCP occurs by biotic as well as abiotic factors of the natural environment. These factors influence the degradation of MCP and act in tandem and complement one another in the microenvironment (Singh *et al.,* 2003). Compared to the potential disadvantage of conventional methods, remediation using microorganisms has been considered an attractive, potentially convenient, effective, low-cost, and eco-friendly method (Liu *et al*., 2009; Lei *et al.,* 2005).

Soil microflora were one of the best eco-friendly agents for detoxification of organophosphates.

Microorganisms use pesticides as nutrients for their growth. Pure cultures can detoxify the pesticides into different metabolites like *P*-nitrophenol, diethyltriphiosphoric acid, *p*-aminophenol, dimethyl phosphate, orthophosphate, etc. (Anantha *et al.*, 2019). The objective of this study is to isolate and characterize monocrotophos degrading bacteria in the area contaminated with monocrotophos compound and use them as a tool to decontaminate.

MATERIALS AND METHODS

Chemicals. Standard analytical grade solution of MCP was procured from the agrochemical shop in

Tirunelveli district, Tamil Nadu. All the other chemicals employed in the present study were of analytical grade and obtained from Himedia.

Soil sample collection. The soil sample was collected from the contaminated area and sprayed with MCP. Collect the sample was stored in airtight polythene bags and stored at 4˚C till use.

Enrichment technique for isolation of MCP degrading microbes. As reported by (Harish *et al.,* 2013) For enrichment of soil sample to obtain pesticide degrading bacterial strains, 10 gm of mixed soil sample was put aseptically in a sterile conical flask, and 10 mL of 1000 ppm Monocrotophos (MCP) solution was added in it and mixed properly by a sterile glass rod. The flask was kept at room temperature in static conditions for five days. After incubation, soil suspensions were prepared in sterile distilled water by serial dilution method from the soil sample. 1 mL aliquots from 10^{-6} to 10^{-9} dilution were inoculated on a Mineral Salt Medium plate containing 1000 ppm MCP by spread plate technique. Plates were incubated for 24 hours in an incubator at 37˚C. After incubation well-isolated colonies were further inoculated on an MSM plate containing 1000 ppm MCP by streak plate method to obtain a pure colony. These plates were incubated for 24 hours in an incubator at 37˚C. Only 1 well-grown bacteria colony was obtained on 1000 ppm MCP containing mineral salt medium was used for further study.

Determination of 16S rRNA gene sequence of bacterial isolates:

Genomic DNA Isolation: Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

Agarose Gel Electrophoresis for DNA Quality and Quantity check. The quality of the DNA isolated was checked using agarose gel electrophoresis.1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until the bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using a Gel documentation system (Bio-Rad).

PCR Amplification and Purification of 16S rRNA Gene. In PCR 30 cycles of amplification were performed which resulted in a very large amplification of DNA. The partial 16S rRNA included, PCR mix contained 6.6µlD/W, 1.9µl 5X sequencing buffer, 0.3µl forward primer, o.3µl reverse primer, 0.2µl sequencing mix, 1µl exosap treated PCR product, 5µl of 2X phire master mix, 1µl DNA and sterilized distilled water. The PCR reaction included the following steps: Initial denaturation of 2 min at 94˚C (preheating) followed by 25 cycles were run on a thermal cycler, each comprising 1 min at 94˚C (denaturation), 1 min at 52˚C (annealing), and 1.5min at 72˚C (extension), followed by a final extension of 10 min at 72˚C. identification of strain was done by 16S rRNA sequence analysis. Finally, to assess the phylogeny of the isolate, a BLASTN analysis (16S rRNA sequence) was carried out through Genbank.

Screening of monocrotophos (MCP) degrading bacteria: For screening of potent MCP degrading bacteria, isolated bacteria were inoculated in a mineral salt medium (MSM). Growth of isolated pure bacteria maintained on Nutrient agar slant above was scrapped by sterile wire loop in presence of 1mL sterile distilled water. Obtained sterile distilled water suspension of culture was subjected to a spectrophotometer to read its absorbance at 600nm. The inoculum density was adjusted to OD600 = 1. From this culture suspension having OD value 1 at 600nm, 1mL of was inoculated aseptically in a flask containing 100 mL MSM broth having 1000 ppm MCP. After inoculation, the flask was incubated in shaking condition at 150 rpm at 37˚C for 7 days.

Screening of potent MCP degraders from the isolated culture was done based on their ability to degrade the 1000 ppm MCP in MSM broth using HPLC analysis. To extract the MCP residues, culture broth obtained after incubation from all the flasks was centrifuged (REMI Heavy Duty Cooling Centrifuge) at 15,000 rpm at 4˚C for 15 minutes. The cell-free supernatants obtained were then used to estimate the residual MCP present in it by high-performance liquid chromatography (HPLC). Degradation was calculated from the retention time of the test sample corresponding to the control sample.

RESULT AND DISCUSSION

Insecticides are crucial to increasing agricultural productivity, but only 1% of pesticides are effective in eliminating pests; the remaining 99% penetrate ground and surface water, causing environmental pollution that harms human health (Battaglin andFairchild 2002). As a result, certain persistent pesticides were outlawed, while others underwent modification without harming the environment. However, a lot of health issues are being brought on by organophosphates (Sogorb *et al.,* 2004). Organophosphates make up a significant and frequently utilized group of the numerous types of pesticides currently in use around the world, making up more than 36% of the global market (Kavikarunya and Reetha 2012). The microbial flora and fauna of soil are the primary target of pesticide use. However, only a few studies have been done to explore the pattern of soil degradation of these hazardous chemicals (Gundi and Reddy 2006). A majority of soils are contaminated by naturally occurring harmful or toxic elements, and pesticides to some or more extents (Sannino and Gianfreda 2001). Additionally, several species of bacteria have been isolated from different environments that degrade organophosphorus compounds in laboratory cultures and soil (Buvaneswar *et al.,* 2016).

The present study aims to isolate pollutants-tolerant and pollutants-resistant bacteria from the respective contaminated sites. As microorganisms such as bacteria are important in bioremediation, this experiment was conducted to identify the bacterial strain from organophosphate-contaminated soil capable of degrading organophosphate. First, for the study soil

samples were collected and it was serially diluted. Mineral salt medium and broth were prepared. A diluted soil sample is inoculated onto a mineral salt agar Petri dish supplemented with MCP as a carbon source at 37°C. From the Petri dish, an isolated colony was picked and later inoculated onto a broth containing 1000ppm MCP for further study. Pure colony plates (Fig. 1) show gram-staining pictures of the isolated gram-negative and short rod-shaped bacteria. They are morphologically round, white to cream pigmented bacteria. Following 4-6 subcultures, pure, isolated, and single colonies were obtained. From the microorganism, genomic DNA and partial 16S rRNA sequences were isolated. A 16S rRNA gene containing 539 nucleotides has been identified (Fig. 2). In the case of 16S rRNA, BLASTn was performed (Fig. 3 and 4 showed 99% identity to the partial 16S rRNA gene from *Cronobacter sakazakii* (ON248143).

Following the discovery of bacteria to determine a relationship between bacteria's capacity to break down pesticides and how long they can survive in a media containing monocrotophos. In comparison to an uninoculated bacterial suspension designated as the control, the MCP degradation efficiency of isolates cultivated in 100 ml of Mineral Salt medium containing 1000 ppm MCP and 1 ml of culture suspension is assessed. The flask was incubated for 7 days at 37°C and 150 rpm while being shaken (Fig. 5). Finally, MCP degradation was determined with the use of HPLC. Figure 6 showed that MCP was biodegraded at a retention time of 2.137 minutes, while bacteria have a retention time of 2.393 minutes. Using HPLC over a period of 7 days with the *Cronobacter sakazakii* (ON248143) strain, the capacity of MCP to be used as a carbon source was examined. some new peaks were also seen in addition which might be due to metabolic products of MCP degradation. This indicates complete detoxification of MCP by *Cronobacter sakazakii* (ON248143). Conversely, Gundi and Reddy (2006) noticed a significant decrease in the monocrotophos in sterile soils. The results were concurrent with the study of Bhalerao and Puranik (2009); Jain *et al.* (2014) which also correlated degradation of MCP by reduction of its peak as compared to that in the control chromatogram. (Jia *et al.,* 2006) reported that the addition of paracoccus sp. M-I (10^6 CFU/g) to fluvo-aquic soil and a high-sand soil containing MCP (50 mg/kg) resulted in a higher degradation rate than that obtained from non-inoculated soil.

Similar to this, *Cronobacter muytjensii* strain GH10 was able to break down 92.5 percent of the pesticide (diazinon) organophosphate (El-Sayed *et al*., 2018). The fungicide TBZ5, which is made up of *Enterobacter sakazakii* sp., demonstrated the highest rate of microbial breakdown, according to (Sehnem *et al.,* 2010)(43.46 mg L^{-1}). In addition, *Enterobacter cloacae* strain EAM 35, which can break down organochlorine insecticides, was described by (Shahid *et al.*, 2021). Other researchers have also retrieved the diverse Enterobacter species from various soil environments and described them using cutting-edge methods and 16S rRNA partial gene sequence analysis (Umar Mustapha *et al*., 2020; Zhang *et al*., 2020; Ramya and Vasudevan 2020; Khalifa *et al.*, 2016). Through the manipulation of suitable microbial strains for environmental development, studies on pesticide biodegradation provided significant knowledge for the creation of effective technologies. Hence the study concludes that *Cronobacter sakazakii* (ON248143) proved itself to be the most efficient tool for the degradation of MCP(Organophosphate).

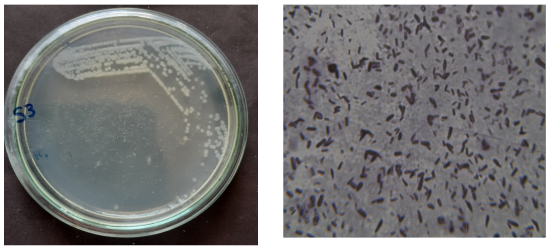


Fig. 1. Isolated Colony – *Cronobacter sakazakii.*

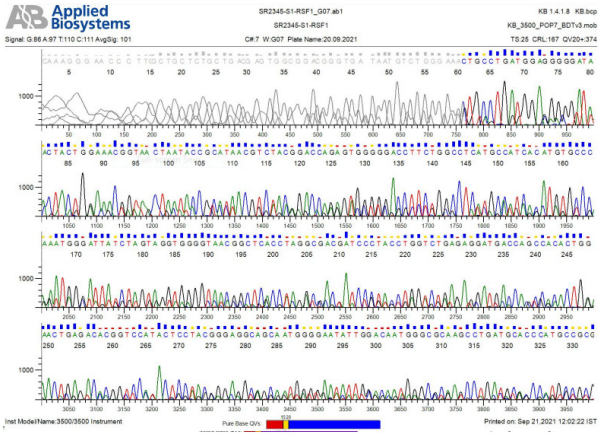


Fig. 2. Arrangement of 16S RRNA Sequence of *Cronobacter sakazakii.*

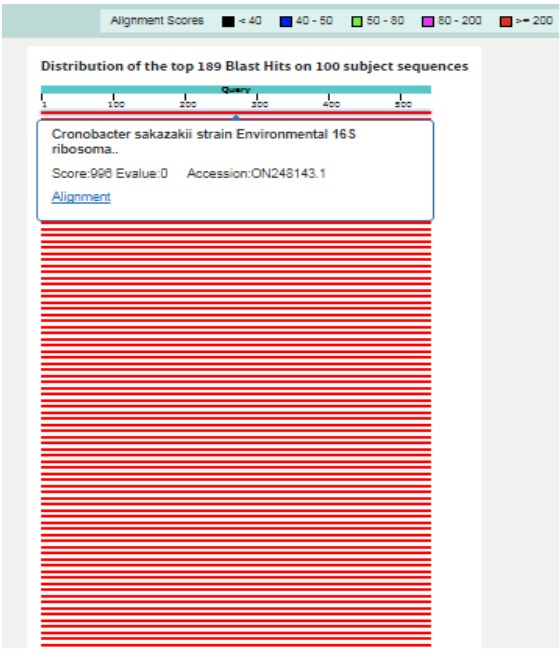


Fig. 3. Graphical Summary of the Blast Query Sequence.

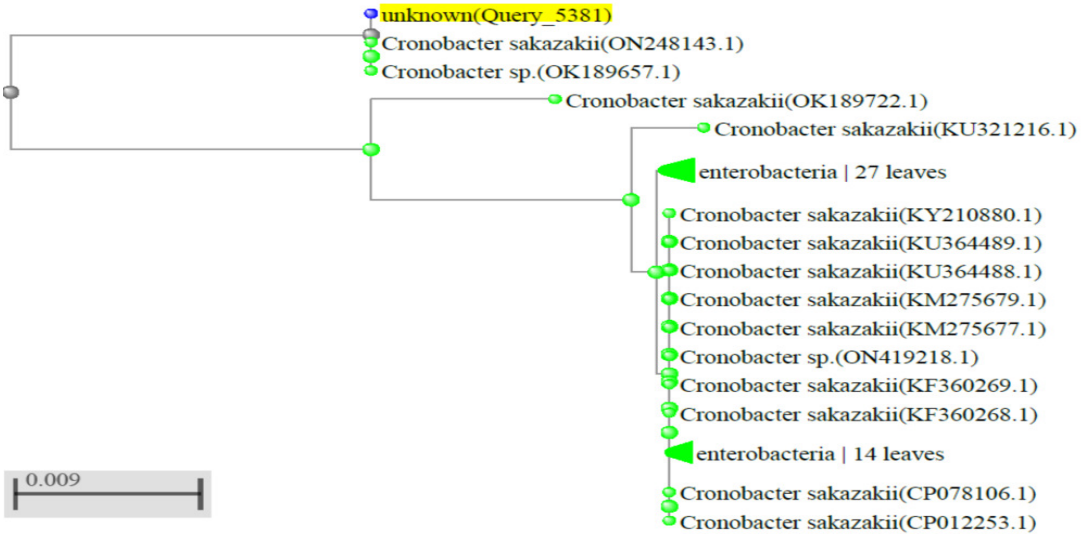
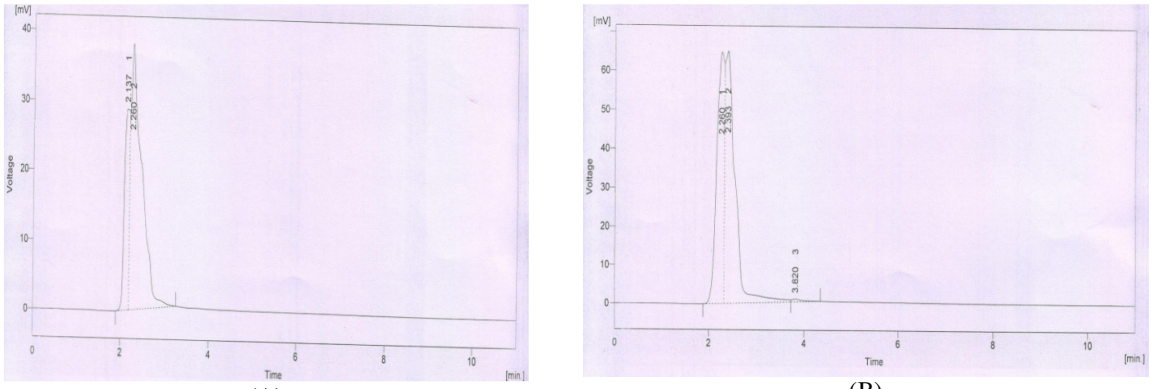


Fig. 4. Phylogenetic analysis by BAST for *Cronobacter sakazakii* (ON248143).

Fig. 5. After 7 Days of Incubation *Cronobacter sakazakii* Degrading MCP (1000ppm) on MSM Media.



(A) (B)

Fig. 6. HPLC Chromatograms for Control (A) & *Cronobacter sakazakii* Degrading Monocrotophos (B).

CONCLUSIONS

With this increased awareness, research has recently focused on the fate of pesticides in soils and health risks due to their transfer and accumulation in plants. Our study reveals that isolated bacterial strains growing in contaminated sites can withstand high levels of monocrotophos in the soil. The potent isolated bacteria in Ops biodegradation were molecularly identified as *Cronobacter sakazakii*(ON248143) by 16S rRNA gene sequence. New strain *Cronobacter sakazakii*(ON248143) proved that is a good tool in the bioremediation process in MCP contaminated soil.

Acknowledgment. This work was fully supported by my supervisor and the Department of Zoology, Rani Anna Government College for (w), Tirunelveli, Tamil Nadu India.
Conflict of Interest. None.

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How to cite this article: M. Chidambarapriya, P. Vanitha Pappa and K. Madhubala (2023). Molecular profiling of a novel bacteria *Cronobacter sakazakii* (ON248143), degrading organophosphate (MCP) pesticide from contaminated soil of agricultural land. *Biological Forum – An International Journal, 15*(4): 584-589.