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A novel approach for enhancing bacterial strains' Nitrobenzene degradation rate

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ABSTRACT

This study presents a novel approach that could efficiently enhance the rate of the strains' nitrobenzene biodegradation by utilizing the synergistic effect of organic reductants and a co-substrate. The authors found that the rates of strains' nitrobenzene biodegradation were increased along with the enhancement of the electron-donating power of organic additives. Combining the electron-donating power of organic reductants acting as catalyst with the ability of a co-substrate to increase the biomass of the strains improved the rate of nitrobenzene biodegradation. Employing the new approach, the rates of nitrobenzene biodegradation of the five targeted strains (*Staphylococcus carnosus* S12, *Bacillus amyloliquefaciens* YX0, *Bacillus subtilis* YX3, *Bacillus cereus* Y10, and *Bacillus cereus* YX2) were enhanced from 8.4%, 16.4%, 23.8%, 11.1% and 8.3% in a salt medium up to 85.1%, 88.6%, 95.8%, 60.5%, and 58.6%, respectively. The process described in this research may offer a protocol useful for enhancing the strains' biodegradation rate of other nitroaromatic compounds.

1. Introduction

Bioremediation is the use of microorganisms to remove pollutants from the soil or water. As an environmental remediation technology, it has great potential due to its economic and ecological advantages (Gavrilescu, 2005). A large number of organic compound-degrading microorganisms have been isolated to date. However, it is extremely difficult, time consuming, labor intensive, and costly to isolate highly efficient biodegrading microorganisms. Therefore, a method capable of efficiently enhancing the biodegradation rate of microorganisms with limited biodegradation ability will be valuable.

Nitroaromatic compounds are ubiquitous aquatic contaminants because of their widespread use as pesticides, munitions, pharmaceuticals, and industrial chemical intermediates (Lin et al., 2013; Zeng et al., 2012). Nitrobenzene has been chosen in the research as a model nitroaromatic compound to establish a novel method for enhancing the biodegradation rate of nitroaromatic compound by microbiological means. Because of the strong electron affinity of the nitro group (Huang et al., 2012), nitrobenzene requires harsher conditions than benzene to be degraded by microorganisms. Previous studies on the chemical reaction of nitroaromatic compounds demonstrated that electron donors of reducing agents (bisulfide, polysulfides, and Fe^{2+}) could contribute to the reduction of nitrobenzene and other nitroaromatic compounds (Klausen et al., 1995; Klupinski et al., 2004; Naka et al., 2006; Schmidt et al., 2010). These results are consistent with the recent study by Narayan Pradhan (Pradhan et al., 2002), showing that electron transfer resulted in nitroaromatic compound reduction. Microbial biodegradation is also a type of oxidation-reduction (or redox) reaction (Hambrick et al., 1980) that involves a transfer of electrons between two species. There have been reports on the enhancement of nitrobenzene bioremediation through the use of inorganic reductants as electron donors (Luan et al., 2009; Roy et al., 2013; Wang et al., 2011). However, few reports have been published on the use of organic reductants for enhancing the biodegradability of nitrobenzene by microorganisms despite the fact that both organic reductants and inorganic reductants have electron-donating groups. Furthermore, the fact that a large number of organic reductants in nature can be screened represents a significant advantage.

The cometabolic removal of toxicants is also a well-established method to enhance their biodegradation by increasing the biomass of microorganisms (Joshi et al., 2010; Sahinkaya and Dilek, 2006; Tarighian et al., 2003). Biodegradable organic compounds

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can be categorized into primary substrates (growth substrates) and co-metabolized species (non-growth substrates) (Saéz and Rittmann, 1993; Wen et al., 2011). If the co-metabolism of co-substrates and the catalysis of organic reductants have a synergistic effect on enhancing the rate of nitrobenzene biodegradation, this phenomenon could be utilized to effectively enhance nitrobenzene biodegradation rate.

In the present study, five target strains (*Staphylococcus carnosus* S12, *Bacillus amyloliquefaciens* YX0, *Bacillus subtilis* YX3, *Bacillus cereus* Y10 and *Bacillus cereus* YX2), which degraded 8.4%, 16.4%, 23.8%, 11.1% and 8.3% of 200 mg L^{-1} nitrobenzene, respectively, in mineral salts medium (MSM) in 5 days, were selected as candidates. It was first investigated how organic oxidizing agents (succinic acid and citric acid) and reducing agents (ascorbic acid, mannitol and Fe^{2+}) affect the rate of nitrobenzene removal by the five target strains. Then, four compounds (sucrose, glucose, peptone, and peptone plus yeast powder) were screened to identify the optimal co-substrate to enhance the rate of nitrobenzene biodegradation by the strains. To this end, experiments were set up with the co-substrate as the growth substrate and the organic reductants as the catalyst enhancing the five target strains' nitrobenzene biodegradation rate.

2. Materials and methods

2.1. Strains

The soils used for the isolation of bacteria were collected from typical farmland of Chongqing and Wuhan, China, and from the activated sludge of Henan, China. One gram of the farm soil or activated sludge was added to 50 mL of lysogeny broth medium (LB) in a 250 mL sealed flask, which was supplemented with nitrobenzene to a final concentration of 200 mg L^{-1}, and the cultures were incubated on a rotary shaker (150 rpm) at 28 °C for 7 days. Each culture was diluted and spread onto solid lysogeny broth medium (SLB). SLB plates were incubated at 28 °C for 4–5 days. Colonies appearing under the same conditions were sub-cultured. Culture purity was confirmed by microscopic examination. Finally, the pure colonies were transferred to a mineral salt medium (MSM), which contained 200 mg L^{-1} nitrobenzene. To investigate their ability to degrade nitrobenzene under aerobic conditions, five nitrobenzene-degrading strains that could degrade no more than 23.8% of 200 mg L^{-1} nitrobenzene in 5 days were selected as candidates. A phylogenetic tree of the 16S rDNA of the strains compared with the sequences available in the GenBank database is shown in Table 1.

2.2. Experimental design

Strains were pre-cultivated in the LB at 28 °C and 200 r min^{-1}. One milliliter of LB-grown cells in the late exponential phase was centrifuged at 8900 g for 10 min. The harvested cells were washed twice with 0.1 M phosphate buffer (pH 7.0), re-suspended in 1 mL of MSM and inoculated in 49 mL MSM in a 250 mL sealed flask. The flask was supplemented with 200 mg L^{-1} nitrobenzene. The cultures were incubated on a rotary shaker (150 r min^{-1}) at 28 °C. Uninoculated media with the same concentration of nitrobenzene served as controls. Samples were withdrawn after 5 days for measurements of nitrobenzene, nitrite, ammonia and cell mass.

Next, the effect of organic reductants on the nitrobenzene biodegradation rate by the five strains was evaluated. Each 250 mL glass serum vial was supplemented with 200 mg L^{-1} nitrobenzene, 49 mL of MSM, and 1 mL of LB-grown cell suspension at the late exponential growth phase along with 0.02% succinic acid, 0.02% citric acid, 0.02% ascorbic acid or 0.02% mannitol (%, w/v). Samples were withdrawn to analyze the residual nitrobenzene after 5 days. Uninoculated media with the same concentration of nitrobenzene served as controls.

The effect of the co-substrates on the nitrobenzene biodegradation rate by the five strains was also investigated. Each 250 mL flask was supplemented with 200 mg L^{-1} nitrobenzene, 49 mL of MSM and 1 mL of LB-grown cell suspension along with 1% sucrose, 1% glucose, 1% peptone or LB (1% peptone + 0.5% yeast powder; %, w/v). Uninoculated media with the same concentration of nitrobenzene served as controls.

Finally, we studied how the co-substrate (peptone) and different organic reductants work synergistically to affect nitrobenzene biodegradation by the five target strains. Each 250 mL flask was supplemented with 1% (%, w/w) peptone, 200 mg L^{-1} nitrobenzene, 49 mL MSM and 1 mL LB-grown cell suspension along with 0.02% succinic acid, 0.02% citric acid, 0.02% ascorbic acid or 0.02% mannitol (%, w/w). Uninoculated media with the same concentration of nitrobenzene served as controls. Samples were withdrawn to analyze residual nitrobenzene after 5 days.

Each experiment was replicated five times.

2.3. Analytical methods

For HPLC analysis, samples were filtered through a Syrasep polytetrafluoroethylene syringe filter (0.2 μm) and then analyzed. Nitrobenzene was measured using HPLC (Agilent 1260, Wilmington, DE, USA) equipped with an Agilent Extend-C18 column (150 mm × 4.6 mm). The analysis was performed with a flow of methanol/water (v/v, 7:3) at a rate of 1.0 mL min^{-1}. The absorbance wavelength for nitrobenzene was 280 nm, and the cell density was calculated based on the OD_{600} value with reference to a calibration curve constructed with scalar dilutions of a cell suspension. The ammonia was detected by Nessler's reagent colorimetric method and nitrite concentrations were analyzed by using diazotization method. (Administration, 2007).

Table 1
Identification and comparison of bacterial isolates from different locations.

Isolate	GenBank Accession Number	Reference Strain	% identity	Isolation Source
Staphylococcus carnosus lihwang S12(S.c S12)	KM093862	*Staphylococcus carnosus* subsp. *carnosus* TM300	99%	the vegetable garden of Chongqing
Bacillus amyloliquefaciens lihwang YX0(B.a YX0)	KM403448	*Bacillus amyloliquefaciens* LFB112	99%	the farm soil of Wuhan
Bacillus subtilis lihwang strian YX3 (B.s YX3)	KM403447	*Bacillus subtilis* strain HDYM-28	99%	the active sludge of Henan
Bacillus cereus lihwang Y10 (B.c Y10)	KM093861	*Bacillus cereus* F837/76	99%	the cornfield of Chongqing
Bacillus cereus lihwang YX2 (B.c YX2)	KM093863	*Bacillus cereus* strain MT6	99%	the vegetable garden of Chongqing