Iranian a of Environmental Technology
1st Year, Vol. 1, No. 2, Autumn & Winter 2015
**Received:** June 2, 2015/ **Accepted:** August 26, 2015. **Page:** 21- 34

# Isolation and characterization of phenol degrading bacteria from Midok copper mine at Shahrbabk provenance in Iran

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## Abstract

Phenol is one of the major aromatic pollutants among variety of toxic compounds. Phenol is a man- made as well as a naturally occurring aromatic compound and an important intermediate in the biodegradation of natural and industrial aromatic compounds. To clean up the aromatic contaminated sites a bioremediation method is considered as an economical and safe approach for the environment. In this study, 10 phenol- degrading bacterial strains were isolated from three sites at Midok copper mine in Shahrbabak. These sites include: Magnetit site (WG), Hematit site (WH) and Near Hematit site (NH). Total heterotrophic and phenol degrading bacteria were quantified with most probable number (MPN) and colony forming unit (CFU) methods. The results of this study show that the (WG) site of the mine have the highest phenol degrading bacteria. Totally 10 phenol degrading bacteria were isolated from three sites in Midok copper mine. Then, five bacterial strains were selected according to high growth rate and phenol degradation. Finally two strains named isolates P62 and 69P were selected for analysis of *16S rRNA* sequences. Strain P62 belongs to *Pseudomonas putida*AHBP62 and strain P69 is related to *Arthrobacter scleromae*AHB69P that has capability degradation of 600 (ppm) phenols in 7 days. By using these degradative bacteria in contaminated mine sites the environmental pollution can be managed.

**Key words:** Degrading Bacteria, Phenol, Midok copper mine, *Pseudomonas putida*

## Highlights

- Ten phenol degrading bacteria were isolated from three sites in Midok copper mine at Shahrbabak.
- Two prevalent phenol degrading strains were identified as *Pseudomonas* and *Arthrobacter*.
- This is the first report on isolation of aromatic degrading bacteria from mine environment.

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## Introduction

The environment has been contaminated by different toxic compounds that enter into environment with variety of ways especially through industrial discharges. Last few decades, an array of foreign compounds has been introduced into the environment due to the industrial revolution. Among all those aromatic compounds, phenol and their derivatives are known as a common constituent of soil contaminated from many industries including oil refineries, pharmaceutical, petroleum, textiles and coal refining (1). Phenols are compounds with ArOH formula which are extremely toxic and found in different form or together with other elements. Simple phenol is liquid or solid with low melting point, but its boiling point is high because of hydrogen bonds. Phenol is slightly solvable in water due to its ability to make hydrogen bounds with water (9 gram in 100 ml water) (2). Several approaches are available for phenol removal such as coagulation, adsorption on activated carbon and advanced oxidation process; in comparison to these chemical approaches, biological treatment is more effective and less expensive. Currently, biodegradation of aromatic compounds received a great attention from many people from industries and researchers due to their toxicity and refractory (3). The use of microorganisms to degrade aromatic pollutants is an interesting low- cost approach for the treatment of industrial sewage disposal, contaminated sediments, soil and groundwater. Typically, biodegradation is practiced by providing favorable environmental conditions in order to stimulate the removal of pollutants (4). Many aerobic bacteria are capable of using aromatic compounds as their sole carbon source and energy. Aerobic degradation of phenol with pure cultures especially *Pseudomonas putida* has been widely studied (5). Phenol biodegradation studies with the bacterial species have resulted in bringing out the possible mechanism and also the enzyme involved in the process (6). This strain is capable of utilizing phenol as a sole carbon source. In microbial degradation of phenol under aerobic conditions, degradation is initiated by oxygenation in which the aromatic ring is initially monohydroxylated by a mono oxygenase phenol hydroxylase at an ortho position, to form catechol. This is the main intermediate stage resulting from metabolism of phenol by different microbial strains. Depending on the type of strain, the catechol then undergoes a ring cleavage which can occur either at the ortho position, initiating the ortho pathway that leads to the formation of succinate and acetyl- CoA, or at the meta position, initiating the meta pathway that leads to the formation of pyruvate and acetaldehyde (7 & 8).

The aim of the present research is isolation of microbial strains from soils of three regions of Midok copper mine from Shahrbabk province in Iran**.** Some characteristics of the isolates were determined, such as the morphology, the capability to grow and degradation of phenol, hydrophobicity and extracellular emulsifying activity. The novelty of this research is that this is the first report for isolation of phenol degrading bacterial strains from mine environment in Iran.

## Material and Method

**Sampling:** For isolation of phenol degrading bacteria wastewater and soils samples were collected from three contaminated stations in Midok copper mine at Shahrbabk (Iran): Magnetit site (WG), Hematit site (WH) and Near Hematit site (NH). The difference between these three regions is that in the Magnetit site mine wastewater was discharged but at Hematit site oil contamination was detected and Near Hematit site have metal pollution. The range of phenol contamination in these three regions is as follow: WG 580 (ppm), WH 530 (ppm) and NH 440 (ppm). Soils samples (200 g) and wastewater samples (300 ml) were taken from 1 to 10 cm below the surface of land using a sterile knife. The samples were collected into sterile jars, placed on ice, and immediately transported to the laboratory for further analysis (Fig. 1) (9).

**Enumeration of heterotrophic and phenol- degrading bacteria in collected samples:** Total heterotrophic and phenol degrading bacteria were quantified in collected samples with most probable number (MPN) and colony forming unit (CFU) methods. For enumeration of heterotrophic and phenol degrading bacteria, bacteria present in the soil or wastewater samples were serially diluted and plated (100 µl) on Nutrient Agar (NA) and Bushnell Hass agar (BH) media, respectively. All plates were incubated at 30±1°C. After two days, the numbers of colonies were counted (9). Results were expressed as colony forming units per one gram of soil (CFU $gr^{-1}$).

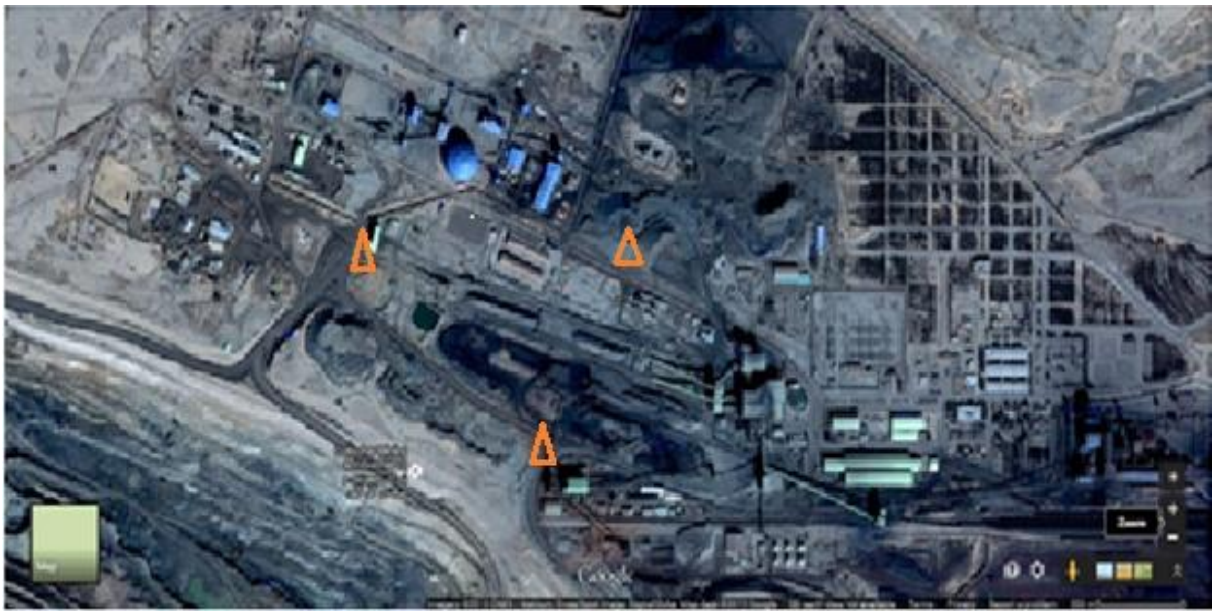

Fig. 1- Location of the sampling sites in Midok copper mine Shahrbabak- Iran (Satellite position which was taken from Google).

**Screening and isolation of phenol-degrading bacteria:** The Bushnell Hass (BH) medium with 0.02% (v/v) of phenol as sole carbon source and energy were used for isolation of phenol- degrading bacteria. BH medium contained (per liter of distilled water): 0.2 g $MgSO_4.7H2O$, 0.02 g $CaCl_2.$ $2H2O$, 1.0 g $KH_2PO_4$, 1.0 g $K_2HPO_4$, 1.0 g $NH_4NO_3$, 0.05 g $FeCl_3.$ For solid media, Bacto Agar (Difco) (15 g/l) was added to the solution (10). Portion of soils (5 g) were added to Erlenmeyer flasks containing 100 ml of medium and the flasks were incubated for 10 days at 30 ± 1ºC on rotary shaker (180 rpm, INFORS AG, Switzerland). Then 5 ml aliquots were removed to fresh medium. After a series of two further subcultures, inoculums from the flask were streaked out and phenotypically different colonies purified on BH agar medium. Phenotypically different colonies obtained from the plates were transferred to fresh medium with and without phenol to eliminate autotroph and agar- utilizing bacteria. The procedure was repeated and isolates only exhibiting pronounced growth on phenol were stored in stock media with glycerol at- 20ºC for further characterization (10).

**Identification of isolates**

**Biochemical characterization:** To identify and characterize the isolated bacteria, biochemical tests such as Gram staining, oxidation/fermentation, production of acid from carbohydrates, oxidase test, catalase test and production of gas were carried out according to the Bergey's manual to identify Bacteriology (11).

**Molecular identification:** Analysis of *16S rRNA* was performed to the taxonomic characterization of isolated strains. Total DNA extraction of bacterial strains was carried out with CTAB method (12). The bacterial *16S rRNA* loci was amplified using forward domain specific bacteria primer, Bac27- F (5′-AGAGTTTGATCCTGGCTCAG- 3′) and universal reverse primer Uni- 1492R (5′-TACGYTACCTTGTTACGACTT- 3′). The amplification reaction was performed in a total volume of 25 µl consisting, 2 mM $MgCl_2$ (1 µl), 10X PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 µl), 2 mM each dNTP (2 µl), 0.15 mM each primer (1 µl), 1U (0.5 µl) taq DNA polymerase (Qiagen, Hilden, Germany) and 2 µl of template DNA (50 p). The distill water was added for remaining of reaction (15 µl). Amplification for 35 cycles was performed in a thermal cycler Gene Amp 5700 (PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was 95°C for 5 min (1 cycle); 94°C for 1 min and 72°C for 2 min (35 cycles); and 72°C for 10 min after the final cycle (13). PCR products were sequenced by Macrogen (Korea). Similarity rank from the Ribosomal Database Project RDP and FASTA Nucleotide Database Query were used to determine partial *16S rRNA* sequences to estimate the degree of similarity to other *16S rRNA* gene sequences (14). Analysis and phylogenetic affiliates of sequences was performed as previously described protocols. A phylogenetic tree was constructed according to the neighbor-joining method with MEGA 4.1 software (15).

**Measure of growth rate and phenol degradation:** Bacterial isolates were grown at 30±1ºC for 7 days on rotator shaker (180 rpm INFORS AG, Switzerland). Growth curves of the isolates were routinely assessed indirectly by turbidity measured as optical density ($O.D_{600nm}$) in a UV–visible spectrophotometer (Shimadzu UV- 160, Japan) (16). The phenol removal assay was carried out using 2, 4- dichloro- quinon- 4- chloroimide dyes (Gibb's reagent). In this method, 150 ml medium was centrifuged (6000 g for 10 min), 30 ml $Na_2HCO_3$ and 20 ml Gibb's reagent were then added to the supernatants, and the color that developed was read at 630 nm (17).

**Measure of emulsification activity and Bacterial adhesion to hydrocarbon (BATH):** The emulsification activity ($E_{24}$) was determined by the addition of hexadecane, to the same volume of cell free culture broth, mixed with a vortex for 2 min and left to stand for 24 h. The emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm). Bacterial adhesion to hydrocarbon was performed according to the procedure explained by Pruthi and Cameotra (18).

**The effect of different concentrations of phenol on growth of selected bacterial strains**: To study the effect of different phenol concentrations on the growth of bacterial strains BH medium was supplemented with various concentrations of phenol (0.02, 0.03, 0.04, 0.05 and 0.06%). The flasks were incubated for 7 days at 30± 1°C on a rotary shaker, operating at 180 rpm (Shaker INFORS AG, Switzerland). Growth was routinely assessed indirectly by turbidity measured as optical density ($OD_{600\ nm}$) in a UV- visible spectrophotometer (Shimadzu UV- 160, Japan) (19).

## Results

**The quantity of heterotrophic and phenol degrader bacteria in collected samples:** The distribution of heterotrophic and phenol degrading bacteria were different in three collected sample zones. The results for this quantification were shown in Table (1) and (2). As shown in these tables most of the (WG) site has the highest communities of phenol degrading and heterotrophic bacteria, although less heterotrophic and phenol degrading bacteria existed in (NH) site.

Table 1- Quantity of heterotrophic and phenol degrading bacteria (MPN)

| Sample | MPN- phenol degrading bacteria | MPN- heterotrophic bacteria |
|---|---|---|
| WG | $15\times10^{4}$ | $21\times10^{11}$ |
| NH | $43\times10^{2}$ | $2\times10^{10}$ |
| WH | $15\times10^{4}$ | $11\times10^{12}$ |

Table 2- Quantity of heterotrophic and phenol degrading bacteria (CFU $gr^{-1}$)

| sample | CFU $10^{-3}$ heterotrophic bacteria | CFU $10^{-4}$ heterotrophic bacteria | CFU $10^{-5}$ heterotrophic bacteria | CFU $10^{-1}$ Phenol degrading bacteria | CFU $10^{-2}$ Phenol degrading bacteria | CFU $10^{-3}$ Phenol degrading bacteria |
|---|---|---|---|---|---|---|
| WG | $75\times10^{4}$ | $7\times10^{5}$ | $5\times10^{6}$ | $19.8\times10^{3}$ | $12\times10^{4}$ | $6\times10^{5}$ |
| NH | $6\times10^{4}$ | $2\times10^{5}$ | $1\times10^{6}$ | $1\times10^{3}$ | $5\times10^{3}$ | $3\times10^{4}$ |
| WH | $58\times10^{4}$ | $9\times10^{5}$ | $2\times10^{6}$ | $18\times10^{3}$ | $7\times10^{4}$ | $38\times10^{4}$ |

**Isolation and identification of phenol-degrading bacteria:** After two weeks of screening ten phenol- degrading bacteria were isolated from enrichment soil cultures that established at 30 ± 1ºC. The results show that all isolated bacteria can utilize initial phenol concentration (200 ppm). Between these isolates, five strains (named: P62, P63, P64, P67, and P69) have high growth rate on 400 (ppm) of phenol that were selected for further study. These strains were first classified by biochemical tests. The results of biochemical tests were shown in Table (3). As shown in this table, majority of isolates were Gram negative and all isolates did not have motility. From these isolated strains P62 and P69 had sufficient growth at high concentrations of phenol (600 ppm) and were selected as prevalent strains. Molecular identification of these two prevalent isolates was performed by amplification and sequencing the *16S rRNA* gene and comparing them to the database of known *16S rRNA* sequences. The molecular identification shows that these two isolated bacteria (strains P62 and P69) belong to *Pseudomonas putda* (P62) and the strain P69 is related to *Arthrobacter scleromae* (Fig. 2). The phylogenetic tree of these two strains were shown in (Fig. 3) and (Fig. 4).

Table 3- Chemical tests. symbol used: +: Positive response,-: Negative response

| Strain name | GRAM STAIN | CATALASE | OXIDASE | O | F | NITRATE | CITRATE | S | I | M | TSI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| P62 | _ | + | + | + | _ | + | + | _ | _ | _ | Alkali /alkali |
| P63 | _ | + | _ | _ | _ | _ | + | _ | _ | _ | acid/ alkali |
| P64 | + | + | + | _ | + | + | _ | _ | _ | _ | acid/ acid |
| P67 | _ | + | + | _ | _ | + | + | _ | _ | _ | Alkali/ alkali |
| P69 | + | _ | _ | + | + | + | + | _ | _ | _ | acid/ alkali |

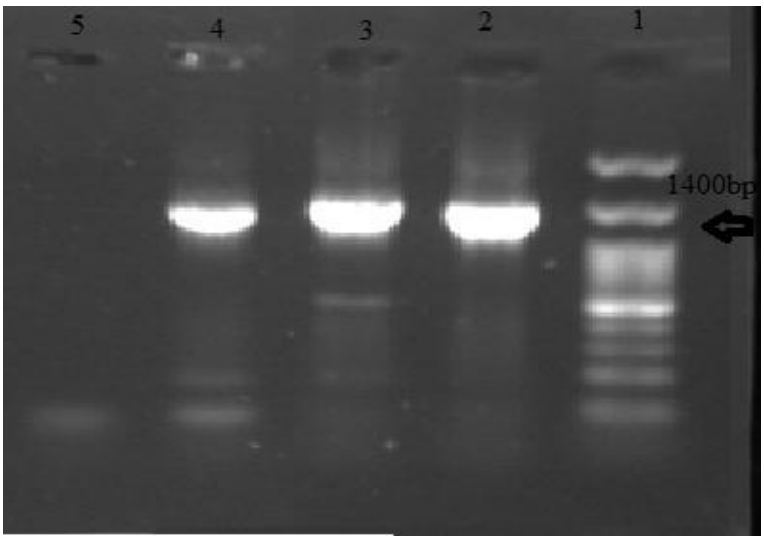


Fig. 2- Gel electrophoresis of the PCR product from Strain 62p and 69p. Lane 1 is 100 bp DNA ladder size marker; lane 2, positive control (*Pseudomonas putida* strain W30), lane3, strain 62p, lane 4, strain 69p, lane 5, negative control ($H_2O$).

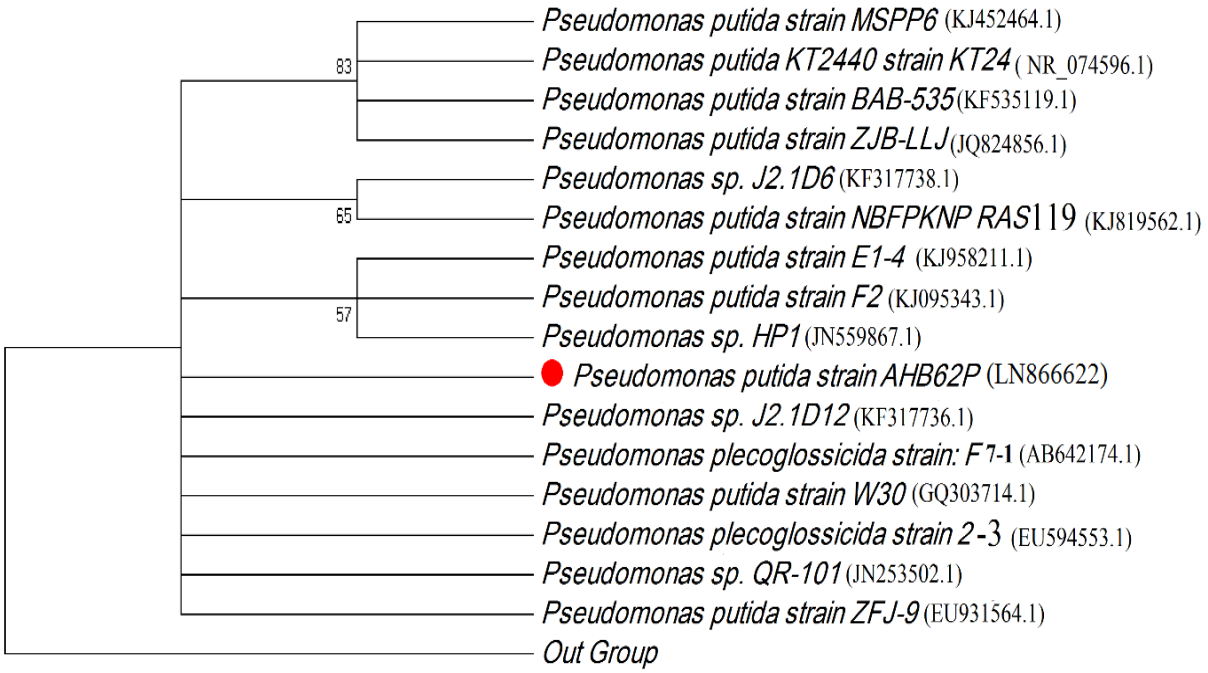


Fig. 3- Phylogenetic tree showing the inter- relationships of strain AHB62P with the most closely related type strains of the genus *Pseudomonas* inferred from sequences of *16S rRNA* gene. *Vibrio anguillarum* (X16895.1) was used as an out group. The tree was generated using the neighbor- joining method. Bootstrap values are expressed as a percentage of 1000 replications, are given at the branching point. The accession number of each strain is shown in parenthesis.

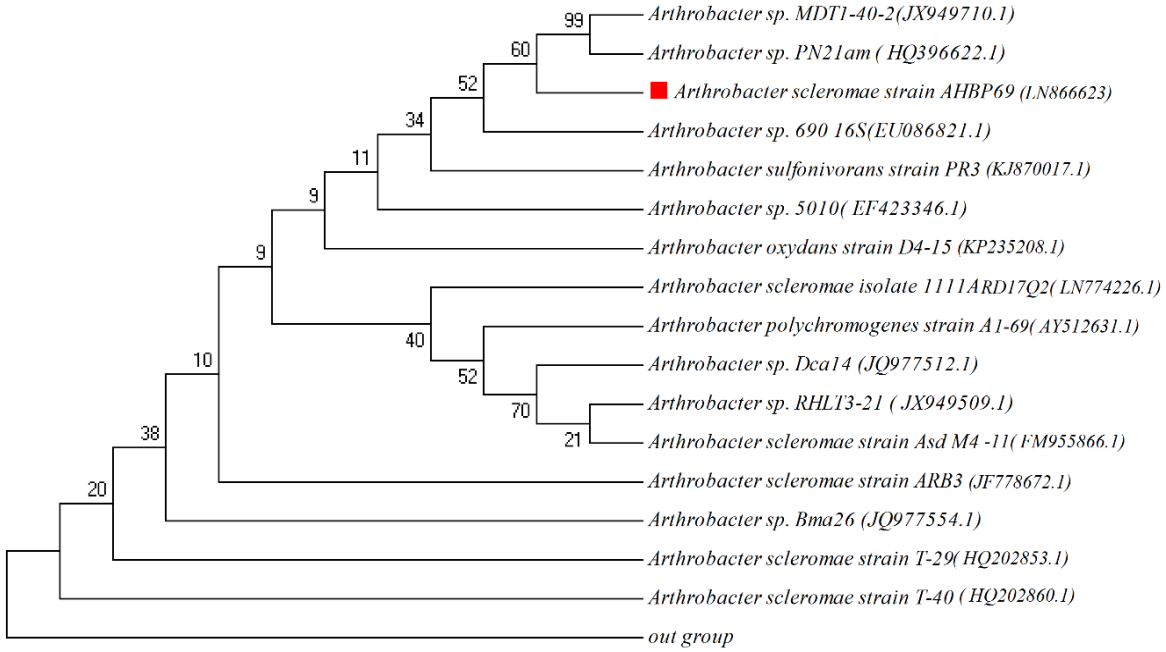


Fig. 4- Phylogenetic tree showing the inter- relationships of strain *Arthrobacter scleroma*e strain AHBP69 with the most closely related type strains of the genus Arthrobacter inferred from sequences of *16S rRNA* gene

**Growth rate and phenol degradation by isolated strains:** The results for phenol degradation and growth rate of all isolated bacteria were presented in Table (4) and Fig. (5). As shown in this figure all isolated bacteria reach 0.8 OD after 7 days and approximately degraded 50 percent of phenol in culture medium. But 3 strains have showed high growth rate and phenol biodegradation (62P, 64 P and 69P) at 500 (ppm). Finally 2 strains (62P and 69P) showed high growth rate and phenol biodegradation (95 and 98%) at (600 ppm) of phenol concentration (Fig. 6).

Table 4- Qualitative degradation, Rate of phenol removal (%) and Value of O.D600.

| Strain | Qualitative degradation | Rate of phenol removal (%) | Value of O.D600 |
|---|---|---|---|
| 62p | +++ | 48 | ·/67 |
| 63p | +++ | 52 | ·/716 |
| 64p | +++ | 95 | ·/878 |
| 67p | +++ | 56 | ·/56 |
| 69p | ++ | 56 | ·/525 |

**Emulsification activity ($E_{24\%}$) and BATH values in isolated strains:** For screening of the prevalent bacteria and elimination of weak strains these two tests were carried out. The results for these two tests were presented in Table (5). As shown in this table the highest percentage of BATH between all isolates related to 63P strain (75%) and the highest percentage of emulsification activity ($E_{24}$) attributed to 64P strain (30%).

Table 5- Emulsification activity (E24%) and Cell surface hydrophobicity (BATH%)

| Strain | Emulsification activity (E24%) | Cell surface hydrophobicity (BATH%) |
|---|---|---|
| 62p | 4.6 | 56 |
| 63p | 23 | 75 |
| 64p | 30 | 0.12 |
| 67p | 4.6 | 38 |
| 69p | 7.6 | 25 |

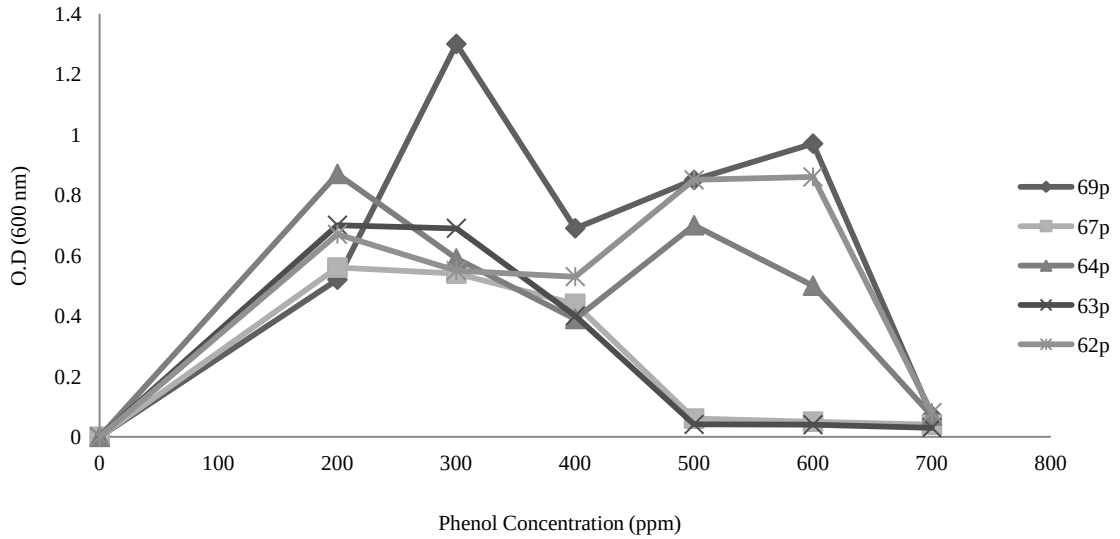


Fig. 5- growth curve bacteria strains in different concentration of phenol

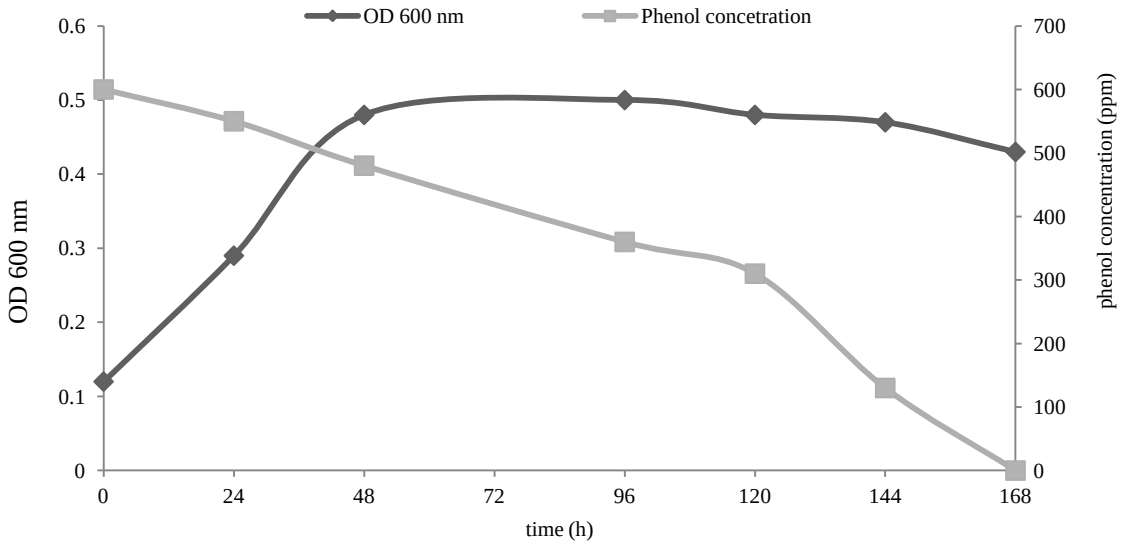


Fig. 6- Growth (as O.D. 600 nm) and phenol removal in tube culture of *Arthrobacter scleromae* strain AHB 69P at 30 C in (600 ppm) phenol concentration with gibes method during 168 h.

**The effect of different concentrations of phenol on the growth rate of selected bacterial strains**: The effect of different concentrations of phenol on the growth of selected bacterial strains show that all bacteria can utilize three concentrations of phenol (200, 300 and 400 ppm). Although, three strains showed growth and phenol biodegradation (62P, 64P and 69P) up to 500 (ppm) concentration. Finally only two strains (62P and 69P) could degrad high concentration of phenol (600 ppm) after 7 days of incubation (Fig. 6).

## Discussion and Conclusion

Industrial wastewater was selected to isolate phenol degrading bacteria due to high probability of the presence of bacteria having potentiality of degrading organic compounds (20). Isolation of phenol degrading bacteria from industrial waste/ effluent is well documented. Due to the presence of phenol in industrial wastewater, the persistent bacteria are often well adopted (21). Members of the genus *Pseudomonas* are reported for degradation of a variety of organic compounds. A lot of experiments performed previously, demonstrate members of genus *Pseudomonas* as the best degrader of phenol and phenolic compounds *Pseudomonas putida* and many other *Pseudomonas* strains are identified and characterized for phenol tolerance and degradation (20 & 21). Also phenol degrading bacteria were isolated of Siberia soils by Koutny *et al* (19). They concluded that a dominant species in phenol degradation is *Pseudomonas* particularly *putida* in these soils (21). Phenol concentrations of 300 (ppm) and 200 (ppm) were applied to isolate phenol degrading bacteria respectively by Wantanabe *et al*. and Whitely *et al* (22 & 23). Emtiazi et al isolated Twenty- five aerobic phenol-degrading bacteria from environmental

samples were collected from various sites in Isfahan can degrade phenol at 200 (ppm) concentration. Also *Pseudomonas putida* strains C5 and D6 showed maximum growth (as O.D. at 600 nm) (24).

The wide varieties of microorganisms that can aerobically degrade phenol include pure bacterial cultures such as: *Arthrobacte*r, *Pseudomonas fluorescens* (25- 27). Zheng et al reported that the microbial strain *Pseudomonas aeruginosa* HSD38 is able to degrade up to 500 (ppm) of phenol below the detection level but unable to tolerate more than 700 (ppm) of initial concentration of phenol (28). Gonzales et al showed a degradation capacity of more than 90% of 500 (ppm) phenol by *Pseudomonas putida* ATCC 17484 (29). Koutnay et al isolated some phenol- degrading bacteria and also identified them as genus *Pseudomonas* sp (30). Annadurai et al reported that *Pseudomonas pictorum* immobilized on activated charcoal is an excellent possibility for the reduction of treatment cost of phenol. They reported that the immobilized system is able to degrade up to 99% of initial concentration of phenol till 600 (ppm) (31). Karigar et al studied ability of *Arthrobacter citreus*, isolated from a hydrocarbon contaminated site, to consume phenol as the sole carbon source (32). Yang and Lee reported that the microbial strain *Pseudomonas resinovorans* isolated and studied in their investigation was unable to tolerate an initial concentration of phenol more than 600 (ppm) (33). Agarry et al studied the bioremediation potential of an indigenous *Pseudomonas fluorescence* in batch culture using synthetic phenol in water in the concentration range of (100 – 500) (ppm) as a model limiting substrate. The effect of initial phenol concentration on the degradation process was investigated. Phenol was completely degraded at different cultivation times for the different initial phenol concentrations. Increasing the initial phenol concentration from 100 mg/L to 500 mg/L increased the lag phase from 0 to 66 hours and correspondingly prolonged the degradation process from 84 hours to 354 hours (34). Vidyavathi *et al* reported phenol degradation by *Nocardia* that resulted in complete degradation of phenol (100 ppm) within 96 hours. The bioremediation potential of an indigenous *Pseudomonas fluorescence* was studied in batch culture using synthetic phenol in water in concentration range of (100- 500) mg/L as a model limiting substrate (35). Li et al. investigated the growth kinetics of a psychrotroph, *Pseudomonas putida LY1*, while growing on phenol as a sole carbon and energy source. This bacterium could completely biodegrade 200 mg/L phenol across a temperature range from 2.5 to 35$^{o}$C, with an optimum temperature of 25$^{o}$C (36). In the current study among the isolates, *Pseudomonas putida* strain AHB62P and *Arthrobacter scleromae* strain 69P were found to be more efficient could degrade phenol up to 500 (ppm) and 600 (ppm), respectively. This isolation can remove phenol completely after 168 hours incubation. Phenol at high concentration is toxic to microorganisms, plants and animals including human beings (37). In

spite of the physical and chemical methods in use, the microbial degradation of toxic xenobiotics is gaining importance (38). Naturally occurring 62P and 69P are found to be well suited to phenol degradation process. Therefore, the phenol degradability of the isolates can be maintained and used at large scale treatments. This research is the first report for isolation of phenol degrading bacterial strains from mine environment in Iran, and the results of this research show that different mines in Iran have good potentiality for isolation of pollutant degrading bacteria.

In this research, 5 various colonies were isolated based on specific morphologic characteristics in order to study phenol degradation and 2 various colonies were selected for molecular detection. 2 bacteria were effective degradation for (600 ppm) phenol concentration belong *Pseudomonas putida*strainAHB62P and *Arthrobacter scleromae* strain AHB69P. In this study two was identified as an efficient strain for phenol biodegradation. This strain showed a good capacity for adaptation with increased concentrations of phenol.

**Acknowledgement**

This work was financially supported by Shahid Bahonar University of Kerman. All authors had equal role in design, work, statistical analysis and manuscript writing. The authors declare no conflict of interest.

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# جداسازی و شناسایی باکتری‌های تجزیه کننده فنل از معدن مس میدوک شهربابک

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## چکیده

فنل ساده‌ترین ترکیبات آروماتیکی است که دارای ویژگی جهش‌زایی بوده و از مهم‌ترین راه‌های ورود این ترکیبات به خاک پساب کارخانجات، صنایع و معادن‌است. باکتری‌ها و قارچ‌ها قادر به تجزیه این ترکیبات به عنوان منبع کربن وانرژی هستند. باکتری‌ها با داشتن آنزیم‌های منواکسیژناز و دی اکسیژناز این ترکیبات را به مشتقات دی هیدرودی ال وکاتکول تبدیل می‌کنند. اکسیداسیون بعدی این مواد باعث تبدیل آن‌ها به دی اکسیدکربن و آب می‌شود. در این پژوهش، از سه نقطه از معدن مس میدوک شهربابک نمونه‌برداری و ۱۰ سویه باکتری جداسازی شد که توانایی رشد در غلظت (۲۰۰ppm) را داشتند. با افزایش غلظت ۵ سویه توانایی رشد را داشتند که آزمون‌های بیوشیمیایی روی آن‌ها انجام شد. در نهایت، دو سویه توانایی تجزیه فنل را در غلظت (۶۰۰ppm) داشتند که برای شناسایی مولکولی انتخاب شدند. این سویه‌ها *Pseudomonas putida strain* AHB62P و *Arthrobacter scleromae* strain AHB 69P بودند.

**واژه‌های کلیدی:** باکتری تجزیه کننده، فنل، معدن مس میدوک

* نویسنده مسؤول مکاتبات
تاریخ دریافت: ۱۳۹۴/۰۳/۱۲- تاریخ پذیرش: ۱۳۹۴/۰۶/۰۴