Molecular, Biochemical, and Phenotypic Identification of Phenol and Cresol Degrading *Acinetobacter baumanni* Strain Selected from Al-Rumaila Oil Well

Noor Mohammad Ali[1], Aamal Ghazi Mahdi Al-Saadi[1]

[1]M.Sc., Ecology Department/ College of science, University of Al -Qadisiyah, Diwaniyah 58001, Iraq,

Abstract

Background: phenol and cresol are dangerous compounds that have harmful effects on aquatic life, plants and, many other organisms. Therefore, it is necessary to effectively eliminate these compounds to protect the environment.

Aim: The aim of this study is to select and characterizebacterial strain(s) from oil contaminated soils and testing its ability for biodegrading phenol and cresol.

Methods: Biochemical tests were performed in addition to morphological and molecular detection through 16S rRNA analysis to identify the selected strain after growing on Mineral salts medium (MM) containing phenol or cresol . The residual concentration of phenol and cresol was analyzed over the time period by means of the 4-aminoantipyrine assay .

Results: the selected strain was identified as Acinetobacter baumannii strain HILLA-1, and it was able to remove 100% of the phenol at all the used concentrationsafter 48 h. This strain was also able to degrade 100% of cresol at concentrations (25,50,75,100,200 ppm /L) and99.37%,88.69%,and 94.73% at concentrations (300,400,500 ppm /L) respectivelyafter 48 h.

Conclusion: The selected and identified Acinetobacter baumannii strain HILLA-1 can provide a putative source for bioremediation of phenol and cresol contaminated environments.

Keywords: *Acinetobacter baumanni,phenol, cresol, Biodegradation, 16S rRNA*

Introduction

A large number of organic compounds have been released to the environment since the beginning of the industrial revolution. These compounds are chemically manufactured and many of them are discarded as waste [1]. Phenol is one of the most common toxic environmental pollutants, and it is classified as a dangerous substance to human health and other organisms (EPA, 2003) because many of phenolic compounds possess the ability to survive and not disintegrate in the environment. They also have the ability to move. biotransform, and bioaccumule in human and animal tissues as well as increasing their concentration in the food chain biomagnification [11]. Phenol is an aromatic compound consisting of a hydroxyl groupbound to a benzene ring, and its chemical formula is (C6H6O).It is highly toxic and found in various formulations or associated with other compounds (EPA, 2003). It is a liquid or solid with a low melting point, but it has a high boiling point due to the hydrogen present in it. It is soluble in water due to its ability to form a hydrogen bond with water. It is found in the wastes of many industrial processes, such as oil refineries, cooking factories, industrial resins manufacturing, petroleum-based processing plants, pharmaceuticals, plastic industries, and dye industries [4]. Its use has a greatly polluted wide range of soil, rivers

,and waste. Phenolic compounds have harmful effects on aquatic life, plants and many other organisms [27]. The natural sources of phenol that were formed as a result of forest fires and the natural emission of phenol when asphalt was used as an adhesive in cities [15]. On the other hand, cresol is a colorless viscous liquid that is used as an intermediate in the production of other chemicals. Its chemical formula is C7H8O. Methyl phenol is an aromatic organic compound that hasa variable melting point that is determined by ambient temperatures. It is involved in various fields in the manufacture of pesticides, petroleum products, dyes, and also in the pharmaceutical industries [5]. It is very toxic to humans if inhaled or swallowed, even in very low concentrations. It often results in problems such as eye, mouth, throat and skin irritation, vomiting, liver and heart damage, paralysis, coma, and sometimes death [30] . Residues of phenol and its derivatives can be removed from the environment through a combination of physical and chemical treatments such as adsorption, osmosis, adsorption, photo catalysis, and electrolyte oxidation, but these treatments are impractical due to the high cost and formation of other toxic compounds [31] Therefore, biological methods have received more attention than physical and chemical methods because many different bacteria are known to use phenolic compounds as the only sources of carbon and energy [32]. The degradation of phenol and its derivatives has been studied by bacteria. A large number of different bacterial species have been found to have the ability to biodegrade phenolic compounds such as Acinetobacter spp. In the current study, an Acinetobacter baumannii strain that has the ability to biodegrade phenol and cresol at high concentrations was selected from oil contaminated soil of Al-Rumaila oil well and identified based on phenotypic, biochemical, and molecular approaches. The Biodegradation rates of phenol and cresol by the selected strain were also reported in this study.

Materials and Methods

Sample collection

(20 samples) collected from soil contaminated with petroleum products from various places. Samples were collected in sterile glass bottles and transferred to thelaboratory for the required tests.

Growth media

Mineral salts medium (MM)was used for selection. It consists of the following g / L materials, according to [16] Mgso 4.7H2o, (0.1g) NH4 (2SO4), (0.01g) NaCl,(0.01g) CaCl2, (0.45g) K2HPO4, (0.002g) Fecl3. The materials were dissolved in a liter of distilled water and mixed well to ensure that all the materials weredissolved and sterilized for 20 minutes at 121 ° C and the pH was adjusted (7).

Selection of phenol and cresoltolerantbacterial strains

A weigh of 5 g of each soil sample was added to 50 ml of liquid mineral salts medium (MM). Phenol or cresolwas added at a concentration of (25 mg /L) and (25 ppm/L), respectively as the sole source of carbon and energy. The flasks then wereincubated for 5 days at 30 ° C [31]. 0.1 ml of the bacterial suspension was transferred to MM agar medium containing the same concentrations of phenol and cresol and incubated for 5 days at 30 ° C . This step was repeated two times to obtain pure colonies and to ensure that the isolated bacterial species are capable of degrading phenol and cresol .

The ability of the bacterial isolates to degrade phenol and cresol compounds Standard titration curves were prepared forboth compounds by preparing standard solutions for each of them. The assay of testing the ability of the isolated strain to degrade phenol(25,50,75,100,200,300,400,500 mg/l) and cresol (25,50,75,100,200,300,400,500 ppm/l) was performed as mentioned in (Barwick& Vicki 2003). Then using the absorption values on length 460nm to builda calibration curve for the two compounds using an Excel program to obtain the mathematical equation in order to calculate the concentrations of the remaining phenol and cresol compounds. After the growing of bacteria to assess their efficiency on length 600nm in the biodegradation process This assay was performed twice to confirm the degradation ability of the isolated strain to phenol and cresol twice to confirm the degradation of the isolate to phenol and cresol by4-aminoantipyrine assay. Identification of the Phenol-cresol degrading Bacterium The isolated bacterial strains was identified based on morphological characteristics on MacConkeyagar,Vitek 2 compact system biochemical tests(which was

used according to the manufacturer's instructions), and sequence analysis of the 16S rRNA gene DNA extraction and 16S rRNA sequencing Genomic DNA was extracted from the selected Acinetobacterbaumanniibacterial isolate using the PrepTM Genomic DNA MiniKit provided by Macrogen / Korea according to the manufacturer's instructions. DNA amplification was performed by a PCR master mix containing 3 µl forward and reverse primers, 14 µl nuclease-free water, 5 µlTemplate DNA, 25 µl Master Mix. 16S rRNA gene was amplified from the DNA genome by PCR using the following forward and reverse primers for 16S rRNA respectively5 ´AGAGTTTGATCCTGGCTCA - 3 ´ 5 ´GGTTACCTTGTTACGACTT -3. The polymerase chainreaction was carried out under the following conditions: Initial denaturation at 95 ° C for 120 seconds followed by denaturation at 95 ° C for 30 seconds, followed by annealing at 53 ° C for 30 seconds, followed by elongation at a temperature of 72 ° C for 150seconds and the final elongation score of 72 for 300seconds, respectively.

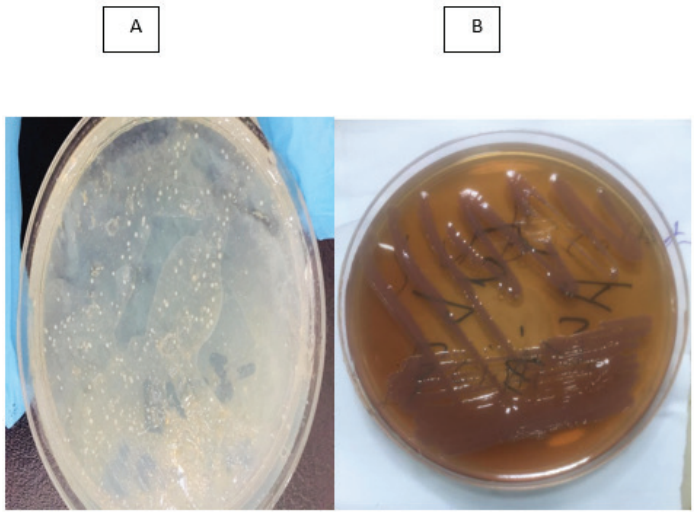
DNA sequencing analysis

The 16S rRNA PCR product of *Acinetobacter baumannii* strain HILLA-1 was extracted and sent to Bioneer (Korea) to perform nitrogenous base sequencing. The base sequence of the selected strain was registered in the NCBI GenBank records , and its accession number is MT032339.1.Using the BLASTn tool, Homologous sequences were attained from the NCBI database. Homology to*Acinetobacter baumannii* strain HILLA-1were chosen with accession numbers:MK027249.1,MN636473.1, MN623687.1, MN175924.1, MN175925.1, MN175923.1, MN175922.1, MN175921.1, MN175920.1, MN173945.1 Sequences alignment and phylogenetic tree analysiswere done using Mega X software. The phylogenetic tree was drawnbased onneighbor-joining method.

Results

Characterization of the selected phenol/Cresol strain

An ***Acinetobacterbaumanni*** strain, obtained in the current study, was isolated from oil contaminated soil of Al-Rumaila oil well. The strain was selected using *a MM medium containing phenol or cresol then characterized after culturing* on MacConkey agar plates. The colonies appeared small, round, convex and mucous, with a light cream color as shown in Figure 1. The colonies were purified and identified using Gram's stain which showed *Gram-negative bacterium in a form of semi-spherical bacilli*. The selected strain was further subjected to biochemical identification using Vitek 2 compact system as shown in table 1.



Figure(1). Phenotypic characteristics of the isolated bacterial strain growing on solid mineral salts medium (A) and MacConkeyagar medium (B).

Table 1: biochemical properties of the isolated strain using Vitek 2 compact system.

2	AlaPhe-Pro-ARYLAMIDASE	-	3	ADONITOL	+
4	L-PYrrolydonyl-ARYLAMIDASE	+	5	L-ARABITOL	+
7	D-CELLOBIOSE	+	9	BETA-GALACTOSIDASE	+
10	H2S PRODUCTION	-	11	BETA-N-ACETYL-GLUCOSAMINIDASE	-
12	GlutaylArylamidasepNA	-	13	D-GLUCOSE	+
14	GAMMA-GLUTAMYL-TRANSFERASE	+	15	FERMENTATION/GLUCOSE	+
17	BETA-GLUCOSIDASE	+	18	D-MALTOSE	+
19	D-MANNITOL	+	20	D-MANNITOL	+
21	BETA-XYLOSIDASE	+	22	BETA-Alanine arylarnidasepNA	-
23	L-Proline ARYLAMIDASE	-	26	LIPASE	-
27	PALATINOSE	+	29	Tyrosine ARYLAMIDASE	+
31	UREASE	+	32	D-SORBITOL	+
33	SACCHAROSE/SUCROSE	+	34	D-TAGATOSE	+
35	D-TREHALOSE	+	36	CITRATE(SODIUM)	+
37	MALONATE	+	39	5-KETO-D-GLUCONATE	-
40	L-LACTATE alkalinisation	+	41	ALPHA-GLUCOSIDASE	-
42	SUCCINATE alkalinisation	+	43	Beta-N-ACETYL-GALACTOSAMINIDASE	-
44	ALPHA-GALACTOSIDASE	+	45	PHOSPHATASE	+
46	Glycine ARYLAMIDASE	-	47	ORNITHINE DECARBOXYLASE	+
48	LYSINE DECARBOXYLASE	+	53	L-HISTIDINE assimilation	+
56	COUMARATE	+	57	BETA-GLUCORONIDASE	-
58	O/129 RESISTANCE(comp.vibrio.)	+	59	Glu-Gly-Arg-ARYLAMIDASE	-
61	L-MALATE assimilation	+	62	ELLMAN	-
64	L-LACTATE assimilation	-			

Analysis of 16S rRNA gene sequence

16S rRNA fragment was amplified from the gDNA of the ***Acinetobacter baumanni*** isolate and detected with about 1498bp as shown in Figure 2. The sequence of 16S rRNA gene of the isolated strain of the current study can be found under the accession number MT032339.1 at NCBI–GenBank with the name of *Acinetobacter baumannii* strain HILLA-1. The BLAST results showed that the base sequence of the 16S rRNA of *Acinetobacter baumannii* strain HILLA-1 was 98.94% identical to *Acinetobacter baumannii* strain B18(Accession number MK027249.1) and 98.82% identical to *Acinetobacter baumannii* strains with Accession numbers: MN636473.1, MN623687.1, MN175924.1, MN175925.1, MN175923.1, MN175922.1, MN175921.1, MN175920.1, MN173945.1 (Table 2).

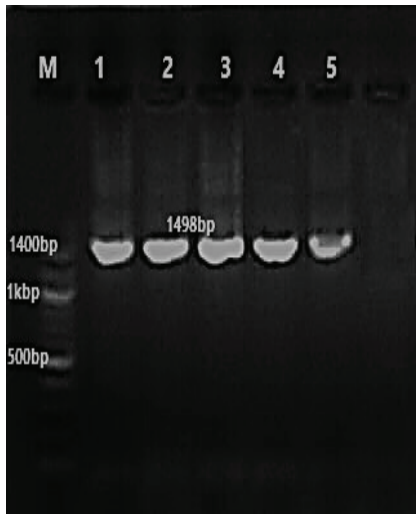


Figure 2: 16S rRNA PCR fragment obtained from the characterized *Acinetobacter baumannii* strain HILLA-1 . M: 2000 bp marker of DNA , Lane 1: The target amplification of 16S rRNA gene (size 1498bp).

Table 2 : Homology sequence identity for 16S rRNAgene of*Acinetobacterbaumannii* strain HILLA-1

No.	Accession number	Name of species sequence	Identity%
1	MK027249.1	Acinetobacterbaumannii strain B18 16S ribosomal RNA gene, partial sequence	98.94
2	MN636473.1	Acinetobacterbaumannii strain Charmo6 16S ribosomal RNA gene, partial sequence	98.82
3	MN623687.1	Acinetobacterbaumannii strain VGM2 16S ribosomal RNA gene, partial sequence	98.82
4	MN175924.1	Acinetobacterbaumannii strain DSM 1762 16S ribosomal RNA gene, partial sequence	98.82
5	MN175925.1	Acinetobacterbaumannii strain DSM 1924 16S ribosomal RNA gene, partial sequence	98.82
6	MN175923.1	Acinetobacterbaumannii strain DSM 1918 16S ribosomal RNA gene, partial sequence	98.82
7	MN175922.1	Acinetobacterbaumannii strain DSM 1923 16S ribosomal RNA gene, partial sequence	98.82
8	MN175921.1	Acinetobacterbaumannii strain DSM 1676 16S ribosomal RNA gene, partial sequence	98.82
9	MN175920.1	Acinetobacterbaumannii strain DSM 1675 16S ribosomal RNA gene, partial sequence	98.82
10	MN173945.1	Acinetobacterbaumannii strain rY32 16S ribosomal RNA gene, partial sequence	98.82

Phylogenetic tree analysis

Figure 3 shows the phylogenetic relationship analysis using the sequenced 16S rRNA gene from *Acinetobacter baumannii* strain HILLA-1with all sequences listed in Table 2. The results revealed that *Acinetobacterbaumannii* strain HILLA-1is closely related to *Acinetobacterbaumannii strain* rY32.

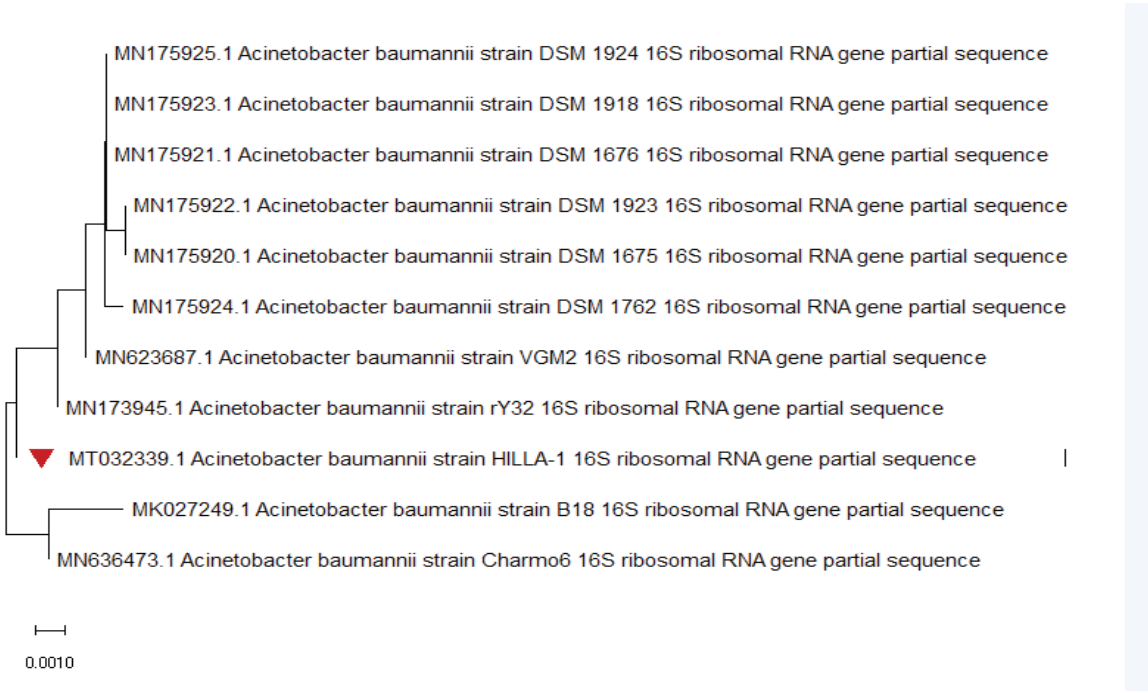


Figure 3: Phylogenetic relationship of the sequenced 16S rRNA gene from *Acinetobacterbaumannii* strain HILLA-1, accession number (MT032339.1). Analysis was done by neighbor –joining method.

The ability of ***Acinetobacter baumannii*** **strain HILLA-1**to degrade phenol and cresol in different concentrations

Results showed that Acinetobacterbaumannii strain HILLA was capable of degrading phenol completely by 100% for all concentrations (25,50,75,100,200,300,400, 500 mg / L)after 48 hours of incubation, as shown in Table (3),withan increase in the rate of bacterial growth during the incubation period, as shown in Figure 4. Furthermore, the results revealed that the biodegradation ability of Acinetobacterbaumannii strain HILLA to cresol for concentrations (25,50,75,100,200 ppm /L) was100% while the concentrations (300,400,500 ppm /L) are 99.37%,88.69%,and 94.73% respectively after48hrs as shown in Table (4) with an increase in the vital growth rate of isolate as in Figure 5

Table 3: Biodegradation rates of Acinetobacter baumanniistrain HILLA-1for Phenol

Acinetobacterbaumannii							
	The remaining of phenol after degradation			% of Degradation after			Mean Degradation% /hr
Start concentration of phenol mg/l	4 hrs.	24hrs.	48hrs.	4 hrs.	24hrs.	48hrs.	
500	376.11	121.66	0	24.778	75.668	100	3.65
400	362.77	88.33	0	9.3075	77.9175	100	
300	221.66	42.77	0	26.11333	85.74333	100	
200	131.66	35	0	34.17	82.5	100	
100	98.88	0	0	1.12	100	100	
75	70.22	0	0	6.373333	100	100	
50	35	0	0	30	100	100	
25	17.22	0	0	31.12	100	100	

Table 4:*Biodegradation rates of Acinetobacterbaumannii***strain HILLA-1** *for* **Cresol**

Acinetobacterbaumannii							
	The remaining of cresol after degradation			% of Degradation after			Mean Degradation% /hr
Start concentration of cresol ppm/l	4 hrs.	24hrs.	48hrs.	4 hrs.	24hrs.	48hrs.	
500	479.66	74.11	26.33	4.068	85.178	94.734	2.19
400	391.88	45.22	45.22	2.03	88.695	88.695	
300	271.88	33	1.88	9.373333	89	99.37333	
200	183	38.55	0	8.5	80.725	100	
100	96.33	47.44	0	3.67	52.56	100	
75	73	29.66	0	2.666667	60.45333	100	
50	50	16.33	0	0	67.34	100	
25	15.44	0	0	38.24	100	100	

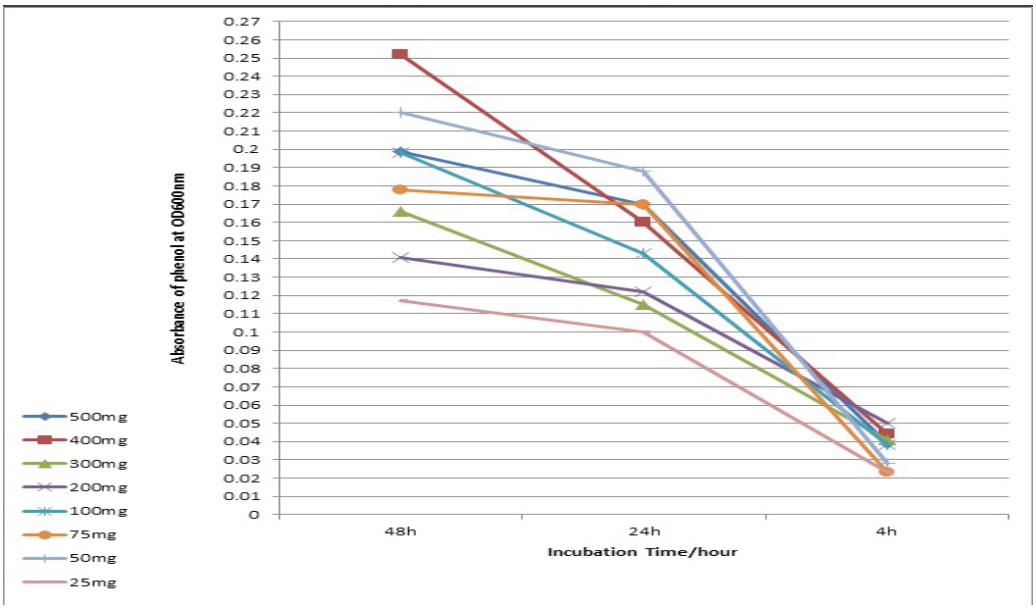


Figure 4:Growth curve of *Acinetobacterbaumannii*strain HILLA-1 in presence of different phenol concentrations

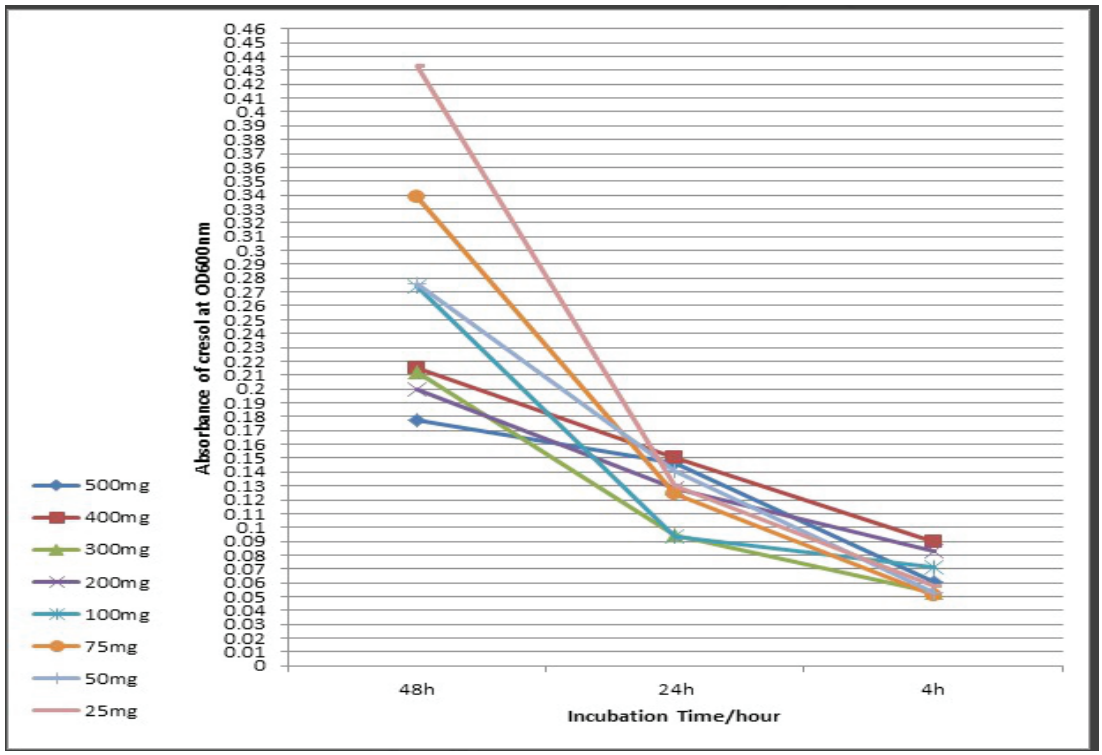


Figure 5: Growth curve of *Acinetobacter baumannii* strain HILLA-1in presence of different Cresol concentrations

Discussion

Petroleum compounds and its derivatives polluting the environment threaten human health and many other living organisms present in land and marine systems, so getting rid of pollution by oil vehicles is very important at various levels . Biological treatment is one of the most important techniques available in removing pollution as it is less dangerous than other types of treatments. It is a physical and chemical purification of the environment from pollutants by using microorganisms naturally present in such environments because of their ability

to break down dangerous compounds into less toxic compounds.

Conclusion

We have selected and identified a local *Acinitobacterbaumannii* strain, which degrades phenol and cresol as the only carbon sources. Our strain showed a high efficiency in breaking down even high concentrations of phenol (500 mg/L),and cresol (500 ppm/L). Therefore, *Acinetobacterbaumannii* strain HILLA-1may be taken into consideration as a helpful biotechnological means for the treatment of diverse environments contaminated with phenol or cresol.

Financial Disclosure: There is no financial disclosure.

Conflict of interest: None to declare.

Ethical Clearance: All experimental protocols were approved and all experiments were carried out in accordance with approved guidelines.

References

1. Adams D, DW Ribbons. The metabolism of aromatic ring fission products by Bacillus stearothermophilus IC3. General Microbiology. 1988; 134: 3179-3185
2. Adav S, Chen M, Lee D, Ren N. Degradation of phenol by aerobic granules and isolated yeast Candida tropicalis. Biotechnology and bioengineering, 96(5), 844-852.
3. Adav SS, MY Chen, DJ Lee, NQ Ren. Degradation of phenol by Acinetobactorstrain isolated from aerobic granules. Chemosphere. 2007; 67: 1566-1572
4. Afzal M, Iqbal S, Rauf S, Khalid Z. Characteristics of phenol biodegradation in saline solutions by monocultures of Pseudomonas aeruginosa and Pseudomonas pseudomallei. Journal of Hazardous Materials. 2007; 149(1): 60-66
5. Ahmed, SA, Mohd AS, Noorliza M, Mohd Y, Nor AS. Isolation , Identification and characterization of Elevated phenol Degrading Acinetobacter sp. Strain AQ5 NOL1 . Australain Journal of Basic and Applied Sciences. 2011; 5 (8) : 1035 – 1045
6. Al-Khalid T, El-Naas M. Aerobic biodegradation of phenols: a comprehensive review Critical Reviews in Environmental Science and Technology. 2012; 42(16): 1631-1690.
7. Bai, J., J-P. Wen, H-M. Li and Y. Jiang. Kinetic modeling of growth the biodegradationof phenol and m-cresol using Alcaligenesfaecalis. Process Biochemistry. 2007; 42: 510-517
8. Barwick V. Preparation of calibration curves. A Guide to Best Practice. Valid Analytical Measurement Programme. LGC. National Measurement System Chemical and Biological Metrology Website**.** 2003.
9. Beshay U, D Abd-El-Haleem, H Moawad, S Zaki. Phenol biodegradation by free andimmobilizedAcinetobacter. Biotechnology Letters. 2002; 24: 1295-1297.
10. Dong F, LL Wang, CW Wang. Molecular cloning and mapping of phenol degradation genes from Bacillus stearothermophilus FDTP-3and their expression in Escherichia coli. Applied and Environmental Microbiology. 1992; 1992: 2531-2535
11. Dos Santos V L. de Souza Monterio A, Braga DT, Santoro MM. Phenol degradation by Aureobasidiumpullulans FEB isolated from industrial effluents. J. Hazard Mater. 2009;161: 1413-1420.
12. Duffner, F.M., U. Kirchner, M.P. Bauer and R. Muller.(2000) . Phenol/cresol degradation by thethermophilic Bacillus thermoglucosidasius A7: cloning and sequence analysis of five genes involved inthe pathway. Gene, 256: 215-221
13. Fernando, T. and Aust SD (1994) Biodegradation of toxic chemicals by white rot fungi. In : Biological degradation and bioremediation of toxic chemicals. Chaudhry- GR. Ed. (Discoorides Press, Portland, or), pp. 386-387.
14. Gallego A, Gemini V, Fortunato M, Dabas P. Degradation and detoxification of cresols in synthetic and industrial wastewater by an indigenous strain of Pseudomonas putida in aerobic reactors. Environmental Toxicology: An International Journal. 2008; 23(6): 664-671.
15. Ghadhi SC, Sangodkar UM. Potentials of Pseudomnascepacia PAA in bioremediation of aquatic wastes containing phenol. Proceeding of National symposium frontiers in applied and environmental microbiology 1995; 11-13.
16. Goulding C, Gillen C, Bolton E. Biodegradation

of subatituted benzenes. J. Appl. Bacteriol. 1988; 65:1-5

17. Hutchison DH, Robinson C. A microbial regeneration process for granular activatedcarbon II regeneration studies. Water Research. 1990; 24: 1217-1223
18. Kotturi G, CW Robinson, WE Inniss. Phenol degradation psychrotropic strain ofPseudomonasputida. Applied Microbiology and Biotechnology. 1991; 34: 539-543
19. Kumaran P, YL Paruchuri. Kinetics of phenol biotransformation. Water Research. 1996; 31; 11-22
20. Leonard D, ND Lindley. Carbon and energy flux constrains in continuous cultures ofAlcaligenseutrophus grown on phenol. Microbiology. 1998; 144: 241-248
21. Milo RE, FM Duffner, R Muller. Catechol 2,3-dioxygenase from thermophilic, phenol-degrading Bacillus thermoleovorans strain A2 has an expected low thermal stability. Extremophiles. 1999; 3:185-19
22. Monteiro A, R Boaventura, AE Rodrigues. Phenol biodegradation byPseudomonasputida DSM 548 in a batch reactor. Biochemical Engineering Journal. 2000; 6: 45-49
23. Paller G, RK Hommel, HP Kleber. Phenol degradation by AcinetobactercalcoaceticusNCIB 8250. Basic Microbiology. 1995; 35: 325-335
24. Pessione E, C Giunta. Acinetobacterradioresistens metabolizing aromatic compounds.II. Biochemical and microbiological characterization of strain. Microbios. 1997; 89: 105-11
25. Tibbles B, Baecker A. Effect of pH and inoculum size on phenol degradation by bacteria isolated from landfill waste. Environmental Pollution. 1989; 59(3): 227-239.
26. Wang Y, Y Tian, B Han, H Zhao, J Bi, BL Cai. Biodegradation of phenolby free and immobilized Acinetobacter sp. strain PD12. Journal of Environmental Sciences. 2007; 19; 222-225
27. Wei G, Yu J, Zhu Y, Chen W, Wang L. Characterization of phenol degradation by Rhizobium sp. CCNWTB 701 isolated from Astragaluschrysopteru in mining tailing region. Journal of Hazardous Materials. 2008; 151(1): 111-117.
28. Yang C, C Lee. Enrichment, isolation, characterization of phenol-degrading Pseudomonasresinovorans strain P-1 and Brevibacillus sp. Strain P-6. International Biodeterioration and Biodegradation. 2007; 59: 206-210
29. Ying W, Ye T, Bin H, ZHAO H. Biodegradation of phenol by free and immobilized Acinetobacter sp. strain PD12. Journal of environmental sciences. 2007; 19(2): 222-225.
30. Nair CI, Jayachandran K, Shashidhar S. Biodegradation of phenol. African Journal of Biotechnology.2008 ; 7(25): 4951-4958
31. Marrot B, Barrios-Martinez A. Biodegradation of high phenol concentration by activated sludge in an immersed membrane bioreactor. Biochemical Engineering Journal. 2006; 30(2): 174-183.
32. Singh S, Singh B, Chandra R. Biodegradation of phenol in batch culture by pure and mixed strains of Paenibacillus sp. and Bacillus cereus. Pol J Microbiol. 2009; 58(4): 319-325.