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Short Communication

Isolation and Degradation Characteristics of a Cold-Resistant Nitrobenzene-Degrading Strain from River Sediments

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Abstract

A cold-resistant bacterium (strain GYT1) for degrading nitrobenzene was isolated from sediment in the Wei-he River by cultivation, domestication, enrichment, screening, and purification. Strain GYT1 is observed as a gram-negative bacterial strain of short pole shape according to the analysis of its morphology and physiological-biochemical characteristics. Strain GYT1 is identified as *Methylobacillus* sp. Lap based on the similarity analysis of its 16S rDNA gene sequence with the sequences logged in RDP database and the GenBank. *Methylobacillus* sp. Lap has never been reported to be able to degrade nitrobenzene. The degradation characteristics show that strain GYT1 can grow with nitrobenzene as the sole source of carbon and nitrogen. Nitrobenzene can be degraded completely when nitrobenzene concentration is less than 711µg/L at 12±0.5ºC, with 15ml inoculation volume of strain GYT1 and pH 6.3-7.2. The degradation process meets the first-order reaction dynamics. According to the results of GC-MS analysis, nitrobenzene can be degraded into small molecule substances of non-toxicity or low-toxicity, and finally into CO_2 and H_2O by multi-step biochemical reactions under the action of enzyme catalytic metabolism.

Keywords: nitrobenzene, river-sediments, dominant bacteria, biodegradation, microorganisms

Introduction

Nitrobenzene ($C_6H_5NO_2$, or NB), a typical representative of NO_2-Aromatic compounds, is widely used as the raw materials for the manufacture of aniline, azobenzene, and dyes. Nitrobenzene is usually discharged into the environment with wastewater or due to accidental leakage. Because of the difficulty of biodegradation, nitrobenzene has become one of the most common pollutants in rivers, groundwater, and soil [1, 2]. Many studies have shown that nitrobenzene and its derivatives are highly toxic and mutagenic for animals and humans.

In general, the biological treatment of nitrobenzene has advantages over physicochemical methods because it involves fewer chemical agents, equipment, and less secondary pollution [3, 4]. Microbial degradation of nitrobenzene has been studied extensively in the past. Researchers have discovered several microorganisms capable of degrading nitrobenzene such as *Pseudomonas* sp, *Staphylococcus* sp, *Streptococcus, Bacteroides distasonis*, *Bacteroides merdae*, *streptococus*, *klebsiella-pneumoniae, corynebacterium,* and *C. perfringens* [5-7]. However, most of the previous studies consider room-temperature bacteria. Less attention has been paid to cold-resistant bacteria. In fact, groundwater temperature is around 12ºC with the annual change of <0.1ºC [8-11]. At this temperature, most microorganism growth and

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metabolism are in the inhibitory state, whose degrading ability of organic matter has also been greatly inhibited [12-14].

It is of significance to find bacterial species with advantages such as wide availability, high environmental endurance, and strong degradation capacity. Microorganisms in the river suffer nitrobenzene chronically, thus nitrobenzene-degrading bacteria with special characteristics can be expected in the biological degradation process. Therefore, the sediments from the Wei-he River, China, were chosen to isolate the bacteria capable of degrading nitrobenzene. In this study, a novel cold-resistant bacterial strain that utilizes nitrobenzene as the sole source of carbon and nitrogen was isolated. Optimal growth conditions, substrate utilization, and the dynamics of nitrobenzene biodegradation were investigated. Nitrobenzene metabolites were analyzed by gas chromatography-mass spectrometry (GC/MS2010, Shimadzu Corporation) and a unique degradation process was observed.

Materials and Methods

Source of Bacteria

A wet sediment was collected as the bacterial source in February 2013 (with the environment temperature under 12ºC) using a Pedersen dredge from the upper 10-20 cm layer of a typical section (N39º45’, E108º56’) of the Wei-he River, China. The bacterial source was then carried to the laboratory under the conditions of 4ºC in a refrigerator.

Chemicals

Nitrobenzene standard (1000 mg/L) was purchased from Beijing Chemical Reagent Co., Ltd. Methanol and n-hexane (chromatographic pure solvents) were purchased from the American TEDIA Company. All the other chemical reagents used in the experiments are of analytical reagent grade.

Main Culture Media

The Liquid medium contained 1L inorganic salt solution (2g/L KH_2PO_4, 3.5g/L $K_2HPO_4 \cdot 12H_2O$, 0.02g/L $FeSO_4$, 0.5g/LNaCl, 0.1g/L $CaCl_2$, 0.2g/L $MgSO_4 \cdot 7H_2O$) and 4 ml of trace elements solution (0.2g/L $ZnSO_4$ 0.3g/L$CoCl_2 \cdot 6H_2O$, 0.03g/L H_3BO_3, 0.04g/L $CuSO_4 \cdot 5H_2O$, 0.1g/L $NiSO_4 \cdot 6H_2O$, 0.4g/L $MnCl_2 \cdot 4H_2O$, 0.1g/L $Na_2MoO_4 \cdot 2H_2O$). The pH value was adjusted to 7.0±0.1 by adding filter-sterilized 10% HCl or 10% NaOH. Nitrobenzene was added as the sole source of carbon and nitrogen, the concentrations of which varied in different experimental settings. The resulting medium was sterilized under a 121ºC stream for 20 min [15].

The solid medium was obtained by adding 18 g/L nutrient agar to the liquid medium. It was also sterilized under a 121ºC stream for 20 min.

Cultivation of Bacteria

The sediment of the Wei-he River (500 mL) were placed in a 2500 mL sterilized suction flask containing 2000 mL liquid medium with 100 µg/L nitrobenzene, which was cultured in a constant-temperature shaking incubator (BS-1E, China) at 12±0.5ºC and 120 r/min for seven days to obtain the microbial suspension solution.

Domestication of Bacteria

500 mL of the microbial suspension solution was inoculated into a 2500 mL sterilized suction flask containing 2000 mL liquid medium, which was domesticated in a constant-temperature shaking incubator at 12±0.5ºC and 120 r/min. During this process, the concentration of nitrobenzene (which is the sole source of carbon and nitrogen) was adjusted to 200, 400, 600, 800, 1000, and 1200 µg/L every five days for domestication of the bacteria. The mixed bacteria solution for nitrobenzene degradation was obtained after continuous domestication to 90 days.

Isolation and Purification of Bacteria

The mixed bacteria solution (1 mL) was inoculated into a screw cap tube containing liquid medium with 512 µg/L nitrobenzene, which was cultivated in constant-temperature shaking incubator at 12±0.5ºC and 120 r/min for 120 h. The bacteria of obvious growth was inoculated to a solid medium and cultivated for 24 h. The single colony growing on the solid medium was purified three times by plate scribing and separating, Finally, six bacteria strains taking nitrobenzene as the sole source of carbon and nitrogen for growth were obtained and named GYT1~GYT6 respectively.

Screening of Bacteria

The cultivation solution of each strain was inoculated into a 200 mL sterile solution containing 512 µg/L nitrobenzene, at this time the bacteria concentration was 1.5×10^9/mL, which was cultivated in a constant-temperature shaking incubator at 12±0.5ºC and 120 r/min for 120 h. The dominant strain (GYT1) attaining the highest degradation efficiency was screened by measurement of the residual nitrobenzene.

Influencing Factors of Nitrobenzene Degradation by Strain GYT1

For all the following experiments, the nitrobenzene degrading process by strain GYT1 was carried out in 200 mL sterile Erlenmeyer flasks in a constant-temperature shaking incubator at 12±0.5ºC and 120 r/min. The pH values were adjusted by adding filter-sterilized 10% HCl or NaOH. The bacteria concentration was 1.5×10^9/mL.The concentration of the residual nitrobenzene was periodically measured.

The effect of inoculation volume of strain GYT1: Strain GYT1 of different inoculation volumes were cultured in liquid media containing 512 μg/L nitrobenzene with pH set to 7.0±0.1. (The optimal inoculation volume turns out to be 15 ml. See the Results and Discussions section).

The relationship between strain GYT1 growth and nitrobenzene degradation: Strain GYT1 of 15 ml inoculation volume was cultured in a liquid medium containing 512 μg/L nitrobenzene with pH adjusted to 7.0±0.1. In the real experiments, we concurrently performed multiple replications of this experiment for measurements of the residual nitrobenzene at different time points.

The effect of additional carbon sources: strain GYT1 of 15 ml inoculation volume was cultured in liquid media containing 512 μg/L nitrobenzene with different additional carbon sources such as glucose, lactose, sucrose, and starch. The concentration of all the additional carbon sources was 0.2 mg/L [16]. The pH value was set to 7.0±0.1. The degradation results were compared with the original case (only nitrobenzene as carbon source) and the blank (no source at all) case.

The effect of additional nitrogen sources: strain GYT1 of 15 ml inoculation volume was cultured in liquid media containing 512 μg/L nitrobenzene with different additional nitrogen sources such as ammonium nitrate, ammonium sulfate, ammonium chloride, and ammonium acetate. The concentration of all the additional nitrogen sources was 0.5 mg/L [17]. The pH value was set to 7.0±0.1. The degradation results were compared with the original case (only nitrobenzene as nitrogen source) and the blank (no source at all) case.

The effect of the pH value: strain GYT1 of 15ml inoculation volume was cultured in liquid media containing 512 μg/L nitrobenzene with different pH values.

The effect of the initial nitrobenzene concentration: strain GYT1 of 15 mL inoculation volume was cultured in liquid media containing different nitrobenzene concentrations within the range of 110 to 1106 μg/L with pH set to 7.0±0.1. The half-lives and correlation coefficients of degradation at different nitrobenzene concentrations were computed, and the degradation products were analyzed qualitatively.

Identification of Strain GYT1

We cultivated strain GYT1 in a liquid medium containing 512 μg/L nitrobenzene. Strain GYT1 at the logarithmic growth phase was then inoculated to a solid medium also containing 512 μg/L nitrobenzene and cultivated for 24 h. The morphology and size of strain GYT1 after staining by Gram were observed under a MOTIC Digital biological microscope (DMBA400-P).

The 16S rDNA gene of strain GYT1 was obtained using a commercial genomic DNA extraction kit (SK1201-UNIQ-10, Shanghai Sangog Biotech Co., Ltd., China). The gene was amplified by polymerase chain reaction (PCR) using Taq polymerase. The PCR amplification was done with universal primers 7F:5'-CAGAGTTTGATCCTGGCT-3' and 1540r:5'-AGGAGGTGATCCAGCCGCA-3'. The products of the PCR amplification were purified and sequenced at Shanghai Sangog Biotech Co., Ltd., China.

The 16SrDNA gene sequence of strain GYT1 was aligned with the sequences logged into the RDP database (http://rdp.cme.msu.edu/index.jsp) and the GenBank from National Center for Biotechnology Information (NCBI) by BLAST. The phylogenetic tree of strain GYT1 was constructed by the Neighbor-Joining method using MEGA software (version 4.1). The homology analysis of the gene sequence was done by ClustelX1.8

Measurements of Nitrobenzene and Cell Concentration

N-hexane of 5 ml was added into a 40 ml brown reagent bottle with screw cover (Scientific Specialties Service Inc, Dikma) containing 10 ml nitrobenzene solution. The bottle was kept in a constant temperature rotating shaker (HZQ-C, China) at 200 r/min for 20 min and then in ultrasonic waves at frequency of 28 kHz for 10 min for the extraction of Nitrobenzene. 1 μl of the upper layer extract was then measured at GCMS-2010.

The GC conditions were as follows: the chromatographic column was a RTX-WAX from Shimadzu (30m×0.25mm×0.25mm). The temperature of the column was maintained at 50ºC for 3 min, and then increased to 230ºC at the rate of 5ºC/min, and finally maintained at 230ºC for 5 min. The temperature of the injection port and gasification were both at 230ºC. The injection volume was 1 μL at split-less mode. Helium was used as carrier gas (purity 99.999%).

The MS conditions were as follows: the energy was from a 70eV EI source of electron bombardment. The ion source temperature was 200ºC. The interface temperature was 230ºC. The mass scan range was 40-450 m/z.

The cell concentration was measured by Visible Spectrophotometer [18].

Results and Discussion

Screening of the Dominant Degradation Bacteria of Nitrobenzene

The degradation rates of the six strains (GYT1-GYT6) of nitrobenzene are shown in Fig. 1. The result shows that the degradation rates are significantly different. The degradation rate of strain GYT1 attained more than 98%, while the other strains only between 65% and 80%. This difference is due to the different biochemical properties of the bacteria species themselves [19, 20]. Nitrobenzene of 500 μg/L can be almost fully degraded by strain GYT1 within 120 h, which shows that the high-efficiency cold-resistant strains of nitrobenzene degradation can be isolated by the cultivation and domestication of the mixed microorganism from Wei-he River sediment. This

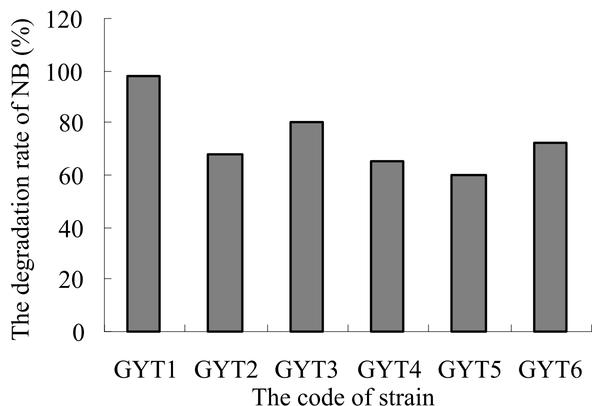

Fig. 1. The degradation rates of six strains.

demonstrates the feasibility of the bioremediation of the nitrobenzene-polluted rivers and groundwater by cold-resistant strains.

Morphology and Identification of Dominant Strain GYT1

After strain GYT1 was cultured on the solid medium for 24 h, the colony was circular and milky white. The diameter of the colony is less than 0.3cm. The surface and edge are smooth and translucent. There is a slight bump in the middle. Strain GYT1 is observed as gram-negative bacterium and exhibited a short pole shape.

The length of the PCR amplification product of strain GYT1 is 1,540 bp. The gel electrophoresis pattern is shown in Fig. 2. The 16SrDNA nucleotide sequence is aligned with the sequences logged in RDP database and the GenBank. The phylogenetic trees of strain GYT1 are constructed as shown in Fig. 3. The homology analysis of nucleotide sequence shows that strain GYT1 attains 84%, 98%, 100%, and 97%, respectively, with the homology of *Methylobacillus glycogenes*, *Methylomonas methylovora, Methylobacillus* sp. Lap, and *Methylobacillus flagellatus*. Therefore, strain GYT1 is identified as *Methylobacillus* sp. Lap.

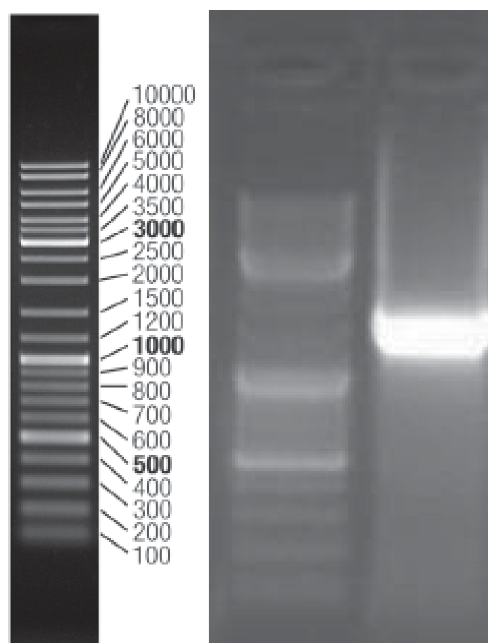

Fig. 2. The electrophoresis pattern of strain GYT1.

Effect of Strain GYT1 Inoculation Volume

When the inoculation volumes are 5 mL, 10 mL, 15 mL, 20 mL, and 30 mL (bacterial concentration: 1.5×10^9/mL), respectively, the degradation at different inoculation volumes are shown in Fig. 4. The results show that nitrobenzene degradation is accelerated with the inoculation volumes. When the inoculation volume is 5 mL, it takes 168 h to degrade nitrobenzene completely. When the inoculation volume is above 15 mL, it only takes 120 h. This shows that increasing the inoculation volume can shorten the time of degradation. However, the inoculation volume cannot see unlimited increases to accelerate the degradation because strain GYT1 would lack nutrition if the inoculation volume is too large, which restrains the growth and metabolism of strain GYT1. We can see that an inoculation volume of more than 15 mL has no significant improvement on degradation. Thus the appropriate inoculation volume is 15 mL.

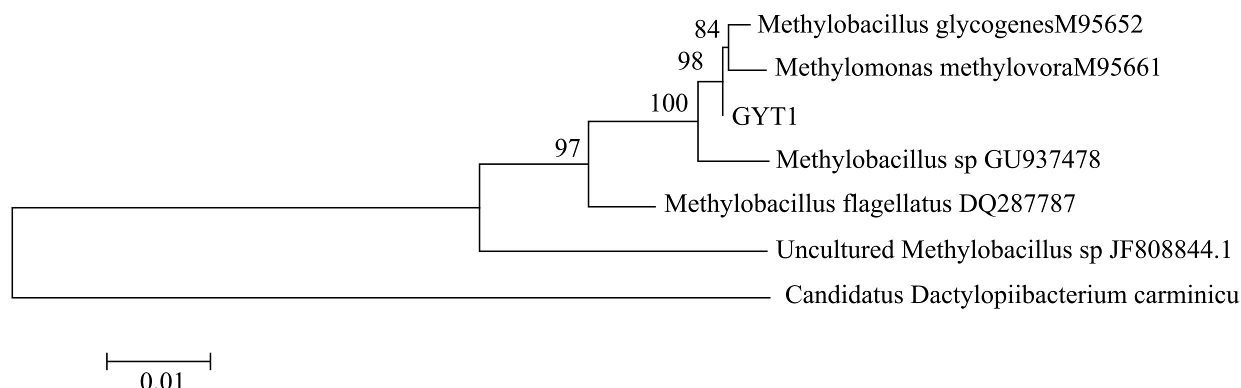

Fig. 3. The 16Sr DNA gene phylogenetic trees of strain GYT1.

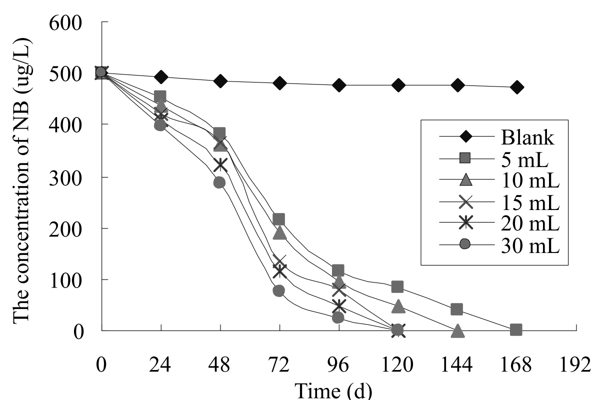

Fig. 4. Effect of GYT1 inoculation volume.

The Relationship between Strain GYT1 Growth and Nitrobenzene Degradation

The curves of strain GYT1 growth and nitrobenzene degradation are shown in Fig. 5. The results show that the concentration of strain GYT1 increases as the concentration of nitrobenzene decreases. This suggests that strain GYT1 can grow with nitrobenzene.

Effect of Additional Carbon Sources

Degradation by different additional carbon sources is shown in Fig. 6. The results show that glucose, lactose, and sucrose can accelerate nitrobenzene degradation due to the microbial co-metabolism [21], while starch has little effect on degradation. Although the nitrobenzene degradation is accelerated with additional carbon sources, all the degradation rates attain 100% after 120 h. This indicates that strain GYT1 can make use of nitrobenzene as the sole carbon source for growth.

Effect of Additional Nitrogen Sources

Degradation by different additional nitrogen sources is shown in Fig. 7. The results show that different additional nitrogen sources have little effect on nitrobenzene degradation. All the degradation rates attain 100% after 120 h except acetate ammonium, whose degradation rate is only 83% after 120 h. The pH value is 9.2 after reaction. This is because the reaction solution becomes alkaline after adding acetate ammonium, which restrains the growth and metabolism of strain GYT1, thus decreasing the degradation rate. This indicates that strain GYT1 can make use of nitrobenzene as the sole nitrogen source for growth.

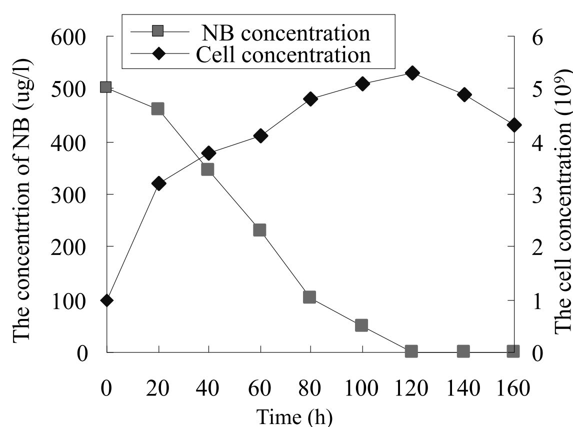

Fig. 5. The curves of strain GYT1 growth and nitrobenzene degradation.

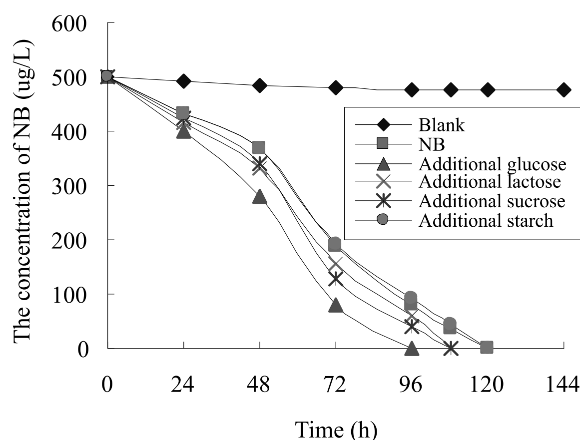

Fig. 6. Effect of additional carbon sources.

Effect of pH Value

The degradation curves of strain GYT1 of nitrobenzene at different pH values are shown in Fig. 8. The result shows that H^+ concentration plays a very important role in the growth and metabolism of strain GYT1 in the degradation course. When the pH value ranges from 6.3 to 7.2, the degradation rate of nitrobenzene becomes closed to 100% after120 h. When the pH value is above

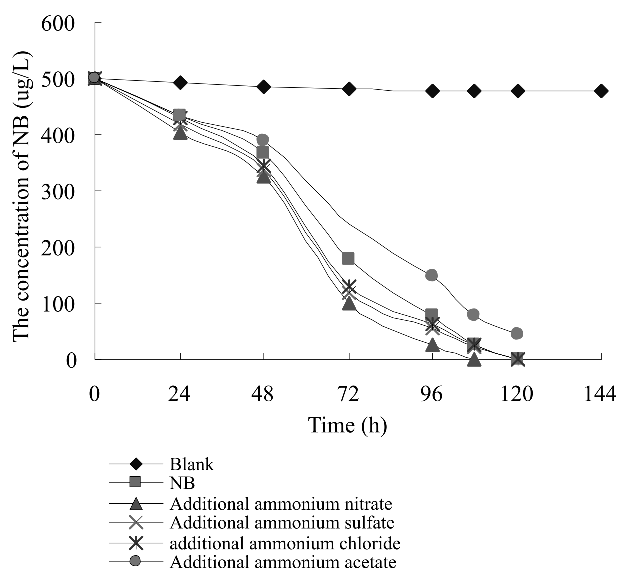

Fig. 7. Effect of additional nitrogen sources.

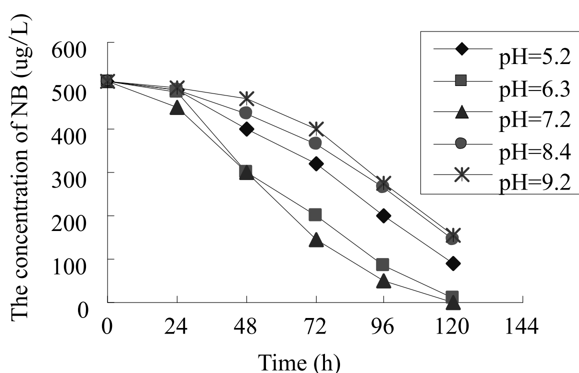

Fig. 8. Effect of pH value.

7.2, the degradation ability of strain GYT1 is restrained. For example, when the pH values are 8.4 and 9.4, respectively, the degradation rates are 72.1% and 69.5% after 120 h. This is because a higher pH value can cause the change of membrane charge of the microbial cells, which affects the ionization effect of nitrobenzene and the activity of enzyme in the process of microbial metabolism [22-23]. In addition, the higher pH value can also change the toxicity of nitrobenzene, which impacts the bacteria's capability to use sources of carbon and nitrogen [24]. On the contrary, when the pH value is less than 6.3, the redox electric potential is decreased and the logarithmic growth period of strain GYT1 is shortened [25]. When the pH value is 5.2, the degradation rate is 82.8% after 120 h. Thus, the ideal pH value for strain GYT1 growth ranges from 6.3 to 7.2.

Effect of Initial Nitrobenzene Concentrations

The degradation curves of different initial nitrobenzene concentrations are shown in Fig. 9.

The results show that nitrobenzene degradation is divided into two stages: adaptation and rapid degradation. Nitrobenzene concentration decreases slowly during the adaptation period, which is mainly because the growth of strain GYT1 is inhibited by the toxicity of nitrobenzene. A higher initial nitrobenzene concentration would result in a longer adaptation period of below 512 μg/L, when the adaption period is around 24 h; above 512 μg/L the adaption period is around 48 h. Nitrobenzene is degraded rapidly during the rapid degradation period. Nitrobenzene degradation rates attain 100% within 120 h when the nitrobenzene concentrations are within 110-512 μg/L, while it needs 144 h when the nitrobenzene concentration is 711 μg/L. When the nitrobenzene concentration reaches 1106 μg/L after 168h, still 92% of nitrobenzene is degraded. In fact, a higher nitrobenzene concentration results in higher stimulation and a longer degradation period for the bacterium because the increase of nitrobenzene concentration provides more nutrition for the bacterium. However, once the concentration exceeds a certain value, it would harm the growth of the microorganisms due to the toxicity, which decreases their degradation ability [26]. From this experiment, we conclude that strain GYT1 has a good degradation effect with initial nitrobenzene concentration ranging from 110 to 1106 μg/L. Therefore, strain GYT1 can be used to repair the rivers and groundwater polluted by nitrobenzene with concentrations of less than 1200 μg/L in practice.

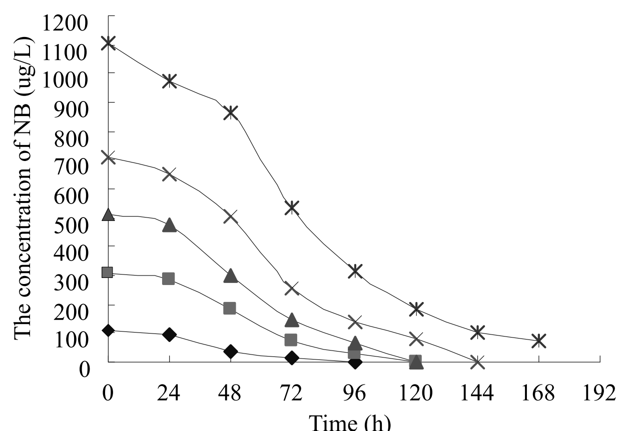

Fig. 9. Effect of initial nitrobenzene concentrations.

The time-varying nitrobenzene concentrations are fitted by zero-order degradation dynamics equation ($C = -kt + B$), first-order degradation dynamics equation ($\text{In}C = \text{In}C_0 + kt$) and second-order degradation dynamics equation ($1/C = -kt + B$), respectively. The results show that the degradation process of nitrobenzene meets the first-order reaction dynamics equation under different initial concentrations. The dynamics equation, half-life, and correlation coefficients of nitrobenzene biodegradation are shown in Table 1.

From Table 1 we can observe that the dynamics equations under different concentrations all have high correlation coefficients, which indicates that the first-order reaction equation can well describe the nitrobenzene degrading process by strain GYT1. The half-life of nitrobenzene biodegradation increases from 21.93 h to 35.91 h as the nitrobenzene concentration increases from 110 to 711 μg/L. This indicates that nitrobenzene has not met the demand from strain GYT1, i.e., nitrobenzene is still in the unsaturated state relative to cell concentration.

Table 1. The dynamics equation, half-life, and correlation coefficients of biodegradation.

Initial concentrations of nitrobenzene (μg/L)	Dynamics equation	Half-life (h)	Correlation coefficient (R^2)
110	lnC=-0.0316t+4.9604	21.93	0.9261
306	lnC=-0.0247t+6.0507	28.06	0.9160
512	lnC=-0.0224t+6.5243	30.94	0.9223
711	lnC=-0.0193t+6.8454	35.91	0.9476
1106	lnC=-0.0170t+7.3574	40.76	0.9475

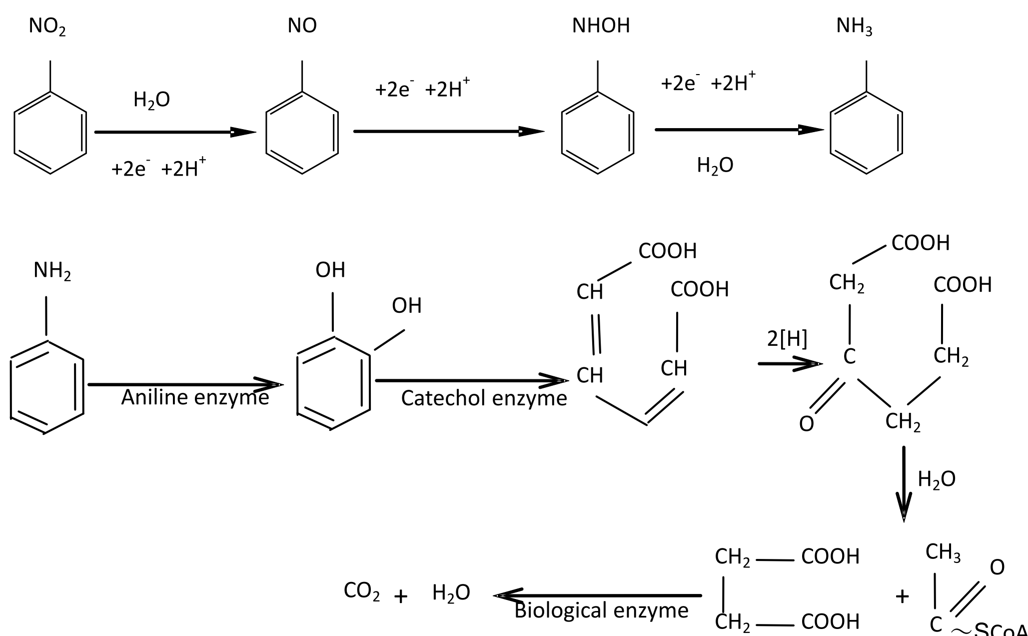

Fig. 10. The possible process of nitrobenzene anaerobic biodegradation.

When the concentration of nitrobenzene is 1106 μg/L, nitrobenzene meets the demand well. However, due to the toxicity of nitrobenzene this high nitrobenzene concentration results in strong inhibition on the growth of strain GYT1, which extends the half-life to 40.76 h.

Qualitative Analysis of Nitrobenzene Degradation Products

In the processes of nitrobenzene biodegradation, the density of the electron cloud on the benzene ring decreases because the nitro on nitrobenzene can attract electrons. Therefore, nitrobenzene is prone to reduction reaction [27]. Under the quantitative catalytic of biological enzyme [28], firstly nitrobenzene is reduced into aniline ($C_6H_5NH_2$), and then $C_6H_5NH_2$ continues to be degraded into phenols such as catechol ($C_6H_4(OH)_2$). Finally $C_6H_4(OH)_2$ can be metabolized by either ortho-position (o-) or meta (m-). In our experiment, we found that $C_6H_4(OH)_2$ is metabolized by the ortho-position (o-), i.e., $C_6H_4(OH)_2$ is cut between the two hydroxyl groups. According to the results of GC-MS analysis, the intermediate metabolites such as succinct acid, acetyl-CoA, and other small molecule substances of non-toxic or low-toxicity [29, 30] are produced by the multi-step biochemical reactions of phenols. Then these intermediates are further degraded into CO_2 and H_2O under the action of enzyme catalytic metabolism. The possible process of nitrobenzene degradation is shown in Fig. 10.

Conclusions

Strain GYT1 isolated from the Wei-he River sediments is identified as *Methylobacillus* sp. Lap. Strain GYT1 can take nitrobenzene as the sole source of carbon and nitrogen for growth. Nitrobenzene can be degraded completely within 144 h when nitrobenzene concentration was less than 711 μg/L at 12 ± 0.5ºC, with 15ml inoculation volume of strain GYT1 and pH 6.3-7.2. The degradation process meets the first-order reaction dynamics. Nitrobenzene can be degraded into small molecule substances of non-toxicity or low-toxicity and finally into CO_2 and H_2O by multi-step biochemical reactions under the action of enzyme catalytic metabolism.

The cold-resistant strain capable of degrading nitrobenzene has been rarely studied. In this paper, the nitrobenzene degrading microorganisms are studied at 12±0.5ºC. In nature, many organic pollutants may be present at even lower temperatures, thus the cold-resistant degrading microorganisms lower than 12ºC should be further studied. In addition, the degradation mechanism and degradation products of nitrobenzene require further study.

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