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Full Length Research Paper

A study on nitrogen removal efficiency of *Pseudomonas stutzeri* strains isolated from an anaerobic/anoxic/oxic wastewater treatment process

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In order to improve the nitrogen removal efficiency in an anaerobic/anoxic/oxic treatment plant, a strain with high nitrification and denitrification capability was isolated from a specific anaerobic/anoxic/oxic treatment process. The characteristics of isolate were experimentally analyzed. By using the nitrogen balance method, the total nitrogen loss was calculated to be 40.1% (w/w) when the carbon source was citric acid with a C/N ratio of 5. Meanwhile, the isolated strain was identified by 16S rDNA to be a *Pseudomonas stutzeri* with a similarity of 99%. Varying the initial TN, the C/N, the pH value and the ambient temperature in the reaction system, the efficiency of nitrogen removal was studied. The results showed that the highest efficiency occurred when the C/N was 12, the pH value was 7 and the temperature was 32°C. The results were also compared to the practically monitoring data coming with a good agreement. Consequently, it is viable to improve the nitrogen removal efficiency by varying the reaction conditions.

Key words: Anaerobic/anoxic/oxic treatment process, reaction condition, denitrification, nitrification, nitrogen removal, *Pseudomonas stutzeri.*

INTRODUCTION

Nitrogen biological removal is normally carried out by different bacteria groups in two steps. Firstly, ammonium is aerobically removed by autotrophic bacteria and changed into nitrate and nitrite ($NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$), which is referred to as nitrification. Secondly, the nitrate and the nitrite are anaerobically converted to N_2 by heterotrophic bacteria ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$,), which is called denitrification (Robertson et al., 1983). Usually, it is claimed that only the autotrophic nitrifying bacteria can perform an efficient nitrification and the denitrification can only occur under a strictly anaerobic condition. However, Robertson had found that the *T. pantotropha* was capable of both heterotrophic nitrification and aerobic denitrification with the N_2 as the final product (Robertson et al., 1988). These bacteria are easy to be found in different waste water treatment systems. In theory, the nitrogen removal efficiency of treatment processes is determined by the biological reactions of bacteria. Therefore, the reaction conditions also strongly influence the treatment process efficiency. Consequently, the most efficient reaction conditions, which actually lead to the working conditions of a waste water treatment plant due to the highest efficiency acquired, is worthy to be experimentally found out. So far, several different bacteria found to heterotrophically nitrify as well as to aerobically denitrify, such

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as the *Paracoccus denitrificans,* the *Alcaligenes faecalis,* the *Comamonas* sp. and the *Diaphorobacter* sp., which had been isolated by researchers (Kim et al., 2005; Lin et al., 2007). The effects of reaction conditions on the nitrogen removal efficiency were also studied. In accordance with the literatures, it is summarized that the efficiency increases strongly as the ratio of C/N increases when the value of C/N is less than 10; the highest efficiency is obtained at 25 - 35°C and under a pH value of 6 - 8; as the initial total nitrogen increases, the nitrogen removal efficiency decreases (Bernat et al., 2007; Khardenavis et al., 2007; Munch et al., 1996; Niel van et al., 1992; Chen et al., 2003). The *Pseudomonas stutzer* was also reported with an aerobic denitrification and nitrification capability (Su et al., 2001). However, there is no reported experiment which has been conducted to study the effect of reaction conditions on the nitrogen removal efficiency of the *P. stutzeri*.

In this paper, a strain was isolated from an anaerobic/anoxic/oxic tank. The tank has been built in a waste water treatment plant in Guangzhou. As the nitrogen in the inlet water strongly varies, the effect of the treatment process is mainly determined by the nitrogen removal efficiency. So it is much worthy to figure out the most efficient reaction conditions for the bacteria in the tank. Initially, in order to study the bacteria in it, the strain isolated was experimentally analyzed and its characteristics were studied. After which, the efficiency of the bacteria in nitrification–denitrification process was investigated under different reaction conditions. By this way, the most efficient working condition of the system was obtained.

MATERIALS AND METHODS

Enrichment and isolation of strain

The sludge obtained from the anaerobic/anoxic/oxic treatment system was inoculated into an enrichment liquid medium, so called enrichment. The bacteria in the sludge grew in a temperature-controlled shaker at 32°C with a 150 r/min rotating speed (Ahn, 2006). The inoculation process was repeated every four days. After 28 days, the enrichment liquid was spread in lines by using a platinum loop on a medium plate which was made of the nitrification with 1.5% (w/v) nutrient agar. Then purified strains were isolated by repeated spreading for 3 times. The isolate was stored in independent medium plates. Finally, the isolate was inoculated in the characterization medium to assess the efficiency of utilizing NH_4^+-N and NO_3^--N (or NO_2^--N) as the nitrogen source.

Characterizing the isolated strain

Sodium citrate was used as carbon source, with a C/N ratio of 5. The operating temperature was 32°C and the pure oxygen was applied as the oxygen source. The initial concentrations of different substrates were (mg/l): NH_4^+-N, 104.7; NO_2^--N, 0.0; NO_3^--N, 0.3; TN, 115.1; and C/N, 5. The bacterial suspension (inoculums with concentration of 5% w/v or v/v) of the isolated strain was inoculated to a 200 ml sterilized medium by using a 500 ml sealed flasks. The upper part of the flask was charged with oxygen at a purity of 98% (v/v) under a 100 kPa pressure. The pH value of the medium was 7 initially, and the bacteria in the flask were incubated under 150 r/min and 32°C conditions for 96 h. Samples taken from the flasks were measured at an interval of 8 h from the beginning of incubation. DO, pH, dry weight, NH_4^+-N, NO_2^--N, NO_3^--N and total nitrogen were recorded. Gas left in the flasks was injected into the 20% (w/w) H_2SO_4 for measuring the NH_3.

Phenotypical and chemotaxonomical characterization

The Gram staining and the agar plate streaking were applied to obtain the microscopic features of the isolated strains. The cell size and the morphology of the isolated strains were determined microscopically by using a microscope. The DNA extraction box, the PCR and the DGGE were used to obtain the pure DNA. Then the 16S rDNA was used to identify the strain. The F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and the R1522 (5'-AAGGAGGTGATCCAGCCGCA-3') were used as the upstream and downstream primer respectively to amplify their 16S rDNA. The amplification was conducted by PCR. Finally, the 16S rDNA sequence obtained was compared to the microorganisms in the Genbank (http://www.ncbi.nlm.nih.gov/BankIt).

Varying the reaction conditions

In order to find out the most efficient reaction conditions, four different parameters were varied during the experiments, which were the initial TN, the ratio of C/N, the pH value and the ambient temperature. The reference condition was that the initial total nitrogen was 115.0 mg/l, the C/N was 5, the pH value was 7 and the ambient temperature was 32°C. The sodium citrate was used as the carbon source. Varying one of the parameters, the experimental process was repeated with the total nitrogen loss measured when the experiment lasted for 96 h after inoculation. Meanwhile, the effect of reaction conditions on the efficiency of the nitrogen removal was studied. In this case, LTN (the total nitrogen in the supernatant fluid of bacterial suspension) and the TN of bacterial suspension were tested to calculate the nitrogen lost in the flask. The amount of the nitrogen gas generated in the experiment was calculated to be the nitrogen lost in the flask (Joo et al., 2005).

For measuring the efficiency of nitrification and denitrification, NH_4Cl in the medium was chosen as the main nitrogen source. The only organic nitrogen source in the medium was EDTA as the other nitrogen source. The nitrification rate was calculated in terms of the reduction of NH_4^+-N, NO_2^--N, and NO_3^--N concentration in the suspensions. The denitrification rate was estimated based on the combination of the decrease of TN, the decrease of NH_4^+-N concentration, the increase of NO_2^--N concentration and the variation of NO_3^--N concentration. The nitrogen balance was applied to calculate the total nitrogen loss in the medium.

Analytical methods

The bacterial suspension was centrifuged at a speed of 6000 r/min for 10 min. The supernatant fluid was used to measure the NO_3^--N. The total nitrogen in the liquid and in the cell pellet was measured, respectively. Biomass nitrogen was the total nitrogen in the cell pellet. The Biomass nitrogen and Organic nitrogen was calculated by using the following expressions:

(1) TN = LTN +Biomass nitrogen;
(2) LTN = NH_4^+-N + NO_3^--N + NO_2^--N + Organic nitrogen.

where initial total nitrogen was determined by the ultraviolet adsorption and ascorbic acid reduction method. The pH value was determined by pH test papers. The DO was measured with

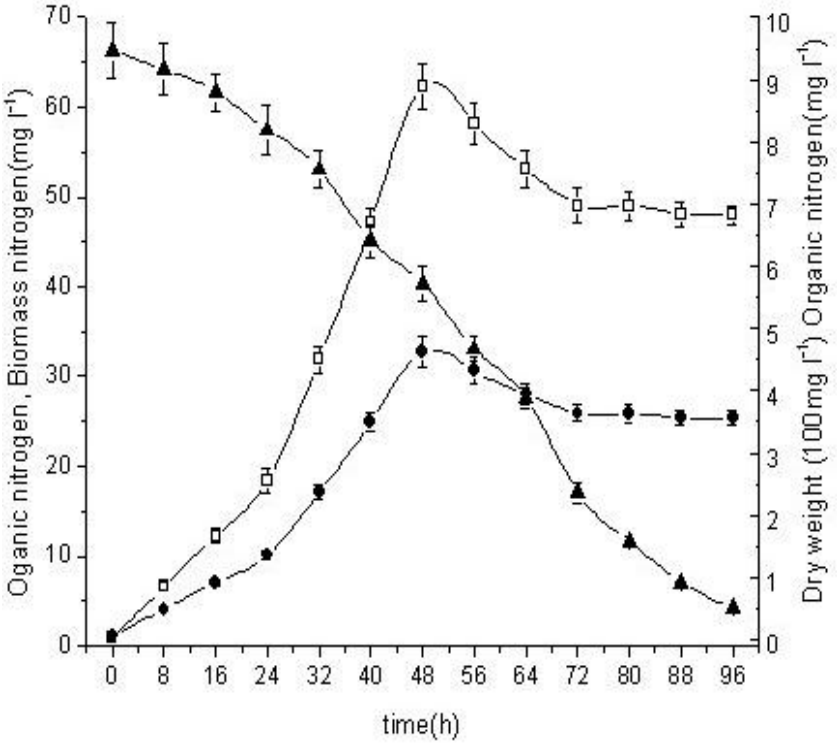

Figure 1. Changes in Dry weight, Biomass nitrogen and Organic nitrogen of WYT1 under reference condition (● Dry weight; □ Biomass nitrogen; ▲ Organic nitrogen).

dissolved oxygen meter. The dry cell weights were converted from OD600 values by using a linear regression equation which was Dry weights (g/l) = 0.49OD. Estimation of NH_4^+-N was made by a Nessler's reagent method monitoring the absorbency at 420 nm. Nitrate was measured by an ultraviolet adsorption and alkaline potassium persulfate digestion method (UV). Nitrite was measured by N-(1- naphthalene)- ethylene method monitoring the absorbency at 540 nm.

Statistical analysis

The standard error of each mean data was calculated by using the equations as follow:

$$\varepsilon = s/n^{0.5} \quad (3)$$

$$S = \sqrt{\frac{\sum_{i=1}^{n} (m_i - M)^2}{n-1}} \quad (4)$$

where n is the number of measurements, m_i are the data and M is the average value. Each experiment runs in triplicate. A flask with medium and without bacteria is used as a blank sample.

RESULTS AND DISCUSSION

Isolation of the strain

A specific strain named WYT1 was experimentally isolated. The isolate is a rod with a diameter of 0.5 mm and is 1.0 to 3.0 mm long. It is Gram negative. The colony of is opaque, yellow and round.

Variation of Dry weight, DO and pH value

The dry weight, the Organic nitrogen and the Biomass nitrogen under the reference condition are presented in Figure 1. As shown in the Figure 1, the dry weight increased rapidly during the first 48 h after the medium was inoculated. When the dry weight reached a peak of 0.46 g/l, the concentration of Biomass nitrogen reaches the highest value of 62.3 mg/l. On the other hand, the Organic nitrogen decreased much fast in the whole process until it gets to about 0.5 mg/l.

The DO in the medium varied between 1.0 and 1.2 mg/l. The lowest DO, 1.0 mg/l, appeared at 72 h after inoculation. Then the DO increased again and ended up with a highest value of 1.2 mg/l. The pH value in the medium maintains between 6.5 and 7.5. The lowest pH value of 6.5 appeared simultaneously with the lowest DO. At that moment, NO_3^--N in the experiment reached its highest value, which indicated that the nitrification caused the decreases of pH value and DO.

Characteristics of the isolated strain

The NH_4^+-N, NO_2^--N, NO_3^--N, LTN and TN under the

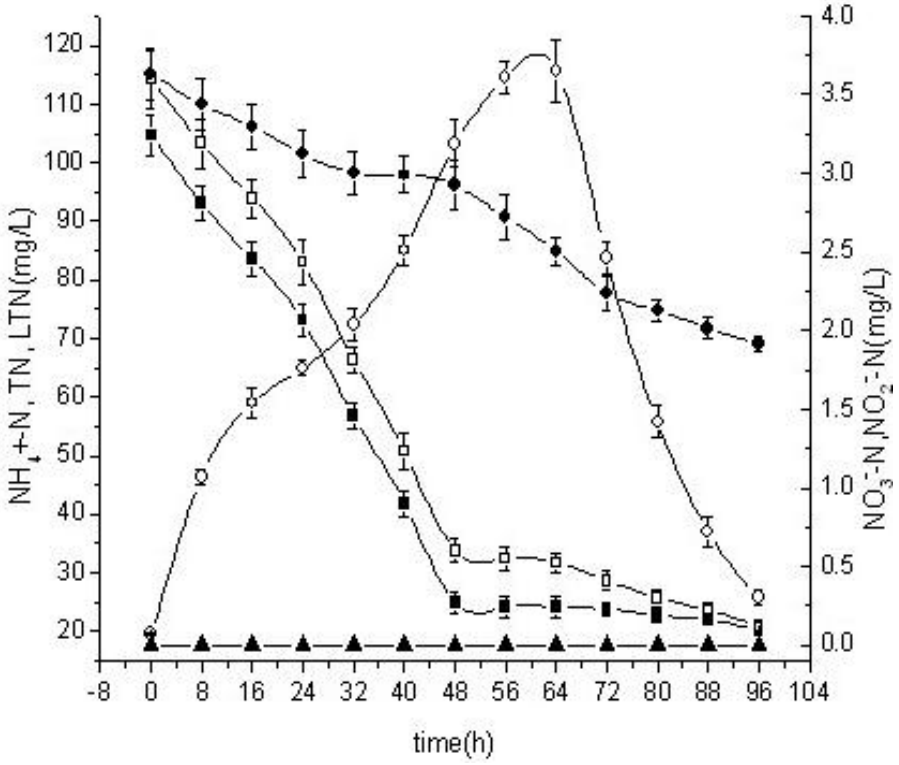

Figure 2. Changes in NH_4^+-N, LTN, TN, NO_2^--N, NO_3^--N of WYT1 under reference condition (■ NH_4^+-N, □ LTN, ● TN, ▲ NO_2^--N, ○ NO_3^--N)

reference condition are shown in Figure 2. During the growth period of the bacteria, the concentration of NH_4^+-N decreased sharply. As the main nitrogen source in the liquid was NH_4^+-N, the LTN also rapidly decreased. The Biomass nitrogen increased at its fastest speed due to the same reason. The concentration of NO_3^--N increased during the same period because of the nitrification. In the first 48 h, 80.4 mg/l LTN was consumed and the NO_3^--N concentration increased to 3.2 mg/l, however only 19.0 mg/l TN concentration was reduced. The results showed that the nitrogen was used to synthesize the bacteria at the initial stage rather than generating nitrogen by nitrification-denitrification.

The NO_3^--N increased strongly without any nitrite build-up throughout the experiment. This result has also been reported by others (Joo et al., 2005). As shown in Figure 2, the 23.0% (w/w) decrease of dry weight during the last 48 h showed that the cells started to break down. The organic nitrogen was decomposed and converted to NH_4^+-N or NO_3^--N, which was faster than the organic nitrogen dissolution. The consumption rate of nitrogen in the nitrification-denitrification process was much faster than the rate of the organic nitrogen generation. Therefore, the break down of bacterial cells did not cause an increase of the organic nitrogen and the LTN. However, the decreasing rate of NH_4^+-N was slowed down due to the conversion of organic nitrogen to NH_4^+-N.

During the growth period of the bacteria (the first 48 h), the concentrations of NH_4^+-N, LTN and NO_3^--N decreased much rapidly. After which, the removal rate of NH_4^+-N was 76.2% (w/w). Then the decrease slowed down until the removed NH_4^+-N reached about 80.7% (w/w). The trend of LTN decrease was similar with NH_4^+-N. The final removal rate of LTN in the medium was 81.6% (w/w). The concentration of NH_4^+-N in a 20% (w/w) H_2SO_4 solution was maintained at 0 mg/l, so the NH_4^+-N decrease in medium was cause by bacterial.

Taxonomic identification of the isolated strain

The 16S rDNA sequence of WYT1 was submitted to the GenBank databases under accession no. FJ768951. The isolated strain was identified by 16S rDNA to be a species of *P. stutzeri* with a similarity of 99%.

Nitrogen removal efficiency under different reaction conditions

As shown in Table 1, although the total loss of LTN increased, the removal rate of LTN decreased as the initial TN increased. The removal rate increased strongly with the increase of C/N when the value C/N was less

Table 1. Removal rate of LTN under different reaction conditions.

Initial TN (mg/l)	**15**	**30**	**45**	**75**	**115**
Removal rate of LTN (%)	96.0	94.2	93.1	85.9	81.6
C/N	**2**	**5**	**8**	**10**	**12**
Removal rate of LTN (%)	65.5	81.6	89.4	92.6	92.8
pH	**4**	**6**	**7**	**9**	**10**
Removal rate of LTN (%)	45.7	65.7	81.6	79.4	70.1
Temperature (°C)	**5**	**15**	**25**	**32**	**37**
Removal rate of LTN (%)	51.7	65.2	71.4	81.6	79.5

than 8. On the other hand, when the value of C/N was greater than 8, increasing C/N led to little improvement in the removal rate of LTN. Therefore, it is not cost-effective to enhance the LTN removal rate by simply increasing the ratio of C/N as long as its value exceeds 8. It can also be found in Table 1 that the maximum removal rate of LTN was obtained under the pH value of 7 and the acidic environment had more significant impact on the bacteria compared to the alkaline environment where the pH value was much greater than 7. Meanwhile, the removal rate increased as the ambient temperature increased and reached the peak value at 32°C. It slightly decreased when the ambient temperature increased from 32 to 37°C. The maximum TN removal efficiency of waste water treatment plant occurs at an ambient temperature of about 35°C (Yu et al., 2008). The experiment results agree well with the monitoring data from the plant. That means the essential characteristic of the treatment process could be presented by WYT1. As a result, the way by which the efficiency of the plant is improved is deduced as: increase of the C/N to 8, maintaining the pH value at 7 and increase of the LTN of the inlet water to be 60 mg/l.

The denitrification efficiency of WYT1 is higher than the *T. pantotropha ATCC 35512* isolated by Su in 2001. The denitrification efficiency of *T. pantotropha ATCC 35512* in 72 h was 27.29% (Su et al., 2001).

In conclusion, the results showed that the WYT1 was capable of concurrent aerobic nitrification-denitrification and was identified to be a species of *P. stutzeri*. The maximum nitrogen removal rate was obtained at 32°C when the pH value equalled to 7. The experiment results agree well with the monitoring data of the plant, which means the essential characteristic can be presented by WYT1. Meanwhile, several ways by which the efficiency of the waste water treatment process can be improved is deduced in terms of the experiment results.

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