INTRODUCTION

Microbial contamination of water is a significant health threat which is an important issue for drinking water supplies,\(^1\) so that disinfecting drinking water supplies is considered to be an important advance in public health.\(^2\) Recently, there has been an increasing interest in the potential use of engineered nanomaterials for treatment of polluted water.\(^3,4\)

ABSTRACT

Aims: The present study reports the antibacterial efficiency of starch-stabilized nano scale zero-valent iron (S-NZVI) particles on *Escherichia coli*.

Materials and Methods: NZVI was synthesized using NaBH\(_4\) and FeSO\(_4\).7H\(_2\)O, and characterized by scanning electron microscopy, as well as X-ray diffraction. The effects of concentration, contact time, dissolved oxygen, and stabilization were tested. *E. coli* was determined by the pour plate method.

Results: The results revealed that the complete inactivation (100%) of *E. coli* was occurred at using 100 mg/l of NZVI after 30 min under anaerobic condition. The inactivation efficiency was decreased in an aerobic condition. When NZVI concentration increased to 500 and 1000 mg/L, complete inactivation was achieved under both anaerobic and aerobic condition. In general, *E. coli* inactivation efficiency using NZVI was strongly dependent on the contact time and the concentration of NZVI particles with its maximum efficiency at 500 mg/L within 120 min. Stabilization-NZVI by starch did not improve its bactericidal activity and the inactivation of *E. coli* by stabilized nanoparticles required higher concentration compared to that by nonstabilized nanoparticles.

Conclusion: The present study showed that nonstabilized Fe\(^0\) nanoparticles have higher bactericidal efficiency than that of S-NZVI. This investigation also suggests that NZVI can be used as an effective and strong agent for antimicrobial applications.

Key words: *Escherichia coli*, inactivation, stabilized nanoparticles, starch, zero-valent iron
waters. Antimicrobial nanoparticles could overcome critical challenges associated with traditional chemical disinfectants such as harmful disinfection by-products (DBPs) (e.g., carcinogenic trihalomethanes, BrO₃) and they could enhance performance of the existing technologies such as ultraviolet inactivation of viruses, solar disinfection of bacteria, biofouling-prone membrane filtration, and advanced oxidation processes. It can also abate concerns of water quality degradation within distribution networks and in large centralized water treatment systems. A variety of nonmaterial including nano-Ag, nano-ZnO, nano-TiO₂, nano-Ce₂O₃, carbon nanotubes, and fullerenes have been proven as an effective microbicide with the lowest rate of DBPs formation.

Nano scale zero-valent iron (NZVI) is one of the first generations of nano scale technologies which has been used in the environmental remediation and water treatment. NZVI is able to remove a wide range of contaminants such as arsenic (As), chromium (Cr⁶⁺), lead (Pb²⁺), chlorinated solvents including perchloroethylene and trichloroethylene and polychlorinated biphenyls. Several studies have also shown the potential applicability of NZVI in inactivating and removing bacteria and viruses. Previously, Diao and Yao have shown that Fe⁰ nanoparticles inactivated *Bacillus subtilis* var. niger and *Pseudomonas fluorescens* bacteria. Kim *et al.* have demonstrated the inactivation of MS2 coliphage (MS2) by nano zero-valent iron (ZVI) particles in an aqueous solution. Auffan also found that Fe⁰ nanoparticles induce intracellular oxidative stress toward *Escherichia coli* via generating reactive oxygen species (ROS), which causes membrane damages and cell death. It was indicated in another study that the release of Fe²⁺ ion and disturbance of the enzymatic function is responsible for the antimicrobial activity of nano Fe particles.

Since magnetic nanoparticles (e.g., NZVI) can be separated from water by a relatively low magnetic field, they could be used as a platform to develop multifunction nano composite materials. This would enable both chemical disinfection and photocatalytic destruction of waterborne pathogens while enhancing the retention of nano materials. It has been shown that NZVI particles (NZVIPs) have more antimicrobial activity than other iron-based nanoparticles. Rapid degradation of more contaminations is feasible using NZVI because it’s large surface areas and higher surface reactivity, which would provide the best performance for contamination removal.

Despite these benefits, NZVI has a strong tendency to agglomeration, because of van der Waals and magnetic attraction among magnetic nanoparticles, which is an unfavorable property for remediation. In addition, uncoated nanoparticles are very susceptible to some environmental agents such as pH, temperature, electrolytes, and solvent. It has been suggested that surface modification of ZVI nanoparticles by stabilizers helps to produce smaller particles with a controlled shape which are more mono-dispersed.

Although several studies have shown the antimicrobial properties of NZVI, little is done on comparison between bacterial effect of starch-SNZVIPs and non-S-NZVI. In the present work, the bactericidal efficiency of starch-S-NZVI was studied in comparison with bare NZVI (b-NZVI) (none stabilized nanoparticles). Starch was used as a cost effective and environment-friendly stabilizer. The Gram-negative *E. coli* (ATCC strain 8739) was used as the bacteria in this study.

### MATERIALS AND METHODS

**Synthesizing starch-stabilized nano scale zero-valent iron particles**

The starch-S-NZVIPs and b-NZVIPs were synthesized according to the procedure used by Keenan *et al.* via aqueous phase reduction of ferrous sulfate (FeSO₄·7H₂O) by sodium borohydride (NaBH₄) at room temperature.

Briefly, stabilized Fe⁰ nanoparticles were synthesized by adding 100 mL of 0.5 M NaBH₄ (MERKCO/Germany) in drop-wise fashion to a three neck flask containing 0.14 M solution of FeSO₄·7H₂O (MERK KGaA/Germany) (1:1 volume) and 0.2% (w/w) starch as a stabilizer with continuous mixing. To ensure efficient use of the reducing agent and prevent NZVI particles from being oxidized, the reactions had to be performed in anaerobic conditions. Accordingly, N₂ gas was bubbled to remove the dissolved oxygen (DO) during the synthesis. Once all of NaBH₄ solution was added, the sample was continuously stirred for 30 min to complete the reaction and ensure uniform growth of iron nanoparticles. The NZVI was synthesized via the following reaction:

$$2\text{Fe}^{2+} + \text{BH}_4^- + 3\text{H}_2\text{O} \rightarrow 2\text{Fe}^{0} + \text{H}_2\text{BO}_3^- + 4\text{H}^+ + 2\text{H}_2$$

The prepared solution was centrifuged (jouan-b, 11) at 2500 rpm for 5 min then the supernatant was decanted. After the nanoparticles had been washed with ethanol for 3 times, they were centrifuged, and the supernatant was decanted. Finally, the samples stored and dried in vacuum (iemierson-c55jxrl-420-5) overnight. b-NZVIPs (nonstabilized nano particles) were prepared through the same procedure as were S-NZVIPs without using starch.

**Analytical method**

In order to specify structure and composition of freshly synthesized NZVIPs, X-ray diffraction (XRD) analysis was carried out on a Siemens D5000 (Germany) diffractometer using Cu-Kα radiation (40 kV, 30 mA, λ = 0.15418 nm). The Samples were scanned for a 2θ range of 20-85°. Crystalline size of the nanoparticles was measured using Sherrer formula from the line broadening of XRD peak.
Surface morphology and size of the NZVIPs were characterized by scanning electron microscopy (SEM) on a Hitachi S 4160 model (Japan) instrument.

Microbial agent’s preparation

*Escherichia coli* (ATCC strain 8739) as the more precise indicator of microbial pollution was employed in this study. Bacteria suspension was obtained following the same procedures described by Lee *et al.*[^15^]. *E. coli* was inoculated on petri dishes with tryptic soy broth agar (Merck KGaA/Germany) and grown at 37°C for 18 h. The bacteria were obtained by centrifugation at 1000 g for 10 min; which were then washed twice with 50 mL of 150 mM phosphate buffer saline (PBS, pH = 7), and re-suspended in 50 mL of PBS to prepare the bacteria stock solution.[^15^]. The resultant bacterial suspension had an *E. coli* concentration of approximately 2-3 × 10⁵ CFU/mL.

Inactivation experiments

The microbial suspensions were diluted to about 10³ CFU/mL. NZVIP concentration of 5, 10, 50, 100, 500, and 1000 mg/L were tested in this study. The mixture samples were shaken at 250 rpm under room temperature for 5, 15, 30, 60, 120 min.

*Escherichia coli* was determined via the pour plate method. The number of colony forming units (CFUs) was counted on nutrient agar plates after they were incubated at 37°C for 18-24 h. Samples were plated in triplicate to ensure fidelity of the results. Colony counts were reported as the average value over three samples.

All the experiments were conducted under both anaerobic conditions (N₂ gas bubbled prior to initiation of the experiments to remove DO, and the test tubes were capped with parafilm) and aerobic conditions (exposed to the atmosphere).[^15^]

The following equation was used to evaluate bactericidal activity of the NZVIPs:

\[
\text{Inactivation rate (\%) = } \frac{\text{CFU}_{\text{control}} - \text{CFU}_{\text{exposed}}}{\text{CFU}_{\text{control}}} \times 100
\]

where CFU\textsubscript{control} represents the number of CFUs in the absence of NZVIPs, and CFU\textsubscript{exposed} indicates of the CFUs after doing the experiment with NZVIPs on nutrient agar plates.

The effect of parameters including contact time, NZVI concentration, DO, and stabilization on the bacteria removal efficiency was examined using starch-stabilized and bare Fe⁰ nanoparticles. The experiments were performed by applying 360 samples in triplicates.

Statistical analysis of the data

Data were summarized in mean (± standard deviation). Data distribution was checked for normality by one-sample Kolmogorov–Smirnov test. Since the normality was supported by this test, a parametric statistical test was used to assess the mean effect of concentration and DO. In addition, a three-way analysis of variance was performed to find their interactions. In the case of significant effects, *post-hoc* was applied to confidence intervals by implementing Bonferroni correction. All the analyses were performed using Minitab 16.

\textit{P} < 0.05 at a confidence level of 95% were considered statistically significant.

RESULT

Characterization results

X-ray diffraction and SEM results of Fe⁰ nanoparticles in this study are shown in Figures 1 and 2. According to XRD analysis, the synthesized crystals were ZVI. As shown in the figure, characteristic main diffraction peak at 2θ = 44.7° confirmed crystallization of Fe⁰ nanoparticles. The calculated crystalline size for Fe⁰ nanoparticles by Sheerer formula was found 10 nm.

![Figure 1: X-ray diffraction pattern of synthesized starch-stabilized nano scale zero-valent iron](image1)

![Figure 2: Scanning electron microscopy image of synthesized starch-stabilized nano scale zero-valent iron](image2)
SEM image for nanoparticles of Fe<sup>0</sup> is presented in Figure 2 which indicates that the stabilized synthesized NZVIPs were spherical in shape with an average diameter of about 50-100 nm.

**Escherichia coli inactivation**
Statistical analysis indicated that effects of the nanoparticles concentration (P < 0.001), contact time (P < 0.001) and oxidation status (P < 0.001) on the bacterial inactivation were significant.

**Effect of nano scale zero-valent iron concentration on the inactivation efficiency**
Figures 3a and b and 4a and b illustrate inactivation efficiency of *E. coli* after being treated with different concentration of S-NZVI and b-NZVI within different contact times. It is apparent from these figures that there was a direct relationship between inactivation efficiency of *E. coli* and nanoparticles concentration under both aerobic and anaerobic conditions, and increasing concentration of NZVI resulted in higher inactivation efficiency. Figure 3a illustrates that the inactivation efficiency increased from 50% to 100% as the S-NZVI concentration increased from 5 to 500 mg/L. According to Figure 3b, the removal efficiency of *E. coli* was about 26% using S-NZVI at 5 mg/L for 30 min; however, it reaches 100% when the S-NZVI concentration exceeded 100 mg/L. At the highest concentrations of NZVI (500, 1000 mg/L), a strong bactericidal effect (100%) was observed; even during a short contact time (5 min) [Figures 3 and 4]. Statistical analysis indicated that there was no significant difference between 1000 mg/L of NZVI within 5 min of exposure time versus 5 mg/L of that after 60 min of exposure (P = 0.163).

**Effect of contact time**
Bactericidal activity of NZVI was tested by culturing samples after 5, 15, 30, 60, and 120 min of treatment. In general, there was a positive correlation between inactivation rate and contact time. Figures 3a and b and Figure 4a and b show that there has been an abrupt increase in *E. coli* inactivation, after 30 min of exposure. At higher contact times, a small dosage of nanoparticles was able to effectively inactivate *E. coli*. For example, required concentration of NZVI to complete removal of bacteria population was 1000 mg/L after 5 min, whereas it was reduced to 50 mg/L when contact time increased to 120 min [Figure 4a].

From the data in Figures 3a and 4a, we can see that in anaerobic condition, stabilized and bare Fe<sup>0</sup>-NPs with the treatment time period of 120 min were capable to considerably reducing bacterial growth (80-90%) at their lowest concentrations (5 and 10 mg/L), and eventually increase it to 100% at higher concentrations (100, 500 mg/L).
According to the statistical analysis, no significant difference was found between exposure time of 30 and 60 min ($P = 0.26$), also 60 and 120 min ($P = 0.21$).

**Effect of dissolved oxygen**

Based on statistical analysis, there was a significant difference between aerobic and anaerobic conditions ($P < 0.05$).

Figure 5a and b depict a comparison between inactivation efficiency of nanoparticles at two conditions: Aerobic and anaerobic. As shown in Figure 6, inactivation efficiency of b-NZVIPS and S-NZVIPS was decreased in comparison with those of anaerobic condition.

In aerobic condition, the required concentration of nanoparticles for complete inactivation (100%) was about 10 times higher than that of the anaerobic condition. According to statistical analysis, there was no significant difference between the obtained efficiency with 100 mg/L in the anaerobic condition or 1000 mg/L in aerobic condition ($P = 1$).

In anaerobic conditions, concentration of 50 mg/L was sufficient to inhibit 100% bacterial growth after 1 h of treatment; whereas it was decreased to 65% under aerobic conditions [Figure 5b]. Figure 5a and b show that the presence of oxygen hindered inactivation of *E. coli* so that a negligible bactericidal activity (%) was resulted after a short mixing time (5 and 15 min). In the presence of oxygen, highly efficient inactivation of bacteria (90-100%) was only achieved with concentrations exceeding 500 mg/L after 60 min of shaking.

**Effect of stabilization**

Table 1 shows a statistic comparison between NZVI and S-NZVI at the equilibrium levels of concentrations. This table shows that there was no significant difference between NZVI and S-NZVI with exceptions at 100 and 10 mg/L.

Figure 6a compares the inactivation efficiency by stabilized and b-NZVI under aerobic condition. As can be seen, stabilization of NZVI reduced its toxicity toward *E. coli*, when exposed to the same concentration of nanoparticles within the same exposure time.

**Table 1: Statistic comparison between NZVI and S-NZVI**

| NZVI concentration (mg/L) | S-NZVI concentration (mg/L) | $P$  | $t$  |
|---------------------------|----------------------------|------|------|
| 5                         | 5                          | 1    | 0.6  |
| 10                        | 10                         | 0.00 | 5.59 |
| 50                        | 50                         | 0.79 | 1.84 |
| 100                       | 100                        | 0.009| 3.77 |
| 500                       | 500                        | 0.9  | 1.6  |
| 1000                      | 1000                       | 1    | 0.54 |

NZVI: Nano scale zero-valent iron, S-NZVI: Stabilized nano scale zero-valent iron
Figure 6b compares the results obtained from S-NZVI and b-NZVI under anaerobic condition. It is apparent from this figure that at low concentration (10 mg/L), b-NZVI shows higher inactivation efficiency than stabilized nanoparticles. However, similar efficiency was resulted using both nanoparticles as NZVI concentration increased to 100 and 500 mg/L.

**DISCUSSION**

The results suggested that the cytotoxicity of nanoparticles was strongly depended on NZVIPs concentration which was consistent with the previous reports. This result may be due to increasing number of nanoparticle attachments to the membrane and deepening intracellular penetrability of nanoparticles at higher concentrations. In anaerobic conditions, as nanoparticles concentration increased, a considerable reduction was observed in the bacterial growth. This result could be attributed to the residual ratio of nonoxidized NZVI. Once initial oxygen was consumed in the oxidation reaction, the residual NZVI plays a role in the bactericidal activity.

Our results indicated that there was an increase in E. coli inactivation within increasing contact time under aerobic condition. In contrast, Lee et al. found that inactivation rate of E. coli decreased over time under air saturation. Increasing exposure time could enhance the chances of particle-bacterial cell interaction; it can also increase accessibility of the Fe$^0$ surface for bacteria through keeping oxidation products suspended and preventing from oxide film formation on Fe$^0$. This operation may explain the relatively good correlation between shaking time and inactivation rate of E. coli under aerobic conditions.

The results of this study showed that inactivation efficiencies of both b-NZVIPs and S-NZVIPs were lower at the presence oxygen when compared to anaerobic conditions. This finding was in agreement with those of Lee’s indicating that the presence of DO significantly reduces the antimicrobial activity of nano-Fe$^0$. This result might be associated with rapid oxidation of Fe$^0$ into Fe$^{+3}$ by DO resulting NZVI surface to be coated by an iron oxide layer. Considering the fact that physical contact of cells with the ZVI nanoparticles plays an important role in disruption of cell membranes and generation of ROS, one may deduce that NZVI surface coating with an iron oxide layer might contribute to decrease its antimicrobial activities. There are, however, other possible explanations. A possible explanation might be that under aerobic conditions, E. coli secretes enzymes like superoxide dismutase (SOD). It has been reported in the literature that SOD as a superoxide radical scavenger, prevents ROS including superoxide, peroxide hydrogen, and hydroxyl radicals from being formed by suppressing the subsequent Fenton reaction. Chang et al. suggested that adding SOD to silver nanoparticle suspensions, reduced toxicity of these particles toward E. coli membranes. Another possible explanation is that in the presence of oxygen, various types of Fe-oxide such as magnetite and maghemite can be formed at the particle surface that these compounds are less toxic than Fe$^0$.

Even recent evidences have suggested that oxidation of iron can affect its biological impacts on bacteria by controlling ion release. Results obtained from the stabilization of nanoparticles indicated that inactivation efficiency of bare particles was higher than that of stabilized particles. They also showed that stabilization of nanoparticles with 0.2% (w/w) of starch reduced the rate of E. coli inactivation. This finding was consistent with that of Voladker’s who found that starched copper nanoparticles were less toxic than cupric ions. In addition, Zhou et al. studies suggested that carboxymethyl cellulose-coating decreases toxicity and oxidizing capacity of NZVI toward bacteria Agrobacterium. Li et al. also found that NZVI modification by polymer decreased its inactivation efficiency from 5.2 to 0.2 logs. Considering the fact that generation of ROS is one of the hypothesized mechanisms for inactivation of bacteria with NZVI, generating intracellular ROS and subsequently decomposing protein functional groups would require close proximity of nanoparticles to the cells. Starch might have protected E. coli cells in coated nanoparticles, reducing interactions of ROS or nanoparticles with cells. This causes a reduction in the physical disruption of cell structures; especially at low concentration and short contact time.

One of the most probable antimicrobial mechanisms in metal nanoparticles is a physical disruption of cell structures. Therefore, coating nanoparticles by stabilizers may contribute to weaken nanoparticles attachments to the bacteria surface via reducing contact area between nanoparticles and bacteria which may lead to attenuate cell membrane degradation.

Pan et al. showed that encapsulation of magnetite nanoparticles changes surface reactivity through a diffusion barrier toward contaminants. It has also been shown that humic acid and synthetic polymers decreased NZVI toxicity against E. coli by surrounding the nanoparticles surface and limiting their adhesion to the bacteria. Although stabilization may cause a reduction in the interactions between nanoparticles as well as alteration of aggregation pattern, its toxicity is alleviated by keeping nanoparticles from interaction with bacteria. In addition, a previous study has suggested that free ions could lead damages to DNA, proteins, and cell wall while they enhance penetrability of nanoparticles inside the cell in which slower release of iron ions from the capped nanoparticles occurred. While NZVI toxicity may be mediated through a release of Fe$^{+3}$, it is reasonable to assume that the close proximity of NZVI to the bacteria would increase its toxicity potential.
CONCLUSION

This study set out to determine the effect of starch-S-NZVIPs on E. coli. However, application of starch as the stabilization agent did not enhance inactivation efficiency of NZVIPs. The results of this investigation have revealed that generally the starch-SNZVIPs and b-NZVIPs have strong bactericidal activity against E. coli.

According to the statistical results, the concentration of NZVIPs and contact time were the most effective parameters in the bactericidal activity of NZVI. In addition, the results showed that bacteria removal using bare nanoparticles under anaerobic condition was more effective than that of S-NZVI under aerobic conditions. Nanoparticles surface coating with such compounds as iron oxide might reduce the potential toxicity of nano-Fe₀ while limiting its antimicrobial activities. This is one of the NZVI limitations deals with in disinfection application. Thus, additional research is required to overcome this drawback.

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Conflicts of interest

There are no conflicts of interest.

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