Journal of Petroleum Science and Technology, Vol. 2, No. 2, 2012, 58-62

Research Note

Biodegradation of Phenol by Newly Isolated Phenol-degrading Bacterium *Ralstonia* sp. Strain PH-S1

A. Tabib, A. Haddadi*, M. Shavandi, M. Soleimani, and P. Azizinikoo
Department of Biology, Faculty of Science, Karaj Branch, Islamic Azad University
haddadi@kiau.ac.ir

Abstract
A newly phenol-degrading bacterium, identified as Ralstonia sp. strain PH-S1, was isolated from oil-contaminated soil in Khark Island. It was isolated by a multistep enrichment and screening technique on mineral medium (MM) containing 100 mg.l^{-1} of phenol as the sole source of carbon. The bacterium was able to degrade up to 1100 mg.l-1 of phenol but the cell growth decreased with higher concentrations of phenol. The PH-S1 strain grew well in the pH range of 4 to 9 and in the temperature range of 30 to 40 °C. Different concentrations of NaCl ranging from 10 to 20% on the growth of bacteria was studied and it was found that this strain was able to grow well in 10% NaCl; but, higher concentrations of NaCl decreased the growth of the strain.
The laboratory scale results indicated the potential application of the strain in the treatment of low saline industrial wastewaters. However, further investigations are required to confirm the ability of the strain.

Keywords: Isolation, Ralstonia , Phenolic Compounds, Industrial Wastewaters

Introduction
Aromatic compounds are among the most prevailing and constant pollutants in the environment [1]. One of the pollutants in the wastewater of chemical and petroleum industries which is highly toxic to living organisms is phenol. Phenol is toxic upon ingestion, contact, or inhalation and is lethal to living organisms even at a concentration of as low as 5 mg.l^{-1} [2, 3]. As it is fatal to fishes at 0.1 ppm [4] and can create serious problem to human beings and animals through food chain, its removal from industrial wastewater is necessary and the degradation of such hazardous compounds has thus far been a challenging task for the pollution scientists. 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 [5].

Saline wastewaters created by many industrial processes raised serious concerns about treatment of these hazardous wastewaters [6, 7]. Organic compounds of high-salinity industrial wastewaters can be removed by using halophilic bacterial cultures without need to first decrease the salt concentration.

Typical nonhalophilic bacteria grow well in media containing NaCl concentrations below 1% w/v (10 g.l^{-1}). Bacteria with growth optima in NaCl concentrations in excess of 1% are defined as halophilic, and are further categorized into three groups according to the salinity of their growth optima: slightly halophilic (1–3% w/v), moderately halophilic (3–15% w/v), or extremely halophilic (>15% w/v) [8].

Many phenol biodegradation studies involve using nonhalophilic species [9–11], while capabilities of halophilic phenol degrading bacteria are less investigated.
The present paper deals with the degradation of phenol by a newly isolated moderately halophilic bacterium isolated from soils of Khark Island in the south of Iran.

Materials and Methods
Microorganisms Isolation and Growth Conditions
Khark Island located in south of Iran. The samples were transferred into 100 ml sterile mineral medium (MM) containing 100 mg.l^{-1} of phenol as the sole source

of carbon and energy. The composition of MM was K_2HPO_4=1.5 g, KH_2PO_4=0.5 g, NaCl=5.0 g, NH_4Cl=3.0 g, $MgSO_4$=0.3 g, $CaCl_2$= 0.01 g, and $FeCl_3$=0.01 g in 1000 ml of distilled water. The pH was adjusted to 6.8±0.2. The flasks were incubated at 30 °C with shaking at 110 rpm in the dark in order to avoid photo-destruction of phenol. Growth was determined as absorbance at 600 nm.

After four transfers, serial dilutions of the cultures were prepared and spread on nutrient agar. The plates were incubated at 30 °C for 3 days. Colonies were isolated and purified from this medium. To verify the phenol degradation activity of these isolates, they were again inoculated in MM containing 100 mg.l^{-1} of phenol and absorbance at 600 nm was determined one more time.

Growth of bacteria was determined at different temperatures ranging from 30 to 40 °C and at different pH values ranging from 4 to 9 in the presence of 100 mg.l^{-1} phenol.

Effect of Different Phenol Concentrations on the Growth of the Culture

Isolated strains were tested for their ability to tolerate different concentrations of phenol in a mineral medium (MM) supplemented with phenol at a concentration of 200, 300, 500, 700, 900, 1100, and 1200 mg.l^{-1} at 30 °C with shaking at 110 rpm in the dark. Inoculum was phenol-adapted cells grown in the presence of 100 mg.l^{-1} phenol. Growth was determined as absorbance at 600 nm.

Ability of the bacteria to use various aromatic compounds as sole source of carbon were tested with MM containing 100 mg.l^{-1} of the m-cresol, 3-nitro phenol, and 4-chloro phenol. Growth was determined as absorbance at 600 nm.

Measurement of Phenol Residual Concentrations

The residual phenol concentration was measured at every 24-hrs interval by 4-aminoantipyrine spectrophotometric method [12].

Effect of Different NaCl Concentrations on the Growth of Bacteria

Effect of different concentrations of NaCl ranging from 10 to 20% on the growth of bacteria was surveyed.

16S rDNA Sequence Analysis

After the partially characterization of isolated strain based on the morphological and physiological characteristics and biochemical tests (according to Bergey's manual) the genomic DNA was extracted using a phenol-chloroform extraction protocol [13]. The 16S rRNA gene was amplified using universal primers including forward primer, i.e. 9F (GAGTTTGATYMTGGCTCAG) and reverse primer, i.e. 1541R (AAGGAGGTGWTCCARCC), and PCR product was sequenced. Nucleotide sequences of each 16S rRNA gene were aligned using respective sequences of allied reference strains.

Results and Discussion

Isolation of Microorganisms

After extensive screening procedure, seven morphologically different bacterial strains were isolated; but, among all of the tested strains 2 isolates were able to efficiently degrade phenol as the sole source of carbon and energy (PH-S1 and PH-S2). As demonstrated in Figure 1 the strain PH-S1 showed better growth characteristics.

On nutrient agar plate strain PH-S1 showed beige, smooth, glistening with entire margin, dome-shaped, mucus colonies, while the colonies of strain PH-S2 were white, rough, non-spreading, and dome-shaped.

The PH-S1 strain grew well in the pH range of 4 to 9 and in the temperature range of 30 to40 °C.

Phenol Biodegradation

Strain PH-S1 was incubated in MM along with 200 to 1200 mg.l^{-1} phenol as the sole source of carbon to identify the tolerance limit of bacteria to phenol. As shown in Figure 2, the PH-S1 strain was able to degrade phenol up to a concentration of 1100 mg.l^{-1} in 6 days at 0.5% NaCl. While the phenol concentration exceeded 1100 mg.l^{-1}, growth of the bacteria was decreased and then stopped. These results are similar to that obtained by Nilotpala et al.; they isolated a serratia strain that was able to degrade phenol up to a concentration of 1050 mg.l^{-1} but in 11 days and in the presence of 0.05 % NaCl.

Growth curve and phenol biodegradation curve of PH-S1 strain in a medium containing 100 mg.l^{-1} phenol is illustrated in Figure 3. The growth of the strain was started after a short delay and after proceeding for several hours the bacteria entered the log phase. The highest growth occurred 72 hours after inoculation and right after it stationary phase began. As the results indicated, in spite of little or no loss of phenol during the lag phase, a rapid biodegradation of phenol was seen with an increase in the biomass.

The strain was also able to decompose 100 mg.l^{-1} of the m-cresol but it could not utilize 3-nitro phenol and 4-chloro phenol as the sole source of carbon and energy.

Study of Bacterial Growth in the Presence of the Different Concentrations of NaCl

Growth of PH-S1 strain in NaCl concentrations ranging from 10 to 20% was studied. As shown in Figure 4, this strain can grow well in 10% NaCl but a higher concentration of NaCl decreases its growth. These finding are comparable to that of Bernet et al.; they reported five bacterial cultures which were capable of biodegrading phenol from 50 mg.l^{-1} to below the detection limit of 2 mg.l^{-1} in the presence of 10% NaCl. One of these cultures was able to degrade phenol at an initial concentration of up to 320 mg.l^{-1} [8].

As some industrial wastewaters have higher concentrations of salts, the treatment of saline wastewaters created by many processes is of environmental concerns and while desalination by chemical and physical methods are expensive, microbial biodegradation can be an effective and economically viable method for the treatment of these contaminants. According to Bernet et al. [8], bacteria with growth optima in 3-15% w/v NaCl concentrations are defined as moderately halophilic; so the PH-S1 strain, as a moderately halophilic phenol-degrading bacterium, can be a good candidate for the treatment of low saline wastewaters.

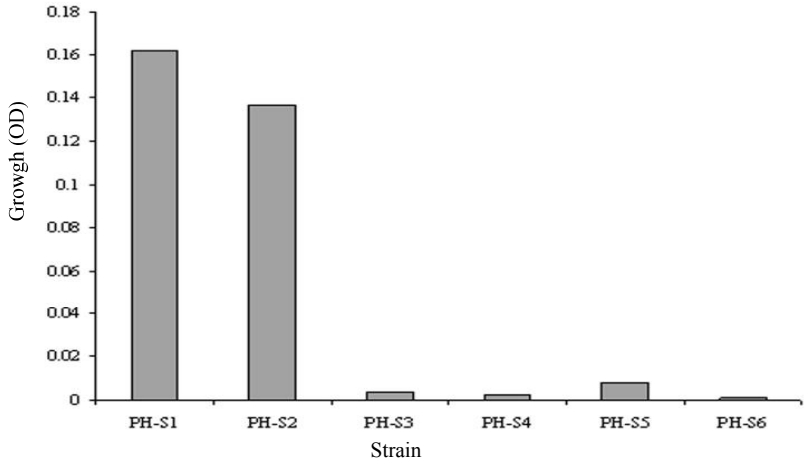

Figure 1: Growth of Ralstonia sp. strain PH-S1 at a phenol concentration of 100 mg.l^{-1}

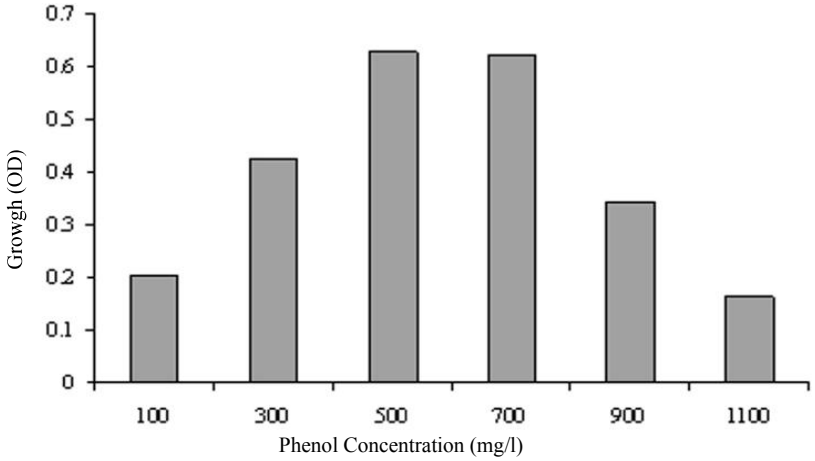

Figure 2: Growth of Ralstonia sp. strain PH-S1 at different concentrations of phenol

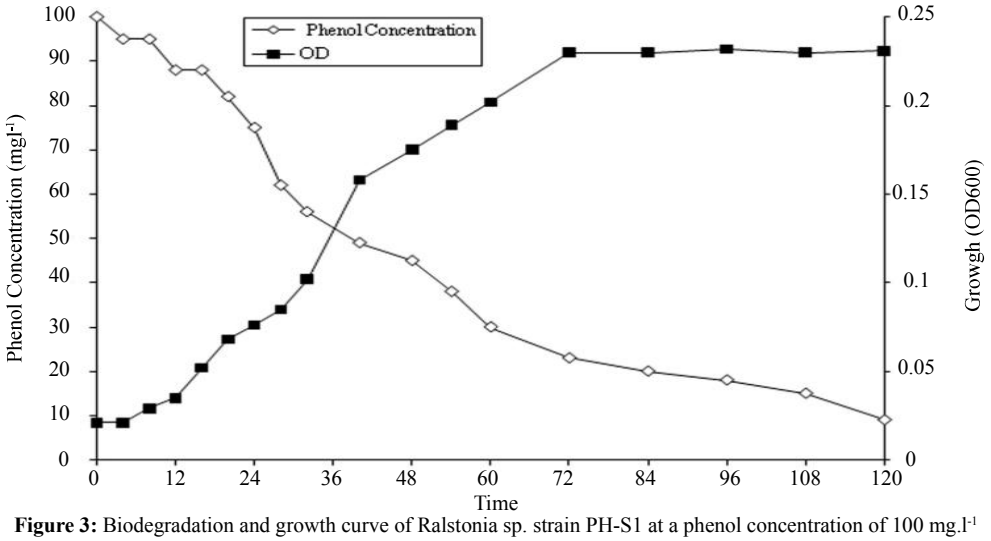

Figure 3: Biodegradation and growth curve of Ralstonia sp. strain PH-S1 at a phenol concentration of 100 mg.l^{-1}

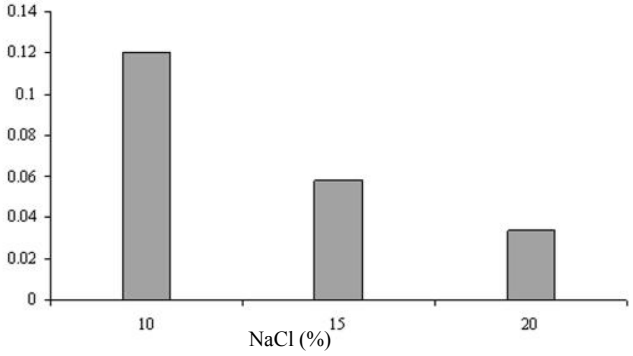

Figure 4: Growth of ralstonia sp. strain PH-S1 at different concentrations of NaCl

Phylogeny Analysis of 16S rRNA Gene

Sequencing of 16S rDNA genes has been very useful for the recognition of isolated bacteria. To identify PH-S1 isolate, gene encoding 16S rRNA were amplified by PCR and sequenced as described above. We found that PH-S1 isolate was classified in the Ralstonia genera; blast analysis demonstrate that the similarity between 16S rDNA sequences of PH-S1 and uncultured Ralstonia sp. clone S-34, Ralstonia pickettii strain TA, and Ralstonia sp. M22 were 99%, 99%, and 98% respectively. So the bacterium was identified as Ralstonia sp. strain PH-S1 using 16S rDNA sequence analysis. The sequence of the 16s rRNA gene of the strain has been submitted to genbank under accession number of JN543508.

Conclusion

The identification of bacterial genera capable of degrading phenolic compounds demonstrated the dominance of genus Pesudomonas, which is may be because of its spread distribution in soils. Other genera of bacteria including Agrobacterium, Burkholderia, Acinetobacter, Ralstonia, Klebsiella, Bacillus, and Rhodococcus were illustrated as the degraders of phenolic compounds [14]. In this study, a slightly holophilic bacteria was isolated from the oil-contaminated soil of Khark Island. We concluded that PH-S1 strain could degrade phenol up to a concentration of 1100 $mg.l^{-1}$. Higher initial concentrations of phenol had an inhibitory effect on the growth of bacteria. It was also able to degrade m-cresols, but it could not degrade nitro- and chloro- phenols. This bacterium grew well in the pH range of 4 to 9 and in the temperature range of 30 to 40 °C. The bacterium was identified as Ralstonia sp. strain PH-S1 by the phylogeny analysis of 16S rRNA gene. The ability of the strain to degrade concentrations of phenol as high as 1100 $mg.l^{-1}$, to withstand a wide pH range, and to degrade cresol as a second prevailing phenolic contaminant and having slight halophilic characteristics make it a good candidate for the bio-treatment of industrial effluents containing phenol.

Acknowledgements

We are thankful to Mr. Bakhtiari and Miss Asadi for their helpful suggestions.

References

[1]. Jong Su S., Young Soo K., and Qing X., "*Bacterial Degradation of Aromatic Compounds*", Int. J. Environ. Res. Public Health, Vol. 6, pp. 278- 309, 2009.

[2]. Alon N., Ron A., Eyal Z., Stefan R., and Charles L., "*Phenol Biodegradation by Corynebacterium Glutamicum Encapsulated in Electrospun Fibers",* Journal of environmental protection, Vol.3, pp.164-168, 2012.

[3]. Kumaran P., and Paruchuri Y., "*Kinetics of Phenol Biotransformation*", Water Research, Vol.31, pp. 11-22, 1996.

[4]. Nuhoglu N., and Yalcin B., "*Modeling of Phenol Removal in a Batch Reactor"*, Biochemistry, Vol.40, pp.1233-1239, 2005.

[5]. Gurusamy A., Lai Yi. L., and Jiunn-Fwu L., "*Biodegradation of Phenol by Pseudomonas Pictorum on immobilized with Chitin*", African Journal of Biotechnology, Vol.6, No.3, 296-303, 2007.

[6]. Mahiuddin M., Fakhroddin A., and Al-Mahin A., "*Degradation of Phenol via Meta Cleavage Pathway by Pseudomonas fluorescens PU1*", Microbiology, Vol.56, pp.1-6, 2012.

[7]. Saravanan P., Pakshirajan K., and Prabirkumar S., "*Biodegradation of Phenol and M-Cresol in a Batch and Fed Batch Operated Internal Loop Airlift Bioreactor by Indigenous Mixed Microbial Culture Predominantly Pseudomonas sp.*", Bioresource Technology, Vol.99, pp.8553–8558, 2008.

[8]. Butani N., Parekh H., and Salyia V., "*Biodegradation of Phenol by a Bacterial Strain Isolated from a Phenol Contaminated Site in India*", Research Journal of Environmental sciences, Vol.1, pp.46-49, 2012.

[9]. Ventosa A., and Nieto JJ., "*Biotechnological Applications and Potentialities of Halophilic Microorganisms*", World J Microbiol Biotechnol., Vol.11, pp.85–94, 1995.

[10]. Woolard C., and Irvine R., "*Treatment of Hypersaline Wastewater in the Sequencing Batch Reactor*", Water Reserch, Vol.29, No.4, pp.1159–68, 1995.

[11]. Brent M, Tomas W., and David R., "*Kinetics of*

Phenol Biodegradation in High Salt Solutions", Water Research, Vol.36, pp.4811–4820, 2002.
[12]. Ellis T., Smets B., and Grady J., "*Effect of Simultaneous Biodegradation of Multiple Substrates on the Extant Biodegradation Kinetics of Individual Substrates*", Water Environment Reserch, Vol.70, No.1, pp.27–38, 1998.
[13]. Kumaran P., and Paruchuri Y., "*Kinetic of phenol biotransformation*", Water Reserch, Vol.31, No.1, pp.11–22, 1997.
[14]. Leonard D., Youssef B., Destruhaut C., Lindley ND., and Queinnec I., "*Phenol Degradation by Ralstonia Eutropha: Colormetric Determination of 2-Hydroxy-Muconate Semialdehyde Accumulation to Control Feed Strategy in Fedbatch Fermentations*", Biotechnology and Bioengineering, Vol.65, No.4, pp.407–15, 1999.
[15]. Eaton A., Clesceri L., and Greenberg A., "*Standard Methods for the Examination of Water and Wastewater*", 19th Ed., American Public Health Association Inc., 1995.
[16]. Saldana G., Martinez-Alcantara V., Vinardell J., Bellogin R., Ruiz- Sainz J., and Balatti P., "*Genetic Diversity of Fast-Growing Rhizobia that Nodulate Soybean*", Arch. Microbiol., Vol.180, pp.45–52, 2003.
[17]. Pradhan N., and Ingle A.O., "*Mineralization of Phenol by a Serratia plymuthica Strain GC Isolated from Sludge Sample*", International Biodeterioration and Biodegradation, Vol.60, pp.103-108, 2007.
[18]. Gehong W., Jianfu Yu., Yuhua Zh., Weimin C., and Wang L., "*Characterization of Phenol Degradation by Rhizobium sp.CCNWTB 701 Isolated from Astragalus Chrysopteru in Mining Tailing Region*", Journal of Hazardous Materials, Vol.151, pp.111–117, 2008.