

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

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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

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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 °C 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 K₂HPO₄, 2.25 g KH₂PO₄, 1 g (NH₄)₂SO₄, 0.2 g MgCl₂.6H₂O, 0.1 g NaCl, 0.02 g FeCl₃.6H₂O and 0.01 g CaCl₂ (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 °C 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 (OD₆₀₀) 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/ μL 10	0.3	0.4	0.12 μM for general detection 0.16 μM for P.Putida detection
	Reverse	Pmol/ μL 10	0.3	0.4	12 μM for general detection 6 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(°C)
Initial Denaturation	-	10	94
Step 1 (5 cycles)	Denaturation	1	94
	Annealing	1	58
	Extension	1	72
Step 2 (5 cycles)	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 °C
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).

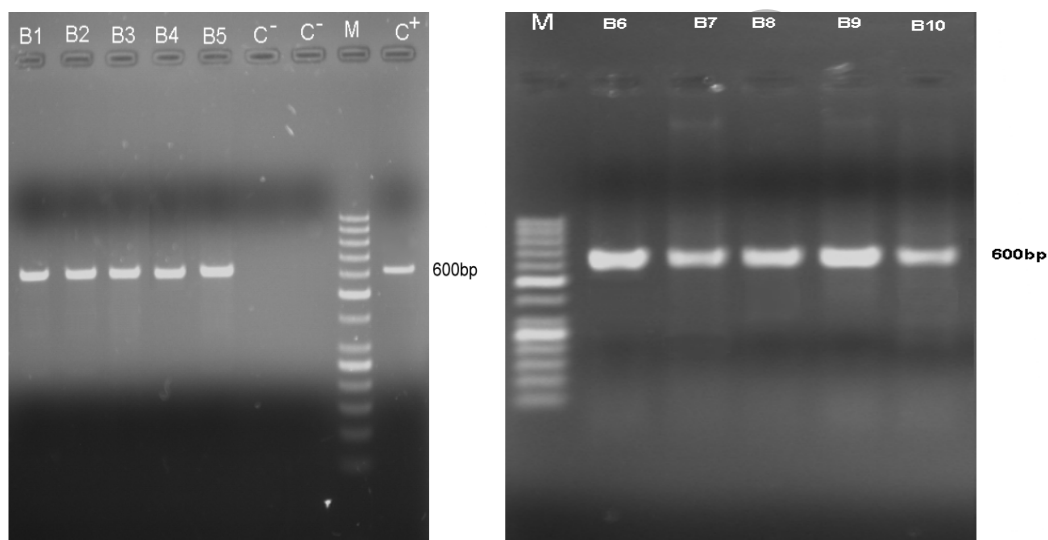


Fig. 1: PCR amplification of LmPH fragments for general detection of phenol-degrading bacteria by pheU primers (M= DNA size marker, B1 to B10 = Bacteria DNA, C⁺= Positive control, C⁻= Negative control)

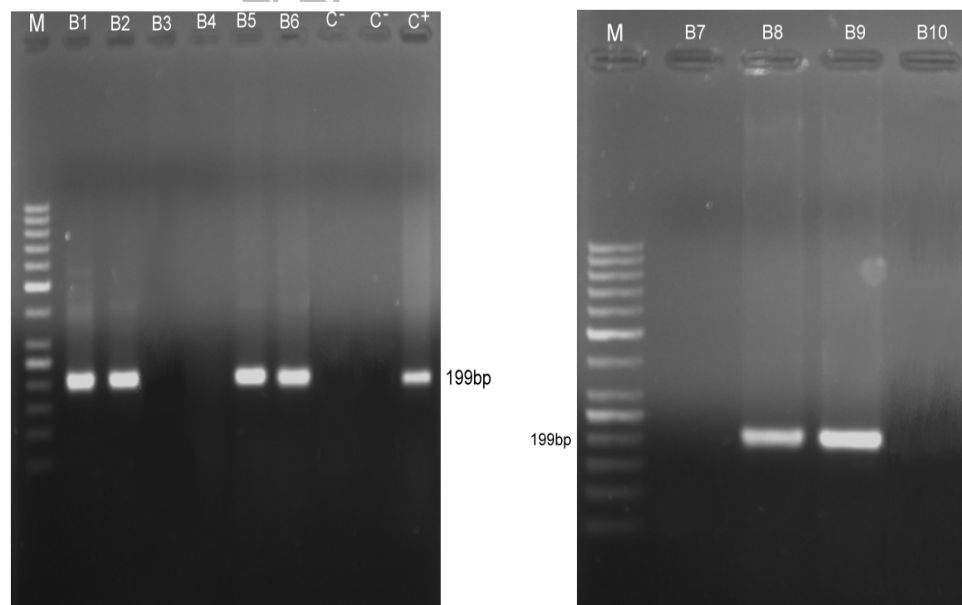


Fig. 2: PCR amplification of DmpN gene for detection of *Pseudomonas Putida* by DmpN primers (M= DNA size marker, B1 to B10 = Bacteria DNA, C⁺= Positive control, C⁻= Negative control)

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).

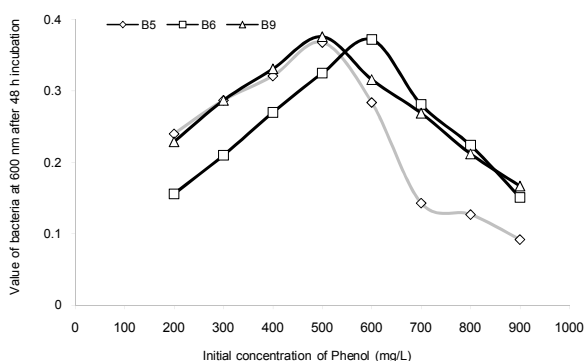


Fig. 3: Growth rate of dominant phenol-degrading bacteria after 48 h. incubation

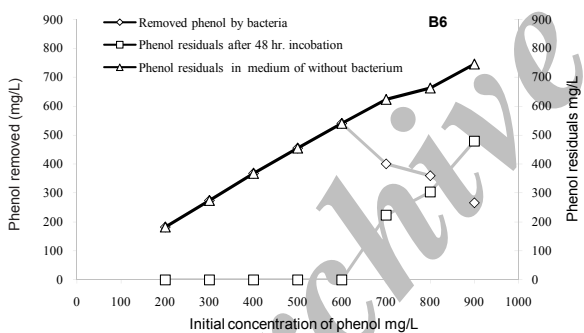


Fig. 4: Phenol removed by dominant isolated bacteria after 48 h. incubation

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 be responsible for encoding phenol hydrolysis, based on DmpN gene.

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