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Biodegradation of 4-Chlorophenol by *Providentia sp. CJ-3* isolated from contaminated soil sediment

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Abstract

A bacterium was isolated from soil sediment contaminated with 4-chlorophenol (4-CP) by enrichment culture technique. The isolated bacterium was identified as *Providencia CJ3* on the basis of rDNA gene analysis. *Providencia CJ3* convincingly degraded 4-CP as evidenced by the IR spectra of organic extract of the spent medium. The IR absorption spectrum of organic extract showed absence of peaks at 3200-3600 cm^{-1} and 675-870 cm^{-1} region represents the degradation of an aromatic ring. The TLC analysis of organic extract showed the accumulation of a metabolite with an R_f value of 0.53 that corresponded to hydroquinone on co-chromatography. The cell free extract subjected to SDS-PAGE electrophoresis, revealed induction of several proteins during 4-CP biodegradation. The activity of chlorophenol-NADPH-Oxidoreductase was assayed and the specific activity was found to be 0.84μmoles/min/mg of protein.

Keywords: rDNA gene analysis, *Providencia sp.* 4-chlorophenol, Biodegradation, SDS-PAGE, IR spectra

Introduction

Chlorophenols represent an important source of soil contaminants as they are widely used as bactericides, insecticides, herbicides, and fungicides. 4-Chlorophenol (4-CP), a representative of *p*-substituted phenols has been listed as a priority pollutant [15]. It is also distributed widely in the environment as an end-product of reductive dechlorination of polychlorinated phenols [16]. 4-CP is used as a disinfectant in homes, farms, hospitals, and as an antiseptic for root canal treatment [17].

Chlorophenols are produced commercially as well as during disinfection of wastewater or drinking water with chlorine [1]. They are also produced during the bleaching of wood pulp with chlorine [3]. The chloroperoxidase-mediated chlorination of natural organic matter does contribute to the levels of chlorophenols that are found in surface water [6]. Chlorophenols have been measured in city air at concentrations of less than a part per trillion of air. National Occupational Exposure Survey (NOES) has estimated that humans are exposed to 4-CP at work [12]. The bioaccumulation potential

of 4-CP, was reviewed and based on bioconcentration values and log octanol/water partition coefficients, all chlorophenols are potent enough to accumulate in aquatic organisms [10]. Thus, removals of such contaminants from environment by adopting economy methods are required.

Microbial bioremediation has become more attractive than the traditional methods for decontamination due to convenience, lower cost and minimal impacts on the environment [9]. The objective of this study is to study the biological detoxification of 4-chlorophenol by soil bacteria. Here we report the biodegradative pathway of 4-CP operating in *Providencia sp.*

Materials and methods

Media and growth conditions

The bacterium was cultured in a liquid Mineral Salt Medium (MSM) comprising of (g/L) of $NaHCO_3$ (2.0), NH_4Cl (2.0), NaCl (2.0), $MgSO_4$ (0.4), K_2HPO_4 (0.4), $NaNO_2$ (2.0), Yeast extract (0.02%) and peptone (0.04%). 4-CP (0.2% w/v) was incorporated as the growth substrate after autoclaving the medium. The bacterial growth was monitored at 660 nm with a spectrophotometer at regular intervals by withdrawing a known volume of the medium aseptically. Bacterium was maintained as liquid cultures by transfer to fresh medium weekly.

16S rDNA sequence and phylogeny analysis

Briefly PCR was performed using the extracted genomic DNA of the bacterial sample. The PCR product was purified and analyzed by the DNA Sequence analyzer. Given sample passed pre sequencing QC by gel electrophoresis. Then two sequencing reactions were performed. The primer used for sequencing was 16SF_079 and 16SR_077. BLAST analysis using BLAST server was performed on the consensus sequence of reverse and forward sequences. The similar sequences were aligned using ClustalW software. The alignment files were used to construct the phylogenetic tree using Phylodraw.

Bacterial degradation of 4-Chlorophenol

The biodegradation of 4-CP was monitored by distinct change in its absorption maxima (λ_{max} 286nm) using UV-Visible spectrophotometer. The degradation rate of 4-CP was determined by culturing the bacterium in 100ml MSM supplemented with 4-CP (0.2% w/v). The decrease in concentration of the 4-CP was also determined at regular time intervals as per the Standard Methods for the Examination of Water and Wastewater [2].

Extraction and characterization of metabolites

Metabolites that accumulated in the spent medium during the growth of bacteria on 4-CP as substrate were isolated by solvent extraction. The spent medium after acidification with dilute HCl (0.1M) was extracted with diethyl ether (1:3 v/v) three times. Later the resulting extract was dried over anhydrous sodium sulphate and evaporated to dryness. The residue obtained was dissolved in methanol and characterized by TLC.

The TLC analysis involved the co-chromatography of the isolated (extracts) and authentic compounds with readymade commercial plates (Alugram sil G 0.20mm thick layer, Macherey-Nagel GmbH & Co Germany) using ethyl acetate: benzene (9:15 v/v) as the solvent system [7], [8].

IR spectra of intermediate compounds

The bacteria in their log growth phase were collected by centrifugation. The metabolites that accumulated in the spent medium were isolated by solvent extraction method. The spectrum of control and degraded sample

was recorded with IR spectrophotometer and then both spectra were compared.

Protein induction during the degradation studies

Bacterial isolate was grown on 4-CP and cells were harvested in their log growth phase. The pellet was collected by centrifugation and washed repeatedly with 50mM sodium phosphate buffer of pH-7.0 and suspended in 0.1M potassium phosphate buffer. The cells were disrupted by sonicator (Vibracell, Sonics and Materials CT USA Model VC 130 PB 50MHz) for a total of 3 minutes under ice cold conditions. Afterwards the cell debris and undisrupted cells were removed by centrifugation at 10,000rpm for 15 minutes; resulting supernatant (20µg protein) was used to assess the protein induction profile by SDS-PAGE. The protein profile obtained from cells grown on nutrient broth served as the control.

Enzyme assays

Preparation of cell free enzyme extract

The bacterial isolate was grown in the presence of 4-CP and the resulting cells were harvested in their log growth phase. The cells obtained were repeatedly washed with 50mM sodium phosphate buffer, pH-7.0 and finally suspended in 0.1M KH_2PO_4 buffer, (pH-7.2) containing 1mM ascorbic acid, 10% acetone, 10% glycerol and 1mM dithiothreitol and sonicated with a cell disrupter (Vibracell, Sonics and Materials CT USA Model VC 130 PB 50MHz) for a total of 3 minutes under ice cold conditions. After sonication, the cell debris and unbroken cells were separated by centrifugation at 10,000rpm for 15 minutes. The resulting supernatant was used as the source of enzyme. The protein in the enzyme preparation was estimated according to FC method [11].

Chlorophenol-NADPH-Oxido-Reductase assay

The Chlorophenol-NADPH-Oxidoreductase activity in the cell free extracts was measured in a reaction mixture (5 ml) containing 25mM phosphate buffer (pH 7.2), 0.17mM NADPH, 0.04mg of enzyme; the reaction was initiated by adding 0.08mM 4-CP [8]. Enzyme activity was monitored by noting the decrease in absorbance at 340nm due to the substrate dependent oxidation of NADPH at 25^{o}C. One enzyme unit is defined as the amount of enzyme which in presence of chlorophenol causes the oxidation of 1µmol NADPH per minute.

Results

Isolation of 4-CP degrading micro-organism

The bacteria degrading 4-chlorophenol (4-CP) were recovered from soil contaminated with chlorophenol by adopting enrichment culture technique. Most potent and efficient chlorophenol degrading bacteria that utilize 4-CP were further purified by adopting standard microbial procedures and characterized to be *Providencia sp. CJ-3.* The biochemical characteristics of the isolated bacterium were listed in Table.1.

Table 1. Characteristics of *Providencia sp CJ-3*.

Organism	Characteristics
Providencia sp. CJ 3	Gram negative, Motile rods, flagellated, catalase positive, oxidase positive, Indole negative, Methyl red negative, Voges-proskauer positive, starch hydrolysis positive, H_2S production positive, citrate negative, urease positive, ferment sucrose, glucose, Optimal growth temperature of 35-37^{o} C.

Table 2: Blast Similarity Searches for r-DNA obtained from environmental bacterial isolate (*Providencia sp. CJ-3*).

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
HQ455043.1	*Providencia sp. CJ-3* 16S rRNA gene, partial sequence	1027	1027	91%	0.0	84%
HM347050.1	*Providencia sp. DGC-5* 16S rRNA gene, partial sequence	1027	1027	91%	0.0	84%
AY870456.1	*Providencia sp. UTDM314* 16S rRNA gene, partial sequence	1027	1027	91%	0.0	84%
HQ407257.1	*Providencia rettgeri strain T82* 16S rRNA gene, partial sequence	1016	1016	91%	0.0	84%
HM854245.1	*Providencia rettgeri strain KTH-7* 16S rRNA gene, partial sequence	1016	1016	91%	0.0	84%
HQ009881.1	*Providencia rettgeri strain KPE62307H* 16S rRNA, partial sequence	1016	1016	91%	0.0	84%

Table 3: Pairwise evolutionary distance for rDNA (label-1) from 4-CP degrading bacterium compared with database entries (label-2).

Label-1	Label-2	Distance
seq_consensus _RDNA-BCBUCCI	gi|306035140|gb|HM854245.1|	0.173500
seq_consensus _RDNA-BCBUCCI	gi|300675908|gb|HM347050.1|	0.160760
seq_consensus _RDNA-BCBUCCI	gi|313586338|gb|HQ455043.1|	0.169080
seq_consensus _RDNA-BCBUCCI	gi|62084729|gb|AY870456.1|	0.173580
seq_consensus _RDNA-BCBUCCI	gi|310751459|gb|HQ407257.1|	0.176300
seq_consensus _RDNA-BCBUCCI	gi|305862885|gb|HQ009881.1|	0.164620

16S rDNA sequence and phylogeny analysis

The isolated bacterium was subjected to rDNA phylogenetic analysis. Based on the result of rDNA sequence and phylogeny analysis (Fig.1), the isolated bacterium was identified as *Providencia sp. CJ-3*. The blast similarity searches for various ribosomal RNA gene accessions from *Providencia sp. CJ-3* represented close match (E=0). The pairwise evolutionary distance derived from ClustalW established that the isolated bacterium is *Providencia sp. CJ-3* (Table.2 & 3).

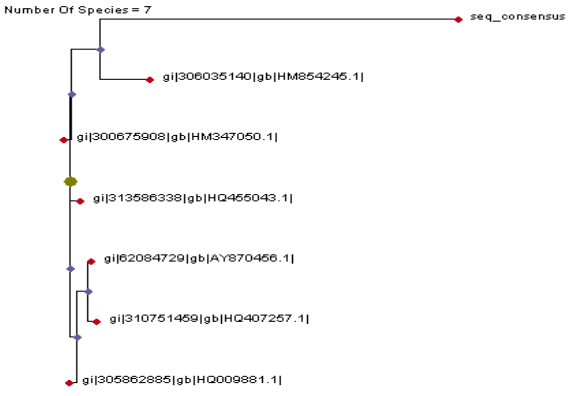
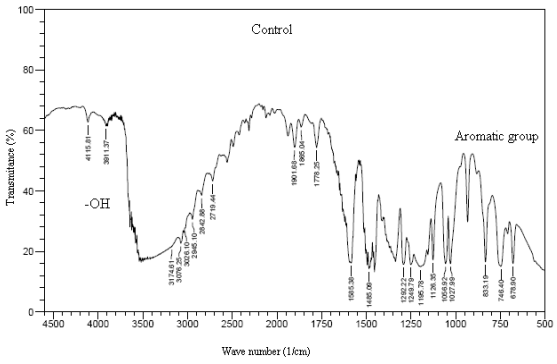
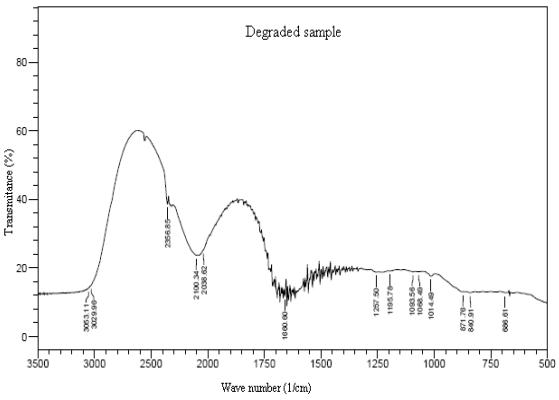


Figure 1: The phylogenetic tree for 4-Chlorophenol degrading environmental bacterial isolate.



a)



b)

Figure 2: Infrared spectrum of control (a) and organic extracts of spent medium (b) during biodegradation of 4-CP by *Providencia sp CJ-3.*

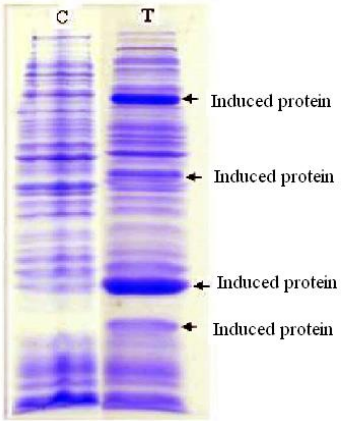


Figure 3: SDS-PAGE profile of proteins induced in *Providencia sp. CJ-3* metabolizing 4-chlorophenol (T) and Nutrient broth (C).

Degradation of 4-chlorophenol by Providencia sp. CJ-3

The metabolic fate was investigated by including 4-CP as a sole source of carbon and energy in to the growth medium. The growth response of the bacterium was monitored as an increase in OD at 660nm. The growth curve indicated that the exponential growth phase was between 20-25 days. The degradation of 4-CP was also monitored by distinct change in its absorption maxima (λ_{max} 286nm) consequent to microbial growth and it was noticed that the 4-CP concentration greatly decreased with increasing incubation periods (data not shown)

The organic extract of the control and degraded sample was subjected to IR spectrum analysis. The IR spectrum of the control showed peaks for hydroxy (–OH) group at 3200-3600 cm^{-1}region and for aromatic ring the absorption was in the region of 675-870 cm^{-1}. The IR absorption spectrum of the degraded sample showed absence of peaks for hydroxyl group as well as for aromatic ring of 4-CP; represents the degradation of an aromatic ring (Fig.2).

Characterization of metabolites

Metabolites that accumulated in the spent medium during the growth of *Providencia sp. CJ-3* on 4-CP were isolated and characterized. The TLC analysis demonstrated the accumulation of a metabolite with an R_f value of 0.53 that corresponded to hydroquinone on co-chromatography.

This result established that hydroquinone was one of the intermediary metabolite during the degradation of 4-CP by *Providencia sp. CJ-3.* The involvement of this intermediate in the catabolism was further supported by its utilization studies in replacement cultures (data not shown).

Protein induction profile during the biodegradation of 4-CP by Providencia sp. CJ-3

The SDS-PAGE profile of (Fig.3) induced proteins during the biodegradation of 4-CP by *Providencia sp. CJ-3* revealed the induction of several proteins different from control indicating the adaptation of the bacteria to the 4-CP and its metabolism by these proteins.

Enzyme activities in Providencia sp. CJ-3 cell free extracts

The metabolic pathway of 4-CP operating in *Providencia sp. CJ-3* was characterized by the detection and assay of the participating enzymes.

Providencia sp. CJ-3 degraded 4-CP, by expression of Chlorophenol-NADPH-Oxidoreductase enzyme. The presence of this enzyme in *Providencia sp. CJ-3* cell free extracts was confirmed by assay. The specific activity of the enzyme was found to be 0.84μmoles/min/mg of protein.

The SDS-PAGE electrophoretograms after separation of proteins in the cell free extracts showed the expression of several proteins that could be enzymes involved in 4-CP degradation. This fact was supported by our observation that when these bacteria degrade 4-CP readily when transferred from nutrient broth to MSM supplemented with 4-CP.

Discussion

The biodegradation of 4-CP has been investigated by many workers. Anaerobic dehalogenation of 4-CP, a common intermediate of polychlorophenol degradation, by mixed cultures was reported by [13], [14] investigated, *Arthrobacter chlorophenolicus* A6, a 4-CP-degrading strain, was degrading 4-CP *via* hydroxyquinol, during aerobic microbial degradation. In comparison, complete degradation of 4-CP by a laboratory transfer of a methanogenic enrichment from Bayou Chico took 10 months [5]. Transformation of 2,4-dichlorophenol (2,4-DCP), 4-CP and phenol was enhanced in sulphate-limiting conditions with average 47.7% TCP reduction compared to 11.6% in sulphate-enriched administered reactors monitored for 56 days [4].

In the current work we have concentrated on the biodegradation pathway of 4-CP in *Providencia sp. CJ-3*. The IR spectrum, TLC and enzyme analysis are all suggesting that *Providencia sp. CJ-3* biodegrades 4-chlorophenol successively in step by step degradation process. The SDS-PAGE protein profiles of *Providencia sp. CJ-3* revealed the induction of several proteins that may help adaptation of the bacteria to 4-CP and its metabolism by the new proteins.

Conclusion

The observation that bacteria can use 4-CP may have important implications for the natural attenuation and bioremediation of contaminated aquifers. The isolated *Providencia sp. CJ-3* was capable of metabolizing 4-CP under aerobic condition. The metabolic versatility of this type in bacterial diversity has raised interest in the research community to look for such suitable bacteria for potential use in biotechnological applications.

Conflict of interest: None

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