Pesticides Bioremediation Potentials of Bacterial Isolates of Contaminated Spot

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Abstract: Bioremediation approach to clean up pollution is a rapidly changing and expanding area of environmental biotechnology. Bacterial consortium capable of degrading atrazine and methyl-parathion was isolated from a mud waste of a gas station. Five isolates were screened and classified differentially according to shape, colony type, gram reaction and spores formation as D1, D2, D3, D4, and D5. Isolates were able to grow in mineral salt medium supplemented with paraoxon or methyl-parathion (until 250 ppm) as a sole carbon source. D1 and D5 isolates are more efficient for degrading 70–85% of the initial dose of paraoxon (250 ppm) after incubation for 48 hrs. Also, D1 and D2 showed highest efficiencies among the other isolates by achieving more than 40% degradation of tested methyl-parathion. Moreover, the five isolates have been tested for degrading atrazine at 0.06 and 0.12 mM and showed capabilities for utilizing atrazine as a sole nitrogen source. D1 has shown higher atrazine-degradation rates in atrazine-containing minimal media than other isolates. Current investigation has indicated that D1 degrade as high as 96% of atrazine after incubating for 28 days, which introducing D1 as a nominee for bioremediation programs. The molecular identification of D1 was performed by amplification of 16S rDNA gene and sequencing of the purified PCR product. Based on the obtained data, isolate D1 was designated as *Bacillus* sp. D1 with GenBank accession no. KJ545610. Finally, the current bacterial isolates have potential as a bioremediation tool in contaminated areas for different pesticides categories.
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1. Introduction

Many agricultural dealerships' and pesticide mix-load sites' soils and associated groundwater have contained such extremely high concentrations of pesticides, fertilizers and other organic compounds used in pesticide formulations that they have been designated as point sources of contamination (Habecker, 1989; Ames and Hoyle, 1999). During the twentieth century, the explosive development and use of different types of pesticides especially OP's and herbicides, and it is predicted that this trend would most likely continue as new applications for these compounds are discovered. Their widespread use has caused severe environmental pollution, since pesticides applied in agricultural areas do not remain at their target sites, but often enter aquatic environments via soil percolation, air drift or surface runoff (Andersona and Hunta, 2003). Although most developed countries established bans and restrictions on the use of several OP's and atrazine during the 1970s and 1980s, they are still being used in certain countries for agricultural and public health purposes because of their low cost and versatility as pest control (Xue *et al.,* 2006). Moreover, misuse, remaining and banned pesticides are still need for controlling (Richins *et al.,* 1997). Remediating pesticides contamination problems as well as dealing with the huge stocks of banned pesticides became one of the most public concerns in Egypt as well as worldwide. Traditional methods of pesticide decontamination that officially approved by EPA such as chemical treatment, incineration or excavation for dealing with pesticides contamination problem become inadequate (Omenn, 1992). Bioremediation technology is now proved as a cleanup technology because it is thought to be a low cost and environmental-friendly technique alternative to chemical treatment procedure for solving this problem (Singh *et al,.* 2006a; Chirnside *et al.,* 2009). The effective biotechnological main approaches to bioremediation are environmental modification, such as, biostimulation, bioaugmentation, and genetically engineered microorganisms (GEM's). Those three main approaches are used extensively at different efficiency for treating pesticide contamination problem (Singh *et al.,* 2006b and Wang *et al.,* 2013). However, isolate microorganisms from contaminated places considered as the first step for initialization the three previous mentions approaches. Isolation of microorganisms for using as bio-remediating agents

have been used by several authors (Qingyan, *et al.,* 2008; Malghani, *et al.,* 2009; and Wang 2013). For OP's decontamination (Mansee *et al.,* 2004; and Ortiz-Hernández and Sánchez-Salinas, 2010). Also, using microorganisms for remediating herbicides have been examined by many researchers (Qingyan, *et al.,* 2008; Chirnside *et al.,* 2009; Lima *et al.,* 2009 and Satsuma 2009).

Isolation and characterization of bacteria from polluted area have been conducted for bioremediation issue in many articles (Qingyan *et al.,* 2008; Lima *et al.,* 2009; Malghani *et al.,* 2009; Fuentes *et al.,* 2010). Using of single bacteria consortium for decontamination pesticides was taken a part of researchers interest (Mansee *et al.,* 2004; Chirnside *et al.,* 2009; Fuentes *et al.,* 2010) while others focusing on using single sp. (Qingyan *et al.,* 2008; and Cycon *et al.,* 2009). Moreover, Omotayo *et al*. (2011) used traditional and *in situ* enrichment using porous Bio-Sep beads fortified with atrazine were used to obtain mixed consortia of atrazine-degrading bacteria. The results indicate that *in situ* enrichment with Bio-Sep beads might be a viable method to cultivate atrazine-degrading bacteria not currently represented in existing culture collections. Thus, the detection of known atrazine-catabolic genes in soil or bacteria from tropical African contaminated systems is an indication of the likely global distribution of these important s-triazine genes. Wang *et al,* (2013) used a previously isolated atrazine-degrading bacterium, *Arthrobacter* sp. strain DAT1, to investigate its potential of remediating soil with heavy contamination. There bioremediation process could achieve a high removal rate in 2-3 days which provide some new insights toward bioremediation of heavily atrazine-contaminated soil.

The current study aims to isolate bacteria from polluted spot (petroleum gas station) and assaying its efficiency for degradation methyl-parathion, paraoxon and atrazine.

2. Material and Methods

Chemicals

Methyl-parathion 99.5% (dimethyl- *p*-nitrophenyl phosphorothioate) and Paraoxon 98% (diethyl- *p*-nitrophenyl phosphate) were purchased from Chem. Service, Inc, West Chester, PA, USA. Atrazine 98% (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) was obtained from El-Helb Company. Nutrient broth (NB), buffer phosphate (sodium monobasic phosphate, sodium dibasic phosphate and sodium citrate pH 8.0), and one liter of the basic salt media (nonselective media) contained (0.05 g of $MgSO_4$, 5 $x10^{-3}$ g of $MnSO_4$, 0.01 g of $CaCl_2$, $5x10^{-4}$ g of $CuSO_4$, $1x10^{-3}$ g of $FeSO_4$, 0.037 g of $(NH_4)_2SO_4$, 0.04 g of K_2HPO_4, and 4 g of glucose) were used.

Atrazine medium (minimal medium) contained 1.6 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.2 g of $MgSO_4$. $7H_20$, 0.1 g of NaCl, 0.02 g of $CaCl_2$, 1 g of sucrose, 1 g of sodium citrate, 2.5 ml of atrazine stock solution (2 mM), 20 ml of a salt stock solution, and 20 ml of a vitamin stock solution was dissolved in one liter of distilled water. The salt stock solution contained 2.5 g of EDTA; 11.1 g of $ZnSO_4$; 5.0 g of $FeSO_4$; 1.54 g of $MnSO_4$. H_20; 0.4 g of $CuS0_4$. $5H_20$; 0.25 g of Co $(NO_3)_2$ $6H_20$; 0.18 g of $Na_2B_40_7$-10 H_20; and 5.0 ml of concentrated H_2SO_4 to retard precipitation of salts, dissolved in one liter of distilled water. The vitamin stock solution prepared by dissolving the following gradients in one liter of distilled water: 5 mg of thiamine-HCl, 2 mg of biotin, 2 mg of folic acid, 10 mg of nicotinamide, and 10 mg of pyridoxine-HCl. Solution acidity was adjusted to pH 7.3. U buffer (10 mM sodium phosphate, 0.1 mM $MgSO_4$ pH 7) was also used.

Bacteria Isolation

Different isolates of bacteria were isolated from mud samples that were collected from wastes of a petroleum gas station in west sectors of Alexandria, Egypt. Loopful from solution prepared by diluting 1 gm of each sample in 9 ml DW, was streaked onto the nonselective media incubated at 30^o C for 2-3 days. The growing bacterial colonies were selected and purified for further identification. Five different bacterial isolated were observed (D_1, D_2, D_3, D_4 and D_5) based on cultural characteristics including shape of bacterial cells, sporulation, colony type and gram stain, according to Bergey's manual of systematic bacteriology (**Table1**).

Table 1. Microscopically examination of bacterial isolates from wastes of a petroleum gas station

Isolates	Characteristics			
	Shape of cells	Type of colony	Gram reaction	Spores
D1	Rod	Single	Positive	present
D2	Rod	Aggregate	Negative	Negative
D3	Cocci	Single	Positive	Negative
D4	Rod	Aggregate	Negative	Negative
D5	Rod	Single	Positive	Negative

Assaying of Isolates Detoxification Capabilities

OP's

Efficiency of the five bacteria isolates for organophosphorus degradation was assayed according to Mulbry *et al,* (1998). One colony of each of isolated strains was grown in 5ml nutrient broth media (NB) and shaked at 37°C, 250 rpm on an orbital incubator-shaker along for 48 hrs.. Growth was observed by solution turbidity intensity. Then one ml

of cell suspension was transferred to 50 ml of the same liquid media and repeated shaking under the aforementioned conditions. Finally, 3 ml of resulted cell suspension was transferred to 150 ml of the same media, in 250 ml flask and shaken at the previous conditions. Cells were harvested by centrifugation at 4°C, 5000 rpm for 10 min. Cells then washed with 40 ml of 150 mM buffer phosphate pH 8.0 twice and re-centrifuged at the same conditions. The resulted pellets were weighted and then diluted in 10ml of buffer phosphate.

Ten microlitter of the harvested bacteria suspension were added to a serial of the tested OP's compounds solutions ranged from 10 to 250 µg ml^{-1}, prepared in 50 mM buffer phosphate, pH 8.0. Three replicates were carried out for each concentration, as well as for control which prepared by incubated bacteria cells in OP's free buffer solution. The replicates were incubated at 37°C for 48 hrs. and then analyzed for producing *p*-nitrophenol by measuring optical densities spectrophotometrically at 410 nm as an indication for OP's degradation and the efficiency of isolated strains.

Atrazine

Bacteria isolates were grown as described by García-Gonzalez *et al.,* (2003) with certain modification for assaying atrazine degradation. One colony of isolated bacteria was inoculated in 3 ml of minimal medium, with ammonium chloride (4 mM) as the sole nitrogen source and shaking overnight at 30°C. Cells were harvested by centrifugation at 4°C, 5000 rpm for 15 min, washed with 10 mM phosphate-buffered saline three times and re-centrifuged at the same conditions. The obtained pellets were re-suspended in minimal medium with atrazine as a nitrogen source. Cultures were shaken overnight at 30°C and re-centrifuged at the same previous conditions. The resulted pellets were washed three times with U buffer and re-suspended in the same buffer to an OD_{600} of 0.25.

One ml of the harvested bacterial suspension was incubated with atrazine (either 0.06 or 0.12 mM). Three replicates was carried out for each concentration and incubated at 30°C for different time intervals 1, 6, 24 hrs.; 7, 14, 21 and 28 days, as well as three replicates for control that prepared by incubated tested concentration of atrazine in bacterial suspension free buffer solution. One milliliter of each sample was centrifuged immediately for 3 min at 4°C, 5000 rpm. The remaining atrazine concentration was measured spectrophotometrically at 221 nm (A_{221}) of the supernatants.

Molecular characterization of D1 isolate.

The genomic DNA was extracted from D1 isolate using Thermo Scientific GeneJET Genomic DNA Purification Kit. The amplification of the 16S rDNA gene was performed using universal primers F-Start (5'-AGAGTTTGATCMTGGC TCAG-3') and End-R (5'-TACGGYACCTTGT TACGACT-3') to give a PCR product of approximately 1400 bp. The PCR mixture was composed of 100 ng of genomic DNA, 30 pmol of each primer, 200 µM of dNTPs, 1U of Taq polymerase and 10 µl of 10X PCR reaction buffer, the reaction volume was adjusted to 50 µl in 0.5 ml Eppendorf tube. The PCR amplification conditions were performed by an initial denaturation step at 94°C for 10 min followed by 30 denaturation cycles at 94°C for 1 min, annealing at 56 °C for 1 min and an extension at 72 °C for 1 min followed by a final extension step at 72°C for 10 min. The PCR products were purified using PCR purification kit (GeneJET PCR Purification Kit, Thermo Scientific) and the two strands of each amplicon were sequenced using chain terminator method (Bioneer Company, Korea).

Sequences were then subjected to similarity BLAST searches (www.ncbi.nlm.nih. gov/blast) and the closely related sequences from GenBank were detected. The phylogenetic relationships of the experimental isolates and closely related species were analyzed using the multi-sequence alignment program (MEGA 5.1)

The selected sequences were aligned using CLUSTALW module integrated into MEGA5.1 package (Tamura *et al*., 2011). Sequence comparison and phylogenetic analyses of the partial sequencing of 16S rDNA were performed using the software MEGA5.1 (Tamura *et al*., 2011). Neighbor-joining (Saitou and Nei, 1987) with Kimura 2-paramter (Kimura, 1980) method were used for computing the evolutionary distance, thousand bootstraps replicates were used to build the corresponding phylogenetic trees.

3. Results and discussion

Isolation of bacteria from mud sample collected from the gas station in Alexandria introducing five different bacterial isolates symbolized as D1, D2, D3, D4, and D5. Those isolates have been recognized differently as mentioned in materials and methods section and illustrated in Table1. Data show that those five isolates are classified differentially according to shape of cells, colony type, Gram reaction and spores formation. Assaying of their capabilities for degrading different targets of pesticides was carried out in the next section.

Isolates capabilities for OP's Degradation

Assaying the capability of isolates for degrading OP's was carried out by measuring its magnitude for paraoxon degradation (Mansee *et al.,* 2004). **Figure1** illustrates the degradation percentages of different paraoxon concentrations when incubated with each of

the five isolates. Bacterial isolates show almost the same trend with different intense for degrading paraoxon at the different concentration. D1 and D5 isolates are more efficient for degrading paraoxon than other isolates. Those isolates are degrading >70 and 85% of 250 ppm of paraoxon, respectively after 48 hrs. of incubation. Such percentages of degrading paraoxon are highly promising and oriented the efforts to assaying the efficiency of those isolates for degrading the OP pesticide methyl parathion.

Metal content of Lake Mariout water samples

Data in Table 1 reveal that Ni was recorded in relatively high level ranging from 2.949 to 6.997 mg/l by average of 4.62 mg/l compared to other tested metals. Average concentrations of Al, Mn, Pb and Fe were detected in low values (1.34, 0.42, 0.27 and 0.3 mg/l, respectively). Other elements such as Cu, Cd, Cr, Zn and Co were below the limit of detection.

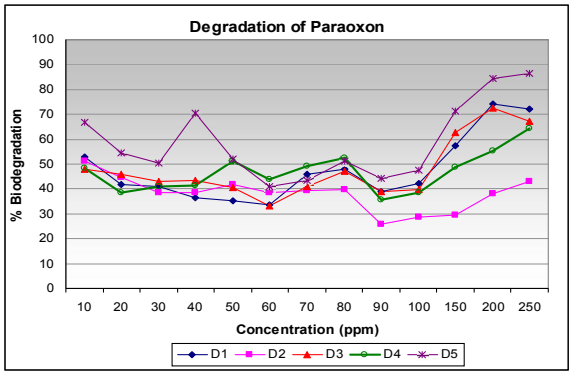

Figure 1. Efficiency of bacterial isolates of mud samples for biodegradation of Paraoxon

Efficiency of Isolates for methyl Parathion Degradation

The five bacterial isolates efficiencies for degrading methyl parathion have been assayed and presented in **Figure 2.** Isolates were incubated for 48 hrs. with series of methyl parathion concentrations (10-250 ppm) before measuring their degradation percentages. Degradation percentages of 10 ppm methyl parathion are ranged between 30-70 % for the five tested isolates. D1 and D2 isolates showed highest efficiencies among the other tested isolates by achieving more than 40% degradation percentages for most of tested methyl parathion concentrations. Those two isolates are promising for using as bioactive agents for degrading methyl parathion and other OP's as well. The present investigation showing the ability of isolates for OP's biodetoxification are in agreement with the result of Ortiz-Hernandez *et al.* (2001) who isolated a methyl-parathion degrading consortium from agriculture soils in central Mexico, showing a capability for using methyl-parathion as the only carbon source. Also, Bhadbhade *et al.* (2002) used *Arthrobacter atrocyaneus*, *Bacillus megaterium*, and *Pseudomonas mendocina* for bioremediation of wastewater containing Monocrotophos (MCP) an organophosphorus insecticide during manufacture. They used of pure cultures for bioremediation of effluent containing MCP appears to be the first trail of such attempt. Siddavattam *et al.* (2003) supported the present results by collected several bacterial strains isolated from soil samples of diverse geographical regions which can use organophosphate pesticides as a source of carbon. All these organisms synthesize an enzyme called parathion hydrolase, and in each case the enzyme is encoded by a gene (*opd* gene) located on a large indigenous plasmid. Moreover, Bano and Musarrat (2003) isolated and characterized phorate degrading soil bacteria of environmental and agronomic significance. The HPLC analysis of the organophosphorus pesticide, phorate in bioaugmented soil revealed its complete disappearance within 40 days. Other studies were reported the activity of bacterial strains, which isolated from soil, water or contaminated sites in bio degradation of OP's compounds Mansee *et al.* (2004), investigated the capabilities of various bacterial isolates from specified contaminated areas along Marriute Lake in Alexandria for paraoxon degradation. Ghanem *et al.* (2007) isolated a chlorpyrifos- degrading bacterial strains from an activated sludge sample collected from the Damascus waste water treatment plant, Syria. They determined the ability of the isolated strains to degrade the tested OP. Also Li *et al.* (2008) for exploration of various chlorpyrifos-degrading bacteria to clean-up the pollutant is of immense importance supported our results that introducing different isolates from different contaminated site in the pesticide company for degrade OP's. Lakshmi *et al.* (2008) reported on the diversity of microbes capable of degrading chlorpyrifos and the potential of these degraders towards the in situ bioremediation of chlorpyrifos-contaminated soils.

Isolates Efficiencies for Atrazine Biodegradation: *0.06 mM of Atrazine*

Capabilities of the five gas station mud waste bacterial isolates have been tested for degrading the herbicide; atrazine at 0.06 mM (**Figure3**). Atrazine degradation percentages up to 24 hrs. weren't pronounced. The degradation efficiencies were increased with increasing the incubation time with all strains. Atrazine degradation percentage after 4 weeks of incubation with isolates, reached to its maximum values (55-96%) for the five isolates. D1 isolate proof itself among the other isolates as the highly potent isolate for degrading 0.06 mM atrazine after 4 weeks.

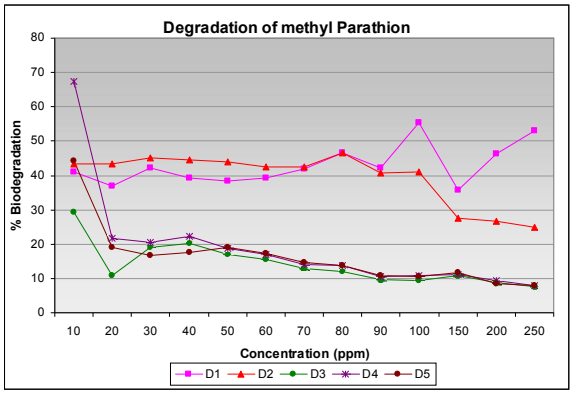

Figure2. Efficiency of bacterial isolates of mud samples for biodegradation of methyl Parathion

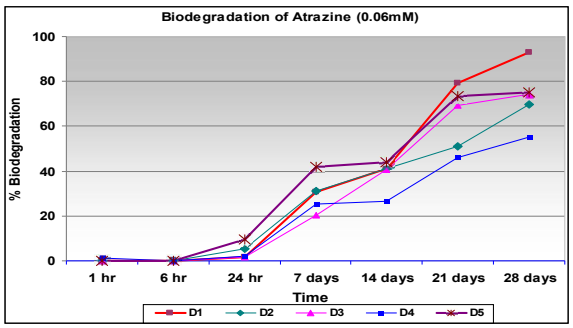

Figure3. Efficiency of bacterial isolates from mud samples for bioremediation of 0.06mM Atrazine

0.12 mM of Atrazine

Degradation of atrazine at 0.12 mM as a result of incubation with the five bacterial isolates along for 28 days is presented in **Figure 4.** Atrazine degradation percent was increasing with increasing incubation intervals and showing same previous trend (0.06 mM). After 28 days of incubation, the degradation percentages were ranged from 50-80%. D1 isolate found to be the most effective isolate for degrading atrazine at tested intervals.

The results obtained were parallel to those reported by different researchers (García-Gonza´lez *et al.,* 2003 and Qingyan *et al.,* 2008). They isolated different species of bacteria from different contaminated sites that capable of utilizing herbicide atrazine as a sole nitrogen source for growth. Qingyan *et al.,* (2008) was in agreement with our results when isolated a bacterial strain (AD26) from an industrial wastewater sample by enrichment culture. A bioremediation trial of contaminated soil has indicated that AD26 can degrade as high as 98% of atrazine contained in soil (300 mg/kg) after incubating for 20 days, nominating this strain as a good candidate for use in bioremediation programs. Also, Chirnside *et al.* (2009) isolated a selected microbial consortium (SMC) capable of degrading two specific herbicides, alachlor and atrazine from a pesticide-contaminated mix-load site soil. On the contrary to the present results, they observed an initial fast degradation rate of atrazine was followed by a much slower, more gradual degradation rate period that lasted about 35 days.

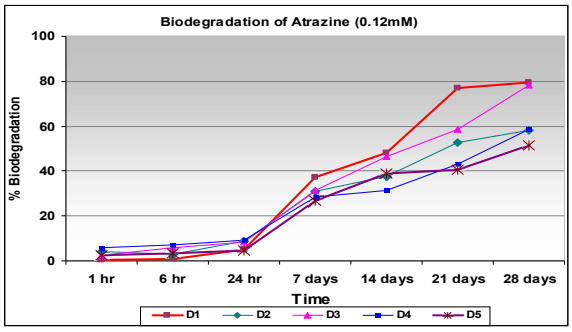

Figure4. Efficiency of bacterial isolates from mud samples for bioremediation of 0.12mM Atrazine

Isolation of microbial community from a model river ecosystem (microcosm) and investigate its capabilities for mineralizing atrazine was introduced by Satsuma (2009). Two strains were successfully isolated from such community and identified as *Nocardioides* and *Pedomicrobium*. The first strain showed capabilities to degraded atrazine to cyanuric acid while *Pedomicrobium* sp could mineralize cyanuric acid alone. Thus, he cited that the coexistence of these two community members functionally serves to completely biodegrade atrazine. This could be explaining the slowdown of atrazine degradation rate after certain intervals as a result of producing the cynuric acid metabolite. Wang *et al.* (2010) introduced an *Arthrobacter* sp. strain HB-5 from industrial waste water that exhibited faster atrazine degradation rates in atrazine-containing mineral media than the well-characterized atrazine degrading bacteria isolate *Pseudomonas sp.* ADP. Zhang *et al.* (2012) investigate metabolic ability and individual characteristics of an atrazine-degrading consortium DNC5 isolated from corn-planted soil. The characterization of the individual metabolic characteristics and the mutualism of the cultivable members in the consortium DNC5 were conducted. They found the consortium is a new combination of isolates in an atrazine degrading consortium.

Wang and Xie (2012) isolated a bacterial strain (DAT1) capable of utilizing atrazine as a sole nitrogen source for growth from an agricultural soil in China. The strain DAT1 was identified as an *Arthrobacter* species. The strain demonstrated a very high efficiency of atrazine biodegradation with a broad optimum pH and temperature ranges and could be

enhanced by addition of both carbon and nitrogen sources, suggesting its huge potential for remediation of atrazine-contaminated sites. The current study recommended using isolates from contaminated sites for bioremdiating different pollutants especially pesticides which is supported by other investigators. Further studies for identification and recognized the isolates are taken place in our coming tasks.

Molecular characterization of the most potent strain D1

The molecular identification of D1 isolate was performed by amplification of 16S rDNA gene and sequencing of the purified PCR product. The obtained sequences were compared to the 16S rDNA database in the GenBank. The results showed that the D1 16S rDNA gene was 99% similar to *Bacillus subtilis* 22 with accession no. of FJ435215.1.

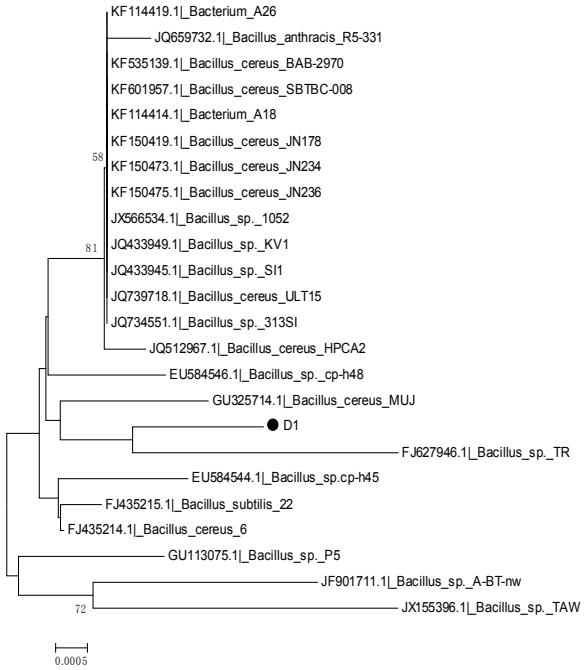

Figure.5. Neighbor-joining tree represents phylogenetic relationship isolate D1 based on 16S rDNA partial sequences isolated in this study. Evolutionary distances were calculated using the Kimura 2 model using MEGA5.1 software. The numerals show the results of the bootstrap analysis values from 1000 replicates (only bootstrap values above 50% were shown).

The phylogenetic relationships of the D1 isolate and closely related species were analyzed using the multisequence alignment program (MEGA 5.1) and the results were presented in phylogenetic tree (**Figure 5**). Based on the obtained data, isolate D1 was designated as *Bacillus* sp. D1 and submitted to the GenBank under accession no. KJ545610. The results of identification of D1 as *Bacillus* sp. was supported by different authors work (Bhadbhade *et al.,* 2002; and Thabit and EL-Naggar 2013). They isolate, identify and used different *Bacillus* spp. for bioremediation different pesticides including OP's and herbicides.

4. Conclusion

Decontamination of the two problematical pesticides: atrazine and methyl-parathion was conducted by five bacterial isolates from wastes of gas station. Identification of the most effective isolate (D1) in the present study designated as *Bacillus* sp. D1. Using those isolates showing a good potentials for bioremediation of pesticides and could open a chances for using bacteria isolates from stressed contaminated spots for helping in solving some of environmental contamination problems.

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