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Isolation and identification of a bacterial strain from soil for bioremediation of phenol for pollution control

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Phenol is one of the most widely distributed environmental pollutant found in industrial wastewater capable of causing harm to flora and fauna. These hazardous compounds can be removed from the nature by various ways but one of the most efficient methods is bioremediation. In order to isolate most potent bacterial strain from soil sample, was collected from the hospital area and enriched regularly with 500 ppm phenol for 10 days. After serial dilution isolated colonies were transferred to slant. Screening was done to isolate most potent strain. Residual phenol concentration was estimated spectrophotometrically. The strain marked as P108 was found having almost 98.08% of phenol removal efficiency in 24 h at 37°C temperature and pH 6.8. Morphological, Biochemical, 16S rDNA Sequence and Phylogenetic Analysis was conducted to characterize and identify the strain. It was found that the isolated *Pseudomonas* sp. P108 strain have a significant similarity with *Pseudomonas otitidis* strain MCC10330. The isolated strain was acclimatized in minimal salt media (MSM) for about 2 months in 700 ppm phenol concentration for better efficiency.

Keywords: Phenol, bioremediation, enrichment, *Pseudomonas otitidis*, Phylogenetic Analysis.

1. Introduction

From chemical, pharmaceutical and oil refinery industry many aliphatic and aromatic hydrocarbons are released which are very hazardous for the nature[1]. One of the pollutants is phenol which can damage the gastrointestinal tract. It can cause irritation of respiratory tracts and muscle tremors. It can also cause damage of liver, kidney and nervous system. Phenol is very much hazardous to the aquatic ecosystems[2]. So it is mandatory that the phenol and phenolic wastes should be treated properly before disposal into the nature[2,3]. As per Central Pollution Control Board (CPCB), the maximum permissible level of phenol in inland water is ~1 ppm[4–6]. The safety limit of phenol in drinking water should not be more than 1 mg/L as per World Health Organization (WHO)[7–9].

Phenol can be treated by methods like adsorption, chlorination, ozonation as well as many physicochemical methods[2,10] but due to high cost of these methods and production of toxic intermediates, biological methods like biosorption, biodegradation, bioaccumulation etc. involving bacteria, algae, fungi etc. are necessary[2]. Studies have been done on biodegradation of phenol using *Pseudomonus* sp. NBM11 which was able to degrade up to 1000 ppm phenol completely in the temperature ranging between 30°C–32°C and pH 6.8–7.211. *Pseudomonas aeruginosa*, isolated from industrial soil was found to remove catechol[12].

The current study has been carried out aiming to isolate the most potent bacterial strain to remove phenol and the identification of the isolated strain.

2. Materials and methods

Since the bacterial strains were collected in plate culture in the form of their individual colonies and then transferred to the slant culture, therefore it was necessary to prepare the medium for slant and plate culture. Moreover, for the selection of most potent bacterial strain for phenol removal from aqueous solution, a fermentation medium was prepared. The same fermentation medium was used for preparing the in-

oculum. The composition of the plate, slant culture medium, fermentation and inoculum medium are described in 2.1 and 2.2 respectively.

(2.1) Preparation of medium for plate and slant culture:

The medium composition of slant and plate culture was (g/L) – Peptone : 5.0; Beef extract : 3.0; Agar : 30; pH : 6.8–7.2. All the constituents except agar were dissolved in water and pH was adjusted to 6.8–7.2 using dilute hydrochloric acid and dilute sodium hydroxide. Then the agar was added and the whole solution was kept in boiling water bath until the whole agar melted. 5 ml of the hot medium was transferred to each of the test tubes and were cotton plugged, wrapped with brown paper and sterilized at 121°C for 15 min. 16 ml of molten medium was taken in each of the several test tubes to use in plate dilution technique and sterilized as usual.

(2.2) Preparation of medium for inoculum and fermentation of the selected strain:

Medium composition for preparation of inoculum as well as fermentation was – Peptone: 0.5%; Yeast extract: 0.2%; Beef extract: 0.1%; pH: 6.8–7.2. The medium was prepared by dissolving the weighed amount of those constituents in water. pH was adjusted to 6.8–7.2 using dilute hydrochloric acid and dilute sodium hydroxide. Then 50 ml medium was distributed in each of the 250 ml Erlenmeyer flasks. However all the flasks were cotton plugged and wrapped with brown paper.

All the flasks were then sterilized at 121°C for 15 min.

(2.3) Isolation and screening of most potent bacterial strain:

The bacterial strain capable of removing phenol was isolated by soil enrichment and serial dilution-plate count method. The soil sample collected from hospital area was enriched with 500 ppm phenol for 10 days. After serial dilution and plate count isolated colonies were transferred in individual slant. Total 29 isolated colonies were transferred to slant, out of which 18 slants were obtained finally.

Screening was done to select most potent strain. Each of the isolated colonies were transferred to liquid medium. It was incubated at 37°C for 24 h in presence of 500 ppm phenol. After 24 h the broth was centrifuged. The cell mass was discarded. The clear supernatant was used for spectrophotometric estimation of residual phenol content.

(2.4) Estimation of residual phenol content:

Residual phenol concentration was measured in spectrophotometer at 510 nm wavelength followed by 4-Aminoantipyrine Method[13]. Residual phenol content was calculated from the standard curve made with the known concentrations of phenol.

(2.5) Identification of the isolated bacterial strain:

As per Bergey's Manual of Determinative Bacteriology[14] the morphological and biochemical characterization of the isolated bacterial strain was done. 16S rDNA Sequence and Phylogenetic Analysis of isolated strain was performed as per standard method.

3. Results and discussion

(3.1) Isolation and screening of most active bacterial strain:

To identify the most active bacterial strain for phenol removal, cultures of selected eighteen (18) well-developed isolates from soil enriched sample were grown in nutrient agar medium. About eighteen (18) isolates were cultivated three times in different sets of experiment. Among the eighteen (18) isolates, one with the maximum phenol removal potential has been reported here. The colony size, colony pigmentation, colony form, colony margin and elevation of colonies of eighteen (18) samples of isolates, marked as P1, P6, P7, P15, P22, P23, P30, P31, P32, P38, P40, P50, P66, P72, P95, P106, P108 and P111 respectively are shown in Table 1. The isolated P108 shows the maximum phenol removal potential of 98.08% for an initial phenol concentration of 500 ppm and it was selected for further study.

(3.2) Colony and growth characteristics of the isolate P108:

Experimental results of colony and growth characteristics of strain P108 have been shown in Table 2 and Table 3. For the purpose of identification of the isolated pure culture of strain P108 simple staining and gram staining was done. The microscopic image of the bacterial smear after staining has been shown in Fig. 1 and Fig. 2. The image demonstrated that the cell retain the safranin stain only, thereby appear in pink colour indicates that the bacteria is Gram-

Table 1. Screening of the most potent bacterial strain

Sample strain number	Colony size	Colony pigmentation	Colony form	Colony margin	Colony elevation	% of phenol removal
P1	Moderate	White	Circular	Serrate	Flat	83.16
P6	Small	Light yellow	Circular	Entire	Raised	81.35
P7	Small	White	Circular	Serrate	Unbonate	80.15
P15	Small	White	Circular	Serrate	Flat	83.76
P22	Small	White	Circular	Serrate	Flat	83.46
P23	Moderate	White	Circular	Entire	Flat	83.16
P30	Moderate	White	Circular	Serrate	Flat	90.53
P31	Moderate	White	Circular	Entire	Unbonate	84.69
P32	Large	White	Irregular	Serrate	Flat	91.22
P38	Moderate	White	Irregular	Serrate	Flat	89.41
P40	Moderate	White	Circular	Undulate	Flat	87.34
P50	Moderate	White	Irregular	Serrate	Flat	88.69
P66	Moderate	White	Circular	Entire	Flat	95.85
P72	Moderate	White	Irregular	Serrate	Flat	96.96
P95	Large	Reddish yellow	Circular	Serrate	Flat	95.88
P106	Large	White	Irregular	Serrate	Flat	97.92
P108	Large	White	Circular	Serrate	Flat	98.08
P111	Moderate	White	Circular	Serrate	Flat	97.14

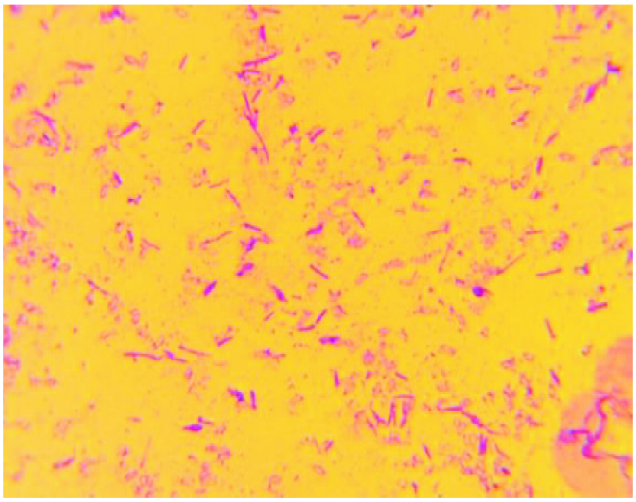

Fig. 1. Simple staining of the strain P108.

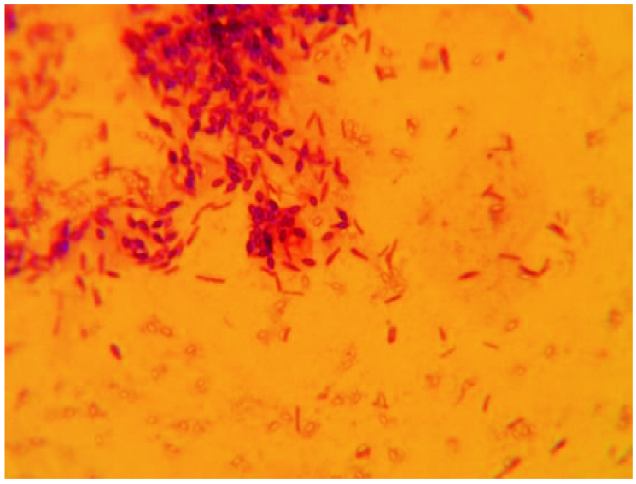

Fig. 2. Gram staining of the strain P108.

negative. Different biochemical tests such as ammonia from arginine, arginine used as a sole source of energy, nitrate reduction test, catalase reaction test, indole formation test, litmus milk test, Voges-Proskauer test, carbohydrate fermentation test, urease test, starch hydrolyses test and few growth tests in different medium were performed by using isolated pure culture of strain P108 and results are given in Table 4.

(3.3) 16S rDNA sequence and phylogenetic analysis of isolated strain P108:

The nucleotide homology and phylogenetic analysis was carried out with the 1430bp 16S rDNA gene sequence of the isolated strain P108 (Gel image of the 16S rDNA amplicon is shown in Fig. 3) and it was found that the isolated *Pseudomo-*

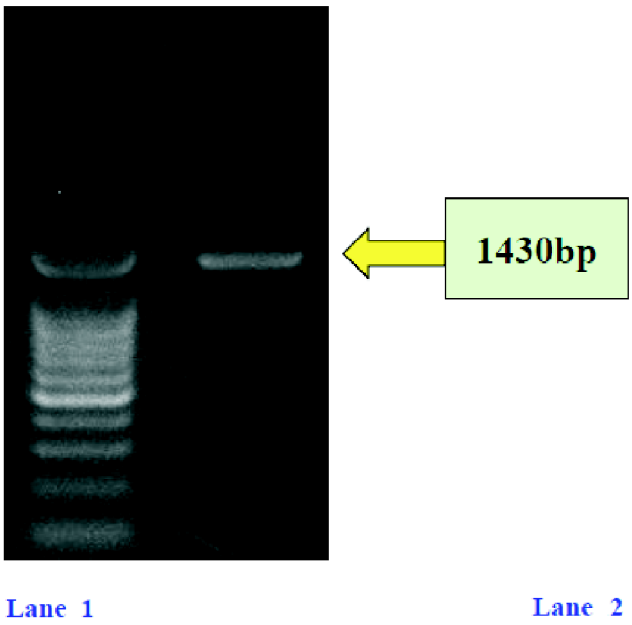

Fig. 3. Gel image of 16S rDNA amplicon of isolate P108. [Lane 1: DNA Marker and Lane 2: 16S rDNA amplicon Band].

nas sp. P108 strain (Gen Bank accession number NR_043289.1) have a significant similarity with *Pseudomonas otitidis* strain MCC10330. Identity analysis on the EZ taxon server[15] revealed that the 16S rDNA gene sequence had closest similarity (99.71%) with the gene sequence of the type strain of *Pseudomonas otitidis* strain MCC10330 refer in Table 5. The phylogenetic position based on the NJ algorithm of the isolate P108 having NCBI Gene Bank accession number NR_043289.1 has been shown in the dendogram (Fig. 4).

Table 2. Colony characterization of the isolated bacterial strain

Colony characteristics	P108
a. Size	>1 mm
b. Opacity	Translucent
c. Surface growth	Smooth
d. Edge	Diffuse
e. Consistency	Good
f. Pigmentation	Nil

Table 3. Growth characterization on slant

Colony characteristics	P108
a. Opacity	Translucent
b. Surface growth	Smooth
c. Consistency	Diffuse
d. Color	Good
e. Pigmentation	Nil

Table 4. Biochemical characteristics of isolate P108

Parameters		Characteristics of P108	
1.	Ammonia from Arginine	Positive	
2.	Arginine used as sole source of energy	Positive	
3.	Nitrate reduction	Positive	
4.	Catalase reduction	Negative	
5.	Carbohydrate fermentation	Acidity	Gas formation
	a. Fructose	Negative	Negative
	b. Arabinose	Positive	Negative
	c. Galactose	Negative	Negative
	d. Xylose	Negative	Negative
	e. Glucose	Positive	Negative
	f. Lactose	Negative	Negative
	g. Raffinose	Negative	Negative
	h. Sucrose	Negative	Positive
	i. Maltose	Negative	Negative
	j. Dextrin	Negative	Negative
	k. Salicin	Negative	Negative
	l. Mannitol	Negative	Negative
	m. Glycerol	Negative	Negative
	n. Inositol	Negative	Positive
6.	Indole formation	Positive	
7.	Litmus milk test	Positive	
8.	Starch hydrolysis test	Negative	
9.	Urease test	Negative	
10.	Voges – Proskauer test	Negative	
11.	Growth under anaerobic condition	Negative	
12.	Growth at different temperatures	Positive	
13.	Growth at extreme pH and NaCl conc.	Positive	

Table 5. Results of the identity analysis of strain *P. seudomonas* sp. P108 strain based on the EzTaxon server in relation to the pairwise similarity with other strains

SL. No.	Similar Species According to Rank	NCBI Accession No.	Pairwise Similarity (%)
1.	*Pseudomonas otitidis* strain MCC10330	NR_043289.1	99.71%
2.	*Pseudomonas aeruginosa* strain DSM 50071	NR_117678.1	98.55%
3.	*Pseudomonas aeruginosa* strain ATCC 10145	NR_114471.1	98.53%
4.	*Pseudomonas resinovorans* strain ATCC 14235	NR_112062.1	98.32%
5.	*Pseudomonas aeruginosa* strain NBRC 12689	NR_113599.1	98.53%
6.	*Pseudomonas guezennei* strain RA26	NR_114957.1	99.47%
7.	*Pseudomonas stutzeri* ATCC 17588 = LMG 11199	NR_103934.2	97.53%
8.	*Pseudomonas alcaligenes*	NR_117827.1	97.66%
9.	*Pseudomonas stutzeri* strain VKM B-975	NR_116489.1	97.39%
10.	*Pseudomonas aeruginosa* strain DSM 50071	NR_026078.1	97.31%
11.	*Pseudomonas alcaligenes* strain NBRC 14159	NR_113646.1	97.64%
12.	*Pseudomonas stutzeri* strain NBRC 14165	NR_113652.1	97.50%
13.	*Pseudomonas alcaligenes* strain IAM 12411	NR_043419.1	97.10%
14.	*Pseudomonas pseudoalcaligenes* strain Stanier 63	NR_037000.1	97.09%
15.	*Pseudomonas stutzeri* strain CCUG 11256	NR_118798.1	97.56%

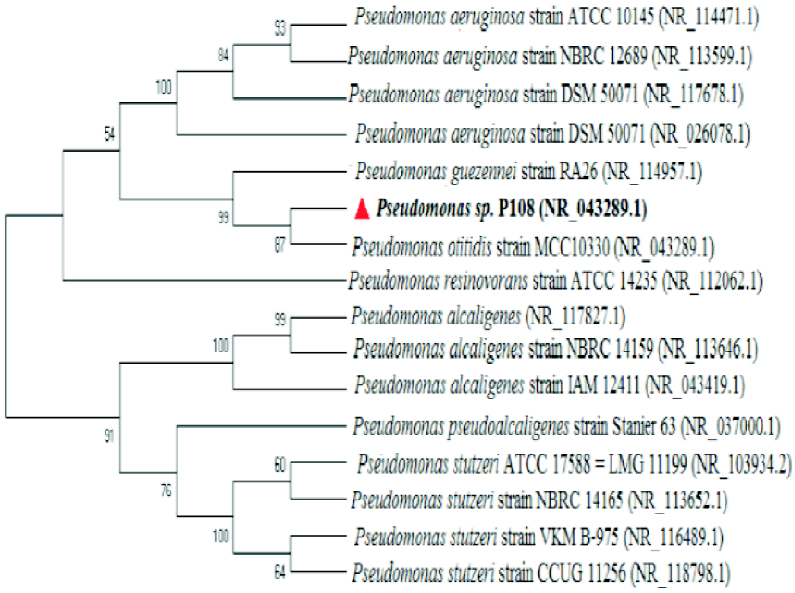

Fig. 4. Unrooted phylogenetic tree based on 16S rDNA sequences obtained by the Neighbor-Joining (NJ) method showing the position of *Pseudomonas* sp. P108 strain among its Phylogenetic Neighbors. NCBI Accession Numbers are provided in Parentheses.

Conclusion

A bacterial strain was isolated from phenol enriched soil and the isolated strain was capable to remove 98.08% phenol from liquid medium. Morphological, biochemical and phylogenetic assay were performed for the identification of the strain. It was found that the isolated *Pseudomonas* sp. P108 strain have a significant similarity with *Pseudomonas otitidis* strain MCC10330. Further for the better efficiency of phenol removal the strain was acclimatized in Minimal Salt Medium (MSM) for 2 months.

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