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Assessment of phenol biodegradation capacity of indigenous bacteria isolated from sewage treatment plant

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Among different xenobiotics, phenol is a man made as well as a naturally occurring aromatic compound and an important intermediate in the biodegradation of natural and industrial aromatic compounds. The investigation was undertaken to isolate, characterize and exploit phenol degrading bacteria from sewage treatment plant (STP) (artificial ecosystem having diverse group of bacteria which are adapted to different aromatic pollutant and capable to degrade xenobiotic aromatic organic pollutants). Out of five different phenol degrading bacteria, one potent strain (IS-3) was identified as *Citrobacter freundii* with maximum degradation capacity of 1000 ppm phenol in three days under *in vitro* studies. Phenol degradation performance was greatly influenced by different physical factors like incubation temperature, supplemented glucose, nitrogen source, NaCl and pH of the growth medium. The maximum phenol degradation was observed at incubation temperature of 33°C, 7.5 pH of the medium, 0.1 gl^{-1} of NaCl, 0.25 gl^{-1} of glucose and 0.25 gl^{-1} of ammonium sulphate. We report in this study that the identified potential strain (IS-3) can be used for treatment of phenol contaminated waste water maintaining above the mentioned optimum factors for faster degradation of the phenol contaminated industrial effluents.

Key words: Xenobiotics, phenol, bio-degradation.

INTRODUCTION

With the advent of modern technology and industrialization, contamination of the natural resources by chemicals has become a serious concern. A huge quantity of xenobiotic compounds especially phenolic compounds are introduced into the ecosystem every day due to the production of agricultural, industrial and pharmaceuticals products. These phenolic compounds are often found in waste water from coal-gasification,

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coke-oven batteries, refinery and petrochemical plants and other industries, such as synthetic chemicals, herbicides, pesticides, antioxidants, pulp and paper, photo developing chemicals, etc. (Kavita and Palanivelu, 2004; Jayachandran and Kunhi, 2008). Phenols released into the environment from different industries are of high concern because of their potential toxicity. Many of the phenolic compounds are carcinogenic and are included in the U.S. Environmental Protection Agency's priority pollutant lists (Zhang and Wiegel, 1990; Neumaan et al., 2004) and these toxic and xenobiotic chemicals cause challenging problems for the environment due to recalcitrant in nature. According to WHO (1994), the concentration of phenol has been prescribed as 1 µgl^{-}1 in drinking water. Therefore, the removal of such chemicals from industrial effluents is of great concern. The annual production of phenol is around 8.9 million tonnes in 2012 which is again expected to be increased to 10.7 million tonnes in 2016 (Anonymous, 2015). Phenol and their derivatives are aromatic organic compound and resistant to natural biodegradation and persist in the environment for long period (Juang et al., 2006). Current methods for removing phenols from wastewater include hybrid process (Bodalo et al., 2008) like electro catalytic degradation (Wang et al., 2009), adsorption on to different matrices, chemical oxidation, solvent extraction or irradiation (Spiker et al., 1992). Most of these processes are expensive and also generate other toxic byproducts (Ajay et al., 2004). One of the cheapest possible solutions to resolve phenol contamination problem is bioremediation using vast and diverse microbes (Basak et al., 2014). Biodegradation is a viable bioremediation technology for organic pollutants by using microorganisms to remove or detoxify toxic or unwanted chemicals in an environment. A goal of bioremediation is to transform organic pollutants into harmless metabolites or mineralize the pollutants into carbon dioxide and water (Rittman and McCarty, 2001). Work on phenol biodegradation has been reported by using pure and mixed cultures of bacteria which are able to thrive on high concentration of phenol (Gonzalez et al., 2001; Shen et al., 2009; Basak et al., 2014). Wang et al. (2007) isolated a new phenol-degrading bacterium *Acinetobacter* sp. strain PD12 from activated sludge and reported that there still need to isolate new phenol-degrading bacteria that can grow at elevated concentration of phenol. Besides these, the other optimum physical conditions like temperature, pH, additional substrate supplementation on biodegradation efficiency of naturally occurring microbial strains can also provide further insight into the bioremediation process. Therefore, the present investigation was carried out to isolate and to characterize phenol degrading indigenous bacteria from sewage treatment plant (STP) and to study degradation kinetics of phenol degradation under different growth conditions (pH, incubation temperature, additional nutrient sources, salt amendment and additional different carbon sources).

MATERIAL AND METHODS

Site description and sample collection

To isolate potent bacteria with phenol degrading capacity, sewage water sample was collected from sewage treatment plant of Saidpur, Patna, Bihar, India (latitude 25°34' 31" N and longitude 85°05'52" E). The initial phenol concentration of the sewage water (before treatment) was 453 ppm. The waste water was stored at 4°C in a closed and previously sterile bottle for further analysis.

Growth medium and isolation of phenol degrading bacteria

Indigenous bacteria of sewage waste water were isolated in mineral salt agar medium supplemented with phenol concentration of 200 mg l^{-1}. The compositions of mineral salt medium (MSM) in g l^{-1} were KH_2PO_4 (0.42), K_2HPO_4 (0.375), $(NH_4)_2SO_4$ (0.244), NaCl (0.015), $CaCl_2.2H_2O$ (0.015), $MgSO_4.7H_2O$ (0.05) and $FeCl_3.6H_2O$ (0.054). Sewage water sample was serially diluted, spread plated and incubated at 30°C.

Morphological characterization

The isolated colonies of each pure strain were streaked upon solid agar plates for detailed morphological characterization. The colonial shape, color, texture and opacity were recorded following standard protocol (Pelczar and Reid, 1958). Based on morphological difference, five different bacterial strains were isolated. Gram nature of isolates has been carried out using Gram staining and individual cellular morphology in presence and absence of phenol were studied using scanning electron microscope (Eichorst et al., 2007). The pure cultures were maintained in serum bottles in mineral salt medium (MM1) containing 200 ppm phenol.

Screening of potential phenol degrading bacteria

In this method, best phenol degrading isolate was enriched by continuously supplying small portions of phenol to the medium. All the five isolates were spread and grown in the MM1 media plates enriched with 200, 500, 800 and 1000 ppm of phenol. The isolate which survived in the maximum concentration of phenol exposure was selected for further study.

Characterization and identification of the phenol resistant isolate

The isolates were characterized based on their morphology by following Bergey's Manual of Systematic Bacteriology (2001), biochemical characteristics, metabolic versatility for different carbon substrate utilization and Gram's staining. Motility was assessed by direct microscopic observation during growth and by testing the ability of the strains to migrate from the point of inoculation through semisolid agar (0.3%) plates containing 20 mM succinate (Adler, 1966). The isolates were verified by using BIOLOG plates (BIOLOG, Hayward, CA, USA), Analytical Profile Index (API) scheme for characterization.

BIOLOG identification of bacteria

Bacteria demonstrating consistent biodegradation capacity for

Figure 1a. Biolog MicroTitre Ecoplate for bacterial identification.

phenol were selected for confirmation of its identification using the Microlog™ system (BIOLOG, Inc., Hayward, CA). Single colonies were obtained by streaking on media and the following steps were performed in the process of identification: (i) bacteria were streaked onto BIOLOG universal growth (BUG) agar medium (BIOLOG, Inc.); (ii) approximate bacterial number was quantified by serial dilution and plate technique and 150 µl of the bacterial solution were pipetted into each of the 96 wells in the BIOLOG microplates (Figure 1a); (iii) the plates were incubated at 30°C for 16–24 h and then read with an automated ELISA plate reader (BIOLOG, Inc.) and also assessed visually and identified to species level.

Estimation of phenol biodegradation

Residual phenol concentration was measured following 4-aminoantipyrine colorimetric method based on the standard methods for the examination of water and wastewater (APHA, 1998) with little modifications (wavelength 500 nm). The analytical procedure included the following chemicals *viz.* 0.5 N sodium bicarbonate, 0.6% (w/v) 4-aminoantipyrine, 2.4% (w/v) potassium ferrocynate. For quantification of phenol in broth cultures, 2 ml broth culture was withdrawn in a micro-centrifuge tube and centrifuged at 12000 rpm for 3 min. 20 µl of this supernatant was added with 80 µl distilled water to make 1:5 dilution. These were then used for phenol estimation by antipyrine method (APHA, 1998).

Optimization of physical factors for phenol degradation by the isolates and data analysis

The optimum physical factors such as pH of the medium, temperature, carbon source, NaCl and ammonium sulphate (as nutrient source) were evaluated for maximum phenol degradation. Phenol degradation at incubation temperature from 22 to 40°C with constant initial concentration of phenol (200 mg l^{-1}) and neutral pH in absence of carbon was carried out. Similarly, other parameters were kept constant. To see the effect of different pH level on phenol biodegradation, five different pH level (6, 6.5, 7, 7.5 and 8) was maintained. For optimization of glucose as a carbon source, the culture was kept at 30°C with neutral pH and four different glucose concentrations (no glucose, 0.25% glucose, 0.5% of glucose and 1% glucose). The residual phenol concentration was measured at time slots of 24, 48, 72 and 96 h after inoculation with pure isolates. Each level of factors was replicated three times. Data generated from the experiment was analysed statistically using statistical software INDOSTAT (version 8.0).

RESULTS AND DISCUSSION

Characterization and screening of phenol degrading isolates

In our study, the treated sewage water and sediment of Saidpur STP of Patna, India was used for isolation of bacterial strain. Initially, a total of ten strains were isolated among which five isolates (IS-6, IS-7, IS-8, IS-9 and IS-10) showed identical characteristics. The remaining five isolates (IS-1, IS-2, IS-3, IS-4 and IS-5) showed distinct colony morphology and preliminarily chosen for phenol degrader (Table 1). These five bacterial strains were found capable to use phenol as a sole source of carbon and energy. All the isolates except IS-3 were found as single celled, mono-cocci. IS-3 was found as single celled, rod shaped Gram-negative bacilli. Scanning Electron Microscopic (SEM) view confirmed that cell size of IS-3 varied from 2-5 µM in length. During exponential phase, the cells of IS-3 were found motile by means of several peritrichous flagella (Figure 1b). Motility of IS-3 was least to negligible in the cultures at stationary phase. IS-3 was found as most efficient in terms of phenol degradation as compared to other strains (IS-2, IS-1, IS-4 and IS-5). The phenol degradation rate of IS-3 (18 $mgl^{-1}h^{1}$) was almost thrice than IS-1 and IS-2 and twice than IS-4 and IS-5 (Figure 2). As IS-3 is a native and indigenous bacterial strain isolated from phenol polluted sewage waste water, hence, higher degradation capacity for xenobiotic compounds corresponds to earlier

Table 1. Characterization of phenol degrading isolates of sewage treatment plant waste water (Saidpur, Patna, Bihar, India).

Strain	Colony morphology (in MM1 media)	Cell morphology	Flagella	Optimum pH	Phenol tolerance (ppm)	Generation time (h)
IS-1	Orangish, circular	Mono-cocci	polar	6.5	1000	12
IS-2	Light creemish, circular, transperaent	Mono-cocci	Polar	7.0	1000	12
IS-3	Dirty creemish, circular, transparent	Rod	Peritrichus	7.5	1800	9.5
IS-4	White, circular, wrinkled, opaque	Mono-cocci	Peritrichus	7.0	1200	11
IS-5	Light orangish, small circular, opaque	Mono-cocci	Polar	7.0	1500	12

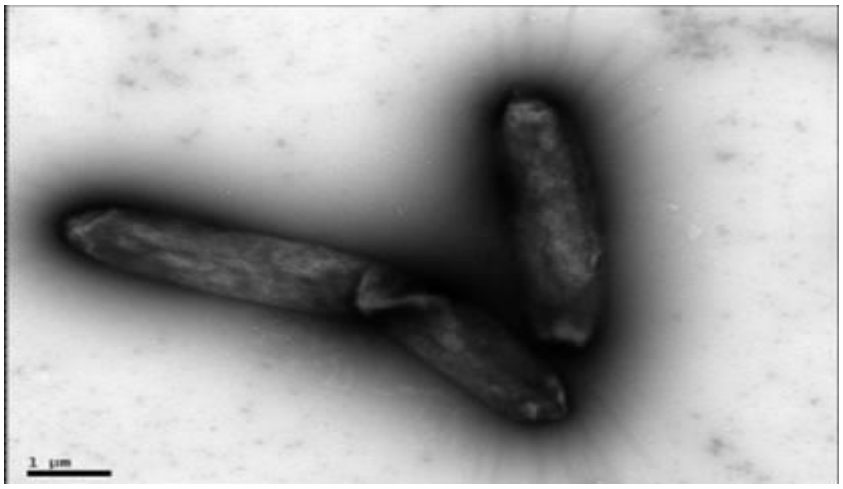

Figure 1b. Scanning electron microscopic (SEM) view of IS-3 (*Citrobacter freundii*).

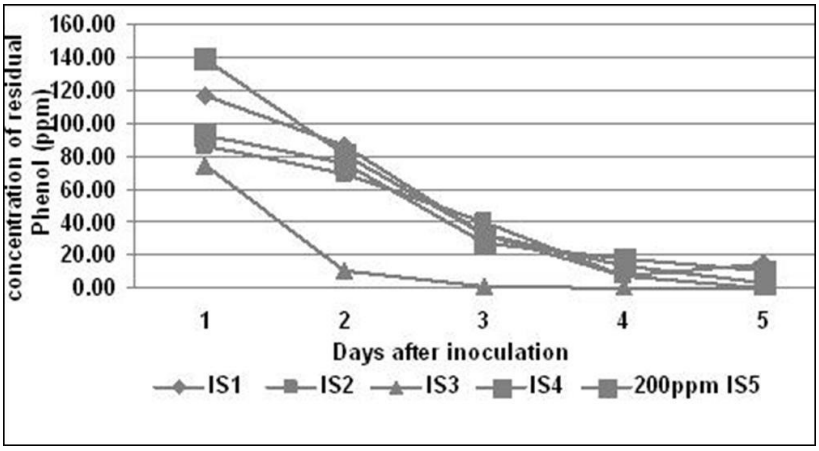

Figure 2. Degradation kinetics of phenol (initial enrichment of 200 ppm) by five different isolates (All the results are average of 3 replications).

Table 2. Metabolic versatility for different carbon substrate utilization pattern (foot print) of the isolates studied by BIOLOG.

Carbon substrates	Isolates				
	IS-1	IS-2	IS-3	IS-4	IS-5
Pyruvic acid methyl ester	+	-	-	+	-
Tween 40	+	-	+	-	-
Tween 80	+	-	+	-	-
α-Cyclodextrin	+	+	+	+	+
Glycozen	+	+	-	-	-
D-Cellobiose	-	-	+	-	+
α -D-lactose	-	-	+	-	+
β- Methyl-D-Glucoside	+	-	-	-	-
D-Xylose	+	+	+	+	-
i-Erythritol	-	-	+	+	+
D-Mannitol	-	-	+	+	+
N-Acetyl-D-Glucosamine	-	-	-	-	-
D-Glucosamine Acid	-	-	-	-	-
Glucose-1-Phosphate	-	-	+	+	-
D,L-α-Glycerol Phosphate	-	-	+	+	-
D-Galactonic acid γ-Lactone	+	-	+	+	+
D-Galacturonic Acid	+	+	+	+	+
2-Hydroxy Benzoic Acid	+	+	+	+	+
4-Hydroxy Benzoic Acid	+	+	+	+	+
γ-Hydroxybuteric Acid	+	-	+	+	+
Itaconic Acid	-	-	+	+	+
α-Ketobutyric Acid	+	-	+	+	+
D-Malic Acid	-	-	+	+	+
L-Arganine	+	-	+	-	+
L-Asparagine	-	-	+	-	+
L-Phenylalanine	-	-	+	+	+
L-Serine	+	-	-	-	-
L-Threonine	+	-	+	+	+
Glycyl-L-Glutamic Acid	+	-	-	-	-
Phenylethyl-amine	+	+	+	+	+
Putrescine	+	-	+	-	+

Symbols: +, positive reaction; - negative reaction.

Table 3. Metabolic versatility for different aromatic compound utilization for carbon and energy by the isolates under aerobic conditions.

Aromatic carbon substrates	Strains				
	IS1	IS2	IS3	IS4	IS5
Phenol	+	+	+	+	+
p-Cresol	+	-	+	+	+
4-OH benzylalcohol	-	-	+	-	-
4-OH benzylaldehyde	-	-	+	+	-
4-OH benzoate	-	-	+	-	-
Protocatechuate	+	+	+	-	-
Benzoate	+	+	+	+	+
Benzyl alcohol	+	-	+	+	+
Benzylaldehyde	+	-	+	+	+
Phenylacetate	+	+	+	+	+
4-OH phenylacetate	+	-	+	+	+
Cinnamic acid	-	+	-	+	+
Hydrocinnamic acid	-	-	+	-	+
Phenylalanine	+	-	+	+	+
Tyrosine	+	-	-	-	-
2-Aminobenzoate	+	-	+	+	+
Toluene	-	-	+	-	-
Benzene	+	+	+	+	+
Tryptophan	_	+	+	+	-
2-aminophenol	+	+	+	+	+
4-aminophenol	+	+	+	+	+

All compounds were tested at a concentration of 200 ppm under aerobic conditions, +, positive reaction; -, negative reaction.

adaptation to phenolic compounds present in the waste water. Buitron and Gonzalelez (1996) also reported that previously adapted microbes perform faster degradation than the others. As sewage treatment plant is an artificial ecosystem where diverse group of bacteria are found and which have an exposure for diverse group of xenobiotics are capable to degrade a large group of xenobiotic aromatic organic pollutants. Hence, in treated sewage waste water, chances for presence of already adapted and acclimatized phenol degrading bacteria are quite more. Even on similar assumptions, the presence of indigenous microorganisms in contaminated soil (with industrial oil) was chosen as due to high probability of the presence of and acclimatized microorganisms (Nuhoglu and Yalcin, 2005).

Metabolic versatility

As IS-3 showed good performance in terms of phenol degradation in comparison to other isolates, this strain was tested for their metabolic versatility by judging their ability to utilize different carbon substrates by using BIOLOG GN-III plates to identify the isolate at species level (Tables 2 and 3). Biolog's powerful carbon source utilization technology accurately identifies environmental and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" from discrete test reactions performed within a 96 well microplate. The scope of the 96 assay reactions, coupled with sophisticated interpretation software, delivers a high level of accuracy that is comparable to molecular methods. Stefanowicz et al. (2006) also reported the efficiency for identification of bacterial strains up to species level. Results of metabolic foot print for utilization of simple sugar to complex aromatics carbon substrates are summarized in Table 2. Among the five isolates, IS-3 had shown more catabolic versatility in terms of utilization of more diverse and complex carbon substrate utilization.

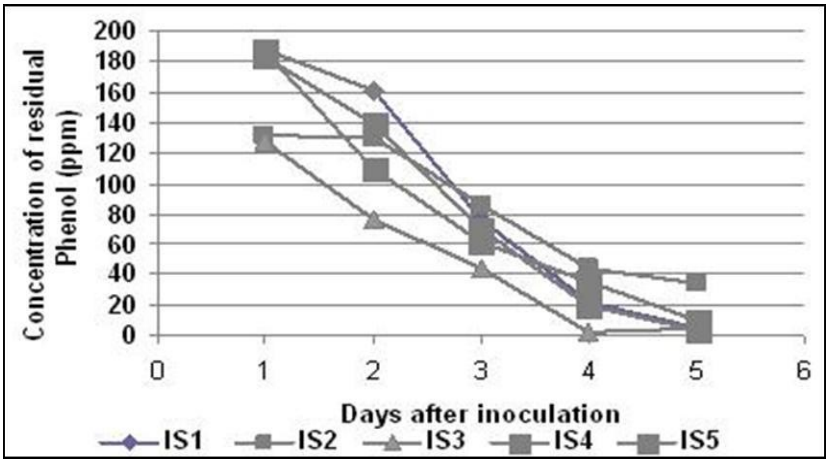

Figure 3. Degradation kinetics of residual phenol (initial enrichment of 500 ppm) by STP isolates (All the results are average of 3 replications).

Carbon substrate utilization foot print was matched with the inventory and IS-3 was confirmed as *C. freundii*. According to Bergey's manual of systematic Bacteriology (2001), 96% of results showed the similarity in characteristics with *C. freundii*. Various phenol degrading micro-organisms including bacteria, yeast, fungi and algae have been reported previously (Yang and Humphrey, 1975; Alcocer et al., 2007; Dong et al., 2008; Banerjee and Ghosal, 2010). Among them, bacteria are of specific importance. Bacteria (*Pseudomonas* sp., *Acinetobacter* sp.), yeast (*Pleurotutus ostreatus, Candida tropicalis, Trichosporon cutaneum* and *Phanerochaete chrysosporium*) and fungi (*Fusarium flucciferum* and *Aspergillus fumigates*) can degrade phenol and among algae, *Ochromonas danica* can degrade phenol (Ariana et al., 2004). Although, there are several report about diverse group of microorganisms belong to several genera for phenol degradation (Kennes and Lema, 1994; Bandyopadhyay et al., 1998; Annadurai et al., 2007; Varma and Gaikwad, 2009; Basak et al., 2014) but there are no published report on *Citrobacter* sp. for phenol degradation. While the isolated strains were tested for their ability to mineralize or transform a large variety of simple aromatic compounds under aerobic conditions, similar trends was noticed for IS-3 (Table 3).

Degradation kinetics of phenol and screening of potent phenol degrading isolate

There were five distinct colonies identified after serial dilution. Their morphological features are presented in Table 1. Although, all of the five isolates were found capable of degrading phenol but IS-3 was found significantly different in phenol degradation capacity. Responses of all of the isolates were significantly different from each other while tested in 200 ppm of initial enrichment of phenol in the media. Out of different isolates, IS-3 degraded 200 ppm of initial enrichment of phenol completely after 72 h of inoculation [Critical difference (CD) =4.09] (Figure 2). The IS-2 was also able to degrade completely within 96 hrs. At 500 ppm of initial enrichment of phenol, IS-3 was able to degrade it completely in 96 h (Figure 3). After 5 days of inoculation of IS-2 in MM1 broth containing 500 ppm of phenol, residual phenol concentration of 40 ppm was found. After 5 days of inoculation, IS-1 and IS-4 in MM1 broth containing 800 ppm of initial enrichment of phenol had residual phenol concentration between 100 to 150 ppm (CD=3.93; Figure 4). At 1000 ppm of initial enrichment of phenol, IS-3 was able to degrade it completely within 5 days and the rest of the isolate had shown residual concentration of phenol in between 400 to 600 ppm (CD=3.16; Figure 5). Among all isolates these, IS-3 was found very much potent to degrade phenol at all initial enrichment irrespective of lower to higher concentration (200-1000 ppm). Overall, IS-3 was found faster and most efficient degrader which can be exploited for further bioremediation studies. Acclimatization of the microorganisms and the bacteria which are adaptive in general overcomes the substrate inhibition problems that normally occurred in phenol biodegradation at high concentration (Lob and Tar, 2000).

Optimization of physical factors for efficient degradation of contaminated phenol present in waste water

Effect of incubation temperature on phenol biodegradation by Citrobacter freundii

C. freundii was able to grow and degrade at temperature ranging from 22-40°C (Figure 6a). However, maximum

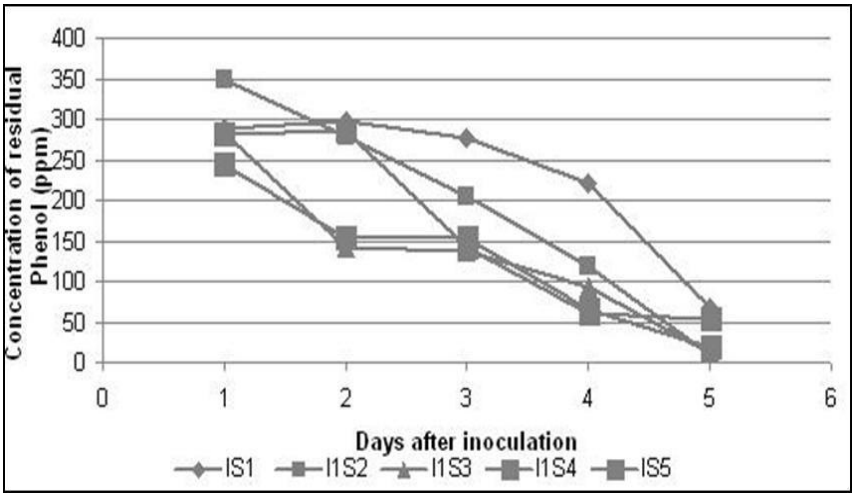

Figure 4. Degradation kinetics of residual phenol (initial enrichment of 800 ppm) by STP isolates.

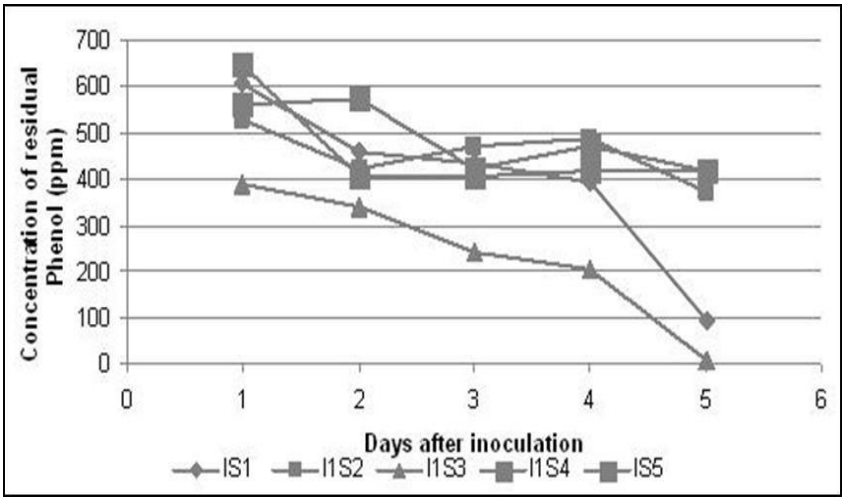

Figure 5. Degradation kinetics of residual phenol (initial enrichment of 1000 ppm) by isolates.

phenol degradation (420 ppm) was noticed at 33°C and 24 h after inoculation. Even at 35 and 37°C, bio-degradation was significantly ($p<0.05$) higher while compared with biodegradation rate with lower incubation temperature (22, 25, 28 and 30°C). As incubation temperature increased from 33°C onwards, a declining trend in the degradation of phenol was noticed. Although in all incubation temperature except 33°C, complete phenol (initial enrichment of 500 ppm) was utilized as sole source of carbon and energy within five days after incubation, but degradation kinetics was quite slower in both lower and higher temperature regimes (except at optimum point) while after 72 h at 22 and 40°C the residual concentration of phenol was 252.57 and 180.98 ppm, respectively. This result corroborates with previous report of phenol degradation by *Pseudomonas* sp. (Polymenakou and Stephanou, 2005) but contradict the findings of Rosa et al. (2004) where 30°C was optimum for biodegradation of phenol. At elevated temperature exposure than the optimum one, showed decreasing trend in the biodegradation capacity. Similar trends had been reported by Gurusamy et al. (2007) for *Pseudomonas pictorum.*

Effect of growth medium pH on phenol biodegradation by *Citrobacter freundii*

To see the effect of pH on phenol degradation by IS-3 (*Citrobacter freundii*), five different pH level (6, 6.5, 7, 7.5

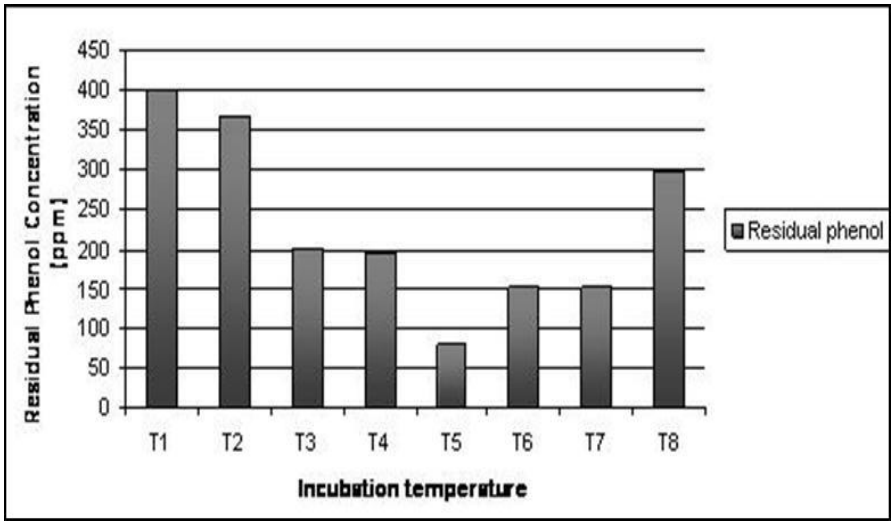

Figure 6a. Effect of incubation temperature on phenol degradation by Citrobacter freundii (IS-3). Initial pH of the growth medium was maintained 7 and medium was enriched with 500 ppm of phenol (T1: 22°C; T2: 25°C; T3: 28°C; T4: 30°C; T5: 33°C; T6: 35°C; T7: 37°C; T8: 40°C).

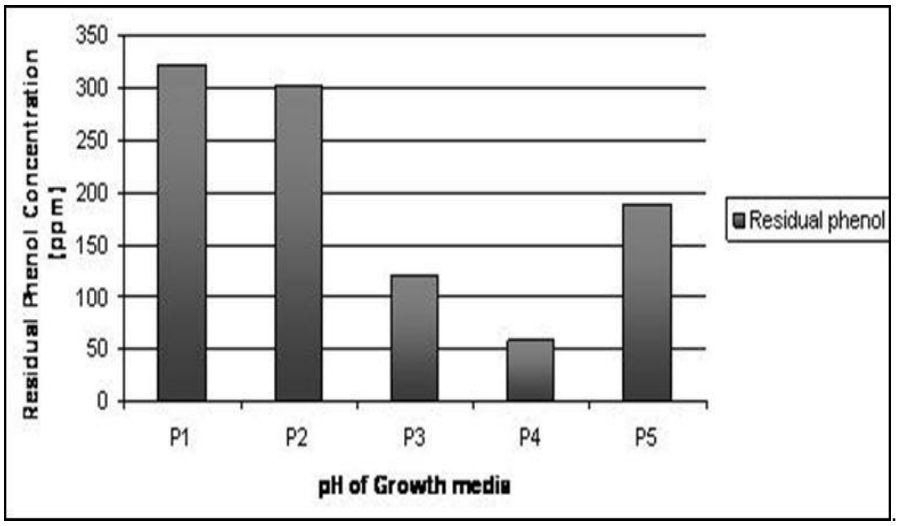

Figure 6b. Effect of pH of the growth medium on phenol degradation by *Citrobacter freundii* (IS-3). Incubation temperature for the culture was maintained at 30°C and medium was enriched with 500 ppm of phenol (P1:6; P2:6.5; P3:7; P4:7.5; P5:8).

and 8) were adjusted. Phenol degradation was greatly influenced by initial culture pH (Figure 6b). Out of 500 ppm, maximum phenol degradation (440 ppm) was noticed at pH level of 7.5 which later on was confirmed as the optimum pH level for highest biomass or cell growth for *Citrobacter freundii* under 30°C and absorbance was taken at 600 nm. *Citrobacter* belongs from Enterobacteriaceae family and the strain which was isolated from STP waste water, favoured slightly alkaline pH range. Although in neutral pH range a significant and good degradation (380 ppm in 24 h after inoculation) rate was noticed but was significantly less ($p<0.05$) than the degradation observed at 7.5 pH level. Though growth of the isolate was not suppressed completely at pH 6 but the level of degradation (180 ppm at 24 h after inoculation) was significantly lower than pH 7, 7.5 and 8. However, phenol degradation rate was found statistically at par for pH 6 and 6.5. Suhaila et al. (2010) also reported highest phenol degradation, which was associated with the highest growth of *Rhodococcus*

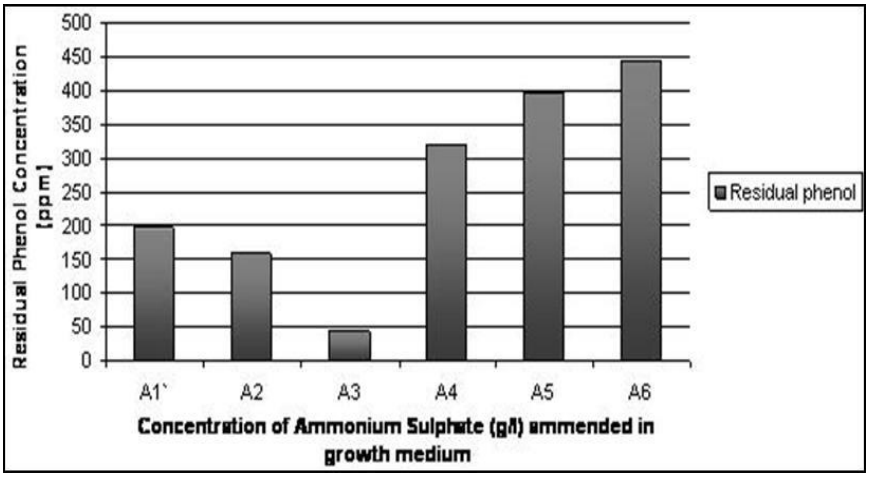

Figure 6c. Effect of augmentation of the growth media with different concentration of Ammonium Sulphate as extra nutrient source on phenol degradation by *Citrobacter freundii* (IS-3). Initial temperature and pH of the growth medium was 30°C and 7, respectively and medium was enriched with 500 ppm of phenol (A1:0 g/l); A2:0.10 g/l; A3:0.25 g/l; A4:0.50 g/l;A5:0.75 g/l; A6:1 g/l).

UKM-P at neutral pH range of 7 to 7.5, where all the phenol present in the culture (0.5 gl^{-1}) was degraded. Higher pH (>8) inhibited phenol degradation. At pH higher than 8, growth of *Rhodococcus* UKM-P was slightly inhibited, which resulted into incomplete degradation of phenol present in the culture (Suhaila et al., 2010). Similarly for *Citrobactor*, growth was slightly inhibited from pH 8 onwards.

Effect of nitrogen source on phenol biodegradation by *Citrobacter freundii*

To see the effect of external nutrient source of nitrogen on phenol degradation by *C. freundii*, ammonium sulphate was amended at different level and results are presented in Figure 6c. Among different amendment level, 0.25 gl^{-1} of ammonium sulphate depicted highest (456 ppm) degradation of phenol out of 500 ppm. Although, further higher level of amendment encouraged a good growth of bacterial biomass, but significantly suppressed phenol degradation. At 1 gl^{-1} of enrichment with ammonium sulphate had contributed only 56 ppm phenol degradation. This trend was almost comparable with 0.5 and 0.75 gl^{-1} enrichment with ammonium sulphate as nitrogen source. Our result contradict the findings of Suhaila et al. (2010) where highest degradation of phenol by *Rhodococcus erythropolis* was obtained at 0.4 and 0.8 gl^{-1} of amendments of ammonium sulphate. This may be because of the fact that our isolated bacteria was different from the one reported by Suhailia et al. (2010). The microbes are versatile in nature. Even similar species isolated from two different habitats may behave differently in their metabolic activity (Martin dos Santos et al., 2008).

Effect of glucose concentration on phenol biodegradation by *Citrobacter freundii*

Phenol degradation capacity by isolated *C. freundii* was examined at different level (no glucose, 0.25, 0.5 and 1 gl^{-1}) of glucose amended in the growth media and residual phenol was estimated at 24 hr after inoculation and incubated at 30°C (Figure 6d). The result envisages highest phenol degradation (426 ppm out of 500 ppm) at 0.25 gl^{-1} of glucose. The degradation rate had dropped down to 32% at 0.5 gl^{-1} of glucose concentration. This drastic decrease in the degradation rate may be due to presence of glucose in the media which is a simple sugar and easily available to the inoculum. When easily available substrates are present bacteria try to avoid oxidation and utilization for complex substrates like phenol present in media. Similarly, further increase in the glucose concentration suppressed phenol degradation kinetics and only 67 ppm out of 500 ppm of phenol was degraded at 24 h after inoculation. In earlier study, Kar et al. (1996) showed the effect of glucose on phenol degradation and the results indicate that when a mixed substrate (phenol and glucose) was used, phenol acclimatized population showed initial preference for phenol to glucose concentration. But our results are in contradiction with the report of Kar et al. (1996). From this study it is clear that higher concentration of glucose curtailed down utilization of phenol as a sole source of carbon and energy. This result corroborates with earlier works done by Suhaila et al. (2010). A glucose concentration of 0.5% repressed the induction of phenol

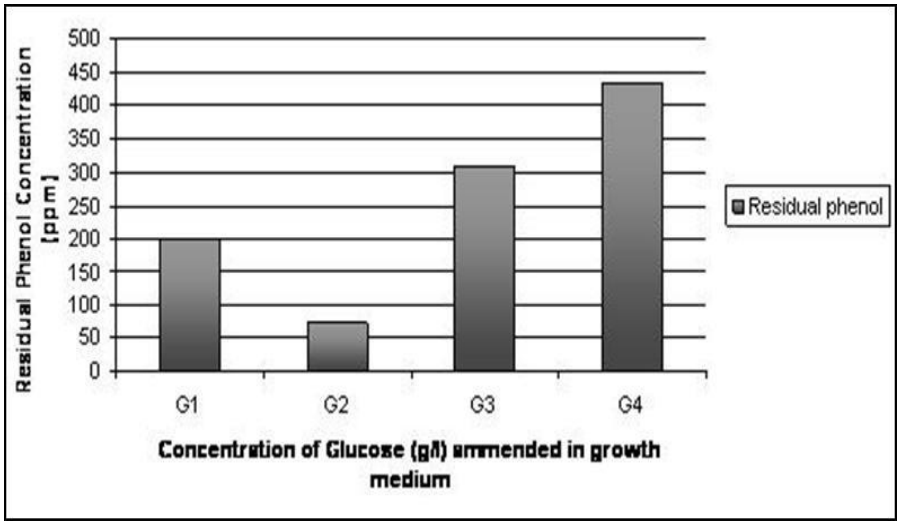

Figure 6d. Effect of amendment with different concentration of glucose on phenol degradation by *Citrobacter freundii* (IS-3). Initial temperature and pH of the growth medium was 30C and 7, respectively and medium was enriched with 500 ppm of phenol (G1:0 g/l; G2:0.25 g/l; G3: 0.50 g/l; G4:1 g/l).

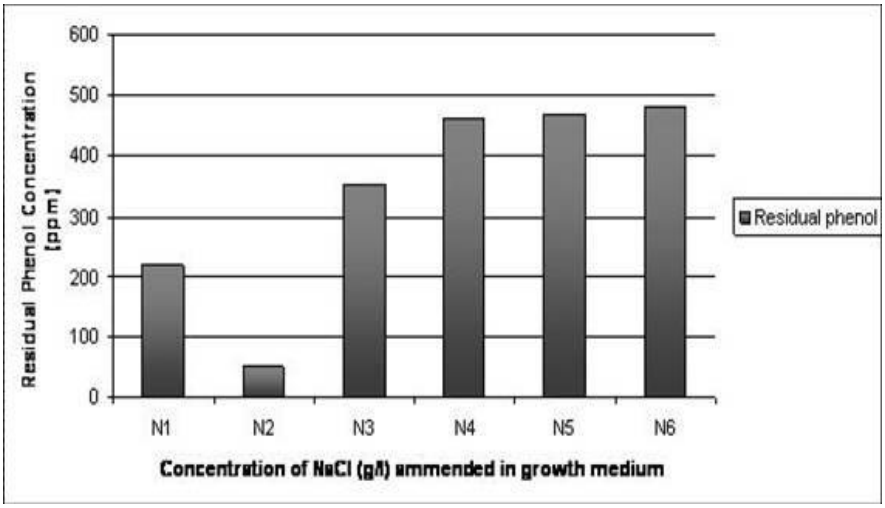

Figure 6e. Effect of amendment with different concentration of NaCl on phenol degradation by *Citrobacter freundii* (IS-3). Initial temperature and pH of the growth medium was 30^{o}C and 7, respectively and medium was enriched with 500 ppm of phenol (N1 0 g/l; N2 0.1 g/l; N3:0.25 g/l; N4: 0.50 g/l; N5:0.75 g/l; N6:1 g/l)

oxidation though glucose and did not fully repress utilization of phenol. Similar kind of results was obtained by Khaled (2006).

Effect of different level of NaCl concentration on phenol biodegradation by *Citrobacter freundii*

A result pertaining to effects of different NaCl concentration on phenol degradation is presented in Figure 6e. The highest degradation of phenol (449 ppm out of 500 ppm that is 89%) was noticed in 0.1% NaCl level. A little increase in NaCl level (0.25 gl^{-1}) had revealed a significant suppression in the degradation rate. Similar kinds of trends were also reported by Suhaila et al. (2010). Higher phenol degradation at lower concentration of NaCl may be attributed towards encouraged bacterial growth with small NaCl amendment. Further increase in the NaCl level upto 1 gl^{-1} had suppressed phenol degradation to a negligible status. This repression in the degradation may be attributed to inhibitory effect on bacterial growth as NaCl

at higher concentration poses preservative or antimicrobial effect. Even though the effects of other factors on phenol biodegradation was studied vastly (Chakraborty et al., 2010) but we report here the effects of NaCl amendment on phenol degradation by phenol-acclimatized pure culture.

Conclusion

The study reveals that IS-3 (*C. freundii*) was highly capable of degrading phenol when the initial enrichment of phenol varied from lower (200 ppm) to higher (1000 ppm) concentration and moreover this strain showed tolerance for phenol at higher level (1800 ppm). This strain degrades effectively and rapidly the phenol present in the water sample. Phenol degradation performance was greatly influenced by different physical factors like incubation temperature, supplemented glucose, nitrogen source, NaCl and growth medium pH. From this investigation, optimum factors were observed as 33°C (incubation temperature), 7.5 (pH of the medium), 0.1 gl^{-1} of NaCl, 0.25 gl^{-1} of glucose and 0.25 gl^{-1} of ammonium sulphate as external amendment for bio augmentation. Members of the genus *Citrobacter* are known pollutant degrader and from this study it is clearly revealed that a slight manipulation or alteration or optimization of the physical factors may enhance the phenol degradation capacity greatly. Hence, this strain (IS-3) is a potent candidate for utilization in the waste water treatment for phenol contaminated effluent from different industries as well as bioremediation and restoration of a degraded site.

Conflict of interests

The authors did not declare any conflict of interest.

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