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Original Research

Identification and Nitrogen Removal Characteristics of a Denitrifying Strain

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Abstract

The nitrogen (N) removal characteristics of denitrifying bacterium LZ-14 were investigated in this study. Extensive carbon sources can be used for LZ-14, with acetate as the most effective one. The strain LZ-14 grew fast at a range of 20ºC to 35ºC, and exhibited high N-removal efficiency. Nitrogen removal mainly was performed in the growth phase of 12-36 hr with an amount of nitrite accumulation that was reduced completely in the following 24 hr. The strain LZ-14 was identified as *Pseudomonas* by PCR amplification and homology analysis of 16S rDNA sequence. Strain LZ-14 was inoculated in simulative constructed wetlands (SCWs) to enhance pollutant removal in pilot-scale, which showed a significant effect on COD_{Cr} and total nitrogen (TN) removal.

Keywords: denitrifying bacteria, nitrogen removal, 16S rDNA, bio-enhancement, *Pseudomonas* sp.

Introduction

The emission of nitrogen pollutants is increasing rapidly with the development of industry and agriculture in China. Sewage discharged randomly has caused serious contamination in both groundwater and surface water. Currently, although the worsening trend of water pollution has been curbed effectively by a large number of wastewater treatment works, nitrogen concentration in the effluent is still high after secondary treatment.

The nitrogen-containing contaminants discharged into waters will not only impair water quality and damage fisheries development, but also harm human health. The lethal dose of ammonia dissolved in water for most fish is 1 mg/L [1], and the nitrite in stomach leads to a carcinogenic effect as it could combine with amine or amide to form nitrosamine compounds [2]. So it is necessary to take efficient methods to eliminate N-pollutants from water completely. Biological treatment is considered to be the most economical and effective mechanism for wastewater treatment [3]. It usually involves nitrification and denitrification, and the denitrification could reduce nitrate to nitrogen gas.

The constructed wetlands technology, an energy-saving and sustainable method for wastewater treatment [4], could be used to treat various types of wastewaters such as municipal and industrial wastewaters [5], urban and agricultural runoff [6-8], and acid mine drainage [9, 10]. Some research has shown that the removal rates of constructed wetlands on organic matter, suspended solids, and bacterial contamination could reach 90%; the ammonia-N removal rate also is quite high, but total nitrogen removal is not satisfactory, which is generally only 40%-60% [11-13]. With pre-treatment facilities and a large-enough treatment area, 90% of nitrogen could be removed [14, 15], but that also would entail high cost and extended time. Besides, with the load increasing, the treatment efficiency of constructed wetlands decreases [16]. In a constructed wetlands ecosystem, the removal of total nitrogen mostly depends on ammonification, nitrification, and denitrifica-

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tion, of which biodenitrification contributes 40.9% of total nitrogen removal [17]. In the biodenitrification process, the nitrate instead of oxygen acts as the electron acceptor for respiratory electron transport. Via intermediate nitrite, nitric oxide and nitrous oxide, finally nitrate is reduced to nitrogen gas [18]. Because of the excellent nitrogen removal ability of biodenitrification, improving the role of denitrifying bacteria to increase the nitrogen treatment efficiency in constructed wetlands has become a significant issue today.

The strain LZ-14 was isolated from the top soil sampled from the rhizosphere of *Arundo donax* L. in a Xinxue River wetland constructed by our lab. This strain has been screened as a micro-aerobic and facultative heterotrophic denitrifying bacterium [19]. The effects of pH and C/N rate on the denitrification activity and growth of LZ-14 have been investigated by Chen [19]. The aim of this study was to identify this strain and investigate its other denitrifying characteristics, including the effect of different carbon sources, temperatures, and dissolved oxygen (DO) concentrations on denitrification activity. At last, simulative constructed wetlands (SCWs) were constructed in pilot-scale to investigate the influence of bio-enhancement with strain LZ-14 on treating Xinxue River water.

Materials and Methods

Materials

Strain and Culture Media

The strain LZ-14 (Genbank access number: FJ588910) used in this study was isolated by our lab. The denitrifying medium (DM) contained: 2 g CH_3COONa, 0.4 g KH_2PO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, and 1 g KNO_3 per liter (pH 7.0-7.2). The composition of isolation medium (IM) was 2 g CH_3COONa, 15 g peptone, 3 g yeast extract, 1 g glucose, 6 g NaCl, 12 g agar, and 1.5 g KNO_3 per liter (pH 7.0-7.2).

Soil, Wastewater, and Plant Samples

Soil samples and *Phragmites* used during this experiment were taken from the Xinxue River-constructed wetland located in Jining city Shandong Province China (E: 116º, N: 35º). The artificial wastewater was composed of CH_3COONa, KH_2PO_4, KNO_3 and tap-water. The natural wastewater was sampled from the polluted Xinxue River in spring. The wastewater qualities are shown in Table 1.

Microstructure

The strain was characterized by Gram staining. Cell and colony morphology of the strain were detected using a microscope (CX31, OLYMPUS, Japan).The microscopic characteristics of LZ-14 also were studied using transmission electron microscopy (TEM: JEOL 1230, Japan).

Table 1. Chief pollutants of the wastewater samples.

	COD (mg/L)	TN (mg/L)	NO_3^--N (mg/L)
Xinxue River water	33.0	4.67	3.9
Artificial wastewater	142.98	17.2	15.3

Denitrification Capacity and Growth Curve

Sterile DM (100 mL) in 250 mL flask was inoculated with a clone of strain LZ-14 and incubated stably at 30ºC for 12 h. The activated bacteria suspension (ABS) was obtained with the density of 3.6×10^{10} cell/mL. ABS (10 mL) was fed into 200 mL of sterile DM and then incubated anaerobically at 30ºC. During incubation, the growth of cells was tested periodically by measuring the optical density of the culture broth at 600 nm (OD_{600}) using a spectrophotometer (UV-2450, Shimadzu, Japan). Meanwhile, the concentration of TN also was measured according to the standard method [20].

Test of Denitrification Characteristics

Carbon Source and Nitrogen Source

To investigate the effects of different carbon sources on the denitrification activity of LZ-14, different C-compounds were separately used as the carbon source of the sterile C-free DM (including sodium acetate, sodium citrate, potassium sodium tartrate, glucose, sucrose, peptone, starch, ethanol, cellulose, and acetamide). The concentration of the carbon source in DM was 5 g/L. This study was operated in 250 mL flasks, each with 100 mL DM. The bacterium cells from 10 mL of ABS were centrifuged (10,000 rpm, 10 min), washed, and inoculated into the 100 mL of DM. Then the bacterium was anaerobically cultured at 30ºC for 3 days. The NO_3^--N removal efficiencies were calculated at last.

The adaptability of LZ-14 to different nitrogen was investigated in 250 mL flasks with the sterile N-free DM, but with different nitrogen sources: potassium nitrate, sodium nitrite, acetamide, ammonium chloride, and urea. The concentration of the nitrogen source in DM was 1 g/L. The strain cells from 10 mL of ABS were added into 100 mL of DM after being centrifuged (10,000 rpm, 10 min) and washed. Then the strain was cultured anaerobically at 30ºC for 3 days. During incubation, strain growth was detected by measuring the OD_{600} of the culture solution.

Temperature and Dissolved Oxygen (DO)

Six culture temperatures (10, 20, 25, 30, 35, 40ºC) were set to investigate the effect of different temperatures on the denitrification activity and growth of strain LZ-14. Then the OD_{600} and NO_x^--N (NO_3^--N+NO_2^--N) concentration of the DM were detected.

Four culture conditions with different dissolved oxygen levels: static and sealed (sealed by rubber), static, 100 r/min, and 160 r/min shaking (sealed by eight-sterile gauze) [21] were set up to investigate the effect of different DO concentrations on the denitrification activity of LZ-14. The cultures were kept at 30ºC for 2 days, and nitrogen removal was detected.

Identification by 16S rDNA Sequence Analysis

LZ-14 cells were added into 100 μL distilled water, after resuspension and incubation at 100ºC for 7 min, the mixed material was centrifuged at 12,000 r/min for 5 min at 4ºC. The supernatant was collected as the DNA template for PCR amplification. Bacterial universal primers used for PCR were: 27 F (5P-AGAGTTTGATCCTGGCTCAG-3P) and 1492 R (5P-GGTTACCTTGTTACGACTT-3P) [22]. PCR reaction mixtures (25 μL) contained 2 μL DNA template, 2 μL dNTPs, 0.2 μL Taq polymerase, 1 μL of each primer, 12.5 μL 2×PCR buffer, and 8.8 μL ddH_2O. Reaction cycle parameters included an initial denaturation step of 5 min at 94ºC, followed by 30 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 55ºC, elongation for 1 min at 72ºC, with a final extension step of 10 min at 72ºC. The size and yield of PCR products were checked by agarose gel electrophoresis. The PCR products were collected from the agarose gel.

Sequence analysis of the PCR products was done by ShengGong Bioengineering Co., Ltd. in Shanghai. The 16S rDNA sequences were compared with all accessible sequences in GenBank databases using the BLAST server. The target sequences and all the related sequences were analyzed using Clustalx1. 83 and Mega 4 servers. A phylogenetic dendrogram was constructed using neighbour-joining program to determine the evolutionary status of LZ-14.

Pilot-Scale Enhancement by Strain LZ-14 on SCW

To investigate the effect of bio-enhancement on wastewater treatment in constructed wetland, several SCWs were constructed with plastic buckets (diameter: 0.5 m, height: 0.8 m) in laboratory. Soil was filled into plastic buckets to a depth of 15 cm. Each bucket was planted with 6-8 *Phragmites* communis. Wastewater was fed into each bucket to the depth of approximately 5-10 cm. The experiment was started when the *Phragmites* communis sprouted new leaves from the roots. The ABS was inoculated into the buckets according to 5% (v/v) of the wastewater. The wastewater contamination removal under static condition was investigated. The bucket without ABS was used as the control experiment. Xinxue River water and artificial wastewater were used as raw samples in this study. The effect of bio-enhancement on treating the two kinds of wastewater was investigated by detecting the concentrations of COD_{Cr}, TN, nitrate and nitrite during operation.

Analytical Methods

The measurements of TN, nitrate, nitrite and COD_{Cr} concentrations were carried out according to the standard methods (EPAC, 2002) [20].

Results and Discussions

Morphological Characteristics

The strain was bacilliform and gram-negative. The colony of LZ-14 was khaki, round, middle convex, smooth edge, translucent, glossy, and dense. There were some dense granular materials in the cells (Fig. 1), which might be related to the denitrification action of the strain as described by Sinninghe Damsé [23].

Denitrification Activity and Growth Curve

The growth pattern of strain was indicated by changes in OD_{600} of the culture broth in Fig. 2. Apparently, an adap-

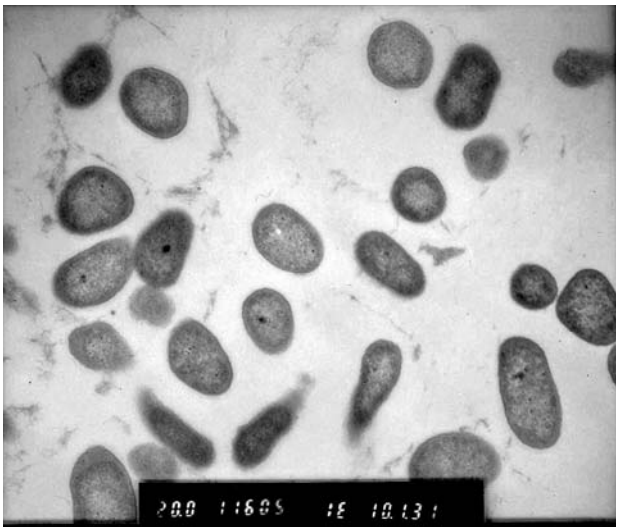

Fig. 1. TEM photo of strain LZ-14 (×20,000).

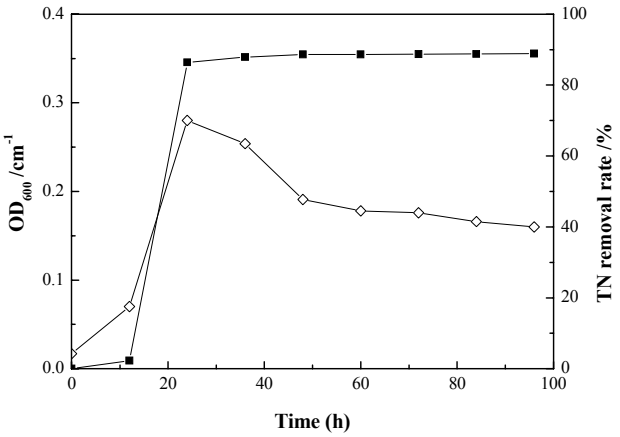

Fig. 2. Growth curve and TN removal of strain LZ-14.
◇ – OD600; ■ – TN removal rate.

Table 2. Gas release and NO_3^--N removal of strain LZ-14 under different carbon sources.

Carbon	Sodium acetate	Sodium citrate	Potassium sodium tartrate	Glucose	Sucrose	Peptone	Starch	Ethanol	Cellulose	Acetamide
Gas	++	++	–	+	–	+++	–	–	–	–
NO_3^--N removal rate (%)	100.0	83.1	96.4	98.7	100.0	100.0	100.0	100.0	61.9	96.5

The number of bubbles: (++++) > (+++) > (++) > (+) > (–) = zero

tive phase was needed for LZ-14, during this phase cells grew slowly and only a small amount of nitrogen was removed. During 12-24 hr, cells grew rapidly and TN was removed noticeably. After 24 hr, because of the lack of carbon, nitrogen, and other nutrients in the medium, the strain entered the decline phase gradually. The TN removal rate finally remained at 95%. The increase of TN removal rate was consistent with the accumulation of strain biomass. Denitrification action of LZ-14 performed mainly during the logarithmic growth phase (12-24hr). TN removal rate increased from 15% to 90%.

Test of Denitrification Characteristics

Test of Carbon Source and Nitrogen Source

As shown in Table 2, strain LZ-14 could use a variety of carbon sources. With each of the tested carbon sources, LZ-14 expressed high nitrate reduction ability. When sodium acetate, sodium citrate, glucose or peptone was used, there was an obvious gas release. It was indicated that nitrate was reduced to a gas compound and eliminated from water. With the other carbon sources, there was no gas produced, whereas the removal rates of nitrate were all favorable. It was indicated that the denitrification process was incomplete with those carbon sources. The nitrate was just reduced to nitrite.

LZ-14 could adapt to the five tested nitrogen sources. When urea was used, the maximum biomass of the strain was achieved (OD_{600}, 0.271). It is worth noting that some strains are unable to use nitrite for their growth because of its toxicity [24]. But the strain LZ-14 achieved similar biomass when either nitrate (OD_{600}, 0. 124) or nitrite (OD_{600}, 0.129) was used. The diversity of nitrogen utilization indicated that the strain was able to adapt various types of wastewater with different nitrogen sources.

Effect of Temperature on Growth and Denitrification

Temperature is an important factor for bacterial growth. The majority of denitrifying bacteria grow well at 20-35ºC. In this study, the temperature of 10ºC was too low for LZ-14 to grow. When the temperature remained above 20ºC, the strain grew steadily, and obtained the maximum biomass at 30ºC. The biomass decreased when temperature was above 30ºC (Fig. 3).

The denitrification efficiency of LZ-14 varied slightly when the temperature changed from 20 to 40ºC. Between 20ºC and 35ºC nitrogen was removed completely. The NO_x^--N removal efficiency reached 92.3% at 40ºC, while nearly no nitrogen removal was discovered below 10ºC. This study showed the consistency between the strain growth and nitrogen removal. With the rise of temperature, cells reproduced rapidly into a large population, and the denitrification process took place just at this growth stage of LZ-14. On the one hand, the assimilation of cells consumed some nitrogen; on the other hand, the nitrate respiration that provided energy for cell reproduction removed most of the nitrogen. In one word, the nitrogen removal by LZ-14 mainly occurred in its growth phase.

Effect of DO on Denitrification

Denitrifying bacteria are known to be micro-aerobic, the strictly anaerobic denitrifying bacterium has not been discovered [13]. In general, denitrifying bacteria would prefer aerobic respiration when the O_2 concentration exceeds a certain level, and they take nitrate respiration when O_2 falls below a certain concentration. Different types of strain have different critical concentrations of O_2. The effect of different DO concentrations on nitrogen removal by LZ-14 was shown in Table 3 (initial NO_3^--N 140.3 mg/L). Under static and anaerobic conditions LZ-14 achieved 100% of nitrogen removal. With the increase of DO concentration, the nitrogen removal efficiency decreased gradually. This result indicated that the higher DO concentration inhibited the denitrification of LZ-14. The aerobic environment would prevent nitrate reduction with the direct expression of nitrite accumulation and the lower nitrogen removal rate. In order to avoid the accumulation of nitrite and achieve ideal nitrogen removal, the anoxic environment was recommended for the application of LZ-14.

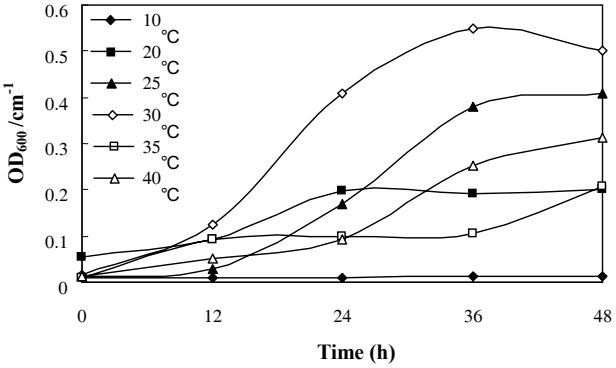

Fig. 3. Growth curve of strain LZ-14 under different temperatures.

Table 3. Influence of DO on nitrogen removal.

Culture conditions	Static and sealed	Static	100 r/min oscillation	160 r/min oscillation
Remaining NO_3^--N (mg·L^{-1})	not detected	17.3	20.8	20.5
Remaining NO_2^--N (mg·L^{-1})	not detected	68.5	80.4	98.5
NO_x^--N removal rate (%)	100	38.8	27.9	22.3

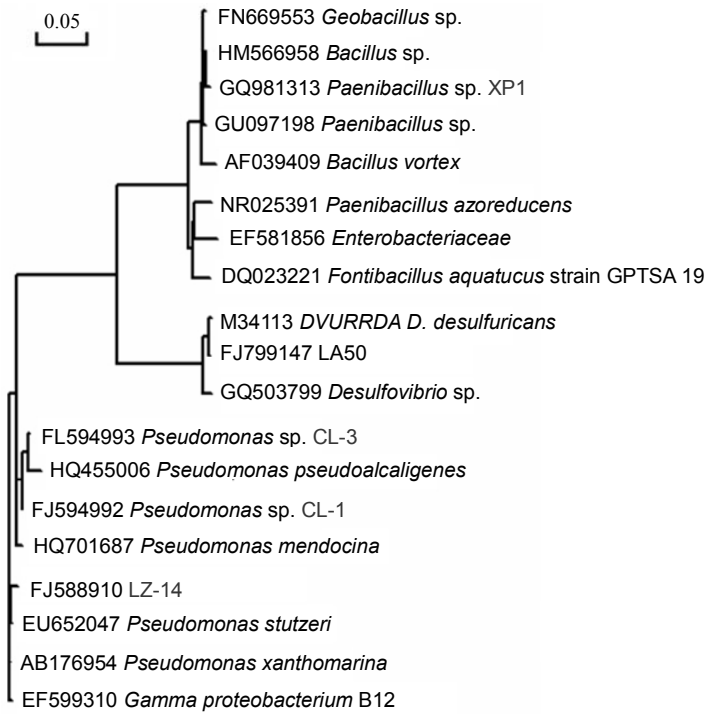

Fig. 4. Phylogenic tree on the 16S rDNA sequence.

Identification of Strain LZ-14

Approximately 1,387 nucleotides were sequenced from 16S rDNA amplification and used to construct a phylogenetic dendrogram with related strains (Fig. 4). The strain LZ-14 was close to *Pseudomonas stutzeri,* according to the phylogenetic analysis. Thus it was determined that LZ-14 is affiliated with the *Pseudomonas* genus.

The denitrifying bacteria are involved in *Pseudomonas*, *Paenibacillus*, *Paracoccus*, *Alcaligenes*, and *Rhodococcus,* etc. [25]. The *Pseudomonas* genus contains different species of denitrifying bacteria. As a model organism, *Pseudomonas stutzeri* has been used to investigate denitrification from 1983 [26]. With suitable medium (about 150 mg N^{15}/L), about 77.9% of nitrate was removed by *P. stutzeri* within 12 hr; with synthetic wastewater (about 15 mg N^{15}/L), only 36.4% nitrate was removed by *P. stutzeri* in 6 days [27]. The denitrifying efficiency of the strain LZ-14 was up to 90% within 24 hr and the N-removal rate remained at 90%-95% (with DM: about 140 mg N/L). It was obvious that the denitrifying activity of strain LZ-14 was higher than some other *P. stutzeri*.

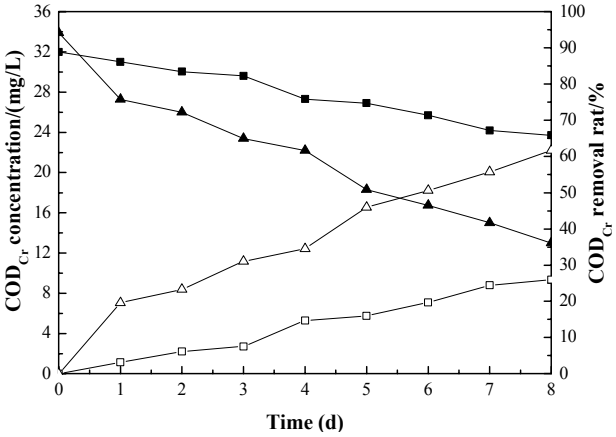

Fig. 5. Variation of COD_{Cr} over time in Xinxue River water inoculated with LZ-14.
■ – COD_{Cr} of control test without inoculation of strain
▲ – COD_{Cr} of experimental group inoculated with strain
□ – COD_{Cr} removal rate of control group
△ – COD_{Cr} removal rate of experimental group

Enhancement Treatment on Xinxue River Water

COD_{Cr} Removal

The COD_{Cr} is mainly removed by micro-organism degradation and soil adsorption in constructed wetland. In this study the initial COD_{Cr} value of the Xinxue River water was about 32.0 mg/L, which was almost the average level during one year. In the control test, the COD_{Cr} decreased slowly from 32 mg/L to 24 mg/L within 8 days, and only 25% of COD_{Cr} was degraded by the indigenous microorganisms and plants (Fig. 5). However, the COD_{Cr} of the inoculated SCWs met the grade III Surface Water Quality Standard of China (below 20 mg/L) within 5 days, and got 61.7% of removal rate within 8 days. By the addition of LZ-14 the COD_{Cr} removal rate increased 35.7%. That revealed the significant effect of bio-enhancement by LZ-14 on COD_{Cr} removal in constructed wetland.

Nitrogen Removal

This test indicated that the nitrogen removal was greatly improved by bio-enhancement (Fig. 6). The TN concentration of the control experiment decreased slightly in the first 2 days. Afterward it declined quickly from 4.3 mg/L to 3.05 mg/L within the next 2 days, and remained at approximatly 3.0 mg/L until the end. With the addition of the strain, the TN concentration consistently declined from 4.7 mg/L to 0.8 mg/L within 8 days, obtaining 83% of removal rate.

Figs. 6 b and c showed that the major nitrogen in Xinxue River water was nitrate (approximately 70%). On the one hand, nitrate is the easy-to-use nitrogen source for microbe and plant growth. It could be partly removed by the assimilation of microbe and plant [28, 29]. On the other hand, nitrate is the electron acceptor of the denitrification process. It could be removed from water by denitrifying bacteria. An obvious improvement in nitrate removal was observed in the inoculated SCWs. Generally, the transient accumulation of denitrification intermediates is widespread, and the nitrite accumulation occurs first [30]. However, there was no evident nitrite accumulation throughout this test. The nitrite was constantly removed from the initial 0.1 mg/L to the final 0.006 mg/L with the addition of the strain. It revealed that the strain LZ-14 has high activity of nitrite reduction. Thus the addition of strain LZ-14 in constructed wetland would not harm the quality of the river water.

In addition, the effect of bio-enhancement on treating the artificial wastewater in SCWs also was investigated in pilot-scale. About 76.8% of COD_{Cr} and 64% of TN were removed in the control SCW without inoculation of strain LZ-14 within 8 days. While the removal rates of COD_{Cr} and TN in the inoculated SCWs were 82.5% and 95%, respectively (not shown in figure). The advancement of contaminants elimination by bio-enhancement was remarkable.

The Xinxue River passes through the Xinxue River-constructed wetland and flows into Nansi Lake. The Nansi Lake water is expected to meet the grade III surface water quality standard of China (GB 3838-2002) (TN below 1.0 mg/L, COD below 20 mg/L). However, the average quality of the river water (COD about 35 mg/L, TN about 5 mg/L) does not meet the standard now. Thus, the Xinxue River water must be further treated before flowing into Nansi Lake. Research was conducted to investigate the treatment efficiency of SCWs on Xinxue water with the addition of strain LZ-14. Generally, the water quality of the Xinxue in spring could represent the annual average level. Consequently, the river water sampled in spring was used in this study. In the rainy season, this river receives a great deal of runoff carrying abundant contamination, and the water quality will deteriorate. Thus the artificial wastewater was used to simulate the seriously polluted river water in

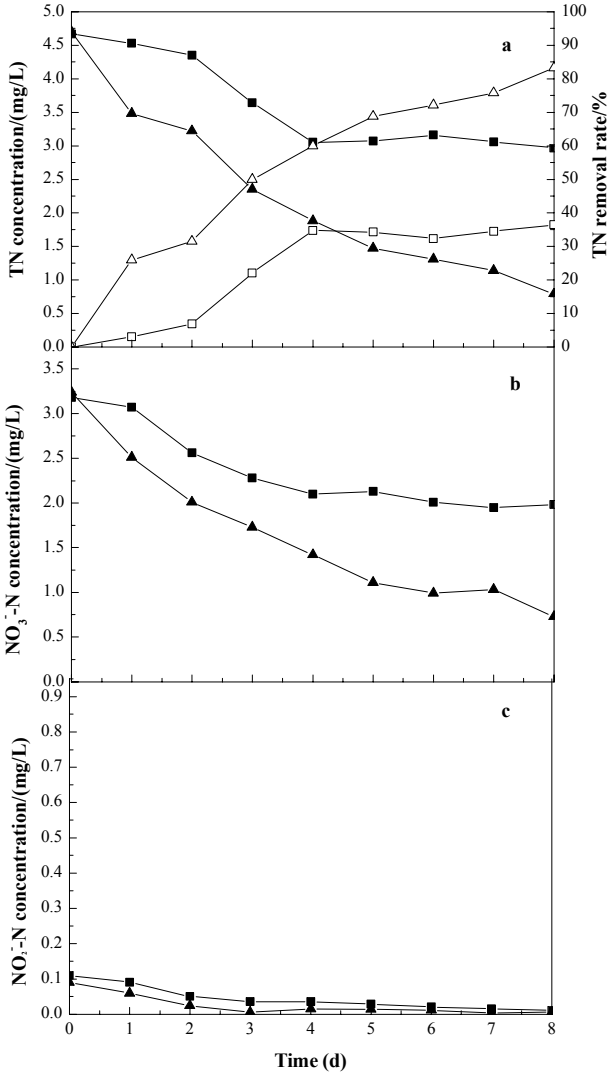

Fig. 6. Variation of different forms of nitrogen concentrations over time in Xinxue River water inoculated with LZ-14. △ – TN removal rate in experimental group inoculated with strain; □ – TN removal rate in control group. ▲ – (a,b,c), nitrogen concentration in experimental group inoculated with strain; ■ – (a,b,c), nitrogen concentration in control group.

the rainy season. For the river water, the COD_{Cr} and TN were reduced to 13 mg/L and 0.79 mg/L, respectively, after bio-enhancement treatment. But without bio-enhancement, COD_{Cr} and TN were only reduced to 23.7 mg/L and 2.97 mg/L, respectively. For the synthetic wastewater, COD_{Cr} and TN were reduced to 18.5 mg/L and 0.94 mg/L, respectively, by bio-enhancement treatment. While without bio-enhancement COD_{Cr} and TN concentrations were 33.12 mg/L and 6.17 mg/L, respectively, at the end of the study. The results showed that both the low-load natural river water and the high-load synthetic wastewater could meet grade III surface water quality standards after bio-enhancement treatment. This study indicated that the bio-enhancement by strain LZ-14 was efficacious for the annual river water.

Conclusions

1. Strain LZ-14 is an efficient denitrifying bacterium. During the denitrification process there are many dense granular materials in the cells. Those dense materials may be related to denitrification action. Further research is needed to show the exact relation between denitrification and those dense granular materials. By phylogenetic analysis, strain LZ-14 was identified as *Pseudomonas* genus.
2. Strain LZ-14 could use not only the soluble easy-biodegraded organic compounds (such as methanol, ethanol), but also the hard-biodegraded organic matters (such as starch, protein). The wide range of carbon sources of strain LZ-14 ensured that it could adapt to different environments.
3. The appropriate temperature for growth and denitrification of LZ-14 is 20-35ºC. Strain LZ-14 is sensitive to oxygen; increasing DO concentration resulted in a decrease of denitrification efficiency in this study. Thus, the practical implication of strain LZ-14 should be under anoxic conditions.
4. The removal efficiencies of TN and COD_{Cr} in SCWs were enhanced by the addition of strain LZ-14. COD_{Cr} and TN removal rates of the Xinxue River water increased by 31.7% and 46.8%, respectively, with the addition of the strain. The COD_{Cr} and TN removal rates of the artificial wastewater increased by 5.7% and 30.8%, respectively, compared with its control test.

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