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Isolation and Characterization of *Bacillus subtilis* MF375213.1 and *Bacillus axarquiensis* MF375110.1 From Used Petroleum Oils at Assiut City, Egypt

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ABSTRACT

Petroleum oil is the main pollutant specially in petroleum countries producers. In This research study looked for bacterial strains have ability to degrade petroleum oily pollutants. Two bacterial isolates from 23 isolates have high ability to degrade engine oil were characterized and identified. The two bacterial isolates have been identified phenotypically and genotypically methods, as *Bacillus subtilis* strain BS1 and *Bacillus axarquiensis* strain BA1, these deposited in national center for biotechnology information (NCBI Gen Bank) under the Accession numbers MF375213.1 and MF375110.1 respectively. Two isolated strains have ability to grow in minimal medium containing petroleum oil with maximum bacterial growth was determined by spectrophotometry. *Bacillus subtilis* growth exhibited 1.7 OD at 600nm after 2 days of incubation time whereas *Bacillus axarquiensis* exhibited 1.14 OD at 600 nm after 3 days of incubation time with minimal medium containing 2% petroleum oil.

INTRODUCTION

Petroleum-based products are the major source of energy for industry and daily life. Used motor oil is the brown-to-black oily liquid removed from a motor vehicle, with increasing demands of fossil fuel energy, extensive exploration of natural sources has caused number of large scale accidental spills of crude oil and resulted in environmental disasters (Darsa *et al.,* 2014), Lubricant oil from pump motors, automobile engines and engines of boats and ships constitute an important pollutant of water and soil ecosystems, leading to devastating damage to the aquatic ecosystem at times of accidents. There are wide varieties of microorganisms in water and soil known to utilize petroleum hydrocarbons as an energy source and degrade them (Gopinath *et al.,* 2015). Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment (Rahman *et al.,* 2003; Brooijmans *et al.,* 2009). It has long been a popular bioremediation process in case of oil or petroleum contaminated soil (Beaudin *et al.,* 1996; Van Gestel *et al.,* 2003). Compost is rich in carbon, organic nitrogen, phosphorous and mineral compounds required for growth of microorganisms helping to degrade the oil (Joo *et al.,* 2007). Several bacterial species have been identified as having the ability for oil degradation. In general, *Bacillus* sp. has been identified as petroleum hydrocarbon degrader Ghazali *et al.,* 2004) and is known as naphthalene and pyrene degrader (Ron and Rosenberg, 2001; Zhuang *et al.,* 2002). *B. axarquiensis, B. thuringiensis* and *B. cereus* isolates able to degrade the aromatic fraction of the crude petroleum oil under both non-saline and saline conditions (Lobna, *et al.,* 2017).

MATERIALS AND METHODS

Collection of samples

Used motor oil was obtained from automobile workshops. The used oils were collected in well washed and airdried plastic bottles.

Isolation of petrol degrading bacteria

The collected oil samples were serially diluted up to 10^7 and 0.1 mL from the dilutions 10^5 and 10^6 were spread plated on mineral salt medium (magnesium sulphate 0.2 g, calcium chloride 0.02 g, mono potassium phosphate 1 g, di potassium phosphate 1 g, ammonium nitrate 1 g and ferric chloride 0.05 g) containing 2.5% of petrol (Bushnell, and Haas, 1941), the plates were incubated at 37°C for 24 hrs. (cited in Darsa *et al.,* 2014), and from the developed colonies, one was selected for further experiments.

Biodegradation studies

One hundred mL of minimal medium (dextrose 1 g, ammonium sulphate 1 g, dipotassium phosphate 7 g, monopotassium phosphate 2 g, sodium citrate 0.5 g and magnesium sulphate 0.1 g) with petrol and one mL inoculum from the overnight culture maintained in nutrient broth during the logarithmic phase were added to 250 mL Erlenmeyer flasks which were subjected to a period of four days. Flasks were incubated in a shaker at 30 °C at 100 rpm. After specified time, flasks were taken out. The ability of the isolated strain to degrade petrol was studied by determining the parameter optical density (OD).

Identification of petrol selected bacterial degrading strains

Phenotyping Identification

Gram staining and biochemical tests such as catalase, oxidase, Voges proskaeur, and indole production, sporulation test were carried out for strain identification (Holt *et al.,* 1994).

Genotyping identification by 16S rRNA gene

DNA Extraction

The genomic DNA of *Bacillus* strains was isolated using modification method from Sambrook (Sambrook, *et al.,* 1989).

Identification by 16S Ribosomal RNA gene

PCR Amplification according to Sambrook (Sambrook, *et al.,* 1989) The PCR amplification reactions were performed in a total volume of 50 μL. Each reaction mixture contained the following solutions will prepared: 2 μL of DNA (40 ng), 1 μL of 10 pmol forwarded 16S-rRNA primer (5′-AGAGTTTGATCMTGGCTCAG -3′); 1 μL of 10 pmol reverse 16S rRNA primer (5′-AACTGGAGGAAGGTGGGAT -3′); 0.8 μL of 12.5 mM (dNTP's); 5 μL of PCR buffer included MgCl2, and 0.2 μL Taq polymerase (1 Unit) and water-free DNAse and RNAse were added up to 50 μL.The PCR apparatus was programmed as follows: 3 min denaturation at 95 ∘C, followed by 35 cycles that consisted of 1 min at 95 ^{o}C, 1 min at 58∘ C, and 1 min at 72 ^{o}C, and a final extension was 10 min at 72 ^{o}C. The products of the PCR amplification were analyzed by agarose gel electrophoresis (2%). 2.6.3. PCR Cleanup and 16S rRNA Sequencing. The PCR products were cleaned up for DNA sequencing following the method described by Sambrook et al. 1989. Automated DNA sequencing based on enzymatic chain terminator technique, developed by Sanger (Sanger *et al.,* 1977).

Phylogenetic Analysis:

Similarity analysis of the nucleotides was performed by BLAST searches against sequences available in GenBank. For phylogenetic tree construction, multiple sequences were obtained from GenBank and the alignments were performed using clustal W program. A phylogenetic tree was built using MEGA 5 software (Tamura *et al.,* 2011).

RESULTS AND DISCUSSION

Strains Isolation and Identification

Identification by Morphological and Biochemical Tests

A total of 23 pure cultures of spore-forming bacteria were isolated and purified which obtained from different samples collected from Assiut city, Egypt. All isolates shown the ability to degrade petroleum oil. Two *Bacillus* isolates have high ability to degrade petroleum oil purified and gained the names *Bacillus* sp. *BS1*, and *Bacillus* sp. *BA1*.

The morphological and physiological characteristics of the isolated strains were compared with the data from Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994) revealed that *Bacillus* sp. *BS1* and *Bacillus* sp. *BA1* are matching with those of *B. subtilis* group. In previous articles on taxonomy, species included in the *B. subtilis* group. The two strains are aerobic, motile, Gram-positive rods, and spore forming. The results of biochemical tests of the *Bacillus* sp. *BS1* and *Bacillus* sp. *BA1* indicated that they are related to *B. subtilis*, and *B. axarquiensis* which summarized in Table 1.

Bacillus axarquiensis (a.xar.qui.en9sis. N.L. adj. masc. *axarquiensis* pertaining to Axarquia, the Arabic name for the region surrounding the city of Ma´laga in southern Spain), It is characterized by catalase-positive and oxidase negative. It reduces nitrate aerobically. Starch, Tween 20, Tween 80, DNA, gelatin, casein and lecithin are hydrolysed. Citrate is used as sole carbon and energy source. Haemolysis, Voges–Proskauer test, dihydroxyacetone production, H_2S from cysteine, O-nitrophenyl b-D-galactopyranoside (ONPG) hydrolysis, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are positive in the results (Cristina *et al.,* 2005).

Tab 1. physiological and biochemical tests for the selected strains.

Name of test	*BS1*	*BA1*
Gram stain	Gram-positive	Gram-positive
Shape	rod	rod
Motility	+	+
Spore formation	+	+
vogues Proskauer	+	+
Methyl red	+	+
Citrate utilization	+	+
Indole production	-	-
Catalase	+	+
Oxidase	+	-
Nitrate reduction	+	+
Lactose fermentation	+	-

\+ = Positive - = Negative

Identification by 16S Ribosomal RNA (rRNA)

The DNA was purified and analyzed by gel electrophoresis, the quality of the DNA for each sample has been identified for further investigation. The amplified 16S rRNA gene from the DNA of *Bacillus* sp. *BS1* and *Bacillus* sp. *BA1* was determined using 2% agarose gel. The size of the amplified fragments was determined by using size standard (Gene ruler 100bp–3000bp DNA ladder). The PCR products were visualized under UV light and photographed using gel documentation system. Approximately 1200bp of 16S rRNA gene was amplified.

The PCR products were purified and sequenced using 16S forward primer. The sequences of *Bacillus* sp. *BS1* and *Bacillus* sp. *BA1* were deposited in national center for biotechnology information (NCBI GenBank) under the Accession numbers (MF375213, MF375110), respectively. The basic local alignment search tool (BLAST) algorithm was used to retrieve for homologous sequences in GenBank. The *Bacillus* sp. BS1 revealed 99% identity to *Bacillus subtilis* while *Bacillus* sp. *BA1* revealed 99% identity to *Bacillus axarquiensis*. Based on the morphological, biochemical, and molecular

characteristics, the *Bacillus* sp. BS1 and *Bacillus* sp. BA1 were designated as *Bacillus subtilis* BS1 and *Bacillus axarquiensis* BA1, respectively. A phylogenetic tree based on the comparison of 16S rRNA sequences of reference strains was constructed. The phylogenetic analysis was performed with 1200bp sequences using the software MEGA 5 (Tamura *et al.*, 2010).

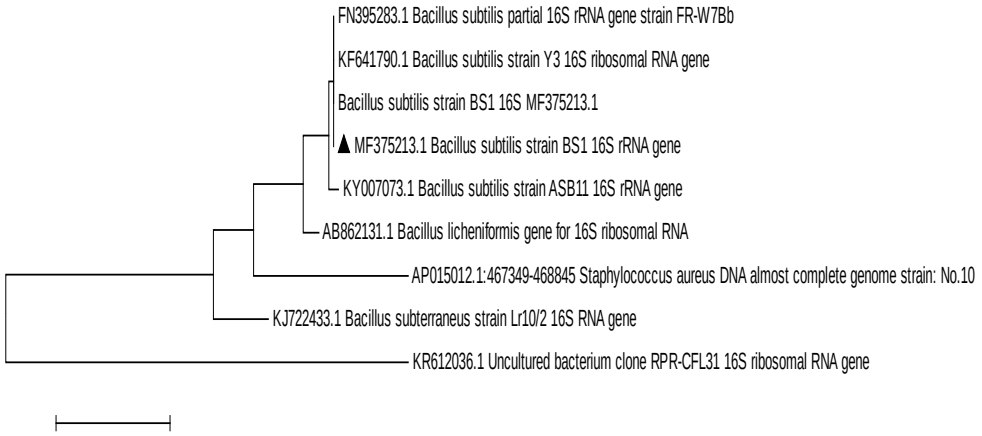

Fig. 1: Phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain BS1 among members of the genus *Bacillus* species.

DNA nucleotide sequence of *16S rRNA* gene of BA1 strain that was amplified by PCR and partially sequenced for *Bacillus subtilis* (MF375213.1) and nucleotide sequence was the following:

```
   1 acatgcaagt cgagcggaca gatgggagct tgctccctga tgttagcggc ggacgggtga
  61 gtaacacgtg ggtaacctgc ctgtaagact gggataactc cgggaaaccg gggctaatac
 121 cggatgcttg tttgaaccgc atggttcaaa cataaaaggt ggcttcggct accacttaca
 181 gatggacccg cggcgcatta gctagttggt gaggtaatgg ctcaccaagg caacgatgcg
 241 tagccgacct gagagggtga tcggccacac tgggactgag acacggccca gactcctacg
 301 ggaggcagca gtagggaatc ttccgcaatg gacgaaagtc tgacggagca acgccgcgtg
 361 agtgatgaag gttttcggat cgtaaagctc tgttgttagg gaagaacaag taccgttcga
 421 atagggcggt accttgacgg tacctaacca gaaagccacg gctaactacg tgccagcagc
 481 cgcggtaata cgtaggtggc aagcgttgtc cggaattatt gggcgtaaag ggctcgcagg
 541 cggttcctta agtctgatgt gaaagccccc ggctcaaccg gggagggtca ttggaaactg
 601 gggaacttga gtgcagaaga ggagagtgga attccacgtg tagcggtgaa atgcgtagag
 661 atgtggagga acaccagtgg cgaaggcgac tctctggtct gtaactgacg ctgaggagcg
 721 aaagcgtggg gagcgaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg
 781 ctaagtgtta gggggtttcc gcccccttagt gctgcagcta acgcattaag cactccgcct
 841 ggggagtacg gtcgcaagac tgaaactcaa aggaattgac gggggcccgc acaagcggtg
 901 gagcatgtgg tttaattcga agcaacgcga agaaccttac caggtcttga catcctctga
 961 caatcctaga gataggacgt ccccttcggg ggcagagtga caggtggtgc atggttgtcg
1021 tcagctcgtg tcgtgagatg ttgggttaag tcccgcaacg agcgcaaccc ttgatcttag
1081 ttgccagcat tcagttgggc actctaaggt gactgccggt gacaaaccgg gagaaagttg
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1201 agaa
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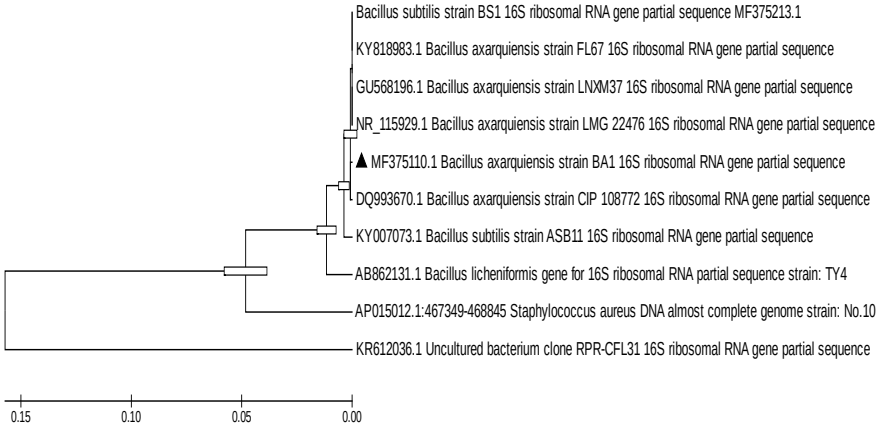

Fig. 2: Phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain BA1 among members of the genus *Bacillus* species.

DNA nucleotide sequence of 16S rRNA gene of BA1 strain that was amplified by PCR and partially sequenced for *Bacillus axarquiensis* (MF375110.1) and nucleotide sequence was the following:

```
   1 acatgcgagt cgagcggaca gatgggagct tgctccctga tgttagcggc ggacgggtga
  61 gtaacacgtg ggtaacctgc ctgtaagact gggataactc cgggaaaccg gggctaatac
 121 cggatgcttg tttgaaccgc atggttcaaa cataaaaggt ggcttcggct accacttaca
 181 gatggacccg cggcgcatta gctagttggt gaggtaatgg ctcaccaagg caacgatgcg
 241 tagccgacct gagagggtga tcggccacac tgggactgag acacggccca gactcctacg
 301 ggaggcagca gtagggaatc ttccgcaatg gacgaaagtc tgacggagca acgccgcgtg
 361 agtgatgaag gttttcggat cgtaaagctc tgttgttagg gaagaacaag taccgttcga
 421 atagggcggt accttgacgg tacctaacca gaaagccacg gctaactacg tgccagcagc
 481 cgcggtaata cgtaggtggc aagcgttgtc cggaattatt gggcgtaaag ggctcgcagg
 541 cggttcctta agtctgatgt gaaagccccc ggctcaaccg gggagggtca ttggaaactg
 601 gggaacttga gtgcagaaga ggagagtgga attccacgtg tagcggtgaa atgcgtagag
 661 atgtggagga acaccagtgg cgaaggcgac tctctggtct gtaactgacg ctgaggagcg
 721 aaagcgtggg gagcgaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg
 781 ctaagtgtta gggggtttcc gccccttagt gctgcagcta acgcattaag cactccgcct
 841 ggggagtacg gtcgcaagac tgaaactcaa aggaattgac gggggcccgc acaagcggtg
 901 gagcaggtgg tttaattcga agcaacgcga agaaccttac caggtcttga catcctctga
 961 caatcctaga agataggacg tcccccttcgg gggcagagtg acaggtggtg catggttgtc
1021 gtcagctcgt gtcgtgagat gttgggttaa gtcccgcaac gagcgcaacc cttgatctta
1081 gttgccagca ttcagttggg cactctaagg tgactgccgg tgacaaaccc gaggaaaggt
1141 gggg
```

Biodegradation of petroleum oils

Changes in the optical density of medium during the four days of treatment of petrol by *Bacillus subtilis and Bacillus axarquiensis* strain are illustrated in Figs. 3, 4. Optical density values exhibited an increase during the initial period of treatment up to 3 days and afterwards showed a decline. Maximum bacterial growth of *Bacillus subtilis* and *Bacillus axarquiensis* in medium with petrol oil were 1.7 OD at 600 nm with 2% petroleum oil after 2 days incubation time and 1.14 OD at 600 nm with 2% petroleum oil after 3 days of incubation time respectively.

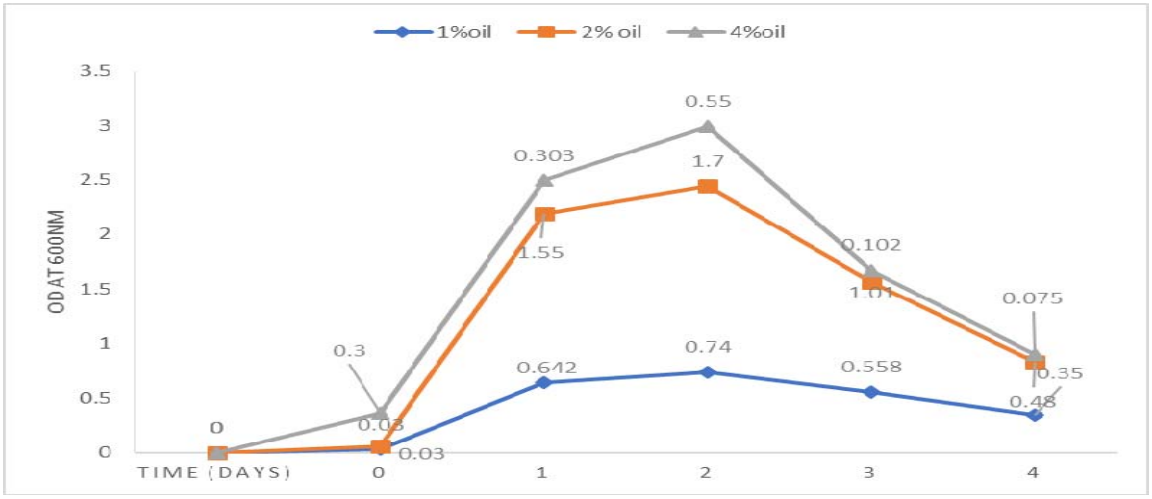

Fig. 3: Changes in the optical density of the culture medium during the degradation of petrol by the bacterium *Bacillus subtilis.*

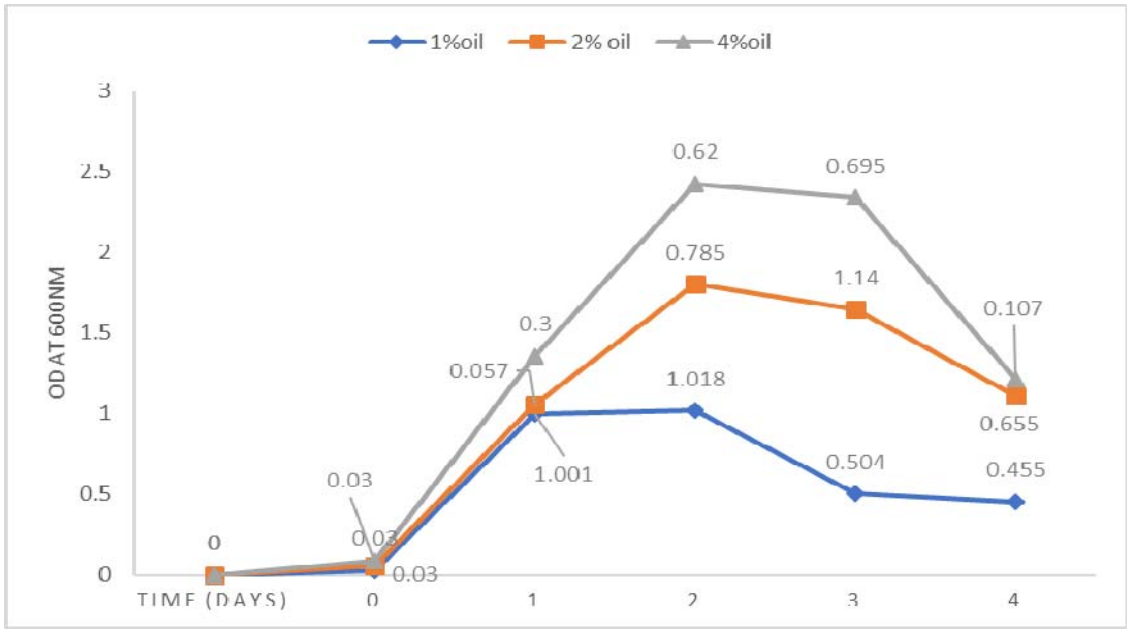

Fig. 4: Changes in the optical density of the culture medium during the degradation of petrol by the bacterium *Bacillus axarquiensis*

Bacteria can degrade carbon compounds in petroleum oil as a necessary source of energy (AMSA, 2004). There are many different varieties of hydrocarbons and over millions of years' bacteria have evolved catalytic enzymes that are specific for degradation reactions. Some of the simpler compounds can be degraded by a very wide variety of bacteria but the ability to degrade hydrocarbons is found in fewer species. No single bacterium can make all the different enzymes, instead; each kind of bacterium specializes in a few hydrocarbons as preferred food sources (Owaid, 2008).

CONCLUSION

According to data of this research study, two bacterial strains isolated and identified, have ability of biodegradation of petroleum oil, the two given strains identified as *Bacillus subtilis* , *Bacillus axarquiensis* and they can be used as active agents in petroleum degradation process. Further study needed to imply of the identified strains in field of contaminant water by oil.

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