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Biodegradation of phenol by *Klebsiella oxytoca* isolated from oil contaminated environments

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ABSTRACT

The degradation of phenol by microorganisms in the soil is an important method through which these materials are removed from the environment, thus reducing environmental pollution. The aim of this study is to select and characterize bacterial strain(s) from oil contaminated soils and testing the ability for biodegradation of phenol. In this study, two *Klebsiella oxytoca* isolates were selected from petroleum contaminated soils using mineral salts medium supporting with phenol as the only carbon source. The isolates were identified by microscopic, morphological, and biochemical approaches , and their capability of biodegrading phenol was analyzed by 4-aminoantipyrine assay. The results showed that the selected *Klebsiella oxytoca* isolates were able to remove 100% of phenol even at the highest used concentration(500 mg / L).The results of the current study indicate the possibility of using *Klebsiella oxytoca* in bioremediation of phenol contaminated environments.

1. Introduction

Contamination of soils with oil hydrocarbons are a significant global environmental concern which has gained community consideration in recent decades. Individual actions are the most important aspect responsible for the leakage of hydrocarbons from agricultural or manufacturing products. Even though oil is one of the predominant resources of energy to sustain the profitable and communal improvement of the country, oil products have turn out to be of the most significant types of organic contaminants resulting from massive leakage of subversive storeroom tanks and unintentional discharges during transferring and clearance **(USEPA, 2011)**. This is why oil pollution accidents have become global phenomena and have caused serious environmental problems such as the introduction of toxic compounds into food sources and changes in the physical and chemical properties of soil **(Atanasković *et al.*, 2016)**. A large number of organic compounds have been released into the environment. These compounds are produced chemically, and many of them are disposed of as waste.Phenol is one of the most dangerous toxic contaminants to the environment, and it is one of the most common pollutants, which is classified as harmful to human health and to other plants and animals **(EPA, 2003)**.Phenol is an aromatic compound formed by a hydroxyl group connected to benzene ring with a chemical formula (C_6H_6O).It is highly toxic and present in different formulations or associated with other compounds **(EPA, 2003)**. Phenol is a liquid or a solid with a low melting point, but it has a high boiling point due to the hydrogen in its structure. It is soluble in water due to its ability to form a hydrogen bond with water. Phenolic complexes are vital to numerous manufacturing.

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They can be detected in the waste of several industrialized routes, such as oil strainers, food preparation industrial units ,pharmaceutical, polymer and stain production **(Fernando and Aust, 1994).** Their massive use has polluted a wide range of soil, rivers, industrial waste and wastewater. Phenolic composites encompass harmful possessions on marine living organisms, plants, and lots of other species. At some stages of biotransformation these compounds are capable of providing inhibitory effects. Therefore, to protect the environment and preserve human healthiness , it is important to effectively remove phenolic compounds **(Afzal *et al.*,2007)**. Phenol residues can be removed from the environment by a combination of physical and chemical treatments. However, these treatments are impractical due to the high cost and the formation of other toxic compounds. For example, as a result of chlorination, phenol turns into another toxic compound called chlorophenol **(Wei *et al.*, 2008)**.Therefore, biological techniques have acknowledged more attention than physical or chemical procedures because diverse microorganisms are recognized to utilize phenols as the only sources of carbon and energy **(Marrot *et al.*,2006)**. It is found that different bacterial strains are capable of biodegrading phenolic compounds, including *Klebsiella oxytoca*. Many research reported that this bacterium has the capacity to degrade phenol **(Singh *et al.*, 2009)** .In this study, two *Klebsiella oxytoca* strains, which have the capability of biodegrading high concentrations of phenol were selected from Electric generator soil and gas station soil , and identified based on phenotypic, and biochemical tests.

2.Materials and methods

2.1 Sample collection

Twenty samples were collected from various locations of soil polluted with petroleum products .The soils were saved in sterilized glass bottles, and transported to the lab for the tests to be performed .

2.2 Growth media

Mineral salts medium (MM) was used for selection .It consists of the following materials:(0.02 g) Mgso 4.7H2o, (0.1 g) NH4 (2SO4), (0.01 g) NaCl, (0.01 g) CaCl2, (0.45 g) K2HPO4, (0.002 g) Fecl3**(Shawabkeh *et al.*, 2007)**.The materials were dissolved and mixed well in a liter of distilled water to assure all the materials soluble and sterilized at 121 ° C for 20 minutes and pH 7. Phenol was added then at the required concentration to the medium as the only source of carbon.

2.3 Selection of phenol tolerant bacterial strains

Five grams of each sample of soil were added to(50 ml) of liquid mineral salts medium(MM). Phenol was added at a concentration of 25mg/L as a primary concentration. the flasks were then incubated at 30 ° C for 5 days **(Wei *et al.*, 2008)**. (0.1 ml) of the bacterial suspension was transported to the MM agar medium containing the same phenol concentrations and incubated at 30 ° C for 5 days . This step was performed two more times to get isolated colonies and to confirm that the purified strain can grow on the medium and use phenol as carbon source .

2.4 Identification of the Phenol degrading Bacteria

The isolated bacterial strains were identified based on microscopic and phenotypic identification on MacConkey agar as well as Vitek 2 compact system biochemical tests. The diagnostic set contains 47 pits inoculating with bacterial suspension at the age of 24 hours and incubating for 24 hours, then the device records the color changes resulting from bacterial growth **(Goulding et al., 1988) , (Pincus, 2006).**

2.5 The ability of the bacterial isolates to degrade phenol compound

Increasing concentrations of phenol ,including 50, 75 ,100,200,300,400,500mg/L were used to test the ability of the selected bacterial isolates for phenol biodegradation. The assay was performed as described in **(Koneman *et al.*, 1997)** .A standard titration curve was set for phenol , then the absorption values at 420 nm were used to design a calibration graph for phenol compound using an Excel program to obtain the mathematical equation .Titration of 4-amino antipyrine was used to measure the concentrations of the remaining phenol compound by spectrophotometer on wavelength 460

nmafter bacteria have grown(Prichard&Barwick,2003) ,and the wave length of 600 nm was also recorded to determine bacterial growth curve. This assay was conducted twice to confirm the results.

3.Results and Discussion

3.1 Bacterial selection and Identification

A total of 20 petroleum polluted soil samples from various locations were screened for bacterial strains capable of growing and utilizing phenol as a sole carbon source. Two *Klebsiella oxytoca* isolates were selected after culturing on MM medium supporting with phenol. One isolate was from electric generator soil and the second was from gas station soil. Microscopic examination showed Gram-negative bacilli sticky, mucoid colonies, (Figure 1). The selected strains were further subjected to biochemical identification using Vitek 2 compact system as shown in table (1). The characters are identical to the global identification characteristics of *Klebsiella spp* (**Endimiani *et al.*, 2009).**

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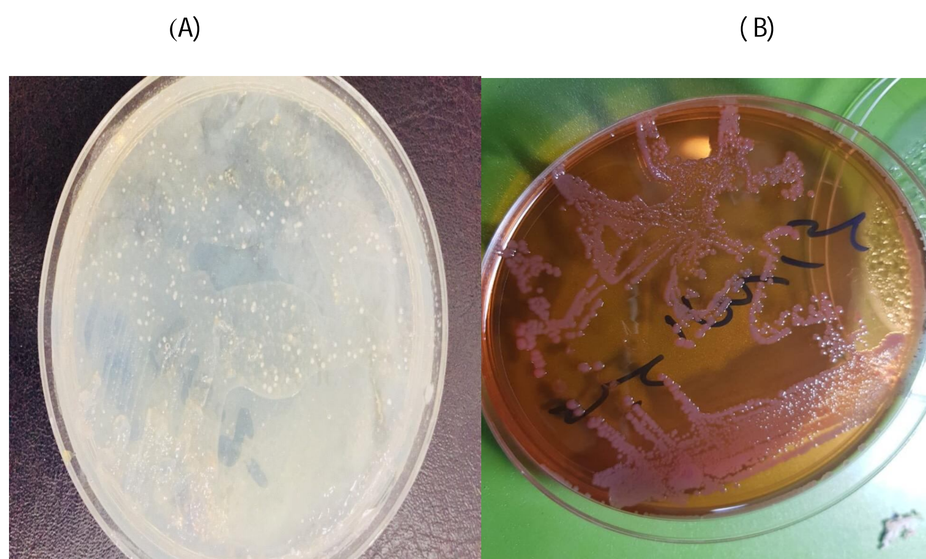
(A) (B)



Figure(1). Phenotypic characteristics of the isolated *Klebsiella oxytoca* growing on solid mineral salts medium (A) and MacConkey agar medium (B)

Table 1: Biochemical properties of the isolated *Klebsiella oxytoca* using Vitek 2 compact system

	Test name	results		Test name	results
2	Ala-Phe-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	-			

+: positive reaction, -: negative reaction

3.2 The ability of *Klebsiella oxytoca* strains to degrade phenol in different concentrations

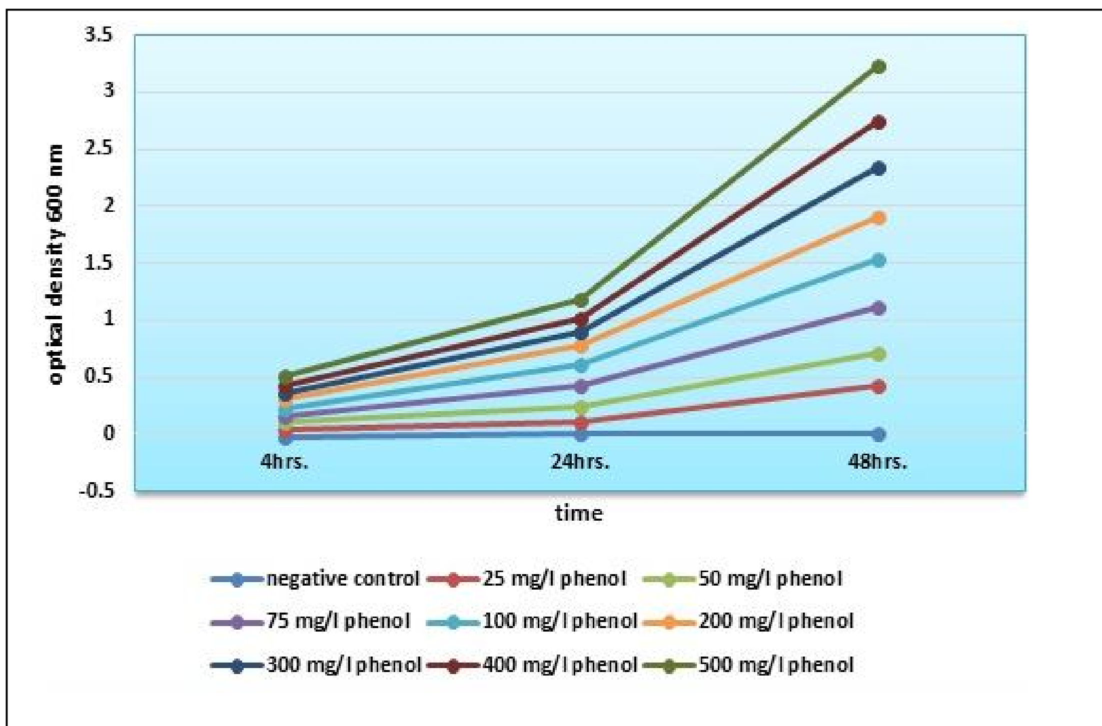
Results showed that *Klebsiella oxytoca* strains were capable of degrading phenol completely by 100% for all the used concentrations (25,50,75,100,200,300,400, 500 mg / L) after 48 hours of incubation, as shown in Table (2), with an increase in the rate of bacterial growth during the incubation period, as shown in Figure2. Hydrocarbon compounds and their environmental-polluting derivatives are dangerous on human health and many other living organisms found in land and aquatic environments, so it is very important at different levels to get rid of oil vehicle emissions. Biological treatment is one of the most effective methods available for pollution reduction as it is less harmful than other treatments. It is utilizing microorganisms naturally found in such habitats because of their ability to break down harmful compounds into less toxic compounds. The target of using bacteria isolated from places previously polluted with petroleum products is to have a high ability to absorb these compounds and thus to promote the hydrocarbon biodegradation process . Different methods have been used to remove phenols, but utilization of biodegradation techniques are widely favored due to their low costs and the probability of complete mineralization **(Afzal *et al.*,2007)**. In the current research, *Klebsiella oxytoca* strains were isolated and characterized from oil polluted environments and their derivatives, and their capacity to degrade phenol compounds as the sole source of carbon was investigated. The results showed that *Klebsiella oxytoca* bacteria had a100% decomposition efficiency of phenol for all concentrations in addition to an increase in the biological growth of bacteria. Thus, a complete removal of phenol from the medium was obtained after the end of the specified time period. Other studies have also indicated the ability of *Klebsiella oxytoca* strain to degrade phenols **(Shawabkeh *et al.*, 2007)**.

4. Conclusion:

In this study, two *Klebsiella oxytoca* strains were selected and characterized. The isolated strains showed a high ability to degrade phenol at concentrations up to 500mg/L indicating the possibility of using *Klebsiella oxytoca* in biological treatment of environments contaminated with phenol and its derivatives to reduce the toxicity of these substances and thus reduce their danger to living organisms.

Table (2) Biodegradation rates of phenol by *Klebsiella oxytoca*

Klebsiella oytoca							
	The remaining of phenol after degradation			% of Degradation			Mean Degradation% /hr
Start concentration of phenol mg/l	4 hrs.	24hrs.	48hrs.	4 hrs.	24hrs.	48hrs.	3.22
500	405	193.88	0	19	38.776	100	
400	201.66	70.55	0	49.585	17.6375	100	
300	203.34	107.22	0	32.22	35.74	100	
200	192.11	81.66	0	3.945	40.83	100	
100	94.37	94.11	0	5.63	94.11	100	
75	75	50.55	0	0	67.4	100	
50	43.88	13.88	0	12.24	27.76	100	
25	12.77	12.77	0	48.92	51.08	100	



Figure(1): Growth curve of *Klebsiella oxytoca* in presence of different phenol concentrations

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