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Expression, purification and substrate specificities of 3-nitrotoluene dioxygenase from *Diaphorobacter* sp. strain DS2

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ABSTRACT

3-Nitotoluene dioxygenase (3-NTDO) is the first enzyme in the degradation pathway of 3-nitrotoluene (3-NT) by *Diaphorobacter* sp. strain DS2. The complete gene sequences of 3-NTDO were PCR amplified from genomic DNA of *Diaphorobacter* sp., cloned, sequenced and expressed. The 3-NTDO gene revealed a multi component structure having a reductase, a ferredoxin and two oxygenase subunits. Clones expressing the different subunits were constructed in pET21a expression vector system and overexpressed in *E. coli* BL21(DE3) host. Each subunit was individually purified separately to homogeneity. The active recombinant enzyme was reconstituted *in vitro* by mixing all three purified subunits. The reconstituted recombinant enzyme could catalyse biotransformations on a variety of organic aromatics.

1. Introduction

Nitrotoluenes are excessively used in the production of pesticides, drugs, dyes and explosives. These compounds are recalcitrant pollutants [1] and are hazardous to all life forms [2,3]. Several bacterial strains have been reported to utilize 2-NT [4,5], 3-NT [6,7], 4-NT [8–10] nitrobenzene [11], dinitrotoluenes [12,13] as their sole carbon and nitrogen source. Degradation pathways for different nitroaromatics that are present in some of these bacterial strains have been reviewed recently [1]. The degradation process of nitroaromatics is initiated by the action of dioxygenase enzymes. Nitroarene dioxygenase enzyme systems add two oxygen atoms to the benzene ring simultaneously with concomitant release of a nitrite moiety. These are multicomponent enzyme systems. The oxygenase component is the main catalytic subunit whereas the reductase and the ferredoxin subunits are the parts of electron transfer system [14]. Quite a few bacterial nitroarene dioxygenase system genes have been cloned, expressed and studied like nitrobenzene dioxygenase (NBDO) from *Comamonas* sp. strain JS765 [15], 2-chloronitrobenzene dioxygenase from *Pseudomonas stutzeri* strain ZWLR2-1 [16], 2-nitrotoluene dioxygenase (2NTDO) from *Acidovorax* sp. JS42 [17], dinitrotoluene dioxygenase (DNTDO) from *Burkholderi acepacia* R34 [18], and *Burkholderia* sp. strain DNT [19]. Genetic and biochemical characterization of nitroarene dioxygenases have not only given insights into their mechanism but also revealed novel routes for the creation of better biocatalysts [20,21].

Diaphorobacter sp. strain DS2 was recently reported by us to utilize 3-NT as its sole source of carbon, nitrogen and energy [7]. We could also PCR amplify the 3-NTDO gene from the genomic DNA of *Diaphorobacter* species strain DS2 [22]. Here we report the overexpression, purification, reconstitution, substrate specificity and biotransformation of different aromatics by 3-nitrotoluene dioxygenase from the *Diaphorobacter* sp. strain DS2.

2. Material and methods

Phusion® high-fidelity DNA polymerase purchased from Finzymes was used for PCR amplification. Restriction enzymes, T-4 DNA ligase, and primers were purchased from New England Biolabs Company, Bangalore Genei and Bioserve Biotechnologies (Pvt.) Limited respectively. All the chemicals were purchased from Qualigens and of highest purity.

2.1. Bacterial strains, plasmids and media

Diaphorobacter sp. strain DS2 (MTCC No. 11718) was grown as previously reported [7]. *E. coli* DH5α was used for transformation and maintenance of the recombinant plasmids. *E. coli* BL21 (DE3) was used as a host system for expression of recombinant proteins. pET-21a (Novagen) was used as an expression vector. Complete gene sequence of 3-nitrotoluene dioxygenase gene from strain

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DS2 has been determined and already submitted to the Genbank with the accession No. KC691252 [22].

2.2. Construction of expression clones

We constructed pET21a based plasmids for the overexpression of reductase subunit (*mntAa*) with six histidine residues at C-terminus. 1 kb gene was amplified by using primers 5′GCGAATACATATGGAACTGGTAGTAGAACCCCTC3′ and 5′CCGCTCGAGGACACCGATGGGATAGAACGC3′ and ligated at Nde I and XhoI site into pET21a to produce pDS17. To express ferredoxin subunit (*mntAb*), a pair of primers 5′GCGAATACATATGAGCGAGAACTGGATCGACGCC3′ and 5′AAGGAAAAAAGCGGCCGCTTAGTCCAGCTTGAGCATCACGC3′ were used to amplify with NdeI and NotI restriction sites and ligated to pET21a which was previously treated with same set of enzymes to produce pDS2. Similarly oxygenase of 3NTDO (*mntAcAd*) was expressed by using primers 5′GCGAATACATATGAGTTACCAAAACTTAGTGAGTGAAGCAGG3′ and 5′CCGCTCGAGTCACAGGAAGACCAACAGGTTGTG′. Purified PCR amplified fragment was treated with NdeI and XhoI and ligated into pET21a (pre-digested with same set of restriction enzymes and purified) to produce pDS21. These constructs were then transformed into *E. coli* DH5α cells and screened for expression under IPTG induction. Enzyme assays were done as previously reported [4]. Typically, the reaction mixture contains 50 mM MES buffer (pH-6.8), 0.3 mM NADH, 0.3 mM ferrous ammonium sulfate, supernatant (from bacterial cell lysates: alone or in combination) and 1 mM of 3-NT while 3-NT was not added in control experiments. The tube was incubated at 30 °C, at 150 rpm for 2 h. 20 μl of sulfanilamide solution and NEDA solution was added to 200 μl of assay mixture absorbance was recorded at 545 nm after 30 min incubation.

2.3. Cell growth and enzyme purification

BL21(DE3)(pDS17), BL21(DE3)(pDS21) and BL21(DE3)(pDS2) were maintained in LB media plates containing 100 μg/ml ampicillin. For enzyme purification, larger culture LB medium was inoculated with 1% (v/v) of overnight grown culture. When OD at 600 nm was between 0.6 and 0.8, the culture flasks were cooled on ice. The flasks were induced with 0.1 mM IPTG (from stock of 1 M). 0.01% ferrous ammonium sulfate (from stock of 10%) and 0.01% Cysteine (from stock of 10%) was also added to the culture. The flasks were then incubated at 16 °C for 16 h. The cells were harvested by centrifugation (4000*g*, 4 °C and for 10 min), washed with phosphate buffer and resuspended in lysis buffer. The combination of lysis buffer is different for different enzyme components (Table 1).

2.4. Purification of reductase

E. coli BL21(DE3)(pDS17) cell pellets were suspended in lysis buffer (Table 1), sonicated (total time 8 min. 20 s on, 30 s off). The cell debris was removed by centrifugation at 17,000*g* for 45 min at 6 °C. The protein was purified by using a nickel affinity column (10 ml), pre-equilibrated with buffer A (50 mM sodium phosphate buffer, 0.3 M NaCl and 15 mM imidazole, pH-8.0). The enzyme was eluted with buffer A containing 250 mM imidazole and concentrated using ultrafiltration membrane of 30 K.

2.5. Purification of ferredoxin

E. coli BL21(DE3)(pDS2) cell pellets were suspended in lysis buffer containing 50 mM potassium phosphate pH-7 and other components as mentioned in Table 1. The cell extracts after centrifugation were applied to a DEAE-cellulose column (4.4 by 27 cm 300 ml approx.) that had been pre-equilibrated with 600 ml PGD (50 mM phosphate buffer pH 7, 5% glycerol, 0.5 mM DTT) buffer. The column was washed with 900 ml of same buffer at flow rate of 45 ml/h. The bound proteins were eluted with a linear gradient from 0 to 0.8 M KCl (total gradient volume of 1000 ml) at the same flow rate. Fractions exhibiting ferredoxin activity were pooled and concentrated by ultrafiltration using a 10 K cutoff. This was followed by a 30% ammonium sulfate precipitation. The supernatant solution was applied to a bio gel P-60 gel filtration column (1.6 by 75 cm), pre-equilibrated with 50 mM phosphate buffer pH-7.0. The protein was eluted, active fractions pooled and concentrated.

2.6. Purification of oxygenase

E. coli BL21(DE3)(pDS21) cells were suspended in lysis buffer (Table 1). The cell extracts were applied to a DEAE-cellulose column (4.4 by 27) containing approximately 300 ml bed volumes pre-equilibrated with 600 ml MGD (50 mM MES buffer pH 6.8, 5% glycerol, 0.5 mM DTT) buffer. The bound proteins were eluted with linear gradient from 0 to 0.8 M KCl (total gradient volume of 1000 ml). Fractions exhibiting oxygenase activity were pooled and concentrated by ultrafiltration system as described for ferredoxin. After a 20% ammonium sulfate precipitation the supernatant was applied to a butyl-Sepharose column (1.6 by 23, 50 ml). The bound proteins were eluted with a linear gradient from 0.85 to 0 M ammonium sulfate (total gradient volume of 500 ml). Fractions exhibiting oxygenase activity were combined and concentrated as mentioned above.

Cytochrome *c* oxidoreductase assay was used to analyze the reductase activity as reported previously [23], with a difference that the reaction mixture contained 87 μM horse heart cytochrome *c* (Sigma) and 300 μM NADH. The reaction was started by addition of NADH and monitored using absorbance at 550 nm. One unit of activity was defined as the amount of protein that was required for reducing 1 μM of cytochrome *c* per min. The activity was also determined in presence of 1 μM FAD and in presence of ferredoxin component separately.

2.7. Enzyme activity

Ferredoxin, oxygenase and reductase components were exchanged to the same buffer (50 mM MES, pH 6.8) using 3, 100 or 30 KDa centricon system respectively. Oxygenase and ferredoxin activity were calculated by measuring nitrite released method [4]. The assay mixture contained purified reductase and ferredoxin components of 15 and 150 μg respectively. The reaction was initiated by addition of appropriate amount of oxygenase component (100–400 μg) incubated at 30 °C for 5 min with stirring.

2.7.1. Flavin determination

The concentration of FAD present in the reductase was determined by UV–vis spectroscopy (JASCO V550) [24] and fluorescence spectroscopy [25] (Varian Cary eclipse fluorescence spectrophotometer). Typically, after heating the protein at 100 °C for 5 min, the precipitated protein was removed by centrifugation (10,000*g* for 5 min at 4 °C) and the supernatant was analyzed. Using excitation set at 450 nm the emission was recorded at 525 nm. An appropriate calibration curve was made using 0.05 mM stock solution of FAD in water.

Protein concentration was determined by the Bradford method [26] using bovine serum albumin as the standard. Iron content was determined by using Ferene S (Sigma) as described by Zabinski

Table 1
Purification of recombinant 3NTDO components.

Components of 3NTDO[a]	Amount of protein (mg)[b]	Activity[c]		Specific activity (%)	Purification fold
		Units/mg	Units		
Reductase[a]					
Crude cell extract	55	7.78	428[a]	100	–
Ni-affinity chromatography	3.7	49.8	184.26	43	6.4
Ferredoxin[a]					
Crude cell extract	1195	10.53	12583[b]	100	–
DEAE	520	23.2	12064	95	2.2
Bio-gel P-60	108	71.42	7675	60	6.78
Oxygenase[a]					
Crude cell extract	1245	12.5	15562[d]	100	–
DEAE	279	15.6	4359[d]	28	0.8
Butyl Sepharose	60	52.6	3157[d]	20	4.2

[a] Lysis buffer used (i) for reductase: 50 mM sodium phosphate pH-8.0, 0.3 M NaCl, 5% ethanol, 5% glycerol, 1 mM dithiothreitol (DTT). Phenylmethylsulfonyl fluoride (PMSF-0.2 mM), DNase (0.01 mg/ml) and lysozyme (0.25 mg/ml); (ii) for ferrodoxin (50 mM potassium phosphate pH7 and other additives are same as for reductase except NaCl); (iii) for the oxygenase subunits (50 mM MES buffer pH-6.8 and other additives are same as for ferredoxin);
[b] Protein estimation by bradford assay.
[c] One unit was defined as amount of the enzyme required for release of 1 nmol of nitrite per min.
[d] One unit of activity was defined as the amount of protein that was required for reducing 1 μM of cytochrome *c* per min.

et al. [27] and the amount of acid-labile sulfide was determined as described by Chen and Mortenson [28].

2.8. Molecular weight determinations

LC/ESI-MS confirmed the molecular weight of the reductase, the ferredoxin and the oxygenase subunits. 5 μl of protein sample (5 μg) was injected onto a C8 analytical column that was attached to an Agilent 1100 HPLC system coupled with HCT Ultra ETD II Ion trap ESI-mass spectrometer (BrukerDaltonics, Bremen, Germany. Data were analyzed using the software Data Analysis 4.0 (Bruker-Daltonics, Bremen, Germany). This experiment was performed at the proteomics facility of Molecular biophysics unit at Indian Institute of Sciences, Bangalore (India).

2.9. Biotransformation studies

The reactions were performed in a 250 ml flask containing 50 ml volume of the reaction mixture. The reaction mixture composition was same as the one used for enzyme assay. After 1 h, the reaction mixture was acidified (pH 5–6) with dilute HCl. The transformation products were extracted from the reaction mixture with ethylacetate, dried over anhydrous sodium sulfate and concentrated in vacuo. This concentrated sample was analysed by GC–MS and NMR. Conditions for GC–MS analysis were the same as reported earlier [7].

3. Results

In previous work we cloned and sequenced a PCR amplified 4997 base pair DNA region and identified putative reductase, ferredoxin, oxygenase large and small subunits with its regulatory sequence based on its sequence homology to genes in the GenBank [22]. The first step in the degradation of 3-NT is catalyzed by the 3-NTdioxygenase enzyme system. Here we show that different pET21a constructs expressing each subunit separately on purification and reconstitution does indeed catalyze the nitrite removal from 3-NT.

3.1. Reconstitution of active recombinant enzyme

Activity of whole recombinant dioxygenase enzyme was determined by observing nitrite release from 3-NT. Crude extract from *E. coli* with only pET-21a vector and extracts from cells expressing only reductase, ferredoxin and oxygenase subunits alone or in combination of any two subunits did not show any nitrite release separately. Active enzyme was obtained only when the crude extract from all cells expressing three different subunits separately was mixed together. So different subunits were purified to a single band on an SDS page (Fig. 1) and mixed together to reconstitute the active recombinant dioxygenase enzyme system.

The reductase molecular weight was about 36 kDa including six histidine residues at C terminus reconfirmed by ESI-LCMS (Fig. S2A). Solution of this protein was deep orange in color showed absorption maxima at 271, 397, 451, 491(shoulder) and 545 nm (shoulder) (Fig. S1A). Iron and acid labile contents of reductase confirmed the presence of iron and sulfur. Each mole of the reductase contained 0.4–0.5 mol of FAD as determined by both UV spectroscopy and fluorescence spectroscopy. Addition of 1 μM FAD in the assay mixture stimulated its activity and increased the activity by 1.7-fold. Some loss of FAD occurred during the purification of

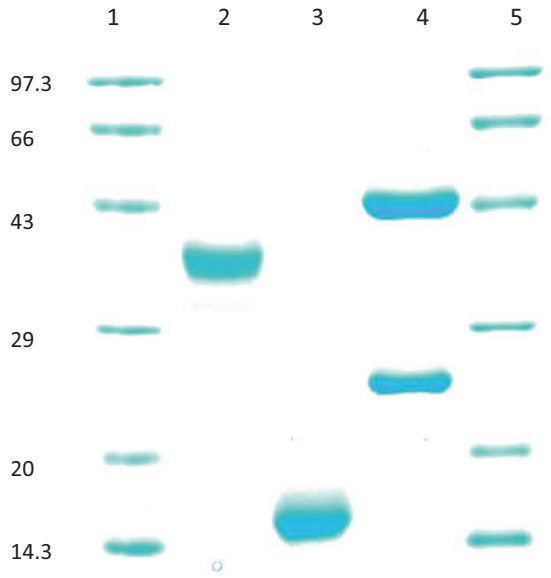


Fig. 1. Coomassie blue stained SDS–PAGE of purified components of 3-nitrotoluene dioxygenase. Lanes 1 and 5: Molecular mass standards (numbers at left are the molecular masses in kDa) Lane 2, Reductase; Lane 3, Ferredoxin; Lane 4, Oxygenase. Each lane was loaded with 25 μg of protein.

reductase. Similarly addition of ferredoxin unit also enhanced its activity.

Ferredoxin subunit was purified in three steps process using anion exchange chromatography, ammonium sulfate precipitation and gel filtration chromatography. The purified protein was dark brown in color and showed absorption maxima at 326, 456 and 580 nm (shoulder) (Fig. S1B). ESI-LCMS of this subunit showed its molecular weight at 11.35 kDa (Fig. S2B) which was in good agreement with molecular weight calculation obtained from its deduced aminoacids. SDS–PAGE analysis revealed that it was a monomeric protein like other reported nitroarene dioxygenase systems.

Oxygenase subunit was purified using a two step procedure involving ion exchange and hydrophobic interaction chromatography (Table 1). The purified protein gave two bands of α and β subunits in SDS–PAGE. The molecular mass (by ESI-LCMS) showed that the α subunit was 51 kDa and β subunit was 22.9 kDa. This matches with molecular mass obtained by calculation of aminoacids of both subunits (Fig. S2C). Rieske [2Fe–2S] center was confirmed by UV–vis spectroscopy and iron and acid-labile sulfur concentration. The U.V. absorption spectrum of the purified oxygenase showed maxima at 566 (shoulder), 462 and 334 nm (Fig. S1C).

3.2. Substrate specificity of recombinant 3NTDO with different compounds

3-NTDO activity was determined by the measurement of nitrite released from 3-nitrotoluene and other aromatic substrates. Even 3-nitrotoluene was not converted completely into methylcatechols as we could detect 7% of 3-nitrobenzylalcohol. The relative substrate specificity for different substrates was calculated considering activity of 3-NTDO for 3-NT to methylcatechols as 100% (Table 2). The enzyme specificity was observed to be highest for 3-NT whereas, 2,4-DNT was the least preferred substrate. The activity with 2-NT and 4-NT was only 71% and 33% respectively (in comparison to 3-NT), that confirmed its preference towards 3-NT. The intensity of the pink color during nitrite detection from different nitroaromatic substrates in 5 min is shown in Fig. 2.

Table 2
Relative substrate specificity of recombinant 3NTDO with different nitroaromatic compounds.

Sl. No.	Substrate	% Relative activity
1	2-Nitrotoluene	71
2	3-Nitrotoluene	100
3	4-Nitrotoluene	33
4	Nitrobenzene	78
5	2,4-Dinitrotoluene	5
6	2,6-Dinitrotoluene	84
7	2-Chloronitrobenzene	80
8	3-Chloronitrobenzene	33
9	4-Chloronitrobenzene	15
10	4-Chloronitrotoluene	27

3.3. Biotransformation of different substrates by recombinant 3NTDO

A number of aromatic compounds were tested for their hydroxylation by recombinant reconstituted 3-NTDO (Table 3). 3-Methylcatechol (58%) was the preferred product over 4-methylcatechol (42%) from 3-NT. A major difference was observed when 4-NT was used as substrate. About 60% product was found to be a dead end product 2-methyl-5-nitrophenol. The remaining 40% was 4-methylcatechol. With 2-nitrotoluene 9% of 2-nitrobenzylalcohol was formed as a very minor product. The remaining 91% was converted into 3-methylcatechol with release of nitrite. Chlorocatechols were observed as products with chloronitrobenzene substrates. Similarly like other reported nitroarene dioxygenases, 3NTDO also functions as a monooxygenase. The conversion of toluene to benzyl alcohol as a major product (92%) with formation of *o*-cresol in very minor amount (8%) is also observed.

4. Discussion

Several dioxygenase enzyme systems have been reported prior to this work [29]. Attempts were made to express the whole 3-NTDO gene (with all its components in one stretch) in a pET-21a vector (this paper). In this effort only reductase and ferredoxin subunits could be expressed. Further, a new construct was created by removing remnants of truncated ORFs [22] and by moving the reductase unit just before ferredoxin failed to express ferredoxin. Therefore different subunits were expressed separately in *E. coli*. When oxygenase was expressed with a *His*-tag at C terminus of the small subunit, the enzyme had no activity. The SDS–PAGE analysis showed that oxygenase small subunit was not present in crude cell extract (Fig. 3, lane 3) whereas was present in the cell pellet (Fig. 3, lane 4). Since the oxygenase forms a hetero-hexamer ($\alpha_3\beta_3$ as reported for other dioxygenases of NDO family of Rieske non heme iron dioxygenases) [14], it may be possible that six histidines present at C terminus of small subunit interfere with dimer formation.

In a separate recombinant oxygenase construct (pDSO5, oxygenase was cloned at NotI site rather than NdeI, prepared but not used for purification) where 25 extra amino acids were present at N terminus of oxygenase large subunit (Fig. 3, lane 1) did not alter the activity. Similarly attachment of His-tag to C terminus of the ferredoxin made it inactive where as 16 extra aminoacids at N terminus did not make it inactive. Hence oxygenase and ferredoxin subunits were expressed without the His-tag and purified separately. Addition of FAD (1 μM final conc.) to the enzyme assay mixture of the reductase stimulated activity and enhanced it by 1.7-fold. This may be because of loss of FAD from its binding site during purification. Loss of FAD during purification was also reported in the case of reductase$_{NAP}$ from *Pseudomonas* sp. strain NCIB 9816-4 [30] and reductase$_{TOL}$ [31] where as reductase$_{2NT}$ [32] and reductase$_{BPH}$ [33] was purified without significant loss of FAD. Oxygenase subunit has been reported to lose its some of the active site mononuclear ferrous iron during purification [29]. There are reports of increase in turnover number on the addition

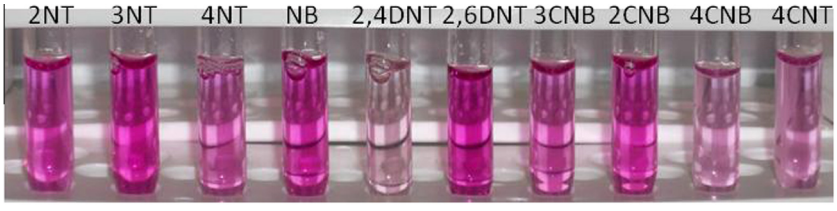
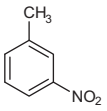
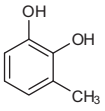
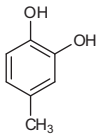
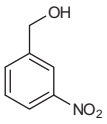
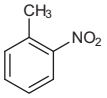
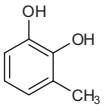
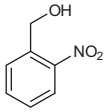
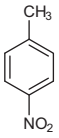
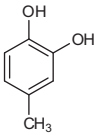
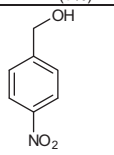
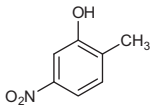
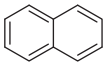
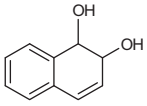
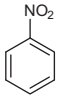
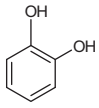
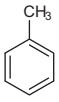
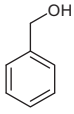
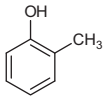
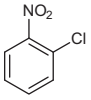
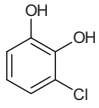
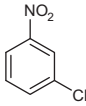
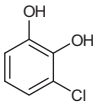
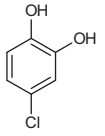


Fig. 2. Nitrite release from different nitroaromatic substrates by 3-NTDO. 2NT: 2-nitrotoluene; 3NT: 3-nitrotoluene; 4NT: 4-nitrotoluene; NB: nitrobenzene; 2,4DNT: 2,4-dintrotoluene; 2,6DNT: 2,6-dinitrotoluene; 3CNB: 3-chloronitrobenzene; 2CNB: 2-chloronitrobenzene; 4CNB: 4-chloronitrobenzene; 4CNT: 4-chloronitrotoluene.

Table 3
Products formed from various compounds by recombinant 3-NTDO. Product ratios were determined from integration of GC–MS total ion current chromatograms.

Substrate	Products formed by 3 NTDO		
 3-nitrotoluene	 3-methyl catechol (54%)	 4-methyl catechol (39%)	 3-nitrobenzyl alcohol (7%)
 2-nitrotoluene	 3-methyl catechol (91%)	 2-nitrobenzyl alcohol (9%)	
 4-nitrotoluene	 4-methyl catechol (34%)	 4-nitrobenzyl alcohol (3%)	 2-methyl-5-nitrophenol (63%)
 naphthalene	 naphthalene cis-1-2-dihydrodiol		
 nitrobenzene	 catechol		
 toluene	 benzyl alcohol (92%)	 o-cresol (8%)	
 2-chloro nitrobenzene	 3-chloro catechol		
 3-chloro nitrobenzene	 3-chloro catechol (8%)	 4-chloro catechol (92%)	

of ferrous iron to the enzyme assay [34]. Ferrous ammonium sulfate was therefore included in the enzyme reaction mixture of oxygenase subunit.

The first intermediate of the 3-NT degradation pathway was not detected during 3-NT degradation by *Diaphorobacter* sp. strain DS2 [7]. As shown in Table 3 that recombinant enzyme transforms 3-NT to catechols as major products where as with whole bacterial cell of strain DS2, these catechols were not detected. This showed that we were able to detect only those intermediates which were dead end products and released into the medium because of non utilization by the microbe [7]. It also showed that why very less biomass increase was observed with 4-NT because a very less fraction of 4-NT was converted to 4-methylcatechol which could be utilized by the microbe for growth. 2-Methyl-5-nitrophenol and 4-nitrobenzyl alcohol did not serve as substrate for biomass growth.

All the nitroarene dioxygenases share a high level of deduced amino acid identity and oxidize similar compounds but their substrate oxidation profiles are different. Like 2NTDO cannot use 2,4-

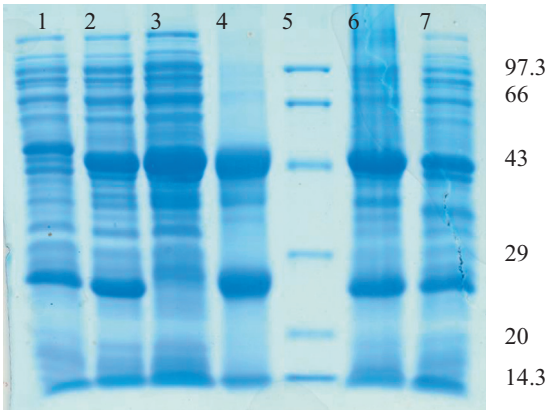


Fig. 3. SDS–PAGE showing different constructs expressing oxygenase. Lane 1: pDSO5 (oxygenase cloned at NotI and XhoI site of pET21a having 25 aminoacids extra at *N*-terminus;); Lane 2: pDSFDO (ferredoxin and oxygenase cloned in pET21a); Lane 3: pDS6; (His tagged at C-terminus of oxygenase small subunit) supernatant; Lane 4: pDS6 cells; Lane 5: marker; Lane 6: pDS21 (oxygenase used for purification); Lane 7: pDS4 (reductase cloned in pDSFDO construct). The gel was stained with coomassie blue.

DNT as substrate while NBDO releases nitrite from 2,4-DNT, even though both 2NTDO and NBDO share very high level of amino acid identity in their catalytic alpha subunit. 3-NTDO shares 90–92% sequence identity with NBDO and 2NTDO. NBDO has shown more than 2-fold greater activity towards 3-nitrotoluene [15]. NBDO produces only 4-methylcatechol from 3-NT where as 3-NTDO prefers 3-methylcatechol over 4-methyl catechol (this work). This activity was reported earlier for 2NTDO [35]. When 4-NT was used as substrate, about 60% product was found to be 2-methyl-5-nitrophenol. This product was also observed with whole bacterial cell of strain DS2 and also reported from *Acidovoarx* sp. strain JS42 [4] but not with other reported dioxygenase enzymes. Similarly, benzyl alcohol was observed as a major product with toluene as a substrate with our enzyme while NBDO converts toluene to toluene *cis*-2,3-dihydrodiol [15]. 3-NTDO converts naphthalene to *cis* 1,2-dihydronaphthalenediol (this study). Similar type of activities was reported by other nitroarene dioxygenases like 2NTDO [36], NBDO [15] and DNTDO [19]. It is a well accepted fact that all the nitroarene dioxygenases have their origin from NDO like systems. During protein evolution of nitroarene dioxygenases, the enzyme might have gained the ability to remove the nitro group while retaining its activity to hydroxylate naphthalene [37]. Mutations in one enzyme and its amino acid role cannot be extended to other nitroarene dioxygenases. Thus every oxygenase can be considered to be a truly different system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.113.

References

[1] K.S. Ju, R.E. Parales, Nitroaromatic compounds, from synthesis to biodegradation, Microbiol. Mol. Biol. Rev. 74 (2010) 250–272.
[2] C. Watanabe, T. Egami, K. Midorikawa, Y. Hiraku, S. Oikawa, S. Kawanishi, M. Murata, DNA damage and estrogenic activity induced by the environmental pollutant 2-nitrotoluene and its metabolite, Environ. Health Prev. Med. 15 (2010) 319–326.
[3] M. Kulkarni, A. Chaudhari, Microbial remediation of nitro-aromatic compounds: an overview, J. Environ. Manage. 85 (2007) 496–512.
[4] B.E. Haigler, W.H. Wallace, J.C. Spain, Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42, Appl. Environ. Microbiol. 60 (1994) 3466–3469.
[5] S.I. Mulla, R.S. Hoskeri, Y.S. Shouche, H.Z. Ninnekar, Biodegradation of 2-nitrotoluene by *Micrococcus* sp. strain SMN-1, Biodegradation 22 (2011) 95–102.
[6] X.J. Tian, X.Y. Liu, H. Liu, S.J. Wang, N.Y. Zhou, Biodegradation of 3-nitrotoluene by *Rhodococcus* sp. strain ZWL3NT, Appl. Microbiol. Biotechnol. 97 (2013) 9217–9223.
[7] D. Singh, G. Ramanathan, Biomineralization of 3-nitrotoluene by *Diaphorobacter* species, Biodegradation 24 (2013) 645–655.
[8] B.E. Haigler, J.C. Spain, Biodegradation of 4-nitrotoluene by *Pseudomonas* sp. strain 4NT, Appl. Environ. Microbiol. 59 (1993) 2239–2243.
[9] W. Rhys-Williams, S.C. Taylor, P.A. Williams, A novel pathway for the catabolism of 4-nitrotoluene by *Pseudomonas*, J. Gen. Microbiol. 139 (1993) 1967–1972.
[10] T. Spiess, F. Desiere, P. Fischer, J.C. Spain, H.J. Knackmuss, H. Lenke, A new 4-nitrotoluene degradation pathway in a *Mycobacterium* strain, Appl. Environ. Microbiol. 64 (1998) 446–452.
[11] S.F. Nishino, J.C. Spain, Oxidative pathway for the biodegradation of nitrobenzene by *Comamonas* sp. strain JS765, Appl. Environ. Microbiol. 61 (1995) 2308–2313.
[12] S.F. Nishino, G.C. Paoli, J.C. Spain, Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene, Appl. Environ. Microbiol. 66 (2000) 2139–2147.
[13] R.J. Spanggord, J.C. Spain, S.F. Nishino, K.E. Mortelmans, Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp, Appl. Environ. Microbiol. 57 (1991) 3200–3205.
[14] D.T. Gibson, R.E. Parales, Aromatic hydrocarbon dioxygenases in environmental biotechnology, Curr. Opin. Biotechnol. 11 (2000) 236–243.
[15] D.J. Lessner, G.R. Johnson, R.E. Parales, J.C. Spain, D.T. Gibson, Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765, Appl. Environ. Microbiol. 68 (2002) 634–641.
[16] H. Liu, S.J. Wang, J.J. Zhang, H. Dai, H. Tang, N.Y. Zhou, Patchwork assembly of nag-like nitroarene dioxygenase genes and the 3-chlorocatechol degradation cluster for evolution of the 2-chloronitrobenzene catabolism pathway in *Pseudomonas stutzeri* ZWLR2-1, Appl. Environ. Microbiol. 77 (2011) 4547–4552.
[17] J.V. Parales, A. Kumar, R.E. Parales, D.T. Gibson, Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42, Gene 181 (1996) 57–61.
[18] G.R. Johnson, R.K. Jain, J.C. Spain, Origins of the 2,4-dinitrotoluene pathway, J. Bacteriol. 184 (2002) 4219–4232.
[19] W.C. Suen, B.E. Haigler, J.C. Spain, 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase, J. Bacteriol. 178 (1996) 4926–4934.
[20] T. Leungsakul, B.G. Keenan, H. Yin, B.F. Smets, T.K. Wood, Saturation mutagenesis of 2,4-DNT dioxygenase of *Burkholderia* sp. strain DNT for enhanced dinitrotoluene degradation, Biotechnol. Bioeng. 92 (2005) 416–426.
[21] B.G. Keenan, T. Leungsakul, B.F. Smets, T.K. Wood, Saturation mutagenesis of *Burkholderia cepacia* R34 2,4-dinitrotoluene dioxygenase at DntAc valine 350 for synthesizing nitrohydroquinone, methylhydroquinone, and methoxyhydroquinone, Appl. Environ. Microbiol. 70 (2004) 3222–3231.
[22] D. Singh, A. Kumari, G. Ramanathan, 3-Nitrotoluene dioxygenase from *Diaphorobacter* sp. strains: cloning, sequencing and evolutionary studies, Biodegradation (2013), http://dx.doi.org/10.1007/s10532-013-9675-9.
[23] T. Ueda, M.J. Coon, Enzymatic oxidation. VII. Reduced diphosphopyridine nucleotide-rubredoxin reductase: properties and function as an electron carrier in hydroxylation, J. Biol. Chem. 247 (1972) 5010–5016.
[24] L.G. Whitby, A new method for preparing flavin-adenine dinucleotide, Biochem. J. 54 (1953) 437–442.
[25] S.G. Mayhew, J.H. Wassink, Determination of FMN and FAD by fluorescence titration with apoflavodoxin, Methods Enzymol. 66 (1980) 217–220.
[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[27] R. Zabinski, E. Munck, P.M. Champion, J.M. Wood, Kinetic and Mossbauer studies on the mechanism of protocatechuic acid 4,5-oxygenase, Biochemistry 11 (1972) 3212–3219.
[28] J.S. Chen, L.E. Mortenson, Inhibition of methylene blue formation during determination of the acid-labile sulfide of iron–sulfur protein samples containing dithionite, Anal. Biochem. 79 (1977) 157–165.
[29] D.J. Ferraro, L. Gakhar, S. Ramaswamy, Rieske business: structure-function of Rieske non-heme oxygenases, Biochem. Biophys. Res. Commun. 338 (2005) 175–190.

[30] B.E. Haigler, D.T. Gibson, Purification and properties of NADH-ferredoxinNAP reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816, J. Bacteriol. 172 (1990) 457–464.
[31] V. Subramanian, T.N. Liu, W.K. Yeh, C.M. Serdar, L.P. Wackett, D.T. Gibson, Purification and properties of ferredoxinTOL. A component of toluene dioxygenase from *Pseudomonas putida* F1, J. Biol. Chem. 260 (1985) 2355–2363.
[32] R.E. Parales, R. Huang, C.L. Yu, J.V. Parales, F.K. Lee, D.J. Lessner, M.M. Ivkovic-Jensen, W. Liu, R. Friemann, S. Ramaswamy, D.T. Gibson, Purification, characterization, and crystallization of the components of the nitrobenzene and 2-nitrotoluene dioxygenase enzyme systems, Appl. Environ. Microbiol. 71 (2005) 3806–3814.
[33] C.L. Yu, W. Liu, D.J. Ferraro, E.N. Brown, J.V. Parales, S. Ramaswamy, G.J. Zylstra, D.T. Gibson, R.E. Parales, Purification, characterization, and crystallization of the components of a biphenyl dioxygenase system from Sphingobium yanoikuyae B1, J. Ind. Microbiol. Biotechnol. 34 (2007) 311–324.
[34] W.C. Suen, D.T. Gibson, Isolation and preliminary characterization of the subunits of the terminal component of naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816-4, J. Bacteriol. 175 (1993) 5877–5881.
[35] K.S. Ju, R.E. Parales, Control of substrate specificity by active-site residues in nitrobenzene dioxygenase, Appl. Environ. Microbiol. 72 (2006) 1817–1824.
[36] J.V. Parales, R.E. Parales, S.M. Resnick, D.T. Gibson, Enzyme specificity of 2-nitrotoluene 2,3-dioxygenase from *Pseudomonas* sp. strain JS42 is determined by the C-terminal region of the alpha subunit of the oxygenase component, J. Bacteriol. 180 (1998) 1194–1199.
[37] K.S. Ju, J.V. Parales, R.E. Parales, Reconstructing the evolutionary history of nitrotoluene detection in the transcriptional regulator NtdR, Mol. Microbiol. 74 (2009) 826–843.