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Heterotrophic ammonium removal characteristics of an aerobic heterotrophic nitrifying-denitrifying bacterium, *Providencia rettgeri* YL

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Abstract

Bacterium *Providencia rettgeri* YL was found to exhibit an unusual ability to heterotrophically nitrify and aerobically denitrify various concentrations of ammonium (NH_4^+-N). In order to further understand its removal ability, several experiments were conducted to identify the growth and ammonium removal response at different carbon to nitrogen (C/N) mass ratios, shaking speeds, temperatures, ammonium concentrations and to qualitatively verify the production of nitrogen gas using gas chromatography techniques. Results showed that under optimum conditions (C/N 10, 30°C, 120 r/min), YL can significantly remove low and high concentrations of ammonium within 12 to 48 h of growth, respectively. The nitrification products hydroxylamine (NH_2OH), nitrite (NO_2^-) and nitrate (NO_3^-) as well as the denitrification product, nitrogen gas (N_2), were detected under completely aerobic conditions.

Key words: *Providencia rettgeri* YL; heterotrophic ammonium removal; aerobic denitrification

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Introduction

Increases in ammonium-nitrogen levels in water from key sources such as industrial, agricultural, urban and domestic wastewater can cause numerous environmental and health problems if not treated adequately. In municipal treatment systems, the most widely used and cost effective treatment method involves the use of autotrophic nitrifiers and heterotrophic denitrifiers to ultimately convert nitrogenous compounds to nitrogen gas (Sinha and Annachhatre, 2006; Andrade *et al*., 2007; Khardenavis *et al*., 2007). Disadvantages to such systems are that the nitrification process tends to be time consuming and demands large expanses of space to house separate aerobic and anaerobic tanks (Khin and Annachhatre, 2004). In addition, autotrophs are sensitive to high loads of ammonium and carbon concentrations (Joo *et al*., 2005) which restrict their application to systems containing high ammonium such as agricultural and industrial wastewaters.

More recent studies have highlighted the existence of some bacteria such as *Paracoccus denitrificans* (formerly known as *Thiosphaera pantotropha*), *Pseudomonas stutzeri* and *Alcaligenes faecalis* that are not only capable of performing heterotrophic nitrification but also have the phenomenal ability to denitrify their nitrification products under aerobic conditions (Ludwig *et al*., 1993; Moir *et al*., 1995; Zhao *et al*., 1998; Takayuki *et al*., 1998; Su *et al*., 2006). These reports are common on simultaneous nitrification and denitrification (SND) whereby some bacteria have been found to perform nitrification and denitrification concurrently in a single reactor in which aerobic conditions tend to vary based on carefully controlled oxygen gradients (Münch *et al*., 1996; Zeng *et al*., 2005; Khardenavis *et al*., 2007).

In this study, an unusual occurrence of ammonium and nitrogen removal was detected in the bacterium *Providencia rettgeri* YL which was newly isolated in a membrane bioreactor (MBR) in our laboratory (Lin *et al*., 2005). YL shows the ability to heterotrophically nitrify various concentrations of ammonium in aerobic conditions. Although nitrite and nitrate were only slightly detected, hydroxylamine and nitrogen gas were clearly identified during a single process of aerobic ammonium removal. YL is therefore considered to bear ammonium removal characteristics that are substantially different from conventional nitrification and denitrification concepts. Although research on species of *Providencia rettgeri* in application to ammonium removal in wastewater treatment is currently rare, more recent information about the ability of YL to heterotrophically remove ammonium has been documented in the research of Huang *et al*. (2008) and Lin *et al*. (2009).

The aim of this study was to further investigate the heterotrophic ammonium removal ability and factors affecting the optimum performance of *Providencia rettgeri* YL, and to determine its potential application in biological wastewater treatment systems.

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1 Materials and method

1.1 Microorganism

Providencia rettgeri YL was isolated from an MBR conducted in our laboratory, it showed the ability of simultaneous nitrification and denitrification (SND). Pure isolates were obtained from the system by plating on peptone-meat extract (PM) agar. The PM liquid medium consisted of 10 g/L peptone, 10 g/L beef extract and 5 g/L sodium chloride, which was distributed evenly into 250 mL shaking flasks. Agar powder (2%) was added to each PM sample and sterilized at 120 Pa for 30 min. Pure isolates were later grown in basic medium and stocked in 25% glycerol at –25°C for long-term storage, and sealed in sterilized 1.5 mL centrifuge tubes at 4°C for routine experiments.

1.2 Growth and storage medium

Synthetic wastewater was used as the basic medium for storage, cultivation and subsequently for studies on heterotrophic ammonium removal (at C/N mass ratio 10). The mixture was prepared using (g/L): $Na_2HPO_4{\cdot}12H_2O$ 21.5, KH_2PO_4 0.91, NaCl 4, glucose (anhydrous) 2, NH_4Cl 0.08, and 30 mL of each trace solution (pH maintained between 7–7.5). The trace solution consisted of the following components (g/L): $MgSO_4$ 6.42, $MnSO_4$ 0.63, and H_3BO_3 3.36. Each solution was distributed evenly using 250 mL shaking flasks and inoculated for experimental analysis.

1.3 Shaking culture experiment

Cultures, incubated in basic medium at 30°C and 120 r/min for 48 h using an orbital shaking incubator (HZQ-F160, Harbin Donglian Electronic & Technical Development Ltd., China), were centrifuged at 9000 r/min for 8 min using a high-speed centrifuge (FULGOR GL-20B, Shanghai FULGOR Analytical Apparatus Ltd., Co., China), and then the supernatant was discarded. The remaining bacteria were washed with purified water (sterilized at 120 Pa, 30 min) for 2 times. Final bacterial samples (diluted to 5–10 mL) were evenly distributed (1000–2000 μL) into purified experimental medium. The effect of C/N on ammonium removal was examined by adjusting the ratio to 5, 10, 20 and 30 and fixing the amount of ammonium chloride as the nitrogen source at 120 mg/L. To observe the effects of dissolved oxygen (DO) concentration on ammonium removal, adjustments were made to shaking speed levels which were changed to 60, 80 and 120 r/min with low to high shaking speeds represented conditions of low to high DO concentrations. The effect of temperature was determined by adjusting temperatures to 10, 20, 25, 30, 35, and 40°C. The effect of different ammonium concentrations on the ammonium removal efficiency was conducted by adjusting initial ammonium concentration to 40, 180 and 300 mg/L to represent the conditions of low, medium and high concentrations respectively. Samples were taken periodically to examine changes in growth, ammonium concentration and detection of intermediates.

1.4 Gas detection experiment

Basic cultures of high concentration ammonium (NH_4^+-N) at C/N ratio 10 (50 mL each) were inoculated with purified samples of YL (50 mL) and sealed in air-tight 100 mL serum bottles. The medium and headspace were subsequently evacuated and aerated with pure oxygen at constant pressure (0.3 MPa) for 8–10 min and injected with the volume equivalent of 2000 μL of helium. Tests to detect the changes in N_2 and O_2 respectively were conducted using gas chromatography (GC-14B, Shimadzu, Japan) at 12 h intervals for a total of 48 h. Gas samples were extracted using a 100 μL air-tight glass syringe. The carrier gas Ar had a flow rate of 20 mL/min. Column, injector and detector temperatures were 80, 100 and 110°C, respectively.

1.5 Analytical methods

Growth of the bacteria was monitored by measuring the optical density ($OD_{600\,nm}$) using a spectrophotometer (UV-1200 Unico, Shanghai Instruments Co., Ltd., China). The concentrations of ammonium (NH_4^+-N), nitrification products hydroxylamine (NH_2OH), nitrite (NO_2^-), nitrate (NO_3^-), denitrification product (N_2) and glucose were determined as follows. Ammonium was analyzed by the water quality-determination of ammonium-spectrophotometric method with salicylic acid (GB7481-87). NH_2OH was analyzed indirectly by the spectrophotometric determination of Fe^{3+}. NO_2^- and NO_3^- were analyzed using N-(1-naphthalene)-diaminoethane photometry and phenol disulphonic acid methods, respectively. Total organic carbon (TOC) and total nitrogen (TN) were analyzed using a TOC/TN analyzer (N/C3000ChD, Jena, Germany).

2 Results and discussion

2.1 Morphological and physiological characteristics

Providencia rettgeri YL was found to be Gram-negative and strictly aerobic. The cells of YL were observed by scanning electron microscopy and were identified as short non-motile and rod-shaped (1.0 × 0.5 μm) (Fig. 1). Based on the morphological, biochemical and 16S rRNA gene sequence analysis, strain YL was recognized as a strain of *Providencia rettgeri* and was thereafter named *Providencia rettgeri* YL.

2.2 Heterotrophic ammonium removal

2.2.1 Effect of ammonium concentration

Heterotrophic ammonium removal ability by YL was observed when ammonium chloride and glucose were used as nitrogen and organic carbon sources respectively. In the basic, medium growth was observed and an initial concentration of 84.63 mg/L NH_4^+-N was reduced by 99.9% to 0.05 mg/L within just 12 h of growth. Nitrification products NH_2OH-N, NO_2^--N and NO_3^--N were detected during the removal process within 24 h of incubation. TOC and TN decreased by 73% and 79%, respectively, with final TN being 18.8 mg/L in 48 h. In aerated shaking cultures

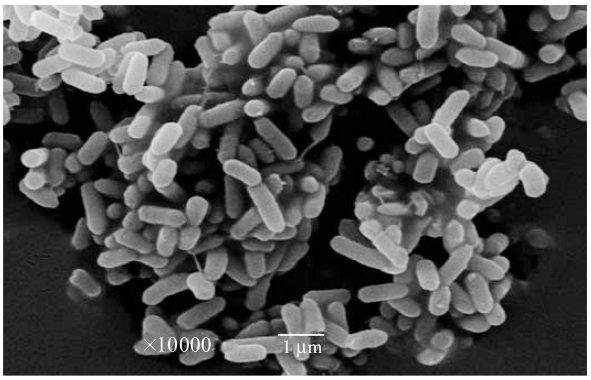

Fig. 1 Scanning electron microscopy picture of *P. rettgeri* strain YL (× 10000).

maintained at 30°C and 120 r/min, concentrations of 40, 180 and 300 mg/L NH_4^+-N were observed for ammonium removal. Figure 1 shows the changes in NH_4^+-N concentration during a 48-h growth period. NH_4^+-N was removed 98% and 100% within 12 and 24 h at 40 and 180 mg/L NH_4^+-N, respectively, while at 300 mg/L over 48 h was needed for almost complete removal.

The results showed that although the removal process was slower in higher concentrations, it was clear that YL was not inhibited by high concentrations of ammonium. These findings proved that YL not only possessed a ability to remove ammonium and reduce TN but also has a capacity to effectively reduce ammonium at higher concentrations than autotrophic nitrifiers, and it could be a beneficial feature for applications in industrial or agricultural wastewater treatment systems where ammonium concentrations tend to be high.

2.2.2 Heterotrophic growth

Changes in growth and TOC for different concentrations of ammonium are shown in Fig. 3. At a fixed ratio of C/N 10 there was a gradual decline in TOC concentration corresponding to the increase in growth for each concentration of NH_4^+-N. Low NH_4^+-N concentrations resulted in lower optical densities as opposed to increased concentrations where high optical densities were recorded, indicating higher levels of growth. Growth was therefore considered to be directly affected by the availability of organic carbon and nitrogen sources. Similar growth patterns were observed at concentrations of 180 and 300 mg/L and cell proliferation occurred after 8 h of incubation. However, at 180 mg/L changes in growth were minimal after 24 h while at 300 mg/L optical density readings continued to increase gradually. This indicated that a higher concentration require a longer incubation period to allow for full consumption of higher NH_4^+-N concentration.

2.2.3 Detection of nitrogen gas

The gradual reduction in TN concentration during the NH_4^+-N removal process (Fig. 2a) suggested the possible occurrence of NH_4^+-N conversion to intracellular nitrogen and N_2 by the process of aerobic denitrification which has been known to occur with some species of bacteria such as *Alcaligenes faecalis* (Joo *et al*., 2005). To

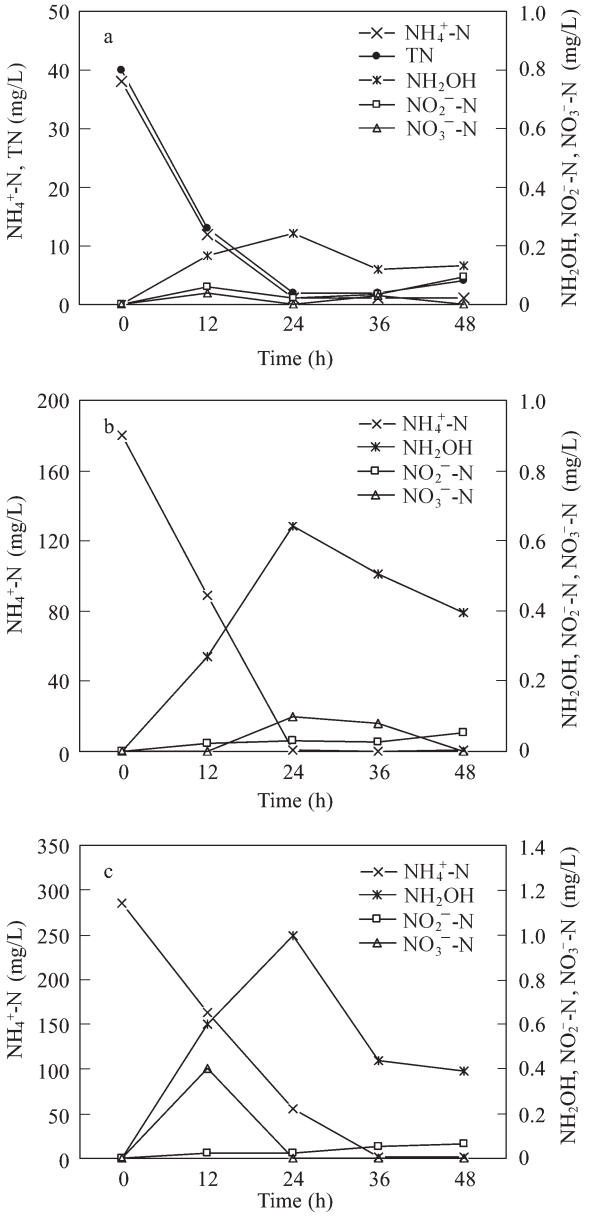

Fig. 2 Heterotrophic ammonium removal by *P. rettgeri* YL. Initial NH_4^+-N concentration: (a) 40 mg/L; (b) 180 mg/L; (c) 300 mg/L. Production of the intermediates NH_2OH, NO_2^- and NO_3^- are shown for each concentration (a–c).

verify the possible occurrence of aerobic denitrification, gas chromatographic tests were conducted to identify the production of N_2 by YL. Figure 4 shows that from an original concentration of 180 mg/L of ammonium, N_2 levels increased by an average of 51.1% within a 48 h period and O_2 levels fell from 100% to 44% of the original volume. These results (combined with those in Figs. 2 and 3) not only indicate the heterotrophic nature of YL and its ability to effectively remove NH_4^+-N, but aerobic denitrification also.

2.3 Production of intermediates

Figure 2 demonstrates the occurrence of intermediates during the ammonium removal process in different concentrations of ammonium. The production of NH_2OH-N, NO_2^--N and NO_3^--N was consistent with that of known

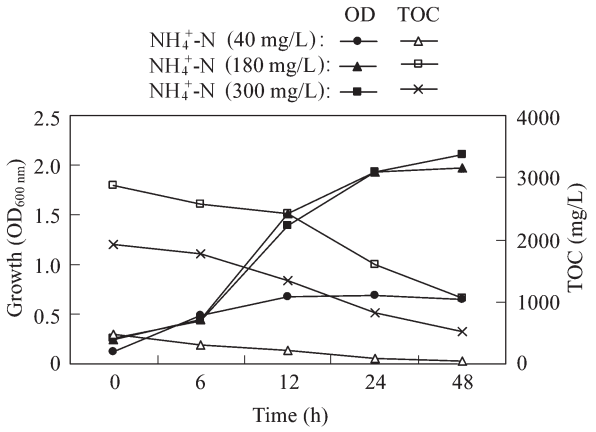

Fig. 3 Changes in growth and total organic carbon (TOC) at 40, 180 and 300 mg/L NH_4^+-N at C/N 10, 30°C and shaking speed of 120 r/min.

heterotrophic nitrification processes where: ammonium → hydroxylamine → nitrite → nitrate (Khin and Annachhatre, 2004). Hydroxylamine was produced at consistently higher concentrations than nitrite and nitrate within 0–6 h of NH_4^+-N removal with peak NH_2OH-N occurring in 12 h to 24 h which then gradually decreased. Increase in NH_2OH-N corresponded well with increase NH_4^+-N concentrations with peak levels being 0.24, 0.64 and 1 mg/L NH_2OH-N at 40, 180 and 300 mg/L, respectively. This showed that NH_2OH production was directly related to changes in NH_4^+-N concentrations and is therefore a prominent feature in the ammonium removal process by YL. This differed from productions of NO_2^--N and NO_3^--N which were consistently low regardless of the changes in initial NH_4^+-N concentrations. This low detection of nitrite and nitrate is however not unusual as there have been reports that some heterotrophic nitrifiers do not accumulate very high levels of nitrite or nitrate (Robertson *et al*., 1989, van Niel *et al*., 1992). It is also possible that the transfer to nitrite and nitrate may have been too rapid to allow for adequate detection, which is common in some cases of ammonium removal (Wang *et al*., 2007) and therefore cannot be ignored as a significant feature of the ammonium conversion sequence of YL.

In light of the results and with the known detection of N_2 a possible pathway for ammonium removal by YL was

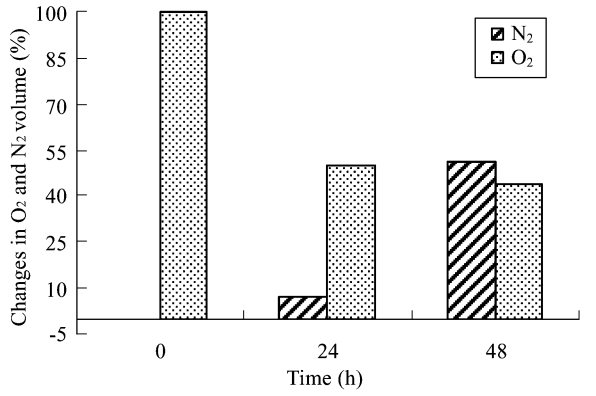

Fig. 4 Percentage changes in O_2 and N_2 levels from an original loading of 180 mg/L NH_4^+-N at C/N 10, 30°C, and 120 r/min.

suggested. It is proposed that ammonium is initially oxidized to hydroxylamine then to nitrite followed by nitrate which is then reduced to nitrite and ultimately nitrogen gas is produced. The suggested sequence of conversion is diagrammatically illustrated in Fig. 5 and closely follows the research of Richardson *et al*. (1998). However, further research is still needed to confirm the precise heterotrophic nitrogen removal pathway by YL.

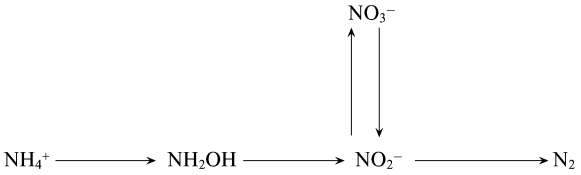

Fig. 5 Proposed pathway for nitrogen removal by *P. rettgeri* YL.

2.4 Factors affecting ammonium removal

2.4.1 C/N ratios

The effect of C/N ratio on total ammonium removal using glucose-C medium containing an initial concentration of NH_4^+-N 120 mg/L was examined in shaking culture (Fig. 6a). At C/N ratios of 10, 20 and 30, NH_4^+-N was completely consumed within 12 h of growth, while at C/N ratio of 5 consumption of NH_4^+-N ended between 10–15 h at a concentration of 30.5 mg/L. This was mainly due to the rapid utilization of the carbon source which was consumed by 80% within 12 h and ultimately affected ammonium removal efficiency (Figs. 6a and 6b). At C/N 10 the rate of NH_4^+-N removal was relatively balanced with glucose reduction, being a total of 77% while at C/N ratios 20 and 30 excess amounts of carbon (54% and 64%, respectively) remained in solution after NH_4^+-N had been completely depleted.

The results not only show the ability of YL to remove NH_4^+-N under various C/N ratios, but also a low supply of carbon results in the reduce NH_4^+-N removal ability while higher carbon ratios may cause a high residual carbon level in the medium. The most balanced ratio was C/N 10.

2.4.2 Shaking speeds and temperatures

The effect of shaking speed and temperature on ammonium removal from 80 mg/L NH_4^+-N during a 48-h growth period at 30°C was investigated (Table 1). Ammonium removal was effectively achieved at a shaking speed of 120 r/min with an average removal percentage up to 99%. At speeds of 60 and 80 r/min ammonium removal was significantly low and allowed the average NH_4^+-N removal percentage only 33% and 51%, respectively.

Studies have shown that the level in DO concentration in any solution is strongly affected by variations in shaking speed (McDaniel *et al*., 1968; Ho and Chou, 2000; Joo *et al*., 2005; Qi *et al*., 2006). In a control experiment conducted in our laboratory the relationship between mixing speed and DO was evident (Fig. 7). With increased shaking speeds there was a corresponding increase in DO concentration.

In relation to results in Table 1, it could be deduced

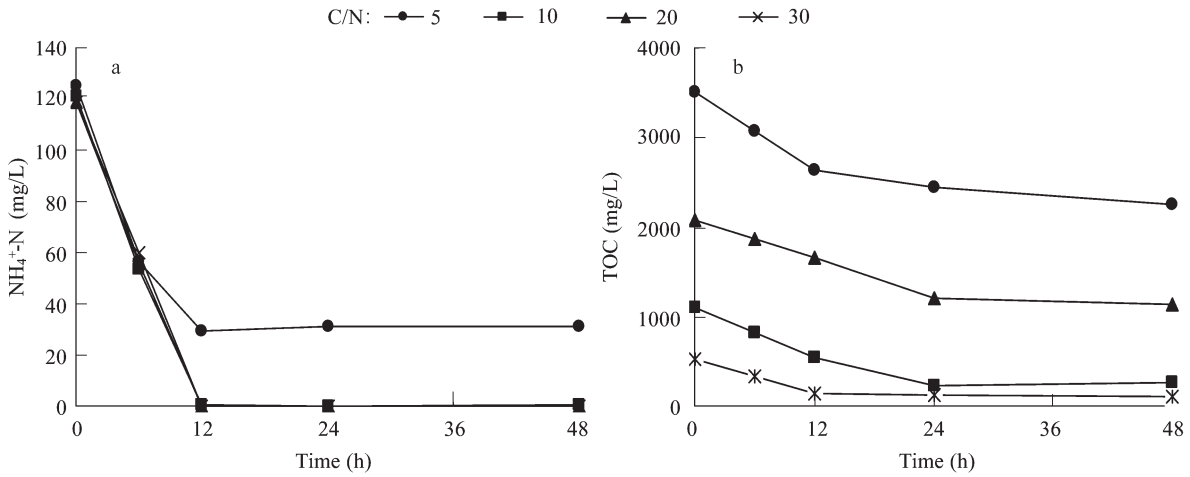

Fig. 6 NH_4^+-N removal by *P. rettgeri* YL (a) and corresponding changes in TOC (b) at different C/N ratios.

Table 1 Ammonium removal by *P. rettgeri* YL at different speeds and temperatures in a shaking culture experiment

Shaking speed (r/min)	Temp. (°C)	Initial NH_4^+-N (mg/L)	Final NH_4^+-N (mg/L)	NH_4^+-N removal (%)
60	30	77.56	52.02	33
80	30	77.56	37.98	51
120	30	84.68	0.98	99
120	10	79.45	71.78	9.65
120	20	80.29	0	100
120	25	82.93	0	100
120	30	80.29	0	100
120	35	80.97	1.79	97.8
120	40	80.35	80.13	0.27

Glucose was used as carbon source; C/N = 10.

that there exists a strong relationship between DO and ammonium removal efficiency by YL. The ammonium removal by YL was least efficient at a shaking speed of 60 r/min, resulting in only a 60% removal. However, with each increase in shaking speed there was a marked improvement in the ammonium removal efficiency. Ammonium removal by YL was therefore strongly affected by oxygen availability and therefore dependent on aerobic conditions.

Ammonium removal efficiency is affected by significant changes in temperature (Table 1). At 30°C, ammonium removal occurred rapidly with 100% NH_4^+-N being removed within 12 h of incubation. Removal patterns were similar at 20 and 25°C. However, the growth and removal were slow during the first 12 h after a rapid increase. At temperatures of 10 and 40°C, there was no significant change in ammonium concentration. Highest optical densities for growth occurred at 25 and 30°C with only slightly lower level at 20°C, but there was almost no change in growth at 10 and 40°C.

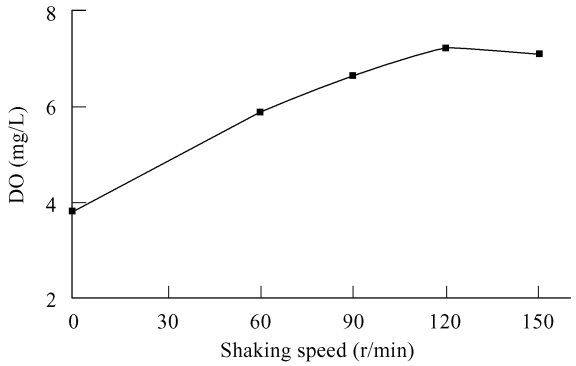

Fig. 7 Relationship between dissolved oxygen and shaking speed.

3 Conclusions

In summary, the bacterium *P. rettgeri* YL has the ability to remove ammonium by means of a single process of heterotrophic nitrification. The removal ability is not inhibited by higher concentrations and occurs best under conditions of C/N 10, 30°C and 120 r/min. During removal, the intermediate products hydroxylamine, nitrite and nitrate are detected but nitrite and nitrate are produced in consistently lower concentrations. Based on the production of intermediates and detection of nitrogen gas, *P. rettgeri* YL was considered to bear characteristics similar to known heterotrophic nitrifying-aerobic denitrifying bacteria. In light of results, a possible pathway for ammonium removal was suggested as being ammonium to hydroxylamine to nitrite, and then nitrate is reduced to nitrite and eventually to nitrogen gas. However, to confirm these findings the further research is necessary to determine the precise ammonium removal pathway of YL.

Acknowledgments

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