Research Article

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Optimizing Suitable Conditions for the Removal of Ammonium Nitrogen by a Microbe Isolated from Chicken Manure

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Abstract: Strain C was isolated from chicken manure, and its phenotypic characteristics were gram-stain negative, yellow-pigmented, aerobic bacterium, heterotrophic, non-motile, chemoorganotrophic, non-gliding as well as non-spore-forming. A 16S rRNA gene sequence analysis showed that strain C occupied a distinct lineage within the family of the genus *Chryseobacterium*, and it shared highest sequence similarity with *Chryseobacterium solincola* strain 1YB-R12 (80%). The new isolate has been studied for removing ammonium-nitrogen ($\text{NH}_4\text{-N}$) and the optimization of suitable conditions. The strain C was able to degrade over 42.8% of $\text{NH}_4\text{-N}$ during its active growth cycle. Experimental study of the effect of temperature and pH on $\text{NH}_4\text{-N}$ removal showed that the temperature and pH optima for $\text{NH}_4\text{-N}$ removal were 30–35°C and 4–8, respectively. The results indicated that strain C shows a potential application for wastewater treatment.

Keywords: *Chryseobacterium*; nitrate; temperature; pH; wastewater

1 Introduction

Ammonium-nitrogen ($\text{NH}_4\text{-N}$) is the main component of nitrogen in wastewater from the livestock and poultry industries, and its content in wastewater is very high

[1]. $\text{NH}_4\text{-N}$ removal from bodies of water was affected by ammonia volatilization, nitrification, and dissimilatory nitrate reduction to ammonium, anaerobic ammonia oxidation, plant and microbial uptake in addition to substrate adsorption [2–4]. For these, the nitrification by microbes is one of the most economical and ecological processes for $\text{NH}_4\text{-N}$ removal from wastewater [5,6], and bio-treatment is an effective and low-cost biotechnology for degrading $\text{NH}_4\text{-N}$ content in wastewater [7,8].

$\text{NH}_4\text{-N}$ can be degraded traditionally by both autotrophic-nitrifying and heterotrophic-nitrifying microbes [9,10]. Some heterotrophic-nitrifying bacteria, such as *Acinetobacterium baumannii* [11], *Candida rugosa* [12], *Candida krusei* [13], and *Pichia farinosa* [14] can convert $\text{NH}_4\text{-N}$ into nitrite ($\text{NO}_2\text{-N}$) or nitrate ($\text{NO}_3\text{-N}$). In recent studies, most bacteria included *Alcaligenes faecalis* [15], *Pseudomonas stutzeri* [16], and *Rhodococcus species* [7], which are capable of nitrification as well as aerobic denitrification.

In aquatic and terrestrial ecosystems, most members of genus *Chryseobacterium* can be widely found and distributed; some *Chryseobacterium* species are pathogenic for both humans and animals [17], such as *Chryseobacterium meningosepticum*, *Chryseobacterium indologenes*, *Chryseobacterium balustinum*, *Chryseobacterium scophthalmum*, and other strains; some members of the *Chryseobacterium* family are confirmed to be a crucial bacterial group associated with plants [18,19], as these strains of the plant-associated species of *Chryseobacterium* can promote plant growth [20,21]. Some reports also described the compound-accumulation and resistance ability of *Chryseobacterium* strains, such as *C. solincola* [22] and *C. polytrichastri* [23]. For instance, *C. Solincola* can bioaccumulate heavy metals, which may have great application potentials for *in situ* bioremediation of heavy metals-contaminated water or soil systems [22]. However, these *Chryseobacterium* have only been isolated from water and soil, and few

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researchers have paid attention to livestock and poultry manures though they contain abundant bacteria. There may be *Chryseobacterium* in livestock and poultry manures. At present, the bacteria were reported to be most capable of ammonium nitrogen degradation, including *Bacillus* sp. [24,25], *Staphylococcus* sp. [26], *Pseudomonas* sp. [27,28], and so on. However, there is little investigation and information on the effect of *Chryseobacterium* species on inorganic nitrogen, especially $\text{NH}_4\text{-N}$, which could broaden the application of *Chryseobacterium*. Furthermore, the capability of *Chryseobacterium* species to carry out heterotrophic nitrification has not been studied thus far.

The aim of the present study was to determine the $\text{NH}_4\text{-N}$ removal capacity of candidate organisms isolated from chicken manure. We could identify a bacterium on the basis of phenotypic properties and phylogenetic distinctiveness. We also studied the effect of temperature and pH on the removal capacity of ammonium-nitrogen of these species.

2 Materials and methods

2.1 Medium

To isolate the target bacteria and promote its growth as well as inhibit the growth of other bacteria, an enrichment medium of the following specifications was used: 5.0 g Glucose, 2.0 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g NaCl, 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{K}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 0.4 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1L distilled water, pH 7.2-7.4. The isolation medium, which constituted enrichment medium with 2% agar, and the selection medium were used for isolating and purifying individual bacterial colony members. The selection medium was made up of the following: 5.0 g Glucose, 0.6 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g NaCl, 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{K}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 0.25 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 L distilled water, pH 7.2. Lysogeny broth medium, as seed culture medium, was used for strain preservation as follows: 10 g tryptone, 10g NaCl, and 5.0 g yeast extract in 1 L distilled water. Ammonia nitrogen medium (ANM) was used for investigating the capacity to degrade $\text{NH}_4\text{-N}$. Each liter of ANM (pH 7.2-7.4) contained 5.0 g Glucose, 0.7 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g NaCl, 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{K}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 0.25 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

2.2 Isolation of strain C

Samples of fresh chicken manure were obtained from a broiler poultry farm in the Shandong province, China.

2g of chicken manure samples were added to 100 ml of enrichment medium in a 250-ml flask, supplemented daily with 5% $(\text{NH}_4)_2\text{SO}_4$ (w/w%), and incubated on a rotary shaker at 180 rpm and 30°C for 7 days. The culture was diluted and spread onto isolation medium. Isolation medium plates were incubated at 30°C over two days and the colonies were sub-cultured in selection medium under the same conditions. Line separation and purification of the colonies was performed repeatedly on selection medium. The pure colonies were preserved in lysogeny broth medium. Microscopic examination was performed to confirm the culture purity. Finally, the strain C was isolated.

2.3 Identification of strain C

The colony morphology, pigmentation, growth characteristics and phenotypic characterization for strain C were observed following a two day incubation period at 30°C. Genomic deoxyribonucleic acid (DNA) was extracted from strain C, and 16S rRNA genes were amplified by PCR using two primers: 5'-AGAGTTTGATCCTGGCTCAG-3', forward; 5'-AAGGAGGTGATCCAGCCGCA-3', reverse. The result of PCR amplification of 16S rRNA genes as well as sequencing of purified PCR products were compared to the GenBank database to search for a homologous sequence.

2.4 Biodegradation experiments

2.4.1 $\text{NH}_4\text{-N}$ degradation during the growth of strain C

Strain C was inoculated in 100 ml of Lysogenic broth medium in a 250-ml flask for one day, and 5 ml of Lysogenic broth medium was put in a 250-ml flask on a rotary shaker at 180 rpm at 30°C. 1 ml of bacterial fluid was withdrawn each 6 h, and its optical density (OD_{600}) was analyzed to monitor the growth of strain C. Strain C was transferred to ANM, which contained approximately 192.1 mg/L ammonia nitrogen ($\text{NH}_4\text{-N}$), to evaluate its capacity to degrade $\text{NH}_4\text{-N}$ under aerobic conditions. Bacterial counts, OD_{600} , and $\text{NH}_4\text{-N}$ of the samples were measured. The bacterial counts used the plate count method; and OD_{600} and $\text{NH}_4\text{-N}$ in ANM were measured using an ultraviolet-visible (UV-Vis) spectrophotometer (UV-8000S, METASH, China). $\text{NH}_4\text{-N}$ concentrations were analyzed periodically using the Nessler's reagent colorimetry method.

Figure 1: The preliminary result and conventional plate streaking of Strain C.

2.4.2 Effect of temperature and pH on NH₄-N degradation

The effects of temperature (20°C, 25°C, 30°C, 35°C, 40°C at pH 7.2 and 180 rpm) and pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 at 30°C and 180 rpm) on NH₄-N degradation were investigated as described above. The incubator was set at a constant temperature, and the pH was adjusted using NaOH or HCl.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results

3.1 Identification of strain C

According to cell morphology analysis (Figure 1), a round ivory opaque, moist and smooth micro-colony generated on the isolation medium plate. Rod-shaped cells of strain C were observed under the light microscope. The phenotypic characteristics of strain C are shown in Table 1. Strain C was gram-negative bacillus, not-motile, and aerobic, and could use glucose sucrose and mannitol, but not sorbitol, melibiose, lactose, arabinose, inositol, rhamnose, and amygdalin, indicating that strain C is a heteromorphic strain. Nitrates were not reduced to other forms of nitrogen. Ammonia production was absent, and tests for indole and H₂S production, ornithine dearboxylase and citrate utilization were negative, but gelatin liquefaction was positive.

The 16S rRNA gene sequence of the amplified and sequenced strain C is shown in Figure 2. The results indicated that levels of 16S rRNA gene sequence similarity between strain C and strains of recognized species of the

Table 1: Physiological and biochemical characteristics of strain C.

Test item	Result	Test item	Result
Gram staining	-	Lactose fermentation	-
Cell shape	Rod	Citrate utilization test	-
H ₂ S production	-	Indole test	-
Motility	No	Nitrate reduction	-
Aerobism	+	Ammonia production	-
Oxidase	+	Gelatin liquefaction	+
Glucose fermentation	+	Ornithine decarboxylase antizyme	-
Sucrose fermentation	+	Arabinose fermentation	-
Mannitol	+	Inositol	-
Sorbitol	-	Rhamnose	-
Melibiose	-	Amygdalin	-

genus *Chryseobacterium* were 99%; the highest sequence similarity was 80% with the *Chryseobacterium solincola* strain 1YB-R12 [17]. Phylogenetic analysis confirmed that strain C was a member of the genus *Chryseobacterium*, and it could form a monophyletic clade with the *Chryseobacterium solincola* strain 1YB-R12 (Figure 2).

3.2 NH₄-N degradation during the growth of strain C

Strain C was in an adaptive phase from 0 h to 12 h, and the logarithmic growth phase of the bacterium was 12-24 h (Figure 3). Strain C reached maximum biomass values

Figure 2: Phylogenetic position of strain C and its closest relatives in the genus, *Chryseobacterium*, based on 16S rRNA gene sequences.

Figure 3: Growth curves of strain C.

at 30 h (OD₆₀₀ = 3.526, Log(CFU) = 7.412), after which the biomass maintained a stable growth trend for several hours-the plateau phase. Beyond 42 h, strain C entered the decline phase. Hence, optimal biomass of strain C was observed between 25h and 36h.

As shown in Figure 4, the degradation of ammonium nitrogen (NH₄-N) was incomplete after 48h. Only 42.8% of the initial NH₄-N was removed by the end of this experiment. However, NH₄-N concentrations decreased from 0h to 36h, followed by a slight increase at 48h. Meanwhile, nitrate nitrogen (NO₃-N) had formed, and its concentrations increased over time. NO₃-N concentrations

increased rapidly until the 24h, and then increased slowly from the 24h to the 48h.

3.3 The change of NH₄-N and OD₆₀₀ under different temperature and pH

The effect of temperature on the NH₄-N removal capacity and OD₆₀₀ of strain C is shown in Figure 5. Between 20 and 40°C, NH₄-N removal and OD₆₀₀ were parabolic, with the highest removal rate (30.98%) and the maximum value (3.47) of OD₆₀₀ at 30°C during 1d. The growth of strain C was given a rating of 'good' at 25-40°C (optimum, 30-35°C). At 20°C, the NH₄-N removal rate was approximately 10% during 1 d; between 20°C and 30°C, NH₄-N removal rates increased with increasing temperature. However, NH₄-N removal rates decreased with an increase in temperature as the temperature rise to more than 35°C. Contrary to this, OD₆₀₀ was greatest between 30 and 35°C (3.470-3.473). Temperatures below 30°C, experienced an increase in cell density of strain C, while increasing temperatures demonstrate that the biomass yields of strain C were enhanced by greater temperatures. This trend stopped at temperatures greater than 35°C.

Increasing the pH from 4 to 9, resulted in similar variations in NH₄-N removal and OD₆₀₀ (Figure 6). First, both values increased, then decreased with increasing pH values. The NH₄-N removal rate ranged from 19.82% to 38.38%, and OD₆₀₀ ranged from 0.80 to 3.60 between pH

Figure 4: The change of NH₄-N removal with time.

Figure 5: The effect of temperature of NH₄-N removal and OD₆₀₀ by strain C.

Figure 6: The effect of pH of ammonium nitrogen removal and OD₆₀₀ by strain C.

4 to 9. The strain C removed NH₄-N within the pH range of 4 to 8, exceeding 28% degradation. The NH₄-N removal rates by strain C were more than 31.08% during the pH of 4–7. At a pH of 5, the NH₄-N removal rate and OD₆₀₀ reached

optimal values, followed by a fluctuating decrease with increased pH.

4 Discussion

According to morphological characteristics and the 16S rRNA gene sequence, strain C was identified as a *Chryseobacterium* strain, and the growth curves suggested that the strain C could utilize glucose as a sole carbon source, but that it was insufficient for driving the growth of strain C (Figure 3). Strain C was also found to degrade NH₄-N.

4.1 NH₄-N degradation

Under aerobic conditions, NH₄-N degradation is believed to follow oxidative pathways [3]. The oxidative pathway is classified by the formation of a nitrite and nitrate [29,30]. NH₄-N degradation by strain C ultimately produced nitrate (Figure 4), but the amount of nitrate was less than the amount of NH₄-N consumed, which indicated that some NH₄-N was adsorbed by cells or transformed into nitrite under the experimental conditions [7]. The degradation of NH₄-N was not complete. Due to an insufficient carbon source, strain C could not utilize the carbon source to generate energy to drive metabolic reactions, which inhibited NH₄-N degradation. Some C strains died, and the adsorbed NH₄-N to it was desorbed, which led to a slight increase in NH₄-N concentrations. Overall, the data demonstrated that the NH₄-N degradation of strain C followed an oxidative pathway, with glucose being one of the limiting factors in NH₄-N removal. The data also showed that the function of strain C was the same as nitrifying bacteria, and thus strain C had the potential to degrade NH₄-N.

Other bacteria were found to degrade NH₄-N. Some bacteria isolated from livestock wastewater samples and membrane bioreactors, were able to remove NH₄-N and total nitrogen from wastewater by the utilization of nitrite and nitrate as nitrogen sources, such as *Acinetobacter* sp. [31], *Bacillus methylotrophicus* [25], and *Klebsiella pneumoniae* [32], *Acinetobacter* sp. [31], and *Bacillus methylotrophicus* L7 [25] and so on. This was due to them being heterotrophic nitrification-aerobic denitrification bacteria. *Rhodococcus* sp. and *Acinetobacter* sp. were reported to degrade 100% of 50 mg/L NH₄-N and 90.8% of 97.19 mg/L NH₄-N after 24h, respectively. L7 had the highest tolerance for NH₄-N among three strains

(*Rhodococcus sp.*, *Acinetobacter sp* and L7), degrading 36% of 1121.24 mg/L $\text{NH}_4\text{-N}$ in water after 108h. Another $\text{NH}_4\text{-N}$ -degrading bacterium, *Acinetobacter baumannii* strain YX3 [11], removed 90.69% of 148.48 mg/L $\text{NH}_4\text{-N}$ and 84.65% of $\text{NH}_4\text{-N}$ (148.48 mg/L) converted to $\text{NO}_3\text{-N}$ under sufficient carbon source. In this study, strain C degraded 42.8% of 192.1 mg/L $\text{NH}_4\text{-N}$ in 48h due to an insufficient carbon source. The three strains mentioned above were isolated from wastewater, and they adapted to a water environment with a high pollution level, so their ability to degrade $\text{NH}_4\text{-N}$ was higher than strain C's. Moreover, strain C could not degrade $\text{NO}_3\text{-N}$, which may have led to the accumulation of $\text{NO}_3\text{-N}$, which in turn affected the ability to remove $\text{NH}_4\text{-N}$.

4.2 Effect of temperature and pH on $\text{NH}_4\text{-N}$ removal

The $\text{NH}_4\text{-N}$ removal rate was lowest at 20°C, indicating that lower temperatures inhibited strain C's ability to remove $\text{NH}_4\text{-N}$ (Figure 5). However, the decrease in $\text{NH}_4\text{-N}$ removal rates at temperatures higher than 35°C showed that higher temperature also suppressed $\text{NH}_4\text{-N}$ removal. The greatest OD_{600} was observed at 30–35°C indicating that the cell density of strain C was largest at this temperature range. This explains the reason why the highest $\text{NH}_4\text{-N}$ removal rate was also observed at this temperature range. Below 30°C, the cell density and biomass yields of strain C increased with temperature. Higher temperatures caused some strain C to die, while excessive temperatures (> 35°C) could lead to thermal passivation of cellular enzymes of strain C [33], leading to the decrease of the biomass yields. This process is irreversible, which may result in a massive death of strain C, and only a small number of surviving microorganisms. Thus, the growth of strain C was significantly affected by temperature. According to national standards “fecal harmless health standard” (GB7959-87), the compost temperature was generally greater than 50°C during the manure fermentation process, which probably inhibited the growth of strain C. Therefore, strain C was not suitable for the application of fermented manure to improve $\text{NH}_4\text{-N}$ degradation. However, the temperature of water in wastewater treatment is generally less than 40 °C [34], suggesting that this strain may be used for enhanced wastewater treatment.

$\text{NH}_4\text{-N}$ removal was also affected by the pH of wastewater. Different types of wastewater, such as industrial wastewater and agricultural wastewater, vary in pH because of their different sources and content. Previous studies demonstrated that most bacteria can

remove $\text{NH}_4\text{-N}$ within a narrow pH range (6.0-8.5), and highly acidic or alkaline conditions have negative effects on bacterial activity, limiting their ability to remove pollutants from wastewater [35-37]. In this study, due to insufficient carbon source, only 38.4% of $\text{NH}_4\text{-N}$ was removed at pH 5, while between pH 4 and 7 stronger microbial activity was evident, which promoted $\text{NH}_4\text{-N}$ removal. This indicates that strain C could grow in a slightly acidic and neutral environment. Low bacterial growth above a pH of 8 was probably the result of the loss of water cells due to excessive negative ionic attraction and exchange. Above pH 8, less degradation of $\text{NH}_4\text{-N}$ was detected, which indicated that alkaline (pH 8–9) conditions inhibited strain C growth. Strain C's negative response to low pH might be diminished by the presence of tryptone in the lysogenic broth medium which could improve the acid tolerance [37-39]. Thus, strain C was crucially found to be a pH-resistant bacteria.

5 Conclusion

Strain C bacterium was isolated from chicken manure. 16S rRNA gene sequence analysis results indicated that strain C belongs to a family of Chryseobacterium and it can degrade ammonium nitrogen. It can remove 45% of ammonium-nitrogen during the active growth cycle. This indicates that strain C may be promising for treating highly ammonium-contaminated wastewater, especially from the livestock and poultry industries as well as from the food industry as they are high-carbon-source which enhances the growth of this strain.

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