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Genetic and metabolic profiling of pentachlorophenol utilizing bacteria from agricultural soil irrigated with pulp and paper mill effluent

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ABSTRACT

Pulp and paper mill is a source of major environmental pollutants generating industries which include pentachlorophenol a highly chlorinated aromatic compound. PCP degrading bacterial isolates obtained from pulp and paper mill effluent contaminated site were characterised using amplified ribosomal DNA restriction analysis of 16S rRNA gene. Based on cluster analysis the representative 13 isolates were identified by partial 16S rRNA gene sequencing. These PCP degrading bacterial isolates were classified into four major bacterial lineages, α, β, γ-*Proteobacteria* and *Firmicutes*. The PCP_{max} and IC_{50} values of these isolates ranged from 100 to 300 mg PCP/l and 125 to 375 mg PCP/l respectively. *Pseudomonas aeruginosa* (PCP1) and *Pseudomonas* sp (PCP42) exhibited maximum PCP_{max} andIC_{50} value. Degradation of PCP by these isolates at their respective PCP_{max} ranged from 67.25% to 72.98% as analysed by the HPLC. Metabolic substrate usage of the isolates was evaluated using the BIOLOG™ ECO plates and unique carbon substrate usage profiles were observed. PCP degrading isolates, *Azospirillum* (PCP13 and PCP16) and *Inquilinus limosus* (PCP 27) were reported for the first time, these might represent new chlorophenol-degrading taxa.

Key words : BIOLOG, Culturable diversity, Pentachlorophenol utilizing bacteria, Pulp and paper mill effluent

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 (Singh and Thakur 2006). Paper mill generates as low as 1.5 m^3 of effluent per ton to as high as 60 m^3 tonne of paper produced (Asghar *et al*. 2007). The safe permissible limit of PCP in water is 0.30 μg/l (US EPA 1999). However, in our country, the large units of pulp and paper mills discharge their effluent, having residual PCP in high concentrations (> 80 mg/l effluent), in local water ways (Raj *et al*. 2005).

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 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

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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 functionally characterized and identified by 16S ribosomal RNA gene restriction fragment length polymorphism (RFLP) patterns, partial 16S rRNA gene sequencing, tolerance to PCP and capacity to utilize it as carbon source.

MATERIALS AND METHODS

The effluent from the Century Pulp and Paper Mill (CPM), LalKuan, Uttarakhand, 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 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.

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 extractable K as per methods described by Page *et al.* (1982).

All isolation steps were performed using mineral salts (MSM) medium containing 50 µg PCP/ml by following the procedure described in Karn *et al.* (2010a). From each of the sample single colonies were picked based on morphological differences after 24 hr of incubation at 30°C. The selected colonies were purified and maintained on MSM agar slants having PCP (50 µg/ml). The purified isolates were further screened for their ability to grow at 100, 150, 200, 250 and 300 ppm of PCP in the growth medium.

Genomic DNA extraction from the isolates showing growth above 100 ppm PCP was carried out by the method described by Pospiech and Neumann (1995). 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). Approximately 1 µg of PCR-amplified 16S rRNA gene fragments were restricted with endonucleases *Hha* I, *Rsa* I and *Bst*U I separately at 37°C for overnight and resolved by electrophoresis in 2.5% agarose. Strong and clear bands were scored for similarity and clustering analysis using the software, NTSYS-PC2 package (Numerical taxonomy analysis program package, Exeter software, USA). Similarity among the strains was calculated by Jaccard's coefficient, and dendrogram constructed using UPGMA method (Nei and Li 1979).

Representative bacterial isolates were selected based on cluster analysis of ARDRA for 16S rRNA gene sequencing. Purified 16S rRNA gene from these 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 3130 × l 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 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 (Thompson *et al.* 1994). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). Tree topologies were evaluated through bootstrap analysis of 1000 data sets by MEGA 4.0 package (Tamura *et al.* 2007).

The identified isolates were examined for carbon substrate utilizationby using the BIOLOG^{TM} ECO automated identification system (Hayward, California). Each well of the BIOLOG^{TM} ECO micro-titre plates were inoculated as per the manufacturer's instruction with 125 µl of cell suspension (10^8 cells/ml) and were incubated at 30°C in sealed plastic bags for 48 hr. Substrate oxidation was measured with a micro-titre plate reader at 590 nm. Clustering was based on binary data (usage/non-usage) for each of the 31 substrates. Data were submitted to cluster analysis using a simple matching coefficient (S_{SM}) (Sokal and Michener 1958) and clustering was achieved by the unweighted-pair-group method of association (UPGMA) (Sneath and Sokal 1973).

Growth kinetics of the representative isolates was studied using microbial growth kinetic analyzer (Bioscreen^{Tm}, Labsystems, Finland). Growth curve of all the 13 isolates was drawn from the data obtained through Bioscreen^{Tm} and specific growth rate (µ) of each of the isolate was calculated by the equation, $\mu = [(\log_{10} N2 - \log_{10} N1)\ 2.303)]/\ (t2 - t1)$, where t2-t1 is the minimum doubling time of population. The µ value was calculated as the highest rate along the exponential portion of the growth curve. The concentration of PCP supporting highest value of specific growth rate was designated as PCP_{max}. The concentration of PCP causing 50% reduction in specific growth rate was designated as IC_{50}.

PCP degradation potential of representative isolates at their respective PCP_{max} concentration was analyzed using HPLC by following the procedures as described earlier by Chandra *et al.* (2006). The dried PCP extracted sample was dissolved in 1.0 ml acetonitrile (HPLC grade) and a 10 µl sample was analyzed with a Waters 515 HPLC equipped with a UV-VIS (Waters-2487) detector set at wavelength of 254 nm using reverse phase C-18 column (size 250 × 4.6 mm) with particle size 5 mm was used to carry out separation. The isocratic mobile phase was acetonitrile and water in the ratio of 70:30 (v/v) and flow rate was 1 ml/min. A PCP and TeCH standard was run under the same conditions. The percent PCP utilization was estimated by measuring the peak area.

RESULTS AND DISCUSSION

Physicochemical characteristics of soil samples

The soil was sandy loam texture with pH 8.16; electrical conductivity (EC), 0.73 dS/m; organic carbon (OC), 0.95%; available N, 64.85 kg/ha; Olsen P, 16.86 kg/ha and extractable K, 130.62 kg/ha. The soil of control site also had sandy loam texture, pH, 7.30; electrical conductivity (EC), 0.30 dS/m; organic carbon (OC), 0.81%; available N, 63.09 kg/ha; Olsen P, 12.57 kg/ha and extractable K, 168.56 kg/ha.

Isolation, screening and phylogenetic analysis of bacterial isolates

By enrichment of soil samples with mineral salt medium containing 50 ppm of PCP as sole carbon source, 82 isolates tolerant to PCP were picked and screened for the growth at higher concentrations of PCP. Out of 82 isolates, only 45 isolates could tolerate and grow at PCP concentration 100 to 300 ppm. The tolerance level of PCP of selected isolates is shown in Table 1.

Restriction digestion of 16S rRNA gene using three endonucleases (*Hha* I, *Rsa* I and *Bst*U I) yielded 7 to 9 distinct restriction patterns for each enzyme. About 2 to 8 restricted fragments of varying sizes were common to each of the restriction patterns. Cluster analysis of combined 16S rRNA gene restriction pattern based on Jaccard's similarity index, grouped all the 45 isolates under 13 distinct groups. Representative isolate from each of the 13 groups were selected for sequencing.Moyer *et al.* (1996) reported *Hha* I, *Rsa* I, and *Bst*U I to be the best enzymes for RFLP pro?ling when performed virtually using a local bacterial sequence database.

The 16S rRNA gene sequences of the representative isolates were submitted to NCBI GenBank database under accession numbers HM439388, HM439399, HM439401-402, HM439406, HM439408, HM439412-413, HM439416, HM439420-421, HM439425, and HM439427.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 four major lineages of domain Bacteria; the α, β and γ-*Proteobacteria* and *Firmicutes* (Fig 1). Table 1 shows the nucleotide identity percentages of individual isolates to the closest identified phylogenetic neighbor in the NCBI database as compared by partial 16S rRNA gene sequences.

The four isolates of á-*Proteobacteria*, matched with sequences of *Azospirillum* sp. (PCP13 and PCP16), *Inquilinus limosus* (PCP27) and *Ochrobactrum anthropic* (PCP31). Although *Azospirillum* isolates were earlier reported to degrade phenol and benzoate in presence of yeast extract (Barkovskii *et al.* 1995), but this is the first report of PCP degradation by *Azospirillum.* The Isolates PCP13 and PCP16 had 96 and 95% sequence similarity to *Azospirillum* sp.

Table 1 Phylogenetic position and growth kinetics of the isolates and their percent pentachlorophenol degradation

Strain	Accession no.	Name	Identity %	$^{1}PCP_{max}$ (mg/l)	$^{2}\mu$ at PCP_{max}	$^{3}IC_{50}$ (mg/l)	µ at IC_{50}	%PCP degradation
PCP1	HM439387	*Pseudomonas aeruginosa*	99	300	0.46 (± 0.02)^{4}	350	0.20 (± 0.02)	72.91 (± 1.68)
PCP2	HM439388	*Pseudomonas putida*	98	200	0.29 (± 0.03)	275	0.14 (± 0.03)	65.03 (± 3.80)
PCP13	HM439399	*Azospirillum* sp	96	100	0.58 (± 0.04)	150	0.25 (± 0.04)	72.98 (± 2.73)
PCP15	HM439401	*Burkholderia* sp	98	100	0.77 (± 0.04)	200	0.38 (± 0.03)	72.90 (± 1.62)
PCP16	HM439402	*Azospirillum* sp	95	100	0.38 (± 0.01)	125	0.18 (± 0.02)	69.76 (± 2.63)
PCP20	HM439406	*Cupriavidus* sp	98	100	0.35 (± 0.08)	150	0.19 (± 0.03)	72.75 (± 1.88)
PCP22	HM439408	*Stenotrophomonas* sp	98	150	0.54 (± 0.02)	200	0.23 (± 0.02)	71.93 (± 2.46)
PCP27	HM439412	*Inquilinuslimosus*	98	150	0.38 (± 0.07)	250	0.22 (± 0.02)	67.25 (± 1.88)
PCP28	HM439413	*Stenotrophomonasmaltophilia*	99	150	0.48 (± 0.03)	200	0.18 (± 0.03)	72.93 (± 2.59)
PCP31	HM439416	*Ochrobactrumanthropi*	99	200	0.58 (± 0.06)	250	0.31 (± 0.05)	72.36 (± 3.00)
PCP37	HM439420	*Pseudomonas mendocina*	99	200	0.36 (± 0.01)	300	0.15 (± 0.04)	72.98 (± 0.32)
PCP38	HM439421	*Bacillus* sp	97	200	2.30 (± 0.20)	225	1.30 (± 0.17)	72.97 (± 2.59)
PCP42	HM439425	*Pseudomonas* sp	95	300	0.33 (± 0.05)	325	0.14 (± 0.04)	72.07 (± 1.94)

$^{1}PCP_{max}$: Concentration of PCP supporting maximum growth, $^{2}\mu$: Specific growth rate, $^{3}IC_{50}$: concentration of PCP at which growth reduced by 50%, ^{4} Figures in parenthesis is the standard deviation form mean

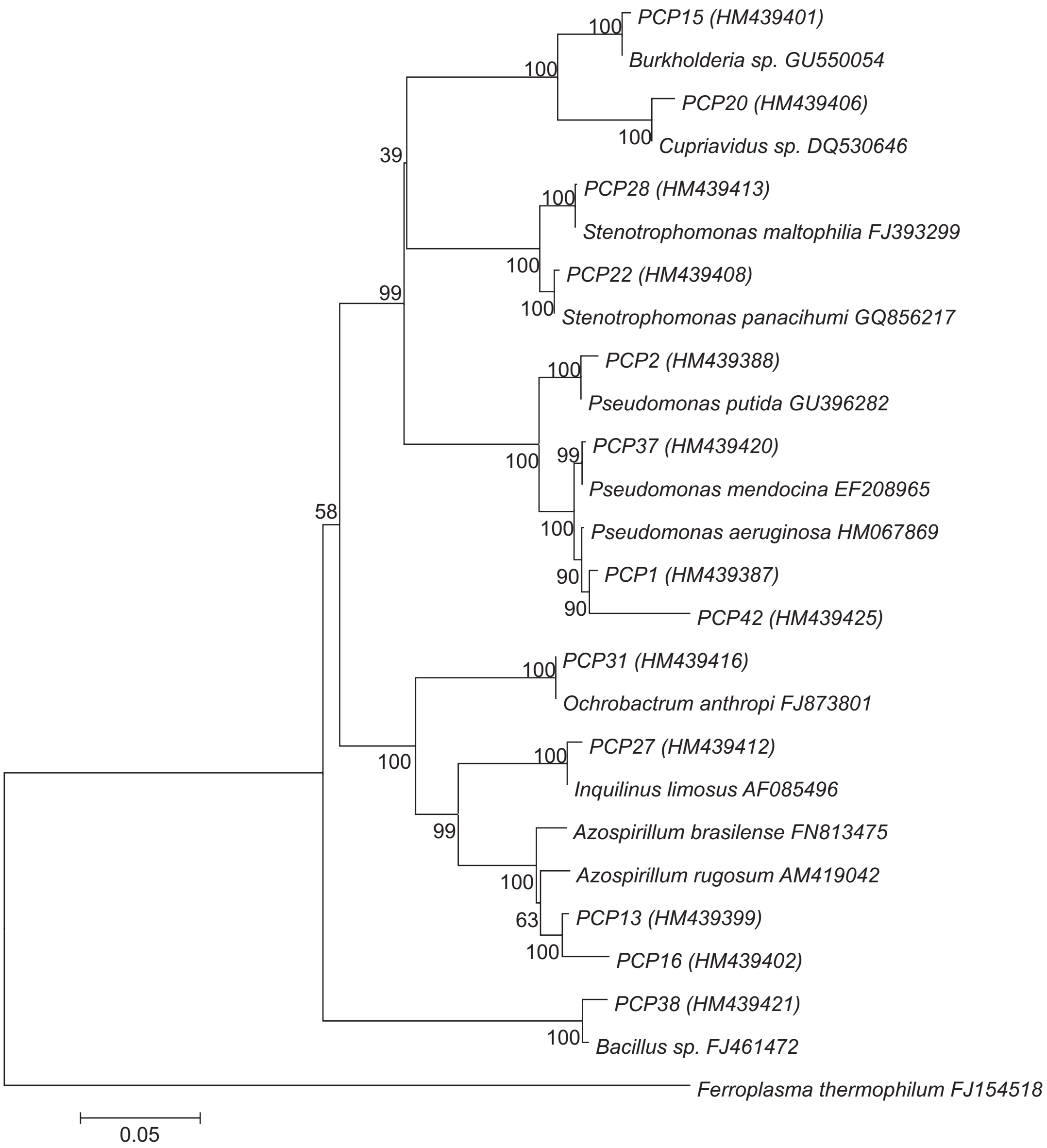

Fig 1 Phylogenetic tree based on the 16S rRNA gene sequences of PCP degrading isolates and their closest phylogenetic relatives. The numbers on the tree indicate the percentage of bootstrap sampling derived from 1000 replicates.16S rRNA gene sequences of *Ferroplasma thermophilum* was used as out-group.

showing that these isolates could be novel species. *Azospirillum* strains with ability to degrade aromatic compounds indicate that bacteria of this genus could be used to develop plant-bacterial systems for bioremediation of soils polluted with PCP. *Ochrobactrum anthropi* was reported to degrade chlorophenol (Muller *et al*. 1998), whereas no such report is available regarding *Inquilinus limosus.*Therefore this could also be a new PCP degrading bacterial species.

Two isolates of β-*Proteobacteria*, matched with sequences of *Burkholderia* sp (PCP15) and *Cupriyavidus* sp. (PCP20). The subdivision of β-*Proteobacteria* includes several genera those have ability to degrade xenobiotic compounds (Azaizeh *et al*. 2011). *Burkholderia* and *Cupriyavidus* are well-known bacterial genera for degrading chlorinated aromatic compounds (Webb *et al.* 2010,Sánchez and González 2007). In a recent study by Cáliz *et al*. (2011), they found dominance of *Burkholderia* in TCP- and PCP-polluted soil microcosms. Whereas six β-*Proteobacteria* isolates were classified into *Pseudomonas aeruginosa* (PCP44), *P. putida* (PCP2), *Stenotrophomonas* sp. (PCP22), *S. maltophila* (PCP28), *P. mendocina* (PCP37), and *Pseudomonas* sp (PCP42). *Pseudomonas* is a well-known PCP degrading genera reported to degrade high concentration of PCP (Karn *et al.* 2010b, Kaoa *et al*. 2005). Chlorophenol degradation is also well known in *Stenotrophomonas* (Qiao *et al.* 2007). The single isolate of *Firmicutes* phyla was identified as *Bacillus* sp. (PCP38), which is reported to degrade high concentration of PCP up to 300 mg/l (Chandra *et al*. 2006).

Carbon substrate utilization

Clustering analysis of 13 isolates based on use/non-use of 31 substrates studied by the BIOLOG^{TM} system is shown in Fig 2. At a 62% similarity level all 13 isolates were clearly separated in 2 groups (A and B) (Fig 2). Group A had 12 isolates all from *Proteobacteria*, whereas remaining one isolate was grouped separately and belonged to *Firmicutes*. Different carbon substrates such as methyl-D-glucoside, D-galactonic acid lactone, i-erythritol, 2-hydroxy benzoic acid, L-phenylalanine, tween 80, D-mannitol, 4-hydroxy benzoic acid, L-serine, cyclodextrin, N-acetyl-D-glucosamine, hydroxybutyric acid, L-threonine, and glycogen are the key carbon sources which differentiated *Proteobacteria* group to *Firmicutes*. The substrate usage patterns for the isolates revealed a broad variability, isolate PCP38 (*Bacillus* sp.) from group B used the least, i.e. only 11 substrates, which is about 35.48% of the total 31 substrates tested. Whereas PCP28 (*Stenotrophomonas maltophilia*) from group A used the maximum number is amounting to 28 substrate, 90.32% of the total substrates. Relating BIOLOG^{TM} experimental findings to the ecology of PCP degrading bacteria is challenging, but because some of the substrate usage was exclusive to isolates cultured from a specific collection site, one could hypothesize that metabolic activity is a reflection not just of the species, but is also a reflection of the specific environmental conditions.

Microbial growth kinetics and HPLC analysis

All the 13 isolates showed growth at different concentration of PCP ranging from 100 to 375 mg/l and the specific growth rate at PCP$_{max}$ and IC$_{50}$ concentration of PCP is given in Table 1. Out of 13 isolates, *Pseudomonas aeruginosa* (PCP1) and *Pseudomonas* sp (PCP42) were the best in PCP tolerance among other isolates. Both the isolates, PCP1 and PCP42, had their specific growth rate maximum at 300 ppm of PCP in the medium (Table 1). They also showed maximum degradation of 72.91 and 72.07 respectively (Table 1) as evident by the conversion of PCP to tetrachloro-para-hydroquinone (TeCH). Garg *et al*. (2012) reported a strain of *Pseudomonas* which was extremely tolerant to excessively higher concentration of PCP (1400 mg/l). Four isolates, *Azospirillum* sp. (PCP13 and PCP16), *Burkholderia* sp

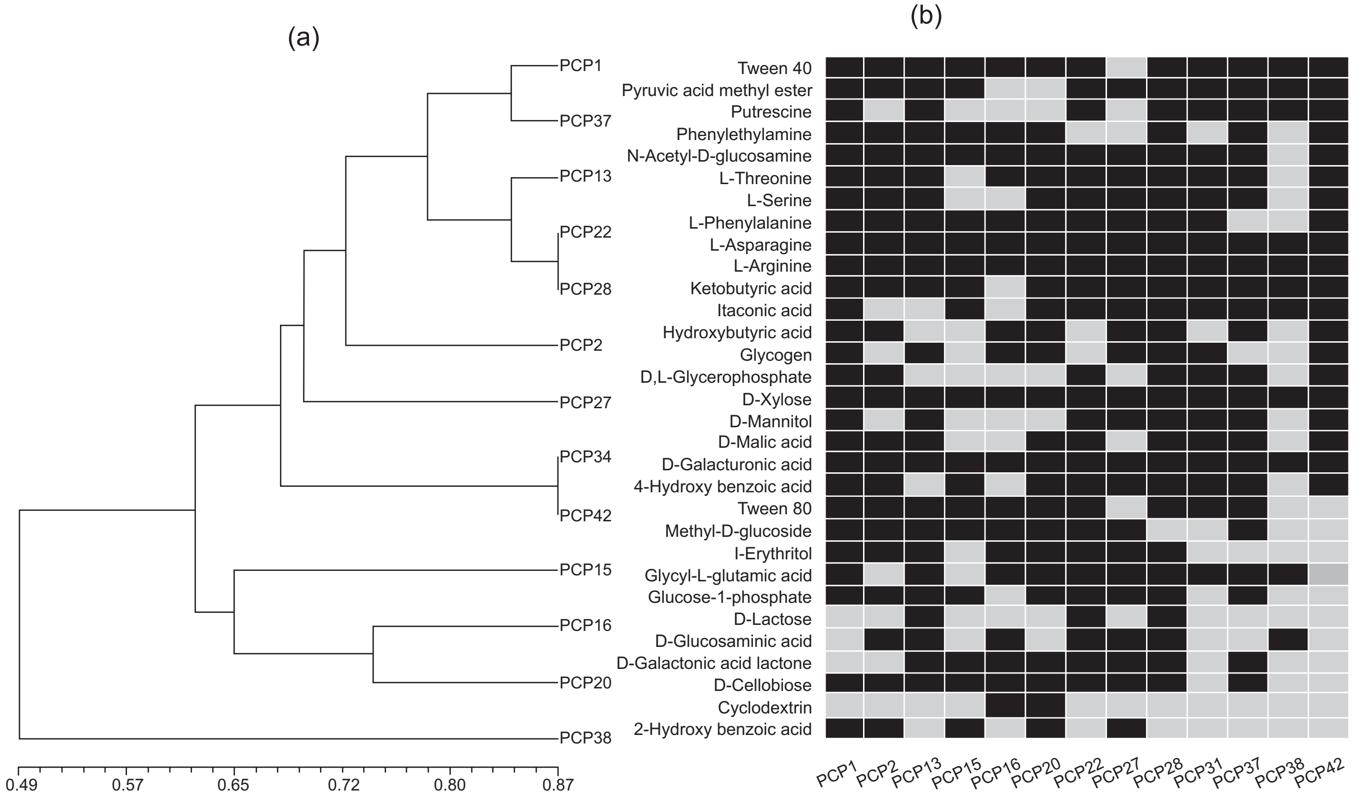

Fig 2 Cluster analyses of carbon substrate usage PCP utilizing bacterial isolates as obtained by BIOLOG^{TM}. (a) Dendrogram showing clustering of 13 representative bacterial isolates based on simple matching (S_{SM}) coefficient and unweighted-pair group method with average (UPGMA) clustering. (b) Carbon source usage of representative isolates via BIOLOG^{TM} ECO assay plates. Black boxes represent statistically significant substrate usage at any point during experiments.

(PCP15), and *Cupriavidus* sp (PCP20) showed minimum $\mathrm{PCP}_{\max}$ (100 mg/l) with percentage degradation of 72.98, 69.76, 72.90 and 72.75 respectively (Table 1). The IC_{50} values were 25 to 75 mg/l more than the respective $\mathrm{PCP}_{\max}$ values of the isolates, being maximum (350 mg/l) for *Pseudomonas aeruginosa* (PCP1) and minimum (125 mg/l) for *Azospirillum* sp. (PCP16) (Table 1). As an uncoupler of oxidative phosphorylation, PCP is toxic to microorganisms (Escher *et al*. 1996). Our results are in agreement with Männistö *et al*. (1999), where it is reported that 100 mg/l PCP had no effect on growth of most of the bacterial isolates. It was also observed that as the concentration of PCP was increased from $\mathrm{PCP}_{\max}$ values the specific growth rate (μ) decreased and generation time (g) increased.

In 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 300 ppm obtained in this study can be used for developing consortium for degrading PCP in contaminated soils.

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REFERENCES

Anandarajah K, Kiefer P M, Donohoe B S and Copley S D. 2000. Recruitment of a double bond isomerase to serve as a reductive dehalogenase during biodegradation of pentachlorophenol. *Biochemistry* **39**(18): 5 303–11.

Asghar M N, Khan S and Mushtaq S. 2007. Management of treated pulp and paper mill effluent to achieve zero discharge. *Journal of Environmental Management* **88:** 1 285–99.

Azaizeh H, Castro P M L and Kidd P. 2011. Biodegradation of organic xenobiotic pollutants in the rhizosphere. *Organic Xenobiotics and Plants* 8: 191–215.

Barkovskii A L, Korshunova V E and Pozdnyacova L I. 1995. Catabolism of phenol and benzoate by *Azospirillum* strains. *Applied and Soil Ecology* **2**(1): 17–24.

Cáliz J, Vila X, Martí E, Sierra J, Cruañas R, Garau M A and Montserrat G. 2011. Impact of chlorophenols on microbiota of an unpolluted acidic soil: microbial resistance and biodegradation. *FEMS Microbiology Ecology* **78**: 150–64.

Chandra R, Ghosh A, Jain R K and Singh S. 2006. Isolation and characterization of two potential pentachlorophenol degrading aerobic bacteria from pulp paper effluent sludge. *Journal of General and Applied Microbiology* **52**(2): 125–30.

Chandra R, Raj A, Yadav S and Patel D K. 2009. Reduction of pollutants in pulp paper mill effluent treated by PCP-degrading bacterial strains. *Environmental Monitoring and Assessment* **155**: 1–11.

Edwards U, Rogall T, Blocker H, Emde M and Böttger E C. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research* **17:** 7 843–53.

Escher B I, Snozzi M and Schwarzenbach P. 1996. Uptake, speciation, and uncoupling activity of substituted phenols in energy transducing membranes. *Environmental Science and Technology* **30**: 3 071–9.

Garg S K, Tripathi M, Singh S K and Singh A. 2012. Pentachlorophenol dechlorination and simultaneous Cr^{6+} reduction by *Pseudomonas putida* SKG-1 MTCC (10510): characterization of PCP dechlorination products, bacterial structure, and functional groups. *Environmental Science and Pollution Research* DOI 10.1007/s11356–012–1101–z.

Jensen J. 1996. Chlorophenols in the terrestrial environment. *Reviews of Environmental Contamination and Toxicology* **146**: 25–51.

Kao C M, Chai C T, Liu J K, Yeh T Y, Chen K F and Chen S C. 2004. Evaluation of natural and enhanced PCP biodegradation at a former pesticide manufacturing plant. *Water Research* **38**(3): 663–72.

Kao C M, Liu J K, Chen Y L, Chai C T and Chen S C. 2005. Factors affecting the biodegradation of PCP by *Pseudomonas mendocin*a NSYSU chloride release. *Journal of Hazardous Materials* **124**: 68–73.

Karn S K, Chakrabarty S K and Reddy M S. 2010a. Characterization of pentachlorophenol degrading *Bacillus* strains from secondary pulp-and-paper-industry sludge. *International Biodeterioration and Biodegradation* **64**: 609–13.

Karn S K, Chakrabarty S K and Reddy M S. 2010b. Pentachlorophenol degradation by *Pseudomonas stutzeri* CL7 in the secondary sludge of pulp and paper mill. *Journal of Environmental Sciences* 22(10): 1 608–12.

Männistö M K, Tiirola M A, Salkinoja-Salonen M S, Kulomaa M S and Puhakka J A. 1999. Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater. *Archives of Microbiology*171: 189–97.

Moyer C L, Tiedje J M, Dobbs F C and Karl D M. 1996. A computersimulated restriction fragment length polymorphism analysis of bacterialsmall-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Applied and Environmental Microbiology* **62**: 2 501–7.

Muller R H, Jorks S, Kleinsteuber S and Babel W. 1998. Degradation of various chlorophenols under alkaline conditions by Gram-negative bacteria closely related to *Ochrobactrum anthropi*. *Journal of Basic Microbiology* **38**(4): 269–81.

Muthukumar T and Vediyappan S. 2010. Comparison of arbuscular mycorrhizal and dark septate endophyte fungal associations in soils irrigated with pulp and paper mill effluent and well-water. *European Journal of Soil Biology* **46**(2): 157–67.

Nei M and Li W H. 1979. Mathematical-model for studying genetic-variation in terms of restriction endonucleases. *Proceedings of National Academy of Sciences USA* **76:** 5 269–73.

Page A L, Miller, R H and Keeney D R. 1982. *Methods of Soil Analysis*, 2nd edn. Amercen Society of Agronomy, Madison, WI, USA.

Pospiech A and Neumann B. 1995. A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends in Genetics* **11**: 217–8.

Qiao C L, Liu Z and Yang C. 2007. Biodegradation of p-nitrophenol and 4-chlorophenol by *Stenotrophomonas* sp. *FEMS Microbiology Letters* **277**(2): 150–6.

Raj A, Chandra R and Patel D K. 2005. Physicochemical characterization of pulp and paper mill effluent and toxicity assessment by a tubicid worm *Tubifex tubifex*. *Toxicology International* **12**: 109–88.

Saitou N and Nei M. 1987. The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–25.

Sánchez M A and González B. 2007. Genetic characterization of 2,4,6-trichlorophenol degradation in *Cupriavidus necator* JMP134. *Applied and Environmental Microbiology* **73**(9): 2 769–76.

Singh P and Thakur I S. 2006. Colour removal of anaerobically treated pulp and paper mill effluent by microorganisms in two steps bioreactor. *Bioresource Technology* **97**: 218–23.

Sneath P H A and Sokal R R. 1973. *Numerical Taxonomy: The Principles and Practice of Numerical Classification*. W H Freeman, San Francisco.

Sokal R R amd Michener C D. 1958. A statistical method for evaluating systematic relationships. *Univ Kansas Sci Bull* **38**: 1 409–38.

Tamura K, Dudley J, Nei M and Kumar S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1 596–9.

Thompson J D, Higgins D G and Gibson T J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4 673–80.

Tripathi B M, Kaushik R, Kumari P, Saxena A K and Arora D K. 2011. Genetic and metabolic diversity of streptomycetes in pulp and paper mill effluent treated crop fields. *World Journal of Microbiology and Biotechnology* **27**(7): 1 603–13.

Ugurlu M, Gurses A, Dogar C and Yalcin M. 2007. The removal of lignin and phenol from paper mill effluents by electrocoagulation. *Journal of Environmental Management* **87**: 420–8.

US EPA.1999. Search in ecotoxicology database system (U.S. EPA MED-Duluth) http://www.epa.gov/medecotx/ecotox home.htm.

Webb B N, Ballinger J W, Kim E, Belchik S M, Lam K S, Youn B, Nissen M S, Xun L and Kang C. 2010. Characterization of chlorophenol 4-monooxygenase (TftD) and NADH: *FAD oxidoreductase (TftC) of Burkholderia cepacia* AC1100. J*ournal of Biological Chemistry* **285**: 2 014–27.