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Key Enzymes in the Nitroglycerin Degradation Pathway

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Abstract

Nitroglycerin (NG), widely used in explosives, propellants and pharmaceuticals, can be found in contaminated groundwater and soil and poses potential hazards to the environment. *Arthrobacter sp*. JBH1 was the first bacterial isolate able to grow on nitroglycerin (NG) as the sole source of carbon and nitrogen. The initial reaction and some of the enzymes involved in the NG transformation pathway were proposed, but the genes that encode the initial enzymes and the mechanism of release of the third nitrate group were unknown. In order to rigorously establish the degradation pathway, four old yellow enzyme (OYE) homologs from the genome of JBH1, and a glycerol kinase homolog, MngP, involved in the lower NG transformation pathway were overexpressed and purified from *E. coli*. The enzyme assays showed that the old yellow enzyme, PfvA, was 8 times more effective than PfvC in catalyzing the initial step of NG transformation. In addition to 1,2-dinitroglycerin (1,2-DNG), 1,3-DNG was a major denitration product. PfvA could also catalyze the transformation of both DNG isomers to 1-MNG and/or 2-MNG. 1-MNG was then subject to phosphorylation by MngP. Another key finding of this study is that the phosphorylated 1-MNG is a substrate for PfvC, which can catalyze removal of the last nitro group to produce glycerol-3-phosphate that enters central metabolism.

Keywords: Nitroglycerin Degradation; Hazards; Environment; Mammals

Introduction

Nitroglycerin (NG) has been used globally as an explosive for over 100 years [1]. It is toxic to fish (LD50=1 mg/L) and mammals (30-1300 mg/kg) [2]. NG and its partially denitrated products are widely distributed in groundwater and soil at explosives manufacturing and handling facilities in the U.S. where they pose potential hazards to the environment [3-8]. High concentrations of NG and its partially denitrated products have created intense selective pressure which has led to the evolution of microbes able to tolerate and degrade NG [3]. Various bacteria can metabolize NG via reductive elimination of nitrite and production of 1,2-dinitroglycerin (1,2-DNG), 1,3-dinitroglycerin (1,3-DNG), 1-mononitroglycerin (1-MNG), and 2-mononitroglycerin (2-MNG). The enzymes involved in the denitration process are nitrate ester reductases, including *Enterobacter clocae* PB2 PETN reductase (Onr) [4], *Agrobacterium radiobacter* GTN reductase (NerA) [5,6], *Bacillus subtilis* flavin oxidoreductase (YqjM) [7], and *Pseudomonas putida* xenobiotic reductases

(XenA and XenB) [3]. They form part of the Old yellow Enzyme (OYE) family and share similarity in structure and function.

The catalytic mechanisms and ligand binding properties of OYEs have been studied extensively [8-14]. The crystal structure reveals that the single subunit OYE consists of eight stranded α/β- barrels. The structure is quite similar to those of some other flavoproteins such as trimethylamine dehydrogenase, glycolate oxidase and flavocytochrome b2 [15], which evolved from a common ancestral flavoprotein. These flavoenzymes incorporate a flavin mononucleotide (FMN) non-covalently bound in the active site. FMN binds to the protein via eight hydrogen bonds involving Thr-37, Gln-114, Arg-348 and Arg-243. NAD(P)H can stack on FMN and form hydrogen bonding with His-191, Asn-194, and Tyr 375 [9]. When catalyzing NG transformation, OYE forms hydrogen bonds with the terminal nitrate ester via His-191 and Asn-194, and with secondary nitrate groups via Tyr-196 [13]. The active site amino acids are highly conserved in the OYE family and mutation of these amino acids results in decreased binding affinity or NG reduction rate [8,13]. The regioselectivity of the denitration of NG varies among OYEs from different species due to diversity of the amino acids in the active site of the enzymes. For example, OYE isolated from brewer's bottom yeast produced 1,2-DNG and 1,3-DNG from NG in a ratio of 1:1.7, which indicated a preference for the C-2 over C-1 or C-3 nitrate [13]. An even stronger preference for the C-2 nitrate ester group was observed in pure cultures of *Agrobacterium radiobacter*, with a 1,2-DNG to 1,3-DNG ratio of 1:8 [16]. It is worth noting that 1,2-DNG and 1-MNG have chiral centers, which is an indication that the enzymes involved in NG transformation are enantioselective and multiple enzymes may be required for the NG transformation pathway [17-19].

Arthrobacter sp. strain JBH1 isolated by Husserl, et al. [20] is so far the only bacterial strain capable of growing on NG as the sole source of carbon and nitrogen. The sequential denitration of NG by JBH1 results in transient appearance of 1,2-DNG and 1-MNG, catalyzed by a flavoprotein, believed to be (PfvC), a member of the OYE family (NADPH oxidoreductase; EC 1.6.99.1) (Figure 1), and accumulation of trace amounts of 2-MNG, which cannot be degraded by JBH1. Meah, et al. [21] reported that another old yellow enzyme (OYE; EC 1.6.99.1), sharing 29 % identity in amino acid sequence with PfvC, produced both 1,2-DNG and 1,3-DNG from NG, and 2-MNG was further produced from 1,2-DNG. In the previous study with JBH1, however, 1,3-DNG was not detected although it can serve as a carbon and nitrogen source for growth. The above observations suggest that the enzymes catalyzing the denitration of NG are highly specific, since the degradation pathway does not seem to involve 1,3-DNG or 2-MNG. The degradation pathway in JBH1 differs from those observed for other pure cultures, in which both 1,2-DNG and 1,3-DNG are produced [22,23]; some strains even show a strong preference for the nitrate ester at C-2 over C-1 [16,24]

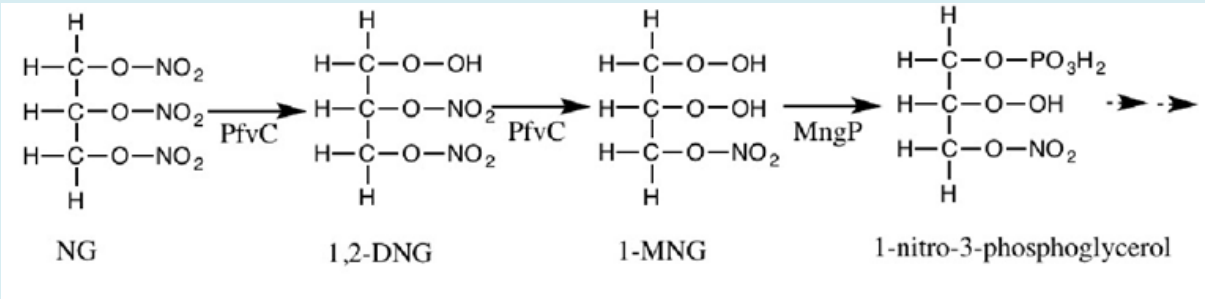

Figure 1: The NG transformation pathway proposed by Husserl, et al. [25]. NG was exclusively transformed to 1, 2-DNG by an old yellow enzyme, PfvC, which also catalyzed the transformation of 1,2-DNG to 1-MNG.

In Figure 2, the structural information on OYEs provides a good reference for the prediction of the activity and function of any given OYE homolog [9,13]. There are 4 OYE homologs (*pfvA*, *pfvB*, *pfvC*, and *pfvD*) in the JBH1 genome. A local sequence alignment of a well-conserved segment among the OYEs reveals that Asn-194, His-191, Tyr-196 and Thr-37 are also conserved in PfvA and PfvD (Figure 2). Therefore, in this study we tested the hypothesis that other OYEs in JBH1, especially PfvA and PfvD, play a role in the removal of nitrate ester groups of NG.

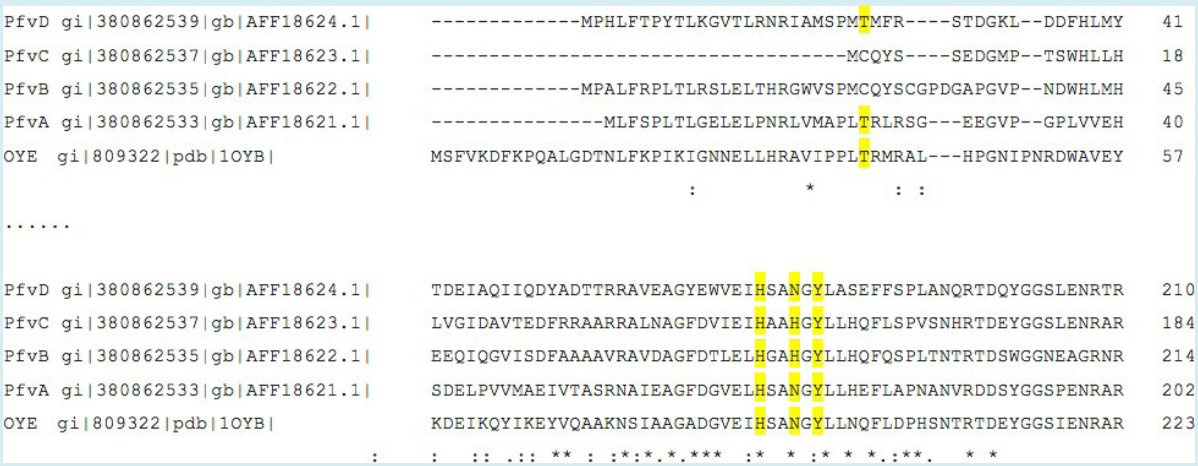

Figure 2: Local sequence alignment of a well-conserved segment among the OYEs in JBH1 and Brewer's yeast OYE. Conserved amino acids at the active site are highlighted.

The genes encoding OYEs in JBH1 were previously cloned and overexpressed in *E.coli* and the activities of the enzymes towards NG, DNG and MNG were tested in cell extracts [25]. Disappearance of NG was observed in the control carrying empty pET24 plasmids, suggesting that an *E.coli* nitroreductase was also active towards NG. *E.coli* clones expressing the genes of the 4 OYEs (*pfvA*, *pfvB*, *pfvC* and *pfvD*) only showed slightly faster rates of NG removal. The reaction kinetics and product composition were not studied, and the enzyme that catalyzed the denitration of NG was identified as PfvC based on the fact that it produced the highest amount of 1-MNG at the end of the reaction. Three other OYEs were excluded from the pathway because their production of 1-MNG in crude cell extracts was similar to that of the control [25]. The above preliminary observations did not rigorously establish the roles of the enzymes involved in NG degradation in JBH1, and many questions remained about the pathway. First, the NG to 1-MNG conversion is a two-step process. NG is first converted to 1,2-DNG, which is further converted to 1-MNG. Either step could be a rate-determining step for different OYEs. Therefore, it is possible that multiple enzymes with different regiospecificities are involved in different stages of NG transformation by JBH1. Second, background reductase activity in *E.coli* must be eliminated to establish a rigorous mass balance and evaluate the contributions of each of the 4 OYEs in catalyzing the denitration process. Overall, it is necessary to consider all OYE homologs in JBH1, examine their expression levels, compare their activities towards NG, 1,2-DNG and 1,3-DNG, and evaluate their roles in NG transformation in JBH1.

Previous work provided preliminary evidence that 1-MNG transformation was accomplished by an ATP-dependent glycerol kinase homolog, MngP (ATP: glycerol-3-phosphotransferase; EC 2.7.1.30) [25]. Glycerol kinase can catalyze the formation of glycerol-3-phosphate by transferring a phosphate group from ATP to glycerol [26-28]. A similar mechanism was proposed for 1-MNG phosphorylation [25]. In preliminary experiments LC/MS analysis revealed a product with the same molecular weight as the phosphorylation product of 1-MNG, 1-nitro-3-phosphoglycerol, which was not subject to further transformation in MngP-catalyzed reactions. Stoichiometric production of 1-nitro-3 phosphoglycerol was not established, and additional studies are therefore needed to understand how phosphorylation facilitates the subsequent assimilation of 1-nitro-3-phosphoglycerol in JBH1 and how the last nitrate ester group is removed to achieve mineralization of NG. Nitrite release from 1-MNG by *E.coli* overexpressing MngP suggests that a nitroreductase with broad substrate specificity may be involved in the denitration. The phosphate group on C-1 may increase the binding affinity toward NG by formation of hydrogen bonding with amino acids in the active center of the enzyme, and therefore make the reduction of the C-3 nitrate ester possible. We hypothesize that one or multiple OYEs (PfvA, PfvB, PfvC or PvfD) in JBH1 could be catalyzing the removal of the final nitrate ester group of 1-nitro-3-phosphoglycerol and producing glycerol-3-phosphate, which can readily enter central metabolism.

To address the above questions and to complete the NG transformation pathway, we examined the expression levels of the 4 OYEs and MngP in JBH1 cells and established the pathway by cloning, overexpression and purification of the key enzymes believed to be involved, validating the pathway *in vitro*.

Materials and Methods

Chemicals

NG, 1,2-DNG, 1,3-DNG, 1-MNG and 2-MNG standard solutions in acetonitrile used for enzyme essays were purchased from Cerilliant (Round Rock, TX). NG for JBH1 growth was synthesized as described previously [29].

Analytical

NG, 1,2-DNG, 1,3-DNG and 1-MNG were quantified using an Agilent 1100 high performance liquid chromatograph (HPLC) equipped with supelco LC-18 column (250 X 4.6mm, 5 um). Methanol-water (50% [vol/vol]) was used as the mobile phase at a flow rate of 1 ml/min and absorbance was monitored at 210 nm. Nitrite was quantified by a colorimetric method 4500-NO_2^-B [30]. Total protein was quantified using a Micro BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL).

Strains and Growth Conditions

JBH1 cells were grown on mineral medium supplemented with NG (0.26mM) at room temperature as previously described [25]. *Escherichia coli* containing expression plasmids were grown on Luria-Bertani broth (LB) supplemented with kanamycin (50mg/L) and chloramphenicol (30 mg/L) at 37°C or room temperature.

Cloning of Genes

To eliminate the rare codons in *mngP*, the sequence was optimized for expression in *E.coli* (Invitrogen Life Technologies, Carlsbad, CA). *pfvA*, *pfvB*, *pfvC*, *pfvD* and *mngP* were amplified from genomic DNA of JBH1 by PCR (Table 1). The purified PCR products were ligated into NdeI and XhoI sites of the pET-28a vector to include a 6×His-tag (Invitrogen Corp, Carlsbad, CA). The recombinant plasmids were transformed into *E.coli* DH5α (New England BioLabs, Ipswich, MA) to maintain the plasmid or into *E.coli* Rosetta 2(DE3) competent cells (Novagen) for over expression.

Strain, plasmid or primer Strains	Description or sequence
Arthrobacter sp. strain JBH1	Utilize NG as sole source of carbon and nitrogen
Escherichia coli Rosetta 2 (DE3)	F^- *ompT hsd*SB(rB– mB–) *gal dcm* (DE3) pRARE2[3] (Cam^R); overexpression host
Plasmids	
pET28a	Kanr; 5,369-bp overexpression vector
pPfvA	Kanr; pET28a with pfvA from Arthrobacter sp. strain JBH1
pPfvB	Kanr; pET28a with pfvA from Arthrobacter sp. strain JBH1
pPfvC	Kanr; pET28a with pfvC from Arthrobacter sp. strain JBH1
pPfvD	Kanr; pET28a with pfvD from Arthrobacter sp. strain JBH1
pMNGP	Kanr; pET28a with mngP from Arthrobacter sp. strain JBH1
PCR Primers	
pfvA-F	5'-ATT CAC CAT ATG ATG CTG TTT TCC CCG TTG
pfvA-R	5'- ATA TAT CTC GAG TTA GCC CGC GTA CGC
pfvB-F	5'- AGA CCT CAT ATG ATG TGC CAG TAC TCC TC
pfvB-R	5'- TAT ATC GAA TTC TCA GTC CCC CTT AGC CT
pfvC-F	5'-AAC G|AA TTC GTG CCG GCA CTG TTC CGG
pfvC-R	5'-AAC A|AG CTT AAA CGA ATG CCG GGG CAC
pfvD-F	5'-AGA CTA CAT ATG ATG CCG CAT CTC TTC AC
pfvD-R	5'- ATA TAT CTC GAG TCA TAA GCC GTC AGA GG
pMNGP-F	5'- AGA ATA GAA TTC GTA CCA GTG AGC GAC TAC
pMNGP-R	5'-AGA ATA AAG CTT GGC CAC GTC CTC GT

Table 1: List of strains, plasmids, PCR primers and RT-PCR primers used in this study.

RNA Isolation and Quantitative Real-Time PCR

For isolation of total RNA, JBH1 grown on ¼ MSB supplemented with NG (0.26 mM) or LB were harvested during exponential growth phase and RNA was extracted using TRI Reagent according to the manufacturer's protocol (T9424, Sigma, St. Louis, MO). Residual genomic DNA was removed using a TURBO DNA-free kit (Invitrogen). Reverse transcription was performed with an Applied Biosystems High Capacity RNA-to-cDNA Kit using 630 ng of DNase-treated RNA. Oligonucleotide primers (Table 2) were designed using the Primer Express 3.0 software (Applied Biosystems). Each RT-PCR reaction mixture (20 uL) contained cDNA (14ng), forward and reverses primers (250 nM), and 2×*Power* SYBR Green PCR Master Mix (10uL, Applied Biosystems, Carlsbad, CA). RT PCR was performed on an ABI 7500 Fast Real-Time PCR System using the manufacturer's protocol. RT-PCR data was analyzed by the comparative CT method (i.e. $2^{-\Delta\Delta CT}$ method) described previously [31]. The differences in expression of *pfvA*, *pfvB*, *pfvC*, *pfvD* and *mngP* in JBH1 grown on NG compared to those in cells grown on LB was determined. The constitutively expressed 16S rRNA gene was used as an internal control, due to the consistency of its expression level when cells were grown in minimal medium containing NG or in LB.

Target Gene	***pfvA***			***pfvC***			***pfvD***			***mngP***		
Replicate	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
CT mean_NG grown	27.68	27.55	27.43	28.26	28.11	27.93	26.18	26.05	26.16	27.64	27.50	27.530
CT mean_LB grown	27.06	27.02	27.02	27.69	27.83	27.75	25.30	25.51	25.30	27.36	27.44	27.60
Fold Change	1.09	1.24	1.37	1.13	1.48	1.60	0.91	1.24	0.99	1.63	1.88	2.14
Fold Change_Mean	1.23			1.40			1.05			1.67		
Standard Deviation	0.14			0.24			0.17			0.26		
Internal Control Gene	**16S rRNA**											
Replicate	**1**	**2**	**3**									
CT mean_NG grown	15.96	15.88	16.09									
CT mean_LB grown	15.21	15.03	15.23									

Table 2: RT-PCR raw data. All experiments were performed in triplicate. Fold change due to NG treatment $2^{-\Delta\Delta CT}=2^{-[(CT\ target\ gene-CT\ 16S\ rRNA)NG\ grown-(CT\ target\ gene-CT\ 16S\ rRNA)LB\ grown]}$.

Overexpression and Purification of Protein

Single colonies from the transformation plate were transferred into 2mL of LB with kanamycin (50 mg/L) and chloramphenicol (30 mg/L). The cultures were incubated at 37°C overnight with shaking, transferred into 200 mL of fresh LB/antibiotic medium at a ratio of 1:50 then incubated at 37 °C with shaking until the OD_{600} reached 0.6-0.8. IPTG was added to a final concentration of 0.4 mM and cells were incubated for 12 hours at room temperature. Cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS) (Cold Spring Protocols) once and resuspended in lysis buffer (6 mL, 1× PBS, 0.3 M NaCl, 10 mM imidazole; pH=7.4). The washed cells were lysed with a French pressure cell at 20,000 lb/in^2, and cell debris was removed by centrifugation (40,000 × g, 4°C, 2 hours). The supernatant was collected for Ni-NTA column purification. Buffers used in purification are described as follows. Ni-NTA column purification buffers include Binding Buffer (1× PBS, 0.3 M NaCl, 10 mM imidazole; pH=7.4), Wash Buffer 1 (1 × PBS, 0.3 M NaCl, 20 mM imidazole; pH=7.4), Wash Buffer 2 (1×PBS, 0.3 M NaCl, 40mM imidazole; pH=7.4) and Elute Buffer (1× PBS, 0.3 M NaCl, 250 mM imidazole; pH=7.4). Appropriate elution buffers and a 1-mL HisTrap HP column was used to collect the target proteins according to the manufacturer's protocol (GE Healthcare, Pittsburgh, PA). Excess salt and imidazole in the eluted fractions were removed with an Amicon ultra centrifugal filter devices (Membrane NMWL=30 kDa) and the final total salt concentration was less than 200uM.

Enzyme assays

His-tags were removed from the proteins by incubation with thrombin (EMD Millipore, Billerica, MA) at 4°C overnight. Enzyme assays were conducted at 30°C. PfvA and PfvC were assayed in PBS buffer containing protein (25-100 ug /ml) and NG (70 uM). An NADPH regenerating system consisting of NADP (1 mM), glucose-6-phosphate (5 mM), and glucose-6-phosphate dehydrogenase (5 Unit/mL) was added to start the reaction. Samples were collected and analyzed by HPLC at appropriate intervals for NG, DNG, and MNG. MngP was assayed in PBS buffer containing MngP (25-100 ug/

ml), MNG (70 uM), ATP (300 uM) and MgCl2 (750 uM). The intermediates were analyzed at appropriate intervals by HPLC as described above or LC-MS by the Bioanalytical Mass Spectrometry Facility at Georgia Institute of Technology (Atlanta, GA) [25].

Results

Regulation of expression of Key Enzymes in Response to NG

The NG transformation pathway in JBH1 appeared to be constitutive based on the fact that NG transformation by resting cells of JBH1 was not affected by growth substrate [25]. The previous study does not provide any evidence about how the activities of the 4 OYE homologs (*pfvA*, *pfvB*, *pfvC*, and *pfvD*) and the glycerol kinase homolog (*mngP*) are affected when JBH1 is exposed to NG. Our working hypothesis was that sequential denitration of NG in JBH1 requires the involvement of multiple enzymes with different regiospecificities. To investigate the regulation of the enzyme(s) that catalyze the NG denitration, comparative RT-PCR was performed with cells grown on NG or LB and harvested during the exponential growth phase to determine the relative expression levels of the related genes (Table 2).

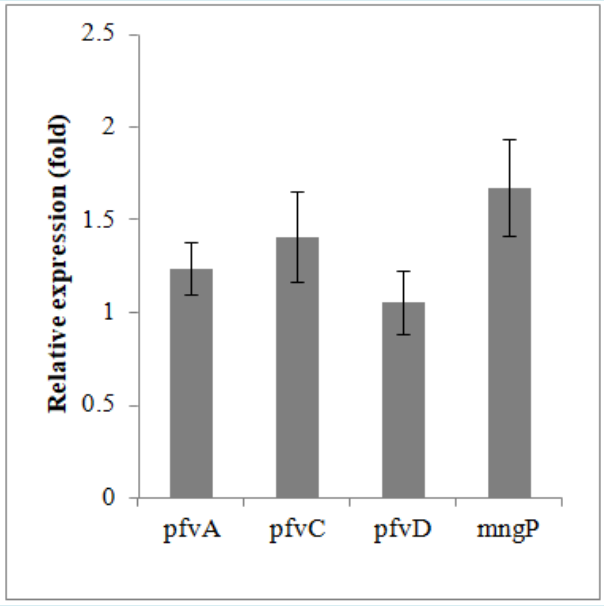

Figure 3: Comparison of the OYE gene expression in JBH1 grown in the absence and presence of NG. Relative expression levels were calculated with respect to the 16S rRNA gene.

Figure 3 summarizes the fold change of the expression levels of the 4 genes during growth on NG as compared to LB. The expression of *pfvB* was not detected in either NG or LB-grown cells and was not plotted in the figure. *pfvA*, *pfvC*, *pfvD* and *mngP* showed modest increase in expression levels when grown on NG (1.23 ± 0.14, 1.40 ± 0.24, 1.05 ± 0.17, and 1.67 ± 0.26 respectively) compared to growth on LB. A student t-test was performed on the data to determine whether the differences in expression level were statistically significant. Only *mngP* had a tvalue greater than t-critical ($\alpha = 0.05$), which suggested that the *mngP* gene was upregulated in NG grown cells. *pfvA*, *pfvC* and *pfvD* seemed to be constitutively expressed in JBH1 cells.

Overexpression and Purification of the Key Enzymes

The purified proteins showed correct sizes on the SDS-PAGE gels (Figure 4). When the activity of purified OYEs was tested PfvB and PfvD did not catalyze denitration of NG. Therefore, denitration of NG by *E. coli* cell extracts overexpressing the two enzymes in previous studies [25] was probably due to background reductase activity in *E. coli*. On the other hand, PfvA and PfvC catalyzed rapid transformation of NG with concomitant nitrite release. The result suggested that one or both of the enzymes were responsible for the initial steps in NG transformation in JBH1 (Figure 5).

Optimization of the *mngP* gene sequence by elimination of rare codons greatly improved its overexpression level in *E. coli*. The activity of MngP was verified using 1-MNG as the substrate. 1-MNG was rapidly transformed by MngP without the release of nitrite. Reaction mixtures without added ATP or $MgCl_2$ did not transform 1-MNG.

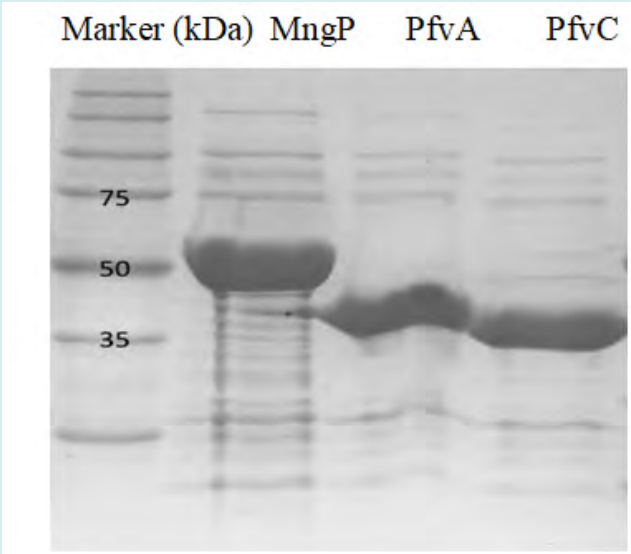

Figure 4: SDS-PAGE of MngP, PfvA and PfvC after thrombin cleavage.

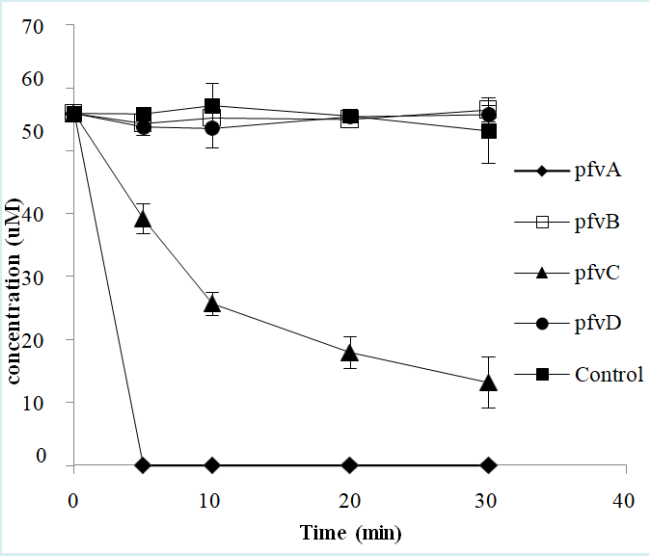

Figure 5: Comparison of activities of purified flavoproteins using NG as substrate. Protein concentrations were 50 ug/L.

Transformation of NG by PfvA and PfvC

Dinitration of NG is required in the overall process of NG mineralization. In order to determine the product distribution of the denitration reactions, purified enzymes PfvA and PfvC were incubated with NG. 1,2-DNG and 1,3-DNG were first produced from NG in PfvA and PfvC catalyzed reactions (Figure 6). In previous studies 1,3-DNG accumulation was not observed during growth of JBH1 on NG, or during transformation of NG by cell extracts of *E.coli* overexpressing *pfvC* [20,25]. When NG was depleted, DNG concentrations started to decrease, with the appearance of 1-MNG and 2-MNG. 2-MNG accounted for 2.1 % of the starting material in PfvA catalyzed reactions, and 6.9% in reactions catalyzed by PfvC. MNG isomers were not transformed further by PfvA or PfvC under the conditions tested. There was no transformation of NG or DNGs in the controls in which no enzymes were added (data not shown).

JBH1 produces a distinct array of metabolites during NG degradation: exclusively 1,2-DNG, 1-MNG and trace amounts of 2-MNG, whereas 1,3-DNG, a common denitration product from NG, does not accumulate in the medium [20,25]. Therefore, studies on the properties and reaction kinetics of PfvA and PfvC were conducted to better understand the degradation of NG by JBH1. The specific activity of PfvA at 30°C was 0.38±0.06 umol/min/mg, about 8 times higher than that of PfvC which had a specific activity of 0.05±0.01 umol/min/mg. PfvA and PfvC catalyzed reactions with NG as the initial substrate resulted in different product compositions. When NG was depleted, the ratio of 1,3-DNG to 1,2-DNG was about 5:1 in the reaction catalyzed by PfvA, while the ratio was about 1:1 by PfvC. Assuming that the denitration process was irreversible, and that the conversion from NG to DNG isomers was much faster than the conversion from DNG to MNG isomers, the ratios of the mixture of DNG isomers suggested that in PfvA catalyzed reactions, the reduction of the secondary nitrate was about 10 times faster than the primary nitrate, because statistically, the odds of removing the primary nitrate was twice that of the secondary nitrate. On the other hand, PfvC had a much smaller preference in the removal of the secondary nitrate than the primary nitrate. The finding that PfvA was much more effective in removing the secondary nitrate ester group of NG (assuming similar expression levels in wild type cells), indicates that instead of PfvC, PfvA is likely to be the physiological enzyme that catalyzes the initial denitration of NG.

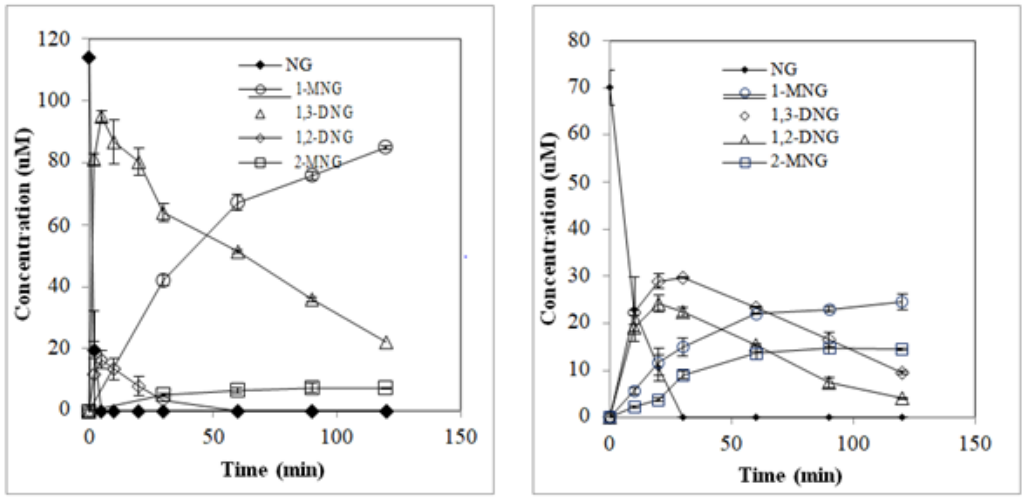

Figure 6: NG transformation by PfvA (A) and PfvC (B).

Transformation of DNG by PfvA and PfvC

The elimination of the first nitrate ester group of NG by each purified OYE produced 1,3-DNG and to a lesser extent, 1,2-DNG (Figure 7). To establish the product distribution of the reduction of the second nitrate ester group, the transformation of DNGs by PfvA and PfvC was investigated. Both enzymes transformed 1,3-DNG to 1-MNG. PfvA had a faster reaction rate with 1,2-DNG than PfvC. In addition, the two OYEs yielded different product distributions in the 1,2-DNG reduction reactions. PfvA transformed 1,2-DNG to 1-MNG and 2-MNG in the ratio of 1:1, while the ratio was 1:2 in reactions catalyzed by PfvC. The fact that these two OYEs had similar specific activities towards the DNG substrates suggests that both of them are involved in the denitration of DNG in JBH1.

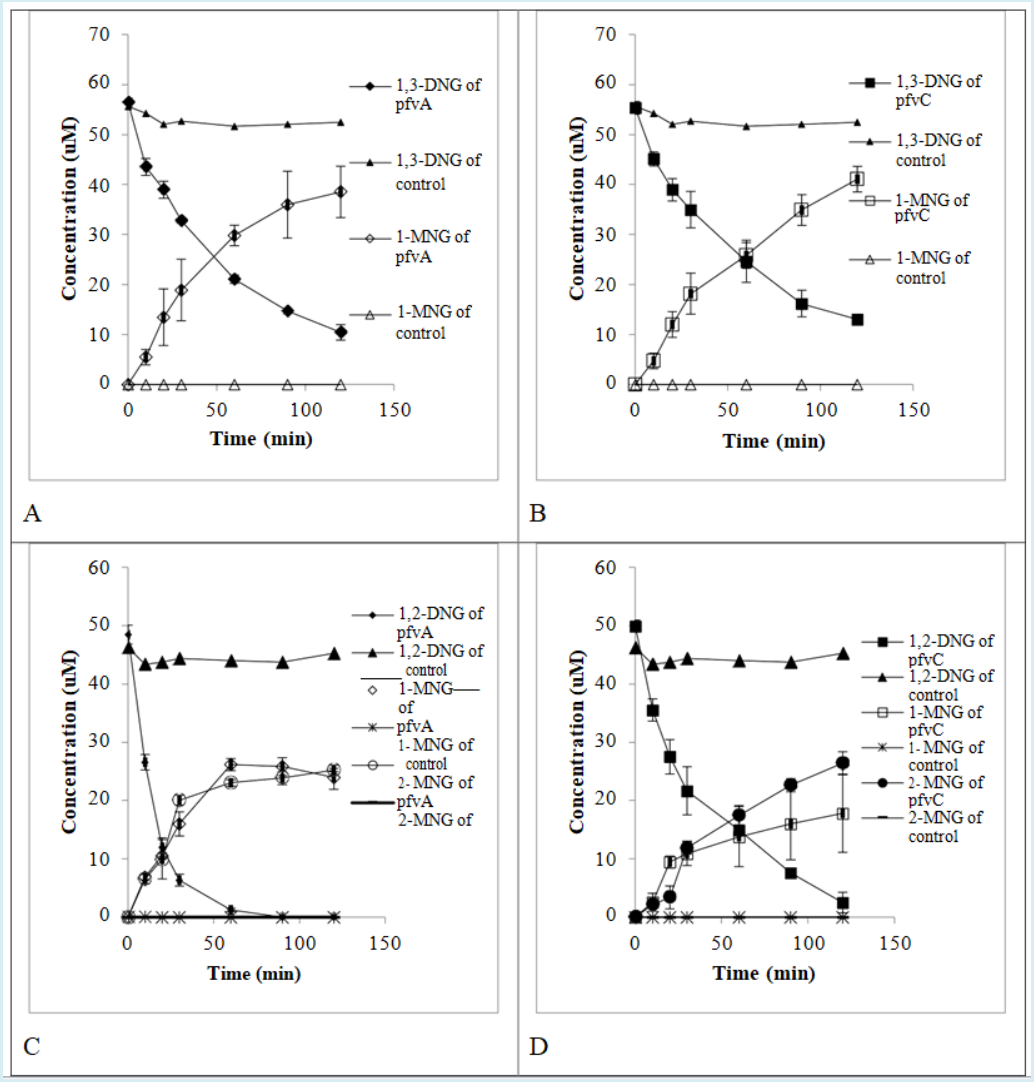

Figure 7: 1,3-DNG transformation by (A) PfvA and (B)PfvC, and 1,2-DNG transformation by (C) PfvA and (D)PfvC.

Transformation of 1-MNG by MngP

Glycerol kinase (MngP) substrate specificity was tested with 1-MNG and 2-MNG. 2-MNG was not transformed by purified MngP. This observation was consistent with previous results showing that JBH1 could not grow on 2-MNG and provided an explanation why traces of 2-MNG accumulated in culture medium during growth of JBH1 on NG [20]. MngP catalyzed the rapid transformation of 1-MNG with initial specific activity of 0.11± 0.02 umol/min/mg protein. However, the reaction of 1-MNG became very slow when about half of 1-MNG was transformed, even when excess enzyme was added (Figure 8). The reaction catalysed by glycerol kinase is highly enantioselective [32]. Since 1-MNG

is a chiral molecule and the commercially available 1-MNG is a racemic mixture, the result suggests that the enantiomers had a ratio of about 1:1, and one enantiomer is favored during the reaction catalyzed by MngP. The reaction with the other enantiomer was negligible, therefore leaving about half of the 1-MNG in the reaction mixture. When MngP catalyzed the transformation of 1-MNG produced during the reduction of NG, results were similar, indicating that PfvA and PfvC produce a racemic mixture of 1-MNG. With the disappearance of 1-MNG, LC/MS revealed the accumulation of a more polar compound, whose molecular weight was consistent with that of 1-nitro-3-phosphoglycerol (MW=215.7). Due to the lack of a standard compound, its concentration was not calculated; however it was possible to estimate the concentration based on the concentration of nitrite released as described in the next section.

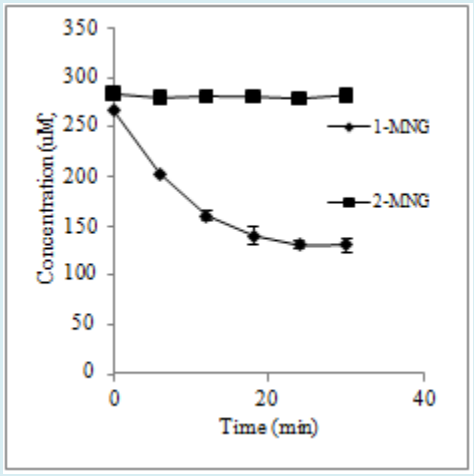

Figure 8: Transformation of 1-MNG and 2-MNG by MngP.

Transformation of 1-nitro-3-phosphoglycerol by PfvA and PfvC

The phosphorylation of 1-MNG could facilitate the denitration of the molecule by OYE either by causing more favorable binding with the enzyme or by changing the electronic properties of the substrate. To test the hypothesis, PfvA or PfvC (0.62 uM) were added to the reaction mixtures after the MngP-catalyzed transformation of 1-MNG. After PfvC was added the 1-nitro-3- phosphoglycerol concentration decreased and nitrite was released (Figure 9). The result indicates that PfvC can catalyze the removal of the third nitrate ester group from the phosphyrylated intermediate. Furthermore, LC/MS analysis revealed a compound with a molecular weight of 172 and the mass spectral properties were identical to those of glycerol 3-phosphate. The mass balance for glycerol 3-phosphate revealed 80 % recovery of carbon and 103 % recovery of nitrogen as nitrite. There was no nitrite release or glycerol-3-phosphate production in PfvA catalyzed reactions (data not shown).

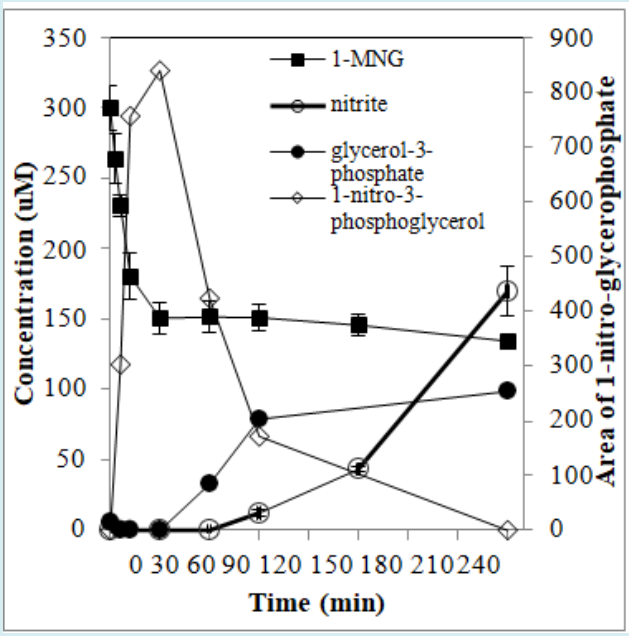

Figure 9: 1-MNG removal by MngP and subsequent reactions. PfvC was added at 30 min.

Discussion

Reductive denitration of NG by nitroreductases [23] is widespread among bacteria but productive metabolism supporting growth is rare. JBH1 is able denitrate 1-MNG and use NG as both carbon and nitrogen sources. The present study established the mechanism of the denitration of 1-MNG, and the reactions leading to it according to the proposed pathway (Figure 10). The results suggest that multiple OYEs are involved in the NG catabolic pathway. RT-PCR results revealed that PfvA and PfvC had similar expression levels in JBH1 cells growing on NG, however, purified PfvA had a much higher activity towards NG than PfvC. Both PfvA and PfvC catalyzed the denitration of 1,3-DNG with similar activities, whereas PfvA was more active toward 1,2-DNG. Therefore, it is reasonable to assume that PfvA is the main enzyme responsible for initial attack on NG. Its preference for the secondary nitrate ester group would lead to the dominant production of 1-MNG, which could be degraded by JBH1, while leaving trace amounts of 2-MNG as a dead-end product, and therefore maximizing the utilization of NG as a carbon and nitrogen source. Although PfvD was also expressed at a comparable level, it did not exhibit any activity towards NG or its transformation products. One possibility is that PfvD has other functions in the cells. It is also possible that heterologous expression and purification resulted in formation of an inactive enzyme which could have failed to fold properly.

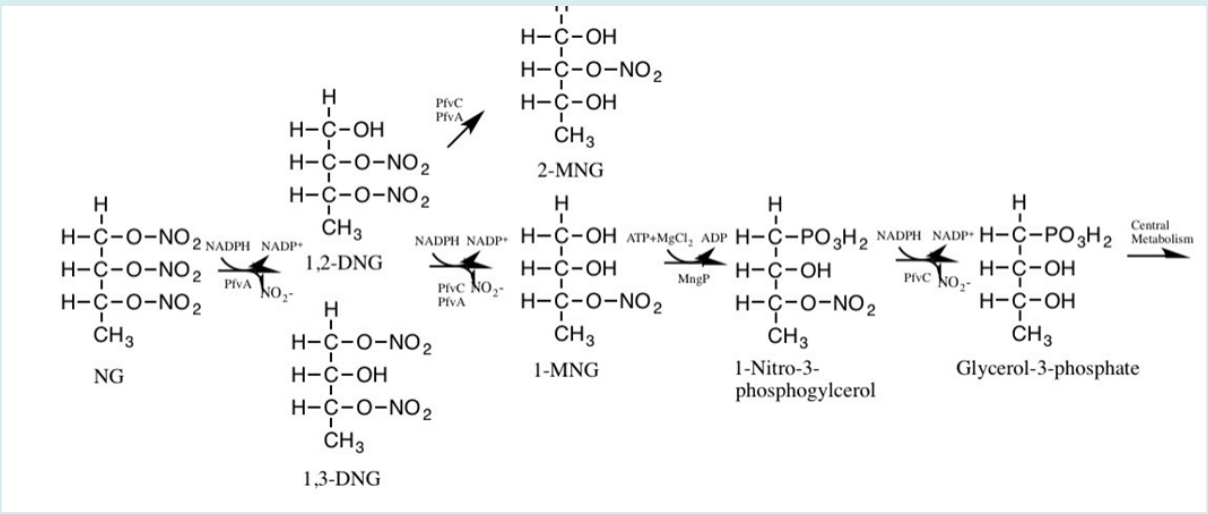

Figure 10: Proposed pathway of NG transformation.

Previous structural studies of OYE provide an explanation why the two OYEs differ greatly in their activities towards NG and DNG. Asn-194 and His-191 around the active site of OYE are crucial for the hydrogen bonding with phenolic compounds [33], the stability of the charge-transfer complexes [33], and interactions with the nicotinamide ring of NADPH [34]. Mutations involving H191N/N194H of OYE1 from brewer's bottom yeast decreased the binding affinity and charge transfer absorbance [34]. Basic local alignment searches of the protein sequence of PfvA and PfvC against brewer's yeast OYE showed that Asn-194 and His-191 as well as other highly conserved amino acids are conserved in PfvA, however, there is an N194H substitution in PfvC, which might be responsible for the much lower specific activity of PfvC toward NG.

In OYE catalyzed reactions with NG, the secondary nitrate ester group is preferred, due to the fact that the electron-withdrawing properties of the primary nitrate esters on both sides of the secondary nitrate ester group made it a stronger electrophile and thus more reactive [13,35]. On the contrary, because there was an electron-donating group –OH on the C-3 position next to the C-2 nitrate of 1,2-DNG, the nitrate ester group attached to C-1 was removed faster in PfvA catalysed reactions, and therefore more 2-MNG accumulated than 1-MNG.

The key finding of this study is the production of glycerol 3-phosphate from the OYE-catalyzed reaction with 1-nitro-3-phosphoglycerol, which is produced from the MngP catalyzed phosphorylation of 1-MNG. Previously, 1-MNG had been considered resistant to transformation by OYE as observed in JBH1. Here we showed that recruitment of *mngP* has made it possible for the old yellow enzyme PfvC to remove the last nitrate ester group [25]. The expression of *mngP* was upregulated when the strain was exposed to NG; however, it was not clear whether the presence of NG or its metabolites induced the expression. The phosphorylated product is a direct substrate of PfvC. It is likely that the phosphate group on C-3 can form a hydrogen bond with Tyr-375, which is conserved in PfvC but not PfvA, and therefore stabilizes the charge-transfer complex and facilitates the denitration at C-1. The denitration product, glycerol-3-phosphate, is an intermediate of glycolysis that can be converted to pyruvate and thus allow NG to serve as a carbon and energy source [36].

The observations that MngP could not catalyze the transformation of 2-MNG, and could only transform a single enantiomer of 1-MNG are consistent with previous indications that glycerol kinase (EC 2.7.1.30) is both regiospecific and stereoselective [32,37]. The inability of JBH1 to grow on 2-MNG indicates that enzymes able to productively metabolize this compound have not been incorporated in the pathway yet. On the other hand, 1-MNG was completely degraded by JBH1 cells; therefore there must be other enzymes in JBH1 that play a role in the degradation of the alternate enantiomer of 1-MNG. Further studies are underway to establish the degradation mechanisms of the remaining 1-MNG enantiomer.

Taken with previous work on JBH1, this research indicates that NG biodegradation can be robust and complete. The presence of JBH1, or similar bacteria, can support effective natural attenuation or bioremediation of NG. Earlier studies with column experiments demonstrated that bio-augmentation with JBH1 resulted in a more efficient removal and mineralization of NG in porous media with high organic

carbon content [38]. Phytoremediation of NG has drawn considerable attention recently [39-42]. Studies by French et al. that showed transgenic tobacco plants carrying PETN reductase genes could convert TNT to less toxic metabolites, and enhance the denitration of NG, DNG and MNG [43]. The discovery of the molecular mechanism of NG degradation provides the basis for the development of transgenic plants incorporating the highly specific genes for NG denitration.

References

1. Spain JC, Hughes JB, Knackmuss HJ (2000) United States. Air Force. Office of Scientific Research; United States. Defense Threat Reduction Agency. Biodegradation of nitroaromatic compounds and explosives; Lewis Publishers: Boca Raton.

2. Urbański T (1964) Chemistry and technology of explosives; Macmillan: New York.

3. Blehert DS, Fox BG, Chambliss GH (1999) Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. J Bacteriol 181(20): 6254-6263.

4. French CE, Nicklin S, Bruce NC (1996) Sequence and properties of pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2. J Bacteriol 178(22): 6623-6627.

5. Marshall SJ, Krause D, Blencowe DK, White GF (2004) Characterization of glycerol trinitrate reductase (NerA) and the catalytic role of active-site residues. J Bacteriol 186(6): 1802.

6. Snape JR, Walkley NA, Morby AP, Nicklin S, White GF (1997) Purification, properties, and sequence of glycerol trinitrate reductase from *Agrobacterium radiobacter*. J Bacteriol 179(24): 7796-7802.

7. Fitzpatrick TB, Amrhein N, Macheroux P (2003) Characterization of YqjM, an old yellow enzyme homolog from *Bacillus subtilis* involved in the oxidative stress response. J Biol Chem 278(22): 19891-19897.

8. Xu D, Kohli RM, Massey V (1999) The role of threonine 37 in flavin reactivity of the old yellow enzyme. Proc Natl Acad Sci USA 96(7): 3556-3561.

9. Fox KM, Karplus PA (1994) Old yellow enzyme at 2-angstrom resolution: overall structure, ligand-binding, and comparison with related flavoproteins. Structure 2(11): 1089-1105.

10. Karplus PA, Fox KM, Massey V (1995) Structure-function relations for old yellow enzyme. FASEB J 9(15): 1518-1526.

11. Massey V (1995) Introduction: flavoprotein structure and mechanism. FASEB J 9(7): 473.

12. Niino YS, Chakraborty S, Brown BJ, Massey V (1995) A new old yellow enzyme of *Saccharomyces cerevisiae*. J Biol Chem 270(5): 1983-1991.

13. Meah Y, Brown BJ, Chakraborty S, Massey V (2001) Old yellow enzyme: Reduction of nitrate esters, glycerin trinitrate, and propylene 1,2-dinitrate. Proc Natl Acad Sci USA 98(15): 8560-8565.

14. Avellaneda H, Arbeli Z, Teran W, Roldan F (2020) Transformation of TNT, 2,4-DNT, and PETN by *Raoultella planticola* M30b and *Rhizobium radiobacter* M109 and exploration of the associated enzymes. World J Microbiol Biotechnol 36(12): 190.

15. Breithaupt C, Strassner J, Breitinger U, Huber R, Macheroux P, et al. (2001) X-ray structure of 12-oxophytodienoate reductase 1 provides structural insight into substrate binding and specificity within the family of OYE. Structure 9(5): 419-429.

16. White GF, Snape JR, Nicklin S (1996) Biodegradation of Glycerol Trinitrate and Pentaerythritol Tetranitrate by *Agrobacterium radiobacter*. Appl Environ Microbiol 62(2): 637-642.

17. White GF, Snape JR (1993) Microbial cleavage of nitrate esters: defusing the environment. J Gen Microbiol 139(9): 1947-1957.

18. Muller TA, Kohler HPE (2004) Chirality of pollutants-effects on metabolism and fate. Appl Microbiol Biot 64(3): 300-316.

19. Bennett BM, McDonald BJ, Nigam R, Simon WC (1994) Biotransformation of organic nitrates and vascular smooth muscle cell function. Trends Pharmacol Sci 15(7): 245-249.

20. Husserl J, Spain JC, Hughes JB (2010) Growth of *Arthrobacter sp Strain* JBH1 on nitroglycerin as the sole source of carbon and nitrogen. Appl Environ Microb 76(5): 1689-1691.

21. Meah Y, Massey V (2000) Old yellow enzyme: Stepwise reduction of nitro-olefins and catalysis of aci-nitro tautomerization. Proc Natl Acad Sci USA 97(20): 10733-10738.

22. Meng M, Sun WQ, Geelhaar LA, Kumar G, Patel AR, et al. (1995) Denitration of glycerol trinitrate by resting cells and cell-extracts of *Bacillus thuringiensis/cereus* and *Enterobacter agglomerans*. Appl Environ Microb 61(7): 2548-2553.

23. Blehert DS, Knoke KL, Fox BG, Chambliss GH (1997) Regioselectivity of nitroglycerin enitration by flavoprotein nitroester reductases purified from two *Pseudomonas* species. J Bacteriol 179(22): 6912-6920.

24. Marshall SJ, White GF (2001) Complete denitration of nitroglycerin by bacteria isolated from a washwater soakaway. Appl Environ Microbiol 67(6): 2622-2626.

25. Husserl J, Hughes JB, Spain JC (2012) Key enzymes enabling the growth of *Arthrobacter sp Strain* JBH1 with nitroglycerin as the sole source of carbon and nitrogen. Appl Environ Microb 78(10): 3649-3655.

26. Anderson MJ, DeLabarre B, Raghunathan A, Palsson BO, Brunger AT, et al. (2007) Crystal structure of a hyperactive *Escherichia coli* glycerol kinase mutant Gly230-Asp obtained using microfluidic crystallization devices. Biochemistry 46(19): 5722-5731.

27. Charrier V, Buckley E, Parsonage D, Galinier A, Darbon E, et al. (1997) Cloning and sequencing of two *Enterococcal* glpK genes and regulation of the encoded glycerol kinases by phosphoenolpyruvate dependent, phosphotransferase system-catalyzed phosphorylation of a single histidyl residue. J Biol Chem 272(22): 14166-14174.

28. Yeh JI, Charrier V, Paulo J, Hou LH, Darbon E, et al. (2004) Structures of enterococcal glycerol kinase in the absence and presence of glycerol: correlation of conformation to substrate binding and a mechanism of activation by phosphorylation. Biochemistry 43(2): 362-373.

29. US Army (1984) Military Explosives, TM 9-1300-214 Department of the Army, Washington, DC.

30. United States (1971) Environmental Protection Agency. Water Quality Office. Methods for chemical analysis of water and wastes; Environmental Protection Agency, Analytical Quality Control Laboratory; for sale by the Supt. of Docs., U.S. Govt Print Off, Cincinnati.

31. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3(6): 1101-1108.

32. Crans DC, Whitesides GM (1985) Glycerol kinase: substrate specificity. J Am Chem Soc 107(24): 7008-7018.

33. Abramovitz AS, Massey V (1976) Interaction of phenols with old yellow enzyme - Physical evidence for charge-transfer complexes. J Biol Chem 251(17): 5327-5336.

34. Brown BJ, Deng Z, Karplus PA, Massey V (1998) On the active site of old yellow enzyme: role of histidine 191 and asparagine 194. J Biol Chem 273(49): 32753-32762.

35. Toogood HS, Gardiner JM, Scrutton NS (2010) Biocatalytic Reductions and Chemical Versatility of the old yellow enzyme family of flavoprotein oxidoreductases. Chemcatchem 2(8): 892-914.

36. Straathof AJJ (2014) Transformation of biomass into commodity chemicals using enzymes or cells. Chem Rev 114(3): 1871-1908.

37. Crans DC, Whitesides GM (1985) Glycerol kinase: synthesis of dihydroxyacetone phosphate, sn-glycerol-3-phosphate, and chiral analogs. J Am Chem Soc 107(24): 7019-7027.

38. Husserl J, Hughes JB (2013) Biodegradation of nitroglycerin in porous media and potential for bioaugmentation with *Arthrobacter sp strain* JBH1. Chemosphere 92(6): 721-724.

39. Kumar S (2013) Phytoremediation of explosives using transgenic plants. J Pet Environ Biotechnol Suppl 4: 11127.

40. Panz K, Miksch K (2012) Phytoremediation of explosives (TNT, RDX, HMX) by wild-type and transgenic plants. J Environ Manage 113: 85-92.

41. Van Aken B (2009) Transgenic plants for enhanced phytoremediation of toxic explosives. Curr Opin Biotechnol 20(2): 231-236.

42. Rylott EL, Bruce NC (2009) Plants disarm soil: engineering plants for the phytoremediation of explosives. Trends Biotechnol 27(2): 73-81.

43. French CE, Rosser SJ, Davies GJ, Nicklin S, Bruce NC (1999) Biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase. Nat Biotechnol 17(5): 491-494.