# Isolation and Molecular Determination of Phenol Degrading Bacteria

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**Abstract**

To determine phenol contaminated soils, forty five phenol-degrading bacteria were isolated from soil and wastewater samples in phenol agar after several subcultures in phenol broth. All isolates utilized 0.2 g/l phenol and were identified as *Pseudomonas sp.* PCR amplification of LmPH and *dmpN* genes showed that all 45 isolated strains contained LmPH genes and 12 strains were *Pseudomonas putida*. The LmPH gene were detected in contaminated and uncontaminated soil, although microtiter MPN methods showed that the quantity of phenol-degrading bacteria in contaminated soil was more than in uncontaminated soil. The soil contamination might be identified by PCR of some genes; however the presence of phenol in all studied soil showed positive reaction in contaminated and uncontaminated natural soil. The results indicated that the phenol degrader bacteria are distributed in all soils.

**Key word**: PCR, Phenol, Phenol hydroxylase, MPN soil and Phenol-degrading bacteria.

# جداسازي و تشخيص مولكولي باكتريهاي تجزيه كننده فنل

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چكيده

جهت شناسايي خاك هاي آلوده به فنل ، چهل و پنج باكتري تجزيه كننده فنل از نمونه هاي خاك و پساب پس از كشت در محيط فنل آگار و فنل براث جداسازي شد. تمام ايزوله ها ، ۰/۲ گرم فنل در ليتر را مصرف نموده و به عنوان گونه هاي سودوموناس شناسايي شدند. تكثير ژن هاي LmPH و dmpN نشان داد كه تمام ايزوله ها حاوي ژن هاي LmPH و دوازده سويه سودوموناس پوتيدا بودند. ژن LmPH در خاك آلوده و غير آلوده شناسايي شد. اگر چه روش ميكروتيتر MPN نشان داد كه تعداد باكتريهاي تجزيه كننده فنل در خاك آلوده بيشتر از خاك غير آلوده بود. آلودگي خاك با شناسايي بعضي ژن ها به روش PCR قابل شناسايي

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بود بهر حال حضور فنل در تمام خاك هاي مورد مطالعه موجب واكنش مثبت در اك طبيعي آلوده و غير آلوده مي شود. نتايج نشان داد كه باكتري هاي تجزيه كننده فنل در تمام خاك ها پخش هستند.

واژه هاي كليدي

## Introduction

In recent years there has been an increase in the environmental release of harmful chemicals. Currently several commercialized chemical, biological, physical and thermal processes are employed for degradation of chemical compounds such as phenol. Biological process is attractive because mixed population of microorganisms break down or transform organic pollutant to innocuous substance (Annaduraia et al., 2002; Sal et al., 2001).

Phenol and its derivatives are one of the major hazardous compounds in industrial wastewater and for this reason biodegradation of phenol has attracted attention. In phenol biodegradation, phenol hydroxylase catalyzes the initial step of microbial phenol degradation by insertion of oxygen into the aromatic ring providing catechol. The catechol is then broken by the *ortho* or *meta* fission pathway (Neujahr and Gaal 1973; Wagner et al., 1999). Phenotypic and genetic analysis of bacterial population in environmental samples is becoming increasingly important in microbial ecology research, because of their potential role in bioremediation of polluted sites (Heinaru et al., 2000; Whiteley et al., 2001). The polymerase chain reaction (PCR) is a very powerful and sensitive analytical technique applied with success to environments such as soil or sediment. A number of PCR-based methods for detection of catabolic genes in soil have been developed. They provide information on in situe condition without cultivation or activation of indigenous microbial population (Hallier et al., 1996).

Few studies on the molecular detection of phenol-degrading bacteria in soil and wastewater have been published (Watanabe et al., 1998a). The aim of this study was the application of PCR for detection of phenol-degrading bacteria in environmental samples such as soils, contaminated soils and wastewater. In addition, the direct detection of phenol-degrading bacteria in environmental samples was another goal of this study.

## Materials and Methods

### Sampling and media:

For isolation of phenol-degrading bacteria, soil and wastewater samples were collected from various sites in Isfahan located at the center of Iran. Samples included soil contaminated with oil hydrocarbon, oil refinery wastewater, coal tar waste water and activated sludge taken from Isfahan south refinery.

The synthetic phenol broth medium used was based on the mineral salts medium containing (per liter): 2.25 g of $KH_2PO_4$, 2.25 g of $K_2HPO_4$, 1 g of $(NH_4)2SO_4$, 0.2 g of $MgCl2.6H_2O$, 0.1g of NaCl , 0.02 g of $FeCl3.6H_2O$, 0.06 g of bromothymolblue and 0.01 g of $CaCl_2$, pH 6.8-7. These synthetic medium were supplemented with phenol as the sole carbon source at a concentration of 3 mM (Watanabe et al., 1998b). Phenol broth was solidified with 15 g/l agar to obtain phenol agar medium. All media were sterilized at 121°C for 15 minutes (all chemical were obtained from Merck and Sigma, Germany and USA).

### Isolation and selection of phenol degrading bacteria:

One gram of soil or one milliliter of wastewater sample was inoculated into phenol broth medium. After 7 days, 5 ml of this medium was sub cultured into fresh phenol broth medium. After four subsequent subcultures (cultures were incubated at 30°C on a rotary shaker at 180 rpm) into phenol broth media, cultures were purified on phenol agar. Identification of strains were performed by biochemical tests according to Bergey's Manual of Determinative of Bacteriology (Holt et al., 1998).

### Growth rate and phenol removal assay:

Growth rate of the isolates was routinely assessed indirectly through turbidity measurement at 600 nm using a UV-visible spectrophotometer (Shimadzu UV-160 Japan). The cell free supernatants were analyzed for residual phenol using the photometric method for phenol estimation. The method was based on rapid condensation of phenol with 4-aminoantipyrene, followed by oxidation with potassium ferricyanide under alkaline conditions to give a red wine colored antipyrine dye. Absorbance of the dye was measured at 500 nm (Celsseri et al., 1983).

### MPN enumeration of phenol-degrading bacteria in soil:

The soil samples (1 g) were diluted in phosphate

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buffer ($K_2HPO_4$ 1 g/l and KH2PO4 1 g/l, pH 7.0), and mixed using a wrist shaker for 30 min. Microtiter plate wells were inoculated with 200 µl of sterile phenol broth medium. Ten fold serial dilutions were prepared from the soil extracts and each of the plate wells was inoculated with 50 µl of each dilution. A piece of wet filter paper was placed on top of each microtiter plate lid to reduce water loss from the well. The plates were incubated for 48 hr at room temperature, after which 50 µl of filter sterilized TTC (Triphenyl Tetrazolium Chloride) was added to each well. In positive wells TTC is reduced to an insoluble formazan that deposits intracellularly as a red precipitate. Positive wells were scored after an overnight, incubation with TTC at room temperature. A computer program (Klee et al., 1993) was used to calculate the MPN for each soil sample (Brian et al., 1996; Johnsen et al., 2002).

**DNA extraction:**

DNA was extracted from the pure culture of each isolated strain by boiling method. In this method, bacteria were dissolved in double distilled water and boiled for 1 min, then centrifuged at 10000 × g for 10 min and 2 µl of aliquots phase was removed as template DNA for PCR reaction.

Total DNA was extracted from 5 g of wet soil by using the method described by Zhou et al (Zhou et al., 1996) with the following modification:

1. Soil samples were mixed vigorously with 14 ml extraction buffer (100mM Tris-HCl pH=8 + 100 mM EDTA pH=8 + 1.5 M NaCl + %1 CTAB) for 1 hr at 30°C.

2. Precipitation was carried out with 0.6 volume of isopropanol at room temperature for 4 hr.

DNA purification was conducted with sephadex G-200 columns. The quality and quantity of the extracted DNA were checked by measuring UV absorption spectrum (Sambrook et al., 1989).

**PCR condition:**

**(a) Universal primers:**

DNA fragment for the largest subunit of multicomponent phenol hydroxylase (LmpHs) was amplified using one pair of degenerate primers (Selvaratnam et al 1995). The sequences of primers were as follow:

pheUf: 5′-CCAGG(C/G)(C/G/T)GA(G/A)AA(A/G)GAGA(A/G)GAA(G/A)CT-3′

pheUr 5′-CGG(A/T)A(G/A)CCGCGCCAGAACCA-3′

Amplification was performed using 25 µl PCR mixture containing 2 mM $MgCl_2$, 10 × PCR reaction buffer (200 mM Tris, 500 mM KCl), 2 mM each dNTP, 0.15 mM each primer and 1U taq DNA polymerase. Target DNA was amplified in a thermal cycler (Eppendorf AG 22331 Hamburg) using the following program:

Step 1, 10 min at 94°C; step 2, five cycles consisting of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; step 3, five cycles consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C; step 4, 25 cycles consisting of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; step 5, 10 min of extension at 72°C (Futamata et al., 2001).

**(b) Specific primer:**

For the detection of *P. putida* among isolates a 199-bp region located within the *dmpN* gene (a gene from the *dmp* operon) was amplified using two primers (Watanabe et al 1998a). This operon encodes phenol hydroxylase enzyme. The sequences of primers were as follow:

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Table 1: Biochemical tests for identification of *P. putida*

| Biochemical tests | *P. putida* |
|---|---|
| 1.Gram stain | - |
| 2. Aerobic growth | + |
| 3. Anaerobic growth | - |
| 4. Oxidation-fermentation (O/F) | O+/F- |
| 5 Acid production from: | |
| 5-1. Glucose | |
| 5-2. Fructose | + |
| 5-3. Xylose | + |
| 5-4. Maltose | + |
| 5-5. Lactose | - |
| 5-6. Manitol | - |
| 5-8. Sucrose | - |
| 6. Oxidase | - |
| 7. Catalase | + |
| 8.Urease | + |
| 9. H2S production | d |
| 10. Simon citrate | + |
| 11. Gelatin hydrolysis | + |
| | - |

DMPN1 :( 5′-ATC ACC GAC TGG GAC AAG TGG GAA GAC C-3′)
DMPN2: (5′-TGG TAT TCC AGC GGT GAA ACG GCG G-3′)

A total volume of 25 µl of PCR mixture contained 2 mM MgCl2, 10 × PCR reaction buffer (200 mM Tris, 500 mM KCl), 1 mM of each dNTP, 0.15 mM of each primer and 1U taq DNA polymerase. Target DNA was amplified in a thermal cycler (Eppendorf AG 22331 Hamburg) with initial denaturation of the target DNA at 94 °C for 2 min, follow by 40 cycles of three-step PCR amplifications consisting of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 1 min and primer extension at 72 °C for 1 min. Samples were incubated at 72 °C for 5 min at the end of amplification cycles to complete the extension reaction (Selvaratnam et al., 1997). PCR products were electrophoresis on a 2 % horizontal agarose gel (Sigma Chemical Co., St. Louis, Mo.). Gels were stained in a solution of ethidium bromide and visualized with a UVP UV transilluminator (UVP Inc., San Gabriel, Calif).

## Results

### Characterization and identification of isolated phenol utilizing bacteria:

Forty-five bacteria isolated from environmental samples were identified by biochemical tests according to Bergy's Manual of Determinative Bacteriology. It was observed that all isolates were Gram-negative, motile rods, oxidase and catalase positive, capable of growth aerobically but not anaerobically and were positive for production of acid from glucose. These results showed that all isolated strains belonged to the Pseudomonas sp. Further biochemical tests for identification of *P. putida* were carried out; the results are shown in table (1).

### Growth rate and phenol removal by isolates:

The phenol degradation rates of whole cells were determined by monitoring the decrease in phenol concentration in media. The data in figure (1) shows that most of the isolates could utilize 0.2 g/l of phenol in 24 hours, leaving only 0.05 g/l of residual phenol.

### General detection of phenol-degrading bacteria:

By using the pheUf and pheUr primers LmpH fragment from 45 isolated strains was successfully amplified. The pheUf and pheUr PCR positive strains could grow on phenol as the sole carbon source. The presence of this gene in these strains allows them to grow on phenol. Figure (2) shows the gel electrophoresis profile of the PCR product from these strains.

### Identification of phenol-degrading *Pseudomonas putida*:

Among the 45 isolated strains, *dmpN* gene was amplified from 12 strains by using DMPN1 and DMPN2 primers. According to the results, these strains were identified as *P. putida* (Selvartan et al 1998). The remaining 33 strains were considered not to be *P. putida*. Figure (3) shows gel electrophoresis of the PCR products from *P.* putida strains that harbor dmpN gene.

### Direct detection of LmPH gene in soil:

Crude DNA was extracted from 5 g of wet soil under three conditions: 50 g of wet soil was contaminated with phenol, 50 g of clay soil was contaminated with phenol and inoculated with phenol-

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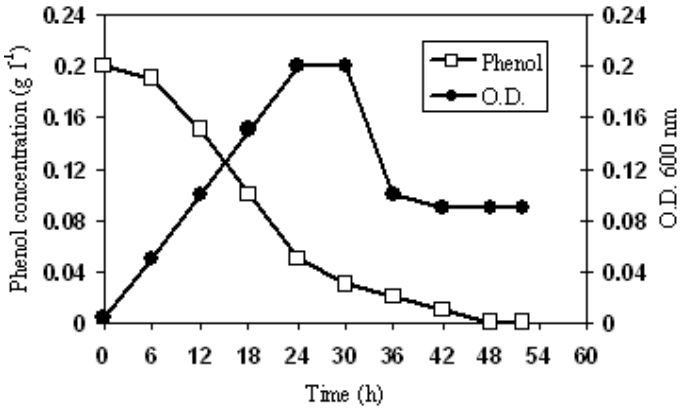


**Figure 1: The rate of growth and removal of phenol assayed for D6 isolated strain (the culture were incubated at 30°C). The strains reached maximum growth at 24 hours and the remaining of phenol was 0.05 g/l after 24 hours. And phenol was removed completely after 48 hours.**

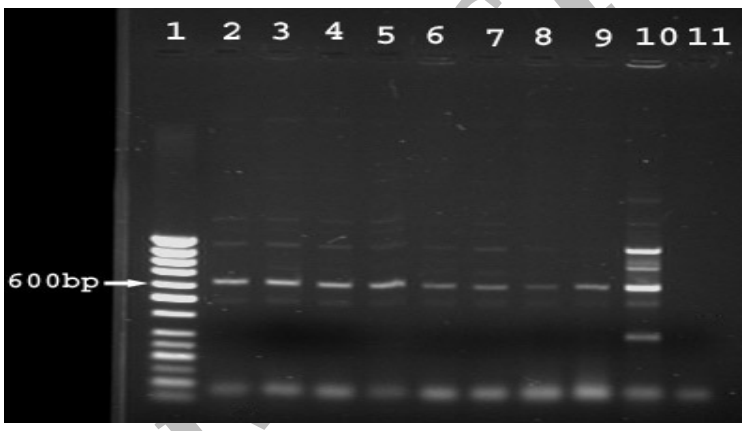


**Figure 2: Gel electrophoresis profile of the PCR product from phenol-degrading strains with universal primers. Lane1: 50bp DNA ladder size marker, lane 2: control positive (Pseudomonas putida ATCC 11172), lane 3-10: isolated strains, lane 11: negative control (H2O).**

**Table2: Estimation of the number of phenol-degrading bacteria in soil by the MPN method.**

| | Contaminated soil with phenol | uncontaminated soil | Contaminated soil inoculated with phenol-degrading bacterium |
|---|---|---|---|
| Number of phenol-degrading bacteria per one gram of soil | 2.6 × 106 | 1 × 105 | 6 × 105 |

degrading strains and 50 g of this clay soil was pristine soil (uncontaminated). PCR was performed with the pheUf and pheUr primers set. The 624-bp product was detected by gel electrophoresis (figure 4). It was observed that the LmpH fragment was successfully amplified by pheUf and pheUr primers from all soil samples, and there was no difference between contaminated and non contaminated soil. But MPN data showed that the number of phenol-degrading bacteria in contaminated soil was higher than in pristine soil. These results indicated that phenol-degrading bacteria are distributed in the environment, but in soil contaminated with phenol the quantity of these bacteria is higher than in uncontaminated soils (table 2).

**Specificity of PCR:**

PheUf /pheUr primers amplified DNA of phenol-degrading strains only, and PCR-product was not found

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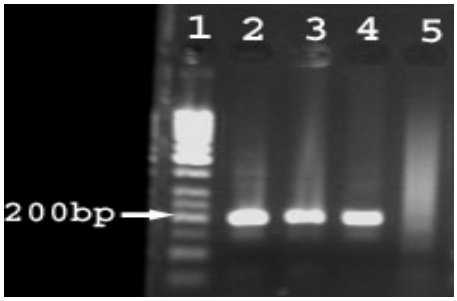


**Figure 3: Gel electrophoresis profile of the PCR product from phenol-degrading strains with specific primers for P. putida, lane1: 50bp DNA ladder size marker, lane 2: control positive (Pseudomonas putida ATCC 11172), lane 3 and 4: isolated strains, lane 5: negative control (H2O).**

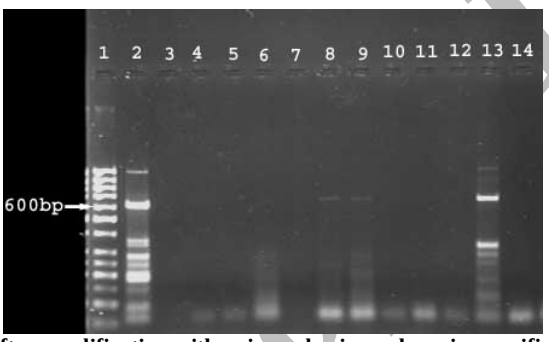


**Figure 4: PCR product obtained after amplification with universal primers by using purified DNA obtained from soil, lane 1: 50bp DNA ladder size marker, lane 2: control positive (Pseudomonas putida ATCC 11172), lane 2-7 and 11-12: unpurified DNA, lane 8: contaminated soil with phenol, lane 9: non contaminated soil, lane 13: contaminated soil inoculated with isolated phenol-degrading bacterium, lane 14: negative control (H2O).**

with any of the non phenol-degrading species (figure 5). In PCR reaction with DMPN1/DMPN2 primers, a 199-bp product was obtained from the *P. putida* phenol-degrading strains (figure 6) and there was no PCR-product formed from other phenol-degrading strains or non phenol-degrading species.

**Discussion**

In this study, 45 phenol-degrading bacterial strains isolated from 5 soil and 3 wastewater samples were identified as *Pseudomonas sp* by using traditional procedures. But using pheUf and pheUr universal primers, all strains produced a 624-bp PCR product. This shows that all 45 phenol-degrading isolates had the largest subunit of multicomponent phenol hydroxylase (LmPH gene) and they could be detected by using universal primers (Watanabe et al 1998a)

Direct detection of the LmPH gene by PCR in soil was another aim of this study that was carried out on extracted DNA from soil. The results showed that phenol-degrading bacteria are distributed in the environment because phenol is released to environment by degradation of plant lignocelluloses compounds and this induces population of phenol-degrading bacteria. But in soil contaminated with phenol, the quantity of these bacteria is higher than in non contaminated soil. Koutny et al (2003) showed that phenol-degrading bacteria were present in pristine soil of south Siberia that was not contaminated with any chemical pollutant. In this study, we showed that genes involved in phenol degradation were present in uncontaminated soil, thus our results confirms the results obtained by Koutny et al (2003).

Identification of phenol-degrading *P. putida* by using specific PCR primers (DMPN1 and DMPN2) was another aim of this study (Selvartane et al 1998). The *dmpN* gene codes for phenol hydroxylase, an enzyme involved in the conversion of phenol to catechol (Nordlund et al., 1990). Twelve of the 45 strains had a 199-bp PCR product considered as *P.putida*. This isolated phenol-degrading bacterium was identified by traditional procedure as well. Selvaratnam et al (1995) developed a reverse transcriptase PCR for monitoring *dmpN* expression in activated sludge. Greatest *dmpN* expression was observed 15 minutes after maximum phenol concentration was reached in the reactor and 15 minutes after the start of aeration. Decreased phenol concentration in the reactor caused reduction of *dmpN* expression (Selvaratnam et al., 1995).

Hallier et al (1996) developed a PCR method for

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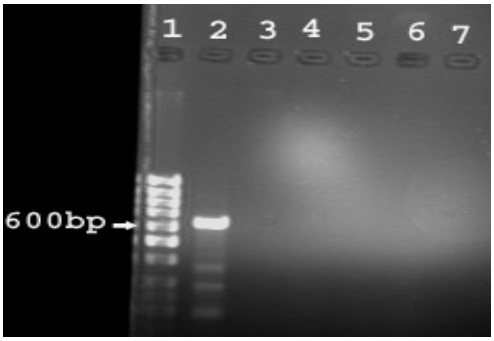


**Figure 5: Specificity of universal primers, the product obtained from M1 strain phenol-degrading bacterium (lane 2).**

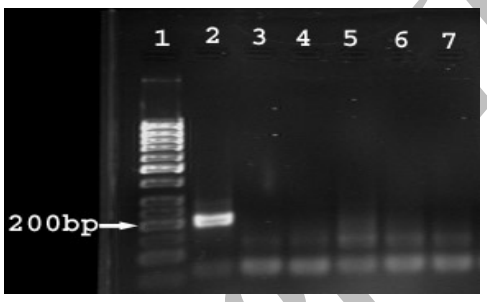


**Figure 6:** Specificity of specific primers, the product obtained from *P. putida* phenol-degrading D6 strain (lane 2).

detection of the toluene degradation gene *xylE* in soil. The detection limit of the method was 10 bacteria in 100 mg of soil. Watanabe et al (1998) isolated DNA from phenol-digesting activated sludge and amplified the partial fragment of the 16s rDNA and the gene encoding the largest subunit of multicomponent phenol hydroxylase (LmPH) by PCR. They concluded that, the genotype and phenotype of the functionally dominant phenol-degrading population in activated sludge were much different from the genotypes and phenotypes of the representive phenol-degrading bacteria characterized previously in several laboratories (Watanabe et al., 1998a).

Futamata et al (2001) compared the sequences of the LmPHs. They found that LmPHs formed three phylogenetic groups I, II and III. They designed universal and group-specific PCR primers and analyzed the phenol/trichloroethylene degrading population in contaminated soil. It was found that aquifer soil harbored diverse genotypes of LmPH and the group-specific primers successfully amplified LmPH fragment affiliated with each of these groups. The universal PCR primers pheUf and pheUr for all LmPH genes were also designed. LmPH fragment could be amplified by using these primers from all 13 of the phenol-degrading bacteria while the combination of the group-specific primers with the universal primers allowed the specific amplification of each group of LmPH (Futamata et al., 2001).

In conclusion our study showed that the universal primers pheUf and pheUr could detect all 45 phenol-degrading bacterial strains isolated from environmental samples, and that the quantity of phenol-degrading bacteria in phenol contaminated soil was more than in uncontaminated soil. Specific PCR-primers DMPN1 and DMPN2 could detect phenol degrading *P. putida.*

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