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SUMMARY OF THE THESIS

Nitroaromatic compounds constitute a major group of xenobiotics involved in global pollution. Biodegradation is the most effective and desirable remediation technique available to restore the existing contaminated sites. The present study encompasses two major aspects. Firstly, biochemical analysis of degradation pathways of nitroaromatic/chloronitroaromatic compounds by bacteria as well as studies on bacterial chemotaxis toward these compounds. Secondly, understanding the physiological responses elicited in degrading bacteria upon exposure to different aromatic compounds. Two of the bacterial strains *Burkholderia* sp. SJ98 and *Arthrobacter protophormiae* RKJ100 isolated earlier in our laboratory have been explored for their metabolic potential in this investigation.

Burkholderia sp. SJ98 was isolated from pesticide contaminated soil and was characterized for the degradation of *p*-nitrophenol, 4-nitrcatechol, *o*-nitrobenzoate and *p*-nitrobenzoate. In addition to nitroaromatics degradation, the organism was chemotactic toward these compounds. Furthermore, it was found to be chemotactic toward some nitroaromatic compounds which were not metabolized by this strain. Recently, in our laboratory, a 6.9 kb fragment of DNA has been cloned and sequenced from *Burkholderia* sp. SJ98 which harbours genes involved in *p*-nitrophenol degradation. Seven open reading frames were identified in the cloned fragment, out of which two significantly matched with benzenetriol dioxygenase and maleylacetate reductase and were designated as *pnpC* and *pnpD*, respectively. In this study, with an aim to characterize the gene product of *pnpC*, the ORF was PCR amplified and cloned in the expression vector pET-28c, and transformed into *E. coli* BL21. Upon induction with IPTG the overexpressed protein designated as BtD (benzenetriol dioxygenase enzyme, putative product of ORF *pnpC*) was purified by Ni-NTA affinity chromatography. Molecular weight of BtD was found to be 32 kDa by SDS-PAGE. The native molecular weight was determined by gel filtration chromatography and was found to be 68 kDa. This clearly indicated that the protein was a homodimer. BtD from *Burkholderia* sp. SJ98 was specifically active towards benzenetriol among various structurally similar compounds tested as substrate. The structural information available from CD spectra showed that BtD has a mixed secondary structure which was stable

over the range of 50-60°C. The pH and temperature optima for purified BtD were found to be 6.8 and 30°C respectively. BtD in the elution buffer was stable up to the concentration of 2 mg ml^{-1}; therefore, stability of BtD was checked in various buffers. It was found to be soluble up to the concentration of 16 mg ml^{-1} in 20 mM NaCl, 20 mM Tris-Cl, pH 8.0. Attempts were also made to crystallize the protein molecule. Despite the formation of microcrystals under certain conditions, those were not suitable for X-ray diffraction. Biochemical understanding of the *p*-nitrophenol degrading pathway enzyme would help in designing enzyme-based bioremediation technologies in future.

As mentioned above *Burkholderia* sp. SJ98 is also chemotactic toward some nitroaromatic compounds which do not serve as sole carbon and energy source. Experiments were performed to determine whether these nitroaromatic compounds are transformed by the strain in presence of alternate carbon source. Based on the chromatographic and spectroscopic data the transformed product(s) of *o*-dinitrobenzene and 2,5-dinitrophenol were found to be *o*-nitroaniline and 2-amino-5-nitrophenol respectively. As strain SJ98 tested positive toward various nitroaromatic compounds, its chemotactic ability was further tested towards chloronitrobenzoates by drop plate assay and swarm plate assay. The results obtained from these plate assays exhibited the migrating rings of bacteria in case of 4-chloro-2-nitrobenzoate, 5-chloro-2-nitrobenzoate and 2-chloro-4-nitrobenzoate. The chemotactic behaviour of strain SJ98 was also quantitatively analyzed by capillary assay. It was observed that the strain was chemotactic toward 4-chloro-2-nitrobenzoate, 5-chloro-2-nitrobenzoate, and 2-chloro-4-nitrobenzoate with chemotaxis index of 12, 14 and 20, respectively. Experiments were also carried out to determine whether these chloronitrobenzoates were transformed by *Burkholderia* sp. SJ98. The results obtained from GC and HPLC studies indicated the formation of some metabolites such as 3-hydroxyanthranilic acid in case of 4-chloro- and 5-chloro-2-nitrobenzoate, and protocatechuate in case of 2-chloro-4-nitrobenzoate degradation. This finding suggested that strain SJ98 could transform these chloronitrobenzoates in presence of an alternate carbon source (10 mM sodium succinate).

Another aspect of the present study was to check the degradation of some toxic aromatic compounds at higher concentrations by *Arthrobacter protophormiae* RKJ100 and *Burkholderia* sp. SJ98. Strain RKJ100 was found to grow on higher concentrations

of *o*-nitrobenzoate and *p*-hydroxybenzoate, and strain SJ98 was able to grow on higher concentration of *p*-nitrobenzoate. Initially, strain RKJ100 grew up to 30 mM *o*-nitrobenzoate, whereas growth was observed up to 200 mM when the cells grown at 30 mM (induced cells) were used as inoculum. Similarly, uninduced cells of strain RKJ100 were capable of growth up to 50 mM *p*-hydroxybenzoate whereas the induced cells could grow up to 150 mM *p*-hydroxybenzoate. Strain SJ98 was able to grow up to 20 mM *p*-nitrobenzoate.

In addition, this work aimed to understand the physiological adaptations of bacteria in response to nitroaromatic compounds. The comparative fatty acid profile of Gram-positive bacterium *Arthrobacter protophormiae* RKJ100 grown on higher concentrations of *o*-nitrobenzoate and *p*-hydroxybenzoate showed changes in the fatty acid content of the organism. There was an overall decrease in the anteiso/iso ratio of fatty acids of the strain during growth on *o*-nitrobenzoate and *p*-hydroxybenzoate as compared to the succinate grown cells. This modification may help strain RKJ100 to maintain its membrane fluidity when exposed to higher concentrations of *o*-nitrobenzoate or *p*-hydroxybenzoate. The cell surface hydrophobicity of strain RKJ100 also increases during growth on higher concentrations of *o*-nitrobenzoate and *p*-hydroxybenzoate as evident from BATH assay. Furthermore, in order to check whether there are changes in morphology of the cells of strain RKJ100, scanning electron microscopy was performed. It was observed that sodium succinate grown cells were larger (2.74±0.42μm) in size as compared to the cells grown on *o*-nitrobenzoate (1.48±0.44μm) and *p*-hydroxybenzoate (1.46±0.36 μm). Decrease in cell size and increased surface/volume ratio in *A. protophormiae* RKJ100 grown on higher concentrations of *o*-nitrobenzoate and *p*-hydroxybenzoate might be the putative mechanism for its tolerance.

The adaptive response toward toxic aromatic compounds also triggers significant variation in whole cell protein expression. One-dimensional protein profiling was carried out with cells of strain RKJ100 grown on *o*-nitrobenzoate and *p*-hydroxybenzoate. Catalase has been identified as one of the major stress proteins in strain RKJ100 which was induced as a result of oxidative stress imparted by nitroaromatic compounds. A molecular chaperone FKBP-type peptidyl-prolyl isomerase domain protein was expressed suggesting its involvement in protein folding during nitroaromatics stress. Another protein induced in strain RKJ100 was identified

as cytochrome c550 that might inhibit the toxicity of nitroaromatic compounds or its degradation metabolites.

Besides understanding the adaptive responses in Gram-positive bacterium, another Gram-negative bacterium *Burkholderia* sp. SJ98 was chosen for further investigation. Comparative fatty acid profile indicated some changes in the fatty acid content of strain SJ98 during growth on nitroaromatic compounds. Nevertheless, how such changes in fatty acid profile influence altered membrane fluidity has not been established so far. On the other hand, 2D-PAGE analysis demonstrated overexpression of some proteins in strain SJ98 during its growth on different nitroaromatic compounds when compared to succinate grown cells. Catalase was identified as a stress protein induced in strain SJ98 as found in case of strain RKJ100. Another protein identified was GroEL which was induced during growth of strain SJ98 on *p*-nitrophenol. Although GroEL is known as a heat shock protein, it has also been reported to be induced by 2,4-dichlorophenoxyacetic acid (a chloroaromatic pesticide) in *Burkholderia* sp. YK-2. An outer membrane protein was identified but its specific function was not assigned. Two other proteins did not show considerable homology with any of the proteins reported and therefore, they were assigned as hypothetical proteins.

In situ bioremediation at heavily contaminated sites could be severely restricted since the heavy load of contamination might impede the growth of bacteria due to their inability to tolerate the higher concentration of pollutants. *A. protophormiae* RKJ100 and *Burkholderia* sp. SJ98 that degrade nitroaromatic compounds at high concentrations could be used as a model organism(s) for bioremediation of heavily contaminated sites. The present study offers an insight to understand the molecular basis of such adaptive responses. Consequently, this may further be exploited to construct genetically modified organisms with vast metabolic potential and unique growth/tolerance ability to higher concentrations of aromatic compounds.