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Characteristics of pyridine biodegradation by a novel bacterial strain, *Rhizobium* sp. NJUST18

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ABSTRACT

Pyridine is one of the most widespread heterocyclic industrial contaminants. Due to rather tough safe level, thorough purification of wastewater containing this eco-toxicant is required. In this study, a novel pyridine-degrading bacterium, strain NJUST18, was isolated from the soil contaminated by pyridine and identified as a member of genus *Rhizobium*. The biodegradation assays suggested that strain NJUST18 could utilize pyridine as the sole source of carbon and nitrogen, at initial concentration as high as 2600 mg l^{-1}. Pyridine depletion, biomass increase, TOC reduction, pH increase, and NH_4^+ release during pyridine biodegradation indicated that pyridine could be mineralized by strain NJUST18. Pyridine degradation at high initial concentrations or high initial pH values demonstrated that this biodegradation process was both pH and NH_4^+ dependent. Release of NH_4^+ into the alkaline medium led to the formation of free ammonia (NH_3) accompanied by the delayed pyridine degradation. High concentration of NH_3 generated weakened pyridine biodegradation. A neutral to slightly alkaline pH was crucial for high strength pyridine degradation by NJUST18. *Rhizobium* sp. NJUST18 could degrade relatively high concentration of pyridine, offering bright prospects for bioremediation of pyridine contaminated environment.

Keywords: Pyridine; Nitrogen heterocyclic compound; Biodegradation; *Rhizobium*; Free ammonia

1. Introduction

Pyridine, which is recognized as the parent of a series of chemicals, is widely used in manufacturing of dye, herbicides, pesticides, pharmaceuticals, etc. Due to its recalcitrant, persistent, toxic, teratogenic, and carcinogenic nature, it constitutes a danger for human and other living organisms [1]. In addition, with an N atom incorporated into the ring system, its water solubility is markedly enhanced. Thereby, the bio-availability is increased with an accelerated risk to the environment. For these reasons, the presence of pyridine in wastewater is severely regulated [2].

There is a interest of removing it from contaminated sites. Many treatment technologies have been developed for pyridine removal from contaminated environment. Among them, physico-chemical methods, such as adsorption, incineration, and oxidation, have been proven to be cost and energy intensive [3]. Biological

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treatment, which is often environmental friendly and cost effective, has turned out to be a favorable alternative [4]. However, the heterocyclic structure of pyridine makes it recalcitrant to microbial degradation and persistent in nature. Bioaugmentation of biodegradation systems with specific microbes could be an effective method to enhance pyridine removal.

Up to date, several works have been reported to isolate new and efficient microbial species for pyridine biodegradation. These microorganisms include genera *Arthrobacter* [5], *Bacillus* [2], *Lysinibacillus* [6], *Nocardiodes* [7], *Paracoccus* [8–10], *Pseudomonas* [3,11], *Rhodococcus* [1], *Shinella* [4], *Shewanella* [2], and *Streptomyces* [12]. However, no work has been reported on biodegradation of pyridine by *Rhizobium*. In addition, due to the limited number of collections of such microorganisms, knowledge about pyridine biodegradation is yet limited. Consequently, it is important to screen new bacterial strains indigenous to sites contaminated by pyridine for its biodegradation.

In addition to the isolation of microbes capable of degrading pyridine, an understanding of the interplay between the biotic and abiotic factors is also important. However, up to now, most of the reports have focused on the biodegradation characteristics and biodegradation pathways. Optimized process parameters aiming at a successful application for the bioremediation of contaminated environments are still lacking. In the considered pyridine wastewater treatment system, pH suffers from important variations due to NH_4^+ release. pH is strongly suspected to be a key parameter for treatment control regarding NH_3/NH_4^+ equilibrium control. However, many recent studies about pyridine biodegradation have not paid any attention to this issue.

In this study, a novel pyridine-degrading strain, *Rhizobium* sp. NJUST18, was isolated from pyridine contaminated soil. *Rhizobium* sp. NJUST18 reported here seems to be the first documented rhizobial bacterium isolate that has the nature of strength to degrade pyridine. The objective of this study was to characterize pyridine degradation performance of NJUST18, and to investigate the effect of some abiotic factors on pyridine degradation. Particularly, both pH and NH_4^+ dependence of pyridine degradation was estimated, aiming at an effective bioremediation strategy.

2. Materials and methods

2.1. Growth medium and cultivation conditions

Enrichment and selection of strain capable of degrading pyridine was performed in mineral salt medium (MSM) supplemented with pyridine. Each liter of MSM contained 1.529 g $Na_2HPO_4{\cdot}12H_2O$, 0.372 g KH_2PO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.05 g $CaCl_2$, and 10 ml SL-4. In MSM, $Na_2HPO_4{\cdot}12H_2O$ and KH_2PO_4 served as the phosphate buffer (7 mM, pH 7.0). Pyridine at desired concentration was added into MSM as the sole carbon and nitrogen source. The composition of SL-4 was according to Shen et al. [13]. Store culture was maintained by periodical transfer onto mineral salt agar plates supplemented with 1,000 mg l^{-1} pyridine and was stored at 30˚C for further study. For long-term maintenance, pyridine-degrading strain was stored in 20% glycerol at −80˚C in an ultra-low temperature freezer.

The cultures were incubated in liquid MSM on a rotary shaker at 180 rpm and 30˚C. The biodegradation experiment was conducted using a series of 150 ml Erlenmeyer flasks as batch reactors. Each flask contained 50 ml liquid MSM supplemented with pyridine at desired concentration.

2.2. Micro-organism cultivation and isolation

Soil samples used to isolate the pyridine-degrading bacteria were collected between June and August 2010 from the sites contaminated by pyridine in Nanjing Redsun Co. Ltd. Twenty samples were randomly collected in sterile containers from 5 cm below the land surface. The average pH of the soil samples was 7.6, while the average total potassium, total phosphorus, total nitrogen, and organic matter were determined to be 21.8, 3.1, 1.5, and 31.4 g kg^{-1}, respectively. Soil samples were air-dried, and then mixed and ground in an agate mortar to pass through a 2-mm nylon sieve. About 2 g ground soil sample described above was inoculated into 50 ml liquid MSM supplemented with 500 mg l^{-1} pyridine and incubated on a rotary shaker for the enrichment of pyridine-degrading bacteria. Seven days later, 2 ml cultures were transferred into 50 ml fresh medium. After three times of successive transfers, the diluted suspensions (10^{-5}–10^{-9}) were spread onto the MSM plates containing 1,000 mg l^{-1} pyridine. Pyridine-degrading bacterial colonies were screened out and purified with streak plate method for three times. Finally, pure colonies were transferred into liquid MSM containing 1,000 mg l^{-1} pyridine to confirm their ability to metabolize pyridine under aerobic condition.

Finally, four pyridine-degrading strains were isolated. A bacterial isolate designated NJUST18, which exhibited the highest pyridine biodegradation ability, was further identified based on morphological, physiological, biochemical tests, and genospecies.

2.3. Strain identification

The morphological, physiological, and biochemical features were used to characterize the strain NJUST18. The strain was further confirmed by 16S rRNA sequence analysis. The RNA was extracted from fresh cells grown in the liquid MSM. 16S rRNA gene was amplified with the primers F8 (5′-AGAGTTTGA TCCTGGCTCAG-3′) and R1522 (5′-AAGGAGGT GAT CCAGCCGCA-3′). Reaction mixture contained each primer (1 μl), deoxy-nucleotide triphosphates (dNTPs, 1 μl), 10× reaction buffer (5 μl), Taq polymerase (0.5 μl), DNA template (1 μl), Mg^{2+} solution (5 μl), and sterile water (35.5 μl) to achieve a final volume of 50 μl. PCR was performed under the following conditions: initial denaturation at 94˚C for 5 min; 30 cycles of 94˚C for 1 min, 56˚C for 1 min, and 72˚C for 2 min; and a final extension step at 72˚C for 7 min. After thermocycling, the sample was loaded on a DNA analyzer according to the manufacturer's instructions. The sequence was deposited in the GenBank database under accession no. JN106368. Nucleotide sequence similarity was analyzed using BLAST (National Center for Biotechnology Information Databases).

2.4. Inoculum preparation for pyridine biodegradation

Luria-Bertani (LB) medium was inoculated with pure NJUST18 and incubated on a rotary shaker at 180 rpm and 30˚C until the bacteria grew into the logarithmic phase (about 48 h after inoculation). For the acclimation of inocula, 1,000 mg l^{-1} pyridine was added into LB medium. The bacteria were harvested by centrifugation at 6,000 × *g* for 5 min, then resuspended, and washed three times with 100 ml MSM. At last, the bacterial deposit was resuspended by vortexing and diluted with MSM to an optical density at wavelength 600 nm (OD_{600}) of about 1.5. The bacterial suspension was immediately employed as the inoculum in the following biodegradation experiment.

2.5. Pyridine biodegradation

The experiments for pyridine biodegradation were conducted with pure NJUST18. The inoculum prepared as described above was then transferred into each experimental flask containing the prepared MSM with 5% (v/v) inoculum size.

In order to investigate the effect of pyridine concentration on pyridine degradation and biomass growth, inocula of NJUST18 strain were inoculated into the MSM containing 600, 1,000, 1,600, 2,000, and 2,600 mg l^{-1} pyridine, respectively, at initial pH of 7.0. Various pHs (6.0–10.0) of MSM containing 1,000 mg l^{-1} pyridine were prepared to determine the optimum pH for pyridine removal by NJUST18. MSM containing 1,000 mg l^{-1} pyridine, supplemented with different concentration of glucose (500, 1,000, 2,000 mg l^{-1}), was used to study the effect of additional carbon source on pyridine degradation. At initial pH of 7.0, MSM containing 1,000 mg l^{-1} pyridine, supplemented with different concentration of ammonium chloride (500, 1,000, 2,000 mg l^{-1}), was used to study the effect of NH_4^+ on pyridine degradation.

Pyridine concentration, cell growth, TOC concentration, pH variation, and NH_4^+-N release during the incubation process were monitored. Non-seeded flasks were used as the control to evaluate pyridine removal due to volatilization. Each result was reported as an average of three independent experiments, with maximum deviations from the average (error bars) indicated.

2.6. Analytical methods

Pyridine in MSM was quantified by HPLC. The mobile phase was a mixture of 70% methanol and 30% water pumped at a flow rate of 1.00 ml min^{-1}. NH_4^+-N concentration was analyzed by Nessler's reagent colorimetric method (China NEPA standard methods, GB 7479-87). TOC was measured using a Germany Elementar vario TOC analyzer. Before TOC, pyridine, and NH_4^+-N concentration analysis, water samples were passed through a 0.22 μm filter.

Biomass produced during the biodegradation process was monitored by optical density (OD_{600}). As NJUST18 exhibited strong self-flocculation/aggregation ability, before determination of OD_{600}, it was necessary to resuspend/deflocculate the growth media by vortexing for 10 min. Cell dry weight 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_{600} and the corresponding cell dry weight. The OD value was then converted to dry cell mass using the calibration curve obtained by plotting dry biomass weight per liter against OD_{600} of the suspension.

3. Results and discussion

3.1. Pyridine degradation performance of NJUST18

Four indigenous bacterial strains able to grow on pyridine as the sole carbon and nitrogen source were isolated from the soil contaminated by pyridine. The strain which exhibited the best pyridine biodegradation ability was named after NJUST18.

In order to evaluate its pyridine degradation performance, strain NJUST18 was inoculated into MSM containing 1,000 mg l^{-1} pyridine and incubated aerobically on a shaker at 180 rpm and 30°C. As shown in Fig. 1(a), about 1,000 mg l^{-1} pyridine could be degraded completely after 102 h. Correspondingly, biomass increased to 475.80 ± 23.91 mg l^{-1} from 49.14 ± 0.02 mg l^{-1} (as shown in Fig. 1(a)). Biomass yield coefficient $Y_{X/S}$ (dry weight of biomass/weight of pyridine consumed) was found to be about 0.46 at initial pyridine concentration of 1,000 mg l^{-1}. During the degradation period, for the negative controls without inoculation, a small portion of pyridine was lost because of volatilization. However, variation of the pyridine concentration in the negative controls was not remarkable (Fig. 1(a)).

As shown in Fig. 1(b), NH_4^+-N concentration increased to 97.94 ± 8.07 mg l^{-1} when pyridine was undetectable in the MSM after 102 h, with about 54.94 ± 4.53% of nitrogen in the pyridine ring converted into NH_4^+-N. Thereafter, the concentration of NH_4^+-N did not decrease clearly within the following 1 day (data not shown). As was reported by other researchers, nitrogen in the pyridine ring was often transformed into NH_4^+ during pyridine biodegradation [4,8,9]. Therefore, the release of NH_4^+ was a key evidence for pyridine ring cleavage. For the carbonous transformation, TOC, initially from pyridine-C, was decreased similarly, indicating a majority of pyridine-C was mineralized into CO_2. Within 102 h, TOC concentration was decreased from 657.26 ± 5.35 mg l^{-1} to 98.57 ± 12.78 mg l^{-1}, with TOC removal ratio of about 85.00% (Fig. 1(b)). Low residual TOC concentration observed in this study suggested that intermediate metabolite leakage was not evident in the presence of NJUST18.

Besides NH_4^+-N release, pH variation was another key indicator for pyridine biodegradation. In the pyridine biodegradation system reported by Bai et al. [4,8], the value of pH was always decreasing slightly during pyridine biodegradation, indicating that some acid intermediates might be produced. However, pH increased sharply immediately after pyridine was completely degraded, probably due to acid intermediates depletion and NH_4^+ generation. What is different is that, in this study, pH was always increasing during the whole biodegradation process, although phosphate buffer (7 mM) was used. At the time of complete exhaustion of pyridine, pH increased from initial 7.0 to final 8.76 ± 0.06. The continuous increase of pH indicated continuous and rapid generation of NH_4^+, indicating that pyridine ring is firstly cleaved between the C2 and N [14]. Unfortunately, identification of metabolic products during pyridine biodegradation has not been successful in this study. This might be

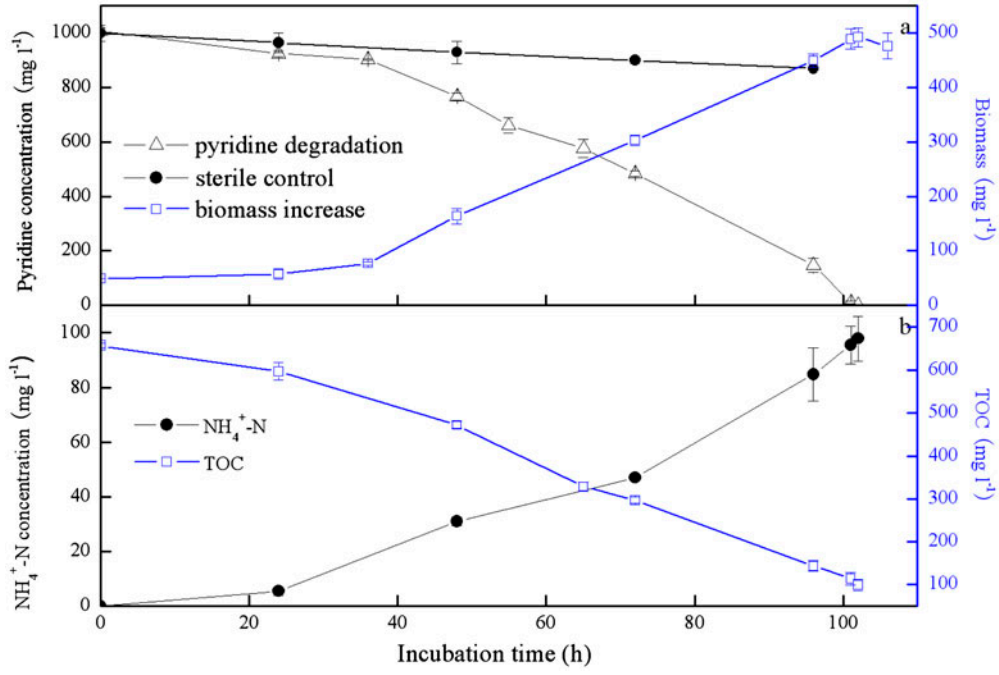

Fig. 1. Pyridine depletion and biomass increase (a), TOC reduction and NH_4^+ release (b) at the presence of NJUST18. Sterile control indicates the incubations without inoculation.

due to very low concentrations of metabolic products in the experiments, which needed further investigation.

From the above data about pyridine depletion, biomass growth, TOC decrease, pH increase, and NH_4^+ formation profiles during pyridine biodegradation by NJUST18, it could be inferred that pyridine could be mineralized by this strain. Therefore, NJUST18 was chosen for further study.

3.2. Characterization of strain NJUST18

Basic morphological, physiological, and biochemical characteristics of NJUST18 were investigated for strain identification. Colonies of NJUST18 appeared circular and white with smooth surface during growth on MSM plates for 96 h. As was indicated by transmission electronic microscope of NJUST18, NJUST18 cell was motile and rod shaped with 2–3 μm in length and 0.8–0.9 μm in width. NJUST18 was a rod-shaped bacterium with flagellum, exhibiting strong self-flocculation/aggregation ability. It was negative in tests such as Gram staining, catalase, oxidase, urease, starch hydrolysis, casein hydrolysis, and gelatin hydrolysis.

For further identification of NJUST18, the partial 16S rRNA sequence was determined. The phylogenetic analysis was carried out based on the 16S rRNA sequence (comprising 1,381 nucleotides). NJUST18 was closely related to *Rhizobium* sp. R-24658 (GenBank accession no. AM084043.1) and *Rhizobiales* bacterium D11-28.1 (GenBank accession no. AM403228.1), with 99% sequence identity.

In combination with the morphological, physiological, biochemical tests, and genospecies, the isolated strain NJUST18 was tentatively identified as *Rhizobium* sp. and named after *Rhizobium* sp. NJUST18.

3.3. Effect of initial pyridine concentration on pyridine degradation and cell growth

For inhibitory type substrate such as pyridine, substrate concentration plays an important role on both substrate degradation and cell growth. As Fig. 2(a) shows, in the MSM with initial pH of 7.0, NJUST18 strain was capable of completely degrading pyridine at concentration as high as 2,600 mg l^{-1}. At initial pyridine concentrations of 600, 1,000, 1,600, 2,000, and 2,600 mg l^{-1}, complete degradation was achieved within 75, 102, 204, 220, and 240 h, respectively (Fig. 2(a)). Correspondingly, the biomass reached maximum of 345.9 ± 15.4, 491.7 ± 17.6, 619.9 ± 57.9, 780.6 ± 28.1, and 964.9 ± 43.5 mg l^{-1}, respectively, at the time of complete exhaustion of pyridine (Fig. 2(b)). Biomass yield coefficient ($Y_{X/S}$) was found to be 0.51, 0.46, 0.33, 0.35, and 0.34, respectively. Considerable decrease in the values of $Y_{X/S}$ was observed with the increase in pyridine concentrations. Similar results of decreasing $Y_{X/S}$ with increasing pyridine concentration in inhibitory region were also reported in the literature [4,8]. It was because more energy was required to overcome the effect of substrate inhibition at high pyridine concentrations. In addition, at high pyridine concentrations, more pyridine was lost because of volatilization.

The lag phase was observed at all concentrations. In addition, the lag phase was extended with the increase of initial pyridine concentrations, although well-acclimatized inocula were used, demonstrating positive correlation between cell biomass and pyridine degradation. Similar phenomenon was observed by others in the pyridine biodegradation systems [2,4,8], which could be attributed to the toxicity and the recalcitrance of pyridine. At low initial concentrations, NJUST18 degraded pyridine completely and rapidly. At high initial concentrations, such as 1,600, 2,000, and 2,600 mg l^{-1}, although complete degradation could be achieved, the biodegradation process was rather slow. It could be observed that towards the end of substrate consumption curve, there was a region of relatively reduced substrate removal rate. These results suggested that the toxicity of pyridine and its transformation products towards NJUST18 strain existed. Improvement of pyridine degradation at high concentrations by NJUST18 through optimizing process parameters was a subject for further investigation.

3.4. Effect of initial pHs on pyridine degradation

The pH values had a significant effect on the degradation efficiency of xenobiotics [15,16]. Fig. 3 illustrated the effect of various pHs (5.0–10.0) on pyridine degradation in MSM. Pyridine degradation was optimal at an initial pH of 7.0 and 8.0, with pyridine degradation accomplished within 102 and 105 h, respectively. At an initial pH of 5.0 and 6.0, prolonged lag phase was observed, probably due to the inhibitory effect of acidity on the activity of intracellular enzyme of bacteria. Poor pyridine degradation performance at low pH was also observed by Li et al. [12]. However, after the prolonged lag phase at initial pH of 5.0 and 6.0, accelerated pyridine degradation by NJUST18 was achieved within 126 and 122 h, respectively. At initial pH of 9.0, prolonged lag phase was not observed, however, delayed pyridine biodegradation was observed at the end of the biodegradation process. At initial pH of 10.0, pyridine removal was rather slow, accompanied with both prolonged lag

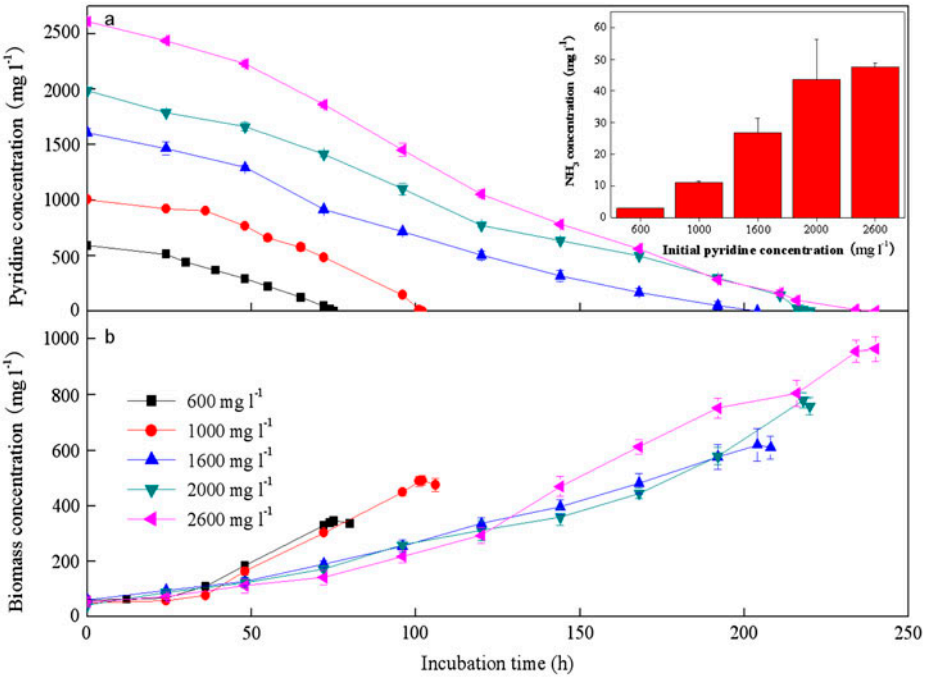

Fig. 2. Effect of initial pyridine concentration on pyridine degradation (a) and biomass increase (b). The inset showed the calculated NH_3 concentrations at different initial pyridine concentrations when pyridine was completely exhausted.

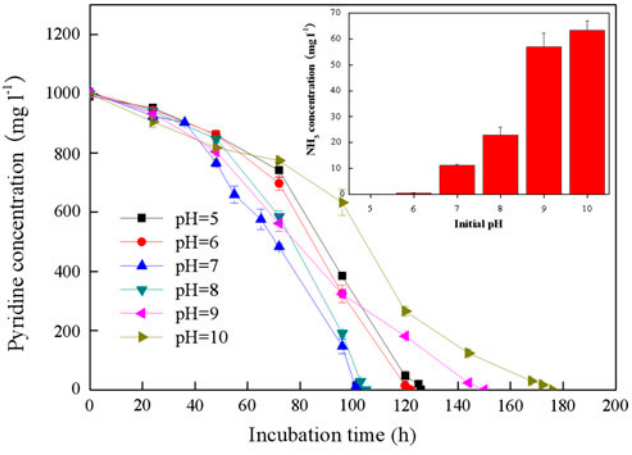

Fig. 3. Effect of initial pHs on pyridine degradation. The inset showed the calculated NH_3 concentrations at different initial pHs when pyridine was completely exhausted.

phase and delayed biodegradation at the end of the biodegradation process. These results indicated that a neutral to slightly alkaline pH might be suitable for pyridine degradation by NJUST18.

3.5. *Effect of additional* NH_4^+ *on pyridine degradation*

As was indicated previously, release of NH_4^+ was a key indicator for pyridine biodegradation [4,8,9]. However, at high initial pyridine concentrations (1,600, 2,000, and 2,600 mg l^{-1}), with the release of NH_4^+ and increase of pH, pyridine biodegradation seemed to be slowed down (Fig. 2(a)). In order to prove the relevance between pyridine biodegradation and NH_4^+ release, the effect of additional NH_4^+ on pyridine degradation was investigated.

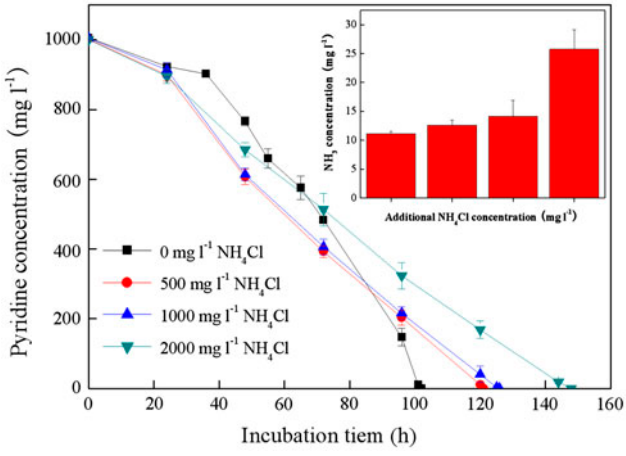

Fig. 4. Effect of additional NH_4^+ on pyridine degradation. The inset showed the calculated NH_3 concentrations at different additional NH_4Cl concentrations when pyridine was completely exhausted.

From Fig. 4, it could be inferred that addition of NH_4^+ had negative effect on pyridine degradation. With the addition of NH_4^+, pyridine degradation was delayed. In addition, with the increase of additional NH_4^+ concentrations, longer incubation time was needed for complete pyridine biodegradation. About 1,000 mg l^{-1} pyridine could be completely degraded within 102 h without the additional NH_4Cl; however, with the addition of 2,000 mg l^{-1} NH_4Cl, the incubation time for complete degradation of 1,000 mg l^{-1} pyridine was extended to 148 h. Thus, it could be inferred that pyridine biodegradation was NH_4^+ dependent. Release of NH_4^+ played a key role during pyridine biodegradation by NJUST18.

3.6. Role of NH_3 in pyridine degradation

As was indicated previously, an increasing trend in pH was observed during the whole pyridine biodegradation process by NJUST18. pH increase during pyridine degradation at high initial pHs and high initial pyridine concentrations could be the main reason for delayed biodegradation at the end of the biodegradation process (Figs. 2 and 3). However, the presence of NH_4^+ had negative effect on pyridine biodegradation (Fig. 4). Thus, it could be inferred that NH_4^+ released during pyridine degradation could be another reason for delayed pyridine biodegradation. Several studies have demonstrated great pH dependence on the extent of toxicity exerted by NH_4^+ [17,18]. The researchers have led to the proposal that the toxic effect of NH_4^+ was due to free ammonia (NH_3).

The NH_4^+-NH_3 balance could be described as follows:

$$NH_4^+ \leftrightarrow NH_3 + H^+$$

pH mainly controlled the NH_3/NH_4^+ equilibrium. Accordingly, the NH_4^+-NH_3 balance equation as a function of pH (Eq. (1)) was used for NH_3 concentration calculation using the experimental data obtained for total NH_4^+ concentration and pH [19].

$$[NH_3] = \frac{[NH_4^+]}{1 + 10^{pK_a - pH}} \quad \text{or} \quad NH_3\ (\%) = \frac{100}{1 + 10^{pK_a - pH}} \tag{1}$$

where pK_a was the acid–base ionization or dissociation constant, $[NH_3]$ was free ammonia (NH_3) concentration (mol l^{-1}), and $[NH_4^+]$ was NH_4^+ total concentration (mol l^{-1}). We used here $pK_a = 10.053 \times 0.032 \times T$ [19] with T = temperature (°C). Since pH and NH_4^+ concentrations were monitored in the experiments, it was possible to compute the NH_3 concentration during pyridine biodegradation by NJUST18.

As shown in Fig. 3, with the initial pH varied from 5.00 to 10.00, NH_3 concentration (calculated based on NH_3-N) increased from 0.08 ± 0.03 to 63.37 ± 3.57 mg l^{-1}, at the time of complete exhaustion of pyridine. As was indicated by Lay-Son and Drakides [19], nitrification process was inhibited at NH_3 concentration as low as 0.1 mg l^{-1}. In the nitrification process reported by Park and Bae [20], inhibition constants (K_i) of NH_3 on nitrite oxidation and ammonium oxidation were found to be 46 and 290–1,600 μM, respectively. High NH_3 released during anaerobic digestion of livestock wastes was also widely known to inhibit methanogenic micro-organisms and result in low methane production [18]. Yang et al. [21] reported the inhibitive effect of NH_3 to the formation of aerobic granules. The results showed that aerobic granules formed only when the NH_3 concentration were less than 23.5 mg l^{-1}, and nitrification was completely inhibited at NH_3 concentration greater than 10 mg l^{-1} [21]. The calculated NH_3 concentrations in this study, especially at initial pH of 9.0 and 10.0, which were 56.93 ± 5.35 and 63.37 ± 3.57 mg l^{-1}, respectively, were high enough compared with the threshold NH_3 concentrations reported in the literature [18–21]. It could be inferred that pyridine degradation process could be influenced strongly by pH through the NH_3/NH_4^+ equilibrium. For pyridine biodegradation at high initial pH values, the high NH_3 concentrations when pyridine was completely exhausted seemed to be a determining factor for delayed biodegradation at the end of the biodegradation process by NJUST18.

At high initial pyridine concentrations, it was observed that towards the end of substrate consumption curve, there was also a region of relatively reduced pyridine removal rate. Mathur et al. [2] attributed the reduced pyridine removal rate at the end of pyridine degradation to the deficit of oxygen and fall in pH of the medium. However, in our work, the aeration provided by shaking the flasks was able to keep the oxygen concentration sufficient, with deficit of oxygen avoided. In addition, continuous increase of pH during the biodegradation process was observed in this study. At initial pH of 7.0, with the initial pyridine concentrations of 600, 1,000, 1,600, 2,000, and 2,600 mg l^{-1}, pHs of medium at the end of biodegradation process increased to 8.38 ± 0.04, 8.76 ± 0.06, 9.06 ± 0.08, 9.08 ± 0.18, and 9.29 ± 0.05, respectively. With the increase of initial pyridine concentrations, NH_4^+-N release increased from 57.49 ± 1.61 to 158.72 ± 16.88 mg l^{-1}. Correspondingly, NH_3 concentrations increased from 2.89 ± 0.14 to 47.62 ± 1.28 mg l^{-1} (Fig. 2(a)). At high initial pyridine concentrations (especially 1,600, 2,000 and 2,600

$mg\,l^{-1}$), NH_3 concentrations were high enough to affect the pyridine consumption kinetics adversely.

The negative effect of NH_3 on pyridine biodegradation by NJUST18 could also be observed in Fig. 4. As the additional NH_4Cl concentrations increased from 0 to 2,000 $mg\,l^{-1}$, NH_3 concentrations increased from 11.14 ± 0.37 to $25.77 \pm 3.34\,mg\,l^{-1}$, accompanied by longer incubation time needed for complete pyridine degradation.

3.7. Effect of additional carbon source on pyridine degradation

As was described previously, NJUST18 could utilize pyridine as the sole carbon and nitrogen source. However, presence of readily biodegradable carbon source could have a significant effect on the degradation of recalcitrant compounds such as pyridine [22,23]. As indicated in Fig. 5, degradation of pyridine was enhanced with the addition of 500 $mg\,l^{-1}$ glucose. Degradation of pyridine was slightly inhibited when 1,000 $mg\,l^{-1}$ glucose was added into the medium. However, pyridine biodegradation was severely delayed in the presence of 2,000 $mg\,l^{-1}$ glucose. It was because usage of glucose as an additional substrate was beneficial for biomass growth. When low concentration of additional carbon source was added, enhanced biomass growth was beneficial for pyridine degradation. However, with the presence of abundant additional carbon source, pyridine degradation could be inhibited by competition of nutrition, such as oxygen and trace elements. Thus, the effect of additional carbon source on pyridine degradation was concentration dependent.

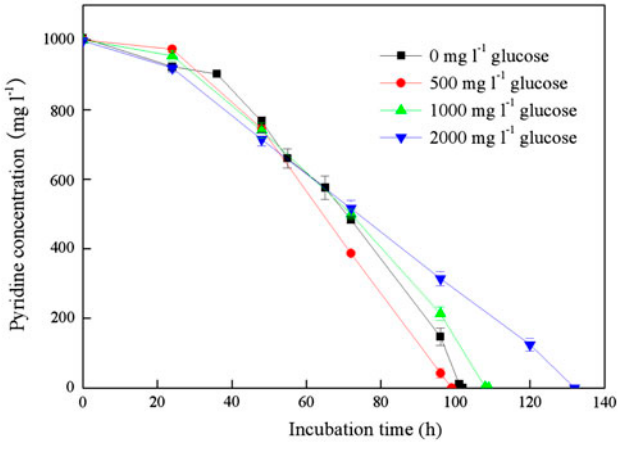

Fig. 5. Effect of additional glucose on pyridine degradation.

3.8. Implication

The concept of bioaugmentation, i.e. adding highly efficient degrading bacteria or enzyme into wastewater treatment, has attracted increasing interest, since biodegradation of some refractory pollutants, such as pyridine, could be improved through bioaugmentation [3,8]. For the bioaugmentation of pyridine treatment system, the key issue was the isolation of pyridine degrader with high efficiency. Currently, several bacteria have been discovered to have capability of pyridine biodegradation. However, biodegradation of pyridine by *Rhizobia* has not been reported up to now. *Rhizobia*, as soil nitrogen fixing bacteria, have now emerged as a promising candidate for recalcitrant pollutant bioremediation. For example, some bacteria belonging to *Rhizobia* have been reported to utilize phenol and polychlorinated biphenyls [24,25]. As they were naturally exposed to a range of aromatic exudates of roots and thus might be proven to possess interesting aromatic catabolic pathways and capabilities [24].

Rhizobium sp. NJUST18 reported in this study was able to mineralize pyridine at initial concentration up to 2,600 $mg\,l^{-1}$, which was an advantage for treatment of pyridine containing wastewater at relatively high concentrations. However, most reported micro-organisms, such as *Shewanella putrefaciens* and *Bacillus sphaericus*, were capable of completely degrading pyridine at concentrations lower than 1,000 $mg\,l^{-1}$ [1,2,12]. In a completely mixed activated sludge process seeded with *Pseudomonas pseudoalcaligenes-KPN*, an increase to influent pyridine of 400 $mg\,l^{-1}$ resulted in reactor failure to degrade pyridine [3]. These results indicated excellent pyridine degradation ability of *Rhizobium* sp. NJUST18.

Alkaline condition did not favor pyridine biodegradation. pH increase during pyridine biodegradation delayed pyridine biodegradation at high concentrations. Besides pH sensitivity, NH_4^+ release was also crucial for high concentration of pyridine biodegradation by NJUST18. At high initial pHs or high initial pyridine concentrations, delayed pyridine biodegradation was perhaps due to the high concentration NH_3 generated. Thus, both pH increase and NH_4^+ release during pyridine biodegradation by NJUST18 were important factors that needed to be considered when making a remediation strategy. One possible solution, which would be useful in continuous bioreactor for pyridine biodegradation, was adding nitrifying bacteria. In a nitrification process, transformation of NH_4^+ into less harmful nitrite or nitrate occurred with pH decrease. We are currently investigating whether NH_4^+ generated during pyridine biodegradation by NJUST18 can be removed from MSM by *Nitrobacter* strains. In addition, in the industrial process, in order

to optimize the operation conditions for pyridine biodegradation, it appears that pH should be controlled and adjusted continuously in the aeration systems to stay low NH_3 level.

An assessment of the literature on pyridine biodegradation showed a predominance of papers with the focus of interest mainly on the degradation pathway, whereas papers with technological orientation were in the minority. Therefore, further work will be focusing on the engineering aspects of *Rhizobium* sp. NJUST18 in continuous treatment reactors. The role and performance of *Rhizobium* sp. NJUST18 under open conditions should be emphasized during our further research.

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