Fazlurrahman Khan (2011). *Biochemical and Molecular studies on microbial degradation of polychlorinated aromatic compounds and their nitro substituted derivatives.* Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.

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SUMMARY OF THE THESIS

The study presented in this dissertation deals with various aspects of biochemical, physiological and molecular characterization of bacterial degradation of polychlorinated nitro substituted aromatic compounds such as pentachloronitrobenzene (PCNB). The majority of the studies carried out involved the isolation and characterization of PCNB degrading aerobic bacteria, pathway elucidation and some of the pathway gene(s) characterization. Initially, the isolation of PCNB degrading bacteria, the enrichment study was performed under both anaerobic as well as aerobic conditions. Before isolating single aerobic bacteria by enrichment, comparative studies of PCNB degradation were performed under both anaerobic and aerobic conditions in soil samples by mixed bacterial culture. The degradation of PCNB under anaerobic conditions by mixed enriched bacterial cultures showed the reductive transformation of PCNB into pentachloroaniline (PeCA), pentachlorobenzene (PeCB), tetrachloroaniline (TeCA), tetrachlorobenzene (TeCB), trichloroaniline (TrCA), trichlorobenzene (TrCB), dichloroaniline (DCA), dichlorobenzene (DCB), chloroaniline (CA), chlorobenzene (CB); however, there was oxidative degradation of PCNB under aerobic conditions resulting in the formation of pentachlorophenol (PCP), tetrachlorohydroquinone (TeCHQ) and 2, 6-dichlorohydroquinone (2, 6-DiCHQ). CA and CB remains as a dead end product in the anaerobic enriched soil sample even at 300 days of incubation. However, there was complete degradation of produced TeCHQ and 2, 6-DiCHQ in the aerobic enriched soil sample. The rate of PCNB degradation under aerobic conditions by mixed bacterial cultures was also higher as compared to degradation under aerobic conditions. Thus, the comparative studies of PCNB degradation confirm that aerobic degradation of PCNB is better and more efficient as compared to the anaerobic degradation.

The mixed bacterial population from both anaerobic as well as aerobic enriched soil sample having the peculiar reductive and oxidative degradation properties were characterized by T-RFLP. The bacterial community structure analysis showed that the anaerobic enrichment of PCNB led to enrichment of a bacterial community dominated by sulfur and/or sulfate reducing bacteria belonging to genera *Desulphovibrio*, *Desulfotalea*, *Desulfonauticus*, *Desulfovibrio*, and *Desulfitobacterium*. Another enriched bacterial species belonged to genus *Chlorobium*. Above strains have not been previously reported for metabolic activity on PCNB and/or on transformation intermediates.

However, during present study, the enrichment of these bacterial species indicates metabolic potential that has never been described previously. However, aerobic enrichments showed the evolution of diverse bacterial communities belonging to a number of genera including *Pseudomonas*, *Bacillus*, *Sphingomonas*, *Alicyclobacillus*, *Hyphomicrobium* and *Flavobacterium*.

From aerobic enriched soil sample the screening and isolation of single aerobic bacteria having potential for degradation of PCNB was performed. After screening, finally 10 strains were isolated which showed the metabolic activity on PCNB. These 10 isolates were characterized by physiological, morphological, biochemical and 16S rRNA gene sequencing. Further attempts have been also made to elucidate the degradation pathways of PCNB from these bacterial strains. Among these 10 isolates, the strain *Pseudomonas putida* IMT3 was selected for the detailed study. The strain IMT3 utilizes PCNB as the sole carbon and energy source and degrades PCNB by oxidative mode with the formation of PCP, TeCHQ and DiCHQ metabolites by the release of nitrite and chloride. The strain IMT3 also utilizes 2-C-4-NP, PNP and 4-NC as the sole carbon and energy sources. Thus, the biochemical characterization of strain IMT3 was also carried out to elucidate the catabolic pathway involved in degradation of PNP and 2C4NP. The results obtained with analytical studies on samples collected from growth studies and resting cell studies clearly demonstrated that the degradation of 2-C-4NP and PNP in strain IMT3 was initiated by oxidative release of nitrite ions with the formation of chlorohydroquinone, hydroquinone from 2-C-4NP and hydroquinone from PNP. Based on the biochemical properties, the strain IMT3 was subjected to molecular characterization of PCNB, 2-C-4NP and PNP degradation. Further, the molecular characterization of PCNB, 2C4NP and PNP degradation in strain IMT3 was carried out by genomic library construction and genome sequence.

Total 3000 cosmid clones have been selected to perform the functional screening on PCNB compound. From 3000 cosmid clones, six cosmid clones were found functionally positive and among these, few clones transform PCNB to PCP and few into PeCA. The genome fragment from all six clones was corner sequenced with T_3 and T_7 specific primers and the sequence (~1 kb) from both ends of insert and analyzed by doing BLAST search with the strain IMT3 genome sequence. Based on the sequence analysis, a Xenobiotic reductase (XenA) gene was found to be present in clone no. 50. Finally, the XenA (1.1 kb) has been amplified and cloned into the expression vector. Initially, it was

assumed that the XenA may also show the reductase activity for the transformation of PCNB to PeCA, unfortunately there was no such activity of XenA on PCNB compound. However, it showed the reductase activity on coumarin, 2-amino-4-nitrophenol, 2, 4-dinitrophenol, 2, 6-dinitrophenol and 2, 6-dinitrotouene.

The genome sequence analysis of strain IMT3 showed that it has a PCP 4-monooxygenase and also the entire pnp gene cluster of PNP degradation pathway. The strain IMT3 degrades PCNB by oxidative mode with the formation of PCP, TeCHQ and 2, 6-DiCHQ as intermediates. Similarly, strain IMT3 also degrades 2-C-4-NP and PNP oxidatively via the formation of chlorohydroquinone and hydroquinone as the intermediates. It is well known that the PCP 4-monooxygenase from *Sphingomonas* species UG30 plays a role in the oxidative removal of chloro group from PCP and nitro group from PNP and 4-NC. Similarly, PNP-monooxygenase oxidatively removes the nitro as well as the chloro group from the nitroaromatic and chloroaromatic compounds. Thus it was also assumed that, the PCP 4-monooxygenase and PNP-monooxygenase may also have same oxidative removal of the nitro group from PCNB compound. To examine the role of PCP 4-monooxygenase gene from strain IMT3 on PCNB and PCP compounds, it was cloned, expressed and functionally characterized. There was no oxidative removal of the nitro group from PCNB by PCP 4-monooxygenase; however, it showed the oxidative removal of the chloro group from PCP only.

Furthermore, the whole pnp gene cluster (pnpA, pnpB, pnpC, pnpD, pnpE, pnpF and pnpG) obtained by the genome sequence of strain IMT3 was cloned; however, the detailed study was carried out only for PnpA. PnpA is a single component monooxygenase having broad substrate range activity on the *para*-nitro and *para*-chloro substituted phenolic compounds such as PNP, 2-C-4-NP, 4-nitro catechol, pentachlorophenol, 2, 4-dichlorophenol, 3, 4-dichlorophenol, 2, 4, 5-trichlorophenol, 2, 4, 6-trichlorophenol and 4-chlorophenol. The hydroxylation activity of PnpA takes place on the electron withdrawing groups such as nitro and chloro present at the *para* substituted phenolic compounds. This is the first report of a single component pnp-monooxygenase showing broad substrate specificity on the *para*-chloro and *para*-nitro substituted phenols. However, PnpA had no activity on the 1, 3, 5, 6-TeCNB, TNT, 2, 4-DNT, and 3, 5-dichloronitrobenzene. The phylogenetic relationship of PnpA of strain IMT3 demonstrated that it is located in the same branch of known *p*-nitrophenol monooxygenase as compared to other tested nitro and chloroarene monooxygenase. This

implies that the PnpA from strain IMT3 and other known *p*-nitrophenol monooxygenase evolved from same origin.

Similarly, a novel catechol 2, 3-dioxygenase (CatD) from strain IMT3 was successfully cloned, which has properties to degrade 4-NC by the action of a novel catechol 2, 3-dioxygenase. The strain IMT3 could transform 4-NC by both un-induced as well as induced cells. The novel CatD has also ring cleaving activity on 3-methylcatechol, 4-methyl catechol, catechol, 4-chlorocatechol, 4, 5-dichlorocatechol and 3, 4-dichlorocatechol. The dendrogram analysis of the catechol 2, 3-dioxygenases showed that it is clustered with chlorocatechol 2, 3-dioxygenase of *Pseudomonas fluorescens*. Thus catechol 2, 3-dioxygenase of strain IMT3 belongs to a different group of catechol 2, 3-dioxygenase. An important finding of this study is that the degradation of 4-NC is independent of substrate/inducer exposure conditions.

An *Escherichia coli* IMT7 isolated from the enriched soil sample was found to transform PCNB to PeCA by reduction of the nitro group into the amino group. Further, the molecular characterization of gene involved in the reduction of nitro group of PCNB compound was carried out; since it is known that nitroreductase is the reductase enzyme present in the *E. coli* and reduces various nitroaromatic compounds. The genome sequence of *E. coli* BL21-Gold (DE3) available in the NCBI data base containing nitroreductase was used to design a primer set for PCR amplification of nitroreductase gene. A 723 base pair nitroreductase gene was amplified from *E. coli* IMT7 and cloned in expression vector by Gateway Cloning Technology. Further, expression (26.8 kDa) and functional characterization were carried out and showed the reduction of PCNB to PeCA; 4-chloro-2-nitrophenol to 2-amino-6-chlorophenol; 2, 6-dinitrophenol to 2-amino-6-nitrophenol; 2, 6-dinitrotoluene to 2-methyl-3-nitroaniline.