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BIODEGRADATION OF PCB CONGENER (2, 4 DICHLOROBIPHENYL) BY *MICROCOCCUS SP.* ISOLATED FROM TRANSFORMER OIL CONTAMINATED SOIL

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ABSTRACT

The bacterium was isolated from transformer contaminated soil and was identified as *Micrococcus* sp. based on their biochemical characteristics. The 0.2% of 2,4-Dichloro biphenyl (2,4- DCB) was included in the growth medium of isolated bacterium as carbon source to study its biodegradation potential. The growth of the bacterium was monitored turbidometrically by reading absorbance at 660nm. The appearance of yellow metabolite, 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (HOPDA), during the growth of *Micrococcus* sp. on2,4- DCB as a substrate, showed maximum absorption at 398nm with no significant chloride release. The degradation study was monitored by GC-MS analysis; GC-MS of control 2,4 DCB with retention time 15.712min was compared to Ionization mass spectra fragmentation pattern stored in computer library ofNIST-MS Type Version -2, 2008. GC-MS of ethyl acetate extract of spent medium showed 91% reduction of 2,4- DCB with chlorobenzoic acid as one of the major intermediary metaboliteand was identical to the GC-MSof chlorobenzoic acid as per computer library of NIST-MS Type Version -2, 2005. The reduction in the substrate concentration was calculated as percentage of degradation by determining the net decrease in Electron capture detection.

KEYWORDS: 2,4- dichloro biphenyl; *Micrococcus* sp,; GC-MS; 2-hydroxy-6-oxo-6phenyl-hexa-2, 4-dienoic acid; chlorobenzoic acid.

INTRODUCTION

Chemicals released from the Industries pollute and contaminate the surrounding land.An extensive amount of these toxic compounds released into the environment are widely distributed, mainly in aquatic and soil systems (Seeger and Pieper 2009). Polychlorinated biphenyls are among one notorious contaminant. Polychlorinated biphenyls (PCBs) are members of chlorinated organic chemicals which may theoretically contain 209 different congeners (Passatore et al. 2014). They are persistently showing their presence in the list of top 10 most toxic priority pollutants of the US Agency for Toxic Substances and Disease Registry (ATSDR) (ATSDR 2011; Meggo and Schnoor 2013).

PCBs are hydrophobic with high affinity for adsorption to soil particles and for bioaccumulation in lipids and biomagnification (Nwinyi and obinna, 2010) causing hepato and immunotoxicity, carcinogenesis, affecting endocrine organs and reproduction in humans (Safe et al. 1997) and animals (Flora et al. 1991). PCBs have recently been categorized as carcinogen class I by the International Agency for Research on Cancer (IARC) (Lauby-Secretan et al. 2013). Further, PCB accumulates in fatty tissues and can be transmitted in breast milk and across placenta (Nwinyi and obinna 2010). To avoid further expansion of pollution, the United Nations Environment Programme Global Treaty adopted Stockholm convention (which consider PCB as one of the Industrial POPs) stipulated that the elimination of all liquids and equipment containing PCBs would be completed by 2028 (UNEP 2002).

Conventional technology like incineration and chemical methods are available which is not cost effective and additionally release dioxins to environment. Currently research has been focussed on development of biological methods which is cost effective and eco-friendly. Several bacteria capable of utilizing PCB as carbon source have been reported, including *Pseudomonas*, *Burkholderia*, *Comamonas*, *Cupriavidus*, *Sphingomonas*, *acidovorax*, *Rhodococcus*, *Corynebacterium* and Bacillus (Leigh, 2006, Sakai *et al*., 2005, Seto *et al*., 1995). Chlorobenzoic acids are common products of aerobic degradation of PCB (Bedard and Haberl 1990).

Although a number of aerobic bacteria able to transform PCB have been characterized, information about final metabolites released after biodegradation are limited. The aim of this study was to investigate the ability of isolated bacterial strain to degrade PCB congener 2, 4-dichlorobiphenyl (2,4 DCB) and to study intermediate products released in PCB biodegradation by GC-MS analysis.

MATERIAL AND METHODS

Collection of soil sample

Five soil and river sediment samples (three from transformer polluted soil and two from river sediments) near Industrial area, Bangalore, India, (12º59' N and 78º 35' E) were collected and used for the bacterial isolation.

Chemicals

Analytical grade high purity 2,4- dichlorobiphenyl (2,4 DCB)(PCB-7) (Sigma Aldrich, USA) was used in the present study. All solvents used in the present study are of analytical grade.

Isolation of bacterium

The soil sample (1g) was mixed with 100ml distilled water and incubated on rotary shaker (110rpm/30ºC). After thirty minutes, the sample was centrifuged (10,000rpm/10min). The pellet was inoculated to 100ml of Seubert substrate mineral medium (SMS medium) (Seubert 1960). The SMS medium was prepared by dissolving K_2HPO_{4-} (6.3g/L), KH_2PO_4 (1.82/g), NH_4NO_3 (1.00g/L), $MgSO_4.7H_2O$ (0.20g/L), $CaCl_{2.}H_20$ (0.10g/L), $NaMoO_42H_2O$ (0.06g/L), $MnSO_4.H_2O$ (0.06g/L) and $FeSO_4$ $7H_2O$ (0.10g/L) in one litre of distilled water. The medium was supplemented with 2,4 DCB (100ppm) as carbon source for the initial enrichment (140rpm/min/35º C/7d). 10% of enrichment culture was transferred to 100ml of SMS medium supplemented with 100ppm of substrate and enrichment was repeated once in 7 days for 8 weeks. Colonies were isolated after plating serially diluted enrichment sample on the solid SMS medium with 2, 4 DCB (100ppm) and simultaneously the diluted enrichment sample was also inoculated to Luria Bertani (LB) agar medium. One of the bacterial isolates showing potential 2,4 DCB degradation was selected and used for further study. The bacterial isolate was further subjected to biochemical tests.

Bacterial degradation of 2, 4-DCB

Isolated bacterium was inoculated to 100ml of SMS/100ppm of 2,4 DCB. The growth of the bacteria was monitored at regular intervals of time at 600nm. Uninoculated media was served as control. Growth of isolate was monitored by quantification of the substrate using UV-VIS spectrophotometer at λ_{max} of its maximum absorption (247nm). Alternatively, solvent extraction of the spent medium was analysed for percentage of degradation and intermediate metabolites production by GC-MS.

Measurement of chloride

The quantity of chloride released in the spent medium during biodegradation of 2,4 DCB by Micrococcus sp. was estimated according to Mohr's argentometric method. (kushalatha et al. 2012).

GC-MS study

The intermediate metabolites accumulated in the spent medium during growth of bacterium was obtained by acidification of the medium with HCl (0.1M), followed by ethyl acetate (1:3v/v) extraction. Later, the resulting extract was dried over anhydrous sodium sulphate and evaporated to dryness. The residue was mixed with methanol and further characterized by GC-MS.

Optimization ofGC chromatogramwas performed in Thermo TRACE GC ULTRA instrument usingTR-5 GC column(30m L x 0.25mm ID x 0.25um)packed with 5% Phenyl ethyl polysiloxane. Operation parameters: The column was operated from 40º C to 280º Cwith flow rate of 1.3ml/min with injection volume of 1.5µL. The GC-mass spectrum experiment was carried out by splitting of outlet of GC column coupled with Thermo DSQ II Quadrapole mass spectrometer equipped with chemical ionization source (CI) with electron multiplier detector. The detector gain was $1.00X10^{\wedge 5}$ (Multiplier voltage: 1479V),the ion source maintained at 220º C. Scan Mass ranges from 50*m/z* to 500*m/z* with positive polarity was used..

RESULTS

Soil and river sediment samples were collected near Industrial area, Bangalore, India and were used for the bacterial isolation. The isolated bacterial species was non motile, Gram positive cocci arranged in tetrads, showed yellow coloured colony (Fig.1). The biochemical characteristics of the isolated organism were catalase positive, oxidase positive, Indole negative, citrate negative, and the organism utilised 5% sodium chloride for growth. Further, glucose utilization without gas production was observed. The organism was unable to utilise maltose, sucrose and lactose. All the above characters supported that, the isolated organism was *Micrococcus* species (Holt *et al*., 1994).

The isolated *Micrococcus* sp. utilised 2,4 DCB; was indicated by measuring its growth turbidometrically (Fig.2). Alternatively the degradation rate of 2,4 DCB was also monitored at 247nm (absorption maximum) at regular intervals (Data not shown). The appearance of yellow metabolite, 2-hydroxy-6-oxo-6phenyl-hexa-2, 4-dienoic acid (HOPDA), during the growth of *Micrococcus* sp. on 2,4 DCB as a substrate, showed maximum absorption at 398nm with no significant chloride release.

GC-MS of control 2,4 DCB with retention time 15.712min and Ionization mass spectra fragmentation pattern stored in computer library of NIST-MS Type Version -2, 2008 isshown (Fig. 2a and 2b).

GC-MS of ethyl acetate extract of *Micrococcus* sp. after degradation showed 91% reduction of 2,4-DCB (Fig. 3a and 3b) with chlorobenzoic acid as of the major intermediatory metabolite (Fig. 4a). The above mass spectrum data were identical to the mass spectrum of chlorobenzoic acid as per computer library of NIST-MS Type Version -2, 2008 (Fig. 4b).

The reduction in the substrate concentration was calculated as percentage of degradation by determining the net decrease in Electron capture detection. Other metabolites detected in GC-MS with respective retention time were shown in Table 1. Proposed pathway for biodegradation of 2,4-DCB by *Micrococcus sp.* is shown in (Fig 5).

Tables and figures

Table 1: Metabolites released during biodegradation of 2,4-DCB by *Micrococcus* sp detected in GC-MS analysis.

RT	Highest Peak	*m/z* values and intensities	Library match
12.483	24.68	145,173,204	chlorobenzoic acid
15.678	29.46	152,222	2,4-dichlorobiphenyl
18.263	30.38	74, 87	Hexaadecanoic acid
19.962	39.17	41,55,74,83,96	o-octadecanoic acid

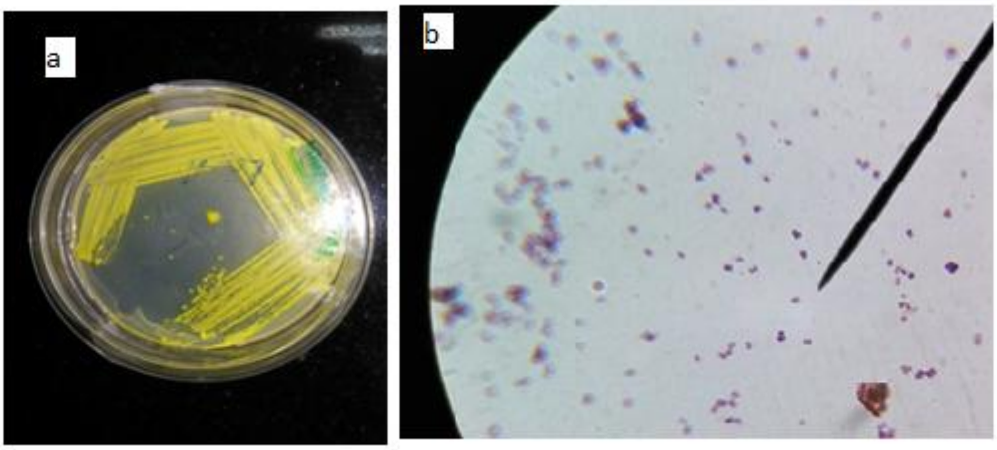


Fig. 1: The apperence of a) isolated soil bacteria on streak plate and b) the gram positive nature of the isolated bacteria.

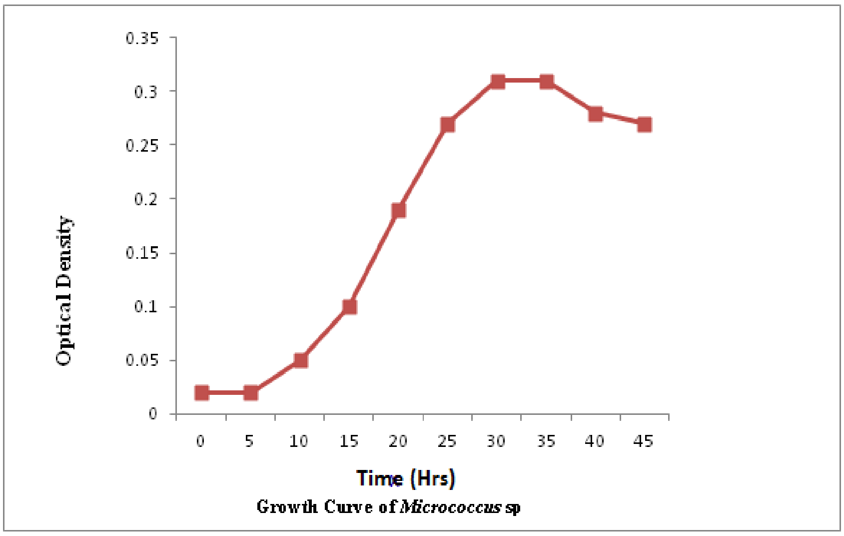


Fig 2: Growth Curve of *Micrococcus* sp. on 2,4-dichlorobiphenyl (2,4 DCB)

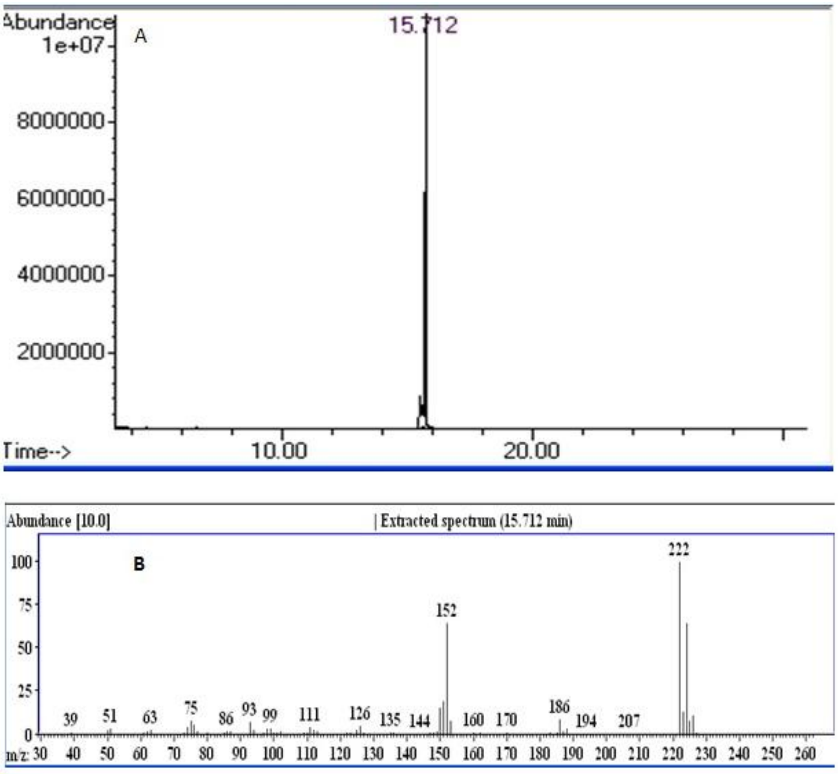
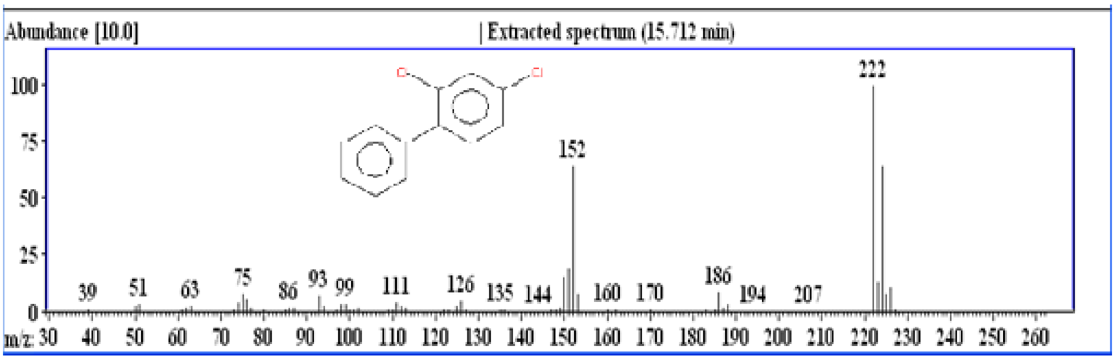
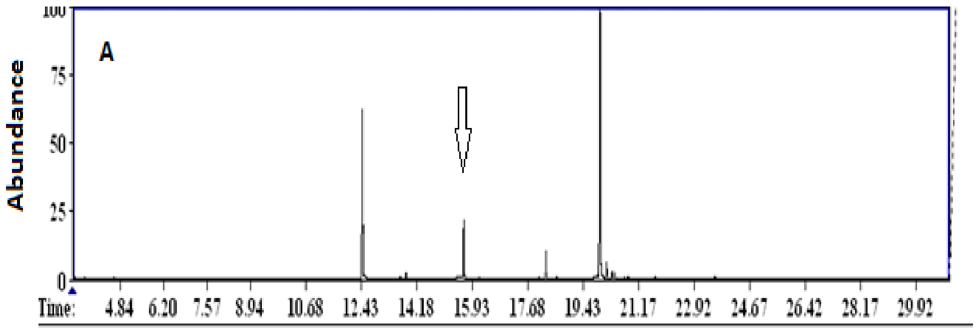


Fig 2a. A) GC-MS of standard 2,4-DCB (Peak eluting at 15.712min) B)Mass Ionization spectrum of standard 2,4-DCB.



2b. Computer library of NISTMS search for the peak15.712min showing 2,4-DCB.



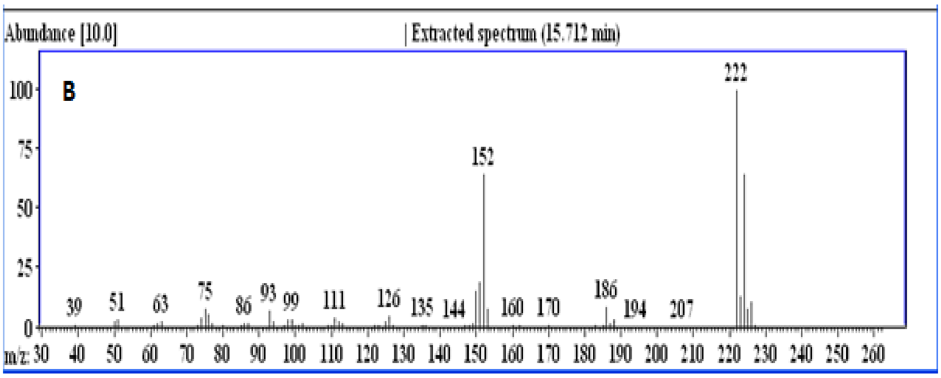
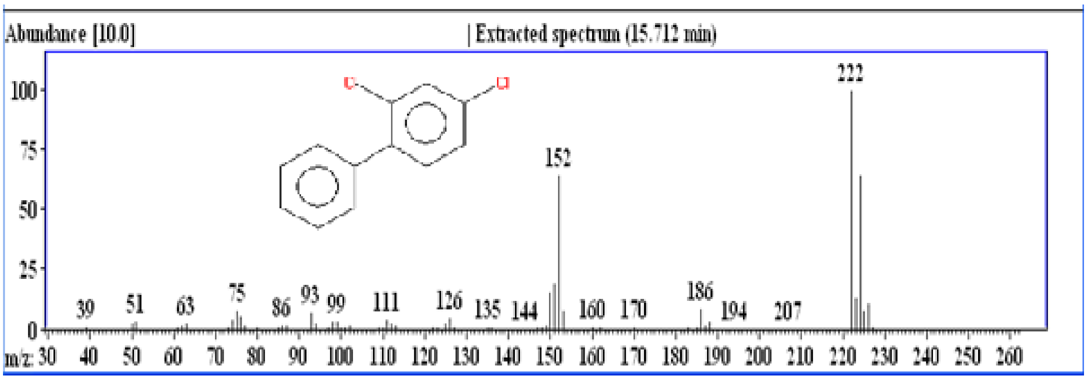


Fig 3a: A)GC-MS of ethylacetate extract oftheMicrococcus sp grown in the presence of 2,4- DCB andB)Mass Ionization spectrum of peak eluting at RT 15.712min of 2,4-DCB (*Note*: reduction in the peak of the substrate indicated by arrowmark).



3b. Computer library of NISTMS search for the peak15.712min showing2,4-DCB.

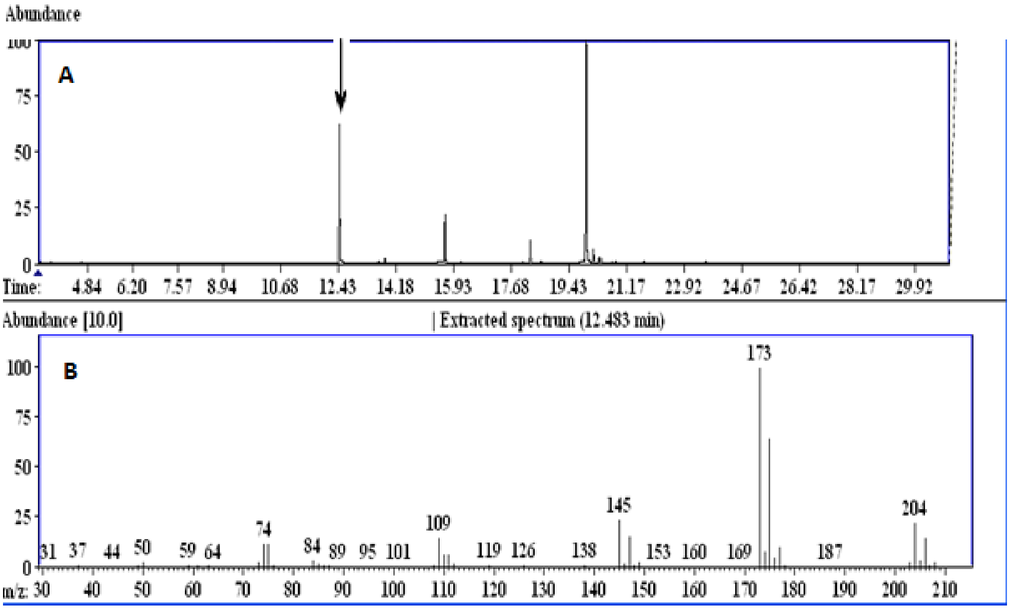
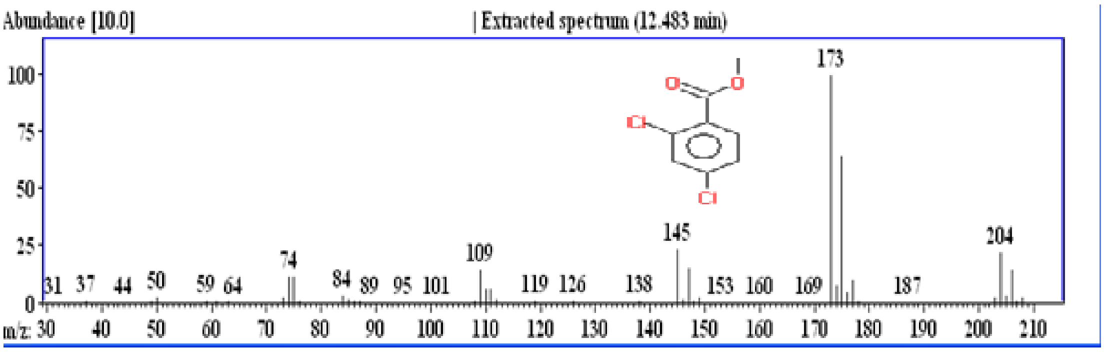


Fig. 4a. A)GCMS of ethylacetate extract of the *Micrococcus* sp grown in the presence of 2,4-DCBand important metabolite eluting at RT12.483 min is chlorobenzoic acidand B) Mass Ionization spectrum of metabolite eluting at RT 12.483 min is chlorobenzoic compound.



4b. Computer library of NISTMS search for the peak12.483min showing 2,4 dichlorobenzoic acid.

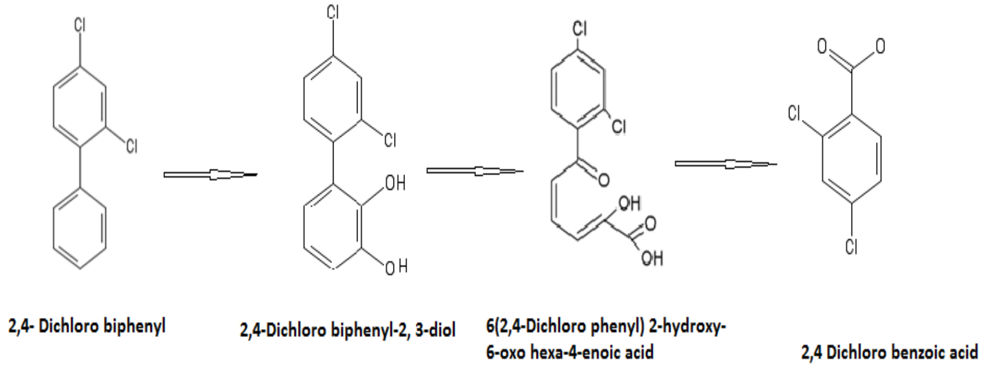


Fig 5. Proposed path way for biodegradation of 2,4-DCB by *Micrococcus sp.*

DISCUSSION

Bacterial isolate *Micrococcus* sp. was isolated from transformer oil polluted soil. Upon utilization of 2, 4 dichlorobiphenyl (2,4-DCB) an increase in turbidity and production of yellow colour showing absorption maximum at 398nm indicated the production of 2-hydroxy-6-oxo-6phenyl-hexa-2, 4-dienoic acid (HOPDA), a *meta* cleavage product. This observation suggests that the pathway for catabolism of 2,4-DCB proceeded via HOPDA. These results are in agreement with the report of Commandeur and Parsons (1990); Maltseva (1999); Borja et al. (2005); Somaraja et al. (2013). Further, Shobha and Gayathri (2015) also reported that biodegradation of 2,4 dichlorobiphenylby *pseudomonas* sp. released chlorinatedHOPDAin the growth medium (Shobha and Gayathri 2015) and cause pause in PCB degradation by accumulation of HOPDA hydrolase (Seah et al. 2001) and protoanemonium (Skiba et al. 2002) which has several negative impact on PCB biodegradation.

Accumulation of chlorobenzoic acid (CBA) indicated incomplete transformation of 2,4 DCB. This is also supported by the absence of chloride release in spent medium during degradation of 2,4 DCB by *Micrococcus* sp. Generally, PCB degrading organism yield CBA as an end product (Rodrigues et al. 2006). Several workers also reported the principle route of PCB degradation in most prokaryotes appears to involve 2,3 dioxygenase attack at an unsubstituted ring or at chlorobiphenyl rings that have at least one pair of adjacent unchlorinated carbons at positions 2,3 (or 5,6) (Bedard et al. 1987; Kobayashi et al. 1996).

Based on the study conducted the proposed pathway for biodegradation of 2,4 DCB by *Micrococcocus sp.* was depicted in fig.5.

Several reports showed that the accumulated chlorinated compounds are highly toxic and also does not support growth of bacterial culture (Havel and Reineke, 1993). Further, Havel and Reineke, 1992 assumed the formation of a toxic compound from 4-CBA by the natural microflora lead to rapid die off of biphenyl metabolizing bacterial strain.Many investigator reports, inhibition of dichlorobiphenyl degradation in presence of dichlorobenzoic acid (Stratford et al. 1996; Vrana et al. 1996).

Although *Micrococcus sp*. degraded 2,4 DCB, accumulation chlorobenzoic acid limits its use in bioremediation of PCBcontaminatedsites. Since, Industrial effluents contain a variety of xenobiotic compounds, including PCB, diverse chlorobenzoic acid and the mixture of xenobiotic compounds, use of the consortium with ability to degrade accumulated CBA is perhaps efficient for *in situ* biodegradation of xenobiotic compounds.

Conflict of interest

The authors of this article declare that there is no conflict of interest and they do not have any financial gain from it.

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