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Simultaneous heterotrophic nitrification and aerobic denitrification at high initial phenol concentration by isolated bacterium *Diaphorobacter* sp. PD-7☆

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ABSTRACT

A strain capable of phenol degradation, heterotrophic nitrification and aerobic denitrification was isolated from activated sludge of coking-plant wastewater ponds under aerobic condition. Based on its morphology, physiology, biochemical analysis and phylogenetic characteristics, the isolate was identified as *Diaphorobacter* sp. PD-7. Biodegradation tests of phenol showed that the maximum phenol degradation occurred at the late phase of exponential growth stages, with 1400 mg·L^{-1} phenol completely degraded within 85 h. *Diaphorobacter* sp. PD-7 accumulated a vast quantity of phenol hydroxylase in this physiological phase, ensuring that the cells quickly utilize phenol as a sole carbon and energy source. The kinetic behavior of *Diaphorobacter* sp. PD-7 in batch cultures was investigated over a wide range of initial phenol concentrations (0–1400 mg·L^{-1}) by using the Haldane model, which adequately describes the dynamic behavior of phenol biodegradation by strain *Diaphorobacter* sp. PD-7. At initial phenol concentration of 1400 mg·L^{-1}, batch experiments (0.25 L flask) of nitrogen removal under aerobic condition gave almost entirely removal of 120.69 mg·L^{-1} ammonium nitrogen within 75 h, while nitrate nitrogen removal reached 91% within 65 h. Moreover, hydroxylamine oxidase, periplasmic nitrate reductase and nitrite reductase were successfully expressed in the isolate.

1. Introduction

Phenol is a characteristic contaminant in many industrial effluents and waste wasters [1]. It is recommended that human exposure to phenol does not exceed 20 mg on average per day. Besides, phenol is toxic to fish and lethal at concentrations of 5–25 ppm [2]. Therefore, treatment of phenol effluents is critical to maintaining human and wildlife environments. Several methods have been reported for removal of phenol from waste waters, including solvent extraction [3], ozonization [4], and biological degradation [5]. Biological treatment is considered advantageous over the other methods because of its environmental friendliness, lower cost, economic and practical viability as it mineralizes phenol and forms less hazardous byproducts [6,7].

Since nitrate commonly occurs in many phenolic waste waters, simultaneous degradation of phenolics and removal of nitrogen offer an attractive option. Bacteria capable of combining heterotrophic nitrification and aerobic denitrification have been investigated as potential microorganisms in soils and wastewater treatment systems [8,9]. Because of their high growth rate, these microorganisms have many advantages for the removal of nitrogen: (1) less acclimation problems; (2) procedural simplicity, with simultaneous nitrification and denitrification; and (3) less buffer quantity needed, because alkalinity generated during denitrification can partly compensate for its consumption in nitrification [10]. Some groups of heterotrophic nitrification and aerobic denitrification bacteria, such as *Alcaligenes faecalis*, *Bacillus* sp., and *Providencia rettgeri*, have been isolated [11–13]. There are many differences between bacteria with the ability for nitrification and denitrification [14]. Generalization of the biochemical mechanisms is still difficult due to the limitation of the number of tested species [15]. Therefore, further investigation on a broader range of species is necessary.

The objectives of this study are to isolate a newly stable and metabolically versatile strain for simultaneous heterotrophic nitrification and aerobic denitrification with high initial phenol as a sole source of carbon, to investigate the intrinsic kinetics of cell growth and phenol degradation of the isolate, and to determine the character of enzyme activities for phenol biodegradation, heterotrophic nitrification and aerobic denitrification. This study is of particular importance to efficient phenolic wastewater treatment where simultaneous removal of nitrogen and phenol is desired.

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2. Materials and Methods

2.1. Sample preparation, growth medium and culture condition

Activated sludge was collected from coking-plant wastewater ponds in Taiyuan City, Shanxi Province, China in April 2012 and used to isolate the strain for simultaneous phenol degradation and heterotrophic nitrification–aerobic denitrification.

The strain was grown and enriched in LB medium (L^{-1}) containing 10 g tryptone, 10 g soudium chloride, and 5 g yeast extract leaching powder with initial pH 7.2–7.4. The composition of basal inorganic medium (B) for biodegradaion study of phenol and aerobic nitrification was as follows (L^{-1}): 0.5 g NH_4Cl, 0.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2$, 0.5 g K_2HPO_4, and 1 ml of trace mineral solution. The trace mineral solution contained (L^{-1}) 2.86 g H_3BO_3, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.08 g $CuSO_4{\cdot}5H_2O$, 2.03 g $MnSO_4{\cdot}4H_2O$, and 1.26 g $Na_2MoO_4{\cdot}2H_2O$ (pH value of 7.2–7.4). Giltay and nitrite (GN) medium [16] at pH 7.2–7.4 contained (L^{-1}) 1.0 g KNO_3 and certain concentration of phenol, to which 5.0 ml of 1% alcoholic solution of bromothymol blue, 1.0 g KH_2PO_4, 1.0 g $MgSO_4{\cdot}7H_2O$, 0.2 g $CaCl_2$, and 0.05 g $FeCl_3$ were added. The medium (C) used for denitrification and nitrate reduction study contained (L^{-1}) 1 g $NaNO_3$ rather than 0.5 g NH_4Cl, other compositions were the same with medium (B). Phenol, as a sole source of carbon, was added to the medium as needed, and then autoclaved at 121 °C for 30 min. The degradation was conducted in 250 ml Erlenmeyer flasks ($n = 3$) sealed with autoclaved gauzes at 30 °C in a rotary shaker with a speed of 180 $r{\cdot}min^{-1}$. Solid media were prepared from growth media with an addition of 2% agar. All chemicals were of analytical grade.

2.2. Enrichment and isolation of bacteria

A 5 ml mixed bacterium liquid was added to autoclaved 100 ml inorganic medium (B), adjusted pH value to 7.2–7.4 with NaOH(aq) and HCl(aq) as neutral conditions to favor the growth of bacteria, and then incubated on a rotary shaker (180 $r{\cdot}min^{-1}$) at 30 °C in an Erlenmeyer flask. After cultivation for 3 days, the culture was periodically sampled and tested for the presence of nitrite and residual phenol. When nitrite was detected and phenol was degraded, 5 ml of the enrichment suspension was transferred to 100 ml of fresh inorganic medium and incubated for another 3 days. Ammonium removal was also measured to screen the bacterial cultures. The most promising culture (with the best ammonium and phenol removal) was spread onto agar LB medium plates using the dilution plate method and incubated at 30 °C for 72 h. Isolated colonies were individually recultured on agar plates to obtain pure strains. The capability of aerobic denitrification of isolated strains was tested on the solid GN media. The strain PD-7 showed the highest aerobic denitrification activity, as indicated by the color change of GN medium from green to bright blue [16]. This isolate was identified and further tested for phenol degradation and heterotrophic nitrification–aerobic denitrification.

2.3. DNA extraction, PCR amplification and 16S rDNA gene sequence analysis

DNA was extracted from a bacterial suspension (1 ml, OD_{600} ~ 1.2) by using the EZ Spin Column Bacterial Genomic DNA Isolation Kit (Sangon, Shanghai). Micrographs of strain PD-7 were taken with a scanning electron microscope (Quanta200, Holland). The physiological characteristics of PD-7 were examined according to the methods in [17]. 16S rDNA gene was amplified by PCR using the universal primers F27 (5′-AGAGTT TGATCMTGGCTCAG-3′) and R1492 (5′-TAC GGYTACCTTGTTACGACTT-3′). 16S rDNA sequence was compared with that of other strains by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Related sequences were obtained from the GenBank database using the BLAST search program.

2.4. Phenol biodegradation

After the strain was activated twice, the cells in the phases $OD_{600} = 1.217$ were harvested as inocula. 5 ml of this subculture was transfused into 100 ml mineral medium (B) containing varying phenol concentrations over the range from 0 to 200 $mg{\cdot}L^{-1}$ at an interval of 20 $mg{\cdot}L^{-1}$, 200–600 $mg{\cdot}L^{-1}$ at an interval of 100 $mg{\cdot}L^{-1}$, and 600–1400 $mg{\cdot}L^{-1}$ at an interval of 200 $mg{\cdot}L^{-1}$. Samples were periodically taken for determining biomass and phenol concentrations.

2.5. Measurements of heterotrophic nitrification and aerobic denitrification with shake flask experiments

Strain PD-7 was separately cultivated in basal media B and C at 30 °C with a shaking speed of 180 $r{\cdot}min^{-1}$. Centrifugation was conducted (4 °C, 15 min) after 72 h of cultivation and then the pellets were washed with sterilized water. Centrifugation and washing were repeated three times to purify the bacterial suspension. To start experiments, different basal media (B and C, 100 ml each) and bacterial suspension (5 ml) were placed into conical flasks (250 ml) and cultivated at 30 °C and 180 $r{\cdot}min^{-1}$. During incubation, the cultures were sampled periodically to determine cell optical density (OD_{600}) and then centrifuged (4 °C, 15 min) to obtain supernatants for determination of phenol concentration, ammonium, hydroxylamine, nitrite and nitrate.

2.6. Enzyme activity

Enzyme activities about phenol degradation were spectrophotometrically determined in cell-free extracts using quartz cuvettes of 1 cm path length. Cells grown in different exponential stages in basal medium (B) were harvested and centrifuged at 7500 $r{\cdot}min^{-1}$ for 10 min. After being washed twice with 0.1 $mol{\cdot}L^{-1}$ phosphate sodium buffer (pH 7.2) and resuspended in the same buffer, the cell pellet was disrupted by ultrasonication for 5 min, and then cell debris were removed by centrifugation of the homogenized cell suspension at 12000 $r{\cdot}min^{-1}$ and 4 °C for 20 min. The cleared supernatant was immediately used for assays of enzyme and total protein. The phenol hydroxylase activity depended on the presence of NADPH and was assayed spectrophotometrically according to NADPH absorbance at 340 nm [18]. Catechol 1,2-dioxygenase and 2,3-dioxygenase activities were spectrophotometrically determined following the formation of *cis*,*cis*-muconic acid and 2-hydroxymuconic semialdehyde at 260 and 375 nm. The former was monitored in the presence of 1 $mmol{\cdot}L^{-1}$ EDTA and 33 $mmol{\cdot}L^{-1}$ Tris–HCl at pH 7.2 and 10 μl enzyme extract with the total volume of 2 ml. The reaction was started by adding 0.1 $mmol{\cdot}L^{-1}$ catechol. The latter, 2,3-dioxygenase, was evaluated by measuring the formation of 2-hydroxymuconic semialdehyde at 375 nm. The reaction mixture contained 0.1 $mmol{\cdot}L^{-1}$ catechol, 50 $mmol{\cdot}L^{-1}$ Tris–HCl at pH 7.2 and 10 μl enzyme extract, within a total volume of 2 ml [19].

Three reaction mixtures I, II and III were prepared for detecting the hydroxylamine oxidase (HAO), nitrate reductase (NAR) and nitrite reductase (NIR), respectively. The disappearance of hydroxylamine from reaction mixture I in the presence of cell-free extract was taken as a measure of HAO activity. Reaction mixture I in 10 ml contained enzyme extract, 0.11 $mmol{\cdot}L^{-1}$ cytochrome *c* and 10 $mmol{\cdot}L^{-1}$ Tris–HCl buffer (pH 7.2), and the reaction was initiated by the addition of hydroxylamine. The production of nitrite from nitrate in the presence of cell-free extract in reaction mixture II was taken as a measure of NAR activity. Reaction mixture II in 20 ml contained enzyme extract, 0.2 $mmol{\cdot}L^{-1}$ NADH and 10 $mmol{\cdot}L^{-1}$ potassium phosphate buffer (pH 7.2), and the reaction was started by the addition of $NaNO_3$. The reduction of nitrite from reaction mixture III in the presence of cell-free extract was taken as a measure of NIR activity. Reaction mixture III in 20 ml contained enzyme extract, 0.2 $mmol{\cdot}L^{-1}$ NADH and 10 $mmol{\cdot}L^{-1}$ potassium phosphate buffer (pH 7.2), and the reaction was started by the addition of $NaNO_2$. Total protein concentration in