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ARTICLE

Molecular Identification and Characterization of *Pseudomonas* sp. NCCP-407 for Phenol Degradation Isolated From Industrial Waste

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Abstract Phenol is a toxic pollutant found in effluent of numerous industries and its elimination is a foremost challenge. The utilization of bacteria plays a crucial role in phenol bioremediation. For isolation of phenol degrading bacteria, sample was collected from industrial waste and enriched in mineral salt medium (MSM) contained 300 mg/L phenol. The strain was identified based on 16S rRNA gene analysis as *Pseudomonas* species and the phylogenetic analysis affiliated the strain with *Pseudomonas monteilii* (AF064458) as the most closely related species. Phenol tolerance of the strain in MSM supplemented with various concentrations of phenol indicates that the strain NCCP-407 can grow best at 750 mg L^{-1} phenol. The strain showed complete degradation of 750 mg L^{-1} phenol in 56 hours when supplement as a sole source of carbon and energy with the average degradation rate of 28 mg L^{-1} h^{-1}. The doubling time was recorded approximately as 12.49 h^{-1}. The present study suggests that this strain is efficient in phenol degradation and can be used in treatment of wastewater containing phenol.

Keywords biodegradation · bioremediation · industrial waste · phenol · *Pseudomonas* · 16S rRNA gene

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Introduction

Environmental pollutant in water and in food chain is the focal dilemma globally (Lowry et al., 2009). Rapid acceleration of pollutant put our ecosystem in severe danger (El-Naas et al., 2009). Among these, phenol is a characteristic pollutant released to the environment by many industries such as pharmaceutical, lather, textiles and petrol refining (Bandhyopadhyay et al., 2001; Yemendzhiev et al., 2008; Cao et al., 2011). Phenol has detrimental effects on humans, plants, animals, microorganisms and aquatic life (Das and Santra, 2012). Intake of phenol (~1 g kg^{-1}) is fatal to humans and animals (Rocha et al., 2007). Phenol is also lethal to aquatic life in the range of 5-25 mg L^{-1} (Nuhoglu and Yalcin, 2005).

EU Directive defined permissible concentration of phenol in drinking water as 0.5 μg L^{-1} (EEC, 1980). Japan ministry of Health and welfare defined a norm of 5 μg L^{-1} ten times higher than that defined by EU Directive (MH&W, Japan, 2000). The US Environmental Protection Agency set the standard of 1 μg L^{-1} for phenol in drinking water (US EPA, 2007). However, up to 53 mg L^{-1} of phenol has been determined in effluent (ATSDR, 2011) and in other report, this quantity reached to 10–17,500 mg L^{-1} (Veeresh et al., 2005 Carbajo et al., 2010).

Different techniques and methods to remove phenol from wastewaters are put into practice including coagulation, chemical oxidation, solvent extraction and adsorption. However, all these methods are not environmental friendly due to the production of toxic secondary intermediates, high cost and health risk to workers (Carmona et al., 2006; Zhai et al., 2012). Currently, bioremediation is receiving much desirability owing to its environmentally friendly nature and being economical as compared to other physiochemical methods (Fialov et al., 2004). The widespread presence of phenol in environment, permits many microorganisms including bacteria, fungi and algae to withstand high concentration of phenol and utilize phenol as primary source of carbon and energy (Kotresha and Vidyasagar, 2008; Park et al., 2012). Members of many

bacterial genera have been isolated and evaluated for phenol tolerance and degradation. Aerobic biodegradation of phenol is reported using various bacteria from different genera. These genera includes *Rhodococcus* (Rehfuss and Urban, 2005), *Pseudomonas* (Polymenakou and Stephanou, 2005), *Xanthomonas* (Lowry et al., 2009), *Bacillus* (Banerjee and Ghoshal, 2010), *Stenotrophomonas* (Zaki, 2006), *Comamonas* (Arai et al., 1998), *Streptococcus* (Mohite et al., 2010), *Gulosibacter* (Zhai et al., 2012) among others.

Objectives of the present study were to isolate and characterize indigenous bacterial strain from industrial waste that can tolerate and degrade the toxic concentration of phenol. In addition, molecular characterization of this strain was also carried out.

Materials and Methods

Isolation and enrichment. All chemicals used in these experiments were of reagent grade and supplied by Merck (Germany) and Sigma Chemicals Co. (USA). Phenol of 99% purity was used throughout the experiments. For isolation of phenol degrading bacteria, samples were collected from industrial waste surrounding the industrial area of Islamabad, Pakistan. Mineral Salt Medium (MSM) broth supplemented with phenol was used for isolation of bacteria. MSM was prepared according to the method described by Zhang et al. (2013). The MSM contained 0.5 g KH_2PO_4 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2$, 0.1 g NaCl, 0.2 g NH_4NO_3, 1.0 g $MnSO_4 \cdot H_2O$, and 0.01 g $Fe_2(SO_4)_3$. The pH was adjusted to 7 with NaOH and HCl solutions. Sample was enriched in MSM supplemented with 300 mg L^{-1} of phenol in 250 mL Erlenmeyer flask and placed on rotary shaker at 120 rpm at 25°C for 3 days. Subsequently, 2–3 drops of enriched sample was spread on MSM agar plates supplemented with 200 mg L^{-1} of phenol and incubated at 28°C. After few days, isolated colonies were re-streaked on MSM agar containing 200 mg L^{-1} of phenol to obtain a purified culture of the strains. The same process was repeated several times for isolation of pure culture. Colony morphology of the stain was observed. The strain was designated as NCCP-407 and preserved at −80°C in MSM broth containing 35% glycerol (final concentration).

Identification of the strain. The strain was identified based on 16S rRNA gene sequence analysis following the method described earlier (Ahmed et al. 2007). For this purpose, the strain was grown on Tryptic Soy Agar (TSA, Difco, USA) plate by incubating at 28 and DNA of the strain was extracted by colony polymerase chain reaction (PCR) method. 16S rRNA gene was amplified in thermal cycler (Applied Biosystems, Veriti, USA) using forward primer 9F (5'-GAG TTT GAT CCT GGC TCA G-3') and reverse primer 1510 (5'-GGC TAC CTT GTT ACG A-3'). The amplification of 16S rRNA gene was performed with the following conditions: 1 cycle of pre-denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, primers annealing at 50°C for 1 min, and extension at 72°C for 1.30 min, and finally extension at 72°C for 5 min. The amplified DNA of 16S rRNA gene was confirmed on agarose gel (0.8%, w/v) and electrophoresis employed for 30 min. Amplified PCR products were purified using purification kit (Qiagen, Germany) according to the manufacturer's procedure. The amplified 16S rRNA gene product was sequenced using forward primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse primer 1492R (5'-ACC TTG TTA CGA CTT-3') from Macrogen, Korea. BioEdit software was used for editing and to achieve assembled consensus sequence. The assembled sequence was subjected to BLAST search on Ez-Taxon Server and DNA Data Bank of Japan, and the sequences of 16S rRNA gene of closely related validly published species were retrieve for phylogenetic analysis of the isolated strain. Alignment of sequences was carried out using Clustal W (version 1.6) (Thompson et al., 1994) and the phylogenetic analysis was performed to determine the evolutionary relationship of the strain with other validly published strains. Phylogenetic tree was constructed using Neighbor-Joining algorithms contained in MEGA-5 software package (Tamura et al., 2011).

Phenol tolerance. Phenol tolerance of the strain was determined at various concentrations of phenol. For this purpose, pre-culture was obtained by growing the strain in 50 mL MSM broth containing 200 mg L^{-1} phenol as a sole source of carbon and energy, incubated at 28°C for 48-72 hours. This pre-culture was used to inoculate MSM broth containing 0, 250, 500, 750, and 1000 mg L^{-1} phenol. Blanks were incubated in parallel under same conditions. Growth of strain was determined using spectrophotometer (IMPLEN, Germany) at 600 nm wavelength at different time intervals.

Phenol degradation. Phenol degrading potential of the strain was determined by inoculating the pre-culture into MSM broth supplemented with 750 mg L^{-1} phenol as a single source of energy and carbon, and incubated at 28°C for a total period of 83 h. Two controls were used in this experiment. One control was MSM broth with inoculum and without phenol and other control was MSM broth supplemented with 750 mg L^{-1} phenol and without inoculum. Growth of the strain was monitored by optical density at 600 nm wave length at different time with respective blank. For quantification of residual phenol and degraded phenol in MSM broth, supernatant was collected at different time interval and subjected to High Performance Liquid Chromatography (HPLC) analysis. For HPLC analysis, samples (culture) at different time interval was collected and centrifuged as 13,000 rpm at 25°C for 5 min. Equal amount of acetonitrile (eluent) was added to the 0.5 mL of centrifugate. The HPLC (PerkinElmer, USA) system used was equipped with binary LC pump 250, LC oven 101, Column C18, LC 295 UV/Vis detector, Interface 900 Series, and TurboChrome Workstation (Ver. 6.1.2.0.1) was used for quantification of phenol. The mobile phase was acetonitrile and water at 60:40 with flow rate of 0.8 mL/min, and UV visible detector was set at 280 nm wavelength.

Statistical analysis. The results of phenol degradation and growth of bacterium in phenol containing MSM with respect to time were subjected to regression analysis using Minitab 16.0 software.

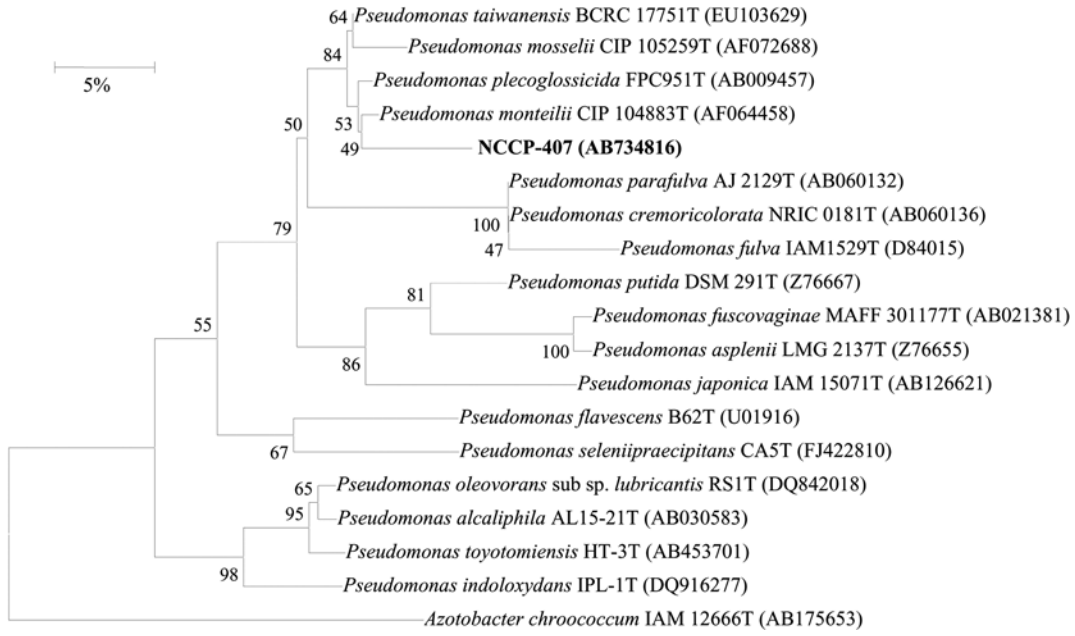

Fig. 1 Phylogenetic tree showing the inter-relationships of strain NCCP-407 with the most closely related type strains of the genus *Pseudomonas* inferred from sequences of 16S rRNA gene. *Azotobacter chroococcum* (AB175653) was used as an out group. The tree was generated using the neighbour-joining method. Bootstrap values are expressed as a percentage of 1000 replications, are given at the branching point. The Bar show 5% sequence divergence. The accession number of each strain is shown in parenthesis.

Results and Discussion

Identification of bacteria. Industrial waste was selected for isolation of phenol degrading bacterium due to high probability of the presence of bacteria having potential of degrading organic compounds. Isolation of phenol degrading bacteria from industrial waste/effluent is well documented (Hamitouche et al., 2012, Pishgara et al., 2012). Due to the presence of phenol in industrial waste, the persistant bacteria are often well adopted (Gonzalez et al., 2001). For determination of morphological characteristics, isolated strain was grown on tryptic soy agar plates for 24 h at 28. It was observed that the colony is circular in shape with entire margins having smooth surface and crate-form elevation. The colony was transparent.

The strain was identified based on 16S rRNA gene sequence analysis. PCR amplification was performed, and amplicons were confirmed by gel electrophoresis. Amplified products were purified using PCR product purification kit (Qiagen, Germany) and sequenced. The obtained sequence was submitted to DNA Data Bank of Japan with the accession number (AB734816). BLAST search was performed on Eztaxon server to retrieve sequences of related strains. Based on 16S rRNA gene sequence analysis it was found that, NCCP-407 belongs to genus *Pseudomonas*. Sequence comparison of the strain with other validly published strains showed that NCCP-407 shared 99.4, 99.33, and 99.19% sequence identity with *Pseudomonas plecoglossicida* (AB009457), *Pseudomonas taiwanensis* (EU103629) and *Pseudomonas monteilii* (AF064458) respectively. Phylogenetic tree was constructed using Neighbor-Joining algorithm contained in MEGA-5 software package, showing the affiliation of the isolated strain with the already published strains (Fig. 1).

Members of the genus *Pseudomonas* are reported for degradation of a variety of organic compounds (Tripathi et al., 2011). A lot of experiments performed previously, demonstrates the members of genus *Pseudomonas* as the best degrader of phenol and phenolic compounds (Collins et al., 2005, Hamitouche et al., 2012). *Pseudomonas aeruginosa* AT2 (El-Sayed et al., 2003), *Pseudomonas fluorescence* (Agarry et al., 2008). *Pseudomonas putida* (Ravikumar et al., 2011) and many others *Pseudomonas* strains are idetified and characterized for phenol tolerance and degradation. Phylogenetic analysis on the basis of 16S rRNA gene sequence confirms that the isolated strain belong to the genus *Pseudomonas*. 16S rRNA gene sequence analysis is the bench mark for bacterial identification becaue of many reasons. All bacteria contain this gene with a little variation due which strains can be allocated to specific genera. The natural function of this gene is not change and the sequence length is enough to get information related to variation (Patel, 2001).

Phenol tolerance. Tolerance of strain *Pseudomonas* NCCP-407 to phenol was determined by inoculating in mineral salt medium supplemented with 0, 250, 500, 750, and 1,000 mg L^{-1} phenol as a sole source of carbon and energy. The strain was incubated at 28°C for a total period of 66 h. At 0 mg L^{-1} phenol no growth was observed, while growth was observed at the rest of given concentrations. At 250 and 500 mg L^{-1}, no lag phase was observed, while at 250 mg L^{-1} log phase was started from the time of

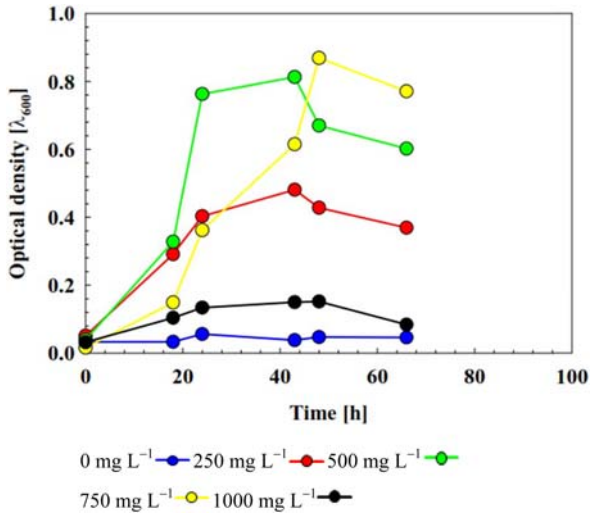

Fig. 2 Tolerance of NCCP-407 to different concentration of phenol grown at 28°C in relation to time scale.

inoculation (initial OD was 0.051) and continued up to 43 hours. Maximum OD of 0.481 showed the highest growth at 43 hours. At 500 mg L^{-1} log phase was started from the time of inoculation (initial OD was 0.039) and continued up to 43 h. Maximum growth was observed at 43 hours of inoculation and the OD was 0.813 at 600 nm. At 750 mg L^{-1}, lag phase was started at time of inoculation with OD of 0.016 and continued up to 18 hours. The log phase at this concentration was started after 18 h of inoculation and continued up to 2 days of inoculation with completion in 30 h. Maximum growth was observed at 48 hours of inoculation and the OD obtained was 0.89 at 600 nm. At 1,000 mg L^{-1}, a negligible growth was observed with OD value not more than 0.153 (Fig. 2).

Higher OD value was obtained at 750 mg L^{-1} of phenol in comparison to other concentrations. As *Pseudomonas* NCCP-407 showed growth at all given concentration of phenol but negligible growth was observed at 1000 mg L^{-1} of phenol indicating toxicity of this concentration. Bacterial growth is inhibited at higher phenol concentration (Lin et al., 2008, Saravanan et al., 2008). *Pseudomonas* NCCP-407 shared 99.4% 16S rRNA sequence identity with *Pseudomonas plecoglossicida* which is reported for m-cresol (92%, 763 mg L^{-1}) degradation (Cao et al., 2011). Similarly, no other phylogenetic neighbor of our strain is reported for phenol degradation.

Phenol degradation. Isolated strain was evaluated for its phenol degrading potential. The strain was grown in MSM supplemented with 750 mg L^{-1} phenol. Optical density at 600 nm was determined from the time of inoculation till stationary phase at different time intervals. Two set of controls were used, one control had MSM with 750 mg L^{-1} phenol without inoculum and the other control had the inoculums but without phenol. The strain was incubated with phenol for 83 hours at 28°C. No lag phase was observed for phenol degradation. Maximum OD (600 nm) value of 0.87 was observed (initial OD value was 0.016) at 53 h of incubation. No

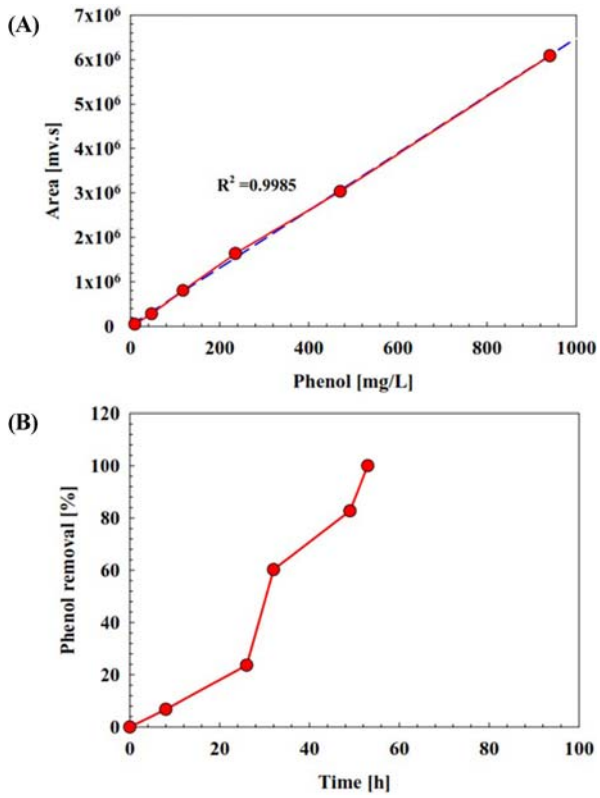

Fig. 3 (A) Peak area of the external standards and regression equation (B) Percentage removal of phenol from Tryptic Soy Broth medium by the NCCP-407.

growth was observed in control without inoculum. Residual phenol in broth was quantified using HPLC. For this purpose, six external standards (9.4, 147.05, 117.64, 235.28, 470.55, and 941.1 mg L^{-1}) of phenol were prepared and analyzed on HPLC. Residual phenol in samples (supernatants) was calculated using the obtained regression equation given in Fig. 3A. No phenol degradation was observed in control having phenol and without inoculum or in control without phenol with inoculum. The strain degraded 6.7, 23.6, 60.2, 82.7, and 100% of phenol (750 mg L^{-1}) in 8, 26, 32, 49, and 53 h respectively (Fig. 3B). Complete degradation of 750 mg L^{-1} phenol was observed in 53 hours with the average degradation rate of 29.0 mg L^{-1} h^{-1}. A decrease in the concentration of phenol is coincided with the increase in growth as indicated by increase in optical density. Maximum phenol degradation was observed at 53 h (Fig. 4). The doubling time at this concentration was recorded approximately as 12.49 h^{-1}. The results obtained from degradation of phenol and growth of the bacterium (with respect to time) in phenol containing MSM was subjected to regression analysis. The model shows highly significant relation with p <0.001. The value of the determination coefficient (R^2=89%) being a measure of goodness of fit to the model indicates that 90% of the total variations in the data are explained by the model (about 5.7% is not explained). Additionally the R^2 adjusted value (90%) is very close to the R^2 value indicating

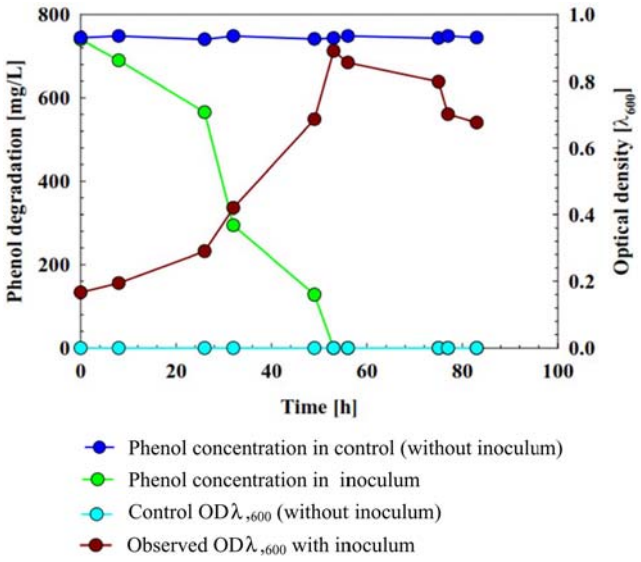

Fig. 4 Degradation of phenol (750 mg L^{-1}) and growth as measured by optical density at 600 nm of NCCP-407.

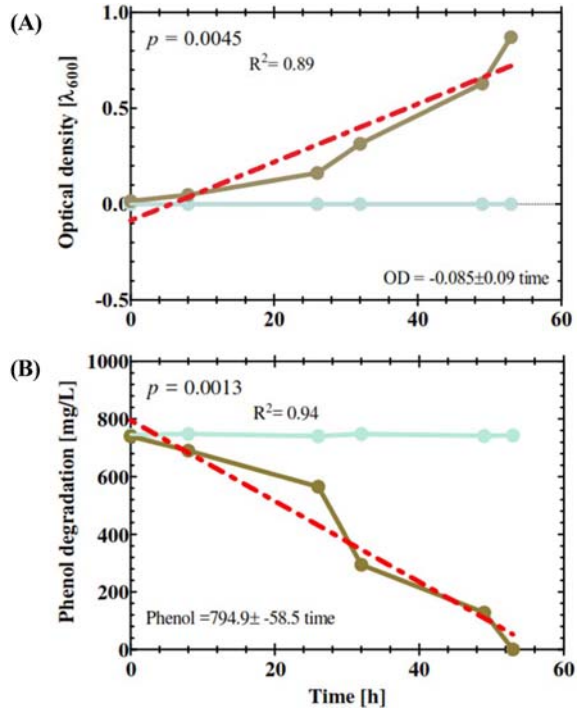

Fig. 5 (A) Regression analysis for the degradation of 750 mg L^{-1} phenol by *Pseudomonas* NCCP-407 with respect to time (B) regression analysis for the growth of *Pseudomonas* NCCP-407 with respect to time in 750 mg L^{-1} phenol as a sole source of carbon and energy.

that the model is accurately described (Fig. 5A). Similarly, the R^2 value for bacterial growth verses time in 750 mg L^{-1} phenol is 82.17% indicating that the model did not explain 10.8% variability in data. The model is highly significant as indicated by p-value (0.0013) (Fig. 5B). There is a strong negative correlation (correlation value = −0.973) present between phenol degrading and growth of the bacterium, which means that increase in bacterial biomass decreases phenol concentration in medium.

Our results obtained from phenol degradation with a little deviation are in accordance with that of Zhu et al. (2012). They reported *Pseudomonas* PCT01 strain which was isolated from wastewater and degraded 750 mg L^{-1} of phenol in 35 hours, while our strain degraded 750 mg L^{-1} of phenol in 53 h. Difference in the time taken for complete removal of phenol by the members of same genus is common. Two *Pseudomonas* sp. PCT01 and PTS02 were evaluated for phenol degradation and it was found that PCT01 degraded 750 mg L^{-1} in 35 hours while in case of PTS02 the process was protracted (Zhu et al., 2012).

Industrial waste is the potential source of phenol degrading bacteria. This work describes *Pseudomonas* NCCP-407 strain efficiency to degrade phenol up to 1,000 mg L^{-1}. The strain showed maximum tolerance to 750 mg L^{-1} phenol and degraded such quantity of phenol in 53 h at pH 7 and 28°C, which is very rapid comparatively with others published strains evaluated for phenol degradation. This work delivers a useful guideline for isolation and characterization of phenol degrading bacteria. The stain could be used in elimination of phenol and phenolic compounds from wastewater.

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