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Isolation of novel bacterial strains from contaminated soils for phenol biodegradation

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ABSTRACT

Isolation and screening of novel strains of bacteria from natural sources for biotransformation studies were investigated. Two strains were isolated from different contaminated soils around lake side soils of Gandigudem, Isnapur and Kazipally in and around Hyderabad, India. These strains were identified at the Institute of Microbial Technology, Chandigarh, India, basing on physical and biochemical characterization assigned MTCC numbers. These two strains were designated as *Kocuria rosea* MTCC 8311 and *Bacillus cereus* MTCC8312. These strains were used for biodegradation studies.

KEYWORDS

Phenol;
Phenol-degrading bacterial strains;
Isolation;
Screening;
Novel bacterial strains;
Soil;
Biodegradation.

INTRODUCTION

Biotechnology employs microorganism as well as higher cells and their active principles with the aim of achieving desirable conversions of various substrates[1]. The use of novel strains in the biodegradation is an important aspect. Phenolic compounds are introduced in the environment in the waste streams of several industrial operations, through its use as biocides or as by-products of other industrial operations, such as pulp bleaching with chlorine, water disinfections or even waste incineration. Phenols have also been used as general-purpose disinfectants, and it has been found that they can also appear as degradation products of other chlorinated xenobiotics[2]. The two major uses of phenol in 2004 were the production of bisphenol-A (48%) and the production of phenolic resins (25%)[3]. Phenol has been identified in at least 595 of the 1,678 hazardous waste sites that have been proposed for inclusion on the EPA National Priorities List (NPL)[4]. It is found extensively in the environment[5]. The contamination of water and soils by phenol and its derivatives is a matter of serious environmental concern due to their toxic effects. Phenol is highly carcinogenic compound recognized by EPA. Prompt removal of these substances from the environment is very important since the toxic effects of phenol are pronounced even at very low concentrations[6]. Case reports of humans exposed to phenol show several adverse effects[7-9].

Although physical and chemical methods are in vogue for removal of phenol and its derivatives, biodegradation methods are less expensive and can potentially degrade these contaminants to innocuous products. Microbial and fungal degradation of phenol and chlorophenols have been reported by several groups [10-16]. The occurrence of phenol in various environmental

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matrices of Hyderabad City is well documented[17,18]. Though biological degradation of phenol by different bacterial species have been extensively investigated, studies on its degradation by pure strain of *Bacillus cereus* is available less in international context and degradation by pure strain of *Kocuria rosea* is not available much in Indian and international context. The objectives of the present study are isolation of novel strains and examine the efficiency of these strains in degrading phenol in spiked garden samples.

EXPERIMENTAL

Materials and methods

Chemicals

Phenol (99% assay) was obtained from Merck chemicals Ltd. All other chemicals used were of highest purity and available commercially.

Sampling

Soil samples were collected from lakesides in the three major industrial estates Gandigudem, Isnapur and Khazipally to isolate efficient phenol degrading strains. A soil profile one to twenty cm under the surface, 300g soil was sampled using a sterile soil kit. Three sub samples were taken from each location and mixed to achieve a composite sample in a sterile plastic bag. These samples were stored in 5^0C until further analysis. All EPA methods are followed for characterization of soils.

Soil analyses procedures

(a) pH

10grms of the soil was weighed and dried in an oven, to this add 100 ml distilled water was added and the solution was stirred. The standard buffer solution of pH 4.0 was taken into a beaker and was inserted in the pH electrode. If the instrument reads a value less or more than 4.0.The instrument is now calibrated and ready for use. The pH of soil solution was then taken.

(b) Bulk density

The sample collected is dried until constant weight is obtained. Put a little dry soil to a measuring cylinder and record the volume. Find out the weight of this volume. A unit volume expressed as g/cm^3.

(c) Specific gravity

It has been directly related to bulk density. It has been used as an index of some aspects of soil quality as in the case of bulk density.

Procedure

Collect the sample and dry it in an oven until we get a constant weight. Fill a preweighed glass bottle of known volume with dried soil and note down its weight. Fill it with distilled water weight and final weight was taken.

(d) Moisture content

An empty Petri plate was weighed (W1 g). A known amount of soil sample was placed on the Petri plate and their combined weight was taken (W_2 g). This was then placed in the hot air oven at 105^0C for about an hour. The Petri plate was then weighed again (W_4). Moisture helps in control of important factors, which are essential to normal plant growth, soil, air and soil temperature.

(e) Alkalinity

10 g of the soil was weighed and 100 ml of distilled water was added to it and shaken well for half an hour. Filtered through wattman filter paper note the volume of filtrate.10ml of the sample extract was taken in a conical flask 2-3 drops of phenolphthalein indicator was added. The color was not changed. To the above sample solution 2-3 drops of methyl indicator was added the color changed to yellow. This was titrated against standardized H_2SO_4. The end point is orange from yellow. The titrations were repeated till two consecutive readings were obtained and the values were recorded.

(f) Chlorides

10 ml of the soil sample extract was taken and to it 2-3 drops of potassium chromate indicator was added. The color changed to yellow. This was then titrated against $AgNO_3$ till the end point red has reached.

(g) Sulfates

5 ml of soil extract is diluted to 50 ml in standard flask to this 25 ml conditioning reagent is added to this a pinch of $BaCl_2$ was added. The spectrophotometric reading was read at 420 nm. The concentration for the sample extract with and without reagents was read.

(h) Organic matter

0.1 g of the soil was weighed and placed in a conical flask to it 10 ml of $K_2Cr_2O_7$ was added, followed by 20 ml of conc.H_2SO_4. The apparatus was kept for digestion for half an hour. To this 200 ml of distilled water was added followed by 10 ml of the H_3PO_4, 0.2 g of NaF and 1 ml of diphenylamine indicator. This was then titrated against 0.05N FAS till the end point dark green color has reached from pale green color through turbid blue. A blank was also was also prepared similarly.

(i) Nitrate

In a conical flask 10 g of soil was taken fallowed by 50 ml extraction reagent then keep for shaking about 15 min, then 0.4 g $Ca(OH)_2$ was added again kept for shaking for 5 min, then 1 g magnesium carbonate is added, filter the content. Dilutions were made in 1:10 v/v ratio (i.e., 5 ml sample in 50 ml dist., water). In to the diluted sample (10 times) 1 ml 1N HCl was added then concentrations were observed in spectrophotometer at 220 nm.

(j) Ca and Mg

In a conical flask 50 g soil followed by 100 ml 1N Ammonium acetate solution, kept in a shaker for 15 min and filtered and volume noted down the content.

Mg hardness: In a conical flask 10 ml soil extract sample, 1 ml ammonia buffer and pinch of EBT indicator was added (pinkish red appears), and titrated against EDTA, pinkish red changed to red, Black was also rundown.

Ca hardness: In a conical flask 10 ml soil extract sample 1 ml NaOH, followed by Mureoxide indicator and titrated against EDTA. Pink colour changed to purple.

(k) Total nitrogen

1 g soil taken in Kjeldahal vials and then 4 g of catalytic mixture and 10 ml Conc., H_2SO_4 was added to vial and then placed in digestion apparatus for 1 h at 350^0C. The flasks were then cooled and distilled in distillation apparatus in alkaline medium and liberated NH_3 was collected in 0.02 N H_2SO_4, then back titrated against NaOH till orange color was obtained. Total nitrogen is calculated using formula.

Phenol analysis by anti pyrine method

Take 100 ml distillate or an aliquot of the distillate containing not more than 0.5 mg phenol and diluted to 100 ml in a 250 ml beaker. Prepare a 100 ml distilled water blank and a series of 100 ml phenol standards containing 0.1, 0.2, 0.3, 0.4, and 0.5 mg phenol. Add 2.5 ml 0.5N NH_4OH solution in each and adjust the pH to 7.9 ± 0.1 with phosphate buffer. Add 1.0 ml 4-aminoantipyrine solution and 1.0 ml $K_3Fe(CN)_6$ solution in each flask and mix well. Measure the absorbance of each after 15 minutes against the blank at 500 nm. Plot absorbance against microgram phenol concentration and draw a calibration curve. Estimate sample phenol from photometer reading by using a calibration curve.

Preparation of mineral media

Mineral media A: It contains 0.2 g magnesium sulphate ($MgSO_4.7H_2O$), 0.05 g of calcium chloride ($CaCl_2.2H_20$), 0.5 g of potassium chloride (KCl) and 1 g of ammonium chloride (NH_4Cl) per liter.

Mineral media B: It contains 2.5 g ferrous sulphate ($FeSO_4.7H_2O$), 1.5 g EDTA, 1.0 g of copper sulphate ($CuSO_4.5H_2O$), 2.5 g of zinc sulphate ($ZnSO_4.4H_2O$), 2.5 g of manganese sulphate ($MnSO_4.4H_2O$), 0.5 g of cobalt chloride ($CoCl_2.6H_2O$) and 1.0 g of boric acid (H_3BO_3) [pH 6.8-7.4]. 1 ml of mineral media B was added to mineral media A and made up to 1 lt. The mineral media are maintained at neutral conditions (pH 6.8-7.4).

Isolation and screening of bacteria

Briefly, 5 g of above three soils was added to 100 ml of nutrient broth in three different flasks aseptically and incubated for 48 h at 25^0C in rotary shaker 50 rpm/min. After 48 h 5 ml supernatant of above culture is again transferred into 3 different flasks containing 100 ml nutrient broth and incubated for 48 h at 25^0C. Five ml of the above supernatants was transferred in to 10 mg/L phenol containing 100 ml mineral media (pH 6.8) in three different flasks and incubated at 25^0C in rotary shaker to eliminate autotrophs and agar utilizing bacteria. This procedure was repeated twice and isolates exhibiting pronounced growth on phenol were stored for further characterization. A series of morphological and biochemical tests were conducted to characterize

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and identify the phenol degrading bacterial cultures (TABLES 1 and 2).

Characterisation of organisms

Physical characterization

Gram staining

Gram staining is a differential staining in which a depolarization step occurs between application of 2 basic dyes or stains. *Procedure*: Prepare a thin uniform smear on clear slide and heat fix gently. Apply crystal violet for 1 min and wash with tap water. Apply iodine solution for 1 min and decolorize with acetone-alcohol for 20-30 sec. Apply Saffronine for 1 min and wash with tap water and air dry. Examine the preparation under the light microscopic.

Biochemical tests

(a) Indole test

SIM or tryptone broths were inoculated with loopful of test cultures in different test tubes. A un inoculated negative control should also be kept. The tubes are incubated at 27^{0}C for 24h. After incubation Kovac's reagent was added and allowed to stand for 1-5 min. The tubes were observed for the dark red colored ring on the surface layer.

(b) Methyl red test

Tubes with MRVP broth are inoculated with Test cultures and an uninoculated negative control should also be kept. These were then incubated at 37^{0}C for 48h. Following incubation add 5-6 drops of methyl red solution into each tube. Observe the tubes for the color change.

(c) Voges proskaver test

Tubes of MRVP broth were inoculated with test cultures. A negative control should also be kept. These tubes are incubated at 37^{0}C for 48h. After incubation Baritt's reagent was added. Tubes are gently shaken to aerate and stand for 1 h. Tubes are then observed for the color change.

(d) Citrate utilization test

The tubes with simmon's citrate agar slants are streaked with test cultures and incubated at 37^{0}C for 48 h. An uninoculated negative control should also be kept. Tubes are observed for color change.

TABLE 1: Characterisation of soil

S.no	Parameter	IS	KZ	GG	Garden soil
1	pH	7.24	6.50	7.40	7.37
2	Bulk Density	1.238	1.161	1.194	1.216
3	Specific Gravity	1.28	1.24	1.30	1.22
4	Moisture content (%)	19.5	18.7	16.4	27.0
5	Alkalinity(mg/g)	0.64	0.61	0.82	1.01
6	Chlorides (mg/g)	0.67	0.56	0.79	0.78
7	Sulphates (mg/g)	0.21	0.56	0.50	0.79
8	Organic Matter (mg/g)	27.16	27.16	20.12	2.15
9	Hardness Ca (mg/g)	0.50	0.14	0.36	0.71
	Hardness Mg (mg/g)	0.02	0.08	0.09	0.15
9	Nitrates	0.32	0.28	0.30	0.92
10	Total nitrogen (mg/g)	21.5	22.3	27.4	39.0

TABLE 2: Morphological and cultural characteristics of the isolates for identification of strain

S.no.	Morphological tests	Strain 1(PL1)	Strain 2(PL2)
	Colony morphology		
1	Configuration	Circular	Circular
2	Margin	Entire	Rhizoid
3	Elevation	Low convex	Umbonate
4	Surface	Smooth & glistening	Dry with central elevated portion like water droplet
5	Pigment	Pastel Orange	-
6	Opacity	Opaque	Opaque
7	Gram staining	+	+
8	Cell shape	Cocci	Rods
9	Size(μm)	Diameter:~1.0μ	Length:3-4? Width:~1.0μ
10	Arrangement	Pairs &tetrads& clusters	Singles, pairs & short chains
11	Vacuoles	-	+
12	Spores	-	+
	Endospore		+
	Position		Central
	Shape		Oval
	Sporrangia bulging		-
13	Motility	-	+

(e) Gelatin liquefaction test

Nutrient gelatin deeps were inoculated with test cultures and incubated at 37^{0}C for 24-28 h. A negative control should also be kept. Following incubation the

tubes are placed in refrigerator at 40ºC for 30 min along with the control. Tubes are then looked for solidification.

(f) Catalase test

The nutrient broth tubes were inoculated with test cultures and incubated at 37^{0}C for 24 h. A Positive control inoculated with the test sample. 3% H_2O_2 is added to the tubes. Tubes were observed for effervescence.

(g) Urease test

Urea agar slants were inoculated with Test cultures and incubated at 37^{0}C for 24 h. A negative control should also be kept. Tubes were observed for color change.

(h) Starch hydrolysis or amylase activity test

Starch agar plates are inoculated by streaking onto plates with different test cultures and incubated at 37^{0}C for 24hrs. A negative control should also be kept. After incubation the starch plates were flooded with iodine solution. The plates were observed for clear zone.

(i) Sulpher reduction test

The SIM agar deeps were inoculated with different test cultures and incubate at 37^{0}C for 4 days. A negative control should also be kept. The tubes were observed for the appearance of darkening along the line of growth.

(j) Nitrate reduction test

The Nitrate broth tubes are inoculated with test cultures and incubated at 37^{0}C for 24 h. A negative control should also be kept. After incubation the tubes were then observed for gas formation.

(k) Ammonification test

Peptone broth tubes were inoculated with test organism and incubated at 37^{0}C for 24 h. A negative control should also be kept. After incubation Nessler's reagent was added into the tubes. The tubes are observed for the brown precipitate.

Effect of pH, temperature and NaCl (%) on growth of isolates PL1, PL2

Effect of pH on growth was studied by adjusting pH of broth ranging from 4-10. Effect of temperature on growth was studied by incubating the culture media at temperatures ranging from 8^{0}C-52^{0}C. Effect of NaCl (%) on growth was studied by incubating the culture media at NaCl (%) ranging from 2-10. After 48 h of incubation, the culture was observed for growth.

Bioaugmentation studies for removal of spiked phenol from the soils

Soil samples were taken in to plastic trays (30 15 cm) and spiked with phenol (1 mg/1g). Subsequently, 5% pure cultures of the two strains and their mixed consortia is inoculatedin these spiked soil samples separately, positive control and control were also maintained simultaneously.

The phenol degradation and direct total microbial count for every one-week for a period of 10 weeks were conducted to evaluate the ability of microorganisms to degrade the contaminants. Soils were mixed once in every three days to provide sufficient air and oxygen. The soils were moistured by the addition of 10 ml of deionised water every 3 days until the end of experiment. Before experiment is conducted the soils are sterilized, by taking soil samples in a plastic bags and placing them in 1 lt beakers covered by aluminium foil and autoclaved.

RESULTS AND DISCUSSION

Three different lake soils were taken, each 5 g of soils were transferred into sterile water taken in sterilized 100 mL conical flasks. The flasks were incubated at room temperature for two to three days. Later, by using micropipette, 1000 µL of suspension was transferred in to 100 mL sterile nutrient broth medium containing conical flasks. These were incubated at room temperature for 2-3 days, by using micropipette, 100 µL of nutrient broth was transferred in to sterile nutrient agar medium (previously added with phenol) containing petri plates. The petri plates were incubated at room temperature for two to three days. The colonies obtained on incubation were subjected to morphological studies, biochemical studies and purity studies. Pure bacterial colonies were used for the study. From pure bacteria growth sub-culturing was done on nutrient agar slants. Those cultures were maintained in the laboratory and used in the entire study.

We could isolate two different bacterial cultures which have been identified as *Kocuria rosea*

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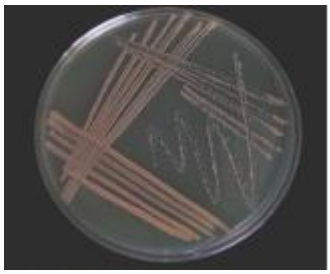

Figure 1: Isolate *Kocuria Rosea* Mtcc8311 Mtcc8312 on nutrient agar

Figure 2: Isolate *Bacillus Cereus* Mtcc8312 MTCC8312on nutrient agar

MTCC8311 and *Bacillus cereus* MTCC8312. These cultures are shown in figures 1 and 2. Since biodegradation of phenol using these bacterial strains have not been reported earlier, Baker et al. 1980; Bollag et al. 1986; Borthwick et al. 1978; Chu et al. 1973; Chun Chin Wang et al. 2000; Colores et al. 1995; Cortes et al. 2002 Used different bacteria and fungi for biodegradation of phenol and chlorophenol. The occurrence of phenol in various environmental matrices of Hyderabad City is well documented Anjaneyulu et al. 2002; CPCB 2002.

Characterization of phenol degrading bacteria

Two isolates namely *Kocuria rosea* (Strain 1) and *Bacillus cereus* (Strain 2) were obtained in the present study by selective enrichment methods using phenol as sole carbon source. Strains 1 and 2 are gram positive. Morphologically, colonies of strain 1 is circular, Entire, low convex ,smooth glistening, pastel orange(pigment), opaque whereas strain 2 exhibited a circular, rhizoid, umbonate, dirty white, opaque and central elevated portion like a water droplet appearance. Apart from this, in these two strains strain1exhibited a cocci and strain 2 exhibited rod shape, both were motile, which was conformed by hanging drop technique. All the two strains tested positive for Voges Proskauer tests, starch hydrolysis, nitrate reduction, Catalase test, citrate utilization, oxidase test, tyrosine degradation and lysine decarboxylase. Growth on macConkey agar, indole test, H_2S production, esculin hydrolysis, urea hydrolysis, arginine dihydrolase, tween 20 hydrolysis, tween 60 hydrolysis, tween 80 hydrolysis, O/F test, ONPG test and hippurate hydrolysis tests are negative. For methyl red test, casein hydrolysis, gelatin hydrolysis, ornithine decarboxylase, tween 40 hydrolysis and phosphatase test strain 1 gave negative result, where as strains 2 gave a positive result. Based on morphological and biochemical characteristics (TABLES 1-4) these strains were identified as: Strain 1 *Kocuria rosea*MTC*C*8311 and Strain 2 *Bacillus cereus* MTCC8312 respectively.

TABLE 3: Physiological characteristics of the isolates

S.no.	Tests	Strain 1(PL1)	Strain 2(PL2)
1.	**Growth at temperatures**		
	8°C	-	-
	15°C	-	-
	25°C	+	+
	30°C	+	+
	37°C	+	+
	42°C	+	+
	52°C	-	-
2.	**Growth at pH**		
	pH 4.0	-	-
	pH 5.0	-	+
	pH 6.0	-	+
	pH 7.0	+	+
	pH 8.0	+	+
	pH 9.0	+	+
	pH 10.0	+	+
3.	**Growth on NaCl (%)**		
	2.0	+	+
	4.0	+	+
	5.0	+	+
	7.0	W	W
	10.0	-	-
4.	Growth under anaerobic condition	-	-

Phenol degradation by pure and mixed cultures in soil samples

From flask culture studies, it is evident that the maximum concentration that could be degraded by these two strains was 1000 mg/L. Hence, garden soil samples were spiked with 1000 mg/L of phenol (worst case scenario) and the above two strains were examined for their efficiency to degrade phenol in soil matrix and the corresponding results are presented in TABLE 4. At the end of 4 weeks, maximum growth in terms of

TABLE 4: Biochemical characteristics of the isolates

S.no.	Biochemical tests	Strain 1	Strain 2
1	Growth on macconkey agar	-	-
2	Iodole test	-	-
3	Methyl red test	-	+
4	Voges proskauer	+	+
5	Citrate utilization	+	+
6	H_2S production	-	-
7	Gas production	-	-
8	Casein hydrolysis	-	+
9	Esculin hydrolysis	-	-
10	Gelatin hydrolysis	-	+
11	Starch hydrolysis test	+	+
12	Urease test	-	-
13	Nitrate reduction	+	+
14	Catalase test	+	+
15	Oxidase test	+	+
16	Lysine decarboxylase	+	+
17	Arginine dihydrolase	-	-
18	Ornithine decarboxylase	-	+
19	Tween 20 hydrolysis	-	-
20	Tween 40 hydrolysis	-	+
21	Tween 60 hydrolysis	-	-
22	Tween 80 hydrolysis	-	-
23	Sulpher test	-	-
24	Phosphatase test	-	+
25	O/F test	-	-
26	ONPG test	-	-
27	Tyrosine degradation	+	+
28	Hippurate hydrolysis	-	-

TABLE 5 : Percentage removal of phenol in soil by *Kocuria rosea* MTCC8311, *Bacillus cereus* MTCC8312 and mixed culture

Weeks	% Removal by *Kocuria rosea* MTCC8311	CFU	%Removal by *B.cereus* MTCC8312	CFU	Mixed	CFU
1	1.8	2.9×10^{9}	3.1	3.0×10^{9}	2.1	3.1×10^{9}
2	27.5	7.6×10^{10}	20	5.8×10^{10}	28.7	8.3×10^{10}
3	66.5	6.2×10^{11}	37.5	8.2×10^{10}	68.5	7.4×10^{11}
4	87.2	7.5×10^{11}	57.2	5.9×10^{11}	88.7	7.8×10^{11}
5	92	9.2×10^{10}	76.4	6.2×10^{11}	93.4	8.9×10^{10}
6	95.8	4.6×10^{10}	82	8.8×10^{10}	97.8	3.9×10^{10}
7	98.5	8.1×10^{9}	98	7.2×10^{10}	99.4	8.4×10^{9}
8	99.62	2.8×10^{9}	98.2	6.5×10^{9}	99.71	3.9×10^{9}
9	99.68	2.2×10^{9}	99.5	4.2×10^{9}	99.73	3.6×10^{9}
10	99.66	2.6×10^{9}	99.6	3.1×10^{9}	99.7	3.2×10^{9}

CFU was observed in mixed culture with a corresponding degradation of phenol by 88.7% followed by *Kocuria rosea* (87.2%) while the degradation of phenol by *Bacillus cereus* (76.4%) was achieved at the end of 5 weeks. Of the two strains used, *Kocuria rosea* showed maximum growth and degradation efficiency at the end of 4th week. However, interestingly all the strains including the mixed consortia completely degraded phenol at the end of 10th week by 99%. Though the maximum time taken for the complete degradation of phenol is 10 weeks it, however, is significantly degraded by the end of 4-5 weeks by all the strains and mixed cultures (TABLE 5).

CONCLUSION

In conclusion the present procedure for the Isolation and screening of novel strains of bacteria from natural sources for biodegradation studies were investigated. Two strains were isolated from different lake soil sources like Gandigudem, Isnapur and Kazipally. These strains were identified and assigned MTCC numbers at the Institute of Microbial Technology, Chandigarh, India based on physical, morphological, biochemical characterization. These two strains were designated as *Kocuria rosea* MTCC8311 and *Bacillus cereus* MTCC8312. These strains were used for biodegradation studies. Which will be an important addition to the present existing procedures. Degradation of phenol in garden soil samples (spiked with phenol (1000 mg/L)) was examined using Kocuria *rosea* and *Bacillus cereus* individually and their mixed consortia. From this study it can be concluded that though some variations were demonstrated in phenol degrading efficiency by pure cultures and mixed consortia. Among the two strains *Kocuria rosea* showed maximum efficiency in phenol degradation because at the end of 5th week it achieved 92 % degradation this indicate less time was required for *Kocuria rosea* followed by *Bacillus cereus* species suggesting that these strains can be applied for remediation of phenol-contaminated soils. The maximum time needed to degrade phenol completely in soil samples was in 10 weeks. Further research is warranted to substantiate the present findings.

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