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Newly Isolated *Pandoraea* sp. Capable of Phenol Biodegradation

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Abstract: The aim of this study was to isolate and characterize new strains capable of phenol bioremediation. A new strain of *Pandoraea* sp. was isolated from Red Sea soil contaminated with hydrocarbon. Morphological and molecular characterization were performed to identify the isolated strain, it was designated as *Pandoraea* sp. phen16 and located in the database under accession number EU549818. The isolated strain could remove 100% of 50 mg phenol L^{-1} in culture as sole carbon source after 3 days of incubation, where 100 mg phenol L^{-1} in culture inhibited the growth, only 15% from total phenol was removed. For further support a PCR product was obtained from amplification of phenol hydroxylase, suggesting the possible existence of the ring-hydroxylating mono-oxygenases genes responsible for phenol degradation.

Key words: Bioremediation, environmental pollution, *Pandoraea* sp., phenol, gram negative bacteria

INTRODUCTION

Wastewaters from fossil fuel, refining, pharmaceuticals and pesticides are the main sources of phenolic pollution. Wastewaters from a refinery are a complex mixture of organic and inorganic compounds, often containing more than one type of phenolic compound. Phenol and cresols are major constituents found in refinery effluents (Berne and Cordonnier, 1995; Farooqi *et al*., 2008). A phenol concentration of 1 mg L^{-1} or greater affects aquatic life (Farooqi *et al*., 2008). Therefore, in most cases stringent effluent discharge limit of less than 0.5 mg L^{-1} is imposed. Many substituted phenols including chlorophenols, nitrophenols and cresols have been designated as priority pollutants by US Environmental Protection Agency (EPA) (Keith and Telliard, 1979). Phenols can be removed by solvent extraction, adsorption, chemical oxidation, incineration and other non-biological treatment methods but these methods suffer from serious drawbacks such as high cost and formation of hazardous by products (Loh *et al*., 2000). Biological degradation is generally preferred due to lower costs and the possibility of complete mineralization microorganisms.

Strains of *Pandoraea* sp. have been isolated from clinical samples. Primarily from respiratory tracts of patients suffering from cystic fibrosis but also from blood and from non clinical sources such as water, sludge, soil and dried milk (Coenye *et al*., 2000; Moore *et al*., 2001). *Pandoraea* sp. (genomospecies 1) described by Coenye *et al*. (2000); originally isolated by Parsons *et al*. (1988). This organism was studied because of its ability to degrade chlorinated aromatic compounds.

Little is known about the degradation of hydrocarbons and other environmental contamination by this organism. Degradation of phenol by *Pandoraea* sp. PG-01 was first reported by Jiang *et al*.

(2004). Jiang *et al*. (2007) co-cultured functionally similar strains, *Pandoraea* and *Rhodococcus erythropolis*, both of which have high phenol-degradaing rates, for degradation of environmentally phenol.

In this study, a new strain of *Pandoraea* sp. was isolated and tested for its ability to utilize phenol as sole carbon source in mineral media. Phenol removal was measured and PCR detection of hydroxylase gene was performed.

MATERIALS AND METHODS

Source of Bacteria

The phenol-degrading bacteria used in this study were isolated from soil brought from Red Sea Area in Egypt in 2004. Soil was collected from 10-15 cm depth near an oil well and stored at refrigerator for further investigations.

Growth Medium and Isolation Conditions

A defined mineral salt media was used in this study contained, per liter of water: 0.02 g $MgSO_4.7H_2O$, 0.1 $(NH_4)_2SO_4$, 0.01 NaCl, 0.01 $CaCl_2$, 0.45 K_2HPO_4 and 0.002 $FeCl_3$. The initial pH was adjusted to 7.0. In case of mineral medium agar plates, agar was added in a concentration of 15 g L^{-1}. Five gram soil was added to 100 mL pre-sterilized mineral media supplemented with 25 g phenol L^{-1}. Cultures were incubated at 30°C for 5 days and examined for turbidity due to bacterial growth. Flasks showed bacterial growth turbidity was used to inoculate mineral agar plates containing 25 g phenol L^{-1} with 100 μL inoculum size for selecting colonies of degrading bacteria. Plates were incubated for 5 days and the growing colonies were tested for their capability of phenol removal.

Colonies which grew on plates were picked out and purified on LB media. Testing their ability for phenol degradation was performed by cultivation on mineral agar plates supplemented with different phenol concentration (25, 50 and 100 mg L^{-1}) which added separately after the sterilization process. Growth was observed and the most potent isolate (s) was chosen for further investigation. The growth was evaluated on MM agar plates based on its bacterial colony forming unit (cfu).

Phenol Degradation and Analysis

Degradation was performed in flasks containing 100 mL MM and 25, 50 and 100 mg phenol L^{-1} was added as sole carbon source, all tests were performed into triplet. Flasks were incubated at 30°C on 200 rpm shaker. The growth was monitored by measuring the turbidity at 600 nm using spectrophotometer and residual phenol was estimated by HPLC analysis.

Phenol concentrations were measured using reverse-phase-high-performance liquid chromatography (HPLC) (Beckman, 126 solvent module, 168 detector). The solvent used was methanol/water/glacial acetic acid (60:38:2, by vol.) then phenol was detected at 275 nm.

Biochemical Identification of the Isolated Strain

The selected isolate was subjected to some biochemical tests for identification, in addition to gram stain test. All tests were performed according to Bergy's Manual determinative bacteriology (Krieg and Holt, 1984). Hemolytic activity was carried out by Carrillo *et al*. (1996) using blood agar plates containing 5% v/v blood with an incubation period of 24-48 h at 30°C. β-Hemolytic activity was detected by formation of a clear zone around the colony.

Table 1: Primers used in this study

Primer	Target	Sequence (5'-3')	T_a(°C)	Expected product size (bp)	Reference
Bact16	16S rDNA gene	AGAGTTTGATCMTGGCTCAG TACGGYACCTTGTTACGACTT	55	1300	Ausubel *et al.* (1999)
PHE	Phenol hydroxylase	GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC CGCCAGAACCA(C/T)TT(A/G)TC	49	206	Baldwin *et al.* (2003)

PCR Amplification of 16S rDNA and Catabolic Genes

Total genomic DNA was extracted from cells of 5 mL LB overnight bacterial culture as described by Coenye *et al*. (2001). PCR reaction was performed in a light cycler Eppendorf PCR machine. The used primers are shown in Table 1. A 1300 bp fragment was obtained by PCR amplification of the 16S rDNA gene in a 50 μL reaction mixture containing around 100 ng of purified strain DNA (Ausubel *et al*., 1999). The methods used for amplification of phenol hydroxylase was as described by Baldwin *et al*. (2003) The reactions were carried out by initial denaturation for 10 min at 95°C followed by 30 cycles of denaturation for 1 min at 95°C and 1 min at the optimum annealing temperature (Table 1) followed by elongation for 2 min at 72°C and a final extension step for 10 min at 72°C.

Amplicons of 16S rDNA was purified using PCR purification kit (Quigen). The purified products was sequenced by the chain terminator method (ABI 3130XL system, DNA technology, Denmark) using the two corresponding PCR primers separately. The resulted DNA sequence of 16S rDNA was phylogenetically analyzed using the BLAST search program and sequences are available in GeneBank under accession numbers EU549818.

RESULTS AND DISCUSSION

Isolation and Selection of Phenol Degrading Strain

A total of 20 bacterial isolate were isolated from sample soil of Red Sea, the isolates were cultivated on plates supplemented with 25 mg phenol L^{-1} in MM media. The growing colonies were tested for their ability to degrade higher concentration of phenol by cultivation on agar plates supplemented by 50 and 100 mg phenol L^{-1}. Four different colonies were able to grow on the plate containing 100 mg phenol L^{-1} and 9 colonies were grown on 50 mg phenol L^{-1} plate (Data not shown). The growing colonies were subjected to gram stain, the gram negative strain No. 16 (phen 16) was chosen for further studies on the ability of phenol degradation was studied.

Biochemical Characterization of Gram Negative Phenol Degrading Isolate

The isolated bacterium was subjected to several biochemical tests for identification and characterization. Data presented in Table 2, showed that the strain was gram negative short rods non spore forming, non nitrate reducing. The strain could not emulsify gelatin agar and hydrolyze skim milk plate. On the other hand, It could ferment glucose and manitol sugar and positive catalase test was detected. By testing its hemolytic activity on blood agar plates, it was found that phenol 16 did not show either α or β-hemolytic activity.

The data obtained were quite similar to bacteria present under family Burkholderiaceae genus *Pandoraea*. Thus the Pandoraea species could be isolated from soil or sludge samples as reported by Coenye *et al*. (2000) and Moore *et al*. (2001). *Pandoraea* sp. was described by Coenye *et al*. (2000); originally isolated by Parsons *et al*. (1988), was studied because of its ability to degrade chlorinated aromatic compounds.

Table 2: Biochemical characterization of phen16 isolate

Test	Reaction
Gram stain	Gram negative
Shape	Motile short rods
Gelatin agar emulsification	-ve
Starch hydrolysis	-ve
Skim milk hydrolysis	-ve
Nitrate reduction	-ve
Glucose fermentation	+ve
Manitol fermentation	+ve
Catalase test	+ve
Blood hemolysis	-ve

-ve: Absence of activity, +ve: Presence of activity

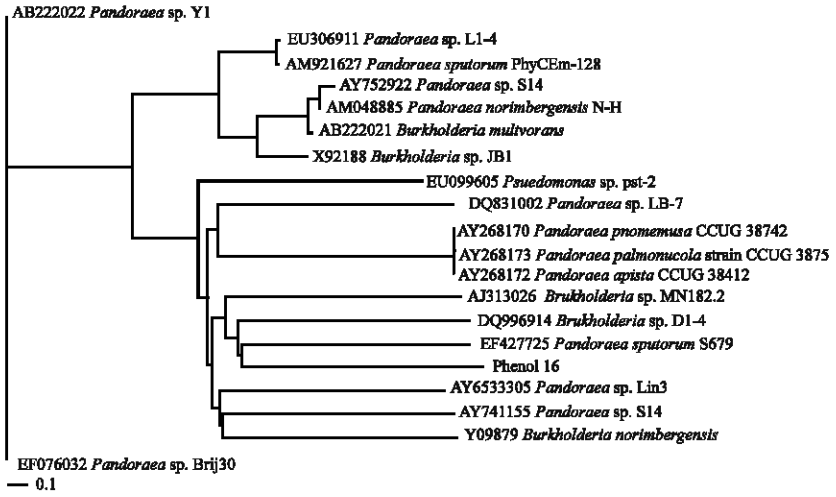

Fig. 1: Phylogenetic position of the isolate phenol 16 based on partial sequencing of the 16S rDNA gene

Amplification of 16S rDNA Gene for Molecular Identification of Phen16 Isolate

In order to obtain complete identification for the new isolate, 16S rDNA gene was amplified by PCR from the extracted chromosomal DNA using bacterial universal primers. The PCR product was then purified and sequenced. The obtained sequence data were aligned against other 16S rDNA sequences presented at the database project (http://www.cme.msu.edu/RDP/ html/index/html; Maidak *et al*. (1994) and Rainey *et al*. (1996). The phylogenetic relationship between the experimental isolate and the closely related species were analyzed by using the multi-sequence alignment program (Bio Edit Sequence Alignment Editor). The resulted phylogenetic tree, in which branch lengths were considered, is presented in Fig. 1. Based on this taxonomic relationship, the closest 16S rDNA gene sequences are those of the genotypes *Pandoraea* sp. with accession number EF076032 and EU306911 with 97% similarity. Therefore, the isolate phen16 was designated as *Pandoraea* sp. phen16 and it is located in the database under accession number EU549818. Genus *Pandoraea* was previously isolated by Demnerova *et al*. (2003) from root zone soil of different plants, they were able to degrade chlorobezoic acid.

Evaluation of Phenol Biodegradation by *Pandoraea* sp. Phen16

The biodegradation capability of the isolated strain was monitored by the cultivation of MM liquid culture supplemented with 50 and 100 mg phenol L^{-1}. The cultures were incubated for 2 days and the OD was recorded together with measuring the percentage of residual phenol. Figure 2 showed

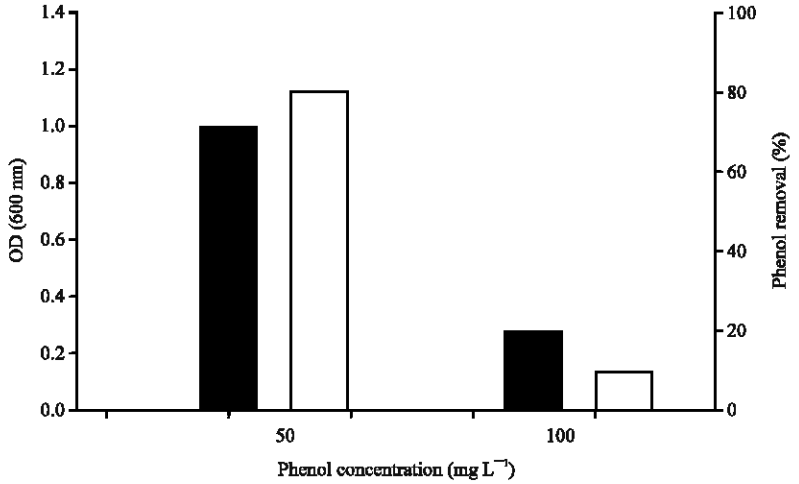

Fig. 2: Cultivation of *Pandoraea* sp. phen16 in liquid MM supplemented with phenol in 50 and 100 mg L^{-1} concentration as sole carbon source and incubated for two days at 30°C

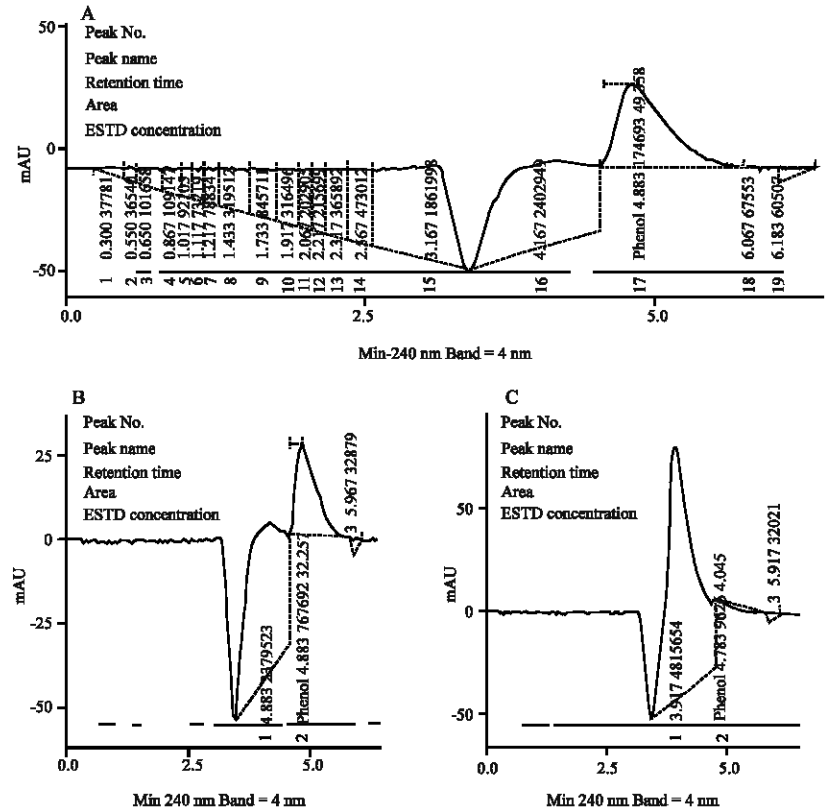

Fig. 3: HPLC chromatogram for phenol in MM liquid culture supplemented by 50 mg L^{-1} phenol as sole carbon source and cultivated with cells of *Pandoraea* sp. phen16. (A) Control culture without cells, (B) After 1 days and (C) After 3 days

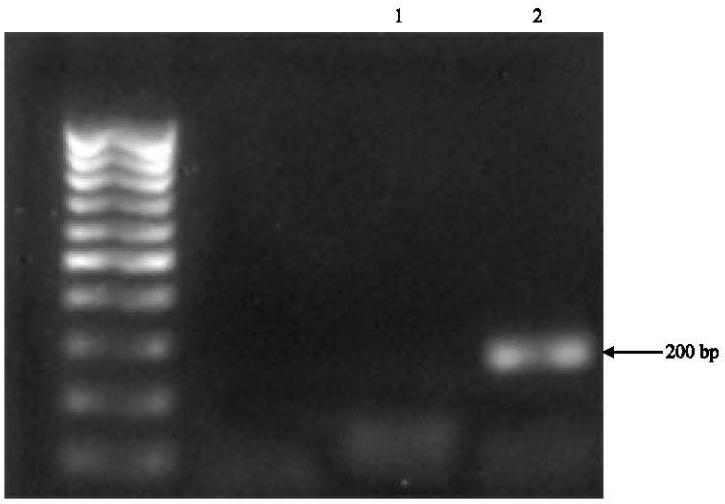

Fig. 4: Agarose gel electrophoresis (1.5%) for PCR analysis of phenol hydroxylase gene using primers pairs PHE Lane 1, negative control; Lane 2, *Pandoraea* sp. phen16, marker used is 100 bp ladder

that *Pandoraea* sp. phen16 was more efficient to remove 50 mg phenol L^{-1} than 100 mg L^{-1}, where, 80% phenol was removed after 2 days of incubation in mM containing 50 mg phenol L^{-1} as sole carbon source. Therefore, 100 mg L^{-1} was found to be an inhibition dose for the isolate growth.

For evaluation of *Pandoraea* sp. phen16 potency to biodegrade phenol, liquid cultures supplemented with 50 mg phenol L^{-1} were inoculated with 0.1% of an overnight culture. Samples were collected from cultures every 1 day for 1 week and phenol was analyzed by HPLC to record the residual phenol concentration. Figure 3 showed the HPLC analysis for the control as well as for culture. Data revealed that the strain could remove 100% of phenol after 3 days of incubation. As it showed by the HPLC analysis that the peak of phenol was disappeared and a new peak was appeared, this peak was seemed to be one of the phenol biodegradation intermediates. Benzoate, catechol, cis-cis-muconate, β-ketoadipate, succinate and acetate have all been identified as intermediates in the biodegradation of phenol (Fedorak *et al*., 1986; Knoll and Winter, 1987).

PCR Amplification of Phenol Hydroxylase Gene

To study and to insure the degradation capability of the strain *Pandoraea* sp. phen16 to phenol, PCR amplification was performed to detect the presence of phenol hydroxylase gene using primers PHE (Table 1). The primers were designed by Baldwin *et al*. (2003), they were chosen from conserved regions in the DNA sequences observed during alignment of ring hydroxylating mono-oxygenases group. Figure 4, showed the presence of PCR product with molecular weight of 200 bp and the absence of this band with the control strain which was not capable of phenol degradation. Baldwin *et al*. (2003), previously reported that phenol monooxygenase PCR product size was around 206 bp, which strongly supported our results that *Pandoraea* sp. phen16 harboring the phenol hydroxylase genes which is responsible for phenol ring cleavage.

CONCLUSION

The unique result of this study is the isolation of genus *Pandoraea* sp. From Red Sea soil. The isolated strain could biodegrade 100% of phenol presented in salt culture media as sole carbon source.

PCR amplification of gene responsible for phenol biodegradation revealed that *Pandoraea* sp. phen16 could perform the degradation through phenol hydroxylating enzymes. We recommend using the isolated *Pandoraea* sp. phen16 together with other hydrocarbon degradating consortia to get-rid from hydrocarbon pollutants.

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