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ORIGINAL PAPER

Aerobic degradation of pyridine by a new bacterial strain, *Shinella zoogloeoides* BC026

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Abstract A new bacterial strain, *Shinella zoogloeoides* BC026, which utilizes pyridine as its sole carbon, nitrogen and energy source, was isolated from the activated sludge of a coking wastewater treatment plant. The BC026 strain completely degraded up to 1,806 mg/l of pyridine in 45.5 h. The optimum degradation conditions were pH 8.0 and temperature 30–35°C. According to product monitoring and genetic analysis, the pyridine ring was cleaved between C_2 and N, resulting in 58% of pyridine-N being directly converted into ammonium. Providing glucose as the extra carbon source, the degradation of pyridine was not affected, while the growth of the strain was promoted, and 41% of pyridine-N was converted into ammonium with a C/N ratio of 35. The ammonium was utilized rapidly by the strain, and a portion of it was transformed into nitrate, then to nitrite, and finally to dinitrogen if enough extra carbon was provided. Considering these characteristics, this strain may accomplish heterotrophic nitrification and aerobic denitrification simultaneously.

Keywords *Shinella zoogloeoides* · Pyridine · Biodegradation · Nitrogen transformation

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Introduction

Pyridine, a representative of nitrogen heterocyclic compounds (NHCs), is often generated by coking plants, pharmaceutical factories and related industries. Due to its toxicity and nauseating odor, discharge of pyridine-containing waste does great damage to human health and the environment. Among various solutions, biological treatment is regarded as a feasible and cost-effective method for clean-up of environments polluted by pyridine and its derivatives [20].

It was first reported in 1914 that pyridine was degraded by some soil microorganisms [32]. Subsequently, some bacterial strains that degrade pyridine under aerobic or anaerobic conditions have been investigated, e.g., *Nocardia* [8], *Micrococcus luteus* [26], *Rhodococcus opacus* [5] and *Pseudomonas* [13, 16]. Some previous research focused on the microbial transformation mechanism of pyridine. So far, at least three metabolic pathways are known. Watson and Cain [32] proposed two different pyridine metabolic pathways in which the pyridine ring was cleaved (1) between the N and the carbon 2 or (2) between the carbon 2 and the carbon 3 by different bacterial strains of *Nocardia* and *Bacillus*, since glutarate semialdehyde and succinate semialdehyde were intermediate products, respectively. Similar results were obtained from many other bacteria. In earlier research, a hydroxylation step for pyridine was not found. But analysis of extracts from the culture broth of *Rhodococcus opacus* and *Arthrobacter crystallopoietes* strains revealed (3) the formation of a number of hydroxylated metabolites during pyridine utilization [7]. Regarding pyridine-N transformation, it was documented that ammonium was released in the course of pyridine biodegradation [5, 32]. Furthermore, the unique potential of *Bacillus coagulans* to reduce N from the aromatic ring to NH_3–N, and

subsequently heterotrophically to NO_2^- and NO_3^-, was demonstrated [30]. Another crucial aspect is how to use these pyridine-degrading bacteria for remediation of contaminated environments. Bioaugmentation by adding pyridine-degrading bacteria [15] or immobilized bacteria [11, 17] to enhance specific biological activity has also attracted increasing interest in recent years.

Reports on the metabolic pathways of pyridine mostly focus on the pyridine-C cycle, but are limited to the pyridine-N cycle in pure culture. Understanding the pyridine-N cycle will help to track the metabolic process from organic N to inorganic N, and also lead to a feasible method of eliminating these N products through biological treatment. This study reports a detailed investigation of pyridine biodegradation by a new bacterial strain, *Shinella zoogloeoides* BC026. In addition, the pyridine-N transformation pathway in two media with different C/N ratios was investigated by product analysis and molecular methods. Some key N intermediates and products were quantified during the biodegradation.

Materials and methods

Media

Three kinds of medium were used in the experiments. Luria-Bertani (LB) medium [24] was used for bacterial enrichment and maintenance. Mineral salt medium (MSM), as described by Kilbane [10] and revised, and MSM plus glucose were used in the biodegradation experiment. Each liter of MSM contained 1.42 g Na_2HPO_4, 1.36 g KH_2PO_4, 0.216 g $MgSO_4 \cdot 7H_2O$, 0.006 g $CaCl_2$ and 1 ml of trace elements solution, and pH was adjusted to 7.5. Each liter of the trace elements solution contained 1.69 g $MnSO_4 \cdot H_2O$, 0.24 g $CoCl_2 \cdot 6H_2O$, 1.16 g H_3BO_3, 0.024 g $Na_2MoO_4 \cdot 2H_2O$, 2.78 g $FeSO_4 \cdot 7H_2O$, 1.15 g $ZnSO_4 \cdot 7H_2O$ and 0.38 g $CuSO_4 \cdot 5H_2O$. Pyridine solution filtered with a 0.2-μm membrane was added to the MSM as the sole carbon, nitrogen and energy source for the bacteria. When required, glucose solution was also added to the MSM as an extra carbon source; 1.9% (wt/ml) of agar was added to the medium to solidify it if agar plates were needed.

Chemicals

Pyridine standard solution was from Chemservice Inc., USA. NH_3–N, NO_2^-–N and NO_3^-–N standard solution were from the China Research Center of Certified Reference Materials. Tryptone and yeast extract were from Oxoid Ltd., UK. Solvents for HPLC analysis and GC/MS analysis were of chromatographic grade. All other chemicals were of analytical grade.

Isolation and enrichment of bacterial strain

Samples of activated sludge were obtained from the coking wastewater treatment plant of Capital Iron and Steel Corp., Beijing, China, and then aerobically domesticated in a reactor supplemented with pyridine from low to high concentration (100–500 mg/l) and several essential trace elements periodically for 60 days. Then 100 ml of the cultivated sample was put into a 500-ml Erlenmeyer flask. In order to deflocculate and mix thoroughly, 2 drops of 0.01% sodium pyrophosphate and several glass beads were added to the sample. After shaking for 30 min at 30°C, 180 rpm, the mixture was centrifuged at $3{,}000 \times g$ for 10 min; 1 g of deposit was transferred into MSM containing 300 mg/l of pyridine and incubated at 30°C, 180 rpm. When pyridine was removed completely from the medium, 5 ml of the culture was transferred to 95 ml of fresh medium. After three successive transfers, the serial dilutions of suspensions (10^{-1} to 10^{-7}) were spread onto the solid MSM with 2% (wt/ml) agar. Pyridine-degrading bacterial colonies were screened out and then purified three times by the streak plate method. Finally, pure colonies were transferred to quinoline-containing liquid MSM to investigate their ability to metabolize pyridine under aerobic conditions. One bacterial strain that utilized pyridine as its sole sources of carbon, nitrogen and energy was selected and named BC026. For maintenance, BC026 was cultivated aerobically in LB liquid medium [24] containing 500 mg/l pyridine and stored in 15% glycerol at −70°C in an ultra-low temperature freezer (Sanyo MDF-382E, Japan).

Identification and characterization of the isolate

The isolated strain was identified by physio-biochemical test (Biolog GN2 MicroPlate) and 16S rRNA sequence analysis. 16S rRNA amplification was described as in a previous study [3], and the sequence was analyzed by an ABI 3730*xl* DNA analyzer (Applied Biosystems, USA). The result was blasted with other published 16S rRNA sequences by using the BLAST program of the National Center for Biotechnology Information (NCBI) database. Selected sequences with the greatest similarity to the isolated sequence were extracted from the GenBank database. Sequence analysis was performed by Bioedit software, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 4.0 [29].

One pure strain sample was sent to the Institute of Microbiology of the Chinese Academy of Sciences for morphological observation. After pre-treatment, the strain BC026 was visualized under a scanning electronic microscope (FEI QUANTA 200, Holland). In addition, the strain was also tested for gram staining, oxygen demand and motility by standard methods.

Inoculum preparation for biodegradation

The inoculum for all experiments was prepared by inoculating the strain BC026 into 300 ml of the LB media with 500 mg/l of pyridine and incubating at 30°C and 180 rpm on a rotary shaker until the bacteria grew into the logarithmic phase. The bacteria were harvested by centrifugation at $3{,}000 \times g$ for 5 min and washed three times with 20 ml of MSM. The bacterial deposit was resuspended by vortexing and diluted with MSM to an optical density at wavelength 602 nm (OD_{602}) of 1–2 (Shimadzu UV2401, Japan). The bacterial suspension was immediately employed as the inoculum in the biodegradation experiment.

Biodegradation of pyridine under different conditions

The biodegradation experiments were performed using different initial pyridine concentrations (100–2,000 mg/l), temperatures (20–35°C) and initial pH values (3.0–10.0). A series of 250-ml Erlenmeyer flasks were used as batch reactors. Each flask contained 100 ml of MSM with a specific concentration of pyridine and inoculum. All flasks were sealed, shaken at 180 rpm, 30°C, and sampled periodically. For analyzing the concentration of pyridine, a portion of sample was filtered through a 0.22-μm membrane. OD_{602} values were measured against time. Sterile controls were also employed in the experiments.

Metabolic pathway of pyridine degradation

The experiments were carried out in a series of 250-ml Erlenmeyer flasks containing 50 ml of MSM or MSM plus glucose, supplemented with pyridine at 400–500 mg/l, sealed with sealfilm and inoculated with pre-cultured bacteria, and shaken at 180 rpm, 30°C. At regular intervals, a flask was taken out for analysis. Then 0.5 ml of growth medium was directly filtered through a 0.22-μm membrane for the analysis of pyridine concentration; 25 ml of growth medium was centrifuged at $5{,}000 \times g$ for 5 min, and the supernatant was filtered through a 0.22-μm membrane for the analysis of NH_3–N, NO_2^-–N, NO_3^-–N, total carbon (TC), total nitrogen (TN), total organic carbon (TOC), inorganic carbon (IC) and possible metabolites. The remaining 24.5 ml of growth medium was analyzed for optical density, pH and DO. The sterile controls without bacterial suspension were also analyzed.

Nitrification and denitrification potential

Three kinds of media were used. The MSM + NH_4Cl + glucose medium was used for the determination of nitrification potential. MSM + KNO_3 + glucose and MSM + KNO_2 + glucose were used to assess the denitrification potential. A series of 500-ml Erlenmeyer flasks, each containing 200 ml of one of the three media, were used. The initial C/N ratios of the three media were all kept at 20:1. NH_3–N, NO_3^-–N and NO_2^-–N were measured in the MSM + NH_4Cl + glucose medium; NO_3^-–N and NO_2^-–N were measured in the MSM + KNO_3 + glucose medium; NO_2^-–N was measured in the MSM + KNO_2 + glucose medium.

Also for further identification of denitrification potential, PCR amplification of nitrous oxide reductase (*nosZ*) gene fragments was conducted as N_2 was very difficult to collect and detect in the batch experiment. Extraction of total DNA from BC026 was as described above. The primers pairs described by Rösch et al. [21] were used to amplify the gene fragments encoding *nosZ* as follows:

nosZ-F: 5′ CGYTGTTC**M**TCGACAGCCAG 3′
nosZ-R: 5′ CATGTGCAG**N**GC**R**TGGCAGAA 3′

Bold-face letters denote degenerate positions. M, A + C; N, A + C + G + T; R, A + G.

Takara *Taq* hot-start polymerase (Takara, China) was used for the PCR reaction. The PCR program was set as described in previous studies [21], with the exception that the denaturation temperature was set at 94°C. Negative controls without a DNA template were done at the same time. The PCR product was separated by 1% agarose gel electrophoresis and stained by SYBR Safe DNA gel stain (Invitrogen, USA). The target DNA fragment was purified and cloned into pGEM-T Easy vectors, and transferred into competent *E. coli* TOP10 for sequencing. The *nosZ* DNA sequence was analyzed for similarity to other published sequences.

Analytical methods

The growth of the bacterial strain was monitored by optical density (OD_{602}). Cell dry weight (CDW, $g\ l^{-1}$) was determined gravimetrically by drying the harvest cells in an oven at 105°C for 24 h after centrifugation and washing with sterilized ddH_2O. A linear equation was found between the value of OD_{602} and the corresponding CDW. So, biomass was determined by converting OD_{602} value to CDW according to the linear equation during the biodegradation experiments.

Pyridine concentration was analyzed by high-performance liquid chromatography (HPLC) (Shimadzu $LC10AD_{VP}$, $SPD10A_{VP}$ UV-Vis Detector; Rheodyne 7725i manual injector; Diamonsil C_{18} reverse-phase column, 250 × 4.6 mm, 5 μm). The solvent system consisted of methol:water (4:1 v/v) delivered at 1.0 ml/min. Pyridine was detected at 254 nm at room temperature. Possible metabolites were analyzed with GC/MS (Agilent 6890N GC/5973 MSD, DB-5MS capillary column, 30 m × 0.25 mm × 0.25 μm),

and the samples were extracted with dichloromethane and dried over anhydrous Na_2SO_4. TC, TN, TOC and IC were analyzed with Multi N/C 3000 (Analytikjena, Germany); NH_3–N, NO_2^-–N and NO_3^-–N concentrations were analyzed by standard methods: the salicylate-hypochlorous acid method, the *N*-1-naphthyl-ethylenediamine method and UV-spectrophotometric determination, respectively [28, 31]. In addition, NH_4^+ was also determined by using the Ammonium-Testkit QUANTOFIX (Sigma-Aldrich, Germany) in order to acquire the approximate range of NH_3–N concentration. pH and dissolved oxygen (DO) values were analyzed by a pH meter (Thermo orion 868, USA) and a DO meter (Thermo orion 3 star benchtop, USA), respectively.

Results

Identification of the BC026

The sequence of the 1,449-bp fragment of the partial 16S rRNA gene of the BC026 was deposited at GenBank. After comparing with similar sequences published in the GenBank database using an online alignment search, the 16S rRNA sequence of the BC026 was 99% identical to those of *Zoogloea ramigera* (X74915) and *Crabtreella saccharophila* (AB238789). Combined with the result of Biolog bacteria automated identification apparatus, the isolated the BC026 strain was identified as *Shinella zoogloeoides*, which was reclassified and renamed from *Zoogloea ramigera* [2] and *Crabtreella saccharophila* [34]. A culture of the strain has been deposited in the China General Microorganism Culture Center (CGMCC; accession no. 2224).

Also, the BC026 strain was a rod-shaped bacterium 1.3–1.8 μm in length and 0.5–0.6 μm in diameter under the SEM. Physiological tests demonstrated that it was a gram negative, aerobic, motile and self-flocculated bacterium.

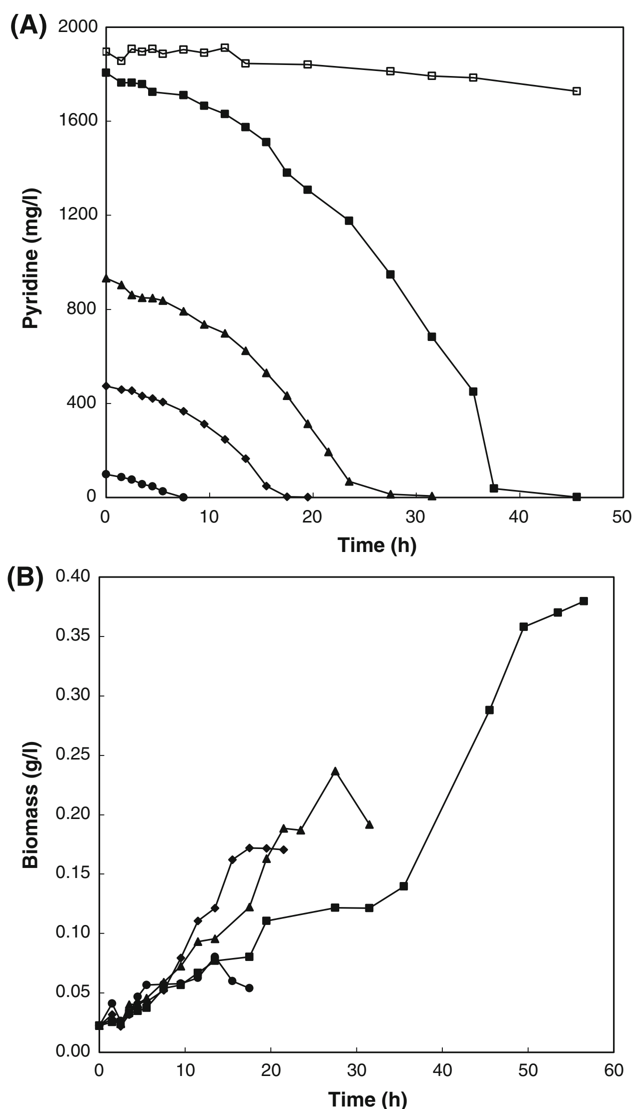

Fig. 1 Pyridine degradation and cell growth of the BC026 with different initial concentrations. *Filled circle* 99 mg/l, *filled diamond* 473 mg/l, *filled triangle* 932 mg/l and *filled square* 1,806 mg/l, *open square* sterile control

Biodegradation of pyridine under different conditions

The strain BC026 almost completely degraded 99, 473, 932 and 1,806 mg/l of pyridine after 7.5, 19.5, 31.5 and 45.5 h (Fig. 1a), while the biomass increased from 0.023 to 0.057, 0.171, 0.192 and 0.288 g/l, respectively (Fig. 1b). Subsequently, BC026 grew rapidly while utilizing pyridine and other intermediate products available in the medium. The growth yield, equaling to the amount of increased CDW divided by the amount of consumed pyridine, was 0.35, 0.32, 0.18 and 0.15 g/g, respectively, from low to high initial pyridine concentrations. The growth yield tended to decline with the increase of initial pyridine concentration.

Further experiments indicated that the BC026 strain could degrade pyridine and grew better in a temperature range of 20–35°C, but not at 37.5°C. The optimum temperature was 30–35°C. The strain also degraded pyridine and grew in an initial pH range of 5.0–9.0, with an optimum at pH 8.0.

Metabolic pathway of pyridine biodegradation

Two media, pyridine–MSM (C/N = 4.3 g/g) and pyridine–MSM plus glucose (C/N = 35 g/g), were used to track the metabolic pathway of pyridine. Figure 2 shows the variation of each factor in the two media over 48 h. The amounts of pyridine volatilization were all less than 5% from the sterile controls in the two media.

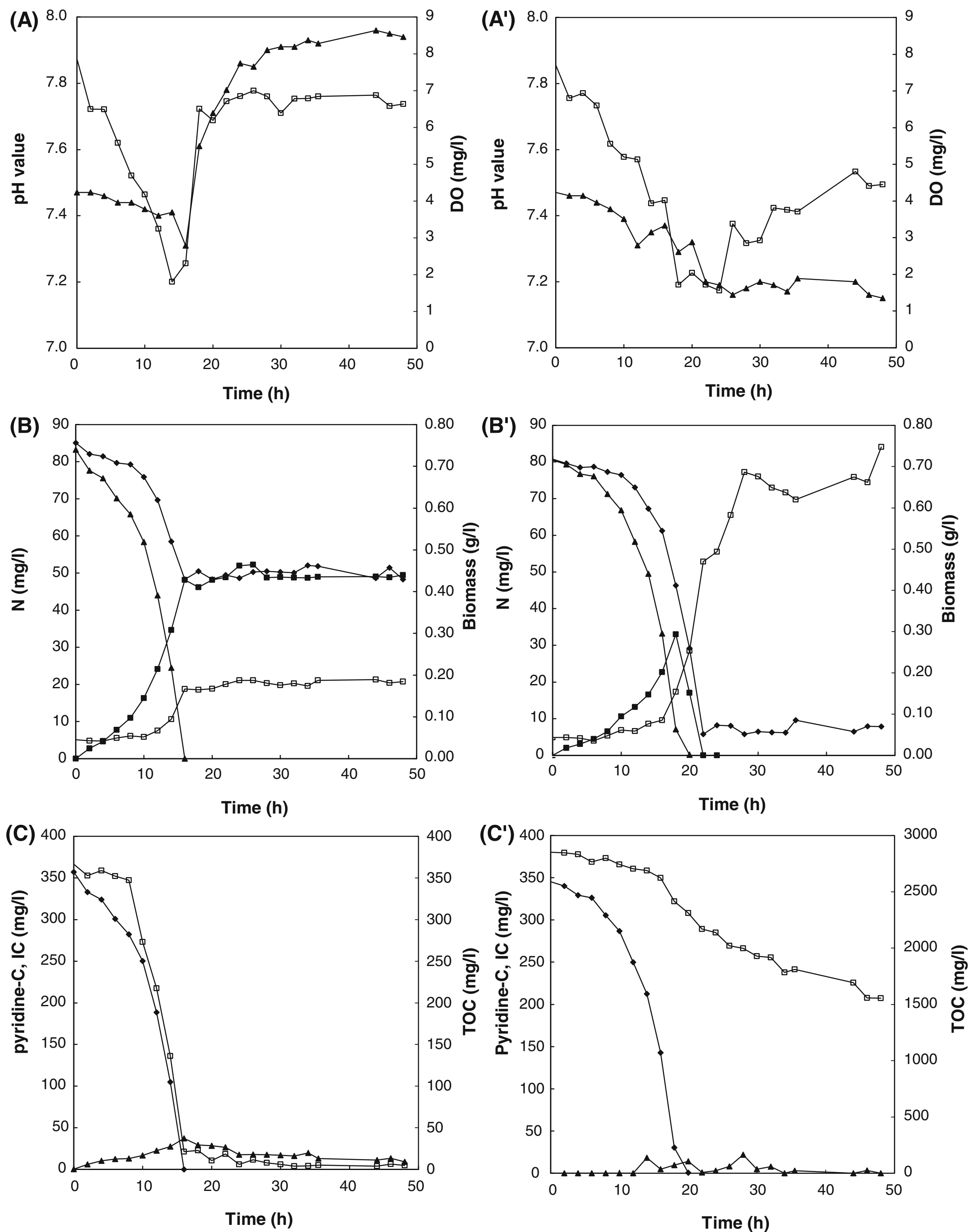

Fig. 2 Biodegradation of pyridine (*left figures*) and pyridine plus glucose (*right figures*) by the BC026. **a** and **a**′ show the variation of pH value and DO. *Filled triangle* pH, *open square* DO. **b** and **b**′ show the N transformation of pyridine. *Filled triangle* pyridine-N, *filled square* NH_3–N, *filled diamond* TN, *open square* biomass. **c** and c′ show the C transformation of pyridine and glucose. *Filled diamond* pyridine-C, *filled triangle* IC, *open square* TOC

1. Biodegradation in pyridine–MSM. In pyridine–MSM, 470 mg/l of pyridine was degraded completely within 16 h, and the BC026 biomass increased continuously from 0.045 to 0.167 g/l (Fig. 2b). The pH value dropped from 7.47 to 7.31, and DO decreased rapidly from 7.86 to 2.31 mg/l (Fig. 2a), reflecting that the BC026 required much oxygen to degrade pyridine. The TN of the solution was equal to pyridine-N since

pyridine was the sole source of nitrogen at the beginning. TN decreased significantly and equaled the total amount of pyridine-N, NH_3–N and other N products in the process of biodegradation (Fig. 2b). At the end of biodegradation, TN nearly equaled the amount of NH_3–N since pyridine-N and other nitrogenous products disappeared. We calculated that 58% of pyridine-N was converted into NH_4^+, resulting in an increase in ammonium concentration from 0 to 48.18 mg/l. TOC, initially from pyridine-C, similarly decreased, and a portion of it was mineralized to CO_2 and H_2O. Lastly, TOC dropped at 4 mg/l (Fig. 2c).

Bacteria growth became stagnant after 16 h, when pyridine was exhausted and no carbon source was available for growth. The pH increased to 7.9–8.0 and DO increased to 6.5–7.0 mg/l since oxygen was no longer utilized by the bacteria. NH_3–N remained at a stable level, as did TN. The concentration of TOC remained at 4 mg/l.

2. Biodegradation in the pyridine–MSM plus glucose. Although glucose was added, the degradation rate of pyridine did not improve over that in pyridine–MSM alone. About 455 mg/l of pyridine was degraded completely within 20 h, while the biomass increased from 0.043 to 0.253 g/l (Fig. 2b′). During this period, the pH value decreased from 7.47 to 7.32, and DO decreased from 7.70 to 1.72 (Fig. 2a′). The NH_3–N concentration increased from 0 to 33.00 mg/l within 18 h and dropped to 17.06 mg/l at 20 h (Fig. 2b′). Forty-one percent of the pyridine-N was transformed into NH_3–N. It was remarkable that the yield of ammonium was lower than that in the pyridine–MSM. This demonstrated that the bacteria consumed more pyridine-N for their growth. The final bacterial biomass (0.253 g/l) also supported this finding. TN decreased from 80.80 to 29.17 mg/l, but was still higher than the ammonium concentration at the end. This indicated that other nitrogenous intermediates were produced during the biodegradation. After pyridine was degraded completely at 20 h, the bacteria continued to grow by utilizing the remaining glucose, NH_3–N and other N sources from 20 to 48 h, resulting in a biomass increase from 0.253 to 0.747 g/l. It was remarkable that NH_3–N was degraded rapidly until it disappeared at 22 h. At the end of experiment (48 h), the pH value decreased to 7.15, and DO increased to 4.45 mg/l. TN decreased to about 6–8 mg/l. The decrease of TOC from 2,850 to 1,556 mg/l and the increase of IC from 0 to 22.0 mg/l indicated that pyridine-C was transformed into CO_2 and other intermediates (Fig. 2c′).

In both media, NO_3^-–N and NO_2^-–N were determined during biodegradation. The concentration of NO_2^-–N was very low, no more than 0.1 mg/l in both media throughout the experiments. The concentration of NO_3^-–N ranged from 0 to 2 mg/l in pyridine–MSM and from 0 to 5 mg/l in pyridine–MSM plus glucose. These results indicated that a little NO_3^-–N was produced in the biodegradation.

No heterocyclic intermediates of pyridine were detected by GC–MS, and no new peak was found in HPLC analysis and UV spectra scanning.

Nitrification of the BC026 strain

The pyridine biodegradation experiment indicated that a portion of NH_3–N might be transformed into NO_3^-–N due to the nitrification of the strain BC026. In order to demonstrate the nitrification ability of BC026, MSM + NH_4Cl + glucose was used as the growth medium. As shown in Fig. 3a, when the initial NH_3–N concentration was 82.48 mg/l, BC026 reduced it completely within 22 h. But when the initial concentration was increased to 178.41 mg/l, 76.5% of NH_3–N was reduced within 22 h, and then the biodegradation entered the stationary phase.

During the biodegradation, BC026 transformed NH_3–N into NO_3^-–N (Fig. 3a). Up to a maximum of 9.77 mg/l NO_3^- was detected in MSM + NH_4Cl (NH_3–N = 82.48 mg/l) + glucose, and 1.92 mg/l NO_3^- was detected in MSM + NH_4Cl (NH_3–N = 178.41 mg/l) + glucose. We also found that 0–0.1 mg/l NO_2^- was detected in the two media. Each experiment was repeated twice, and similar results were obtained.

BC026 grew rapidly, while the concentration of NH_3–N decreased (Fig. 3b), which indicated that BC026 utilized glucose and NH_4^+ as C and N sources for its growth.

Based on the above results, most of the NH_4^+ was utilized by BC026 for its growth, and a little was transformed into NO_2^- and NO_3^-.

Denitrification of the BC026 strain

Products analysis

In order to demonstrate the denitrification potential of BC026, MSM + KNO_3 + glucose and MSM + KNO_2 + glucose were used as media for bacterial growth.

Of the NO_3^-, 89.1% was reduced at 258 h when the initial NO_3^-–N concentration was 52.70 mg/l, and 89.9% was reduced at 402 h when the initial concentration was 101.51 mg/l (Fig. 4a). Some NO_2^- (0–0.06 mg/l) was produced in both media during the biodegradation. Meanwhile, BC026 utilized NO_3^- as the sole N source for its rapid growth (Fig. 4b). These results show that most of NO_3^- was utilized by BC026 for its growth, and a little of it was transformed into NO_2^-. Furthermore, according to the experiment results in the MSM + KNO_2 + glucose, BC026

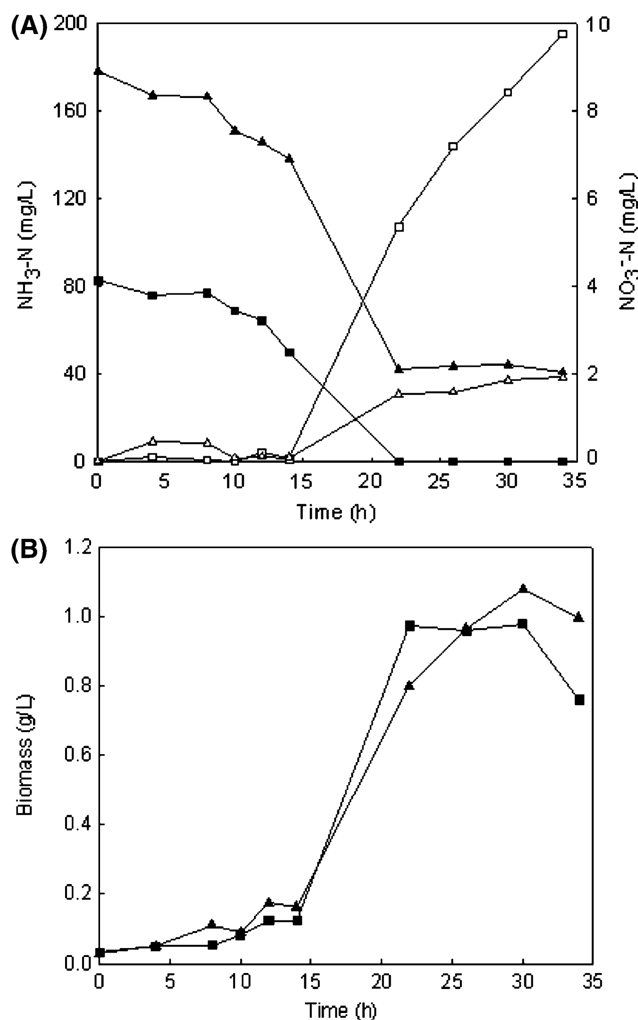

Fig. 3 Utilization of NH_4^+ with two different initial concentrations, 82.48 and 178.41 mg/l, by BC026 (glucose is the carbon source). **a** The transformation of NH_3–N (*filled square*) 82.48 mg/l and (*filled triangle*) 178.41 mg/l and the product of NO_3^- (*open square*) 82.48 mg/l and (*open triangle*) 178.41 mg/l. **b** The growth of BC026 (*filled square*) 82.48 mg/l and (*filled triangle*) 178.41 mg/l

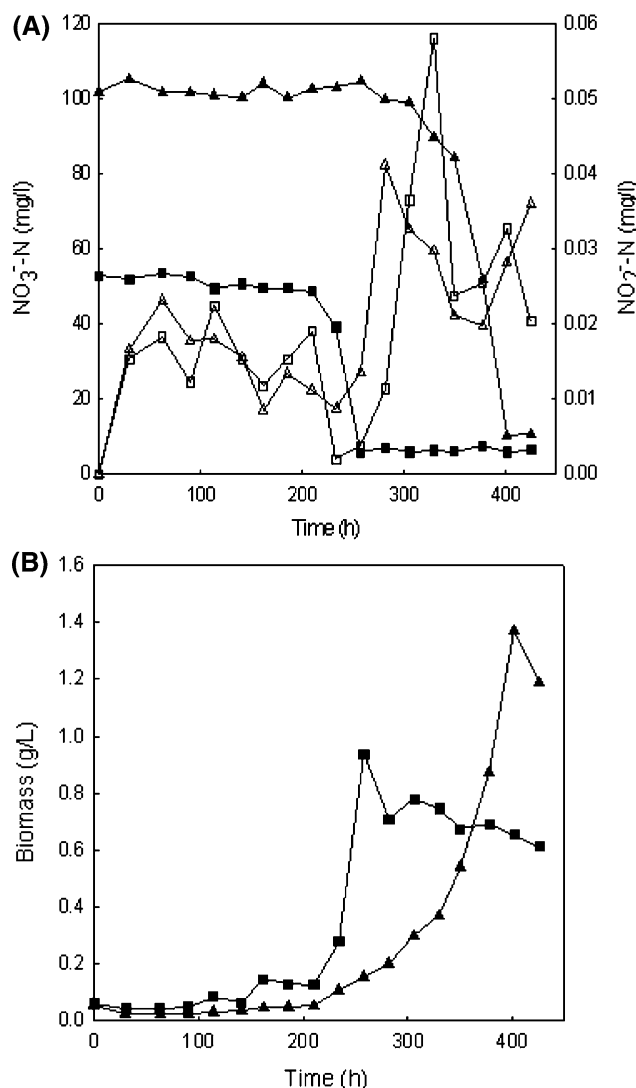

Fig. 4 Utilization of NO_3^- with two different initial concentrations, 52.70 and 101.51 mg/l, by BC026 (glucose is the carbon source). **a** The transformation of NO_3^- (*filled square*) 52.70 mg/l and (*filled triangle*) 101.51 mg/l and the product of NO_2^- (*open square*) 52.70 mg/l and (*open triangle*) 101.51 mg/l. **b** The growth of BC026 (*filled square*) 52.70 mg/l and (*filled triangle*) 101.51 mg/l

could utilize NO_2^- as the sole N source for its growth (Table 1). Since detection of N_2 in minimal amounts is difficult, we applied molecular biotechnology to determine whether BC026 can transform a portion of NO_2^- into N_2.

Functional gene

The *nosZ* gene is often used as a functional marker to identify denitrifying bacteria [19, 25]. In this study, the primer pairs were designed to amplify about 700 bp of fragment from the bacteria according to the former study [21]; 707 bp of the *nosZ* gene fragment was amplified from the BC026 genetic template as shown in Fig. 5. By comparison with the GenBank BLAST program, the *nosZ* gene fragment of the BC026 was 89% identical to those of *Achromobacter cycloclastes* (Y15161) and *Sinorhizobium* sp. PD 12 (DQ377784). The BLAST result indicated that the BC026 may have the potential ability to reduce N_2O to N_2. Based on the above results, the BC026 strain was shown to be a denitrifying bacterium.

Discussion

The bacterium *Shinella zoogloeoides*, called *Zoogloea Ramigera* before 2006, is considered to be partly responsible for flocculation in activated sludge [22] and plays an important role in waste water treatment. Previous studies

Table 1 Denitrification potential of the *Shinella zoogloeoides* BC026

NO_2^-–N (mg/l)		Biomass (g/l)	
0 day	5 day	0 day	5 day
48.01 ± 0.86	0.06 ± 0.01	0.039 ± 0.004	0.490 ± 0.029

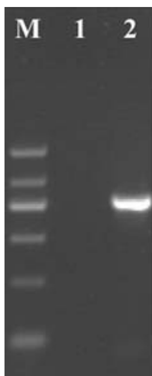

Fig. 5 PCR amplification of the specific fragment of *nosZ* gene from the *Shinella zoogloeoides* BC026. *M* the molecular size markers, with the size of 100, 300, 500, 700, 900 and 1,200 bp from bottom to top. *Lane 1* negative control of *nosZ* gene fragment; *lane 2 nosZ* gene fragment

showed that it degraded a number of organic pollutants [1, 9, 14] and was also a useful biosorbent to remove heavy metals [12, 23, 27]. So far, there are no reports on whether the species can use pyridine and other NHCs as its substrates. Due to its characteristics of self-flocculation, biosorption and biodegradation, further study of this species may help to expand its potential application.

Shinella zoogloeoides BC026, capable of degrading pyridine, was isolated from the activated sludge of a coking wastewater treatment plant. The strain utilized pyridine at high concentrations (up to 1,806 mg/l) as its sole source of carbon, nitrogen and energy. The biodegradation experiment showed that the growth rate and yield of the bacteria declined when the initial pyridine concentration was increased, implying that pyridine is a potential inhibitor. The adaptive pH and temperature conditions for cell growth and pyridine degradation of the strain matched the situation at the coking wastewater treatment plant. This indicates that pyridine pollution might be solved by applying these degrading bacteria in the treatment system.

In the experiment to determine the metabolic pathway of pyridine, two media with different initial ratios of C/N were used. In pyridine–MSM, pyridine was mainly converted to cells, CO_2 and NH_4^+. Since pyridine is an energy-deficient substrate, its C/N ratio is 4.3, the ammonium can not be utilized sequentially by BC026 owing to the available carbon source limitation. The metabolic process stopped after pyridine was exhausted. However, in the pyridine–MSM plus glucose, glucose supplied enough C and energy for the bacteria. The C/N ratio in the solution increased to 35. The strain utilized the carbon source first from pyridine and then from glucose for its growth. Thus, the lower yield of NH_4^+ in pyridine–MSM plus glucose was probably caused by a greater proportion of pyridine-N being used to synthesize phosphoglycerate for bacterial growth. When pyridine was degraded completely, glucose and ammonium were the C and N sources, so the bacteria continued to grow until the ammonium was exhausted.

It was remarkable that pH and DO concentration were two rapid indicators of the process of biodegradation. Specifically, the value of pH always decreased slightly, although NH_4^+ was produced with the degradation of pyridine, suggesting that acid intermediates might also be produced. But when the biodegradation was stopped, the pH increased sharply due to the presence of NH_4^+. The concentration of DO evidently decreased during biodegradation, indicating the rapidity of aerobic degradation and bacteria growth.

In previous studies, the production of ammonium was recognized in many bacterial strains. But other nitrogenous intermediates were seldom referred to. In this study, the appearance of NO_3^-–N indicated that a portion of the NH_4^+ was transformed into NO_3^-, also the amplified experiment in the MSM + NH_4Cl + glucose demonstrated this. Heterotrophic nitrification may be linked to aerobic denitrification, i.e., the nitrate produced by nitrification is converted to dinitrogen via nitric oxide and nitrous oxide [6, 33]. The product analysis indicates that BC026 can utilize NO_3^- as a nitrogen source for growth and transform a portion of NO_3^- into NO_2^-. PCR amplification of the *nosZ* gene indicated that the strain has the potential ability to reduce N_2O produced from NO_2^- to N_2. All molecular studies on the ecology of denitrifying bacteria are based on their products and functional genes [4]. These results demonstrated that BC026 can transform the NO_3^- produced from NH_4^+ into N_2. The proposed pyridine metabolic pathway by the BC026 strain is described in Fig. 6.

According to the GC/MS, UV and HPLC analysis, hydroxyl intermediates were not produced during the degradation of pyridine by BC026. Therefore, pyridine could not be degraded through the pathway proposed by Zefirov et al. [35]. The rapid production of NH_4^+ and acid intermediates indicated that one of two metabolic pathways proposed by Watson and Cain [32] are involved. The pyridine ring is cleaved between the carbon 2 and N, and subsequent deamination to glutaric dialdehyde is followed by successive oxidation to glutarate semialdehyde, glutarate and acetyl coenzyme A. Acetyl coenzyme A may be assimilated

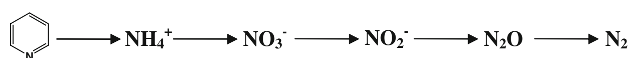

Fig. 6 Proposed N transformation of pyridine by the BC026. Most NH_4^+ was reserved when biodegradation occurred with sole substrate (pyridine). On the contrary, NH_4^+ was transformed completely into the following compounds when supplying enough available C source

via the glyoxylate cycle. This pathway was also described by Rhee et al. [18].

The study discovered the transformation of nitrogen from pyridine by the strain BC026 was in a pathway of heterotrophic nitrification and aerobic denitrification simultaneously. If a proper C/N ratio existed, the pyridine and its intermediates, especially for N products, were degraded completely.

Nucleotide sequence accession numbers

The accession numbers of the isolates 16S rRNA gene and *nosZ* in GenBank are EU346730 and EU346731.

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