RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Effect of temperature, salinity, heavy metal, ammonium concentration, pH and dissolved oxygen on ammonium removal by an aerobic nitrifier

Ying Wang[a], Hu Chen[a], Yu-Xiang Liu[b], Rui-Peng Ren[a], Yong-Kang Lv[a,*]

[a] *Key Laboratory of Coal Science and Technology, Ministry of Education and Shanxi Province,* [b] *College of Environmental Science and Engineering, Taiyuan University of Technology, Taiyuan 030024, Shanxi, China*

** Corresponding author. Tel (Fax):+86 351 6010386; E-mail address: lykang@tyut.edu.cn (Y.-K. Lv).*

Abstract: An aerobic nitrifier WY-01, isolated from coking wastewater, was identified as *Alcaligenes faecalis* by its 16S rRNA gene sequence analysis. It exhibited unusual capability for ammonium removal at initial NH_4^+–N 400 mg/L with low accumulated intermediates, and converted ammonium to N_2 under aerobic conditions. Based on nitrogen removal and enzyme assay, two distinct nitrogen removal pathways were proposed in strain WY-01. Additionally, the effect of different factors on ammonium removal by strain WY-01 was investigated. The results show that efficient removal of ammonium occurred at temperature as low as 10°C, 60 g/L salinity, 8 mmol/L Cu^{2+} or 0.5 mmol/L Zn^{2+} or 1 mmol/L of equivalent Cu^{2+}–Zn^{2+}, initial NH_4^+–N concentration from 50 to 1200 mg/L and pH from 5 to 10. The strong adaptability of strain WY-01 makes it a promising candidate for future application in actual wastewater treatment.

Keywords:

Heterotrophic nitrification, Ammonium, *Alcaligenes faecalis*, Nitrogen removal pathway, Influence factors

1. Introduction

Nitrogen (N) is a necessary nutritional element for plants and animals, but excess nitrogen compounds drained into the natural water cause eutrophication and directly threaten human health. [1-3] Hence, it is necessary to remove nitrogen compounds from wastewater before they are discharged to the environment.

Traditional biological nitrogen removal process, a widely adopted method for nitrogen removal, is normally composed of nitrification by autotrophic nitrifier under aerobic conditions and denitrification by anoxic denitrifiers under anoxic conditions. [4-6] However, the low nitrification rate of autotrophs makes this conventional system time-consuming while the vast space for separate aerobic and anoxic reactors increases wastewater-treatment costs. [7, 8]

As compared with autotrophs, heterotrophic bacteria have several distinct advantages: (1) higher growth rates; (2) higher tolerance to ammonium and organic matter; (3) performing nitrification and denitrification concurrently in single reactor to convert ammonium into nitrogenous gas under aerobic conditions. [5, 9] Moreover, an increasing number of heterotrophic nitrifying bacteria, capable of simultaneous nitrification and denitrification (SND), have been isolated and identified in recent years. [10-13] Therefore, these bacteria make it possible to establish SND reactor for saving operation costs, space and time.

Although these bacteria have shown great potential for nitrogen removal, they usually perform low nitrogen removal efficiency in actual wastewater treatment. [14, 15]

Significant challenges and major problems exist in bacteria themselves, which are vulnerable to external environmental stresses, such as low temperature, [16-18] high salinity, [19, 20] heavy metals, [21, 22] extreme pH, [23] and dissolved oxygen (DO). [24, 25] However, limited efforts have been made to systematically evaluate these parameters on the nitrogen removal, especially for novel strains, and little information is available about microbial responses to the change of external conditions, which is of significance for screening appropriate bacterial and maintaining their activities in actual biological wastewater treatment. Therefore, there is a crucial need to study the effect of external factors on nitrogen removal.

In the present study, a new heterotrophic bacterium, *Alcaligenes faecalis* strain WY-01, was isolated from coking wastewater, which showed excellent ammonium removal ability in extremely high ammonium loads. The possible denitrifying pathway of strain WY-01 was investigated. From the comprehensive evaluation of the impact of different factors on ammonium removal, the isolate showed much higher tolerance to low temperature and high salinity than common nitrifier. The detailed understanding of the characteristics of the microorganism can promote its application in actual wastewater treatment.

2. Experimental

2.1 Medium

The Luria-Bertani medium (LB): peptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L; pH 7.0.

The Selective Medium (SM): $(NH_4)_2SO_4$, 2 g/L, sodium citrate, 5 g/L, $K_2HPO_4•3H_2O$, 0.75 g/L; $NaH_2PO_4•12H_2O$, 0.25 g/L; $MgSO_4•7H_2O$, 0.05 g/L; $FeSO_4•7H_2O$, 0.01 g/L; $MnSO_4•H_2O$, 0.01 g/L; EDTA-2Na, 0.15 g/L; pH 7.0.

The Basal Medium (BM): $K_2HPO_4•3H_2O$, 0.75 g/L; $NaH_2PO_4•12H_2O$, 0.25 g/L; $MgSO_4•7H_2O$, 0.05 g/L; $FeSO_4•7H_2O$, 0.01 g/L; $MnSO_4•H_2O$, 0.01 g/L; EDTA-2Na, 0.15 g/L; pH 7.0. Distilled water was employed to prepare all water phase solutions.

Different amounts of organic carbon and nitrogen sources were added to the BM medium for different experiments. Carbon/nitrogen (C/N) was adjusted to 12 with sodium citrate as the sole carbon source; $(NH_4)_2SO_4$, $NaNO_3$, $NaNO_2$ and $NH_2OH•HCl$ were used in Sections 3.2 and 3.3 (BM-1 to BM-6 in Table 1) and ammonium sulfate was the only nitrogen source in Section 3.4 (Table 2). Solidified media were prepared with the addition of 1.5% (w/v) agar. All the media were autoclaved at 121°C for 20 minutes and all chemicals were of analytical grade.

2.2 Isolation of bacteria

Coking wastewater (10 mL) was added into fresh sterile LB medium (90 mL) in a 250 mL Erlenmeyer flask and the mixture was incubated at 30°C and 120 rpm for two days. Cells were harvested by centrifugation at 6000 rpm for 5 min, washed three times with sterile 0.2% NaCl solution, and then the cell mass was resuspended in 10 mL of sterile 0.2% NaCl solution for inoculation. The resuspended solution was transferred to 90 mL of sterile SM medium, and the mixture was incubated at 30°C and 120 rpm for two days. Gradient dilutions were then conducted, and the bacterial suspensions were

spread onto agar SM medium plates and incubated at 30°C until visible colonies were formed. Then, purified isolates were obtained by repeated streaking on fresh agar SM medium plates (repeated that at least three times). About 30 bacterial colonies were picked, and individually inoculated into sterile SM medium to test their ammonium removal activity. The strain (named WY-01) with highest ammonium removal ability was selected, and stocked in 30% glycerol solution at -80°C for further study.

2.3 Strain identification

The physiological and biochemical characteristics of strain WY-01 were examined according to the methods of Dong and Cai (2001). [26] Genomic DNA was extracted from bacterial suspensions using DNA extraction Kit (Sangon, Shanghai, China). The 16S rRNA gene was then amplified by PCR using the universal primers F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 (5'-TTGGYTACCTTGTTACGACT-3'). PCR products were sequenced by TaKaRa Biotechnology (Dalian) Co., Ltd. Finally, the 16S rDNA sequence of WY-01 was compared with that of other bacteria by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). A phylogenetic tree was built using MEGA 6.0 by neighbor-joining method with 1000 bootstrap replicates.

2.4 Experimental design

BM-1 was used to investigate ammonium removal characteristics of the strain, while the rest (BM 2 - 6) was used to investigate the aerobic nitrogen removal pathway. Besides, the heterotrophic nitrification–aerobic denitrification characteristics of strain

WY-01 were investigated under different environmental factors, including temperature, salinity, heavy metal, initial ammonium concentrations, pH and DO (as shown in Table 2). All of the above experiments were conducted in triplicate in 250 mL Erlenmeyer flasks containing 100 mL of sterile medium. Typically, 1 mL of bacterial suspension (OD_{600} = 1.424) was inoculated into sterile medium, and then cultured at 30°C (except temperature experiment) and 120 rpm (except dissolved oxygen experiment). The temperature was set at 10, 15, 20, 25, 30, 35, 40°C at 120 rpm for temperature experiment and the shaking speed was adjusted to 0, 60, 80, 100, 120, 140, 160, 180 rpm at 30°C for dissolved oxygen experiment. Non-seeded sample experiments were also conducted as controls at the same conditions. During incubation, 10 mL cultures from these batch tests were sampled periodically to determine cell optical density at OD_{600} and then centrifuged (10 min, 10000 rpm) to obtain supernatants for the measurement of ammonium (NH_4^+–N), hydroxylamine (NH_2OH–N), nitrite (NO_2^-–N) and nitrate (NO_3^-–N). All experiments were performed in triplicate.

2.5. Assessment for gas detection

One milliliter of bacterial suspension of strain WY-01 was inoculated into BM-1 or BM-2 (100 mL) in a tightly sealed flask (500 mL). The flask was then fully aerated with high-purity oxygen gas (99.99%). The whole system was cultivated at 30°C and 120 rpm, and the system without the bacterial inoculum was used as a control. Gas samples (400 μL) were collected at the starting and ending points using a gas-tight syringe to detect N_2 and O_2 by gas chromatography equipped with a thermal conductivity

detector.[27]

2.6 Enzyme assay

Cell-free extracts of strain WY-01 were prepared using the ultrasonic method as described by Zhao *et al.*.[28] Hydroxylamine oxidase (HAO) activity was analyzed by the reduction of potassium ferricyanide at 400 nm.[29] Formation of nitrite from nitrate, and reduction of nitrite were taken as a measurement for nitrate reductase (NR), and nitrite reductase (NiR) activity, respectively.[28] Protein concentration in the cell-free extract was determined by the Bradford Protein Assay Kit (Sangon, Shanghai, China). One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the transformation of 1 μmol of substrate per minute. The specific activity (U/mg) was defined as the amount of enzyme units divides by the concentration of protein in mg.

2.7 Analytical methods and calculations

Bacterial growth was monitored at 600 nm using a spectrophotometer (SP-752, Spectrum Shanghai, China). Ammonium was measured by Nessler's reagent photometry at a wavelength of 420 nm. Nitrate was determined by phenol disulfonic acid method at a wavelength of 410 nm. Nitrite was analyzed by N-(1-naphthalene)-diaminoethane photometry method at a wavelength of 540 nm. Hydroxylamine was analyzed by indirect spectrophotometry.[30] Dissolved oxygen (DO) and pH were measured with a DO meter (HI98193, HANNA, Italy) and a pH meter (PB-10, Sartorious, Germany), respectively.

The ammonium removal efficiency formula is $(C_0\text{-}C_t)/C_0 \times 100\%$. The ammonium

removal rate is calculated by $(C_0\text{-}C_t)/t$. C_0 is the initial concentration of NH_4^+–N. C_t is the final concentration of NH_4^+–N at given time. t is the time of strain WY-01 treatment. Each treatment has three repetitions and the results are presented as means ± SD (standard deviation of means). Data were analyzed by one-way ANOVA with Tukey's HSD test ($P < 0.05$) using SPSS Statistics 17.0 software and images were processed by OriginPro 8 software.

3. Results and discussion

3.1 Isolation and identification of strain WY-01

Strain WY-01 is a Gram-negative, non-motile and short rod-shaped bacterium. The colony of strain WY-01 on the plate is yellow, round, opaque and with a smooth surface. The catalase reaction, gelatin liquefaction, starch hydrolysis and oxidase reaction are all positive, while the sugar fermentation, Voges-Proskauer test and indole test are negative. Almost the entire 16S rRNA gene (1434 bp) of strain WY-01 was sequenced, and then the nucleotide sequence was deposited in GenBank under accession number of KM977890. The BLAST results indicate that strain WY-01 is closely related to the genus *Alcaligenes faecalis* with 99% sequence similarity. An N-J phylogenetic tree was constructed on basis of 16S rDNA sequences of WY-01 and some other related strains (Fig. 1). The result further indicates that strain WY-01 is clustered with *A. faecalis*. Therefore, WY-01 is proposed to be an *A. faecalis* species.

3.2 Ammonium removal characteristics of strain WY-01

As depicted in Fig. 2, the growth and ammonium removal characteristics of strain

WY-01 were investigated at 30°C and 120 rpm in 250 mL Erlenmeyer flask systems with an initial NH_4^+–N concentration of about 400 mg/L. During the first 6 hours, WY-01 grows slowly, and no degradation of ammonium is observed. The next 66 h witnesses a dramatic decrease of ammonium from 419.25 ± 12.78 mg/L to 23.74 ± 1.28 mg/L. Interestingly, most of the removal of NH_4^+–N follows a linear trend (at about 5.99 mg NH_4^+–N/L/h) when WY-01 enters its exponential growth phase. Besides, a final 98.14 ± 0.91% of ammonium removal is achieved after incubation of 96 h with an average ammonium removal rate of 4.34 mg NH_4^+–N/L/h.

Nitrification intermediates hydroxylamine, nitrite and nitrate were detected during ammonium removal process (Fig. 2). The concentration of hydroxylamine increases with the decrease of the concentration of NH_4^+–N, reaches a maximum concentration of 10.13 ± 0.47 mg/L at 12 h, and then decreases gradually. Similar to hydroxylamine, the accumulation of nitrite increases during the decline of ammonium concentration and subsequently decreases to zero. However, a low accumulation of nitrate is observed and no decrease is detectable. Thus, the low accumulation of nitrification products, especially nitrite, in the heterotrophic nitrification process makes strain WY-01 superior to some other heterotrophic nitrifying bacteria [5, 13] and prospective for application in the treatment of conventional high-strength ammonium wastewater.

In order to evaluate the aerobic denitrifying performance, N_2 production was tested using gas chromatography in the closed system when strain WY-01 was inoculated in BM-1 medium. After 96 h, 41.65 ± 1.83 mg NH_4^+–N was removed by strain WY-01

with 0.12 ± 0.01 mg nitrate accumulated, and 28.91 ± 1.32% of used NH_4^+–N was finally converted into N_2 in the system, indicating that strain WY-01 has the ability of simultaneous heterotrophic nitrification and aerobic denitrification.

3.3 Aerobic metabolic pathway by strain WY-01

Because hydroxylamine is regarded as a crucial intermediate, involved in a novel metabolic pathway of $NH_4^+ \rightarrow NH_2OH \rightarrow N_2O \rightarrow N_2$, [5, 31] the utilization of hydroxylamine by strain WY-01 was investigated in the BM-2 medium under aerobic conditions. As shown in Fig. 3A, OD_{600} shows an insignificant increase during the whole process. This inability to use hydroxylamine as a N-source for growth is consistent with that of *Acinetobacter calcoaceticus* HNR [28] and *A. faecalis* stain NR, [31] but different from that of *Acinetobacter junii* YB. [11] However, hydroxylamine decreases from 11.54 ± 0.29 mg/L to 0.21 ± 0.01 mg/L and minimal amounts of nitrite (0.92 ± 0.05 mg/L) and nitrate (0.79 ± 0.05 mg/L) accumulate within 24 h. Furthermore, gas detection experiment was also carried out in the closed system when strain WY-01 was inoculated in BM-2 medium for 24 h, and 57.52 ± 1.80% of the consumed NH_2OH–N (1.08 ± 0.04 mg) could be denitrified to produce N_2.

When nitrite is added as the sole N-source in BM-3 medium, OD_{600} shows no growth of strain WY-01 during the whole process (Fig. 3B). Within 72 h, NO_2^-–N decreases from 5.16 ± 0.26 mg/L to 3.59 ± 0.18 mg/L, while NO_3^-–N increases by 1.58 ± 0.08 mg/L. The amount of decreased NO_2^-–N is almost equal to that of increased NO_3^-–N, indicating that both the concentrations of NO_2^-–N and NO_3^-–N are probably under

equilibrium as regulated between heterotrophic nitrification and aerobic denitrification. [32] Interestingly, when nitrite and ammonium are added as a mixed N-source in BM-5 medium, nitrite supplied in the medium could be decreased to zero by strain WY-01 during the ammonium oxidation process (Fig. S1A). This result is different from that of *A. faecalis* strain TUD, which could only denitrify internally-produced nitrification products under aerobic conditions. [33]

As shown in Fig. 3C, an insignificant decrease in OD_{600} is observed when 5 mg/L of nitrate is used as the sole N-source in BM-4 medium. Meanwhile, both NO_3^- decrease and NO_2^- production are not detected, indicating that strain WY-01 is unable to grow and utilize nitrate as the sole N-sources in the medium only containing sodium citrate and nitrate. Furthermore, 5 mg/L of nitrate and 400 mg/L of ammonium were added as a mixed N-source in BM-6 medium to study whether nitrate could be utilized by strain WY-01 under aerobic conditions during the ammonium removal process (Fig. 3D). The concentrations of nitrate increase from 5.28 ± 0.98 mg/L to 6.53 ± 0.94 mg/L in the first 24 hours and then keep stable. These results demonstrate that nitrate could not be utilized by strain WY-01 even induced by ammonium.

In order to further understand the possible heterotrophic nitrogen removal pathway of strain WY-01, a preliminary study on the enzyme activities of HAO, NR and NiR was conducted. Neither NR nor NiR activity in *A. faecalis* strain WY-01 was detectable with NADH as an electron donor, demonstrating that both NR and NiR in strain WY-01 are not responsible for aerobic denitrification to produce nitrogenous gases. However, 0.013

U/mg protein HAO specific activity in strain WY-01 was detected when ferricyanide was used as an electron acceptor, which is in the same order of magnitude as that in *Acinetobacter calcoaceticus* HNR (0.011 U/mg protein),[28] *A. faecalis* strain NR (0.016 U/mg protein)[31] and *Acinetobacter junii* YB (0.0195 U/mg protein).[11] In addition, HAO is responsible for the conversion of hydroxylamine to gaseous nitrogen.[13, 31] Therefore, the presence of HAO provides an additional evidence for the aerobic nitrogen removal pathway of strain WY-01.

In summary, the results show that not only ammonium could be oxidized with low accumulations of nitrate, but also hydroxylamine and nitrite could be utilized by strain WY-01 under aerobic conditions. Moreover, N_2 could be detected in the gas when hydroxylamine or ammonium is separately used as the sole N-source. From the utilization of nitrogen compounds, gas detection and enzyme assay, two different metabolic pathways are proposed for ammonium oxidation in strain WY-01. One is $NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$, the other is oxidizing ammonium to nitrogenous gas directly via hydroxylamine.

3.4 The impact of different factors on ammonium removal by strain WY-01

3.4.1 Temperature

Fig. 4 shows the impact of temperature on ammonium removal ability of *A. faecalis* strain WY-01. At temperatures below 20°C, there exists a long lag phase. The lower the temperature is, the longer the lag phase is (Fig. 4A). Although there is a longer lag phase in strain WY-01 at 10°C, NH_4^+–N could be decreased from 49.21 ± 1.26 mg/L to

1.20 ± 0.07 mg/L in 96 h. The average ammonium removal rate is 0.50 ± 0.03 mg/L/h, higher than that of *microbacterium* sp. strain SFA13 (0.16 mg/L/h).[27] Here, similar results occur at 15°C and 20°C, and the ammonium removal efficiencies reach 98.02 ± 1.40% at 60 h and 96.69 ± 2.87% at 36 h, respectively. As the temperature increases from 25°C to 40°C (Fig. 4B), strain WY-01 grows rapidly and the OD_{600} reaches its maximum in 24 ,18, 12 and 15 h, respectively; meanwhile, almost all of ammonium could be removed with the ammonium removal efficiencies of 97.69 ± 1.95%, 96.97 ± 2.91%, 96.31 ± 1.93% and 95.78 ± 2.87%, respectively. These experimental results indicate that *A. faecalis* strain WY-01 could utilize or convert ammonium during the exponential phase, suggesting that the trend of cell growth is in accordance with ammonium removal (Fig. 4C).

As a result, *Alcaligenes faecalis* strain WY-01 could perform excellent ammonium removal ability at temperature range from 25°C to 40°C. Previous studies revealed that the optimal temperature range of most heterotrophic nitrifying–aerobic denitrifying bacteria is 25 – 37°C. [11-13, 22] However, it is well known that nitrification is strongly inhibited at 10°C or less. [34, 35] In this paper, strain WY-01 could grow and remove ammonium at temperature as low as 10°C, which indicates its potential for removing ammonium at low temperature.

3.4.2 High salinity

Because salinity is an important parameter affecting nitrogen removal, it is necessary to study the influence of salinity on ammonium removal ability of strain WY-01. We

first investigated whether the salinity had effect on the growth of strain WY-01. As shown in Fig. 5A, cells quickly enter the exponential phase after a short lagging growth when the concentration of salinity is not more than 30 g/L, and strain WY-01 grows better in salinity of 20 g/L or 30 g/L than in salinity of 0 g/L or 10 g/L. Moreover, the maximum of OD_{600} with salinity of 20 g/L or 30 g/L is comparable to that with salinity of 40 g/L or 50 g/L. Besides, there is an exponential relationship between the lag phase and the concentration of salinity when the concentration of salinity is between 10 g/L and 60 g/L (Fig. 5B). From Fig. 5A and 5B, the optimal salinity of strain WY-01 is between 20 g/L and 30 g/L, which is consistent with the optimal salinity of most heterotrophic nitrification–aerobic denitrification bacteria. [13, 19, 22]

Obviously, strain WY-01 shows great ammonium removal ability under 50 g/L of salinity, and more than 90% of ammonium removal is achieved within 96 h (Fig. 5C and 5D). This value is much higher than that of *Bacillus methylotrophicus* strain L7 in which 58.70% of ammonium is removed within 0 – 30 g/L NaCl in 108 h. [13] Furthermore, a final 98.29 ± 0.49% of ammonium could be removed in 6 days by strain WY-01 even the concentration of salinity rises to 60 g/L (Fig. S2). As can be drawn from these results, strain WY-01 could grow both without salinity and below 60 g/L of salinity, and realize more than 90% of ammonium removal efficiency. Therefore, strain WY-01 is defined as a halotolerant bacterium. [36]

3.4.3 Heavy metal

Compared with Cu^{2+}, strain WY-01 is much more sensitive to Zn^{2+} as shown in Fig.

6A. Ammonium removal in the solution containing 3 mmol/L Cu^{2+} is 98.19 ± 2.83%, which is similar to the control group. Even when the concentration of Cu^{2+} reaches 8 mmol/L, 68.72 ± 5.34% of ammonium could be removed (Fig. S3A). Conversely, 0.5 mmol/L of Zn^{2+} has a significantly negative effect on ammonium removal efficiency, which is only 42.89 ± 1.28%. Higher concentration of Zn^{2+} in the medium results in lower ammonium removal efficiency: 1.11 ± 0.17% at 5 mmol/L Zn^{2+}, and 0.53 ± 0.04% at 6 mmol/L Zn^{2+} are achieved (Fig. S3B). These results imply that the ammonium removal nearly stops at 5 mmol/L of Zn^{2+}.

In addition, it could be concluded from Fig. 6A that the toxicity of equivalent Cu^{2+} and Zn^{2+} is much less than that of single added Zn^{2+}. When an equimolar mixture of Cu^{2+} and Zn^{2+} is presented together in the medium, the ammonium removal efficiencies increase to 97.69 ± 1.35% at 0.5 mmol/L of Cu^{2+}–Zn^{2+} and 97.54 ± 2.67% at 1 mmol/L of Cu^{2+}–Zn^{2+}. These values are consistent with the control group. While the concentration of Cu^{2+}–Zn^{2+} exceeds 1 mmol/L, the ammonium removal efficiency drops with the increase of concentration of Cu^{2+}–Zn^{2+}. Almost 20.42 ± 3.11% of ammonium removal is obtained by strain WY-01 at 5 mmol/L of Cu^{2+}–Zn^{2+}, which is still higher than that of single added 5 mmol/L of Zn^{2+}. This phenomenon indicates that the toxicity of Cu^{2+}–Zn^{2+} association is not simply superimposed, but much weaker than that of Cu^{2+} plus Zn^{2+}. Similar result is obtained by *Acidithiobacillus caldus* strain BC13, [37] in which the influence of Cu^{2+}–Zn^{2+} association is slightly weaker than that of Cu^{2+} plus Zn^{2+}. Nevertheless, it is quite different from the result of heterotrophic nitrification–

aerobic denitrification bacterium, *Aeromonas* sp. HN-02, to which the toxicity of equivalent Cu^{2+} and Zn^{2+} is much stronger than that of Cu^{2+} plus Zn^{2+}. [22]

For Bacillus sp. PK15, 0.5 mM of Cu^{2+} and Zn^{2+} does not affect its growth and ammonium removal ability. [38] However, strain HN-02 is extremely sensitive to Cu^{2+}, and it could only tolerate 0.5 mg/L of Cu^{2+} (0.0078 mM/L) or 8 mg/L of Zn^{2+} (0.12 mM/L).[22] The study in this paper also shows that strain WY-01 is more sensitive to Zn^{2+} than to Cu^{2+} during ammonium removal process. All these results indicate that effects of heavy metals on different heterotrophic nitrification–aerobic denitrification bacteria are different. Appropriate dosage of heavy metals would not affect the growth of microorganism, but there is no doubt that excess heavy metals would be harmful to microorganism.

3.4.4 Initial ammonium concentration, pH and dissolved oxygen

Fig. 6B shows the ammonium removal performance of strain WY-01 at different initial ammonium concentrations. Over 80% ammonium could be removed at initial ammonium concentration ranging from 50 mg/L to 600 mg/L within 96 h. While the initial concentration of ammonium is over 600 mg/L, the efficiencies of ammonium removal decrease from 90.76 ± 3.63% at 600 mg/L to 7.31 ± 0.29% at 2500 mg/L. Besides, the ammonium removal efficiencies of 52.5 ± 2.1% and 34.84 ± 1.39% are achieved by strain WY-01 with average ammonium removal rates of 6.07 ±0.18 mg/L/h and 4.61 ± 0.19 mg/L/h at 1000 mg/L and 1200 mg/L, respectively. Unlike strain WY-01, *Bacillus subtilis* A1, [39] an aerobic heterotrophic nitrifying–denitrifying

bacterium, is unable to efficiently transform ammonium after 120 h at initial NH_4^+–N concentration higher than 500 mg/L. For another heterotrophic nitrification efficiency–aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7, [13] only 46.40% and 36.50% ammonium is removed within 108 h at initial NH_4^+–N concentration 427 mg/L and 1121 mg/L, respectively, which is much lower than that of strain WY-01 in this study.

The effect of pH on ammonium removal was investigated under aerobic conditions. No significant difference in ammonium removal ($P > 0.05$) is observed at the initial pH of 5 – 10, as more than 90% of ammonium removal efficiency is reached (Fig. 6C). However, there is almost no ammonium decrease at pH of 4 and 11, indicating that strong acidic or alkaline conditions are detrimental to strain WY-01. Thus, strain WY-01 could perform good nitrification ability in neutral or slightly alkaline environment, which is consistent with nearly all reported heterotrophic nitrifying–denitrifying bacterium. [10, 11, 13, 40, 41] These pH values are in accordance with the actual wastewater, which is beneficial for practical applications.

Dissolved oxygen (DO) acts as one of major parameters affecting nitrification and denitrification process. [11, 13, 24] The concentration of DO was controlled by adjusting the rotation speed of the shaker. As shown in Fig. 6D, poor ammonium removal efficiency is obtained in static cultivation ($DO_{0\ rpm} \approx 1.98$ mg/L), which is only 62.41 ± 2.50% within 96 h. The ammonium removal efficiency increases with the increase of shaking speed. However, a slight difference in ammonium removal ($P > 0.05$) is observed when

the shaking speed exceeds 120 rpm ($DO_{120\ rpm} \approx 4.67$ mg/L). Besides, the change trend of ammonium removal rate is consistent with the ammonium removal efficiency. Therefore, the results obtained in this study are coincidental with the previous research.[11, 27]

Taken together, strain WY-01 could grow well and remove ammonium effectively in a broad range of physico-chemical conditions. Seen from Table 3, strain WY-01, which shows the highest tolerance among the relevant strains, could remove ammonium in NH_4^+–N concentration of 2500 mg/L. Additionally, though WY-01 and *Aeromonas* sp. HN-02 are able to grow at low temperatures, WY-01 could utilize ammonium under 60 g/L of salinity. This value is much higher than that for *Aeromonas* sp. HN-02 and comparable to that for *Vibrio diabolicus* SF16. Besides, WY-01 also exhibits stronger tolerance to Cu^{2+} than other strains in the ammonium remove process. Therefore, the extensive adaptability of strain WY-01 to the change of external conditions is of great value for its application in actual biological wastewater treatment.

4. Conclusions

Strain WY-01 was isolated from coking wastewater and identified as *Alcaligenes faecalis*. It exhibited excellent ammonium removal ability at a wide range of ammonium loads, although there were low accumulated intermediates. Under aerobic conditions, strain WY-01 was unable to denitrify nitrite and nitrate, but N_2 could be detected when hydroxylamine was used as a sole nitrogen source. Therefore, there might be two aerobic metabolic pathways for ammonium oxidation in strain WY-01. One is $NH_4^+ \rightarrow$

$NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$, the other is $NH_4^+ \rightarrow NH_2OH \rightarrow N_2$.

Furthermore, the paper emphasized on the effects of different factors on the nitrogen removal, and results indicate that strain WY-01 could grow well in the following culture conditions:

(1) Efficient ammonium removal was retained at 10°C.

(2) WY-01 showed strong ammonium removal ability at salinity below 60 g/L.

(3) WY-01 performed strong resistance to high ammonium concentration.

(4) WY-01 kept active at 8 mmol/L Cu^{2+} or 0.5 mmol/L Zn^{2+} or 1 mmol/L Cu^{2+}–Zn^{2+}. All these results demonstrate that strain WY-01 has a great potential for nitrogen removal from actual wastewater.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (Grant No. 51078252), the Program for International Cooperation Projects of Shanxi Province (Grant No. 2013081020 and 2010081018), Natural Science Foundation of Shanxi Province (2010011016-1), and Science and Technology Development of Shanxi Province (20140321013-04).

Appendix A. supplementary data

Supplementary data associated with this article can be found.

References

1 J. A. Camargo and Á. Alonso, *Environ. Int.*, 2006, 32, 831-849.
2 J. Domagalski, C. Lin, Y. Luo, J. Kang, S. M. Wang, L. R. Brown and M. D. Munn, *Agric. Water Manage.*, 2007, 94, 43-53.
3 Y. F. Zhang and I. Angelidaki, *Water Res.*, 2012, 46, 6445-6453.
4 A. A. Khardenavis, A. Kapley and H. J. Purohit, *Appl. Microbiol. Biotechnol.*, 2007,

77, 403-409.
5 H. S. Joo, M. Hirai and M. Shoda, *J. Biosci. Bioeng.*, 2005, 100, 184-191.
6 R. Zhang, L. Li and J. Liu, *RSC Adv.*, 2015, 5, 40785-40791.
7 I. N. C. Metcalf and H. Eddy, *Wastewater Engineering: Treatment and Reuse, fourth edition*, McGraw-Hill, New York, 2003.
8 T. Khin and A. P. Annachhatre, *Biotechnol. Adv.*, 2004, 22, 519-532.
9 C. Li, J. S. Yang, X. Wang, E. T. Wang, B. Z. Li, R. X. He and H. L. Yuan, *Bioresour. Technol.*, 2015, 182, 18-25.
10 M. S. Zheng, D. He, T. Ma, Q. Chen, S. T. Liu, M. Ahmad, M. Y. Gui and J. R. Ni, *Bioresour. Technol.*, 2014, 162, 80-88.
11 Y. X. Ren, L. Yang and X. Liang, *Bioresour. Technol.*, 2014, 171, 1-9.
12 C. Chen, K. L. Ho, F. C. Liu, M. N. Ho, A. J. Wang, N. Q. Ren and D. J. Lee, *Bioresour. Technol.*, 2013, 145, 351-356.
13 Q. L. Zhang, Y. Liu, G. M. Ai, L. L. Miao, H. Y. Zheng and Z. P. Liu, *Bioresour. Technol.*, 2012, 108, 35-44.
14 J. A. Álvarez, I. Ruiz, M. Gómez, J. Presas and M. Soto, *Bioresour. Technol.*, 2006, 97, 1640-1649.
15 X. Zhou, Y. Li and Y. Zhao, *RSC Adv.*, 2014, 4, 15620-15629.
16 D. Qu, C. Wang, Y. F. Wang, R. Zhou and H. J. Ren, *RSC Adv.*, 2015, 5, 5149-5157.
17 S. Yao, J. Ni, Q. Chen and A. G. L. Borthwick, *Bioresour. Technol.*, 2013, 127, 151-157.
18 S. Yao, J. Ni, T. Ma and C. Li, *Bioresour. Technol.*, 2013, 139, 80-86.
19 Y. Guo, X. M. Zhou, Y. G. Li, K. Li, C. X. Wang, J. F. Liu, D. J. Yan, Y. L. Liu, D. H. Yang and J. M. Xing, *Biotechnol. Lett.*, 2013, 35, 2045-2049.
20 M. Nakano, T. Inagaki, S. Okunishi, R. Tanaka and H. Maeda, *J. Basic Microbiol.*, 2010, 50, 285-289.
21 S. Wang, M. Gao, Z. Wang, Z. She, C. Jin, Y. Zhao and Z. Li, *RSC Adv.*, 2015, 5, 30737-30747.
22 M. X. Chen, W. C. Wang, Y. Feng, X. H. Zhu, H. Z. Zhou, Z. L. Tan and X. D. Li, *Bioresour. Technol.*, 2014, 167, 456-461.
23 A. A. Shapovalova, T. V. Khijniak, T. P. Tourova, G. Muyzer and D. Y. Sorokin, *Extremophiles*, 2008, 12, 619-625.
24 G. Luo, L. Li, Q. Liu, G. Xu and H. Tan, *Bioresour. Technol.*, 2014, 171, 152-158.
25 X. Yang, S. Wang and L. Zhou, *Bioresour. Technol.*, 2012, 104, 65-72.
26 X. Z. Dong and M. Y. Cai, *Manual of Systematic Identification for Common Bacteria*, Science Press, Beijing, 2001.
27 D. Y. Zhang, W. G. Li, X. F. Huang, W. Qin and M. Liu, *Bioresour. Technol.*, 2013, 137, 147-152.
28 B. Zhao, Y. L. He, J. Hughes and X. F. Zhang, *Bioresour. Technol.*, 2010, 101, 5194-5200.
29 S. Otte, J. Schalk, J. G. Kuenen and M. S. M. Jetten, *Appl. Microbiol. Biotechnol.*, 1999, 51, 255-261.
30 D. S. Frear and R. C. Burrell, *Anal. Chem.*, 1955, 27, 1664-1665.

31 B. Zhao, Q. An, Y. L. He and J. S. Guo, *Bioresour. Technol.*, 2012, 116, 379-385.
32 P. Z. Chen, J. Li, Q. X. Li, Y. C. Wang, S. P. Li, T. Z. Ren and L. G. Wang, *Bioresour. Technol.*, 2012, 116, 266-270.
33 E. W. J. Van Niel, K. J. Braber, L. A. Robertson and J. G. Kuenen, *Antoni. Leeuw.*, 1992, 62, 231-237.
34 A. Andersson, P. Laurent, A. Kihn, M. Prévost and P. Servais, *Water Res.*, 2001, 35, 2923-2934.
35 P. Ilies and D. S. Mavinic, *Water Res.*, 2001, 35, 2065-2072.
36 A. Ventosa, J. J. Nieto and A. Oren, *Microbiol. Mol. Biol. Rev.*, 1998, 62, 504-544.
37 J. E. Aston, B. M. Peyton, B. D. Lee and W. A. Apel, *Environ. Toxicol. Chem.*, 2010, 29, 2669-2675.
38 J. K. Kim, K. J. Park, K. S. Cho, S. W. Nam, T. J. Park and R. Bajpai, *Bioresour. Technol.*, 2005, 96, 1897-1906.
39 X. P. Yang, S. M. Wang, D. W. Zhang and L. X. Zhou, *Bioresour. Technol.*, 2011, 102, 854-862.
40 Y. C. Yao, Q. L. Zhang, Y. Liu and Z. P. Liu, *Bioresour. Technol.*, 2013, 143, 83-87.
41 J. M. Duan, H. D. Fang, B. Su, J. F. Chen and J. M. Lin, *Bioresour. Technol.*, 2015, 179, 421-428.
42 M. Ramirez, J. Obrzydowski, M. Ayers, S. Virparia, M. Wang, K. Stefan, R. Linchangco and D. Castignetti, *Sci. World J.*, 2014, 2014, Article ID 901702, 11 pages.

Table 1
List of nitrogen and carbon amounts added to BM (per liter).

Component	Nitrogen and carbon amount					
	BM-1	BM-2	BM-3	BM-4	BM-5	BM-6
Sodium citrate (g)	17.81	2.11	0.22	0.22.	17.81	17.81
NH_4^+–N (mg)	400	-	-	-	400	400
NO_2^-–N solution[a] (mL)	-	-	10	-	10	-
NO_3^-–N solution[b] (mL)	-	-	-	10	-	10
NH_2OH–N solution[c] (mL)	-	10	-	-	-	-

[a] 0.5 g/L NO_2^-–N; [b] 0.5 g/L NO_3^-–N; [c] 0.1 g/L NH_2OH–N. All media were autoclaved at 121°C for 20 min, and filter-sterilized solutions were added separately into the sterile BM medium after cooling.

Table 2
Experimental set.

Section	Factors	Carbon[a]	NH_4^+–N[b]	Variable values
3.4.1	Temperature	2.25	50	T = 10, 15, 20, 25, 30, 35, 40°C
3.4.2	Salinity	4.45	100	NaCl = 0, 10, 20, 30, 40, 50, 60, 70 g/L
3.4.3	Heavy metal	4.45	100	Zn^{2+} = 0, 0.5, 1, 2, 3, 4, 5 mmol/L
				Cu^{2+} = 0, 0.5, 1, 2, 3, 4, 5 mmol/L
				Zn^{2+}–Cu^{2+}[c] = 0, 0.5, 1, 2, 3, 4, 5 mmol/L
3.4.4	Initial ammonium[d]			Initial ammonium concentration = 50, 100, 200, 400, 600, 800, 1000, 1500, 2000, 2500 mg/L
	pH	17.81	400	pH = 4, 5, 6, 7, 8, 9, 10, 11
	DO[e]	17.81	400	Shaking speed = 0, 60, 80, 100, 120, 140, 160, 180 rpm

[a] the amount of sodium citrate added to BM (g/L);

[b] the amount of NH_4^+-N added to BM (mg/L);

[c] n mmol/L Zn^{2+}–Cu^{2+} equals to n mmol/L Zn^{2+} and n mmol/L Cu^{2+};

[d] fixed C/N ratio = 12 in the initial ammonium concentration experiments;

[e] $DO_0 \approx 1.98$ mg/L, $DO_{60\ rpm} \approx 2.36$ mg/L, $DO_{80\ rpm} \approx 3.01$ mg/L, $DO_{100\ rpm} \approx 3.85$ mg/L, $DO_{120\ rpm} \approx 4.67$ mg/L, $DO_{140\ rpm} \approx 5.56$ mg/L, $DO_{160\ rpm} \approx 6.12$ mg/L, $DO_{180\ rpm} \approx 6.48$ mg/L.

Table 3

List of ammonium removal conditions of WY-01 and some relevant strains.

Bacteria	NH_4^+–N (mg/L)	T (°C)	pH	Salinity (g/L)	Cu^{2+} (mmol/L)	Zn^{2+} (mmol/L)
Bacillus methylotrophicus strain L7[13]	1121.24	20 – 37	6 – 9	0 – 40	-	-
Acinetobacter junii YB[11]	1000	20 – 37	5 – 10	-	-	-
Bacillus subtilis A1[39]	500	28	8.0 – 8.2	-	-	-
Vibrio diabolicus SF16[41]	119.77	30	7.5 – 9.5	10 – 70	-	-
Aeromonas sp. HN-02[22]	200	5 – 30	4 – 10	0 – 20	0.0078	0.1231
Bacillus sp. PK15[38]	100	30	7	-	0.5	0.5
Cupriavidus pauculus strain UM1[42]	-	28 – 30	-	-	1	-
WY-01	2500	10 – 40	5 – 10	0 – 60	8	0.5

Figure Caption:

Fig. 1 Phylogenetic tree based on the 16S rRNA gene sequence of *Alcaligenes faecalis* strain WY-01 and other reference sequences. Names of different groups along with the accession numbers are shown in the parentheses.

Fig. 2 The growth and ammonium removal characteristic of strain WY-01 under aerobic culture conditions. Values are means ± SD (Error bars) for three replicates.

Fig. 3. Utilization of nitrogen compounds by strain WY-01 under aerobic culture conditions. hydroxylamine (A), nitrite (B), nitrate (C), nitrate and ammonium (D). Values are means ± SD (Error bars) for three replicates.

Fig. 4 Effects of temperature on the growth and ammonium removal ability of strain WY-01. 10°C – 20°C (A), 25°C – 40°C (B), average removal rate and growth rate (C). Solid symbols, NH_4^+–N; Open symbols, OD_{600}. Values are means ± SD (Error bars) for three replicates.

Fig. 5 Effects of salinity on the growth and ammonium removal ability of strain WY-01. OD_{600} (A), the exponential relationship between the lag phase and salinity (B), NH_4^+–N (C), ammonium removal efficiency and rate (D). Values are means ± SD (Error bars) for three replicates.

Fig. 6 Effects of factors on ammonium removal ability of strain WY-01. heavy metal (A), initial ammonium concentration (B), pH (C), speed (D). Values are means ± SD (Error bars) for three replicates.

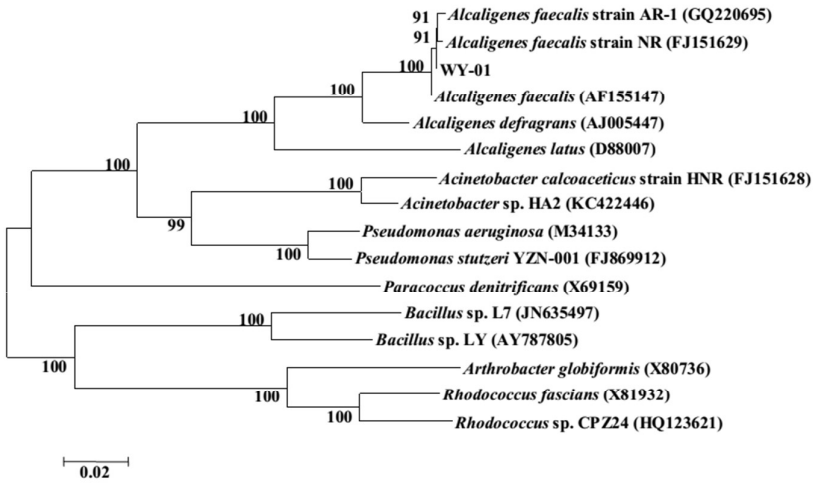

Fig. 1

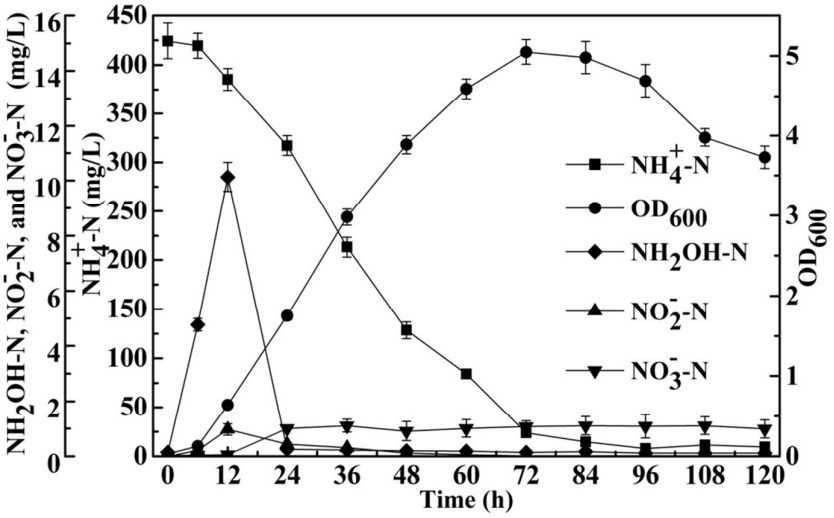

Fig. 2

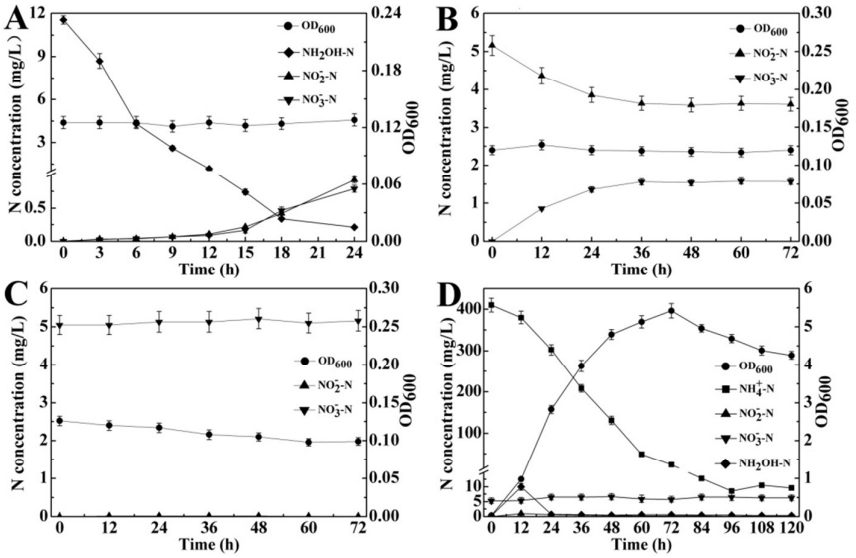

Fig. 3

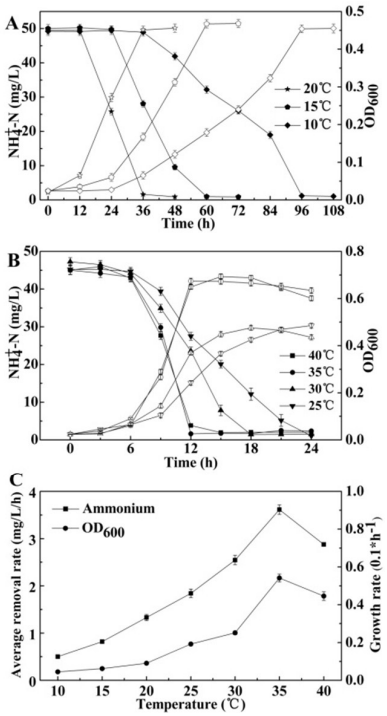

Fig. 4

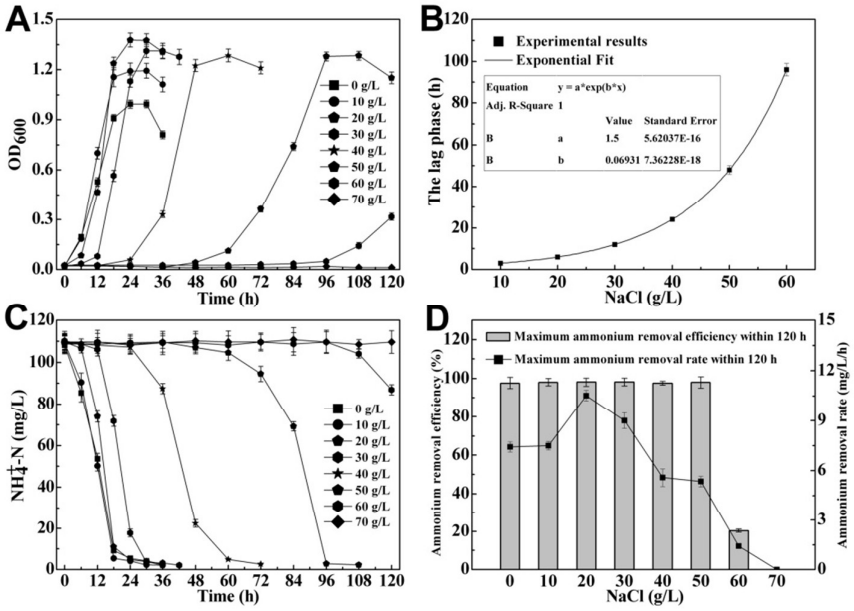

Fig. 5

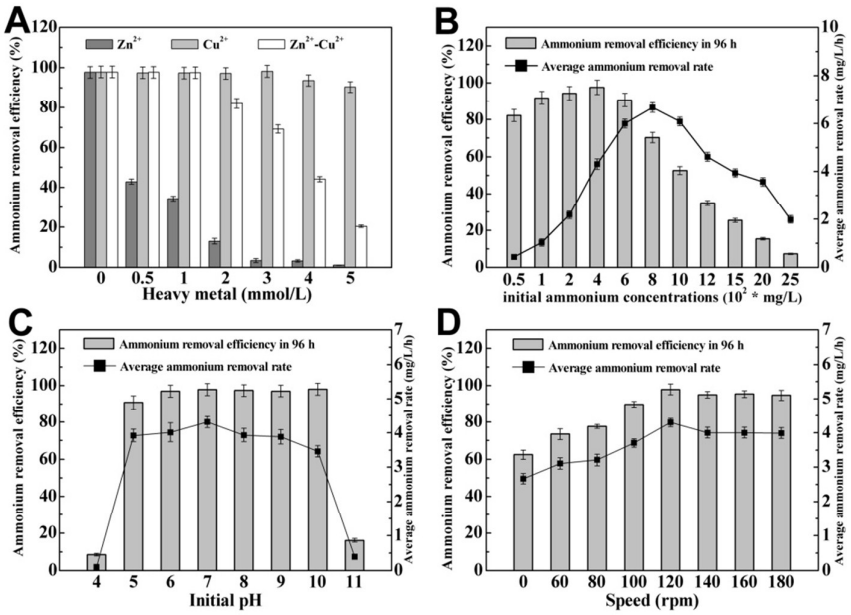

Fig. 6