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RESEARCH ARTICLE

An Identification of Metabolite formed during an Aerobic bacterial degradation of Pentachlorophenol by *Flavobacterium* species

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Abstract

Introduction

India continues to encounter enormous environmental problems and many of them are result of industrial activity. In case of the leather tanning industry, regulations to reduce pollution have been in place since 1986, but measurements of the effluent from the industry still show that the concentrations of chemicals and organic matter are too high. In India, tannery industries have occupied a significant place in economic. It is the 7th major sector of earning foreign exchange in India. Export of leather goods comprises of several leather products of economical values which reclaimed new height of $2.8 billion in 2007-08, comparing with 1965-66 which was $65.5 million and still increasing (Tewari et. al., 2012). There are 3000 major tanneries, which are mainly located at Uttar Pradesh, Karnataka, Delhi, Andhra Pradesh, Punjab, Maharashtra, Kolkata and Chennai (Thakur *et al*., 2001). Due to biocidal property, PCP is used for curving and preservation of leather in tannery industries. PCP is highly toxic and recalcitrant xenobiotic compound due to presence of chlorine atoms along with the benzene ring which lowered down the electron density of benzene ring and renders the compound not to be easily used by organisms as their substrate. With standardized protocol, in present investigation an aerobic bacterial strains were isolated from two different sites PSCS1 and PSCS2 (Kanpur, Jajmau, U.P. India) and screened for their PCP degrading potential by using minimal salt agar medium containing, sodium salt of pentachlorophenol (NaPCP) as sole source of carbon and energy along with bromothymol blue as screening agent. Strains utilizing PCP were characterized morphologically and biochemically. For identification of metabolite during PCP degradation, two types of pathways for aerobic degradation of PCP have been described. One is through formation of chloro-catechols and other is through formation of subsequent hydroquinone. In pathway of chloro-catechols, the subsequent chlorophenols formed are further metabolized via ortho or modified-ortho ring cleavage pathways (Tewari et. al., 2013). In the hydroquinone pathway, subsequent dechlorination leads to formation of hydroquinone, which is subsequently cleaved by *ortho* ring cleavage enzyme. Further, hydroquinone pathway form protonated tetrachlorohydroquinone which is further converted into trichlorohydroquinone and 2,6-dichlororohydroquinone (Sharma et. al., 2009). The HPLC profile of noble metabolite formed during PCP degradation by an aerobic bacterial consortium was studied by justifying the hydroquinone pathway.

Materials and Methods

Isolation and Screening:

The bacterial strains were isolated in nutrient agar medium at 29^0 C for overnight in bacteriological orbital shaker and cultured on screening media of mineral salt agar having a composition of (g l^{-1}): $Na_2HPO_4.2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2; ammonium ferric citrate, 0.01; $Ca(NO_3).4H_2O$, 0.05; $NaNO_3$, 0.085 and trace element solution, 1ml l^{-1} and containing sodium PCP (15 mg l^{-1}) as the sole source of carbon and energy. Bromothymol blue (0.1%) was added as an indicator and the pH was adjusted to 6.5 using 0.1 N NaOH or 0.1 N HCl. Metabolism of PCP was observed as a change in pH of the medium, resulting in the formation of yellow halos around colonies (Thakur et. al., 2002)**.**

Morphological and Biochemical Characterization:

Screened strains were further morphologically characterized on the basis of shape, size, elevation, opacity, colour, spreading nature, margin and textures and biochemically on the basis of gram's test starch test, casein test lipid test citrate test and urease test respectively on MSM agar (Tewari and Shukla, 2012; Andrabi et. al., 2010).

Ring cleavage assessment

A general Rothera test was performed for detection of *ortho* or *meta* ring cleavage. In this test cell lysate (0.5 ml) was incubated with 2-chloro-1,4- benzenediol (0.5 µM) in 2 ml of *tris* buffer (0.02 M; pH 8) for 20 min, followed by addition of ammonium sulphate crystals (1 g), 1% sodium nitropruside solution (freshly prepared) and then ammonia solution (0.5 ml). Tubes were incubated for 1 h at 30^0C in shaking condition. Appearance of a deep violet coloration indicates the *ortho* ring cleavage of the substrate (Chupal et. al., 2005).

Identification of Metabolite

The metabolites formed during degradation of PCP by bacterial consortium were identified as per the method described. As per this method, the sample from the culture flask was centrifuged at 7650 x g for 10 minutes and metabolites present in the supernatant were extracted by dichloromethane (Sharma and Thakur, 2008).

Quantitative assessment of metabolite formed

For quantitative analysis, samples were separated by reverse phase HPLC (STR ODS II column, size 150 x 3.9 mm). The methanol and ammonium acetate buffer (0.01 M, pH 4.8) was used as mobile phase in the ratio of 70:30 v/v, flow rate 1.5 ml/min maintained and detection was performed by HPLC at 224 nm.

Results and Discussion

Isolated strains PSP1, PSP2, PSP3 and PSP4 were grown on MSM agar plates with PCP (15 mg l^{-1}) as the sole source of carbon and energy **(Fig.-1 and Fig.2)**. All isolates were able to metabolize PCP, as shown by their ability to grow and by the change in colour of the bromothymol blue from blue to yellow due to degradation of PCP which produces hydrogen ions. The screened strains were further morphologically characterized on the basis of shape, size, elevation, opacity, colour, spreading nature, margin and textures (**Table-1**) and biochemically on the basis of gram's test starch test, casein test lipid test citrate test and urease test respectively on MSM agar (**Table-2**). The strains showed similarities with the genus *Flavobacterium* species when the results of tests were compared with the Bergey's Manual of Bacteriology (Holt et. al., 1994).

On the basis of appearance of a deep violet coloration indicates the *ortho* ring cleavage of substrate in bacterial consortium. Similar, Rothera test was also performed in different studies for *Pseudomonas aeruginosa* (PCP2) and *Acinetobacter species* (PCP3) confirmed ortho-ring cleavage and was reported that during utilization of PCP as carbon source a stoichiometric amount of chloride was accumulated in the culture broth (Sharma et. al., 2009). HPLC analysis revealed that PCP degradation by aerobic bacterial consortium indicated two new peaks, tetracholorohydroquinone (RT=4.538) and chlorohydroquinone (RT=2.908) after 24 and 48 hrs **(Fig.-3)**. The retention time of the metabolites was identical with standard retention time of these compounds. The peak area of tetracholorohydroquinone was greater compared to chlorohydroquinone after 24 hrs, further the peak area of tetracholorohydroquinone reduced at 48 hrs and that of chlorohydroquinone increased indicating conversion of tetracholorohydroquinone to chlorohydroquinone. This result indicated that the consortium followed the hydroquinone pathway in degradation of PCP. The pathway for PCP degradation reveals additional problems beyond the toxicity of PCP. The intermediates in the degradation pathway are chlorinated hydroquinone, which are toxic for variety of reasons. While, they would be expected to have effects similar to those of PCP on oxidative phosphorylation and membrane properties. There was an additional route for toxicity due to the facile oxidation of hydroquinone to semiquinones and benzoquinones. This process produces superoxide and possibly other reactive oxygen species that can lead to oxidative damage to DNA. Both TCHQ and DCHQ have been shown to cause single-stranded breaks in DNA, predominantly through the formation of reactive oxygen species. Furthermore, benzoquinones that are quite electrophilic can react with variety of cellular nucleophiles, including glutathione,

proteins and DNA. TCHQ has been shown to bind covalently to both DNA and proteins but apparently after oxidation to tetrachlorobenzoquinone (TCBQ). Finally, the redox cycling caused by repeated oxidation of hydroquinone, followed by reduction by glutathione that can depleted the level of this important intracellular reductant. This conversion transform PCP into tetrachlorohydroquinone (TCHQ), trichlorohydroquinone and 2,6-dichlorohydroquinone was also reported and Tetrachloro-p-Hydroquinone (TeCH) was the first intermediate in PCP degradation by *Flavobacterium* species strain ATCC 39723 containing hydroxylase that oxidized PCP to TeCH (Xun and Orser, 1991; Xun et. al., 1992; McCarthy et. al., 1997; Yang and Lee, 2008). Subsequently, they identified the reductive dehalogenation of TeCH to 2,3,6-trichloro-p-hydroquinone and then to 2,6-dichloro-p-hydroquinone in a cell extract with the reduced form of glutathione as the reducing agent under anaerobic conditions. Here they reported that the purification of TeCH reductive dehalogenase that reductively dehalogenated TeCH to trichlorohydroquinone and then to dichlorohydroquinone. The peak of HPLC profile of PCP degradation indicates two new peaks, TeCH and Chq after 6 and 48 hrs and disappearance of PCP was observed, which was accompanied by the appearance of the two new peaks identified as TeCH and Chq. It was observed that peak area of TeCH was greater compared to Chq after 6 hrs. The peak area of TeCH reduced at 48 hrs and that of Chq, increased indicating conversion of TeCH to Chq and utilization of intermediary metabolite of PCP by the bacterial strains was tested (Copley, 2000 and Dercova et. al., 2007). According to earlier reports of PCP degradation pathway in bacteria contains five catalytic enzymes, which are responsible for its mineralization. The bacterial enzyme PCP-4-monooxygenase catalyzes the oxygenolytic removal of the first chlorine from PCP to tetracholorohydroquinone using nicotinamide adenine dinucleotide phosphate (NADPH) as a co-substrate. Tetrachlorohydroquinone is further converted trichlorohydroquinone by reductive dechlorination and subsequently to dichlorohydroquinone was formed by reductive dehalogenase enzyme. Dichlorohydroquinone acts as a precursor for ring cleavage enzyme, which converts it to chloromaleylacetate, an open ring structure (McCarthy et. al., 1997). The similar GC-MS profile of PCP degradation by bacterial consortium and reported peaks of tetrachloro-p-hydroquinone (TeCH) and 2-chloro-1, 4-benzenediol with decrease in the concentration of PCP after 48 hrs. Further a little decrease in PCP concentration with the emergence of a new peak of 2,3,4,6-tetrachlorophenol was also reported. Their results clarified that the nature of pathway followed by bacterial strains consortium able to degrade PCP via hydroquinone pathway (Sharma et. al., 2009). However, in present investigation the consortium converted PCP to tetracholorohydroquinone which was further metabolized to chlorohydroquinone. The consortium of *Flavobacterium* species followed the hydroquinone pathway during PCP degradation and a positive Rothera test for consortium confirmed ortho-ring cleavage.

It was found that four species PSP1, PSP2, PSP3 and PSP4 are screened from site PSCS1 and PSCS2 and screened on MSM agar medium. The morphological and biochemical test revealed that these species sowed similarities with *Flavobacterium* species and was able to degrade pentachlorophenol with result of this degradation new peak was observed during HPLC examination which resembled primary metabolite was tetracholorohydroquinone was reported which further metabolized to chlorohydroquinone. These results clearly justify that an aerobic degradation of Pentachlorophenol follows noble pathway of hydroquinone.

Table 1 – Morphological characterization of PCP degrading bacterial strains screened from tannery sludge:-

Characteristic	PSP1	PSP2	PSP3	PSP4
Shape	C	C	C	C
Size (mm)	0.25	0.05	0.1	0.1
Color	CR	Y	CR	OR
Opacity	T	O	T	O
Texture	NV	NV	V	NV
Spreading nature	N	N	Y	Y
Elevation	F	F	CO	CO
Margin	SR	S	SR	SR

C- Circular, I-Irregular, W-Whitish, CR- Creamish, OR-Orange, P-Pinkish, Y-Yellowish, GW-Grayish white, O-Opaque, T-Transparent, V-Viscous, NV- Not viscous, Y- Yes, N-No, F-Flat, CO-Convex, S-Smooth, SR –Serrated.

Table 2 – Biochemical characterization of PCP degrading bacterial strains screened from tannery sludge:-

Tests	PSP1	PSP2	PSP3	PSP4
Gram staining	+	+	+	+
Starch Test	-	+	-	+
Casein Test	+	-	+	+
Lipid hydrolysis Test	-	+	+	+
Citrate Test	-	-	-	+
Urease Test	+	-	-	+

+ Positive = Present; – Negative = Absent

Fig.1- Screened bacterial Strains PSP1 on MSM Agar plate.

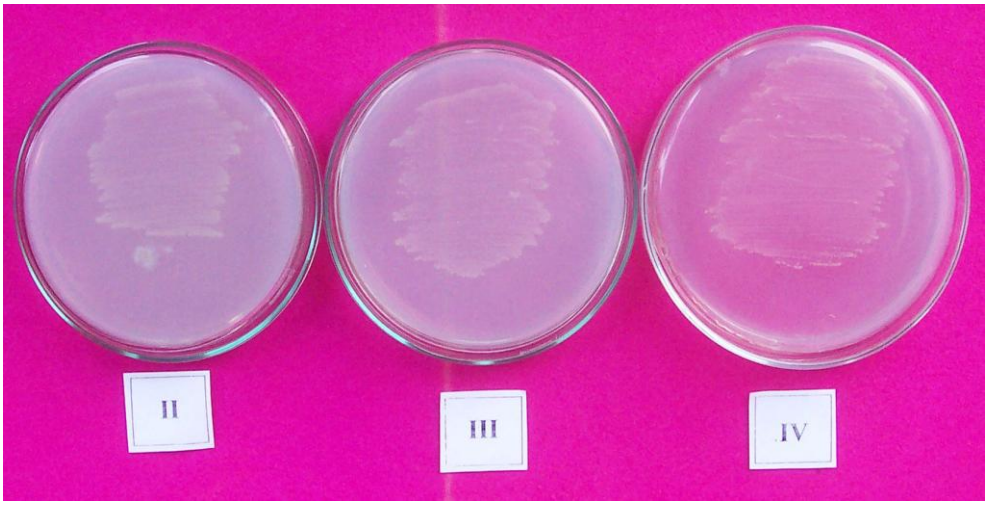

Fig.2- Screened bacterial Strains PSP2, PSP3 and PSP4 on MSM Agar plates.

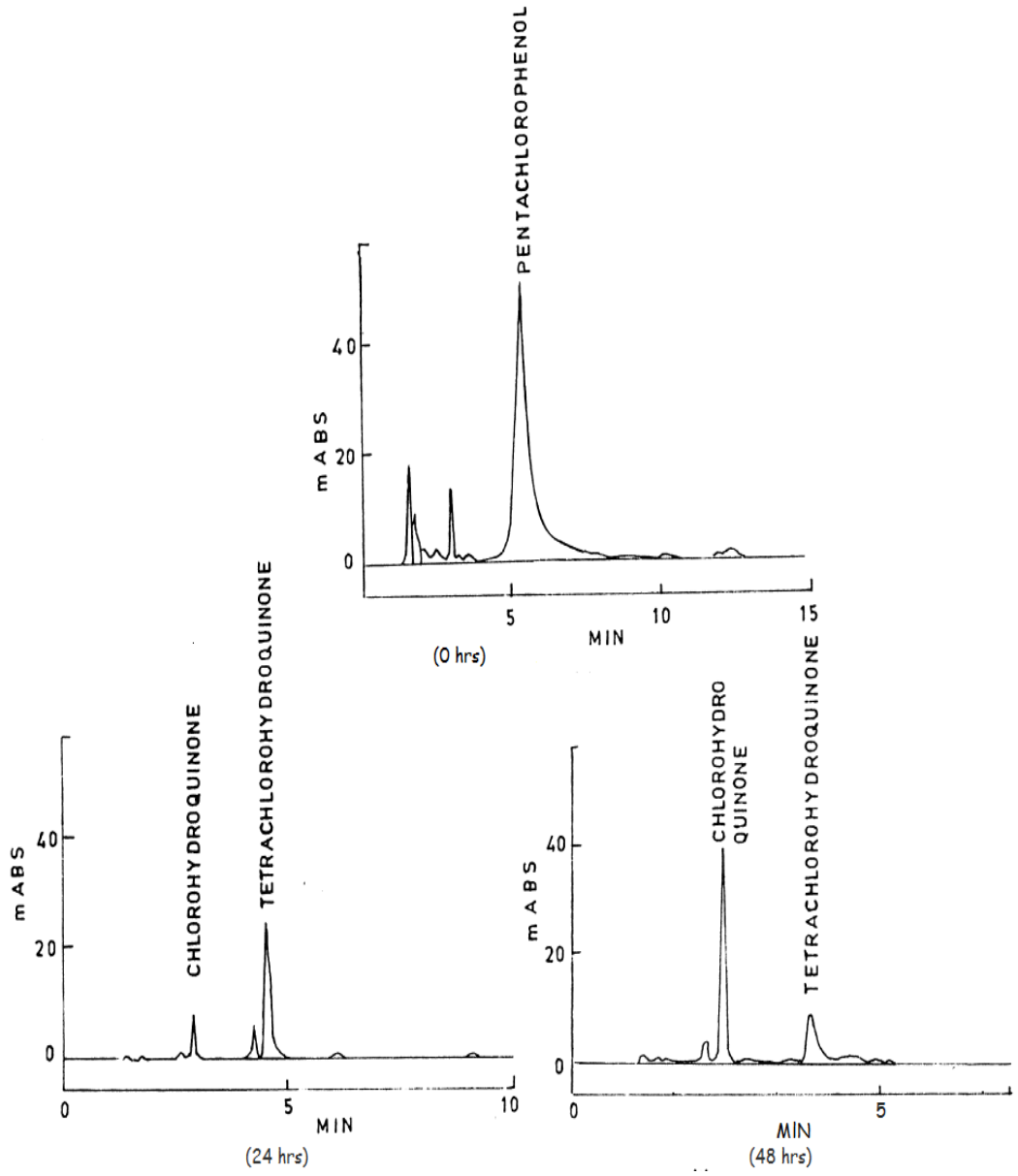

Fig. 3- HPLC profile of metabolites formed during PCP degradation by *Flavobacterium* species consortium in tannery effluent. Tetracholorohydroquinone (RT=4.538) and chlorohydroquinone (RT=2.908) after 24 and 48 hrs respectively.

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