

# Effect of zero-valent iron on *Rhizobium* sp. cells isolated from cadmium-contaminated sites after remediation by zero-valent iron

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**Abstract.** Cadmium contamination found in paddy fields in the Maesot District of Tak Province, Thailand. This area was remediated using 50mg/L of ZVI. The study aimed to isolate and identify soil bacteria in the soil and rice roots and to investigate ZVI's effect on the isolated bacterial cells. The results indicated no significant difference in soil bacteria content before and after remediation at the 95% confidence level. Twelve isolates of nitrogen-fixing bacteria were obtained. Those isolates could grow at high concentrations of 300 mg/L of ZVI. RH17 had a high tolerance for TSA with 300 mg/L of ZVI at only 10 CFU/ml. The effects of ZVI at 150 mg/L on RH17 cells, a small amount of ZVI was observed adhering to the cells' surface and forming giant cells, while at 300 mg/L of ZVI, caused a reduction in growth by 81.0%. The *nifH* gene of RH17 was related to *Rhizobium* sp. strain 5-1-2. The results demonstrated the cadmium remediation process with 50mg/L of ZVI did not affect the cell count of soil bacteria in the paddy field. However, at 150 mg/L or higher, ZVI damaged the isolated *Rhizobium* sp. cell membrane. So, the remediation using ZVI must consider the appropriate concentration.

## 1. Introduction

Toxic and heavy metal contamination in the environment is a long-standing problem that harms humans and the environment. Cadmium contamination in paddy fields in the Maesot District of Tak Province, Thailand, is caused by improper mining practices and inadequate waste management [1]. In the case being studied, it is important to note that there was no decrease in cadmium bioaccessibility over time. Even after four years of cadmium aging in the soil, the bioaccessibility remained very high, accounting for about 60% of the total content in most contaminated soil samples [2]. Traditional techniques, such as excavation and landfilling, are expensive and unsustainable for removing contaminated soil. However, adding immobilizing agents to contaminated soil can reduce metal mobility and bioavailability, making soil amendment cost-effective and less disruptive [3]. One effective agent for reducing heavy metal contamination in soil is zero valent iron (ZVI). It is a low-

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cost amendment that can be used for both reductive and sorptive sequestration of heavy metals [4,5]. ZVI can effectively remediate cadmium-contaminated soil by binding directly to cadmium in soil. Stevenson et al [6] found that iron's ability to bind to cadmium charges effectively reduces cadmium levels in water by up to 80%. However, it should be noted that the ZVI that binds to cadmium can also increase toxicity and significantly reduce microbial activity [7].

Iron is a practical and effective method to bind metal in soil, as stated by Goncalves [8]. However, oxidative stress or the Fenton reaction can adversely affect this method by damaging cell membranes and genetic material. This can result in protein and lipid oxidation, leading to microorganism death or cell rupture [9]. Moreover, short-term exposure to Nanoscale ZVI (nZVI) can reduce freshwater microalgal growth and impact the aquatic environment, as observed in the research by D'ors et al. [10].

In 2020, Khum-in et al [11] successfully reduced cadmium concentration in the Phatat Pha Daeng Subdistrict Mae Sot District, Tak Province, Thailand. They achieved this by using a combination of biochar and zero-valent iron. Community researchers found that the application of biochar with zero-valent iron (BZVI) as a soil amendment reduced cadmium and enriched Fe and Zn in rice grains. In a later study 2023, Khum-in et al [12] indicated that magnet-assisted soil washing followed by soil amendment using ZVI also successfully removed cadmium from soil and reduced cadmium content in rice grains. However, the effects of this remediation process on soil microorganisms have not yet been explored. In rice paddy soil, biological nitrogen fixation is important for sustaining soil nitrogen fertility and rice growth [13]. This study aims to isolate and identify nitrogen-fixing bacteria from soil and rice roots in a cadmium-contaminated area remediated with 50mg/L ZVI. The study also investigates the effects of ZVI on the growth of obtained isolates.

## **2. Materials and methods**

### **2.1 Isolation of soil bacteria related to nitrogen-fixing bacteria and phenotypic characterization**

#### *2.1.1 Bacteria isolation and enumeration*

10 grams of soil contaminated with cadmium and remediated using ZVI were placed in a 250 mL Erlenmeyer flask containing 90 mL of 0.85% NaCl. The control soil samples were taken from the paddy fields before remediation. The mixture was shaken at 120 rpm for 30 minutes. A serial dilution was performed by adding 0.85% NaCl to a dilution of  $10^{-5}$ . The dilution of  $10^{-3}$  to  $10^{-5}$  was then spread onto plates containing Yeast mannitol agar with Congo red (Himedia). These plates were incubated for 2-5 days at 30°C to observe colonies of bacteria and enumeration. After remediation with 50mg/L of ZVI, the rice was grown for 12 weeks. The method used for isolating nitrogen-fixing bacteria from rice roots was based on Mbai FN et al. [14]. The morphology of different colonies was isolated and subcultured in the same media for further study. The number of bacteria before and after remediation were compared at the 95% confidence level.

#### *2.1.2 Nitrogen fixing activity*

Isolated bacteria were cultured in a nitrogen-free mineral medium at 30°C to evaluate nitrogen-fixing activity. The medium's color change was recorded after incubation for 3-7

days. Afterward, the ammonium test kit reagents were added, and the resulting color was recorded by comparing it to the color chart provided [15].

## **2.2 The effect of ZVI on bacterial cells**

### *2.2.1 Survival of bacterial cells in the presence of different concentrations of ZVI*

Isolated bacteria were cultured in Tryptic soy broth and then incubated at 30°C for 48 h. Afterward, centrifugation was performed at 6,000 rpm for 5 min. The cells were washed twice with 150 mM phosphate-buffered saline (pH 7.2) and mixed with 50 mM carbonate buffer (pH 8.0). The concentration of cells was adjusted to approximately  $1.0 \times 10^8$ - $3.0 \times 10^8$  CFU/ml in 25 mL carbonate buffer. These cells were added to tubes containing 50, 100, 150, 200, 250, and 300 mg/L ZVI and then incubated at 30°C for 48 h [16]. The viability of the bacteria was evaluated using a spread plate technique on TSA agar, which was then incubated at 30°C for 48 h.

### *2.2.2 Survival of bacterial cells with varying concentrations in the presence of 300 mg/L of ZVI*

Isolated bacteria were cultured in Tryptic soy broth and then incubated at 30°C for 48 h. The cell concentration was adjusted to approximately  $1.0 \times 10^8$ - $3.0 \times 10^8$  CFU/ml in 25 mL carbonate buffer and then diluted to different cell concentrations (CFU/mL). A variable number of cells with different concentrations were added to the TSA medium with 300 mg/L ZVI. Then, the plates were incubated at 30°C for 24 h, and bacterial growth was observed [17].

### *2.2.3 The effects of ZVI on bacterial cells via Transmission Electron Microscopy*

Isolated bacteria were cultured in Tryptic soy broth and then incubated at 30°C for 48 h. Afterward, centrifugation was performed at 6,000 rpm for 5 min. The cells were washed twice with 0.1M ammonium acetate. The concentration of cells was adjusted to approximately  $1.0 \times 10^8$ -  $3.0 \times 10^8$  CFU/mL. Cells were diluted in 0.1M ammonium acetate to  $10^4$  CFU/mL, treated with 150 and 300 mg/L ZVI, and then shaken at 180 rpm for 60 minutes to distribute the ZVI throughout the solution. The survival of bacteria was checked using the spread plate technique on a TSA medium, and the results were compared with those of the control. Some samples were prepared for study under a Transmission Electron Microscope using negative staining with uranium acetate [18]. The altered cell characteristics were studied at the Science Lab Center, Faculty of Science, Naresuan University.

## **2.3 Phylogenetic analysis of *nifH* gene**

### *2.3.1 DNA extraction, purification, and amplification*

The selected bacteria were grown in Tryptic soy broth under shaking at 30°C for 18 h. The resulting bacterial suspension was then centrifuged at 12,000xg for 12 min. The centrifuged sample was further processed using the Genomic DNA extraction Mini kit, and the extracted DNA was stored at -20°C for further study [19]. DNA amplification with *nifH* genes,

approximately 760 bp of *nifH* gene was amplified by using *nifH1* 5' CTG TTT ACG GCA AGC GCG GTA TCG GCA 3' as forward primer and *nifH2* 5'TCC TCC AGC TCC TCC ATG GTG ATC GG 3' as reverse primer in 50 µL PCR Master mix under DNA amplification conditions. The reaction conditions were an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 30 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min. PCR products were stored at 4°C. Ten microliters of PCR product were examined using gel electrophoresis at a 1.2% agarose gel concentration in 0.5X TBE Buffer, and then the DNA band was measured using UV Light Transilluminators [20].

### 2.3.2 DNA sequencing

The purified PCR product was sequenced by Macrogen Korea and aligned using GenBank's BLAST function and the National Center Biotechnology information on the NCBI website. The data set's evolution tree was inferred using the Maximum Likelihood method based on the Kimura-2-parameter model. Evolution analyses were conducted using Evolutionary Genetics Analysis (MEGA) software version 7 [21].

## 2.4 Statistical analysis

The data analysis was performed by Stata (Stata12.0 Corporation, USA). The One-Way ANOVA was analyzed by comparing multiple groups of LogCFU/g before and after remediation. The P-values < 0.005 were compared with the Greenhouse Geisser epsilon, considered statistically significant.

## 3. Results

### 3.1 Isolation of soil bacteria related to nitrogen-fixing bacteria and phenotypic characterization

The levels of soil bacteria related to nitrogen-fixing bacteria in the soil before and after remediation with ZVI were compared. The results showed that the number of bacteria before remediation was 6.33 log, which was not significantly different from after remediation (6.95 log) at the 95% confidence level. Fifty-six bacterial isolates were found on YMA plates with Congo red, with forty-four originating from soil and eight from roots. Among these, sixteen were identified as gram-positive and forty as gram-negative bacteria. Microscopic analysis revealed that fifty-one isolates had rod-shaped cells, while three had filamentous shapes, and two had rod shapes with rounded ends. From the catalase and cytochrome oxidase synthesis tests, it was found that fifty-two bacterial isolates exhibited catalase synthesis, while only four isolates showed negative results. The cytochrome oxidase test showed positive results for thirty-five isolates and negative results for twenty-one isolates (Table 1).

**Table 1.** Morphological and biochemical characteristics of 56 bacteria isolated from soil and root samples.

Samples	Gram's		Shape			Catalase		Oxidase	
	+	-	rod	bacilli	hyphae	+	-	+	-
Soil	12	36	44	2	2	44	4	28	20

Rice root	4	4	7	-	1	8	-	7	1
Total	16	40	51	2	3	52	4	35	21

Twelve bacterial isolates had biochemical properties similar to those of nitrogen-fixing bacteria, *Rhizobium* sp. Out of these twelve, ten were found in soil (RH5, RH6, RH12, RH17, RH22, RH23, RH25, RH29, RH34, and RH42), and two were found in roots (RH2R and RH7R) (Table 2).

**Table 2.** The morphology and biochemistry of isolated bacteria were compared to *Rhizobium* sp. to determine the amount of fixable nitrogen. \* The study conducted by Yu et al., 2011 [15].

Isolates	Morphological and biochemical test				The amount of fixable nitrogen
	Gram's	Cell shape	Catalase	Oxidase	
<i>Rhizobium</i> spp. *	-	Rod	+	+	2*
RH5	-	Rod	+	+	<0.25
RH6	-	Rod	+	+	1.50
RH12	-	Rod	+	+	<0.25
RH17	-	Rod	+	+	<0.25
RH22	-	Rod	+	+	0.25
RH23	-	Rod	+	+	<0.25
RH25	-	Rod	+	+	0.25
RH29	-	Rod	+	+	0.25
RH34	-	Rod	+	+	<0.25
RH42	-	Rod	+	+	<0.25
RH2R	-	Rod	+	+	<0.25
RH7R	-	Rod	+	+	0.25

### 3.2 The effect of ZVI on bacterial cells

All twelve isolates that gave similar physiological and biochemical test results to bacteria in nitrogen-fixing bacteria were tested for resistance to ZVI at concentrations of 50-300 mg/L. After conducting the test, it was discovered that all twelve isolates survived at the maximum concentration of 300mg/L ZVI, even when their cell concentration was  $1.0 \times 10^8$  CFU/mL. This was in contrast to *Escherichia coli* DH5 $\alpha$  and *Escherichia coli* ATCC25922, which are sensitive to heavy metals and antibiotics and could only survive at a maximum ZVI concentration of 300mg/L.

The survival of bacterial cells with varying concentrations when exposed to ZVI at 300 mg/L. This study discovered that ZVI could prevent the growth of bacteria. Among the several bacteria tested, three isolates (RH5, RH6, and RH2R) could withstand a concentration of 300 mg/L of ZVI at a cell concentration of  $10^3$  CFU/mL. Moreover, at a cell concentration of  $10^4$  CFU/mL, four other isolates (RH17, RH22, RH25, and RH34) exhibited tolerance to ZVI (Table 3). Out of four isolates, RH17 was identified as the fastest grower. Consequently, RH17 was chosen for further studies.

**Table 3.** The concentration of bacterial cells that can be grown on TSA medium with 300mg/L of ZVI.

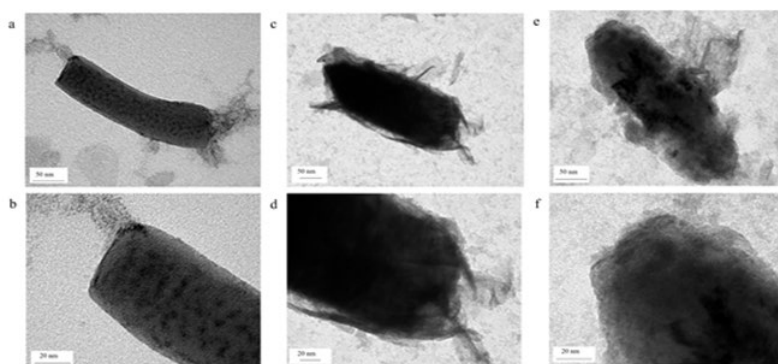
Isolates	Cell concentrations (CFU/mL)
<i>Escherichia coli</i> DH5 $\alpha$	$10^4$
<i>Escherichia coli</i> ATCC25922	$10^4$

RH6, RH7, RH2R	10 <sup>3</sup>
RH12, RH23, RH29, RH42, RH7R	10 <sup>2</sup>
RH17, RH22, RH25, RH34	10

The effects of ZVI on bacterial cells were analyzed via Transmission electron microscopy. RH17 was grown in a Tryptic soy broth with 150 mg/L of ZVI. After being shaken at 180 rpm for 60 minutes, the growth decreased by 71.16%. A slight adhesion of ZVI to the cell surface increased cell size, leading to the formation of more giant cells compared to the control group. In contrast, the growth increased by 13% in the control group, and the cells remained in good condition. However, their cell membranes began to deteriorate when bacterial cells were grown in a TSB medium with 300 mg/L of ZVI. ZVI was found to stick to the cell membrane, and the growth decreased by 81.0% (Table 4, Figure 1).

**Table 4.** Growth of isolated bacteria RH17 in TSB medium with and without added ZVI after 60 minutes of incubation.

ZVI conc. (mg/L)	Cell Number (CFU/mL)		Percent (%)	
	Control	After 60 min	Increased	Decreased
0	1.00x10 <sup>4</sup>	1.13x10 <sup>4</sup>	13	-
150	1.00x10 <sup>4</sup>	2.83x10 <sup>3</sup>	-	71.76
300	1.00x10 <sup>4</sup>	1.90x10 <sup>3</sup>	-	81.00



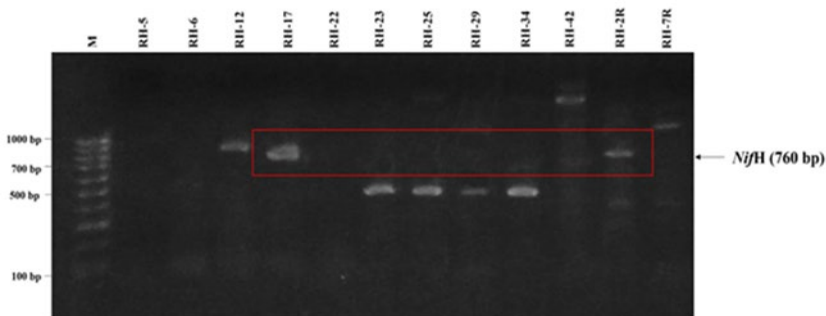
**Fig. 1.** shows the bacterial cells of the RH17 observed under a Transmission electron microscope (TEM) after being grown for 60 minutes in a TSB medium with and without the addition of zero-valent iron.

- a-b Characteristics of bacterial cells in TSB medium
- c-d Characteristics of bacterial cells in TSB medium containing 150 mg/L ZVI
- e-f Characteristics of bacterial cells in TSB medium containing 300 mg/L ZVI

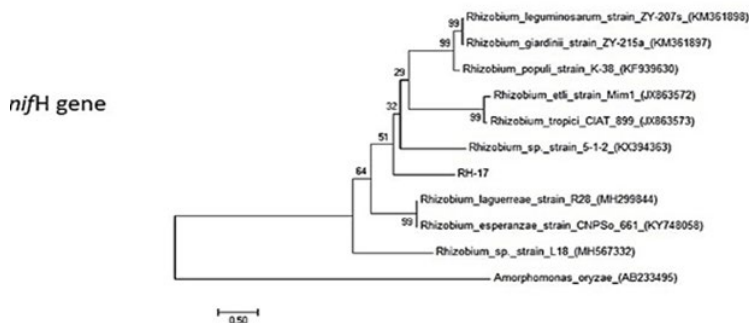
### 3.3 Phylogenetic analysis of *nifH* gene

After amplifying *nifH* genes through a polymerase chain reaction, three isolates (RH12, RH17, and RH2R) displayed a genetic material band of approximately 760 bases (Figure 2). To evaluate the phylogenetic analysis of *nifH* gene, the purified product was sequenced by Macrogen Korea. The nucleotide sequence was aligned using the BLAST function of GenBank and the National Center Biotechnology information on the NCBI website (<http://ncbi.nlm.nih.gov>). The data set was inferred using the Maximum Likelihood method based on the Kimura-2 parameter model. Evolution analyses were conducted using

Evolutionary Genetic Analysis (MEGA) software version 7 [21]. The phylogenetic analysis of the *nifH* gene RH17 is shown in Figure 3.



**Fig. 2.** The band of genetic material that appears after amplifying the *nifH* gene through polymerase chain reaction.



**Fig. 3.** Molecular phylogenetic analysis by Maximum Likelihood method. The Maximum Likelihood method based on the Kimura-2 model inferred the evolutionary history. The three with the highest log likelihood (-6645.8643) are shown. The percentage of trees in which the associated taxa clustered is shown next to the branches. The heuristic search's initial tree (s) were obtained automatically using the Maximum Parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+ 3rd+noncoding. All positions containing gaps and missing data were eliminated. There was a total of 626 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

## 4. Discussion

The amounts of soil bacteria related to nitrogen-fixing bacteria before and after soil remediation with ZVI 50 mg/L in the Phatat Pha Daeng Mae Sot District, Tak Province, Thailand, were not significantly different. Therefore, bacteria can grow and multiply at 50 mg/L ZVI. So, it is possible to conclude that ZVI at this concentration did not affect the growth of soil bacteria. This could be explained by the fact that these isolated bacteria had a high tolerance to ZVI because, in an appropriate environment, they have a high cadmium concentration. As a result, bacteria have adapted to survive in such conditions by using mechanisms such as expelling metals from the cell or reducing the uptake of metals into the cell [22].

The isolation of fifty-six bacterial isolates with varying colonies resulted from the soil and rice roots in the cadmium remediation area: forty-eight from the soil and eight from rice roots. Fifty-six bacterial isolates' physiological and biochemical characteristics revealed twelve shared characteristics with the nitrogen-fixing bacteria. Of the twelve isolates tested, five could fix nitrogen. These five isolates were RH6, RH22, RH25, RH29, and RH7R. They

can fix nitrogen within the 0.25-1.50 mg/L range. This site in the Maesot District, Tak Province, Thailand, was remediated using magnet-assisted soil washing and soil amendment with 50mg/L ZVI to remove cadmium contamination. After 12 weeks of remediation, the concentration of cadmium in the soil decreased from 41.30±3.50 mg/kg to 34.82±6.27 mg/kg, while the concentration of iron increased from 33.49±0.60 mg/kg to 54.71±1.75 mg/kg [10]. The presence of ZVI during the remediation process may impact the nitrogen-fixing bacteria in the area. Therefore, the next step was to assess the impact of higher iron content on these bacteria.

The effect of ZVI on bacterial cells: It has been found that all twelve bacterial isolates, namely RH5, RH6, RH12, RH17, RH22, RH23, RH25, RH29, RH34, RH42, RH2R and RH7R, can survive at a ZVI concentration of 300 mg/L, even when their cell concentration is  $1.0 \times 10^8$  CFU/mL. Among the bacteria tested, three isolates (RH5, RH6, and RH2R) were able to withstand a concentration of 300 mg/L of ZVI at a cell concentration of  $1.0 \times 10^3$  CFU/mL. In contrast, four isolates (RH17, RH22, RH25, and RH34) could grow at a cell concentration of 10 CFU/mL. It has been shown that these bacteria were highly tolerant to ZVI.

After examining bacterial cells of RH17 using a Transmission electron microscope in a Tryptic soy broth medium with 150 mg/L of ZVI, there was a slight adhesion of ZVI on the cell surface. This increased in cell size, leading to the formation of more giant cells compared to the control group. It was observed that the growth decreased by 71.16%. Bacteria can protect themselves against damage caused by heavy metals in several ways. They can produce proteins that bind to metals outside the cell membrane, excrete heavy metals from cells through efflux transporters, synthesize metal-binding proteins within cells to reduce metal toxicity or create organic substances outside the cell to prevent heavy metals from entering cells. This mechanism enables bacteria to thrive in an environment with high levels of metal [23].

Moreover, it was observed that the characteristics of RH17 bacterial cells were altered when the concentration of ZVI was increased to 300 mg/L. Direct contact with ZVI caused deterioration of the bacterial cell wall. ZVI directly affects the phospholipid fatty acid (PLFA) layer around bacterial genetic material. From the report of cell membrane composition analysis, a study reported that when *Pseudomonas putida* F1 is cocultured with ZVI nanoparticles, there is a change in cis-undersaturated fatty acid to trans isomers when the bacterial cells come into contact with the nanoparticles. This makes the bacterial cell membrane stiffer in the short term instead of being soft [24]. Reports have suggested that entering Fe(II) iron into *Escherichia coli* cells can damage their cell membrane. This can trigger the generation of reactive oxygen species (ROS), such as oxygen or hydrogen peroxide, and ultimately lead to oxidative stress. As a result, the bacterial cells may rupture [25].

The results show that the isolate RH17 was highly tolerant to ZVI, which is different from the research conducted by Chen et al. (2013) [26]. Their study investigated the effect of ZVI on the growth of denitrifying bacteria and the denitrification process. Their findings revealed that only high concentrations of ZVI impacted the growth of microorganisms. When the concentration exceeded 20 mg/L, it affected the growth and activities of bacteria, particularly during the denitrifying process.

Phylogenetic analysis of the *nifH* gene revealed that PCR products of approximately 760 bases were expressed by three nitrogen-fixing bacteria isolates, RH12, RH17, and RH2R. The *nif* gene plays an important role in nitrogenase enzymes found in bacteria and archaea, as they convert nitrogen into ammonia through nitrogen fixation. This gene can be found in many bacteria, such as *Klebsiella pneumonia*, *Azotobacter vinelandii*, and *Pseudomonas stutzeri* [27]. However, some bacterial isolates could fix nitrogen without expressing *nifH*, a nitrogenase Fe protein synthesis gene. This gene is commonly found in Proteobacteria,

Cyanobacteria, Firmicutes, and Actinobacteria [28]. It is possible for bacterial isolates without *nifH* genes to still fix nitrogen due to the presence of other *nif* genes, such as *nifD* and *nifK* [29]. Some bacteria have different combinations of *nif* genes. For example, *Rhodobacter capsulatus*, has *nifAB* operon and *nifU*, while *Azotobacter vinelandii* has *nifE* and *nifN*. It has been reported that *Rhizobium* sp. NGR 234 is missing the *nifH* gene. However, the bacterial isolates RH5, RH6, RH7R, RH22, RH23, RH25, RH29, RH34, and RH42 may use *nif* genes other than *nifH* to fix nitrogen [30].

Three nitrogen-fixing bacteria isolates, RH12, RH17, and RH2R, had the *nifH* gene, and RH17 was highly tolerant to ZVI. After phylogenetic analysis of the *nifH* gene, RH17 was identified as belonging to *Rhizobium* sp. strain 5-1-2 (GenBank Accession Number KX394363.1).

## 5. Conclusions

Twelve isolates of nitrogen-fixing bacteria were obtained from the soil and rice roots in Maesot District, Tak Province, Thailand, which had undergone remediation cadmium by using 50mg/L of ZVI. These isolates could grow at high concentrations of 300 mg/L of ZVI. After exposing RH17 cells to a TSB containing 150 mg/L of ZVI, a small amount of ZVI was observed adhering to the cells' surface and forming giant cells, while at 300 mg/L of ZVI, the deterioration of their cell membrane caused a reduction in growth by 81.0%. The *nifH* gene of RH17 was related to *Rhizobium* sp. strain 5-1-2. Our findings revealed that nitrogen-fixing bacteria have a high tolerance to ZVI, necessitating further research into gene identification and the functional characterization of the nitrogen cycle.

Acknowledgments: This work was financially supported by the National Research Council of Thailand (Number 102/2559).

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