ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/299076859

Detection Of Phenol Degrading Bacteria And Pseudomonasputida In Activated Sludge By Polymerase Chain Reaction

Article *in* Iranian Journal of Environmental Health Science & Engineering · January 2009

CITATIONS
22

READS
78

4 authors, including:

Hossein Movahedian Attar
Isfahan University of Medical Sciences
62 PUBLICATIONS **642** CITATIONS

SEE PROFILE

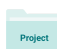
Hassan Khorsandi
Urmia University of Medical Sciences
23 PUBLICATIONS **115** CITATIONS

SEE PROFILE

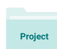
Rasoul - Salehi
Isfahan University of Medical Sciences
175 PUBLICATIONS **1,203** CITATIONS

SEE PROFILE

Some of the authors of this publication are also working on these related projects:



Gene Therapy and Cell therapy for melanoma View project



Evaluation of miR expression in diabetes View project

All content following this page was uploaded by Hossein Movahedian Attar on 05 June 2014.

The user has requested enhancement of the downloaded file.

DETECTION OF PHENOL DEGRADING BACTERIA AND *PSEUDOMONAS PUTIDA* IN ACTIVATED SLUDGE BY POLYMERASE CHAIN REACTION

[1]H. Movahedyan, [*2]H. Khorsandi, [3]R. Salehi, [1]M. Nikaeen

1-Department of Environmental health, Isfahan University of Medical Sciences, Isfahan, Iran
2-Department of Environmental health, Urmia University of Medical Sciences, Urmia, Iran
3-Department of Genetic and Molecular Biology, Isfahan University of Medical Sciences, Isfahan, Iran

Received 6 July 2008; revised 16 May 2009; accepted 30 May 2009

ABSTRACT

Phenol is one of the organic pollutants in various industrial wastewaters especially petrochemical and oil refining. Biological treatment is one of the considerable choices for removing of phenol present in these wastewaters. Identification of effective microbial species is considered as one of the important priorities for production of the biomass in order to achieve desirable kinetic of biological reactions. Basic purpose of this research is identification of phenol-degrading *Pseudomonas Putida* in activated sludge by polymerase chain reaction (PCR) that has high speed and specificity. In this research, 10 various colonies of phenol-degrading bacteria were isolated from municipal activated sludge and the rate of phenol removal and growth rate of these bacteria were assessed in different concentrations of phenol (200 – 900 mg/L). Confirmation of the largest subunit of multicomponent phenol hydroxylase (LmPH) gene and gene coding the N fragment in *Pseudomonas Putida*-derived methyl phenol operon (DmpN gene) through PCR were used for general identification of phenol-degrading bacteria and *Pseudomonas Putida,* respectively. Presence of a 600 bp (base pairs) bond in all of isolated strains indicated that they contain phenol hydroxylase gene. 6 of 10 isolated bacteria were *Pseudomonas Putida* because they produced a 199 bp PCR product by DmpN primers. According to PCR results in this study, the best phenol-degrading bacteria that can utilize 500 – 600 mg/L phenol completely after 48 hours incubation, belong to *Pseudomonas Putida* strains. It is clear that use of isolated bacteria can lead to considerable decrease of treatment time as well as promotion of phenol removal rate.

Keywords: Phenol-Degrading Bacteria, Polymerase Chain Reaction, Wastewater, *Pseudomonas Putida*, Activated sludge

INTRODUCTION

Phenol is an aromatic pollutant, which is present in the wastewater of numerous industries including oil refining, petrochemical, plastic manufacturing, dyestuff, pharmaceutical, production of resin and coke (Prpich and Daugulis, 2005; Gerrard *et al*., 2006; Juang and Tsai, 2006). Although the concentration of phenol in wastewaters varies from 10 to 3000 mg/L (Annadurai *et al*., 2000), effluent standard of phenolic wastewaters is 1 mg/L for discharging to surface waters (Department of the Environment of IRAN, 1994). Thus treatment of phenolic wastewaters is necessary and biological methods are the most appropriate techniques due to mineralization of toxic organic compounds and inexpensiveness (Prpich and Daugulis, 2005). Identification of effective microbial species is considered as one of the important priorities for production of the optimum conditions of biomass in order to achieve desirable kinetic of biological reactions (Noworyta *et al*., 2006). Conventional methods for complete identification of microorganisms in environmental samples are based on culture and differential biochemical and serological tests that are time-consuming and have relatively low sensitivity.

Polymerase Chain Reaction or PCR is a powerful method that allows the species specific detection

Corresponding author: *hassankhorsandi@yahoo.com*
Tel: +98 311 7922667

of organisms in environmental samples based on amplification of DNA specific fragment (Burtscher and Wurtz, 2003). Required time for total PCR steps (DNA extraction, PCR protocol and electrophoresis) is one day (Sambrook and Russell, 2001) 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 (Watanabe *et al*., 1998)

Pseudomonas Putida (P.Putida), a gram–negative, polar flagellated unicellular bacterium, is known to be capable of using aromatic compounds such as phenol as a sole source of carbon and energy. Optimum microbial growth conditions are 30 ºC and pH=6.8 (Seker *et al*., 1997).

Aerobic biodegradation of phenol starts with oxygenation of phenol by phenol hydroxylase enzymes and the genes coding of these enzymes are used for detection of phenol-degrading bacteria by PCR (Selvaratnam *et al*., 1995; Paula *et al*., 2000; Futamata *et al*., 2001).

Since *Pseudomonas Putida* can catalyze recalcitrant organic compounds such as phenol and detection of this bacterium was not carried out by PCR in Iran, in this research detection of *P.Putida* was studied in activated sludge samples of south Isfahan wastewater treatment plant. Besides, the degradation rate of phenol by *P.Putida* was studied.

MATERIALS AND METHODS

Growth media

A basic mineral salts medium (MS medium) was used for the isolation of the organisms. Bacteria were grown at pH=7 on a MS medium containing (per liter) 2.75 g K_2HPO_4, 2.25 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgCl_2.6H_2O$, 0.1 g NaCl, 0.02 g $FeCl_3.6H_2O$ and 0.01 g $CaCl_2$ (Watanabe *et al*., 1998).

Isolation of strains

Phenol–utilizing bacteria were isolated from samples taken from south Isfahan wastewater treatment plant. Samples (10 mL) were inoculated to 250 mL flask containing 100 mL of MS medium supplemented with 200 mg/L phenol. Enrichments were incubated with shaking at 30 ºC and 170 rpm for one week. In the second step, 1 mL of first flask was added to a new flask containing 100 mL of MS medium and incubated in the same condition. In the third step, enriched sample was plated onto solid agar MS/phenol media and then every specific colony was inoculated separately onto nutrient broth and nutrient agar. In this manner pure cultures of phenol–degrading bacteria were isolated ; also, one colony from every plate was taken for bacteria morphology, a number of colonies were used for DNA extraction and one colony was inoculated to MS/phenol media for metabolism study of phenol-degrading bacteria (Saiqa et al., 1998).

Removal of phenol and growth rate of phenol-degrading bacteria

2.5 mL pure culture of phenol-degrading bacteria was added to 10 mg/L MS media in an octal set with increasing concentration of phenol (200 - 900 mg/L) separately. MS/phenol media (without bacteria) and MS/media containing bacteria (without phenol) were also studied. Required samples of the growing cultures were recovered aseptically at 24 hours and 48 hours after inoculation, and then phenol was determined quantitatively by the spectrophotometric method using 4-aminoantipyrine as the color reagent (APHA, 1995) and optical density at 600 nm (OD_{600}) as bacteria growth rate was read by spectrophotometer (Saiqa et al., 1998).

DNA extraction

DNA extraction from the colony of phenol-degrading bacteria was carried out with colony PCR method (Fukui and Sawabe, 2007). The quality and quantity of the extracted DNA were checked by measuring the UV absorption spectrum (Sambrook and Russell, 2001).

PCR conditions

In this study, for general detection of phenol-degrading bacteria based on gene coding LmPH (largest subunit of multi component phenol hydroxylase) and *P. Putida* based on gene coding DmpN (gene coding the N fragment in Pseudomonas Putida-derived methyl phenol operon), DNA templates were amplified by using the Epigradient Thermal Cycler and 25 µL mixtures described in Table 1.

Table 1: PCR reaction mixtures

Constituent		Initial Concentration	Volume (µL) for		Concentration in PCR mixture
			general detection	P.Putida detection	
10x PCR Buffer		200mM Tris-HCl 500mM KCl	2.5	2.5	20mM Tris-HCl 50mM KCl
$MgCl_2$		50mM	1	1	2mM $MgCl_2$
Taq DNA Polymerase		5U/µl	0.2	0.2	1 Unit
dNTP(Mix)		10mM	0.5	0.5	200 µM each dNTP
Primers	Forward	$^{Pmol}/_{\mu L}$ 10	0.3	0.4	0.12µM for general detection 0.16 µM for P.Putida detection
	Reverse	$^{Pmol}/_{\mu L}$ 10	0.3	0.4	0.12µM for general detection 0.16 µM for P.Putida detection
Template DNA		Variable	1	2	Variable
D.D.water (sterile)		-	19.2	18	-
Final volume PCR mixtures			µL 25	µL 25	-

PheU (Phenol hydroxylase encoding gene) primers were used for general (Universal) detection, that PCR product size is 600 bp and nucleotide sequence of these primers is as follows (Futamata et al., 2001):
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´}
DmpN primers with a product size of 199 bp were used for detection of *P. Putida*. Nucleotide sequence of these primers is as follows (Selvaratnam et al., 1995)
DmpNf:{5´- ATCACCGACTGGGACAAGTGGGAAGACC -3´}
DmpNr: {5´-TGGTATTCCAGCGGTGAAACGGCGG-3´}
The PCR conditions used for the general detection (Futamata *et al*., 2001) and detection of *P. Putida* (Selvaratnam *et al*., 1995) have been described in Tables 2 and 3.
Accuracy and precision tests of PCR were carried out by positive (*P. Putida* ATCC 11172) and negative controls.

Table 2: PCR Condition For universal detection of phenol-degrading bacteria (Futamata et al., 2001)

Steps	Sub-step	Time (min)	Temperature(ºC)
Initial Denaturation	-	10	94
Step 1 (5cycles)	Denaturation	1	94
	Annealing	1	58
	Extension	1	72
Step 2 (5cycles)	Denaturation	1	94
	Annealing	1	57
	Extension	1	72
Step 3 (25cycles)	Denaturation	1	94
	Annealing	1	56
	Extension	1	72
Final Extension	-	10	72

Table3: PCR Condition for *P.Putida* detection (Selvaratnam et al.,1995)

Steps	Sub-step	Time (min)	Temperature ºC
Initial Denaturation	-	2	94
Step 1 (40cycles)	Denaturation	1	94
	Annealing	1	50
	Extension	1	72
Final Extension	-	5	72

RESULTS

In this research, 10 various colonies were isolated based on specific morphologic characteristics in order to study phenol degradation and molecular detection. 60 percent of colonies were negative gram Bacilli with deferent colony color and shape; however 20 percent of those were positive gram Bacilli and reminder of colonies were negative gram Cocci.

Results of amplified template DNA for general detection of phenol–degrading bacteria by pheU primers are shown in Fig. 1. Presence of a 600 bp bond in amplified DNA indicated that all of isolated bacteria are phenol- degrading because of having phenol hydroxylase gene. Amplification of template DNA by DmpN primers indicated that 6 bacteria out of 10 isolated, were *P. Putida* due to production of a 199 bp bond in PCR product (Fig. 2).

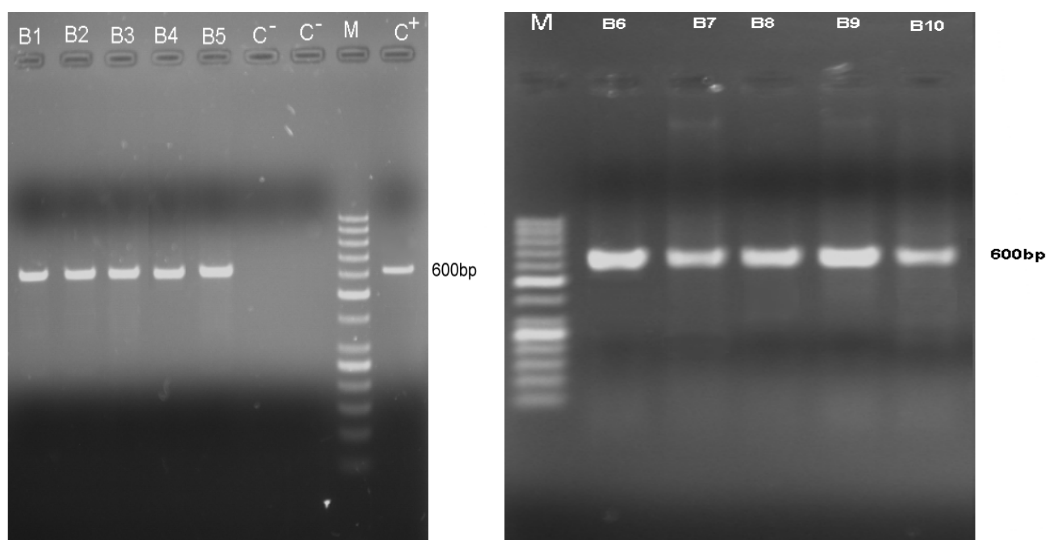


Fig. 1: PCR amplification of LmPH fragments for general detection of phenol-degrading bacteria by pheU primers (M= DNA size marker, B1 to B10 = Bacteria DNA, C^+= Positive control, C^-= Negative control)

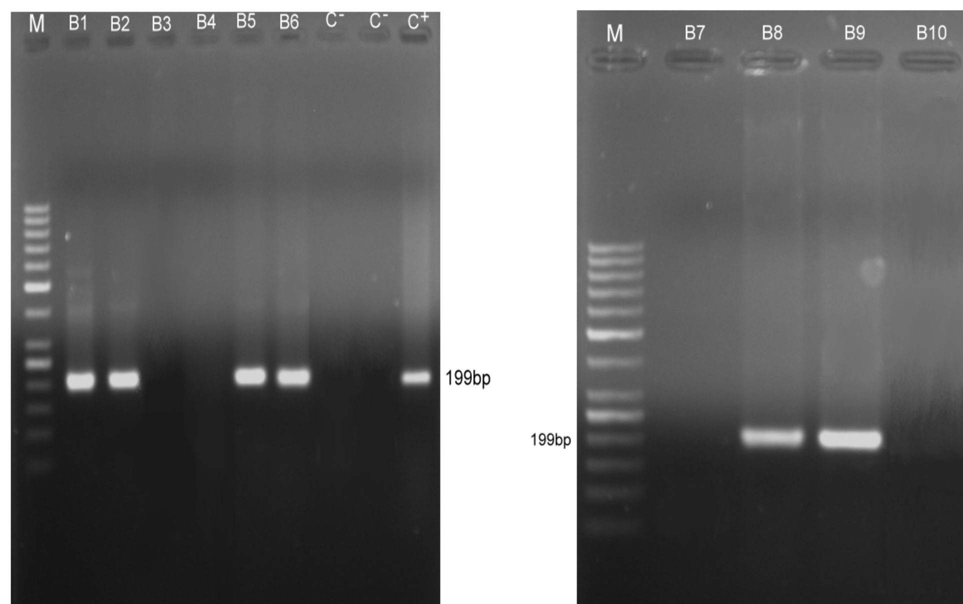


Fig. 2: PCR amplification of DmpN gene for detection of *Pseudomonas Putida* by DmpN primers (M= DNA size marker, B1 to B10 = Bacteria DNA, C^+= Positive control, C^-= Negative control)

According to PCR results in this study, the best phenol–degrading bacteria that utilize completely 500 – 600 mg/L phenol after 48 hours incubation belong to *P.Putida* strains. Rate of phenol removal correlates with growth rate of isolated bacteria, so that high growth of dominant bacteria happened in phenol initial concentration of 500 to 600 mg/L (Fig. 3 and 4).

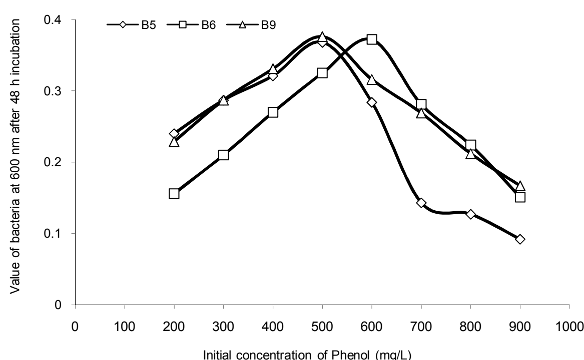


Fig. 3: Growth rate of dominant phenol-degrading bacteria after 48 h. incubation

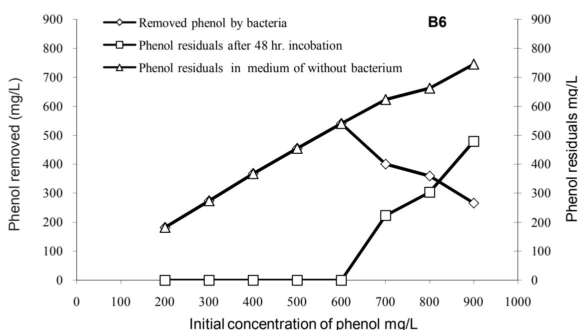


Fig. 4: Phenol removed by dominant isolated bacteria after 48 h. incubation

DISCUSSION

Phenol hydroxylase is responsible for converting phenol to catechol, which is the initial and rate – limiting step in phenol-degrading pathways (Zhang *et al*., 2004). Both single–component and multicomponent types of this enzyme have been identified (Futamata *et al*., 2001; Zhang *et al*., 2004) that among them, multicomponent enzymes are considered the major ones in the environment (Futamata *et al*., 2001). The multicomponent phenol hydroxylase has been used as a molecular marker to assess the function and diversities of phenol–degrading bacteria in the environment (Futamata *et al*., 2001; Zhang *et al*., 2004). Genotypic grouping of the phenol–degrading bacteria based on the amino acid sequences of the LmPH genes were correlated with physiological groupings based on their kinetic properties such as half – saturation constant (low K_s, moderate K_s and high K_s). *Pseudomonas Putida* belongs to high K_s group which some of them could tolerate phenol up to 1000 mg/L (Watanabe *et al*., Nov. 1998; Futamata *et al*., 2001; Wael *et al*., 2003; Zhang *et al*., 2004) .In this study, results of purified bacteria culture in MS/phenol media with 200 to 900 mg/L of phenol showed that bacteria B5, B6 and B9 can remove phenol up to 500 to 600 mg/L completely after 48 hours incubation.

We identified like Selvaratnam et al.(1995) *P. Putida* based on DmpN gene, which encodes phenol hydroxylase in this species. Amplification of extracted DNA by DmpN primers indicated that dominant phenol–degrading bacteria such as B5, B6 and B9 belong to *P. Putida* species with high K_s.

It is clear that with using dominant phenol–degrading bacteria such as B5, B6 and B9 may lead to the promotion of phenolic wastewater treatment. *P. Putida* was identified (similar to Selvaratnam et al.,1995) to the responsible for encoding phenol hydrolysis, based on DmpN gene.

ACKNOWLEDGEMENTS

The authors would like to thank vice chancellor for researches of Isfahan University of Medical Sciences for financial support of this project. We would like to thank of genetic laboratory the of Isfahan Medical faculty, especially G. Amini for kind helps.

REFERENCES

Annadurai, G., Rajesh Babu, S., Mahesh, K.P.O., Murugesan, T., (2000). Adsorption and bio-degradation of phenol by chitosan-immobilized Pseudomonas Putida (NICM 2174). Bioprocess Engineering, **22**: 493-501.

APHA, AWWA, WEF, (1995). Standard Methods for the Examination of Water and Wastewater. 19th, New York.

Burtscher, C., Wuertz, S., (2003). Evaluation of the use of PCR and reverse transcriptase PCR for detection of pathogenic bacteria in biosolids from anaerobic digestors and aerobic composters. Applied and Environmental Microbiology, **68** (8): 4618-4627.

Department of the Environment of IRAN (1994). Wastewaters

effluent standards. Tehran, Iran, Department of the Environment of Iran Press.

Fukui, Y., Sawabe, T., (2007). Improved one-step colony PCR detection of Vibrio harveyi , Microbes & Environments, **22**: 1-10.

Futamata, H., Harayama, S., Watanabe, K., (2001). Group-Specific Monitoring of phenol hydroxylase Genes for a functional assessment of phenol-stimulated Trichloroethylene Bioremediation. Applied and Environmental Microbiology, **67**(10): 4671-4677.

Gerrard, A.M., Junior, J.P., Kosteckova, A., Paca, J., Stiborova, M., Soccol, C.R., (2006). Simple models for the continuous aerobic biodegradation of phenol in a packed bed reactor. Brazilian Archives of Biology and Technology, **49**(4): 669-676.

Juang, R.S., Tsai, S.Y., (2006). Enhanced biodegradation of mixed phenol and sodium salisylate by Pseudomonas Putida in membrane contactors. Water Research, **40**: 3517-3526.

Noworyta, A., Trusek-holowina, A., mielczarski, S., (2006). An integrated pervaporation-biodegradation process of phenolic wastewater treatment. Desalination, **198**: 191-197.

Paula, M., van Schie, Lily Y. Young., (2000). Biodegradation of phenol mechanism and applications. Bioremediation journal, **4**: 1-18.

Prpich, G.P., Daugulis, A.J., (2005). Enhanced biodegradation of phenol by a microbial consortium in a solid- liquid two phase partitioning bioreactor. Biodegradation journal , **16**: 329-339.

Saiqa, A., Roberto Fernandez-Lafuente, Don, A., Cowan, (1998). Meta-pathway degradation of phenolics by thermophilic bacilli. Enzyme and microbial Technology, **23**: 462-468.

Sambrook, J., Russell, DW., (2001). In vitro Amplification of DNA by the Polymerase Chain Reaction in: Molecular Cloning A Laboratory Manual (Vol. 2) , 3rd. Ed., Cold Spring Harbor Laboratory Press, New York, pp. 8.1

Seker, S., Beyenal, H., Satin, B., Tanyolac, A., (1997). Multi-substrate growth kinetics of Pseudomonas Putida for phenol removal. Appl Microbiol Biotechnol, **47**: 610-614.

Selvaratnam, S., Schoedel, B.A., McFarland, B.L., Kulpa, C.F., (1995). Application of reverse transcription PCR for Monitoring expression of the catabolic dmpN Gene in a phenol- degrading sequencing batch reactor. Applied and Environmental Microbiology , **61**(11): 3981-3985.

Wael, S., El-Sayed, Mohamed, K., Ibrahim, Mohamed Abu-Shady, Fawkia El-beih, Naoya Ohmura, Hiroshi Saiki, Akikazu Ando, (2003). Isolation and characterization of phenol – catabolizing bacteria from a coking plant. Biosci. Biotechnol. Biochem., **67**(9): 2026-2029.

Watanabe, K., Yamamoto, S., Hino, S., Harayama, S., **(**Apr. 1998). Population dynamics of phenol-degrading bacteria in activated sludge determined by gyrB-Targeted quantitative PCR, Applied and Environmental Microbiology , **64**(4): 1203-1209.

Watanabe, K., Teramoto, M., Futamata, H., Harayama, S., (Nov. 1998). Molecular Detection, Isolation, and physiological characterization of functionally dominant phenol- degrading bacteria in activated sludge. Applied and Environmental Microbiology , **64**(11): 4396-4402.

Zhang, X., Gao, P., Chao, Q., Wang, L., Senior, E., Zhao, L., (2004). Microdiversity of phenol hydroxylase genes among phenol – degrading isolates of alcaligenes sp. From an activated sludge system. FEMS Microbiol Lett. , **237**(2): 369-375.

View publication stats