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Metal-Ion-Assisted Biodegradation of Sulfamethoxazole by Multidrug-Resistant *Atlantibacter hermannii* Isolated from Hospital Wastewater

Sadiya Khatun, Md. Ashique, Sanzida Sultana, Deri Damien Hagidok, Md. Akhtar-E-Ekram, Md. Salah Uddin, Md. Abu Saleh*, Shahriar Zaman*

Microbiology Laboratory, Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

* Correspondence: szaman@ru.ac.bd (Shahriar Zaman)
saleh@ru.ac.bd (Md. Abu Saleh)

Abstract: The widespread release of sulfonamide antibiotics into aquatic environments has become a major environmental and public health concern due to their persistence and contribution to antimicrobial resistance dissemination. In the present study, a multidrug-resistant (MDR) bacterial isolate capable of degrading sulfamethoxazole (SMX) was recovered from hospital wastewater collected in Bangladesh and identified as *Atlantibacter hermannii* by 16S rRNA gene sequencing. Antibiotic susceptibility testing revealed resistance to 13 out of 14 tested antibiotics, including sulfamethoxazole, confirming its MDR phenotype. The biodegradation potential of the isolate was evaluated in mineral salts medium supplemented with SMX under different physicochemical and metal-ion conditions. Among the tested transition metals, iron supplementation produced the greatest enhancement of bacterial growth and presumed SMX degradation, followed by manganese, whereas copper markedly inhibited bacterial activity. Optimization experiments demonstrated that 5 mM $FeSO_4$ was the optimal iron concentration for degradation. Combined Fe and Mn supplementation further improved degradation efficiency, with the Fe:Mn molar ratio of 1:2 yielding the highest growth response. pH optimization revealed that alkaline conditions (pH 8) were most favorable for Mn-mediated degradation. Ethanol co-supplementation enhanced Fe-mediated degradation up to 0.5% (v/v), but completely inhibited Mn-mediated degradation, suggesting mechanistic differences between iron- and manganese-associated pathways. Collectively, these findings provide the first evidence of SMX biodegradation by *Atlantibacter hermannii* and demonstrate the importance of transition metal optimization in enhancing antibiotic bioremediation. The study highlights the potential application of metal-assisted bacterial systems for the treatment of sulfonamide-contaminated hospital wastewater in resource-limited settings.

Keywords: *Atlantibacter hermannii*; sulfamethoxazole; biodegradation; hospital wastewater; iron supplementation; manganese

Academic Editor: Dr. Sudhangshu Kumar Biswas

Received: 25 April 2026
Revised: 28 May 2026
Accepted: 05 May 2026
Published: 05 July 2026

1. Introduction

Sulfonamide antibiotics are among the most widely used synthetic broad-spectrum antimicrobials in human medicine, veterinary practice, and aquaculture worldwide [1].

Sulfamethoxazole (SMX) is a common and heavily monitored sulfonamide that has been found in surface waters, groundwater, and hospital effluents across various geographic regions, usually at concentrations from nanograms to several micrograms per liter [2].

In Bangladesh, the situation is especially difficult. Hospital wastewater from tertiary-care facilities in major urban centers, including Dhaka, is rarely treated with advanced methods before being released into receiving water bodies [3, 4]. As a result, pharmaceutically active compounds like SMX enter rivers such as the Buriganga, Turag, and Padma, which are primary sources of water for millions of people, posing significant ecological and public health risks. Hospital wastewater represents a hotspot for the proliferation of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), driven by the selective pressure of sub-inhibitory antibiotic concentrations [5, 6].

Multidrug-resistant (MDR) members of the family *Enterobacteriaceae* have been repeatedly isolated from clinical and environmental samples across South Asia. In Bangladesh, a high burden of MDR gram-negative pathogens has been documented in hospital settings, compounded by limited infection-control infrastructure and the widespread availability of over-the-counter antibiotics [7]. The emergence of MDR environmental isolates capable of utilizing antibiotic residues as metabolic substrates, therefore, represents a convergent challenge at the intersection of antimicrobial resistance surveillance and wastewater bioremediation.

Atlantibacter hermannii (formerly *Escherichia hermannii*) is a gram-negative facultative anaerobe within the *Enterobacteriaceae* family [8, 9] occasionally associated with opportunistic infections in immunocompromised hosts [10]. Although several *Enterobacteriaceae* species have been reported to partially degrade sulfonamide compounds under established laboratory settings [11], the biodegradation ability in *A. hermannii* has not been characterized to date. Moreover, the role of transition metal ion cofactors, particularly iron (Fe), manganese (Mn), and copper (Cu), in modulating sulfonamide catabolism by environmental bacteria remains unclear, as does the influence of physicochemical parameters such as pH and co-substrate availability.

Bioremediation via indigenous bacterial communities offers a cost-effective and environmentally compatible strategy for removing pharmaceutical contaminants from wastewater streams [12, 13] which is particularly relevant to low and middle-income countries such as Bangladesh, where advanced oxidation process infrastructure remains limited.

Metal ion supplementation, especially with Fe^{2+}/Fe^{3+}, can potentiate bacterial antibiotic degradation through Fenton-type chemistry, generating reactive oxygen species (ROS) that promote oxidative fragmentation of recalcitrant sulfonamide structures [14, 15, 16]. Co-substrate addition, including low molecular weight alcohols, may further augment oxygenase-dependent catabolic pathways or, conversely, quench radical-mediated mechanisms depending on the metal system employed [17, 18]. Systematic optimization of these parameters is therefore essential before scale-up of any bacterium-based SMX biodegradation process.

The present study was conducted to address these gaps using a novel MDR *A. hermannii* isolate recovered from hospital wastewater in Bangladesh. Together, these findings provide a mechanistic framework for the rational design of metal-ion-assisted bioremediation systems targeting sulfonamide-contaminated hospital effluents in resource-constrained settings.

2. Materials and Methods

2.1. Chemicals and Materials

Sulfamethoxazole was obtained from commercially available Cotrim DS tablets (each containing 800 mg of sulfamethoxazole and 160 mg of trimethoprim as active pharmaceutical ingredients), purchased from a local pharmaceutical store (Rajshahi, Bangladesh). Mineral salts medium (MSM) components: KH_2PO_4, K_2HPO_4, NH_4Cl, NaCl, $MgSO_4.7H_2O$, and $CaCl_2$ were of analytical grade and procured from Sigma-Aldrich (St. Louis, MO, USA). Trace element solution was prepared from $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, and $Na_2MoO_4.2H_2O$. Luria-Bertani (LB) broth and LB agar were used for bacterial enrichment and isolation, and Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) was used for antibiotic susceptibility testing. Antibiotic disks for the Kirby-Bauer disc diffusion test were procured from Bioanalyse® (Ankara, Turkey), and absolute ethanol used was of standard laboratory grade. All solutions were prepared using sterile distilled water (dH_2O).

2.2. Sample Collection and Isolation of Bacteria

Hospital wastewater samples were aseptically collected in July 2025 from a drainage channel adjacent to Rajshahi Medical College, Bangladesh, using sterilized containers. Samples were transported to the laboratory within two hours at ambient temperature and pre-filtered through Whatman No. 1 filter paper to remove debris. 1 mL of pre-filtered wastewater was inoculated into 50 mL of sterile Luria-Bertani (LB) broth (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) and

incubated at 37°C with shaking (120 rpm) for 24 hours. The culture was spread onto LB agar plates after performing a serial dilution up to 10^{-8} and incubated (37°C, 24 h). Following incubation, distinct colonies were purified by repeated streaking. Pure isolates were stored in 50% glycerol at 4°C for short-term storage and at −80°C for long-term storage. All procedures were performed under aseptic conditions in a UV-sterilized laminar airflow cabinet.

2.3. Antibiotic Susceptibility Testing

The assessment of antibiotic resistance was conducted using the Kirby-Bauer disc diffusion technique [19]. Bacterial inoculum density was adjusted to match the 0.5 McFarland standard before plating. The antibiotic discs (Bioanalyse®) tested included penicillin (P), amoxicillin (AX), cefuroxime (CXM), ceftazidime (CAZ), cefotaxime (CTX), cefixime (CFM), aztreonam (ATM), gentamicin (CN), ciprofloxacin (CIP), doxycycline (DO), erythromycin (E), vancomycin (VA), chloramphenicol (C), and sulfamethoxazole (SMX). Plates were incubated at 37°C for 24 hours. Following incubation, zones of inhibition were measured and interpreted as susceptible, moderately susceptible, or resistant according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

2.4. Molecular Identification by 16S rRNA Gene Sequencing

Genomic DNA was isolated from a single colony suspended in 20 µL of TE buffer. The suspension was heated at 95°C for 10 minutes in a thermocycler, then centrifuged at 6,000 rpm for 2-3 minutes. The clear supernatant was used as the DNA template. The 16S rRNA gene was amplified by PCR using universal primers 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1510R (5′-GGCTACCTTGTTACGA-3′) [20]. The reaction started with an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation (94°C for 1 minute), annealing (50°C for 1 minute), and extension (72°C for 1.5 minutes). A final extension step was performed at 72°C for 5 minutes. Amplification of 16S rRNA gene was confirmed to be 0.8% agarose gel. PCR products were sent to ICDDR, B (Dhaka, Bangladesh) for Sanger sequencing. The obtained sequences were compared with the accessions in the National Center for Biotechnology Information database (NCBI) using the nBLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [21]. The identified bacterial sequences were then deposited in GenBank.

2.5. Mineral Salts Medium Preparation

The mineral salts (MS) medium used in all degradation experiments was prepared in distilled water and contained KH_2PO_4 (1.5 g/L), K_2HPO_4 (1.5 g/L), NH_4Cl (1.0 g/L), NaCl (0.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.2 g/L), and $CaCl_2$ (0.02 g/L). It was further supplemented with 1 mL per liter of a trace element solution comprising $FeSO_4{\cdot}7H_2O$ (1.0 g/L), $MnSO_4{\cdot}H_2O$ (0.5 g/L), $ZnSO_4{\cdot}7H_2O$ (0.1 g/L), $CuSO_4{\cdot}5H_2O$ (0.05 g/L), $CoCl_2{\cdot}6H_2O$ (0.05 g/L), and $Na_2MoO_4{\cdot}2H_2O$ (0.05 g/L).

2.6. Preparation of Sulfamethoxazole Stock Solution

A single Cotrim DS tablet was finely ground using a sterilized ceramic mortar and pestle. The resulting powder was transferred to a sterile glass beaker and dissolved in 20 mL of sterile distilled water under continuous magnetic stirring. The suspension was then gravity-filtered through sterile Whatman No. 1 filter paper to remove excipients and any remaining undissolved particles. The clear filtrate was collected as the stock SMX solution and stored at 4°C in a sterile amber bottle to minimize photodegradation. The stock was passed through a 0.22 µm syringe filter to ensure sterility before its addition to the culture medium.

2.7. SMX Biodegradation Experiments

All degradation experiments were carried out in 15 mL sterile test tubes containing MS medium supplemented with SMX as the only organic substrate. The isolated bacterial strain was pre-cultured in LB broth at 37°C with shaking (120 rpm) for 24 hours, then harvested by centrifugation (6,000 rpm, 10 min), washed twice with sterile phosphate-buffered saline (PBS, 0.1 M, pH 7.0), and resuspended in MS medium. An inoculum volume corresponding to an initial OD_{600} of around 0.05 was added to each experimental tube. To differentiate between biological and chemical degradation, all experiments included uninoculated controls (abiotic) and inoculated controls without metal supplementation (biotic). Bacterial growth, used as an indicator of SMX utilization and degradation, was measured spectrophotometrically at OD_{600} on Days 3, 5, and 7 of incubation.

2.7.1. Effect of Individual Metal Ions on SMX Degradation

To assess the impact of individual metal ions on SMX degradation, ferrous sulfate ($FeSO_4{\cdot}7H_2O$), manganese sulfate ($MnSO_4{\cdot}H_2O$), and copper sulfate ($CuSO_4{\cdot}5H_2O$) were added separately to MS medium-SMX cultures at a concentration of 5 mM. Control cultures without metal additives were maintained under the same conditions, and degradation was measured on Days 3 and 7.

2.7.2. Optimization of Iron Concentration

To identify the optimal iron concentration for SMX degradation enhancement, $FeSO_4 \cdot 7H_2O$ was added to MS-SMX cultures at three different concentrations, e.g., 3 mM, 5 mM, and 8 mM. OD_{600} was recorded on Days 3, 5, and 7 to assess dose-dependent effects on bacterial growth and SMX degradation.

2.7.3. Effect of Combined Fe and Mn Ions (Ratio Optimization)

The combined effect of Fe^{2+} and Mn^{2+} supplementation was assessed by adding both metals simultaneously to MS-SMX cultures at a total concentration of 5 mM, in five different molar ratios: Fe:Mn = 1:1, 1:2, 1:3, 2:1, and 3:1. Stock solutions of $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$ were prepared in sterile distilled water and added aseptically. OD_{600} was measured on Days 3 and 5.

2.7.4. Effect of pH on SMX Degradation

The influence of medium pH on SMX degradation was evaluated under Fe and Mn supplementation. MS medium was prepared and adjusted to pH 2, 4, 6, and 8 using sterile 1 M HCl or 1 M NaOH before inoculation. OD_{600} was measured at Days 3 and 5 to determine the pH optimum for maximal degradation.

2.7.5. Effect of Ethanol Co-Substrate Supplementation

The influence of ethanol as an alternative carbon source and metabolic co-substrate was assessed by adding absolute ethanol (99.5% v/v) to Fe- and Mn-supplemented MS-SMX cultures at final concentrations of 0.1%, 0.5%, and 1.0% (v/v). OD_{600} was measured on Days 3 and 5. Separate experiments were conducted for Fe-ethanol and Mn-ethanol combinations to distinguish their individual effects.

3. Results

3.1. Isolation and Colonial Characterization of the Bacterial Strain

Hospital wastewater samples were enriched in LB broth, serially diluted up to 10^{-8}, and cultured on LB agar. Following incubation at 37°C for 24 h, distinct bacterial colonies were obtained. The selected isolate formed circular, convex, smooth, moist, and glistening colonies with entire margins and creamy-white pigmentation, resembling the typical morphology of Enterobacteriaceae members. The uniform colony characteristics observed after repeated subculturing indicated a pure isolation suitable for subsequent molecular characterization.

3.2. Molecular Identification by 16S rRNA Gene Sequencing

Molecular identification was performed by PCR amplification and Sanger sequencing of the 16S rRNA gene using universal primers 9F and 1510R. The resulting sequence was subjected to nucleotide BLAST analysis against the NCBI GenBank database. The query sequence exhibited 93.92% similarity to *Atlantibacter hermannii* strain CIP 103176 (GenBank Accession No. CIP 103176), with the highest alignment score among all retrieved matches. Based on this taxonomic assignment, the hospital wastewater isolate was designated *Atlantibacter hermannii* strain CIP 103176 (**Figure 1**). Phylogenetic placement within the genus *Atlantibacter*, a relatively recently described taxon within the family Enterobacteriaceae, was thus confirmed. The sequence was subsequently deposited in GenBank for public access.

3.3. Antibiotic Susceptibility Profile

The antibiotic susceptibility profile of the isolate was determined by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar against a panel of 14 clinically relevant antimicrobial agents (**Table 1**). The isolate exhibited resistance to 13 of the 14 antibiotics tested, including representatives of multiple drug classes: tetracyclines (doxycycline, DO), phenicols (chloramphenicol, C), fluoroquinolones (ciprofloxacin, CIP), penicillins (penicillin G; amoxicillin, AX), third-generation cephalosporins (ceftazidime, CAZ; cefotaxime, CTX; cefixime, CFM), second-generation cephalosporins (cefuroxime, CXM), monobactams (aztreonam, ATM), glycopeptides (vancomycin, VA), macrolides (erythromycin, E), and the test sulfonamide sulfamethoxazole (SMX). No zone of inhibition was detected around the disks for any of these agents.

The only antibiotic to which the isolate demonstrated sensitivity was gentamicin (CN; 10 μg), which produced a clear zone of inhibition of 18 ± 0.8 mm, interpreted as susceptible according to standard CLSI breakpoints. Crucially, the complete absence of an inhibition zone around the SMX disk (25 μg) indicated intrinsic resistance or acquired tolerance to this sulfonamide, simultaneously establishing its potential utility as a sole carbon/nitrogen source in subsequent biodegradation experiments (**Figure** 2). The multidrug-resistant (MDR) phenotype of this isolate, spanning at least six antibiotic classes, underscores the clinical relevance of its environmental reservoir and its robustness as a candidate organism for bioremediation applications under selective antibiotic pressure.

Table 1: Antibiotic susceptibility profile of the *Atlantibacter hermannii* isolate determined by the Kirby-Bauer disk diffusion method. Zones of inhibition are expressed as mean ± SD (mm) where applicable. Interpretations follow standard CLSI breakpoint criteria. N = 3 independent replicates.

Antibiotic	Code	Disc Conc. (µg)	Zone of Inhibition (mm)	Interpretation
Gentamicin	CN	10	18 ± 0.8	Sensitive (S)
Sulfamethoxazole	SMX	25	0	Resistant (R)
Doxycycline	DO	30	0	Resistant (R)
Chloramphenicol	C	30	0	Resistant (R)
Ciprofloxacin	CIP	5	0	Resistant (R)
Penicillin	P	10	0	Resistant (R)
Amoxicillin	AX	30	0	Resistant (R)
Ceftazidime	CAZ	30	0	Resistant (R)
Cefotaxime	CTX	30	0	Resistant (R)
Cefixime	CFM	5	0	Resistant (R)
Cefuroxime	CXM	30	0	Resistant (R)
Aztreonam	ATM	30	0	Resistant (R)
Vancomycin	VA	30	0	Resistant (R)
Erythromycin	E	15	0	Resistant (R)

3.4. Effect of Individual Metal Ion Supplementation on SMX Degradation

To assess the influence of transition metal ions on bacterial SMX degradation, ferrous sulfate ($FeSO_4{\cdot}7H_2O$), manganese sulfate ($MnSO_4{\cdot}H_2O$), and copper sulfate ($CuSO_4{\cdot}5H_2O$) were each supplemented individually to MS-SMX cultures at a fixed concentration of 5 mM. Bacterial growth, used as a proxy for SMX utilization, was monitored spectrophotometrically at OD_{600} on Days 3 and 7.

Iron supplementation yielded the most pronounced stimulatory effect, with OD_{600} rising from 0.665 on Day 3 to 1.510 on Day 7. This marked growth enhancement suggests that Fe^{2+}/Fe^{3+} participates in Fenton-type reactions, generating reactive oxygen species (ROS) that contribute to oxidative fragmentation of the SMX molecule, thereby increasing substrate bioavailability for the bacterium. Manganese supplementation also enhanced degradation relative to the unsupplemented control, achieving OD_{600} values of 0.632 and 0.651 at Days 3 and 7, respectively, though the modest increase between the two time points suggests a plateau in degradative activity by Day 3. In contrast, copper supplementation yielded markedly lower growth (OD_{600} = 0.050 on Day 3, rising to 0.352 by Day 7), consistent with the well-documented cytotoxic effects of elevated Cu^{2+} concentrations on bacterial physiology. The rank order of stimulatory efficacy was Fe > Mn > Cu, with iron identified as the most effective individual metal ion supplement for augmenting SMX biodegradation (**Figure 3**).

Figure 1: NCBI BLAST alignment summary showing sequence similarity of the isolate to *Atlantibacter hermannii* strain CIP 103176. The query coverage and identity scores are presented as returned by the BLAST algorithm.

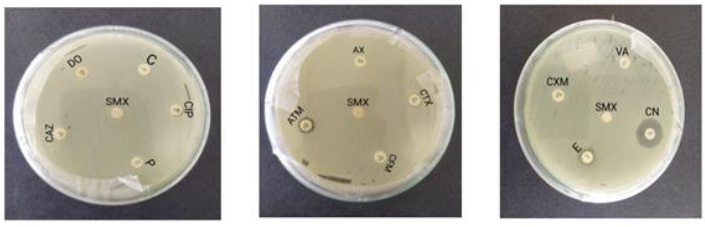

Figure 2: Representative Mueller-Hinton agar plates from the Kirby-Bauer disk diffusion assay illustrating the antibiotic susceptibility pattern of the isolate. Only the gentamicin (CN) disk yielded a visible zone of inhibition; no zone was observed around the sulfamethoxazole (SMX) disk.

3.5. Optimization of Iron Concentration for SMX Degradation

Given the superior performance of iron in Section 3.4, the optimal iron concentration was determined by supplementing MS-SMX cultures with $FeSO_4{\cdot}7H_2O$ at three concentrations (3, 5, and 8 mM) and monitoring OD_{600} on Days 3, 5, and 7. A dose-response relationship was observed, with 5 mM Fe yielding consistently the highest OD_{600} across all time points (Day 3: 0.660; Day 5: 0.810; Day 7: 1.520). The 3 mM treatment produced a moderate but progressive increase in OD_{600} (0.350 → 0.485 → 0.646), whereas the 8 mM treatment, despite achieving an OD_{600} of 1.420 by Day 7, exhibited lower values than the 5 mM group at all time points, indicative of partial growth inhibition at higher iron concentrations. This biphasic response is consistent with Michaelis-Menten-type kinetics, wherein substrate/cofactor concentrations beyond the optimal

level may suppress enzymatic or metabolic activity through product inhibition or oxidative stress. Based on these data, 5 mM Fe was selected as the optimal concentration for all subsequent experiments (**Figure 4**).

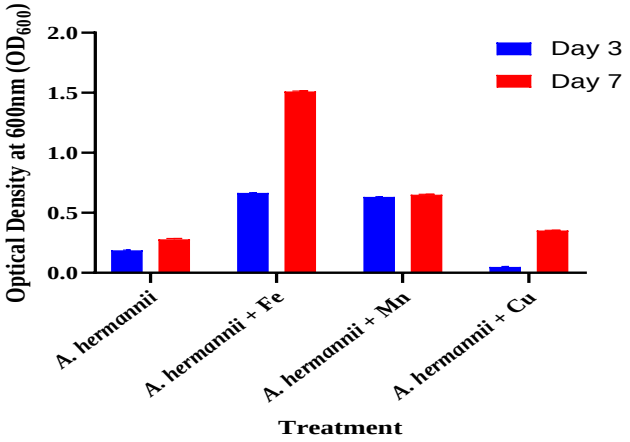

Figure 3: Optical density at 600 nm (OD_{600}) of *Atlantibacter hermannii* cultures supplemented with individual metal ions (Fe, Mn, Cu) at 5 mM in MS-SMX medium, measured on Days 3 and 7. Error bars represent the standard deviation of triplicate measurements.

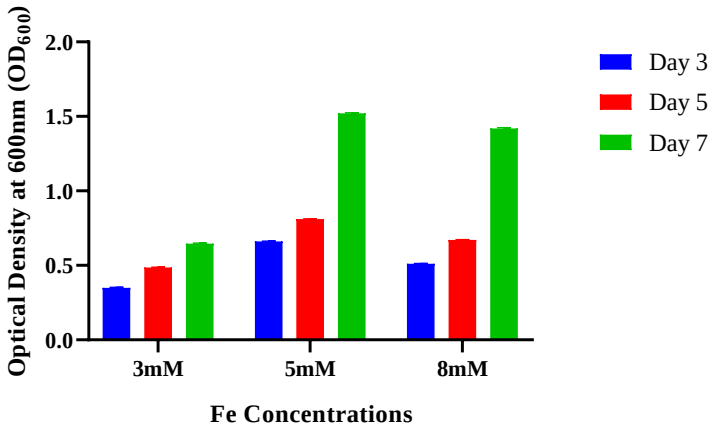

Figure 4: Growth kinetics of *Atlantibacter hermannii* at three iron concentrations (3, 5, and 8 mM) in MS-SMX medium, measured on Days 3, 5, and 7 as OD_{600}. The 5 mM treatment consistently yielded the highest growth response.

3.6. Effect of Combined Fe and Mn Supplementation: Molar Ratio Optimization

To evaluate potential synergistic interactions between iron and manganese, both metal ions were co-supplemented at a total concentration of 5 mM in five distinct molar ratios (Fe:Mn = 1:1, 1:2, 1:3, 2:1, and 3:1). OD_{600} was measured on Days 3 and 5 to assess short-term degradation kinetics. Among all ratios tested, the Fe:Mn ratio of 1:2 produced the highest growth responses (Day 3: OD_{600} = 0.580; Day 5: OD_{600} = 0.770), indicating superior support for bacterial SMX catabolism. Ratios with higher iron proportions (2:1 and 3:1) produced intermediate responses (OD_{600} = 0.36-0.39 on Day 3; 0.57-0.61 on Day 5), while excess manganese (1:3) also failed to exceed the 1:2 optimum (**Figure 5**).

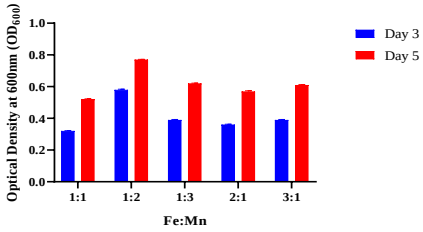

Figure 5: Effect of Fe:Mn molar ratio on bacterial growth (OD_{600}) in MS-SMX medium at Days 3 and 5. The 1:2 Fe:Mn ratio produced the maximum growth response, indicating optimal synergistic stimulation of SMX degradation.

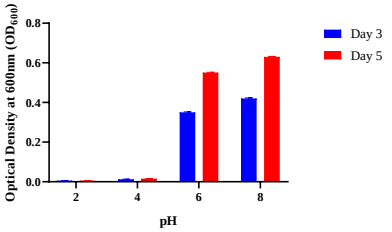

Figure 6: OD_{600} of *Atlantibacter hermannii* in Mn-supplemented MS-SMX medium at pH values of 2, 4, 6, and 8, assessed on Days 3 and 5. Optimal degradation was observed at pH 8.

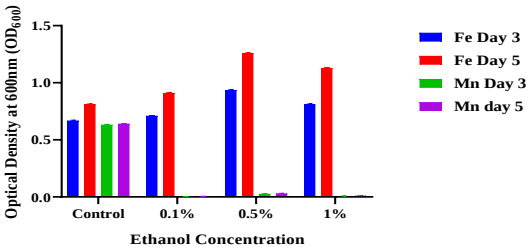

Figure 7: Comparative OD_{600} of *Atlantibacter hermannii* cultures co-supplemented with Fe or Mn and ethanol (0.1, 0.5, 1.0% v/v) in MS-SMX medium. Ethanol enhanced Fe-mediated degradation up to 0.5%, while it completely abrogated Mn-mediated degradation at all concentrations tested.

3.7. Effect of Medium pH on SMX Biodegradation

The influence of initial medium pH on SMX biodegradation by the isolate was evaluated across four pH values (2, 4, 6, and 8) under Mn supplementation, with OD_{600} recorded on Days 3 and 5. The highest bacterial growth was consistently observed at pH 8, with OD_{600} values of 0.420 and 0.630 on Days 3 and 5, respectively. Accordingly, pH 8 was identified as the optimal condition for Mn-mediated SMX biodegradation in this system (**Figure 6**).

3.8. Effect of Ethanol as a Co-Substrate on SMX Biodegradation

The influence of ethanol as an exogenous co-substrate and alternative carbon source on SMX biodegradation was examined by supplementing Fe-supplemented and Mn-supplemented MS-SMX cultures with absolute ethanol at three final concentrations (0.1%, 0.5%, and 1.0% v/v). OD_{600} was recorded on Days 3 and 5. In Fe-supplemented cultures, ethanol co-

supplementation enhanced bacterial growth and presumed SMX degradation in a concentration-dependent manner up to 0.5% (v/v), with OD_{600} values of 0.710 (Day 3) and 0.910 (Day 5) at 0.1% ethanol, rising to 0.930 and 1.250 at 0.5% ethanol. Increasing ethanol to 1.0% resulted in a slight reduction in growth (OD_{600} = 0.810 and 1.130 on Days 3 and 5, respectively), suggesting mild inhibitory effects at higher concentrations. In contrast, ethanol co-supplementation in Mn-supplemented cultures failed to produce measurable growth or SMX degradation under any concentration tested (**Figure 7**).

4. Discussion

To the best of our knowledge, the present study reports the first documented characterization of *Atlantibacter hermannii* as a multidrug-resistant environmental isolate capable of utilizing sulfamethoxazole (SMX) as a sole carbon and nitrogen source, where transition metal ion supplementation, pH, and co-substrate availability influenced its degradative capacity. The isolation of this organism from hospital wastewater, a recognized hotspot for the co-selection of antibiotic resistance and catabolic versatility, and its subsequent phenotypic and genotypic characterization provide a meaningful contribution to the expanding literature on sulfonamide biodegradation by members of the Enterobacteriaceae.

The colonial and biochemical features of the isolate matched those previously described for the Enterobacteriaceae family [22]. Molecular identification through 16S rRNA gene sequencing classified the strain as *Atlantibacter hermannii*, a relatively new classification that was formerly named *Escherichia hermannii* [8]. The 93.92% sequence similarity to the reference strain CIP 103176 is within an acceptable range for species-level identification using the 16S rRNA marker, although this value is close to the inter-species threshold and may indicate natural variation within the species or minor sequencing errors typical of Sanger technology.

The antibiotic susceptibility profile showed a high level of resistance, with the isolate resistant to 13 out of 14 tested agents across six pharmacological classes, i.e., tetracyclines, phenicols, fluoroquinolones, β-lactams (penicillins and cephalosporins), monobactams, glycopeptides, and macrolides. This resistance pattern meets the established criteria for multidrug resistance (MDR), which requires non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories, according to the ECDC guidelines [23]. The intrinsic resistance to vancomycin, a glycopeptide antibiotic targeting the peptidoglycan D-Ala-D-Ala terminus, which is absent in Gram-negative bacteria, aligns with the outer membrane barrier characteristic of this group [24]. Likewise, resistance to penicillins and macrolides in Enterobacteriaceae is often caused by chromosomally encoded β-lactamases and efflux pump systems respectively [25], although molecular confirmation of resistance genes in this isolate awaits genomic analysis. Notably, the isolate was only sensitive to gentamicin, a bactericidal aminoglycoside that targets the 30S ribosomal subunit. This aligns with patterns observed in other MDR Enterobacteriaceae from clinical wastewater, where aminoglycosides tend to remain effective despite widespread resistance to other drug classes [26]. Importantly, the lack of SMX inhibition confirmed the organism's resistance phenotype and indicated SMX as a suitable substrate for biodegradation experiments. The co-occurrence of sulfonamide resistance and degradative capacity is understood as a dual evolutionary strategy: resistance mechanisms reduce intracellular SMX accumulation, while catabolic pathways provide the advantage of utilizing a toxic environmental molecule as a nutrient [7, 8].

The evaluation of individual metal ion supplementation on SMX degradation yielded a clear rank order of efficacy: Fe > Mn > Cu. Iron supplementation at 5 mM produced the most pronounced enhancement of bacterial growth, with OD_{600} rising from 0.665 on Day 3 to 1.510 on Day 7. This stimulatory effect is mechanistically consistent with Fenton chemistry, wherein Fe^{2+} reacts with endogenous or exogenous H_2O_2 to generate hydroxyl radicals (-OH) that are highly reactive oxidants capable of non-specific electrophilic attack on aromatic SMX residues, thereby facilitating initial ring-opening or sulfonamide bond cleavage that renders the molecule amenable to subsequent enzymatic mineralization [29]. The involvement of iron in oxidative co-metabolic degradation of sulfonamides has been documented for other bacterial systems; Hruska and Franek (2012) demonstrated that Fe^{2+}-mediated Fenton oxidation substantially augmented the biodegradation of sulfadiazine by activated sludge consortia, results broadly consonant with those reported here [30].

Manganese supplementation yielded intermediate growth enhancement and appeared to plateau rapidly between Days 3 and 7, suggesting saturation of the Mn^{2+}-dependent catalytic capacity. Manganese peroxidase-like mechanisms and Mn^{2+}-catalyzed superoxide-driven redox cycling have been proposed in the oxidative transformation of persistent micropollutants, although the contribution of Mn compared to Fe is consistently lower in aerobic heterotrophic systems [31].

Copper, by contrast, markedly suppressed bacterial growth at 5 mM, consistent with its well-established cytotoxicity: excess Cu^{2+} disrupts membrane integrity, displaces Fe-S cluster cofactors in metabolic enzymes, and promotes uncontrolled intracellular oxidative stress [32]. The low but non-zero OD_{600} observed by Day 7 in copper-supplemented cultures suggests

partial metal tolerance or sequestration is developing over time; however, this concentration is clearly supra-optimal for co-degradation applications.

The dose-response optimization of iron concentration identified 5 mM $FeSO_4$ as the optimal supplementation level, producing the highest OD_{600} across all measurement time points, while both 3 mM (sub-optimal) and 8 mM (partially inhibitory) produced inferior degradative outcomes. This biphasic response is classically indicative of a hormetic or Michaelis-Menten-type cofactor dependency, whereby inadequate Fe^{2+} limits ROS generation below the threshold required for efficient SMX co-oxidation, while excess Fe^{2+} drives nonproductive oxidative stress, Fenton overactivity, or direct enzyme inactivation. Analogous biphasic iron dose-response relationships have been reported in bacterial degradation of chlorinated and sulfonated aromatic compounds, lending support to the mechanistic interpretation advanced here [33].

When iron and manganese were co-supplemented at a fixed total concentration of 5 mM, the optimal Fe:Mn molar ratio of 1:2 yielded the highest bacterial growth, surpassing all other ratios including 1:1, 1:3, 2:1, and 3:1. This result shows that when both metals are added together, manganese helps break down SMX much more effectively than expected from its effect alone. This suggests the two metals work together synergistically, rather than just adding their individual effects. A plausible mechanism is a coupled redox cycle in which Fe^{2+} generates •OH through Fenton reactions, while Mn^{2+} participates in superoxide dismutation or acts as a cofactor for SMX-oxidizing peroxidases, together enabling a broader oxidative attack on the SMX structure than either metal alone. The slight inhibition observed at the 3:1 Fe:Mn ratio, despite higher iron content, aligns with the 8 mM iron optimization data and further supports the idea that excess iron, even within a combined system, is counterproductive. To our knowledge, systematic molar ratio optimization of Fe:Mn binary metal supplementation for sulfonamide biodegradation in a defined isolate system has not been previously reported, representing a novel methodological contribution of this work.

The pH optimization experiments showed that no SMX biodegradation occurred at pH 2, 4, and 6 with manganese supplementation. Bacterial growth and SMX breakdown were observed only at pH 8. This pronounced alkaline preference is consistent with several converging biological and chemical factors. At acidic pH, protonation of bacterial membrane phospholipid head groups disrupts membrane integrity [34], inhibits electron transport chain function [35], and suppresses the activity of key catabolic enzymes responsible for SMX ring hydroxylation [36], specifically, flavin-dependent mono- and dioxygenases whose catalytic activity is characteristically pH-sensitive [28]. Additionally, at pH below neutrality, Mn^{2+} undergoes protonation-driven speciation changes that reduce its participation in oxidative redox chemistry [37], further diminishing catalytic capacity. The pH 8 optimum identified here is broadly consistent with findings from other sulfonamide-degrading bacterial studies, i.e., Song et al. (2021) reported peak SMX biodegradation at pH 7.2 by *Sphingobacterium mizutaii* LLE5 [38], while studies of activated sludge consortia have similarly noted suppression of sulfonamide degradation under acidic conditions [39]. The complete absence of growth even at pH 6, a condition conventionally considered near-neutral, was noteworthy and may reflect either the pH sensitivity of the *Atlantibacter* strain or the specific dependence of the Mn-mediated catalytic pathway on mild alkaline conditions.

The ethanol co-substrate experiments produced a mechanistically informative dichotomy: whereas ethanol substantially enhanced SMX degradation in Fe-supplemented cultures (optimal at 0.5% v/v), it completely abrogated measurable growth in Mn-supplemented cultures at all concentrations tested. In Fe-supplemented systems, ethanol likely functions as an auxiliary carbon source augmenting the cellular reducing power ($NADH/FADH_2$) required by the flavin-dependent oxygenase's catalyzing SMX oxidation, while simultaneously supporting biomass accumulation and sustaining metabolic activity. The mild growth inhibition observed at 1.0% ethanol is consistent with solvent-induced membrane perturbation at elevated concentrations, a well-documented phenomenon in Enterobacteriaceae [40]. The complete inhibition of Mn-mediated SMX degradation by ethanol at all tested concentrations is most parsimoniously explained by the capacity of ethanol to act as a hydroxyl radical scavenger. Ethanol reacts rapidly with •OH (rate constant ≈ 1.9×10^9 $M^{-1}s^{-1}$), effectively competing with SMX for the ROS generated by Mn-mediated redox cycling, thereby starving the degradative pathway of the chemical oxidants essential for SMX transformation [41]. This interpretation is reinforced by the absence of equivalent inhibition in Fe-supplemented cultures, where enzymatic oxygenase pathways, rather than ROS-dependent chemical oxidation, are proposed to predominate. The divergent responses to ethanol in Fe- and Mn-supplemented systems thus constitute indirect mechanistic evidence for pathway-specific distinctions between iron- and manganese-potentiated SMX biodegradation, a finding with practical implications for the rational design of metal-amended bioremediation systems.

Considered collectively, these findings establish a coherent experimental framework for metal ion-augmented biodegradation of SMX by an MDR environmental bacterium. The sequential identification of optimal metal identity,

concentration, molar ratio, pH, and co-substrate conditions provides a degree of process characterization seldom achieved in single-isolate biodegradation studies and offers a rational basis for prospective scale-up design. The clinical and environmental significance is underscored by the escalating global burden of sulfonamide contamination of hospital effluents and receiving water bodies, an issue of substantial public health concern given sulfonamides' established role in the selection and dissemination of sulfonamide resistance genes (sul1, sul2) across environmental microbiomes [42], [43].

5. Limitations

Several limitations of the present study merit transparent acknowledgement. The isolate was obtained from a single hospital wastewater sampling site, and conclusions regarding the prevalence or ecological distribution of SMX-degrading *Atlantibacter hermannii* in broader environmental matrices cannot be drawn without expanded geographic and source sampling. Furthermore, bacterial growth (OD_{600}) was used as a proxy for SMX degradation; while this is a well-established approach in biodegradation screening studies, direct quantification of SMX removal by HPLC or LC-MS/MS, alongside identification of metabolic intermediates, is necessary to confirm and quantify degradative efficiency at the molecular level. The 16S rRNA gene, while universally applicable, has limited discriminatory power at the species level for certain taxa, and whole-genome sequencing would provide a definitive taxonomic assignment alongside insights into the genetic basis of SMX catabolism and resistance. The resistance determinants underlying the MDR phenotype were characterized by disk diffusion alone; molecular confirmation of resistance genes (e.g., sul1, sul2, blaCTX-M, qnr) by PCR or whole-genome analysis is required to elucidate the specific mechanisms. All experiments were conducted under controlled laboratory conditions, and the translational applicability of the optimized parameters to the physicochemical heterogeneity of real wastewater environments remains to be established. Finally, the absence of in vivo or ex situ mesocosm validation currently limits conclusions to bench-scale findings.

6. Future Directions

Building on these findings, several critical research directions are necessary. Whole-genome sequencing and functional genomic annotation of the *Atlantibacter hermannii* isolate would help identify SMX-catabolizing gene clusters, resistance determinants, mobile genetic elements, and horizontal gene transfer mechanisms. High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) should be used to directly measure SMX degradation rates, determine rate constants, and map the metabolic pathway by identifying key intermediates. Reactive oxygen species quantification assays, including EPR spin-trapping and fluorescent ROS probes, would enable direct experimental validation of the proposed Fenton and Mn-mediated radical mechanisms, shifting from mechanistic inference to experimental confirmation. Expanded environmental monitoring across multiple hospital wastewater systems, agricultural runoff, and effluent-receiving surface waters would clarify how common SMX-degrading *Atlantibacter* species are and their ecological importance. Pilot-scale continuous-flow bioreactor experiments using the optimized parameters identified here would provide essential data for scaling up for engineering applications. Transcriptomic and proteomic analyses under optimal degradation conditions would reveal differentially expressed catabolic enzymes, guiding potential synthetic biology improvements for SMX degradation pathways. Lastly, combining comparative genomics and machine learning approaches may speed up the discovery of new SMX-degrading organisms and enable predictive modeling of degradation efficiency in diverse environmental conditions.

7. Conclusion

This study characterizes a multidrug-resistant *Atlantibacter hermannii* strain isolated from hospital wastewater as a capable SMX-degrading organism whose biodegradative activity is substantially enhanced by iron supplementation, optimally at 5 mM, and further augmented by synergistic Fe: Mn co-supplementation at a 1:2 molar ratio under alkaline conditions (pH 8). The mechanistic divergence between iron- and manganese-mediated degradation pathways, revealed by differential ethanol sensitivity, provides a conceptually significant insight into the distinct biochemistry of metal-potentiated sulfonamide biodegradation. These findings collectively advance the scientific basis for the development of metal-augmented, bacterium-based bioremediation strategies targeting sulfonamide micropollutants in hospital and clinical wastewater streams, contributing to broader global efforts to mitigate the environmental dissemination of antimicrobial resistance.

Author Contributions: Conceptualization, S.K., S.Z., and M.A.S.; methodology, S.K., M.A., S.S., D.D.H.; validation, M.A.E.E., M.S.U., S.Z., and M.A.S.; formal analysis, S.K., M.A., S.S., D.D.H.; investigation, S.K., M.A., S.S., D.D.H.; resources, M.A.E.E., M.S.U., S.Z., and M.A.S.; writing—original draft preparation, S.K., M.A., S.S.; writing—review and editing, M.A.S., and S.Z.; supervision, S.Z., and M.A.S.; All authors have read and agreed to the published version of the manuscript.

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.
Competing Interests: The author declares no conflict of interest.
Funding: The research was supported by a grant (PROJECT ID: SRG-223529) provided by the Ministry of Science and Technology, Government of the Peoples' Republic of Bangladesh.
Ethical Approval: The manuscript does not contain experiments using humans and animals.

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