H2Open Journal

IWA PUBLISHING

H2Open Journal Vol 7 No 2, 149 doi: 10.2166/h2oj.2024.103

Prospecting the biodegradation of ciprofloxacin by *Stutzerimonas stutzeri* R2 and *Exiguobacterium indicum* strain R4 isolated from pharmaceutical wastewater

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ABSTRACT

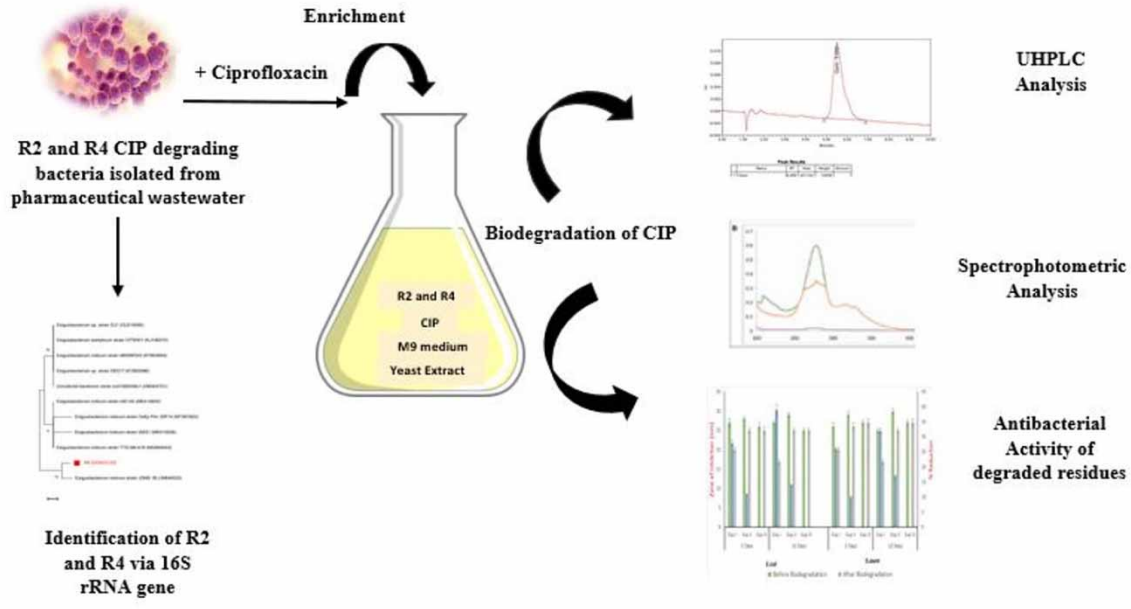
Ciprofloxacin (CIP), an emerging micro-pollutant antibiotic, poses an environmental threat due to its resistance to high-temperature decomposition, aiding antibiotic resistance spread. Conventional degradation generates toxic byproducts, while biodegradation offers an efficient and eco-friendly means to eliminate CIP. In this study, ciprofloxacin-degrading strains were isolated from pharmaceutical wastewater using an enrichment technique. Isolated strains R2 and R4 were identified as *Stutzerimonas stutzeri* and *Exiguobacterium indicum*, respectively, based on their 16S rRNA gene sequence. Ciprofloxacin degrading potential of these strains was tested in shake flask fermentation and quantified using spectrophotometric assays and ultra-high-performance liquid chromatography (UHPLC). UHPLC analysis revealed that in co-metabolism, R2 achieved 51 and 77% degradation, and R4 achieved 60 and 68% after 5 and 10 days of incubation. When CIP served as the only carbon source, R2 degraded it by 23 and 35%, while R4 degraded it by 19% and 28 in 5 and 10 days, respectively. Spectrophotometric analyses produced congruent results with UHPLC. Notably, in co-metabolism, R2 and R4 achieved 66 and 88% degradation within the 5 days. Moreover, the degraded residues displayed reduced antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. In conclusion, both strains show potential for degrading CIP, aiding in mitigating pharmaceuticals and environmental pollutants.

Key words: biodegradation, ciprofloxacin, co-metabolism, pharmaceutical wastewater, ultra-high-performance liquid chromatography

HIGHLIGHTS

- Bacterial strain R2 and R4 was isolated from pharmaceutical wastewater for biodegradation of CIP.
- Spectrophotometric and UHPLC analysis was performed to estimate the CIP degradation.
- Biotransformation of CIP was higher in co-metabolism compared to direct metabolism.
- 66% and 88% of total biodegradation occurred within the first five days of incubation.
- Degraded residues of CIP have weaker antimicrobial potential than standard.

GRAPHICAL ABSTRACT



INTRODUCTION

As the global population grows and industrialization and urban development continue, there is a rising demand for an improved quality of life. Various industries have emerged to meet human needs, yet their operations often generate wastewater, bringing about significant challenges (Rashid *et al.* 2021; Bhat *et al.* 2022; Othmani *et al.* 2022). This worldwide issue encompasses various industrial effluents that pose severe health risks, such as cancer, reproductive disorders, organ failure, and antibiotic resistance. Some of the key contributors to this problem include effluents from fertilizer, food and dairy processing, petrochemical, textile, and pharmaceutical industries (Rashid *et al.* 2020; Rashid *et al.* 2021). Among pharmaceutical compounds, antibiotics are important pharmaceutical compounds widely used in human and veterinary medicine (Baralla *et al.* 2021). Regardless of their benefits, antibiotics constitute an important class of emerging micropollutants of concern, because of their ecotoxicity and destructive biological effects (Miran *et al.* 2018; Thelusmond *et al.* 2018; Holanda *et al.* 2019). Since the preponderance of antibiotics is not fully metabolized by humans and animals, a substantial amount of administered antibiotics is released into the environment via anthropogenic waste (Feng *et al.* 2019). Besides, pharmaceutical and hospital wastewater also contain a significant concentration of toxic refractory compounds that persist in the environment for a long period (Leng *et al.* 2016; Nguyen *et al.* 2018). Recently, antibiotic pollution has garnered significant attention due to the presence of antibiotics in the environment, which exerts pressure favoring the survival of resistant bacteria. These bacteria pose a major public health concern due to the increased occurrence of associated infections (Singh *et al.* 2017; Manage 2018; Lalitha *et al.* 2019; Baralla *et al.* 2021; Mutuku *et al.* 2022). According to the Global Water Research Coalition's (GWRC) list of pharmaceuticals, ciprofloxacin (CIP), a broad-spectrum fluoroquinolone antibiotic that inhibits the key enzymes involved in DNA replication, including topoisomerase IV and DNA gyrase, was prioritized due to its potential threats to the water cycle and aquaculture. About 70% of the expelled CIP remains unmetabolized when released into the environment, and it has a half-life of up to 3,466 days in soil (Cycon *et al.* 2019; Feng *et al.* 2019; Cai *et al.* 2022). Hence, it has been selected as the model antibiotic for this study.

Ciprofloxacin is commonly found in the environment with concentrations ranging from 5.3 to 119.8 μg/kg in agricultural soil, 45.49 mg/kg in manure, 426 mg/kg in sewage sludge, 6.5 mg/L in freshwater ecosystem, and 6.5–31 mg/L in pharmaceutical wastewater (Nguyen *et al.* 2018; Feng *et al.* 2019). The distinctive physicochemical features, electronegativity, and C–F bond stability of fluorine enhance the antibacterial potential of CIP and transform it into a highly recalcitrant compound (Feng *et al.* 2019).

Ciprofloxacin can be removed by employing various physiochemical processes such as photodegradation, electrochemical oxidation, and advanced oxidation (Feng *et al.* 2019). However, these approaches are costly and

sometimes ineffective, thus limiting their large-scale application. Furthermore, inefficient CIP degradation can produce intermediates with a toxicity similar to or higher than the parent molecule, resulting in secondary pollution and harmful consequences (Feng *et al.* 2019).

Using microorganisms for the remediation of CIP is an alternate method. Bioremediation, a process that utilizes microbes, can effectively break down hazardous substances into less toxic or nontoxic substances. Compared to other technologies, this method is generally 60–70% more cost-effective (Singh *et al.* 2017; Sadaf *et al.* 2022). The bacterial strains *Thermus thermophiles* and *Bradyhizobium sp.GLC_01* have been reported for their use in bioremediation of CIP (Nguyen *et al.* 2018; Pan *et al.* 2018). There is still very limited information available about the biodegradation of CIP by individual bacterial strains or consortiums of bacterial strains. Hence, developing processes for complete and faster degradation of CIP is a critical need.

This study aims to isolate CIP-degrading bacteria from pharmaceutical wastewater. The strain was acquired by enrichment and isolation procedures, and their genotype were determined using 16S rRNA gene-based sequencing. After 16S rRNA gene-based sequencing the strains were identified as *Stutzerimonas stutzeri* R2 and *Exiguobacterium indicum* strain R4 members of the genus *Stutzerimonas*, formerly known as *Pseudomonas stutzeri*, gram-negative and rod-shaped, exhibit aerobic, chemoorganotrophic traits without spore formation. They play a vital role in biodegrading complex compounds across various environments (Lalucat *et al.* 2022; Mulet *et al.* 2023). Members of the genus *Exiguobacterium*, gram-positive and motile, can adapt aerobically or anaerobically based on oxygen availability. Isolated from diverse environments like hot springs, wetlands, and river water, these bacteria are known for their role in biodegrading complex compounds (Pandey 2020). The findings of this study revealed that both strains have the ability to degrade CIP. These results can contribute to eliminating antibiotics from the environment and have the potential to be utilized in designing engineering processes aimed at removing CIP from soil and water.

MATERIALS AND METHODS

The untreated wastewater samples contaminated with ciprofloxacin were taken from local pharmaceutical industries of Islamabad, Pakistan, and carried to Applied and Environmental Microbiology lab at Quaid-i-Azam University Islamabad on the same day. The rationale for selecting pharmaceutical wastewater, rich in ciprofloxacin, is because bacteria in this environment might have evolved to degrade CIP due to constant exposure. Additionally, contaminated soils, water bodies receiving pharmaceutical waste, or ecosystems contaminated with CIP are valuable sources to isolate ciprofloxacin-degrading bacteria. Bacterial strains were isolated using an enrichment technique, selectively promoting the growth of ciprofloxacin-degrading bacteria in the presence of ciprofloxacin within the growth media, while inhibiting the growth of other microorganisms. After isolation, the R2 and R4 bacterial strains were investigated for degradation of ciprofloxacin. The degradation of the CIP was analyzed by UHPLC and spectrophotometry. The antibacterial potential of intermediates formed after the degradation of CIP by R2 and R4 was also evaluated.

Isolation and identification of CIP-degrading bacterial strain

To isolate CIP-degrading bacteria, an enrichment assay was performed with some modifications to a previously described method (Feng *et al.* 2019). Pharmaceutical wastewater samples, used to enrich CIP-degrading bacterial community, were collected from various local pharmaceutical industries of Islamabad, Pakistan. All these samples were mixed, and 1 mL of mixed samples was incubated at 37 °C into 100 mL Erlenmeyer flask containing 50 mL of M9 medium ($Na_2HPO_4.7H_2O$ (1.28 g/L), KH_2PO_4 (0.3 g/L), NaCl (0.05 g/L), and NH_4Cl (0.1 g/L) supplemented with yeast extract as additional C source and ciprofloxacin hydrochloride (98% purity). At three day intervals, this culture was transferred to the fresh M9 medium. The progressive acclimation procedure involved gradually reducing the additional C source and increasing the concentration of CIP, from 1 to 0.25 g/L and from 50 to 100 mg/mL, respectively. Following this process, the culture was 10-fold serially diluted in normal saline and plated on M9 agar containing 100 mg/L CIP. After incubation, bacterial colonies with varying morphologies from the serially diluted plates (ranging from 10^{-4} to 10^{-7}) were isolated and preserved in 40% glycerol for further studies.

The Invitrogen kit by Thermo Fisher Scientific was used to extract the genomic DNA bacterial isolates R2 and R4 as per the manufacturer's instructions. Several other extraction protocols, such as phenol–chloroform extraction, CTAB-based methods, and commercial kits from different manufacturers, are available and have been employed in various studies. This specific kit was chosen due to its documented effectiveness in extracting

genomic DNA from bacterial cultures. The universal primers including 5′-AGAGTTTGATTCTGGCTCAG-3′ (27F, Forward primer) and 5′-GGTTACCTTGTTACGACTT-3′ (1492R, reverse primer) were used for the amplification of genes encoding 16S rRNA by PCR. Moreover, the PCR reaction mixtures consisting of dNTPs, Taq polymerase, buffer, forward and reverse primers, DNA template, and Milli-Q-water were used. After optimizing, the thermocycler was programmed to repeat 30 cycles with pre-heating carried out at 95 °C for 5 min, denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s, and final polymerization at 72 °C for 10 min. Afterward, gel electrophoresis was used to validate the final PCR product. The gene sequencing was performed by Lab Genetix (Pakistan). Using the NCBI BLAST database, the query sequence was compared with other available sequences in the library to find its resembling sequences. The phylogenetic tree was constructed by the neighbor-joining method using MEGA11 software. The 16S rRNA sequence of the R2 and R4 strain were deposited in the GenBank under accession numbers OQ256706 and OQP423123, respectively.

Biodegradation of CIP in batch reactors

The batch experiment was run to evaluate the biodegradation of CIP by test strains R2 and R4 under different experimental conditions described as follows. In experiment I, active bacterial cells were inoculated in the M9 medium containing CIP and yeast extract as additional C source (co-metabolism). In experiment II, active bacterial cells were inoculated in M9 medium containing CIP as a sole carbon source. Experiment III served as a control without bacterial cells to assess CIP degradation through abiotic factors. In all experiments, 100 mg/L of CIP was added, and the flasks were incubated at 37 °C with continuous agitation at 150 rpm. Samples were collected for analysis after 5 and 10 days to evaluate the biodegradation of CIP (Nguyen *et al.* 2018).

Analytical procedures for determination of CIP degradation

The degradation of the CIP was studied by ultra-high-performance liquid chromatography (UHPLC) and spectrophotometry. The selection of UHPLC and spectrophotometry was based on their accuracy, sensitivity in analyzing ciprofloxacin degradation, and available resources. The existence of alternative techniques such as mass spectrometry, HPLC coupled with mass spectrometry, or other chromatographic methods is acknowledged. Spectrometric determination of the remaining CIP was done by using a Lambda 25 UV/VIS spectrometer. A standard curve was generated using different concentrations of CIP (10–90 μg/mL) at 278 nm wavelength as λ_{max} to calculate CIP concentration.

Further CIP concentration was also estimated by Waters UHPLC system equipped with the C18 column (4.6 mm × 150 mm) having a PDA detector and at λ278 nm wavelength. The mobile phase consisted of solution A (0.025 M phosphoric acid adjusted with triethylamine at pH 3) and acetonitrile (13:87). The mobile phase was run at the flow rate of 1.0 mL/min with a sample injection volume of 10 μL. The residual CIP was estimated using the following formula

$$\text{Residual CIP} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard}$$

Antibacterial assay of the degraded products

The antibacterial potential of intermediates formed after the degradation of CIP by R4 was evaluated against *Staphylococcus aureus* and *Escherichia coli* on Muller Hinton agar by disc diffusion method (Singh *et al.* 2017).

Statistical analysis

In this study, statistical analyses including mean and standard deviation were computed using Microsoft Excel 2013. Statistix 8.1 software was used to analyze the significant difference in ciprofloxacin removal among three different treatments. This assessment was conducted through a two-way analysis of variance (two-way ANOVA), followed by the Tukey HSD post hoc test. Values were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Isolation and identification of CIP-degrading bacterial strain

S. stutzeri R2 and *E. indicum* R4 two of the seven bacterial strains, isolated from pharmaceutical wastewater by enrichment and acclimation method, which showed resistance against CIP. Acclimatization to a considerably high CIP concentration and a low proportion of secondary C sources may improve bacterial adaptability to

antibiotic resistance as well as its potential to biodegrade pollutants. Furthermore, the adaptation of bacteria to high CIP concentration may cause considerable alterations in the diversity and structure of the microbial communities. Constant exposure to a rather high proportion of CIP might be the primary driving factor for the evolution and enzyme induction in CIP-degrading bacteria (Feng *et al.* 2019). Such biotransformation features might emerge and propagate throughout microbial communities when microbial progeny acquires these functional gene alleles from their parents. Moreover, favorable co-substrates, which are auxiliary compounds or substrates present in the environment, can enhance the biodegradation process of a specific target compound, thereby fostering greater diversity within the microbial community (Eliasson *et al.* 2023). After enrichment with CIP, the strains R2 and R4 were isolated with the ability to grow on the solid M9 medium containing 100 mg/L of CIP.

The 16S rRNA gene sequences of strain R2 (1,052 bp, Genbank accession number: OQ256706) and R4 (1,062 bp, Genbank accession number: OQ423123) revealed their relationship with genus *Stutzerimonas*, 98% similarity to *S. stutzeri* strain ISLP10 (MF125023), and genus *Exiguobacterium*, displaying 99.81% similarity to *E. indicum* JDM5 1B (JN644520), respectively. Furthermore, a phylogenetic tree was constructed based on their 16S rRNA gene sequences, allowing for a comparison with other related bacteria, as illustrated in Figures 1 and 2.

The genus *Stutzerimonas*, recently proposed within the *Pseudomonadaceae* family, includes species that were previously classified under the genus *P. stutzeri*, specifically those belonging to the *P. stutzeri* phylogenetic group (Gomila *et al.* 2022; Mulet *et al.* 2023). The phenotypic characteristics of genus *Stutzerimonas* include gram-negative, rod-shape, and non-spore-forming, aerobic, strictly oxidative, and chemoorganotroph. The phenotypic characteristics of the genus Stutzerimonas include being gram-negative, rod-shaped, non-spore-forming, aerobic, strictly oxidative, and chemoorganotrophic. Members of this genus have been isolated from a wide range of environments, including soil, plants, wastewater, marine water, sediments ranging from 11,000 meters in depth to intertidal shores, as well as from oil-contaminated sand, hydrothermal vents, and sludge (Lalucat *et al.* 2022). Members of the *Stutzerimonas* (formerly *P. stutzeri*) genus have been studied for their significant role in the biodegradation of various complex compounds, including agrochemicals (Xu *et al.* 2023), cyanide, phenol (Singh *et al.* 2018), benzo[a]pyrane (Kumari & Chandra 2023), as well as antibiotics like sulfamethoxazole and chloramphenicol (Yang *et al.* 2021).

Furthermore, the species of genus *Exiguobacterium* are a gram-positive, motile, and non-spore-forming. They can also be either aerobic or anaerobic, depending on the availability of oxygen and growth conditions. The members of this genus have been isolated from a wide range of environments, including hot springs, glaciers, rhizospheres, food processing plants, wetlands, river water, and plastic dumped soil (Chauhan *et al.* 2018; Maroof *et al.* 2022). The member of the genus *Exiguobacterium* have been investigated to play an important role in the biodegradation of a diverse range of complex compounds, viz., pesticides, heavy metals, 4-chloroindole, and triphenylmethane dye (Pandey 2020). *Exiguobacterium undae* strain DR14 and *Exiguobacterium sibiricum* strain DR11 have been reported to show promising biodegradation potential against polystyrene (Chauhan *et al.* 2018). *Exiguobacterium* Sp. strain LM-IK2 isolated from plastic dumped soil was found to degrade low-density polyethylene (Maroof *et al.* 2022). Furthermore, bacteria like *Bradyrhizobium*, *Thermus thermophilus*, and *Geobacillus thermoleovorans* are metabolically active microorganisms that are capable of degrading CIP originating from various sources. Meanwhile, *S. stutzeri* R2 and *E. indicum* R4 represent newly identified genera with the potential to efficiently degrade CIP. These strains, isolated in the current study, could be harnessed for use in diverse wastewater treatment systems, such as trickling filters, to remove the antibiotic CIP from wastewater.

CIP degradation in batch reactors

The removal of CIP in three different batch reactors including (I) co-metabolism, (II) utilization of CIP (100 mg/L) as sole carbon source, and (III) control (antibiotics in the media without any microorganism) were analyzed through spectrophotometer and UHPLC. In the spectrophotometric analysis of R2 (Figure 3) and R4 (Figure 4), it was observed that, in Experiment I, there was a 51% degradation of CIP achieved by R2 and 60% removal of CIP achieved by R4 after 5 days of incubation (Figures 3 and 4). These percentages increased to 77 and 68% for R2 and R4, respectively, after 10 days of incubation in Experiment I. In Experiment II, the degradation of CIP was 23% for R2 and 19% for R4 after 5 days, which increased to 35% for R2 and 28% for R4 after 10 days. However, the degradation of CIP in Experiment III (the control reactor) was negligible. Notably, R2 and R4 achieved 66

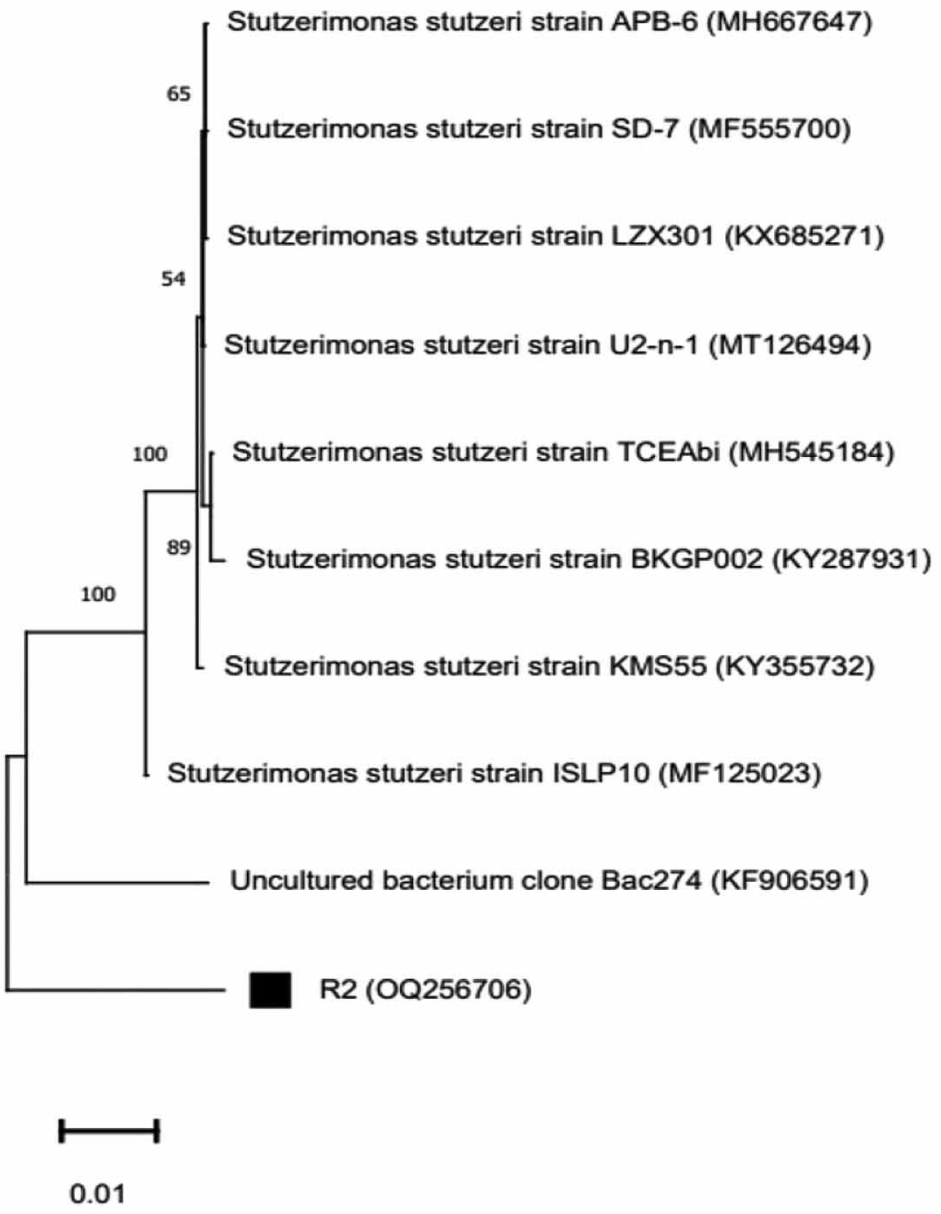


Figure 1 | The phylogenetic tree of bacterial strain R2 was constructed based on the 16S rRNA gene sequences by the neighbor-joining method using MEGA11 software. The bootstrap values are shown at branch points and are based on 1,000 resampled datasets. The scale bar represents 0.01 substitutions per nucleotide position.

and 88% degradation, respectively, within the initial 5 days of incubation. It is probable that the decline observed in the later stage of CIP degradation rate occurred due to the accumulation of degradation products that might have inhibited microbial activity, alongside lower nutrient or substrate levels. These conditions possibly impacted its biodegradation capacity. These findings align with previous research, indicating that limitations in substrate availability and the accumulation of toxic intermediates significantly affected microbial growth and degradation of the target contaminant. Higher nutrient levels favored better performance (Hong *et al.* 2020).

The confirmation of CIP removal was also obtained through UHPLC analysis, which exhibited a similar pattern with the spectrophotometric analysis. After 5 days of incubation, CIP biodegradation rates were 51 and 25% for R2 and 60 and 20% for R4 in Experiments I and II, respectively. After 10 days of incubation, CIP removal increased to 77 and 34% for R2 (Figure 5) and 69 and 30% for R4 (Figure 6) in Experiments I and II, respectively. In Experiment III for both R2 and R4 (the control reactor), the removal of CIP remained negligible.

To the best of our knowledge, this is the first report for the species of *Stutzerimonas* and *Exiguobacterium sp.* involved in CIP degradation and appears to be a promising candidate for the degradation of CIP. In the present

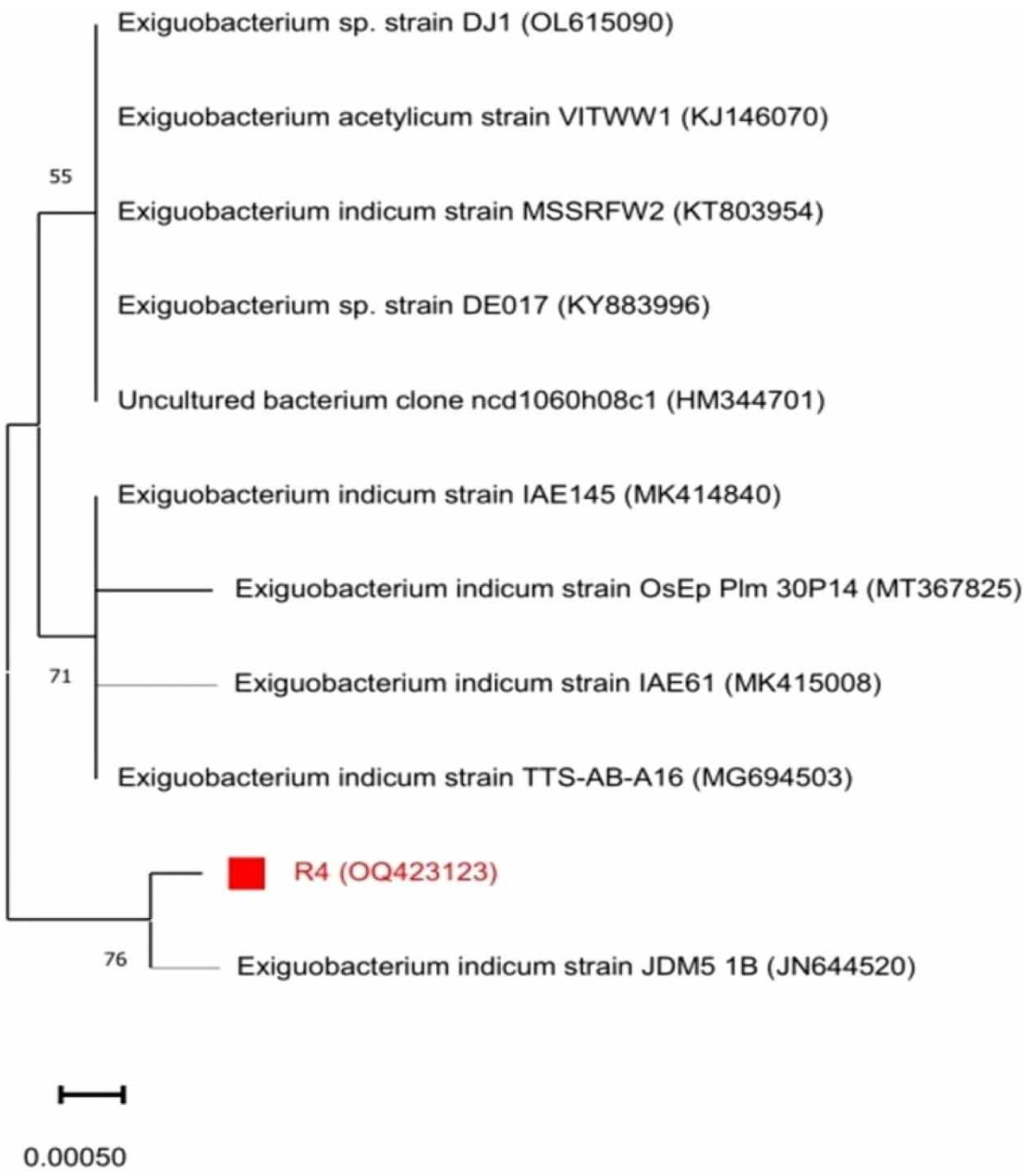


Figure 2 | The phylogenetic tree of bacterial strain R4 was constructed based on the 16S rRNA gene sequences by the neighbor-joining method using MEGA11 software. The bootstrap values are shown at branch points and are based on 1,000 resampled datasets. The scale bar represents 0.005 substitutions per nucleotide position.

study, the degradation efficiency of CIP by *S. stutzeri* R2 is higher than that of *E. indicum* R4. The notable difference in the degradation rates of CIP between *S. stutzeri* and *E. indicum* can be attributed to various factors. *S. stutzeri* demonstrated a higher degradation rate, possibly due to its possession of specialized enzymes. Moreover, more favorable growth conditions or more effective adaptation might have contributed to its superior performance compared to *E. indicum*. Further exploration into enzyme profiling and metabolic pathways would be beneficial in understanding the underlying reasons for the substantial disparity in CIP degradation rates between these two bacterial species. These findings align with previous research demonstrating that favorable growth conditions or more effective adaptation significantly influenced the degradation efficiencies of cefalexin by two other strains, *Rhizobium sp.* (CLX-2) and *Klebsiella sp.* (CLX-3) (Tian *et al.* 2023). However, it was also observed that the degradation rate of both the isolates, *S. stutzeri* R2 and *E. indicum* R4, is greater than previously reported values by other organisms. *Bradyhizobium sp.GLC_01* degrades only 26.4% CIP at the concentration of

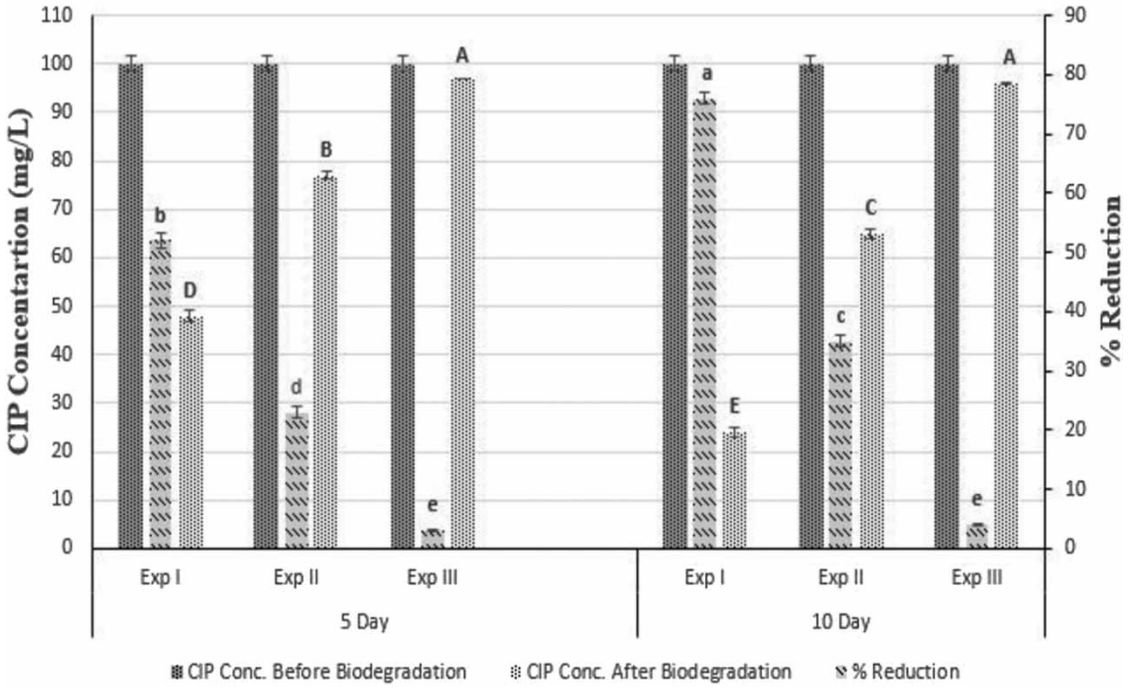


Figure 3 | Spectrophotometric analysis (278 nm) of CIP degradation by incubating 100 mg/L CIP with *Stutzerimonas stutzeri* R2 for 5 and 10 days. Experiments I, II, and III represent the degradation of CIP in the presence of an additional carbon source (co-metabolism), CIP as the sole carbon source, and degradation without inoculation of R4 active cells (control), respectively. Error bars represent the standard deviation. Data with different capital letters indicate the significant differences ($p < 0.05$) of CIP residual among treatments. Data with lowercase letters indicate the significant differences ($p < 0.05$) of percentage reduction among treatments.

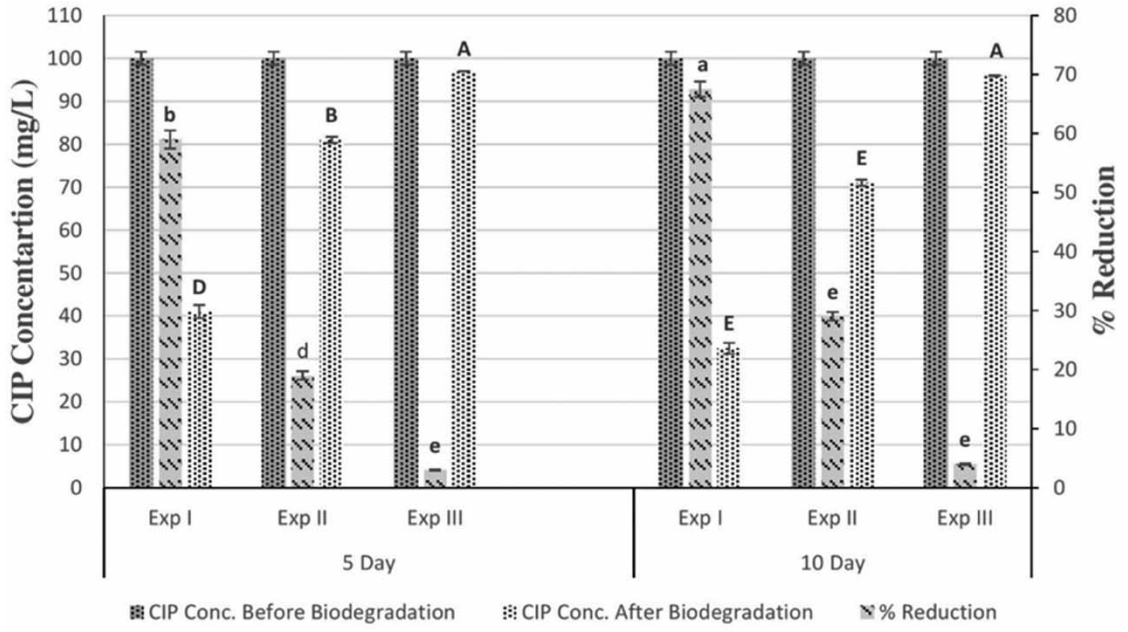


Figure 4 | Spectrophotometric analysis (278 nm) of CIP degradation by incubating 100 mg/L CIP with *Exiguobacterium indicum* R4 for 5 and 10 days. Experiments I, II, and III represent the degradation of CIP in the presence of an additional carbon source (co-metabolism), CIP as the sole carbon source, and degradation without inoculation of R4 active cells (control), respectively. Error bars represent the standard deviation. Data with different capital letters indicate the significant differences ($p < 0.05$) of CIP residual among treatments. Data with lowercase letters indicate the significant differences ($p < 0.05$) of percentage reduction among treatments.

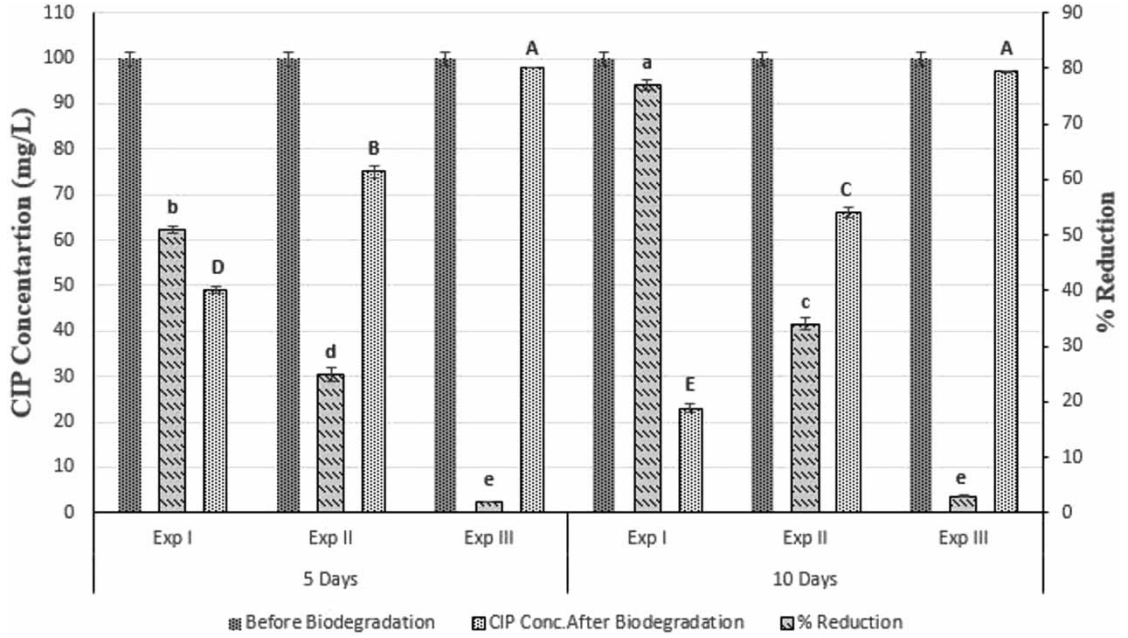


Figure 5 | UHPLC analysis of CIP after biodegradation by *Stutzerimonas stutzeri* R2. This was achieved by incubating 100 mg/L CIP with *Stutzerimonas stutzeri* R2 for durations of 5 and 10 days. Experiments I, II, and III represent the degradation of CIP in the presence of an additional carbon source (co-metabolism), Experiment II assessed CIP degradation when it was the sole carbon source; and Experiment III served as the control, with no inoculation of active R4 cells, respectively. The error bars in the Figure indicate the standard deviation. In the data, treatments that are denoted with different capital letters indicate significant differences ($p < 0.05$) in CIP residual levels among them, while treatments with lowercase letters indicate significant differences ($p < 0.05$) in the percentage of reduction among them.

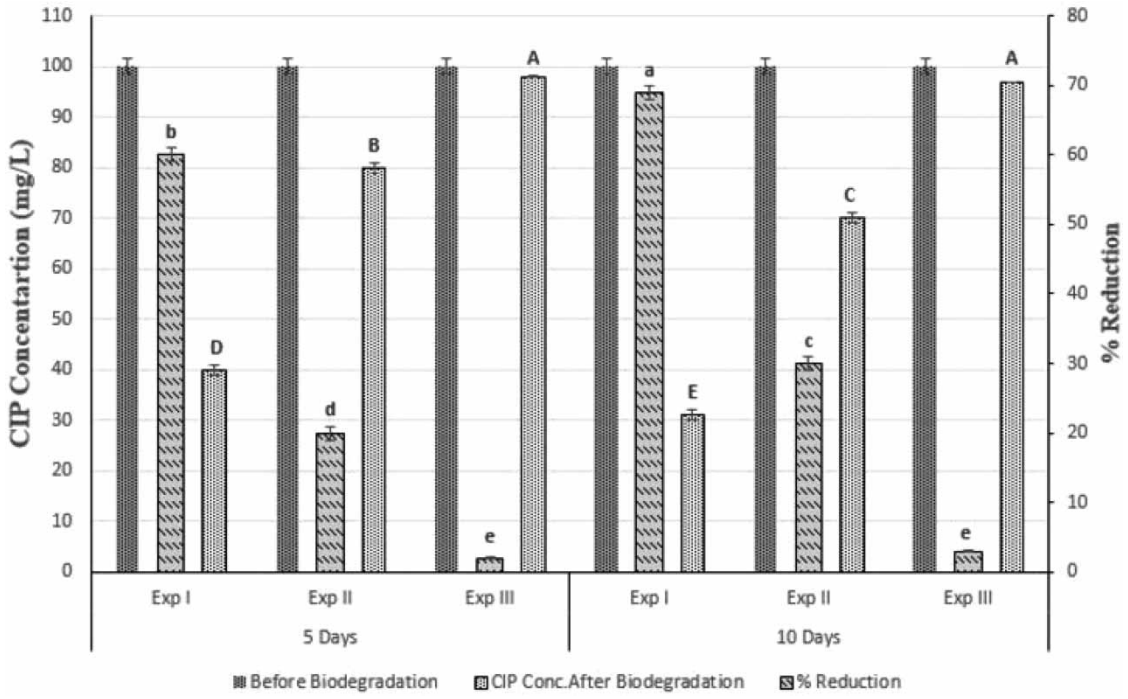


Figure 6 | UHPLC analysis of CIP after biodegradation by *Exiguobacterium indicum R4.* This was achieved by incubating 100 mg/L CIP with *E. indicum R4* for durations of 5 and 10 days. Experiments I, II, and III represent the degradation of CIP in the presence of an additional carbon source (co-metabolism), Experiment II assessed CIP degradation when it was the sole carbon source; and Experiment III served as the control, with no inoculation of active R4 cells, respectively. The error bars indicate the standard deviation. In the data, treatments that are denoted with different capital letters indicate significant differences ($p < 0.05$) in CIP residual levels among them, while treatments with lowercase letters indicate significant differences ($p < 0.05$) in the percentage of reduction among them.

10 mg/L (Nguyen *et al.* 2018). After 11 days of incubation, freshwater microalgae *Chlamydomonas mexicana* removed only 13% of 2 mg/L CIP (Xiong *et al.* 2017). *Thermus thermophiles*, a thermophilic bacterial strain, degraded 55% of CIP (Pan *et al.* 2018).

The findings revealed that the biotransformation of CIP via co-metabolism was higher as compared to direct metabolism in which CIP was used as a sole carbon source. In this study, the addition of co-substrates boosted the growth of microorganisms that degrade pollutants, resulting in the rapid degradation of CIP. The additional substrates could have enabled microbial cells to mitigate CIP toxicity by providing sufficient nutrients. Consequently, this production of more biocatalysts capable of reacting with CIP could lead to faster degradation of CIP through energy-driven co-metabolism. These results are in accordance with those reported previously that showed almost complete biodegradation of CIP by the XG consortium upon supplementation of co-substrate (Feng *et al.* 2019).

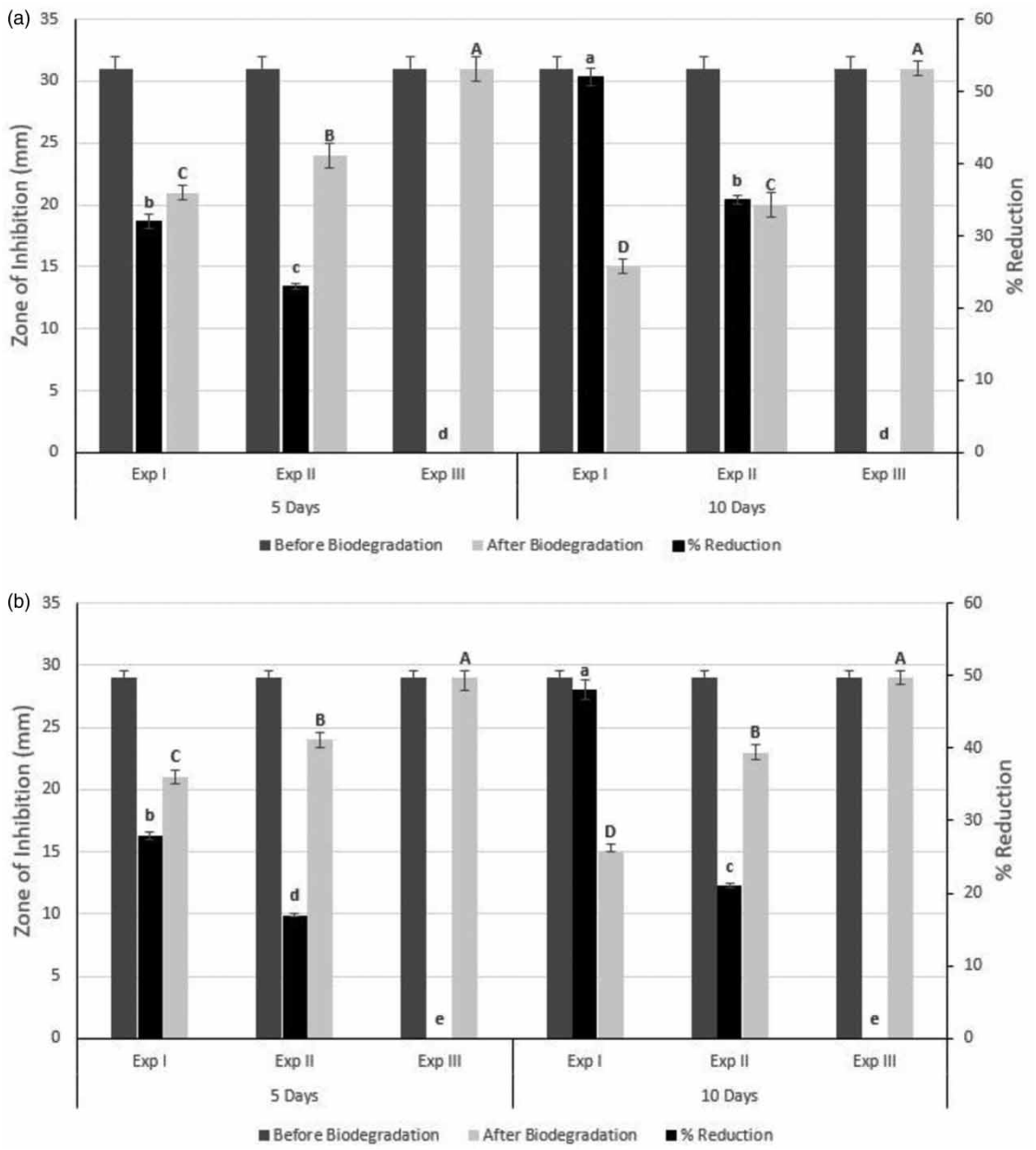


Figure 7 | Antibacterial activity of CIP before and after biodegradation by R2. (a) Antibacterial against *E. coli* and (b) antibacterial activity against *S. aureus*. Error bars represent the standard deviation. Data with different capital letters indicate the significant differences ($p < 0.05$) in the zone of inhibition of degraded products of CIP among treatments. Data with lowercase letters indicate the significant differences ($p < 0.05$) of percentage reduction in antibacterial activity among treatments.

The auxiliary organic feed not only assists the microbial cells in their growth, proliferation, and maintaining viability but also stimulates certain non-specific enzymes that help in the co-metabolic biodegradation of environmental contaminants. Organic compounds play a pivotal role by serving as vital nutrient sources, supplying carbon, energy, and metabolic substrates necessary for microbial growth, proliferation, and maintenance of cellular viability. Additionally, certain organic compounds can act as inducers, stimulating the production of enzymes not only crucial for their own breakdown but also aiding in the co-metabolic degradation of environmental pollutants. However, while these compounds generally support microbial functions, there exists the potential for adverse effects, including toxicity or inhibition at higher concentrations, alterations in microbial community dynamics, and the possibility of reducing the efficiency of contaminant degradation pathways

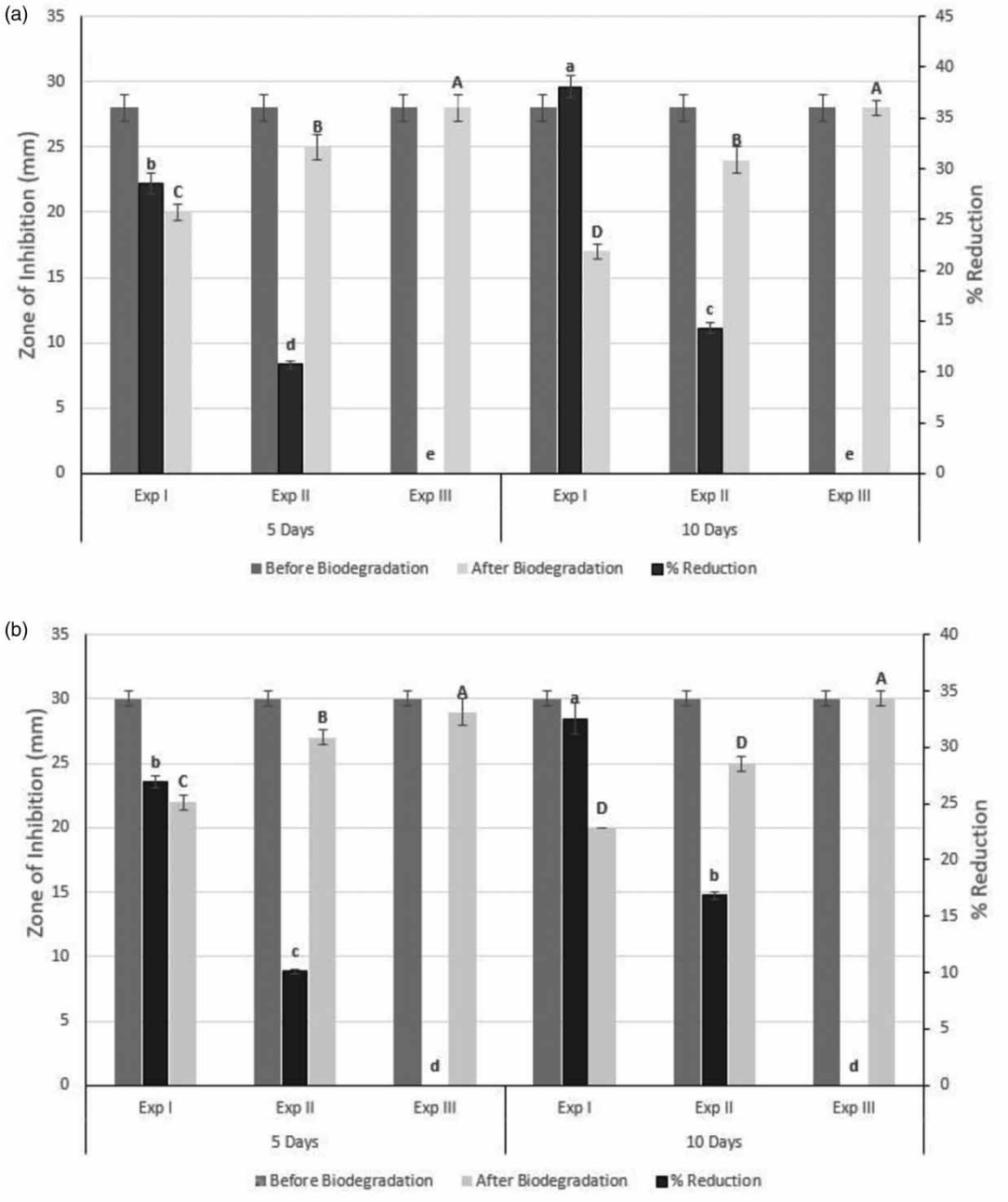


Figure 8 | Antibacterial activity of CIP before and after biodegradation by R4. (a) Antibacterial against *E. coli* and (b) antibacterial activity against *S. aureus*. Error bars represent the standard deviation. Data with different capital letters indicate the significant differences ($p < 0.05$) in the zone of inhibition of degraded products of CIP among treatments. Data with lowercase letters indicate the significant differences ($p < 0.05$) of percentage reduction in antibacterial activity among treatments.

through misdirected resource utilization (Luo *et al.* 2014; Feng *et al.* 2019). In the natural environment, CIP co-exists with a variety of rapidly biodegradable substrates, and therefore co-metabolic degradation potentially plays a key role in the bioremediation of pseudo-persistent pharmaceutical pollutants (Kiel & Engesser 2015; Murphy 2016).

In the current investigation, the addition of co-substrate promoted the growth of CIP-degrading bacteria, *S. stutzeri* R2 and *E. indicum* R4, leading to the rapid biotransformation of CIP. The co-substrate might have allowed the bacterial cells to substantially reduce the toxicity of CIP by providing sufficient nutrients, resulting in the production of biocatalysts that can potentially interact with CIP, hence triggering its biodegradation (energy-driven co-metabolism). Nevertheless, current research has established the practical significance of the requisite carbon sources as co-substrates for improved degradation of CIP by bacteria. Furthermore, the type of co-substrate utilized may be an important element in influencing CIP degradation efficiency (Maia *et al.* 2014; Pan *et al.* 2018).

Antibacterial assay of degraded residues

Degraded CIP was tested for antimicrobial activity against *E. coli* and *S. aureus*. The antibacterial potency of CIP degradation products, formed after 5 and 10 days of incubation with *S. stutzeri* R2 and *E. indicum* R4, was found to be weaker than that of the CIP standard.

It decreased to 32 and 23% for R2 and 29 and 11% for R4 against *E. coli* and it reduced up to 28 and 17% for R2 and 27 and 10% for R4 against *S. aureus* in experiments I and II, respectively after 5 days of incubation. However, after 10 days of incubation decline in antibacterial activity of degraded products was 52% and 35 for R2 and 38 and 14% for R4 against *E. coli* and 48 and 21% for R2 and 32 and 17% for R4 against *S. aureus* for experiments I and II, respectively. In the case of control for both R2 and R4, there was no decline in antibacterial activity against both test strains after the incubation of 5 and 10 days as illustrated in Figures 7 and 8, respectively.

It was observed that an increase in the incubation time of CIP with *S. stutzeri* R2 and *E. indicum* R4 reduced the antibacterial potential of its degraded products. The decline in antibacterial activity may be attributed to the possibility that during biodegradation, ciprofloxacin undergoes chemical modifications or breaks down into metabolites that no longer possess the original antibiotic properties. These metabolites might exhibit altered chemical structures or modified functional groups, resulting in a diminished antibacterial efficacy. These outcomes are supported by a previous study that reported the decline in the antibacterial potential of CIP at an average of 26.3% by fungus *Pleurotus ostreatus* upon incubation of 14 days (Singh *et al.* 2017). These findings are also noteworthy because they indicate that treating CIP effluent with *S. stutzeri* R2 and *E. indicum* R4 may effectively decline its antibacterial properties, thus, potentially reducing the emergence of antibiotics resistance bacteria in the environment.

CONCLUSIONS

The presence of pharmaceutical pollutants in the environment has crucial impacts on the structure and function of the ecosystem and the emergence of antibiotic-resistant bacteria (Dąbrowska *et al.* 2018). The study concludes that the bacterial strains *S. stutzeri* R2 and *E. indicum* R4, which were isolated from pharmaceutical wastewater, exhibited considerable potential for degrading ciprofloxacin. It was observed that after 10 days of incubation, *S. stutzeri* R2 remove 77% of CIP, a higher degradation rate compared to the 68% demonstrated by *E. indicum* R4. Importantly, it was noticed that the biotransformation of CIP was found to be higher in co-metabolism compared to direct metabolism (using CIP as a sole carbon source). When CIP was used as the sole carbon source, the degradation of CIP was 35% for R2 and 28% for R4 after 10 days. Additionally, the products generated after degradation of CIP were having reduced antibacterial activity against test bacterial strains as detected from their zone of inhibition. It was noted that prolonging the incubation time of CIP with *S. stutzeri* R2 and *E. indicum* R4 led to a decrease in the antibacterial potency of its degraded products. The study's findings hold considerable importance. Therefore, it can be concluded that *S. stutzeri* R2 and *E. indicum* R4 are promising candidates for introducing into wastewater treatment systems like trickling filters to remove CIP from pharmaceutical and hospital wastewater before entering the natural environment. This proactive measure might mitigate the proliferation of antibiotic-resistant bacteria in natural environments.

ACKNOWLEDGEMENTS

The authors acknowledge the National Institute of Health Islamabad for providing UHPLC analyses facility.

AUTHORS CONTRIBUTIONS

Conceptualization: Q.A., S.A., and M.B.; Methodology: Q.A., S.A., and R.Z.; Formal analysis and investigation: Q.A., R.Z., and W.S.; Results analysis: Q.A., R.Z., G.M., and T.I.; Writing – original draft preparation: Q.A.; Writing – review and editing: Q.A., S.A., M.B., S.K., and W.S.; Funding acquisition S.A.; Resources: S.A., and M.B.; Supervision: S.A.

FUNDING STATEMENT

This study was funded by the Higher Education Commission of Pakistan. Q.A. was awarded the HEC Indigenous Scholarship granted by the Higher Education Commission of Pakistan.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Baralla, E., Demontis, M. P., Dessì, F. & Varoni, M. V. 2021 An Overview of antibiotics as emerging contaminants: Occurrence in bivalves as biomonitoring organisms. *Animals* **11**, 3239. doi:10.3390/ani11113239.

Bhat, S. A., Bashir, O., Haq, S. A. U., Amin, T., Rafiq, A., Ali, M., Américo-Pinheiro, J. H. P. & Sher, F. 2022 Phytoremediation of heavy metals in soil and water: An eco-friendly, sustainable and multidisciplinary approach. *Chemosphere* **303**, 134788. doi.org/10.1016/j.chemosphere.2022.134788.

Cai, Y., Yan, Z., Ou, Y., Peng, B., Zhang, L., Shao, J., Lin, Y. & Zhang, J. 2022 Effects of different carbon sources on the removal of ciprofloxacin and pollutants by activated sludge: Mechanism and biodegradation. *Journal of Environmental Science (China)* **111**, 240–248. doi:10.1016/j.jes.2021.03.037.

Chauhan, D., Agrawal, G., Deshmukh, S., Roy, S. S. & Priyadarshini, R. 2018 Biofilm formation by exiguobacterium sp. DR11 and DR14 alter polystyrene surface properties and initiate biodegradation. *RSC Advances* **8**, 37590–37599. doi:10.1039/C8RA06448B.

Cycon, M., Mrozik, A. & Piotrowska-Seget, Z. 2019 Antibiotics in the soil environment-degradation and their impact on microbial activity and diversity. *Frontiers in Microbiology* **10**, 338. doi:10.3389/fmicb.2019.00338.

Dąbrowska, M., Muszyńska, B., Starek, M., Żmudzki, P. & Opoka, W. 2018 Degradation pathway of cephalosporin antibiotics by in vitro cultures of Lentinula edodes and Imleria badia. *International Biodeterioration & Biodegradation* **127**, 104–112. doi.org/10.1016/j.ibiod.2017.11.014.

Eliasson, K. A., Singh, A., Isaksson, S. & Schnürer, A. 2023 Co-substrate composition is critical for enrichment of functional key species and for process efficiency during biogas production from cattle manure. *Microbial Biotechnology* **16**, 350–371. doi.org/10.1111/1751-7915.14194.

Feng, N. X., Yu, J., Xiang, L., Yu, L. Y., Zhao, H. M., Mo, C. H., Li, Y. W., Cai, Q. Y., Wong, M. H. & Li, Q. X. 2019 Co-metabolic degradation of the antibiotic ciprofloxacin by the enriched bacterial consortium XG and its bacterial community composition. *Science of The Total Environment* **665**, 41–51. doi:10.1016/j.scitotenv.2019.01.322.

Gomila, M., Mulet, M., García-Valdés, E. & Lalucat, J. 2022 Genome-based taxonomy of the genus stutzerimonas and proposal of S. frequens sp. nov. and S. degradans sp. nov. and emended descriptions of S. perfectomarina and S. chloritidismutans. *Microorganisms* **10**, 1363. doi:10.3390/microorganisms10071363.

Holanda, F. H. E., Birolli, W. G., Morais, E. D. S., Sena, I. S., Ferreira, A. M., Faustino, S. M. M., Grace Da S. Solon, L., Porto, A. L. M. & Ferreira, I. M. 2019 Study of biodegradation of chloramphenicol by endophytic fungi isolated from Bertholletia excelsa (Brazil nuts). *Biocatalysis and Agricultural Biotechnology* **20**, 101200. doi:10.1016/j.bcab.2019.101200.

Hong, X., Zhao, Y., Zhuang, R., Liu, J., Guo, G., Chen, J. & Yao, Y. 2020 Bioremediation of tetracycline antibiotics-contaminated soil by bioaugmentation. *RSC Advances* **10**, 33086–33102. doi:10.1039/D0RA04705H.

Kiel, M. & Engesser, K.-H. 2015 The biodegradation vs. biotransformation of fluorosubstituted aromatics. *Applied Microbiology and Biotechnology* **99**, 7433–7464. doi:10.1007/s00253-015-6817-5.

Kumari, B. & Chandra, R. 2023 Benzo [a] pyrene degradation from hydrocarbon-contaminated soil and their degrading metabolites by Stutzerimonas stutzeri (LOBP-19A). *Waste Management Bulletin* **1**, 115–127. doi.org/10.1016/j.wmb.2023.07.006.

Lalitha, N., Patil, P. K., Rajesh, R. & Muralidhar, M. 2019 Usage of pleurotus ostreatus for degradation of oxytetracycline in varying water salinities in brackishwater aquaculture system. *Journal of Coastal Research* **86**, 138–141. doi:10.2112/SI86-021.1.

Lalucat, J., Gomila, M., Mulet, M., Zaruma, A. & García-Valdés, E. 2022 Past, present and future of the boundaries of the Pseudomonas genus: Proposal of Stutzerimonas gen. nov. *Systematic and Applied Microbiology* **45**, 126289. doi:10.1016/j.syapm.2021.126289.

Leng, Y., Bao, J., Chang, G., Zheng, H., Li, X., Du, J., Snow, D. & Li, X. 2016 Biotransformation of tetracycline by a novel bacterial strain Stenotrophomonas maltophilia DT1. *Journal of Hazardous Materials* **318**, 125–133. doi:10.1016/j.jhazmat.2016.06.053.

Luo, W., Zhu, X., Chen, W., Duan, Z., Wang, L. & Zhou, Y. 2014 Mechanisms and strategies of microbial cometabolism in the degradation of organic compounds–chlorinated ethylenes as the model. *Water Science and Technology* **69**, 1971–1983. doi.org/10.2166/wst.2014.108.

Maia, A. S., Ribeiro, A. R., Amorim, C. L., Barreiro, J. C., Cass, Q. B., Castro, P. M. & Tiritan, M. E. 2014 Degradation of fluoroquinolone antibiotics and identification of metabolites/transformation products by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A* **1333**, 87–98. doi:10.1016/j.chroma.2014.01.069.

Manage, P. M. 2018 Heavy use of antibiotics in aquaculture; emerging human and animal health problems–a review. *Sri Lanka Journal of Aquatic Sciences* doi:10.4038/sljas.v23i1.7543.

Maroof, L., Khan, I., Hassan, H., Azam, S. & Khan, W. 2022 Microbial degradation of low density polyethylene by Exiguobacterium sp. strain LM-IK2 isolated from plastic dumped soil. *World Journal of Microbiology and Biotechnology* **38**, 197. doi:10.1007/s11274-022-03389-z.

Miran, W., Jang, J., Nawaz, M., Shahzad, A. & Lee, D. S. 2018 Biodegradation of the sulfonamide antibiotic sulfamethoxazole by sulfamethoxazole acclimatized cultures in microbial fuel cells. *Science of The Total Environment* **627**, 1058–1065. doi:10.1016/j.scitotenv.2018.01.326.

Mulet, M., Gomila, M., Lalucat, J., Bosch, R., Rossello-Mora, R. & García-Valdés, E. 2023 Stutzerimonas decontaminans sp. nov. isolated from marine polluted sediments. *Systematic and Applied Microbiology* **46**, 126400. doi.org/10.1016/j.syapm.2023.126400.

Murphy, C. D. 2016 Microbial degradation of fluorinated drugs: Biochemical pathways, impacts on the environment and potential applications. *Applied Microbiology & Biotechnology* **100**, 2617–2627. doi:10.1007/s00253-016-7304-3.

Mutuku, C., Gazdag, Z. & Melegh, S. 2022 Occurrence of antibiotics and bacterial resistance genes in wastewater: Resistance mechanisms and antimicrobial resistance control approaches. *World Journal of Microbiology & Biotechnology* **38**, 152. doi:10.1007/s11274-022-03334-0.

Nguyen, L. N., Nghiem, L. D. & Oh, S. 2018 Aerobic biotransformation of the antibiotic ciprofloxacin by Bradyrhizobium sp. isolated from activated sludge. *Chemosphere* **211**, 600–607. doi:10.1016/j.chemosphere.2018.08.004.

Othmani, A., Kadier, A., Singh, R., Igwegbe, C. A., Bouzid, M., Aquatar, M. O., Khanday, W. A., Bote, M. E., Damiri, F. & Gökkuş, Ö. 2022 A comprehensive review on green perspectives of electrocoagulation integrated with advanced processes for effective pollutants removal from water environment. *Environmental Research* **215**, 114294. doi.org/10.1016/j.envres.2022.114294.

Pan, L. J., Li, J., Li, C. X., Tang, X. D., Yu, G. W. & Wang, Y. 2018 Study of ciprofloxacin biodegradation by a Thermus sp. isolated from pharmaceutical sludge. *Journal of Hazardous Materials* **343**, 59–67. doi:10.1016/j.jhazmat.2017.09.009.

Pandey, N. 2020 Exiguobacterium. In: *Beneficial Microbes in Agro-Ecology*. Academic Press, pp. 169–183. doi.org/10.1016/B978-0-12-823414-3.00010-1.

Rashid, T., Iqbal, D., Hazafa, A., Hussain, S., Sher, F. & Sher, F. 2020 Formulation of zeolite supported nano-metallic catalyst and applications in textile effluent treatment. *Journal of Environmental Chemical Engineering* **8**, 104023. doi.org/10.1016/j.jece.2020.104023.

Rashid, T., Sher, F., Hazafa, A., Hashmi, R. Q., Zafar, A., Rasheed, T. & Hussain, S. 2021 Design and feasibility study of novel paraboloid graphite based microbial fuel cell for bioelectrogenesis and pharmaceutical wastewater treatment. *Journal of Environmental Chemical Engineering* **9**, 104502. doi.org/10.1016/j.jece.2020.104502.

Sadaf, S., Singh, A. K., Iqbal, J., Kumar, R. N., Sulejmanović, J., Habila, M. A., Américo-Pinheiro, J. H. P. & Sher, F. 2022 Advancements of sequencing batch biofilm reactor for slaughterhouse wastewater assisted with response surface methodology. *Chemosphere* **307**, 135952. doi.org/10.1016/j.chemosphere.2022.135952.

Singh, S. K., Khajuria, R. & Kaur, L. 2017 Biodegradation of ciprofloxacin by white rot fungus Pleurotus ostreatus. *3 Biotech* **7**, 69. doi:10.1007/s13205-017-0684-y.

Singh, U., Arora, N. K. & Sachan, P. 2018 Simultaneous biodegradation of phenol and cyanide present in coke-oven effluent using immobilized Pseudomonas putida and Pseudomonas stutzeri. *Brazilian Journal of Microbiology* **49**, 38–44. doi:10.1016/j.bjm.2016.12.013.

Thelusmond, J.-R., Kawka, E., Strathmann, T. J. & Cupples, A. M. 2018 Diclofenac, carbamazepine and triclocarban biodegradation in agricultural soils and the microorganisms and metabolic pathways affected. *Science of the Total Environment* **640**, 1393–1410. doi:10.1016/j.scitotenv.2018.05.403.

Tian, J., Chen, C., Lartey-Young, G. & Ma, L. 2023 Biodegradation of cefalexin by two bacteria strains from sewage sludge. *Royal Society Open Science* **10**, 220442. doi:10.1098/rsos.220442.

Xiong, J.-Q., Kurade, M. B., Kim, J. R., Roh, H.-S. & Jeon, B.-H. 2017 Ciprofloxacin toxicity and its co-metabolic removal by a freshwater microalga Chlamydomonas mexicana. *Journal of Hazardous Materials* **323**, 212–219. doi:10.1016/j.jhazmat.2016.04.073.

Xu, C., Cui, D., Lv, X., Zhong, G. & Liu, J. 2023 Heterogeneous distribution of carbofuran shaped by Pseudomonas stutzeri biofilms enhances biodegradation of agrochemicals. *Environmental Research* **229**, 115894. doi.org/10.1016/j.envres.2023.115894.

Yang, F., Jian, H., Wang, C., Wang, Y., Li, E. & Sun, H. 2021 Effects of biochar on biodegradation of sulfamethoxazole and chloramphenicol by Pseudomonas stutzeri and Shewanella putrefaciens: Microbial growth, fatty acids, and the expression quantity of genes. *Journal of Hazardous Materials* **406**, 124311. doi:10.1016/j.jhazmat.2020.124311.

First received 6 November 2023; accepted in revised form 13 January 2024. Available online 24 February 2024