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Research Article

High nitrate removal by starch-stabilized Fe⁰ nanoparticles in aqueous solution in a controlled system¹Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran²Microbiology and Biotechnology Research Group, Research Institute of Petroleum Industry, Tehran, Iran³Department of Civil and Environmental Engineering, Faculty of Environment, University of Tehran, Tehran, Iran⁴Chemical, Polymeric and Petrochemical Technology Research Division, Research Institute of Petroleum Industry, Tehran, Iran

This study was conducted to investigate biodenitrification efficiency with starch-stabilized nano zero valent iron (S-nZVI) as the additional electron donor in the presence of S₂O₃ in aqueous solutions, under anaerobic conditions. The main challenge for nZVI application is their tendency to agglomeration, thereby resulting in loss of reactivity that necessitates the use of stabilizers to improve their stability. In this study, S-nZVI was synthesized by chemical reduction method with starch as a stabilizer. The synthesized nanoparticles were characterized by TEM, XRD, and FTIR. Transmission electron microscopy (TEM) image shows S-nZVI has a size in the range of 5–27.5 nanometer. Temperature and S-nZVI concentration were the important factors affecting nitrate removal. Biodenitrification increased at 35°C and 500 mg/L of S-nZVI, in these conditions, biodenitrification efficiency increased from 40.45 to 78.84%. Experimental results suggested that biodenitrification increased by decreasing initial nitrate concentration. In the bioreactor biodenitrification rate was 94.07% in the presence of S-nZVI. This study indicated that, Fe²⁺ could be used as the only electron donor or as the additional electron donor in the presence of S₂O₃ to increase denitrification efficiency.

Keywords: Biological denitrification / Nanoscale zero-valent iron / Nitrate / Starch / *Thiobacillus denitrificans*

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1 Introduction

Nitrate contamination in water supplies has become the main challenge because of the environmental problem and a potential risk to human health. Use of physical and chemical denitrification methods is limited to energy costs and post-treatment processing while biodenitrification is cost effective and environmentally friendliness [1, 2]. The obligate chemolithoautotrophic bacterium *Thiobacillus denitrificans* is well known for its ability to couple the oxidation of various sulfur and reduced iron compounds to denitrification [3]. Therefore in this study, *Thiobacillus denitrificans* as an autotrophic denitrifier microorganism has been used. Biodenitrification using only electron donor such as n-ZVI [4], ferrous ions [5], elemental sulfur or reduced sulfur compounds [6] is very well acknowledged, thus in this study, the

combined process of *Thiobacillus denitrificans* and S-nZVI as the additional electron donor was applied to increase nitrate removal from aqueous solution. In this process, the S-nZVI produces electron donors, such as H₂ and Fe²⁺ via the S-nZVI corrosion. Autotrophic denitrification of *Thiobacillus denitrificans* by S₂O₃ as sole electron donor is slow process, so the formation of Fe²⁺ can enhance the growth of microorganisms, as well as biodenitrification rate by serving as the additional electron donor, which significantly benefits autotrophic denitrification.

nZVI was applied to increase the reaction efficiency of nitrate reduction. As an electron donor for nitrate, nZVI could promote microbial removal of nitrate through the reactions indicated in Eqs. (1)–(2) [7].

Zero-valent iron nanoparticles tend to condense quickly that leads to loss of their reactivity. Therefore, dispersion of Fe⁰ nanoparticles is a critical step to improving their reaction efficiency. Various particle stabilizing agents have been employed including resin, starch, and carboxymethyl-cellulose [8–10].

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Abbreviations: FTIR, Fourier Transform Infrared; S-nZVI, Starch stabilized nano zero valent iron; TEM, Transmission Electron Microscopy; XRD, X-Ray Diffraction

Starch is a nontoxic, biodegradable, and inexpensive substance that can be used as an effective dispersant for iron nanoparticles [9]. In this research, S-nZVI was synthesized in order to overcome problems related to nZVI, such as instability and agglomeration in aqueous medium. Although a few studies have been investigated the efficiency of n-ZVI nanoparticles on biodenitrification [11–13] but the role of S-nZVI as the additional electron donor on biodenitrification by *Thiobacillus denitrificans* under different environmental factors has been questioned. For this reason, the current study was conducted to determine if combined process of *Thiobacillus denitrificans* and S-nZVI would be better in increasing autotrophic denitrification than free cell and would be increase the rate of biodenitrification.

The objective of this study was to investigate following issues:

(i) The first goal of this paper was to determine, clarify and quantify the role of S-nZVI as the additional electron donor in the biodenitrification process by *Thiobacillus denitrificans* in order to assess its feasibility for nitrate reduction. (ii) Investigation the impact of various influencing factors, such as temperature, S-nZVI concentration and initial nitrate concentration on nitrate removal under anaerobic conditions. (iii) Evaluation of nitrate concentration in the bioreactor. (iv) Investigation of samples by TEM, X-Ray Diffraction (XRD) and Fourier Transform Infrared (FTIR) for understanding of the interaction mechanisms.

2 Materials and methods

2.1 Culture media and cultivation

Thiobacillus denitrificans (strain DSMZ NO. 12475) was purchased from German Collection of Microorganism and Cell Cultures. The temperature was maintained to 30°C and the pH was adjusted to 7. *Thiobacillus denitrificans* was grown in the basal salt medium (BSM) under anaerobic conditions and unshaken in incubator. In order to achieve growth curve, bacterial concentration and nitrate removal was measured, while the initial nitrate concentration was 300 mg/L. Bacterial concentration was measured using dry-weight method. Thus, culture was harvested by centrifugation, was dried, and bacterial concentration was measured before the start of the experiments.

The utilized BSM in this study contained (per liter): 1.8 g KH_2PO_4 , 1.2 g Na_2HPO_4 , 0.1 $(\text{NH}_4)_2\text{SO}_4$, 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 0.02 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.02 MnSO_4 , 0.5 g NaHCO_3 , 2.16 g KNO_3 . Culture medium was composed of 90% (v/v) of BSM and 10% (v/v) of trace element solution. The trace element solution was composed of (g/L): EDTA (0.5), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.01), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.03), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.03), H_3BO_3 (0.2), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.02). All the chemical used in experiments were of chemical grade.

2.2 Synthesize of S-nZVI

In this study S-nZVI was synthesized using the conventional liquid-phase method by reducing the ferric ion with potassium borohydride [14]. A ferric ion solution produced by dissolving ferric chloride hexahydrate (6 g) in an ethanol/water solution

with ratio of 1/3 and was stirred at 250 rpm. Water-soluble starch (2 g) was added to the solution and was stirred for 30 min. Subsequently, a freshly prepared KBH_4 solution (5.4 g of KBH_4 in 30 mL ethanol and 20 mL water solution) was added dropwise into the solution and constantly was stirred for 60 min. The whole process described above was performed under inert atmosphere of N_2 gas with vigorous stirring, to avoid the oxidation of S-nZVI. The formed suspension was filtered and the black nanoscale was washed three times with distilled water, pure ethanol, and acetone. Following this, S-nZVI samples were stored in sealed bottles in a dry environment.

2.3 Characterization and analytical method

Cell interactions with nanoparticles, morphology, and size of nanoparticles were analyzed by TEM (Philips CM 200, 200 kV TEM, ATM 2k × 2k CCD camera).

Structure and crystal phase of S-nZVI was measured by a Philips X-ray Diffractometer PW1840 with Cu-K α radiation (0.154 nm).

In order to analyze the characteristics of synthesized S-nZVI nanoparticles, FTIR spectra were used for identify surface groups and determine the chemical bonds. KBr pellets containing 1.5 mg sample and 15 mg KBr were prepared for FTIR measurement (Bruker, Tensor 27, FT-IR spectrometer). Spectra were recorded in the range 450–4000 cm^{-1} . The atomic absorption spectroscopy (AAS) was taken using a Hitachi Spectrophotometer 100–10 for measuring the percent of iron in S-nZVI.

The experimental samples were gathered after 48 h and cell growth detected by measuring nitrate removal and cell concentration. The experimental samples were centrifuged at 5000 rpm for 20 min. Nitrate concentration was analyzed with the nitrate kit by chromotropic acid method [15].

The nitrate removal rate was calculated using the following Equation (Eq. (3)):

$$\text{Removal rate efficiency (\%)} = 100 (C_1 - C_2) / C_1 \quad (3)$$

In above equation C_1 is the initial nitrate concentration (mg/L), C_2 is the concentration of nitrate after treatment (mg/L).

2.4 Statistical analysis

Design-expert software and response surface methodology were used for statistical optimization for denitrification process using S-nZVI and biodenitrification in the presence of S-nZVI. The two most important process parameters were temperature and S-nZVI concentration, these were designed as X_1 and X_2 respectively. The coded values for these variables were set at 3 levels –1 (minimum), 0 (central) and +1 (maximum). The Box-Behnken method was chosen to represent the number of experiments in order to analyze the interaction between the parameters and optimize the effective process variable. The number of experiments designed by Box-Behnken at 3 levels was 13 runs including 3 repeated experimental runs at the central point to eliminate errors and curvature.

2.5 Experimental set-up

Three types of batch experiments were performed: biotic denitrification (*Thiobacillus denitrificans*), abiotic denitrification (S-nZVI only), and *Thiobacillus denitrificans*-inoculated experiments amended with S-nZVI.

The autotrophic denitrifying activity of *Thiobacillus denitrificans* was carried out in vials of 40 mL with a BSM volume of 36 mL. Under these conditions, S_2O_3 will be the only electron donor available for the cells. To evaluate the effect of temperature on the autotrophic denitrifying activity of microorganism, the vials were incubated at different temperatures.

To evaluate the effect of reaction temperature and S-nZVI concentration on nitrate removal rate by S-nZVI only, 0.05, 0.5 and 1 g/L of S-nZVI was added to vials of 100 mL. Under these conditions Fe^{2+} will be the only electron donor available for nitrate reduction.

In order to prove the effects of temperature and S-nZVI concentration on biodenitrification with S-nZVI in the presence of S_2O_3 , *Thiobacillus denitrificans* and the S-nZVI were added in 36 mL of BSM in vials of 40 mL, then *Thiobacillus denitrificans* and S-nZVI were incubated.

Control experiments (BSM without *Thiobacillus denitrificans*, BSM without S-nZVI, free cells without S-nZVI and S-nZVI without free cell) were carried out in parallel.

In order to obtain anoxic conditions, all the vials were purged with N_2 and sealed to ensure that anaerobic conditions existed. The experimental samples gathered after 48 h, supernatant phase after centrifugation was withdrawn, followed by nitrate concentration was measured.

In this study, all these experiments were carried out in triplicate and the average values of the measurements were used in all analyses.

Based on the above experiments, batch experiments were conducted regarding the effects of reaction temperature and concentration of S-nZVI on nitrate removal efficiency. Batch experiments with various conditions are briefly described as follows: S-nZVI concentration of 50, 500, 1000 mg/L and incubation temperatures of 25, 30 and 35°C, initial nitrate concentration and initial pH was 300 mg/L and 7 respectively.

The continuous experiment was performed to investigate the biodenitrification process in the presence of S-nZVI under optimum conditions in the bioreactor with a working volume of 5 L. The reactor was inoculated with 2 L of sterilized BSM and 10 mL sterilized suspension of S-nZVI and *Thiobacillus denitrificans*. The temperature of operation and S-nZVI concentration were under optimum conditions and initial nitrate concentration and initial pH was 300 mg/L and 7 respectively. To create anoxic conditions, the reactor was filled with N_2 gas in the whole time of the experiment. Samples were taken every 4 h using a sterile syringe, samples were filtered and diluted to low concentration, and nitrate concentration was measured.

Furthermore, in order to identify the effect of initial nitrate concentration on nitrate removal efficiency, the batch experiments were conducted in a vial of 40 volumes, while initial nitrate concentration was 60, 100 and 140 mg/L, initial pH was 7, and temperature and S-nZVI concentration were under optimum conditions.

Figure 1. Growth curve of *Thiobacillus denitrificans*.

Table 1. Results of different temperatures on biodenitrification

Run	Temperature (°C)	Nitrate removal (%)
1	35	40.45
2	30	37
3	25	16.36

3 Results and discussion

3.1 Strain growth and biodenitrification

The relationship between cell concentration and nitrate concentration in the batch experiments is shown in Fig. 1. The solution was contained 300 mg/L of nitrate, at 30°C under anaerobic conditions. Nitrate concentration decreased with time going on. After 10 h in the incubation period, cell concentration and nitrate removal efficiency rapidly increased during the logarithmic phase. Cell concentration was approximately 1400 mg/L at 48 h.

In the study conducted by Krishna and Philips, nitrate removal efficiency increased by increasing cell concentration [16]. As shown in Fig. 1, in general, the remaining concentration of nitrate declined in response to increasing the concentration of cell. In biological nitrate removal process, *Thiobacillus denitrificans* used S_2O_3 (one of the components of BSM) as the only electron donor for nitrate reduction.

3.1.1 Impact of temperature on biodenitrification

Three batch experiments were carried out to evaluate the temperature dependence of chemolithotrophic denitrification coupled to S_2O_3 oxidation by *Thiobacillus denitrificans*. Temperature strongly affected the rate and efficiency of chemolithotrophic denitrification. The effect of different temperatures on nitrate removal is shown in Table 1. By reducing the temperature from 35 to 25°C, biodenitrification reduced from 40.45 to 16.36%. This was due to the fact that optimal cell growth and nitrate reductase activity done at a higher temperature [17]. Biodenitrification is very sensitive to temperature, but only few studies reported that the effects of different temperature on thiosulfate-driven denitrification by *Thiobacillus denitrificans* [18,19]. Vasiliadou *et al.*, proved that higher temperature enhanced growth and denitrification performance of microorganisms [20]. The result of other study showed that nitrate reduction rate increased as temperature increased from 20 to 35°C [21]. Hosono *et al.*, indicated that biodenitrification of *Thiobacillus denitrificans* with

Figure 2. Transmission electron microscopy images. (A) S-nZVI. (B) S-nZVI-amended cell.

FeS₂ as sole electron donor is a slow process and 40% nitrate removal efficiency was observed after seventy days at 25°C [22].

Result of this study demonstrated that the rate of biodenitrification continuously increased as the temperature increased and the activity of the microorganisms influenced by temperature. Investigation of results of current study showed that maximum nitrate removal efficiency observed at high temperature since all metabolic activity of microorganism and participation of enzymes enhanced in high temperature.

3.2 Characterization of metal nanoparticle

The morphology, size and distribution of nanoparticles, determined by TEM, as seen in Fig. 2A, S-nZVI has a spherical black core with a light gray shell around the core, that indicated nZVI was covered by starch [23]. The nanoparticles were spherical in shape with the size ranging from 5 to 35 nm. TEM images were obtained after incubation of *Thiobacillus denitrificans* and 500 mg/L S-nZVI. As shown in Fig. 2B, cell shape of *Thiobacillus denitrificans* is bacilli and when exposed to S-nZVI, the cell morphology was unchanged and the cell membrane was covered by a layer of S-nZVI due to the small size and high surface area of nanoparticles [24].

The XRD pattern of S-nZVI only is presented in Fig. 3A. The apparent peak at $2\theta = 44.9^\circ$ indicated that existence of S-nZVI in freshly prepared nanoparticles. The apparent peak at $2\theta = 44.9^\circ$ consistent with the result of study conducted by Liu *et al.* [12]. The crystallite size of the S-nZVI synthesized in this study

Figure 3. (A) X-ray diffraction pattern of S-nZVI. (B) FTIR spectra of S-nZVI.

was estimated at 27.5 nm from X-ray line broadening using the Scherer formula.

To characterize the modification of the nanoparticles by the starch molecules, FTIR spectra were performed. FTIR Spectra of S-nZVI is presented in Fig. 3B. The bonds at 3411.83 cm⁻¹ and 1639.72 cm⁻¹ were ascribed to OH stretching vibration which suggests the formation of FeOOH layer on S-nZVI nanoparticles [25]. The FTIR spectra of S-nZVI show C-H stretching absorption around 2922.80 cm⁻¹ [26]. The bond at 1375.42 cm⁻¹ was attributed to C-O-H bending. The peaks at 1159.26 and 1010.42 cm⁻¹ were assigned to C-O and C-C stretching vibration of C-O-H bending. This observation is similar to that obtained by Jiao *et al.* [27]. So we conclude the surface of fresh nanoparticle is cover by the starch molecules.

The weight percent of iron in the sample was determined by atomic absorption spectroscopy (AAS), where the Fe content was detected by dilution of powder material in acid that was 22%.

3.3 Development of regression equations and statistical analysis

The correlation between temperature, S-nZVI concentration, and nitrate removal efficiency was examined using Box-Behnken. Table 2 shows the design of experiments as well as the experimental results.

The final equations in terms of coded and actual factor for response are expressed in Tables 3 and 4. In general p- values less than 0.05 was categorized as significant effects.

Table 2. Comparison the removal of nitrate between S-nZVI only and S-nZVI- amended cell

Run	Temperature (°C)	S-nZVI (mg/L)	Denitrification by S-nZVI (%)	Denitrification using S-nZVI-amended cell (%)
1	25	0.05	2.5	18.21
2	35	0.05	12.4	42.12
3	25	1	26.6	35.3
4	35	1	70.42	71.43
5	25	0.5	12.92	36.8
6	35	0.5	57.6	78.84
7	30	0.05	9.4	40.3
8	30	1	43.89	63.2
9	30	0.5	33.57	68.4
10	30	0.5	32.4	67.5
11	30	0.5	33.27	65.7
12	30	0.5	32.73	66.4
13	30	0.5	33.68	67.7

Table 3. Evaluation of nitrate removal by S-nZVI only

Term	F-value	P-value
A-A	57.67	0.0001
B-B	80.78	0.0001
A-B	0.1060	0.0099

Final equation for removal of nitrate by S-nZVI in a volume of 100 mL.
(A and B were temperature and S-nZVI concentration, respectively).
 $R1 = 31.11 + 16.54A + 19.57B + 8.68A \times B$
 R-Squared = 0.9431.

Table 4. Evaluation of biodegradation in the presence of S-nZVI

Term	F-value	P-value
A-A	173.60	0.0001
B-B	74.16	0.0001
A-B	11.26	0.0122
A ²	5.41	0.0529
B ²	53.22	0.0002

Final equation for biodegradation with S-nZVI as the additional electron donor in the presence of S₂O₃ in a volume of 40 mL (A and B were temperature and S-nZVI concentration, respectively).
 $R2 = 68.62034 + 21.34667 \times A + 13.95167 \times B + 6.65750 \times A \times B - 5.55621 \times A^2 - 17.42121 \times B^2$
 R-Squared = 0.9800.

According to above tables, suggested that temperature, S-nZVI concentration, and the interaction between temperature and S-nZVI concentration were effective on nitrate removal.

Under optimum conditions (temperature at 34.65°C and 0.955 mg/L S-nZVI) nitrate removal using S-nZVI only, was 71.69% while under optimum conditions (temperature at 35°C and 0.03 mg/L S-nZVI) biodegradation with S-nZVI as the additional electron donor in the presence of S₂O₃ was 80.10%.

3.4 Effect of S-nZVI concentration and temperature on nitrate removal efficiency

As shown in Table 2, biodegradation increased after adding S-nZVI into the cell system under anaerobic conditions. By adding S-nZVI to the cell system, *Thiobacillus denitrificans* not only used S₂O₃ as an electron donor, but also served Fe²⁺ as electron donor. As shown (Table 2), under optimum conditions, 70.42% nitrate was removed by S-nZVI only, while 78.84% nitrate was removed, when S-nZVI was added to cells. We think chemical oxidation of S-nZVI to Fe²⁺ may have had positive effect on biodegradation, because *Thiobacillus denitrificans* use Fe²⁺ as the only electron donor or as the additional electron donor in the presence of S₂O₃, furthermore the formation of Fe³⁺ is faster in the presence of S₂O₃. Similar results obtained in the study conducted by Straub *et al.* [28]. They demonstrated that anaerobic nitrate dependent microbial oxidation of ferrous iron and formation of Fe³⁺ occurred only in the presence of acetate. It is possible that the addition of S-nZVI to the cell, not only stimulated the activity of autotrophic microorganisms but also could stimulate the activity of competing between microorganism and S-nZVI in order to nitrate reduction. This is the first study on the impact of S-nZVI on biodegradation and information, in this case, is very little but, the results of experiments showed that *Thiobacillus denitrificans* used Fe²⁺ as the additional electron donor in the presence of S₂O₃.

Also Fig. 4A shows the response surface plot for denitrification by S-nZVI only. The response surface plot implied that with increasing concentration of S-nZVI, more nitrates were removed. In this study, three different dosages of S-nZVI (50, 500, 1000 mg/L) was used.

As shown in Table 2, by the dosage increasing, the removal efficiency of nitrate become higher and higher, in 50 mg/L S-nZVI dosage at 35°C, after 48 h nitrate removal efficiency reached 12.4%, then increasing S-nZVI dosage of 50 mg/L to 500 mg/L, cause increasing nitrate removal efficiency to 57.6%, and finally in 1000 mg/L S-nZVI dosage at 35°C, after 48 h, nitrate removal reached 70.42%. In the study conducted by Liu *et al.* by increasing concentration of nZVI from 50 mg/L to 1000 mg/L nitrate removal increased from 1% to 2.37% [12]. By lower dosage of S-nZVI, less accumulation of ferrous iron and less nitrate removal occurred. Since the nitrate reduction occurs the surface of S-nZVI nanoparticles, the more dosages of S-nZVI nanoparticles can be provide more active sites of S-nZVI to reduce nitrate [29, 30].

As shown in Fig. 4A, nitrate removal increased when the temperature also increased.

The percentage of nitrate removal was significantly enhanced, showing 26.6, 57.6, and 70.42% nitrate removal at 25, 30, and 35°C, respectively. Results of this study indicated that high temperature favors better denitrification than low temperature. In this study, the enhancement of reduction rate was obtained by increasing the temperature, which can supply the excessive energy necessary to overcome activation energy barrier. On the other hand, the increase in the reactivity of S-nZVI nanoparticles at a high-temperature favors nitrate reaction through S-nZVI nanoparticles [31].

Figure 4B shows the response surface plot for biodegradation with S-nZVI in the presence of S₂O₃. The high removal of

Design-Expert® Software

A

R1

71.24

2.5

X1 = A: A

X2 = B: B

B

Design-Expert® Software

R1

90.43

18.21

X1 = A: A

X2 = B: B

Figure 4. Three dimensional graphs of the response surface. (A) Response surface of nitrate removal by S-nZVI (B) Response surface of biodenitrification with S-nZVI as the additional electron donor in the presence of S_2O_3 .

nitrate using S-nZVI in conjunction with cell system was observed at 35°C. the response surface plot implied that with increasing reaction temperature more nitrates were removed. Straub *et al.*, demonstrated that the growth and activity of Iron- oxidizing denitrifying bacteria at 28°C was faster than at 15°C [28]. High temperature has a positive effect on cell growth and bacteria activity, and consequently increased nitrate removal in S-ZVI amended cells.

According to Fig. 4B in S-nZVI amended cell, by increasing S-nZVI dosage from 50 to 500 mg/L at 35°C, after 48 h, biodenitrification efficiency increased from 42.12 to 78.84%. Shin and Cha [7] evaluated microbial reduction of nitrate in the presence of Fe^{2+} as the electron source. They investigated effect of different temperature (12, 25, 37°C) on biodenitrification in the presence of Fe^{2+} . About 80% of nitrate was reduced in the Fe^{2+} -cell sample within 14 days, at 37°C.

It is possible that this increasing caused by Fe^{2+} that produced through iron corrosion, which could be used as the additional electron donor for *Thiobacillus denitrificans*. But when S-nZVI concentration increased from 500 to 1000 mg/L at 35°C, biodenitrification efficiency decreased from 78.84 to 71.43%. This is due to the fact that although Fe^{2+} could be used as the electron donor by *Thiobacillus denitrificans* and facilitated biodenitrifi-

cation but excess uptake of Fe^{2+} cause oxidative damage to the cell [32,33]. In contrast, adding 500 mg/L of S-nZVI to the cell not only promoted bacterial growth but also enhanced biodenitrification. In other study by addition of 500 mg/L nZVI in to cells, 70.49% of nitrate was removed [12]. When products of oxidation of metal nanoparticles are increased, these products cause the collapse of the membrane and oxidative stress and thereby inactivates microorganism and decreased activity of microorganism and biodenitrification efficiency [34,35]. There is little information on the microbial toxicity of S-nZVI nanoparticles and published studies are restricted to investigation of effects on single bacterial strains [14,35]. The result of this study indicated that *Thiobacillus denitrificans* was resistant microorganism and only influenced by the high concentration of ions which produced by oxidation of S-nZVI.

3.5 The performance of bioreactor on denitrification

The bioreactor was operated under continuous conditions. First, sterilized synthetic medium containing 300 mg/L NO_3^- and 10 mL sterilized suspension of S-nZVI and *Thiobacillus denitrificans* was added to the bioreactor. Specific denitrification

Figure 5. The performance of the bioreactor for biodenitrification with S-nZVI in the presence of S_2O_3 .

conditions were as follows: initial medium pH of 7, temperature, and S-nZVI concentration was under optimum conditions (35°C and 0.75 g/L respectively). Also to create anaerobic conditions, bioreactor was under the flow of N_2 gas with sterile filter in the whole time of the experiment. In order to evaluate the biodenitrification with S-nZVI in the presence of S_2O_3 in the bioreactor, samples were gathered every 4 h. In Fig. 5, biodenitrification in the presence of S-nZVI is shown in the bioreactor at different times.

The comparative results between the bioreactor and batch systems showed biodenitrification performance was higher in the bioreactor than the batch system. In the batch conditions, biodenitrification with S-nZVI in the presence of S_2O_3 was 80.10% under the optimum conditions after 48 h, while biodenitrification performance under optimum conditions in the bioreactor was 92.07% after 48 h and 94.07% after 52 h. Since the nitrate concentration of the samples didn't change very much, the reaction was stopped after 52 h. Since *Thiobacillus denitrificans* activity is very pH sensitive, the pH in the bioreactor was properly controlled by adding alkalinity in the bioreactor, while there was no control of pH in the batch system. Fajardo *et al.*, [36] reported autotrophic denitrification by *Thiobacillus denitrificans* in a sequencing batch reactor with nitrate removal efficiency of 67%. Virkutyte and Jegatheesan [37] applied hydrogenotrophic bacteria and Fe^{2+} ions to treat nitrate contamination water in the electrochemical reactor and 94.8% of nitrate was removed in 48 h in their study. However, this is the first study on the impacts of S-nZVI nanoparticles on biodenitrification in the bioreactor. Although further testing must be conducted to determine the main reason for this increasing in nitrate removal efficiency in the bioreactor than the batch experiment, the preliminary experiments conducted in this study indicated that the control of pH may be a significant reason for high nitrate removal efficiency in the bioreactor. However, information, in this case, is little, hence a systematic study is required to elucidate, to what extent, could S-nZVI affect biodenitrification efficiency in the bioreactor.

3.6 Effect of initial nitrate concentration on denitrification

Three different initial nitrate concentrations were employed to assess the capacity of *Thiobacillus denitrificans* to tolerate nitrate concentrations in the presence of S-nZVI. The effect of initial

Table 5. Effect of initial nitrate concentration on nitrate removal

Run	Initial nitrate concentration (mg/L)	Denitrification by S-nZVI only (%)	Biological denitrification with S-nZVI modified cell (%)
1	60	75	88.34
2	100	74.2	86.25
3	140	73	83.29

nitrate concentration on denitrification rate is shown in Table 5. Conditions were as follows: initial medium pH of 7, reduction time 48 h, temperature and S-nZVI concentration was 35°C and 500 mg/L respectively.

By reducing concentration of nitrate, denitrification activity of S-nZVI increased. When nitrate concentration reduced from 140 to 60 mg/L, denitrification activity of S-nZVI rose from 73 to 75%. Decreasing initial nitrate concentration was efficient on nitrate transfer on the surface of nanoparticles [38]. By increasing initial nitrate concentration from 60 to 140 mg/L biodenitrification efficiency with S-nZVI in the presence of S_2O_3 decreased from 88.34 to 83.29%. This indicates that a high nitrate concentration resulted in longer lag phases, and consequently a decreasing biodenitrification rate eventuated [39]. In the study occurred by Sun *et al.*, [40] by increasing initial nitrate concentration, nitrate removal efficiency decreased, which is consistent with the results of this study. The results indicating two things: firstly, by increasing nitrate concentration, rate of oxidation of S-nZVI is decreased, consequently less nitrate is removed. Secondly, it is possible by increasing initial nitrate concentration the amounts of Fe^{2+} is reduced to lowest levels and it leads to decrease of nitrate removal efficiency [41, 42].

4 Concluding remarks

Biodenitrification with S-nZVI as the additional electron donor in the presence of S_2O_3 has been carried out in batch systems and the bioreactor. Under optimum conditions, aqueous solutions were treated with removal efficiencies 80.10% in batch systems and 94.07% in the bioreactor. Results indicated that application of starch as the stabilization agent prevents the aggregation Fe^0 nanoparticles which causes high nitrate removal efficiency. Autotrophic denitrification in aqueous solutions could be effectively reduced nitrate to N_2 gas by corrosion mechanisms in the presence of S-nZVI. Results of both batch and continuous experiments showed that *Thiobacillus denitrificans* used S_2O_3 and Fe^{2+} as an electron donor. Appropriate control of operational temperature, S-nZVI dosage and initial nitrate concentration was needed in order to maintain a reduction of nitrate. Characterization of S-nZVI was confirmed by TEM, XRD, and FTIR. Finally, TEM analysis shows size and distribution of S-nZVI on the cells. XRD and FTIR confirmed structure and modification of nanoparticles. Future investigations are needed, such as detection of intermediates in biodenitrification in the presence of S-nZVI and other influencing factors such as pH and hydraulic retention time.

Practical application

This article shows for the first time a practical means to increase the biological denitrification in the presence of S-nZVI in aqueous solution, which was achieved through controlling temperature and S-nZVI concentration under optimum conditions. The application of S-nZVI is a significant and novel advance that will improve the rate of biodenitrification and will increase nitrate removal efficiency, which is the main environmental challenge. This work, therefore, provided a step forward toward increasing the biodenitrification in the presence of S-nZVI in a batch system and the bioreactor, which increase the practical applicability of the S-nZVI for biodenitrification in aqueous solution.

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5 References

- [1] Ghafari, S., Hassan, M., Aroua, M. K., Bio- electrochemical removal of nitrate from water and wastewater – a review. *Biore-sour. Technol.* 2008, 99, 3965–3974.
- [2] Karnasios, K. A., Vasilidou, I. A., Pavlou, S., Vayenas, D. V., Hydrogenotrophic denitrification of potable water. A review. *J. Hazard. Mater.* 2010, 180, 20–37.
- [3] Beller, H. R., Anaerobic, nitrate-dependent oxidation of U (IV) oxide minerals by the chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Appl. Environ. Microbiol.* 2005, 71, 2170–2174.
- [4] Choe, S. H., Ljestrland, H. M., Khim, J., Nitrate reduction by zero-valent iron under different pH regimes. *Appl. Geochem.* 2004, 19, 335–342.
- [5] Hansen, H. C. B., Guldberg, S., Erbs, M., Koch, C. B., Kinetics of nitrate reduction by green rusts- effects of interlayer anion and Fe (II): Fe (III) ratio. *Appl. Clay Sci.* 2001, 18, 81–91.
- [6] Soares, M. I. M., Denitrification of groundwater with elemental sulfur. *Water Res.* 2002, 36, 1392–1395.
- [7] Shin, K. H., Cha, D. K., Microbial reduction of nitrate in the presence of nanoscale zero- valent iron. *Chemosphere* 2008, 72, 257–262.
- [8] Ponder, S. M., Darab, J. G., Mallouk, T. E., Remediation of Cr (VI) and Pb (II) aqueous solutions using supported, nanoscale zero-valent iron. *Environ. Sci. Technol.* 2000, 34, 2564–2569.
- [9] He, P., Zhao, D. Y., Preparation and characterization of a new class of starch-stabilized bimetallic nanoparticles for degradation of chlorinated hydrocarbon. *Environ. Sci. Technol.* 2005, 39, 3314–3320.
- [10] Xu, Y., Zhao, D., Reductive immobilization of chromate in water and soils using stabilized iron nanoparticles. *Water Res.* 2007, 41, 2101–2108.
- [11] Jiang, C., Liu, Y., Chen, Z., Megharaj, M., Naidu, R., Impact of iron-based nanoparticles on microbial denitrification by *Paracoccus* sp. Strain YF1. *Aquat. Toxicol.* 2013, 142–143, 329–335.
- [12] Liu, Y., Li, Sh., Chen, Z., Megharaj, M., Naidu, R., Influence of zero-valent iron nanoparticles on nitrate removal by *Paracoccus* sp. *Chemosphere.* 2014, 108, 426–432.
- [13] Jiang, Ch., Xu, X., Megharaj, M., Naidu, R., Chen, Z., Inhibition or promotion of biodegradation of nitrate by *Paracoccus* sp. In the presence of nanoscale zero-valent iron. *Sci. Total Environ.* 2015, 530–531, 241–246.
- [14] Lee, C., Kim, J. Y., Lee, W. I., Nelson, K. L. et al., Bactericidal effect of zero- valent iron nanoparticles on *Escherichia coli*. *Environ. Sci. Technol.* 2008, 42, 4927–4933.
- [15] APHA. *Standard Methods for the Examination of Water and Wastewater*, 20th Edn., American Public Health Association. Washington, DC 1998.
- [16] Krishna, N. D. V. N. S., Philips, L., *Thiobacillus denitrificans* immobilized biotrickling filter for NO₂ removal. *Clean Techn Environ Policy.* 2005, 7, 285–293.
- [17] Shi, L. N., Zhang, X., Chen, Z. L., Removal of chromium (VI) from wastewater using bentonite-supported nanoscale zero-valent iron. *Water Res.* 2011, 45, 886–892.
- [18] Claus, G., Kutzner, H. J., Physiology and kinetics of autotrophic denitrification by *Thiobacillus denitrificans*. *Appl. Microbiol. Biotechnol.* 1985, 22, 283–288.
- [19] Oh, S. E., Kim, K. S., Choi, H. C., Cho, J. et al., Kinetics and physiological characteristics of autotrophic denitrification by denitrifying sulfur bacteria. *Water Sci. Technol.* 2000, 42, 59–68.
- [20] Vasiliadou, I. A., Karanasios, K. A., Pavlou, S., Vayenas, D. V., Experimental and modelling study of drinking water hydrogenotrophic denitrification in packed-bed reactors. *J. Hazard Mater.* 2009, 165, 812–824.
- [21] Chen, D., Yang, K., Wang, H., Effects of important factors on hydrogen-based autotrophic denitrification in a bioreactor. *Desalination Water Treat.* 2014, 57, 1–8.
- [22] Hosono, T., Alvarez, K., Lin, I. T., Shimada, J., Nitrogen, carbon, and sulfur isotopic change during heterotrophic (*Pseudomonas aureofaciens*) and autotrophic (*Thiobacillus denitrificans*) denitrification reactions. *J. Contam. Hydrol.* 2015, 183, 72–81.
- [23] Yuvakkumar, R., Elango, V., Ragendran, V., Kannan, N., Preparation and characterization of zero valent iron nanoparticles. *J. Nanomater. Biostruct.* 2011, 6, 1771–1776.
- [24] Xiu, Z. M., Jin, Z. H., Li, T. L., Mahendra, S. et al., Effects of nano-scale zero-valent iron particles on a mixed culture dechlorinating trichloroethylene. *Biore-sour. Technol.* 2010, 101, 1141–1146.
- [25] Madhavi, V., Prasad, T. N. V. K. V., Reddy, B. R. et al., Con-junctive effect of CMC-zero-valent iron nanoparticles and FYM in the remediation of chromium-contaminated soils. *Appl Nanosci.* 2014, 4, 477–484.
- [26] An, B., Liang, Q., Zhao, D., Removal of arsenic (V) from spent ion exchange brine using a new class of starch bridged mag-netite nano particles. *Water Res.* 2011, 45, 1961–1972.

- [27] Jiao, C., Cheng, Y., Fan., W., Li, J., Synthesis of agar-stabilized nanoscale zero-valent iron particles and removal study of hexavalent chromium. *J. Environ. Sci. Technol.* 2015, 12, 1603–1612.
- [28] Straub, K. L., Benz, M., Schink, B., Widdel, F., Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl Environ Microbiol.* 1996, 62, 1458–1460.
- [29] Liao, C. H., Kang, S. F., Hsu, Y. W., Zero-valent iron reduction of nitrate in the presence of ultraviolet light, organic matter and hydrogen peroxide. *Water Res.* 2003, 37, 4109–4118.
- [30] Anotai, J., Liao, C. H., Ruangchanikom, C., Nitrate removal by Fe⁰/CO₂ process using an innovative continuous flow reactor. *J Environ Eng Manage.* 2010, 20, 77–84.
- [31] Ahn, S. C., Oh, S. Y., Cha, D. K., Enhanced reduction of nitrate by zero-valent iron at elevated temperatures. *J. Hazard. Mater.* 2008, 156, 17–22.
- [32] Alowitz, M. J., Scherer, M. M., Kinetics of nitrate, nitrite, and Cr (VI) reduction by iron metal. *Environ. Sci. Technol.* 2002, 36, 299–306.
- [33] Diao, M. H., Yao, M. S., Use of zero-valent iron nanoparticles in inactivating microbes. *Water Res.* 2009, 43, 5243–5251.
- [34] Daniel-da-Silva, A. L., Trindade, T., *Biofunctional Composites of Polysaccharides Containing Inorganic Nanoparticles. Advances in Nanocomposite Technology Book.* 2011, pp. 307–347.
- [35] Kim, J. Y., Park, H. J., Lee, C., Nelson, K. L. et al., Inactivation of *Escherichia coli* by nanoparticulate zero valent iron and ferrous ion. *Appl. Environ. Microbiol.* 2010, 76, 7668–7670.
- [36] Fajardo, C., Mosquera- Corral, A., Campos, J. L., Mendez, R., Autotrophic denitrification with sulphide in a sequencing batch reactor. *J. Environ. Manage.* 2012, 113, 552–556.
- [37] Virkutyte, J., Jegatheesan, V., Electro-Fenton, hydrogenotrophic and Fe²⁺ ions mediated TOC and nitrate removal from aquaculture system: different experimental strategies. *Bioresour Technol.* 2009, 100, 2189–2197.
- [38] Li, C. W., Chen, Y. M., Yen, W. S., Pressurized CO₂/zero valent iron system for nitrate removal. *Chemosphere.* 2007, 68, 310–316.
- [39] Liu, Y., Li, G., Chen, Z. L., Megharaj, M., Naidu, R., Removal of nitrate using *Paracoccus* sp. YF1 immobilized on bamboo carbon. *J. Hazard. Mater.* 2012, 229–230, 419–425.
- [40] Sun, Y. P., Li, X. Q., Zhang, W. X., A method for the preparation of stable dispersion of zero valent iron nanoparticles. *Colloids and Surfaces A: Physicochem. Eng. Aspects.* 2007, 308, 60–66.
- [41] Hsu, J. C., Liao, C. H., Weil, Y. L., Nitrate removal by synthetic nanoscale zero-valent iron in aqueous recirculated reactor. *Sustain Environ Res.* 2011, 21, 353–359.
- [42] Karimi, E., Jeffries, C., Yazdian, F., Akhavansepahy, A., Hatamian, A., Rasekh, B., Rashedi, H., Omid, M., Ebrahim-Habibi, M. B., Ashrafi, S. J., DBT desulfurization by decorating *Rhodococcus erythropolis* IGTS8 using magnetic Fe₃O₄ nanoparticles in a bioreactor. *Eng. Life Sci.* 2017, 17, 528–535.