Heterotrophic nitrification and aerobic denitrification using pure-culture bacteria for wastewater treatment
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ABSTRACT

Due to the high water demand and unsustainable water resource, wastewater reclamation and wastewater treatment prior to discharge have become current important issues. Various treatment technologies, such as biological processes, have been improved as alternatives. In this study, the biological nitrogen removal system using pure-culture Bacillus licheniformis was developed and used as an internal treatment unit in an aquarium to improve the effluent quality for water reuse. The efficiencies for NH4-N and total nitrogen (TN) removal and the quality of treated water verified the occurrence of heterotrophic nitrification and aerobic denitrification; the nitrification rate was 0.84 mg/L-h and the denitrification rate was 0.62 mg/L-h. The maximal NH4-N and TN removal efficiencies were approximately 73% at the influent NH4-N of 30 mg/L. However, the other competitive heterotroph of Pseudomonas sp. was observed, which resulted in dramatically decreasing efficiencies and an enlarged ratio of carbon consumption and nitrogen removal. Although the overall performance of the B. licheniformis system was lower than the system using mixed-culture nitrifying and heterotrophic denitrifying microorganisms, the advantages of the B. licheniformis system were ease of operation and the fact that it is a land-limited treatment system. The research is ongoing to enhance performance and maintain excellent efficiency in a long-term operation.

Key words | Bacillus licheniformis, biological nitrogen removal, heterotrophic nitrification and aerobic denitrification, wastewater treatment

INTRODUCTION

Over the past decade, the demand for fresh water has drastically increased with rapid growth in population, global climate change and growing scarcity of surface water and groundwater resources. The lack of fresh water has accelerated efforts to improve the current treatment technology for reclamation of wastewater, such as domestic wastewater. The concept of water reclamation is to remove a high pollutant concentration in the wastewater to an acceptable level, and then reuse the water for other purposes, such as agricultural irrigation, surface water restoration, groundwater recharge and industrial manufacturing processes (Maryam & Buyukgungor 2011). One of the most important pollutants is ammonium-nitrogen (NH4-N) which is commonly found in many wastewater sources including household, industry, landfill and aquaculture. In the meanwhile, the discharge of NH4-N wastewater to the environment causes a poor quality of water resource and consequently affects water pollution from eutrophication.
The nitrogen contamination including NH₄-N and its oxidized forms of nitrate (NO₃-N) and nitrite (NO₂-N) cannot be removed by common filtration, and the consumption of such contaminated water has negative impacts to human health, in particularly infants and pregnant women. The maximal level of nitrogen in safe drinking water has been set at 1.5 mg/L as NH₄-N, 1.0 mg/L as NO₂-N and 11.3 mg/L as NO₃-N (World Health Organization (WHO) 2001). Similarly, the standard limitation of nitrogen in the reclaimable water is established and dependent on the type of water reuse, for example 45 mg/L as total nitrogen (TN) for restricted irrigation (i.e., orchards, industrial crops and fodder) and 30 mg/L as TN for unrestricted irrigation (i.e., public parks and urban uses) (Massachusetts Institute of Technology (MIT) 2012).

Various advanced technologies have been proposed for water reclamation and reuse, especially for nitrogen removal; membrane filtration and biological processes are examples (Shrimali & Singh 2001). Due to the limit of using membranes, in terms of scaling and treatment capacity, the application of membrane filtration for wastewater treatment is inappropriate in some areas. On the other hand, the biological nitrogen removal process includes an aerobic condition in which nitrification occurs by autotroph (NH₄-N to NO₃-N conversion) and an anoxic condition to allow denitrification by heterotroph (NO₃-N to nitrogen gas conversion). The nitrification and denitrification processes are difficult to jointly operate in a single reactor, due to the low growth rate of autotrophic nitrifying microorganisms and the different environments for achieving nitrifying and denitrifying activity. Recently, the simultaneous nitrification and denitrification (SND) system was developed to achieve nitrogen removal using either immobilized or suspended sludge. The system required a low dissolved oxygen (DO) of about 0.3–0.8 mg/L during the treatment, and the coexistence of nitrifying and denitrifying microorganisms was found (Pochana & Keller 1999; Peng & Qi 2007). To enhance the nitrification and denitrification rates, the SND system was operated via an intermittent air supply for providing the aerobic and anoxic conditions. The rates of nitrification and denitrification reached 18 and 6 mg/L-h, respectively (Khanitchaidecha et al. 2015).

The significant factor for success in SND was an abundance of organic carbon. This is because complete denitrification cannot occur when the organic carbon is deficient. In the theoretical denitrification equation, 1.08 mole of organic carbon (i.e., methanol) is required to remove a mole of NO₃-N (Tchobanoglous & Burton 1991). However, in the practical operation of nitrification and denitrification, the ratio of organic carbon and nitrogen contents was reported in the range of 7.5–11.1 (Zhao et al. 2013). Excessive organic carbon can cause carbon contamination in the water, as measured in BOD (biochemical oxygen demand) value, which significantly affects the reclaimable water quality. The high performance of nitrogen removal at a low C/N of 2 was found (Le et al. 2015); however, the operation under low C/N encouraged a long retention time due to the decreasing denitrification rate.

Heterotrophic nitrification and aerobic denitrification is newly discovered and increasing the interest in alternative nitrogen removal. This process is capable of nitrification and denitrification simultaneously under aerobic conditions, thus the NH₄-N can be aerobically converted to nitrogenous gas. The great advantages of the process include: (i) procedural simplicity; (ii) less acclimation problem; and (iii) less buffer requirement (Su et al. 2015). However, the study of nitrogen removal via heterotrophic nitrification and aerobic denitrification has been limited to the batch experiment to identify the best operating condition (i.e., initial pH, temperature and NH₄-N concentration) and the certain groups of heterotrophic nitrifying-aerobic denitrifying microorganisms (Joo et al. 2005; Guo et al. 2015). Various microorganisms of Paracoccus denitrificans, Alcaligenes faecalis, Microvirula aerodenitrificans, Acinetobacter and Bacillus which were isolated from soils and wastewater treatment systems have been found to have capability in heterotrophic nitrification and aerobic denitrification (Takenaka et al. 2007; Yao et al. 2015). Of the above microorganisms, the Bacillus species is advantageous over the others and is the key in the wastewater treatment system. This is because Bacillus can consist of aerobes and facultative anaerobes which live in a wide range of habitats, thus a large volume of Bacillus in the treatment system is easily isolated from the environment. In addition, Bacillus is non-toxic and tolerant to temperature, pH and salt conditions.

The objective of this study was to determine the ability of Bacillus licheniformis (B. licheniformis) to remove NH₄-N, based on its heterotrophic nitrification and aerobic
denitrification. The system containing B. licheniformis carriers was started up by step-wise increasing the initial NH$_4$-N from 10 to 50 mg/L, and then continuously operated at the high NH$_4$-N concentration of 40 mg/L. The organic carbon was maintained at the C/N ratio of 3.5. The efficiencies of NH$_4$-N removal and TN removal, including NH$_4$-N, NO$_3$-N and NO$_2$-N, were used to indicate the system performance. Both values were compared to the system using cultivated sludge from a wastewater treatment plant. The purpose of this study was to demonstrate the potential of the heterotrophic nitrification and aerobic denitrification process to enable the reuse of wastewater as an alternative water resource for human activity. The outcome of this study can reduce the demand on natural water resources.

**MATERIALS AND METHODS**

**Cultured bacteria preparation**

*B. licheniformis* was selected as the heterotrophic nitrifying-aerobic denitrifying microorganisms, and its activity was determined in this study. The pure-culture *B. licheniformis* from Thailand Institute of Scientific and Technological Research was cultivated in the nutrient broth, according to the following procedure: (i) prepare the nutrient broth by dissolving 3 g of beef extract and 5 g of peptone in 1 L of distilled water; (ii) adjust the pH of the nutrient broth to 7.0 and then sterilize at 121°C, 15 psi for 15 min; and (iii) add *B. licheniformis* into the nutrient broth, and then keep it at 37°C for 24–48 hours.

**Experimental set-up**

Porous materials of approximately 2 cm diameter were used as carriers for bacteria attachment. The porous materials were cleaned and sterilized in an autoclave for 2 hours. Then, the porous materials were dipped in the concentrated *B. licheniformis* at 37°C for 24 hours and immediately transferred to the reactor (*B. licheniformis* reactor). The schematic diagram of the *B. licheniformis* reactor is presented in Figure 1; it consisted of a 5-L reactor, *B. licheniformis* attached materials (10% of reactor volume), air pump, water peristatic pump, influent and effluent tanks. The reactor represented an aquarium which generates nitrogen wastewater from protein metabolism in fish, fish feces and microbial degradation of food residues. The *B. licheniformis* attached materials referred to an internal treatment system to reduce the nitrogen concentration and avoid water pollution from nitrogen discharge. Further, the *B. licheniformis* attached materials can reduce the effluent volume and improve the effluent quality satisfactorily for water reuse.

The nitrogen wastewater (influent) was prepared with the following chemicals (per 1 L); 0.15 g of NH$_4$Cl, 0.48 g of NaHCO$_3$, 0.02 g of KH$_2$PO$_4$ and 0.48 g of CH$_3$COONa. The influent was continuously fed to the reactor with a flow rate of 3 L/d. Air was continuously supplied to maintain a high DO of ~5 mg/L. During the start-up, the NH$_4$-N concentration was step-wise increased from 10 to 50 mg/L for bacteria adaptation, as summarized in Table 1. Further, another reactor (mixed-culture reactor) was also set up in accordance with the above procedure. However, the mixed-culture nitrifying-denitrifying sludge was attached on the porous materials, instead of *B. licheniformis*. The mixed-culture sludge was taken from a successful nitrification and heterotrophic denitrification system which was operated for over 3 months. The mixed-culture reactor was operated under continuous aeration (similarly to the *B. licheniformis* reactor) and intermittent aeration for the best performance.

**Water quality analysis**

The nitrogen removal ability of *B. licheniformis* was measured by two parameters: NH$_4$-N removal efficiency and TN removal efficiency. The reduction of NH$_4$-N concentration between the influent and effluent was only relevant for the NH$_4$-N removal
efficiency (refers to nitrification). However, the reduction of all nitrogen forms including NH₄-N, NO₂-N and NO₃-N were relevant for the TN removal efficiency (refers to nitrification and denitrification). The concentrations of NH₄-N, NO₂-N and NO₃-N were measured by using phenate, colorimetric and ultraviolet spectrophotometric screening methods in accordance with the standard methods for the examination of water and wastewater (American Public Health Association (APHA) 1998). The organic carbon content was determined as total organic carbon (TOC) using a TOC analyzer (HACH, IL530 TOC-TN). The pH and DO were regularly measured using a pH meter (Eutech Instruments) and DO meter (CyberScan DO 110 Model).

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\text{NH}_4\text{-N removal efficiency (\%)} = \left(1 - \frac{\text{NH}_4\text{-N}_{\text{effluent}}}{\text{NH}_4\text{-N}_{\text{influent}}} \right) \times 100
\]

(1)

\[
\text{TN removal efficiency (\%)} = \left(1 - \frac{\text{NH}_4\text{-N}_{\text{effluent}} + \text{NO}_2\text{-N}_{\text{effluent}} + \text{NO}_3\text{-N}_{\text{effluent}}}{\text{NH}_4\text{-N}_{\text{influent}} + \text{NO}_2\text{-N}_{\text{influent}} + \text{NO}_3\text{-N}_{\text{influent}}} \right) \times 100
\]

(2)

**Microbial analysis**

The pure culture of dominant bacteria was isolated according to the following procedure: (i) randomly take biomass samples from the porous materials; (ii) isolate on the nutrient broth by spread plate and streak plant techniques and keep at 37°C for 24–48 hours; and (iii) mix the pure culture solution with glycerol 50% and direct to a private company for further molecular analysis.

**RESULTS AND DISCUSSION**

The nitrogen removal system using *B. licheniformis* was started up by increasing the influent NH₄-N concentration from 10 to 30 mg/L. The step-wise increasing of the concentration protected the *B. licheniformis* from NH₄-N shock loading. At the low NH₄-N concentration of 10 mg/L, around 7.5 mg/L of NH₄-N remained in the effluent, whereas the NO₃-N and NO₂-N were 0.5 and <0.5 mg/L, respectively. The NH₄-N removal was 34.2% which was slightly greater than the TN removal of 30.9%. Although the NH₄-N and TN removal efficiencies were relatively low in this state, the results confirm that *B. licheniformis* can consume NH₄-N as a nitrogen source for its microbial metabolisms. At the higher NH₄-N of 20 and 30 mg/L, the NH₄-N and TN removal efficiencies increased and reached stable values of 65–66%. The effluent NH₄-N was in the range of 6 to 10 mg/L, whereas low NO₂-N and NO₃-N of <0.5 mg/L were observed. Figure 2(b) shows that the efficiencies of NH₄-N and TN removal were similar values, which can be explained by the fact that the oxidized NH₄-N was completely denitrified during the high DO via heterotrophic nitrification and aerobic denitrification. The nitrogen removal pathway is suggested to be as follows: NH₄⁺ → NH₂OH → NO₂ → NO₃, then NO₃ → NO₂ → N₂ (Lei et al. 2016). During the start-up, the highest NH₄-N and TN removals of 73% were achieved at NH₄-N of 30 mg/L. The ability of heterotrophic nitrifying–aerobic denitrifying microorganisms was determined in literature using *Alcaligenes faecalis* and *Acinetobacter* sp. (Shoda & Ishikawa 2014; Su et al. 2015), and the excellent efficiency of 90–100% was obtained. The possible reasons for the lower removal efficiency compared to the previous studies are that: (i) different bacteria species were used; (ii) the system was operated
under continuous mode with high NH₄-N feeding; and (iii) the experimental conditions including C/N, pH and initial microbial concentration were different.

The ability of *B. licheniformis* for consuming NH₄-N and NO₃-N was evaluated in two batch tests: (i) NH₄-N of 40 mg/L with aeration and (ii) NO₃-N of 40 mg/L with aeration. The results are shown in Figure 3(a) and 3(b), the NH₄-N and NO₃-N were linearly reduced with the rates of 0.84 and 0.62 mg/L-h, during 24 hours. The NH₄-N consumption rate was higher than the NO₃-N consumption rate because *B. licheniformis* was familiarized with NH₄-N feeding during start-up. In this study, the NH₄-N consumption represented the nitrification ability and the NO₃-N consumption under aerobic conditions referred to the denitrification ability. Therefore, the nitrification and denitrification rates of *B. licheniformis* were lower than the autotrophic nitrification and heterotrophic denitrification rates, which were 4.23 and 4.15 mg/L-h, respectively, as reported in a previous study (Zeng *et al.* 2005).

The influent NH₄-N was increased to the highest concentration of 40 mg/L during day 16–30, as shown in Figure 2(a). Two ranges of removal efficiency were observed; around 56–67% in day 16–24, and around 25–44% in day 25–30. Figure 2(a) and 2(b) show that the efficiencies of NH₄-N and TN removals immediately decreased from 65–66% at the NH₄-N of 30 mg/L to 56% at the NH₄-N of 40 mg/L, and both NH₄-N and TN efficiencies were in the range of 56–67% for 10 days. Later, the NH₄-N and TN removal efficiencies sharply decreased to 25% and maintained efficiencies of 25–44% until finishing the experiment. During this state, the high effluent NH₄-N was found, whereas the NO₃-N and NO₂-N were nearly zero. This can be explained by the maximal capacity of this treatment system being reached, since the influent NH₄-N was controlled at 30 mg/L. Therefore, some NH₄-N at the higher concentration was untreated and then contaminated the effluent. In this study,
the highest performance was only 73% for nitrogen removal and the maximal treatment capacity was approximately 132 mg N/L-day, which was insufficient to reduce the high nitrogen contamination to the acceptable level. The limits of this study are due to: (i) the low initial bacteria concentration; (ii) no synergy effect from variety of bacterial community; and (iii) too high initial NH4-N for effective heterotrophic nitrification and aerobic denitrification.

The ratio of carbon consumption and nitrogen removal during the treatment was calculated and is presented in Figure 4. The value was approximately 0.6 at the first period of start-up (NH4-N of 10 mg/L), when B. licheniformis was unfamiliar to the NH4-N consumption. The ratio was raised to 1.2–1.5 at the higher NH4-N of 20 and 30 mg/L, achieving the best performance and abundance of active B. licheniformis. However, the ratio of carbon consumption and nitrogen removal was increased to be in the range of 2.7–3.1 in the later period of NH4-N 40 mg/L, together with the decreasing nitrogen removal efficiencies. This is possibly because of the rapid growth of competitive heterotrophic microorganisms which consumed the carbon content for their microbial metabolisms. In the meanwhile, less nitrogen was removed via such heterotrophic microorganisms. This assumption was verified by molecular analysis for the biomass attaching on the porous materials. According to the 16S rRNA gene sequence data, the results showed that Pseudomonas became the major species in the system (99% similarity; GenBank accession number: EU487511.2), instead of the B. licheniformis which was rich in the start-up. Pseudomonas was defined as heterotrophic and the aerobic growth rate was much higher than the anoxic growth rate (Koike & Hattori 1973). Although some species of Pseudomonas such as Pseudomonas stutzeri and Pseudomonas aeruginosa had the ability to denitrify NO3-N, the anoxic condition of no oxygen was necessary (Carlson & Ingraham 1985). Since Pseudomonas sp. was easily found in the environment, it was able to contaminate the B. licheniformis system via the air and water supply.

The performance of the B. licheniformis reactor for nitrogen removal was compared to the reactor using mixed-culture nitrifying and heterotrophic denitrifying microorganisms. At the operating condition of initial NH4-N 40 mg/L and continuous air supply, the B. licheniformis reactor obtained around 55.2% for both NH4-N and TN removals (From Figure 2(a)). On the other hand, the NH4-N removal efficiency reached 100% in the mixed-culture reactor, however there was almost zero TN removal. The oxidized NH4-N mainly remained in NO3-N form, as illustrated in Figure 5(a) and 5(b). Since the heterotrophic denitrification required an anoxic condition to transfer the NO3-N to gaseous nitrogen, the high DO of ~5 mg/L during the treatment prevented the denitrification from occurring. Later, the condition of the mixed-culture reactor was changed to the intermittent air supply: 2 hours aeration and 2 hours non-aeration in a cycle. From Figure 5(a), the effluent NO3-N concentration was sharply reduced to 7.1 mg/L and some NO2-N of 9.6 mg/L was found. The TN removal efficiency was immediately increased to 51.6% and continued to 72.2% at the maximum. However, the efficiency of NH4-N removal slightly dropped to approximately 78.2% during the intermittent aeration. Since the NH4-N reduction referred to nitrification and the NO3-N reduction referred to denitrification, the ability for nitrification was greater than that for denitrification in the mixed-culture reactor. From the DO measurement, the aerobic DO concentration of 5 mg/L decreased to around 1.5–2.0 mg/L in the non-aeration period. Therefore, the denitrification activity was suppressed by the remaining oxygen.

From all experimental results, at the initial NH4-N of 40 mg/L, the B. licheniformis reactor obtained the best performance of 66.5% for NH4-N and TN removal, while the mixed-culture reactor reached the highest efficiencies of 82.8% and 72.2% for NH4-N and TN removal, respectively. However, the latter reactor required specific conditions including the aeration period for nitrification and the
The performance of pure-culture heterotrophic nitrifying-aerobic denitrifying in nitrogen removal was investigated in this study. The system containing B. licheniformis was started up and operated at various NH₄-N concentrations of 10–40 mg/L and high DO of 5 mg/L. The efficiencies of NH₄-N and TN removal reached 65–66% at NH₄-N of 20 and 30 mg/L, which consequently decreased to 56–67% at the highest NH₄-N of 40 mg/L, due to the limit of treatment capacity. The effluent contained 18 mg/L as NH₄-N and <0.5 mg/L as NO₃-N and NO₂-N. On the other hand, good NH₄-N and TN removal efficiencies of 78.2% and 72.2% were found in the mixed-culture system of nitrification and heterotrophic denitrification, however this required the specific condition of intermittent aeration which became the system drawback. Further, the decreasing number of B. licheniformis and abundant Pseudomonas sp., in the B. licheniformis reactor indicates that the nitrogen removal system via heterotrophic nitrification and aerobic denitrification needs to be improved before using as the treatment system for wastewater and reclaimable water.

CONCLUSION

The performance of pure-culture heterotrophic nitrifying-aerobic denitrifying in nitrogen removal was investigated non-aeration period for effective denitrification. Therefore, the mixed-culture reactor cannot be applied to nitrogen sources which need to maintain high oxygen such as aquaculture and aquariums. On the other hand, the significant advantages of the B. licheniformis reactor are ease of operation and the fact that it is a land-limited treatment system. Although the new biological process of heterotrophic nitrification and aerobic denitrification using B. licheniformis was unsuccessful in providing acceptable effluent in this study, enhancing its performance for water reuse and reclama- tion remains a research challenge.

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