BIODEGRADATION OF NITROAROMATIC COMPOUNDS:

INTERRELATIONSHIPS BETWEEN CATABOLISM OF

NITROPHENOLS AND NITROBENZENE

By

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Doctor of Philosophy

In

Biology

Waterloo, Ontario, Canada, 2000

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THESIS ABSTRACT

This thesis describes biological mechanisms of degradation of nitroaromatic compounds and more specifically the interrelationships between biodegradative metabolism of nitrophenols (NPs) and nitrobenzene (NB). An initial study on conversion of NB to NPs by electron beam provided the impetus for the biodegradative study.

Electron beam irradiation of aqueous solutions containing 15-30 mg/L of NB at 60kGy dose removed 78% of the contaminant. Three mono-NPs were detected as byproducts of electron beam treatment of NB. A mixed culture which degraded both NPs and NB was enriched from municipal activated sludge using a mixture of three mono-NPs as sole carbon, nitrogen and energy sources. Bacterial growth and degradation rate could be increased by supplementing the medium with 0.1% YE. The mixed culture degraded both the residual NB and the NP products in the electron beam treated samples, and this observation led to the conceptual design of a two-stage electron beam microbial process for degradation of NB. Percentage removal of NB in this two-stage treatment, increased with increasing electron beam dose.

Three groups of bacteria were isolated from the mixed culture after intensively sub-culturing on the NPs as the growth substrates. Strains of group A ($2NP^-3NP^-4NP^+$) were *Pseudomonas* species, which grew on 4-NP, but not on 2-NP or 3-NP. Strains of group B ($2NP^+3NP^+4NP^-$) were also *Pseudomonas* species, which grew on 2-NP and 3-NP, but not on 4-NP. Strains of group C ($2NP^-3NP^+4NP^-$), grew on 3-NP, but not on 2-NP or 4-NP. One of the two strain types was identified as *Variovorax paradoxus*. Degradation of 2- and 4-NP produced nitrite. Only 3-NP-grown cells of strains of Group B and C, degraded NB and produced ammonia from both NB and 3-NP.

Pseudomonas putida 2NP8, a typical strain of group B, was investigated for mechanism and interrelationship of NB and NP metabolism. 2-NP was degraded with production of nitrite. Degradation of 3-NP resulted in the formation of ammonia. Cells grown on 2-NP did not degrade NB. A specific NB degradation activity transforming NB into ammonia, was induced by 3-NP in this strain. The ammonia release mechanism in *P. putida* 2NP8, was investigated by characterizing production of metabolites from NB. Nitrosobenzene and hydroxylaminobenzene were detected as metabolites of NB

degradation by the 3-NP-grown cells, indicating a cometabolism of NB catalyzed by the 3-NP nitroreductase-initiated enzyme system.

Biotransformation products of hydroxylaminobenzene and aminophenol, produced by 3-NP-grown cells of *Pseudomonas putida* 2NP8, were characterized. Ammonia, 2-aminophenol, 4-aminophenol, 4-benzoquinone, *N*-acetyl-4-aminophenol, *N*-acetyl-2-aminophenol, 2-aminophenoxazine-3-one, 4-hydroquinone, and catechol were produced from hydroxylaminobenzene. Ammonia, *N*-acetyl-2-aminophenol, and 2-aminophenoxazine-3-one were produced from 2-aminophenol. All of these metabolites were also found in the NB transformation medium, and this demonstrated that they were metabolites of NB transformation via hydroxylaminobenzene. Production of 2-aminophenoxazine-3-one indicated that oxidation of 2-aminophenol via imine occurred. Rapid release of ammonia from 2-aminophenol transformation indicated that hydrolysis of the imine intermediate was the dominant reaction. The low level of 2-aminophenoxazine-3-one indicated that formation of this compound was probably due to a spontaneous reaction accompanying oxidation of 2-aminophenol via imine. 4-Hydroquinone and catechol were reduction products of 2- and 4-benzoquinones.

The degradation pathway of 3-NP in *P. putida* 2NP8 was postulated, based on NB transformation products. This mechanism suggested that 1,2,4-trihydroxylbenzene is the dioxygenase ring-cleavage substrate in 3-NP metabolism

The 3-NP-grown cells of *Pseudomonas putida* 2NP8 had wide substrate range in metabolizing the nitroaromatic substrate through to ammonia production. When thirty nitroaromatic compounds were tested as substrates, all were quickly degraded except 4-NP, 2,4-di-NP, 2,4,6-tri-NP, 2-nitrobenzoic acid and 2-nitrofuran. Ammonia production from most of the nitroaromatic substrates appeared to be stoichiometric. Metabolites more hydrophobic and hydrophilic than the nitroaromatic substrates were observed during transformation and the metabolites exhibited retention time patterns similar to those observed in the NB biotransformation. A pathway, similar to that for NB transformation, was proposed for degradation of nitroaromatic substrates into ammonia via a hydroxylamino aromatic compound, aminophenol, quinone monoimine and quinone. An apparently constitutive enzyme activity, oxidizing nitrobenzyl alcohol and nitrobenzaldehyde into nitrobenzoic acids, was also observed. This system manifested

low oxidizing activity toward 2-nitrobenzyl alcohol. The cells also reduced nitrobenzaldehyde into the corresponding alcohol product. Degradation of nitrobenzyl alcohol into ammonia in the 3-NP grown cell media occurred either before or after oxidation of the alcohol group.

The 2-NP-induced enzyme system of *Pseudomonas putida* 2NP8 transforms 2-NP into nitrite and the 3-NP-induced system transforms 3-NP into ammonia. When thirty nitroaromatic substrates with one, two or three nitro substitutions, were tested for their capacity to induce a nitrite-releasing activity only 2-NP and 4-Cl-2-NP were found to be the inducers. When the thirty compounds were tested as substrate of a 2-NP-induced enzyme system, only 2-NP and 4-Cl-2-NP were substrates. This contrasted with the very broad substrate specificity of the 3-NP-induced enzyme system. This strain transformed 4-Cl-2-NP into a dead-end metabolite, which was believed to be 4-chlorocatechol.

ACKNOWLEDGEMENTS

To my parents, even though they do not understand a single word of my Thesis, but they are so proud of me. I remember tears of my mother when I left home. Now I am going home. Can you see the ' new clothes' I am wearing, mother? I thank my brothers and sisters for their support.

Many thanks to those I am counting but I have not named here!

Life is "synthesis and degradation". This thesis tells a story about 'degradation' of chemicals, done by bugs. Enjoy!

ABBREVIATIONS:

EB: electron beam

AOP: advanced oxidation process

AP: aminophenol

APX: 2-aminophenoxazine-3-one

HAB: hydroxylaminobenzene

HPLC: high performance liquid chromatography

NAs: nitroaromatic compounds

NB: nitrobenzene

NOB: nitrosobenzene

NP: nitrophenol

NPs: a mixture of three mono-nitrophenols

OD: optical density

TLC: thin-layer chromatography

TMS: trace mental solution

YE: yeast extract

YPS: yeast extract (0.1%)- bacto peptone (0.1%)-sodium chloride (0.05%) medium

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1. INRODUCTION

1.1. General aspects of degradation

Complete degradation of industrial toxic compounds using chemical or biological processes results in carbon dioxide production through an oxidation reaction with or without involvement of oxygen. Incomplete degradation results in partly oxidized carbonic compound and loss of hydrogen. Chemical methods use active 'oxygen'. Biodegradative processes, usually involving aerobic oxidation or anaerobic digestion are cellular enzymes-assisted processes. Cells gain energy and intermediates for growth and cell maintenance. Aerobic metabolism occurs when oxygen is available for aerobic bacteria. Anaerobic respiration occurs when oxygen is not available but where an oxidant other than oxygen, such as sulfate or carbon dioxide, is used

Disappearance of a compound does not necessarily mean that it is degraded. It may be chemically modified or transferred to another phase through absorption, evaporation, and diffusion. The method for measuring 'degradation' is crucial to determine what kind of transformation has occurred. The degradation process, used to remove a hazardous and toxic compound, should produce non-toxic products, preferably carbon dioxide and water as mineralized products. This contrasts with a process in which a compound is transformed into a more toxic product, which is termed as 'bioactivation'.

Two types of compounds may be discerned in terms of their biodegradability. The first type, consisting of organic compound of biological origin, such as glucose, amino acids and fatty acids, are easily biodegradable and are common nutrients for biological organisms. The second, consisting of fossil-originated hydrocarbon compounds that are not direct substrates for primary and central biological metabolism, are more recalcitrant to biological degradation. Accidental spills of these compounds generally need special remedial technologies to speed up their degradation. Chemical, biological and physical methods have been investigated for their disposal.

Chemical oxidation introduces oxygen atoms into the hydrocarbon molecule, thus called oxygenation. Thermodynamically, reactions between hydrocarbons and oxygen release energy, but reactions between ground state dioxygen and ground state organic compounds do not occur at room temperature. Combustion of gasoline in automobiles needs initialignition to

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to prime the reaction, releasing heat to propagate the combustion. For a dilute aqueous solution of an organic compound two strategies are utilized for oxidation: the material is treated with singlet-form (excited state, high energy) oxygen species, such as hydroxyl radicals, which can react rapidly with organic compounds under ambient temperature; or an oxygenation is conducted under high temperature and high pressure.

Biological oxidation is an ambient degradation process. Two types of oxygen incorporation reactions are known: 1) addition of water to a double bond; 2) direct oxygenation from dioxygen. The first type is seen in the central metabolism of nutrient chemicals containing labile chemical bonds (for example C-O-H, C-N-H) such as in sugars, amino acids and fatty acids (Armstrong 1989). These compounds are oxidized through transfer of H, or electrons to biochemical electron acceptors such as $NAD(P)^+$ or FAD^+, and the oxygen is introduced into the metabolizing substrate from water, not oxygen. Heterotrophic organisms have finely-evolved metabolic systems to transform these labile compounds, and to use them as carbon and energy source. Enzymes catalyze removal of hydrogen from the labile bond to form reduced co-enzymes which pass the electrons through a membrane electron transfer system, to oxygen (aerobic metabolism) or other oxidant (anaerobic metabolism). The electron flow creates across-membrane proton concentration gradients, which drive synthesis of high-energy molecules. The hydrogen attached to the carbon backbone is gradually replaced by oxygen from water molecules and carbon dioxide is released.

The second type of biological oxidation, direct oxygenation, is observed in the oxygenation of inert C-H bonds of hydrocarbons (Hayaishi 1974; Nozaki et al. 1982). For example, oxygenase catalyzes incorporation of the oxygen atom from dioxygen into aliphatic hydrocarbon produce alcohol, which is then oxidized through dehydrogenase-catalyzed loss of hydrogen into central metabolic intermediates (in the tricarboxlic acid cycle). Biological oxidation does not necessarily begin by oxygenation, and for those substituted synthetic hydrocarbons, removal of the substitution may accompany formation of substrates for dehydrogenase (Nakazawa et al. 1996).

Increase in halogen, nitro group and other electrophilic substitutions decreases the tendency of hydrocarbons to be oxidized. These electrophilic group-substituted compounds

are more resistant to both chemical and biological oxidation than their non-substituted counterparts.

1.2. Chemical oxidation methods

1.2.1. Degradation of hydrocarbons with oxygen species

Singlet oxygen or free oxygen radical can react with a wide range of chemicals including hydrocarbons. Singlet oxygen or free radicals can be produced from dioxygen, hydrogen peroxide and water molecules. Triplet dioxygen reacts with an unpaired electron in other radicals or transition metal ions with partially filled d-orbitals (Spiro and Stigliani 1996) and produces singlet oxygen or oxygen radical. In sunshine-exposed surface water, the dissolved organic materials absorb sunlight and can transfer this energy to oxygen and convert triplet oxygen into singlet oxygen molecules (Haag and Hoigné 1986). The $^1O_2$ can be transformed into superoxide ion (O_2^-) and hydroxyl radical through interaction with sunlight, unsaturated oranic matter and transitional metal ions (Cooper et al. 1988). These oxygen species can initiate a cascade of free radical reactions with hydrocarbons leading to mineralization. Hydroxyl radical is a very strong and nonselective oxidant and can react with various chlorinated and non-chlorinated contaminants in aqueous phase at reaction rates of 10^7 to $10^{10} M^{-1} s^{-1}$ (Haag and Yao 1992). Efficient generation of hydroxyl radicals is the basis of chemical oxidation degradation technologies.

1.2.2. Technologies generating oxygen radicals from oxygen and/or water

The ambient temperatures processes, in which hydroxyl radical is mostly derived from photolysis of ozone and hydrogen peroxide are called "advanced oxidation processes (AOP)" (Galze et al. 1992; Carey 1992; Venkatadri and Peters 1993; Takiyama et al. 1994). Technologies producing hydroxyl radicals may be separated into five groups based on the substrates and energy sources used.

1.2.2.1. Dye-sensitized photo-oxidation

Dye-sensitized photo-oxidation requires oxygen, a visible light source and photoactive material such as methyl blue (Acher and Rosenthal 1977). The dye absorbs and transfers light energy to either organic material or oxygen molecules. The receiving organic material reacts with triplet oxygen. Receiving oxygen molecules in the ground state (triplet form) are raised to singlet form, capable of oxidizing organic material. This photo-oxidation is important in

removal of organic matters in natural aquifers in strong sunlight regions, and has been investigated for sewage disinfection (Acher et al. 1990) and degradation of chlorophenols (Li et al. 1992). Removal of dyes from the treated effluent could be troublesome.

1.2.2.2. Photo-mediated lysis of hydrogen peroxide or decomposition of ozone

Photo-mediated lysis of hydrogen peroxide or decomposition of ozone produces hydroxyl radicals (Staehelin and Holgné 1985). Ozonation, without photo irradiation, only selectively and incompletely degrades organic compounds, but photo-radiation assisted ozonation can completely degrade various compounds and has been used to successfully treat water polluted by halogenated aliphatics, simple aromatic compounds and cholorinated aromatics (Cater et al. 1992; Zeff and Barich 1992; Venkatadri and Peters 1993).

1.2.2.3. Fenton's reaction

Hydroxyl radical is produced through a Fenton–like reaction of hydrogen peroxide with transition metals with unpaired electrons, such as ferrous ions or its oxide, at acidic to keep the metal ions from precipitation (Carey 1992; Venkatadri et al. 1993). Goethite, the most common crystalline iron oxyhydroxide [$Fe(OOH)_2$] mineral in soil and sand, reticulated iron, and Fe (III) function similarly to transition metal ions, facilitating hydrogen peroxide treatment of soils polluted by chlorinated compounds (Carey 1992; Pignatello and Chapa 1994; Ravlkumar and Gurol 1994; Takemura et al. 1994; Watts et al. 1993).

1.2.2.4. Semiconductor-catalyzed photo-decomposition of water

Semiconductor-catalyzed photo-decomposition of water uses a semiconductor catalyst such as TiO_2 (which is stable and economic) to absorb illuminated light to eject electrons from the catalyst surface and produce a positive hole. The positive hole oxidizes water molecules or hydroxyl ions, producing hydroxyl radicals, or directly oxidizes pollutants (Richard and Boule 1994). Electrons (Glaze et al. 1993) and secondary superoxide ions or hydrogen radicals also participate in degradation and can reduce highly halogenated compounds. This process is not sensitive to pH or colour compounds (Pruden and Ollis 1983; Pelizzetti et al. 1988; Matthews 1986; Matthews 1990; Halmann et al. 1992; Hidaka et al. 1994; Hidaka and Zhao 1992).

1.2.2.5. Water decomposition through high-energy particle bombardment

Water decomposition through high-energy particle bombardment involves production of hydroxyl radicals, hydrogen radicals, and solvated electrons using γ-Rays from

radioactives, or high-energy electrons from electron generators and accelerators. Electron beam-mediated degradation of water pollutants (Cooper et al. 1992a,b, Cooper et al.1993a, b; Nickelsen and Cooper 1992) or air pollutants (Prager et al. 1995) has been demonstrated. Ozone can enhance degradation efficiency by electron beam irradiation (Gehringer et al. 1992). The method is advantageous in that oxidative and reductive species are involved (Buxton 1987) since the latter improves removal of highly oxidized compounds. Electron beam is safer than radioactive and has many industrial applications (Kurucz et al. 1991; Frank 1995). A 1.5MeV electron accelerator facility in Miami was investigated for removal of various pollutants (Kurucz et al. 1991).

1.2.3. Factors affecting radical-based chemical degradation technology

The hydroxyl radical is the main decomposer of chemicals in wastewater treatment, with concentration of hydroxyl radical determining chemical oxidation rate. Energy input, amount of catalyst, concentration of radical-precursor and medium conditions affect production of free hydroxyl radicals. Unless an excess of free hydroxyl radicals is generated, degradation is a function of concentration of both radicals and the pollutant. Degradation efficiency is affected by hydroxyl radical scavengers (such as carbonate ions, nitrate ions) and total dissolved organics.

1.2.4. Chemical oxidation reaction mechanisms

Chemical oxidation reaction mechanisms mainly involve hydroxyl radical-induced oxygenation of organic chemicals leading to formation of carbon dioxide and water. Hydroxyl radicals may abstract H-atoms from saturated alkanes and produce water and other carbon radicals, which in return combine with either oxygen molecules or another hydroxyl radical, or react by addition to unsaturated compounds producing oxygenated products. These two types of reactions lead to mineralization of hydrocarbons.

1.3. Biological metabolism of synthetic hydrocarbons

Microorganisms have evolved the capacity to oxidize and grow on petroleum, coals and fossil-fuel derived organic compounds such as polymeric materials, solvents, pharmaceuticals, agricultural chemicals, dyes and explosives, which are widely distributed in the environments.

1.3.1. Oxygenation of hydrocarbon skeleton structure.

For non-substituted aliphatic (Watkinson and Morgan, 1990; Murrell, 1994; Beilen et al. 1994) or aromatic hydrocarbons (Smith, 1990; Pothuluri and Cerniglia 1994; Harayama and Timmis 1992), the first step of their biodegradation is activation of oxygen and production of alcohol or catechol, mediated by mono- and di-oxygenase. Monooxygenases insert one oxygen into hydrocarbon and produce a water molecule (Lipscomb 1994; Van Berkel and Müller 1990), in reactions involving oxygenation of aliphatic groups, epoxidation of alkenes, monohydroxylation of monophenols and biological lactonation of ketones (Watkinson and Morgan 1990; Trudgill 1990; Smith 1990; Powlowski and Shingler 1994). Dioxygenase catalyzes insertion of two oxygens into aromatic hydrocarbons (Butler and Mason 1997; Nason and Cammack 1992; Lipcomb and Orville 1992).

Most oxygenases have transition metal ions at the reactive sites, which have unpaired electrons in their outmost orbital, and dioxygen is activated into oxidative species by bonding to these metal ions (Harayama and Timmis 1992; Jassen and Witholt 1992; Kok and Neidle 1992; Lipscomb 1994; Butler and Mason 1997; Mason and Cammack 1992; Lipscomb and Orville 1992). For hydroxylating oxygenase electron transfer is involved in the activation of dioxygen. NAD(P)H is the electron donor to the dioxygen, and the initial hydroxylation consumes NAD(P)H (Mason and Cammack 1992). Hydroxylating oxygenase has two components: a reductase system and an oxygenase component. The reductase system is a single flavin protein with a bonded or separate iron-sulfur cluster. The flavin group on the reductase first accepts electron from NAD(P)H and becomes a two-electron reservoir. The electrons are then transferred, one by one, to the [2Fe-2S] cluster. The oxygenase component is also an iron-sulfur protein, and has a catalytic haem (for P450 oxygenase) or non-haem iron Fe(II). The oxygenase iron accepts electron from the reductase iron and then combines with dioxygen and produces oxidative oxygen species.

1.3.2. Bacterial growth on hydrocarbon.

The biochemical basis of hydrocarbons supporting bacterial growth is that the bacteria which grow on the hydrocarbons can convert them into C_2 or C_4 products, which are utilized in the TCA cycle for growth or energy production.

Monooxygenase-initiated oxygenation of aliphatic compounds produces alcohol which is converted into a fatty acid (Watkinson and Morgan 1990). For alkene bonds, acid is formed

via monooxygenase catalyzed epoxidation, hydrolysis into diol and oxidation of this diol. Monoterpenes are converted to fatty acid via ring cleavage reactions involving initial oxygenation of methylene group into alcohol, dehydrogenation of alcohol into ketone, monooxygenase-catalysed baeyer-Villiger reaction of ketone into lactone, followed by hydrolysis (Trudgill 1990). Carboxylic acid is further converted into acetyl group via a beta-oxidation pathway or other alternative route. Methane is metabolized by bacterial monooxygenase-catalyzed oxygenation with subsequent oxidation into formaldehyde, which is either assimilated via the serine pathway or further oxidized into carbon dioxide (Murrel 1994; Lipscomb 1994).

Degradation of chlorinated aliphatic hydrocarbons as substrates for bacterial growth begins with dechlorination and subsequent production of alcohol or acid (Janssen and Witholt 1992; Belkin 1992; Fetzner 1998; Slater et al. 1995; Lee et al. 1998).

Although different aromatic compounds are degraded via different pathway, they are all converted into 1,2- or 1,4-dihydroxyl compounds as substrates of ring-cleaving dioxygenases (Williams and Sayers 1994; Smith 1990; Harayama and Timmis 1992). The dihydroxyl aromatic compounds are converted to C_2 and C_4 TCA cycle intermediates via different aromatic ring-cleavage pathways.

Certain anaerobic bacteria also metabolize some aromatic compounds into C_2 and C_4 TCA cycle intermediates to obtain carbon for growth, but these aromatic compounds are limited to those with hydroxyl, amino, and carboxyl substituents, such as phenol, 3,4,5-trihydroxybenzoate, and benzoic acid (Heider and Fuchs 1997; Gibson and Harwood 1994). Transformation of these aromatic compounds begins by reductase-catalyzed reduction of the benzene ring, followed by ring cleavage.

1.3.3. Removal of non-carbon substituents

Removal of non-carbon substitution groups via oxygenative, substituitive, and reductive mechanisms is key to degradation of synthetic organic compounds, facilitating their use as growth substrates. Some bacteria use the chlorinated compounds as terminal electron acceptor of anaerobic respiration, producing dead end products.

Even though presence of the electrophilic substituents decreases the tendency of hydrocarbons to be oxidized, some bacteria have evolved the capacity to use oxygenase to

initiate degradation of certain substituted aromatic compounds. Removal of the substitutent from the aromatic ring, as a result of the oxygenase reaction, was observed in some of the oxygenase-involved degradation of aromatic compounds (Reineke 1994; Fetzner 1998; Hale and Wiegel 1994; Harayama and Timmis 1992). The initial dioxygenase attacks the 1,2 position relative to the substitution, and forms a cis-dihydrodiol adduct. The substituent is spontaneously released as an anion (with a H^+), producing a catechol as a substrate for further ring-cleavage metabolism. Two electrons are consumed for this energy-consuming conversion. A substitutive strategy has been reported for removal of halogen substituent in both aliphatic and aromatic compounds. The chlorine of 1,2-dicholoroethane are replaced by two hydroxyl groups from water through hydrolytic enzymes in bacteria with 1,2-dichloroethane as growth substrate (Janssen et al. 1994). Reductive removal of electrophilic substituent has been observed in both aerobic and anaerobic degradation of chlorinated and nitro compounds, and the chloro group is replaced by hydrogen. Thus the reductive removal of chlorine is an energy-consuming reaction (Fetzner 1998). The substitutent may not be removed from the substituted compound in the initial degradation reaction, but from an intermediate. Dechlorination from the intermediates, rather than in the initial reactions was observed in degradation of dichlorobenzene and other chlorinated compounds (Harayama and Timmis 1992; Schlömann 1994; Reineke 1994; Hale et al. 1994).

Specific aspects of removal of nitro groups are described section 1.5.2.

1.3.4. Cometabolism

Some enzymes, which are induced to degrade simple growth substrate in certain bacteria, have relaxed substrate selectivity and attack a wide range of substituted synthetic hydrocarbons. This phenomenon is called cometabolism, or fortuitous metabolism or co-oxidation (Janssen and Witholt 1992; Hanson and Brusseau 1994; Belkin 1992). Cometabolism allows the removal of the chloro-substituents and produce more biodegradable compounds. TCE is degraded by methane monooxygenase (Henry and Grbić-Galić 1994). TCE is oxidized into a TCE epoxide, and the latter is spontaneously rearranged via hydrolysis into carbon monoxide, formate, dichloroacetate, and glyoxylate. Polycyclic aromatic hydrocarbons and chlorinated compounds are degraded by extra-cellular ligninase (lignin peroxidase and mangnese peroixidase) (Hammel 1992; Fernando and Aust 1994). While

PCBs hardly supports bacterial growth as carbon source, bacteria with biphenyl as growth substrate could cometabolize certain PCB congeners (Furukawa 1994; Brenner et al. 1994).

1.4. Combination of degradation technologies

Many high-profile toxicants are recalcitrant to biological oxidation. These compounds may not be degraded via a single biological process due to their high concentration; toxicity to bacterial cells; low bioavailability, or the lack of biotransforming enzyme, and process combinations have been investigated for their mineralization.

1.4.1. Combination of an aerobic degradation with an anaerobic process

Aerobic degradation degrades lowly substituted hydrocarbons, but does not degrade compounds with many electrophilic substitutions. Anaerobic processes can reduce the polychlorinated compounds, producing lowly chlorinated compounds as dead-end products. A combination of anaerobic process with aerobic pretreatment facilitates mineralization of the compounds with many electrophilic substitutions (Field et al. 1995). Using a reductive anaerobic process to remove a chlorine is a promising approach to convert the substrate into lowly chlorinated products amenable to aerobic degradation (Lee et al. 1998) as has been shown for PCB degradation (Abramowicz and Olson 1995).

1.4.2. Mixed culture degradation

Chemical pollutants on real sites are exposed to the metabolic activity of mixed cultures. A compound, which may not be shown to be mineralized by any specific strain, may be mineralized in the environments through sequential degradation by multiple strains.

1.5. Degradation of aromatic compounds

1.5.1. Chemical oxidation of aromatic compounds

Non-selective addition of hydroxyl radicals on the benzene ring was demonstrated for phenol, catechol, resorcinol, and p-hydroquinone during electron treatment of benzene (Nickelson et al. 1992, 1994), with production of common chemical oxidation aromatic ring cleavage products such as formaldehyde, acetaldehyde and glyoxal. Phenol, catechol, hydroquinone, p-benzoquinone, muconic acid are degradation intermediates of a semiconductor-catalyzed degradation of benzene (Hashimoto et al. 1984; Pelizzetti et al.

1988). Catechol, hydroquinone, muconic acid, maleic acid, fumaric acid, glyoxal, glyoxylic acid, oxalic acid and formic acid were degradation products of phenol by photo-enhanced ozonation. (Takahashi 1990) and semiconductor-catalysed photo-oxidation (Richard and Boule 1994). The oxygen molecule could react with the radical intermediates and participate in ring opening (Getoff 1996).

Photo-oxidation of 2,4-dinitrotoluene caused aromatic ring cleavage and produced nitrate ions. Fenton's reagent degraded nitrobenzene (NB) via three mononitrophenols 1,2,4-benzenetriol, hydroquinone, hydroxy-4-benzoquinone, 4-benzoquinone. 4-Nitrocatechol was detected as fenton's regent degradation product from 4-nitrophenol (Lipczynska-Kochany 1991). Semiconductor-catalysed photo-oxidation of NB produced monohydroxy-, dihydroxy-and trihydroxy-nitrobenzene with nitrohydroquinone, and parabenzoquinone as intemediates (Minero et al. 1994; Maillard-Dupuy et al 1994). Nitrosobenzene, isomers of dinitrobenzenes and phenol were also observed and indicated complicated reactions including a reductive degradation through electron or hydrogen radicals. Low molecular weight chemicals such as acetate and formate, and mineralized products such as carbon dioxide, nitrate ions, nitrite ion and ammonium ion were found (Minero et al. 1994; Maillard-Dupuy et al. 1994). These reactions demonstrated a ring-opening degradation mechanism through both addition of hydroxyl radicals and reduction through reductive species, such as electron and hydrogen radicals. Production of hydroquinone, nitrocatechol, trihydroxybenzene, aliphatics, nitrate and nitrite ions, ammonia was also reported for TiO_2-catalysed photo-oxidation of 4-nitrophenol (Dieckmann and Gray 1996).

Radioactive-induced degradation of NB also produced mononitrophenol and aminophenols (Kuruc et al. 1994; Cechova et al. 1987; Cefova et al. 1986). Since similar degradative species are produced in both semiconductor-catalysed and irradiation-assisted decomposition of water, similar degradation mechanism should be involved for NB.

1.5.2. Biological metabolism of aromatic compounds

All aerobic bacteria, which grow on aromatic compounds, via different peripheral initial reactions, convert these aromatics into common 1,4- or 1,2-dihydroxylated aromatic compounds (Houghton and Shanley 1994; Schlöm 1994; Williams and Sayers 1994; Smith 1990; Harayama and Timmis 1992). There are two types of ring-cleavage pathway for 1,2-dihydroxyl aromatic compounds: ortho-cleavage dioxygenase catalyzes insertion of two

producing 2-hydroxymuconic semialdehyde. For 1,4-dihydroxyl aromatic compounds, the cleavege occurs between a hydroxyl group and a neighboring carbon, producing 4-hydroxymuconic semialdehyde (Spain and Gibson 1990). The ring-cleaved products are further converted to C_2 or C_4 TCA cycle intermediates by hydration of double bond, oxidation of alcohol group and a reversed aldolase reaction.

1,2,4-Benzenetriol is a common degradation intermediate in degradation of phenolic compounds with multiple substitutents (Chamberlain and Dagley 1968; Chapman and Ribbons 1976; Daubaras et al. 1996; Haigler et al. 1999; Joshi and Gold 1993; Latus et al. 1995; Rieble et al. 1994; Sze and Dagley 1984; Valli et al. 1992; Zaborina et al. 1995). The benzene ring of 1,2,4-benzenetriol is cleaved via an ortho- or a meta-cleavage pathway.

The aromatic compounds with electronphilic substitutent are converted into 1,4 or 1,2 dihydroxyl products and converge to the above central ring-cleavage meatabolism (Winter and Zimmermann 1992; Commandeur and Parsons 1990; Harayama and Timmis 1992). The removal of substituents in the initial reaction or from the intermediates is key to their metabolism to produce TCA cycle intermediates and support bacterial growth, and the strategy for removal of the substituent may be oxygenolic, substitutive or hydrolytic as mentioned above.

Anaerobic mineralization of some aromatic compounds, such as phenolics, amines and benzoate, were reported (Heider and Fuchs 1997; Gibson and Harwood 1994). Key steps involved are: reduction of the benzene ring and production of olefinic compound, addition of water to the double bond to produce alcohol, oxidation of the alcohol and splitting of the ring via a reversed aldolase reaction producing a acetyl-CoA which enters the TCA cycle substrate. Phenol and aniline was degraded via initial conversion into benzoyl-CoA. Aromatic rings of 1,3,5-trihydroxybenzene or 1,3-dihydroxybenzene could be directly reduced.

1.6. Biological metabolism of nitroaromatic compounds.

Nitroaromatics have widespread applications as solvents, manufacturing raw materials for dyes, pharmaceuticals and explosives. Nitroaromatic compounds collectively account for almost 10 percent of total chemical sales (Hartter 1984). Biodegradation of nitroaromatics has

been reviewed (Marvin-Sikkema and de Bont 1994; Spain 1995a,b; Gorontzy et al 1992; Higson, 1992), and three main strategies were described for bacteria to initiate degradation of mono-, di-, or tri-nitrated aromatics. Oxygenase, benzene ring reductase and nitroreductase (type I) have been reported for bacteria to initiate degradation of nitroaromatic compounds. The first two initial reactions produce nitrite and the third produces ammonia.

1.6.1. Oxygense-initaited metabolism

Despite the electron deficiency of nitroaromatic compounds due to the presence of the nitro group, many aerobic bacteria are capable of attacking the aromatic ring of mono- and di-nitroaromatic compounds with specific oxygenases producing 1,2 or 1,4-dihydroxylated aromatic compounds. This pathway was observed in degradation of 2-nitrophenol (NP) (Zeyer and Kearney 1984), 4-NP (Hanne et al. 1993; Spain and Gibson 1991), dinitrophenols (Bruhn 1987; Ecker et al. 1992), NB (Nishino and Spain 1995), 2-nitrotoluene (Haigler et al. 1994), and 1,3-dinitrobenzene (Dickel and Knackmuss 1991). The dioxygenase-catalyzed removal of nitro group from aromatic ring involves three steps: 1) oxygen molecule activation by 2e {or H⁻ in NAD(P)H)}; 2) addition of two 'OH's to the 1 and 2 position of aromatic ring relative to nitro group, forming a cis-cis-dihydrodiol; 3) release of a nitrite anion and formation of a catechol product (for dioxygenase). The catechol is then subjected to ring-cleavage metabolism.

1.6.2. Nitroreductase-initiated metaboilsm

Under both aerobic and anaerobic conditions non-specific nitroreductases in certain microorganism catalyze reduction of the electrophilic nitro group of some nitroaromatics and amines as dead-end reduction products (Blasco and Castillo 1993; Bryant and McElroy 1991; Cerniglia and Somerville 1995; Glaus et al 1992; Gorontzy et al. 1993; Oren et al. 1991; Schackmann and Muller, 1991). Some aerobic bacteria use nitroreductase (type I) to initiate degradation of nitroaromatic compounds as growth substrate, and produce ammonia. This nitroreductase-initiated aerobic degradation was found in degradation of NB (Nishino and Spain 1993b; Park et al. 1999) and 4-nitrobenzoate (Groenewegen et al. 1992), 4-nitrotoluene and 3-NP (Meulenberg et al. 1996; Schenzle et al. 1997,1999). Two approaches have been described for ammonia-release from hydroxylamine intermediates in this nitroreductase-initiated aerobic degradation of nitroaromatics: (a) the hydroxylamine intermediate is rearranged into aminophenol and then ammonia is released after the aminophenol is cleaved

by an dioxygenase; (b) conversion of arylhydroxylamine into 1,2-dihydroxyl aromatic compounds by proposed hydrolytic hydroxylaminolyases. Nishino and Spain (1993b) observed the first approach in the NB degradation pathway of a *Pseudomonas pseudoalcaligenes* and a similar pathway was observed in degradation of NB in *P. putida* (Park et al. 1999), degradation of 4-nitrotoluene in a *Mycobacterium* strain (Spiess et al. 1998, He and Spain 2000). Groenewegen et al (1992) reported the second route in degradation of 4-nitrobenzoate in *Comamonas acidovorans* NBA-10 and this pathway was also described for degradation of 4-nitrotoluene in *Pseudomonas* sp (Haigler and Spain 1993; Rhys-Williams et al. 1993) and degradation of 3-NP in *P. putida* B2 (Meulenberg et al. 1999).

There were few reports that bacteria grew on polynitrated compounds as nitrogen source via reduction into amine. Boopath et al. (1993) reported that an anaerobic bacterium, *Desulfovibrio* sp. B strain, converted 2,4,6-trinitrotoluene into toluene via mono-, di- and tri-aminotoluene, and produced ammonia as nitrogen source for bacterial growth. The ammonia-release mechanism from the above polynitrated compounds still remains poorly characterized.

1.6.3. Aromatic ring reductase initiated metabolism via the meisenheimer complex

For polynitrated aromatic compounds, such as di- or tri-nitrophenols and toluene, a direct reduction of the nitrated benzene ring by H⁻ under aerobic conditions with the release of nitrite was reported (Lenke and Knackmuss 1992; Lenke et al. 1992; Vorbeck et al. 1994). Knackmuss' group (1992a,b) first proposed a nitrite release mechanism via a hydride-meisenheimer complex in degradation of 2,4-dinitrophenol and 2,4,6-trinitrophenol (picric acid) with *Rhodococcus erythropolis*.

Rieger et al (1999) described formation of a hydride-meisenheimer complex in degradation of 2,4,6-trinitrophenol biodegradation using *Rhodococcus erythropolis*. Rajan et al. (1996) described mineralization of picric acid in *Nocardioides simplex*, and Ebbert et al. (1999) identified a F_{420}-dependent enzyme system, which catalyze hydride transfer from NADPH to picric acid to form a hydride-meisenheimer complex in this strain. Formation of this hydride-meisenheimer complex was also found as an intermediate in mineralization of 2,4,6-trinitrotoluene (Vorbex et al. 1994) by 4-nitortoluene-grown cells of a *Mycobacterium* strain, a strain which was previously isolated on 4-nitrotoluene and which degraded 4-nitrotoluene via a nitroreductase-initiated pathway (Spiess et al. 1998).

1.7. Thesis objectives and results summary

Biodegradation of substituted synthetic hydrocarbons necessitates the removal of the substitution, especially electrophilic groups, which decrease biodegradability (Klopman et al. 1995; Boethling et al. 1994; Howard et al. 1992; Kameya et al. 1995). Advanced oxidation processes (AOP) degraded aromatic compounds mainly through hydroxyl radical oxidation reaction, and production of hydroxyl products and other low molecular weight biodegradable products that are more supportive to bacterial growth. In this research the feasibility of combining electron beam pretreatment with biodegrdation to degrade a model compound, NB was explored and the subsequent metabolism of mixtures of the NP products and NB was characterized. The specific goals of this research are: to enrich microbial cultures from activated sludge which are able to degrade both nitrobenzene and its degradation products, nitrophenols; to identify the microorganism which degrades NB and/or NP; to study the biochemical metabolism involved in NB and NP degradation by pure cultures. These goals were achieved and the results were presented in chapter 2 to 7.

We used moderate doses of electron beam radiation to degrade aqueous NB produce a mixture of NB and nitrophenols. To enrich the cultures that degrade both NB and NP, we used NB and/ or NP as the sole carbon and nitrogen sources for growth of bacteria in the activated sludge. We obtained a mixed culture which degraded both NP and NB by using the mixture of three mono-nitorphenols as the sole carbon and nitrogen sources (Zhao and Ward 1999, Chapter 2 of this thesis). Through intensive subculturing, we isolated three groups of bacteria from this mixed culture: group A, $2NP^-3NP^-4NP^+$ where growth was observed on 4-NP), but not on 2- or 3-NP; group B, $2NP^+3NP^+4NP^-$ where growth was observed on 2-NP and 3-NP, but not on 4-NP, and group C, $2NP^-3NP^+4NP^-$ where growth was observed on 3-NP, but not on 2- or 4-NP (Chapter 3). Strains of group A and B belonged to *Pseudomonas* sp. One strain of group C was identified as *Variovorax paradoxus*. Only 3-NP-grown cells of strains of Group B and C, degraded NB, and produced ammonia from both NB and 3-NP. We investigated the NB degradation mechanism by a typical strain of group B, *Pseudomonas putida* 2NP8, and observed that a 3-NP-induced enzyme system cometabolize NB into ammonia via nitroso and hydroxylaminobenzene (Zhao and Ward 2000, Chapter 4 of this thesis). We observed production of aminophenol, 4-benzoquinone, 2-aminophenoxazine-3-

one, 4-hydroquinone, catechol, and ammonia production from hydroxylaminobenzene, and this allowed us to conclude that the hydroxylaminobenzene was first converted to 2- or 4-aminophenol, and that ammonia was released via an oxidation of aminophenol into benzoquinone monoimine with subsequent hydrolysis into benzoquinone (Zhao et al. 2000, Chapter 5 of this thesis). Based on these results, we proposed that degradation of the growth substrate 3-NP involved conversion into 3-hydroxylaminophenol, rearrangement into aminophenolic compounds, followed by oxidization into monoimines with subsequent hydrolysis into quinones and reduction into 1,2,4-benzenetriol (Zhao et al. 2000, chapter 5 of this thesis). We further investigated the substrate selectivity of the 3-NP-induced enzyme system in *P. putida* 2NP8 and found that a wide range of nitroaromatic compounds were transformed into ammonia (Chapter 6 of this thesis). However, 2-NP-grown cells of this strain manifested a narrow degradation selectivity, and only 2-NP and 4-Cl-2-NP among the 30 nitroaroamtic compounds were transformed into nitrite (Chapter 7 of this thesis).

2. MICROBIAL DEGRADATION OF NITROBENZENE AND MONO-NITROPHENOL BY BACTERIA ENRICHED FROM MUNICIPAL ACTIVATED SLUDGE [a]

2.1. Abstract

Using a mixture of three mono nitrophenols as sole carbon, nitrogen and energy sources, mixed cultures were enriched from municipal activated sludge to degrade both nitrophenols and nitrobenzene. Bacterial growth and degradation rate could be increased by supplementing the medium with 0.1% YE. Microorganisms were isolated from the nitrophenols enrichment, and they were identified as strains of *Comamonas testosteroni* and *Acidovorax delafieldii*. These strains showed broad degradation ability toward nitrophenols and nitrobenzene.

2.2. Introduction

Nitroaromatic compounds are very important industrial chemicals, which collectively account for almost 10 percent of total chemical sales (Hartter 1984). Nitrobenzene (NB) is one of the fastest growing end use products (Richards 1996) and Nitrophenols (NPs) and NB are acutely toxic (Hartter 1984)

The nitro group is a strong electron-withdrawing group, which causes nitroaromatic compounds to be resistant to aerobic biodegradation (Field et. al. 1995). Biodegradation of nitroaromatics has been extensively reviewed in recent publications (Marvin-Sikkema and Bont 1994; Spain 1995). Reported half lives for NB in environments such as surfacewater, ground water, soil and activated sludge ranged from two to more than 625 days (Mackey et al. 1995), illustrating the persistence of this chemical. Anaerobic biodegradation, which occurs in the natural environment, produces dead-end amines and mutagenic nitroso compounds (Field et al. 1995). The combination of anaerobic and aerobic degradation has been investigated for degradation of NB, and resulted in the removal of 95% of 102 mg/l NB in a system with a hydraulic retention time of 24 hours (Aziz et al. 1994). In sucrose produced acidogenic

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conditions, acid was produced by anaerobic metabolism of sucrose, and caused the pH to drop to 3-4. Under these conditions of anaerobic metabolism, NB was reduced to aniline. The aerobic degradation of 4-nitrophenol (4-NP) is relatively common (Mackey et. Al. 1995), but there are very few reports on the degradation of 2-NP, or 3-NP (Schenzle et al. 1997; Zeyer and Kearney 1984; Meulenberg 1996). Degradation half-lives of these molecules in surface water and soil may be up to 10 days (Mackey et al. 1995).

Some aerobic bacteria capable of degrading NPs and NB have been isolated, despite the recalcitrance of these compounds to biodegradation. *Pseudomonas* species (Heitkamp et al. 1990, Nishino and Spain 1993a), *Moraxella* species (Spain and Gibson 1991) and *Arthrobacter aurescene* and *Nocardia* sp (Hanne et al 1993) were reported as 4-NP degraders. One strain of *Pseudomonas putida* degraded 2-NP and 3-NP (Zeyer and Kearney 1984) and was recently reported to grow on NB (Meulenberg et al. 1996). *Ralstonia eutropha*, a degrader of 2,4-D, was found recently to degrade 3-NP and transform NB into dead-end aminophenols (Schenzle, et al 1997). *Pseudomonas pseudoalcaligenes* (Nishino and Spain 1993b) and a *Comamonas* species were isolated from NB contaminated sites as NB degraders (Nishino and Spain 1995). The *Pseudomonas* species could also transform NPs. To the best of our knowledge, simultaneous aerobic removal of 2-NP, 3-NP, 4-NP and NB by pure or mixed culture has not been reported.

We have previously shown ability of electron beam technology to remove NB from the aqueous phase (Lubicki et al. 1997). However, the removal efficiency decreased significantly as the concentration of NB was reduced. Three mono NPs were also found in the electron beam treated NB solution. We are exploring the possible use of a combination of electron beam pretreatment and biodegradation to remove NB. Since mild electron beam treatment of NB produces a mixture of NPs and residual NB, we sought to develop an enrichment culture containing degraders of both NB and NPs. In this paper, we present our results of enrichment of both NB and NPs degraders from municipal activated sludge and degradation of NB and NPs by enriched mixed cultures.

2.3. Material and Methods

2.3.1. Media

Basic salt liquid medium contained (g/l): KH_2PO_4, 1; Na_2HPO_4 $12H_2O$, 7; ferric citrate, 0.04; $CaCl_2$ $2H_2O$, 0.1; $MgSO_4$ $7H_2O$, 0.3; pH 7.35. Trace metals solution (TMS) (mg/l): $FeCl_3$ $6H_2O$, 162; $ZnCl_2$ $4H_2O$, 14.4; $CoCl_2 2H_2O$, 12; $Na_2MoO_4 2H_2O$, 12; $CaCl_2 2H_2O$, 6; $CuSO_4 5H_2O$, 1900; H_3BO_4, 50; HCl, 0.44 mole. Basic salts/TMS medium was basic salts medium supplemented with TMS (3ml/ml). Basic/TMS/YE medium was the basic salts medium supplemented with TMS (3ml/L) and sterile yeast extract (YE) (1g/L). YPS medium (g/l): YE, 10; Bacto peptone, 10; NaCl, 5. Nitroaromatics, TMS and YE were added into autoclaved liquid media before incubation or before pouring plates.

2.3.2. Culture enrichment and biodegradation tests

Five milliliters of fresh activated sludge was inoculated into 50 ml of YPS medium in a 250ml Erlenmeyer flask and incubated on an orbital shaker at 180 rpm, room temperature (26°C) for 24 h. A 10% inoculum was transferred into fresh YPS medium and incubated under the same conditions for 24 h. Then 5ml was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of basic salts medium containing 20mg/l of 2-NP, 3-NP and 4-NP, and was again incubated at 26 °C, 180rpm. After 96 h, the yellow colour disappeared and the same amount (20 mg/l) of each NP isomer was added. After colour disappearance, 10 ml culture was transferred into fresh NPs basic salts medium and the NP (20 mg/l) supplement and colour removal step was repeated three times. The disappearance of yellow colour indicated the degradation of NPs because the pH of the medium is 7.36 and 2-NP and 4-NP should give a yellow colour at this pH (3-NP only shows a very faint yellow colour at 20 mg/l). The relationship between colour removal and degradation of NPs was confirmed by HPLC analysis. The NPs enrichment culture was then re-grown in NPs-supplemented basic salts/TMS/YE medium under the same conditions to get more growth.

The fresh enrichment was streaked on the basic salts/TMS/YE agar plates containing corresponding nitroaromatic compounds. Colonies were isolated and streaked on YPS agar plates again. Pure cultures were collected from the agar plates. The following procedure was followed for pure culture degradation. Biodegradation was carried out by first inoculating one loop of fresh pure culture cells on YPS agar into 5 ml of YPS liquid media and incubating for

24 h and then inoculating 1 ml into 9 ml basic salts/TMS/YE medium containing nitroaromatics. The OD of the inoculated culture was around 1.0. NB and/or NPs stock solutions were added into the media to achieve the designed concentration. The bottles used for biodegradation were 40 ml amber glass with the Teflon/silicone septa lined screw caps. The caps were loosened during shaking to facilitate aeration. The degradation was stopped after shaking for 48 or 96 h.

Growth and biodegradation studies were conducted at room temperature, with 200 rpm shaking on an orbital shaker. Biomass concentration was measured in diluted cultures in a Shimadzu UV-120-02 spectrophotometer (Shimadzu Corporation, Kyoto) at 600nm in a 1cm light path. Cultures were centrifuged and the cell pellets were suspended in water to give OD recordings of <0.5. NPs enrichment was maintained and grown in the NPs-supplemented basic salts/TMS media. Frequent addition of NPs immediately after losing yellow colour is necessary to get more biomass for inoculation. The freshly grown NPs-free (colourless) culture was used as inoculum for the mixed culture degradation test. NPs mixture biodegradation was carried out in 250ml flasks. Whereas any cultures containing NB were in loose capped amber glass, 40 ml vials with Teflon/silicone septa lined screw caps.

2.3.3. Nitrophenol and nitrobenzene analysis

Samples from biodegradation tests were subjected to centrifugation at 9000 g for 3 minutes to remove the cells and 0.9 ml of supernatant was transferred into 4 ml of amber glass vial with screw tightened and Teflon-lined caps. Then 0.1 ml of 0.4N HCl solution was added to NP-containing samples (not necessary for analysis of NB). Ethyl acetate, 1 ml, was added and the mixture vortexed for 1 min. The organic layer liquid was collected and used directly for HPLC analysis. When NB and NPs were added to killed cells (boiled for 1 min) and subsequently extracted, greater than 95% of the substrates was recovered. Consequently it was concluded that the cells do not retain NB or NPs and do not influence analytical results. The HPLC analysis was performed on a 3.9 X 300mm μBondapak™ C18 column (Waters, Milford, MA). The apparatus consisted of two Shimadzu LC-600 pumps, a Shimadzu SPD-6A UV spectrophotometric detector and a sample injector 7125 (all components are from Shimadzu Corporation, Kyoto). Sample (15 μl) was injected and eluted with methanol and milliQ water (0.1% acetic acid). For NP solutions, solvents were delivered at the rate of 0.22

(methanol) and 0.78 (milliQ water) ml/min. For NB samples, solvents were delivered at the rate of 0.55(methanol) and 0.45 (milliQ water) ml/min. Compounds were monitored at UV A_{254nm}. The linear equations were used for analyses of every NP isomer and NB. Samples with the concentration of more than 6mg/L were diluted. Analytical data were obtained in duplicate.

2.4. Results and discussions

The initial objective was to find bacteria that had the capability to degrade both NB and NPs. Three enrichment approaches were taken with the following series as enriching substrates respectively: (a) 60 mg/l of NPs (concentration of each NP isomer is 20 mg/l), (b) 60 mg/l of NB, (c) both 60 mg/l of NB and 60 mg/l of NPs. In the case of (b) and (c), in addition to the 60 mg/l NB added, a small vial containing 1 ml of NB was fixed inside the flask as a NB reservoir, because NB is known to volatize. A similar NB feeding procedure has been employed by other researchers, including Nishino and Spain (1993b). This NB feeding procedure was only used in these preliminary enrichment studies. Because of toxicity of the serial substrates, little or no bacterial growth occurred by enriching in a basal salts media. Fresh activated sludge (from the municipal wastewater treatment plant, Waterloo, Ont), was first enriched on YPS medium and then inoculated into the basic salt medium. Nitroaromatics were used as the sole carbon and nitrogen sources.

NPs were found to be able to support bacterial growth as the sole carbon and nitrogen sources and were removed after four days. No NP disappearance occurred in the non-inoculated control flask. The biomass increased by repeated feeding of NPs into the degrading liquid media. NP removal was observed visually by disappearance of the yellow colour and then confirmed by HPLC analysis. The time needed for complete removal was shortened once the cultures were acclimated (Table 2.1). When the concentration of NPs was increased from 60 up to 120 and 240 mg/l, NP removal times by acclimated cultures were extended to 42 h and 192 h, respectively.

In a two-month incubation there was no apparent bacterial growth in the NB medium or in the mixed NB/NPs medium. There was no loss of NPs in the NB/NPs medium. NB appeared to be a poor substrate for activated sludge bacteria, and with a mixture of NPs and NB, NB suppressed growth of the bacteria on NPs.

In order to test the ability of the NPs enriched mixed culture to tolerate NB, a series of NB concentrations (0-5.7 mg/l) was initially added into the NPs-supplemented basic salt medium. When these media were inoculated with the NP acclimated mixed cultures and incubated under standard conditions, bacterial growth was visually observed. Disappearance of NPs occurred after 23 h (Table 2.2). The 23 h culture acclimated with 5.7 mg/l of NB was used to inoculate NP-supplemented media containing a series of 5.7-118 mg/l of NB. For this and the following series of tests, an inoculation rate of 5% was always used and resulted in an initial OD_{600} of 0.02-0.03. NP removal in the presence of up to 22.8 mg/l of NB was observed also at 23 h, and NP removal in the presence of 35.4-118 mg/l of NB was observed at 33 h. This 33-hour culture was used to inoculate NPs-supplemented media containing a higher NB concentration ranging from 227 to 1180 mg/l of NB, and in each case the time for NP removal was determined. No removal of NP was observed when more than 457 mg/l of NB was added. Growth and NP disappearance in the NPs supplemented basic salts/TMS media inoculated with NPs (24 h) enriched mixed culture, was characterized. Individual NPs were quantified by HPLC. The results are presented in Figure 2.1. Biomass OD increased from 0.23 to 0.38 in 30 h, at which 94% of total NPs were transformed. 4-NP was preferentially transformed, followed by 2-NP and 3-NP. When YE (0.1%) was added to the NPs supplemented basic salts/TMS media, a dramatic increase in cell growth (0.23 OD to 0.90 OD in 18 h) and increased rates of NP degradation were observed (Figure 2.2).

The three NPs were utilized as the nitrogen sources for soil bacterial growth (Bruhn et al. 1987). To the best of our knowledge, there has been no report on the simultaneous aerobic degradation of the three NPs as the sole carbon, nitrogen and energy sources, even though degradation of 4-NP was observed in soil or fresh water (Hanne et al 1993; Heikamp et al 1990) and that of 2-NP, 3-NP was reported in soil (Zeyer and Kearney 1984).

NB (8 mg/l) supplemented basic salts/TMS media with or without YE supplement were inoculated with NPs-enriched culture and incubated under standard conditions. Biomass and NB degradation was monitored over time (Figure 2.3). In the absence of YE, growth and NB disappearance were very low. In the presence of 0.1% YE, a rapid burst of growth and NB transformation was observed in the first 30h. The cessation of NB disappearance when growth ceases suggests that NB degradation relies on growth and is a cometabolic process, which

would also result in production of intermediates which may be toxic and may contribute to cell lysis.

The effect of NP on NB disappearance and vice-versa was also examined. NPs were added to the NB-supplemented solution without YE followed by inoculation with NPs enriched culture and incubated under standard conditions. NB, NPs and biomass were monitored over time (Figure 2.4). NP supplement increased the rate of disappearance of NB (compare to Figure 2.3). The presence of NB decreased the rate of degradation of total NPs, specifically 2-NP. Supplementation with a small amount of YE led to a dramatic increase in biomass OD (0.30 to 1.70 in 34.5 h) and a rapid degradation of NB and NP. Degradation of 8 mg/l of NB was also found to be incomplete and only 50% of NB degradation was achieved. YE addition accelerated degradation but did not change the final concentration of substrates.

The influence of NP and NB concentration on their degradation by the NPs enriched culture was investigated in the basic salts/TMS/YE media (Table 2.3). With 5mg/l each of 2-NP, 3-NP and 4-NP and 2mg/l of NB, all nitroaromatics were completely degraded, but at high concentrations, the degradation was incomplete.

Degradation of NB by acclimation of activated sludge with other nitroaromatics has been reported without microbial chracterization, such as, acclimation with aniline and pyridine (Gomólka and Gomólka 1979); degradation of NB and aniline (Patil and Shinde 1988) or 2,4-diNP (Patil and Shinde 1989); degradation of NB, 4-NP and 4-nitroaniline (Janeczko and Gaz 1984); degradation of 4-NP and chlorinated mono or diNB (Kwiatkowski and Ostrowska 1984). Transformation of NPs by cells of *Pseudomonas pseudoalcaligenes* grown on NB was reported, but the strain could not grow on NPs (Nishino and Spain 1995). There are no other reports on the aerobic degradation of NB by NPs degraders, or on the simultaneous aerobic degradation of NB, 2-NP, 3-NP and 4-NP by pure or mixed culture.

The NPs enriched culture was plated out on NPs supplemented basic salts/TMS/YE agar media and incubated for three days at room temperature. Colonies appeared quite similar. Twenty three of the largest colonies were randomly isolated, inoculated into the basic salts/TMS/YE liquid media containing 2 mg/l of NB and 5 mg/l of 2-NP, 3-NP and 4-NP, and incubated for 4 days. Most isolates showed broad degradative ability to both NB and each isomer of NP in the mixture medium. Three out of four were found to be *Comamonas*

testosteroni, which may or may not be identical, while one was identified as *Acidovorax delafieldii* (Table 2.4).

A *Comamonas* species isolated from NB contaminated wastewater (Nishino and Spain 1995) was able to grow on NB. *Comamonas testosteroni* was shown to degrade PCBs (Dercová et al. 1993), PAH mixtures (Goyal and Zylstra 1996), aryl sulfonate (Oppenberg et al. 1995) and chloro- and methyl phenol (Hollender et al. 1994), but has not been shown to possess the ability of degrading both NB and NPs. *Acidovorax delafieldii* strains were also reported as degraders of aromatic compounds (Dercová et al. 1993; Goyal and Zylstra 1996; Oppenberg et al. 1995; Hollender et al. 1994), but never reported as NB or NPs degraders. The significance of degradation of NB by degraders of phenolics can be estimated by the fact that phenolic compounds are often found as the chemical or biological transformation products of the non-hydroxylated parent compounds (Carey 1992). The degradation mechanism of NB and NPs in this enriched culture is being addressed at the pure culture level, and toxicities of these chemicals and other factors to microbial growth and biodegradation are being evaluated.

2.5. Acknowledgment

Table 2.1. Acclimation of YPS-grown activated sludge culture with NPs and influence of NP concentration on NPs removal by the acclimated culture

Inoculum	NPs concentration (mg/l)[a,b]	Time of NPs removal[c] (h)
Acclimation of activated sludge with NPs		
Unacclimated culture[d]	60	96
Above 96 h culture[d]	60	31
Above 31 h culture[d]	60	21

[a] Ratio of 2-NP: 3-NP: 4-NP = 1: 1: 1

[b] NPs (99%, purchased from Sigma, St Louis, MO) stock solutions were prepared at a concentration of 10 mg/ml in Methanol (HPLC grade, 99.8%, obtained from EM Science, Gibbstown, NJ)

[c] NP removal was observed visually at the point of disappearance of the yellow colour and was then confirmed by HPLC analysis.

[d] Inoculum rate: 5%; incubation media: basal salts/TMS.
Unacclimated culture: activated sludge culture pre-grown in YPS medium.

Table 2.2. Influence of NB concentration on NPs removal by the NPs enrichment[1]

Inoculum (mixed culture)			NB[2] conc. (mg/l)	Time for NP removal(h)
Enriching procedure				
NPs(24h) → NPs + 5.7mg/l NB(23h) → NPs +118 mg/l NB(33h)				
√			0-5.7	23
	√		5.7-22.8	23
	√		35.4-118	33
		√	227	168
		√	≥ 457	no degradation

1 NP/conc(mg/l): 2-NP/20; 3-NP/20; 4-NP/20

[2] NB: 99% pure, purchased from British Drug House, Toronto, Ont. Stock solution was
prepared by dissolving 1.00 ml of NB in 20 ml of methanol to give a concentration of
60 mg/ml.
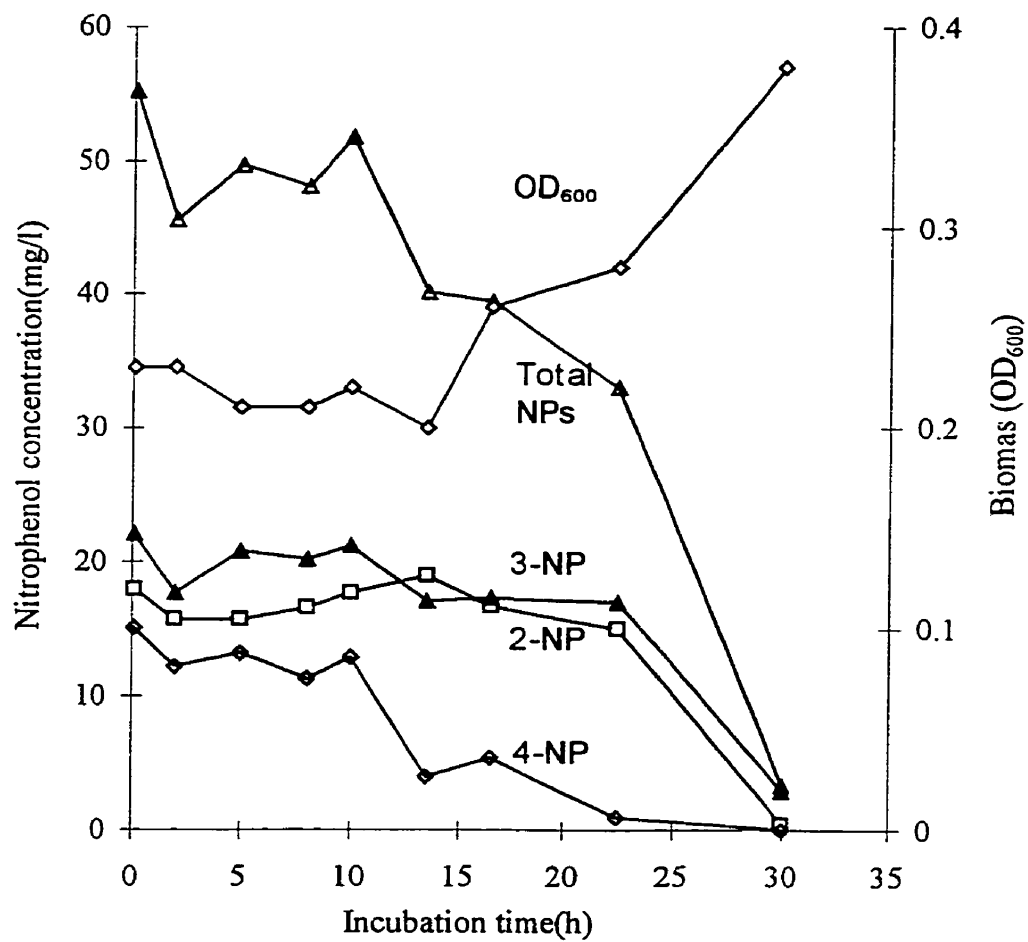
Figure 2.1   Degradation of three mono-nitrophenols mixture by NPs enrichment (no YE)
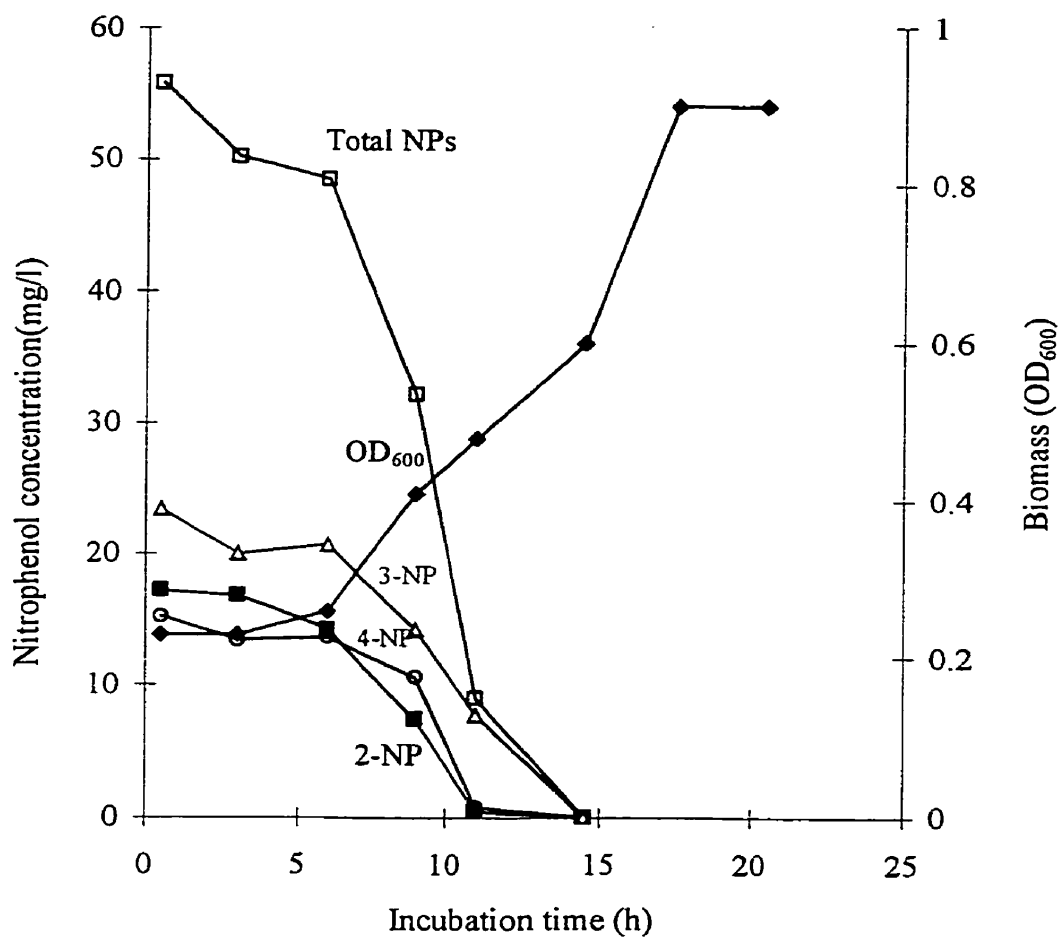
Figure 2.2   Degradation of three mono-nitrophenols mixture by NPs enrichment (0.1%YE)
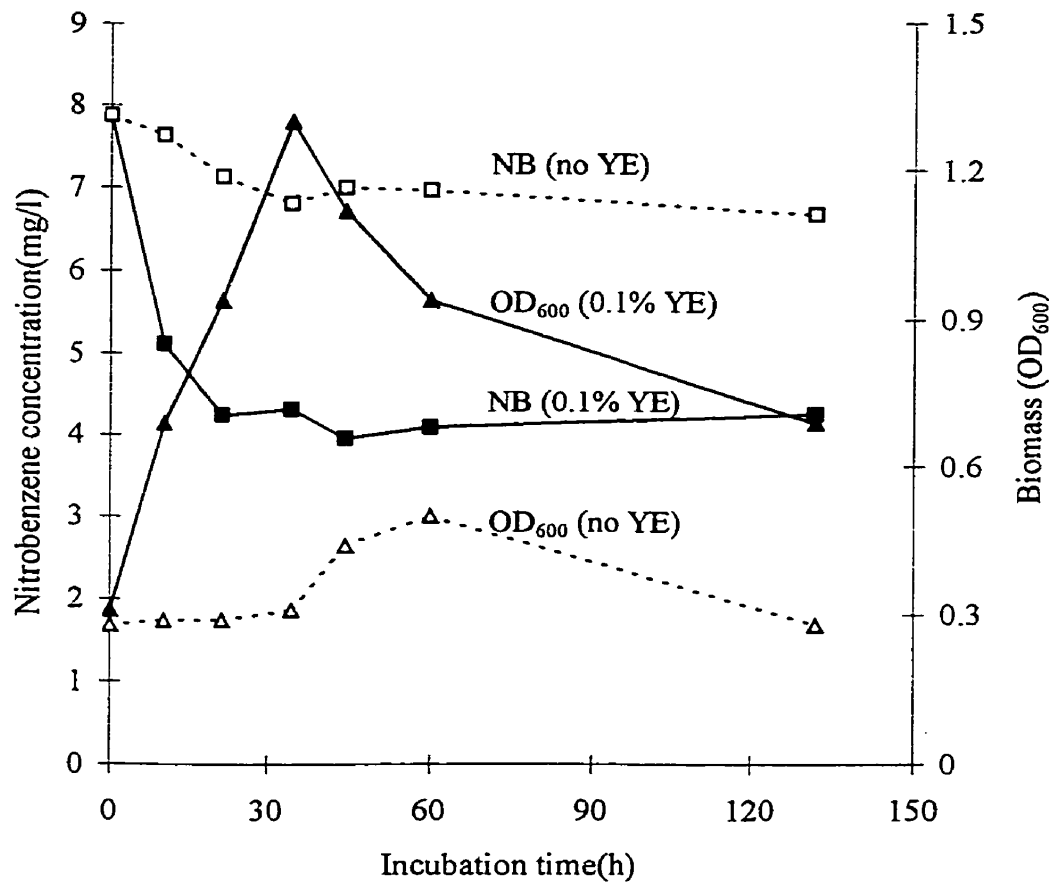
Figure 2.3   Biodegradation of nitrobenzene by NPs enriched mixed cultures in the absence of NPs
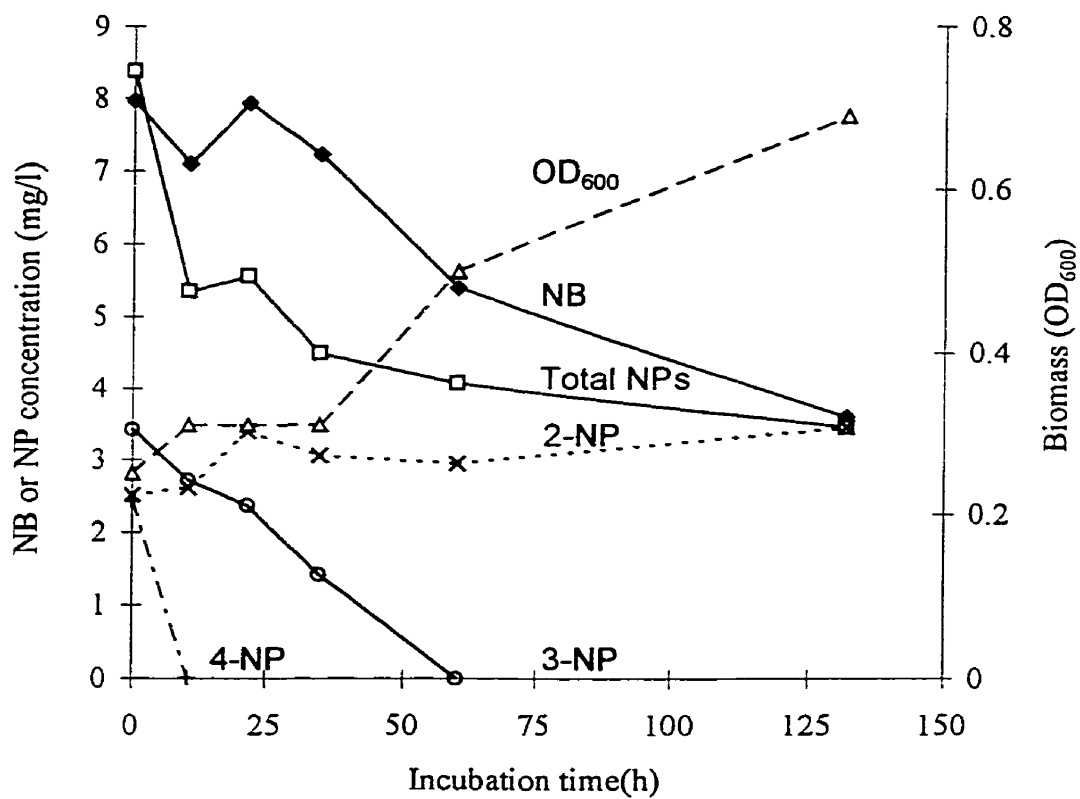
Figure 2.4 Degradation of nitrobenzene by NPs enriched mixed culture in the presence of NPs

Table 2.3. Influences of NP and NB concentration on degradation by the NPs enrichment(inoculation: NPs-grown culture, OD=0.26)

NB and NPs conc. (mg/l)		Degradation (after 48 h, %)		
NPs*	NB	NB	4-NP, 3-NP	2-NP
60	2	15	99	58
60	10	47	99	51
60	30	24	99	27
15	2	92	99	99
15	10	68	99	99
15	30	36	99	99
0	2	92		
0	10	66		
0	30	65		

* Ratio of 2-NP : 3-NP : 4-NP = 1:1:1

Table 2.4. Degradation of NPs and NB mixture by isolates from the NPs enrichments and their identifications [1,2]

Isolates	Degradation (%)			
	4-NP	3-NP	2-NP	NB
Comamonas testosteroni NPs-4	98	54	38	35
Comamonas testosteroni NPs-13	32	41	53	49
Acidovorax delafieldii NPs-15	55	29	37	44
Comamonas testosteroni NPs-24	69	40	55	46

[1] Inoculation is 24 h YPS grown cells and initial OD is 0.8-1.0; Medium: 2mg/L NB and 5mg/L 2-NP, 3-NP,4-NP, 0.1%YE, 4days

[2] Isolates were identified using MIDI-Microbial Identification System based on fatty acid

3. PROCESS FOR DEGRADATION OF NITROBENZENE: COMBINING ELECTRON BEAM IRRADIATION WITH BIOTRANSFORMATION[a]

3.1. Abstract

Electron beam irradiations of aqueous solutions containing 15-30 mg/L of nitrobenzene at 60kGy dose removed 78% of the contaminant. Three mono-nitrophenols were detected as byproducts of electron beam treatment of nitrobenzene. A mixed culture enriched on a mixture of 2-, 3- and 4-nitrophenol degraded both the residual nitrobenzene and the nitrophenol products. Percentage removal of nitrobenzene increased with increasing electron beam dose. This observation led to the conceptual design of a two-stage electron beam microbial process for degradation of nitrobenzene. Three groups of pure isolates were characterized from the mixed culture based on their abilities to grow on corresponding nitrophenol substrates: group A, $2NP^{-}3NP^{-}4NP^{+}$; group B, $2NP^{+}3NP^{+}4NP^{-}$ and group C, $2NP^{-}3NP^{+}4NP^{-}$. Bacteria that grew on 3-NP transformed nitrobenzene into ammonia in the electron beam treated nitrobenzene samples.

3.2. Introduction

Nitrobenzene (NB) is one of the fastest growing end use synthetic products of benzene, world demand of which was estimated to be growing at an annual rate of 3.1% (Richard 1996). NB has been used to produce aniline, and as an organic solvents, and has been widely dispersed in water and soil causing great environmental concern (Hartter 1984; Howard 1989). The strong electron-withdrawing property of the nitro group of NB causes resistance to aerobic biodegradation (Mackey et al. 1995). NB and some of its transformation metabolites, such as nitrosobenzene, hydroxylaminobenzene and aniline, exhibited toxic and mutagenic effects on biological systems (Cho and Lindeke 1988; Gorontzy and Blotevogel 1993; Mansuy et al. 1978; Schackmann and Muller 1991; Thompson et al. 1983). Exploration of effective methods for its degradation represents an important research challenge.

Advanced oxidation processes (AOP) are water treatment processes implemented at ambient temperature which involve generation of highly oxidative chemical species as decomposers of organic chemicals (Carey 1992; Glaze et al. 1992). These AOP processes are not very efficient in generating hydroxyl radicals and are sensitive to the presence of hydroxyl radical scavengers. Longer irradiation or reaction durations are needed to treat wastewater with high concentrations of pollutants and radical scavengers (Berg et al. 1994). AOP pretreatments have led to enhanced biodegradation of some aromatic compounds such as PCBs (polychlorinated biphenyls) and PAHs (polynuclear aromatic hydrocarbons) (Aronstein et al. 1995; Carberry et al. 1995; Martens et al. 1995).

Radiation is a source of high-energy particles, which decompose water molecules into hydroxyl radicals, hydrogen atoms and electrons (Buxton 1987). Degradation of NB by radioactive irradiation has been reported, but such methods are a cause of public concern (Cechova et al. 1987; Kuruc et al. 1994). An electron beam, consisting of high-speed electrons, is considered to be a safer and more effective method for removal of environmental pollutants from aqueous solution (Cooper et al. 1992a,b; Frank 1995; Geoff et al. 1996; Kurucz et al. 1991; Nickelsen et al. 1994; Wang et al. 1994).

A mixed culture enriched on a mixture of 2-, 3- and 4-nitrophenol (NP) was previously reported to degrade both NP and NB, but satisfactory removal of NB (92%) was only achieved at low concentrations of the contaminant (2mg/L) (Zhao and Ward 1999). The objective of this paper is to investigate the application of a combination of electron beam pretreatment with biodegradation to transform high concentrations of NB.

3.3. Materials and methods

3.3.1. Chemicals and stock solutions

2-, 3- and 4-NP were purchased from Sigma (St Louis, Mo). NB was obtained from BDH (Toronto, Ont) (99%). Methanol was obtained from EM Science (Gibbstown, NJ) (HPLC grade, 99.8%). Stock solutions: 2-NP, 3-NP, 4-NP and NB were dissolved in methanol to give a concentration of 10mg/ml.

3.3.2. Electron beam degradation of aqueous solutions of NB

Aqueous (in deionized water) solutions containing 15 mg/L and 30 mg/L of NB were subjected to electron beam treatment. The low voltage electron beam facility located in the high-voltage lab in the Department of Electrical Engineering, University of Waterloo was used for these tests (Lubicki et al. 1997). A schematic diagram of this electron beam apparatus is presented in Figure 3.1. A 10 μm boron nitride window was used, which allowed more than 90% transmission of the electron beam for an applied voltage of about 100 kV. 2 L of liquid sample was circulated to pass the electron beam window at a fixed rate of 1 L/min. 100 kV voltage was applied with the electron current of 0.1 to 0.3 mA. The circulating time and the current controlled the dose applied to the sample. Total dose: D= I(current)V(voltage)T(circulation time)/F (flow rate). If V=100 kV, I=0.2 mA, FR=1 Kg/sec, t=10 min, D=0.2 mA X 100kV X (10 X 60) sec =12 kGy. The electron beam was stopped after certain duration to take a sample for analysis of NB and products. To increase electron beam dose, the electron beam gun was re-primed to radiate the flowing NB solution for additional time as required.

3.3.3. Media

Basic salts liquid medium contained (g/l): KH_2PO_4, 1; Na_2HPO_4 $12H_2O$, 7; ferric citrate, 0.04; $CaCl_2$ $2H_2O$, 0.1; $MgSO_4$ $7H_2O$, 0.3; pH 7.35. Trace metals solution (TMS)(mg/l): $FeCl_3$ $6H_2O$, 162; $ZnCl_2$ $4H_2O$, 14.4; $CoCl_2$ $2H_2O$, 12; Na_2MoO_4 $2H_2O$, 12; $CaCl_2$ $2H_2O$, 6; $CuSO_4$ $5H_2O$, 1900; H_3BO_4, 50; HCl, 0.44 mole. Basic salts/TMS medium were basic salts medium supplemented with TMS (3 ml/L). Basic salts/TMS/YE medium was the basic salts medium supplemented with TMS (3ml/L) and sterile yeast extract (1g/L). YPS medium (g/l): yeast extract, 10; Bacto peptone, 10; NaCl, 5. Nitroaromatics, TMS, yeast extract were added into autoclaved liquid media before incubation or before pouring plates. The medium was autoclaved at 120°C for 30 min.
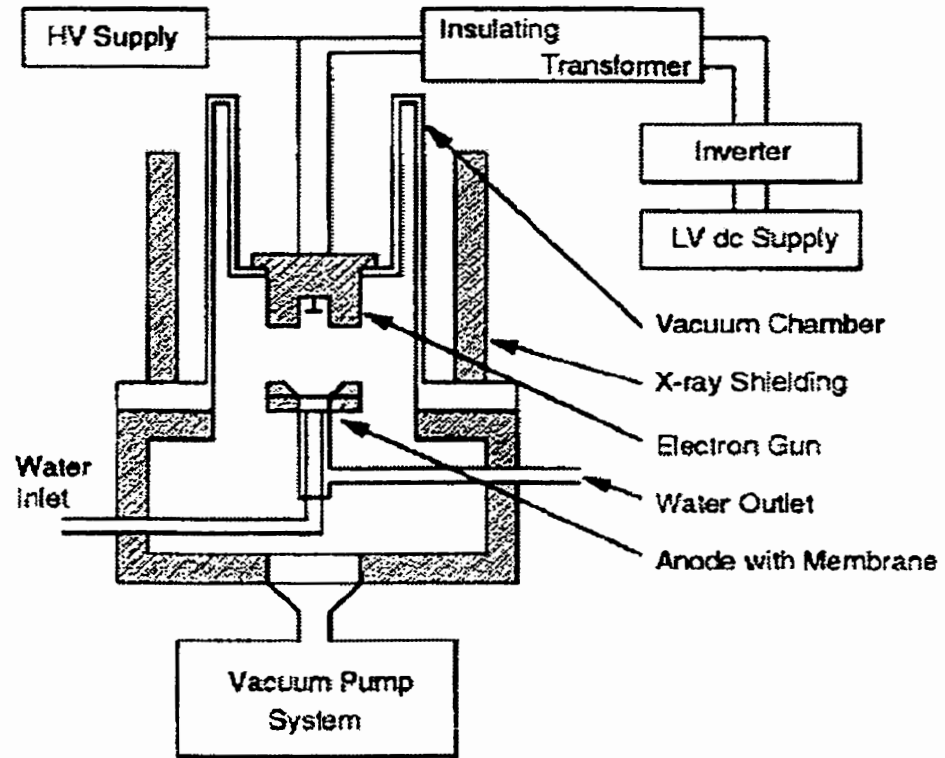
Figure 3.1. Schematic diagram of electron beam apparatus (LV: low voltage source; HV: high voltage source)

3.3.4. Biodegradation of electron beam pretreated NB samples

After incubating the NPs enrichment in basic salts/TMS/YE medium containing 20 mg/L of 2-, 3- and 4-NP for 24 h, the cells were recovered by centrifugation (8832 g) from 1 ml of liquid culture and were suspended into 1 ml of saline. Then the saline cells suspension was inoculated into 10 ml of the degradation medium, which was composed of 2 ml of five times concentrated basic salts/TMS/YE medium and 8 ml electron beam treated NB solution. The medium was incubated on a rotary shaker at 180 rpm, 26°C for 48 h.

3.3.5. Isolation of pure cultures from a mixed culture enriched on a mixture of 2-, 3- and 4-NP from municipal activated sludge

A mixed culture enriched from the municipal wastewater treatment plant (Waterloo, Ont), with a mixture of 2-, 3- and 4-NP as the sole carbon and nitrogen sources, was reported previously (Zhao and Ward 1999). It was maintained in the basic salts/TMS media supplemented with 20 mg/l of 2-, 3- and 4-NP. To isolate pure cultures from this mixed culture, it was incubated in the basic salts/TMS media supplemented with either individual NP or a mixture of three NPs as the sole carbon, nitrogen and energy source(s). When NP had disappeared, the culture was transferred into fresh media of the same composition. This process was repeated for up to fifteen times to enrich bacteria growing on the corresponding NP substrate(s) of interest. Bacterial growth was observed during this enrichment process by the increase in turbidity of liquid media. The liquid enrichments were streaked onto fresh YPS agar plates and colonies were chosen after 72 h incubation at 26 °C. Isolates were purified by restreaking them onto fresh YPS agar plates twice. NP degradation capacity of isolates was evaluated by inoculating the isolates, freshly grown on YPS, into basic salts media supplemented with individual NP or a mixture of 2-, 3- and 4-NP. Removal of 2-NP and 4-NP was indicated by the disappearance of their yellow colour and was confirmed by HPLC. Removal of 3-NP and NB was monitored by HPLC analyses. Growth of pure culture on NP was indicated by an increase in biomass (OD_{600nm}) in the basic salts media supplemented with corresponding substrate(s). Nitrite was measured according to US EPA method 354.1 (EPA 1979). Nessler's regent (VWR scientific products, West Chester, PA) was used to qualitatively test for ammonia.

3.3.6. Degradation of NP and NB by pure cultures

To assess NP degradation capacity of pure cultures, freshly YPS-grown cells of isolates were inoculated into 2.5 ml of basic salts/TMS media supplemented with individual NP (20 mg/L), or a mixture of three NPs (each isomer is 20 mg/L), with or without 0.1% of yeast extract. The production of nitrite and ammonia was analyzed in the yeast extract-free degradation media.

To study biotransformation of NP and NB by resting cells, cells grown in the presence or absence of 3-NP were harvested in the following way. The inoculated 5ml of basic salts/TMS/YE media supplemented with 20 mg/L of 3-NP were incubated overnight. These cultures were transferred into 500-ml flasks containing 100 ml of media of the same composition. During a 26-h incubation, yeast extract and 3-NP were fed at the following times {time(h)/YE(%)/3-NP(mg/L)}: 7/0.1/20; 14/0/20; 22/0.1/20; 25/0/20. The culture was centrifuged (16,300 g, 15 min) and the cell pellet was washed with 50 ml of the phosphate buffer (25 mM, pH 7.3) once. The harvested cells were re-suspended in 6 ml of saline. Cells grown on yeast extract alone were also harvested by parallel incubations of bacteria in the 3-NP-free media. 0.3 ml of cell suspensions were added into 2.5 ml of phosphate buffer (25 mM, pH 7.3) in 20-ml of vials with the caps loosened to maintain aerobic conditions. Cell density (OD_{600nm}) in these biotransformation media ranged from 1.2 to 3.5. The concentration of individual NP or NB was 20 mg/L. The biotransformation reactions were conducted at 26 °C on a rotary shaker at 180 rpm for 2 h.

3.3.7. Bacterial identification

MIDI fatty acid method (MIS; Microbial ID Inc. [MIDI], Newark, Del.): The medium for fatty acids analyses was Trypticase soy broth agar. Fatty acid composition was analyzed by a standard gas chromatoraphic (GC) analysis. Isolate identification was based on the similarity between the fatty acids GC profile of the organism and those in the database. This method requires a minimum similarity index of 0.3 with a minimum of 0.1 between the first identification and any secondary identification.

Identification by GN MicroPlate of Biolog system (Biolog, Inc., Hayward, CA)(Bochner 1989): The isolate was grown on TSA agar plate (Trypticase® Soy Agar, Becton Dikinson and company, Cockeysville, MD) at 30°C for 46 h. Agar-free cells were suspended

in sterile saline by gently picking up cells from the agar using a wet sterile swab. 150 µl of cell suspension with transparency of 49% (required range for Biolog system: 48.5-53%), measured by specific Biolog spectrophotometer and glass tubes, was inoculated into every well of the 95-tests GN MicroPlate panel. The MicroPlate was incubated at 30°C for 24 h, and the pattern of the colour reaction on the panel was scanned for comparison with those of typical gram negative bacteria in the database. Identification was positive only when a similarity index of 0.5 as minimum for 24 h incubation was found between the carbon source profile of the unknown strain and those of typical strains in the library of the Biolog system.

Biochemical tests and motility of isolates were done according to conventional methods.

3.3.8. HPLC analysis

The HPLC analysis was performed on a 3.9 X 300mm µBondapak™ C18 column (Waters, Milford, MA). The apparatus consists of two Shimadzu LC-600 pumps, a Shimadzu SPD-6A UV spectrophotometric detector and a sample injector 7125 (all components are from Shimadzu Corporation, Kyoto). Samples (15 µl) were injected and eluted with methanol and milliQ water (0.1% acetic acid). For NP solutions, solvents were delivered at the rate of 0.22 (methanol) and 0.78 (milliQ water) ml/min. For NB samples, solvents were delivered at the rate of 0.55(methanol) and 0.45 (milliQ water) ml/min. Compounds were monitored at UV A_{254nm}. The linear equations obtained from standard samples were used for the quantitative analyses of every NP isomer and NB. Dilution was needed for samples with the concentration of more than 6 mg/L. All the analytical data were obtained in duplicates.

Samples from biodegradation tests were subjected to centrifugation at 8832 g for 3 minutes to remove the cells. To analyze NPs, the aqueous sample was acidified to pH 2-3 and then extracted with ethyl acetate. 0.9 ml of supernatant or electron beam treated solution was mixed with 0.1 ml of 0.4 N HCl solution in a 4-ml of amber glass vial with screw tightened and Teflon-lined caps. Ethyl acetate, 1 ml, was added and vortexed for 1 min. The ethyl acetate layer was injected into the HPLC column for analyses of 2-NP and NB. To analyze low concentration of 3- or 4-NP in the electron beam treated samples, the ethyl acetate extract was concentrated by drying under nitrogen and the residue was re-dissolved into smaller amount of methanol. When standard nitroaromatics (>0.5 mg/L) were added to the killed cells (boiled for

1 min), 95% of them were recovered from the aqueous phase under the same analytical conditions, therefore we concluded that the cells do not retain NB and NPs (Zhao and Ward 1999). HPLC detection limit: 2- and 3-NP, 0.25 mg/L; 4-NP, 0.5 mg/L; NB, 0.05 mg/L.

3.4. Results

3.4.1. Electron beam treatment of NB

The effect of electron beam treatment of an aqueous solution of NB was determined as a function of total electron beam dose. Initial NB concentrations were 15 and 30 mg/L. NB concentration decreased as electron beam doses increased (Figure 3.2). Percentage removal rates were the same at both NB concentrations. At dose rates of 40and 60 kGy removal rates were 60% and 78%, respectively.

As a result of the electron beam irradiation of NB, a yellow color was produced in the aqueous solution suggesting a conversion of NB to yellow nitrophenolic compounds. Production of 2-, 3-, and 4-NP was confirmed by HPLC analysis (Figure 3.2). At the point of maximum NP production, concentration of 2-NP was greater than the combined concentrations of 3- and 4-NP. Total NPs were also determined and up to 3.5mg/L of total NPs was accumulated in the 30 mg/L NB sample. Total NPs also diminished as a result of increasing electron beam dose rate.

3.4.2. Biodegradation of electron beam treated NB sample by a mixed culture

A mixed culture, capable of degrading NP and low concentrations of NB (Zhao and Ward 1999), was used to treat a NB solution after it was treated with different electron beam doses. After the electron beam pretreatment at 60 kGy dose and biological degradation, the concentration of NB was reduced from 15 mg/L of NB to below the analytical detection limit of 50 µg/L (Figure 3.3). In the uninoculated controls and controls with killed cells, no removal of NB and NP was observed. Killed cells did not retain NB or NP under the experimental conditions and did not affect their analysis.
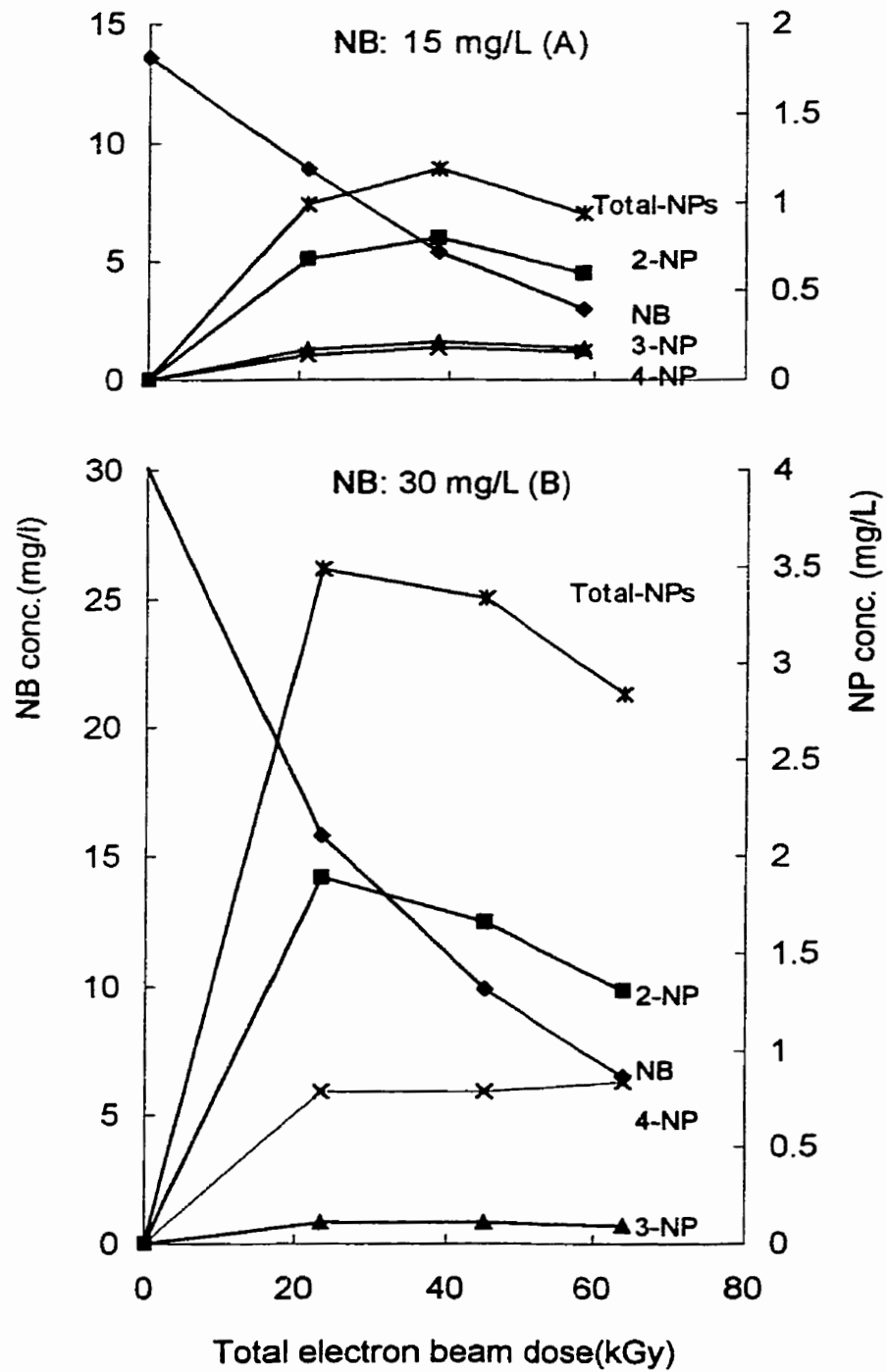
Figure 3.2. Removal of NB and formation of NP during electron
beam irradiation of aqueous solution
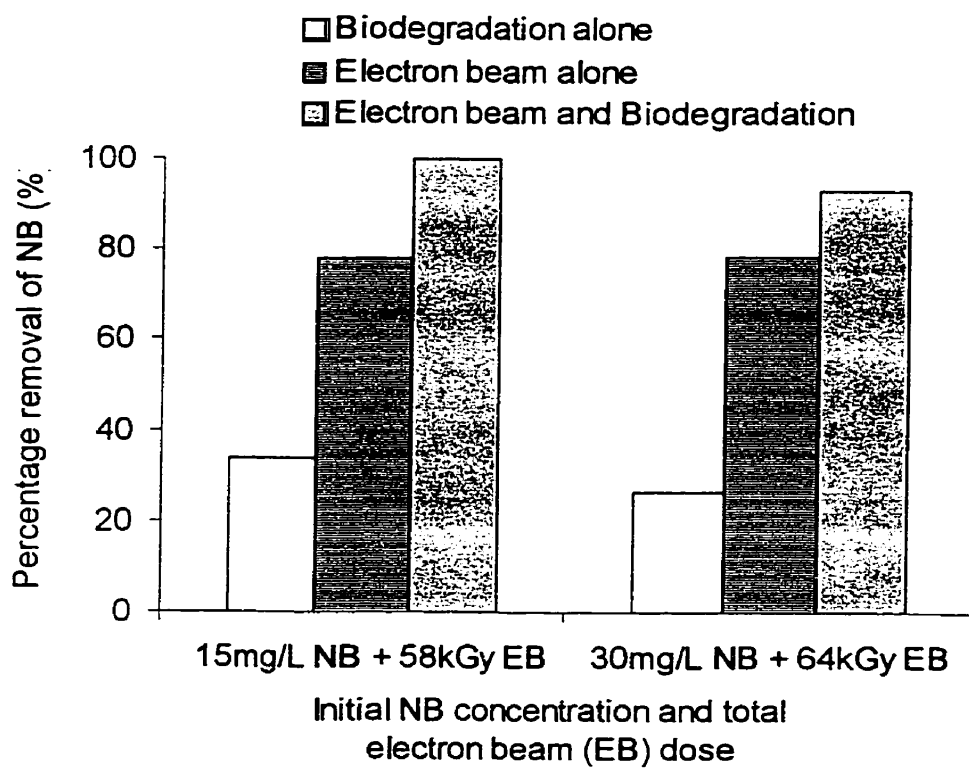
Figure 3.3. Enhancement of NB degradation by electron
beam pretreatment.

3.4.3. Isolation and characterization of NP-degrading pure cultures

Nitroaromatics-degrading pure cultures were isolated after the mixed culture was intensively sub-cultured on either individual NP or a mixture of three NPs as the sole carbon and nitrogen source(s). All strains were gram negative rods and positive in both catalase and oxidase enzyme activity. They all grew on YPS media with an optimal temperature of 30°C. These strains were classified into three groups according to their growth substrate selectivity on NP.

Group A was designated as $2NP^-3NP^-4NP^+$ as growth was observed on 4-NP, but not on 2- or 3-NP. The bacteria were slightly yellow colored and were non-motile. They oxidized but did not ferment glucose and fructose. Fatty acids profiles showed that the closest match for their identification was *Pseudomonas* sp. The similarity indexes of these 4-NP degraders with *P. putida* were high, ranging from 0.70 to 0.9. Nitrite was produced from 4-NP degradation.

Group B was designated as $2NP^+3NP^+4NP^-$ based on their growth substrate selectivity. They were motile and oxidized but did not ferment glucose and fructose. Fatty acids profiles analyses indicated that they were close to *Pseudomonas* sp. The similarity indexes of some of these strains with *P. putida* were very high (>0.9), while others manifested high similarity indexes with *P. fluorescens* (>0.84). Biolog substrate profiles analyses also showed they were close to *Pseudomonas* sp. Their Biolog similarity indexes with *P. putida* were more than 0.66. Therefore we concluded that these strains belonged to the genus Pseudomonas. Nitrite was produced from 2-NP and ammonia was produced from 3-NP.

Group C ($2NP^-3NP^+4NP^-$) grew on 3-NP, but not on 2- or 4-NP. The strains were non-motile, and did not oxidize or ferment glucose and fructose. One strain, bright yellow in color, exhibited a similarity index of more than 0.8 with *Variovorax paradoxus* using both the MIDI and Biolog methoods. Therefore we identified it as *Variovorax* sp. Another strain, C20, had white and mucoid colonies. It grew well at 37°C but little at 20°C. Its favored Biolog substrates were organic acids and amino acids but not carbohydrates except for a weak reaction on D-fructose. Its closest similarity indexes to strains in the Biolog and MIDI database were <0.3, suggesting there were no matching cultures in the database. Further data are needed for its identification. This strain degraded 3-NP with release of ammonia.

3.4.4. Biodegradation of NB by NP degrading pure cultures

Removal of NB by pure cultures of the three isolate groups was investigated. Resting cells of group A (*2NP⁻3NP⁻4NP⁺*) did not show significant degradation capacity toward NB. Group B (*2NP⁺3NP⁺4NP⁻*) and C (*2NP⁻3NP⁺4NP⁻*) showed NB degradation activity. Thus isolates which could grow on 3-NP degraded NB.

Transformation activities of NB and NP by resting cells of strains from group B and C (2NP8, C23 and C20) were tested individually in the cell suspension with cells grown in media containing YE in the presence or absence of 3-NP. The most important information observed in Table 3.1 is that each strain when grown on 3-NP had the ability to completely transform NB with release of ammonia. The results showed some differences in the degradation pattern of NPs. Cells grown in the absence of 3-NP did not degrade NB. In a detailed characterization of the metabolic process for NB transformation by 2NP8 (Zhao and Ward 2000), we have identified the transformation intermediates and confirmed that the stoichiometric release of ammonia occurred. These results suggested that the 3-NP-degrading strains present in the mixed culture had the capacity to degrade the residual NB in the electron beam treated samples.

3.5. Discussion

A novel NB degradation process combining electron beam irradiation with biological treatment has been described. Transformations involved in this two-step NB degradation process in summarized in Figure 3.4. To the best of our knowledge, use of an electron beam process to degrade nitroaromatic compounds has not been reported previously.

Chemical degradation of NB and production of NP has been observed when an aqueous solution of NB was treated with Co^{60} (Cechova et al. 1987; Cefova et al. 1986), TiO_2 (Dieckmann and Gray 1996; Maillard-Dupuy et al. 1994; Minero et al. 1994), or Fenton's reagent (Lipczynska-Kochany 1991, 1992a, 1992b). Degradation of 4-NP mediated by TiO_2 or Fenton's reagent was also described.

Table 3.1. Biotransformation substrate selectivity of resting cells of 3-NP degraders grown on yeast extract in the presence or absence of 3-NP.

Bacteria		3-NP in growth medium	Cell density (OD$_{600nm}$)	Biotransformation		
Group	Strains			Substrate tested	% substrate transformed	Nitrogen form released
B (2NP⁺ 3NP⁺ 4NP⁻)	*Pseudomonas* sp. 2NP8	Yes	1.3	NB	100	NH$_3$
				2-NP	100	NH$_3$, NO$_2^-$
				3-NP	100	NH$_3$
				4-NP	0	-
		No	1.25	NB	0	-
				2-NP	0	-
				3-NP	0	-
				4-NP	0	-
C (2NP⁻ 3NP⁺ 4NP⁻)	*Variovorax paradoxus* C23	Yes	2.8	NB	100	NH$_3$
				2-NP	100	NH$_3$
				3-NP	100	NH$_3$
				4-NP	100	NH$_3$
		No	3.4	NB	0	-
				2-NP	0	-
				3-NP	0	-
				4-NP	0	-
	C20	Yes	1.8	NB	100	NH$_3$
				2-NP	100	NH$_3$
				3-NP	100	NH$_3$
				4-NP	100	NH$_3$
		No	2.8	NB	0	-
				2-NP	0	-
				3-NP	100	NH$_3$
				4-NP	0	-

Our results showed that the percentage of NB removal at 15 or 30 mg/L was the same when the solutions were treated with same dose of electron beam. Cooper et al (1993a,b) described that the electron beam dose for 90% removal of other contaminants, such as polyhalogenated methane and ethylenes, at various concentrations of pollutants was also the same. Electron beam was reported to be used for removal of micro pollutants at the ppb (µg/L) level in drinking or groundwater (Cooper et al. 1992 a,b). The decrease in removal efficiency per dose of electron beam irradiation was apparent as pollutant concentration decreased (Figure 3.2, this report; Cooper et al. 1992a,b).

High concentrations of NB are toxic to bacteria, such as for *Pseudomonas putida*, where the toxic threshold for cell multiplication was reported to be 7mg/L (Verschueren, 1983). We characterized the toxicity of NB to nitroaromatics-degrading bacteria in municipal activated sludge and observed only a 50% removal of NB by this nitroaromatic-degrading mixed culture when the NB concentration was more than 10 mg/L (Zhao and Ward 1999). NB concentrations in aniline plant wastewater were reported to be as high as 2010 mg/L (Patil and Shinde 1988). Pretreatment to reduce the concentration is necessary prior to a biological treatment. The process reported here first reduced NB concentration by conversion to NP and then used a mixed culture to degrade the NP products and NB, achieving complete removal of NB (15 mg/L). Without electron beam pretreatment, the NPs-enrichment only could degrade low concentration of NB (2 mg/L) (Zhao and Ward 1999). NPs are more biodegradable compounds and are more supportive of bacterial growth in municipal activated sludge than NB (Zhao and Ward 1999). Using NPs degraders to degrade NB avoids the difficulty of enriching NB degraders on NB.

Combinations of AOP and biodegradation have been reported to remove priority pollutants with enhancement of biodegradation (Aronstein et al. 1995; Lee and Carberry 1992; Martens and Frankenberger 1995). Co^{60} γ-rays (Taghipour and Evans 1996) or high-energy electron beam (Wang et al. 1994) irradiation improved the biodegradation of the recalcitrant chlorinated compounds in pulp and paper mill effluent.

Bacterial strains isolated from the mixed culture, identified as being similar to *Pseudomonas* sp and *Variovorax* sp, could degrade NB and NPs in the electron beam treated samples. *Pseudomonas* species have been reported as degraders of nitroaromatic compounds

(Marvin-Sikkema and de Bont 1994; Spain 1995). An oxygenase-initiated pathway with the release of nitrite was described for degradation of 4- and 2-NP (Spain 1995; Zeyer and Knearney 1984). Detection of nitrite from 2- or 4-NP degradation by pure cultures isolated in this report suggests a similar oxygenase-initiated degradation pathway. For those bacteria reported to grow on 3-NP as the sole carbon and nitrogen sources, the nitroreductase was found to initiate degradation of 3-NP with production of ammonia (Meulenberg et al. 1996; Schenzle et al. 1997). Our observation of ammonia production from degradation of 3-NP by the three 3-NP degraders was consistent with these literature results. To the best of our knowledge, production of ammonia from 3-NP degradation by 3-NP-degrading bacteria, and nitroaromatics degradation activity in *Variovorax* sp have not been reported previously.

Only cells grown on 3-NP substrate degraded NB. The mechanism of NB transformation by 3-NP-degrading pure cultures is currently being investigated.
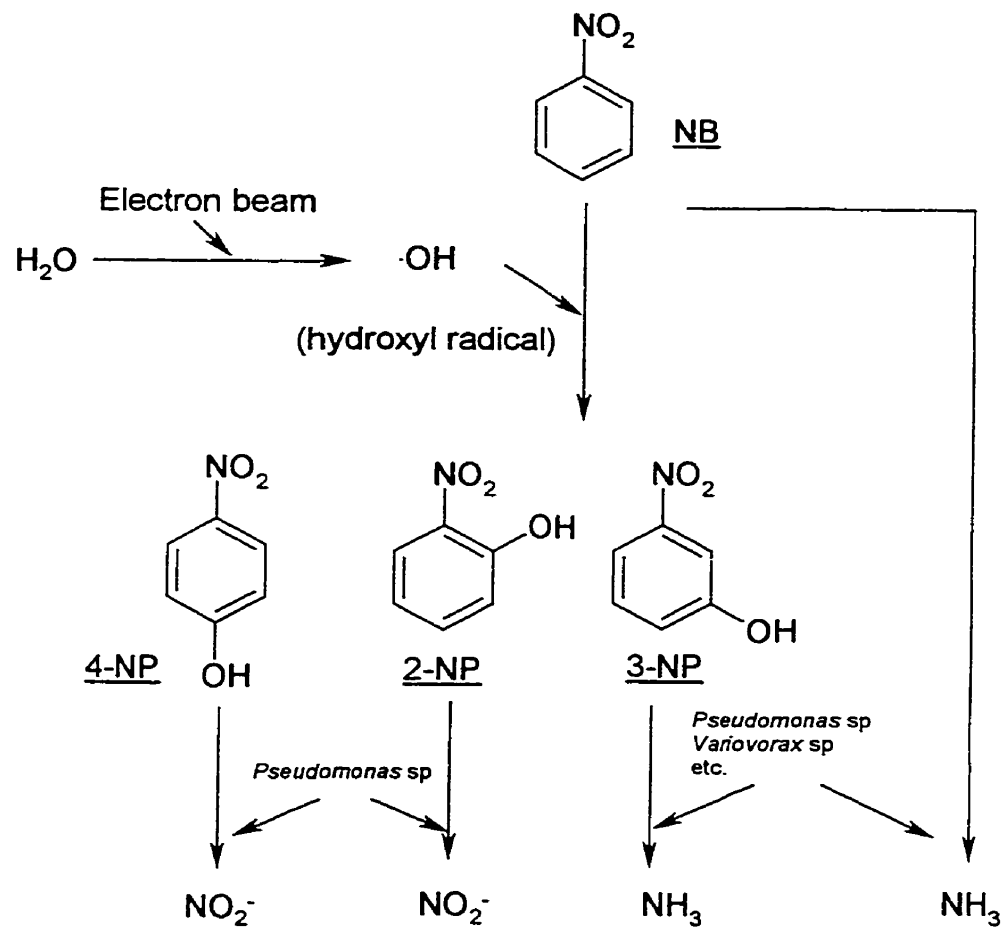
Figure 3.4. A two-step NB degradation process: combining electron beam irradiation with biological treatment.

4. COMETABOLIC BIOTRANSFORMATION OF NITROBENZENE BY 3-NITROPHENOL DEGRADING *PSEUDOMONAS PUTIDA* 2NP8[a]

4.1. Abstract

A strain of *Pseudomonas putida* (2NP8) capable of growing on both 2-nitrophenol and 3-nitrophenol, but not on nitrobenzene, was isolated from municipal activated sludge. 2-Nitrophenol was degraded by this strain with production of nitrite. Degradation of 3-nitrophenol resulted in the formation of ammonia. Cells grown on 2-nitrophenol did not degrade nitrobenzene. A specific nitrobenzene degradation activity was induced by 3-nitrophenol. Ammonia, nitrosobenzene and hydroxylaminobenzene have been detected as metabolites of nitrobenzene degradation by the cells grown in the presence of 3-nitrophenol. These results indicated a NB cometabolism mediated by 3-nitrophenol nitroreductase.

4.2. Introduction

Nitroaromatics have widespread applications as solvents, manufacturing raw materials for dyes, pharmaceuticals and explosives. Among the nitroaromatics, nitrobenzene (NB) is one of the fastest growing end use products of benzene, world demand for which is growing at an annual rate of 3.1% and will reach 30.6 million metric tons in 2000 (Richard 1996). It is acutely toxic (Hartter 1984; Richard 1996) and is a priority pollutant (Keith and Telliard 1979). Due to the strong electron-withdrawing property of the nitro group, it is resistant to aerobic biodegradation (Mackey et al. 1995).

Biodegradation of nitroaromatics has received much attention recently (Gorontzy et al. 1994; Higson 1992; Marvin-Sikkema and de Bont 1994; Spain 1995). Attack on the nitro group attached to the benzene ring is usually the key step in its metabolism. Under both aerobic (Schackmann and Muller 1991) and anaerobic conditions (Gorontzy et al. 1994), the electrophilic nitro group of nitroaromatics can be subjected to fortuitous reduction by unspecific reductase with amines as dead end and toxic products. In spite of the resistance of nitroaromatic compounds to aerobic degradation, aerobic bacteria growing on them have been isolated from their contaminated sites. Two main strategies were found in these bacteria to

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initiate degradation of mono or di-nitrated aromatics. Oxygenase-initiated release of nitrite occurred on degradation of 2-nitrophenol (NP) (Zeyer and Kearney 1984), 4-NP (Hanne et al. 1993; Spain and Gibson 1991), dinitrophenols (Bruhn 1987; Ecker et al. 1992), NB(Nishino and Spain 1995), 2-nitrotoluene (Haigler et al. 1994), and 1,3-dinitrobenzene (Dickel and Knackmuss 1991). A nitroreductase initiated nitro group reduction leading to formation of ammonia was commonly found for degradation of NB (Nishino and Spain 1993; Park et al. 1999) and 4-nitrobenzoate (Groenewegen et al. 1992) and 3-NP (Meulenberg et al. 1996; Schenzle et al. 1997).

For multinitrated aromatic compounds, such as di- or tri-nitrophenols and toluene, a direct reduction of the nitrated benzene ring by hydrogen ions with the release of nitrite was reported under aerobic conditions (Duque et al. 1993; Lenke and Knackmuss 1992a; Lenke et al. 1992b; Vorbeck et al. 1994). Anaerobic degradation of trinitrotoluene (TNT) with production of ammonia as a nitrogen source for growth of Desulfovibrio sp. was reported (Boopathy et al. 1993).

While NB-degrading pure cultures have been isolated from nitroaromatics contaminated sites, NB tends to be persistent in the environment. Estimated half-life of NB in the environments such as surface water, ground water and soil ranged from two days to as high as more than 625 days (Mackey et al. 1995). Important factors key to biodegradation of nitroaromatics include induction of a nitro group removing enzyme and growth of the bacterium producing this enzyme. Inertness and the electron deficiency of the nitrated benzene ring would not favor aerobic degradation and bacterial growth, but substituents such as hydroxyl groups on the benzene ring would improve aerobic biodegradability according to the well-documented structural and biodegradability relationship (Klopman et al. 1992; Boethling et al. 1994). It is also known that some enzymes have relaxed substrate selectivity (Marvin-Sikkeman and de Bont 1994; Delgado et al. 1992), and this led us to postulate that nitro-group attacking enzymes of NP-degrading bacteria might effect NB degradation. Therefore we set out to explore degradation of NB by a NP degrading pure culture.

In this paper we report the isolation of a strain of Pseudomonas putida (2NP8), capable of growing on 2-NP and 3-NP, from municipal activated sludge. NB did not support growth of this strain either as a carbon or nitrogen source, however cells grown in the presence of 3-NP effected co-metabolic degradation of NB with release of ammonia. A

nitroreductase-initiated route for NB degradation in the 3-NP grown cells was demonstrated by identification of nitrosobenzene (NOB) and hydroxylaminobenzene (HAB) as metabolites from NB.

4.3. Materials and methods

4.3.1. Chemicals

2-NP, 3-NP, 4-NP, nitrosobenzene (NOB) were purchased from Sigma (St Louis, Mo). NB was obtained from British Drug House (Toronto, Ont) (99%). Methanol was obtained from EM Science (Gibbstown, NJ) (HPLC grade, 99.8%). Hydroxylaminobenzene (HAB) was prepared according to literature method (Furniss et al. 1989) and its structure was confirmed by melting point and UV spectrum.

4.3.2. Media

Stock solutions of NPs: 2-NP, 3-NP and 4-NP were dissolved in methanol to give a concentration of each isomer of 10mg/ml. NB stock solution (1L) contained 1ml of NB dissolved in methanol to give a concentration of 60mg/ml. Basic salts liquid medium contained (g/l): KH_2PO_4, 1; Na_2HPO_4 $12H_2O$, 7; ferric citrate, 0.04; $CaCl_2$ $2H_2O$, 0.1; $MgSO_4$ $7H_2O$, 0.3; 3ml trace metal solution; pH 7.35. Trace metals solution (mg/l): $FeCl_3$ $6H_2O$, 162; $ZnCl_2$ $4H_2O$, 14.4; $CoCl_2 2H_2O$, 12; $Na_2MoO_4 2H_2O$, 12; $CaCl_2 2H_2O$, 6; $CuSO_4 5H_2O$, 1900; H_3BO_4, 50; HCl, 0.44 mole. Unless otherwise noted, the basic salts medium contained 20 mg/L of each of three NP isomers: 2-NP, 3-NP and 4-NP (NPs basic medium) or single NP isomer (NP basic salts medium). Yeast extract (YE), 0.1%, was added into the above NP(s) basic salts medium to form NP(s)/YE basic salts medium. YPS medium contained (g/l): YE, 10; Bacto peptone, 10; NaCl, 5. Nitroaromatics, Sterile TMS and YE were added into autoclaved liquid media before incubation or before pouring plates. Agar media contained 2% agar. Media were autoclaved at 120°C for 30min.

4.3.3. Bacterial isolation and growth

Enrichment of the nitrophenols (NPs)-degrading mixed culture from the activated sludge of the municipal wastewater treatment plant (Waterloo, Ont) was reported earlier (Zhao and Ward 1999). The mixed culture enriched on NPs was maintained in NPs basic salts medium. The fresh culture grown in NPs/YE basic salts medium overnight was inoculated into 50 ml of 2-NP or NPs or 3-NP basic salts medium in a 250 ml clear glass Erlenmeyer

flask. Bacterial incubation was conducted at room temperature, shaken on an orbital shaker at 200 rpm. The overnight culture was transferred into fresh NP(s) basic salts medium. After subculturing twelve times within two weeks, the cultures were streaked on YPS agar plates and the isolated colonies were re-streaked on YPS agar to assure purity. Pure cultures were maintained on YPS agar plates. Degradation of NP or NB by isolates was assessed by inoculating fresh cells into NP or NB basic salts medium. Growth of *Pseudomonas putida* 2NP8 on NP as sole carbon or nitrogen source was conducted by first growing in 2-NP/YE or 3-NP/YE basic salts media, and then cells were harvested and washed to be used as inocula. All tests of *P. putida* 2NP8 related to growth on NB or NP or biodegradation of NP or NB by growing cells were conducted in 50 ml of basic salts media in foam-plugged 250 ml clear glass Erlenmeyer flasks on an orbital shaker at 200rpm, 26 °C. Bacterial growth was monitored by detecting OD at 600nm in a 1 cm light path or by measuring cell counts.

Bacterial identification was performed by the standard MIDI fatty acid method (MIS; Microbial ID Inc. [MIDI], Newark, Del.). The medum for fatty acids analysis was Trypticase soy broth agar and fatty acid composition was analyzed by a standard gas chromatographic(GC) analysis. Isolate identification was based on the similarity between the fatty acids GC profile of the organism and those in the database. This method requires a minimum similarity index of 0.3 with a minimum of 0.1 between the first identification and any secondary identification.

4.3.4. Preparation of P. putida 2NP8 cells grown in the presence of 3-NP and degradation of NB and nitrosobenzene (NOB)

P. putida 2NP8, maintained on YPS agar, was inoculated into 5 ml of YPS liquid medium and grown for 24h. Unless otherwise noted, the strain was grown in the following steps in clear glass Erlenmeyer flasks at 26 °C, 200 rpm on an orbital shaker. This culture was transferred into 50 ml 3-NP/YE basic salts medium in a 250ml flask and grown overnight. All of the 50ml culture was transferred into 375ml of 3-NP/YE basic salts medium in a 2L flask. In the medium, 3-NP and YE concentration were 20 mg/L and 0.1%, respectively. After 5h of shaking, the same amounts of 3-NP and YE were fed to the medium to facilitate growth for an additional two hours. Then the same amount of 3-NP alone was fed to further induce 3-NP degrading enzymes for 1 hour. Final cell density was 1.6 (OD 600nm, 1cm light path). Cells were harvested by centrifuging at 16,300g for 15 min and washed with 100ml of sterile

phosphate buffer (KH_2PO_4, 1g/l; Na_2HPO_4 $12H_2O$, 7g/L; pH, 7.35). Freshly prepared cells were used immediately for biotransformation of NB, NOB or NP. A similar procedure was used to prepare 2-NP-grown cells for biotransformation of NB and NP (Table 1.). Unless otherwise mentioned, the bottles used for NB and/or NP biodegradation were 40ml amber glass with the Teflon/silicone septa lined caps. The 3-NP freshly grown cells from the above 375 ml medium were suspended in 12 ml phosphate buffer, and dispensed 1 ml of it into 9 ml phosphate buffer containing different concentrations of NB. Final cell density was 3.5 (OD 600nm, 1cm light path). The screw caps of bottles were always loosened to maintain aerobic conditions. The bottles were incubated on an orbital shaker at 200 rpm and 26°C. For measuring initial rates of NB removal, 0.5-1ml of sample was taken at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0h. NB and NP were determined by HPLC.

4.3.5. Analysis of nitrite and ammonia

Nitrite was measured according to US EPA method 354.1 (EPA 1979). Ammonia was quantitatively analyzed by L-glutamate dehydrogenase and NADPH (Sigma diagnostics ammonia reagent, Sigma, St. Louis, MO). Nessler's reagent (VWR scientific products, West Chester, PA) was used to qualitatively test ammonia.

NP, NB and metabolite analysis by HPLC: Samples from biodegradation tests were subjected to centrifugation at 9000 g for 3 min to remove the cells and 0.9 ml of supernatant was transferred into 4 ml of amber glass vial with screw tightened and Teflon-lined caps. Then 0.1 ml of 0.4 M HCl solution was added to samples (not necessary for analysis of NB). Ethyl acetate, 1 ml, was added and the mixture was vortexed for 1 min. The organic layer liquid was collected and used directly for HPLC analysis. The HPLC analysis was performed on a 4.6 X 250mm ZORBAX SB-C18 column (Hewlett Packard, purchased from Chromatographic Specialties Inc, Brockville, Ontario, Canada). The apparatus consisted of two Shimadzu LC-600 pumps, a Shimadzu SPD-6A UV spectrophotometric detector and a sample injector 7125 (all components are from Shimadzu Corporation, Kyoto). Sample (15 μl) was injected and eluted with methanol and milliQ water (0.1% trifluoroacetic acid). Solvents were delivered at the rate of 0.5 (methanol) and 0.5 (milliQ water) ml/min. Compounds were monitored by their UV absorbance at 254nm. More sensitive quantitative analysis of NOB was also analyzed at 306nm. Under these conditions, the compounds were eluted at the following retention times (min): NOB, 14.2; NB, 11.7; 2-NP, 11.2; 3-NP, 8.8; 4-

NP, 8.2; HAB, 3.9. The retention time may vary according to the column conditions. The linear equations were used for the quantitative analyses of every NP isomer, NB and NOB. All the analytical data were obtained in duplicates.

UV spectrum was recorded on a SPD-M10A Shimadzu diodearray detector (Kyoto, Japan). Both samples of NB degradation by the 3-NP-grown resting cells and the authentic NB, NOB and HAB samples were run under the above same HPLC conditions and the UV spectra were recorded. UV spectrum peaks of the standard compounds were (wavelength, nm): NOB: 283, 306; HAB: 236, 281.

4.3.6. Experimentation and analysis

All growth and degradation tests were conducted in duplicate flasks and samples were also analyzed in duplicate. Duplicate samples from the same flask exhibited variations of less than 5%, and results between duplicate flasks showed variation of less than 10%. Data are expressed as the averages of these determinations.

4.4. Results

4.4.1. Isolation and characterization of Pseudomonas putida 2NP8

NPs were used to enrich bacteria with NB degradation activity from a municipal activated sludge. The mixed culture degrading both NP and NB was obtained, and NP degrading pure cultures were isolated after intensely subculturing it on individual NP or a mixture of NPs. One strain (2NP8) was isolated by subculturing the mixed culture on 2-NP as the sole carbon and nitrogen source. Similar strains were also isolated by intensively subculturing this mixed culture on the mixture of NPs or 3-NP as the sole carbon and nitrogen source. Strain 2NP8 grew well at 20°C and 30°C on YPS medium, and had a slightly yellowish color. It was motile and contains both oxidase and catalase activity. The strain oxidised glucose and fructose, but did not ferment these substrates anaerobically. This strain was identified as *P. putida* biotype B (similarity index: 0.932) by MIDI fatty acid method. Therefore it was designated as *Pseudomonas putida* 2NP8.

This strain grew on both 2-NP and 3-NP as sole carbon and nitrogen sources as demonstrated in Figure 4.1. Nitrite was stoichiometrically released as 2-NP disappeared and biomass increased in the basic salt medium, likely suggesting an initial oxygenase-initiated degradation mechanism for 2-NP. Disappearance of 3-NP was accompanied by increase in biomass and accumulation of ammonia in the medium. The concentration of ammonia produced in the 3-NP medium was approximately half the initial 3-NP concentration. In the inoculated control medium without 3-NP only insignificant amounts of ammonia were detected. This suggested production of ammonia was the result of 3-NP degradation and indicated an initial reductive pathway for 3-NP degradation.
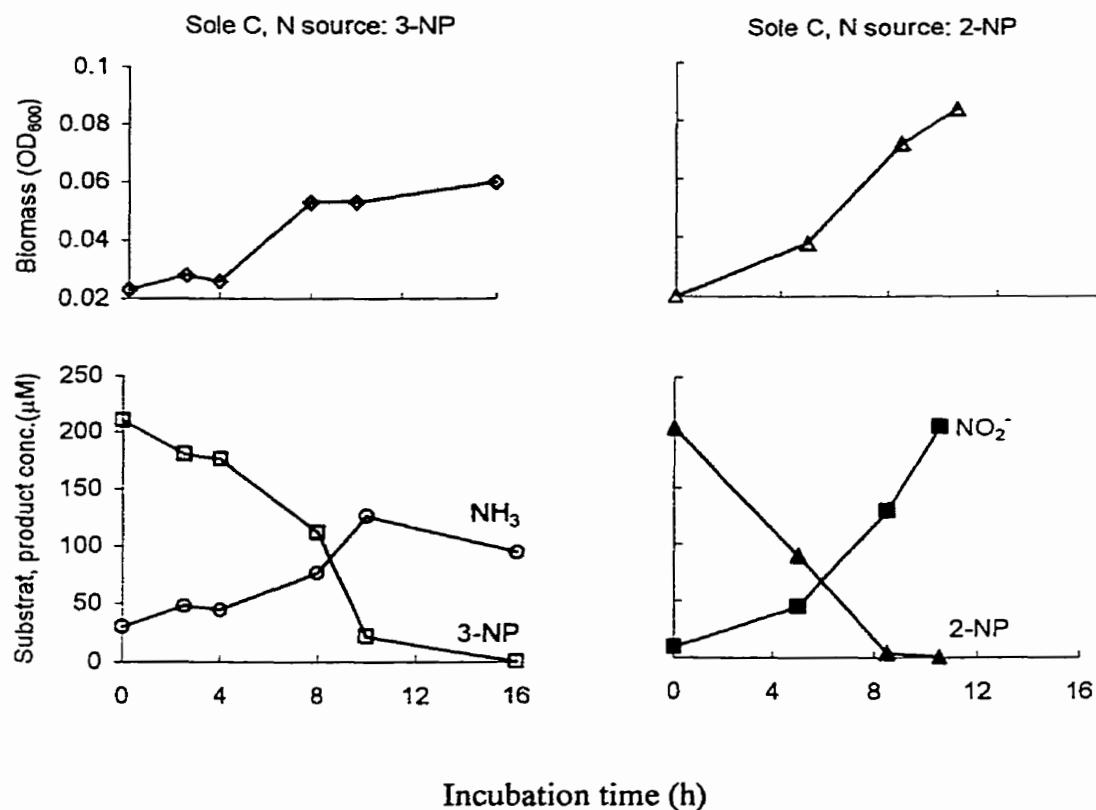
Figure 4.1. Growth of *P. putida* 2NP8 on 2-NP or 3-NP as the sole carbon, nitrogen and energy sources.

Washed cells pre-grown on 2-NP or 3-NP were inoculated into 50 ml basic salts medium supplemented with 2-NP or 3-NP and incubated on an orbital shaker (200rpm, 26 °C). Control was set as inoculated media without NP. In the control media, only insignificant amount of ammonia, no nitrite, and no biomass increase was detected.

This strain was also able to remove NB, but did not grow on NB. In the basic salts medium supplemented with 2-NP, 3-NP, 4-NP and NB, NB was degraded as 2-NP and 3-NP disappeared (Figure 4.2). 4-NP was not removed from the medium. Addition of YE greatly shortened the NP and NB degradation time from nearly 40h to less than 10 h (Figure 4.2). YE was presumed to stimulate bacterial growth, thus raising the rate of NP and NB removal. Growth and removal of NB in the absence of NP was also monitored in a basic salts medium supplemented with YE. Similarly, a rapid increase of OD_{600nm} from 0.065 to 0.67 within the initial 13 hours was observed during a 40-h incubation, but a lower NB removal (44%) was achieved compared to those in the media in thepresence of a mixture of NPs (84%, YE added; 77%, no YE). NB degradation leveled off as growth ceased.

4.4.2. NB degradation activity is induced by 3-NP

In order to determine the relationship between NB and NP degradation, cells grown in the presence of different substrates were tested for their degradation selectivity toward NP and NB. The results of NP and NB transformation by resting cells within 1.5h are presented in Table 4.1. Cells grown on 2-NP only exhibited the ability to remove 2-NP and 3-NP. Cells grown on 3-NP degraded NB, 2-NP and 3-NP. Cells grown on YE or glucose plus ammonium in the presence of NB, showed relatively little NB degradation activity within 1.5h. This indicated the presence of 3-NP induced NB degradation activity in *P. putida* 2NP8.

Other carbon or nitrogen sources were tested for their ability to induce NB degradation activity. Different carbon or nitrogen sources were added to the basic salts medium inoculated with uninduced YPS grown cells, and remaining NB concentration was monitored over 24 h. The results are presented in Figure 4.3. Compared to the medium containing NB alone, addition of glucose, citrate and succinate did not enhance NB degradation even though bacterial growth was observed. Addition of ammonium chloride caused a decrease in biomass and delayed NB degradation. Inclusion of 3-NP in the medium did not increase bacterial growth but significantly improved NB degradation, further suggesting an active NB degradation activity was induced by 3-NP. This 3-NP-induced enzyme activity was presumed to attack NB as a co-substrate. The observed lower level NB degradation (50%) in the growing cell medium without 3-NP, including the basic salts medium containing NB alone, is indicative of a constitutive NB degradation activity, which we think is related to the longer incubation period under growth conditions.

4.4.3. Effect of NB concentration on initial rate of NB degradation by the cells grown in the presence of 3-NP

The effect of NB concentration on initial degradation rate of NB by the whole cells grown in the presence of 3-NP was measured. The cells were suspended in the phosphate buffer (pH 7.35) and were incubated aerobically at 26 °C. Initial NB removal rates were measured. The results are presented in Figure 4.4. NB degradation rate decreased with an increase in NB concentration, approaching zero at 2000μM.

4.4.4. Production of metabolites of NB degradation by cells grown in the presence of 3-NP

Both oxygenase and nitroreductase-initialized NB degradation has been reported (Spain 1995). In order to determine how 3-NP induced cells of *P. putida* 2NP8 to degrade NB, degradation of NB at an optimal NB concentration (370μM, in phosphate buffer, pH7.35) by freshly harvested 3-NP grown cells was conducted with analyses for metabolites. Degradation of NB was indicated by the appearance of a yellowish-brown color in the degradation medium within an initial 3h period. The colour disappeared after 4 h. As shown in Figure 4.5, complete removal of NB was observed within 2h. Compared to NB removal in growth media as shown in Figure 4.2 and 4.3, quicker removal was achieved in this resting cell transformation medium due to higher density (3.5 OD) of cells fully induced by 3-NP. Qualitative production of ammonia was observed by using Nessler's reagent, and quantitative analysis of ammonia was performed by enzymatic analysis using glutamate dehydrogenase. Resting cells in phosphate buffer exhibited a slow release of ammonia in the absence of NB, and this endogenous ammonia release was subtracted from the values observed in the actual NB degradation sample. Stoichiometric release of ammonia was observed and this indicated complete degradation of NB. No nitrite was detected in the degradation medium. Ammonia production suggested that the cells grown in the presence of 3-NP initiated NB degradation by a nitroreductase mechanism. This is consistent with the observation that ammonia was also produced from 3-NP degradation by *P. putida* 2NP8 (Figure 4.1).
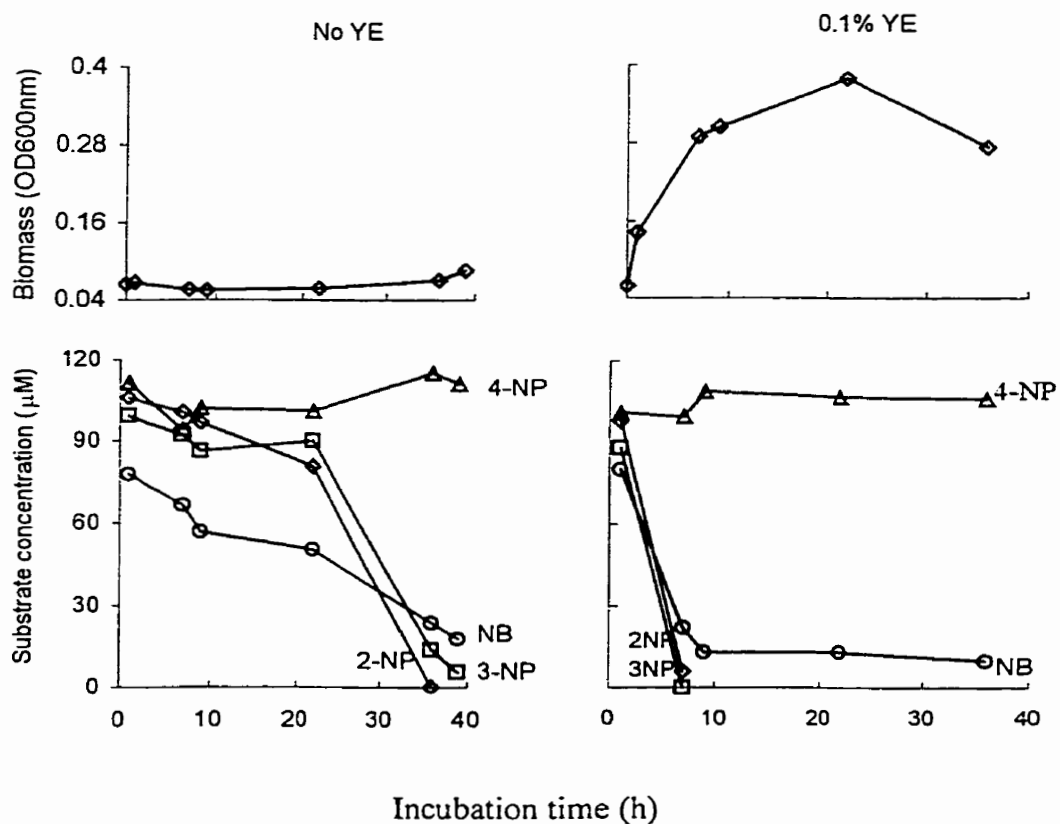
Figure 4.2. Degradation of a mixture of NP and NB by *P. putida* 2NP8.

Cells pre-grown on YPS agar were inoculated into 50ml basic salts medium containing a mixture of 2NP, 3-NP, 4-NP and NB and incubated on an orbital shaker (200rpm, 26 °C) with or without (0.1% yeast extract.

Table 4.1. Degradation substrate selectivity of *P. putida* 2NP8 resting cells.

Growth substrates	Degradation (%)^d			
	2-NP	3-NP	4-NP	NB
2-NP (YE)^a	100	4	0	0
3-NP(YE)^a	94	100	0	100
NB(YE)^b	12	22	19	0
Glucose and $(NH_4)_2SO_4$^{c}	28	22	19	0

$^{a, b, c, d}$: General growth or reaction conditions are given in material and methods. concentrations: nitroaromatics, 20 mg/L; YE, 0.1%; glucose, 0.1%; $(NH_4)_2SO_4$, 0.1%.

^{a} : after growth in NP/YE basic salts medium, NP was fed to further induce enzyme;

$^{b, c}$:growth time, 10h;

^{d}:cells density, 3.5 (OD600nm, 1cm light path); medium, phosphate buffer (27 mM, pH: 7.35); incubation, 200rpm (orbital shaker) at 26°C; incubation time, 1.5h.
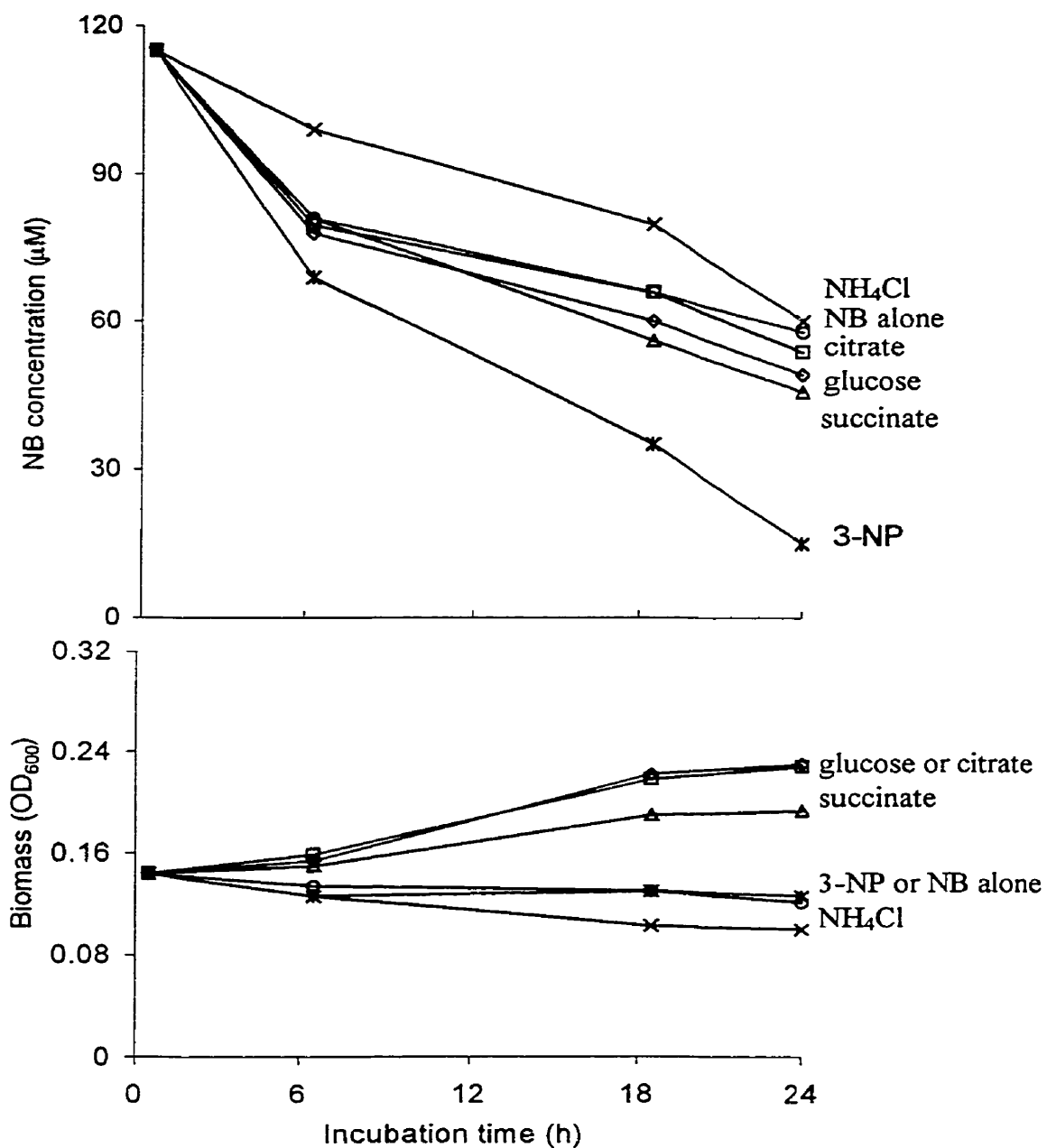
Figure 4.3. Effect of carbon or nitrogen sources on NB degradation by *P. putida* 2NP8.

Cells pre-grown on YPS agar were inoculated (0.15 OD_{600nm}) into 50ml NB basic salts medium which was supplemented with 0.1% other carbon nutrients (glucose, succinate, citrate), or nitrogen nutrients (0.01% NH_4Cl, 20 mg/L 3-NP), or with no supplement.
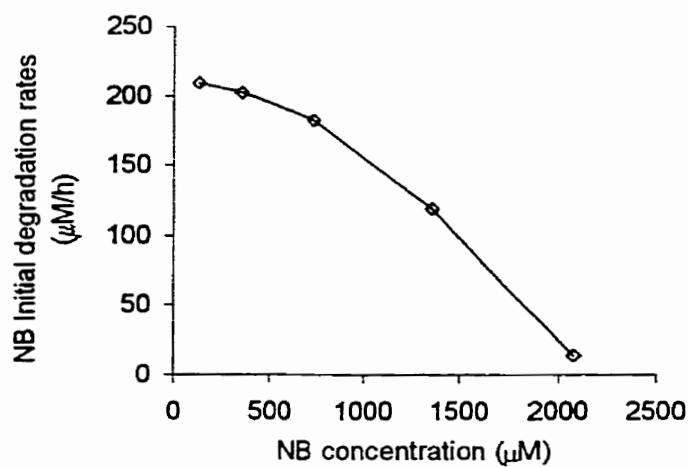
Figure 4.4. Effect of NB concentration on its initial degradation rate by resting cells of *P. putida* 2NP8 grown in the presence of 3-NP.

Degradation conditions: OD_{600nm}, 3.5; phosphate buffer, 27 mM, pH 7.35; temperature, 26 °C; shaken at 200rpm; incubation time, 2h. Initial rates were measured within 0.5-2h (NB concentration, measuring time: 134μM, 0.5h; 358-748μM, 1h; 1374-2114μM, 2h)
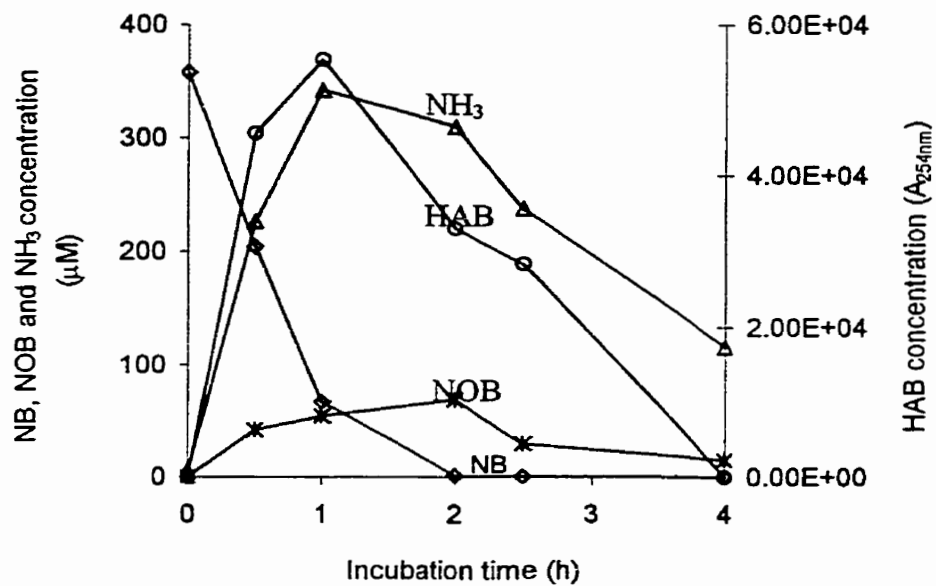
Figure 4.5. NB metabolism by *P. putida* 2NP8 resting cells grown in the presence of 3-NP.

Cells grown in the presence of 3-NP were washed and suspended in 10 ml phosphate buffer (pH 7.35, 27 mM, 3.5 OD_{600nm}) and incubated on an orbital shaker (200 rpm) at a temperature of 26°C. NB, NOB, HAB, and NH_3 were monitored with time.

Metabolites were further analyzed by reverse-phase HPLC. Under experimental conditions, NB has a retention time of 11.8. Two new peaks were found at 14.2min (compound I) and 3.9 min (compound II). Compound I had the same retention time as authentic nitrosobenzene (NOB). When NOB standard sample was added into the ethyl acetate extraction of NB biodegradation sample, no new peak appeared on HPLC analysis, but the peak of compound I was enhanced. When the peak was scanned for its UV spectrum, it has the same UV spectrum (two peaks at 283nm and 306nm) as the authentic NOB. The identification of NOB suggested involvement of 3-NP nitroreductase, which is plausible since 3-NP was degraded with production of ammonia.

Compound II accumulated as NB disappeared from the reaction mixture with the formation of NOB. This compound was proposed as hydroxylaminobenzene (HAB), an immediate downstream nitro reduction reaction product from NOB. In order to confirm this, HAB was prepared from NB and zinc powder according to the literature method (Furniss et al. 1989) and the structure of the HAB product was confirmed by its melting point and UV spectrum (two peaks at 236nm and 280nm). This chemically synthesized product had the same HPLC retention time and UV spectrum (two peaks at 236nm and 280nm) as the unknown compound II.

The detection of NOB and HAB and ammonia clearly demonstrated an initial reductive metabolism of NB. This supported the involvement of an 3-NP-induced 3-NP nitroreductase enzyme system.

4.4.5. Degradation of NOB by resting cells grown in the presence of 3-NP

While the nitroso compound was generally proposed to be the first intermediate of chemical or biochemical nitro group reduction, detection of NOB in the medium of NB biodegradation was seldom reported. In this research, we observed NOB production peak as NB removal proceeded (Figure 4.5). The decline of NOB concentration suggested it was reductively transformed. In order to test if NOB was a possible substrate of the 3-NP-induced enzyme system, it was added into the medium containing fresh or killed cells grown in the presence of 3-NP, and its concentration was monitored over time. NOB rapidly disappeared from the fresh cell medium within 1h (Figure 4.6). However, no NOB removal was observed in the killed cell medium over two hours. HAB formation (detected as a retention time of

3.9min on HPLC) was also observed as NOB degradation proceeded in the fresh cell medium. These results suggested that NOB and HAB were intermediates of NB degradation by the 3-NP induced enzymes.

4.4.6. Does NB support bacterial growth of P. putida 2NP8 ?

Quantitative production of ammonia from NB biotransformation (350μM NB, 2h, Figure 4.5) by cells (initial cell amount: 3.5 OD600nm) grown on 3-NP suggested that NB might be able to support bacterial growth as a carbon or nitrogen source.

Growth of P. putida 2NP8 on NB was investigated in the following media: a) NB as the sole carbon and nitrogen source; b) NB as a nitrogen source and citrate as a carbon source. Washed cells grown on 3-NP were inoculated (0.05 OD 600nm) into the NB basic salts medium with and without citrate. NB disappearance was observed (NB loss in the medium without inoculation was insignificant) and NB was fed in increments upon its removal. Similar results were observed for both situations (Figure 4.7). Little or no bacterial growth (OD 600nm) was observed as NB was removed. A low concentration (50μM) of ammonia production was detected even when significant removal of NB was achieved. The low ammonia-releasing activity may be because of either a low level of initial enzyme (0.05 OD of cell added) or a toxic effect of NB and its transformation intermediates. Addition of citrate as a carbon source did not increase growth, NB biotransformation and ammonia production. Under both situations, NB degradation ceased after NB was fed four times. This suggested the loss of existing NB-degrading enzyme activity. Failure of growth of this strain on citrate as a carbon source might be caused by toxicity of high concentration of NB to the relatively low density (0.05 OD600nm) of inoculated cells. This toxicity might be related to the toxic NB degradation metabolites such as NOB and HAB as identified in the above tests. Thus NB was not found to be a sole nitrogen and carbon source or a sole nitrogen source for growth of P. putida 2NP8 in this test.

Bacterial growth in the basic salts media supplemented with a carbon source such as glucose alone was observed in the presence or absence of NB when washed cells grown on YPS used as inoculum (Figure 4.3). This could be because the inoculated cells contained enough nitrogen for further growth. Therefore, growth in these carbon-supplemented NB media inoculated with YPS grown cells (Figure 4.3) did not prove that NB was a nitrogen source for growth. The reason for the observed NB disappearance in these carbon-supplemented NB media (Figure 4.3) suggested a constitutive NB degradation activity, which was likely related to an incubation over a long period, since little NB removal was observed in the resting cells within 1.5h (Table 4.1).

A strain of *P. putida* utilizing both 2-NP and 3-NP was previously reported to grow on NB as a carbon source (Meulenberg et al.1996). To determine if NB is a carbon source of *P. putida* 2NP8, cells grown on YPS were inoculated into basic salts medium supplemented with 0.1% ammonium sulfite and with concentrations of NB ranging from 0 to 2.7 mM. Bacterial growth over a period of 82 h was not enhanced by inclusion of NB at any concentration under these conditions. NB concentration change was not monitored in these tests, but NB removal was likely, especially at low NB concentration, due to constitutive NB degradation activity as suggested in Figure 4.3. Thus, it was concluded this strain does not grow on NB as a carbon source.

The above results confirmed that NB is neither a carbon nor a nitrogen source for bacterial growth, even though cells grown on 3-NP transformed NB into ammonia. Transformation of NB is a cometabolic activity of the 3-NP degrading system of this strain.
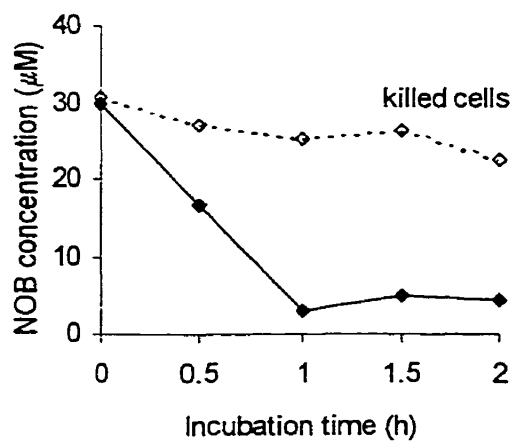
Figure 4.6. Degradation of nitrosobenzene by 3-NP-grown resting cells of *P. putida* 2NP8. Degradation conditions: 10ml phosphate buffer (pH 7.35, 27 mM) contained a cell density of 2.6 OD 600nm, incubated at 200 rpm, 26 °C. Cells were killed by boiling for 1 min.

Incubation time (h)
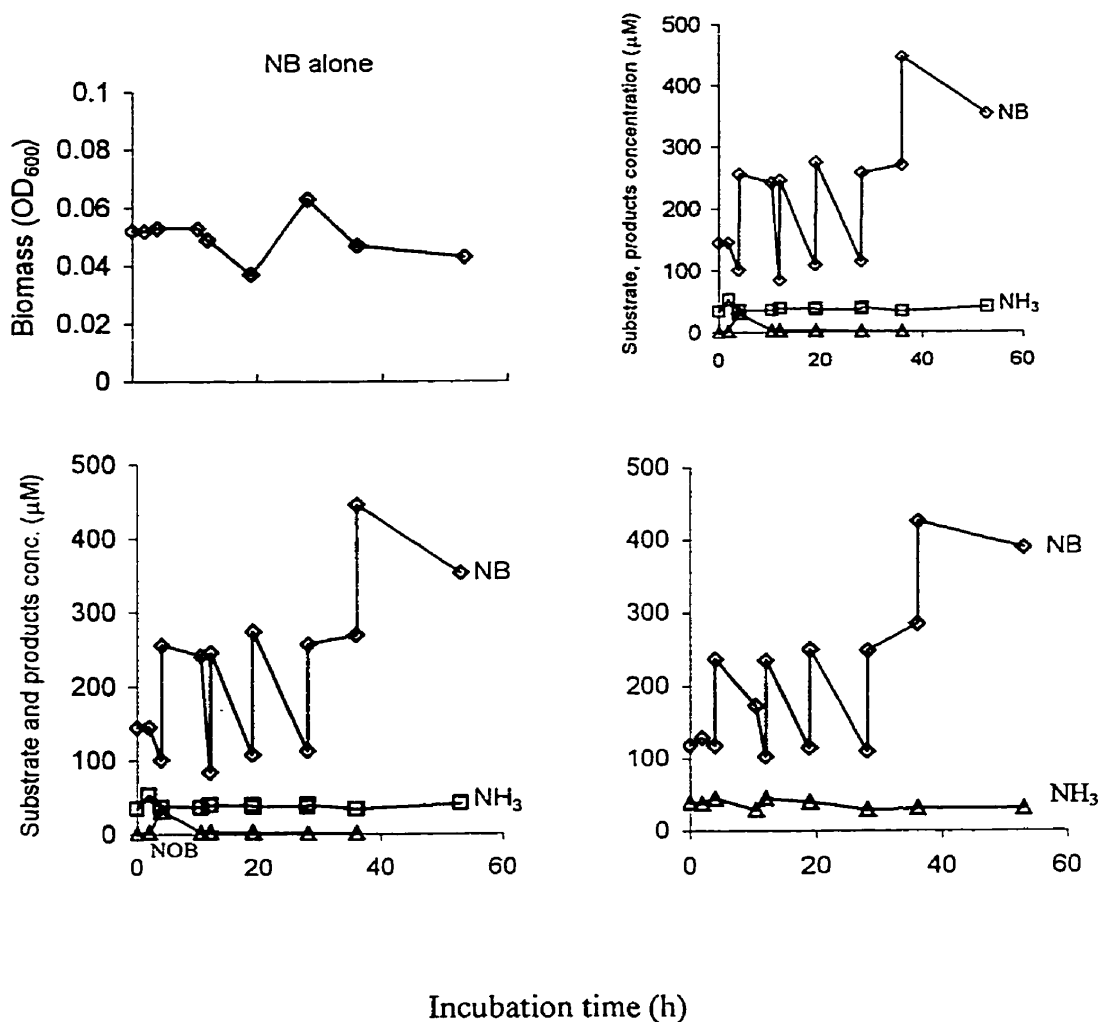
Figure 4.7. NB degradation and growth of *P. putida* 2NP8 (with 3-NP-grown cells as inoculum).

3-NP-grown cells were washed and inoculated into 50 ml of NB basic salts medium in the absence or presence of citrate (0.1%), and were incubated on an orbital shaker (200 rpm, 26 °C). NB was fed during the incubation and concentration of OD600nm, NB, NH_3 and NOB were measured.

4.5.   Discussion

P. putida 2NP8, utilizing both 2-NP and 3-NP as growth substrates, was isolated from municipal activated sludge, not known to have been polluted by nitroaromatic compounds. It metabolized NB with production of ammonia when grown on 3-NP. A strain of *Pseudomonas putida* growing on both 2-NP and 3-NP was previously reported (Zeyer and Kocher 1988; Zeyer and Kearney 1984; Meulenberg et al. 1996). Although biotransformation of NB by 3-NP-degrading enzymes was observed during investigation of 3-NP metabolism by a strain of *Ralstonia eutropha*, aminophenols were described as dead end products (Schenzle et al. 1997).

So far all bacteria growing on 3-NP have been reported to metabolize 3-NP through an initial reduction of the nitro group leading to production of ammonia. Liberation of a nitrite group from 3-NP was only observed in 3-nitrotoluene cometabolism by a PCB degrader, *Pseudomonas putida* OU83 (Ali-Sadat et al. 1995), in which 3-NP was an intermediate. Our results showed that the enzymes induced by 3-NP in *P. putida* 2NP8 converted NB into NOB, HAB and ammonia. This suggested that a 3-NP nitroreductase was the initial enzyme of NB metabolism leading to production of ammonia.

Bacteria growing on NB have been isolated from NB contaminated sites. Both the oxygenase-initiated route with the release of nitrite by a strain of *Comamonas* (Nishino and Spain 1995) and the nitroreductase-initiated route with release of ammonia by strains of *Pseudomonas* (Nishino and Spain 1993) were reported. NP degradation by a NB dioxygenase was mentioned by Nishino and Spain (Nishino and Spain 1995). None of above strains was reported to grow on 3-NP.

Nitroso and hydroxylamino aromatic compounds have been reported as degradation metabolites of a few nitroaromatic compounds. Formation of 4-chloronitrosobenzene and 4-chlorophenylhydroxylamine were observed as metabolites of 4-chloronitrobenzene by a yeast strain of *Rhodosporidium*, and amines were observed as dead end products (Corbett and Corbett 1981). 4-Nitrobenzoate is a carbon and energy growth substrate of a strain of *Comamonas acidovorans*, and 4-hydroxylaminobenzoate and ammonia was detected as metabolites from 4-Nitrobenzoate, but 4-nitrosobenzoate was not successfully identified (Groenewegen et al. 1992). Nitroso and hydroxylamino aromatic compounds were proposed as reductive intermediates of 3-NP (Meulenberg et al. 1996), and NB (Nishino and Spain

1993) and chloronitrobenzenes (Park et al. 1999), but direct detection of these compounds in biodegradation of 3-NP and NB was not reported.

Nishino and Spain (1993) reported that 2-aminophenol was an intermediate of NB reduction by a *P. pseudoalcaligenes*, and release of ammonia was achieved only after a dioxygenase-catalyzed ring-opening of 2-aminophenol. Both 2- and 4-aminophenol from NB were observed as dead end products by a 3-NP-degrading enzyme system in *Ralstonia eutropha* JMP 134(Schenzle et al. 1997). Release of ammonia from 3-NP by *R. eutropha* JMP 134 was shown to occur after conversion of 3-hydroxylaminophenol into aminohydroquinone and no ammonia formation was recorded during transformation of 3-hydroxylaminophenol under anaerobic condition. For 4-nitrobenzoate, however, ammonia was reported to be released from hydroxylaminobenzoate before ring-opening with formation of 3,4-dihydroxybenzoate under oxygen-limited or anaerobic condition and a hydroxylaminolyase activity (Groenewegen et al. 1992) was proposed. Similarly a hydroxylaminolyase activity was indicated during 3-NP metabolism by *Pseudomonas putida* B2 (Meulenberg et al. 1996), and 1,2,4-benzenetriol was observed as a 3-NP degradation intermediate under anaerobic condition. No hydroxylaminolyase was ever reported in metabolism of NB. For *P. putida* 2NP8, the mechanism of ammonia production from HAB and downstream degradation remains to be investigated.

Observation of ammonia production from NB degradation by 3-NP-induced enzyme activity clearly indicated a substantial degradative metabolism of NB. However, we did not observe any growth of strain 2-NP8 on NB as either carbon or nitrogen sources. Addition of citrate as a secondary carbon source did not improve bacterial growth in cultures inoculated with 3-NP-grown cells. Accumulation of NOB and HAB, during NB degradation by 3-NP-grown cells, suggested that these metabolites might be toxic to bacterial growth. For example, they have been reported to combine with protein and nucleic acid (Gorrod and Damani 1985), It is possible that this strain lacks the critical enzymes for downstream metabolism of HAB leading to formation of growth precursors.

Thus our results demonstrate that the NP-degrading system has the capacity to transform NB into ammonia. This cometabolic transformation is initialized by a 3-NP induced nitroreductase in the cells grown in the presence of 3-NP. The partial NB degradation pathway is shown in Figure 4.8.
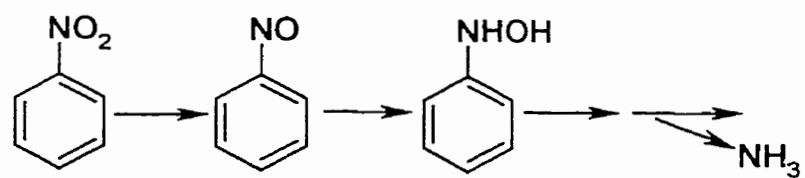
Figure 4.8. Proposed partial pathway of NB metabolism by 3-NP-grown cells of *P. putida* 2NP8.

4.6. Acknowledgements

5. BIOTRANSFORMATION OF HYDROXYLAMINOBENZENE AND AMINOPHENOL BY *PSEUDOMONAS PUTIDA* 2NP8 CELLS GROWN IN THE PRESENCE OF 3-NITROPHENOL[a]

5.1. Abstract

Biotransformation products of hydroxylaminobenzene and aminophenol produced by 3-nitrophenol-grown cells of *Pseudomonas putida* 2NP8, a strain grown on 2- and 3-nitrophenol, were characterized. Ammonia, 2-aminophenol, 4-aminophenol, 4-benzoquinone, *N*-acetyl-4-aminophenol, *N*-acetyl-2-aminophenol, 2-aminophenoxazine-3-one, 4-hydroquinone, and catechol were produced from hydroxylaminobenzene. Ammonia, *N*-acetyl-2-aminophenol, and 2-aminophenoxazine-3-one were produced from 2-aminophenol. All of these metabolites were also found in the nitrobenzene transformation medium, and this demonstrated that they were metabolites of nitrobenzene transformation via hydroxylaminobenzene. Production of 2-aminophenoxazine-3-one indicated that oxidation of 2-aminophenol via imine occurred. Rapid release of ammonia from 2-aminophenol transformation indicated that hydrolysis of the imine intermediate was the dominant reaction. The low level of 2-aminophenoxazine-3-one indicated that formation of this compound was probably due to a spontaneous reaction accompanying oxidation of 2-aminophenol via imine. 4-Hydroquinone and catechol were reduction products of 2- and 4-benzoquinones. Based on these transformation products, we propose a new ammonia release pathway via oxidation of aminophenol to benzoquinone monoimine and subsequent hydrolysis for transformation of nitroaromatic compounds by 3-nitrophenol-grown cells of *P. putida* 2NP8. We propose a parallel mechanism for 3-nitrophenol degradation in *P. putida* 2NP8, in which all the possible intermediates are postulated.

5.2. Introduction

Toxic nitroaromatic compounds tend to be reduced by biological systems in the environment due to electron deficiencies on the nitrogen atom or the benzene ring (Cerniglia et al. 1995; Marvin-Sikkema and de Pont 1995; Preuß and Rieger 1995; Spain 1995).

Arylhydroxylamine is one of the common intermediate products during nitro-group reduction. Hydroxylamines are both reductants and oxidants that attack biomolecules and have highly toxic, carcinogenic, and mutagenic effects on biological systems and human tissues (Corbett and Corbett 1995; Mansuy and Beaune 1978).

The previously described routes for metabolism of arylhydroxylamines, which are involved in nitroreductase-initiated degradation of nitroaromatic compounds, include (i) a two-electron reduction process that produces dead end amines, (ii) the Bamberger rearrangement-like reaction which leads to production of 2-aminophenol (2-AP) or 4-AP (Nishino and Spain 1993; Park wt al. 1999; Schenzle et al. 1997), and (iii) conversion into a 1,2-dihydroxyl aromatic product by hydroxylaminolyase (Groenewegen et al. 1992; Haigler and Spain 1993; Meulenberg and de Pont 1995; Rhys-Williams 1993).

Only ammonia release from nitroaromatic compounds avoids production potentially toxic amines in the environment. The following two ammonia release processes during nitroreductase-initiated aerobic degradation of nitroaromatic compounds have been described: (i) ammonia release via ring fission of AP and (ii) ammonia release before ring fission (conversion of arylhydroxylamine into 1,2-dihydroxyl aromatic compounds by proposed hydrolytic hydroxylaminolyases). Nishino and Spain (1993) observed the first process in the nitrobenzene (NB) degradation pathway of *Pseudomonas pseudoalcaligenes*. and Groenewegen et al. (1992) reported the second process in the 4-nitrobenzoate degradation pathway in *Comamonas acidovorans* NBA-10.

Two pathways have been described for degradation of 3-nitrophenol (3-NP), and both of them are initiated by nitroreductases. Meulenberg et al. (1996) reported that *Pseudomonas putida* B2 converts 3-NP to 1,2,4-benzentriol and ammonia and proposed that a hydroxylaminolyase activity is responsible for the process. Schenzle et al. (1997) observed a Bamberger rearrangement type conversion of 3-hydroxylaminophenol to aminohydroquinone during degradation of 3-NP in *Ralstonia eutropha* JMP 134 and did not investigate the ammonia release mechanism further. We isolated *P. putida* 2NP8 growing on 2-NP and 3-NP. 3-NP-grown cells of this strain aerobically released ammonia from both the growth substrate, 3-NP, and a cometabolizing substrate, NB. We observed hydroxylaminobenzene (HAB) production during NB transformation by 3-NP-grown cells of *P. putida* 2NP8. To shed light on the ammonia release mechanism in this strain, HAB transformation was investigated

because of the instability of the metabolites of the growth substrate, 3-NP. In study this, we characterized products obtained from HAB and AP transformation by 3-NP-grown cells of strain 2NP8 and obtained evidence that ammonia was released via oxidation of aminophenolic intermediates into imines and subsequent hydrolysis.

5.3. Materials and methods

5.3.1. Sources of chemicals

Benzoquinone, hydroquinone, and catechol were obtained from Sigma (St. Louis, Mo.); 2-AP, 4-AP, N-acetyl-2-AP, and N-acetyl-4-AP were obtained from Aldrich (Milwaukee, Wis.); and high-performance liquid chromatography (HPLC) grade methanol was obtained from EM Science (Gibbstown, N.J.). HAB was prepared by using a previously described method (Furniss et al. 1989). Other reagents were 99% pure.

5.3.2. Media

3-NP was dissolved in methanol to obtain a concentration of 10 mg/ml. We have previously described the basic salts medium used (Zhao and Ward 1999). 3-NP basic salts medium contained 20 mg of 3-NP per liter in the basic salts medium. The latter medium was supplemented with 0.1% yeast extract (YE) to obtain 3-NP/YE basic salts medium. YPS medium contained (per liter) 10 g YE, 10 g of Bacto peptone, and 5 g of NaCl. 3-NP, sterile trace metal solution, and YE were added to autoclaved liquid media. Agar media contained 2% agar. Media were autoclaved at 120°C for 30 min.

5.3.3. Preparation of cells grown in the presence of 3-NP and degradation of HAB and AP

P. putida 2NP8, a strain isolated by members of our group from municipal activated sludge (Waterloo, Ontario) (Zhao and Ward 1999), was maintained on YPS agar. Unless otherwise noted, the strain was grown in 250-ml clear glass Erlenmeyer flasks at 26°C and 200 rpm on an orbital shaker. A fresh YPS culture (5 ml) was inoculated into 50 ml of 3-NP/YE basic salts medium grown overnight, and then transferred into 375 ml of 3-NP/YE basic salts medium in a 2 liter flask. After 5 h 3-NP (20 mg/liter) and YE (0.1%) were added. After another 2 h 3-NP (20 mg/liter) was added, and the preparation was incubated for 1 h. The final optical density at 600 nm (OD_{600}) (1-cm light path) was 1.6. Cells were harvested by centrifugation at 16,300 X g for 15 min and were washed with 100 ml of sterile phosphate buffer (1 g of KH_2PO_4 per liter, 7 g of $Na_2HPO_4 \cdot 12H_2O$ per liter; pH 7.35). The cells were

used immediately for biotransformation of HAB and AP. The bottles used for HAB and/or AP biodegradation experiments were 40-ml amber glass bottles with the Teflon-silicone septum lined caps. Freshly grown cells that were suspended at an OD_{600} of 3.5 (1-cm light path) in phosphate buffer, containing different concentrations of NB were incubated on an orbital shaker at 200 rpm and 26°C. The caps of the bottles were loosened to maintain aerobic conditions.

5.3.4. Preparation of N-acetyl-2-AP and 2-aminophenoxazine-3-one (APX) from 2-AP

Five grams (wet weight) of *P. putida* 2NP8 cells grown on 3-NP was harvested from 1.2 liters of 3-NP/YE medium as described above. During incubation for 22 h, YE and 3-NP were added at the following times: 0.2% YE and 42 mg of 3-NP per liter at 7 h; 0.1% YE and 25 mg of 3-NP per liter at 17 h; 0.05% YE and 25 mg of 3-NP per liter at 20 h; and 25 mg of 3-NP per liter at 21 h. Washed cells were suspended in 1 liter of sodium phosphate buffer (25 mM, pH 7.3) supplemented with 150 mg of 2-AP in a 2 liter foam plugged clear glass flask and incubated at 26°C on an orbital shaker at 200 rpm for 24 h. A yellow colour appeared after 4 h and developed into a brown colour as incubation proceeded. A brown precipitate formed at a later stage, and 2-AP had completely disappeared from the medium after 24 h. The coloured compound was separated from both the supernatant and the cell pellet by centrifugation of biotransformation medium at 16,300 X g for 15 min.

The supernatant was extracted with 400 ml of ethyl acetate and was dried with anhydrous sodium sulfate. The extract was concentrated under a vacuum in a rotary evaporator at room temperature (26°C), and the residue was further evaporated to dryness under nitrogen gas. The solid was extracted with 3 ml of methanol and filtered. A brown powder (7 mg) was obtained. The filtrate was injected in batches (injection volume, 0.1 ml) into an SB-C18 HPLC column. The following elution program was used: 0 to15 min, 30% methanol; 15 to30min, 70% methanol. Two main products were collected at 5 to 10min (*N*-acetyl-2-AP) and at 24 to 25 min (APX). Fractions were pooled and concentrated at 26°C and dried under nitrogen gas. Three milligrams of solid was obtained from the 5 to10-min sample with a single HPLC peak at 8.6 min. Only 1 mg of brown powder was obtained from the 24- to 25-min sample.

An air-dried pellet was crushed into powder, and ethyl acetate was used to extract metabolites from the powder; this was followed by extraction with a mixed solvent

(methanol-ethyl acetate-chloroform, 2:2:1, vol/vol/vol), and 150 ml of extract was obtained. The extract was concentrated by rotary vacuum evaporation at 26°C. The total amount of the brown product obtained from both the supernatant and the cell pellet was 113.8 mg. This brown powder was separated by using a dry column (32.5 by 2.5 cm) that was packed with Silica Gel 60 (70-230 mesh; EM Reagents, E. Merck, Darmstadt, Germany) and dried overnight in an 80°C oven; acetone-chloroform-cyclohexane (5:17.5:17.5, vol/vol/vol) was used as the eluant, and 38.2 mg of solids was obtained. Purity was examined by performing silica gel thin-layer chromatography (TLC) with three mixed solvents (Table 5.1). We observed minor product that was light yellow in methanol, but it was not identified because of the small amount present.

5.3.5.   Analysis of metabolite

HAB and its metabolites were analyzed by using a ZORBAX SB-C18 HPLC column (4.6 by 250 mm; Chromatographic Specialties, Brockville, Ontario, Canada). We have previously described the HPLC instruments, general procedures, and methods used for NB and 3-NP analysis (Zhao and Ward 1999). For HAB and AP and their metabolites, biotransformation samples were centrifuged at 9000 X g for 3 min, and 15 µl protions of supernatant were injected and eluted with methanol and MilliQ water. Compounds were monitored at 254 nm.

UV-visible spectra of both metabolites and authentic samples were recorded with a model SPD-M10A diodearray detector (Shimadzu, Kyoto, Japan) by using the HPLC analytical conditions described above. An Si 250F plate (5 by 20 cm; J. T. Baker Chemical Co., Phillipsburg, N.J.) was used for TLC analysis. All spectra (mass spectra, infrared spectra; ^{1}H nuclear magnetic resonance spectra) of the metabolites were recorded by using standard instruments.

Ammonia was analyzed qualitatively by using Nessler's reagent (VWR Scientific Products, West Chester, Pa.) and quantitatively by using L-glutamate dehydrogenase and NADPH (Sigma diagnostic ammonia reagent; Sigma).

5.4. Results

5.4.1. 3-NP induced transformation of NB

3-NP-grown cells of *P. putida* 2NP8, which were used throughout this study, transformed 3-NP and NB, and ammonia was released at rates of 280 and 230 $\mu M \cdot h^{-1}$ (pH, 7.3; OD_{600}, 3.5), respectively. Uninduced cells grown on glucose and ammonium sulfate exhibited lower rates of activity with 3-NP (60 $\mu M \cdot h^{-1}$) and no activity with NB. No transformation activity with either NB or 3-NP was observed in cells grown on YE alone. These results demonstrated that the transformation activity with 3-NP and NB was induced by 3-NP. Our preliminary experiments established that 3-NP-grown cells metabolized NB to ammonia via HAB.

5.4.2. Transformation of HAB into AP

We used an approach similar to the approach described by Schenzle et al. (1997) to investigate aerobic transformation of HAB by resting cells of *P. putida* 2NP8 grown on 3-NP. The extracellular metabolites of HAB transformation were analyzed by HPLC. By comparing the UV spectra and HPLC retention times with the UV spectra and HPLC retention times of authentic compounds, we found that 2-AP and 4-AP were initial metabolites of HAB, and this findings was similar to the finding of Schenzle et al. (1997). We also observed 4-benzoquinone, 4-hydroquinone, and catechol in the HAB transformation medium (Table 5.2). We observed decomposition of HAB in phosphate buffer containing no cells or dead cells (pH 7.3). Mulvey and Waters (1977) reported that the disappearance of HAB could be due to disproportionation. We observed no peaks under our experimental HPLC conditions. We did not find the metabolites produced from cell-mediated transformation of HAB in the decomposing HAB phosphate buffer that did not contain live cells.

5.4.3. Biotransformation of AP

To investigate how ammonia is released, we characterized transformation products of AP formed by 3-NP grown cells. Rapid appearance of a yellow colour and accumulation of a dominant product with an HPLC retention time of 8.6 min indicated that transformation of 2-AP occurred. The initial rate of removal of 2-AP was 220 $\mu M \cdot h^{-1}$ (OD_{600}, 3.5; pH 7.3). In the control medium containing dead cells, little removal of 2-AP and no yellowish colour were observed within 6 h, even though prolonged (48-h) incubation did result in a light yellowish

colour. Using the HPLC retention time and UV spectrum of this compound, we identified it as a transformation product formed from HAB and NB (Table 5.2); this suggested that the compound is a common metabolite.

Transformation products formed from 2-AP were prepared by performing transformation experiments with a high concentration of 2-AP (150 mg/liter) and then extracting the aqueous phase with ethyl acetate and purifying it on a preparative silica gel and by HPLC. The compound that had a retention time of 8.6 min was a white powder. TLC and spectral data for it are presented in Table 5.1. On the basis of the spectra, we established that the compound was N-acetyl-2-AP. We purified the yellow substance from a 2-AP transformation preparation and obtained a brown powder by extraction with ethyl acetate from both the aqueous phase and the cell pellet and by dry silica gel chromatography. We obtained 45.2 mg of the brown powder (31% yield [mol/mol]) from a 24-h preparation obtained from 150 mg of 2-AP substrate. This material produced a single peak on TLC and HPLC gels and UV peaks at 235 and 438 nm (Tables 5.1 and 5.2). On the basis of its ESI spectra, mass spectra, and nuclear magnetic resonance spectra, we determined that this compound was APX (Table 5.1). The aqueous phases of HAB and NB transformation preparations were analyzed by HPLC to determine whether APX was present; and production of trace amounts of APX from both HAB and NB was clearly observed.

4-AP is unstable in aerobic solutions. Corbett (1969, 1970, 1978, 1979) reported that a mild oxidant, ferriccyanide was able to rapidly oxidize 4-AP in an aqueous medium, which formed 4-benzoquinone monoimine, and that this was followed by rapid hydrolysis, which formed 4-benzoquinone and ammonia. The presence of 4-benzoquinone in the HAB biotransformation medium containing 3-NP-grown cells in this study showed that oxidation of 4-AP leading to release of ammonia occurred.

Identification of N-acetyl-2-AP in the 2-AP biotransformation medium led us to consider the possibility that N-acetyl-4-AP might also be a metabolite of 4-AP. By comparing the UV spectrum and HPLC retention time with the UV spectra and HPLC retention times of authentic compounds, we identified N-acetyl-4-AP in the HAB degradation medium containing 3-NP grown cells (Table 5.2).

Table 5.1. Silica gel TLC and spectral data for N-acetyl-2-AP and APX

Compound	R_f (X100) with the following solvents:			^{1}H nuclear magnetic resonance spectrum (ppm)[a]	Mass spectra (m/z)			Infrared spectrum (cm^{-1})
	Acetone-chloroform-cyclohexane (10:35:35, vol/vol/vol)	Benzene-methanol (30:5, vol/vol)	Hexane-ethyl acetate (30:15, vol/vol)		ESI mass spectrum[c]	EI mass spectrum[d]	High-resolution EI mass spectrum	
N-Acetyl-2-AP	27	71	47	8.61(broad peak, s, 1H); 7.41(broad, s,1H); 7.1(t, 1H); 7.0 (d, 1H); 6.9 (d, 1H; 6.8 (t, 1H); 2.25 (s, 3H)	ND[b]	151(M⁺), 109, 80,	ND	3401, 3300-2300, 1657, 1594, 1537, 1451, 766
APX	33	42	58	7.7(d,1H); 7.4(m, 3H); 6.5(s, 1H), 6.3(s, 1H); 6.15(broad, s, 2H)	213 (M+H)⁺	212(M⁺), 186, 185, 144, 130,	212.059 (C$_{12}$H$_8$N$_2$ O$_2$)	3309, 1585

[a] The solvents used were CDCl$_3$ for N-acetyl-2-AP and d^6-acetone for APX.
[b] ND, not determined.
[c] ESI, Electronspray ionization.
[d] EI, electron impact.

Table 5.2. Identification of metabolites on the basis of HPLC retention times and UV spectra[a]

Metabolite	% of methanol in eluting solvent	Retention time (min)		UV λ_max	
		Unknown	Authentic chemical	Unknown	Authentic chemical
2-AP	30	5.9-6.8	5.9-6.8	228,283	227, 283; 229, 281(14,16)
4-AP	30	5.3	5.3	233, 303	233, 303 (47)
4-benzoquinone	30	5.5-5.6	5.5-5.6	257	251
N-acetyl-4-AP	30	4.4-4.5	4.4-4.5	245	247 (15)
N-acetyl-2-AP	30	7.9-8.6	7.9-8.6	207,240,280	207, 242, 283 (47)
Catechol	30	7.6	6.9-7.8	272	277 (47)
4-hydroquinone	30	3.7-3.9	3.7-3.9	224,291	224,294; 224,293(47)
APX	60	9.8-10.2	9.8-10.2	235, 438	435 (32)
HAB	30	7.3-7.4	7.3-7.4	236, 280	236, 280
Nitrosobenzene	60	7.7-7.9	7.7-7.9	283, 306	283, 306

[a]HPLC was performed with ZORBAX SB-C18 column (4.6 by 250 mm) by using a mixture of methanol and water as the eluting solvent (flow rate, 1 ml/min). All UV spectra were recorded at 254 nm in aqueous methanol. The UV spectra of authentic chemicals determined in methanol. All of the UV spectrum profiles of metabolites were the same as the profiles of authentic chemicals.

5.4.4. Time course for quantitative metabolite production from HAB

The time course for production of metabolites from 459 µM HAB is shown in Figure 5.1. Based on the initial HAB concentration of 459 µM, the yield (on a mole equivalent basis) of 4-AP and its derivatives (including *N*-acetyl-4-AP and 4-benzoquinone) was 13%, the yield of 2-AP and its derivatives (including *N*-acetyl-2-AP and APX) was 10%, and the yield of ammonia was 30%. A trace amount of nitrosobenzene, an oxidation product of HAB, was also detected in media with or without live cells. 4-Benzoquinone and *N*-acetyl-2-AP were major metabolites of HAB transformation formed by resting cells grown on 3-NP. Even though 4-AP is the first product of HAB transformation, production of 4-AP appeared to occur later than production of 4-benzoquinone and *N*-acetyl-4-AP. This might have been due to the instability of 4-AP in the aerobic transformation medium and to rapid conversion of 4-AP into its derivatives. Instability during aerobic analytical tests might also have contributed to the observed delay in 4-AP production.

HAB was unstable in buffer containing no cells or killed cells, and it had a half-life of 20 min. The rest of the initial amount of HAB in Figure 5.1 probably disappeared due to the disproportionation reaction described by Mulver and Waters (1977), and the products of this side reaction could not be detected under the analytical conditions used.

5.4.5. Quantitative transformation time course for 2-AP and NB

To quantitatively characterize biotransformation of AP and NB, time courses for transformation of 2-AP and NB were determined. During biotransformation of 2-AP, one-half of the substrate was converted into ammonia, and the rest was converted into *N*-acetyl-2-AP (Figure 5.2A). While a strong yellow colour was produced, quantitative analysis revealed that only 0.1% (mole equivalent) of 2-AP was converted into APX. The initial rates of ammonia and APX production were 73 and 0.10 µM $\cdot$ h^{-1}, respectively. Release of ammonia was 730 times faster than formation of APX.
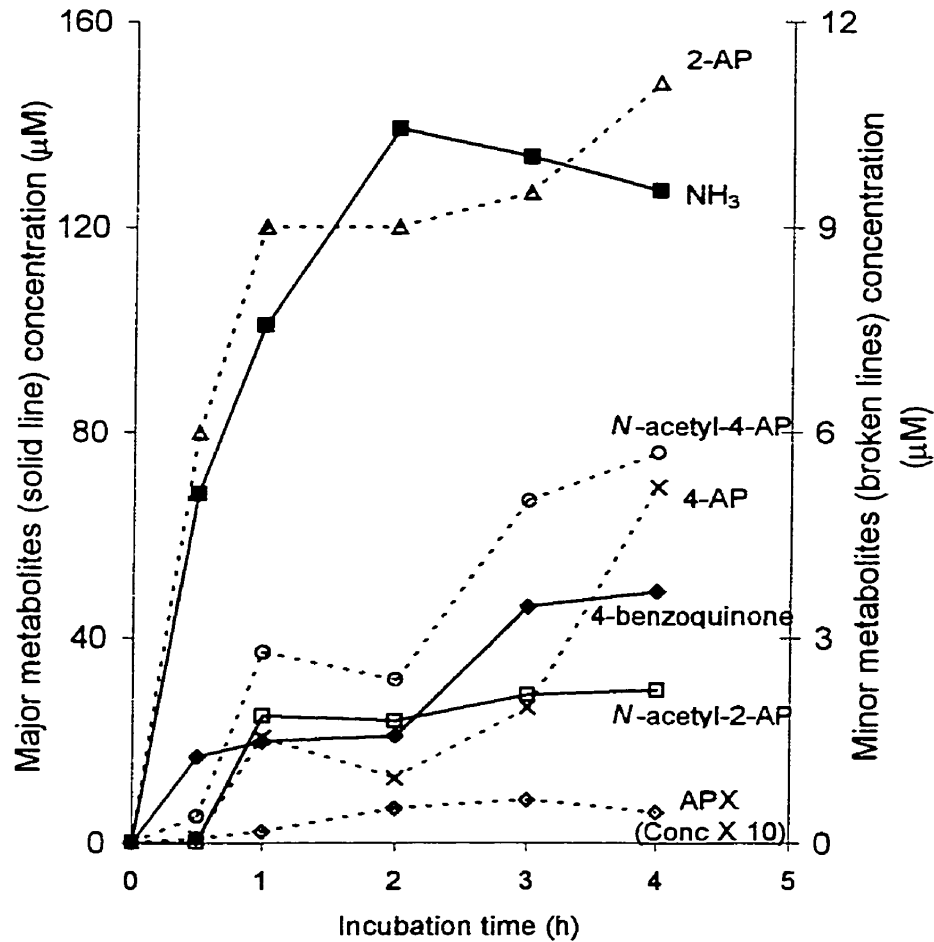
Figure 5.1. Quantitative analyses of metabolites produced from HAB by cells of *P. putida* 2NP8 grown on 3-NP. The reaction medium contained 50 mg HAB per liter, cells (OD_{600}, 3.5), and 20 ml of 50 mM phosphate buffer (pH 7.30). Biotransformation was performed in a 40 ml screw-cap amber vial on a rotary shaker at 150 rpm and 26°C. The cap was loosened to maintain aerobic conditions.
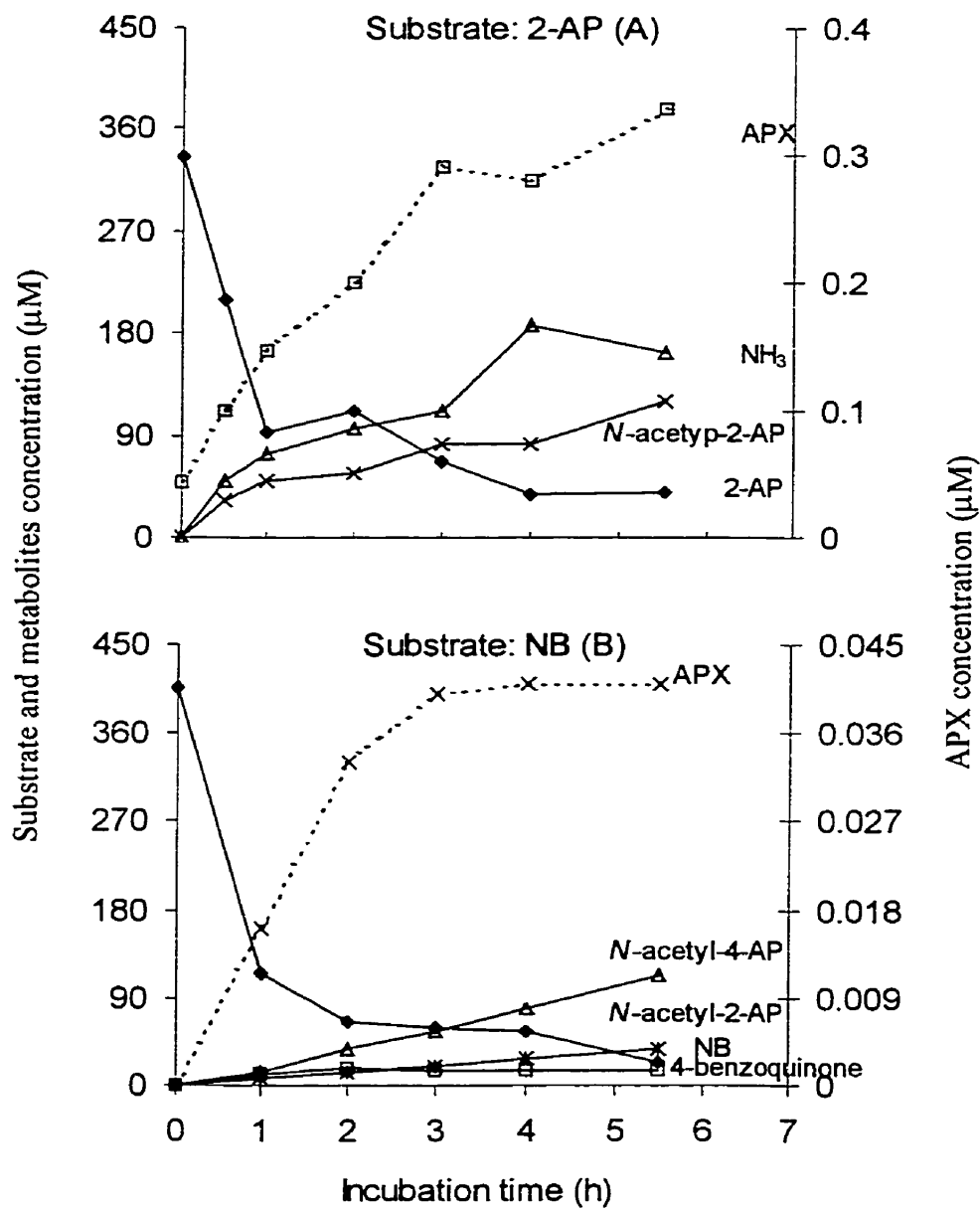
Figure 5.2. Quantitative analyses of metabolites obtained from transformation of 2-AP (A) and NB (B) by cells of *P. putida* 2NP8 grown on 3-NP. The biotransformation and HPLC conditions were the same as those described in the legend to Figure 5.1.

Approximately stoichiometric production of ammonia from NB transformation by 3-NP-grown cells was observed under optimal transformation conditions. To retard transformation and favor metabolite accumulation, a higher concentration of NB (406 μM) and a lower level of aeration were used in this test. Quantitative analyses revealed that the organic metabolites produced 28% N-acetyl-4-AP, 9.3% N-acetyl-2-AP, 3.7% 4-benzoquinone and 0.01% APX (Figure 5.2B). The rest of NB was transformed into ammonia. Only trace amounts of nonacetylated 4-AP and 2-AP were detected as transient products due to either instability or rapid conversion of these intermediates. Production of APX from NB was much less than production of APX (0.1%, mole equivalent) from 2-AP. Quantitative time courses for NB transformation confirmed that AP and the oxidation products 4-benzoquinone and APX were products of NB transformation by 3-NP-grown cells.

5. 5.   Discussion

Based on identification of the transformation products of HAB and AP, we propose a pathway leading to ammonia release from HAB by 3-NP-grown cells of *P. putida* 2NP8 (Figure 5.3). Our results revealed a new mechanism of ammonia release through oxidation of AP to imine, followed by hydrolysis, for transformation of nitroaromatic compound.

Corbett (1969, 1970, 1978, 1979) reported that 4-AP was oxidized to 4-benzoquinone monoimine, but the monoimine product could not be isolated because of it was rapidly hydrolyzed in the aqueous buffer into quinone. We observed both 4-benzoquinone and 4-AP in the HAB and NB transformation media, and this indicated that oxidation of 4-AP led to release of ammonia. Compared to 4-AP, 2-AP is relatively stable in aqueous solutions and oxidation of 2-AP requires a stronger oxidant. Chemical (Maruyama et al. 1996; Simandi et al. 1987; Szeverenyi et al. 1991) or enzymatic (Barry et al. 1988; Barry et al. 1989; Subba Rao et al. 1967; Yano et al. 1991) oxidation of 2-AP with production of APX has been described in many reports, and it has been proposed that 2-benzoquinone monoimine is the first oxidation product of 2-AP, which leads to production of APX. Nogami et al (Nogami et al. 1975) investigated reactions of 2-benzoquinone monoimine in aqueous media and observed the following two reactions involving the imine: (i) hydrolysis into ammonia and 2-benzoquinone and (ii) coupling with another molecule of 2-AP and formation of APX through two addition reactions and two oxidation reactions. These authors reported that the optimal pH for hydrolysis was 6 to 8. The pH of the 2-AP biotransformation medium in our tests (7.3) fell in this range. Because both imine and 2-AP are needed for formation of APX, only the presence of an excess amount of 2-AP favours APX formation which means that hydrolysis is a dominant reaction at low concentrations of 2-AP. This is consistent with our findings that less than 0.1% APX was produced in the 2-AP biotransformation medium and a much lower yield of APX was obtained in the NB and HAB media (Figure 5.1 and 2B) than in the 2-AP medium (Figure 5.2A). Therefore, we propose that 2-AP oxidation to imine and the subsequent hydrolysis are the dominant reactions during NB metabolism.
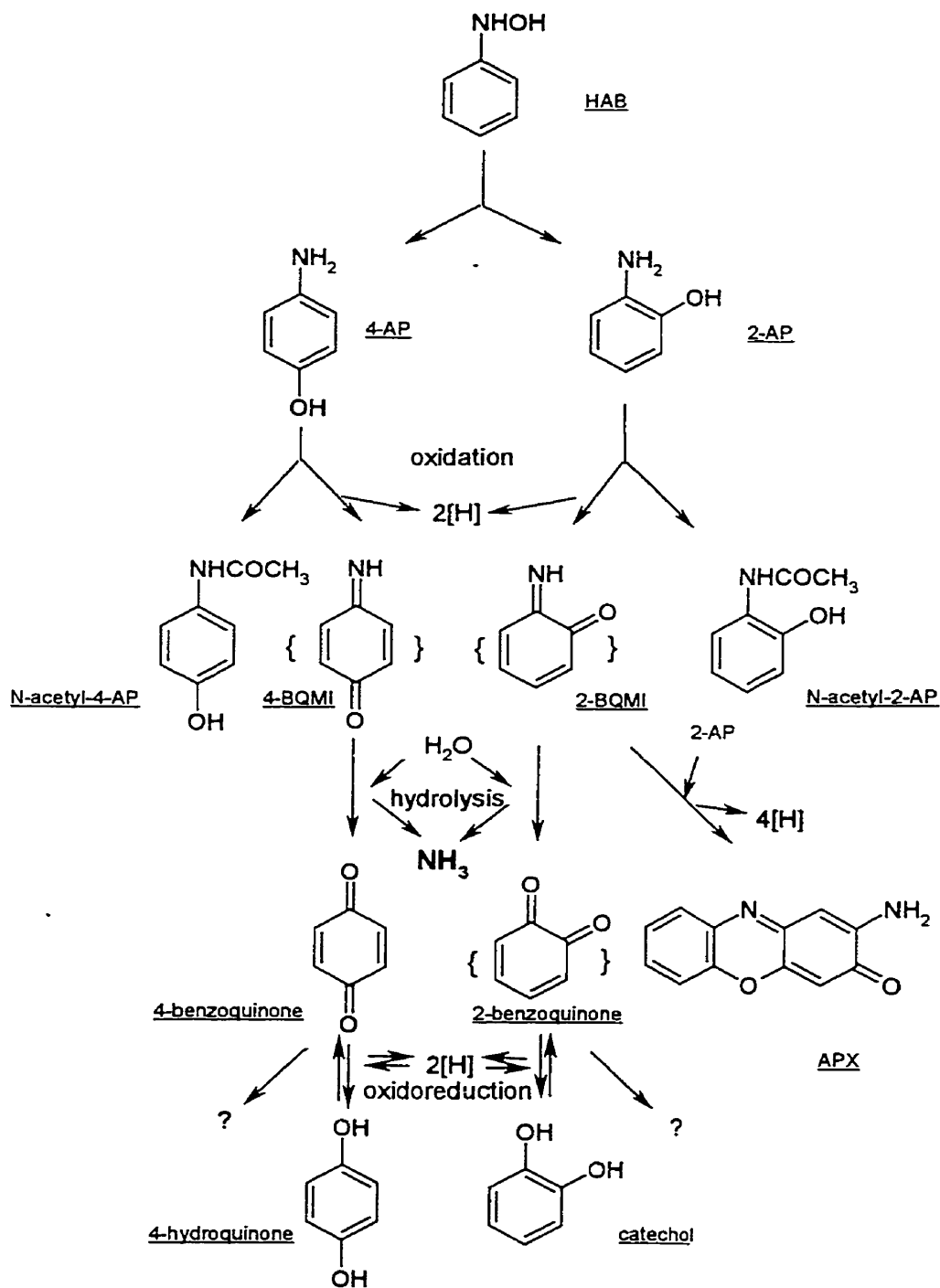
Figure 5.3. Proposed route of HAB biotransformation in *P. putida* 2NP8 cells grown on 3-NP. BQMI, benzoquinone monoimine. Brackets indicate unidentified compounds.

Corbett (1979) reported that 4-benzoquinone disappeared rapidly from an aqueous medium, and this explained the low yield of 4-benzoquinone. Many authors (Brown 1967; Musso 1967; Nogami et al. 1975; Ternay 1979) have found that 2-benzoquinone is very reactive and more unstable than 4-benzoquinone, especially at a low concentration. This explains our failure to identify 2-benzoquinone in the reaction media. The catechol and 4-hydroquinone detected in the transformation media are reduction products of benzoquinones.

The link between formation of APX and 2-AP oxidation via imine has been established in various studies. Chemical oxidation of 2-AP has been reported to produce either APX (Simandi et al. 1996; Szeverenyi et al. 1991; Yano et al. 1991) or the azo product 2,2'-dihydroxyazobenzene, as the sole product (3,13) or a mixture of APX and the azo product (Maruyama et al. 1996; Simandi et al. 1987). The mechanisms leading to formation of the azo product or APX have been reported to be different (Benedini et al. 1985; Simandi et al. 1996; Subba Rao et al. 1967; Yano et al. 1991). A specific phenoxazinone synthase that catalyzes formation of APX and APX analogs from aminophenols is present in microorganisms, plants, animals, and humans (Barry et al. 1989; Ogawa et al. 1983; Savage and Prinz 1977). Enzymes with phenoxazinone synthase activity have been identified by other researchers as oxidative enzymes; these enzymes include catalase (Barry et al. 1989; Ogawa et al. 1983), hemoglobin in human erythrocytes (Tomoda et al. 1984), tyrosinase (Toussaint and Lerch 1987), and a copper-containing oxidase (Barry et al. 1989). In all studies of production of APX from 2-AP, imine was considered the first intermediate during chemical or enzymatic oxidation of 2-AP. In this study, 3-NP-grown cells transformed 2-AP at a rate of 220 $\mu M \cdot h^{-1}$ and ammonia was released simultaneously at a rate of 73 $\mu M \cdot h^{-1}$. Based on the APX concentration in the extracellular aqueous phase, the initial rate of APX production (0.10 $\mu M \cdot h^{-1}$) was as much as 2,200 and 730 times slower than the disappearance of 2-AP and the release of ammonia, respectively. Killed cells did not transform 2-AP. This indicated that biological oxidation of 2-AP into imine occurred along with subsequent hydrolysis as the dominant reactions. The reaction in which APX is formed is probably a spontaneous reaction that accompanies oxidation of 2-AP, and we could not conclude that a specific phenoxazinone synthase is involved. *P. putida* 2NP8 is an oxidase- and catalase-positive strain, and oxidase and catalase may play a role in oxidation of 2-AP and formation of APX. This ammonia

release mechanism is different from the mechanisms observed for 2-AP metabolism in *Pseudomonas* sp. strain AP-3, as described by Takenaka et al (Takenaka et al. 1998) and in *P. pseudoalcaligenes* JS45, as described by Nishino and Spain (Nishino and Spain 1993); in these organisms ammonia is released after dioxygenase cleavage of the aromatic ring.

Our results also provided information concerning the mechanism of ammonia release from 3-NP, a growth substrate and inducer of NB transformation activity in strain 2NP8. Cells induced by 3-NP transformed 3-NP, NB, and 2-AP at similar initial rates (280, 230, and 220 μM · h^{-1} respectively). Uninduced cells grown on glucose-ammonium sulfate exhibited activity toward 3-NP of 60 μM · h^{-1}. Uninduced cells grown on YE alone exhibited no activity toward 3-NP. Neither of these types of cells transformed NB. These observations indicated that the enzyme(s) that transformed NB was induced by 3-NP. Our conclusion was also supported by the results of Schenzle et al. (1997), who reported that 3-NP-induced cells of *R.. eutropha* JMP 134 converted both 3-hydroxylaminophenol and HAB via a Bamberger rearrangement. We propose a parallel 3-NP degradation pathway in which all of the possible intermediates are postulated based on HAB transformation (Figure 5.4). 3-Hydroxylaminophenol, the reduction product produced by 3-NP nitroreductase, would be converted to two possible products, aminohydroquinone and 4-aminocatechol, via ortho and para Bamberger rearrangements, respectively. Both aminohydroquinone and 4-aminocatechol should be oxidized into imines more easily than AP is oxidized into imines because of the presence of an additional hydroxyl group (1969, 1970, 1978, 1979). Only 1,2,4-benzenetriol can be expected if hydrolysis of imines and subsequent reduction of the quinones occur. Meulenberg et al. (1996) identified 1,2,4-benzenetriol as an intermediate of nitroreductase-initiated 3-NP transformation by *P. putida* B2 under anaerobic conditions. Schenzle et al. (1997) described aminohydroquinone as an intermediate of 3-NP nitroreductase-initiated 3-NP transformation by *R.. eutropha* JMP134 under anaerobic conditions. All of these results are consistent with our proposed 3-NP degradation mechanism. Our proposed mechanism for 3-NP degradation, which was based on evidence obtained from transformation of the 3-NP analog NB, needs to be confirmed by direct studies of 3-NP metabolism, and we are currently exploring ways to do this.

Figure 5.4. Proposed route of 3-NP biotransformation in cells of *P. putida* 2NP8. All intermediates were postulated based on HAB biotransformation by 3-NP-grown cells.

AP is toxic to bacteria (Blum and Speece 1991; Musaev et al. 1984; Nendza and Seydel 1990; Thompson et al 1983), and detoxification activity in *P. putida* 2NP8 was clearly indicated by presence of acetylated amines. These compounds are known to be important in microbial detoxification and have been widely observed during nitroreductase-initiated degradation of nitroaromatic compounds (Gilcrease and Murphy 1995; Noguera and Freedman 1996; Park et al. 1999; Schackmann and Muller 1991; Schenzle et al. 1997; Tweedy et al. 1970). APX is an analog of the toxic compound actinomycin, which combines with DNA and inhibits RNA synthesis (Hollstein 1974). The effect of APX on growth has toxicological significance.

6.   SUBSTRATE SELECTIVITY OF A 3-NITROPHENOL-INDUCED METABOLIC SYSTEM IN *PSEUDOMAS PUTIDA* 2NP8 TRANSFORMING NITROAROMATIC COMPOUNDS INTO AMMONIA UNDER AEROBIC CONDITIONS[a]

6.1.   Abstract

The 3-nitrophenol-grown cells of *Pseudomonas putida* 2NP8 had wide substrate range in metabolizing the nitroaromatic substrate through to ammonia production. All the thirty nitroaromatic compounds were quickly degraded except 4-nitrophenol, 2,4-dinitrophenol, 2,4,6-trinitrophenol, 2-nitrobenzoic acid and 2-nitrofuran. Ammonia production from most of nitroaromatic substrates appeared to be stoichiometric. Metabolites more hydrophobic and hydrophilic than the nitroaromatic substrates were observed during transformation and the metabolites exhibited retention time patterns similar to those observed in the nitrobenzene (NB) biotransformation. A pathway similar to that for NB transformation was proposed for degradation of nitroaromatic substrates into ammonia via a hydroxylamino aromatic compound, aminophenol, quinone monoimine and quinone. We also observed an apparently constitutive enzyme activity oxidizing nitrobenzyl alcohol and nitrobenzaldehyde into nitrobenzoic acids. This system manifested low oxidizing activity toward 2-nitrobenzyl alcohol. The cells also reduced nitrobenzaldehyde into the corresponding alcohol product. Our results showed that degradation of nitrobenzyl alcohol into ammonia in the 3-NP grown cell media occurred either before or after oxidation of the alcohol group.

6.2.   Introduction

Three types of metabolic pathways have been reported for the initial utilization of nitroaromatic compounds, as the carbon and/or nitrogen sources, by aerobic bacteria. The first type is mono- or di-oxygenase-initiated release of nitrite and production of 1,2 or 1,4-dihydroxylated aromatic compounds. Knackmuss' group (Dickel et al. 1991; Ecker et al. 1992), Spain and his co-workers (Haigler et al. 1994; Jain et al. 1994; Nishino and Spain 1993a; Nishino and Spain 1995; Spain and Ginson 1991; Spanggord et al. 1991; Suen and Spain 1993), Hanne et al (1993) and Zeyer et al (1984, 1988, 1986) described this pathway in

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[a] This paper has been revised to Applied and Environmental Microbiology. Co-author is Owen Ward.

degradation of mono- or di-nitrated aromatic compounds in certain bacterial strains. The second pathway type is nitroreductase-catalyzed reduction of nitroaromatic compounds into hydroxylamino aromatic compounds with either subsequent rearrangement into aminophenol or direct conversion into 1,2-dihydroxylated aromatic compounds and ammonia. The research groups of de Bont (Groenewegen and de Bont 1992; Groenewegen et al. 1992; Meulenberg et al. 1996), Spain (Haigler and Spain 1993; Nishino and Spain 1993b), Williams (Rhys-Williams et al. 1993), Knackmuss (Schenzle et al. 1997, 1999a, 1999b; Spiess et al. 1998) and Ward (Zhao and Ward 2000; Zhao et al. 2000) reported this pathway in the degradation of 3-NP, nitrobenzene, 4-nitrotoluene and 4-nitrobenzoate in certain bacteria. Knackmuss' group first described a pathway producing a hydride-meisenheimer complex followed by release of nitrite in degradation of 2,4-dinitrophenol and 2,4,6-trinitrophenol (picric acid) in *Rhodococcus erythropolis* (Lenke and Knackmuss 1992; Lenke et al. 1992; Rieger et al. 1999). Rajan et al (1996) described mineralization of picric acid in a *Nocardioides simplex*. Ebert et al (1999) reported that a F_{420} dependent enzyme system in *N. simplex* catalyzed hydride transfer from NADPH to picric acid.

There were few reports that bacteria grew on multinitrated compounds as nitrogen source via reduction into amine. Boopath et al. (1993) reported that an anaerobic bacterium, *Desulfovibrio* sp. B strain, converted 2,4,6-trinitrotoluene into ammonia and toluene via mono-, di- and tri-aminotoluene, and provided nitrogen for bacterial growth. Duque et al (1993) observed that a strain of *Pseudomonas* sp. aerobically removed nitrite from 2,4,6-trinitrotoluene. The ammonia-release mechanism from the above multinitrated compounds still remains poorly characterized. Complete reduction of the nitro group has usually been found as cometabolic reductive events under aerobic conditions (Glaus et al. 1992; Schackmann et al. 1991) or anaerobic metabolism (Gorontzy et al. 1993; Oren et al. 1991) with amines as dead end products in many instances (Bryant and McElroy 1991; Cerniglia and Somerville 1995; Glaus et al. 1992; Gorontzy et al. 1993; Oren et al. 1991; Schackmann and Müller 1991).

The nitroreductase-initiated metabolism was reported for bacteria which utilize 3-NP as growth substrate with production of ammonia. In *Pseudomonas putida* B2, Meulenberg et al described a production of 1,2,4-trihydroxylbenzene from 3-NP via 3-hydroxylaminophenol (Meulenberg et al. 1996) and this was similar to the mechanism described for degradation of

4-nitrobenzoate in *C. acidovorans* NBA-10 (Groenewegen and de Bont 1992; Groenewegen et al. 1992). In *R. eutropha* JMP134, Schenzle et al (1997, 1999a,b) reported conversion of 3-NP into aminohydroquinone via 3-hydroxylaminophenol, without characterization of the ammonia release mechanism from aminohydroquinone.

Recently we observed that 3-NP-induced enzymes in *P. putida* 2NP8 could convert nitrobenzene, a cometabolic substrate, into ammonia, via conversion into aminophenols, subsequent oxidation into imines, and hydrolysis of the imines (Zhao and Ward 2000; Zhao et al. 2000). We postulated that this strain could convert 3-NP, the growth substrate, into hydroxylquinol (1,2,4-benzenetriol) via oxidation of aminohydroquinones into imine, hydrolysis of imine into quinone, and reduction of quinone (Zhao et al. 2000). Our proposal was supported by many reports describing hydroxylquinol as a ring-cleaving dioxygenase substrate in metabolism of wide range of aromatic compounds (Chamberlain and Dagley 1968; Chapman and Ribbons 1976; Daubaras et al. 1996; Haigler et al. 1999; Joshi and Gold 1993; Latus et al. 1995; Rieble et al. 1994; Sze and Dagley 1984; Valli et al. 1992; Zaborina et al. 1995). Our results have shown that catechol was one product of nitrobenzene degradation by the 3-NP-induced enzyme, but that nitrobenzene was not a growth substrate. This is consistent with the results of Latus et al (1995) in *Azotobacter* sp. and Daubaras et al (1996) in *Burkholderia cepacia* AC1100, which claimed that hydroxylquinol dioxygenase does not accept catechol as substrate.

Removal of nitro group from the aromatic ring is crucial to degradation of nitroaromatic compounds. Conversion of NB, a non-growth substrate, into ammonia by these 3-NP-induced enzymes in *P. putida* 2NP8 (Zhao and Ward 2000), encouraged us to speculate that other nitroaromatic compounds might also be degraded substantially into ammonia. In this paper, we investigated the substrate selectivity of this enzyme system and found that the 3-NP-induced enzyme system in *P. putida* 2NP8 had wide substrate range and transformed many nitroaromatic substrates into ammonia.

6.3.   Materials and methods

6.3.1.   Sources of chemicals

Nitroaromatic compounds: Aldrich (Milk, WI); methanol: EM Science (HPLC grade, Gibbstown, NJ).

6.3.2. Media

3-NP and other nitroaromatic compounds were dissolved individually in methanol to give a concentration of 10mg/ml. We have previously described the basic salts media (55-57). NA basic salts medium contained 20 mg/L of individual nitroaromatic compound in the basic salts medium. The latter medium was supplemented with 0.1% yeast extract (YE) to form NA/YE basic salts medium. YPS medium contained (g/l): YE, 10; Bacto peptone, 10; NaCl, 5. Nitroaromatic compound, sterile TMS and YE were added into autoclaved liquid media. Agar media contained 2% agar. Media were autoclaved at 120°C for 30min.

6.3.3. Preparation of cells grown in the presence of 3-NP and degradation of nitroaromatic compounds

P. putida 2NP8, a strain isolated by our group from municipal activated sludge (Waterloo, Ontario) (Zhao and Ward 1999, 2000), was maintained on YPS agar. Unless otherwise noted, the strain was grown in clear glass Erlenmeyer 250-ml flasks at 26°C, at 200 rpm on an orbital shaker. Fresh culture grown on 3-NP/YE (5ml, overnight) was inoculated into 50 ml 3-NP/YE basic salts medium and grown overnight (12 h). The latter culture was transferred into 1 L of 3-NP/YE medium in a 5L flask. After 14h 3-NP (50 mg/L) and YE (0.25%) were added and incubated overnight for 10h. At this time point, more 3-NP (60 mg/L) and YE (0.15%) was added. After 3h, 3-NP (40 mg/L) was added and the latter was incubated for 1 hour. Final cell density was 2.8 (OD 600nm, 1cm light path). Cells (3.85g, wet cells) were harvested by centrifuging at 16,300g for 20 min and washed with 100ml of sterile phosphate buffer (KH_2PO_4, 1g/l; Na_2HPO_4 $12H_2O$, 7g/L; pH, 7.35). Cells were used immediately for biotransformation of nitroaromatic compounds. Bottles used for biodegradation were 40ml amber glass with the Teflon/silicone septa lined caps. Cells, suspended in phosphate buffer (8 mg wet cells per ml) (OD_{600nm} 5.6, 1 cm light path), containing nitroaromatic compounds substrate (50 mg/L), were incubated on an orbital shaker at 200 rpm and 26°C. Caps of bottles were loosened to maintain aerobic conditions. The weight ratio of dry cells to wet cells was 21.7% (wet cells were dried in 85 °C oven, 5 days). The specific 3-NP transformation activity was 22 μM/h/mg (dry cell weight).

6.3.4. Preparation of cells grown on glucose and degradation of nitroaromatic compounds

A similar process was used for nitroaromatic substrate transformation with glucose-grown cells as was used in 3-NP-grown cell transformation except the cells were pregrown in the liquid medium containing 0.1% glucose and 0.1% ammonium sulfate instead of 3-NP.

6.3.5. HPLC analysis of nitroaromatic substrate and metabolites

The nitroaromatic compounds and metabolites were analyzed using a 4.6 X 250mm ZORBAX SB-C18 HPLC column (Chromatographic Specialties, Brockville, Ontario, Canada). We have previously reported the HPLC instruments, general procedures and methods for NB and 3-NP analysis (Zhao and Ward 1999, 2000; Zhao et al. 2000). Biotransformation samples were centrifuged at 9000g for 3 min. For samples of 3-nitroaniline, 4-nitrocatechol, picric acid, nitrobenzylalcohol, nitrobenzaldehyde, supernatants were directly injected for HPLC analysis. For the samples of other nitroaromatic compounds, the HPLC samples were prepared as follows. 0.45 ml of supernatant was transferred into 2 ml of vial with screw tightened and Teflon-lined caps. Then 0.05 ml of 0.4N HCl solution was added to samples. Ethyl acetate, 0.5 ml, was added and the mixture was vortexed for 1 min. The organic layer liquid was collected and used directly for HPLC analysis. Volumes of samples (15μl) were injected and eluted with mixture of 60% methanol and 40% milliQ water (0.1% trifluroacetic acid) at a flow rate of 1 ml/min. Eluting compounds were monitored at 254nm.

Ammonia was analyzed quantitatively with L-glutamate dehydrogenase and NADPH (Sigma diagnostic ammonia regent, Sigma, St. Louis, MO). Nitrite was measured according to US EPA method 354.1 (EPA 1979) (1979).

6.4.   Results

6.4.1. 3-NP-grown cells degraded a wide range of nitroaromatic compounds

We have reported that 3-NP-induced nitroreductase-initiated metabolic activity in P. putida 2NP8, transforming the growth substrate 3-NP and the cometabolic substrate NB into ammonia (Zhao and Ward 2000; Zhao et al. 2000). To investigate substrate selectivity of this 3-NP-induced metabolic pathway in this strain, we tested the removal of 30 nitroaromatic compounds by resting cells pregrown on 3-NP. The concentration of the substrates in the aqueous phase of transformation samples was analyzed. Loss of substrates in controls with

killed cells was negligible for all nitroaromatic compounds and 2-nitrofuran except for 1-nitronaphthalene, 50% of which was retained by biomass wthin 3h. The specific initial removal rates were calculated and the results are presented in Table 6.1.

Overall, our results showed that the 3-NP-grown cells quickly degraded all the nitroaromatic compounds except 2-nitrobenzoic acid, 4-nitrophenol, 2,4-dinitrophenol, 2,4,6-trinitrophenol, and that the rate of removal of 2-nitrofuran was also low. This indicated a wide substrate range of this 3-NP-degrading enzyme system. We found that 2-nitrobenzaldehyde was transformed via oxidation of the aldehyde group into the acid rather than by reduction of the nitro group.

We analyzed the effect of different substitutions at various positions of the aromatic ring and found that the degradation rate was greatly affected by hydroxyl groups located at the 2- or 4-position relative to the nitro group or by a carboxylic group at the 2-position only. Cells showed good degradation ability to all of the 4-substituted nitroaromatics including dinitrotoluenes except where that substituent was a hydroxyl group. Low or no degradation activity was observed toward 4-NP, 2,4-dinitrophenol, and 2,4,6-trinitrophenol. It seemed that the presence of hydroxyl group at the 4-position relative to the nitro group reduced the degradation rate. The hydroxyl group is different from others in that it is acidic and a strong electron donor, thus causing a decrease in the reduction potential of the nitro group, the first enzymatic reaction in the 3-NP-induced enzyme system. All the nitrobenzenes, with substitutions at the 2-position relative to the nitro group, were degraded except those containing aldehyde or carboxyl group. This may be caused by the tendency of the carbonyl bond of the aldehyde or carboxylic group on the benzene ring to form a rigid structure creating steric hindrance to the neighboring nitro group reaction. Other groups at the neighboring position may freely move away from the nitro group. This may avoid steric congestion and interference of the reduction reaction. 2-Nitrophenols had a lower degradation rate, which might be due to the electron donating effect of the hydroxyl group to the neighboring nitro group. Substitutions at the 3-position, including hydroxyl and carboxyl groups, did not reduce the degradation rate. 4-Nitrocatechol, having hydroxyl groups at both the 3- and 4-position, was degraded but at a rate intermediate between the rates observed for 3-nitrophenol and 4-nitrophenol.

It was noteworthy that the nitrated fused aromatic ring compound, 1-nitronaphthalene, was quickly removed. 2-Nitrofuran, a nitrated five-ring hetero aromatic compound was degraded, albeit at a relatively low rate, indicating that the 3-NP-induced specific nitroreductase may be different from the non-specific nitrofuran nitroreductase found in *E. coli* (Bryant and McElroy 1991; Cerniglia and Somerville 1995).

6.4.2. 3-NP-grown cells transformed nitroaromatic compounds into ammonia

We chose the nitroaromatic substrates that manifested high rates of degradation by 3-NP-grown cells, and investigated ammonia production from the nitroaroamtic compounds (Table 6.2). Ammonia-release from many of the substrates appeared to be stoichiometric. Lower ammonia production was observed from 2-NP, 4-nitrocatechol and 3,4-dinitrotoluene.

Because there have been reports on nitrite release via reduction of the aromatic ring (Ebert et al. 1999; Lenke and Knackmyss 1992; Lenke et al. 1992; Rajan et al. 1996; Gieger et al. 1999), we also analyzed nitrite production from these nitroaromatic substrates. We found no nitrite in the 3-NP-grown cell transformation media and excluded this possibility. These results demonstrated that the broad specific activity of the 3-NP-induced enzyme system in *P. putida* 2NP8 was not limited to the initial nitro group reduction step, as reported previously by Schenzle et al. (1997) and Meulenberg et al. (1996) but extended further down to ammonia production.

Table 6.1 Degradation rates (μM/h/mg dcw) of nitroaromatic substrate by 3-nitrophenol grown cells
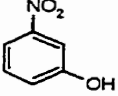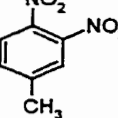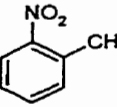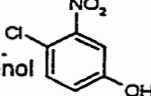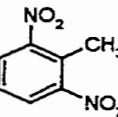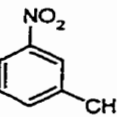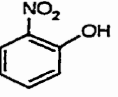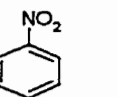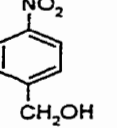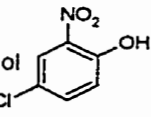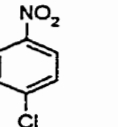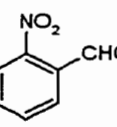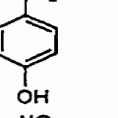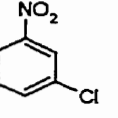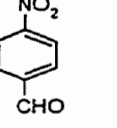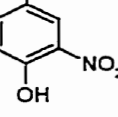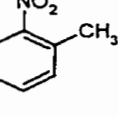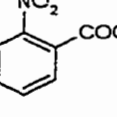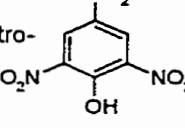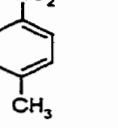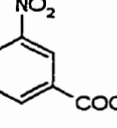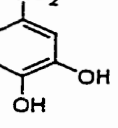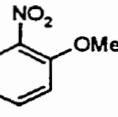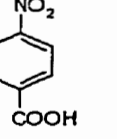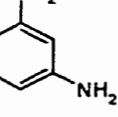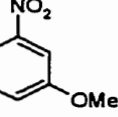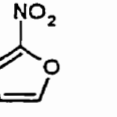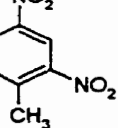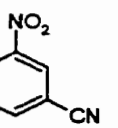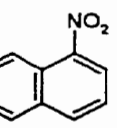
Substrate structure	Rate	Substrate structure	Rate	Substrate structure	Rate
3-nitrophenol	22	3,4-dinitro-toluene	16	2-nitrobenzyl alcohol	20
4-chloro-3-nitrophenol	18	2,6-dinitro-toluene	17	3-nitrobenzyl alcohol	20
2-nitrophenol	14	nitrobenzene	25	4-nitrobenzyl alcohol	20
4-chloro-2-nitrophenol	13	4-chloro-nitrobenzene	19	2-nitro-benzaldehyde	20
4-nitrophenol	6	3-chloro-nitrobenzene	19	4-nitro-benzaldehyde	20
2,4-dinitro-phenol	1	2-nitrotoluene	21	2-nitro-benzoic acid	1
2,4,6-trinitro-phenol	0.2	4-nitrotoluene	22	3-nitro-benzoic acid	17
4-nitro-catechol	12	2-nitroanisole	18	4-nitro-benzoic acid	17
3-nitroaniline	10	3-nitroanisole	20	2-nitrofuran	4
2,4-dinitro-toluene	17	3-nitro-benzonitrile	20	1-nitro-naphthalene	17

Table 6.2. Ammonia production from nitroaromatic compounds after transformation by *P. putida* 2NP8 cells grown on 3-nitrophenol.

Nitroaromatic substrate	Substrate ammount (μmole)	Substrate transformation (%, 3h)	Ammonia produced (μmole)
3-nitrophenol	1.4	100	1.3
4-chloro-3-nitrophenol	1.2	100	1.2
2-nitrophenol	1.4	83	0.4
4-chloro-2-nitrophenol	1.2	99	1.2
4-nitrocatechol	1.3	76	0.6
nitrobenzene	1.6	100	1.4
1-chloro-4-nitrobenzene	1.2	100	1.2
1-chloro-3-nitrobenzene	1.2	100	1.1
2-nitrotoluene	1.4	99	1.4
4-nitrotoluene	1.4	100	1.3
2-nitroanisole	1.3	100	1
3-nitroanisole	1.3	100	0.95
3-nitrobenzoate	1.2	100	1.32
4-nitrobenzoate	1.2	99	1.01
2-nitrobenzylalcohol	1.3	100	0.95
3-nitrobenzylalcohol	1.3	100	1.01
4-nitrobenzylalcohol	1.3	100	1
2-nitrobenzaldehyde	1.3	100	0.06
4-nitrobenzaldehyde	1.3	100	1.1
3-nitrobenzonitrile	1.3	100	0.97
2,4-dinitrotoluene	1.1	100	0.93
3,4-dinitrotoluene	1.1	100	0.62
2,6-dinitrotoluene	1.1	100	1.2
1-nitronaphthalene	1.2	100	0.69

6.4.3. Metabolite production from nitroaromatic compounds when incubated with 3-NP-grown cells

We have reported previously that this strain cometabolized NB into ammonia and produced nitrosobenzene, hydroxylaminobenzene, aminophenol, 2-aminophenoxazine-3-one, N-acetylaminophneol, benzoquinone and hydroquinone and catechol as metabolites (Zhao and Ward 2000; Zhao et al. 2000). As we used a similar procedure to conduct the biotransformations reported in this paper, the cells must have used a similar pathway if ammonia production occurred. We analyzed the positive biotransformation samples for metabolites when we incubated 3-NP-grown cells with the nitroaromatic compounds. The controls contained transformation medium with killed 3-NP-grown cells and live glucose-grown cells. We observed new compounds produced during the transformation (Table 6.3). These compounds strongly absorbed UV at 254nm, thus indicating an unsaturated structure, likely an intact aromatic ring. With aqueous methanol as solvent the reversed C-18 column, used for analysis, would retain hydrophobic compounds (less polar) more strongly than hydrophilic compounds (polar), resulting in the non-polar compounds having higher retention times than the polar compounds. Retention times of metabolites produced from 3-NP grown-cell biotransformations of nitroaromatic substrate are listed in Table 6.3. With 60% methanol as solvent, metabolites from NB transformation were separated into two groups. The first group consists of metabolites, which are more hydrophobic than the substrate, such as nitroso compound. Nitroso compound (Rt, 8.1min) is a major and stable metabolite observed in transformation of nitrobenzene by 3-NP-grown cells of *P. pitida* 2NP8 (Zhao and Ward 2000). The second group consists of metabolites, which are more hydrophilic than the substrate, such as hydroxylaminobenzene, aminophenol, quinone and others (Zhao et al. 2000). The major HPLC peaks with retention times of about 3 min, represent these hydrophilic products formed from further transformation of nitroso compound.

We analyzed the retention time pattern of metabolites produced from transformation of nitrobenzene and other nitroaromatic substrates and found that degradation of nitroaromatic compounds tested in this paper were also consecutive, multistep transformation events consistent with the pathway that we proposed previously (Zhao et al. 2000).

Table 6.3. HPLC retention times of metabolites formed from nitroaromatic compounds when incubated with 3-NP-grown cells of *P. putida* 2NP8

Nitroaromatic substrate	C-18 reversed HPLC column retention time (Rt., min, 60% methanol as solvent)		
	Substrate Rt. (Rt.-s)	Metabolite Rt. (Rt-m)	
		< Rt. (s)[a]	> Rt. (s)[b]
3-nitrophenol (3NP)	5.5	-	-
4-chloro-3-nitrophenol (4Cl3NP)	8.4	-	(16.3)
2-nitrophenol (2NP)	7.0	3.5	7.5, 7.9
4-chloro-2-nitrophenol (4Cl2NP)	12.3	5.2, 8.1	-
4-nitrocatechol (4NC)	4.1	-	-
nitrobenzene (NB)	7.0	2.8, 3.3	8.1 (nitrosobenzene), 10 (2-aminophenoxazine-3-one) (Ref 57)
4-chloronitrobenzene (4ClNB)	11.8	5.4, 7.1, 10.2	-
3-chloronitrobenzene (3ClNB)	13.9	3.3, 3.5, 5.4 (10.6, 12.6)	-
2-nitrotoluene (2NT)	10.3	(3.8)	-
4-nitrotoluene (4NT)	11.0	(5.2, 10.2)	-
2-nitroanisole (2NAS)	5.7	3.2	10.5
3-nitroanisole (3NAS)	10.1	3.5, 8.5	
3-nitrobenzoic acid (3NBA)	5.9	-	6.6, (7.1)
4-nitrobenzoic acid (4NBA)	6.3	2.9	7.3
2-nitrobenzyl alcohol (2NBAL)	4.51	2.7, 3.0, 3.9 (2NBA)	6.8, 7.4, (5.1)
3-nitrobenzyl alcohol (3NBAL)	4.47	2.6, 3.0, 3.5	7.0, 7.6, {6.0 (3NBA), 6.4, 13}
4-nitrobenzyl alcohol (4NBAL)	4.45	2.9, 4.1	6.9, 7.4, 11.8, {6.4 (4NBA), 11.1}
2-nitrobenaldehyde (2NBAD)	4.8	3.9 (2NBA)	-
4-nitrobenzaldehyde (4NBAD)	5.2	2.9	6.4 (4NBA), 6.9, 7.5, 20.4, 22.0, (8.1, 11.0, 11.7)
3-nitrobenzonitrile (3NBN)	4.9	-	10.0
2,4-dinitrotoluene (24DNT)	9.4	3.2, 3.7, (6.5)	11.8, 22.1, (10.6, 12.8)
3,4-dinitrotoluene (34DNT)	7.3	3.8, (6.7)	8.8, 9.3 , (7.0, 8.0)
2,6-dinitrotoluene (26DNT)	9.07	3.7	13.3, (8.6, 8.0, 10.5, 12)
1-nitronaphthalene (1NNT)	19.7	7.1	22.2

Notes and symbols:

-: no peak appeared in this retention time region. Major peaks and minor peaks (in bracket) were given according to the UV absorbance at 254nm. High A_{254nm} should not be always interpreted as high concentration in the media when comparing different compound, thus characterization by absorbance at single wavelength is subjective.

[a]: peaks with smaller retention time than initial substrate indicates hydrophilic metabolite, such as hydroxylamino compound, aminophenol, N-acetylaminophenol, quinones and catechol compounds;

[b]: peaks with larger retention times indicates nitroso or condensed products (dimer); The possible retention time order of metabolites according to their chemical structure, HPLC column and our previous results (56, 57) of nitrobenzene biotransformation,is: Rt. (dimer) > Rt. (nitroso compound) > Rt. (nitroaromatic substrate) > Rt. (hydroxylamino compound) > N-acetylaminophenol~catechol~aminophenol-quinone. Nitrosoaromatic compound has strong absorbance at 254nm, and it was eluted about 1-2min later after the nitroaromatic substrate. The strong peaks at 3min are most likely those of the quinone and aminophenol derivatives.

The metabolite production sequence from nitrobenzene transformation is {compound, (retention time)}: NB (7.0 min)→ metabolite 1 (8.1min, nitrosobenzene)→ metabolite 2 or 3 (2.8-3.3 min, hydroxylamino compound, aminophenol, and quinones etc) (Zhao et al. 2000). We observed similar metabolite formation sequence in transformation of other substrates, such as: 4-nitrobenzoic acid, substrate (6.3min)→metabolite 1 (7.3min)→metabolite 2 (2.9min); 2-nitrophenol, substrate (7.0min) → metabolite 1 (7.9 min) → metabolite 2 (3.5min); 1-nitronaphthalene, substrate (19.7min) → metabolite 1 (22.2min) → metabolite 2 (7.1min). Dinitrotoluene has two nitro groups and one or two of them could be attacked. We observed two major peaks with equal yield, having a slightly larger retention time than the substrate (likely a nitroso product) in transformation of 3,4-dinitrotoluene {substrate (7.3 min) → metabolite 1 (8.8 min) and metabolite 2 (9.3 min)}, thus suggesting that either one of the two neighboring nitro groups, were initially attacked. We observed only one major peak with a retention time slightly larger than the substrate from 2,4-dinitrotoluene {substrate (9.4min) → metabolite 1(11.8 min)} and this suggests that only one of the two nitro groups was preferably selected to be attacked. Since the two nitro groups of 2,6-dinitrotoluene are at positions symmetrical to each other, only one nitroso compound would be produced as a result of the initial reduction. This was confirmed by our observation that only one major metabolite produced with retention time slightly larger than the substrate {substrate (9.07min) → metabolite 1 (13.3 min)}. We observed little metabolite formation from some substrates such as 3-NP and 4-chloro-3-NP, and this may be due to more rapid degradation of the metabolites.

We monitored the biotransformation time-courses of all utilized substrates, twelve of which are presented in Figure 6.1(A-L). Each of these time-courses demonstrated removal of substrate, formation of metabolites and ammonia production.

Figure 6.1 A-L. Times courses of nitroaromatic compounds biotransformation catalyzed by *P. putida* 2NP8 resting cells grown on 3-NP.

Right Y, Metabolites amount, the unit is $A_{254nm}/10,000$ unless otherwise noted.

[a]: Unit of metabolite m3.0 in Figure 6.1G: $A_{254nm}/100,000$.

Biotransformation conditions: 5 ml phosphate buffer (25mM, pH 7.3), aerobic, 150 rpm shaker, 26 °C, 8mg/L wet cells.

HPLC analytical conditions: solvent, 40% ultra pure water (containing 0.1% trifluroacetic acid) and 60% methanol; column, Zorbax SB-C 18 column; flow rate, 1ml/min; UV detector wavelength, 254nm (attenuation, 1).

Symbols: m5.2, metabolite with HPLC retention time of 5.2min; 4Cl2NP, 4-chloro-2-nitrophenol; 3ClNB, 3-chloronitrobenzene; 3NBA, 3-nitrobenzoic acid; 4NBA, 4-nitrobenzoic acid; 3NBAL, 3-nitrobenzyl alcohol; 4NBA, 4-nitrobenzyl alcohol; 4BALD, 4-nitrobenzaldehyde; 24DNT, 2,4-dinitrotoluene; 34DNT, 3,4-dinitrotoluene; 26DNT, 2,6-dinitrotoluene; 3NAS, 3-nitroanisole; 1NNT, 1-nitronaphthalene.
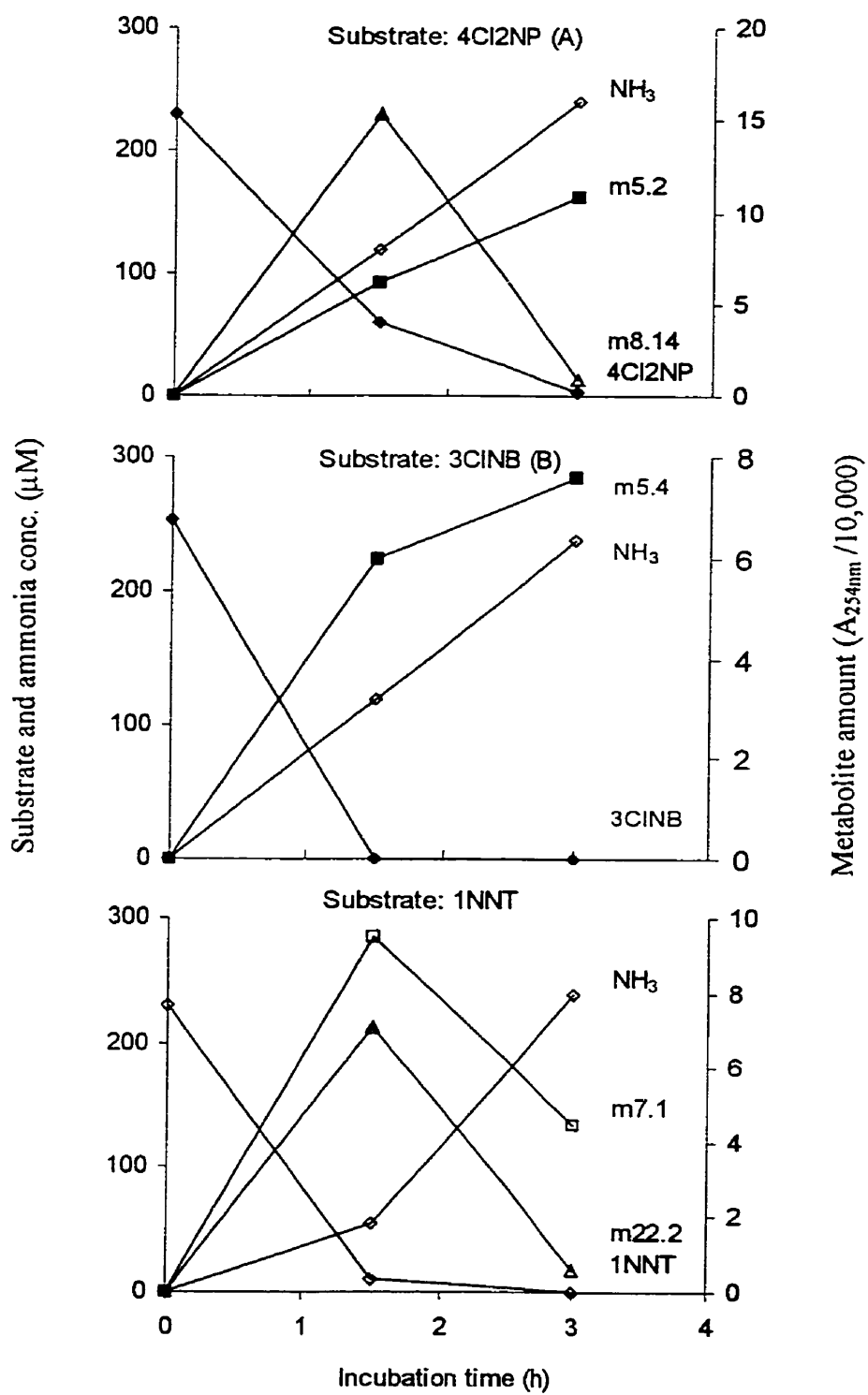
Figure 6.1A-C
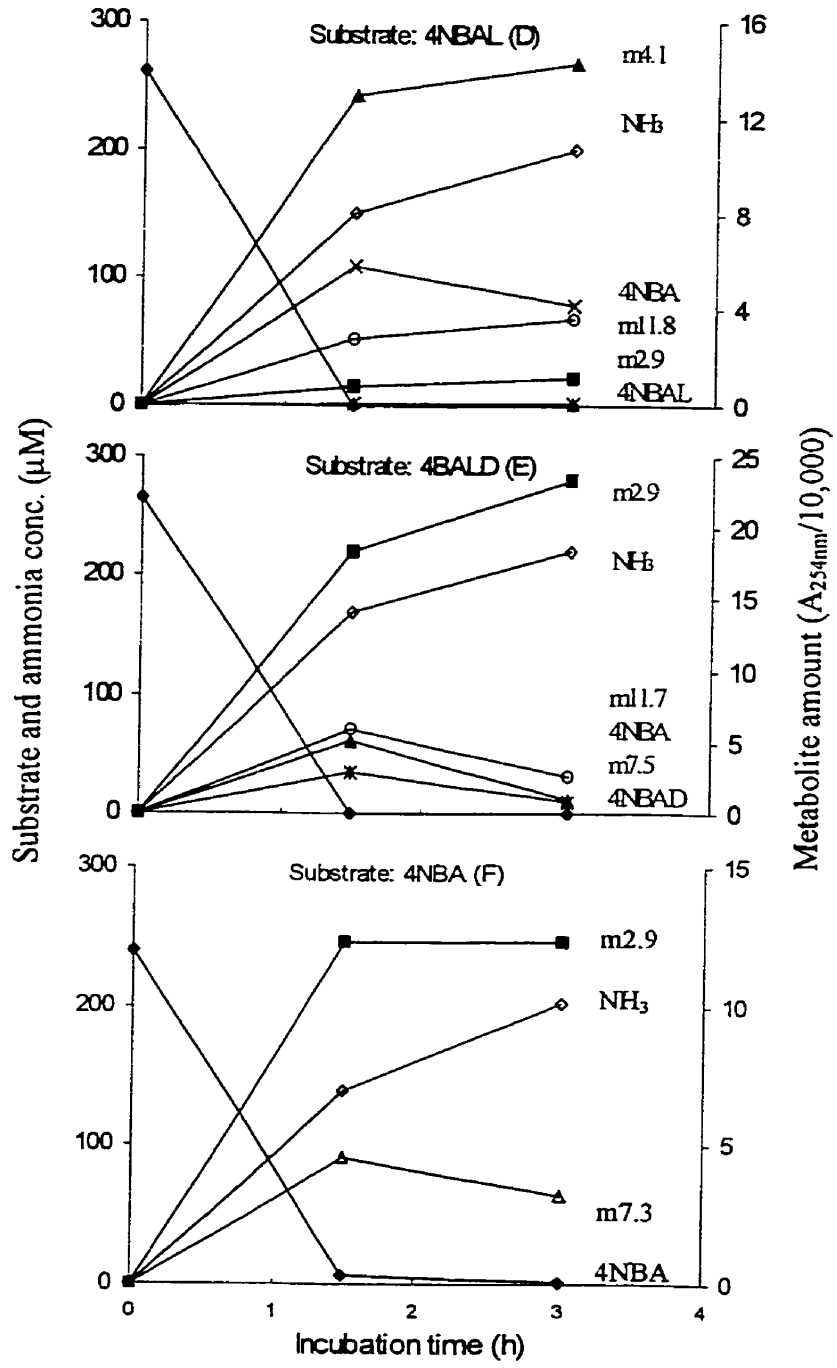
Figure 6.1D-F
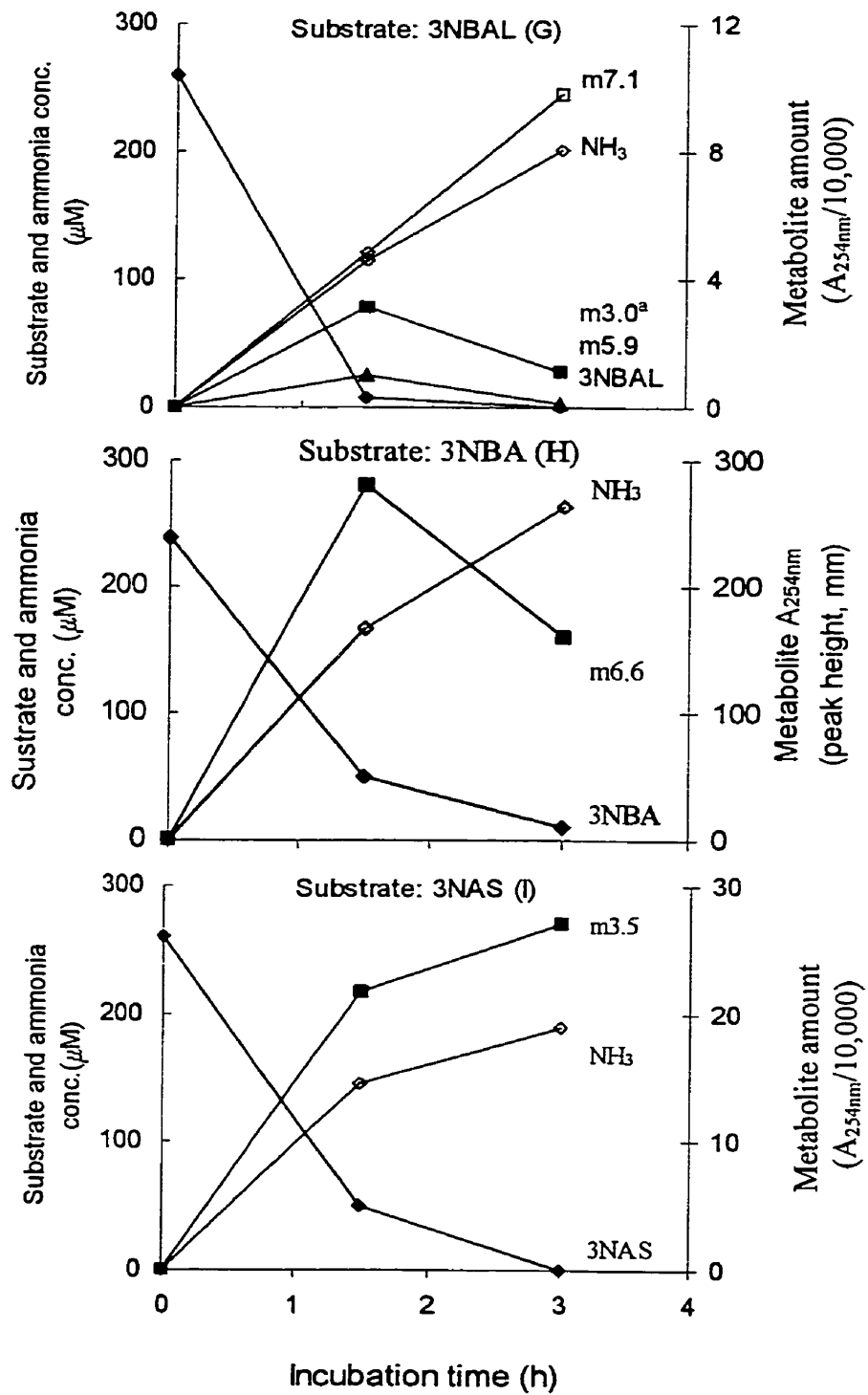
Figure 6.1G-I
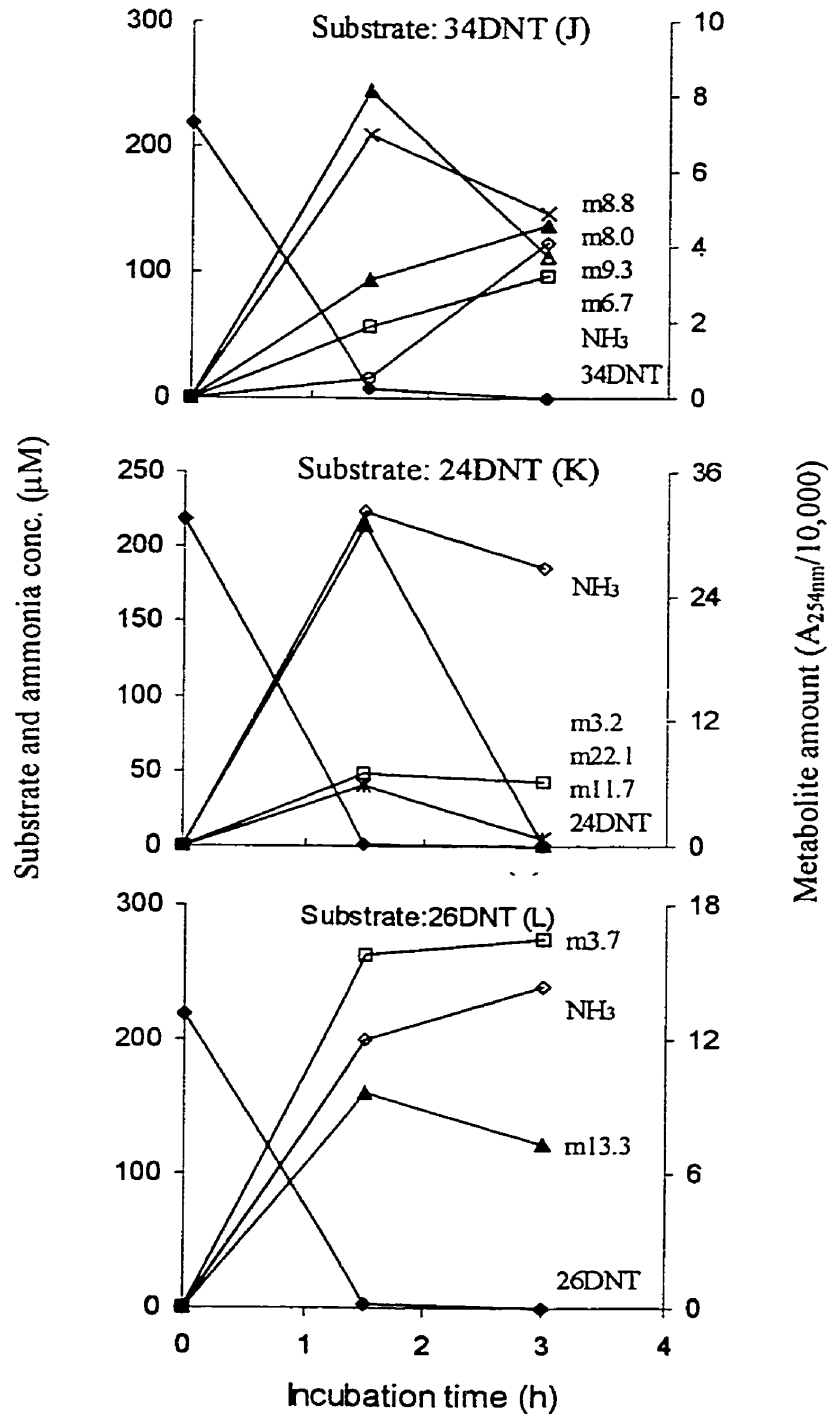
Figure 6.1J-L

6.4.4. Metabolic pathway of nitrobenzyl alcohol, nitrobenzaldehyde and nitrobenzoic acid in 3-NP-grown cells of P. putida 2NP8

In 3-NP-grown cell biotransformation media, we observed production of 50% of 2-nitrobenzoic acid from 2-nitrobenzaldehyde and 3.5% of 4-nitrobenzoate produced from 4-nitrobenzaldehyde. In the control media with live glucose-grown cells, we found the cells both oxidized nitrobenzaldehyde into nitrobenzoic acid and reduced them into nitrobenzyl alcohol. Yield of biotransformation products from the glucose-grown cell media were (substrate: product / yield): 2-nitrobenzaldehyde: alcohol / 40%, acid / 60%; 4-nitrobenzaldehyde: alcohol/0.2%, acid/92%. Thus formation of nitrobenzoic acid from nitrobenzaldehyde in the 3-NP-grown cell media appeared to be due to a constitutive enzyme activity. In the transformation medium with nitrobenzaldehyde and 3-NP-grown cells, a high level of 2-nitrobenzoic acid was observed, but only a low yield of 4-nitrobenzoic acid was observed. This suggested either fast degradation of 4-nitrobenzoic acid following its formation or a direct degradation via initial attack on the nitro group. 2-Nitrobenzoic acid could not be degraded. We did not find an alcohol product from nitrobenzaldehyde using 3-NP-grown cells.

The glucose-grown resting cells also exhibited apparent constitutive enzyme activity degrading 3- or 4-nitrobenzyl alcohol, with little activity on 2-nitrobenzyl alcohol and all other nitroaromatic compounds including nitrotoluenes. Glucose-grown cells slowly oxidized 3- (14%) or 4-nitrobenzyl alcohol (4%) into nitrobenzoate. We observed production of trace amount of nitrobenzoic acid from 3- or 4-nitrobenzyl alcohol in transformation media containing 3-NP-grown cells, indicating a quick degradation of these products by 3-NP-grown cells. A high degradation rate of 3- or 4-nitrobenzyl alcohol in the 3-NP-grown cell media demonstrated that degradation via initial reduction of the nitro group, instead of oxidation of the alcohol group, was the dominant reaction. Since there were reports that a strain of *Pseudomonas* sp. could degrade 4-nitrotoluene via initial oxidation of methyl group, leading to its degradation into ammonia (Haigler and Spain 1993; Rhys-Williams et al. 1993), we analyzed the ability of our strain (*P. putida* 2NP8) to oxidize nitrotoluene into nitrobenzyl alcohol. We found that cells of this strain, grown on glucose or 3-NP, lacked this enzyme activity.

Based on the evidence presented, combined with our previous reports (Zhao and Ward 2000; Zhao et al. 2000), we concluded that this strain transformed the nitroaromatic compounds into ammonia via a pathway similar to that of nitrobenzene degradation. The proposed pathway is given in Figure 6.2A. A proposed mixed pathway for degradation of nitrotoluene, nitrobenzyl alcohol, nitrobenzaldehyde and nitrobenzoic acid by 3-NP-grown cells of *P. putida* 2NP8 is also summarized in Figure 6.2B-D. All nitrobenzyl alcohols, nitrobenzaldehydes except 2-nitrobenzaldehyde were degraded into ammonia with or without initial attack on the aldehyde or alcohol group. We did not test whether 3-nitrobenzaldehyde can be reduced or directly degraded into ammonia, however the aldehyde is obviously an intermediate of oxidation of 3-nitrobenzyl alcohol. Even though oxidation activity of nitrotoluene is missing in the strain, the co-metabolic activity of the 3-NP-grown cells seems to resemble in other respects other specific 4-nitrotoluene degraders (Haigler and Spain 1993; Rhys-Williams et al. 1993) with inducible nitrotoluene oxidation and 4-nitrobenzoic acid reduction activity.
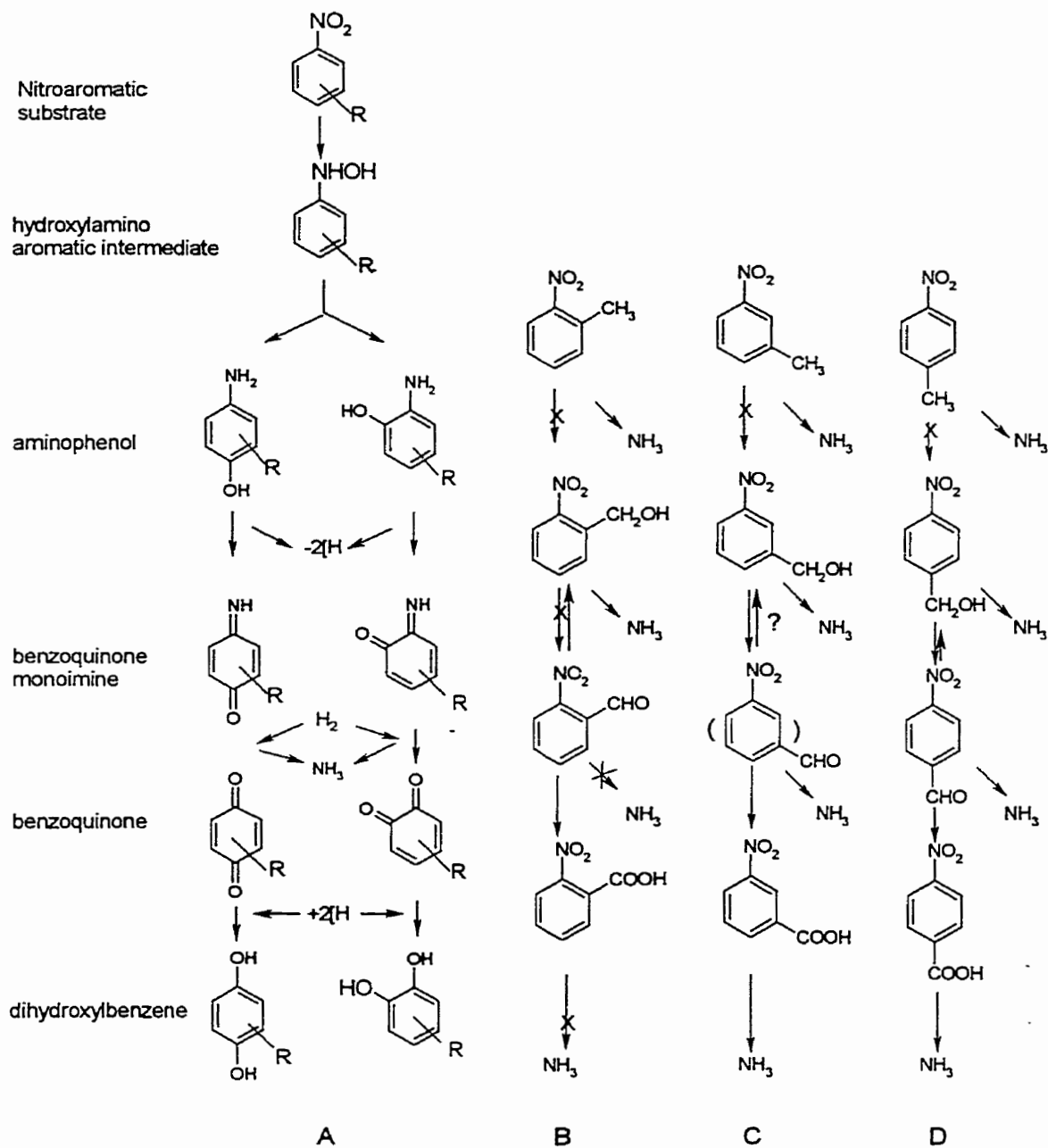
Figure 6.2. Proposed pathway for ammonia-release from nitroaromatic compounds and transformation of nitrobenzyl alcohol in *P. putida* 2NP8. (Bracket indicates unidentified compound. Question mark indicates the reactions not tested.)

6.5. Discussions

In this paper, we demonstrated that the 3-NP-induced enzymatic system for transformation of nitroaromatic compoud has wide substrate range and could convert many substituted nitroaromatic compounds into ammonia.

Two 3-NP-induced enzyme systems have been reported. The initial enzyme in all the 3-NP degrading bacteria with 3-NP as growth substrate was nitroreductase. The initial nitroreductase was found to have wide substrate range in reducing the nitroaroamtic compounds with NAD(P)H (Meulenberg et al. 1996; Schenzle et al. 1997). Meulenberg et al (1996) described a 3-NP-induced enzyme system in *P. putida* B2, and found that the initial 3-NP nitroreductase in cell-free extract had the ability to catalyze reduction of many other nitroaromatic compounds with NAD(P)H as an electron donor. However, neither the cell-free extract nor the intact cells of *P. putida* B2 grown on 3-NP, converted the nitroaromatic and hydroxylaminoaromatic substrates, other than 3-NP, into ammonia. This contrasts with our results in that a wide range of nitroaromatic compounds were transformed into ammonia by the 3-NP-grown cells of *P. putida* 2NP8, thus indicating a different 3-NP degrading system.

Schenzle et al (1997, 1999a, 1999b) reported another 3-NP-degrading enzyme system in *Ralstonia eutropha* JMP134. The initial 3-NP nitroreductase also catalyzed reduction of a wide range of nitroaromatic substrates with NADPH as electron donor (Schenzle et al. 1999a). 3-NP-grown cells of this strain converted 3-NP and 2-chloro-5-NP into ammonia. However, NB was converted into dead end amino phenols (41), rather than ammonia. This 3-NP-induced enzyme is also different from the one we reported in *P. putida* 2NP8.

Both our results in *P. putida* 2NP8 and the results for *R. eutropha* JMP134 reported by Schenzle et al (1999a) showed that the enzyme system did not attack 4-NP, 2,4-dinitrophenol and 2-nitrobenzoate. Meulenberg et al (1996) also observed that the initial enzyme in *P.putida* B2 exhibited a low capacity to reduce 4-NP and 2-nitrobenzoate. Schenzle reported (1999a) that the initial enzyme in *R. eutropha* JMP134 reduced picric acid but not 2-nitrotoluene. We observed the opposite, namely that *P. putida* 2NP8 reduced 2-nitrotoluene but not picric acid.

Both nitroreductase- and oxygenase-initiated (Cerniglia and Somerville 1995; Groenewegen and de Bont 1992; Groenewegen et al.1992; Haigler et al. 1994; Nishino and Spain 1993a,b; Park et al. 1999; Rhys-Williams et al. 1993; spanggord et al. 1991; Spiess et al. 1998; Valli et al. 1992; Zeyer et al. 1986) metabolism has been reported for transformation

of many nitroaromatic compounds tested in this paper. The metabolic pathways described in these reports are different from those we propose for *P. putida* 2NP8.

While nitroreductase-initiated metabolism of nitrobenzene, nitrobenzoic acid and 4-nitrotoluene, as growth substrates, has been reported, little is known about substrate selectivity in bacterial degradation of non-growth-supporting nitroaromatic substrates. Groenewegen et al. (1992) reported nitroreductase-initiated metabolism of 4-nitrobenzoate in *Comamonas acidovorans* NBA-10, and proposed a hydroxylaminolyase that converted the hydroxylaminobenzoate intermediate directly into 3,4-dihydroxylbeozoate and ammonia. Rhys-Williams et al (1993) reported that *Pseudomonas* sp. TW3 used the same mechanism to degrade 4-nitrobenzoate, an intermediate from 4-nitrotoluene transformation by this strain, via oxidation of methyl group. Spiess et al (1998) observed degradation of 4-nitrotoluene in a *Mycobacterium* strain (HL 4-NT-1) via initial reduction of nitro group leaving the methyl group intact. However, this strain transformed the substrate into ammonia via ring-cleavage of 6-amino-3-cresol by aminophenol dioxygenase similar to that reported for nitrobenzene degradation by *P. pseudoalcaligenes* JS45 (Nishino and Spain 1993b). Park et al (1999) reported a nitroreductase initiated transformation of choloronitrobenzene by NB-grown cells of a strain of *P. putida*, with *N*-acetylated-choloroaminophenol (chlorohydroxyacetanilide) as dead end product. Cerniglia et al (1995) reported microbial transformation of nitropolycyclic aromatic hydrocarbons by non-specific nitroreductase and described nitroso, hydroxylamino intermediates and amine, but not ammonia, as metabolites.

Bacteria also used oxygenase to initiate degradation of some of the nitoraroamtic compounds reported in this paper. Haigler et al (1994) described a constitutively expressed dioxygenase in *Pseudomonas* sp. strain JS42, converting 2-nitrotolene into nitrite. For 4-chloro-2-NP, Zeyer et al (1986) reported release of nitrite via a dioxygenase in *P. putida* B2. Spanggord et al (1991) reported that a strain of *Pseudomonas* sp. transformed 2,4-dinitrotoluene into 4-methyl-nitrocatechol by a dioxygenase. Valli et al (1992) reported that the lignin-degrading fungus, *Phanerochaete chrysosporium*, transformed 2,4-dinitrotoluene via a multistep pathway including an initial reduction into amines followed by oxidation into benzoquinones with release of ammonia. Duque et al (1993) isolated a strain of *Pseudomonas* sp. (C1S1) which converted trinitrotoluene into dinitrotoluene, mononitrotoluene, and toluene, with release of nitrite. The mechanism related to nitrotoluene conversion remains unclear.

Ali-Sadat et al (1995) described cometabolic degradation of 3-nitrotoluene in *P. putida* OU83, and found both 3-aminotoluene and nitrite released via 3-NP.

Haigler and Spain (1993) and Rhys-Williams et al (1993) reported that 4-nitrobenzyl alcohol and 4-nitrobenzaldehyde were intermediates in degradation of 4-nitrotoluene by a strain of *Pseudomonas* sp. We detected an apparently constitutive enzyme activity in *P. putida* 2NP8, oxidizing nitrobenzylalcohol and nitrobenzaldehyde into nitrobenzoic acid. We also observed a quick conversion of the substrate into ammonia. There were overlaps in metabolite profile of 4-nitrobenzyl alcohol and 4-nitrobenzaldehyde and 4-nitrobenzoic acid (Table 6.3 and Figure 6.1D, E, F), suggesting common metabolites. Both 4-nitrobenzyl alcohol and 4-nitrobenzaldehyde were converted into metabolites with retention times of 2.9min, 6.4 min (4-nitrobenzoic acid), 7.4min, 6.9min, 11.0min and 11.8min. This in turn suggests a shared upstream pathway, likely the degradation via the aldehyde and the acid. Both 4-nitrobenzoic acid and 4-nitrobenzaldehyde produced metabolites with retention time of 2.9min and 7.3min, again suggesting a shared metabolism from 4-nitrobenzoic acid. There were also differences between metabolite profiles of the three substrates, indicating degradation before they converge to 4-nitrobenzoic acid. At a constant biomass concentration (wet cells, 8 mg/ml), the oxidation product (4-nitrobenzoic acid) yield (1.5h, 5%) from 4-nitrobenzyl alcohol in the glucose-grown cells medium is much lower than 4-nitrobenzyl alcohol degradation yield (1.5h, 100%, Figure 6.1G) in 3-NP-grown cells medium. This suggested that degradation of 4-nitrobenzyl alcohol must have occurred before oxidation and is the dominant reaction. This evidence indicated this strain could convert the substrates into ammonia either before or after they were oxidized into nitrobenzoic acid and these mixed pathway are presented in Figure 6.2B-D.

Haigler and Spain (1993) and Rhys-willams et al (1993) described a complete degradation of 4-nitrotoluene into ammonia via initial oxidation of methyl group and a subsequent nitroreductase-initiated metabolism of 4-nitrobenzoic acid. We observed an apparently constitutive enzyme activity oxidizing nitrobenzyl alcohol into nitrobenzoic acid, but this strain (*P. putida* 2NP8) lacked the methyl group oxygenase. Robertson et al (1992) reported oxidation of nitrotoluenes into nitrobenzyl alcohol by toluene dioxygenase. Delgado et al (1992) reported that an toluene-induced toluene monooxygenase had the ability to oxidize a) nitrotoluene into nitrobenzyl alcohol, b) nitrobenzyl alcohol into

nitrobenzaldehyde, and c) oxidation of nitrobenzaldehyde. They concluded nitrotoluene was not the induction effector of the pathway. The constitutive enzymatic activity in *P. putida* needs to be characterized to understand how it is different from the above toluene and 4-nitrotoluene oxygenase.

We have reported previously that 3-NP-grown cells of *P.putida* 2NP8 transformed nitrobenzene into ammonia via: 1) reduction of nitrobenzene to hydroxylaminobenzene; 2) rearrangement of hydroxylaminobenzene into aminophenol; 3) oxidation of aminophenol into benzoquinone monoimine; 4) hydrolysis of imine into ammonia and quinone (Zhao et al. 2000). Production of ammonia from the nitroaromatic compounds tested in this paper demonstrated all the enzymes in the pathway must have relaxed substrate selectivity, thus allowing reaction of all the intermediates to proceed to release of ammonia (Figure 6.2). Further characterization of the metabolites and enzymes involved in these biotransformations will now be conducted.

7. DEGRADATION OF NITROAROMATIC COMPOUNDS VIA ELIMINATION OF NITRITE IN CELLS OF *PSEUDOMONAS PUTIDA* 2NP8

7.1. Abstract

The 2-nitrophenol-induced enzyme system of *Pseudomonas putida* 2NP8 transforms 2-nitrophenol into nitrite and the 3-nitrophenol-induced system transforms 3-nitrophenol into ammonia. When thirty nitroaromatic substrates with one, two or three nitro substitutions, were tested for their capacity to induce a nitrite-releasing activity only 2-nitrophenol and 4-Cl-2-nitrophenol were found to be the inducers. Likewise when the thirty compounds were tested as substrate of a 2-nitrophenol-induced enzyme system, only 2-nitrophenol and 4-Cl-2-nitrophenol were substrates of the 2-nitrophenol-induced nitrite-releasing enzyme system. This contrasts with the very broad substrate specificity of the 3-nitrophenol-induced enzyme system. This strain transformed 4-Cl-2-nitrophenol into a dead-end metabolite A, which is believed to be 4-chlorocatechol based on its HPLC performance and a proposed oxygenase-initiated pathway.

7.2. Introduction

Three types of enzymes have been reported for aerobic bacteria to initiate degradation of the inert nitroaromatic compounds as their growth substrates: a) oxygenase; b) benzene ring reductase; c) nitroreductase. The first two initial reactions produce nitrite and the third produces ammonia. The benzene ring reductase-initiated degradation of some multinitrated compounds and hydride ion benzene ring reduction form a meisenheimer intermediate as a precursor to nitrite release (Ebert et al. 1999; Lenke and Knackmuss 1992; Lenke et al. 1992; Rajan et al. 1996; Rieger et al. 1999; Vorbeck et al. 1994; Vorbeck et al. 1998). The nitroreductase was the first enzyme in the degradation of some nitroaromatic compounds such as nitrobenzene (Nishino and Spain 1993b; Park et al. 1999; Somerville et al. 1995), 4-nitrotoluene (Suen et al. 1993), 4-nitrobenzoic acid (Groenewegen and de Bont 1992; Groenewegen et al. 1992; Haigler and Spain 1993; Rhys-Williams et al. 1993), 3-nitrophenol (3-NP) (Meulenberg et al. 1996; Schenzle et al. 1997, 1999a, 1999b; Zhao and Ward 1999; Zhao et al. 2000; Zhao and Ward, unpublished results) in certain bacteria grown on these nitroaromatic compounds. In the nitroreductase-initiated metabolism of nitroaromatic

compounds, the ammonia is released via an incomplete reduction of the nitro group into a hydroxylamino group, rather than a complete reduction to amine as observed (Schackmann and Müller 1991) in cometabolism involving unspecific nitroreductase. Although the nitroaromatic compounds are electron deficient due to the presence of the nitro group, many aerobic bacteria are capable of attacking the aromatic ring with specific oxygenase and release nitrite from mono- and di-nitroaromatic compounds (Dickel and Knackmuss 1991; Haigler et al. 1994; Hanne et al. 1993; Jain et al. 1994; Nishino and Spain 1993a, 1995; Spain and Gibson 1991; Spain et al. 1979; Spanggord et al. 1991; Suen and Spain 1993; Zeyer et al. 1986; Zeyer and Kearney 1984; Zhao and Ward, unpublished results).

The oxygenase-catalyzed removal of the nitro group from the aromatic ring involves three steps: 1) oxygen molecule activation; 2) addition of the 'active oxygen' to aromatic ring, forming a non-aromatic and unstable adduct and 3) release of a nitrite anion and formation of a quinone intermediate (for monoxygenase) or a dihydroxy-product (for dioxygenase) (Dickel and Knackmuss 1991; Haigler et al. 1994; Nishino and Spain 1995; Spain and Gibson 1991; Spain et al. 1979; Spanggord et al. 1991; Zeyer and Kocher 1988). The dihydroxyl aromatic compounds are the products of this initial oxygenase-reaction and subjected to ring-cleavage metabolism. Bacteria employ this route to degrade 2- or 4-NP. 4-NP degradation activity has been found in many environments (Hanne et al. 1993; Jain et al. 1994; Nishino and Spain 1993a; Spain and Gibson 1991; Zhao and Ward, 1999 and unpublished results), but there is only one report on bacterial degradation of 2-NP as growth substrate (Zeyer and Kearney 1984). Zeyer and Kearney described a *Pseudomonas putida* B2 degrading 2-NP and 3-NP (36). No strain has been reported to degrade 2-NP alone but not 3-NP. We also isolated a strain of *P. putida* 2NP8 which degraded both 2-NP and 3-NP, has a 3-NP-induced enzyme system capable of converting nitroaroamtic compounds into catechols and ammonia (Zhao and Ward 2000; unpublished results). Zeyer et al. reported that the purified 2-NP oxygenase in *P. putida* B2 had the capacity to degrade some other substituted 2-NP (Zeyer et al. 1986). In this paper we investigated the capacity of thirty mono-, di- and tri-nitroaromatics to induce the 2-NP-degrading enzyme system and to participate as substrates in the reaction.

7.3.   Materials and methods

7.3.1.   Sources of chemicals

Nitroaromatic compounds: Aldrich (Milk, WI); methanol: EM Science (HPLC grade, Gibbstown, NJ).

7.3.2.   Media

2-NP and other nitroaromatic compounds were dissolved individually in methanol to give a concentration of 10mg/ml. We have previously described the basic salts media (Zhao and Ward 1999). NA basic salts medium contained 20 mg/L of individual nitroaromatic compound in the basic salts medium. The latter medium was supplemented with 0.1% yeast extract (YE) to form NA/YE basic salts medium. YPS medium contained (g/l): YE, 10; Bacto peptone, 10; NaCl, 5. Nitroaromatic compound, sterile TMS and YE were added into autoclaved liquid media. Agar media contained 2% agar. Media were autoclaved at 120°C for 30min.

7.3.3.   Preparation of cells grown on 2-NP or glucose and degradation of nitroaromatic compounds

P. putida 2NP8 was inoculated into 2-NP/YE (50ml in 250 ml clear flask) and incubated overnight on a rotary shaker at 200 rpm, at 26 °C. To get more growth, 2-NP (40 mg/L) and YE (0.2%) was then added and the latter incubated for 10h. This culture was transferred to 1 L of 2-NP/YE medium. After a 3-h incubation, 2-NP (50 mg/L) and YE (0.25%) was added and the culture was incubated for 11h (overnight). At this time point 2-NP (40 mg/L) and YE (0.2%) was added. After another 3-h incubation, 2-NP (20 mg/L) was added and the culture was incubated for 1 hour. Final cell density was 2.83 (OD_{600nm}, 1cm light path). Cells (4.58g, wet cells) were harvested by centrifuging at 16,300g for 20 min and washed with 100ml of sterile phosphate buffer (KH_2PO_4, 1g/l; Na_2HPO_4 $12H_2O$, 7g/L; pH, 7.35). Cells were used immediately for biotransformation. The specific 2-NP transformation activity was 22 $\mu M/h/mg$ (dry cell weight). Bottles used for biodegradation were 40ml amber glass with the Teflon/silicone septa lined caps. Cells, suspended in phosphate buffer (8 mg wet cells per ml) (OD_{600nm} 5.6, 1 cm light path), containing nitroaromatic compounds substrate (50 mg/L), were incubated on an orbital shaker at 200 rpm and 26°C. Caps of bottles were loosened to maintain aerobic conditions. The ratio of dry cell versus wet cells was 21.7% (wet cells were dried in 85 °C oven, 5 days).

7.3.4. Degradation of nitroaromatic compounds by growing cells

Fresh culture (0.25ml) of *P. putida* 2NP8 grown in YPS medium (overnight), was inoculated into 5 ml of NA/YE media in 25 ml glass tube, containing different nitroaromatic compounds (20 mg/L). The initial OD_{600nm} was 0.31. The cultures were incubated at 26°C at 200 rpm on an orbital shaker. After 24 h, the OD_{600nm} was increased to 0.7-0.9. Samples were taken for centrifugation and the supernatant was analyzed for concentration of substrate remaining in the media and for nitrite produced.

7.3.5. HPLC analysis of substrates and metabolites

The nitroaromatic compounds and metabolites were analyzed using a 4.6 X 250mm ZORBAX SB-C18 HPLC column (Chromatographic Specialties, Brockville, Ontario, Canada). We have previously reported the HPLC instruments, general procedures and methods for NB and 3-NP analysis (Zhao and Ward 1999 and 2000). Biotransformation samples were centrifuged at 9000g for 3 min. For samples containing 3-nitroaniline, 4-nitrocatechol, picric acid, nitrobenzylalcohol and nitrobenzaldehyde, the supernatants were directly injected for HPLC analysis. For other nitroaromatic compounds, the HPLC samples were prepared as follows: 0.45 ml of supernatant was transferred into 2 ml of vial with screw tightened and Teflon-lined caps. Then 0.05 ml of 0.4N HCl solution was added to samples. Ethyl acetate, 0.5 ml, was added and the mixture was vortexed for 1 min. The organic layer liquid was collected and used directly for HPLC analysis. 15μl volumes of samples were injected and eluted with mixture of 60% methanol and 40% milliQ water (0.1% trifluroacetic acid) at a flow rate of 1 ml/min. Compounds were monitored at 254nm.

Nitrite was measured according to US EPA method 354.1 (EPA 1979).

7.4. Results

7.4.1. Nitroaromatic compound induced nitrite-elimination activity in P. putida 2NP8

We have previously reported that *P. putida* 2NP8 degrades 2-NP via release of nitrite (Zhao and Ward 2000). We therefore surveyed the capacity of this strain to degrade 30 nitroaromatic compounds and to produce nitrite in media containing YE for 24h (Table 7.1). Only 2-NP and 4-Cl-2-NP were degraded with production of significant amounts of nitrite, 61μM from 115 μM of 4-Cl-2-NP and 105 μM from 144 μM of 2-NP. A metabolite (A) with a retention time of 3.3min accumulated in the growth media with 4-Cl-2-NP as substrate. No corresponding metabolite was observed with 2-NP. Based on its UV absorbance at 254nm (almost 3 times the absorbance of the initial substrate, 4-Cl-2-NP), we considered it to be a dead end product or a poor substrate for further metabolism. Zeyer and Kearney have reported oxygenase-initiated nitrite elimination from 2-NP in *P. putida* B2 (Zeyer and Kearney 1984). Our strain seemed similar to *P. putida* B2 in that both 4-Cl-2-NP and 2-NP induced nitrite elimination activity, and 4-Cl-2-NP was a poor substrate for further metabolism.

We also analyzed how the culture degraded other nitroaromatic compounds without releasing nitrite. For nitrobenzaldehydes and nitrobenzyl alcohols, we found that they were transformed via oxidation and/or reduction of aldehyde and alcohol groups and the results were presented in Table 7.2. The dominant reaction of 2- nitrobenzyl aldehyde was the reduction into the alcohol product (75%), but 4-nitrobenzaldehyde was preferably oxidized into the acid (92%). The culture degraded less than 50% of all other nitroaromatic compounds except 3,4-dinitrotoluene (72%) and 1-nitronaphthalene (72%), producing little nitrite. Adsorption of the hydrophobic 1-naphthalene to the biomass attributed partly to the loss of substrate in the media, because 50% of the latter was found to be retained to biomass in a suspension containing 8mg/ml of killed cells and 50 mg/L of the compound. Cells growing on the nitroaromatic substrate other than 2-NP and 4-Cl-2-NP had little capacity to produce nitrite.

Table 7.1. Nitroaromatic substrate removal (Rm.) and nitrite production by growing cells of *P. putida* 2NP8.

Nitroaromatics tested	Substrate conc (μM)	Rm.(%)	NO_2^-conc.(μM)
2-nitrophenol	144	100	105
4-chloro-2-nitrophenol	115	100	61
3-nitrophenol	144	100	1
4-chloro-3-nitrophenol	115	12	3
2,4-dinitrophenol	108	17	4
4-nitrophenol	144	8	3
picric acid	87	0	1
3-nitroaniline	145	40	3
4-nitrocatechol	129	28	4
nitrobenzene	162	6	3
1-chloro-4-nitrobenzene	127	49	8
1-chloro-3-nitrobenzene	127	49	11
2-nitrotoluene	146	18	4
4-nitrotoluene	146	54	5
2-nitroanisole	130	20	3
3-nitroanisole	130	31	16
2-nitrobenzoate	120	21	1
3-nitrobenzoate	120	71	1
4-nitrobenzoate	120	39	17
2-nitrobenzylalcohol	130	9	1
3-nitrobenzylalcohol	130	89	2
4-nitrobenzylalcohol	130	38	3
2-nitrobenzaldehyde	132	100	2
4-nitrobenzaldehyde	132	100	7
3-nitrobenzonitrile	135	32	1
2,4-dinitrotoluene	110	28	0
3,4-dinitrotoluene	110	72	4
2,6-dinitrotoluene	110	25	4
2-nitrofuran	177	16	26
1-nitronaphthalene	115	72	3

Table 7.2 Transformation of nitrobenzaldehyde and nitorbenzylalcohol by growing cells of *P. putida* 2NP8 (substrate concentration, 25 mg/L)

A: transformation of nitrobenzaldehyde via attacking on the aldehyde group

Substrates	Product and Yield (%)			
	Alcohol	Yield	Acid	Yield
2-nitrobenzaldehyde	2-nitrobenzyl alcohol	75	2-nitrobenzoic acid	25
4-nitrobenzaldehyde	4-nitrobenzyl alcohol	8	4-nitrobenzoic acid	92

B: oxidation of nitrobenzyl alcohol into nitrobenzoic acid

Substrates	Acid product	Yield (%)
2-nitrobenzylalcohol	2-nitrobenzoic acid	trace
3-nitrobenzylalcohol	3-nitrobenzoic acid	62
4-nitrobenzylalcohol	4-nitrobenzoic acid	40

7.4.2.   2-NP-grown cells catalyzed degradation of nitroaromatic substrate

2-NP-grown whole cells of *P. putida* 2NP8 exhibited good degradation activity toward 2-NP, 4-Cl-2-NP and two nitrobenzaldehydes and completely removed these compounds within 1.5h. The cells degraded some of 3- and 4-nitrobenzyl alcohols, and 3-nitroanisole. No degradation was observed for mono- or di-nitrotoluene, nitrobenzoic acids, 1-nitronapthalene and other compounds, which were substrates of a 3-NP-induced enzyme system in the same strain (unpublished results). Nitrite production patterns in this 2-NP-grown cell media (Table 7.3) were similar to those observed in the growing cell media incubated with individual nitroaromatic substrates. Nitrite accumulation was quantitative from 2-NP. Nitrite yield from 4-Cl-2-NP is 67%. A significant amount of the metabolite A, with a retention time of 3.3 min, was also accumulated in the medium containing 4-Cl-2NP.

Since the retention time of metabolite A was much smaller than those (11.2min) of the substrate (4-Cl-2-NP) under the reversed phase (C-18 column) separation conditions, we concluded metabolite A was a highly hydrophilic compound, a substantially degraded product from 4-Cl-2-NP. According to the mechanism as proposed by Zeyer and Kearney (Zeyer and et al. 1986; Zeyer and Kearney 1984), the possible product from 4-Cl-2-NP is 4-chlorocatechol. Authentic 4-chlorocatechol was not available for confirmation. The retention time (3.8min) of 4-nitrocatechol, a structural analog to 4-chlorocatechol, was very close to that (3.3min) of metabolite A. The metabolite A amount (A_{254nm} value, 6.5 X 10^5) accumulated in the transformation medium was close to that (2.3 X 10^5, 50 mg/L) of the initial substrate, 4-Cl-2-NP, and that (2.4 X 10^6, 50 mg/L) of 4-nitrocatechol. This metabolite A, presumably 4-chlorocatechol, was a poor substrate for further metabolism. 4-nitrocatechol (Table 7.4), an analog to 4-chlorocatechol, was also not a substrate for the 2-NP-induced enzyme system in the whole cells of this strain, supporting this conclusion.

The 2-NP-grown cells exhibited little capacity (5 μM, 3%) to produce nitrite from nitroaromatic substrates other than 2-NP and 4-Cl-2-NP (Table 7.3). Nitrobenzaldehydes and nitrobenzyl alcohols were degraded via attack on the aldehyde or the alcohol group (Table 7.4). Both 2- and 4-nitrobenzaldehyde were predominantly oxidized into the acids, with a lower conversion of 3-nitrobenzyl alcohol into the acid (Table 7.4).

The glucose-grown cells exhibited little nitrite production activity toward all the nitroaromatic compounds including 2-NP and 4-Cl-2-NP. These uninduced cells exhibited

degradation activity toward nitrobenzyl alcohols, nitrobenzaldehydes. Little transformation activity was observed toward all other nitroaromatic compounds (Table 7.3). The transformation reaction patterns of nitrobenzaldehdye and nitrobenzyl alcohol in the glucose-grown cell media were the same as those in 2-NP-grown cells media (Table 7.4).

7.5. Discussions

In this paper, we observed that only 2-NP and 4-Cl-2-NP induced nitrite producing activity from nitroaromatic compounds by P. putida 2NP8 and that the same compounds were the only substrates. Our findings are consistent with an oxygenase-initiated pathway described by Zeyer et al. (1984, 1986, and 1988) who described that a strain of P. putida B2, capable of growing on 2-NP as the sole carbon and nitrogen source which transformed 2-NP into catechol via release of nitrite. The initial 2-NP oxygenase and the followed ring-cleavage catechol 1,2-dioxygenase in P. putida B2 could be induced by a few other 2-NP with substitution at 4-position relative to hydroxyl group, including 4-Cl-2-NP (Zeyer and Kocher 1988). Zeyer et al (1986) observed that 2-NP oxygenase activity toward 4-Cl-2-NP was only 20% of that toward 2-NP while catechol 1,2-dioxygenase toward 4-chlorocatechol was only 4% of that toward catechol and 4-Cl-2-NP was not a growth substrate. The metabolite A, which we concluded to be 4-chlorocatechol, was found as a product of 4-Cl-2-NP transformation in P. putida 2NP8 and was a poor substrate for further metabolism. Bruhn et al (1988) described that 2-NP and 4-Cl-2-NP were inducers and substrates of a nitrophenol oxygenase in a strain of Pseudomonas sp. N31. Strain N31 converted 4-Cl-2-NP into 4-chlorocatechol and converted 2-NP into catechol, but the latter two products could not be further metabolized due to lack of the catechol dioxygenase activity. 2-NP and 4-Cl-2-NP was only nitrogen source for growth of strain N31. They constructed new strains which were able to use 2-NP and 4-Cl-2-NP as the sole carbon and nitrogen source for growth by transferring into the strain the chlorocatechol metabolic genes from those chloroaromatics-degrading bacteria.

Zeyer et al (1986, 1988) reported that the nitrophenol dioxygenase in P. putida B2 had the capacity to degrade many other 2-NP with substitutions at 4-position relative to the hydroxyl group, but did not report the degradation activity of the enzyme on other nitroaroamtic compounds.

Table 7.3 Nitroaromatic compound removal (Rm.) and nitrite production by resting cells (1.5h).

Nitroaromatic substrate	Conc (μM)	Substrate conversion and nitrite production			
		2-NP-grown cells		Glucose-grown cells	
		Rm.(%)	NO$_2^-$conc(μM)	Rm.(%)	NO$_2^-$conc(μM)
2-nitrophenol[a]	287	100	178	9	12
4-chloro-2-nitrophenol[b]	230	100	71	4	4
2,4-dinitrophenol	217	0	5	0	4
3-nitrophenol	287	15	2	0	5
4-chloro-3-nitrophenol	230	13	2	9	4
4-nitrophenol	287	0	0	16	5
picric acid	174	0	0	0	3
3-nitroaniline	290	0	1	2	4
4-nitrocatechol	258	6	2	5	16
nitrobenzene	325	6	1	3	2
1-chloro-4-nitrobenzene	254	4	3	4	3
1-chloro-3-nitrobenzene	254	5	1	14	3
2-nitrotoluene	291	0	7	45	2
4-nitrotoluene	291	0	1	0	2
2-nitroanisole	261	0	1	11	3
3-nitroanisole	261	25	1	17	3
2-nitrobenzoate	239	0	0	7	3
3-nitrobenzoate	239	0	1	0	1
4-nitrobenzoate	239	0	0	0	1
2-nitrobenzylalcohol	261	0	2	0	4
3-nitrobenzylalcohol	261	26	0	15	3
4-nitrobenzylalcohol	261	22	1	10	5
2-nitrobenzaldehyde	265	100	1	100	2
4-nitrobenzaldehyde	265	100	1	100	5
3-nitrobenzonitrile	270	0	2	0	4
2,4-dinitrotoluene	219	0	1	3	7
3,4-dinitrotoluene	219	0	3	0	5
2,6-dinitrotoluene	219	0	2	0	4
2-nitrofuran	354	0	0	0	0
1-nitronaphthalene	230	0	1	0	3

3 h nitrite production: [a]: 280μM; [b]:158μM.

Table 7.4. Transformation of nitrobenzaldehyde and nitorbenzylalcohol by *P. putida* 2NP8 resting cells (substrate concentration, 50 mg/L)

A: transformation of nitrobenzaldehyde via attacking on the aldehyde group

Substrates	Product and yield (%) by cells grown on different media			
	2-NP-grown (1.5h)		Glucose-grown (1.5h)	
	Alcohol	Acid	Alcohol	Acid
2-nitrobenzaldehyde	40	60	40	60
4-nitrobenzaldehyde	0.2	92	0.2	92

B: oxidation of nitrobenzyl alcohol into nitrobenzoic acid

Substrates	Product yield (%) by cells grown on different media			
	2-NP-grown (1.5h)		Glucose-grown(1.5h)	
	Acid product	Yield	Acid product	Yield
2-nitrobenzylalcohol	2-nitrobenzoic acid	trace	2-nitrobenzoic acid	trace
3-nitrobenzylalcohol	3-nitrobenzoic acid	20	3-nitrobenzoic acid	14
4-nitrobenzylalcohol	4-nitrobenzoic acid	3.5	4-nitrobenzoic acid	5

Oxygenases degrading other nitroaromatic compounds via elimination of nitrite have been described, but none of them accept 2-NP as substrate. Monooxygenase-initiated degradation of 4-NP was found to be present in many bacteria, such as *Moraxella* (Spain and Gibson 1991; Spain et al. 1979), actinmycetes (Hanne et al. 1993) and *Arthrobacter* sp (Jain et al. 1994) and *Pseudomonas* sp (Nishino and Spain 1993a). Haigler et al (1993) and Spanggord et al (1991) described a dioxygenase-initiated degradation of 2-nitrotoluene and 2,4-dinitrotoluene respectively in *Pseudomonas* sp. Ali-Sadat et al (1995) observed nitrite-release from 3-NP, an intermediate from 3-nitrotoluene in *Pseudomonas putida*, without characterizing the mechanism. Our results demonstrated the 2-NP-induced enzymes in *P. putida* 2NP8 did not accept the nitroaromatic compounds other than 2-nitrophenols as inducing or reaction substrates.

Knackmuss' group reported (Ebert et al. 1999; Lenke and Knackmuss 1992; Lenke et al. 1992; Rieger et al. 1999; Vorbeck et al. 1994; Vorbeck et al. 1998) release of nitrite through reduction of benzene ring of trinitroaromatic compounds, such as 2,4-dinitrophenol, 2,4,6-trinitrophenol (picric acid) and 2,4,6-trinitrotoluene in certain bacteria. Because the 2-NP-grown cells of *P. putida* 2NP8, did not attack di- or tri-nitroaromatic compounds, it is unlikely that a benzene ring reductase was involved in elimination of nitrite from 2-NP and 4-Cl-2-NP. Therefore we concluded that an oxygenase activity as described by Zeyer et el (1984, 1986), was likely to be responsible for initial metabolism of the growth substrate, 2-NP, in *P. putida* 2NP8 (Figure 7.1).

From our previous results (Zhao and Ward 2000 and unpublished results; Zhao et al. 2000) and those described here, we have demonstrated that *P. putida* 2NP8 has 3-NP- and 2-NP-induced enzyme systems manifesting broad and narrow specificity toward nitroaroamtic substrates, and producing ammonia and nitrite as end products, respectively.
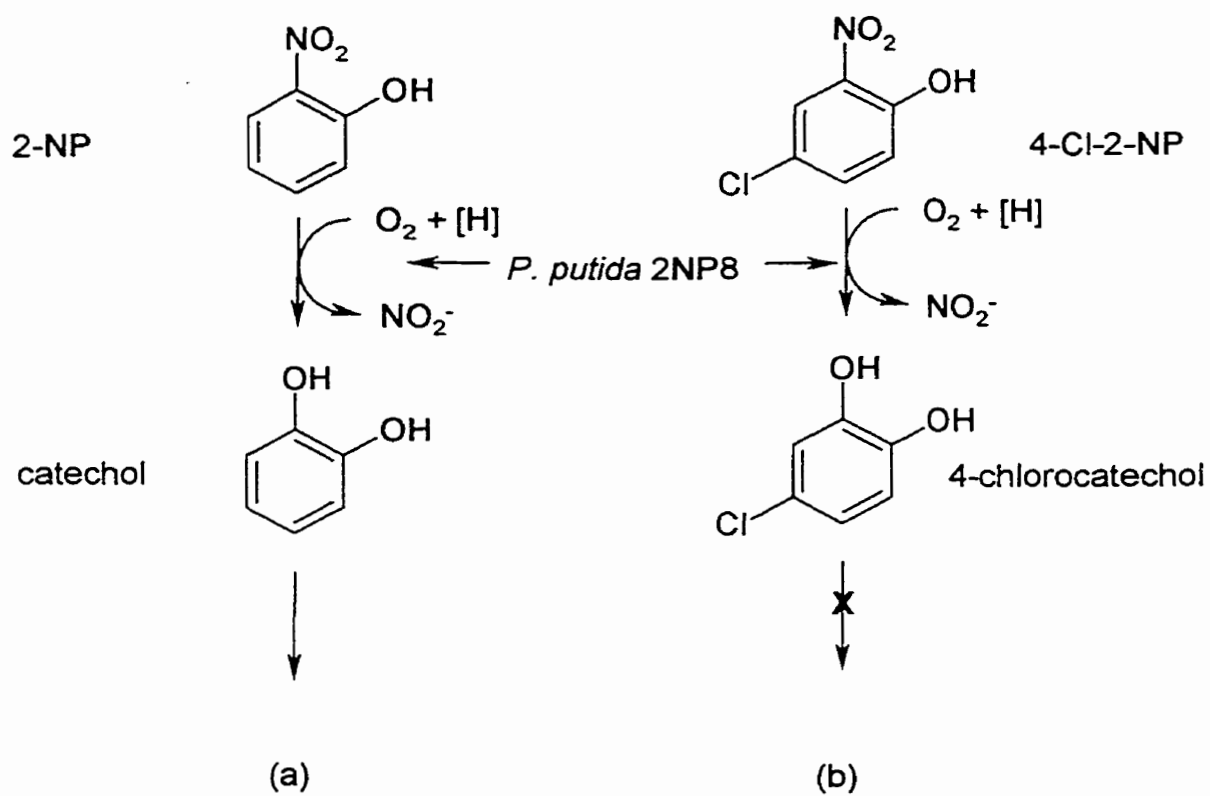
Figure 7.1. Proposed pathway for initial degradation of 2-NP (a) and 4-Cl-2-NP (b) in *P. putida* 2NP8. Catechol was not identified. 4-chlorocatechol was the postulated structure of a metabolite from 4-Cl-2-NP.

8. CONLUSIONS

8.1. Summary of the main findings

An initial study on conversion of nitrobenzene to nitrophenols by electron beam provided the impetus to investigate the interrelationships between biodegradative metabolism of nitrophenols and NB by a mixed culture and pure isolates. The main findings of this study are summarized as follows:

1. Electron beam irradiation of aqueous solution of NB produced three mononitrophenols. A 60 kGy dose of electron beam removed 78% percent of 15-30 mg/L of NB.

2. A mixed culture, isolated from a municipal activated sludge using 2-NP, 3-NP and 4-NP as the sole carbon, nitrogen and energy source, degraded all the mononitrophenols and NB.

3. The mixed culture degraded the nitrophenols and residual NB remained in the electron beam treated sample. A combination of electron beam with biodegradation exhibited better contaminant removal than electron beam treatment or biological degradation alone.

4. Three groups of bacteria were isolated from the mixed culture after intensive sub-culturing on the nitrophenols as the growth substrates. Strains of group A ($2NP^-3NP^-$ $4NP^+$) were *Pseudomonas* species which grew on 4-NP, but not on 2-NP or 3-NP. Strains of group B ($2NP^+3NP^+4NP^-$) were also *Pseudomonas* species, which grew on 2-NP and 3-NP, but not on 4-NP. Strains of group C ($2NP^-3NP^+4NP^-$), grew on 3-NP, but not on 2-NP or 4-NP. One of the two strain types contained in group C was identified as *Variovorax paradoxus*. Degradation of 2- and 4-NP produced nitrite while degradation of 3-NP produced ammonia.

5. Only 3-NP-grown cells of groups B and C transformed NB into ammonia. 2- or 4-NP-grown cells did not transform NB.

6. *Pseudomonas putida* 2NP8, a typical strain of group B, cometabolically degraded NB into ammonia when grow on 3-NP, with nitrosobenzene and hydroxylaminobenzene as intermediates.

7. 3-NP-induced enzymes in *P. putida* 2NP8 transformed hydroxylaminobenzene in the following sequence: hydroxylaminobenzene was converted to 2- or 4-aminophenol; aminophenols were oxidized to benzoquinone monoimine; the imines were hydrolyzed

into quinones and ammonia and the quinones were reduced to hydroquinone and catechol. N-acetylated aminophenols and 2-aminophenoloxazine-3-one were also found as biotransformation products from NB and hydroxylaminobenzene.

8. The degradation pathway of 3-NP in *P. putida* 2NP8 was postulated, based on NB transformation products. An ammonia release pathway via oxidation of aminophenol and the subsequent hydrolysis of imine, was proposed for the first time for degradation of nitroaromatic compounds. This mechanism suggested that 1,2,4-benzenetriol is the dioxygenase ring-cleavage substrate in the 3-NP metabolism.

9. The 3-NP-induced enzyme system in *P. putida* 2NP8, had wide substrate range, and could transform many mono- and di-nitroaromatic compounds into ammonia. Metabolites, similar to those observed in the NB transformation, were observed. 4-NP, 2,4-dinitrophenol, and 2,4,6-trinitrophenol were not substrates for this enzyme system.

10. Only 2-NP and 4-chloro-2-NP, among 30 nitroaromatic compounds examined, were inducers and substrates of the nitrite elimination activity in *P. putida* 2NP8. 4-Cl-catechol was proposed to be a dead end transformation product in this strain.

8.2.   Suggestions for future research

According to the results presented in the thesis, the following aspects of biological degradation of nitroaromatic compounds and electron beam treatment need to be addressed:

1. 1,2,4-Benzenetriol is postulated to be the ring-cleavage substrate in 3-NP metabolism via 3-hydroxylaminophenol and the subsequent transformation into 4-aminocatechol and/or aminohydroquinone. 1,2,4-Benzenetriol was reported in 3-NP degradation in *P. putida* B2 (Meulenberg et al. 1996) while *Ralstonia eutropha* JMP134 transformed 3-NP into aminohydroquinone (Schenzle et al. 1997, 1999a,b). The 3-NP nitroreductase, 3-hydroxylaminophenol mutase, aminophenol oxidase, and 1,2,4-benzenetriol dioxygenase activities in strain *P. putida* 2NP8 need to be characterized to fully describe this pathway. Characterization of 3-NP degradation metabolites is also necessary to validate the proposed 3-NP metabolic pathway.

2. 3-NP is the only nitrogen and carbon source for the pure culture. It will be interesting to investigate how metabolism of a single compound, 3-NP, can meet the demand for growth carbon intermediates, energy and nitrogen source at the same time.

3. Optimization of 3-NP-degradation enzyme biosynthesis and biotransformation conditions for degradation of nitroaromatic compounds into ammonia requires investigation.

4. The 3-NP-induced enzyme system transformed many nitroaromatic compounds into catechol products, which are biodegradable and deemed not to be persistent in the environment. This could provide a remedial solution to some high profile nitroaromatic wastes, such as 2,4,6-trinitritoluene (Rieger and Knackmuss 1995) and nitro-polycyclic aromatic hydrocarbons (Cerniglia and Somerville 1995). Further exploration of degradation of these high-profile nitroaromatics is of special interest.

5. Because of the wide substrate range of this 3-NP-induced enzyme system, elucidation of the 3-NP metabolic genes will be helpful in attempting to understand the regulation and origin of this nitroreductase system.

6. In this research we isolated three types of 3-NP degraders. The 3-NP degrading enzyme activities in two other strains (*Variovorax paradoxus* 3NP23 and unknown strain 3NP20) need to be characterized.

7. Nitroreductase-initiated degradation of NB (Nishino and Spain 1993; Park et al. 1999) and 4-nitrotoluene (Spiess et al. 1998) as bacterial growth substrates has been reported, and the ring cleavage substrates are aminophenols, not catechols. *P. putida* 2NP8 has the genes for metabolism of NB via aminophenol, which was oxidized to hydroquinone and reduced to catechol. This strain seems to have the catechol degradation gene because it metabolized 2-NP via release of nitrite (producing catechol). The following questions remains unanswered: can cells grown on a mixture of 2- or 3-NP mineralize NB? Can this 3-NP degrading strain be engineered to use NB as growth substrate?

8. Cometabolic transformation of NB by the 3-NP-grown cells produced catechol. The wide susbtrate selectivity of the enzyme system suggests that these cells transform many other nitroaromatic compounds into catechol compounds, which are useful fine chemicals and hardly available by chemical methods. Investigation of biotransforamtion of nitroaromatic compounds into catechol compounds will be of interest (Meulenberg and de Pont 1995).

9. One important aspect of the two-step process is the combination of electron beam treatment with biodegradation. Using the electron beam degradation alone to remove all the NB is difficult, but it can significantly decrease the NB concentration to a level suitable to be discharged or to be treated by a subsequent biological system. The results in this thesis

showed that a mixture of three mono-nitrophenols could be used to enrich the culture to degrade both the residual NB and the toxic nitrophenolic products. This raises a question: can we use a mixture of phenolic derivatives of some recalcitrant compounds such as PCBs or PAHs, to enrich cultures that degrade both the phenols and their parent compounds?

A key challenge for degradation of synthetic compounds is to address the initial biodegradation barriers, namely hydrophobicity of the substrate and the presence of electrophilic substituents. Removal of electrophilic substituent and/or increase the polarity and hydrophilicity are two targets to increase the biodegradability (Boethling et al. 1994; Howard et al. 1992). Chemical pretreatment is an important tool for introduction of hydroxyl groups, thereby increasing biodegradability while cometabolism is an important biological method for transforming non-growth-promoting compounds into substrates which can be utilized as microbial nutrients. More attention needs to be focused on both chemical and cometabolic enzyme treatments as methods to degrade those recalcitrant compounds in polluted wastewaters and soils.

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