



Heterotrophic nitrogen removal by a newly isolated microorganism *Oligella* sp. XS68

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Abstract: A new bacterium capable of heterotrophic nitrogen removal was isolated and identified as *Oligella* sp. XS68. The strain exhibited efficient heterotrophic nitrogen removal capabilities, with a low accumulation of nitrification products. Single-factor experiments indicated that efficient nitrogen removal and growth of the strain XS68 occurred with sodium succinate as the carbon source, C/N ratio 15, pH 6.0–9.0, temperature 30–37 °C, and shaking speed 160–200 rpm. The removal efficiency of ammonium nitrogen could achieve 98 % within 96 h when the initial nitrogen concentration was 421.3 mg L⁻¹. These findings demonstrate that XS68 is a promising candidate for nitrogen removal in wastewater treatments.

Keywords: wastewater treatment; biological treatment; nitrification; denitrification; ammonium.

INTRODUCTION

Wastewater with excessive amounts of nitrogen compounds will exert many detriment effects on water environment, because excessive nitrogen can be toxic to aquatic life, cause oxygen depletion and eutrophication.¹ Therefore, the reduction of nitrogen levels in discharged wastewater has become a key concern in water pollution control.² Biological treatment processes are extensively used for nitrogen removal from polluted water, and it is traditionally realized by autotrophic nitrifiers and heterotrophic denitrifiers. Autotrophic nitrifiers oxidize ammonium and convert it to nitrification products (nitrite or nitrate) under aerobic conditions, while heterotrophic denitrifiers utilize those nitrification products and convert them to gaseous denitrification products (nitrogen gas) under anaerobic conditions.³ In the past few decades, especially in recent years, it is refreshing to find that some heterotrophic microorganisms can simultaneously perform both

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nitrification and denitrification under aerobic conditions. Moreover, heterotrophic microorganisms are tolerant to high concentrations of ammonium and organic matter, while the autotrophic microorganisms are generally incapable of surviving in such environments.³ Therefore, ammonium removal by heterotrophic microorganisms seems to be more attractive. Some bacterial species with this capability have been investigated, such as *Acinetobacter*,^{2,4-6} *Alcaligenes*,⁷⁻⁹ *Bacillus*,^{10,11} *Cupriavidus*,¹² *Pseudomonas*,^{2,13,14} *Rhodococcus*¹⁵ and others. These functional bacteria exhibit much higher growth rate than autotrophs and could use various organic substrates.¹² However, it is still necessary to screen more extensive and effective microorganisms to meet the demand for bioremediation of nitrogenous wastewater containing different organic matter. A landfill leachate treatment system with high ammonium concentrations is a good resource for isolating the heterotrophic microorganisms.

In this paper, a new *Oligella* sp. strain XS68 was isolated from landfill leachate and an efficient nitrogen removal performance verified for the first time. The detailed experiments were carried out to investigate the heterotrophic nitrogen removal characteristics of the new isolate under different culture conditions. This study provided a new promising microbial source for treatment of nitrogenous wastewater.

EXPERIMENTAL

Sampling and media

The wastewater samples were collected from landfill leachate treatment plants located in Hangzhou city, Zhejiang Province, China, and used to isolate heterotrophic nitrogen removal microorganism. The values of pH, chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrate (NO_3^--N), and total nitrogen (TN) of the landfill leachate were 7.68 ± 0.33 , 32040 ± 1580 , 1452 ± 326 , 80 ± 27 and 1986 ± 182 mg L⁻¹, respectively. The basal medium used for bacteria isolation and heterotrophic nitrifying was composed of $(\text{NH}_4)_2\text{SO}_4$ 2.0 g L⁻¹, disodium succinate hexahydrate 23.85 g L⁻¹ and a trace element solution 50 mL L⁻¹ with an initial pH of 7.0. The trace element solution consisted of K_2HPO_4 5.0 g L⁻¹, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 2.5 g L⁻¹, NaCl 2.5 g L⁻¹, $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ 0.05 g L⁻¹ and $\text{MnSO}_4\cdot 4\text{H}_2\text{O}$ 0.05 g L⁻¹. Solid medium was prepared from the basal medium with a changed $(\text{NH}_4)_2\text{SO}_4$ concentration of 0.5 g L⁻¹ and an addition of agar 20 g L⁻¹. All chemicals were of analytical grade.

Isolation and identification

A 5-mL wastewater sample was added to 50 mL of autoclaved basal medium in a 250 mL Erlenmeyer flask. The cultures were incubated in a rotary shaker at 30 °C and 180 rpm for 5 days. The cultures (5 mL) were then transferred to fresh basal medium (50 mL) and incubated for another 5 days. After repeating this procedure four times, the resulting cultures were diluted and spread onto solid medium plates to isolate pure colonies. The isolated pure colonies were then inoculated onto fresh solid medium plates. After cultivation for 4 days, Nessler's reagent was added into the plates, and the colonies with large clear zones were selected as potential strains. The selected potential strains were individually tested for nitrogen removal capabilities through shake flask experiments with basal medium. The strain with good nitrogen removal ability was suspended in 25 % glycerol solution and stored at -80 °C.

The 16S rDNA gene of the strain was extracted, amplified by polymerase chain reaction (PCR) using bacterial universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTACGACTT-3'), and sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). The sequence was compared with that of other microorganisms by way of BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>). Sequence alignment was performed using the BLAST Search program. A phylogenetic tree was constructed by MEGA 6.06 using the neighbor-joining method.

Effect of single-factor on nitrogen removal performance

The nitrogen removal performance of the strain XS68 were investigated under different culturing conditions, including carbon source, C/N ratio, initial pH, temperature and rotation speed. For the carbon source experiments, sodium acetate, disodium succinate and trisodium citrate were selected. Each carbon source was added into the basal medium instead of disodium succinate at a C/N ratio of 10. To determine the effects of the C/N ratio, rotation speed, initial pH and temperature on nitrogen removal, various conditions of the C/N ratio (1, 2.5, 5, 10 and 15), the rotation speed (120, 160, 180 and 200 rpm), the initial pH (5, 6, 7, 8 and 9), and the temperature (15, 30, 34 and 37 °C) were investigated. Unless otherwise stated, all the experiments were conducted at an initial (NH₄)₂SO₄ concentration of 2.0 g L⁻¹, C/N 15, initial pH 7.0, culturing temperature 30 °C and shaking speed 160 rpm. During incubation, the cultures were sampled periodically to measure the bacterial growth (optical density at 600 nm, OD₆₀₀), pH and NH₄⁺-N concentration.

Analytical methods

The cell growth of the strain XS68 was measured spectrophotometry at 600 nm (OD₆₀₀). The pH value was measured with a pH meter (PB-10, Sartorius, Germany). NH₄⁺-N, NO₃⁻-N and NO₂⁻-N concentrations were determined according to the methods¹⁶ Nessler's reagent, phenoldisulfonic acid and N¹-1-naphthalenyl-1,2-ethanediamine spectrophotometry, respectively. Hydroxylamine was analyzed according to the method of Frear and Burrell.¹⁷ The intracellular nitrogen (all the nitrogen compounds in the cells) was estimated according to the method described by Yao *et al.*⁶

RESULTS AND DISCUSSION

Isolation and characterization of strain XS68

After a series of enrichment and screening experiments, the strain XS68 with high efficiency for nitrogen removal was isolated. The partial 16S rRNA sequence of the strain XS68 was determined and deposited in the GenBank database with an accession number of KC843430. A neighbor-joining phylogenetic tree was then constructed based on its 16S rRNA sequence, as shown in Fig. 1. The result indicated that the strain XS68 was most closely related to the genus *Oligella*, showing 98 % similarity to *Oligella ureolytica* ATCC 43534 (NR 114553). Combined with the above results, the strain XS68 was proposed to be an *Oligella* species. The strain XS68 may be the first report about the ability of heterotrophic nitrogen removal by this species.

Ammonium nitrogen removal performance of strain XS68

The ammonium nitrogen removal performance in the basal medium by XS68 is given in Table I. The ammonium nitrogen decreased from 421.3 mg L⁻¹ to 226.41 mg L⁻¹ after 24 h of incubation, and the nitrogen removal rate was 8.12 mg L⁻¹ h⁻¹, which was higher than that of *Rhodococcus* sp. CPZ24 (3.4 mg L⁻¹ h⁻¹),¹⁵ *Bacillus methylotrophicus* strain L7 (2.15 mg L⁻¹ h⁻¹)¹¹ and *Vibrio diabolicus* SF16 (2.29 mg L⁻¹ h⁻¹).¹⁸

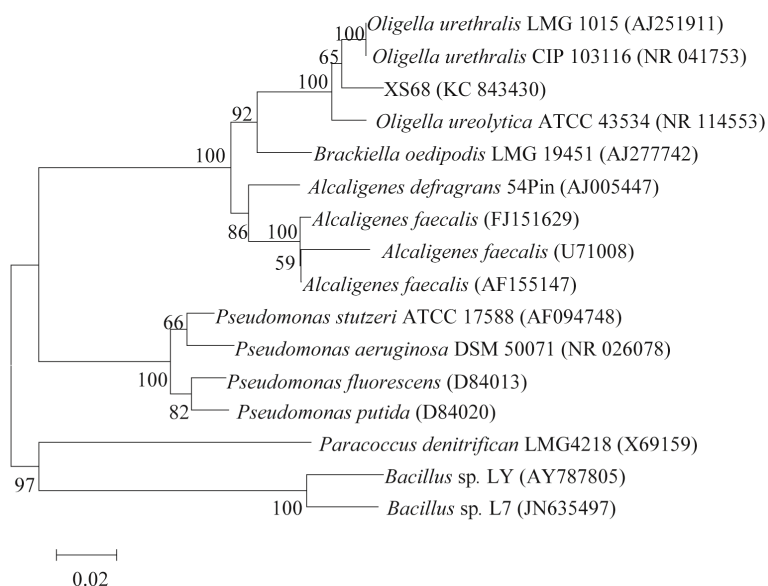


Fig. 1. Phylogenetic tree of *Oligella* sp. XS68 derived from neighbor-joining analysis of partial 16S rRNA gene sequence.

TABLE I. Nitrogen balance of ammonium nitrogen removal by XS68

Cultivation time, h	Concentration of N, mg L ⁻¹					Loss of N, %
	NH ₄ ⁺ -N	NH ₂ OH-N	NO ₂ ⁻ -N	NO ₃ ⁻ -N	Intracellular N	
0	421.30±0.78	0	0	0	0	
24	226.41±0.22	0	0	2.11±0.21	113.16±0.15	40.8±0.20

During the nitrogen removal process, nitrification products including hydroxylamine and nitrite were not detected, but a minor amount of nitrate was detected, which is similar to *Bacillus subtilis* A1,¹⁰ *Acinetobacter calcoaceticus* STB1³ and *Paracoccus versutus* LYM,¹⁹ with which nitrate was the main accumulated nitrification intermediate, but nitrite and hydroxylamine were present in traces or below the detection limit, possibly due to the instability of hydroxylamine and the fast transformation to nitrite to nitrate by heterotrophic nitrification. In addition, the intracellular nitrogen increased to 113.16 mg L⁻¹ due to

the growth of the strain. Analysis of nitrogen balance showed that there was 58.1 % removal nitrogen converted to biomass, 1.1 % was converted to nitrification products (mainly NO_3^- -N), and 40.8 % was lost, which was probably removed in the form of gaseous denitrification products.¹⁹ The putative nitrogen transformation pathway could be $\text{NH}_4^+ \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$, then NO_3^- was denitrified to gaseous products.¹⁹ However to date, the nitrogen removal pathway of heterotrophic nitrifying bacteria under aerobic conditions is not fully understood, and further research is still needed.²

Effect of different factors on ammonium nitrogen removal

Effect of carbon source. Carbon compounds usually serve as energy and electron sources for heterotrophic bacteria.²⁰ Preliminary experiments (OD_{600} was measured after 3 days of growth) showed the strain XS68 grew better in the medium with sodium succinate or sodium citrate as sole carbon source and failed to grow on the medium with sodium acetate, sucrose, glucose, methanol or ethanol as sole carbon source. Then, sodium succinate, sodium citrate and sodium acetate were selected as sole carbon sources to further study their influence on ammonium nitrogen removal and cell growth of XS68, and the results are presented in Fig. 2.

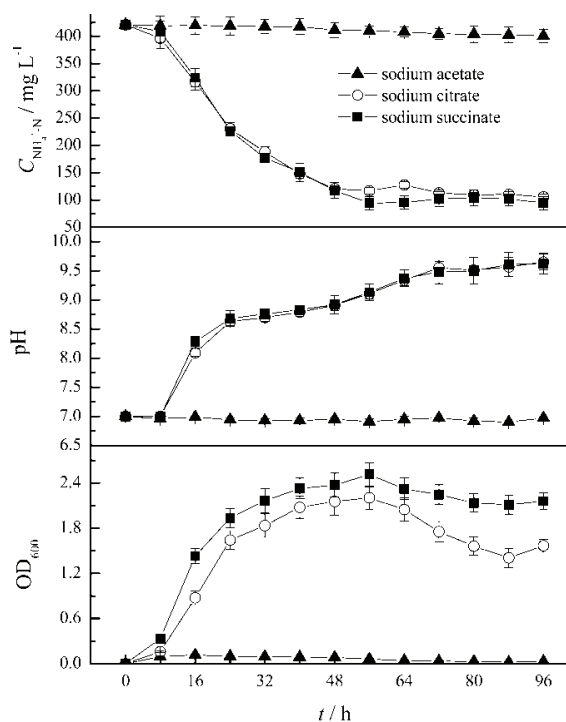


Fig. 2. Effect of carbon source on ammonium nitrogen removal by XS68.

When sodium succinate or sodium citrate were used as the sole carbon source, the strain XS68 exhibited an efficient nitrogen removal ability and growth, presenting removal efficiencies of 77.6 and 72.3 % after 56 h of cultivation, respectively. At this time, the corresponding OD_{600} reached the peaks of 2.5 and 2.2, respectively. Both of the pH values increased from 7.0 to 9.5 after 72-h cultivation. However, the ammonium nitrogen removal ability and cell growth were almost undetected when sodium acetate was used as the carbon source, indicating that this carbon source could not be utilized by the strain XS68. By comparison, the highest nitrogen removal rate was observed in sodium succinate. Therefore, sodium succinate was employed in subsequent research.

Effect of C/N ratio. To determine the effect of different C/N ratios on ammonium nitrogen removal, C/N ratios of 1, 2.5, 5, 10 and 15 were selected with a fixed ammonium nitrogen concentration. The results in Fig. 3 indicate that there was a marked tendency for the ammonium nitrogen removal efficiency, together with OD_{600} to rise with increasing C/N ratio.

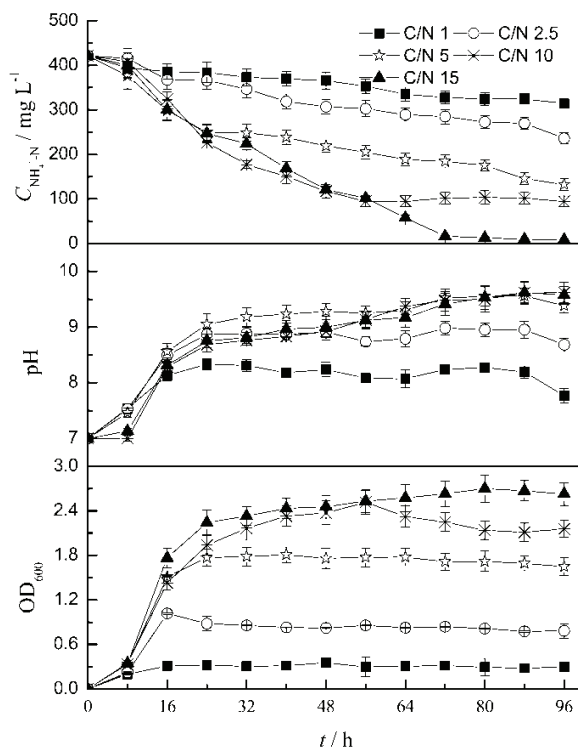


Fig. 3. Effect of C/N ratio on ammonium nitrogen removal by XS68.

At a C/N ratio of 15, a removal efficiency of 96.2 % was presented after 72 h incubation. This demonstrated that the high C/N ratio was beneficial to the

growth of the strain XS68. When the C/N ratio was one, only 25.5 % of ammonium nitrogen was removed after 96 h incubation, the growth of the bacteria was slow and the corresponding pH increased slowly. The low ammonium nitrogen removal rate at the low C/N ratio was mainly due to insufficient carbon supply, which would impair both microbial growth and electron donors for denitrification.^{2,4} Most previous investigations on nitrogen removal by heterotrophic bacteria were conducted at a high C/N ratio.^{2,8,12,21} Finally, C/N 15 was selected for the further experiments.

Effect of rotation speed. As shown in Fig. 4, increasing the rotation speed significantly promoted ammonium nitrogen removal. An obvious improvement in ammonium nitrogen removal was observed when the rotation speed was increased from 120 to 160 rpm.

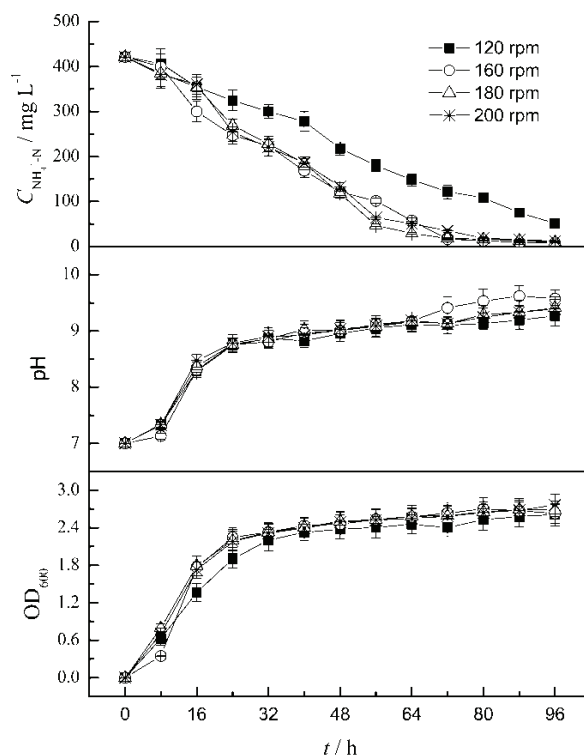


Fig. 4. Effect of rotation speed on ammonium nitrogen removal by XS68.

When the rotation speed was further increased, the nitrogen removal efficiency remained almost at the same level. More than 95 % of ammonium nitrogen was removed after 80 h cultivation at 160, 180 and 200 rpm, whereas only 74 % of the ammonium nitrogen was removed at 120 rpm. The result was similar to that reported by Yang *et al.*² and indicated that ammonium nitrogen removal by

heterotrophic bacteria was strongly dependent on the aerobic conditions.² In addition, the change of rotation speed had little influence on the cell growth. When the rotation speed ranged from 120 to 200 rpm, the pH and OD_{600} of culture medium remained stable. Therefore, the optimum rotation speed for the strain XS68 was selected as 160 rpm.

Effect of initial pH. The effects of initial pH on cell growth and the efficiency of ammonium nitrogen removal of the strain XS68 are shown in Fig. 5. The strain XS68 exhibited a strong ability to adapt to pH variation. When the initial pH was set at 6.0 to 9.0, the final ammonium nitrogen removal efficiency was about 97 %, the final OD_{600} about 2.5 and the final pH value about 9.5 after 96 h of cultivation.

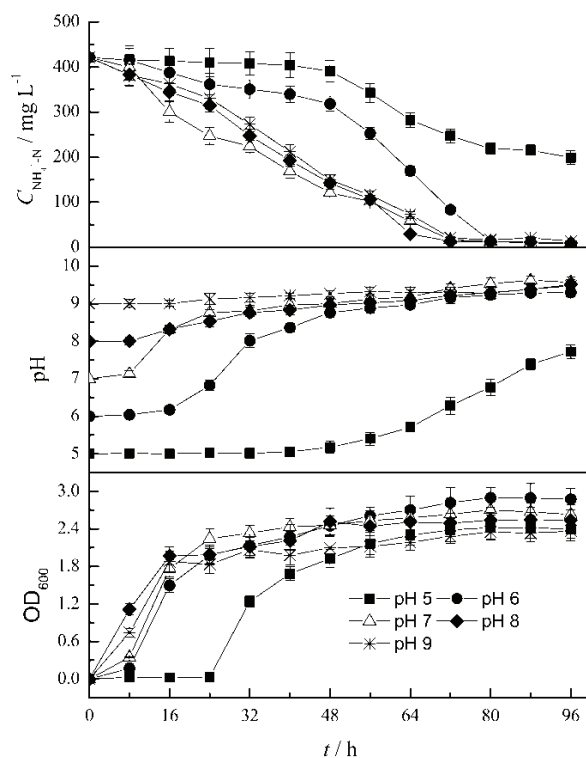


Fig. 5. Effect of initial pH on ammonium nitrogen removal by XS68.

When the initial pH value was 5.0, the ammonium nitrogen removal efficiency was 52.8 % after 96 h of cultivation. Furthermore, the cell growth was slower at the early stage of incubation. This indicated that the acidic environment was detrimental for ammonium nitrogen removal and cell growth, and slightly acidic, neutral and alkaline environments were beneficial for ammonium nitrogen removal. These results were consistent with those for *Acinetobacter* sp. Y1.⁵ This

phenomenon may be caused by more free ammonia contained in the medium in alkaline condition, which is required by ammonia monooxygenase.²² However, Liu *et al.*²³ isolated a nitrifying fungus of *Paecilomyces variotii* from chicken manure that played the biggest role in the removal of ammonium at pH 4.0–7.0. One reason for this discrepancy may be that the optimum pH of most fungi is acidic or neutral – quite different from bacteria.²³ A study of Kim *et al.* showed that the activities of an ammonia-oxidizing microorganism can be inhibited by high amounts of free ammonia.²⁴ It was proposed that the ammonia-oxidizing activities of different nitrifying microorganisms are affected by free ammonia concentration, and pH is a key factor to tune the concentration of free ammonia.

Effect of temperature. Fig. 6 shows that a higher temperature significantly promoted the efficiency of ammonium nitrogen removal and cell growth of the strain XS68.

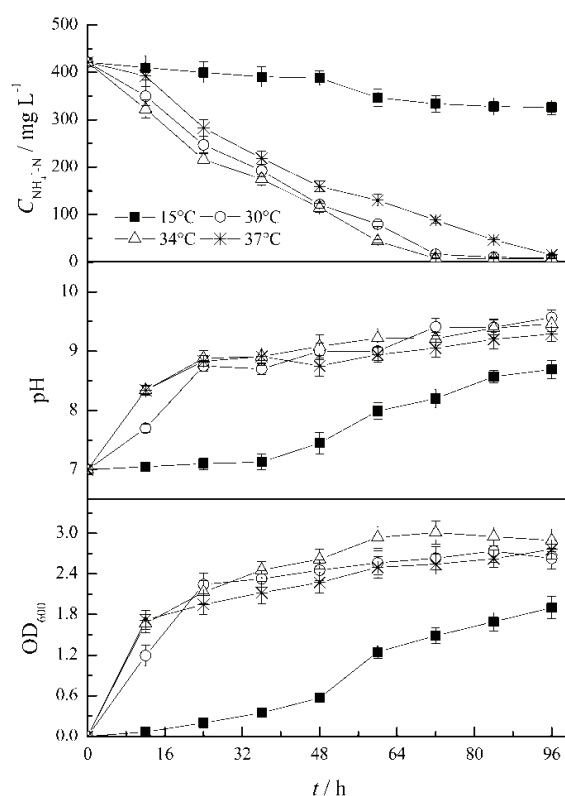


Fig. 6. Effect of temperature on ammonium nitrogen removal by XS68.

The ammonium nitrogen removal efficiencies were all about 98 % within 30–37 °C, presenting the fastest removal rate and cell growth at 34 °C. The pH values all reached about 9.5 after 96 h of cultivation. However, when the tempe-

perature was set as 15 °C, only 22.6 % of ammonium nitrogen was removed after 96 h of incubation, the growth of bacteria was slow and the corresponding pH increased slowly. According to the opinion of Robertson *et al.*, aerobic conversion of ammonium to nitrogen gas is catalyzed by constitutive nitrifying and denitrifying enzymes, the activity of which is most sensitive to variations in temperature.²⁵ Moreover, a low temperature is not favorable to the growth of bacteria, thus causing worse ammonium removal performance.²

CONCLUSIONS

Oligella sp. XS68, a novel bacterium capable of heterotrophic nitrogen removal, was isolated from landfill leachate and identified by phylogenetic analysis. The preferred conditions for heterotrophic nitrogen removal and cell growth of the isolate were sodium succinate as the carbon source, C/N ratio of 15, pH 7.0, temperature of 34 °C, and rotation speed of 160 rpm. Nitrification products including hydroxylamine and nitrite were below the detectable limit, and trace amounts of nitrate were detected during the nitrogen removal process. The strain could remove about 98 % of ammonium nitrogen within 96 h under the preferred conditions at an initial nitrogen concentration of 421.3 mg L⁻¹. All results implied XS68 has a promising prospect of application in biological removal of nitrogen compounds from wastewater.

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ИЗВОД

УКЛАЊАЊЕ ХЕТЕРОТРОФИЧНОГ АЗОТА НОВОИЗОЛОВАНИМ МИКРООРГАНИЗМОМ *Oligella* sp. XS68

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Иzolована је нова бактерија, способна да уклони хетеротрофични азот и идентификована као *Oligella* sp. XS68. Овај сој је показао способност ефикасног уклањања, са малом акумулацијом нитрификационих производа. Експерименти са појединачном променом фактора указали су на ефикасно уклањање азота и раст соја XS68 при условима када је натријум-сукцинат извор угљеника, C/N однос 15, рН 6,0–9,0, температура 30–37 °C, а брзина мућкања 160–200 rpm. Ефикасност уклањања азота је достигала 98 % у року од 96 h када је почетна концентрација била 421,3 mg L⁻¹. Ови резултати показују да је XS68 обећавајући кандидат за уклањање азота у третману отпадних вода.

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