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BIOAUGMENTATION OF *BACILLUS SUBTILIS* FOR THE DEGRADATION OF MONOCROTOPHOS IN AGRICULTURAL SOIL FROM KARNATAKA, INDIA

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ABSTRACT

Monocrotophos is a highly potent organophosphate insecticide. It is preferred by most of the farming communities. But this insecticide is non-biodegradable by natural processes and hence accumulates in the environment. Therefore, an efficient *Bacillus* strain capable of degrading monocrotophos was isolated from the agricultural soil from Mysuru district, Karnataka, India. 25 isolates of *Bacillus subtilis* were isolated from 45 agricultural soil samples. All the isolates were subjected to degradation studies through agar well diffusion method and high performance liquid chromatography method. One among 25 isolates was chosen as the test isolate, based on the results of degradation studies. Further, the isolate was sequenced with species-specific primers for 16S rRNA gene amplification. With this confirmation the isolate was submitted to GenBank. Also the sequence was compared with other bacterial sequences obtained from Gen Bank database BLAST program to construct a phylogenetic tree. The bacterial isolates from the present study exhibited profound capabilities to degrade monocrotophos at a concentration as high as 1000 mg/l in the minimal salt medium. The residue analysis of the insecticide revealed that the isolates could degrade 75% of monocrotophos after the treatment with bacterial inoculants. The present study has revealed the ability of *B. subtilis* to utilize monocrotophos insecticide as its nutrient source which can tolerate up to 1000 mg/l of monocrotophos, leading to its degradation. Thus, the present bacterial strain can be used for the bioremediation of monocrotophos contaminated agricultural soils.

KEY WORDS: Bioaugmentation, Monocrotophos, Biodegradation, *Bacillus subtilis.*

INTRODUCTION

Agriculture plays a crucial role worldwide as the sole source of food to nourish the ever growing human population. In agriculture, pesticides are important defense agents against pests which incur heavy losses during crop production. The role of pesticides is crucial for the survival of agricultural system all over the world. At the same time pesticides are considered to be biorecalcitrants, hardly degradable by natural oxidation process, thus posing a huge threat to the ecosystem (Shardendu *et al.,* 2018). In the past four decades, organo-phosphorus compounds have been extensively used in agriculture all over the world. Organo-phosphorus compounds exhibit a broad spectrum action against pests, and therefore have gained prime importance in the agricultural sector (Abraham and Silambarasan, 2015). Monocrotophos (dimethyl [(E)-4-(methylamino)-4-oxobut-2-en-2-yl] phosphate) is an organo-phosphate insecticide considered to be highly hazardous, yet the most preferred pesticide in India for the cultivation of vegetables (Ravneet and Dinesh, 2019).

In 2013, the World Health Organization (WHO) pointed out that monocrotophos is the primary choice among insecticides, accounting for more than 40% of the insecticides used for the management of pests (Shetty *et al.,* 2013). Monocrotophos rapidly kills the insect pests by its choline-esterase-inhibiting activity (Sidhu *et al.,* 2015). The Environmental Protection Agency (EPA) has

classified monocrotophos as being highly toxic (class I) to the environment and the biosphere itself (Sanger *et al*., 1975; Miller *et al*., 1999). It has been reported that 120 mg of monocrotophos can kill human beings (Ashwin *et al*., 2012).

Verma *et al*, (2014) have reported that monocrotophos is the preferred pesticide for vegetable cultivation among the Indian farmers as it is effective against a wide variety of pests namely, American boll worm, aphid, leaf hopper, grey weevil, spotted boll worm, pink boll worm, trips, white fly known to incur a huge loss to the agricultural crops (Shetty *et al.*, 2013). Monocrotophos is absorbed by soil particles due to its hydrophilic nature. It is highly toxic to the soil microorganisms, birds and animals including humans (Horne *et al.*, 2002; Kodandaram *et al.*, 2013; Srinivasulu *et al.*, 2016).

The toxicity of monocrotophos is mainly through ingestion, inhalation and skin absorption (Jia *et al.*, 2007). Monocrotophos poisoning in humans could be traced through symptoms such as headache, nausea, giddiness, vertigo, vomiting, sweating, lacrimation and excess salivation (Singh and Walker, 2006). It also affects the human respiratory system and nervous system by inhibiting the acetylcholinesterase, an essential enzyme for the normal nerve biochemical signal transmission. Monocrotophos is also known to exhibit carcinogenic effects in the liver and thyroid cells (Shetty *et al.*, 2013). Indian Ministry of State for Agriculture and Farmers Welfare has recommended that monocrotophos should be brought under 'to be phased out pesticide' category. Therefore, the usage of monocrotophos could be restricted in the forthcoming years.

Considering the enormous ill effects of monocrotophos on both soil and living beings, it is essential to find ways to degrade the pesticide residues to protect the environment. Bioaugmentation is a process to improve the degradative capacity of pesticide-contaminated geographical area by introducing specific microorganisms to achieve bioremediation. The main advantage of bioaugmentation is that, the process can be tailored to a specific dominant pollutant prevalent in a specific geographical region (Mariusz *et al.*, 2017). Bioremediation is an excellent method for the degradation of organo-phosphorus insecticides. The process is usually mediated by a specific biochemical pathway inherent among many soil bacteria (Bhalerao and Puranik, 2009; Ahire *et al.*, 2012).

Biodegradation of organo-phosphates like monocrotophos is carried out by the action of bacterial metabolic pathway involving hydrolysis of alkyl and aryl bonds. Removal of hydrogen molecule from the parental hydrocarbon molecule is the key step leading to the detoxification of organophosphorus compounds. This reaction depends on hydrolase enzymes like phosphatase (Kovacs *et al.*, 2016). Phosphatases are by far the best studied enzymes involved in bacterial metabolism of organo-phosphates like monocrotophos. An important step in the mineralization of organophosphorus pesticides is the hydrolysis of the parent molecule (Subhas and Singh, 2003). Once the process of hydrolysis is initiated, the pesticide becomes vulnerable to oxidation, thereby clearing its residues from the environment (Bhadbhade *et al.*, 2002).

Autochthonous bacteria capable of degrading and utilizing the pesticides as their carbon source have been isolated from the agriculture fields and analyzed for their bioremediation activity (Horne *et al.*, 2002; Gundi and Reddy, 2006; Dahm, 2008). Microbes such as *Bacillus, Pseudomonas, Aspergillus, Anabaena* and *Nostoc* have been reported to be able to utilize monocrotophos as a nutrient source leading to partial or complete degradation of the insecticide (Ravneet Kaur and Dinesh Goyal, 2019).

Till date no effective strain has been introduced in the agricultural market for the bioremediation of monocrotophos. The tested bacterial isolate from our research, was capable of utilizing monocrotophos as the sole carbon source for its growth. Hence, the present study is highly appropriate for the management of monocrotophos contaminated agricultural soil. To the best of our knowledge, this is the first potential strain isolated from the geographical region of Karnataka state, India, which can be patented and successfully applied for the bioremediation of monocrotophos contaminated agricultural soil.

MATERIALS AND METHODS

Pesticide stock solution

Monocrotophos (36% soluble liquid) technical grade (manufactured by Insecticides Ltd., Gujarat, India) was purchased from the local pesticide dealers. The working solution was prepared by adding 1mL of monocrotophos in 1000 mL of distilled water to get 1000 mg/L of the solution. This preparation was

used as the experimental dosage.

Sample Collection

Forty-five (45) soil samples contaminated with the monocrotophos insecticide were collected from the agricultural fields of Heggadadevana Kote, a place in Mysuru District, Karnataka, India. The agricultural lands lie between 11° 60′ and 21° 17′ north latitude and 75° 19′ and 77° 77′ east longitudes at an altitude of 610 m from the sea level. The weather at the site is hot and exhibits variable humidity levels ranging from 70% to 85%. The maximum temperature ranges from 35 °C to 38 °C and a minimum between 15 °C and 20 °C. The samples for bacterial isolation were collected from the soil at a depth of 15 cm from the top layer of the soil. The samples were air-dried, aseptically ground, passed through 2 mm sieve and stored in sterile zip-locked polyethylene covers at 4°C until further use.

Isolation of monocrotophos degrading bacterial strain from contaminated soil

The collected soil samples contaminated with monocrotophos were subjected to bacterial isolation procedures (Eliud *et al.*, 2016). One gram of the monocrotophos contaminated soil sample was added to a conical flask with 10 mL of sterile distilled water. The mixture was shaken well and serially diluted from $10^{-1}$and 10^{-5} dilutions. The aliquots of 0.1 mL of the inoculum from the final two dilutions were used to enumerate the isolates by spread plate technique in triplicates and incubated for 24 h at 37 °C.

Well separated individual colonies with different colony morphologies were selected and sub-cultured by streak plate technique on fresh nutrient agar media. The morphological features of the selected isolates were studied using light microscopy. The preliminary identification of the bacterial isolates was based on Bergey's Manual of Determinative Bacteriology (Brady, 1990).

Biodegradation of Monocrotophos

In the present study, the biodegradation of monocrotophos by the isolates is supported by agar well diffusion and high-performance liquid chromatography (HPLC) analysis.

Agar well Diffusion

0.1 mL suspension of 24 h old broth culture of the identified bacteria was swab-inoculated to the nutrient agar plates with the help of a sterile L-shaped glass rod. Wells were created using a sterile cork borer and filled with monocrotophos insecticides of different concentrations of 100, 250, 500, 750, 1000, 1250, 1500 mg/L, respectively. The plates were then incubated overnight at 37 °C and observed after the incubation period of 24 h (Alok Singh *et al*., 2015).

This method helped determine the ability of the isolates to grow in the presence of monocrotophos insecticide. An increase in the growth with no zone of inhibition was expected of the isolates to be considered for bioremediation.

High-Performance Liquid Chromatography (HPLC) analysis

The isolated samples were grown in 250 ml Erlenmeyer flasks in 100 mL of minimal salt medium with monocrotophos at a concentration of 1000 mg/L. These were incubated in a rotary shaker at 150 rpm, at 37 °C for 7 days. Samples were then centrifuged (3000×g) for 15 min at 4 °C. Cell-free supernatants were extracted using equal volume of ethyl acetate. Then, the ethyl acetate layer was pooled out and purified by solid phase extraction in Florisil glass column (15cm×1.5cm) packed with glass wool plug at the bottom and 3g of Florisil (Abhilash *et al.*, 2008; Jaiswal *et al.*, 2019). All the samples were eluted using rotary vacuum evaporator (Shimadzu, Rotation evaporator QR 2005-S, Japan) at 50 °C, 100 rpm. The reconstituted residues of monocrotophos in 2 mL of methanol were quantified by HPLC analysis method. Acetonitrile:water (20:80) was used as the mobile phase at a flow rate of 1 ml/min (Bhadbhade *et al.*,2002).

The residual monocrotophos was quantified spectrophotometrically at 254 nm (Shimadzu UV detector). The concentration of the unused monocrotophos was then calculated as molar absorption coefficient (Beer-Lambert Law).

$$A = \in lc$$

The percentage concentration of monocrotophos degradation (%) was calculated based on the amount of residual monocrotophos.

$$\text{Degradation (\%)} = \frac{\text{(Residual amount in control – residual amount in sample)}}{\text{(Residual amount in control)}} \times 100$$

The biodegradation level was calculated comparing the peak area of the test sample sample with that of the standard sample using

chromatogram (Skripsky and Loosli, 1994; Deng *et al.*,2015; Jaiswal *et al.*, 2019).

Polymerase chain reaction (PCR) and 16S rRNA sequencing

The selected bacterial isolates were identified and characterized based on the results of standard biochemical tests with reference to the Bergey's Manual of Systematic Bacteriology (Singh and Walker, 2006; Sidhu *et al.*, 2015). These were subjected to 16S rRNA gene sequencing. The DNA was extracted and the purified bacterial DNA was used for PCR (Krieg and Holt, 1984; Ahire *et al.*, 2012). The gene encoding 16S rRNA was amplified by PCR using a set of species-specific primers: P3 (forward primer): 52-AGAGTTTGATCA TGGCT CAG-32 and P13 (reverse primer): 52-GGTTAC CTTGTTACGACTT-32 with the following PCR conditions:

The initial denaturation was done at 94 °C for 4 min in the first cycle, and the final denaturation at 94 °C for 30 sec in 30 cycles. Annealing was set at 58 °C for 30 sec and the extension at 72 °C for 45 sec in 30 cycles and the final extension at 72 °C for 8 min in one cycle. PCR amplicon was confirmed by mixing 5 µL of the PCR product from each tube with 1 µL of 6X gel loading dye. Electrophoresis was carried out in 1.2 % agarose gel, with a constant voltage of 5 V/cm for 30 min. The amplified product appeared as a single compact band of the expected size under UV light and was documented by gel documentation system (Bio-Rad). The final confirmation of the bacteria was done using 16S r RNA genome sequencing.

The sequence was compared to other bacterial sequences available in the GenBank database with the support of BLAST program (Brady, 1990; Bhalerao and Puranik, 2009). Multiple sequence alignment was carried out using Clustal Omega. Phylogenetic analyses of the sequences were constructed using the Neighbor Joining (NJ) methods in MEGA 5.2 Version (software package) combined with Bootstrap analysis with 1000 replications.

Based on the results obtained in degradation studies a potential *Bacillus* isolate-1, with a nucleotide sequence coding for the 16S rRNA gene, was deposited in the GenBank database.

RESULTS AND DISCUSSION

Isolation and DNA sequencing of bacterial strains

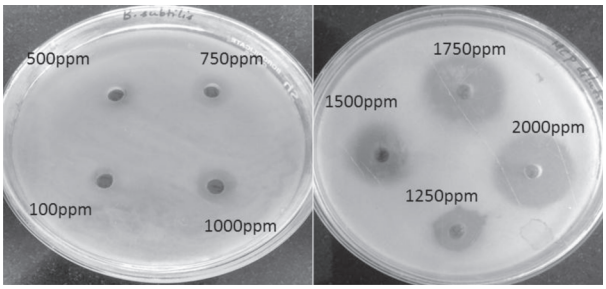
In this study, 45 different agricultural soil samples were screened to obtain 25 *Bacillus subtilis* strains. The isolates were identified based on biochemical and 16S rRNA analysis. *Bacillus subtilis* was found to be endospore forming, gram-positive, motile, and typically rod-shaped bacterium measuring about 4-10 µm in length and 0.25-1.0 µm in diameter. It showed positive biochemical results for catalase, citrate, gelatin hydrolysis, nitrate reduction, Voges-Proskauer and variable oxidase tests. The results were negative for indole, methyl-red and urease tests.

Biodegradation studies

Agar Well Diffusion

The degradation potential of the isolates was observed in different concentrations of monocrotophos in the nutrient medium by agar well diffusion method. The isolate-1 *Bacillus subtilis* PKM1-MG028597 showed a tolerance up to 1000mg/L of monocrotophos with minimal growth and a clear inhibition zone (Figure 1(a) and 1(b)). This capacity of the isolate hints a need for further research on the genetic manipulation of the corresponding gene loci responsible for its enhanced degradation properties at varied environmental conditions. The degradation of monocrotophos with concentration less than 1000 mg/L by the remaining 24 bacterial isolates was tabulated (Table 1).

The degrading capacity of the bacterial isolate provides a dual advantage of pesticide degradation as well as solubilizing soil phosphate (a compound essential for seed setting) thus increasing the bioavailability of phosphate to the crop plants. The *in vitro* investigation of the potency of the bacterial



1(a) 1(b)

Fig. 1(a). Agar well diffusion method: *Bacillus subtilis* species showing resistance with cell growth at 1000 mg/L of monocrotophos pesticide.

Fig. 1(b). Petriplate showing maximum zone of inhibition by *Bacillus subtilis* PKM1-MG028597 with increased pesticide concentrations of above 1250 mg/L of monocrotophos pesticide.

Table 1. Growth of *Bacillus subtilis* strains in the presence of monocrotophos.

Isolate No.	Concentrations of monocrotophos (mg/L)						
	Bacterial Strain	100	250	500	750	1000	1250
Isolate-1	*Bacillus subtilis* -PKM1	+	+	+	+	+	-
Isolate-2	*Bacillus subtilis*2	+	-	-	-	-	-
Isolate-3	*Bacillus subtilis*3	+	+	-	-	-	-
Isolate-4	*Bacillus subtilis* 4	+	-	-	-	-	-
Isolate-5	*Bacillus subtilis*5	+	+	+	+	+	-
Isolate-6	*Bacillus subtilis* 6	+	-	-	-	-	-
Isolate-7	*Bacillus subtilis* 7	+	-	-	-	-	-
Isolate-8	*Bacillus subtilis*8	+	+	-	-	-	-
Isolate-9	*Bacillus subtilis* 9	+	+	+	+	+	-
Isolate-10	*Bacillus subtilis*10	+	-	-	-	-	-
Isolate-11	*Bacillus subtilis*11	+	+	+	+	-	-
Isolate-12	*Bacillus subtilis* 12	+	-	-	-	-	-
Isolate-13	*Bacillus subtilis* 13	+	+	+	-	-	-
Isolate-14	*Bacillus subtilis*14	+	+	+	-	-	-
Isolate-15	*Bacillus subtilis* 15	+	+	-	-	-	-
Isolate-16	*Bacillus subtilis*16	+	+	+	+	-	-
Isolate-17	*Bacillus subtilis* 17	+	+	+	+	+	-
Isolate-18	*Bacillus subtilis*18	+	+	+	+	+	-
Isolate-19	*Bacillus subtilis*19	+	+	+	+	+	-
Isolate-20	*Bacillus subtilis* 20	+	+	+	-	-	-
Isolate-21	*Bacillus subtilis* 21	+	+	+	+	+	-
Isolate-22	*Bacillus subtilis* 22	+	-	-	-	-	-
Isolate-23	*Bacillus subtilis* 23	+	+	+	+	+	-
Isolate-24	*Bacillus subtilis*24	+	-	-	-	-	-
Isolate-25	*Bacillus subtilis* 25	+	+	+	+	+	-

"+"indicates the growth of the isolates in presence of monocrotophos.
"-" indicates no growth of the isolates in presence of monocrotophos.

isolates for degradation studies by agar well diffusion is a type of poisoned food technique. The efficacy was inferred from conventional diffusion tests. This is best suited to validate and compare the *in vitro* activity of bacterial strains for their ability to degrade pesticide, as a standard laboratory technique (Alok Singh *et al*. 2015). In addition, the efficacy of the delivered pesticide concentration and volume could be aptly predicted using agar well diffusion, which is indispensable for the *in situ* trials in the agricultural fields contaminated with pesticides like monocrotophos.

Chromatographic analysis

Monocrotophos degradation by *Bacillus subtilis* PKM1 was confirmed by HPLC analysis. The bacteria isolated from monocrotophos contaminated soil exhibited excellent degradation capabilities. The degradation increased with an increase in the incubation period (Figure 2(a)). Observation was done at specific intervals of time (1st, 2nd, 3rd, 4th, 5th, 6th and 7th day) and the analytical incubation periods revealed a gradual decrease in the concentration of monocrotophos leading to 75% degradation on the seventh day. The percentage results of monocrotophos degradation have been represented diagrammatically (Figure 2(b)).

The peak of the ethyl-acetate extract of the cell free supernatant of *Bacillus subtilis* PKM1 inoculated in minimal salt media was observed at a retention time (t_R) of 3.7 min. A comparison of the peak at 3.7 min of the control sample (with an area of 8542187 absorbance units), against the samples from the 7th day analysis (with an area of 2171291 absorbance units) at the y axis, showed a considerable decrease in the concentration of monocrotophos (shown in supplementary data S1 and S2).

Monocrotophos degradation has been attempted elsewhere in liquid culture medium using different bacteria (Rachna and Veena, 2015), algae (Megharaj *et al.,* 1987) and fungi (Tejomyee and Pravin, 2009; Jain *et al.,* 2014). In this study, the identified isolate *B. subtilis* strain PKM1 showed a maximum efficiency with 75% degradation in 7 days. It is not

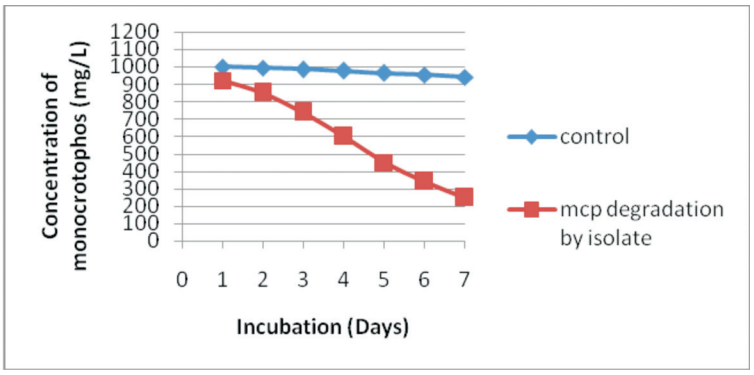


Fig. 2(a). Monocrotophos degradation (in mg/L) by *Bacillus subtilis* PKM1 during 7 days in the minimal salt medium under the shake culture condition.

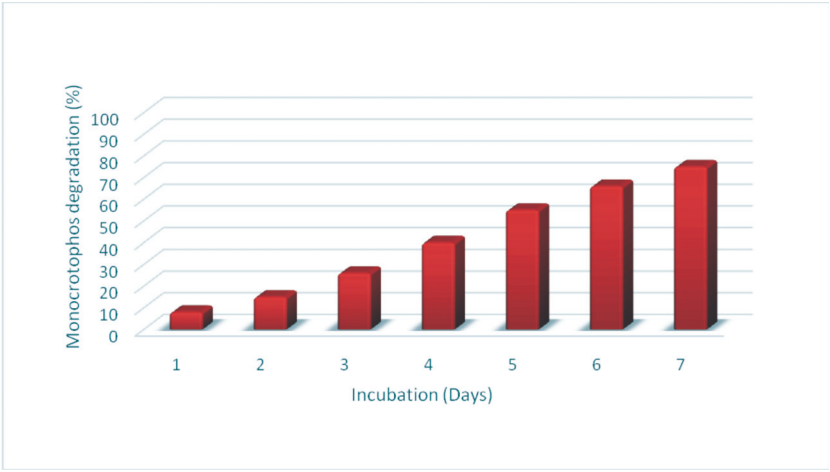


Fig. 2(b). The percentage of monocrotophos degradation by *Bacillus subtilis* strain PKM1 during 7 days in the minimal salt medium under the shake culture condition.

an easy task to simply isolate an organism capable of degrading pesticides like monocrotophos. The bacterial isolate needs to show its activity under different biotic conditions. A major challenge for a bioremediation agent is to survive in competition with other microbes that already exist in the same niche. The antagonistic interactions play a crucial role in the final outcome of the bioaugmentation process. However, the isolate *B. subtilis* strain PKM1 is indigenous to the place where it was isolated; therefore the strain had the advantage of early colonization.

Furthermore, 16S rRNA amplification by PCR confirmed that the isolate was *Bacillus subtilis.* The PCR product was subjected to 16S rRNA sequencing and the sequence was deposited in GenBank. A phylogenetic tree was constructed based on a similar known *Bacillus* species and other related species (Figure 3). The isolate *Bacillus subtilis* strain PKM1 was deposited in GenBank database with the accession number MG028597.

The DNA sequence was found 99% similar to the sequences of *Bacillus subtilis* strain KPA-1: KC833048 and *Bacillus subtilis* strain DSM 10: NR027552. The phylogenetic analysis revealed that *B. subtilis* PKM1: MG028597 shared high similarities with other *B. subtilis* strains. Therefore, a successful PCR amplification of the 16S rRNA and the sequence data from GenBank, in comparison with several species of *Bacillus*, proved that the isolate in our study involved in the monocrotophos degradation was *B. subtilis*.

CONCLUSION

The present study provides a practical solution to reduce the burden of pesticide poisoning in our precious environment. The bacterial isolate *Bacillus subtilis* PKM1 (Genbank accession number: MG028597) showed a degradation of

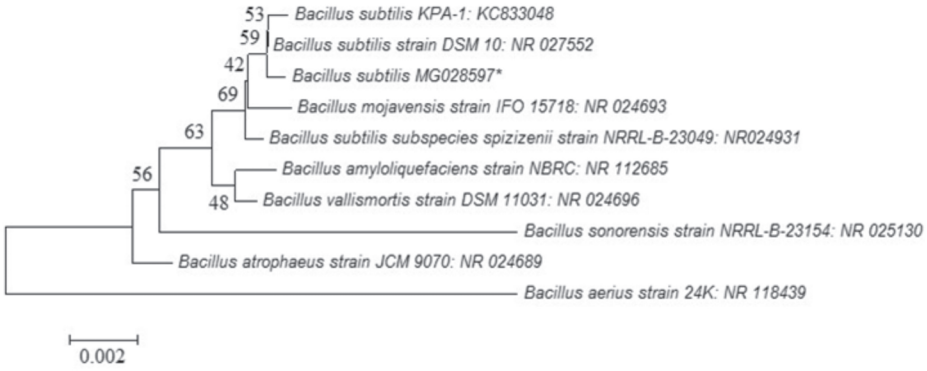
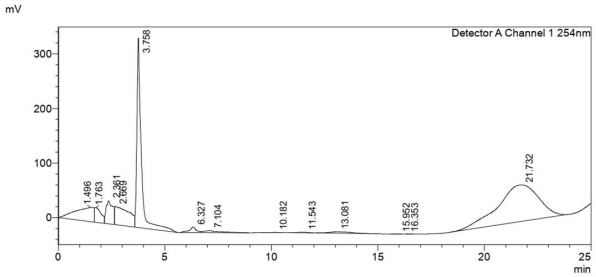
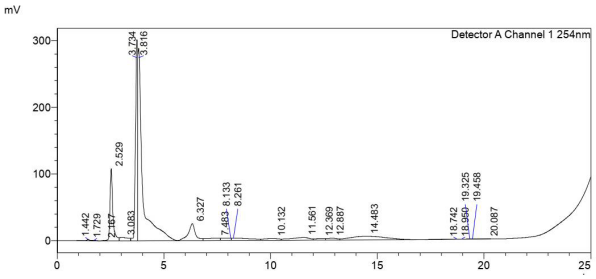


Fig. 3. Phylogenetic tree of *Bacillus subtilis* PKM1-MG028597 isolated from the present study. The scale bar 0.002 indicates substitutes per nucleotide position. The Bootstrap values indicate percentage at all branches.



Supplementary file S1) Chromatogram - Control



Supplementary file S2) Chromatogram analysis after 7 days of *B. subtilis* PKM-MG028597 incubation

monocrotophos in agar well diffusion method and HPLC analysis as reported in our study. It can also provide insight to the degradative capacity of the isolates in order to build a strategy for further studies, to acclimatize them into the natural environmental conditions, thereby augment the pesticide degradation. Microbial degradation can prove efficient, eco-friendly and cost effective to combat the adverse effects of pesticides. Hence the isolation of pesticide degrading organisms from the contaminated soil itself provides a positive approach to study the genetic makeup of the microorganism which allows it to survive in the pesticide contaminated soil, understand its genetic characterization and manipulation which could improve its degradation capacity. The overall results of this study prove that the identified isolate *B. subtilis* could be a promising agent for the degradation of monocrotophos contaminated agricultural soil.

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