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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 carbon source, C/N ratio 15, pH 6.0-9.0, temperature 30-37 °C, and shaking speed 160-200 rpm. The ammonium nitrogen removal efficiency could achieve 98 % within 96 h when the initial nitrogen concentration was 421.3 mgL⁻¹. These findings demonstrate that XS68 was a promising candidate for nitrogen removal in wastewater treatments.

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

INTRODUCTION

The wastewater with excessive amounts of nitrogen compounds will exert many detriments 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 The biological treatment processes are extensively used for nitrogen removal from the polluted water, and it is traditionally carried out 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 carry out both nitrification and denitrification under aerobic conditions. Moreover, the heterotrophic microorganisms are tolerant to high concentrations of ammonium.

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and organic matter, while the autotrophic microorganisms are generally incapable of surviving in such environments. Therefore, ammonium removal by heterotrophic microorganisms seems to be more attractive. Some bacterial species with this capability have been investigated, such as Acinetobacter, Cupriavidus, Pseudomonas, Rhodococcus and others. These functional bacteria exhibit much higher growth rate than autotrophs and could use various organic substrates. 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. The landfill leachate treatment system with high ammonium concentration is a good resource for isolating the heterotrophic microorganisms.

In this paper, a new Oligella sp. strain XS68 was isolated from landfill leachate and verified efficient nitrogen removal performance 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 (NH₄⁻N), nitrate (NO₃⁻-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 (NH₄)₂SO₄ 2.0 g L⁻¹, disodium succinate hexahydrate 23.85 g L⁻¹ and a trace element solution 50 mL⁻¹ with an initial pH of 7.0. The trace element solution consisted of K₂HPO₄ 5.0 g L⁻¹, MgSO₄·7H₂O 2.5 g L⁻¹, NaCl 2.5 g L⁻¹, FeSO₄·7H₂O 0.05 g L⁻¹ and MnSO₄·4H₂O 0.05 g L⁻¹. Solid medium was prepared from the basal medium with a changed (NH₄)₂SO₄ 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 the potential strains. The selected potential strains were individually tested for nitrogen removal capabilities through shake flask experiments with the 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′-TACGT-TACC TTGTTACGACTT-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 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. In 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 C/N ratio, rotation speed, initial pH and temperature on nitrogen removal, various conditions of the C/N ratios (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 initial (NH$_4$)$_2$SO$_4$ concentration 2.0 gL$^{-1}$, 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 (the optical density at 600 nm, OD$_{600}$), pH and NH$_4^+$-N concentration.

**Analytical methods**

The cell growth of the strain XS68 was measured by spectrophotometry at 600 nm (OD$_{600}$). The pH value was measured with a pH meter (PB-10, Sartorius, Germany). NH$_4^+$-N, NO$_3^-$-N and NO$_2^-$-N were determined according to the methods$^{16}$ of Nessler’s reagent, phenol disulfonic acid and N-(1-naphthalene)-diaminoethane spectrophotometry, respectively. Hydroxylamine was analyzed according to the method of Prear and Burrell.$^{17}$ The intracellular nitrogen (all the nitrogen compounds in the cells) was estimated according to the method described by Yao et al.$^{18}$

**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 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. So far, the strain XS68 may be the first report about the ability of heterotrophic nitrogen removal of this species.

**Ammonium nitrogen removal performance of strain XS68**

The ammonium nitrogen removal performance in the basal medium by XS68 was showed in Table I. The ammonium nitrogen decreased from 421.3 mg L$^{-1}$ to 226.41 mgL$^{-1}$ 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
YAN et al. (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\(^{-1}\)).\(^{18}\)

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![Phylogenetic tree of *Oligella* sp. XS68 derived from neighbor-joining analysis of partial 16S rRNA gene sequence](image)

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 of XS68

<table>
<thead>
<tr>
<th>Cultivation time, h</th>
<th>NH(_4^+)-N, mg L(^{-1})</th>
<th>NH(_2)OH-N</th>
<th>NO(_2^) -N</th>
<th>NO(_3^) -N</th>
<th>Intracellular N</th>
<th>Loss of N, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>421.30±0.78</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>226.41±0.22</td>
<td>0</td>
<td>0</td>
<td>2.11±0.21</td>
<td>113.16±0.15</td>
<td>40.8±0.20</td>
</tr>
</tbody>
</table>

During the nitrogen removal process, the nitrification products including hydroxylamine and nitrite were not detected, but minor amount of nitrate was detected, which were similar to *Bacillus subtilis* A1,\(^{10}\) *Acinetobacter calcoaceticus* STB1\(^{3}\) and *Paracoccus versatius* LYM,\(^{19}\) where nitrate was the main accumulated nitrification intermediate, but nitrite and hydroxylamine were trace or below the detection limit, possibly due to the instability of hydroxylamine and fast transformation to nitrite, then to nitrate by heterotrophic nitrification. In addition, the intracellular nitrogen increased to 113.16 mg L\(^{-1}\) due to the growth of 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 NH\(_4^+\) → NH\(_2\)OH → NO\(_2^\) → NO\(_3^\) → NO\(_3^\)-N, then NO\(_3^\) was denitrified to gaseous products.\(^{19}\) But up 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. 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 source to further study the influence on the ammonium nitrogen removal efficiency and cell growth of XS68, and the results were presented in Fig. 2.

![Graph showing the effect of carbon source on ammonium nitrogen removal](image)

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

When sodium succinate and sodium citrate were used as the sole carbon sources, the strain XS68 exhibited efficient nitrogen removal ability and growth, presenting removal efficiency 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 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 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, the C/N ratios of 1, 2.5, 5, 10 and 15 were selected with a fixed ammonium nitrogen concentration. The results in Fig. 3 indicated that there was a marked tendency for the ammonium nitrogen removal efficiency, together with OD_{600}, to rise as the C/N ratio increased.

![Graph showing the effect of C/N ratio on ammonium nitrogen removal of XS68](image)

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

At a C/N ratio of 15, a removal efficiency of 96.2% was presented after 72 h incubation. It demonstrated that the high C/N ratio was beneficial to the growth of the strain XS68. When the C/N ratio was 1, only 25.5% of ammonium nitrogen was removed after 96 h incubation, the growth of bacteria was slow, and the corresponding pH increased slowly. The low ammonium nitrogen removal rate at low C/N ratio was mainly due to the insufficient carbon supply, which would
impair both microbial growth and electron donors for denitrification.\textsuperscript{2,4} Most previous investigations on nitrogen removal by heterotrophic bacteria were conducted at a high C/N ratio.\textsuperscript{2,8,12,21} Finally, C/N 15 was selected for the further experiments.

Effect of rotation speed

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

![Figure 4: Effect of rotation speed on ammonium nitrogen removal of XS68](image)

When the rotation speed increased further, the nitrogen removal efficiency stayed almost the same level. More than 95% of ammonium nitrogen was removed after 80 h cultivation at 160, 180 and 200 rpm, whereas 74% of ammonium nitrogen was only removed at 120 rpm. The result was similar to Yang’s report,\textsuperscript{2} and indicated that ammonium nitrogen removal by heterotrophic bacteria was strongly dependent on aerobic conditions.\textsuperscript{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\textsubscript{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 ammonium nitrogen removal efficiency of the strain XS68 were 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 of about 97%, the final OD$_{600}$ of about 2.5 and the final pH value of about 9.5, were observed after 96 h cultivation.

When the initial pH values were 5.0, the ammonium nitrogen removal efficiency was 52.8% after 96 h cultivation. Also, the cell growth was slower at the early stage of incubation. It indicated the acidic environment was harmful for ammonium nitrogen removal and cell growth, and slightly acidic, neutral and alkaline environment were beneficial for ammonium nitrogen removal. The results were consistent with those of *Acinetobacter* sp. Y1. This phenomenon may be caused by more free ammonia contained in the medium in alkaline condition, which is required by ammonia monoxygenase. However, Liu *et al.* isolated a nitrifying fungus of *Paecilomyces variotii* from chicken manure which 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 Kim’s study has shown that the activities of the ammonia-oxidizing microorganism can be inhibited by high free ammonia.²⁴ It proposed that the ammonia-oxidizing activities of different nitrifying microorganisms were affected by free ammonia concentration, and pH is a key factor to tune the concentration of free ammonia.

Effect of temperature

Fig. 6 showed that higher temperature significantly promoted the ammonium nitrogen removal efficiency and cell growth of the strain 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 were all reached about 9.5 after 96 h of cultivation. However, when the temperature was set as 15 °C, only 22.6% of ammonium nitrogen was removed after 96 h incubation, the growth of bacteria was slow, and the corresponding pH increased slowly. According to Robertson’s opinion, aerobic conversion of am-
Mon ammonium to nitrogen gas is catalyzed by constitutive nitrifying and denitrifying enzymes, whose activity is most sensitive to variations in temperature. Moreover, low temperature is not favorable to the growth of bacteria, thus causing worse ammonium removal performance.

CONCLUSION

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 shaking speed of 160 rpm. The 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 had a promising prospect of application in biological removal of nitrogen compounds from wastewater.

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REFERENCES