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Microbial degradation and metabolic pathway of pyridine by a *Paracoccus* sp. strain BW001

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Abstract A bacterial strain using pyridine as sole carbon, nitrogen and energy source was isolated from the activated sludge of a coking wastewater treatment plant. By means of morphologic observation, physiological characteristics study and 16S rRNA gene sequence analysis, the strain was identified as the species of *Paracoccus*. The strain could degrade 2,614 mg l^{-1} of pyridine completely within 49.5 h. Experiment designed to track the metabolic pathway showed that pyridine ring was cleaved between the C_2 and N, then the mineralization of the carbonous intermediate products may comply with the early proposed pathway and the transformation of the nitrogen may proceed on a new pathway of simultaneous heterotrophic nitrification and aerobic denitrification. During the degradation, NH_3-N occurred and increased along with the decrease of pyridine in the solution; but the total nitrogen decreased steadily and equaled to the quantity of NH_3-N when pyridine was degraded completely. Adding glucose into the medium as the extra carbon source would expedite the biodegradation of pyridine and the transformation of the nitrogen. The fragments of *nirS* gene and *nosZ* gene were amplified which implied that the BW001 had the potential abilities to reduce NO_2^- to NO and/or N_2O, and then to N_2.

Keywords Pyridine · Degradation · *Paracoccus* · Metabolic pathway · Nitrogen transformation

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Introduction

Pyridine and its derivatives, obtained from coal or artificial synthesis, are widely used as industrial solvents, which inevitably enter into environment (Watson and Cain 1975). Large quantities of pyridine were often detected in the wastewater from coking plants, pharmaceutical factories and other relative industries. Due to its toxicity and nauseous odor, discharging of pyridine-containing waste does great damage to human health and environmental quality. Bioremediation is regarded as a feasible method for clean-up of environments polluted with heterocyclic chemicals, in particular, pyridine and its derivatives (Ronen and Bollag 1995).

In 1914, it was first documented that pyridine could be decomposed by some soil microorganisms (Kost and Modyanova 1978). Subsequently, the biodegradation of pyridine has been studied extensively.

A number of bacterial strains were reported to degrade pyridine under aerobic or anaerobic conditions, e.g., *Nocardia* (Houghton and Cain 1972; Watson and Cain 1975), *Corynebacterium* and *Brevibacterium* (Shukla 1973), *Bacillus* (Watson and Cain 1975), *Micrococcus luteus* (Sims et al. 1986), *Rhodococcus opacus* (Brinkmann and Babel 1996), *Pseudomonas* (Mohan et al. 2003; Pandey et al. 2007). Previous studies of those pyridine-degrading strains mainly focused on two points, the mechanism of cleavage of the pyridine ring and the bioaugmentation for pollution treatment.

By studying *Nocardia* and *Bacillus*, Watson and Cain (1975) proposed two metabolic pathways of pyridine degradation that the pyridine ring was cleaved between the N and carbon 2, or between the carbon 2 and carbon 3, as intermediate products of glutarate semialdehyde or succinate semialdehyde was found, respectively. Further investigation on *Azoarcus* showed that the important step in pyridine degradation was N–C_2 ring cleavage (Rhee et al. 1997). In earlier research, hydroxylated pyridine derivatives were not observed during the decomposition of pyridine. In recent decades, GC/MS analysis of extracts of the culture broth of *Rhodococcus opacus* and *Arthrobacter crystallopoietes* strains proved the formation of a number of hydroxylated metabolites during pyridine utilization. Yet, enzyme studies related with the proposed pathways have not been performed (Fetzner 1998). For the transformation of the heteroatom N, it was reported that ammonia was released in the course of pyridine biodegradation (Watson and Cain 1975; Brinkmann and Babel 1996). Furthermore, a unique potential of *Bacillus coagulans* to reduce nitrogen from aromatic ring into NH_3 and subsequently to oxidate NH_3 into NO_2^- and NO_3^- heterotrophically was demonstrated (Uma and Sandhya 1997). However, the mechanism of pyridine degradation lacks for evidences from enzymology and molecular biology. Although functional genes encoding nitrogen transformation has been studied extensively, it is not studied for the nitrogen transformation initiated from pyridine, especially denitrification has not been noticed in previous studies.

The concept of bioaugmentation, i.e., adding highly efficient degrading-bacteria or enzyme for wastewater treatment, has attracted increasing interest, since it can improve the biodegradation of some refractory pollutants. The addition of pyridine-degrading bacteria (Li et al. 2001; Padoley et al. 2006) or immobilized bacteria cells (Rhee et al. 1996; Kim et al. 2006; Tian et al. 2006) to enhance the decomposition of pyridine and its derivatives, has been practised in recent years. Compared to the conventional wastewater treatment systems, the potential ability to degrade toxic chemicals is enhanced either by the dissociative bacteria (Padoley et al. 2006) or by the immobilized cells (Wang et al. 2002).

Successful bioremediation needs not only the knowledge of which microorganism degrade a specific compound, but also the understanding of the pathways involving in biodegradation both at physiological and molecular levels (Jain et al. 2005). In this study, a new pyridine-degrading bacterial strain, the *Paracoccus* sp. BW001, was isolated. Its biodegradation ability and characteristics were studied. Besides, the cleavage position of pyridine ring as well as the transformation of carbon and nitrogen from pyridine ring were tried to investigate by means of intermediate products analysis and genetic characterization.

Materials and methods

Chemicals

Pyridine standard sample was purchased from the Chemservice Inc., USA. NH_3-N, NO_2^--N, and NO_3^--N standard samples were purchased from the China Research Center of Certified Reference Materials. Tryptone and yeast extract were purchased from the Oxoid Ltd., UK. Other chemicals were of analytical grade.

Medium

Two kinds of mediums were used in this study. The Luria-Bertani (LB) medium (Sambrook and Russell 2001) was used for bacteria enrichment and maintenance. The mineral salt medium (MSM), as described by Kilbane (2005) and modified was used in the biodegradation experiment. Each liter of MSM contained Na_2HPO_4, 1.42 g; KH_2PO_4, 1.36 g; $MgSO_4 \cdot 7H_2O$, 0.216 g; $CaCl_2$, 0.006 g; and trace elements solution, 1 ml; and adjusted the pH to 7.5. Each liter of the trace elements solution contained $MnSO_4 \cdot H_2O$, 1.69 g; $CoCl_2 \cdot 6H_2O$, 0.24 g; H_3BO_3, 1.16 g;

$Na_2MoO_4 \cdot 2H_2O$, 0.024 g; $FeSO_4 \cdot 7H_2O$, 2.78 g; $ZnSO_4 \cdot 7H_2O$, 1.15 g; and $CuSO_4 \cdot 5H_2O$, 0.38 g. Pyridine solution filtered with 0.2 μm membrane was added into the MSM as the sole carbon, nitrogen, and energy source for the bacteria. Sometimes, glucose solution was also added into the MSM as the extra carbon source. Agar of 1.9% (w/v) was added into the medium to solidify if agar plates were needed. All the mediums were sterilized at 121°C for 20 min before use.

Bacteria cultivation and isolation

Samples of activated sludge were gathered from the coking wastewater treatment plant of Wuhan Iron and Steel Corporation, Hubei Province, China. Samples of 200 ml were put into a 500-ml Erlenmeyer flask. In order to deflocculate and mix thoroughly, two drops of 0.01% (w/v) sodium pyrophosphate and several glass beads were added, and the mixture was shaken for 30 min at 30°C, 180 rpm. The deposition was collected and centrifuged at 3,000 × *g* for 10 min (Eppendorf 5810R, Germany). The centrifuged deposition of 1 g was transferred into the MSM containing 300 mg l^{-1} pyridine and incubated at 30°C, 180 rpm. When pyridine was disappeared completely from the medium, 5 ml of the culture was transferred to 95 ml of fresh medium. After three times of successive transfers, the diluted suspensions (10^{-2}–10^{-7}) were spread onto the MSM plates. Pyridine-degrading bacteria colonies were screened out and purified with streak plate method for three times. Finally, pure colonies were transferred into pyridine-containing liquid MSM to confirm their ability to metabolize pyridine under the aerobic condition. A bacterial strain with relatively high growth rate and degradative efficiency was obtained and named BW001. For maintenance, the BW001 was cultivated aerobically with LB liquid medium containing 500 mg l^{-1} pyridine and stored in 15% of glycerol at 70°C in an Ultra-low temperature freezer (Sanyo MDF-382E, Japan).

Morphological observation and physiological characterization

One sample of the strain BW001 was sent to the Institute of Microbiology, Chinese Academy of Sciences for morphological observation. After pretreatment, BW001 strain was observed under a scanning electron microscope (FEI QUANTA 200, Holland). Furthermore, the strain was tested for Gram staining, oxygen demand, motility, antibiotic resistance, and nitrogen-fixing capacity.

Bacterium identification by 16S rRNA sequence

Genomic DNA was extracted from the bacteria grown overnight in the LB liquid medium by using TIANamp Bacteria DNA Kit (TianGen, China). Partial fragments of 16S rRNA gene were amplified with the following primer pair (Devereuv and Wilkinson 2004):

Forward 27F: AGAGTTTGATCATGGCTCAG

Backward 1492R: TACGGTTACCTTGTTACGACTT

Polymerase chain reaction (PCR) amplification was carried out with 50 μl of mixtures in 0.2-ml PCR tubes (Axygen, USA), containing TianGen 2× Hotstart *Taq* PCR Mastermix, 25 μl; forward and backward primers (10 pmol), 1 μl, respectively; genomic DNA template, 1 μl; and ddH_2O, 22 μl. A negative control without DNA template was prepared simultaneously. The PCR was performed in a thermocycler (Thermo PXII, USA) with an initial denaturation of the template DNA at 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension performed at 72°C for 15 min and then kept at 4°C. The PCR product was separated by agarose (0.8%) gel electrophoresis. The target DNA fragments were purified by a Qiaquick gel extraction kit (Qiagen, Germany) and cloned into the pGEM-T Easy vectors (Promega, USA) according to the protocols of the manufacturers. The recombinant plasmids were transformed into competent *E. coli* TOP10 and sequenced by universal primers. The result was blasted with other published 16S rRNA sequences, using the BLAST program of the National Center for Biotechnology Information (NCBI) database (Altschul et al. 1990). Selected sequences with the greatest similarity to the isolated sequence were extracted from the GenBank database. Sequence analysis was employed by the software Bioedit and the phylogenetic tree was constructed by using neighbor joining method with the software MEGA 4.0 (Tamura et al. 2007).

Inoculum enrichment for biodegradation

The inoculum for all experiments was prepared by inoculating the strain BW001 in the LB medium with 500 mg l^{-1} of pyridine and incubating at 30°C, 180 rpm on a rotary shaker until the bacteria grew into the logarithm phase. The bacteria cells were harvested by centrifuging at 3,000 × *g* for 5 min. The cells were washed for three times with 20 ml of the MSM. The bacteria deposition was resuspended by vortex, and diluted with the MSM to an optical density at 602 nm (OD_{602}) of 1–2 (Shimadzu UV2401, Japan). The bacteria suspension was employed as the inoculum in the biodegradation experiment immediately.

Biodegradation of pyridine

The experiment was conducted using a series of 250-ml Erlenmeyer flasks as batch reactors. Each flask contained 100 ml of the MSM with a specific concentration of pyridine (in the range of 400–3,000 mg l^{-1}) and same initial amount of the inoculum (about 0.1 OD_{602} unit). The flask that contained the highest concentration of pyridine without the bacteria inoculum was used as a negative control under the same condition. All flasks were sealed with sealfilm and shaken at 30°C, 180 rpm, and sampled periodically. For analyzing the concentration of pyridine, a portion of sample was filtered with 0.22 μm membrane. OD_{602} values were also measured against time.

Metabolic pathway of pyridine degradation

The experiments were carried out in a series of 250-ml Erlenmeyer flasks containing 50 ml of the MSM, supplemented with pyridine at 400–500 mg l^{-1} and inoculated with pre-cultured bacteria. Negative controls were prepared similarly except for the bacteria inoculum. All flasks were sealed with sealfilm and incubated at 30°C, 180 rpm. At regular time interval, a flask was taken out for the analysis. The Medium of 0.5 ml was filtered with 0.22 μm membrane directly for the analysis of pyridine concentration; 24.5 ml were analyzed for optical density, pH, and dissolved oxygen (DO); the rest 25 ml were centrifuged at 5,000 × *g* for 5 min. The supernatant was also filtered with 0.22 μm membrane and the filtrate was detected for possible metabolites and analyzed the concentrations of NH_3-N, NO_2^--N, NO_3^--N, total nitrogen (TN), total carbon (TC), inorganic carbon (IC), and total organic carbon (TOC).

PCR amplification of *nirS* and *nosZ* genes

Extraction of the genomic DNA from the strain BW001 was done as described above. In the genome, two gene fragments encoding cytochrome cd_1-containing nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*) were amplified with the primers as following (Rösch et al. 2002):

nirS-F: 5′-CACGG**Y**GT**B**CTGCGCAAGGGCGC-3′
nirS-R: 5′-CGCCACGCGCGG**Y**TC**S**GGGTGGTA-3′
nosZ-F: 5′-CGYTGTTC**M**TCGACAGCCAG-3′
nosZ-R: 5′-CATGTGCAG**N**GC**R**TGGCAGAA-3′

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

The Takara *Taq* hot-start polymerase (Takara, China) was used for PCR. The PCR program was set as described by Rösch et al. (2002), with the exception that the denaturation temperature was set at 94°C. The negative controls without DNA template were performed at the same time. The PCR products were separated by agarose (1%) gel electrophoresis and stained by SYBR Safe DNA gel stain (Invitrogen, USA). The target DNA fragments were purified and cloned into the pGEM-T Easy vectors. The recombinant plasmids were transformed into the competent *E. coli* TOP10 and sequenced. The *nirS* and *nosZ* DNA sequences were analyzed for the similarity to other published sequences by using BLAST program in the GenBank database.

Analytical methods

Pyridine concentration was analyzed by a high performance liquid chromatography (HPLC) system (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 mobile phase was methanol solution with the volume ratio of 80:20 (methanol:water) at the flow rate of 1.0 ml min^{-1}. Pyridine was detected at 254 nm wavelength. Possible metabolites were analyzed with GC/MS (Agilent 6890 N 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.

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.

TN, TC, IC, and TOC were analyzed by a Multi N/C 3000 (Analytikjena, Germany). NH_3-N, NO_2^--N, and NO_3^--N concentrations were analyzed with standard methods, i.e., salicylate-hypochlorous acid method, *N*-1-naphthyl-ethylenediamine method, and UV-spectrophotometric determination, respectively (State Environmental Protection Administration of China 1989; USEPA 2003). In addition, NH_4^+ was also determined by using Ammonium-Testkit QUANTOFIX (Sigma-Aldrich, Germany) in order to acquire the approximate range of the NH_3-N concentration. pH and DO were monitored by a pH meter (Thermo orion 868, USA) and a DO meter (Thermo orion 3-star benchtop, USA).

Results

Morphological and physiological characterization

As shown in Fig. 1, the strain BW001 was a short rod-shaped bacterium with dimensions of 0.8–1.2 μm in length and 0.5 μm in width without flagellum under the SEM. The results of physiological tests indicated that it was a gram-negative, aerobic, non-motile, and nitrogen-fixing strain, and resistant to cephalosporin, kanamycin, and rifampcin.

Identification of the strain BW001

The sequence of 1,425 bp fragment of the partial 16S rRNA gene of the strain BW001was deposited at the GenBank. After comparing the sequence with those published similar sequences in the GenBank database by an online alignment search, a dendrogram illustrating the results of 16S rRNA analysis using software MEGA 4.0 was constructed as shown in Fig. 2. The result indicated that the partial 16S rRNA sequence of the BW001 was 99% identical to that of *Paracoccus* sp. PCLb-30 (Accession No. AB195172), and all its closest members belonged to the genus of *Paracoccus*. Based on the 16S rRNA gene sequence as well as the morphological and physiological characterization, the isolated BW001 was identified as *Paracoccus* sp.

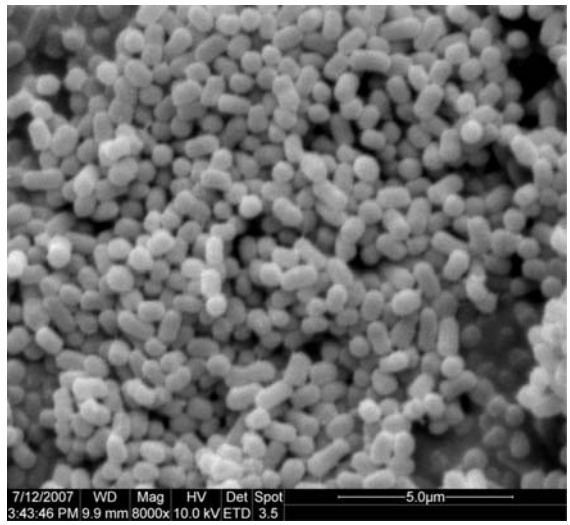

Fig. 1 The image of the *Paracoccus* sp. BW001 by SEM

Biodegradation of pyridine

For the biodegradation experiment with different initial pyridine concentrations, Fig. 3(a) shows the decrease of pyridine and Fig. 3(b) shows the growth of the BW001. Pyridine of 393, 922, 1,919, and 2,614 mg l^{-1} were degraded completely after 15.5, 21.5, 31.5, and 49.5 h, respectively. By utilizing pyridine, the bacteria BW001 with an average initial OD_{602} 0.089 grew rapidly and stabilized at OD_{602} 0.80, 1.70, 3.71 and 2.81 corresponding to the initial pyridine concentration from low to high. The amount of increased cell mass (C_m, the final biomass subtracts the initial biomass) and the apparent yield (Y, the amount of increased cell mass divided by the amount of consumed pyridine) under different initial pyridine concentration (C_0) were calculated and listed in Table 1. Although the biomass of the bacteria increased with increasing initial concentration of pyridine, the apparent yield decreased on the contrary.

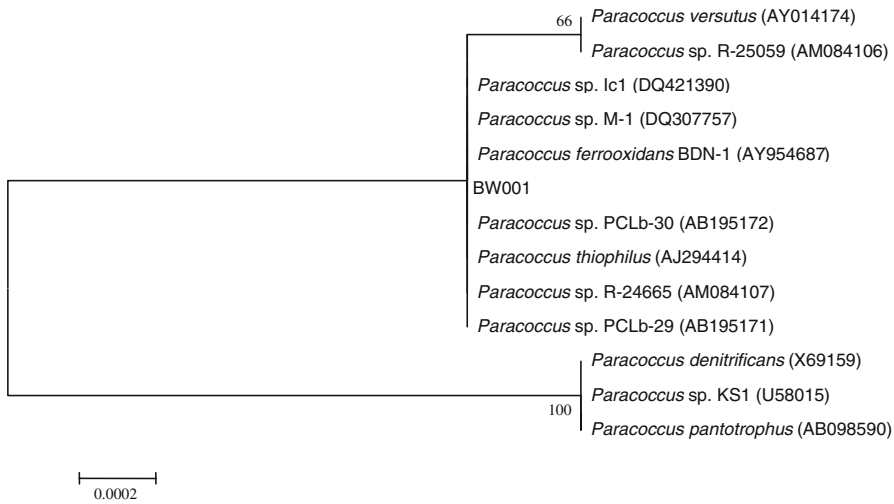

Fig. 2 Phylogenetic tree illustrating the similarity of the *Paracoccus* sp. BW001 to the members of its closest relatives. The numbers on the branch nodes demonstrate the percentages of bootstrap support for the clades based on 1,000 bootstrap resamplings. The scale bar indicates the average numbers of nucleotide substitutions per site. The numbers in the brackets are each strain's GenBank accession in the NCBI

To find the optimal conditions for the cell growth, further biodegradation experiments under different pH (3.0–10.0) and temperatures (20–35°C) were accomplished. The strain could grow better in the pH range of 5.0–9.0 and temperature range of 25–35°C with an optimum at pH 8.0 and 35°C.

Metabolic pathway of pyridine degradation

For the experiments to track the metabolic pathway of pyridine degradation, two mediums including the MSM and the MSM plus glucose were used. Negative controls without the bacteria were also performed for both mediums. During the experiments, the transformation of the carbon and nitrogen from pyridine were detected in 48 h. Figure 4 shows the variation of each item in two mediums. The amounts of pyridine volatilization were all less than 5%.

The results revealed that pyridine biodegradation was not accomplished in one step by the strain BW001, but experienced several phases in two mediums. The degradation of pyridine and the transformation of NH_3 were found to be the key steps. Therefore, according to the variation of pyridine and NH_3-N, the metabolic pathways in the MSM and the MSM plus glucose were divided into two phases and four phases, respectively.

In Fig. 4, sub-figures (a)–(c) show the variation of each item in the biodegradation with the MSM. Phase I was the pyridine degradation period lasting for 17 h. Pyridine of 478 mg l^{-1} was degraded completely and the strain BW001 grew continuously from OD_{602} 0.169 to 1.24. During the biodegradation, the value of pH kept stable around 7.45, and DO decreased rapidly from 7.30 to 1.89 mg l^{-1} reflecting that the BW001 required much oxygen to degrade pyridine. To focus on the nitrogenous transformation, TN of the solution was equal to Pyridine-N since pyridine was the sole source of nitrogen at the beginning; TN decreased obviously and equaled to a total of Pyridine-N, NH_3-N and other nitrogenous products in the process of biodegradation; at the end of the biodegradation, TN nearly equaled to NH_3-N since Pyridine-N and other nitrogenous products were disappeared. It was calculated that 53% of the nitrogen from pyridine was converted into NH_3 resulting in that the NH_3-N concentration was increased from 0 to 45.19 mg l^{-1}. For the carbonous transformation, TOC, initially from Pyridine-C, was decreased similarly and a portion of pyridine was mineralized into CO_2 and H_2O. Finally, the concentration of TOC was dropped at 19 mg l^{-1}. Phase II was a stagnant period of the bacteria growth after 17 h. In this phase, pyridine was exhausted, so there was no available carbon source for the strain BW001, resulting in that the growth of the bacteria was stopped and its density decreased a little. The value of pH increased to 7.98, DO increased above 7.10 mg l^{-1} as oxygen was no longer utilized by the bacteria. NH_3-N maintained at a stable level as the same as that of TN. The concentration of TOC kept at 19 mg l^{-1} as the remnant organic carbon could not be utilized by the bacteria.

In Fig. 4, sub-figures (a′)–(c′) show the variation of each item in the biodegradation with the MSM plus glucose. As same as that in the MSM, phase I

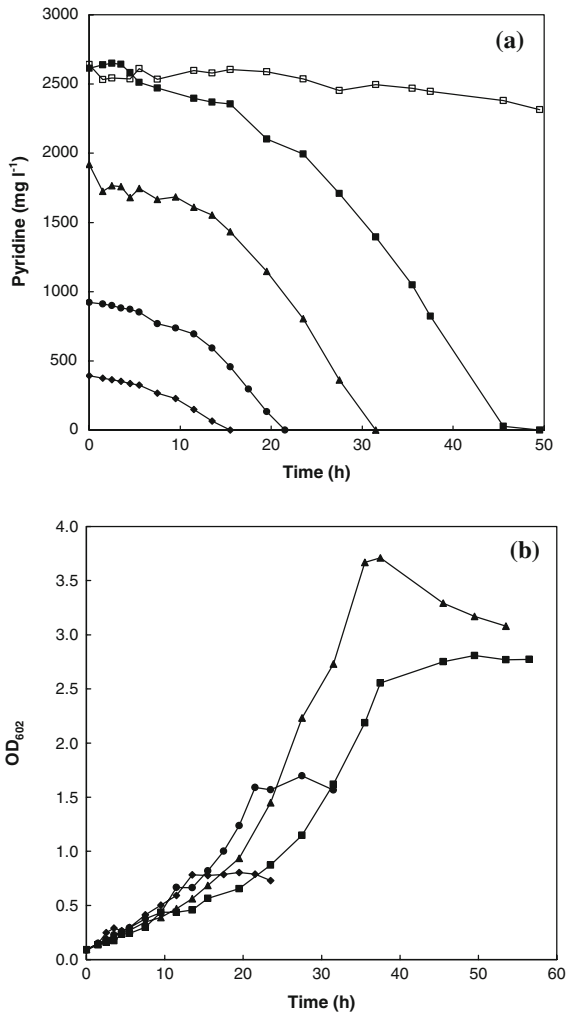

Fig. 3 Pyridine degradation and cell growth of the *Paracoccus* sp. BW001 with different initial concentrations in MSM. (**a**) Pyridine degradation; (**b**) The growth of BW001. (◆) 393 mg l^{-1}, (●) 922 mg l^{-1}, (▲) 1,919 mg l^{-1}, (■) 2,614 mg l^{-1}, and (□) sterile control

Table 1 Growth parameters of the *Paracoccus* sp. BW001

C_0 (mg l^{-1})	C_m (g l^{-1})	Y (g g^{-1})
393	0.224	0.571
922	0.490	0.532
1,919	0.861	0.449
2,614	0.887	0.339

was the pyridine degradation period. The difference was that the degradation rate of pyridine was faster, as 463 mg l^{-1} of pyridine was degraded completely in 13 h; and the growth of the strain BW001 was also faster, as the bacteria density was increased from OD_{602} 0.125 to 1.51. The additional carbon source of glucose promoted the growth of bacteria and the degradation of pyridine. During the biodegradation, the value of pH varied a little from 7.41 to 7.35, which was different from that in the MSM and probably caused by producing excessive acid intermediate; and DO decreased sharply from 7.32 to 0.49 mg l^{-1}, further demonstrating that the degradation reaction in the MSM plus glucose was more intensive than that in the MSM. For the nitrogenous transformation, about 42% pyridine-N was transformed to NH_3-N so that NH_3-N increased from 0 to 34.47 mg l^{-1}. It was remarkable that the yield of NH_3 was lower than that in the MSM demonstrating that the bacteria consumed more pyridine-N for their growth. TN decreased from 84.87 to 35.15 mg l^{-1}, nearly as same as the concentration of NH_3-N at the end. For the carbonous transformation, TOC was consisted of pyridine and glucose, but the existence of glucose did not influence the degradation of pyridine, and the strain BW001 utilized pyridine quickly. The results of TOC and IC indicated that the pyridine-C was transformed to CO_2 and other intermediates. Phase II was the growth stagnant period of the bacteria from 13 to 19 h. The variation trends of all items were the same as those in the MSM. The value of pH increased from 7.44 to 7.70, and DO increased from 6.66 to 7.15. Bacteria density, TN, NH_3-N and TOC were not changed obviously in this phase. Viewing the whole process of biodegradation in the MSM plus glucose, phase II seemed as an adaptation period for the strain to adjust itself to the accumulated NH_3-N since pyridine as the original carbon and nitrogen sources was exhausted. After the adjustment, in phase III, the bacteria restarted growing on glucose and NH_3 as their carbon and nitrogen source, respectively, until 28 h when NH_3-N was exhausted. Bacteria density increased from OD_{602} 1.34 to 1.68. The value of pH decreased to the original level, approximating to 7.4; and DO decreased rapidly, ending at 4.15 mg l^{-1}, which was higher than that in the end of the phase I. TN and TOC kept on decreasing. Phase IV began when glucose became the only energy source for the bacteria at 28 h. The bacteria continued to grow as TOC kept on decreasing and bacteria density was increased from OD_{602} 1.68 to 4.44. Other items, e.g., pH, DO, TN and IC had no significant change.

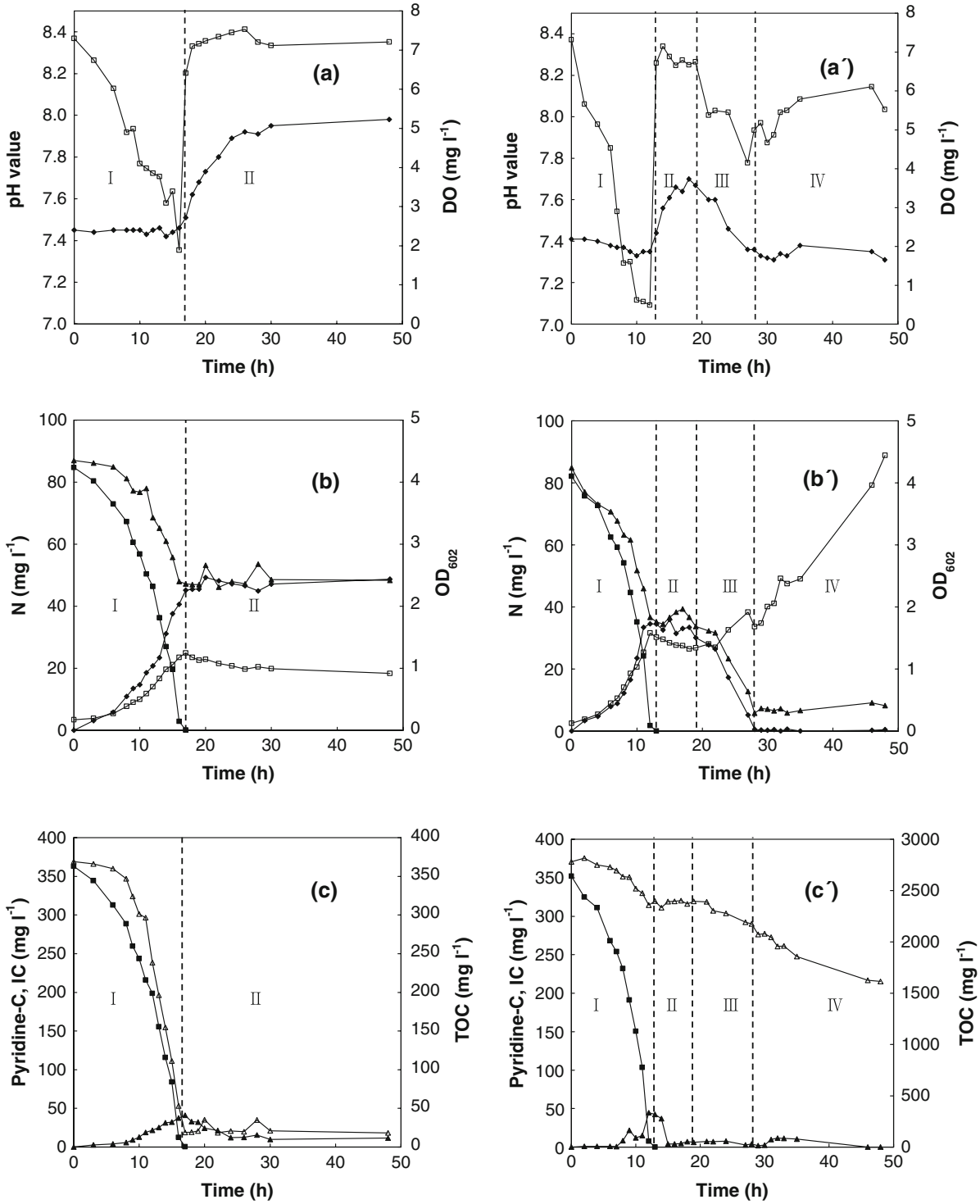

Fig. 4 Biodegradation process of pyridine (left figures) and pyridine plus glucose (right figures) by the *Paracoccus* sp. BW001. Sub-figures (**a**) and (**a′**) show the variation of pH value and DO concentration, (◆) pH, (□) DO; sub-figures (**b**) and (**b′**) show the nitrogenous transformation of pyridine, (■) Pyridine-N, (◆) NH_3-N, (▲) TN, (□) OD_{602}; and sub-figures (**c**) and (**c′**) show the carbonous transformation of pyridine and glucose, (■) Pyridine-C, (▲) IC, (△) TOC

During the biodegradation in above two substrates, NO_2^--N and NO_3^--N were also determined. The concentration of NO_2^--N was very low, no more than 0.1 mg l^{-1} throughout the experiments. The concentration of NO_3^--N ranged from 0 to 4 mg l^{-1}. These results indicated that nitrification occurred during the biodegradation of pyridine by the strain BW001. Other findings were that no heterocyclic intermediates of pyridine were detected by GC-MS, and no new peak was detected by the HPLC analysis and the UV spectra scanning during the biodegradation. It is proved that hydroxyintermediates are not involved in pyridine metabolism by the strain BW001. Such result was also reported by Rhee et al. (1997).

Denitrification potential

According to the nitrogenous transformation and the bacterium identification, the strain *Paracoccus* sp. BW001 was highly suspected as a denitrifying bacterium. All molecular studies on the ecology of denitrifying bacteria are based on functional genes and their products (Bothe et al. 2000). *nirS* gene and *nosZ* gene were often used as functional markers to identify the denitrifying bacteria (Braker et al. 1998; Scala and Kerkhof 1998; Hallin and Lindgren 1999; Rich et al. 2003). The *nir* could transfer NO_2^- to NO and the *nos* could transfer N_2O to N_2 (Wallenstein et al. 2006). Both genes had been proved to be broadly presented in different strains of *Paracoccus* (Baker et al. 1998; Saunders et al. 2000; Throback et al. 2004; Heylen et al. 2006).

Using proper primers (Rösch et al. 2002), a 699 bp *nirS* gene fragment and a 710 bp *nosZ* gene fragment were amplified from the BW001 genetic template as shown in Fig. 5. By the comparison with GenBank BLAST program, the *nirS* gene fragment from the BW001 was 96% identical to that of *Paracoccus denitrificans* (U75413) and *Paracoccus pantotrophus* (AJ401462), and the *nosZ* gene fragment from the BW001 was 90% identical to that of *Paracoccus denitrificans* (X74792). The BLAST results indicated that the strain BW001 had the potential abilities to reduce NO_2^- to NO, and then to N_2.

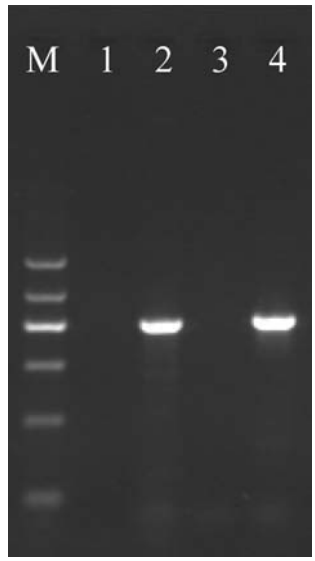

Fig. 5 PCR amplification of the specific fragments of *nirS* and *nosZ* genes from the *Paracoccus* sp. BW001. M, the molecular size markers, with the size of 100, 300, 500, 700, 900, 1,200 bp from bottom to top; lane 1, negative control of *nirS* gene fragment; lane 2, *nirS* gene fragment; lane 3, negative control of *nosZ* gene fragment; lane 4, *nosZ* gene fragment

Based on the above results, further experiments were designed using "MSM + glucose + KNO_3" and "MSM + glucose + KNO_2" as the culture mediums. Five days later, BW001 grew rapidly by utilizing the carbon source from glucose and the nitrogen source from nitrate or nitrite. The concentrations of NO_2^- and NO_3^- were decreased sharply (Table 2). These results identified that the strain BW001 was a denitrifying bacterium.

Discussion

Biodegradation ability of the strain BW001

In recent years, a number of strains of *Paracoccus* have been reported to degrade halobenzoate (Song

Table 2 Denitrification potential of the *Paracoccus* sp. BW001

Nitrogen source	Concentration (mg l^{-1})		OD_{602}	
	0 d	5 d	0 d	5 d
NO_2^--N	55.92 ± 1.45	0.75 ± 0.17	0.060 ± 0.004	1.982 ± 0.188
NO_3^--N	53.30 ± 0.15	1.65 ± 0.60	0.056 ± 0.001	1.838 ± 0.068

et al. 2000), poly (ε-caprolactone) (Horiba et al. 2005) and monocrotophos (Jia et al. 2006). These findings suggested that the genus *Paracoccus* could metabolize many kinds of toxic aromatic compounds. Most *Paracoccus* strains were isolated from sewage activated sludge or contaminated soils, indicating that further study of this genus may benefit to understand the ecological diversity of these organisms and their potential abilities of biodegradation.

Growing in the medium of pyridine-MSM, the BW001 could utilize pyridine with high concentration up to 2,614 mg l^{-1} (much higher than early studies) as sole carbon, nitrogen and energy source. When the initial concentration of pyridine was increased, the growth speed of the bacteria and the yield would decline comparatively as pyridine was a potential inhibitor.

Biodegradation pathway by the strain BW001

In the experiments to track the metabolic pathway of pyridine, two mediums with different ratios of C/N were applied.

In the MSM, pyridine was mainly converted to phosphoglycerate, CO_2, and NH_3 by the bacteria in phase I. Since 53% of nitrogen in pyridine was transformed into NH_3 in the experiment, the rest 47% of nitrogen could be assumed to be assimilated into biomass.

Since pyridine is an energy-deficient substrate as its ratio of C/N is 4.3, the released NH_3 cannot be utilized sequentially by the BW001 when the available carbon source is exhausted in the MSM. So, the metabolism of the bacteria was stopped in phase II.

However, in the MSM plus glucose, glucose supplied enough carbon source and energy for the bacteria. The ratio of C/N in the solution turned to be 33. The metabolism of the strain BW001 was divided into four phases. Phase I was the co-metabolic phase of pyridine and glucose, the degradation speed of pyridine was promoted by glucose obviously. The bacteria utilized carbon not only from pyridine but also from glucose for its growth. By calculation, the utilization ratio between the pyridine-C and glucose-C was about 4.5:1. Thus, the lower yield of NH_3 in the MSM plus glucose was probably caused by that a greater proportion of pyridine-N was synthesized into phosphoglycerate due to the existence of glucose. When pyridine was degraded completely, glucose and NH_3 became the only sources of carbon and nitrogen in the medium, respectively. After an adjustment phase (phase II), the bacteria continued to grow until NH_3 was exhausted (phase III). In the last phase IV, although a small quantity of nitrogen source remained in the medium did not change obviously, the bacteria still grew vigorously by utilizing glucose.

It was remarkable that pH and DO were two quick-indicators reflecting the occurrence of the biodegradation. During the biodegradation, although NH_3 was produced along with the degradation of pyridine, the value of pH was usually stable or slightly decreased, deducing that some acid intermediates might be produced; the concentration of DO decreased evidently, indicating the fierce aerobic degradation and the rapid bacterial growth.

In former studies related with pyridine degradation, the production of NH_3 was recognized in most bacterial strains. But the other nitrogenous intermediates were seldom referred. In this study, the appearance of NO_3^--N and NO_2^--N indicated that a portion of NH_3 was nitrified to NO_3^- and NO_2^- while organic carbon existed. A few strains of the genus *Paracoccus* were reported to be the heterotrophic nitrifiers (Moir et al. 1996a, b; Crossman et al. 1997), which could heterotrophically transfer NH_3 to NH_2OH, and then to NO_2^- by ammonia monooxygenase (AMO) and hydroxylamine oxidase (HAO). Heterotrophic nitrification may be linked to aerobic denitrification, i.e., the nitrite produced by nitrification is converted to dinitrogen via nitric oxide and nitrous oxide (Wehrfritz et al. 1993; Crossman et al. 1997). The PCR analysis and further experiments on denitrification potential also supported this argument. The *nirS* gene and *nosZ* gene were founded in the BW001. In addition, the BW001 could utilize NO_3^- and NO_2^- as nitrogen sources for its growing. These results revealed that the strain BW001 could transform NO_3^- and NO_2^- produced from NH_3 to N_2 at last. The transformation pathway of nitrogen from pyridine was proposed as described in Fig. 6. The enzyme and functional gene for the first step is still obscure, but other enzymes and genes involved in the transformation of NH_3 and following products are already clear.

According to the GC/MS, UV and HPLC analysis, it was clear that hydroxyintermediates were not produced during the degradation of pyridine by the

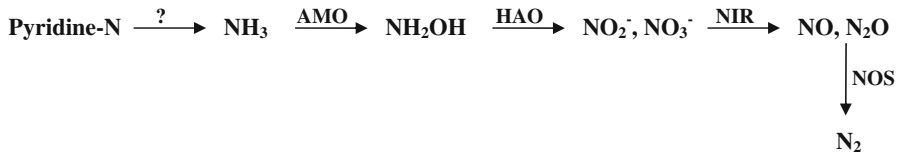

Fig. 6 Proposed transformation pathway of the nitrogen from pyridine. *Note*: 1. Most NH_3-N was reserved when biodegradation occurs with sole substrate (pyridine). On the contrary, NH_3-N was transformed completely to the following compounds if extra carbon source was supplied. 2. The abbreviations above the arrows represent the necessary enzymes relative to the transformation

strain BW001. So, in this study, pyridine was not possible to be degraded in the pathway proposed by Zefirov et al. (1994). It may be performed on one of the metabolic pathways proposed by Watson and Cain (1975), i.e., pyridine ring was cleaved between the C_2 and N, and deaminated to glutaric dialdehyde subsequently, followed by successive oxidation to glutarate semialdehyde, glutarate, and acetyl coenzyme A. Acetyl coenzyme A may be assimilated via the glyoxylate cycle. This pathway was also described by Rhee et al. (1997).

Conclusion

The aerobic biodegradation of pyridine by a new isolated bacterial strain BW001 was investigated. According to the SEM observation, the physiological characteristics and 16S rRNA sequence analysis, the isolated bacteria belongs to the genus of *Paracoccus*. It can utilize pyridine as sole carbon, nitrogen and energy sources, even degrade pyridine with 2,614 mg l^{-1} within 49.5 h. Extra carbon source may promote the degradation of pyridine. During the biodegradation, pyridine ring was cleaved between the C_2 and N at first; then the transformation of nitrogen from pyridine was in a pathway of heterotrophic nitrification and aerobic denitrification simultaneously.

Nucleotide sequence accession numbers

The accession numbers of the isolates 16S rRNA gene, *nirS*, and *nosZ* on GenBank are EU192073, EU192074 and EU192075.

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