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Ochrobactrum anthropi used to control ammonium for nitrate removal by starch-stabilized nanoscale zero valent iron

Jun Zhou, Qianyu Sun, Dan Chen, Hongyu Wang and Kai Yang

ABSTRACT

In this study, the hydrogenotrophic denitrifying bacterium *Ochrobactrum anthropi* was added in to the process of nitrate removal by starch-stabilized nanoscale zero valent iron (nZVI) to minimize undesirable ammonium. The ammonium control performance and cooperative mechanism of this combined process were investigated, and batch experiments were conducted to discuss the effects of starch-stabilized nZVI dose, biomass, and pH on nitrate reduction and ammonium control of this system. The combined system achieved satisfactory performance because the anaerobic iron corrosion process generates H_2, which is used as an electron donor for the autohydrogenotrophic bacterium *Ochrobactrum anthropi* to achieve the autohydrogenotrophic denitrification process converting nitrate to N_2. When starch-stabilized nZVI dose was increased from 0.5 to 2.0 g/L, nitrate reduction rate gradually increased, and ammonium yield also increased from 9.40 to 60.51 mg/L. Nitrate removal rate gradually decreased and ammonium yield decreased from 14.93 to 2.61 mg/L with initial OD_{600} increasing from 0.015 to 0.080. The abiotic Fe^0 reduction process played a key role in nitrate removal in an acidic environment and generated large amounts of ammonium. Meanwhile, the nitrate removal rate decreased and ammonium yield also reduced in an alkaline environment.

Key words | ammonium yield, combined system, nitrate, *Ochrobactrum anthropic*, starch-stabilized nZVI

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INTRODUCTION

Currently, many studies are being carried out involving nitrate removal from water because excessive nitrate present in water can create many serious environmental and human health problems (Foglar *et al.* 2005; Grommen *et al.* 2006; Ghafari *et al.* 2008; Chaplin *et al.* 2009; Zhang *et al.* 2014). Conventional nitrate removal methods contain biological denitrification, chemical reduction, reverse osmosis, and ion exchange (Hwang *et al.* 2011; Martin & Nerenberg 2012; Ensie & Samad 2014).

The nanoscale zero valent iron (nZVI) reduction process as a chemical reduction has attracted a lot of attention due to the fact that nZVI exhibits high and stable reactivity (Song & Carraway 2008; Li *et al.* 2012) for nitrate removal. Although nZVI is effective for nitrate reduction, the major product is the undesirable ammonium (Huang & Zhang 2004; Ryu *et al.* 2011). For this reason, many studies have been conducted to optimize the final products of nitrate removal by nZVI (Biswas & Bose 2005; Liou *et al.* 2005; An *et al.* 2014). An *et al.* (2014) introduced hydrogenotrophic bacteria to an nZVI nitrate reduction system, and reported that the hydrogenotrophic bacteria decreased the Fe^0 reduction reaction and thus decreased the ammonium yield. Shin and coworkers (Shin & Cha 2008) investigated the microbial reduction of the nitrate process in the presence of nZVI, and proved that the combined nZVI–cell system was efficient for the degradation of nitrate. Biswas & Bose (2005) investigated the metallic iron-assisted abiotic nitrate reduction process to minimize the ammonium yield generated by the nZVI reduction reaction, and conducted long-time batch experiments to reveal the mechanisms for nZVI-assisted biological denitrification and less ammonium production.

The hydrogenotrophic denitrification approach refers to the process whereby hydrogenotrophic denitrifying bacteria utilize clean hydrogen as an electron donor, and inorganic carbon as energy to convert nitrate into nitrogen

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gas (Ghafari *et al.* 2008; Karanasios *et al.* 2010). This biological method is of increasing concern because, despite its high efficiency (Karanasios *et al.* 2010), the hydrogen supply is a tough problem as hydrogen is highly explosive. Hydrogen generated from anoxic corrosion of metallic iron may be a satisfactory solution. Sunger & Bose (2009) studied the hydrogenotrophic denitrification process which used hydrogen generated from anoxic corrosion of metallic iron, and discussed the nitrate removal and ammonium control performance of this combined system.

In consideration of the high toxicity of nZVI to microorganisms, there is a need to study the feasibility of using combined autohydrogenotrophic bacteria and starch-stabilized nZVI to treat the nitrate-contaminated water.

As reported, *Ochrobactrum anthropi* was one of the main hydrogenotrophic denitrifying bacteria and exhibited effective denitrification capacity in hydrogen-dependent denitrification reactors (Szekeres *et al.* 2002; Karanasios *et al.* 2010). In the present study, starch-stabilized nZVI was used to remove nitrate-polluted water, and the autohydrogenotrophic bacterium *Ochrobactrum anthropi* B2 was added to control ammonium generated by the abiotic part. The ammonium control performance and cooperative mechanism of this combined process were investigated. The aim of this study was to investigate the optimization of the combined system for nitrate removal, focusing on the effects of starch-stabilized nZVI dose, biomass, and pH on nitrate reduction and ammonium control process.

MATERIALS AND METHODS

Starch-stabilized nZVI preparation

Starch-stabilized nZVI was synthesized by adding 10.0 wt% starch on nZVI, and the nZVI particles were synthesized by the reduction reaction of $FeCl_3 \cdot 6H_2O$ solution and KBH_4 in polyvinylpyrrolidone (surfactant) solution. Ferric chloride hydrate (5.56 g) was dissolved into 70 mL deoxygenated deionized (DI) water and 30 mL ethanol mixed solution (solution A 100 mL), and 0.648 g potassium borohydride was dissolved into 100 mL deoxygenated DI water in another beaker (solution B 100 mL). After 0.5 g polyvinylpyrrolidone was added into solution A, solution B was added dropwise to solution A under continuous stirring conditions (using a mechanical stirrer). The reaction lasted 30 min under vigorously mixed conditions. After that, 10.0 wt% starch was added to stabilize the nZVI in solution. The resulting nZVI particles were then washed with DI water and anhydrous ethanol several times, and then dried under a vacuum environment, which is important for the application of the combined system.

Ochrobactrum anthropi cultivation

The autohydrogenotrophic denitrifying bacterium *Ochrobactrum anthropi* B2 (GenBank: KJ957921) was isolated from our laboratory. *Ochrobactrum anthropi* B2 was isolated from a biofilm reactor, which was used to remove nitrate, and the bacteria were obtained through multiple separation and purification processes. The bacteria possess the ability of autohydrogenotrophic denitrification. The compositions of the bacteria culture media were (mg L^{-1}): $ZnCl_2$ 0.68, $CoCl_2 \cdot 6H_2O$ 0.19, $MnSO_4 \cdot 7H_2O$ 0.12, $NiCl_2 \cdot 6H_2O$ 0.27, $CuCl_2 \cdot 2H_2O$ 0.32, $Na_2MoO_4 \cdot 2H_2O$ 0.36, $MgCl_2 \cdot 6H_2O$ 0.28, H_3BO_3 0.35, $NaHCO_3$ (inorganic carbon source) 550, KH_2PO_4 350, $NaNO_3$ (nitrogen source) 400, pH 7.0–7.5. The bacteria were cultured in a biochemical incubator in order to maintain reactivity.

Batch experiments

In order to investigate the ammonium control performance and cooperative mechanism of this combined system, batch experiments were carried out in Erlenmeyer flasks in an incubator shaker. Twenty millilitres of bacteria solution ($OD_{600} = 0.015$), 1.0 g/L starch-stabilized nZVI, 20 mL nitrate solution (initial nitrate concentration = 100 mg/L) and 160 mL distilled water were added into the Erlenmeyer flask. The pH of the mixed liquor was adjusted to 7.0. Then the Erlenmeyer flask was put into the incubator shaker at 25 °C and 200 rpm.

To investigate the effects of starch-stabilized nZVI dose on the combined system, the starch-stabilized nZVI doses were adjusted to 0.5, 1.0, 1.5, and 2.0 g/L at OD_{600} 0.015 and pH 7.0 condition for 100 mg/L nitrate removal.

The biomass was adjusted to OD_{600} 0.015, 0.030, 0.050 and 0.080 at 1.0 g/L starch-stabilized nZVI and 7.0 pH condition to evaluate the effects of biomass on the combined system.

In order to evaluate the influences of pH on the combined system, the pH of the mixed solutions was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 at OD_{600} 0.015 and starch-stabilized nZVI 1.0 g/L condition for 100 mg/L nitrate removal.

Analytical methods

Total nitrogen, ammonia nitrogen (NH_4^+-N), nitrate (NO_3^--N), and nitrite (NO_2^--N) were determined

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according to *Standard Methods for the Examination of Water and Wastewater* (APHA 2005). The pH was determined using a pH meter (PHS-3C, Kexiao Instrument, China). Biomass was determined by the turbidity (OD_{600}) by means of a spectrophotometer (UV-2802H, Shanghai Optical Company, China). The batch experiments were conducted in duplicate in order to control quality.

RESULTS AND DISCUSSION

Ammonium control performance and cooperative mechanism on nitrate removal

Figure 1 shows the nitrate reduction performance of sole starch-stabilized nZVI, sole *Ochrobactrum anthropi* B2, and the combined abiotic and biotic process. The initial nitrate concentration of 100 mg/L could be rapidly reduced in 8 hours by sole starch-stabilized nZVI, which indicated that the starch-stabilized nZVI exhibited strong reduction capacity. However, 85.09 mg/L ammonium, and no obvious nitrite (below 2.31 mg/L), was generated during this reduction process, meaning that the main and final product of nitrate reduction by starch-stabilized nZVI was ammonium, which was in accordance with the studies of Ryu *et al.* (2011), Liou *et al.* (2006), and Hwang *et al.* (2011). During the sole biotic process, nitrate could be slowly degraded by *Ochrobactrum anthropi* B2, and there was no nitrite (below 1.86 mg/L) or ammonium (below 2.54 mg/L) generated. For the combined *Ochrobactrum anthropi* and starch-stabilized nZVI system, although nitrate removal rates were lower than sole starch-stabilized nZVI, the ammonium yield decreased to 14.90 mg/L with no nitrite accumulation, meaning that the addition of the autohydrogenotrophic bacterium *Ochrobactrum anthropi* could effectively decrease ammonium yield in the process of nitrate removal by starch-stabilized nZVI. The anaerobic iron corrosion process would generate H_2 (Equation (1)) (Biswas & Bose 2005; Shin & Cha 2008; An *et al.* 2014), which was used as an electron donor for *Ochrobactrum anthropi* to achieve the autohydrogenotrophic denitrification process (Equation (2)) to convert nitrate to N_2 (Szekeres *et al.* 2002; Karanasios *et al.* 2010; Xia *et al.* 2010).

$$Fe^0 + 2H_2O \rightarrow H_2 + Fe^{2+} + 2OH^- \quad (1)$$

$$2NO_3^- - N + 5H_2 \rightarrow N_2 + 4H_2O + 2OH^- \quad (2)$$

OD_{600} increased from 0.015 to 0.190 in the combined system, meaning that autohydrogenotrophic bacteria grew well for nitrate degradation, which also proved that *Ochrobactrum anthropi* could harmonically cooperate with starch-stabilized nZVI corrosion for nitrate degradation.

The X-ray diffraction (XRD) spectrum of the combined *Ochrobactrum anthropi* and starch-stabilized nZVI system after reaction are shown in Figure 2. The product detected in the combined system was only Fe^0 at $2\theta = 44.5^\circ$, meaning that the Fe^0 corrosion product in the biological process was non-crystalline. This result was in accordance with the research of An and coworkers (An *et al.* 2014).

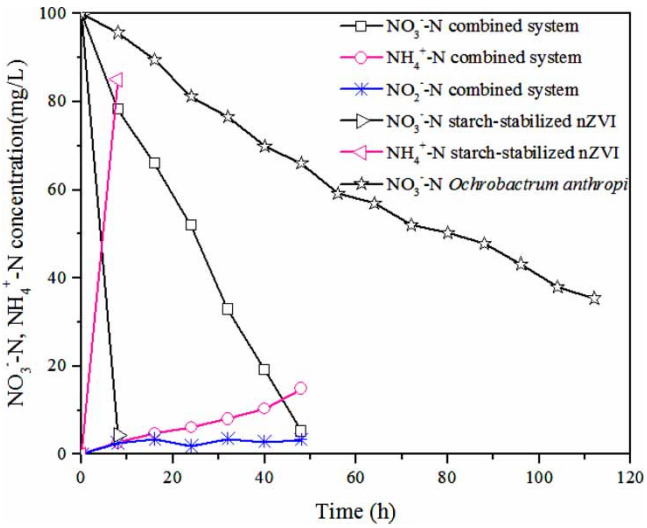

Figure 1 | Performance of the combined system.

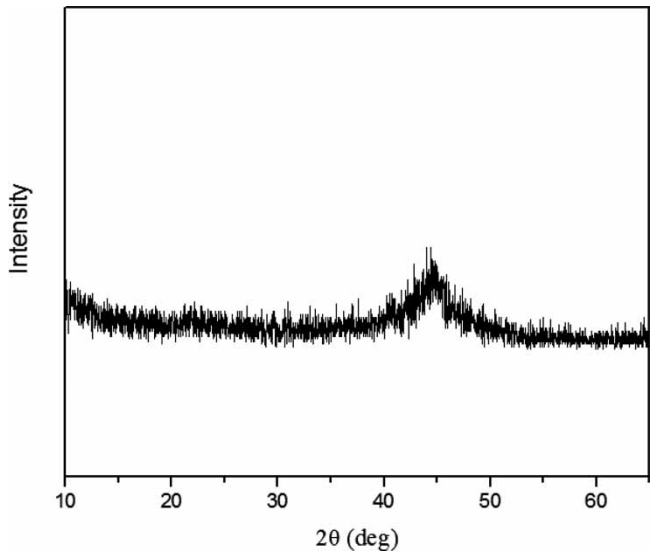

Figure 2 | XRD spectrum of the combined system after reaction.

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Effects of starch-stabilized nZVI dose on nitrate removal

Figure 3 shows the nitrate removal efficiencies of this combined system at different starch-stabilized nZVI doses. When starch-stabilized nZVI dose increased from 0.5 to 2.0 g/L, nitrate reduction rate gradually increased, and ammonium yield also increased from 9.40 to 60.51 mg/L. It can be calculated from Equations (1) and (2) that the theoretical consumption of starch-stabilized nZVI was 1.0 g/L for removal of 100 mg/L $NO_3^- - N$ by this combined method. When the addition of starch-stabilized nZVI was lower than 1.0 g/L, the Fe^0 was not enough for the combined biotic and abiotic reaction, and the residual nitrate was only consumed by the autohydrogenotrophic *Ochrobactrum anthropi*, so that the nitrate removal rate was low. For 1.5 or 2.0 g/L starch-stabilized nZVI, because of the excess of starch-stabilized nZVI, the pure abiotic Fe^0 reduction reaction (Equation (3)) (Biswas & Bose 2005; Shin & Cha 2008) was much more active and played the dominant role in the nitrate removal process.

$$4Fe^0 + NO_3^- + 10H^+ \rightarrow 4Fe^{2+} + NH_4^+ + 2H_2O \quad (3)$$

After reaction, OD_{600} 0.015 changed to 0.213, 0.190, 0.117, and 0.046 under 0.5, 1.0, 1.5, and 2.0 g/L starch-stabilized nZVI conditions, respectively, which also indicated that the biotic reactivity was gradually weakening with increasing biomass. On the other hand, the biotic reaction was slower than the abiotic reaction, so that the nitrate could be rapidly reduced and thus large amounts of ammonium would be generated. Therefore, increasing starch-stabilized nZVI dose could increase nitrate removal efficiency and increase ammonium yield.

Effects of biomass on nitrate removal

Figure 4 presents the nitrate removal and ammonium control performance under different biomass conditions. When initial OD_{600} increased from 0.015 to 0.080, nitrate removal rate gradually decreased, and ammonium yield also decreased from 14.93 to 2.61 mg/L, meaning that the abiotic Fe^0 reduction process was inhibited under higher biomass conditions. When the biomass was increased, more and more bacteria could compete for limited electron donor and nitrogen sources, so that the biotic process had an advantage over the abiotic process as the biomass increased and gradually played a key role in the nitrate removal process. In general, the efficiency of the biotic process was lower than that of the abiotic process, so that the nitrate removal rate under biotic-dominated conditions presented a decreasing tendency with increasing biomass. Meanwhile, ammonia yielded by the abiotic process decreased due to the fact that the biotic process became the dominant pathway for nitrate removal.

Effects of pH on nitrate removal

Figure 5 shows the effects of pH on the combined system for nitrate reduction. When pH increased from 5.0 to 9.0, nitrate removal rate gradually decreased and ammonium yield decreased from 49.32 to 7.24 mg/L. Under an acidic environment, starch-stabilized nZVI corrosion was accelerated (Alidokht *et al.* 2011) and thus enhanced nitrate

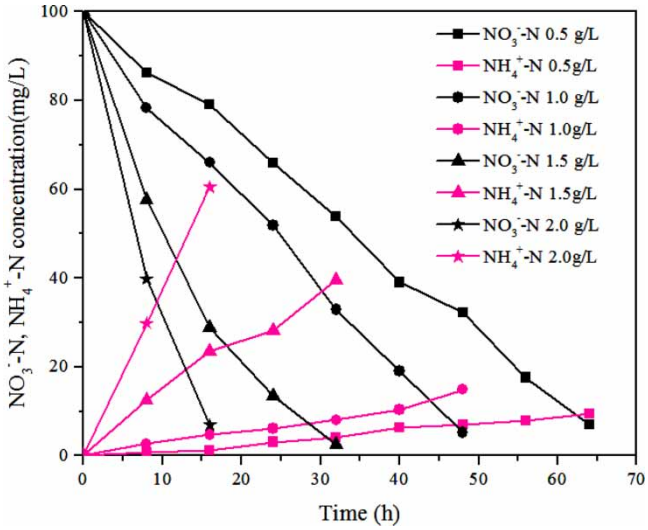

Figure 3 | Effects of starch-stabilized nZVI dose on the combined system.

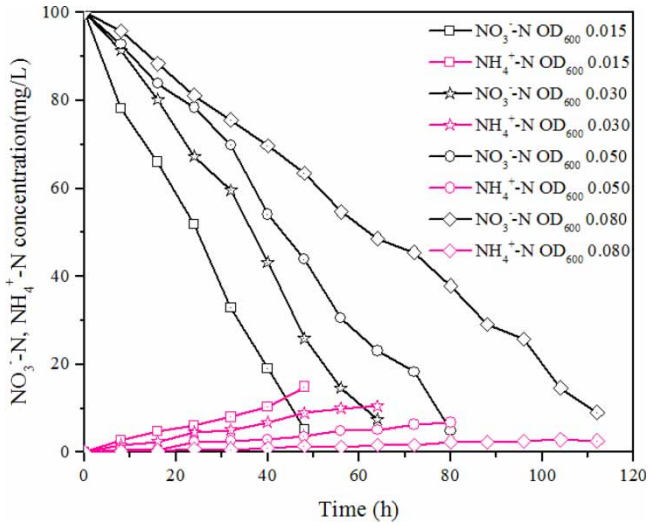

Figure 4 | Effects of biomass on the combined system.

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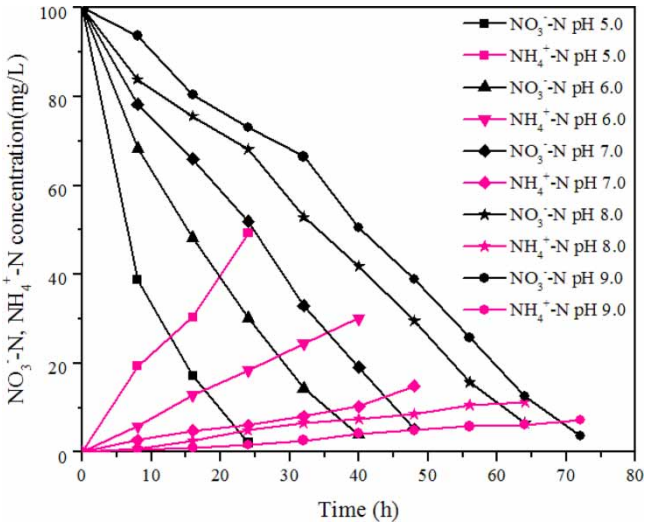

Figure 5 | Effects of pH on the combined system.

removal rate. On the other hand, the anion $NO_3^- - N$ was easily removed by starch-stabilized nZVI due to Fe^0 carrying large numbers of positive charges under acidic conditions (Almeelbi & Bezbaruah 2012). Moreover, Fe^0 could be easily reacted with $NO_3^- - N$ (Equation (3)) in an acidic environment. Compared with the abiotic process, the activity of *Ochrobactrum anthropi* often decreased under acidic conditions and the biotic process would be inhibited (Wang *et al.* 2009; Ghafari *et al.* 2010; Xia *et al.* 2010). Therefore, the abiotic Fe^0 reduction process played a key role for nitrate removal in an acidic environment and generated large amounts of ammonium. In an alkaline environment, for the biotic process, *Ochrobactrum anthropi* always presented weak reactivity for nitrate degradation because most denitrifying bacteria prefer neutral conditions (Vasiliadou *et al.* 2006; Ghafari *et al.* 2010, 2008; Karanasios *et al.* 2010). For the abiotic process, Fe^0 reduction efficiency could be inhibited under alkaline conditions as Fe^0 carried more negative charges and thus decreased the affinity for $NO_3^- - N$. On the other hand, more OH^- would slow down the Fe^0 reduction reaction (Equation (3)) rate for nitrate removal. Therefore, the nitrate removal rate would decrease and ammonium yield also reduce in an alkaline environment.

CONCLUSIONS

The autohydrogenotrophic bacterium *Ochrobactrum anthropi* exhibited excellent performance on the control of the generation of ammonium, which was the by-product of nitrate reduction by starch-stabilized nZVI. XRD demonstrated the corrosion products of nZVI may be non-crystalline. Increasing starch-stabilized nZVI dose could increase nitrate removal efficiency and increase ammonium yield. Higher biomass lowers the reduction of nitrate by nZVI. When pH increased from 5.0 to 9.0, nitrate removal rate gradually decreased and ammonium yield decreased from 49.32 to 7.24 mg/L. The autohydrogenotrophic *Ochrobactrum anthropi* was proved to exhibit effective capacity for ammonium control under appropriate conditions in the process of nitrate removal by starch-stabilized nZVI. These outcomes demonstrate the feasibility of using combined autohydrogenotrophic bacteria and starch-stabilized nZVI to treat the water contaminated by nitrate, and offer a guide for the application of the combined system for nitrate removal. Further investigations are needed to promote the efficiency of nitrate removal.

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