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Research Paper

MOLECULAR CHARACTERIZATION OF BACTERIA CAPABLE OF ORGANOPHOSPHATE DEGRADATION

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Synthetic organophosphorus compounds (OP) are widely used in agriculture to control agricultural and household pests. The excessive use of OP compounds have generated a number of environmental problems such as contamination of air, water and terrestrial ecosystems, harmful effects on different biota, and disruption of biogeochemical cycling. Therefore, there is a need for economical and dependable methods of OPs detoxification from the environment. Conventional methods (chemical treatment, incineration and landfills) are having a number of disadvantages when compared to biological methods. Soil microflora is one of the basic eco-friendly agents for detoxification of pesticides. OP pesticide-degrading bacteria have been isolated by using soil enrichment process with organophosphates. From pesticide enriched soil through serial dilution pure isolated colonies were obtained. For molecular identification of the isolated strains genomic DNA was isolated and analyzed by agarose gel electrophoresis. The 16S rRNA gene was amplified through PCR using forward primer 5' AGAGTTTGAT CCTGGCTCAG 3' and reverse primer 5' GACGGGCRGTGWGTR CA 3'. The amplified product was purified & the gene was sequenced using sangersdideoxy method. The 1330 nucleotide sequence of the gene was compared with known nucleotide sequence using BLASTn and this gene showed 99% similarity with the 16S rRNA gene of the *Pseudomonas putida*. Finally, the phylogenetic analysis was done.To analyze the degradation capability, the phosphate levels are estimated by Fiske-Subbarow method. The phosphate levels are increased due to detoxification of organophosphorus compounds.

Keywords: Organophosphate (OP), *Pseudomonas putida*, BLASTn, Phylogenetic analysis

INTRODUCTION

India is primarily agriculture-based country with more than 60-70% of its population depends on agriculture. As India's population is increasing day by day there is more pressure on the annual food grain production and on minimizing the crop losses (Shroff, 2000). To meet these objectives, the use of more fertilizers to increase the

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productivity of land under cultivation on one hand and providing protective cover to the crops on the other is the most important (Pradnya P Kanekar *et al*., 2004). An organophosphate (OP) is the general name for esters of phosphoric acid. Organophosphates are also the basis of many insecticides, herbicides, pesticides, and chemical warfare agents, it is due to their nerve toxicity to inactivate acetyl cholinesterase enzyme in insects, humans and many other animals (Zhengliu *et al*., 2009, and Compton *et al*., 1997). Organophosphorus insecticides are all esters of phosphoric acid which include aliphatic, phenyl and heterocyclic derivatives (Hassal, 1990). There are currently 140 OP compounds being used as pesticides and as plant growth regulators around the world. Their acute toxicity to non-target organisms, including humans arouses an environmental concern for developing safe, economical, and reliable methods for detoxification/remediation of these compounds (Yu lei *et al*., 2005).

Conventional methods to detoxify OP pesticides mainly rely on chemical treatment, incineration and landfills and all these are having a number of disadvantages like the production of a large volume of acids and alkali that subsequently must be disposed in case of chemical methods, leaching of pesticides into surrounding soil and ground water supplies in case of landfills, toxic emissions into the environment in case of incineration. Compared to the potential disadvantage of conventional methods, remediation using microorganisms has been considered as an attractive, potentially convenient, effective, low-cost and an eco-friendly method (Zhengliu *et al*., 2009, and Yu lei *et al*., 2005). With the developments in biotechnology, new efforts have been emphasized on the use of natural or soil microorganisms for the degradation of pollutants rather than disposal. The signs and symptoms of acute organophosphate poisoning are an expression of the effects caused by excess acetylcholine; they may occur in various combinations and can be manifest at different times. Signs and symptoms can be divided into three groups, i.e., muscarinic effect, nicotinic effect, and central nervous system effect. Those individuals who are exposed to organophosphorus pesticides with pre-existing/ organic diseases of the central nervous system, mental disorders and epilepsy, pronounced endocrine and vegetative disorders, pulmonary tuberculosis, bronchial asthma, chronic respiratory diseases, cardio-vascular diseases and circulatory disorders, gastrointestinal diseases (peptic ulcer), gastroenterocolitis, diseases of liver and kidneys, eye diseases (chronic conjunctivitis and keratitis)/ are at elevated risk from exposure. Even it causes dreadful diseases like Alzheimer's (K. Hayden *et al*., 2010), Chronic fatigue (Tahmaz *et al*., 2003) and have also been related to various modern diseases, including Creutzfeldt–Jakob (CJD) and the Gulf War syndrome. Soil microflora is one of the best eco-friendly agents for detoxification of organophosphates. Microorganisms use pesticides as nutrients for their growth. Pure cultures are able to detoxify the pesticides into different metabolites like *P*-nitrophenol, diethyltriphiosphoric acid, *p*-aminophenol, dimethyl phosphate, orthophosphate, etc. (Pradnya P Kanekar *et al*., 2004). So, our present work is focused on isolation of bacteria from organophosphate enriched soil and its molecular characterization by amplification and sequencing of 16S rRNA gene, phylogenetic tree construction. Finally, analysis of the degradation capability.

MATERIALS AND METHODS

Soil Samples

Organophosphate pesticide enriched agricultural soil samples from different locations in and around Vijayawada were collected in sterile polythene bags and is used in enrichment culture studies.

Preparation of Media

For obtaining pure and isolated colonies nutrient agar and LB Broth media were prepared. Nutrient agar is a microbiological growth medium commonly used for the routine cultivation of non-fastidious bacteria. Nutrient agar typically contains 0.5g Peptone, 0.3 g beef extract/Yeast extract, 1.5 g agar, 0.5 g NaCl make it to 100 ml with distilled water, the pH is adjusted to neutral (6.8) and was sterilized at 121^0C for 20 min in suitable conical flasks. LB broth it consists of 10 g tryptone, 5 g Yeast extract, 10 g NaCl make it to 1lt. and was sterilized at 121^0C for 20 min in suitable conical flasks. Nutrient agar medium is poured in petridishes and allow them to solidify and simultaneously prepare the LB broth test tubes.

Isolation of Microorganism from Soil Sample

Soil samples were serially diluted in sterile water blanks and 0.1ml each dilution sample was spread on agar media surfaces in petridishes and it is incubated for 24hrs at room temperature. Isolated and clear colonies were observed in the petridish. Among the colonies, isolated colony was picked from the petridish and it is inoculated into the LB broth and it is incubated for 24hrs at 35^oC.

Genomic DNA Isolation

1.5 ml of bacterial culture was taken in centrifuge tube. Centrifuged at 10,000 rpm for 2 minutes. The supernatant was discarded. To the pellet 1 ml of distilled water was added and dissolved the pellet completely. Again centrifuged at 10,000 rpm for 2 min. Repeated the same for two times. The supernatant was discarded and to the pellet 100µl of Tris EDTA buffer was added and dissolve the pellet completely in buffer.

Agarose Gel Electrophoresis

Gel electrophoresis is a technique used for the separation of nucleic acid and to monitor the success of nucleic acid isolation procedures, where electrically charged molecules migrate at a rate proportional to their charge-to-mass ratio when placed under electric field. About 0.8g of agarose was weighted and taken in a 100 ml reagent bottle. To this 100 ml of 1X TBE buffer was added and it was heated in microwave oven till agarose melts. The agarose solution was then poured in a gel-casting unit assembled with appropriate comb and it was allowed to get polymerize. When the agarose polymerized, the comb was removed and the gel was kept in electrophoresis tank consisting of 1X TBE buffer. About 2 ml of isolated bacterial genomic DNA was mixed with 2 ml of loading dye (bromophenol blue) and it was loaded in 0.8 % agarose gel. The gel was electrophoresed at 90 volts for about 30 minutes and it was observed on UV transilluminator.

PCR Amplification and Purification of 16S rRNA Gene

The Polymerase Chain Reaction (PCR) is an enzyme catalyzed biochemical reaction in which small amounts of a specific DNA sequences are amplified into large amounts of linear double-stranded DNA (Mullis 1990). In PCR, the specific primers (forward and reverse primers) complementary to known sequences were added

to DNA and the mixture is heated at 94°C for denaturation of DNA (Unzip the double helix). The mixture is then allowed to cool, enabling the primers to anneal to the complementary sequences. A heat stable DNA polymerase was used to make copies of DNA from the original DNA sequences. The DNA was again heat denatured to separate the strand, then cooled to allow the primers to re-anneal and prime the synthesis of additional DNA. 25 cycles of amplification were performed that resulted in a very large amplification of DNA. The 16S rRNA gene was amplified using primers 5' AGAGTTTGAT CCTGGCTCAG 3'and 5' GACGGGCRGTGWGTR CA 3'. PCR mix contained 10X buffer, 100mM dNTPs, 2.52M $MgCl_2$, 2U*Taq*DNA polymerase, 1µl of each (forward and reverse) primers, 2µlof genomic DNA and sterilized distilled water to make a final volume of 25 ml. *Taq*DNA polymerase initiates replication of DNA fragment by using nucleotide bases from dNTPs mixture (A, C, G, T).

The PCR reaction included the following steps; initial denaturation of 2 min at 94°C (Pre heating) 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 for utilization of extra dNTPs in the PCR mixture.

Sequence of 16S rRNA Gene Using Sangers Dideoxy Method

In 1974, Sanger, designed a procedure for sequencing the DNA similar to the natural process of DNA replication. DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of the sequence of all nucleotides. With this knowledge, comparisons are made between homologous genes across species. Identification of strain was done by 16S rRNA sequence analysis.

BLASTn Analysis and Phylogenetic Analysis

Basic Local Alignment Search Tool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the nucleotides of DNA sequences. A BLASTn search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. BLAST finds homologous sequences, not by comparing either sequence in its entirety, but rather by locating short matches between the two sequences, after this first match that BLAST begins to make local alignments. This program, given a DNA query, returns the most similar DNA sequences from the DNA database that the user specifies. Using the results received through BLASTn a phylogenetic tree is created using the BLAST web-page.

Analysis of Organophosphate Degradation by Isolated Bacteria

For analysis of organophosphate degradation flasks with medium and 0.5% organophosphate were incubated at 37°C for 4 days on a shaker at 200 rpm. Control and test reaction was set up without and with inoculums respectively. As a control organophosphate free soil sample was taken and for test 0.5 % of organophosphate was added to the soil sample. After 4 days of incubation

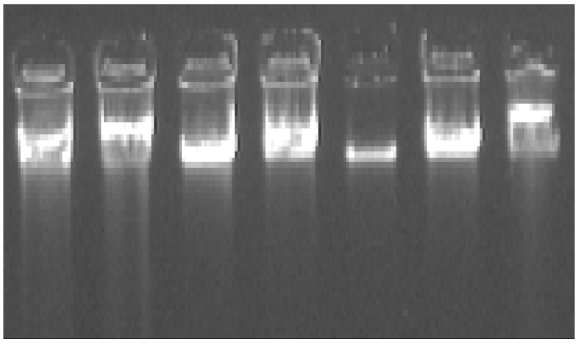
the phosphate levels were estimated using Fiske-Subbaraw method (Jayaram, 2002). From the standards graph the phosphorus content present in the test samples were measured.

RESULTS AND DISCUSSION

To increase the agricultural yield pesticides plays an important role, but only less than 1% of the pesticides are enough to kill the pests, remaining pesticides enter into the ground and surface water and causes environmental pollution (Battaglin and Fairchild, 2002). Thus some of the pesticides were banned and some are modified without damaging to the environment. However, organophosphates are creating a lot of health problems (Sogorb *et al*., 2004). Different kinds of bacteria present in soil and water are capable of degrading many insecticides (Ramanathan M P and Lalithakumari, 1999; El-Deeb B A *et al*., 2000; Bhadhade B J *et al*., 2002; and Chen Y *et al*., 2002).

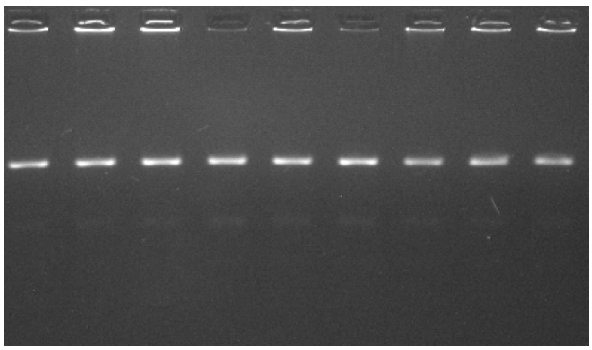
The present investigation involves the isolation of pollutants tolerant and resistant bacteria from the respective contaminated sites. As the microorganisms like bacteria plays an important role in bioremediation, experiment was carried out to identify the bacterial strain from organophosphate contaminated soil capable of degrading organophosphate. First, for the study soil samples were collected and it was serially diluted. Nutrient agar medium and LB broth was prepared. The diluted soil samples are inoculated on to the nutrient agar petridish. An isolated colony was picked from the petridish and later the same colony is inoculated onto the broth. From this pure, isolated and single colonies were obtained after 4-6 times of subculture. The genomic DNA was isolated from the microorganism. Electrophoretic analysis of isolated DNA using 0.8% agarose gel followed by observation on Ultraviolet transilluminator revealed sharp high molecular weight bands of DNA (Figure 1) that indicates the DNA was of good quality and suitable for PCR analysis. The 16S rRNA gene of the

Figure 1: Genomic DNA



isolated organism was amplified using primers 5' AGAGTTTGAT CCTGGCTCAG 3'and 5' GACGGGCRGTGWGTR CA 3'with the help of a thermocycler (Figure 2) and it was purified. The purified 16S rRNA gene was sequenced using dideoxy method (Figure 3). After sequencing it was identified that the 16S rRNA gene consists of 1330 nucleotides (Figure 4). BLASTn analysis was performed for the 16S rRNA gene. The

Figure 2: PCR Product of 16S rRNA Gene



16S rRNA gene showed 99% similarity with the 16S rRNA gene of the *Pseudomonas putida*. Finally a phylogenetic tree was constructed. From the phylogenetic tree (Figure 5) the organism that is able to degrade the organophosphorus compounds were identified as *Pseudomonas putida.* The Fiske-Subbaraw method shows that the amount of phosphate was increased in the sample which was treated with the *Pseudomonas putida*, it is due to the breakdown of organophosphates. So, with this quantitative analysis it is confirm that the organism which was isolated is capable of degrading organophosphorus compounds.

Figure 3: 16S rRNA Gene Sequence Using Sangersdideoxy Method

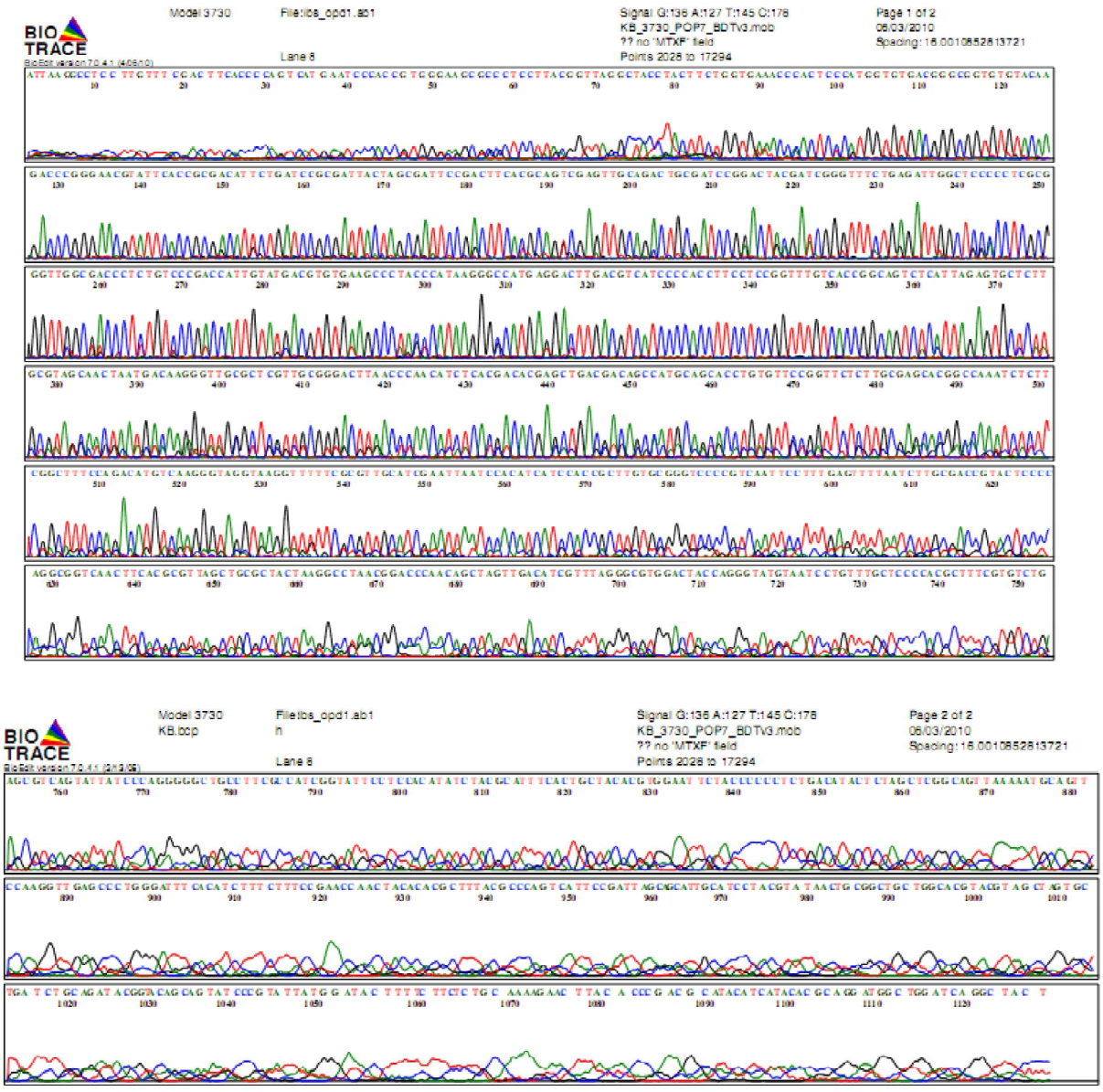
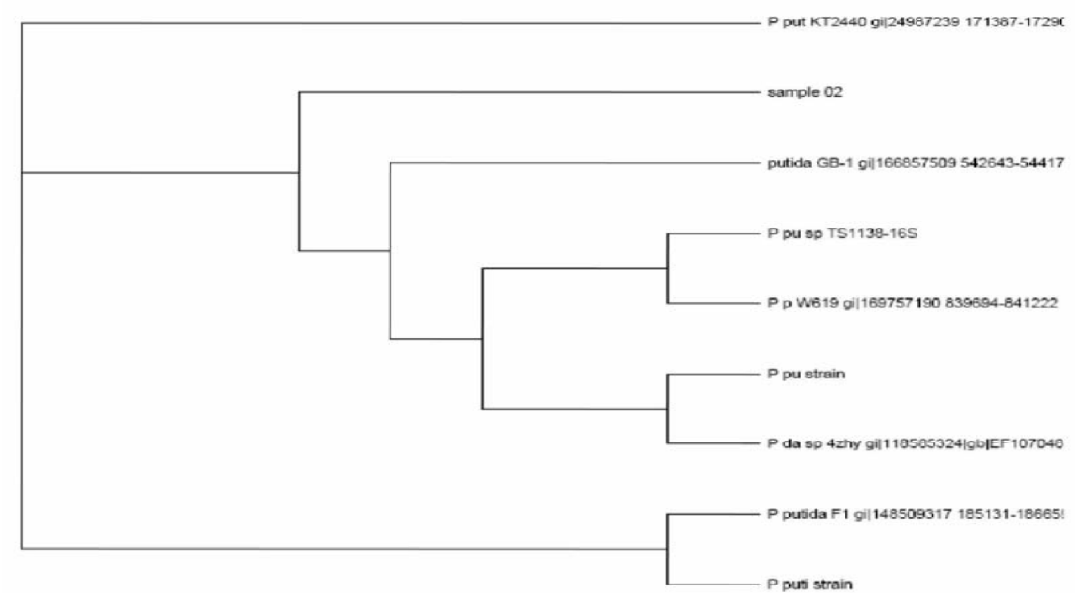


Figure 4: The 16S rRNA Gene Sequence

>OPD_1_ribo_seq

GGTGGGAGCTACCTGCAGTCGACGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGA
GTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTC
GCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT
GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAA
ACACGGTCCAAACTCCTACGGGAGGCAGCAGTGGGGAATTTGCCCATGGGGCGCAAGCCGGATGCAGCC
ATGCGGGGGGGATACATAGGGCCTACGGTTGTAAACCTTCTTTACCGGGAAAGAACTCGGTTAGAGACAT
AAAATTCTTCCGAATGACCTTAACCCGCAAAAAATATACCGCGTTAATTCCATTCCTACCTTCCTCGAAT
ATATTGATGGTGCAAGCTTTAATCACGACTTACAGCGTCGTACACCTCACGACAGGCGGTTTGATCACTC
AGATGTAAAATTCCCCTCGCATCAATCCTGGTAACTTGCATTCGAAAACTGGCCTAGGCTAGCACTCTTG
GTAGAAGGCGGTAAGAAATTCTTCAGCAGAAGCACTGTAAATGCCTAACACATCTTGGACCGAATACCGC
TATGGCGTAATGGCGGCCCTCCTGGACAAACCACTGATCGACTCAAGTAGTCGATAACCCTGGCGCAGCA
CAAAAGGATTTAGAAAACCCTCGGTTAGTCCACCCCTGTAAAACGATAGTCCAAATAGAGGTATGACTGC
CCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC
CTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATC
CTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGAT
GACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGC
GACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCA

Figure 5: Phylogenetic Analysis by BLAST for *Pseudomonas putida*



CONCLUSION

The isolated soil bacteria are capable of degrading organophosphates. Its molecular characterization showed that the bacteria are *Pseudomonas putida.* Instead of going for the chemical methods, the eco-friendly soil bacteria can be used for degradation of organophosphates.

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