Biodegradation of 2,6-dibromo-4-nitrophenol by *Cupriavidus* sp. strain CNP-8: Kinetics, pathway, genetic and biochemical characterization

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**GRAPHICAL ABSTRACT**

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**ABSTRACT**

Compound 2,6-dibromo-4-nitrophenol (2,6-DBNP) with high cytotoxicity and genotoxicity has been recently identified as an emerging brominated disinfection by-product during chloramination and chlorination of water, and its environmental fate is of great concern. To date, the biodegradation process of 2,6-DBNP is unknown. Herein, *Cupriavidus* sp. strain CNP-8 was reported to be able to utilize 2,6-DBNP as a sole source of carbon, nitrogen and energy. It degraded 2,6-DBNP in concentrations up to 0.7 mM, and the degradation of 2,6-DBNP conformed to Haldane inhibition model with $\mu_{\text{max}}$ of 0.096 h\textsuperscript{-1}, $K_c$ of 0.05 mM and $K_s$ of 0.31 mM. Comparative transcriptome and real-time quantitative PCR analyses suggested that the *hnp* gene cluster was likely responsible for 2,6-DBNP catabolism. Three Hnp proteins were purified and functionally verified. HnpA, a FADH\textsubscript{2}-dependent monooxygenase, was found to catalyze the sequential denitration and debromination of 2,6-DBNP to 6-bromohydroxyquinol (6-BHQ) in the presence of the flavin reductase HnpB. Gene knockout and complementation revealed that *hnpA* is essential for strain CNP-8 to utilize 2,6-DBNP. HnpC, a 6-BHQ 1,2-dioxygenase was proposed to catalyze the ring-cleavage of 6-BHQ during 2,6-DBNP catabolism. These results fill a gap in the understanding of the microbial degradation process and mechanism of 2,6-DBNP.

**Abbreviations:** 2,6-DBNP, 2,6-Dibromo-4-nitrophenol; 2,6-DBBQ, 2,6-dibromo-1,4-benzoquinone; 2,6-DBHQ, 2,6-dibromohydroquinone; 6-BHQ, 6-bromohydroxyquinol

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1. Introduction

Halogenated nitrophenols are widely used in the syntheses of agricultural and industrial chemicals and are common aromatic halogenated disinfection by-products (DBPs) during chlorination and chlorination of water [1,2]. Due to their mutagenic, teratogenic and carcinogenic potential, halogenated nitrophenols in the environment have caused great problems for human health and ecosystems security. As an emerging aromatic halogenated DBP, 2,6-dibromo-4-nitrophenoI (2,6-DBNP) has been identified in drinking water [3], swimming pool water [2] and saline wastewater [4]. Previous toxicological studies have demonstrated that 2,6-DBNP was dozens to hundreds of times more cytotoxic than several regulated DBPs by the U.S. Environmental Protection Agency (EPA) [5,6]. Recently, 2,6-DBNP has also been detected in various mollusks from Bohai Sea, China [7], indicating its potential risk to human by bioaccumulation. Therefore, considering the high toxicity of 2,6-DBNP and its wide distribution, removal of this halogenated nitrophenol from the environment is very meaningful.

Although several physico-chemical methods have been applied for halogenated nitrophenol degradation [1,8], they are not cost-effective and are unable to mineralize the pollutants completely. Microorganisms play key roles in the degradation of halogenated nitrophenols in the environment. Currently, bioremediation was considered to be the most cost-effective and environmentally-friendly means for pollution abatement, and it has been successfully used in treatment of numerous aromatics-contaminated environments [9-15]. Therefore, in order to eliminate 2,6-DBNP from the environment by bioremediation, the essential prerequisite is to obtain a microorganism with the ability to degrade this toxicant.

Brominated nitrophenols (BNPs) are structurally analogues of chlorinated nitrophenols (CNPs) which are the most common halogenated nitrophenols. To date, many microorganisms have evolved their capacity to degrade various isomers of mono-chlorinated nitrophenols (MCNPs) including 2-chloro-4-nitrophenol (24-CN) [16-19], 4-chloro-2-nitrophenol [20], 4-chloro-3-nitrophenol [21] and 2-chloro-5-nitrophenol [22]. Noteworthily, the relative position of nitro and chloro significantly affected MCNPs degradation by microorganisms because of electron delocalization on the aromatic ring. Therefore, the enzyme(s) involved in 24-CN degradation were not able to catalyze the transformation of other MCNP isomers [16]. Moreover, even against the same MCNP substrate, microorganisms have evolved different metabolic pathways [1]. The MCNPs-utilizers may also degrade the corresponding mono-brominated nitrophenols (MBNPs), but there is no experimental evidence available. Further, in contrast to MBNPs, dibrominated nitrophenols (DBNPs) are more toxic and more resistant to microbial degradation because of an additional bromine on the benzene ring. Thus, to date there is no data available on the microbial degradation of DBNPs.

Herein, Cupriavidus sp. strain CNP-8 was proved to be able to completely mineralize 2,6-DBNP. The degradation kinetics of 2,6-DBNP by this strain was investigated. Moreover, the genes responsible for 2,6-DBNP catabolism was identified by comparative transcriptome assay by this strain was investigated. Moreover, the genes responsible for 2,6-DBNP catabolism was identified by comparative transcriptome assay. Comparative analysis of the transcriptomes was performed as described previously [27]. Real-time quantitative PCR (RT-qPCR) was carried out using a TransStart Tip Green qPCR SuperMix kit (TransGen). The primers were listed in Table S1. The PCR reactions were performed in triplicates independently. The relative expression levels of the target genes were estimated using the 2^(-ΔΔCt) method [28] with the 16S rRNA gene as an internal control.

2. Materials and methods

2.1. Strains, plasmids, primers and culture conditions

The bacterial strains and plasmids are described in Table 1, and the primers are listed in Table S1. Cupriavidus sp. strain CNP-8 isolated previously [22] has been deposited in the China Center for Type Culture Collection (accession number: M 2017546). Escherichia coli strains were grown in lysogeny broth (LB) (tryptone: 10 g L\(^{-1}\), NaCl: 10 g L\(^{-1}\) and yeast extract: 5 g L\(^{-1}\)) at 37°C. Cupriavidus strains were grown in minimal medium [23] (MM, without CaCl\(_2\)) at 30°C supplemented with 2,6-DBNP. When necessary, 20% LB (v/v) was added into MM to enhance the biomass. Kanamycin (50 mg L\(^{-1}\)) or chloramphenicol (34 mg L\(^{-1}\)) was added to the medium as necessary.

2.2. Biodegradation of 2,6-DBNP

Strain CNP-8 was initially cultivated in MM + LB (4:1, v/v) overnight. The harvested cells were washed thrice and suspended in fresh MM to an initial OD\(_{600}\) of 0.05; followed by addition of 0.3 mM of 2,6-DBNP. The ability of strain CNP-8 to utilize 2,6-DBNP was determined by monitoring the consumption of 2,6-DBNP, together with the growth of cells (OD\(_{600}\)). To study the effect of simple carbon sources on 2,6-DBNP degradation, lactate, succinate or glucose (0.5 and 0.5 g L\(^{-1}\), respectively) was supplemented into MM containing 2,6-DBNP. Whole-cell biotransformation was carried out as described previously [24]. All above experiments were performed in triplicate.

2.3. 2,6-DBNP degradation kinetics

Degradation of 2,6-DBNP from 0.05 to 1.0 mM by strain CNP-8 was performed to estimate the degradation kinetics parameters. Bacterial growth kinetics was modeled by the equation as follows:

\[
\mu = \frac{\ln (X/X_0)}{t - t_0}
\]

\(X\): biomass concentration, \(\mu\): specific growth rate, \(t\): time.

Considering the toxicity of 2,6-DBNP against strain CNP-8, the Haldane’s model with equation as described was used to investigate the kinetics of 2,6-DBNP degradation [25].

\[
\mu = \frac{\mu_{\text{max}} S}{K_c + S + (S^2/K_i)}
\]

\(S\): 2,6-DBNP concentration, \(\mu_{\text{max}}\): the maximal specific growth rate, \(K_c\): the half saturation constant, \(K_i\): the inhibition constant.

The biomass yield was calculated by the equation as follow [26]:

\[
Y = \frac{X - X_0}{S_0 - S}
\]

\(X_{\text{max}}\): the maximal biomass, \(X_0\): the initial biomass, \(S_0\): the initial 2,6-DBNP concentration, \(S\): the 2,6-DBNP concentration when the biomass concentration is maximal.

2.4. Comparative transcriptome and real-time quantitative PCR

The total RNA from 2,6-DBNP-induced and uninduced cells was isolated by an Easy Pure RNA Kit (TransGen, Biotech, China). Then, cDNA was transcribed from total RNA followed by cDNA library sequencing with Illumina HiSeq 2500 system (Novogene Bioinformatics Technology Co., Ltd., Beijing, China). Reads generated by the sequencing machines were cleaned by discarding the adaptor and low-quality sequences. Subsequently, the clean reads were then assembled and mapped to the genome sequence of strain CNP-8. Comparative analysis of the transcriptomes was performed as described previously [27]. Real-time quantitative PCR (RT-qPCR) was carried out using a TransStart Tip Green qPCR SuperMix kit (TransGen). The primers were listed in Table S1. The PCR reactions were performed in triplicates independently. The relative expression levels of the target genes were estimated using the \(2^{-\Delta\Delta C_t}\) method [28] with the 16S rRNA gene as an internal control.

2.5. Protein expression and purification

The hnpA, hnpB, and hnpC genes were amplified from genomic DNA of strain CNP-8 using FastPfu DNA Polymerase (Transgene, China), with primers listed in Table S1. The PCR product was cloned into the NdeI and XhoI digested pET-28a (+), producing expression plasmids.
### Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cupriavidus</em> sp.</td>
<td>CNP-8</td>
<td>2,6-DBNP user, wild type</td>
</tr>
<tr>
<td>CNP-8hnpA</td>
<td>CNP-8 mutant with hnpA gene deleted</td>
<td>This study</td>
</tr>
<tr>
<td>CNP-8hnpA[pRK-hnpA]</td>
<td>hnpA gene was complemented by pRK-hnpA in CNP-8hnpA</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong> strains</td>
<td>BL21(DE3)</td>
<td>BL21(DE3) harboring the expression plasmids was grown</td>
</tr>
<tr>
<td>D358a</td>
<td>BL21(DE3)</td>
<td>The plasmids with correct sequence of inserted genes were</td>
</tr>
<tr>
<td>WM3064</td>
<td>BL21(DE3)</td>
<td>obtained Tris-HCl buffer, NAD(P)H (0.2mM), FAD (0.02mM) and H6-</td>
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<td></td>
<td></td>
<td>HnpB (5μg) and H6-HnpA (30μg), and the enzyme action system contained</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tris-HCl buffer, NADH (0.2mM), FAD (0.02mM),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H6-HnpB (5μg). The Michaelis-Menten kinetics was investigated by increasing FAD from 0.2 to 20μM when NADH was fixed at 200μM and H6-HnpB (5μg).</td>
</tr>
<tr>
<td>Plasmids</td>
<td>pET-28a (+)</td>
<td>Expression vector, Kan^8</td>
</tr>
<tr>
<td>pK18mobpsacB</td>
<td>Gene replacement vector derived from plasmid pK18; Mob^+ sacB^- Km^7</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pRK415</td>
<td>Broad host range vector, Te^8</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pXM19</td>
<td>Source of chloramphenicol resistance gene (cm)</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pET-hnpA</td>
<td>Ndel-Xhol fragment containing hnpA inserted into pET-28a (+)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pET-hnpB</td>
<td>Ndel-Xhol fragment containing hnpB inserted into pET-28a (+)</td>
</tr>
<tr>
<td></td>
<td>pET-hnpC</td>
<td>Ndel-Xhol fragment containing hnpC inserted into pET-28a (+)</td>
</tr>
<tr>
<td></td>
<td>pK18-hnpA</td>
<td>hnpA gene knockout vector containing DNA fragments homologous to the upstream and downstream regions of the hnpA and cm genes</td>
</tr>
<tr>
<td></td>
<td>pRK-hnpA</td>
<td>hnpA gene complementation vector by cloning hnpA into the KpnI/EcoRI restriction site of pRK415</td>
</tr>
</tbody>
</table>

The plasmids with correct sequence of inserted genes were transformed into *E. coli* BL21(DE3). The protein expression and purification was carried out as described previously [29], with some modification. BL21(DE3) harboring the expression plasmids was grown in LB at 37 °C to OD600 of about 0.5, and then induced with 0.2mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 12h at 16°C. The harvested cells were resuspended in binding buffer (50mM sodium phosphate, 150mM NaCl, pH 8.0) and disrupted by ultrasonication. The cellular debris was removed by centrifugation at 20,000 × g for 30 min at 4 °C, and the supernatants were applied to Ni-NTA agarose (GE Healthcare, USA) pre-equilibrated with binding buffer. Agarose column was washed sequentially with binding buffer containing 20, 40 and 80mM imidazole to remove nonspecifically bound proteins. Finally, binding buffer containing 200mM imidazole was used to elute the histag Hnp proteins. The purified proteins were dialyzed against binding buffer to remove imidazole, and their purities and molecular weights were determined by SDS-PAGE. Protein concentration was measured by the Bradford assay [30].

#### 2.6. Enzyme assays

All the enzyme assays were carried out at 30 °C, in 50mM Tris-HCl buffer (pH 7.5) in a total volume of 1ml using a Perkin Elmer Lambda 356 UV/Vis spectrophotometer. The activity of H6-HnpB was determined spectrophotometrically by measuring the decrease of NAD(P) H at 340 nm (ε340 = 6220 M−1 cm−1) [31]. The reaction system contained Tris-HCl buffer, NAD(P)H (0.2mM), FAD (0.02mM) and H6-HnpB (5μg). The Michaelis-Menten kinetics was investigated by increasing FAD from 0.2 to 20 μM when NADH was fixed at 200μM or increasing NADH from 10 to 200μM when FAD was fixed at 20 μM.

For the activity assay of H6-HnpA against 2,6-DBNP, the 1ml reaction system contained Tris-HCl buffer, NADH (0.2mM), FAD (0.02mM), H6-HnpB (5μg) and H6-HnpA (30μg), and the enzyme activity was assayed by monitoring the decrease of 2,6-DBNP at 400 nm. The enzyme kinetics was investigated by increasing 2,6-DBNP from 2.5 to 40μM. The concentration of 2,6-DBNP was determined by HPLC analysis. One unit of enzyme activity was defined as the quantity of enzyme needed to consume 1μmol substrate in 1 min. To identify the products of 2,6-DBNP, the products in the reaction system (10ml) were initially acetylated as described previously [31] and then identified by GC–MS analysis. Because 6-bromohydroxyquinol was not commercially available, its structure analogue hydroxyquinol was used to assay H6-HnpC activity.

The 1ml reaction system contained Tris-HCl buffer, ascorbic acid (1mM), HnpC (2μg) and hydroxyquinol (0.1mM). The activity of H6-HnpC was determined by monitoring the consumption of hydroxyquinol (λmax = 289 nm) and formation of maleylacetate (λmax = 243 nm).

#### 2.7. Gene knockout and complementation

The upstream (800 bp) and downstream (849 bp) fragments of hnpA were amplified from the genomic DNA and the chloramphenicol resistance gene (cm) was amplified from pXM19 (Table 1), with primers listed in Table S1. These fragments were ligated into pK18mobpsacB (pre-digested with EcoRI and HindIII) using In-Fusion HD Cloning Kit (Takara) to yield the suicide plasmid pK18-hnpA. The plasmid was then transformed into mobilizer strain *E. coli* WM3064 (Table 1). The suicide plasmid in WM3064 was transformed to strain CNP-8 by biparental mating [32,33]. The double-crossover recombinant of was screened on LB plates containing sucrose (10%, w/v) and chloramphenicol (34μg/ml), and the mutant strain CNP-8hnpA was confirmed by PCR analysis. hnpA amplified by PCR was digested by KpnI/EcoRI and then ligated into the KpnI/EcoRI-digested pRK415 (Table 1), yielding plasmid pRK-hnpA. It was transformed into WM3064, and then introduced into the mutant strain by biparental mating, yielding complementary strain CNP-8hnpA[pRK-hnpA]. The ability of mutant and complementary strains to utilize 2,6-DBNP was determined by monitoring the substrate degradation and the cell growth.

#### 2.8. Analytical methods

The concentrations of 2,6-DBNP and the acetylated products of 2,6-DBNP were identified by GC–MS analysis as described [31]. The nitrite concentration was determined by colorimetric method as described previously [35]. Bromide ion was determined on ion chromatography (Dionex ICS-3000) equipped with an Ion Pac As14 column (4 × 250, Dionex), with the eluant of NaHCO3 (8 mM) and Na2CO3 (3.5 mM) at a flow rate of 1.0 ml min−1 [36].
Cells), SRP150666, SRP150667 and SRP150670 (three biological replicates of uninduced cells). The nucleotide sequence has been deposited in NCBI under GenBank accession number MH271067. The transcriptome data have been deposited in the Sequence Read Archive database of NCBI with accession numbers SRP150659, SRP150660, SRP150665 (three biological replicates of uninduced cells), SRP150666, SRP150667 and SRP150670 (three biological replicates of 2,6-DBNP-induced cells). The nucleotide sequence has been deposited in NCBI under GenBank accession number MH271067.

3. Results and discussion

3.1. Degradation of 2,6-DBNP by strain CNP-8

As an emerging aromatic halogenated DBP, 2,6-DBNP has been recently identified in various water environment, especially in China [2–4,7]. However, very little is known about the biodegradation process of 2,6-DBNP to data. The time course assay of 2,6-DBNP degradation, together with the growth of CNP-8 and accumulation of nitrite and bromide ions is shown in Fig. 1A. After a delay period of approximately 10 h, strain CNP-8 rapidly degraded 0.3 mM 2,6-DBNP to undetectable levels within 25 h, and the OD_{600} increased from 0.051 to 0.103. 0.57 mM of bromide was released when 2,6-DBNP was degraded completely, indicating that CNP-8 could remove both bromines from 2,6-DBNP. The non-stoichiometric release of nitrite during 2,6-DBNP degradation is owing to that CNP-8 utilized it as the source of nitrogen. No degradation of 2,6-DBNP was observed in a negative control without inoculation of CNP-8 cells. These results revealed that strain CNP-8 can degrade 2,6-DBNP, and utilize it as sole carbon, nitrogen and energy sources for growth. In addition, this strain was also able to degrade 2,6-dichloro-4-nitrophenol, the chlorinated analogue of 2,6-DBNP, and utilize it for cell growth (data not shown). Bio-transformation showed that the 2,6-DBNP-induced CNP-8 (OD_{600} = 2) degrade 0.3 mM 2,6-DBNP within 30 min, while no apparent degradation of 2,6-DBNP was observed by the un-induced cells during this period (Fig. 1B), indicating that the enzymes responsible for 2,6-DBNP degradation were inducible. To our knowledge, CNP-8 is the first strain reported for 2,6-DBNP utilization. The studies on 2,6-DBNP degradation by this strain will greatly improve our understanding of the fate of 2,6-DBNP in the environment.

3.2. Effect of additional carbon on 2,6-DBNP biodegradation

Three simple carbon sources including lactate, succinate and glucose (0.5 and 5 g/L, respectively) was added into MM in the presence of 0.3 mM 2,6-DBNP to determine their effect on 2,6-DBNP degradation by strain CNP-8 (Table S2). All supplemented carbon sources at concentrations of either 0.5 or 5 g/L supported the growth of CNP-8 to different extent. For substrate degradation, addition of lactate, succinate and glucose at concentration of 0.5 g/L was found to accelerate 2,6-DBNP degradation, and succinate supplement resulted in higher degradation rate than other to carbon sources. However, the degradation rate of 2,6-DBNP decreased significantly with addition of 5 g/L carbon sources, although the biomass yield had a remarkable increase. This suggested that addition of easily degraded substrate at low concentration was beneficial for the degradation of 2,6-DBNP by CNP-8, but the excessive addition would cause the delayed degradation of 2,6-DBNP. Similar phenomena have also been reported in the microbial degradation of some other aromatic compounds [25,37]. Therefore, it is our view that addition of low concentration of simple carbon sources is an advisable strategy when CNP-8 was used for bioremediation of 2,6-DBNP-contaminated environments.

3.3. Kinetic studies of 2,6-DBNP degradation

Because strain CNP-8 is the first microorganism degrading 2,6-DBNP, the kinetic parameters for 2,6-DBNP biodegradation was unavailable in the literature. However, this knowledge is very valuable in evaluating the capacity of microorganisms in biodegradation process [16]. In this study, degradation of different concentrations of 2,6-DBNP was carried out to study the effect of initial substrate concentration on CNP-8 growth. This strain degraded 2,6-DBNP completely when the initial concentration was no more than 0.7 mM, and the delay periods of both 2,6-DBNP degradation and CNP-8 growth extended with the increase of 2,6-DBNP concentration (Fig. 2). Neither 2,6-DBNP degradation nor CNP-8 growth was observed when initial 2,6-DBNP was 1 mM (data not shown). These indicated that high concentration 2,6-DBNP could inhibit the growth of strain CNP-8. Indeed, aromatic compounds exhibiting growth inhibition against their utilizers at high concentration was inevitable [16,25,37–39]. Subsequently, Haldane’s growth kinetics model, widely used to depict growth kinetics of inhibitory compounds [37,39,40], was used to determine the kinetic parameters of 2,6-DBNP biodegradation. Experimental and predicted specific growth rates of CNP-8 according to Haldane’s model were shown in Fig. 3. The maximal specific growth rate (μ_{max}) was 0.096 h^{-1} when 2,6-DBNP concentration was about 0.11 mM. Particularly, the specific growth rate increased with an increase of initial 2,6-DBNP to a certain concentration, and then decreased on further increasing 2,6-DBNP concentration, similar with the microbial degradation studies of phenol [25], 4-chlorophenol [39] and 2,4,6-trinitrophenol [40]. The half saturation constant (K_s) and inhibition constant (K_i) for 2,6-DBNP degradation by CNP-8 was 0.05 mM and 0.31 mM, respectively. By comparing the kinetic parameters of 2,6-DBNP with those of a much simpler pollutant 2C4NP [26], CNP-8 had lower substrate affinity and degradation rate for 2,6-DBNP, and higher inhibition by 2,6-DBNP as compared to 2C4NP. This is likely due to that 2,6-DBNP is higher toxic...
and more resistant to microorganism than 2C4NP.

The biomass yield coefficient was estimated according to Eq. (3). The yield coefficient changed from 0.05 to 0.19 mg mg\(^{-1}\) when the initial concentration of 2,6-DBNP varied from 0.05 to 0.7 mM, and seemed to be too low. It is probably attributed to that a larger portion of the molecular weight of 2,6-DBNP was provided by two bromine atoms. The value of yield coefficient changed slightly at low 2,6-DBNP concentrations varying from 0.05 to 0.2 mM. Beyond 0.2 mM, the yield coefficient decreased with the increase of 2,6-DBNP up to 0.7 mM. This is likely due to that more energy coming from 2,6-DBNP degradation was needed to overcome the effect of 2,6-DBNP inhibition. Similarly, decrease of yield coefficient with increase of initial substrate concentration in the inhibitory range have also been reported during the microbial degradation of some other aromatics [16,25]. Overall, although substrate inhibition was inevitable, \(K_i\) was extremely low as compared to \(K_s\) during 2,6-DBNP degradation, indicating that CNP-8 was capable to degrade 2,6-DBNP from the kinetic viewpoint.

3.4. Prediction of 2,6-DBNP catabolic genes by comparative transcriptome analysis and RT-qPCR

Above biotransformation assay showed that the enzymes involved in the catabolism of 2,6-DBNP were inducible; therefore, comparative transcriptome analysis was initially carried out to find the potential genes responsible for 2,6-DBNP degradation. The results revealed that the transcription levels of 25 genes were increased over 30-fold in 2,6-DBNP-induced CNP-8 as compared to the uninduced cells (Table S3). Generally, the genes responsible for catabolism of nitroaromatic compounds are clustered in the genome. Therefore, based on the annotations of the 25 up-regulated genes, a gene cluster containing GM005195 to GM005200 (59 to 648-fold up-regulated) was predicted to be responsible for 2,6-DBNP catabolism. The cluster was named as \(hnp\) cluster, as annotated and outlined in Fig. 4A. Among the enzymes encoded by these \(hnp\) genes, HnpA, HnpB and HnpC showed high identity to the FADH\(_2\)-dependent 2,4,6-trichlorophenol (2,4,6-TCP) monooxygenase (TcpA), putative flavin reductase (TcpB) and 6-chlorohydroxyquinol 1,2-dioxygenase (TcpC), respectively [41]. HnpD is homologous to the malicenolate reductases (PnpF), which was reported to convert 2-chloromaleylacetate to \(\beta\)-ketoadipate via malicenolate [34]. HnpR, a LysR regulatory protein, was proposed to control \(hnp\) gene expression as it is homologous to the transcriptional regulator TcpR in 2,4,6-TCP degradation [42]. To verify the transcriptome results, we analyzed the mRNA levels of four \(hnp\) genes by RT-qPCR. The transcription levels of \(hnpA\), \(hnpB\), \(hnpC\) and \(hnpD\) in 2,6-DBNP-induced cells increased 254-, 160-, 143- and 37-fold, respectively as compared to the uninduced cells (Fig. 4B), similar to the transcriptome data.

3.5. Expression and purification of HnpA, HnpB and HnpC

To further confirm whether \(hnp\) genes are responsible for 2,6-DBNP catabolism, the functions of the enzymes encoded by \(hnpA\), \(hnpB\) and \(hnpC\) were validated experimentally. HnpA, HnpB and HnpC were expressed as N-terminal His\(_6\)-tagged fusion proteins in \(E. coli\) BL21(DE3). All three proteins were substantially soluble in \(E. coli\) grown at 16°C and easy for purification. Approximately 10.8 mg H\(_6\)-HnpA, 12.5 mg H\(_6\)-HnpB and 24.4 mg H\(_6\)-HnpC were, respectively, purified from 1L of culture medium. SDS-PAGE analysis showed that the molecular masses of the denatured H\(_6\)-HnpA, H\(_6\)-HnpB and H\(_6\)-HnpC were approximately 59, 24, and 31 kDa (Fig. 5), respectively, which were consistent with the deduced molecular mass from their amino acid sequence.

3.6. HnpB catalyzes the reduction of FAD

The flavin reductase activity of HnpB was assayed by monitoring the disappearance of the NAD(P)H (\(\lambda_{max}=340\) nm) in the presence of FAD. NADH (\(\lambda_{max}=340\) nm) was rapidly consumed by purified H\(_6\)-HnpB with the production of NAD\(^+\) (\(\lambda_{max}=260\) nm) (Fig. S1A). No NADH consumption occurred when HnpB was omitted from the reaction system (Fig. S1B). HnpB exhibited the maximum FAD-reducing activity (5.4 U mg\(^{-1}\)) in the presence of NADH. Only 30% relative activity was observed when NADH was replaced by NADPH, indicating that NADH was preferred to NADPH as the co-substrate of HnpB. The \(K_m\) values of HnpB for FAD and NADH were 5.4 ± 1.1 and 28.7 ± 4.7 \(\mu\)M, respectively.
3.7. HnpA catalyzes the sequential denitration and debromination of 2,6-DBNP to 6-bromohydroxyquinolin in the presence of HnpB

Purified H6-HnpA alone did not degrade 2,6-DBNP in the presence of NADH and FAD. However, rapid degradation of 2,6-DBNP (λ<sub>max</sub> = 400 nm) occurred with the addition of H6-HnpB, together with consumption of NADH (λ<sub>max</sub> = 340 nm) (Fig. 6A). In addition, the formation of an isobestic point at 280 nm indicated that 2,6-DBNP was transformed to a new product (λ<sub>max</sub> ≈ 250 nm). These results indicated that FADH₂ provided by HnpB is necessary for the activity of HnpA, similar with the reports of the FADH₂-dependent monooxygenase (TcpA) involved in catabolism of 2,4,6-TCP [43]. The maximal specific activity of HnpA against 2,6-DBNP was 0.27 ± 0.05 U mg⁻¹. Enzyme kinetic assays showed that HnpA had a K<sub>m</sub> of 5.3 ± 1.2 μM and a K<sub>cat</sub>/K<sub>m</sub> ratio of 0.09 ± 0.02 μM⁻¹ min⁻¹ for 2,6-DBNP.

E. coli BL21(DE3) carrying pET-hnpA alone was found to degrade 2,6-DBNP, although there is no HnpB (Fig. S2). This is likely due to that HnpA is able to use free FADH₂ coming from reduction of FAD by the indigenous NADH-FAD oxidoreductase in E. coli [44], similar with the reports for several other halogenated aromatics monooxygenases [31,43]. Notably, there is no significant degradation of 2,6-DBNP by BL21(DE3)-pET-hnpA under anaerobic conditions, indicating that O₂ is essential for the catalytic activity of HnpA against 2,6-DBNP (Fig. S2).

For product identification, ascorbic acid (1 mM) was used to prevent the autooxidation of products from 2,6-DBNP catalyzed by HnpA. Two compounds with GC retention times of 17.7 and 22.1 min, respectively, were detected during the GC-MS identification of the acetylated products (Fig. S2). This is likely due to the fact that HnpA is able to use free FADH₂ coming from reduction of FAD by the indigenous NADH-FAD oxidoreductase in E. coli [44], similar with the reports for several other halogenated aromatics monooxygenases [31,43]. Notably, there is no significant degradation of 2,6-DBNP by BL21(DE3)-pET-hnpA under anaerobic conditions, indicating that O₂ is essential for the catalytic activity of HnpA against 2,6-DBNP (Fig. S2).

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Fig. 6. Spectral changes during the transformation of 2,6-DBNP by purified HnpAB (A) and the transformation of hydroxyquinol by purified HnpC (B).

Fig. 7. Identification of the acetylated products of 2,6-DBNP by GC–MS. (A) The gas chromatogram is the extracted ion current chromatogram at m/z 268 ± 0.50 and 204 ± 0.50 from the total ion current chromatogram. (B) The mass spectra of acetylated 2,6-DBHQ. (C) The mass spectra of acetylated 6-BHQ.
H6-HnpC catalyzed the consumption of hydroxyquinol (λ_{max} = 289 nm), together the formation of a product with a λ_{max} of 243 nm, in line with the spectral property of maleylacetate [47]. No apparent degradation of HQ was observed for the negative control (no HnpC). Considering that HnpC has high identity with the 6-CHlorohydroxyquinol (6-CHQ) 1,2-dioxygenase (TeqC), which was reported to transform 6-CHQ to 2-CHloromaleylacetate (2-CMA) [41]. Moreover, hnpC is clustered with hnpA and highly transcribed in 2,6-DBNP-induced CNP-8. These suggested that HnpC is responsible for ring-cleavage of 6-BHQ during 2,6-DBNP degradation (Fig. 4C). The activity of HnpD (maleylacetate reductase) was not characterized in this study. However, previous studies have shown that maleylacetate reductases could catalyze the dechlorination reaction of 2-CMA to generate β-ketoadipate via MA [34,48]. Moreover, hnpD is located downstream of hnpC and up-regulated in 2,6-DBNP-induced CNP-8. Therefore, HnpD was proposed to be responsible for debromination of 2-BMA to β-ketoadipate via MA during 2,6-DBNP degradation (Fig. 4C).

### 3.9. hnpA is essential for strain CNP-8 to utilize 2,6-DBNP

To verify the physiological role of HnpA in 2,6-DBNP catabolism in vivo, an hnpA deletion mutant strain CNP-8ΔhnpA was constructed by homologous recombination. Strain CNP-8ΔhnpA lost the ability to grow on 2,6-DBNP, and hnpA gene complementation of the mutant CNP-8ΔhnpA (CNP-8ΔhnpA[pRK-hnpA]), introduction of the plasmid pRK-hnpA recovered the ability to utilize 2,6-DBNP (Fig. 8). Thus, hnpA1 was necessary for CNP-8 to utilize 2,6-DBNP for growth.

### 4. Conclusions

_Cupriavidus_ sp. strain CNP-8 was the first microorganism capable to utilize 2,6-DBNP. It could degrade 2,6-DBNP in concentrations as high as 0.7 mM, with μ_{max} = 0.096 h^{-1}, K_{s} = 0.05 mM and K_{m} = 0.31 mM. The hnp gene cluster responsible for 2,6-DBNP degradation was identified, and three enzymes were purified and functionally verified. HnpA could catalyze the sequential denitration and debromination of 2,6-DBNP to 6-BHQ in the presence of HnpB. HnpC likely catalyzed the ring-cleavage of 6-BHQ during 2,6-DBNP catabolism. HnpA was found to be essential for CNP-8 to utilize 2,6-DBNP by gene knockout and complementation. This report fills a gap in the understanding of the microbial 2,6-DBNP degradation.