JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Sept. 2016. Vol. **10**(3), p. 2045-2054

Molecular and Biochemical Profiling of Pentachlorophenol Utilizing Bacteria from Pulp and Paper Mill Effluent Irrigated Soil in Northern India

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https://doi.org/10.22207/JPAM.10.3.43

(Received: 06 September 2015; accepted: 11 November 2015)

Pulp and paper mill is a source of major environmental pollutant's generating industries which include pentachlorophenol a highly chlorinated aromatic compound. PCP degrading bacterial isolates obtained from pulp and paper mill effluent contaminated site were characterized biochemically and molecularly. Based on their morphological and biochemical characterization, 22 isolates were selected for their ability to grow at different concentrations of PCP. Out of 22 isolates, 8 isolates *viz.* LK 1, LK 4, LK 39, LK 81, LK 124, LK 141, LK 147 and LK 156 showed significantly higher growth at 100, 300, 500 and 700 ppm of PCP. Growth at 700 ppm reduced significantly due to decrease in pH of the medium to 6.1. These 22 isolates were classified into three major bacterial lineages, α,γ-*Proteobacteria* and *Firmicutes*. Maximum isolates belonged to *Pseudomonas* sp such *P. aeruginosa, P. citronellolis*, *P. putida* and *P. plecoglossicida*. PCP degradation by *Ensifer adhaerens* and *Lysinibacillus fusiformis* is reported for the first time in present study and these might represent new chlorophenol-degrading taxa. *Lysinibacillus fusiformis* (LK 156) could show maximum growth at 300 and 500 ppm of PCP.

Keywords: Pentachlorophenol utilizing bacteria, Pulp and Paper mill effluent, *Lysinibacillus fusiformis*, *Ensifer adhaerens*

Discharge of pulp and paper mill include many environmental pollutants among which Chlorophenols (CPs) are major ones (Chandra *et al*. 2009), which are generated as the by-products when chlorine is used for bleaching of pulp and as water disinfectant. Pentachlorophenol (PCP) and its sodium salt have been widely used as wood and leather preservative owing to their toxic effect on bacteria, mould, algae and fungi (Kaoa *et al*. 2004). Its frequent and widespread use has led to contamination of aquatic and terrestrial ecosystems (Jensen 1996).

Pulp paper industries are the sixth largest effluent generating industries of the world (*Ugurlu et al. 2007*). Since early fifties the number of paper pulp mills in India has increased from 17 to more than 406 in 2008, with simultaneous increase in paper production from 0.13 to 1.9 million tons per annum . Paper mill generates as low as 1.5 m^3 of effluent per ton to as high as 60 m^3 per tonne of paper produced (Asghar *et al*. 2007). The safe permissible limit of PCP in water is 0.30 ìg l^{-1} . However, in our country, the large units of pulp and paper mills discharge their effluent, having residual PCP in high concentrations (> 80 mg l^{-1} effluent), in local water ways .

Pulp and paper mill effluent irrigation to crops is a cheap and attractive option compared to discharge of this effluent into natural waterways (Muthukumar and Vediyappan 2010). Local farmers irrigate their agricultural fields on regularly basis from these water channels and thereby contaminating them with PCP. Being highly chlorinated, PCP is expected to be recalcitrant to aerobic biodegradation as in general, aromatic compounds with higher amounts of chlorine are more resistant to biodegradation (Anandarajah *et al*. 2000). Due to persistence of PCP in soil and

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water environments, both the European and US Environment Protection Agencies have classified PCP as a 'priority pollutant' and have recommended restricted use to minimize its further accumulation and to circumvent toxicity of the ecosystem.

Biological treatment of PCP attracts more attention than physical and chemical methods, because a variety of microorganisms are known to utilize it as their sole carbon source and the reaction products are Cl^- ions, CO_2 and biomass. Several microorganisms possessing the ability to metabolize various industrial pollutants have been isolated from the environment (Tripathi *et al*. 2011). Aerobic PCP degradation by mixed microbial cultures is important since most PCP-contaminated sites are surface soil or sediments which may support growth and activity of aerobic microbial consortia. Bioremediation protocols for soil contaminated with high concentration of PCP can be achieved only by using efficient indigenous PCP degrading microorganisms.

We analysed PCP utilizing bacteria in agricultural soils irrigated with pulp and paper mill effluent discharged from Century Pulp and Paper mill, LalKuan, Uttrakhand, India. Chlorophenol-degrading bacterial isolates were biochemically characterized and identified by partial 16S rRNA gene sequencing.

MATERIALS AND METHODS

Field site and sample collection

The effluent from the Century Pulp and Paper mill (CPM), LalKuan, Uttrakhand, India (79°100E longitude and 29°30N latitude), which is discharged in local waterways is being used as source of irrigation to the sugarcane fields since last 25 years. A field was selected from this site for sampling. A total of 5 composite soil samples were collected at 0-15 cm depth during the month of March using a soil auger. Each composite sample was made of five sub samples, collected from along the zigzag paths (Zigzag sampling) to account for the randomness. The collected soil samples were properly labelled and stored in polythene bags and transported to the laboratory in the insulated container at 4°C.

Soil physico-chemical analysis

The soil samples were analyzed for various physical and chemical characteristics such as texture, pH, electrical conductivity (EC), organic carbon (OC), available N, Olsen P and exchangeable K as per methods described by Page *et al*. (1982). Residual PCP in soil samples was estimated using HPLC as described.

Enrichment of soil samples with PCP

The enrichment of PCP degrading bacteria was carried out as per method described by Karn *et al*. (2010). From each of the soil samples, 10 gm of soil was added in 90 ml of mineral salt (MS) medium having PCP @ 50 µg ml^{-1} (Dams *et al*., 2007). The composition of MSM (in gmL^{-1}) was KH_2PO_4, 0.68; K_2HPO_4, 1.73; $MgSO_4.7H_2O$, 0.1; $CaCl_2.2H_2O$, 0.02; NH_4SO_4, 0.017; and 1 ml of trace metal solution which includes (in mgL^{-1}) $FeSO_4.7H_2O$, 200; $ZnSO_4.7H_2O$, 10.0; $MnSO_4.4H_2O$, 3.0; $NiCl.6H_2O$, 2.0; H_3BO_3, 30.0; $CoCl_2.6H_2O$, 1.0; $ZnCl_2$, 10.0; and EDTA, 2.5. PCP was added to the medium after autoclaving. The pH was adjusted to 7.3 ± 0.2 prior to autoclaving.

The flasks were incubated for 7 days at 30 °C on rotary shaker at 200 rpm. After 7 days of incubation, 10 ml soil suspension was taken from the flask and transferred aseptically to the flask containing fresh MS medium having PCP @ 50 µg ml^{-1} and again incubated for 7 days. This step was repeated for 4 more weeks.

Isolation of PCP degrading bacteria

After 6 weeks of enrichment, the potential PCP degrading bacterial strains were isolated by serial dilution technique on MS medium containing 50 µg PCP ml^{-1}. From each of the sample, single colonies were purified by repeated streaking. All the purified isolates were morphologically characterized based on their colony shape, size, colour, pigmentation, margin and elevation after 48 h of incubation (Seeley and VanDenmark, 1972). Representative morphotypes were purified, sub-cultured and maintained on MS medium agar slants having PCP @ 50 µg ml^{-1}.

Biochemical characterization of the selected isolates

The biochemical characterization of the 22 selected isolates was done in accordance with Bergey's Manual of Systematic Bacteriology. Each pure culture was tested for Gram reaction. The catalase activity was determined based on formation of bubbles in the presence of 3% H_2O_2 solution. Oxidase was performed on paper discs

using tetramethyl-p-phenylenediamine. Nitrate reductase was detected on nitrate agar plates with methyl green as an indicator. Urease test was carried out by method described by Christensen (1946). Urea broth containing phenol red indicator was inoculated with test cultures and incubated for 5 - 6 days at 37 °C. Presence of yellow color indicated the presence of urease. Starch hydrolysis was demonstrated from clearing zones formed around the colonies grown on starch containing agar.

Table 1. Physicochemical properties of soil

Parameters	Average Values[1]
pH	8.16 (±0.1)
EC (dS m^{-1})	0.73 (±0.04)
OC (%)	0.95 (±0.16)
Available N (kg ha^{-1})	64.85 (±10.9)
Olsen P (kg ha^{-1})	16.81 (±0.21)
Exchangeable K (kg ha^{-1})	164.5 (±18.12)
Residual PCP (mg Kg^{-1} soil)	113.34 (±11.36)

[1]Mean of 5 replications and figure in parenthesis are Standard deviation from mean

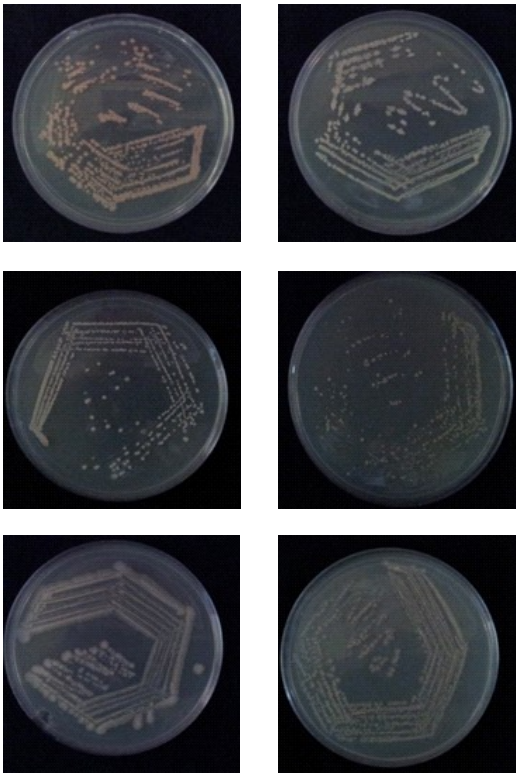

Plate 1. Purified colonies of few selected PCP utilizing bacteria isolated from pulp and paper mill effluent irrigated soil

Growth of selected bacterial isolates at different concentrations of PCP

The biochemically characterized isolates were further screened for their ability to grow at 100, 300, 500 and 700 ppm of PCP in MS broth. Desired concentration of filter sterilized PCP from its stock solution was added to sterile MS broth in order to get final concentration of 100, 300, 500 and 700 ppm. Three replications were maintained for each isolate. The flasks were inoculated with different bacterial isolates individually and incubated at 30 °C at 200 rpm for 48 h. After incubation, pH of the broth was measured. Growth of the cells was measured in terms of total protein. The total protein of the bacterial isolates was

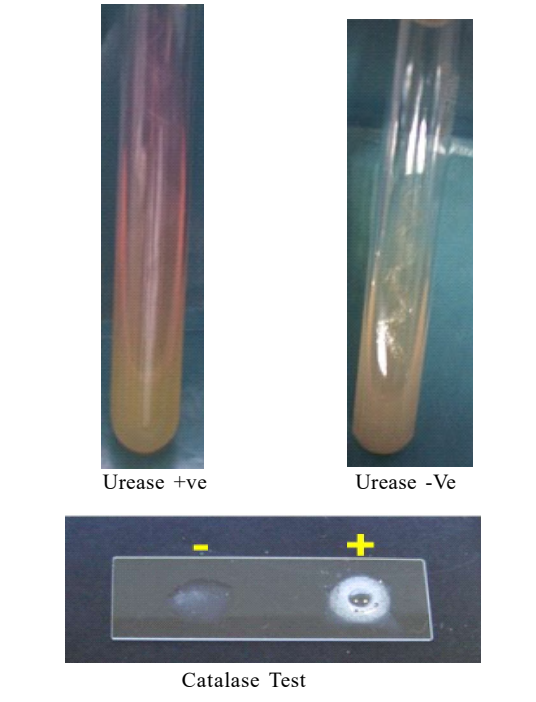

Plate 2. Biochemical characterization of bacterial isolates

estimated by Bradford's method (Bradford, 1976), which involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. When the dye binds to protein, it is converted to a stable unprotonated blue form and is detected at 595 nm using a spectrophotometer.

16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA extraction from the isolates showing growth above 100 ppm PCP was carried out by modified method of Charles and Nester, 1993. Briefly, 1.5 ml overnight grown cultures in TY broth were centrifuged at 12000 rpm for 10 min and washed with 1.5 mL of 0.85% NaCl. Washed pellet was suspended in 0.4 mL Tris-EDTA buffer ($T_{10}E_{25}$). Cell Lysis was done by adding 20 mL of 25% SDS, 50 mL of 1% lysozyme and 50 mL of 5M NaCl followed by incubation at 68 °C for 30 min in a circulatory water bath. Proteins were precipitated by 260 mL of 7.5 M ammonium acetate solution followed by centrifugation at 12000 rpm for 10 min. Supernatant was pipetted out in fresh sterile microfuge tube in which 1mL RNase (4 mg mL^{-1}) was added followed by Incubation at 37 °C for 20 min. Equal volume of chloroform was added and RNA was precipitated by centrifugation for 1 min at 12000 rpm. The top layer containing total cell DNA was pipetted out and precipitated by adding 0.8 vol. of isopropanol followed by incubation on ice for 30 min and pelleted by centrifuging at 10000 rpm for 15 mins. DNA was further washed with 0.5 mL of 70% ethanol and spun down at 10000 rpm for 1 min. Traces of ethanol were removed by air drying the tubes in inverted position. Pure DNA sample was then suspended in 20 mL Tris-EDTA buffer ($T_{10}E_1$) and stored at 4 °C for further use.

The gene encoding 16S rRNA was amplified by PCR using the pair of universal primers pA (5'-AGA GTT TGA TCC TGG CTC AG-3') and pH (5'-AAG GAG GTG ATC CAG CCG CA-3') and conditions described in Edwards *et al*. (1989). 16S rRNA gene was used as a template in cycle sequencing reactions with fluorescent dye-labelled terminators (Big Dye, Applied Biosystems). Both primers pA and pH were used for sequencing and run in 3130xl ABI prism automated DNA sequencer. All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLASTn search. Identification to the species level was determined based on 16S rRNA

Table 2. Morphological characterization of selected bacterial isolates obtained from pulp and paper mill effluent irrigated soil

Strain Number	Colony Colour	Size and Pigmentation	Margin	Elevation	Shape
LK-1	Shiny blackish	Punctiform pigmented	Entire	Flat	Rods
LK-4	Creamy white	Circular non pigmented	Undulate	Convex	Cocci
Lk-5	Off white	Punctiform non pigmented	Lobate	Raised	Rods
LK-23	Off white	Punctiform non pigmented	Entire	Flat	Rods
LK-32	Shiny whitish	Circular pigmented	Lobate	Flat	Rods
LK-39	Creamy white	Irregular pigmented	Curled	Raised	Rods
LK-41	Creamy white	Circular pigmented	Undulate	Flat	Rods
LK-43	Dull	Circular pigmented	Lobate	Umbonate	Rods
LK-47	White	Punctiform non pigmented	Undulate	Umbonate	Rods
LK-51	Creamy white	Punctiform non pigmented	Undulate	Raised	Rods
LK-54	White off	Irregular non pigmented	Entire	Pollinated	Rods
LK-59	Dull mucoid	Irregular non pigmented	Curled	Raised	Rods
LK-60	Creamy white	Circular non pigmented	Entire	Raised	Rods
LK-72	Dull	Irregular pigmented	Undulate	Lobat	Rods
LK-81	Shiny	Punctiform pigmented	Curled	Raised	Rods
LK-124	Creamy white	Irregular non pigmented	Entire	Pollinated	Rods
LK-141	Shiny	Irregular non pigmented	Lobate	Raised	Rods
LK-142	Creamy white	Circular non pigmented	Entire	Convex	Rods
LK-147	Creamy white	Irregular non pigmented	Undulate	Flat	Rods
LK-150	White off	Circular non pigmented	Curled	Raised	Rods
LK-156	Creamy white	Circular pigmented	Entire	Raised	Rods
LK-188	Creamy white	Circular non pigmented	Curled	Flat	Rods

gene sequence similarity (>97%) with that of a prototype strain sequence. Multiple sequence alignment of approx 1500-bp sequences was performed using CLUSTAL W, version 1.8. A phylogenetic tree was constructed using the neighbor-joining method. Tree topologies were evaluated through bootstrap analysis of 1,000 data sets by MEGA 4.0 package .

Data Analysis

Statistical analyses of the data was performed using STATISTICA 10. Analysis of variance and separation of means by least significant differences were performed by using the general linear models (GLM). Unless indicated otherwise, differences were considered only when significant at P = 0.05.

RESULTS AND DISCUSSION

Physico-chemical characteristics of soil samples

The soil was sandy loam in texture with alkaline pH and electrical conductivity of 0.73 dSm^{-1} (Table 1). Soil was having moderate organic carbon content (0.95%) and good in available N (64.85 kg ha^{-1}) and extractable K (130.62 kg ha^{-1}) content but poor in P content (16.86 kg ha^{-1}). Significant amount of residual PCP was present in soil (113.34 mg Kg^{-1}) reflecting the toxic levels of PCP in soil. There is no prescribed set limits for PCP in soil, however, The United States Environmental Protection Agency (EPA) has registered PCP in the list of priority of pollutants and the safe permissible limits of PCP in water is 0.30 µg L^{-1} (US EPA, 1999). The major source of PCP in agricultural soil at the farmer's field at Lal Kuan, Uttrakhand is due to irrigation with water containing effluent discharged from Century pulp and paper mill, Lal Kuan. The PCP is generated as by-product due to bleaching of pulp with chlorine (Vallecillo *et al*., 1999) and is released with effluent in environment.

Isolation of bacterial isolates and their morphological characterization

By enrichment of all the five soil samples with mineral salt medium containing 50 ppm of PCP as sole carbon source, 188 isolates were selected

Table 3. Biochemical Characterization of selected bacterial isolates obtained from pulp and paper mill effluent irrigated soil

Strain Number	Gram reaction	Oxidase	Catalase	Urease	Starch hydrolysis	NO_3 reduction test
LK-1	-ve	+	+	-	+	+
LK-4	-ve	+	+	-	+	-
Lk-5	-ve	+	+	-	+	-
LK-23	-ve	+	+	-	+	-
LK-32	-ve	+	+		+	-
LK-39	-ve	+	+	+	-	+
LK-41	-ve	+	+	+	-	+
LK-43	-ve	+	+	+	-	+
LK-47	-ve	+	+	+	-	+
LK-51	-ve	+	+	+	-	+
LK-54	-ve	+	+	+	-	+
LK-59	-ve	-	+	+	+	-
LK-60	-ve	+	+	+	-	+
LK-72	-ve	+	+	+	-	+
LK-81	-ve	+	+	-	+	-
LK-124	-ve	+	+	+	+	+
LK-141	-ve	+	+	-	+	-
LK-142	-ve	+	+	+	-	+
LK-147	-ve	+	+	+	+	+
LK-150	-ve	+	+	+	-	+
LK-156	-ve	+/-	+	+/-	-	-
LK-188	-ve	-	+	-	+	+

for morphological characterization. These isolates were purified to single colony for morphological characterization. Based on morphological characterization, 22 isolates were selected for further studies (Table 2). All the isolates were found to be rod shaped except LK4 which was cocci. Most of the colonies were whitish in colour, however, variation in white colour was observed from dull, creamy white to off-white. Two colonies were shiny with blackish shade. Margins of colonies varied from circular, irregular to punctiform with entire, curled and undulated margin. Some of colonies were pigmented. The margins of colonies were either flat or raised.

Biochemical Characterization of selected isolates

All the 22 isolates were biochemically characterized and results are shown in table 3. All the isolates were found to be Gram negative except LK 156 which showed variable Gram reaction. Similarly, all the isolates were oxidase positive except LK 156 which showed variable oxidase test results and LK 188 which was oxidase negative. All the isolate were catalase positive and only 12 isolates *viz.* LK 39, 41, 43, 47, 51, 54, 59, 60, 72, 128, 142 and 147 were urease positive. Isolates LK 1, 4, 5, 23, 32, 59, 81, 124, 141, 147 and 188 were able to carry out starch hydrolysis. Nitrate reduction

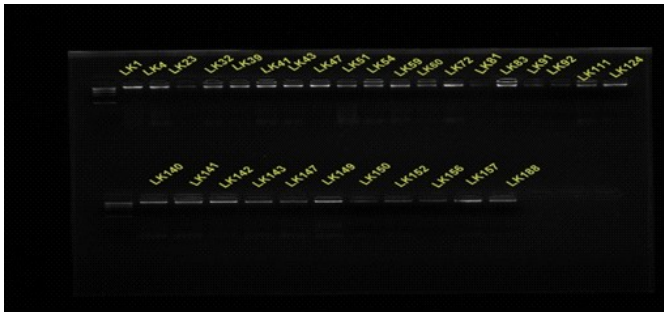

Plate 3. Visualization of genomic DNA isolated from selected bacterial isolates obtained from pulp and paper mill effluent irrigated soil

Table 4. Growth of selected bacterial isolates at different concentration of PCP

Isolate No.	Protein (µg mL^{-1}) at different concentration of PCP			
	100 ppm	300 ppm	500 ppm	700 ppm
LK 1	357.00	625.33	335.50	145.33
LK 4	273.67	500.33	367.00	122.00
LK 5	28.67	145.33	95.33	13.67
LK 7	152.00	185.33	68.67	45.33
LK-32	92.00	175.33	158.67	42.00
LK-39	310.33	543.67	527.00	120.33
LK-41	158.67	208.67	142.00	77.00
LK-43	27.00	477.00	143.67	55.33
LK-47	145.33	162.00	58.67	37.00
LK-51	83.67	283.67	183.67	85.33
LK-54	113.67	413.67	80.33	57.00
LK-59	232.00	182.00	115.33	75.33
LK-60	143.67	193.67	160.33	48.67
LK-72	115.33	265.33	267.00	38.67
LK-81	315.33	148.67	448.67	175.33
LK-124	275.33	595.33	448.67	232.00
LK-141	280.33	582.00	415.33	152.00
LK-142	162.00	420.33	270.33	55.33
LK-147	372.00	552.00	318.67	152.00
LK-150	108.67	375.33	42.00	95.33
LK-156	548.67	782.00	665.33	345.33
LK-188	103.23	58.77	43.48	37.11
LSD $_{(p=0.05)}$	43.13	34.61	38.53	21.79

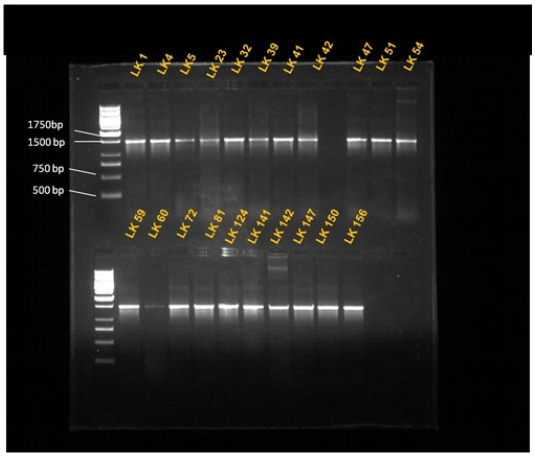

Plate 4. Visualization of PCR amplified 16S rDNA product of selected bacterial isolates obtained from pulp and paper mill effluent irrigated soils

ability was found in isolates LK 1, 39, 41, 43, 47, 51, 54, 60, 72, 124, 142, 147, 150, 188. Based on morphological and biochemical characterization most of the isolates showed resemblance with *Pseudomonas* sp. Based on morphological and biochemical characterization PCP utilizing bacteria was also identified by Shukla *et al*., (2001),Sharma and Thakur, (2008) and Tewari *et al.* (2011). Utilization of PCP in form of sole source of carbon by *Pseudomonas* sp and *Arthrobacter* sp was reported by Shukla *et al*. (2001) and Sharma and Thakur (2002). Sharma and Thakur (2008) characterized the *Pseudomonas* sp from paper mill and studied the potency of the isolated strains for PCP reduction in sequential bioreactor.

Growth of bacterial isolates at different concentrations of PCP

All the 22 isolates were further screened for their ability to grow at 100, 300, 500 and 700 ppm of PCP in MS broth. Growth was measured in terms of protein per ml of broth. All the 22 isolates could grow from 100 to 700 ppm of PCP in the MS broth, however, low growth was observed at 700 ppm (Table 4).

Significant variation was observed in growth as total protein among isolates at all the concentrations of PCP after 48 h of incubation. Out of 22 isolates, 8 isolates *viz*. LK 1, LK 4, LK 39, LK 81, LK 124, LK 141, LK 147 and LK 156 showed significantly higher growth at all the concentrations of PCP (Table 4). Isolate LK 156 showed maximum growth in terms of protein (548.67 µg mL^{-1} at 100 ppm PCP; 782.00 µg mL^{-1} at 300 ppm PCP; 665.33 µg mL^{-1} at 500 ppm PCP and 345.33 µg mL^{-1} at 700 ppm PCP). Isolate LK 5 showed significantly low growth as compared to other isolates. It was observed that all the isolates could grow well at 300 ppm of PCP in medium as compared to 100 ppm of PCP as evident by significantly higher protein at 300 ppm of PCP. Similar observation was observed for few isolates which grew well at 500 ppm of PCP as compared to 100 ppm of PCP (Table 4). The cultures were initially isolated by enrichment method and continuously maintained at 50 ppm of PCP in MA agar slants. When these isolates were grown at higher PCP concentration under shaking condition they exhibited higher growth in terms of protein. It could be due to higher availability of PCP as sole C source in the medium.

Table 5. Nucleotide identity (%) of isolates to the closest phylogenetic neighbour and classification of the isolates

Strain Number	Accession Number	Identity	% Similarity	Classification
LK 1	KF261572	*Pseudomonas aeruginosa*	96%	γ - Proteobacteria
LK 4	KF261573	*Ensifer adhaerens*	98%	α – Proteobacteria
LK 5	KF261574	*Pseudomonas citronellolis*	97%	γ - Proteobacteria
LK 23	KF261575	*P. citronellolis*	99%	γ - Proteobacteria
LK-32	KF261576	*P. citronellolis*	97%	γ - Proteobacteria
LK-39	KF261577	*Pseudomonas putida*	99%	γ - Proteobacteria
LK-41	KF261578	*P. putida*	99%	γ - Proteobacteria
LK-43	KF261579	*P. putida*	99%	γ - Proteobacteria
LK-47	KF261580	*P. putida*	99%	γ - Proteobacteria
LK-51	KF261581	*P. putida*	99%	γ - Proteobacteria
LK-54	KF261582	*P. putida*	99%	γ - Proteobacteria
LK-59	KF261583	*Ochrobactrum* sp	99%	α – Proteobacteria
LK-60	KF261584	*P. putida*	99%	γ - Proteobacteria
LK-72	KF261585	*P. putida*	99%	γ - Proteobacteria
LK-81	KF261586	*Pseudomonas citronellolis*	99%	γ - Proteobacteria
LK-124	KF261587	*P. plecoglossicida*	99%	γ - Proteobacteria
LK-141	KF261588	*P. citronellolis*	99%	γ - Proteobacteria
LK-142	KF261589	*P. putida*	99%	γ - Proteobacteria
LK-147	KF261590	*P. plecoglossicida*	99%	γ - Proteobacteria
LK-150	KF261591	*P. putida*	97%	γ - Proteobacteria
LK-156	KF261592	*Lysinibacillus fusiformis*	99%	Firmicutes: Bacilli
LK-188	KF261593	*Enterobacter* sp	99%	γ - Proteobacteria

The results indicated that PCP concentrations less than 500 ppm in medium were utilized by acclimated culture after 48 h of incubation. However, when the PCP concentration was above 500 ppm the utilization of PCP by the culture was low as indicated by low growth. The aerobic pathway of PCP degradation is: $C6Cl_5OH + 4.5O_2 + 2H_2O \rightarrow 6O_2 + 5HCl$ (Crawford and Crawford, 1996). The equation showed that PCP degradation leads to a decrease in pH. The pH of broth after 72 h of incubation did not decrease significantly in the medium containing PCP at 100 to 500 ppm of PCP and ranged in between 6.8 to 7.0. In contrast, at 700 ppm of PCP, the pH in medium decreased significantly and ranged in between 5.9 to 6.1. It could be the reason for low growth of isolates at 700 ppm of PCP in the medium. Similar observation was observed by Yang et al (2006) where the lag phase of bacterial isolate increased at higher concentration of PCP (>200 ppm) as compared to lower concentrations of <200 ppm and resulted in low growth at >200 ppm of PCP even after 45 h of incubation.

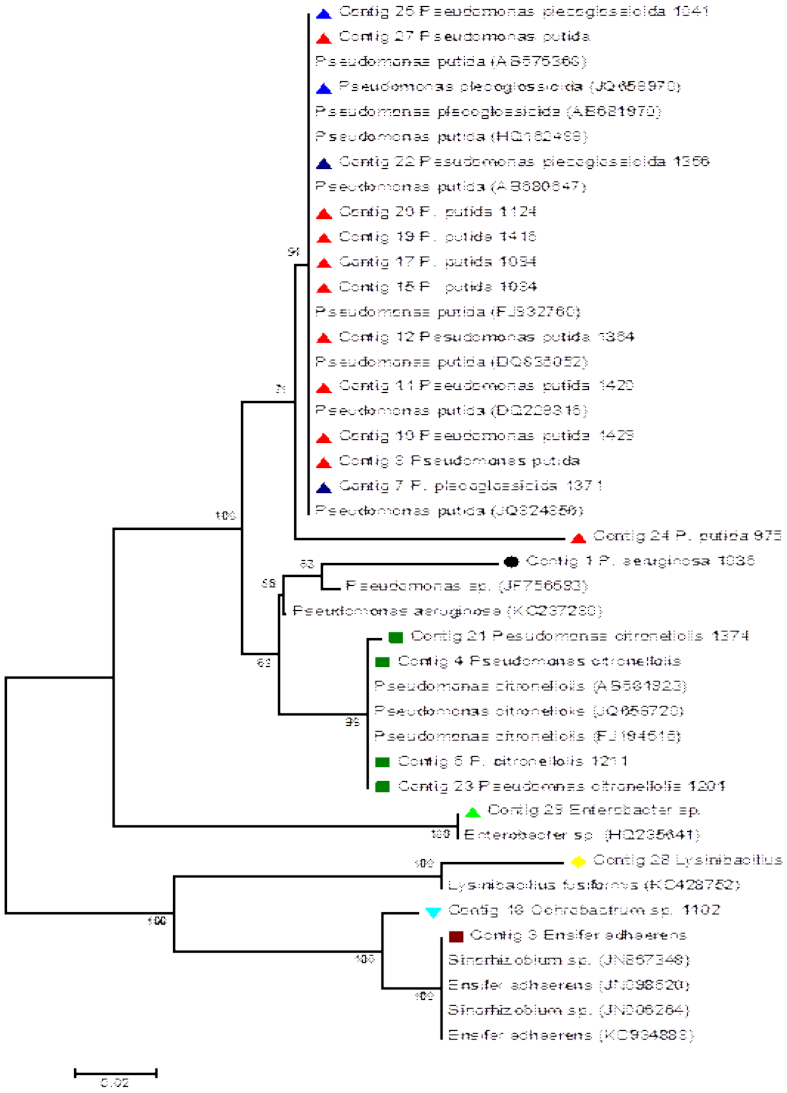

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of PCP degrading isolates and their closest phylogenetic relatives. The numbers on the tree indicate the percentage of bootstrap sampling derived from 1000 replicates

16S rRNA gene sequencing and phylogenetic analysis

From the genomic DNA of all the 22 bacterial isolates 16S rRNA gene was amplified using both forward and reverse primer (pA and pH, respectively). The reverse and forward purified 16S rRNA gene from all the isolates was sequenced. After obtaining the forward and reverse sequences of 16S rRNA gene (approx. 600-700 bp), contigs were made using online software CAP3. All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases by BLASTn search and the identity of isolates is given in table 5. The partial sequences of 16S rRNA gene sequences after analysis were submitted to NCBI GenBank database under accession numbers KF261572 to KF261593 (Table 5). Multiple sequence alignment of approx 1500-bp sequences was performed using CLUSTAL W, version 1.8 and phylogenetic was constructed (Fig 1). The phylogenetic relationships of the isolates as inferred from comparison of partial sequences (approx 1500bp) of the 16S rRNA genes showed that these isolates fell into three major lineages of domain Bacteria; the α,γ-*Proteobacteria* and *Firmicutes*.

The 19 isolates of γ-*Proteobacteria*, matched with sequences of *Pseudomonas aeruginosa* (LK 1), *Pseudomonas citronellolis* (LK 5, LK 81 and LK 141), *Pseudomonas putida* (LK 41, LK 43, LK 47, LK 51, LK 54, LK60, LK 72, LK 142 and LK 150), *Pseudomonas plecoglossicida* (LK 124 and LK 147) and *Enterobacter* sp (LK 188). *Pseudomonas* is a well-known PCP degrading genera reported to degrade high concentration of PCP (Karn *et al* 2010, Kaoa *et al*. 2005). Karn *et al* (2011) also reported degradation of PCP by *Enterobacter* sp isolated from distillery dumpsite using enrichment method. Karan (2011) observed *Enterobacter* sp was able to degrade 70% of PCP at 100 mg L^{-1} in growth medium. In our study, *Enterobacter* sp although could grow at 700 ppm of PC but the growth was low as compared to other isolates (Table 4).

Only 2 isolates *viz. Ochrobactrum* sp (LK 59) and *Ensifer adhaerens* (LK 4) belonged to α – Proteobacteria. *Ochrobactrum anthropi* was reported to degrade chlorophenol (Muller *et al*. 1998), whereas no such report is available regarding *Ensifer adhaerens.* Therefore this could also be a new PCP degrading bacterial genera. The single isolate of *Firmicutes* phyla was identified as *Lysinibacillus fusiformis* (LK 156) which could show maximum growth at 300 and 500 ppm of PCP in MS medium (Table 4). No reports are available in literature about *Lysinibacillus* sp degrading PCP, however, Chandra *et al* (2006) reported degradation of high concentration of PCP up to 300 mgL^{-1} by *Bacillus* sp. Hence, along with *Ensifer adhaerens*, *Lysinibacillus fusiformis* is also reported for the first time in present study.

CONCLUSION

Our results show that the ability to degrade pentachlorophenol is widely distributed among phylogenetically very different bacteria in agricultural soils irrigated with water contaminated with effluent discharged from pulp and paper mill. Bacterial isolates utilizing PCP up to 500 ppm obtained in this study can be used for developing consortium for degrading PCP in contaminated soils.

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