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Reduction of nitro phenols using nitroreductase from *E. coli* in the presence of NADH

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ABSTRACT

The reductions of nitrophenols catalyzed by nitroreductase from *E. coli* in the presence of NADH were investigated in this paper. 4-Aminophenol and 4-hydroxylaminophenol were found in the reductive products of 4-nitrophenol and the maximum reductive ratio was about 83.49% when the reaction time was 70 min; 4,6-dinitro-2-pimelie kelone was found in the reductive products of 2,4-dinitrophenol and the maximum reductive ratio was about 75.28% when the reaction time was 80 min; 2,4-dinitrophenol and 4,6-dinitro-2-pimelie kelone were found in the reductive products of 2,4,6-trinitrophenol and the maximum reductive ratio was about 62.08% when the reaction time was 100 min. The similar reductive ratios of nitrophenols were obtained under aerobic and anaerobic conditions. The results indicated that nitroreductase was an oxygen-insensitive enzyme.

1. Introduction

Nitrophenols are a kind of the most widely used industrial organic compounds. They are frequently used as intermediates in the production of explosives, pharmaceuticals, pesticides, pigments, dye, wood preservatives and rubber chemicals [1–3]. But they become common environmental pollutants because of their toxicity and their resistance to microbial degradation [4–6]. So these compounds are considered as a priority pollutant by the Environmental Protection Agency (EPA) of USA, and its concentration in natural waters is restricted to less than 10 mg/L [7,8].

At present, the widely used treatments of the nitrophenols pollutants are adsorbing, chemical oxidation, precipitation, abstraction, evaporation and incineration. But these chemical and physical methods have a lot of problems such as the multiplicity and the large cost. The biological treatment of pollutants provided safe and effective method as compared with chemical and physical treatment techniques [9–20]. Recently, Zhang et al. found that *Rhodococcus* sp. CN6, isolated from a pesticide industry's effluent-sediment, was able to completely degrade and utilize 100 mg/L *p*-nitrophenol (PNP) as the sole carbon, nitrogen and energy sources for growth in the minimal salt media (MSM) within 12 h [21]. However, the problems in the biological treatment of pollutants are the slow reaction rate and difficulty to find the suitable microorganism. Hence more studies were being concentrated for the development of new methods for nitrophenols degradation. Such as the adsorption of 4-nitrophenol onto Amberlite® IRA-900 modified with metallophthalocyanines [7], low-temperature plasma [22], commercial unmodified and modified activated carbons [23], wet electrocatalytic oxidation (WEO) [24], and electrocoagulation (EC) [25].

Nowadays, researchers have taken more attention on reduction of the nitro compounds catalyzed by enzymes. It can provide green treatment of nitrophenols by using appropriate enzymes. Kitts et al. [26] had reported the reduction of RDX by a type I oxygen-insensitive nitroreductase without providing details on either products or degradation pathways. Whereas Bhushan and Halasz reported the *N*-denitrohydrogenation of RDX by a flavoenzyme, diaphorase [27]. They also reported the reduction of CL-20 catalyzed by nitroreductase in 2004 [28].

In the present study, the reductions of 4-nitrophenol, 2,4-dinitrophenol, and 2,4,6-trinitrophenol catalyzed by nitroreductase from *E. coli* in the presence of NADH were performed. The reductive products were detected by HPLC, GC and MS.

2. Materials and methods

2.1. Chemicals

Nitroreductase from *E. coli.* (purity is 90% by SDS-PAGE) and $NADHNa_2$ (Purity > 98%) were purchased from Sigma Chemical. 4-Nitrophenol, 2,4-dinitrophenol and 2,4,6-trinitrophenol were got from Beijing Chemical Reagent Co., Ltd. All above chemicals were

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of analytical grade. Chromatographic grade acetonitrile was purchased from Fisher Scientific Co., Ltd.

2.2. Biotransformation of nitrophenols

The reactions were performed in 5 mL centrifuge tubes at 37 °C. The reaction mixture consisted of 0.25 mmol/L nitrophenols, 1.0 mmol/L NADH, 50 mmol/L phosphate buffer (pH 7.0), and 50 μg enzyme in a final volume of 1 mL. It was quenched by heating the solution at 90 °C for 30 min.

2.2.1. Determination of NADH oxidation

The oxidation of NADH was measured using UV–vis spectrophotometer on Shimazu UV-1800. Because NADH contains fluazifop loop, it has an absorption peak at 340 nm. After it was oxidized, NAD^+ does not contain this loop, so the decreased value of NADH is direct ration to the amount of NAD^+ formation.

Prepare different concentrations of NADH and use the same buffer without NADH as the blank reference. Determine the NADH value at 340 nm, and draw the standard curve. Then the unknown concentration of NADH can be measured with the determined NADH value.

2.2.2. Determination the concentration of nitrophenols with HPLC and HPLC–MS

A LC-6AD Shimadzu HPLC equipped with DAD detector was used for analysis of the compounds in the final reaction mixture. It was carried out using a ODS C-18 column (with i.d. 4.6 mm × 250 mm), with a linear gradient from 5% to 100% acetonitrile at a flow rate of 1 mL/min.

Mass spectrometry was performed using electro spray ionization (ESI) in negative ion mode with trap control 4.1 control system and MSD Trap Report V2.1 data acquisition system (Agilent, USA). The drying gas temperature was at 325 °C, the nebulizer gas at 30 psi, drying gas at 9 L/min. Full scan mode was used for data collection. Scan range was set at 100–200 *m*/*z*.

3. Results and discussion

The biotransformations of 4-nitrophenol were catalyzed by nitroreductase from *E. coli* in the presence of NADH at pH 7.0 and 37 °C as shown in Fig. 1. The reduction product of 4-nitrophenol is 4-aminophenol, and there is intermediate compound 4-hydroxylaminophenol. The reduction product of 2,4-dinitrophenol is 4,6-dinitro-2-pimelie kelone. The reduction

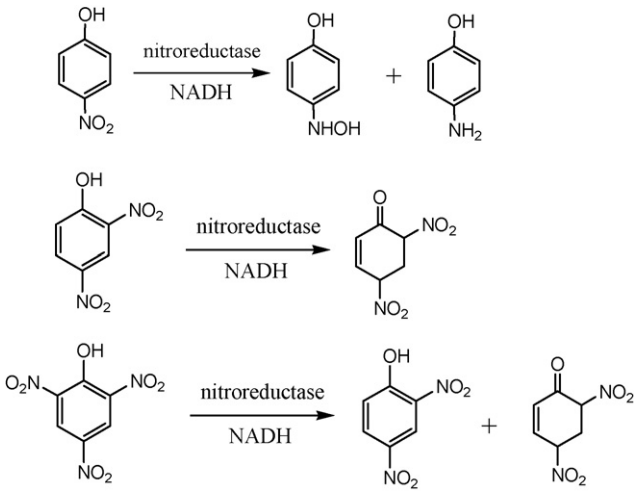

Fig. 1. Reduction of nitrophenols.

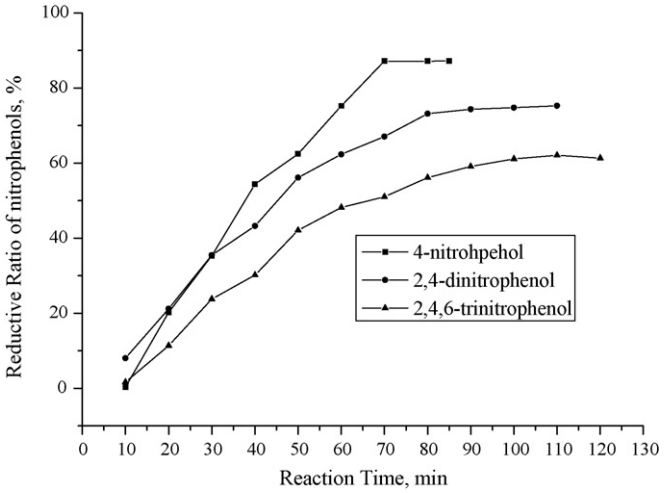

Fig. 2. The relationship between reaction time and reductive ratio of nitrophenols.

product of 2,4,6-dinitrophenol is 2,4-dinitrophenol and 4,6-dinitro-2-pimelie kelone. The similar reductive ratio could be obtained under both anaerobic and aerobic conditions, indicating the involvement of an oxygen-insensitive process. The similar reductive ratios of nitrophenols were obtained under aerobic and anaerobic conditions. The results indicated that nitroreductase was an oxygen-insensitive enzyme.

3.1. Reduction of 4-nitrophenol catalyzed by nitroreductase

The relationship of reaction time and the reductive ratio of 4-nitrophenol, was illustrated in Fig. 2. The maximum biotransformation rate of 4-nitrophenol was about 83.49% when the reaction time was 70 min.

The effect of the concentration of NADH on the reductive ratio of 4-nitrophenol can be seen in Fig. 3. When the concentration of NADH was about 0.5 mM, the reductive ratio was the highest. So the optimum concentration of NADH was as twice as that of 4-nitrophenol.

The reaction products were tested using HPLC, HPLC–ESI-Mass and standard compound. The results showed that 4-nitrophenol decreased obviously. Three compounds, 4-aminophenol, 4-hydroxylaminophenol and NAD were found at the end of the reaction. In the reduction of 4-nitrophenol, 4-hydroxylaminophenol

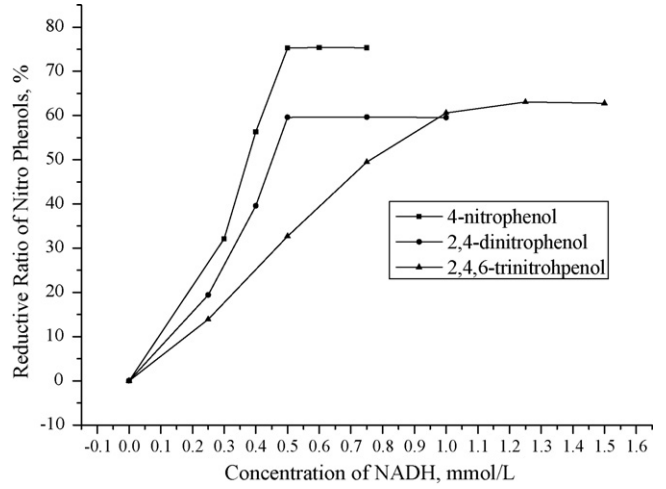

Fig. 3. The relationship between concentration of NADH and the reductive ratio of nitrophenols.

was yielded, and 4-aminophenol was produced for the further reduction. Bryant pointed out that the two-electron transfer reduction proceeded in the transformation from nitro group to amino group [29].

3.2. Reduction of 2,4-dinitrophenol (DNP) catalyzed by nitroreductase

The relationship of reaction time and the reductive ratio of 2,4-dinitrophenol was illustrated in Fig. 2. The maximum reductive ratio of 2,4-dinitrophenol was about 75.28% when the reaction time was 80 min.

We can see the effect of the concentration of NADH on the reductive ratio of 2,4-dinitrophenol in Fig. 3. When the concentration of NADH was about 0.5 mM, the reductive ratio was the highest. So the optimum concentration of NADH was as twice as that of 2,4-dinitrophenol.

The reaction products were tested using HPLC, HPLC–ESI-Mass and standard compound. The results of HPLC showed that 2,4-dinitrophenol decreased obviously, but the new product was not very obvious. We determined 4,6-dinitro-2-pimelie kelone using the method as Behrend described [30].

3.3. Reduction of 2,4,6-trinitrophenol (TNP) catalyzed by nitroreductase

The relationship of reaction time and the reductive ratio of 2,4,6-trinitrophenol was illustrated in Fig. 2. The maximum reductive ratio of 2,4,6-trinitrophenol was about 62.08% when the reaction time was 100 min. The reductive ratio did not have any change after 100 min.

The relationship of concentration of NADH and the reductive ratio of 2,4,6-trinitrophenol was illustrated in Fig. 3. When the concentration of NADH was about 1.0 mM, the reductive ratio was the highest. So the optimum concentration of NADH was as fourfold as that of 2,4,6-trinitrophenol.

The reaction products were tested using HPLC, HPLC–ESI-Mass and standard compound. The results showed that 2,4,6-trinitrophenol decreased obviously. 2,4-dinitrophenol and NAD were found in the final reaction mixture. The biotransformation mechanism of 2,4,6-trinitrophenol was presented in Fig. 1. From the results above, we can draw conclusion: the reduction of nitrophenols catalyzed by nitroreductase was more difficult when the nitryl was more.

If there were more nitro groups in the nitrophenols, the nitrophenol will not be easy to reduce. As the nitro group increases, the maximum reductive ratio decreased, the reduction need more NADH, and need more time for the reaction. As the results shown in Figs. 2 and 3, the maximum reductive ratio of 4-nitrophenol was about 83.49% when the reaction time was 70 min, the maximum reductive ratio of 2,4-dinitrophenol was about 75.28% when the reaction time was 80 min, the maximum reductive ratio of 2,4,6-trinitrophenol was about 62.08% when the reaction time was 100 min.

4. Conclusions

This work developed a new method to reduce 4-nitrophenol, 2,4-dinitrophenol and 2,4,6-trinitrophenl. 4-Nitrophenol was reduced to 4-aminophenol and 4-hydroxylaminophenol; 2,4-dinitrophenol was reduced to 4,6-dinitro-2-pimelie kelone; and 2,4,6-trinitrophenol was reduced to 2,4-dinitrophenol and 4,6-dinitro-2-pimelie kelone. The similar reductive ratios of nitrophenols were observed under both aerobic and anaerobic conditions, indicating the involvement of an oxygen-insensitive process.

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