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Heterotrophic nitrification and aerobic denitrification by the bacterium *Pseudomonas stutzeri* YZN-001

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ABSTRACT

A strain YZN-001 was isolated from swine manure effluent and was identified as *Pseudomonas stutzeri*. It can utilise not only nitrate and nitrite, but also ammonium. The strain had the capability to fully remove as much as 275.08 mg L^{-1} NO_3^-–N and 171.40 mg L^{-1} NO_2^-–N under aerobic conditions. Furthermore, At 30 °C, the utilization of ammonium is approximately 95% by 18 h with a similar level removed by 72 h and 2 weeks at 10 and 4 °C, respectively. Triplicate sets of tightly sealed serum bottles were used to test the heterotrophic nitrifying ability of *P. stutzeri* YZN-001. The results showing that 39% of removed NH_4^+–N was completely oxidised to nitrogen gas by 18 h. Indicating that the strain has heterotrophic nitrification and aerobic denitrification abilities, with the notable ability to remove ammonium at low temperatures, demonstrating a potential using the strain for future application in waste water treatment.

1. Introduction

At present, the removal of nitrogenous substances from industrial and domestic waste waters has received increased attention because of the role these have played in eutrophication of receiving waters. Conventional methods for nitrogen removal include chemical, physical and biological processes (Chen et al., 2003). Recently, nitrogen removed by biological means had been found to be a widely adopted approach for waste water treatment due to its ease of implementation, efficiency, and cost benefit (Khardenavis et al., 2007; Kim et al., 2005). The most common biological processes include nitrification, which oxidises ammonium via nitrite to nitrate by autotrophs under aerobic conditions, and denitrification, which converts nitrite and nitrate to N_2 gas by denitrifying bacteria under anaerobic conditions (Chang et al., in press; Ji and Chen, 2010). Therefore, this process consists of several distinct metabolic steps, and both aerobic and anaerobic conditions must be prepared (Daniel et al., 2009).

For the past few years, certain groups of heterotrophic-nitrifying bacteria such as *Paracoccus denitrification, Thiosphaera pantotropha, Commamonas* sp., *and Alcaligences faecalis* were reported to have heterotrophic nitrification and aerobic denitrification ability, and as such may be used to overcome this problem in waste water treatment systems (Blaszczyk, 1993; Gumaelius et al., 2001; Hooijmans et al., 1990; Joo et al., 2005). These microorganisms could be utilised to reduce the costs associated with maintaining an anoxic tank or to reduce the size of the tank required. The capability of aerobic denitrification under high oxygen atmosphere by *Pseudomonas stutzeri* was demonstrated by Su et al. (2001), with a nitrate removal rate of 0.032 mmol NO_3^-–N g $cell^{-1}$ h^{-1} after a 44-h incubation.

P. stutzeri strains have been classified into three groups based on their mode of denitrification. The first group includes 50% of the isolated strains, which directly reduce nitrate to nitrogen gas without accumulating nitrite. The second group possesses a two-phase denitrification process and encompasses 25% of the isolated strains, and the remaining isolates accumulate nitrite at low concentration (Su et al., 2001). The studies on *P. stutzeri* strains have focused on their ability to remove nitrate and nitrite, and research on the strains ability to remove ammonium removal is rare. Moreover, these strains are typically cultivated at 30 °C; thus, the characterisation of the strains capable of removing nitrogen at low temperatures has not been reported (Hamedaani et al., 2004; Rezaee et al., 2008). Here, we attempted to isolate bacteria from pig manure effluent and determine their ability to remove nitrogen under aerobic and low temperature conditions.

2. Methods

2.1. Isolation and identification of heterotrophic nitrifying bacteria

One litre basic screening medium was prepared by dissolving 0.5 g of $(NH_4)_2SO_4$, 5.95 g of sodium succinate, and 50 mL of a trace elements solution that consists of the following components (per litre): 5 g of K_2HPO_4, 2.5 g of $MgSO_4{\cdot}7H_2O$ and NaCl, 0.05 g of

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$MnSO_4{\cdot}4H_2O$ and $FeSO_4{\cdot}7H_2O$. Ten grams of sludge samples, collected from a piggery waste water treatment system, were added to Erlenmeyer flasks containing 90 mL autoclaved sterile medium and incubated at 30 °C for 1 week. Serial dilutions were then made and spread on solidified basic screening medium containing 2% (w/v) agar and incubated at 30 °C until visible colonies had formed. Separate colonies were chosen and were individually tested for nitrifying activity by growing the culture for 48 h. Initial identification schemes were performed with biochemical tests as suggested by the Bergey's Manual of Systematic Bacteriology and Systematic Determinative Manual of General Bacteria (Garrity et al., 2004). Simultaneously, the molecular taxonomy of the isolates was determined by sequencing their 16S rDNA according to Su et al., 2006.

2.2. Assessment of ammonium oxidation at different temperatures

To observe the effect of incubation temperature on ammonium removal, 100 mL of the basic medium was placed in a 250 mL shaking flask, and a 1% preculture of the isolate was inoculated. The medium was then incubated at five different temperatures of 4, 10, 20, 30 and 37 °C for approximately 36 h. Aliquots of 5 mL were removed periodically for chemical analysis and measurement of cell density.

2.3. Assessment of nitrite and nitrate removal

Two nitrogen compounds, nitrite and nitrate, were used instead of ammonium in the basic medium to elucidate the denitrification process of the isolate. NO_2^-–N and NO_3^-–N were adjusted to 200 and 300 mg L^{-1}, respectively. For each compound, the amount of succinate as the carbon source was changed to adjust the Carbon/Nitrogen(C/N) to 10. Then, 4.5 mL of cell suspension was inoculated into triplicate 250 mL Erlenmeyer flasks with 150 mL sterile medium and was incubated aerobically at 30 °C at 150 rpm about 36 h.

2.4. Estimation of heterotrophic nitrification and aerobic denitrification capability

Fifty millilitres of basal medium was placed in triplicate 125 mL tightly sealed glass serum bottles. These bottles were evacuated, and pure oxygen was fully pressurised into the bottles three times before autoclaving. A bacterial suspension (1% inoculum) of the isolate was inoculated into the triplicate bottles and was incubated at 30 °C. Samples were taken from the bottles periodically to determine OD_{600}, NH_4^+–N, NO_2^-–N, NO_3^-–N, O_2 and N_2 concentrations.

2.5. Assessment of nitrite and nitrate removal in domestic sewage

After a 24-h incubation, Strain YZN-001 was transferred into domestic sewage at dosage of 5%(V/V). Samples were collected at 72 h. The ammonia concentration was then determined by Nessler assay at a wavelength of 420 nm.

2.6. Analytical methods

The growth of isolates was tested by spectrophotometry at a wavelength of 600 nm. In addition, culture samples were centrifuged at 8000 rpm for 10 min to allow for chemical analysis. The ammonia concentration was then determined by Nessler assay at a wavelength of 420 nm. The nitrate concentration was determined using a UV spectrophotometric screening method and by calculating the difference between OD_{220} and $2 \times OD_{275}$. The nitrite concentration was determined by colorimetry at a wavelength of 540 nm according to the State Environmental Protection Administration of China (2002). For oxygen and nitrogen determination, 20 μL of each sample (injection volume) was removed from the headspace of the serum bottles and was analysed by GC/TCD (Kim et al., 2005). The final concentration of oxygen and nitrogen were quantified by the methods proposed by Su et al. (2001).

2.7. Statistical analysis

Data in this experiment was analysed by Microsoft excel and SPSS12.0 software. The nitrification ratio formula is $(C_0–C_n)/h$. C_0 is initial concentration of NH_4^+–N(NO_2^-N or NO_3^-N). C_n is the final concentration of NH_4^+–N(NO_2^-N or NO_3^-N) at n hour. h is the time of YZN-001 treatment. Ammonium removal rate formula in domestic sewage treatment is $(C_0–C_n)/C_0 \times 100\%$. C_0 is initial concentration of NH_4^+–N. C_n is the final concentration of NH_4^+–N.

3. Results and discussion

3.1. Results of isolation and identification of heterotrophic nitrifying bacteria

Four distinct colonies that can utilise ammonia were obtained on the agar plates after a 24-h incubation. Strain YZN-001 was selected based on ammonia removal and nitrite accumulation. YZN-001 was a rod-shaped, Gram-negative, motile, urea hydrolysis-negative, catalase-positive and oxidase-positive bacterium. It grew well on the basic medium and could utilise NH_4^+–N at 4 °C. There was a low accumulation of NO_2^-–N, and it disappeared completely with the growth of strain YZN-001. The nucleotide sequence of the 16S rDNA gene of *P. stutzeri* YZN-001 (1481 bp, Accession No.: FJ869912) was 99% identical to *P. stutzeri* from the NCBI GenBank database. Phylogenetic analysis in NCBI showed that strain YZN-001 Phylogenetic relationship is the most closed with *P. stutzeri* M16-9-2. Morphological and biochemical characteristics, along with the phylogenic identification of the isolate using 16S rDNA sequence analysis, indicate that the bacterium is *P. stutzeri*.

3.2. Growth and ammonia removal at different temperatures

The growth and ammonia removal characteristics of *P. stutzeri* YZN-001 at different temperatures in the basic medium was investigated in shaking cultures, as shown in Fig. 1. Similarly to *Pseudomonas* sp. ASM-2-3 (Kariminiaae-Hamedaani et al., 2004), *P. stutzeri* YZN-001 was able to grow at temperatures between 4 and 45 °C. Although the growth ratio of *P. stutzeri* YZN-001 was low, at 4 °C, it could oxidise 106.30 ± 7.82 mg L^{-1} NH_4^+–N after 2 weeks, and the nitrification ratio of NH_4^+–N was approximately 0.3 mg L^{-1} NH_4^+–N h^{-1} (Fig. 1A). When the temperature increased from 4 to 10 °C, NH_4^+–N was reduced from 109.35 ± 1.09 mg L^{-1} to 2.89 ± 2.21 mg L^{-1} after 72 h, and the nitrification ratio was 1.48 mg L^{-1} NH_4^+–N h^{-1} (Fig. 1B and Table 1). *Pseudomonas* sp. ASM-2-3 could be used for nitrate removal at low temperatures, but its ability to remove ammonium was not reported. The ammonium removal characteristics of *P. stutzeri* YZN-001 at 20, 30 and 37 °C were similar to *Alcaligenes faecalis* No. 4 (Joo et al., 2005). Patterns of ammonium removal at 30 and 37 °C were nearly the same; 110 mg L^{-1} NH_4^+–N was consumed after 18 h, and the nitrification ratio of NH_4^+–N were 5.53 and 5.39 mg L^{-1} NH_4^+–N h^{-1}, respectively. This value was approximately 5 times larger than the value of 1.15 mg L^{-1} NH_4^+–N h^{-1} of *P. alcaligenes* AS-1 (Su et al., 2006). However, at 20 °C, a long period was observed where there was no ammonium removal, and the maximum ammonium nitrification ratio was 4.20 mg L^{-1} NH_4^+–N h^{-1} (Fig. 1C and Table 1). Experimental results indicate that *P. stutzeri* YZN-001 utilises or converts NH_4^+–N during the growing phase, and the OD_{600} of *P. stutzeri* YZN-001 reached the stationary growth phase after 18 h at 20, 30 and