



# Production of 2-amino-5-phenoxyphenol from 4-nitrobiphenyl ether using nitrobenzene nitroreductase and hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45

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**Microbial metabolism of nitroarenes via *o*-aminophenols requires the participation of two key enzymes, a nitroreductase and an hydroxylaminobenzene mutase. The broad substrate ranges of the enzymes suggested that they could be used as biocatalysts for the production of substituted *o*-aminophenols. We have used enzymes from *Pseudomonas pseudoalcaligenes* JS45 for the conversion of 4-nitrobiphenyl ether to the corresponding *o*-aminophenol. Partially purified nitrobenzene nitroreductase reduced 4-nitrobiphenyl ether to the corresponding 4-hydroxylaminobiphenyl ether. Partially purified hydroxylaminobenzene mutase stoichiometrically converted the intermediate to 2-amino-5-phenoxyphenol. The results indicate that the enzyme system can be applied for the production of *o*-aminophenols useful as intermediates for synthesis of commercially important materials. *Journal of Industrial Microbiology & Biotechnology* (2000) 24, 301–305.**

**Keywords:** bacteria; nitroaromatic compounds; aminophenol; biocatalysis; biotransformation

## Introduction

*o*-Aminophenols are important intermediates in the synthesis of common azo dyes and phenoxazinones [3]. They are a key feedstock for the synthesis of polybenzoxazole polymers [4,23]. Substituents carried by the *o*-aminophenol confer on the benzoxazole products properties that are useful in electronic [12,14,28], opto-electronic [2,21], pharmaceutical [18], medical [31], military [6–9,33], and biosynthetic applications [19,32]. Commercially useful substituted aminophenols are difficult to synthesize chemically, therefore, we are seeking a biocatalytic strategy.

Microbes can transform nitroarenes to *o*-aminophenols using two enzymes, a nitroreductase and an hydroxylaminobenzene mutase [15,24,26,30] (Figure 1a). The process has been characterized well in *Pseudomonas pseudoalcaligenes* strain JS45. Nitrobenzene nitroreductase reduces nitrobenzene to hydroxylaminobenzene and a mutase rearranges the intermediate to *o*-aminophenol [24]. The nitroreductase from *P. pseudoalcaligenes* JS45 has been purified and characterized as a 30-kDa flavoprotein requiring NADPH as an electron source [29]. Two genes expressing mutase activity have been cloned into *E. coli* and one enzyme, Hab B, has been partially purified. It is heat stable to 90°C and requires no cofactors to catalyze an intra-molecular rearrangement of the hydroxylaminobenzene to *o*-aminophenol [11]. Preliminary experiments indicated that both the reductase and mutase have relaxed substrate specificities.

Biotransformation assays can be performed in whole

cells or with partially purified enzyme systems. In this study, we investigated whether partially purified enzymes could be used to catalyze the transformation of the model compound, 4-nitrobiphenyl ether, to the corresponding *o*-aminophenol (Figure 1b).

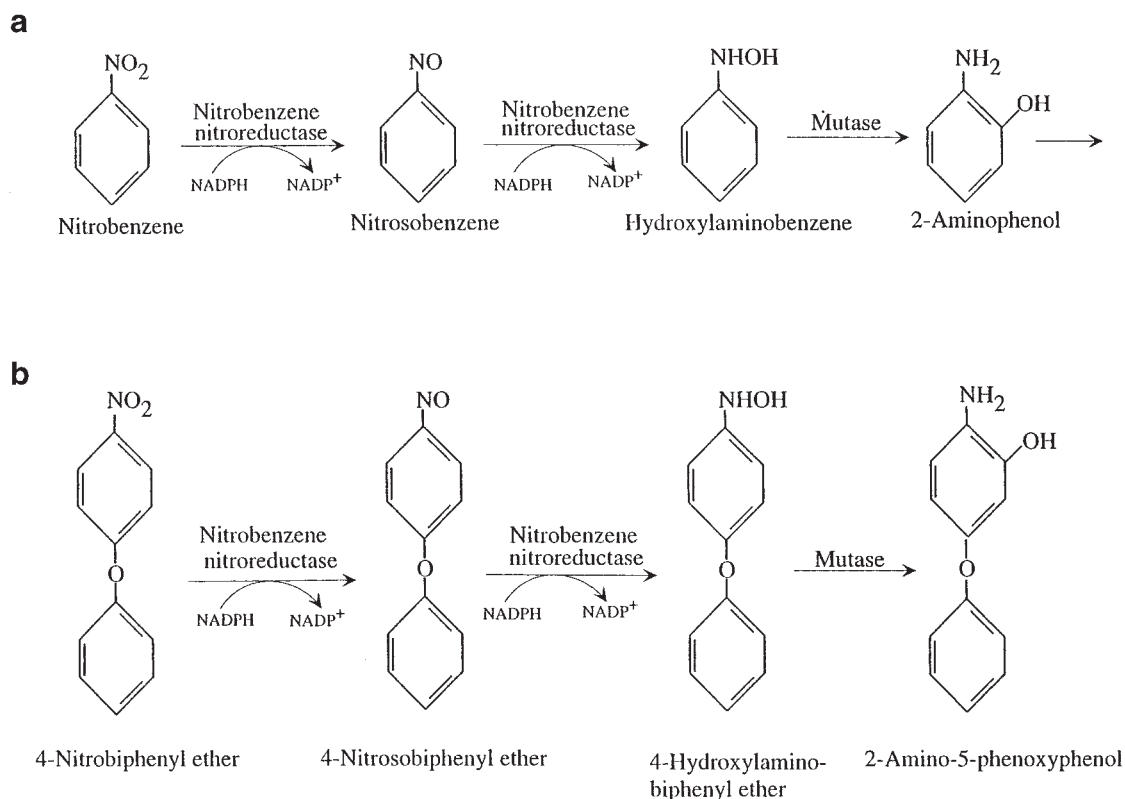
## Materials and methods

### *Partial purification of the enzymes*

*P. pseudoalcaligenes* JS45 was grown and crude cell extracts were prepared as previously described to purify the nitrobenzene reductase [24,29]. The crude extract was loaded on a 100-ml Q-Sepharose Fast Flow column (Pharmacia, Piscataway, NJ, USA, XK-26) previously equilibrated with 150 mM KCl in 20 mM phosphate buffer. Proteins were eluted with a step gradient that began with 100 ml buffer containing KCl (150 mM) and then a linear gradient of 150–300 mM KCl at a flow rate of 2.5 ml min<sup>-1</sup>. The fractions containing nitrobenzene reductase activity, which eluted in the linear gradient between 65 to 80 ml, were pooled, washed three times and concentrated on an Amicon PM-10 membrane and stored in 500- $\mu$ l aliquots at –80°C for use in transformation assays. Partial purification of the Hab B mutase from *E. coli* (pNBZ139) which contains *habB* was performed as previously described [11].

### *Transformation of 4-nitrobiphenyl ether*

Biotransformation for measurement of product accumulation was conducted by incubating 4-nitrobiphenyl ether (30  $\mu$ M) with partially purified reductase (0.57 mg protein ml<sup>-1</sup>) and mutase (0.27 mg protein ml<sup>-1</sup>) in 1 L of phosphate buffer (20 mM, pH 7.0, sparged with argon for 1 h) containing NADPH (200  $\mu$ M). Transformation of 4-nitrobiphenyl ether for end-product purification was performed in



**Figure 1** (a) Initial steps in the biodegradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45. (b) Production of 2-amino-5-phenoxyphenol from 4-nitrobiphenyl ether catalyzed by using the nitrobenzene reductase and hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45.

1 L of phosphate buffer containing NADPH (1 mM), glucose-6-phosphate dehydrogenase (100 units), glucose-6-phosphate (1 mM), 4-nitrobiphenyl ether (360  $\mu$ M) dissolved in ethanol and delivered over 2 h (5 ml final ethanol volume), and nitroreductase (0.18 mg protein added every 30 min). After 2 h the hydroxylaminobenzene mutase (0.261 mg protein) was added to complete the transformation. The reaction mixture was stirred under argon at 22°C and the progress of the reaction was monitored by HPLC. Subsequent additions of enzyme were made as necessary to complete the transformation. At the end of the incubation the reaction mixture was extracted four times with 500 ml of ethyl acetate (sparged with argon). Extracts were dried over sodium sulfate, concentrated under a stream of nitrogen and stored at  $-80^{\circ}\text{C}$  until purified by TLC, as described below.

### Chemicals

All chemicals were analytical grade. 4-Nitrobiphenyl ether and zinc dust were obtained from Aldrich (St Louis, MO, USA). Chemical synthesis and purification of 4-hydroxylaminobiphenyl ether was conducted according to a previously published method [22] with the modification that the 1,4-dioxane (ACS Certified, Fisher Scientific, Pittsburg, PA, USA) was purified by distillation. The conversion efficiency during the chemical reduction of 4-nitrobiphenyl ether to 4-hydroxylaminobiphenyl ether was 93% as determined by HPLC. The product purified by recrystallization decomposed rapidly so the final yield was not determined. The melting point was  $72.8\text{--}73.6^{\circ}\text{C}$  and the  $A_{\text{max}}$

was 245.6 nm in ethanol which compared well to the published results of  $71.0\text{--}74.0^{\circ}\text{C}$  and 246 nm, respectively.

The solubility of 4-nitrobiphenyl ether in 20 mM phosphate buffer was determined by the addition of 200 mg finely crushed 4-nitrobiphenyl ether to a 500-ml equilibrium flask (Ace Glass, Inc, Louisville, KY, USA) containing 400 ml buffer which was continuously stirred and submerged in a  $22^{\circ}\text{C}$  water bath for 5 days. Samples were analyzed daily by UV/Vis spectrophotometry and quantified by high performance liquid chromatography (HPLC). The solution reached equilibrium by the third day.

### Analytical methods

An HPLC equipped with a diode array detector monitoring at  $A_{210}$  (Hewlett-Packard, Wilmington, DE, USA, Model 1040 M) was used to identify and quantitate 4-nitrobiphenyl ether, 4-hydroxylaminobiphenyl ether, and 2-amino-5-phenoxyphenol. Quantification was obtained by generating a five-point linear calibration curve from five single standards of the parent compound (3–100  $\mu$ M) and of 4-hydroxylaminobiphenyl ether (3–54  $\mu$ M) and from duplicate standards of 2-amino-5-phenoxyphenol (2–30  $\mu$ M). The compounds were separated by paired-ion chromatography on a  $C_8$  Spherisorb column (250 mm  $\times$  4.6 mm; Alltech, Deerfield, IL, USA) with 60% methanol and 40% water, both containing 0.5 mM hexane sulfonic acid (low UV Pic B-6 reagent, Waters, Milford, MA, USA), as the solvent system at a flow rate of  $1.2\text{ ml min}^{-1}$ . Capillary gas chromatography/mass spectral (GC/MS) analyses were performed in the splitless mode on a Hewlett-Packard GC

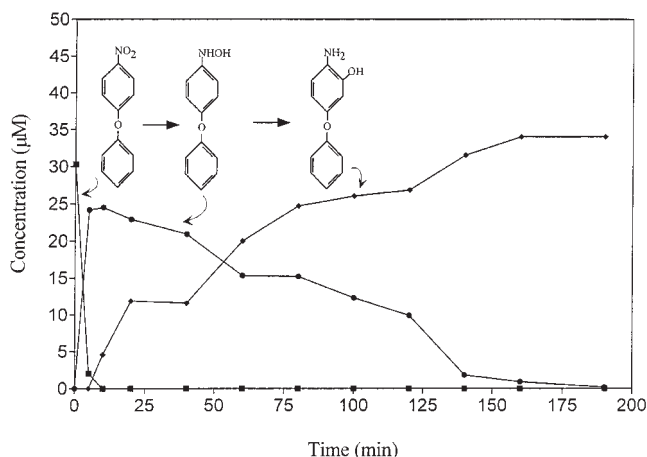
(model 5890) equipped with a mass selective detector (model 5971) and a DB-5 column (J & W Scientific, Folsom, CA, USA, 30 m long  $\times$  0.25 mm ID, 1.0  $\mu$ m film thickness). The aminophenol derivatized with Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Bellefonte, PA, USA) was analyzed by GC/MS. The temperature program began at 100°C and increased 10°C min<sup>-1</sup> to 280°C. The *n*-butylboronic acid derivative [16] was analyzed using an initial temperature of 50°C increasing 20°C min<sup>-1</sup> to 340°C. Nitrobenzene nitroreductase activity was measured spectrophotometrically by following NADPH oxidation as previously described [29].

Thin layer chromatography (TLC) purification of the aminophenol was performed on a 1-mm thick silica plate (PK6F, 60A, Whatman, Clinton, NJ, USA) using a solvent system consisting of a 20:80 mixture of ethyl acetate-hexane under argon. Samples were extracted in ethyl acetate, dried under argon, and stored at -80°C until analyzed by HPLC and GC/MS.

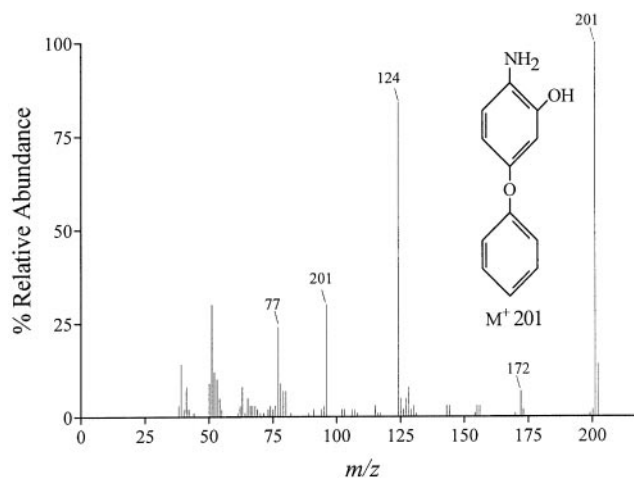
## Results

Partially purified nitrobenzene nitroreductase transformed nitrobenzene and 4-nitrobiphenyl ether at rates of 7.9 and 8.1  $\mu$ mol min<sup>-1</sup> mg protein<sup>-1</sup>, respectively. HPLC analysis of the reaction mixtures initially containing 4-nitrobiphenyl ether (RT 15.8 min) and the nitroreductase yielded a single product whose LC retention time (5.5 min) and UV spectrum were identical to those of the chemically synthesized 4-hydroxylaminobiphenyl ether. The enzymatic conversion was quantitative, 27.3  $\mu$ M 4-nitrobiphenyl ether was converted to 26  $\mu$ M 4-hydroxylaminobiphenyl ether. Attempts to extract the product with ethyl acetate yielded a mixture of compounds detected by GC/MS. The extracts contained compounds tentatively identified as 4-hydroxylaminobiphenyl ether (*m/z* 210), 4-nitrosobiphenyl ether (*m/z* 199) and 4-aminobiphenyl ether (*m/z* 185). The results suggest that the hydroxylaminobiphenyl ether decomposes readily. Attempts to derivatize the unstable hydroxylaminoarene in aqueous solutions [13] were not successful. Therefore further attempts to characterize the compounds in extracts were not performed.

Biotransformation of 4-nitrobiphenyl ether in the presence of partially purified nitrobenzene nitroreductase and mutase led to transient accumulation of hydroxylaminobiphenyl ether and then the stoichiometric accumulation of a single product (Figure 2). Large-scale transformation and purification by TLC yielded 34.6 mg of red crystals (48% yield). The product had a melting point of 118.5–124.9°C. The reported melting point of 2-amino-5-phenoxyphenol is 123.5–125.0°C [20]. We did not attempt to optimize the recovery. The compound remained stable for up to 3 months when stored under argon at -80°C. The end-product was also purified by HPLC and analyzed by GC/MS which revealed a compound with parent ion at *m/z* 201 consistent with the expected mass of 201.23 (Figure 3). The fragment ions at *m/z* 172 (M-29), 124 (M-77), 96 (M-105), and 77 (M-124) were consistent with the losses of CHO, C<sub>6</sub>H<sub>5</sub>, C<sub>7</sub>H<sub>5</sub>O and C<sub>6</sub>H<sub>6</sub>NO<sub>2</sub>. Derivatization with BSTFA yielded a mixture of compounds: the major component had a parent ion at *m/z* 273 consistent with the derivatization of one sub-

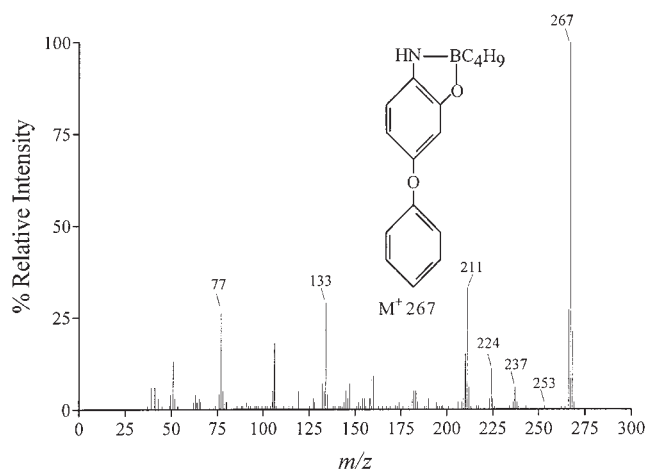


**Figure 2** Transformation of 4-nitrobiphenyl ether during incubation with nitrobenzene nitroreductase and hydroxylaminobiphenyl mutase. Partially purified reductase and mutase were incubated with 30  $\mu$ M 4-nitrobiphenyl ether (■) in 20 mM phosphate buffer (pH 7.0, sparged with argon). The reaction mixture was monitored by HPLC for production of 4-hydroxylaminobiphenyl ether (●) and 2-amino-5-phenoxyphenol (◆).



**Figure 3** Mass spectrum of the end-product from 4-nitrobiphenyl ether transformation.

stituent. The minor component had a parent ion at *m/z* 345, consistent with the expected mass of the compound containing two trimethylsilane moieties. The derivative contained two trimethylsilane groups indicating that the compound contained two available functional groups that could be derivatized. The derivatization pattern is consistent with that of an aminophenoxyphenol. The position of the substituents was verified by derivatization with *n*-butylboronic acid which derivatizes compounds with functional groups in the *ortho*-position, such as catecholamines or catechols [16]. The expected mass of the derivatized product is 266.8 and the GC/MS analysis revealed a product with a parent ion (M<sup>+</sup>) at *m/z* 267 and fragment ions at *m/z* 253 (M-14), 237 (M-30), 224 (M-43), 211 (M-56), 134 (M-133), and 77 (M-190) corresponding to the possible loss of N or CH<sub>2</sub>, C<sub>2</sub>H<sub>6</sub>, C<sub>3</sub>H<sub>7</sub>, C<sub>4</sub>H<sub>8</sub>, C<sub>7</sub>H<sub>8</sub>NOB and C<sub>10</sub>H<sub>13</sub>NO<sub>2</sub>B (Figure 4) which strongly suggests that the end-product is 2-amino-5-phenoxyphenol. 2-Aminophenol, 2-amino-4-nitrophenol and 3-aminophenol were reacted with *n*-butylboronic acid



**Figure 4** Mass spectrum of 2-amino-5-phenoxyphenol derivatized with *n*-butylboronic acid.

for comparison. GC/MS analysis revealed for 2-amino-phenol a peak with a parent ion at  $m/z$  175 (expected is 175) and for 2-amino-4-nitrophenol a peak with a parent ion at  $m/z$  220 (expected is 220). 3-Aminophenol gave a peak with a parent ion at  $m/z$  109 which indicates that it did not react with the derivatizing agent. The results clearly indicate the mutase specifically rearranges 4-hydroxylaminobiphenyl ether to 2-amino-5-phenoxyphenol.

## Discussion

2-amino-5-phenoxyphenol was synthesized from 4-nitrobiphenyl ether by the nitroreductase and mutase enzyme system. The reductase catalyzes the partial reduction of the nitro group in a reaction analogous to the one previously described for nitrobenzene degradation [29]. The mutase catalyzes the rearrangement of the resultant hydroxylamino intermediate to the corresponding *ortho*-aminophenols. The Bamberger rearrangement is well known in organic chemistry [27]. The reaction converts hydroxylaminobenzene to a mixture of almost exclusively 4-aminophenol and traces of 2-aminophenol. In contrast, the enzyme is highly selective for the production of the *ortho*-isomer [24]. Other mutase enzymes catalyze the formation predominantly of *ortho*-isomers [15,30].

The transformation experiments were performed under anaerobic conditions primarily because hydroxylaminoarenes are oxygen-sensitive [13] and 4-hydroxylaminobiphenyl ether also proved to be unstable in air. Measurement by HPLC of hydroxylaminobiphenyl ether required that the vials containing the reactants be sealed to keep the reaction anaerobic. Furthermore, given that nitrobenzene-grown cells synthesize a ring-cleavage dioxygenase inactive in the absence of molecular oxygen, anaerobic conditions were selected when first screening for the catabolic capability of *P. pseudoalcaligenes*. The anaerobic conditions made it less likely that the dioxygenase would transform the desired aminophenol product.

2-Amino-5-phenoxyphenol was previously synthesized chemically as an intermediate in the synthesis of phenoxybenzoxazole used as a fluorescent whitening agent and photosensitizer [20]. Aminophenol synthesis using 4-chloroni-

trobenzene and phenol as starting compounds required three steps and the yield was not reported. The biological production in contrast can be carried out in a single reaction and the conversion is stoichiometric.

The common route for commercial synthesis of aminophenols occurs in two steps, the nitration of phenol followed by reduction of the nitro-group with a metal to make the amine. The influence of the hydroxyl moiety varies with each substrate. For example, for phenol, the substitution is directed preferentially to the *ortho* position but for naphthalene the *para* position is more readily attacked. In either case, yields are very low for mononitration of phenols and the conditions needed are extreme. With the enzymatic reaction, the *ortho* isomer is produced readily in high yield since it is the sole product.

Hydroxylaminoarenes can be produced from the corresponding nitroarene by the action of zinc and ammonium sulfate. Hydroxylaminobenzene for example is relatively stable and can be purified for use in subsequent synthetic reactions. Where the hydroxylamino compound is stable and can be produced in high yield chemically the use of nitroreductases would not be necessary. 4-Hydroxylaminobiphenyl ether proved to be extremely unstable and difficult to purify. Such intermediates can, however, be produced in high yield by the action of nitroreductase enzymes and the rearrangement to the aminophenol can be carried out in the same reactor without purification of the intermediates. Several other enzymes could be used to catalyze the nitroreduction [1,5,10,17,25,34] or the mutase reaction [30]. The catalytic properties of such enzymes have not been evaluated.

We used partially purified enzymes to catalyze the conversion described here. Crude lysates from *E. coli* pNBZ139 would also be effective in the transformation, but partial purification of the enzyme provided a higher specific activity. The disadvantage of such a strategy is the need to regenerate NADPH consumed in the reactions. It is also possible to carry out the transformations using intact cells as the biocatalyst. We are currently working to optimize the conditions for such transformations.

Our interest in aminophenols stems from their use as starting materials for the synthesis of polybenzoxazoles [4]. In some instances, the cost of the polymers is prohibitive because of the high cost of the aminophenol monomers. The strategy described here for production of the model compound 2-amino-5-phenoxyphenol, is being tested for application to a variety of other aminophenols.

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