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Remediation of *Staphylococcus Aureus* in Contaminated Wastewater using Biochar Supported Nanoscale Zero-valent Iron

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Abstract

Over the last decade, green synthesis methods for biochar-supported metallic nanoparticles have gained popularity due to their versatile applications in technology. Addressing concerns about microorganism contamination in wastewater, nano zero-valent iron (nZVI) within biochar (BC) inhibits bacteria through reactive oxygen species and interacting with bacterial membranes. This study examines two distinct materials: rice husk (RH) and lignin (Lig), for the synthesis of nZVI. RH was explored via two routes: (1) Synchronous route where iron salt was loaded into raw RH feedstock, followed by pyrolysis at 300 °C and subsequent carbothermal reduction (CR) of iron at 900 °C (BM-nZVI), and (2) Asynchronous, involving iron salt loading onto pre-pyrolyzed RH-BC with subsequent CR (BC-nZVI). For Lig, the study examined: (1) Synchronous route with nZVI deposition on pre-pyrolyzed (1000 °C) Lig-BC, creating surface-deposited nZVI (Lig-s-nZVI), and (2) Asynchronous route by mixing iron salt with dissolved feedstock to embedded nZVI in a carbonaceous carrier, resulting in engraved nZVI (Lig-eG-nZVI). The characterization of synthesized nZVI particles was obtained by SEM analysis. This study shows to comparatively evaluate the biological responses of *Staphylococcus aureus* (*S. aureus*) to four variations of BC-supported nZVI materials including the pristine BC of each material serving as the control. Initially, pure bacterial cultures were employed to measure inhibition zones using the well diffusion method, revealing prominent inhibition zones for RH-nZVI, while no significant inhibition was observed for Lig-nZVI and pristine BC. The minimum inhibitory concentration of RH-nZVI against *S. aureus* was determined to be 0.02 g mL^{-1}, and optical density at 600 nm (OD_{600}) was assessed. By contrast, BC-nZVI reached higher levels of microbe inactivation than BM-nZVI due to the FeC_4 layers surrounding nZVI particles, potentially hindering BM-nZVI's reactivity. Testing was conducted on hospital and farm wastewater. Gel electrophoresis, Gram's staining, and catalase test were used to identify microbes in wastewater.

Keywords: Antimicrobial activity; wastewater; lignin biochar supported nZVI; rice husk biochar supported nZVI; *Staphylococcus aureus*

Abbreviations

BC - Biochar.
BM - Biomass.
CR - Carbothermal reduction.
DMSO - Dimethyl sulphoxide AR.
LB Agar - Luria Bertani Agar.

Lig - Lignin.
Lig-eG-nZVI - Lignin engraved nano zero-valent iron.
Lig-s-nZVI - Lignin surface deposited nano zero-valent iron.
MIC - Minimum Inhibitory Concentration.
NA - Nutrient Agar.
nZVI - Nano zero-valent iron.
OD_{600} - Optical density at 600 nm.
RH - Rice husk.
S. aureus - *Staphylococcus aureus*.

Introduction

In nanotechnology, nanoparticles (NPs) play a major role and find use in a variety of applications within the field [15, 25]. The term biochar (BC) refers to the porous, carbon-rich charred organic matter which is produced when waste biomass is carbonized under extreme heat and pressure. When nanostructured materials are added to biochar in order to enhance its novelty, the application becomes even more compelling [4].

By exploiting their unique physical chemical and biological properties, metal nanoparticles are being widely used for a wide range of applications in the rapidly developing field of nanobiotechnology, including (1) drug delivery, (2) biosensors, (3) bioimaging, (4) antimicrobial activities, (5) food preservation, etc [19] Recent studies have investigated the antibacterial activity of nano zero-valent iron particles (nZVI), which have been considered an effective antibacterial agent. A number of studies have already shown that the nZVI nanoparticles are more effective against bacteria than other iron-based nanoparticles, as well as being able to inactivate bacteria such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) more effectively in an environment devoid of oxygen compared to one with saturated air [19, 5].

The purpose of this study is to test the efficacy of synthesized biochar supported nZVI nanoparticles against *S. aureus* by using various dosages of the nanoparticles as a bactericidal agent. In this study, the growth inhibition mechanisms, and the microbial inactivation mechanisms of nZVI particles have been discussed in detail in relation to *S. aureus*. It was also found that the sterilization effect and the bioactivity of the RH-BC supported nZVI particles towards pathogenic hospital wastewater and farm wastewater as well as their capability to dispose of these pathogenic microorganisms from actual samples.

S. aureus is a Gram-positive bacterium that belongs to the *Micrococcaceae* family. *S. aureus* forms irregular grape-like clusters (Figure 3.4). The organism grows rapidly under aerobic conditions, forming glistening, smooth, entire, raised, translucent colonies that often exhibit golden pigmentation [13].

Human skin and the nasopharynx are the natural habitats of *S. aureus*. A wide range of human diseases can be caused by *S. aureus*, ranging from minor skin infections to potentially fatal infections such as bacteremia and meningitis. A nosocomial or community-acquired pneumonia, skin infections, soft tissue infections, or bloodstream infections caused by *S. aureus* are among the most common causes of infection [25].

Nanotechnology is a very broad field that includes nanomaterials, nanotools, and nanodevices [15, 14]. Despite this long history, the development of nanotechnology and the production of nZVI have brought new application fields and new challenges for metallic iron. A significant difference exists between the properties of nZVI and bulk iron, and as a result of its relatively low cost, high reactivity, and good adsorption ability, it has become a very important engineered nanomaterial, as well as the most widely used nanomaterial for environmental remediation, such as soil and groundwater treatment [24]. Recently, there has been a lot of interest in nZVI particles in the field of environmental remediation for their ability to remove both organic and inorganic pollutants from aqueous solutions [14].

Despite its particle size, zero-valent iron (Fe^0) is known to be an excellent electron donor. A strong tendency for Fe^0 to release electrons is evident in the presence of water [24].

$$Fe^0 \longrightarrow Fe^{2+} + 2e^-$$

Biochar is a dark, porous solid that is made by thermally decomposing organic materials or carbonizing waste biomass [6, 17, 22]. In a prior work, composites of nZVI and biochar were created, and it was discovered that they have good adsorptive properties for a range of pollutants, including heavy metals [33]. The inclusion of metallic nanoparticles (NPs) into a biomass-based matrix has been the subject of extensive research since the resulting nanocomposites displayed significant antibacterial action against bacteria with unbeatable resistance. However, the nanocomposites have drawbacks, including a lengthy preparation process and a small amount of nanoparticle loading capacity [12]. Rice husk biochar has been shown to have positive effects on soil pH, moisture, Olsen-P, SMBC, SMBP, phosphatase activity, and soil microbiota characteristics at specific time points, but the effects at different time points were not significant. As a result of these changes, biochar treatment provided microbes with better growth conditions [17]. This work reports the inhibition ability of BC-nZVI and lignin-BC-nZVI.

Growth of *E. coli* is studied in a biochemical incubator using biochar composites and silver emended biochar nanocomposites. Ag-biochar composites are used as antibacterial agents because of their strong antimicrobial properties [6]. Antimicrobial and antibacterial properties of nano biochar composites make them effective for treating contaminated water. The product is highly efficient, reusable, and has a long shelf life, so it can be used in a wide range of applications, such as sanitation, medical care, and environmental remediation [27].

Due to their large specific surface area and small particle size, nZVI particles tend to aggregate quickly, which reduces their capacity to extract heavy metals and bacteria [12]. As a porous carrier with a high specific area of carbon based material, BC is inexpensive and obtained by the pyrolysis of biological waste, In the meanwhile, BC is thought to be useful substance for dispersing nZVI and lowering agglomeration [18]. Moreover, is a commonly utilized remediation material for the adsorption and complexation process that remove bacteria.

Nanoparticles have different properties in contrast to their bulk materials because of their occurrence in Nano scale. Metallic nanoparticles are widely used in several fields including the textile industry, food industry, agriculture, health sector, and cosmetics.

Recent advances in nanotechnology have led to the synthesis of several different kinds of metal and metal oxide nanoparticles that can act as antimicrobial agents. A wide range of biomedical applications are possible with these compounds. Among them are textile fabrics, and disinfectants or anti-biofilm agents used in water treatment. It has been proven that nZVI inhibited growth and activity of *E. coli* and *S. aureus* growth and activity [27, 33].

In a recent study, the pre-treatment and disinfection of municipal wastewater using nZVI nanoparticles was examined [27]. As a part of this study, hospital wastewater and farm wastewater were investigated as applications.

Despite the increasing interest in biochar-supported nano zero-valent iron (nZVI) for bacterial remediation, there is a notable lack of comparative studies evaluating the efficacy of rice husk biochar-supported nZVI (RH-BC) and lignin biochar-supported nZVI (Lig-BC) particularly against *Staphylococcus aureus*. This study shows that using different types of biochar, specifically RH-BC and Lig-BC, can enhance the remediation potential of nZVI.

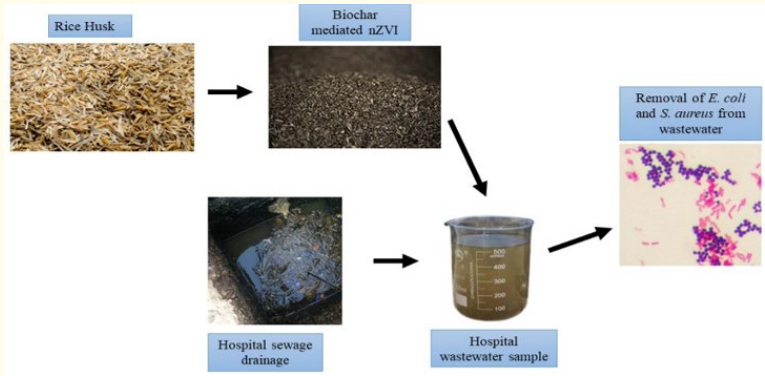

Figure 1.1: Schematic diagram of removal of *E. coli* and *S. aureus* from hospital waste using biochar supported nZVI.

Materials and Methods

Chemicals, Instruments and Apparatus

The reagents used in this study were of analytical grade and were used without further purification. Dimethyl sulphoxide AR (DMSO -99%), hydrogen peroxide (H_2O_2 - 30%) was purchased from HiMedia, Mumbai, India. Distilled water was used in preparation of all solutions.

Nutrient agar (NA; HiMedia, Mumbai, India), Luria Bertani agar (Miller) (LB; HiMedia, Mumbai, India), Nutrient broth (NB, HiMedia, Mumbai, India) were prepared according to the instructions provided by the manufacturer.

Bio safety cabinet (BSC-1500IIA2-X, Jinan Biobase Biotech Co., LTD, Japan), Shaking water bath (GFL D-30938 Burgwedel, Germany), Centrifuge (Thermo fisher scientific, Thermo Electron LED VGmbH Zweigniederlassung Osterode, Am Kalkberg, 37520 Osterode am Harz, Germany), Magnetic stirrer, UV spectrophotometer (6705, Jenway, Ole-parmer Ltd, stone, UK), Analytical Balance (AS 82/220. R2, RADWAG wagi electoniczne, Poland), Pharmaceutical refrigerator (MPR-715F-PE, PHC corporation), Microwave (M1L202B U-therm international (H.K) Limited), PCR machine (VeritiTM 96- Well Thermal Cycler EN61326, Class B, Group 1, Life technologies holdings Pte Ltd Singapore), UV Transilluminator (SN 502007 Labnet international), Incubator (Raypa, Spain).

Biochar supported nano zerovalent iron particles

The lignin and rice husk biochar supported nZVI with dimensions of 0.05-0.1 mm were obtained from Institute of Chemistry Ceylon, Rajagiriya, Sri Lanka and stored in airtight containers.

The synthesis of husk biochar (RHBC) nZVI composites were performed via two routes. In the synthesis of BM-nZVI, the iron salt was loaded into raw feedstock, and the pyrolysis of biomass and carbothermal reduction (CR) of iron were performed synchronously. In contrast, iron salt was loaded onto pre-pyrolyzed RHBC and subjected to subsequent CR during the asynchronous route. When lignin was utilized as the carrier in the synchronous route (Lig-eG-nZVI), the iron salt was mixed with the dissolved feedstock so that nZVI were embedded in the carbonaceous carrier. In contrast, nZVI is deposited on the surface of pre-pyrolyzed lignin biochar in an asynchronous route (Lig-s-nZVI).

Bacteria culture and nano zero valent iron particles

Staphylococcus aureus (ATCC 25923) was purchased from Department of Microbiology, University of Kelaniya, Sri Lanka and stored at -80 °C. The bacterial strains were grown in nutrient broth at 37 °C in the shaking water bath overnight and broth cultures were stored at 1 °C. The liquid medium was sterilized by autoclave at 121 °C for 15 minutes.

For the determination of colony forming units (CFUs), a serial dilution procedure was performed using a stock culture. A 1:10 dilution series was created by adding 1 mL of culture to 9 mL of autoclaved distilled water. Each dilution was then plated on nutrient agar and incubated at 37 °C for 24 hrs. To determine the number of CFUs, colony counts were manually conducted [2].

Biochar-supported nanoparticles and their antibacterial activity

Based on the agar well diffusion method, biochar-supported nZVI particles were tested for their antibacterial activity [4. 27]. In 10 cm petri dishes, 0.1 mL of fresh cultures were added to 20 mL of nutrient agar medium and LB agar medium. A concentration of 0.5 g/mL of each sample was added to wells with a diameter of 6 mm. (Two types of samples were used for each petri dish: Rice Husk biochar-supported nZVI as BC-nZVI and BM-nZVI, and Lignin biochar-supported nZVI as Lig-S-nZVI and Lig-eG-nZVI). The samples were prepared using 50 µL of DMSO. The plates were then refrigerated for 90 minutes to allow the nZVI substance to diffuse. The plates were then incubated at 37 °C for 24 and 48 hours. Using a ruler, the inhibition zone was determined at the end of the incubation period [4, 27]. As a positive control, pristine biochar was used in the antibacterial test.

Analysing the minimum inhibitory concentration (MIC) of biochar-supported nZVI

The MIC is defined as the lowest concentration at which a substance inhibits the growth of microorganisms or other biological entities after 24 hours of incubation [27]. MIC of biochar supported nZVI particles was determined using the well diffusion assay method [4, 27]. The MIC determination was performed for two types of rice husk biochar-supported nZVI particles: BC-nZVI and BM-nZVI. Pour plates were prepared by inoculating 0.1 mL of fresh cultures onto nutrient agar medium in 10 cm petri dishes. The biochar supported nZVI particles were dissolved in 50 µL of DMSO and added to the wells in concentrations of 0.500 g/mL, 0.375 g/mL, 0.250 g/mL, 0.125 g/mL, and 0.020 g/mL. The plates were incubated overnight at 37 °C. Using a ruler, the inhibition zone was determined at the end of the incubation period.

Analysing the optical density assay (OD_{600})

Optical density at 600 nm (OD_{600}) is commonly used to determine the turbidity or concentration of bacterial cultures in a liquid culture medium where the absorption measured at 600 nm. The principle behind it is that the more turbid a bacterial suspension is, the higher the optical density reading at a particular wavelength will be [27]. The following formula was used to calculate the growth of inhibition for the wells used for the antimicrobial test at each extract dilution.

$$Percentage\ inhibition = \frac{ODcontrol - ODsample}{ODcontrol} \times 100\ \%$$

In this study, the optical density assay (OD_{600}) was employed to determine the effectiveness of biochar-supported nano zero-valent iron (nZVI) particles in two different forms: BC-nZVI and BM-nZVI. Using both colony forming units (CFUs) and OD_{600} measurements, the quantity of bacteria in batch culture was determined. To carry out the OD_{600} assay, three test tubes were prepared. The first test tube contained 14.25 mL of nutrient broth along with 0.75 mL of bacteria, serving as the control. The second test tube contained 15 mL of nutrient broth with 0.025 mg of BC-nZVI, while the third test tube contained 15 mL of nutrient broth with 0.025 mg of BM-nZVI. Initially, the absorbance value of the 10-fold diluted samples was measured using a UV-Vis spectrophotometer. Then the test tubes were placed in a shaking water bath at 37 °C for 2 hours, and the absorbance was measured again for the 10-fold diluted samples. This process was repeated every 2 hours for a total duration of 24 hours.

Additionally, to determine the bacterial count, spread plates were prepared at regular intervals of 6 hours for a total duration of 24 hours. For each sample 10-fold serial dilution up to a concentration of 10^5 CFU/mL was carried out. Subsequently, 100 µl of the 10^5 CFU/mL dilution was used to spread on the nutrient agar plates. The spread plates were then incubated at 37 °C for 24 hours. To determine the number of colony-forming units (CFUs), manual colony counts were performed.

Applications of biochar-supported nZVI particles on hospital wastewater and farm wastewater

Hospital wastewater was collected from the National Hospital of Sri Lanka, Colombo and farm wastewater from a farm in Malabe, Sri Lanka. The determination of antibacterial activity for hospital wastewater was also conducted using the agar well diffusion method. Pour plates were prepared by inoculating 0.1 mL of fresh cultures onto nutrient agar medium in 10 cm petri dishes. BC-nZVI and BM-nZVI were added to wells with a 6 mm diameter at a concentration of 0.5 g/mL each. In order to prepare the samples, 50 µl of DMSO were used as the solvent. The plates were refrigerated for 90 minutes to allow the diffusion of the nZVI substance, followed by incubation at 37 °C for 24 hrs. After the incubation period, the inhibition zone was measured using a ruler to determine the antibacterial activity. As a positive control, biochar was used in the antibacterial test.

The above-mentioned procedure was carried out for farm wastewater as well.

Wastewater physiochemical analysis

In addition, some physical and chemical properties of the sample were examined before the biochar-supported nZVI particles were used for treatment [31]. The wastewater pH was determined using a pH meter (pH-3110, Xylem Analytics, Germany). The temperature was determined.

Biochemical Tests for wastewater

Gram's Staining

The Gram's staining test was conducted following the protocol provided by the American Society of Microbiology (ASM) [11].

Catalase test

A few drops of wastewater samples were placed on glass slides and then 3% of hydrogen peroxide was added into it.

DNA extraction by the DNeasy PowerSoil pro Kit

Prior to the DNA extraction, each wastewater sample was centrifuged for 10 minutes at 8,000 rpm. The manufacturer's instructions for the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) were followed to extract DNA from the supernatant (Figure 2.1) [7]. The chromosomal DNA from *S. aureus* was extracted.

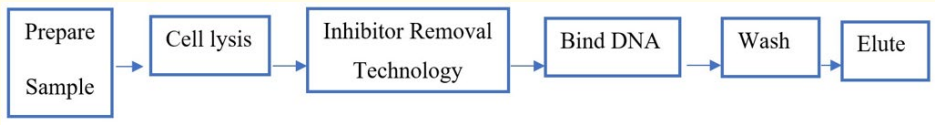

Figure 2.1: DNeasy PowerSoil Kit Procedure.

Polymerase Chain Reaction (PCR) amplification

PCRs were performed in a total volume of 15 µL containing 0.75 µL of extracted DNA from each sample, 1 µL of 10X buffer, 1 µL of $MgCl_2$, 1 µL of dNTP's, 0.5 µL of 27F primer, 0.5 µL of 1492 primer, 0.25 µL of Taq polymer and finally, 10 µL RNase free water (Qiagen, Hilden, Germany). The PCR (4375786, Thermo Fischer Scientific, Singapore) was performed using the following conditions: preheating at 94 °C for 5 minutes proceeded by 40 cycles of denaturing at 94 °C for 30 s, annealing at 56 °C for 45 s, elongation at 72 °C for 45 s, and finally a single step of 72 °C for 5 minutes [8]. The 15 µL PCR products were electrophoresed through a 1.0% agarose gel in TBE buffer (40 mM Tris-base, pH 8.0, and 1 mM Na2EDTA) for 30 minutes a constant voltage of 100V. After electrophoresis, gel was photographed on the UV transilluminator (U1002-230V, Labnet, Taiwan).

Statistical analysis

Experimental treatments were conducted in duplicate, and some data were expressed as mean ± standard deviation (SD). IBM SPSS 22.0 statistical software was used to conduct one-way analysis of variance (ANOVA) for statistical significance with p value < 0.05 [8].

Results and Discussion

Antimicrobial activity

The antimicrobial activity of biochar-supported nZVI against *S. aureus* was evaluated by measuring the diameter of the zones of inhibition formed by the nanocomposites against the bacteria. Among the different types of biochar-supported nZVI tested, rice husk biochar-supported nZVI exhibited significant zones of inhibition, while lignin-supported nZVI showed no zone of inhibition against *S. aureus*.

Figure 3.1.1 and Figure 3.1.2 presents the photographs showing the zones of inhibition formed by rice husk biochar-supported nZVI against *S. aureus* on nutrient agar and LB agar respectively, while Figure 3.1.3 displays the zones of inhibition formed by lignin-supported nZVI against the bacteria. These visual representations provide a clear demonstration of the antibacterial activity of the biochar-supported nanocomposites.

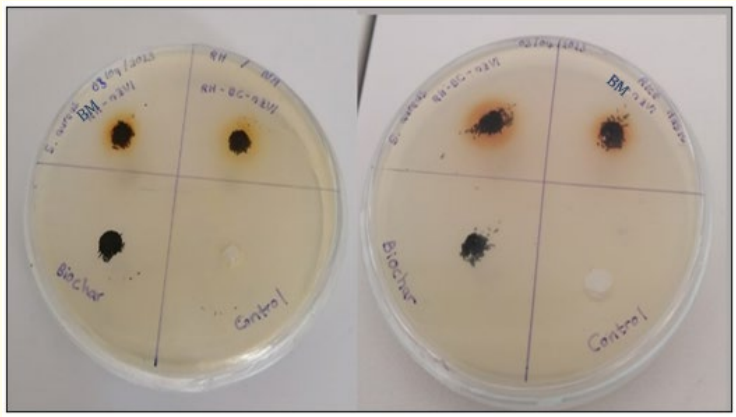

Figure 3.1.1: Zone of inhibition shown by RH-BC supported nZVI against *S. aureus* on LB agar.

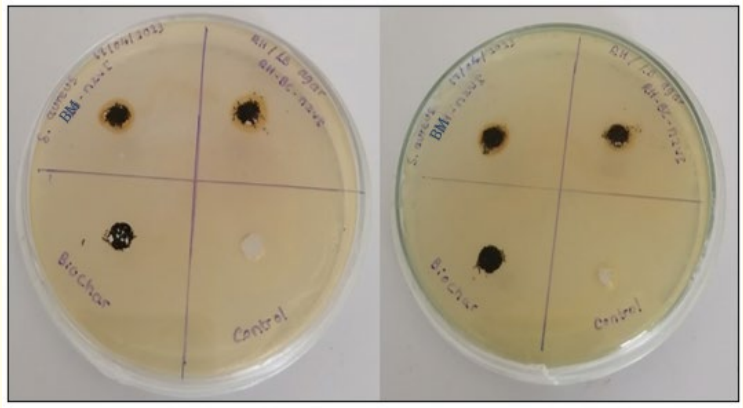

Figure 3.1.2: Zone of inhibition shown by RH supported nZVI against *S. aureus* on nutrient agar.

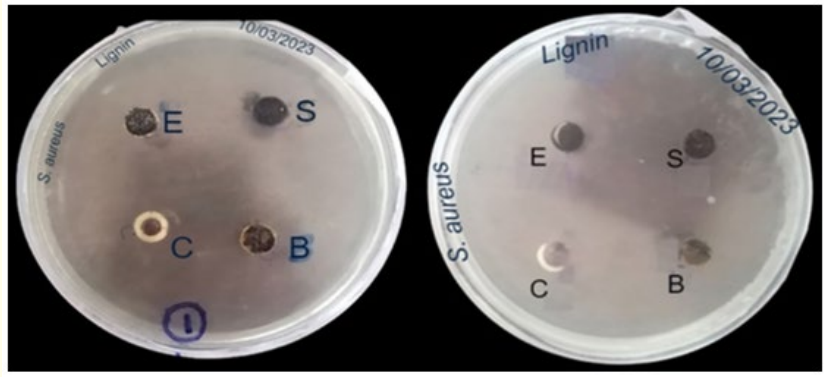

Figure 3.1.3: Zone of inhibition shown by lignin biochar supported nZVI against S. aureus. (E-Lig-eG-nZVI; S-Lig-s-nZVI; B-Biochar: C-Control).

Table 3.1.1 and Table 3.1.2 summarize the zones of inhibition in terms of their diameter exhibited by the biochar-supported nanocomposites against *S. aureus* after 24 hrs and 48 hrs respectively. It serves as a comprehensive overview of the antibacterial performance of the different composites studied.

Among the tested biochar-supported nanocomposites, the BC-nZVI composites demonstrated the highest antibacterial activity against *S. aureus* when grown on nutrient agar.

The observed inhibition of bacterial growth can be attributed to the disruption of cell membranes caused by the nanocomposites, leading to the breakdown of cell enzymes [4]. These results collectively indicate that rice husk-supported nZVI composites possess significant antimicrobial properties. Based on the findings, it can be concluded that Staphylococcus sp., including *S. aureus*, generally thrives in all protein formulations. Among the different media used, nutrient agar supported the highest growth of the tested bacteria [3].

	Zone of inhibition of Rice Husk biochar supported nZVI / mm		*Zone of inhibition of Lignin biochar supported nZVI/ mm*	
Media	***BC-nZVI***	***BM-nZVI***	***Lig-s-nZVI***	***Lig-eG-nZVI***
Nutrient Agar	6.5 ± 0.7	5.0 ± 0.0	NA	NA
LB Agar	4.0 ± 0.0	2.5 ± 0.7	NA	NA

Data are mean ± standard deviations (n=2). The mean is significantly different (p> 0.05) by the Turkey's post-hoc test. Diameter of the well was 6 mm. NA; not active.

Table 3.1.1: Diameter of inhibition zone of different biochar supported nano composites after 24 hrs.

	Zone of inhibition of Rice Husk biochar supported nZVI / mm		*Zone of inhibition of Lignin biochar supported nZVI/ mm*	
Media	***BC-nZVI***	***BM-nZVI***	***Lig-s-nZVI***	***Lig-eG-nZVI***
Nutrient Agar	6.5 ± 0.7	6.0 ± 0.0	NA	NA
LB Agar	5.0 ± 0.0	3.0 ± 0.0	NA	NA

Data are mean ± standard deviations (n=2). The mean is significantly different (p> 0.05) by the Turkey's post-hoc test. Diameter of the well was 6 mm. NA; not active.

Table 3.1.2: Diameter of inhibition zone of different biochar supported nano composites after 48 hrs.

Determination of MIC

For the quantitative measurement of antimicrobial activity of the rice husk biochar-supported nZVI, the MIC was determined by measuring the diameter of their zones of inhibition against the bacteria. Different concentrations of the RH-BC-supported nZVI, including concentrations of 0.500, 0.375, 0.250, 0.125, and 0.020 g/mL, were produced in order to determine the MIC.

Photographs depicting the zones of inhibition formed by BC-nZVI and BM-nZVI against bacteria are provided in Figure 3.2.1 and Figure 3.2.2, respectively. MIC values obtained for both BC-nZVI and BM-nZVI were found to be 0.020 g/mL, indicating their similar inhibitory effects on the target bacteria.

Figure 3.2.1: MIC of BC-nZVI ***Figure 3.2.2:*** MIC of BM-nZVI

(i)0.500 g/mL, (ii) 0.375 g/mL, (iii) 0.250 g/mL, (iv) 0.125 g/mL, and (v) 0.020 g/mL.

To summarize the results in a quantitative manner, Table 3.2.1 presents the diameter of the zones of inhibition produced by BC-nZVI and BM-nZVI against *S. aureus*.

Concentration (g/mL)	*Diameter of the inhibition zone BC-nZVI (mm)*	*Diameter of the inhibition zone BM-nZVI (mm)*
0.500	6.5 ± 0.7	5.5 ± 0.7
0.375	6.0 ± 0.0	4.5 ± 0.7
0.250	3.5 ± 0.7	3.0 ± 0.0
0.125	2.0 ± 0.0	1.0 ± 0.0
0.020	NA	NA

Data are mean ± standard deviations (n=2). The mean is significantly different (p> 0.05) by the Turkey's post-hoc test. Diameter of the well was 6 mm. NA; not active.

Table 3.2.1: Minimal inhibitory concentration of rice husk supported biochar nZVI particles determined by the well diffusion assay with the diameter of the inhibition zone in (mm).

To further contextualize our findings, we compared the MIC values obtained for the proposed nZVI agent with those reported in the literature for other antimicrobial agents. For instance, the antimicrobial activity of CuO/C nanocomposites against K. pneumoniae was evaluated, and the MIC and Minimum Bactericidal Concentration (MBC) were determined [4]. The quantitative values of MIC for the biochar-supported nZVI agent, along with the MIC values reported for different antimicrobial agents in various studies, are presented in Table 3.2.2. This comparison helps establish whether the agents under investigation possess bacteriostatic or bactericidal properties against various pathogens.

By comparing the various concentrations used in this study, it is observed that increasing the concentration of biochar-supported nZVI leads to certain effects. One such effect is the increase in the specific surface area-to-volume ratio of the nanoparticles. As the concentration of nZVI increases, a larger number of nanoparticles are present in the given volume, resulting in a higher surface area available for interaction with the target bacteria [27].

Furthermore, the increase in concentration also leads to the release of a greater number of antibacterial species from the surface of the nanoparticles. The antibacterial activity of biochar supported nZVI is attributed to the release of iron ions, which can react with bacterial cells and inhibit their growth. As the concentration of biochar-supported nZVI increases, a higher quantity of iron ions is released, thereby enhancing the antibacterial effect.

Antibacterial agent	*MIC at 24 h*	*Reference*
Rc/Ag nanocomposites	250 mg/L	[22]
Ag@Biochar Nanocomposite	25 mg/L	[23]
CS–MO nanocomposites	9.8 mg/mL	[24]
Ag-CMPB	312.5 mg/mL	[25]
BC-nZVI	0.02 g/mL	This study
BM-nZVI	0.02 g/mL	This study

Table 3.2.2: Minimal inhibitory concentration (MIC) of biochar supported nanocomposites for *S. aureus* from the literature.

Determination of OD_{600}

Using a spectrophotometer, the OD_{600} of RH-BC supported nZVI was calculated. At various times following the addition of the particles to a suspension of *S. aureus* bacteria, the OD_{600} was determined. The results showed that the OD_{600} of the suspension decreased with time, indicating that the bacteria were being killed by the particles.

Figure 3.3.1 depicts the growth curve of *S. aureus* when exposed to different types of RH-BC-supported nZVI, as measured by optical density at a wavelength of 600 nm. The curve reveals distinct phases of bacterial growth. The lag phase extended for approximately 5 hours, followed by a transition to the exponential phase, which lasted from 6 to 18 hours. After reaching the exponential phase, the growth rate began to plateau, indicating the onset of the stationary phase. The decline in optical density, signaling the death phase, was observed to be gradual after 22 hours.

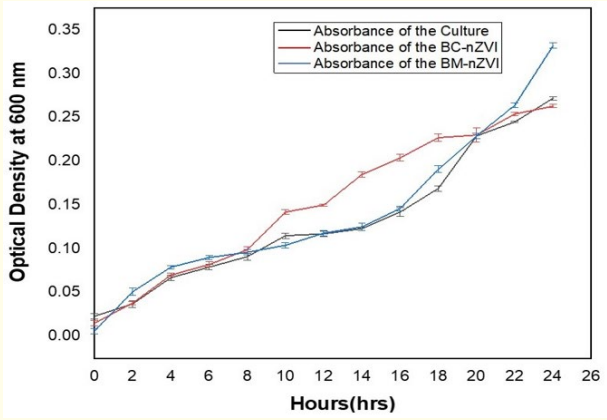

Figure 3.3.1: *S. aureus* and different types of RH-BC-supported nZVI optical density at 600 nm (OD_{600}) growth curve.

Figure 3.3.2 summarizes the distinct phases of growth of *S. aureus*. The results show that the bacterial growth decreases due to the RH-BC supported nZVI while control shows the increase of *S. aureus* community.

By analyzing Figure 3.3.1, Figure 3.3.2, and Table 3.3.1, it can be concluded that the RH-BC-supported nZVI particles demonstrated antimicrobial effects on *S. aureus* over time. Specifically, *S. aureus* exposed to BC-nZVI and BM-nZVI exhibited a decrease in growth of approximately 4.01% and 8.39%, respectively, within a 24-hour period.

The optical density of RH-BC-supported nZVI particles was compared to that of other antimicrobial agents reported in the literature. It is worth noting that high concentrations of nZVI suspension can lead to restricted movement and increased aggregation, which in turn may reduce the relative surface activity and efficiency of inactivation [27]. Furthermore, the nature of the active sites on the particle surface was observed to be influenced by the surrounding environment, as well as the particle size [19, 16, 28].

Under normal environmental conditions, the nZVI particles are typically surrounded by passivation layers consisting of oxides, resulting in a core-shell structure. This shell of oxides provides the nZVI particles with specific properties. However, the composition and thickness of the passivation layers can vary depending on the environmental conditions [23].

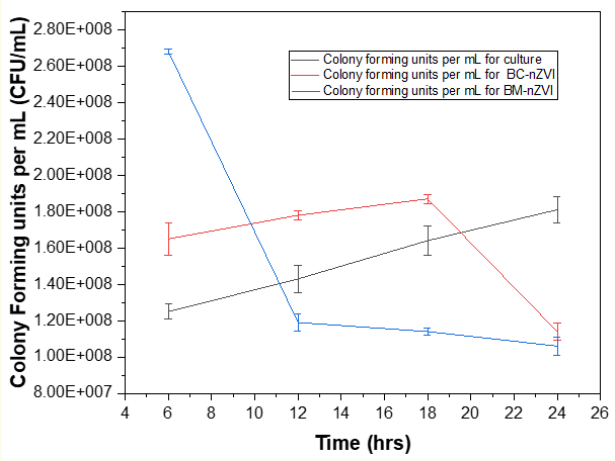

Figure 3.3.2: *S. aureus* Colony Forming Unit per millilitre (CFU/mL) growth curve.

Biochar supported nZVI for hospital wastewater and farm wastewater reclamation

Physiochemical properties

In this study, the physiochemical properties of hospital and farm wastewater were examined prior to undergoing treatment using biochar supported nZVI particles. Table 3.4.1 summarizes the pH and temperature values that were tested.

Sample	***pH***	***Temperature / ^0C***
Hospital Wastewater	6.71	25.0
Farm Wastewater	5.54	25.0

Table 3.4.1: Physiochemical Properties of the Wastewater.

From the literature, it has been established that several environmental factors play a crucial role in influencing the growth rate of bacteria. Among these factors, pH, temperature, and oxygen availability are considered to be the most significant [27, 26, 29].

The pH range of the wastewater samples was determined to assess its suitability for bacterial growth. Temperature is another critical factor that influences the rate of bacterial growth. Higher temperatures generally promote faster microbial activity, while lower temperatures may slow down the process [29]. By evaluating the temperature of the wastewater samples, we can understand its potential impact on the microbial population.

Antibacterial activity on wastewater

The antimicrobial activity of hospital and farm wastewater was evaluated using RH-BC-supported nano zero-valent iron. The diameter of the zones of inhibition formed by the nanocomposites against bacteria was measured to determine the effectiveness of the treatment. Figure 3.4.1 and Figure 3.4.2 depict photographs illustrating the zones of inhibition formed by RH-BC-supported nZVI for hospital wastewater and farm wastewater, respectively. To further analyse the results, Table 3.4.2 summarizes the zones of inhibition in terms of their diameter exhibited by different types of RH-BC-supported nZVI after a 24-hour incubation period.

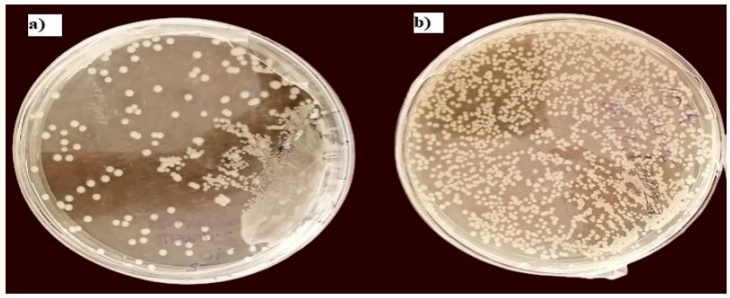

Figure 3.4: Serial dilution spread plate on LB agar a) 10^5 CFU b) 10^4 CFU.

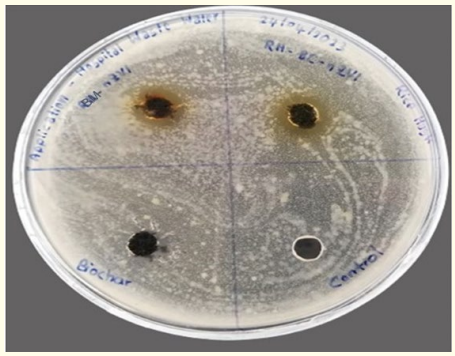

Figure 3.4.1: Zone of inhibition shown by RH-BC supported nZVI for Hospital wastewater.

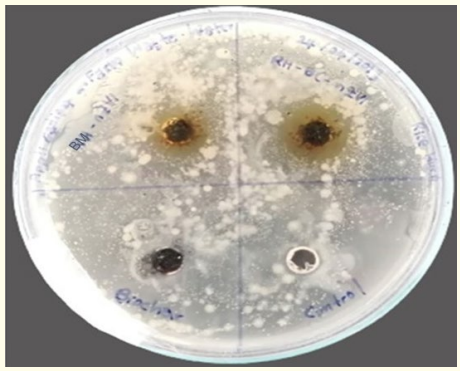

Figure 3.4.2: Zone of inhibition shown by RH-BC supported nZVI for Farm wastewater.

The contamination of water with pharmaceutically active compounds poses a serious threat to ecosystems [1]. These compounds, often present in wastewater, can adversely affect aquatic organisms and disrupt natural ecological processes. It is crucial to address this issue to safeguard the integrity of water resources and protect the ecosystem from the detrimental effects of these contaminants.

In the literature, there are previous studies that have explored the antibacterial activity of wastewater, particularly in relation to specific treatment methods. For example, the use of Ramie BC-nZVI was found to have no negative impact on soil microorganisms. In fact, it was observed that this treatment method could even enhance the abundance and diversity of indigenous bacteria. This beneficial effect is attributed to the ability of Ramie BC-nZVI to reduce the toxic effects of chromium (Cr), alter soil properties, and provide a favourable habitat for the survival of bacteria [31].

Another treatment approach, GAC/nZVI (granular activated carbon combined with nanoscale zero-valent iron), has shown promising results in promoting the degradation of toxic compounds in tetracycline wastewater. This suggests that GAC/nZVI can effectively mitigate the presence of harmful substances in wastewater, thereby reducing their potential impact on the environment [32].

Sample	***Diameter of the Zone of inhibition of BC-nZVI / mm***	***Diameter of the Zone of inhibition of BM-nZVI / mm***
Hospital Wastewater	7.5 ± 0.7	7.0± 0.7
Farm Wastewater	8.5 ± 0.7	5.5 ± 0.7

Data are mean ± standard deviations (n=2). The mean is significantly different (p> 0.05) by the Turkey's post-hoc test. Diameter of the well was 6 mm.

Table 3.4.2: Diameter of inhibition zone of RH-BC supported nano composites for wastewater.

Identification of microbial community

The biochemical analysis of hospital wastewater and farm wastewater was conducted. Specifically, the presence of *Staphylococcus aureus* (*S. aureus*) was investigated using various tests, including Gram's staining, catalase test, and electrophoresis of the DNA extract.

Gram's staining

Gram's staining technique was employed to identify and differentiate the bacterial species present in the wastewater samples. The staining revealed the presence of both Gram-positive and Gram-negative bacteria in the hospital and farm wastewater samples. Upon Gram's staining, both Gram-positive and Gram-negative bacteria were observed in the wastewater samples. The image of the Gram's staining can be seen in Figure 3.4.3.

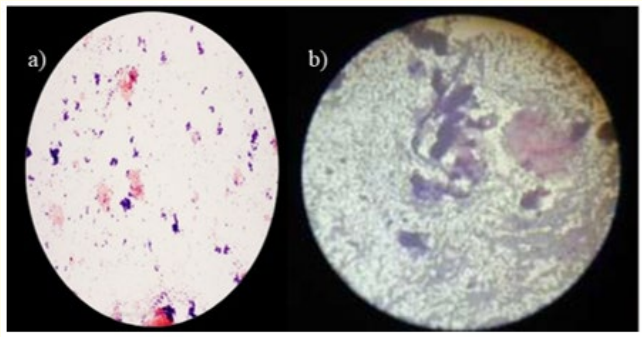

Figure 3.4.3: Gram's staining for a) Hospital wastewater b) Farm wastewater.

Catalase Test

The catalase test was performed to identify the presence of catalase enzyme activity, which is a characteristic feature of *S. aureus*. Positive results were obtained, indicating the presence of *S. aureus* in the wastewater samples. Figure 3.3.3 illustrates the positive catalase test results.

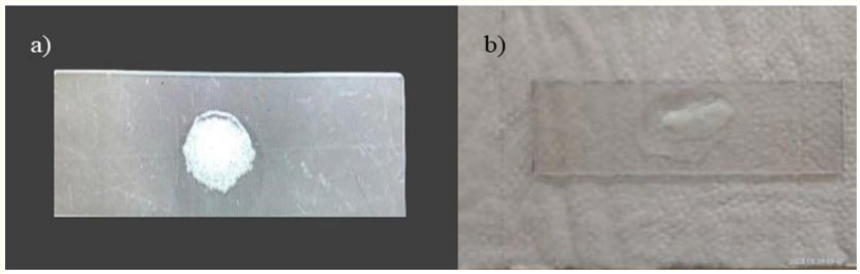

Figure 3.4.4: Catalase Test for a) Hospital wastewater b) Farm wastewater.

Genomic characterization and electrophoresis for DNA extracted

The gel electrophoresis analysis of both hospital wastewater and farm wastewater samples exhibited positive results for the presence of *S. aureus*. This was confirmed by the appearance of distinct bands in the gel corresponding to the expected size range of the target gene region associated with *S. aureus*.

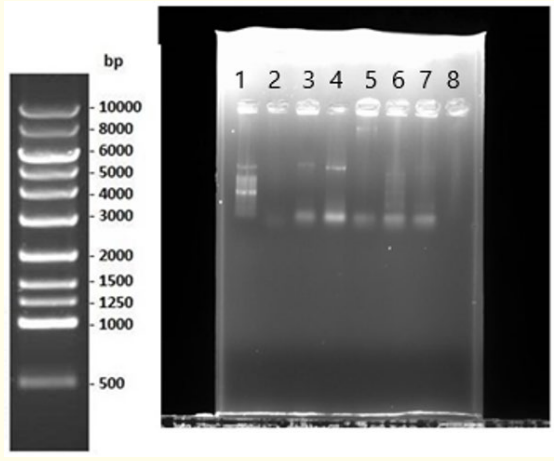

Figure 4.7: Agarose gel image of the isolated colonies. 1 denotes the 1kb ladder and 3,4 denotes farm and hospital wastewater and 5 denotes the *S. aureus* chromosomal DNA.

Antibacterial activity of rice husk supported nZVI

Inactivation mechanisms of RH-BC-supported nZVI particles have been extensively studied due to their potential antibacterial activity. RH-BC possesses inherent antibacterial properties attributed to its chemical composition and structural characteristics. The antibacterial activity of rice husk can be explained by the release of various bioactive compounds such as silica, phenols, and lignin derivatives, which exhibit bactericidal effects [6]. Additionally, the high surface area and porous nature of RH-BC enable physical interactions with bacterial cells, leading to the disruption of cell membranes and subsequent bacterial inactivation [20].

The antibacterial activity of nZVI nanoparticles can be attributed to several mechanisms. Firstly, the nZVI suspension contains iron in the zero-oxidation state (Fe^0) [27]. When exposed to an aqueous environment, Fe^0 undergoes oxidation, resulting in the generation of soluble ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions.

$$Fe^0_{(s)} \rightarrow Fe^{2+}_{(l)} + 2e^-$$

$$Fe^0_{(l)} \rightarrow Fe^{3+}_{(l)} + 3e^-$$

Moreover, the free electrons present in nZVI nanoparticles can react with dissolved oxygen from the atmosphere, leading to the formation of superoxide anion (O_2•-) and super hydroxide radicals (OH•):

$$O_{2\,(g)} + e^- \rightarrow O_2\bullet^-_{(l)}$$

$$H_2O_{(l)} + 2e^- \rightarrow HO_2^-\bullet_{(l)}$$

Furthermore, the superoxide anion ($O_2\bullet^-$) generated can react with hydrogen ions (H^+) to produce the HO_2 radical:

$$O_2\bullet^-_{(l)} + H^+_{(l)} \rightarrow HO_2\bullet_{(l)}$$

Additionally, the released iron species, Fe^{2+} and Fe^{3+}, can undergo redox reactions with dissolved oxygen, leading to the formation of various iron oxides: [11]

$$Fe^{2+}_{(l)} + O_{2\,(g)} \rightarrow FeO_{(l)}$$

$$Fe^{2+}_{(l)} + O_{2\,(g)} \rightarrow Fe_2O_{3\,(l)}$$

$$Fe^{3+}_{(l)} + O_{2\,(g)} \rightarrow Fe_3O_{4\,(l)}$$

Therefore, when RH-BC and nZVI are combined, their hybrid version exhibits enhanced antibacterial properties. The synergistic effects of rice husk biochar's organic compounds, high surface area, and porous structure, along with the antibacterial mechanisms of nZVI, contribute to the superior antibacterial activity observed in this hybrid material.

Conclusion

In conclusion, this study investigated the capability of biochar-supported nano zero-valent iron (nZVI) for the remediation of *Staphylococcus aureus* in hospital and farm wastewater. The antimicrobial activity of biochar-supported nZVI was evaluated by measuring the diameter of the zones of inhibition formed by the nanocomposites against the bacteria. Among the different types of biochar-supported nZVI tested, RH-BC-supported nZVI demonstrated significant zones of inhibition, while lignin biochar-supported nZVI showed no zone of inhibition against *S. aureus*. The minimum inhibitory concentration (MIC) for *S. aureus* was determined to be 0.020 g/mL. These findings have significant implications for the field of bioremediation and water reclamation. The effectiveness of RH-BC-supported nZVI in inhibiting the growth of *S. aureus* suggests its potential application in treating hospital and farm wastewater contaminated with this pathogen. The biochar-supported nZVI nanoparticles offer a green synthesized alternative for the remediation of gram-positive bacteria, including *S. aureus*, and potentially other gram-negative bacteria as well. This study provides a foundational

understanding of the toxic effects of biochar-supported zero-valent iron nanoparticles on *S. aureus* and contributes to the development of sustainable and eco-friendly strategies for water treatment and bacterial control.

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Conflict of Interest

The authors declares that they have no conflict of interest.

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