

## Bioremediation of phenol and naphthalene by *Bacillus* species and *Brachybacterium* species isolated from pharma soil sample

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### ABSTRACT

In this present study, Soil samples were collected from the pharma industries namely Sai Meera pharmaceuticals, Chennai. Samples were screened and totally 10 colonies were isolated in the Mineral Agar medium containing 10 mg of phenol and naphthalene and named as P1 to P10. The screened colonies were enriched in liquid minimal media containing phenol and naphthalene for 5 days. The enriched colonies streaked on the mineral agar plates containing increasing amount of phenol and naphthalene for potential strain. The potential strains were selected and named as P7 and P10. The potential strains were studied for degradation ability using 4-amino antipyrine method and the degradation ability were calculated by colorimetric O.D values at 600nm. Optimization of pH and temperature was carried out for the maximum degradation of chemical by the potential strains. The P7 and P10 strains showed better growth in neutral pH. P7 strain showed good growth at 28°C and 37°C but P10 strain shown better growth at 28°C. Immobilization of the potential strains was carried out for the better and maximum degradation and their degradation ability was calculated by 4-amino antipyrine method. Immobilized cells can be reused for further degradation. The potential strains P7 was identified as *Bacillus* sp and P10 was identified as *Brachybacterium* sp.

**Keywords:** Microbial degradation, pharma industrial soil, phenol and naphthalene, immobilization, *Bacillus* sp, *Brachybacterium* sp and 4- amino antipyrine.

### INTRODUCTION

Chemicals are part of the modern life. They are part of all spheres of human life and used as pharmaceuticals, pesticides, detergents, fertilizers, dyes, preservatives, food additives etc. Pharmaceutical wastes produced on a daily basis may be land filled provided that they are dispersed in large quantities of general waste. Cytotoxic and narcotic drugs, however, should never be land filled, even in small quantities. Mild liquid or semi-liquid pharmaceuticals, such as solutions containing vitamins, cough syrups, intravenous solutions, eye drops, etc. (but not antibiotics or cytotoxic drugs), may be diluted in a large bow of water and discharged into municipal sewers. Petroleum hydrocarbons can be divided into four classes namely saturates, aromatics, the asphaltenes (phenol, fatty acids, ketones, esters and

porphyrines), and the resins (pyridines, quinolines, carbazoles, sulfoxides and amides).

Phenol,  $C_6H_5OH$  or hydroxybenzene, is an aromatic molecule containing hydroxyl group attached to the benzene ring structure. Phenol commonly known as carbolic acid has a molecular weight of 94.11 gm/M 1. It has a melting point of 43°C and forms white to colorless crystals, colourless to pink solid or thick liquid. Phenol is a constituent of coal tar, and is formed during decomposition of organic materials. Ingestion of even small amounts may cause vomiting, circulatory problems, paralysis, convulsions, and coma. Phenol affects the nervous system and key organs, i.e. spleen, pancreas and kidneys.

Naphthalene PAHs including naphthalene (2 rings), fluorene (3 rings), benzo (a) anthracene,

chrysene, fluoranthene, pyrene (4 rings), and benzo (a) pyrene (5 rings). Exposed to naphthalene vapours causes Nausea, vomiting, abdominal pain, and anemia Increased serum bilirubin, met hemoglobin, Heinz bodies, and fragmented red blood cells hematuria, anemia, restlessness, and liver enlargement<sup>2</sup>.

**Microbial degradation** In aerobic respiration, oxygen acts as the electron acceptor. Molecular oxygen is a reactant for oxygenase enzymes and is incorporated into the final products. In anaerobic respiration, different inorganic electron acceptors are released such as NO<sub>3</sub>, SO<sub>4</sub>, SO, CO<sub>2</sub> and Fe<sub>3</sub> during microbial degradation. Following microorganism is responsible for the degradation of phenol and naphthalene. *Acinetobacter* sp., *Arthrobacter* sp.,<sup>3</sup> *Bacillus* sp.,<sup>4</sup> *Micrococcus* sp., *Nocardia* sp. *Pseudomonas* sp.<sup>5</sup> *P. pictorum*- NICM-2077, *Rhodococcus* sp, DCB-p0610 *Fusarium* - F. FE11 *Penicillium* AF2, AF4,<sup>6</sup> *Coprinus* sp. *Candida tropicalis*. Considering the versatile nature of microorganisms as a good source of degrading the wastes, especially the pharmaceutical residues an attempt has been made to understand the utility of microorganisms for the degradation of pharmaceutical waste.

## MATERIAL AND METHODS

### Collection of sample

The soil sample was collected from Sai Meera pharma industry at Chennai. Sample was collected in polythene bag by using pre-sterilized spatula.

### Screening for phenol and naphthalene degrading organism

One gm of soil sample was serially diluted up to 106 dilution, and spreaded on minimal agar medium containing 10 mg/ 100 ml phenol and naphthalene which was added after sterilization, and the plates were kept for incubation at 28°C for 5 days<sup>7</sup>.

### Enrichment of the isolated strains

The 18 hrs selected cultures were prepared in nutrient broth. One ml of this turbid broth was taken and added into 49 ml of liquid mineral medium supplemented with 10 mg of phenol and

naphthalene in a sterile 250 ml conical flask and kept in a rotary shaker at 120 rpm for 5 days<sup>8</sup>.

### Selection of potential phenol and naphthalene degrading strains (Plate method)

Minimal agar medium was prepared with increasing concentration of selected phenol and naphthalene as follows 10 mg/100 ml, 20 mg/100 ml, 30 mg/100 ml, 40 mg/100 ml, and 50 mg/100 ml of enriched cultures were inoculated on minimal agar media. Plates were incubated at room temperature for 48-72 hrs.<sup>8</sup>

### Degradation by free cells

The selected potential isolates were inoculated in 10 ml nutrient broth, after 18 hrs of incubation 5 ml of this nutrient broth was added to 95 ml of minimal media containing different concentrations of phenol and naphthalene individually 30mg/100ml, 40mg/100 ml, 50mg / 100ml. 3 ml of sample were removed every one hour and microbial growth was measured using colorimeter at 600 nm. The aliquot samples were centrifuged at 1000 rpm for 5 min and supernatant was obtained for the study of degradation ability of phenol and naphthalene using 4-amino antipyrine method<sup>4</sup>.

### 4-amino antipyrine method

The phenol and naphthalene compounds reacts with 4-amino antipyrine at pH 7.9 ± 0.1 in the presence of potassium ferricyanide (K<sub>3</sub>Fe (CN) 6) and forms a pink colored antipyrine dye. This dye kept in aqueous solution and the absorbance was monitored at 500 nm.<sup>4</sup>

### Optimization of phenol and naphthalene degradation by potential strain

#### Effect of pH and temperature

The minimal agar medium was prepared with different pH (5, 7 and 9) and different temperature (28°C, 37°C, 45°C) for 48-72 hrs also supplemented with 50mg phenol and naphthalene separately.

### Preparation of immobilized cells

Phenol and naphthalene (0.01%) as a sole source of carbon and kept in rotary shaker at 120rpm for 72 hrs at 30°C. 3gm of sodium alginate was dissolved in 100 ml of distilled water and mixed

thoroughly and autoclaved. Then it was stirred with 3 ml of potential broth culture using magnetic stirrer. Then the final mixture was extruded through a needle into 200 ml of 0.05M  $\text{CaCl}_2$  solution to yield 3 to 4 mm diameter alginate beads. After 20 min, the beads were filtered out and stored in 0.05 %  $\text{CaCl}_2$  solution in the refrigerator.<sup>9</sup>

#### Degradation of phenol and naphthalene by immobilized cells

15 gm of immobilized beads were placed into 100 ml of mineral medium containing the desired amount of phenol (30 mg/100 ml, 40 mg/100 ml, and 50 mg/100 ml). Aliquot samples of the broth culture were taken at regular interval for the study of degradation by 4-amino antipyrine methods.

#### Identification of potential strains

Phenotypic characteristics such as micromorphology (gram staining, capsule staining endospore staining and motility), cultural characteristics (on basal media, differential media and selective media), and biochemical characteristics (catalase, oxidase, IMViC). Based on the results of studied phenotypic characteristics all the selected bacterial isolates are identified with the help of Bergey's Manual of Determinative Bacteriology<sup>10</sup>.

## RESULTS AND DISCUSSION

#### Screening for phenol and naphthalene degrading organism

During the third day of incubation, two colonies from phenol containing plate and three colonies from naphthalene containing plate were observed and the plates were further incubated. After 5 days, another two colonies from phenol containing plate and three colonies from naphthalene containing plate morphologically different colonies were observed. Totally 10 isolates were selected as phenol and naphthalene degrading bacterial colonies and named as strain P1 to P10. The stock cultures for the selected strains were maintained. Rigo and Alegre, used phenol as the sole carbon source in the defined mineral salt medium for the isolation process<sup>7</sup>, in the present study were used as phenol and naphthalene as the sole carbon source it is the screening method for the degradation.

#### Selection of potential phenol and naphthalene degrading strains

The enrichment medium which is used for the enrichment of an isolated strains from mixed cultures which show good variations of species in mixed cultures<sup>8</sup>. In this study, Minimal medium was used for the enrichment purpose. Better growth was observed after five days. After incubation, all the

**Table 1: Selection of potential phenol and naphthalene degrading strains**

S. No.		Strains Phenol (mg)					Naphthalene (mg)				
		10	20	30	40	50	10	20	30	40	50
1.	P1	+	-	-	-	-	+	-	-	-	-
2.	P2	+	-	-	-	-	+	+	-	-	-
3.	P3	+	+	-	-	-	+	-	-	-	-
4.	P4	+	+	-	-	-	+	-	-	-	-
5.	P5	+	+	-	-	-	+	-	-	-	-
6.	P6	+	+	-	-	-	+	-	-	-	-
7.	P7	+++	++	++	++	++	++	++	++	++	++
8.	P8	+	+	-	-	-	+	-	-	-	-
9.	P9	+	-	-	-	-	+	-	-	-	-
10.	P10	+++	++	++	++	++	++	++	++	++	++

'+++' better growth, '++' good growth, '+' growth, '-' no growth

strains namely P1 to P10 shows growth in 10 mg of phenol and naphthalene, but P7 and P10 show maximum degradation against all the concentrations of phenol and naphthalene. From these, P7 and P10 were selected as potential strains and used further studies (Table 1). On serial dilution two colonies from phenol plates and three colonies from naphthalene plates were observed within two days, in the next three days two colonies from phenol and three colonies from naphthalene were isolated. Totally ten colonies were observed on the mineral salt medium.

#### Degradation by free cells (4-amino antipyrine method)

The phenol and naphthalene in different concentration were added to mineral media for degradation by the potential isolates was studied by the colour reaction of chemical (phenol and naphthalene) with potassium ferric cyanide at pH 7.9. The optical density (O.D) value was measured for the change in colour by using colorimeter. For the degradation studies, various concentrations of phenol and toluene in (25 mg / L, 50 mg / L, 75 mg / L and 100 mg / L) were used. It is beneficial for the study of biodegradation to quantify their pollutant effect studied<sup>4</sup>. For this present study, increasing concentration of phenol and naphthalene such as 30 mg/ 100ml, 40 mg/ 100ml and 50 mg/ 100ml of phenol and naphthalene were used for the degradation process. Out of the two strains P7 and P10 were selected as potential strains. (Fig. 1 to 4).

#### Optimization of phenol and naphthalene degradation by potential strain P7 and P10

##### Effect of pH

After incubation, the plates containing both phenol and naphthalene in different pH condition were observed and the better growth was observed by P7 and P10 at neutral pH in the phenol plate and neutral pH in naphthalene plate. (Table 2 & 3).

##### Effect of temperature

After incubation, the plates containing both phenol and naphthalene in different temperature conditions were observed. The strains P7 and P10 indicate better growth in 28°C and 37°C in phenol plate and 28°C in naphthalene plate. (Table 4 & 5).

For the optimization process, different pH, temperature and enzymatic activity of *Serratia plymuthica* isolate from sludge sample reported<sup>11</sup> which shows the optimization of the degradation at certain pH and temperature optimized the cells by applying different pH and temperature for the microbial degradation of phenol<sup>12</sup>. In the same way in the work degradation of phenol by the influence of optimum pH was already reported<sup>13</sup>. In this study P7 and P10 strain show better growth in neutral pH. P7 and P10 strain shown better growth in 28°C and 37°C for phenol degradation. P7 and P10 strain shown better growth in 28°C for naphthalene degradation.

**Table 2: Effect of pH**

Chemical	Strain	pH		
		5	7	9
Phenol(50mg)	P7	+	++	-
	P10	+	++	+

**Table 3: Effect of pH**

Chemical	Strain	pH		
		5	7	9
Naphthalene (50mg)	P7	-	++	+
	P10	-	++	-

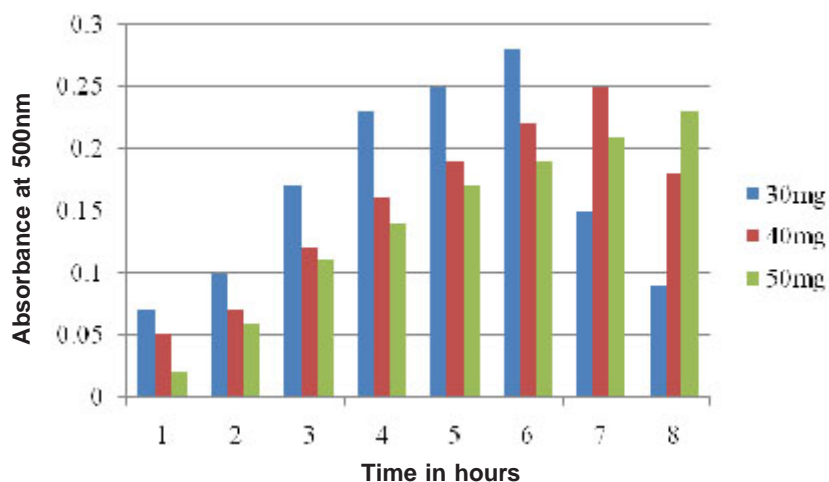
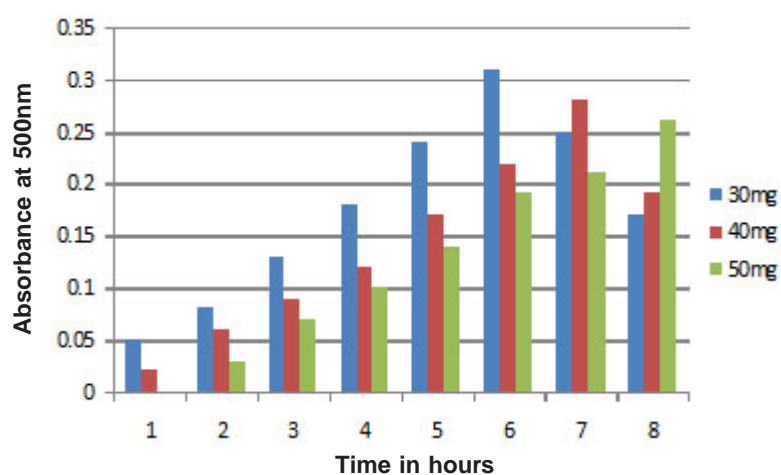
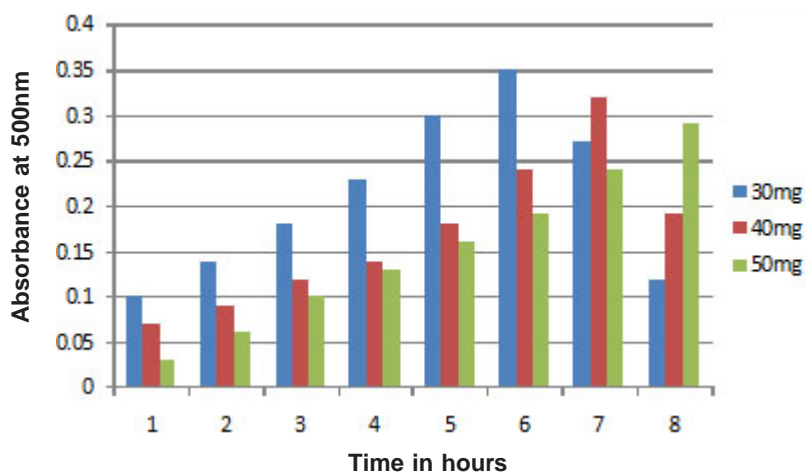
**Table 4: Effect of temperature**

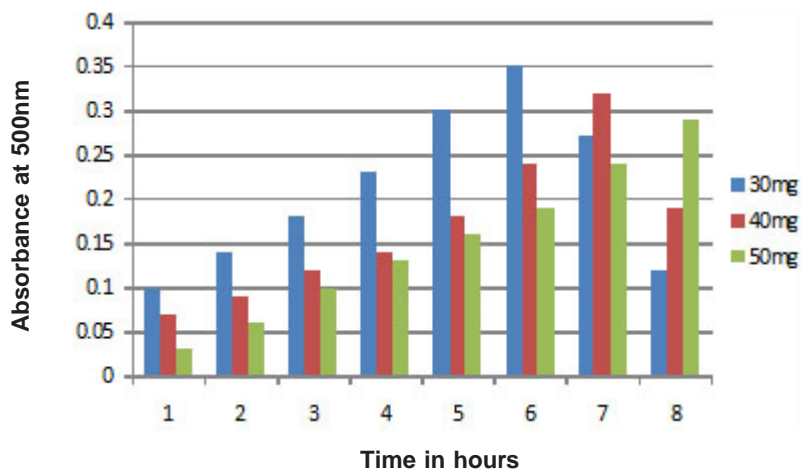
Chemical	Strain	Temperature (°C)		
		28	37	45
Phenol(50mg)	P7	++	++	-
	P10	++	++	+

**Table 5: Effect of temperature**

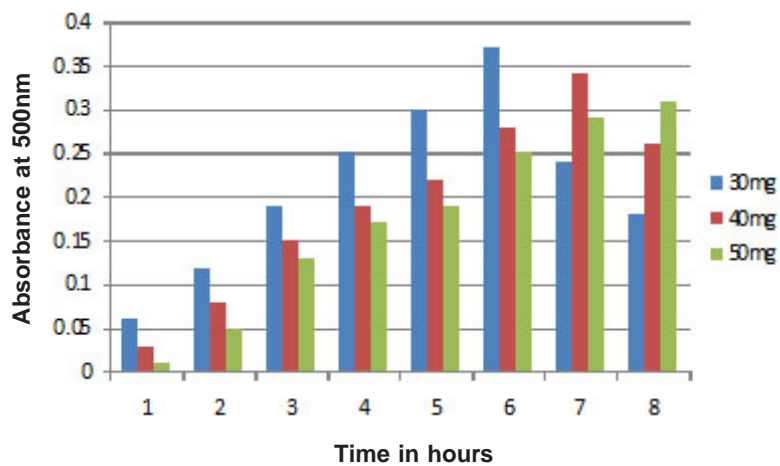
Chemical	Strain	Temperature(°C)		
		28	37	45
Naphthalene (50mg)	P7	++	+	+
	P10	++	+	+

‘++’ good growth, ‘+’ growth, ‘-’ no growth

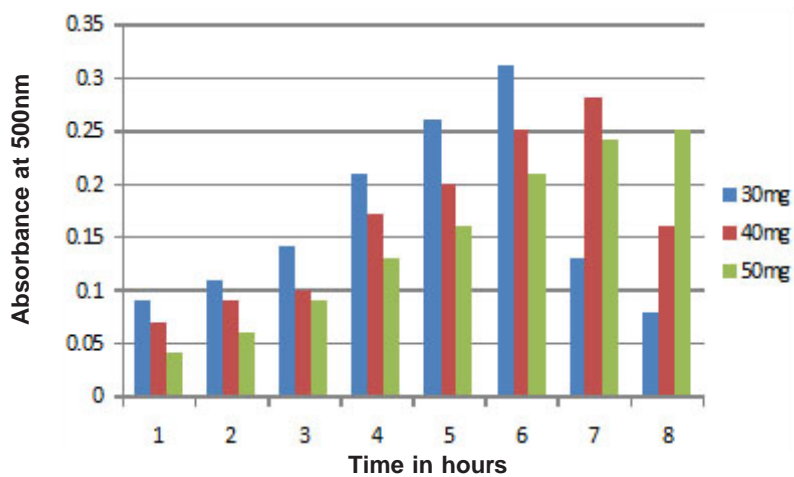
**Graph 1: Phenol degradation by free cells of P7****Graph 2: Naphthalene degradation by free cells of P7****Graph 3: Phenol degradation by free cells of P10**



Graph 4: Naphthalene degradation by free cells of P10



Graph 5: Phenol degradation by immobilized cells of P7



Graph 6: Naphthalene degradation by immobilized cells of P7

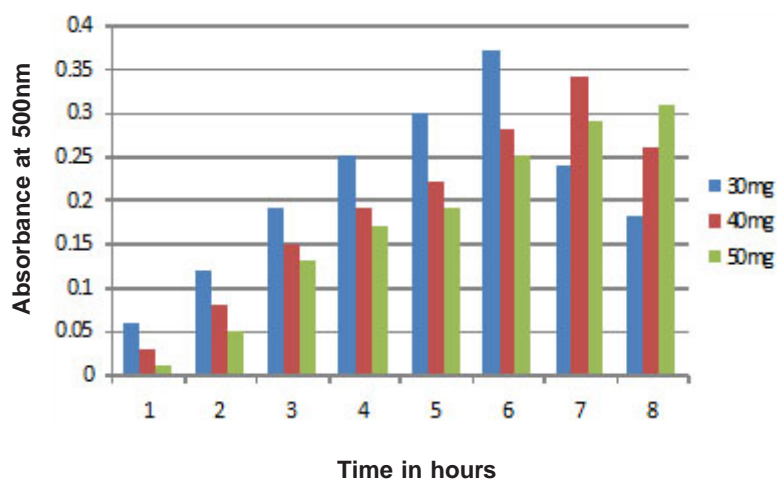
#### Degradation studies by immobilized cells (4-amino antipyrine method)

The degradation of phenol and naphthalene in different concentration were added to mineral media by the potential isolates was studied by the colour reaction of chemical (phenol and naphthalene) with potassium ferric cyanide at pH 7.9. The optical density value was measured for the change in colour by using colorimeter. Recently, for the better advantage in degradation of various chemicals the free microbial cells were immobilized. So that they can be reused for the cycle and compare to free cells these shows on better results. (Figs. 5 to 8) *Arthrobacter* sp is immobilized

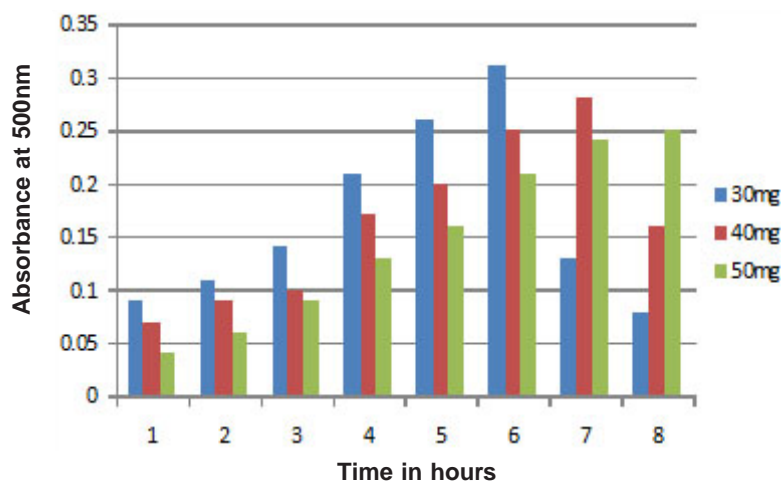
for the phenol degradation studied<sup>14</sup>. Free and alginate entrapped *Pseudomonas* sp is used for the naphthalene biodegradation<sup>15</sup>.

#### Identification of potential strain

Based on the phenotypic studied, the potential strains were identified as *Bacillus* sp (P7). *Brachybacterium* sp (P10). In this study, *Bacillus* (P7) and *Brachybacterium* (P10) are the potential strain for degradation process. In which, *Bacillus* is already reported in the phenol and toluene degradation<sup>4</sup> and *Brachybacterium* is a new strain to degrade phenol and naphthalene.



Graph 4: Naphthalene degradation by free cells of P10



Graph 5: Phenol degradation by immobilized cells of P7

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