



# Optimization of potential phenol degrading *Pseudomonas* strain KP-3 isolated from soil sample and characterization by using Gen III Microlog

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## Abstract

To identify phenol degrading isolate, a phenotypic fingerprint is produced in a characteristic pattern from discrete test reactions performed using Gen III Micro log, a biolog microtitre plate assay. Six strains were isolated from phenol contaminated effluent, KP-1 to KP-6. Screening was done at different concentrations from 0.2 to 1.4 g/l. Relationship between growth and degradation of the isolate suggested that the strain KP-3 could degrade 92.7% of phenol optimally at 36°C, pH 7.5 and 20 g/l of phenol. This study demonstrated that *Pseudomonas* strain KP-3 had strong ability to degrade.

**Key words:** Phenol, biodegradation, *Pseudomonas* strain KP-3, Gen III microlog.

## Introduction

Phenol is a toxic aromatic compound used or produced in many industries and as a result it is released as a regular component of industrial wastewaters (Bonfa *et al.*, 2013). Phenol is a usual and important pollutant found in a variety of effluent streams from chemical industries such as resins, dye stuffs, drug industry, photographic chemical, textile, and pulp and paper industry (Basha *et al.*, 2010). Phenol is found in petroleum products such as creosote and coal tar, and can be released by combustion of wood and auto exhaust (Lefebvre and Moletta, 2006). Phenol present in the soil most likely finds its way into groundwater. Phenol enters into body by ingestion, inhalation and dermal contact. Prolonged exposure to phenol causes cardiovascular diseases while ingestion of liquid products containing concentrated phenol can cause serious gastrointestinal damage and even death (Chakraborty *et al.*, 2010). The use of concentrated phenol to

the body can cause severe skin erosion (Health Protection Agency, 2007). Physical, chemical and biological methods are used to remove phenol from the environment.

Bioremediation using microorganisms is the one of the cheapest and safest solutions. Microbial metal bioremediation is a well planned strategy due to its low cost, high efficiency, and eco-friendly nature (Rajendran *et al.*, 2003; Wasi *et al.*, 2011). The present study is aimed at finding out the most efficient bacterial isolates which can utilize phenol as a carbon source and thereby reduce the phenol concentrations, especially in soil sample. In this study, biodegradation potential of phenol degrading bacterial strains was tested with different concentrations of phenol by isolation and the evaluation of the ability of isolated strains for phenol biodegradation to establish the effects of different parameters on the phenol degradation of isolated bacteria. Characterization of phenol degrading bacteria was done by using a metabolic phenotypic figure print Gen III biolog which is an authorized testing of Gram-negative and Gram-positive bacteria in the same test panel.

## Materials and methods

### Sampling

In this study, soil sample was collected from the Kurmannapalam, Visakhapatnam District. It was placed in sterile bottles and transferred to refrigerator used in lab for enrichment in less than 24 h.

### Sample preparation

The samples stored in the refrigerator at 4°C were used within 7 days from the day of collection for bacteriological analysis. One gram of soil sample was taken in 250 ml flask, suspended in 100 ml of distilled water, mechanically shaken and kept in rest for 10 min to permit heavy particles to settle down. The approximate volume of upper layer of water was taken for bacteriological analysis.

### Media composition

MSM medium (g/L):  $\text{KH}_2\text{PO}_4$  1.5,  $\text{K}_2\text{HPO}_4$  0.5,  $\text{NH}_4\text{Cl}$  0.5,  $\text{CaCl}_2$  0.02,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2, NaCl, 0.5,  $\text{Na}_2\text{SO}_4$  3.0, Yeast Extract 2.0, Nutrient Agar 2.0 and 1 ml trace solution.

### Isolation of microorganisms capable of degradation of phenol

Soil effluent of about 1 ml was suspended in 10 ml of distilled water, stirred well for about 30 min and filtered. From this, 0.1 ml sample was pipetted out and surface spread in each Petri plate containing nutrient agar, nutrient agar + 200 mg/l phenol including the Petri plate containing mineral salt agar medium (MSAM). All the Petri plates were incubated at 37 °C for about a week. Regular observations were made.

### Screening and selection of phenol degrading microorganisms

After incubation, the organisms growing on Petri plates were selected. The pure microorganisms were inoculated in the MSAM media containing phenol (200ppm) as carbon source and tested for their potential to grow on phenol. The strains that have ability to growth at these concentrations were selected. After one week, the well defined colonies were refined by streaking on agar plates containing the same medium by streak plate method.

### Maintenance of phenol-resistant isolates

Isolates transferred were grown on MSAM phenol agar slants and sub-cultured. The isolates which were grown well in MSAM were maintained at 37°C and labelled as KP-1, KP-2, KP-3, KP-4, KP-5 and KP-6.

### Strain selection based on phenol acclimatization

The isolated strains KP1, KP2, KP3, KP4, KP5 and KP6 were inoculated into MSAM containing phenol as carbon source for 48 hours shaking at 150 rpm. After 48 hours, the cell density was determined spectrophotometrically by assessing turbidity at 600 nm. The isolated strain KP3 was grown in MSAM medium by incubating overnight at 37°C on shaker at 150 rpm. The culture was inoculated into MSAM medium with phenol as sole carbon source for 24 hours. Preliminary degrading studies were carried out with inclusion of isolated strain containing different concentrations of phenol, different periods of time, different temperature conditions and different pH values and inoculum volume. The phenol concentrations were determined by assaying the samples at every 24 hrs interval by using UV Spectrophotometer. The residual amount of phenol present in the sample was measured by calorimetric assay by 4-amino antipyrine method.

### 4-Amino antipyrine method

When phenol reacts with 4-Amino antipyrine in the presence of potassium ferricyanide, it forms coloured antipyrine dye in aqueous solution. Freshly inoculated culture of 5 ml was taken and then 5 ml culture was added in 95 ml of phenol broth medium containing 1-3g/L phenol at 12 hours interval and then centrifuged at 1200 rpm for 1 hr. Supernatant sample was collected and sample was prepared for the measurement of optical density of phenol. Phenol analysis was made by measuring at wave length 500 nm using UV spectrophotometer, after colour development by 4-Amino antipyrine method for the examination of sample.

### Biolog microbial identification system and phenotypic microarray technology

GENIII biolog is a software used to identify the microorganism. The new GENIII redox chemistry is significant to the unequalled array of both gram-negative and gram-positive bacteria. The isolated bacteria were recognized by new GEN III Microplates test panel of the Biolog system. The

test panel contains a “Phenotypic Fingerprint” of the microorganism. The test panel consists of 71 carbon sources and 23 chemical sensitivity assays.

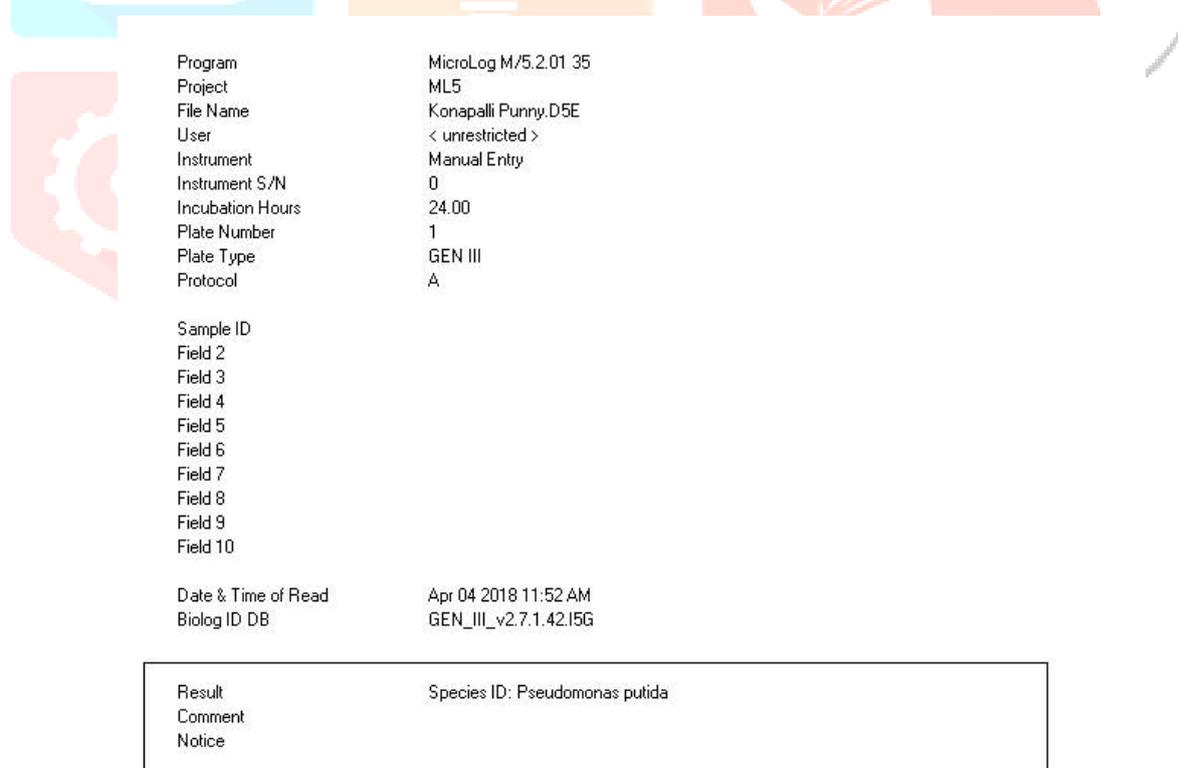
## Results

### Morphological characteristics

Phenol-degrading gram-negative bacterium was isolated and designated as strain KP3. The colony morphology of the strain on the plate of phenolic MSAM medium was convex, yellowish, mucous and with a single edge.

### Biolog GN analysis

An appropriate Biolog GN Micro-plate was used to determine the relative capacity of the substrate (Sangram and Hara Mohan, 2015) utilization by KP3 strain. The result illustrated that the isolate could react strongly or even very strongly with 30 of the 95 carbon substrates, and weakly with the other substrates in nutrition pools of the Biolog GN system after 24 h of incubation. According to Biolog GN identification, the reaction profile was similar to that of *Pseudomonas putida* with maximum comparability (Figure 1).



Rank	PRDB	SIM	DIST	Organism Type	Species
1	0.868	0.657	3.428	GN-Nent	Pseudomonas putida
2	0.113	0.075	4.738	GN-Nent	Pseudomonas syringae pv maculicola
3	0.013	0.008	6.099	GN-Nent	Pseudomonas viridilivida
4	0.006	0.003	6.632	GN-Nent	Pseudomonas asplenii

Figure 1. Detection of isolate by Biolog Gen III micro plate

## Optimization of physiological parameters:

### Effect of contact time

Degradation of phenol at various periods of time by the isolated KP3 strain was studied (Figure 2). It was observed that the percentage of degradation increased from 0 to 75.5 with an increase in contact of time from 24 hours to 96 hours. After 96 hours, the rate of degradation tends to decrease rapidly. Effect of exposure time on the response of pure cultures of bacteria and microbial community to toxicity of phenol was evaluated.

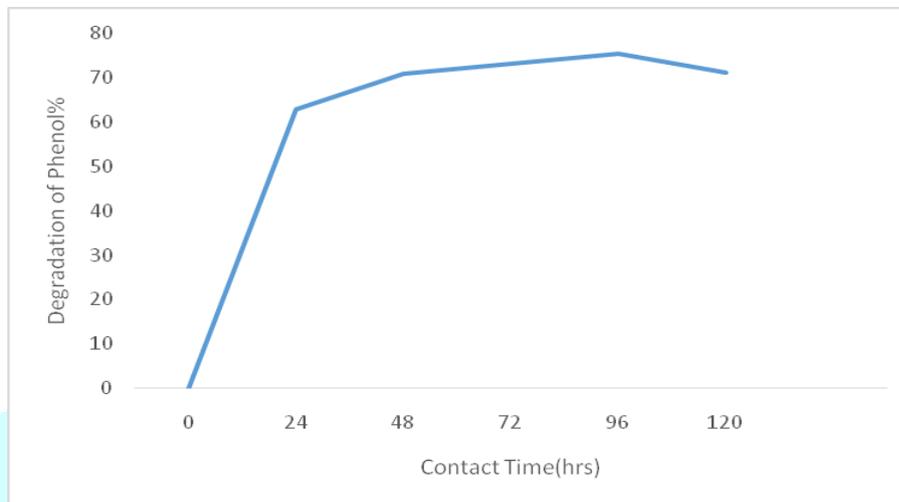


Figure 2. Effect of contact time on % degradation of phenol

### Effect of initial phenol concentration

The results obtained are shown in Figure 3. The percentage of degradation of phenol by isolated KP3 strain decreased from 78.95 to 62.30 with an increase in initial concentration from 200 mg/l to 1400 mg/l. The higher concentration of phenol inhibits cell growth and nucleic acid biosynthesis. The effect of dye concentration is an important consideration for the growth of organisms. The study suggests that the decrease in degradation efficiency might be due to increase in the toxic effect of phenol, with an increase in phenol concentration from 200 mg/l to 1400 mg/l.

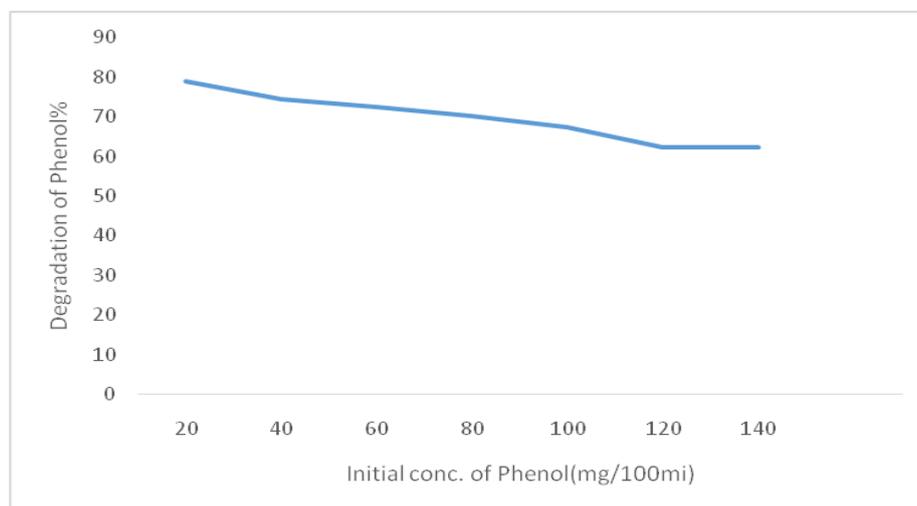


Figure 3. Effect of initial phenol concentration on % degradation of phenol

### Effect of inoculum volume

Degradation of phenol at different volumes of inoculum by the isolated KP3 strain was studied (Figure 4). It was observed that the percentage of degradation increased from 71.1 to 92.25% with an increase in inoculum volume from 1v/v to 5 v/v. The rate of degradation tended to decrease rapidly after inoculum volume 4v/v.

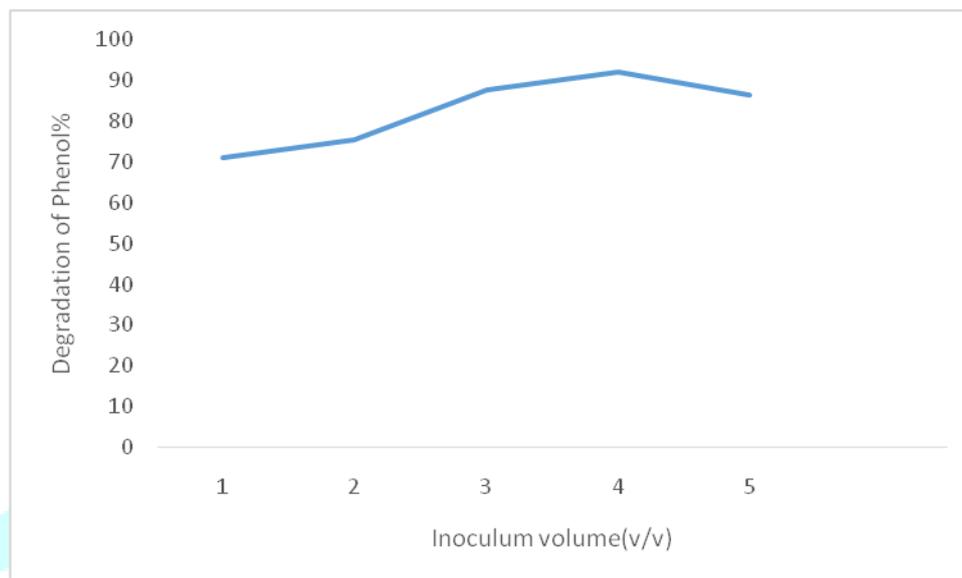


Figure 4. Effect of inoculum volume (v/v) on % degradation of phenol

### Effect of pH

Degradation of phenol at various pH values by the strain was studied (Figure 5). It was observed that the percentage of degradation increased from 72.5 to 93.5 with an increase in pH from 5.5 to 7.5 and decreased from 7.5 to 9.5 with an increase in pH from 6.5 to 7.5.

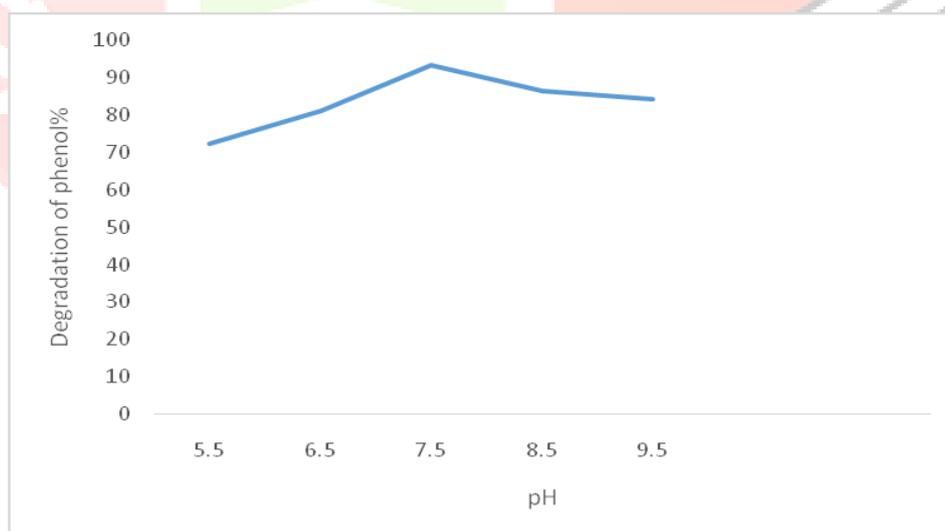


Figure 5. Effect of pH on % degradation of phenol

### Effect of temperature

Degradation of phenol by the strain was studied at different temperatures (Figure 6). Experiments were conducted to determine the effect of temperature on percentage of degradation of phenol with a constant of 96 hrs time at different temperatures (28 to 36°C) with an initial phenol concentration of 200 ppm. It was perceived that phenol degradation activity of the culture increased with an increase in incubation temperature from 28 to 36°C with maximum activity at

36°C. Cells may become metabolically active and capable enough to produce the required enzymes needed for degradation. Further increase in temperature resulted in marginal reduction in degrading ability of the bacterial culture.

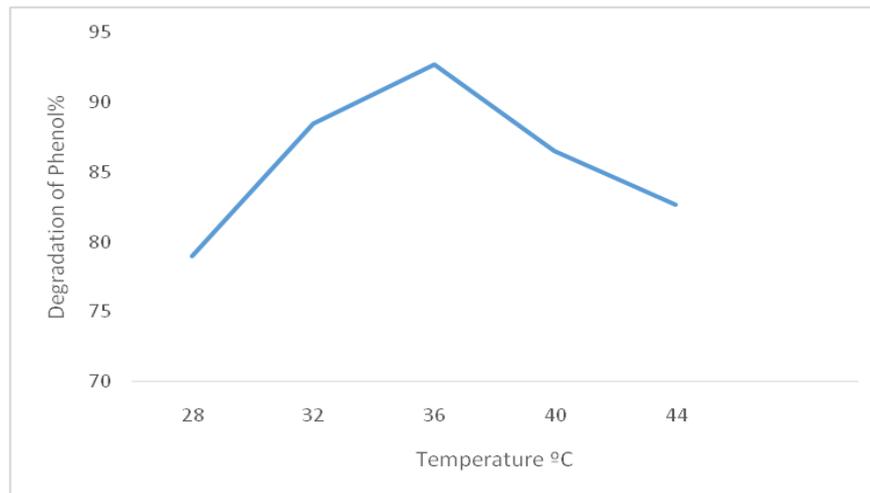


Figure 6. Effect of temperature on % degradation of phenol

### Discussion

Biodegradation is one of the inexpensive methods with no production of high risk by-products (Paul *et al.*, 2005). In this study, *Pseudomonas* strain KP-3 capable of degrading phenol was isolated from soil sample. The isolate used phenol as sole source of carbon and energy for its growth under incubation conditions. The isolate in the phenol medium grew well in which phenol could be degraded almost completely over a period of 96 h of incubation with stable pH. Characterization of isolated strain by Gen III Micro log shows that phenol-degrading bacteria belongs to *Pseudomonas putida* which had high phenol degradation activity. However, the results showed that the characters closely resemble *Pseudomonas syringae pv maculicola*, *Pseudomonas resinovorans*, *Pseudomonas fuscovaginae*. The ideal conditions for growth and phenol degradation of *Pseudomonas* strain KP-3 are more closely related to the field situation. The effect of contact time, initial phenol concentration, pH, inoculum volume and temperature on the rate of phenol degradation by KP-3 strain showed that this strain has the potential for resolving the phenol pollution problem.

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