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Molecular Identification and Characterization of Phenol - Degrading Bacteria from Oil-Contaminated Sea Water

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Biological treatment is one of the considerable choices for removing of phenol as a main pollutant present in the environment. Recently phenol biodegradation has been considered, and marine bacteria are the most important phenol biodegrader. In this study, the phenol degrading bacteria from marine environmental samples were isolated from RasGhareb Red Sea Governorate, Egypt. Isolation and identification of the most effective bacterial species using direct isolation method on mineral salt medium supplemented with 2.0 mM phenol as the sole carbon source are mentioned. The two phenol-degrading bacteria were characterized by morphological, biochemical properties and confirmed by DNA fingerprinting technique. Ribosomal database project consistent with phylogenetic analysis of 16S rRNA gene sequences showed that the strain RGRS-1 has 99% similarity with *Ochrobactrum sp.* strains while RGRS-2 has 98% similarity with *Kocurica camiphilia* strains. The DNA G+C content of strain RGRS-1 was 53 mol% while RGRS-2 was 57 mol%. On the basis of polyphasic analysis, strain RGRS-1 is considered to represent a novel species of the genus *Ochrobactrum*, for which the name *Ochrobactrum sp.* RGRS-1 is proposed. On the other hand, RGRS-2 proposed a name is *Kocurica camiphilia* RGRS-2 .The nucleotide sequences of 16S rRNA gene have been deposited in GenBank under accession numbers: KP221808 and KP221809 for RGRS-1 and RGRS-2 respectively. The aerobic isolates grew on phenol at concentrations ranging from 2-10 mM. The ability of the isolates to degrade phenol make them a potential candidate for use in bioremediation of environments contaminated by such or related compounds.

Key words: Biodegradation, Bioremediation, marine water, phenol, *Ochrobactrum sp.* RGRS-1, *Kocurica camiphilia* RGRS-2.

Phenol and phenolic compounds are synthetic as well as a naturally occurring organic aromatic compounds which are important intermediate in the biodegradation of industrial and natural aromatic compounds[1-3]. Many of these synthetic compounds cause environmental pollution and human health problems as a result of their persistence, toxicity and transformation into hazardous intermediates[4]. As major pollutants, their existence in industrial wastewater of oil refineries, and petrochemical and phenol resin industry plants, has been established[5-7]. Different techniques and methods to remove phenol from wastewaters are put into practice including coagulation, chemical oxidation, solvent extraction and adsorption. However, all these methods are not environmental friendly due to the production of toxic secondary intermediates, high cost and

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health risk to workers[8,9]. Currently, bioremediation is receiving much desirability owing to its environmentally friendly nature and being economical as compared to other physiochemical methods[10]. The basic idea behind bioremediation is to find bacteria that are capable of using the contaminant as carbon and energy source[11]. Biodegradation is the metabolic power of the microorganisms to mineralize the organic pollutant or convert them to less harmful and safe materials. This method is an operational technology to clean non-destructively and also it is favorable in view of preparation because it accelerates the natural biological degradation through optimizing limited conditions[12].

The widespread presence of phenol in environment, permits many microorganisms including bacteria to withstand high concentration of phenol and utilize phenol as primary source of carbon and energy[13,14]. Bacteria able to thrive and grow in the presence of high concentrations of aromatic compounds have been reported[9,15-18]. Apart from their usefulness in bioremediation purposes, they also have many medical applications, including discriminating morphine from codeine, measuring hormonal catecholamines (adrenalin, noradrenaline, and dopamine) and as a potential drug target against human pathogenic fungi[19]. Phenol degrading bacteria mainly investigated in the terrestrial environments and there are a few studies on phenol biodegradation in marine environment[20].

Polymerase Chain Reaction or PCR is a powerful method that allows the species specific detection of organisms in environmental samples based on amplification of DNA specific fragment[21]. Required time for total PCR steps (DNA extraction, PCR protocol and electrophoresis) is one day[22] and it provides higher specificity and sensitivity. Of course the success of PCR for specific detection is highly dependent on the specificity of the nucleotide sequences used as the primers[23].

The aim of this work was to describe isolation and molecular characterization of some phenol-degrading bacteria obtained from oil-contaminated sea water. The results from this investigation would be useful for prediction of the bioremediation mechanisms of these microbial isolates.

MATERIALS AND METHODS

Sampling, Growth Medium and Culture Conditions

Bacterial isolates used in this study were isolated from oil-contaminated sea water which collected from RasGharib-Red Sea, Egypt in April 2014. Seawater samples were collected from a depth of 15 cm in sterile 1000-ml bottles and transported on ice to the laboratory for isolation. Mineral salts medium KMM1[24] was used for isolation of naturally occurring phenol-degrading bacteria. Basal medium KMM1 contained (g/L). NaCl, 2.8; KCl, 6.5; $MgCl_2.6H_2O$, 0.50; $CaCl_2.2H_2O$, 0.10 ; NH_4Cl, 5.6; $NaSO_4$, 1.0 and KH_2PO_4, 1.0. Phenol was added to the media from a 2.0 M stock solution to a final concentration of 2.0 mM. Cultures were incubated on a shaker (100 rpm) at 30°C and monitored for the turbidity increase due to bacterial growth.

Isolation and Selection of Phenol degrading Bacteria

One ml of seawater (1 ml) samples were added to Erlenmeyer flasks containing 100 ml KMMI medium and the flasks incubated for 7 days at 30°C on a rotary shaker operating at 180 rpm.[20]. Then 5-ml aliquots were removed to fresh KMMI broth medium (100 ml). After a series of four further subcultures (each after 2 days at 30°C on the shaker), inoculums from the flasks was streaked out and phenotypically different colonies purified on KMMI agar. Isolates were screened to select for bacteria that can grow rapidly on KMMI plates with phenol as sole carbon source. Individual colonies, which, grew on the plates, were then re-inoculated in fresh KMMI liquid media to ascertain their ability to degrade phenol. Two isolates exhibited relatively faster growth rates than the rest were picked and chosen for further study[25].

Morphological, Biochemical and Physiological Characterizations

Gram-stain was performed and the growths of isolates at different temperatures were done[25]. Enzyme profiles and carbon substrate utilization characteristics were determined using the Biolog assay technique. Highest concentration of phenol at which the isolates) could initiate growth was determined by monitoring the optical density of the culture growing at initial phenol concentrations ranging from 2-10mM. Experiments

were carried out in 250 mL flasks containing 50 mL of KMMI and the test media was inoculated with 100 µL of a 24 h old culture grown in nutrient broth. Cultures were then incubated in a shaker (100 rpm) at 30°C for one week[26]. Isolates were also inoculated into basal KMM1 without the substrates, which served as the control experiment. The tolerance of isolates to different concentrations of NaCl was tested (1, 4, 8 and 10 % (w/v) sodium chloride.

DNA Isolation, PCR Amplification and Sequencing

DNA extraction was done by using the protocol of GeneJETgenomic DNA purification Kit (Thermo K0721) as following manufacture of kit. The PCR amplification of 16S rDNA region was carried out following the manufacture of Maxima Hot Start PCR Master Mix (Thermo K1051). The 16SrDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16SrDNA region. The domain bacteria-specific primer 27F (forward primer) was 5'AGAGTTTGATCMTGGCTCAG3' and universal bacterial primer 1492R (reverse primer) was 5'TACGGYTACCTTGTTACGACTT3'[27]. The PCR reaction was performed with 5µl of genomic DNA as the template, 1µl of 16SrRNA Forward primer, 1µl of 16SrRNA reverse primer 18 µl Water, nuclease-free and 25 µl Maxima® Hot Start PCR Master Mix (2X) in a 50µl reaction mixture as follows: activation of 2 Taq polymerase at 95 °C for 2 minutes, 35 cycles of 95 °C for 1 minutes, 65°C, and 72 °C for 1minutes each were performed, finishing with a 10-minute step at 72 °C. After completion, the PCR products were electrophoresed on 1 % agarose gels, containing ethidium bromide (10mg ml 21), to ensure that a fragment of the correct size had been amplified.

The amplification products were purified with K0701 GeneJET™ PCR Purification Kit (Thermo**).** Afterward, the samples become ready for sequencing in ABI Prism 3730XL DNA sequencer and analysis on GATC Company. Sequencing reaction was performed with the primers 518F 5' (CCA GCA GCC GCG GTA ATA CG) 3' and 800R 5' (TAC CAG GGT ATC TAA TCC) 3' using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Diformamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequence alignment was prepared with DNASTAR software programs (DNASTAR. INC., Madison, Wis.).

Phylogenetic Analysis and Tree Construction

Phylogenetic data were obtained by aligning the nucleotides of different 16S rRNA retrieved from BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), using the CLUSTAL W program version 1.8 with standard parameters. The classifier is trained on the new phylogenetically consistent higher-order bacterial taxonomy (Ribosomal Database Project, RDP Classifier) proposed by Wang[28], (http:// rdp.cme.msu.edu/classifier/classifier.jsp). Phylogenetic and molecular evolutionary analyses were conducted using Mega 6 program. All analyses were performed on a boot strapped data set containing 100 replicates (generated by the program).

Nucleotide Sequence ID

The nucleotide sequences of the 16S rRNA gene of RGRS-1 and RGRS-2 strains have been deposited in the GenBank under accession number**:** KP221808 and KP221809 respectively.

RESULTS AND DISCUSSION

Bioremediation is a low-cost treatment alternative for the clean up of petroleum-contaminated soils and wastewater[11]. Microbial isolates for site remediation may be obtained from a variety of sources. The obvious sources are from the contaminated water itself, where it can be assumed that such species will have adapted to the prevailing ecological condition. Phenol-degrading bacteria exist widely in the environments and they are usually isolated from phenol-contaminated sites. In this study, we isolated two degrading strains, RGRS-1, RGRS-2. These strains were isolated from oil-contaminated sea water, RasGharib-Red Sea, Egypt.

Isolation and Characterization of Phenol-degrading Bacteria

Two bacterial isolates from direct plate method on mineral salt medium supplemented with phenol as a carbon source showed that two isolates coded RGRS-1, RGRS-2 were promising phenol degradation. Therefore, these isolates were

selected for complete identification. The potential bacterial isolates were morphologically and biochemically typified and properties were listed in Table 1.

Table 1. Morphological and biochemical characteristics of the bacterial strains RGRS-1, RGRS-2 which able to grow in a medium where phenol is the sole carbon source

Test	RGRS-1	RGRS-2
Cell Morphology	Rod	Rod
Gram stain	-ve	+ve
Motility	Motile	Nonmotile
Catalase test	+	+
Oxidase test	+	-
Arginine dehydrogenase	+	-
Lysine decarboxylase	+	+
Citrate utilization	-	-
H_2S production	-	-
Urease	-	+
Indole test	-	-
Triple iorn sugar test	Red/yellow	Red/red
Utilization of sugers:		
Gelatin	+	-
Glucose	+	+
Mannitol	+	-
Sorbitol	+	-
Fructose	+	+
Sucrose	+	+
Arabitol	+	-
Rhamnose	-	-
Galactose	+	+
Lactose	-	-
Maltose	+	+
Dextrose	+	+
Glycerol	+	-
Mannose	-	-
Inositol	+	-
Acetic acid	+	+
Starch hydrolysis	+	-
D-Gluconic acid	+	+
Growth at different Temp. (^oC)		
15	+	+
25	+	+
30	+	+
40	+	+
Growth at different pH		
5	+	-
6	+	+
8	+	+
Growth at different concentration of NaCl (%)		
1	+	+
4	+	+
8	+	+
10	+	+

Gram stain indicated that these phenol degrading isolates were rod in shape, RGRS-1 was Gram-negative and motile strain, while RGRS-2 was Gram-positive and nonmotile. Additional characterization of phenol-degrading isolates by classical microbiological tests indicated that these isolates were characterized by positive results using catalase reaction and lysine decarboxylase test while they gave negative results with indole test, citrate utilizations and H_2S production.

The results in Table 1 indicated that, urease reaction was negative with RGRS-1 while oxidase test and Arginine dehydrogenase reaction were positive. On the other hand, Urease reaction was positive with RGRS-2 while oxidase test and Arginine dehydrogenase reaction were negative.

As the characteristics listed in Table 1 showed, isolate RGRS-1 is quite similar to isolate RGRS-2 in all biochemical tests, except the isolate RGRS-2 was not grown at pH 5 and did not utilized Gelatin, Manitol, Sorbitol and Starch. Both strains were capable to grow at different degree of temperature from 15-40 ^oC at pH ranged from 5-8. In step before adding the bacteria to contaminated site, it was important to measure the capability of bacterial isolates to grow at different salt concentrations. The two strains were tolerant to different concentration of NaCl (1-10%). Enan[26] demonstrated that the phenol-degrading bacteria were able to survive in the presence of NaCl concentrations up to 5% to 10%. On the other hand, Venosa and Zhu [29] reported that the rates of hydrocarbon bio-degradation decreased with increasing salinity in the range of 3.3-28.4%.

Qualitative test for these bacterial isolates was performed on a KMMI medium containing phenol as a carbon and energy source. Results illustrated in Figure (1A) show that growth rate of RGRS-1 isolate on different concentrations of phenol (2, 4, 6, 8 and 10 mM) were very slow at the first three days. After the third day growth rate increase markedly at 2 mM followed by 4 mM then 6 mM also decline rapidly after the fourth day at all concentrations. At the highest concentration (8, 10 mM) growth initiated immediately after the third day and begins to decrease after fourth day as shown in Figure (1A). Many researchers stated that bacterial growth was inhibited at higher phenol

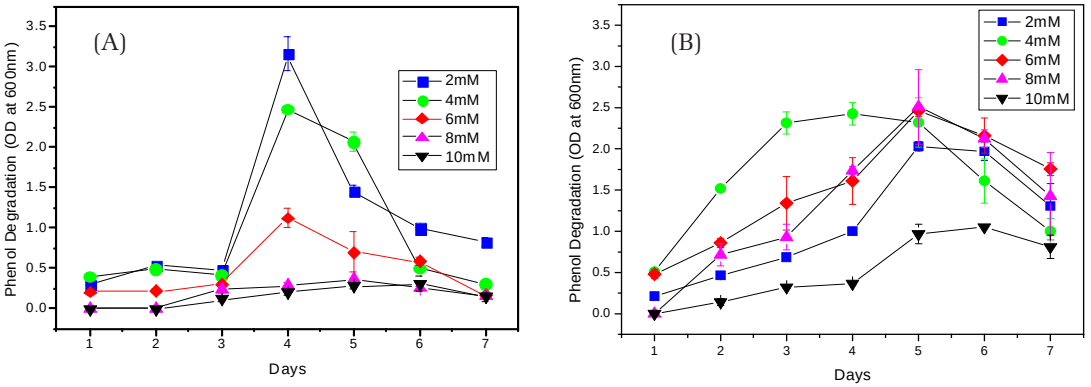

Fig. 1. Time course characteristics for growth of : (A) isolate RGRS-1, (B) isolate RGRS-2 on phenol concentrations (2-10 mM). Growth was determined by measuring the turbidity at 600 nm (OD600).

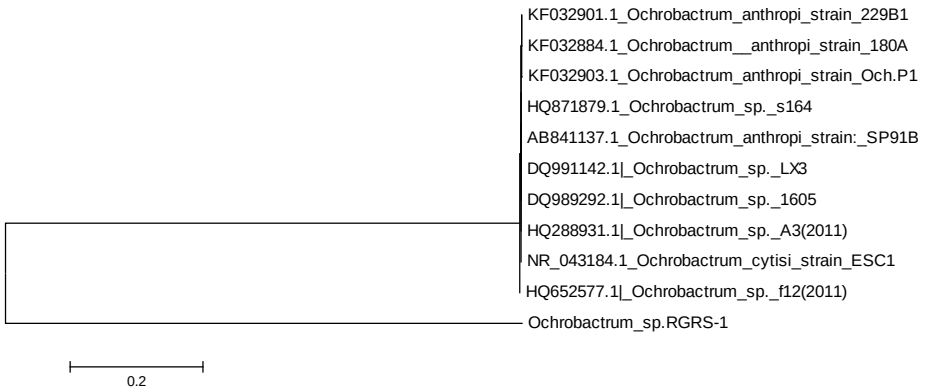

Fig.2. Neighbor-joining phylogenetic representation of the strains and their closest NCBI (BLAST) relatives based on 16S rRNA gene sequences of *Ochrobactrum sp.*RGRS-1 and some known sequences of *Ochrobactrum* species.

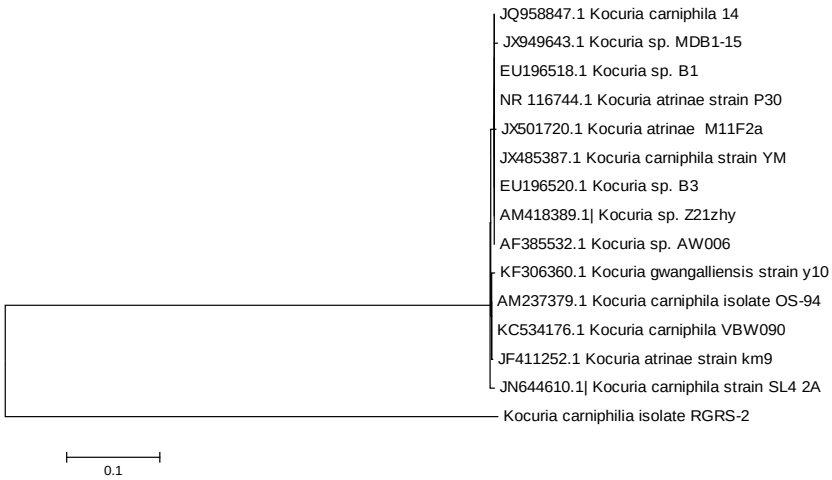

Fig. 3. Neighbor-joining phylogenetic representation of the strains and their closest NCBI (BLAST) relatives based on 16S rRNA gene sequences of *Kocurica camiphilia* RGRS-2 and some known sequences of *Kocurica* sp.

concentration [30,31,11].

In case of RGRS-2 isolate, growth rate was detected at concentration 10 mM, but the growth rate increase with the phenol concentration decrease as show in Figure 1B. After 4 days of growth rate of RGRS-2 isolate increased reach its maximum rate on the fifth day at the concentration 6 and 8mM then the rate of growth decline on the days 6 and 7. On the other hand, the growth rate on the first three days was rapid at concentration (4, 6, 8 mM), but it is slower at the highest concentration (10 mM). Saxena[32] reported that *Acinetobacter* sp. capable of degrading phenol (9.5 mM of phenol concentration) under normal conditions. While Dong[33] showed that the phenol tolerance of the genera *Acinetobacter*, *Pseudomonas* and *Vibrio* were reported to be 7 to 10 mM.

Molecular Characterization of Phenol-degrading Isolates

Sequencing and phylogenetic analysis

Genomic DNA of phenol-degrading bacteria was subjected to a PCR amplification to differentiate identical isolates revealed by biochemical tests. The nucleotide sequence (1651bp and 1263bp) of strains *Ochrobactrum sp.* RGRS-1 and *Kocurica camiphilia* RGRS-2 was matched with the 16S rRNA reported gene sequences in the gene bank database. The database of NCBI Blast (www.ncbi.nlm.nih.gov/BLAST) was used to compare the *Ochrobactrum sp.*RGRS-1 and *Kocurica camiphilia* RGRS-2 with those of member *Ochrobactrum* and *Kocurica* species strains. The results showed that the high sequence similarity species (99%) with *Ochrobactrum sp.* and *Kocurica camiphilia* strains.

The primer pairs' 27F/1492R amplified the fragments of the expected size of the genomic DNA isolated from strains *Ochrobactrum sp.*RGRS-1 and *Kocurica camiphilia* RGRS-2. The primers were successfully used to amplify genomic DNA from the isolated samples. These results are in agreement with those of Edwards[26] who found that these primers are specific for bacteria.

The phylogenetic tree (Fig 1 and 2) showed that strains *Ochrobactrum sp.*RGRS-1 and *Kocuric acamiphilia* RGRS-2 is most closely related to *Ochrobactrum sp*. and *Kocurica camiphilia*. Therefore, it was proposed a name *Ochrobactrum sp.*RGRS-1 and *Kocurica camiphilia* RGRS-2. The nucleotide sequences of 16S rRNA genes have been deposited in GenBank under accession numbers: KP221808 and KP221809 for RGRS-1and RGRS-2 respectively.

The percentage of G+C is one of many general features used to characterize bacterial genomes. The G+C content of the genomic DNA was 53 and 57 mol% for RGRS-1and RGRS-2 respectively was obtained from the phylogenetic analysis. These results were in accordance with those by McCutcheon and Moran[34] who mentioned the percentage composition of the known *bacteria species* can range from 25% to 75% for GC richness.

CONCLUSION

In this study, two degrading bacteria were isolated and identified as the 'Powerful' one able to using phenol as sole sources of carbon and energy were characterized from the red sea oil-contaminated water. The two phenol-degrading bacteria were characterized by morphological, biochemical properties and confirmed by DNA fingerprinting technique. In the continuation of isolation new and efficient microbial strain for phenol degradation, we report here a novel bacterial strain, which is quite capable to utilize phenol as sole source of carbon and energy. The 16S rDNA analysis prove it as a novel strain. This work has provided a useful guideline in evaluating potential phenol biodegraders isolated from sea water environment.

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