Int J Pharm Bio Sci 2013 July; 4(3): (B) 405 - 413

Research Article Biotechnology

International Journal of Pharma and Bio Sciences

ISSN
0975-6299

OPTIMIZATION OF PHENOL BIODEGRADATION BY *PSEUDOMONAS PUTIDA* ISOLATED FROM INDUSTRIAL EFFLUENT

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ABSTRACT

Bioremediation is a rapidly growing technology to solve the pollution problems all over the world. Providing good environmental condition is one of the important parameter for bacterial bioremediation. In this study, we had optimized bioremediation process to enhance the phenol removal from aqueous solution by a newly isolated and biochemically identified bacteria *Pseudomonas putida* from an industry effluent. This bacterium was capable of utilizing phenol as a sole source of carbon and was very efficient for phenol degradation at optimized culture condition. 1000 mg.l^{-1} of phenol was completely degraded at 60th hour of contact time. The optimum pH and temperature for highest phenol degradation was 7 and 35^{0}C respectively. Media was supplemented with different concentrations of Iron (Fe) and Selenium (Se) for the growth of *Pseudomonas putida* and phenol degradation. Maximum phenol degradation of 79.7 % and 80.3% was observed at 0.5mg/l Fe and 0.5mg/l Se respectively. From the results, we conclude that the phenol biodegradation and cultural parameters are interdependent by statistical analysis.

KEYWORDS: **Biodegradation; Phenol; *Pseudomonas putida*; Iron; Selenium; Temperature; pH.**

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INTRODUCTION

Phenol and its derivatives are the most common toxic organic pollutant of various industrial wastewaters and therefore it requires proper treatment before being discharged. Nowadays, bioremediation is used as one of the most common technique for effluent treatment than the conventional methods. The biodegradation method of phenol reduction is universally preferred, because of lower costs and the possibility of complete mineralization (Stephen et al. 2005; Brar et al. 2006; Agarry et al. 2008). Microorganisms are utilizing phenol and its derivatives as sole carbon source and energy at varying concentrations, under optimum conditions. They include several bacteria (Agarry et al. 2008; Liu et al. 2009; Ma et al. 2010; Shetty et al. 2007), a few yeast strains (Fialova et al. 2004; Yan et al. 2005) and mycelial fungi (Santos et al. 2003; Yordanova et al. 2009). Among the microbes, bacteria are considered as one of the important candidates for degradation because its higher capacity to degrade phenol (Kafilzadeh et al. 2010). Aerobic biodegradation of phenol by microorganisms is now well established. In the first step of aerobic degradation, molecular oxygen is used by phenol hydroxylase to form catechol. Catechol can then be degraded via two pathways known as ortho- or meta-pathways. In the ortho- pathway, the aromatic ring is cleaved between the catechol hydroxyls by a catechol 1,2-dioxygenase forming 2-hydroxymuconic semialdehyde. The resulting product is metabolized further to Krebs cycle intermediates. In the meta- pathway, ring fission occurs adjacent to the two hydroxyl groups of catechol. The enzyme catechol 2,3-dioxygenase transforms catechol to 2-hydroxymuconic semialdehyde, which is metabolized further to intermediates of the Krebs cycle (Van Schie and Young, 2000).

Microbial degradation processes result from the combined enzyme, cell, and community based activities of microorganisms whose regulation and kinetics are likely to be highly individualized and variable in space and time. These activities are dependent on environmental factors (Santos et al. 2009). Several external factors can limit the rate of biodegradation of organic compounds. These factors may include temperature, pH, oxygen content and availability, substrate concentration and physical properties of contaminants. Each of these factors should be optimized for the selected organism for the maximum degradation of the organic compound of choice. The optimization of the substrate concentration in phenol biodegradation is particularly important since it inhibits the growth of the organism at higher concentrations (Nair et al. 2008). In the present investigation, we had optimized aerobic phenol degradation by newly isolated *Pseudomonas putida* in aqueous medium and found the degree of linear relationship between the parameters and phenol degradation.

MATERIALS AND METHODS

Isolation and screening of phenol degrading microorganism

Soil samples were collected from a nearby industrial effluent, Thirumalzhasi Industrial Estate, Chennai, Tamil Nadu, India. The isolation of bacterial strains were carried out by serially diluting the soils samples in saline water and subsequently plating on Minimal Salt Medium (MSM) as follows : Na_2HPO_4 (6 g/l), KH_2PO_4 (3 g), NaCl (0.5 g/l), NH_4Cl (1 g/l), $CaCl_2.2H_2O$ (1 M/l) and $MgSO_47.H_2O$ (1 M/l) and Agar 10g/l. 10mg/l of phenol was added to MSM medium as sole carbon source and then incubated at 35^{0}C for 24hrs. Colonies that appeared on the plates indicates phenol degrading bacteria. Further they were picked out and purified by streaking. The colony morphology and microscopic characters were observed under the light microscope. Bacterial isolate SPD-7 showing maximum growth and phenol degradation was identified and further studies were carried out. This single colony was maintained on nutrient agar media incorporated with yeast extract (1.5 g/l), Beef Extract (1.5g/l),

NaCl (5.0g/l), Agar (15g/l), tryptone (5 g/l), KH_2PO_4 (1 g/l), $MgSO_4\ 7H_2O$ (1 g/l), thiamine (1 g/l), glucose (5 g/l) and stored at 4ºC until further use.

Identification and characterization of the strain KDP-8

Morphological, physiological and biochemical characteristics of the potent phenol degrading strain, SPD-7 was determined by the method described in "Bergey's Manual of Determinative Bacteriology" (Holt *et al.*1994).

Batch studies of Phenol degradation

The optimization of media, the effect of contact time (0 to 72hrs), the effect of initial pH (4 to 9), temperature (25^0C to 50^0C), and effect of different concentrations of selenium (0.1 to 1 g/l) and Iron (0.1 to 1 g/l) are carried out in batch studies using shake flaks for the strain, SPD-7. Phenol degradation experiments were carried out in 250 ml shake flask containing 100 ml of cultural medium. The following liquid media (g/l) was used: $(NH_4)_2\ SO_4$ - 0.5, NH_4NO_3-1.0, NaCl-0.5, $MgSO_4.7H_2O$-0.5, K_2HPO_4-1.5, KH_2PO_4-0.5, $CaCl_2$-0.01, $FeSO_47H_2O$-0.01. 1000mg/l phenol used as an initial concentration and sole carbon source for all the experiments. Phenol was filter sterilized separately using 0.2 μm regenerated cellulose membrane filter and added to the sterilized medium after cooling to room temperature (Bai et al., 2007). The medium was inoculated with 5% (v/v) inoculum to initiate the cultivation and degradation of the phenol. The shake flasks were incubated in a temperature controlled orbital shaker maintained at 150 rpm. During the cultivation, 10mL of culture samples were withdrawn periodically for analysis.

Estimation of phenol

Phenol was estimated by photometric method. 4-amino-antipyrene was used as the colouring agent and the absorbance was measured at 510 nm by UV-Visible spectrophotometer (APHA 1992). The percentage degradation of phenol was calculated by the following equation

$$\%\text{ of Degradation} = \left[\frac{Po - Pf}{Po}\right] X\ 100$$

Statistical Analysis

All the data were statistically analyzed to find the coefficient of correlations (Pearson) between variables and amount of phenol degradation by Software - MINITAB Release 12.2. The Pearson product moment correlation coefficient was calculated to measure the degree of linear relationship between the variables. The correlation coefficient assumes a value between -1 and +1. If one variable tends to increase as the other decreases, the correlation coefficient is negative. Conversely, if the two variables tend to increase together the correlation coefficient is positive.

RESULTS AND DISCUSSION

Bacteria isolation and identification

In the present study, Thirumalzhasi Industrial Estate (Tamil Nadu, India) area was selected to collect soil samples from Industrial effluent and isolate the high phenol resistant bacteria because that the site polluted with toxic pollutants might contain indigenous microorganisms due to acclimatization towards toxic pollutants. Soil samples when subjected to serial dilution and subsequently plated on a solid enrichment media yielded nearly 12 distinct bacterial colonies. Among the isolated colonies, four colonies showed maximum phenol degradation. One of the isolate SPD-7 was a potent phenol degrader strain among other strains isolated in this study showing highest phenol degradation and hence it was selected for further studies. Table 1 and 2 shows the results of the morphological, physiological and biochemical characteristics of strain SPD-7. Morphologically SPD-7 showed smooth, convex, round colony with an entire margin on nutrient agar plates. It was a gram-negative rod shape. It could grow on MacConkey agar. Strain SPD-7 tested positive

for oxidase, citrate utilization and Voges Proskauer, dextrose, lactose and maltose test. However, it showed negative results for glucose fermentation, nitrate reduction test, urea hydrolysis, methyl red test and hydrogen sulphide gas production. Based on the results from morphological, physiological and biochemical characteristics, phenol degrading strain SPD-7 was identified as *Pseudomonas putida.*

Table 1
Morphological characteristics of the isolated organism

Morphological Charateristics	Observed results
Configuration	Round
Margin	Entire
Elevation	Convex
Surface	Smooth
Pigments	No colour
Shape	Rod
Motility	+
Gram -reaction	Negative

Table 2
Biochemical characteristics of the isolated organism

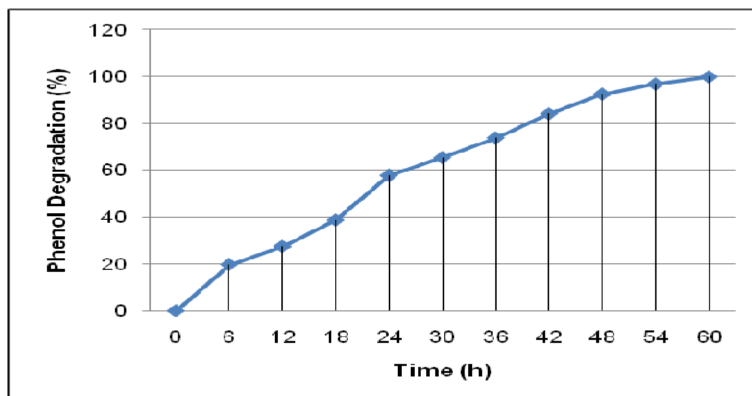
Biochemical Characteristics	Observed Result
Growth on MacConkey Agar	+
Oxidase test	+
Glucose Fermentation	-
Voges Proskauer Test	+
Citrate utilization test	+
Catalase test	+
Indole production test	-
H_2S Production	-
Oxygen tolerance test	Aerobic
Nitrate reduction test	-
Urease test	–
Methyl Red Test	-
Lecithinase	-
Dextrose	+
Lactose	+
Maltose	+

(+) Positive, (-) Negative

Effect of contact time

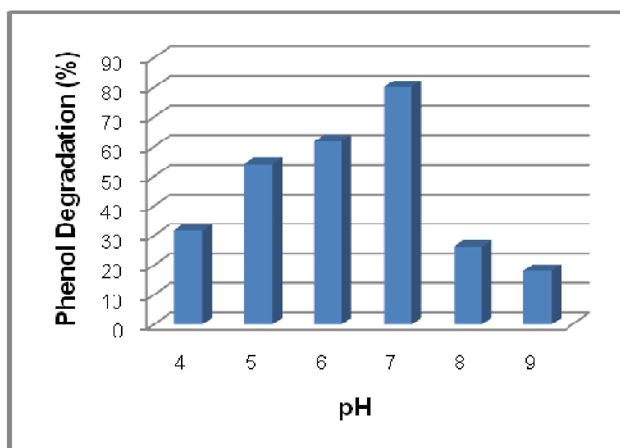
Fig.1 shows the effect of contact time on phenol degradation by newly isolated *P. putida.* 1000 mgl^{-1} phenol was amended with culture media and incubated in the orbital shaker at 150rpm. Samples were withdrawn every 6 hrs for phenol estimation and it was observed that phenol degradation occurred with the increase in the percentage degradation linearly with time. Phenol was completely degraded at 60th hour. Contact time and phenol degradation was interdependent because it showed highly positive correlations value, γ = +0.940. Paraskevi and Euripides (2005) reported that, *Pseudomonas* sp. isolated from the petroleum contaminated soil degrades phenol up to 1300 mg.l^{-1} within 156 hrs. Similarly Chung et al., (2003) also reported that, *Pseudomonas putida* degrades phenol up to 2000 mg.l^{-1} within 156 hrs. Ravikumar *et al.,* (2011) among thirty two morphologically different strains, *Pseudomonas putida, Bacillus* sp and *Pyrococcus horikoshii* was found as better strains for phenol degradation which are isolated from the retting water. In that *Pseudomonas putida* showed the complete degradation of phenol up to 2000 mg.l^{-1} within 144 hrs.

Figure 1
Effect of contact time on phenol degradation



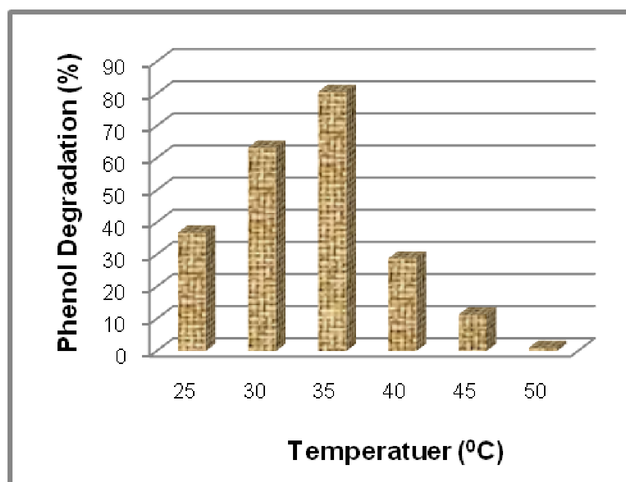
Effect of initial pH
The effect of initial pH on biodegradation provides an insight on the nature of physicochemical interaction between solute in solution and the bacterial cells. The maximum phenol removal was founded to be at pH 7 (Fig.2). The effect of initial pH on the biodegradation of phenol was interdependent. pH above 7 showed negative correlation because the growth of *P. putida* was inhibited, which resulted to incomplete degradation of phenol present in the culture. Most of the organisms cannot tolerate the pH values below 4.0 and above 9.0 as it affects the metabolic pathway and denature the proteins finally proves to be lethal (Annadurai et al. 2000).

Figure 2
Effect of initial pH on phenol degradation



Effect of initial temperature
Newly isolated *P. putida* was able to grow and degrade phenol at a temperature range from 25^{0}C to 40^{0}C but the highest phenol degradation was obtained at 35^{0}C (Fig. 3). Phenol degradation is temperature dependent. Growth of *P. putida* and phenol degradation consequently decreased after increasing the temperature to 35^{0}C. Temperature above 40^{0}C showed negative correlation. At 30°C *Pseudomonas* SA01 had significant degradation potential for the rapid utilization of phenol (Shourian et al. 2009). A study performed by Prieto et al. (2002) reported that the highest degradation of phenol by *Rhodococcus erythropolis* was obtained at 30^{0}C.

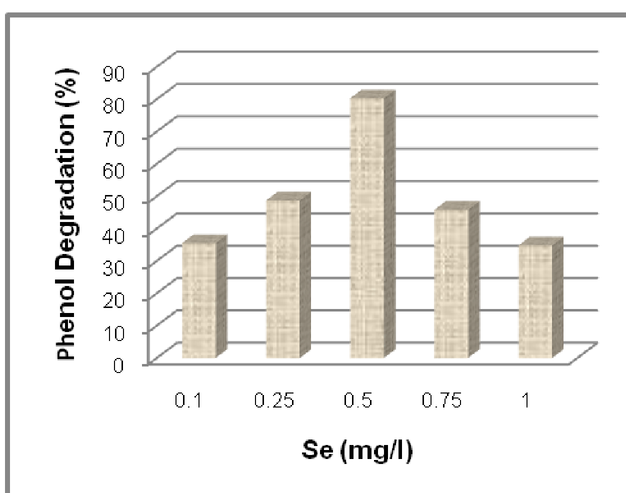
Figure 3
Effect of initial temperature on phenol degradation



Effect of selenium

In the present study, selenium concentration was optimizd to enhance the phenol degradaton by *P.putida.* Maximum phenol removal was obsereved at 0.5g/l Se (Fig.4). When selenium concentration was increased the bacterial growth had decreased, and ultimately this affected the phenol degradation owing to less biomass. The coefficient of correlation between the low concentration of selenium (0.1 to 0.5 g/l) and the percent of phenol degradation showed positive correlation (γ = +0.996), whereas higher concentration (0.75 to 1.0 g/l) showed negative correlation (γ = -1.0). This may be the first attempt to study the requirement of selenium for phenol biodegradation.

Figure 4
Effect of Se on phenol degradation

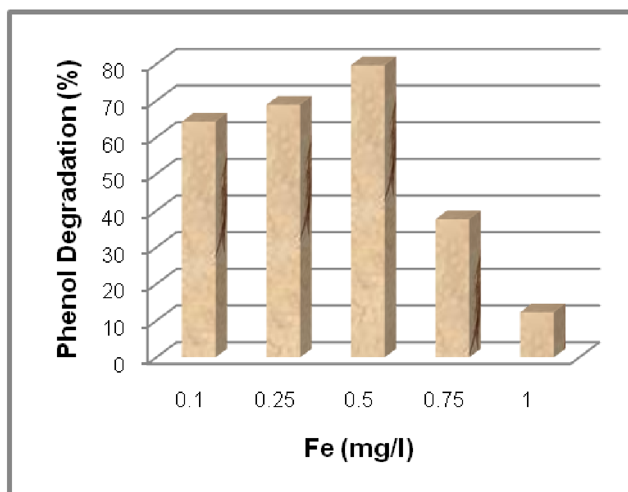


Effect of Iron

The essential elements Cu, Fe, Mn and Zn are required in low concentrations by all kinds of life because they play an important role in metabolic processes taking place in living cells (Botkin and Keller 2005). However, elevated levels of these elemnets are toxic to most organisms (Kaplan, 2004). Different initial concentration of Fe was used to optimize the culture media for phenol degradation. Maximum

growth of *P.putida* and phenol removal was found to be at 0.5 mg/l Fe (Fig.5). The higher Fe concentration like 0.7 and 1 g/l culture affected phenol degradation significantly.

Figure 5
Effect of Fe on phenol degradation



Bacteria *P.putidia* isolated from industry effluent had high phenol degrading ability. 100% of phenol degradation was achieved with an initial concentration of 1000 mg/l phenol at 60th h. This new isolate consumed only 50% time for complete phenol degradation when compared to the existing literature as this strain may be more acclimatized towards toxic pollutants. Hence, this strain could be used to remove phenol from wastewaters. The optimum pH and temperature for the highest phenol degradation was 7 and 35^{0}C respectively. We also studied the requirement of Fe and Se for *P.putida* growth as well as phenol removal. The maximum degradation was observed at 0.5g/l of Fe and Se. In future, we would like to use the same culture conditions to study the enzyme phenol hydroxylase activity and its kinetics.

ACKNOWLEDGMENT

The authors would like to thank The Director, Centre for Research and Development, Bharathiar University, Coimbatore-46 for giving this opportunity. The authors also acknowledge and gratefully thank Dr. Mazher Sultana, Professor and Head, Dept. of Animal Biotechnology, Presidency College, Chennai-5 for her helpful comments and valuable discussions during the tenure of the work. We also thank Ms. M. Narayani for proofreading and language check.

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