Biodegradation Study of a Novel Biphenyl-Degrading Bacterial Isolate GS-008

P. K. Somaraja and D. Gayathri*

Department of Microbiology, Davangere University, Shivagangothri, Davangere-577002, Karnataka, India; gayathridevaraja@gmail.com, somarajas@gmail.com

Abstract

Objectives: Study of biphenyl degrading bacteria from probable PCB contaminated soil at Anjaneya Mill Locality, Davangere, Karnataka, India. Methods: A novel biphenyl degrading bacterial isolate GS-008 was isolated from transformer oil contaminated soil. During Biphenyl catabolic pathway, yellow metabolite developed on addition of 2,3- dihydroxybiphenyl dioxygenase substrate and therefore enriched colonies appeared. Further, the isolate GS 008 was characterized 16S rDNA typing identified as *Stenotrophomonas* sp. Findings: GC analysis revealed that the significant Biphenyl degrading activity of GS-008. The degradation of biphenyl was detected with the production of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) and their extent of production was determined using range of time points. Novelty: GS-008 bacterial isolate is one of the promising isolates to degrade the 2,3- dihydroxybiphenyl various PCB contaminated sites.

Keywords: biphenyl dioxygenase, 4 dienoic acid, 2-Hydroxy 6-oxo 6-phenyl hexa 2, *Stenotrophomonas* sp, Substrate biphenyl;2

1. Introduction

Biphenyls have wide applications in Coal tar, crude oil, and natural gas and have been used in organic synthesis, as food preservatives, heat transfer fluids, in addition to PCB (polychlorinated biphenyls) production. Although their usage has been decreased in recent times, it exists in the environment leading to chemical pollution.

As per toxicological parameters, biphenyl in diet leads to cause disorders of kidney, reduced life span or gall bladder cancer, moreover, it may further result in eye irritation, toxicity to liver or central nervous systems. Efficient microbial inoculation in-situ would be a choice of bioremediation of polluted environment. For the first time bacterial isolates that could utilise biphenyl as single carbon source carbon and energy was reported since four decades. Later on, others reported on biphenyl-degrading bacteria, and their mechanism was elucidated.

Using aerobic conditions by upper pathway through dioxygenation at the 2,3-position is the major catabolic pathway of biphenyl biodegradation. The ring is cleaved to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA, the ring meta-cleavage product), which consequently hydrolyzed to benzoic acid and 2-hydroxy-penta-2,4-dienoate. The genes encoding the enzymes of the biphenyl upper pathway were initially cloned from *Pseudomonas pseudoalcaligenes* KF707 and later from *Burkholderia sp.* LB400, which is now reclassified as *Burkholderia xenovorans*. To recently, four types of bph gene clusters have been cloned. But study on biphenyl/PCBs degradation and bph genes have been mainly limited to the genera *Pseudomonas*, *Burkholderia*, and *Rhodococcus*. Therefore, it is necessary to isolate biphenyl degrading efficient aerobic bacteria and clone novel bph gene to achieve effective bioremediation of the biphenyl/PCBs pollution.

Here we report the isolation of a new biphenyl degrading strain, *Stenotrophomonas* sp. GS-008, and its characterization, including growth on and degradation of biphenyl. Since there is an urgent need for the devel-
opment eco-friendly, sustainable, microbial resource in environmental bioremediation.

2. Materials and Methods

2.1 Soil Samples
Probable PCBs contaminated soil near electric transformer (depth 0–20 cm), was collected from Anjaneya cotton mill, Davanagere, Karnataka state, India.

2.2 Chemicals Used
Biphenyl (>99% purity) were purchased from Sigma-Aldrich (Shanghai, China). All other chemicals were AR (analytical grade) highest purity and was used in the present study.

2.3 Media and Culture Conditions
With Biphenyl (source of carbon) phosphate-buffered mineral salts (PAS) medium (77.5 ml of PA concentrate and 5g of yeast extract to 910 ml of glass-distilled water) was used for the present study. However, for autoclaved 10 ml of sterile PAS 100 salts and molten biphenyl was added. Further, PA concentrate was consisted of K$_2$HPO$_4$ (56.77 g/L), KH$_2$PO$_4$ (21.94 g/L), and NH$_4$Cl (27.61 g/L) while, PAS 100X salts contained MgSO$_4$ (19.5 g/L), MnSO$_4$H$_2$O (5 g/L), FeSO$_4$7H$_2$O (1 g/L), and CaCl$_2$2H$_2$O (0.3 g/L). Further, to avoid precipitation of basic saltsof concentrated H$_2$SO$_4$ (few drops) were added.

2.4 Enrichment through Microcosm Technique
Enrichment through microcosms were done by adding 2 g of sample soil to 18 of 7–10 days, about 10ml of this enriched culture was inoculated to 90ml of PASwith 1g/L biphenylly and incubated (200-250 rpm, 28°C). During the growth, development of yellow colouration indicated the generation of a metabolite 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid.

2.5 Determination of Dioxygenase Activity
In order to select 2,3-dihydroxybiphenyl dioxygenase producing bacterial isolates PAS media, the substrate containing 1 mg/mL of dihydroxybiphenyl with 0.1 mL/mL of acetone [15] was sprayed. Development of yellow zone indicated dioxygenase activity and those isolates were further streaked to 20 times to ensure clonal purity of the isolates.

2.6 16 S rRNA gene Sequencing and Phylogenetic Analysis of Biphenyl Degrading Isolates.
16S rDNA sequence analysis of biphenyl-degrading bacterial isolates was performed by using PCR with eubacterial primers 27f and 1492r for amplifying the 16S rRNA genes. 50 ml of the PCR mixture was prepared with following composition: 0.5 pmol of each primer with 10 ng of DNA template, 10 mM Tris– HCl (pH 8), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM of dNTPs, 1.25 U of Taq polymerase. PCR amplifications were performed in T-gradient thermal cycler (Biometra, Goettingen, Germany) programmed as follows: at 95°C for 5 min of denaturation, and 35 cycles at 94°C for 60s, 55°C for 60s and 72°C for 120s, with a final extension of 72°C lasted for ten minutes. On 0.8 % agarose gel PCR products were resolved and purified using QiaGen QIA quick gel extraction kit according to the manufacturer’s specifications. Using automatic sequence analyzer with ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystem, USA) and with primers mentioned earlier sequence analyses was done. Further, GenBank NCBI using the BLAST server sequence of 16SrDNA amplicons was compared to those to the existing. By using Clustal X program, Multiple sequence alignments and construction of the phylogenetic tree was constructed[16]. Further, Program Mega 5.05 was used for inferring evolutionary history and graphical presentation of the results.

2.7 Preparation of Resting Cells
The potential biphenyl degrading bacterial isolate GS-008 was grown PAS medium(100 mg/L biphenyl) incubated (30°C/150 rpm). Then the cells were centrifuged and again inoculated to PAS medium (OD 1/660 nm). Again, the cells were centrifuged (8,000 rpm/10min/4°C), washed 2X and takenin 0.1 M phosphate buffered saline (pH 7.4) made up 10 mg mL$^{-1}$ (wet cells weight) for various assays or stored under refrigeration until further use.

3. Biodegradation Study of Biphenyl by the Isolate GS-008
The study was conducted using biphenyl degrading isolate GS-008(30-32°C/150 rpm/biphenyl 100 mg L$^{-1}$), and
the concentration of the cells 20 mg L\textsuperscript{-1} was set.

4. Biphenyl Extraction and GC analysis

For biodegradation assays with Biphenyl, 5 mL of the inoculum (3d old culture/ PAS medium) was added inoculated 50 mL of PAS media/biphenyl: 100mg/L. The control contained only assay mixture, with no inoculum. For the analysis, the inoculated flasks were incubated (2w/200 rpm, 28\degree C), then the culture sample was increased high temperature treatment (90\degree C for 10 min) followed by the addition of 20 mL of n-hexane and incubated on rotary shaker (2 h/250 rpm/18\degree C). Further, with 5g of anhydrous Na\textsubscript{2}SO\textsubscript{4} the mixture was centrifuged (4,000g/4 min) and n-hexane layer was collected. Further Gas Chromatography analyses was done Thermo Trace GC system equipped with a FID (Regional medical research center, ICMR, Belagavi, India).

The following temperature range was maintained during the process: 80\degree C for 5 min, 5\degree C min\textsuperscript{-1} to 100\degree C min\textsuperscript{-1} to 280\degree C, followed by a holding time of 5 min. The injector and detector temperature were 280\degree C and the carrier gas was at a constant flow rate of 1.0 ml/min. the injection volume was 1 \mu l and injection was split (50:1). Specific peaks in chromatograms of the sample were measured by comparing with those chromatograms in control samples. The peak area in chromatogram was expressed as % of the corresponding peak in the chromatogram of the control sample for the degraded products.

5. Results and Discussion

5.1 Enrichment and Selection of Biphenyl Degraders

Soil sample collected from sample site having probable PCBs contamination (Anjaneya Mill, Davangere, Karnataka) through microcosm technique bacterial culture was enriched, after at least 20 subcultivation the isolate was purified on PAS biphenyl medium.

During enrichment, production of HOPDA (yellow metabolite), an indication of 2,3-dihydroxybiphenyl dioxygenase release (2,3-DHBD), was recorded in the tested isolate. Initially, HOPDA development resulted in 3 to 5 days during and however, the reaction time was reduced to 24 h during subsequent substrate exposure.

An increase in the biomass of the isolate GS 008 on PAS/biphenyl agar indicated the enrichment of the culture. On further characterization, (colonies turned yellow after spraying with 2,3-dihydroxy biphenyl), confirmed biphenyl-degrading activity.

A total of 15 isolates were obtained, out of which only GS-8 isolate was selected because of their growth abundance in presence of biphenyl. The 16S rDNA nucleotide sequence analysis of GS -008 showed its phylogenetic similarity to *Stenotrophomonas* sp. (Fig. 1).

5.2 Isolation and Identification of *Stenotrophomonas* sp. G-008

The 2,3-dihydroxybiphenyl dioxygenase enzyme encoded by BphC gene produce yellow meta cleavage product by reacting with the substrate 2,3-dihydroxybiphenyl. Accordingly, after incubation of the isolate on PAS agar plates, the substrate was sprayed on the bacterial colonies and the positive colonies were turned into yellow color within 1-3 min indicating the activity of 2,3-dihydroxybiphenyl dioxygenase expressed from bphC gene of bacterial colonies.

The bacterial isolate chosen for the present study GS-008 was Gram negative, motile bacillus and on 16S rDNA sequencing. These sequence comparison with the entries of GenBank (NCBI), the isolate GS-008 showed the highest similarity to the genus *Stenotrophomonas* sp.* The relationship between these isolates are available in the GenBank (NCBI), and phylogenetic tree has been constructed (Fig. 1). Although, study on the genus *Stenoptrophomonas* sp. with regard to biodegradation of xenobiotics are limited, GS-008 isolate was potential in
Biodegradation Study of a Novel Biphenyl-Degrading Bacterial Isolate GS-008

degrading biphenyl, when compared to *Mycobacterium sp.* which transformed 98% of 80 mg/L biphenyl [1].

5.3 GC Analysis for Biphenyl Degradation by the Bacterial isolate GS-008

Bacterial isolate GS-008 with biphenyl degrading ability was analyzed by GC. After seven day culture sample was analysed and the total ion current chromatogram has been presented in Figure 2 b, in comparison with pure biphenyl compound [Fig 2a]. Degradation was analyzed by comparing the RT other related compounds. In addition to this, other unpredicted chlorinated aromatic metabolites have also been found in the extracts. Apart from this, unchlorinated metabolites probably resulted from biphenyl degradation have also been detected. While the isolate GS-008 could degrade biphenyl more rapidly, transforming over 95% of biphenyl by seven days’ time. As such, GS-008 perhaps play a more important role in the degradation of biphenyl and other related xenobiotics/PCB congeners in the environment. Most bacteria isolates capable of degrading biphenyl have the upper pathway with 2,3-dihydroxybiphenyl and its meta-cleavage product (HOPDA) being the key intermediates, the latter of which is then converted to benzoic acid [2-4,11,17-19]. The yellow color of the HOPDA formed often makes it a “signature” intermediate. The maximum absorption wavelength of HOPDA was 435nm at pH 12, but shifted to 336nm at pH 2.5. As the pH decreases, the maximum absorption wavelength also decreases [20]. Results of the present study suggest that HOPDA is likely an intermediate by product of the substrate biphenyl.

6. Conclusion

*Stenotrophomonas sp.* GS-008 showed its ability to degrade a variety of aromatic compounds. Being a strain of *Stenotrophomonas sp.* it expands the biphenyl degradation capability to degrade PCB congeners. Future studies on its genes of biphenyl degradation path way will be useful for better understanding of biphenyl degradation in the environment and to biotechnological applications.

7. References

1. Moody JD, Doerge DR, Freeman JP, Cerniglia CE. Degradation of biphenyl by Mycobacterium sp. strain PYR1. *Applied and Environmental Microbiology*. 2002; 58:364–69.
2. Boehncke A, Koennecker G, Mangelsdorf I, Wibbertmann A. Concise International Chemical Assessment Document No. 6, Biphenyl. World Health Organization, Geneva, Switzerland. 1999.
3. Sandmeyer EE. Aromatic Hydrocarbons. Wiley: New York. 1981.
4. Lunt D, Evans WC. The microbial metabolism of biphenyl. *Biochemical Journal*. 1970; 118: 54–5.
5. Catelani D, Mosselmans G, Nienhaus J, Sorlini C, Treccani V. Microbial degradation of aromatic hydrocarbons used as reactor coolants. *Experientia*. 1970; 26: 922–3.
6. Pieper DH. Aerobic degradation of polychlorinated biphenyls. *Applied Microbiology and Biotechnology*. 2005; 67: 170–91.
7. Furukawa K, Hikaru S, Masatoshi G. Biphenyl dioxygenases: functional versatility and direct evolution. *Journal of Bacteriology*. 2004; 186: 5189–96.
8. Borja J, Taleon D M, Auresenia J, Gallardo S. Polychlorinated biphenyls and their biodegradation. *Process Biochemistry*. 2005; 40: 1999–2013.
9. Erickson BD, Mondello FJ. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl degrading enzyme in *Pseudomonas* strain LB400. *Journal of Bacteriology*. 1992; 174: 2903–12.
10. Hayase N, Taira K, Furukawa K. *Pseudomonas* putida KF715 *bphABCD* operon encoding biphenyl and poly-
chlorinated biphenyl degradation: cloning analysis, and expression in soil bacteria. Journal of Bacteriology. 1990; 172: 1160–4.

11. Shobha KJ, Gayathri D. Degradation of 2,4 dichlorobiphenyl via metacleavage pathway by Pseudomonas spp. Consortium. Current Microbiology. 2015; 70(6): 871–6.

12. Furukawa K, Miyazaki T. Cloning of gene cluster encoding biphenyl and chlorobiphenyl degradation in Pseudomonas pseudoalcaligenes. Journal of Bacteriology. 1986; 166: 392–8.

13. Mondello FJ. Cloning and expression in Escherichia coli of Pseudomonas strain LB400 genes encoding polychlorinated biphenyl degradation. Journal of Bacteriology. 1989; 171: 1725–32.

14. Yang X, Sun Y, Qian S. Biodegradation of seven polychlorinated biphenyls by a new isolated aerobic bacterium (Rhodococcus sp. RO4). Journal Indian Microbiology and Biotechnology. 2004; 31: 415–20.

15. Wagner-Dobler I, Bennasar A, Vancanneyt M, Strompl C, Brümmer I, Eichner C, Grammel I, Moore ERB. Microcosm enrichment of biphenyl-degrading microbial communities from soils and sediments. Applied and Environmental Microbiology. 1998; 64: 3014–22.

16. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research. 1997; 25: 4876–82.

17. Somaraja PK., Gayathri D. Ramaiah N. Molecular characterization of 2-chlorobiphenyl degrading Stenotrophomonas maltophilia GS-103. Bulletin of Environmental Contamination Toxicology. 2013; 91: 148–53.

18. Shobha KJ, Gayathri D, Somaraja PK. Diversity of 2, 4 Dichlorobiphenyl degrading consortium of Pseudomonas isolates GSA and GSb for degradation of Poly Chlorinated Biphenyl congeners. Indian Journal of Biotechnology and Bioinformatics. 2016; 4(3): 1–5.

19. Gayathri D and Shobha KJ. 2,4 Dichlorobiphenyl, a congener of polychlorinated biphenyl degradation by Pseudomonas sp GSA and GSb. Indian Journal of Experimental Biology. 2015; 53(8): 536–42.

20. Masai E, Yamada A, Healy JM, Hatta T, Kimbara K, Fukuda M, Yano K. Characterization of biphenyl scatabolic gene-sol of Gram-positive polychlorinated biphenyl degrader Rhodococcus sp. strain RHA1. Applied and Environmental Microbiology. 1995; 61: 2079–85.

21. Catelanid, Colombi A, Sorlini C, Treccani V. 2-Hydroxy-6-oxo-phenyl hexa-2,4-dienoate: the meta-cleavage product from 2,3-dihydroxy biphenyl by Pseudomonas putida. Biochemical Journal. 1973; 134:1063–6.