IN VITRO SCREENING OF BACTERIAL STRAINS FOR THE REMOVAL OF CIPROFLOXACIN (CPX) FROM WATER

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Abstract. Ciprofloxacin (CPX), is a broad-spectrum fluoroquinolone antibiotic widely used to treat bacterial infections in humans and animals. In recent years, ecological problems caused by the disposal of CPX-waste product have attracted increasing attention. This study aimed to evaluate the ability of different bacteria to remove CPX from water. The ability of five bacterial strains namely; *Acenitobacter* sp., *Acenitobacter lwofii, Bacillus. pumilus, Burkholderia phytofirmans,* and *Mesorihizobium* sp. were examined in this study. Tests were performed on Luria Bertani (LB) as well as on Mueller-Hinton (MH) agar at different CPX concentrations (20-100 mg L^{-1}). All five tested bacterial strains showed growth on LB as well as on MH agar plates at all concentrations between 20 to 100 mg L^{-1} CPX. Three bacterial species: *Acenitobacter lwofii*, *Bacillus pumilus*, and *Mesorihizobium sp.* were reported more resistant to CPX of the five strains tested. In a shake flask study, the strain *B. pumilus* initially showed higher degradation rate (75.53%) followed by *Acenitobacter lwofii* (72.3%), *Mesorihizobium* sp. (70.32), *Burkholderia phytofirmans* (67.10%), and *Acenitobacter sp.* (63.2%) at low CPX concentration (5 mg L^{-1}) in minimal saline media. However, *Mesorihizobium* sp. showed higher degradation efficiency (76.32%) at a higher CPX concentration (10 mg L^{-1}). A group of three bacterial strains, *A. lowfi*, *B. pumilus*, and *Mesorihizobium* sp. showed good CPX degradation rate (95.4%), and achieved higher degradation efficiency than the single strains at 20 mg L^{-1} CPX concentration. Overall, the results suggest that using a combination of bacteria may be a useful tool for the remediation of antibiotic pollution. In addition, this study reveals the ability of previously isolated bacteria to biodegrade CPX as a sole source of carbon, providing new insights into the biodegradation of CPX.
Keywords: *antibiotics, ciprofloxacin-contaminated water, biodegradation, bacterial consortium, remediation*

Introduction

Environmental pollution is one of the most challenging and problematic issues of today. The rapid increase in the world population increases the demand for the world's scarce freshwater resources. Therefore, protecting our water resources and solving

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problems such as toxicity and pollution that can harm people and the environment in the long term has become an important issue (Naveed et al., 2024; Mohamed et al., 2023; Luo et al., 2014). Many micro-pollutants have recently been detected in water, including pharmaceutical compounds, industrial chemicals, personal care products, and various xenobiotic compounds. These pollutants have become a major problem in the world's oceans. Excessive use of antibiotics in humans and veterinary medicine increases their environmental occurrence. Antibiotics are found in surface water, groundwater, and drinking water (Mohamed et al., 2023; Tian et al., 2023; Maghsodian et al., 2022). Antibiotics are designed to resist biodegradation and work effectively at low doses. There has been increasing concern recently about the potential impact of synthetic pesticides that could disrupt important ecological processes supported by organisms, such as food production, the carbon and nitrogen cycle, and the decomposition of pollutants (Girardi et al., 2011; Mousavi et al., 2023; Mu et al., 2023).

Ciprofloxacin is a broad-spectrum antibiotic that affects its target by inhibiting the DNA gyrase, which is known as topoisomerase II and topoisomerase IV (Serizawa et al., 2010; Braetz et al., 2023). DNA gyrase contains subunits A and B. Quinolones such as ciprofloxacin are believed to prevent subunit A from resealing the DNA double-strand; therefore, single-stranded DNA may result in exonucleolytic degradation (Shariati et al., 2022). In most studies, the effect of ciprofloxacin on DNA gyrase has been emphasized; however, a previous investigation has suggested that ciprofloxacin could affect *Mycobacterium smegmatis* cell wall compounds (Shariati et al., 2022). Humans and animals only partially metabolize CPX. In humans, 45-62% of the CPX dose is excreted in urine and 15-25% in feces. It is frequently found in the environment and is genotoxic (Gauba and Saxena, 2023; Shah et al., 2022). Ciprofloxacin can enter the environment via antimicrobial-laced manure and easily make its way to the marine environment via wastewater disposal. Thus, wastewater treatment facilities are significant sources of antibiotic-resistant bacteria that are spread into aquatic environments (Shaker et al., 2022; Xu et al., 2023). As a result, pathogenic bacteria develop resistance to antibiotics, making existing antibiotics ineffective against infectious diseases. Additionally, the presence of antibiotics in wastewater can affect the microbiome of wastewater treatment plants, reducing wastewater treatment efficiency (Mutuku et al., 2022; Serwecińska, 2020).

There are many types of physical processes currently available for removing antibiotics from wastewater, including absorption, digestion, redox, photolysis, hydrolysis, reverse osmosis, and chemical degradation (Lofrano et al., 2017; Ding et al., 2016; Liyanage and Manage, 2018). However, many countries in the world, especially developing countries like Pakistan, cannot or do not have access to such technologies. Bioremediation is a green tool that uses bacteria to eliminate complex hazardous chemicals or make them less toxic or non-toxic. It is the most effective, efficient and environmentally friendly way to control pollution (Akram et al., 2023; Naveed et al., 2023; Khan et al., 2023; Bora et al., 2023; Ercoli et al., 2023; Bora et al., 2020). Microbial biodegradation in sewage sludge and aquatic environments has been reported to be an important method for removing antibiotics (Huang et al., 2021). In the case of CPX, little information is available on bacterial degradation "in situ" and "in vitro". The main aim of this study was to evaluate the ability of bacteria to biodegrade CPX at different concentrations and evaluate its effect on bacterial growth and biodegradation. Furthermore, CPX was chosen as a representative of the fluoroquinolone class because it is widely used as an antibiotic in many parts of the world.

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Materials and methods

Ciprofloxacin (CPX) tablets (250 mg) were purchased from a local pharmacy (Sami Pharmaceuticals, Private Limited, Karachi, Pakistan). Acetonitrile (ACN), methanol, and other HPLC-grade chemicals were provided by Sigma-Aldrich (Germany). Biodegradation of CPX was studied using three types of media: Luria Bertani (LB) medium, low-salt medium (MSM), and Mueller-Hinton medium). All chemicals and cultures media were obtained from Merck, Germany and Sigma-Aldrich, USA.

Bacterial strains

Five bacterial strains, *Burkholderia phytofirmans* PsJN (Sessitsch et al., 2005), *Acenitobacter sp.* CYRH21 (Fatima et al., 2015), *Acenitobacter lwofii* ACRH76 (Fatima et al., 2015), *Bacillus pumilus* C2A1 (Anwar et al., 2009), and *Mesorihizobium sp.* HN3 (Jabeen et al., 2015) were used in this study (*Table 1*). All strains were cultured in LB medium at 37°C and 120 rpm for 24 h. Bacteria were centrifuged at 5000 g for 10 min. To obtain the correct pellets, the washed pellet was suspended in sterile saline (0.90% NaCl) to obtain the desired count as described previously (Sutton, 2011).

Table 1. *Bacterial strains used to study CPX-biodegradation*

IGS type	Bacterial strain's	Niche	References
Ps.JN	*Burkholderia phytofirmans*	Endophyte	Sessitsch et al., 2005
CYRH21	*Acenitobacter sp.*	Rhizosphere	Fatima et al., 2015
ACRH76	*Acenitobacter lwofii*	Rhizosphere	Fatima et al., 2015
C2A1	*Bacillus pumilus*	Endophyte	Anwar et al., 2009
HN3	*Mesorihizobium sp.*	Endophyte	Jabeen et al., 2015

Spread plate method

In-vitro screening of bacterial strains for their CPX-tolerance potential was determined using the spread plate method (Aryal, 2017; Herigstad et al., 2001; Ghaly, 2024; Tsai et al., 2024). In vitro screening of bacteria for CPX tolerance using the plating method. A sequential method was used to measure bacterial survival in the presence of concentrations of CPX on LB agar plates. Using the plating method, 100 µl inoculum of each bacteria was plated on LB media plates containing different concentrations of CPX (20, 40, 60, 80, and 100 mg L−1). All plates were placed in the oven at 37 °C. After 24 h of incubation, check the plates for bacterial growth.

Disc diffusion method

The sensitivity of bacteria to CPX was further confirmed by the Kirby-Bauer disc diffusion method (Hudzicki, 2009). First, streaked sterile soft swabs of strains PsJN, CYRH21, ACRH76, C2A1, and HN3 onto MH agar plates. Allowed the inoculant to dry for 2-3 min. Placed two sterile dishes in the middle, top and bottom of the Petri dish. Used a sterile micropipette to pour 5 µl of CPX solution at different concentrations (e.g. 20, 40, 60, 80 and 100 mg L^{-1}) onto each disk of the MH agar plate and let it sit for 2-3 min to allow it to absorb into the surface. All the Petri plates were placed for incubation (37°C) and the zone of inhibition after 24 h.

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Screening of potent bacterial strains for CPX biodegradation

The CPX degradation capacity of bacterial cells was determined using a previously described method (Alan and Technology, 2019). Briefly, liquid MSM with CPX concentrations of 5, 10, and 20 mg L^{-1} was used as a carbon source and energy for bacterial growth. A suspension containing 10 mL (10^9 cells mL) of each bacterium was poured in 200 mL MSM and kept on a shaker at 35°C and 150 rpm for 12 days. Samples were taken every 4 days of incubation in a sterile falcon tube. Determination of CPX residues in water using high-performance liquid chromatography (HPLC). The optical quality of the collected samples was recorded using a spectrophotometer at 600 nm to measure bacterial growth. Moreover, the suspended bacteria spread onto LB agar plates, confirming that all bacteria in the culture medium survived.

Cross-streak test between co-inoculated strains

All strains were grown on LB agar at 37°C for 24 h. All strains were then plated vertically and at right angles to each other on a freshly prepared LB agar plate and allowed to grow for 24 h. The second colony was placed outside at a 90° angle to the first colony and cultured for 24 h to grow. Observed colony counts and zones of inhibition.

Development of CPX-biodegrading bacterial consortium

Three strains (*Acenitobacter lwofii*. ACRH76, *Bacillus pumilus* C2A1, and *Mesorihizobium* sp. HN3) showing the maximum CPX degradation capacity were selected for bacterial consortium development. The strains were mixed in a proportion of 1:1:1 to form a bacterial consortium (CMAB) (Sutton, 2011).

Analysis of residual of CPX in water

The amount of CPX in water was estimated according to a previously described method (Gezahegn et al., 2019). Briefly, CPX was extracted from aqueous solution using acetonitrile (ACN). Acetonitrile extracts were analyzed using Perkin Elmer HPLC (Germany). A double elution system is used to separate molecules by moving water and acetonitrile through a C18 column. This process acidifies the molecules with 2% phosphoric acid to make them more mobile. The system operates at a flow rate of 0.8 mL/min. Detection using a 275 nm diode array detector adding a sample volume of 15 µl. The temperature was set at 30°C.

Data analysis

Microsoft Excel 2016 was used for all statistical analysis. The mean value ($n = 3$) and their standard errors were calculated for each treatment. Analysis of variance was used to evaluate the treatments, followed by a post-hoc Tukey test ($p \leq 0.05$).

Results and discussion

Screening of CPX-resistant bacterial strains on LB and MH media

All strains tested grew on LB agar plates at all concentrations between 20 and 100 mg L^{-1} CPX (*Fig. 1*). The Kirby-Bauer disk diffusion method was also used to confirm the growth of selected bacteria in the presence of CPX (*Fig. 2*). Resistance to

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the antibiotic CPX is associated with chromosomal changes that affect the synthesis of important enzymes such as DNA gyrase subunits (GyrA and ParC) and topoisomerase IV, leading to reduced synthesis of the drug into the enzyme DNA complex and plasmid-mediated resistance to gene modified bacteria (Hooper and Jacoby, 2016). Other mutations also occur in genes that cause efflux pumps in bacteria (Brar et al., 2020; Jacoby, 2005). We were not able to investigate specific resistance mechanisms in this study; Conversely, we found that increasing concentrations of antibiotics in different media did not inhibit the growth of selected bacteria.

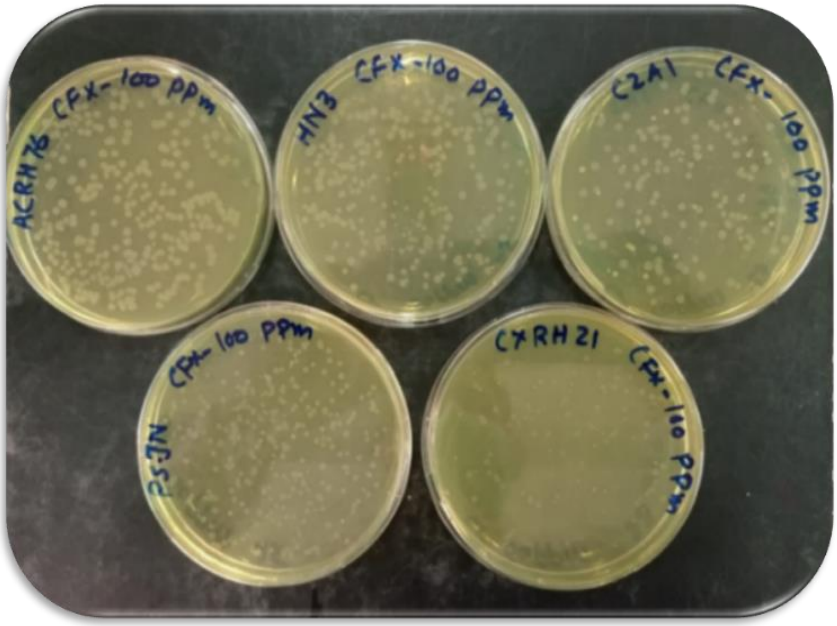

Figure 1. *The growth of five bacterial strains on LB agar plates containing CPX (100 mg L^{-1})*

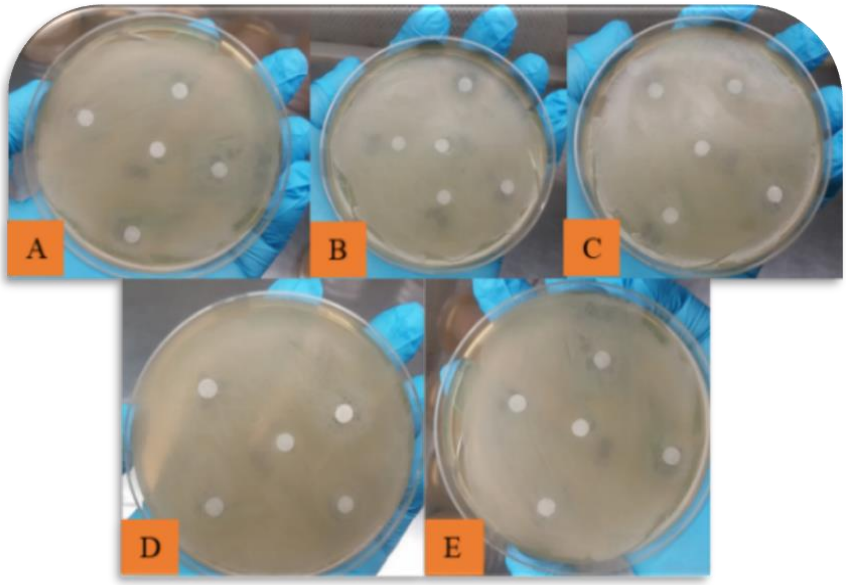

Figure 2. *CPX (100 mg L^{-1}) antibiotic sensitivity test. A76 (A), HN3 (B), C2A1 (C), Ps JN (D), and CYRH21 (E)*

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Screening of CPX-resistant bacterial strains in MSM

All of the bacteria used in this study are capable of degrading CPX. *B. pumilus* strain showed higher initial degradation (75.53%), followed by *Acenitobacter lwofii* (72.3%), *Mesorihizobium sp.* followed (70.32%), *Burkholderia* (67.10%) and *Acinetobacter* (67.10%). (63.2%) at low CPX concentration (5 mg L^{-1}) (*Fig. 3A*). However, Mesorihizobium sp. (HN3) showed better activity (76.32%) at higher CPX (10 mg L^{-1}) as shown in *Figure 3B*. Only 10% of the CPX removed from the flasks in the absence of bacterial inoculation (Control). Natural decay (distortion) of CPX time may be responsible for this. The degradation efficiencies of the bacterial strains were slightly different at these concentrations. This may be because some bacteria digest antibiotics faster than others (Goh et al., 2002; Engin et al., 2023; Rütten et al., 2022).

Figure 3. *Compatibility test*

Development of CPX-biodegrading bacterial consortium

The relationship between selected bacteria was determined as shown in *Figure 4*. In the shake flask study, bacteria tested individually and in combination for the degradation of CPX in MSM (*Fig. 5*). In our study, the combined strains of the three strains showed better CPX degradation ability (95.4%) and achieved more degradation than the single strain. This indicates that the use of mixed bacteria is better for the biodegradation of CPX. Many previous studies have shown that microbial cooperation can lead to the removal of organic pollutants from water (Rusch et al., 2019). According to Liao et al. (2016), Mixed bacteria showed greater removal of CPX from wastewater compared to single bacteria. Similarly, other studies have reported that bacterial communities disrupt CPX more than individual strains (Jia et al., 2018). Cleavage of isoxazole and piperazinyl rings by sulfite reductase and cytochrome P450 (CYP450) enzymes is expected to result in biodegradation of CPX (Jia et al., 2019). Analysis of degradation intermediates by HPLC and LC/MS showed that 100% of CPX could be removed from water due to complete microbial degradation (Li et al., 2021).

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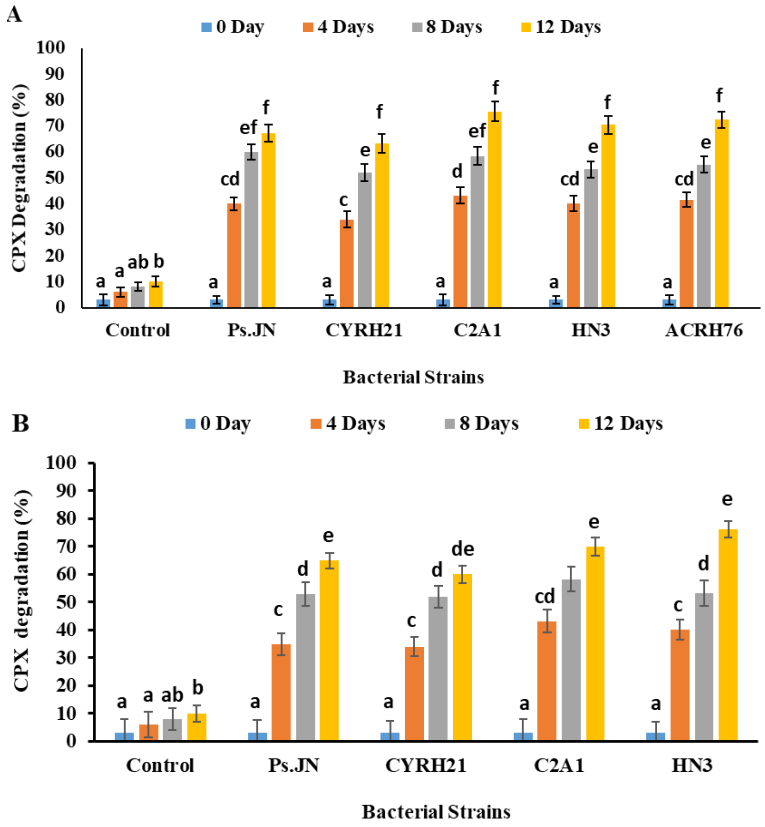

Figure 4. *Degradation (%) of CPX by individual bacterial strains in MSM having 5 mg L^{-1} (A), and 10 mg L^{-1} (B), CPX after 4, 8, and 12 days of incubation period. The bacterial strains, PsJN (Burkholderia phytofirmans), CYRH21 (Acenitobacter sp.), ACRH76 (Acenitobacter lwofii), C2A1 (Bacillus pumilus), and HN3 (Mesorihizobium sp.), used individually. Each value is the mean of three replicates, and the error bars represent the standard deviation. Means followed by the same letters in columns are not significantly different ($p < 0.05$)*

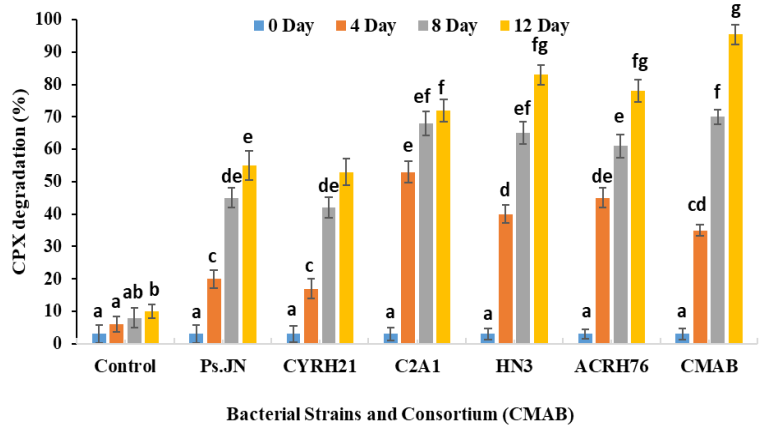

Figure 5. *Degradation (%) of CPX by individual bacterial strains and consortium (CMAB) in MSM having 20 mg L^{-1}, CPX after 4, 8, and 12 days of incubation period. The bacterial strains, PsJN (Burkholderia phytofirmans), CYRH21 (Acenitobacter sp.), ACRH76 (Acenitobacter lwofii), C2A1 (Bacillus pumilus), and HN3 (Mesorihizobium sp.), used individually and in consortium. Each value is the mean of three replicates, and the error bars represent the standard deviation. Means followed by the same letters in columns are not significantly different ($p < 0.05$)*

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Conclusions

Ciprofloxacin, a member of the third-generation antibacterial quinolones, is widely used in the treatment of infections and diseases caused by Gram-negative and Gram-positive bacteria. The study examined that the consortium of three bacterial strains, *A. lowfi*, *B. pumilus*, and *Mesorihizobium sp*., was found more effective in degrading CPX (95.4%) than the single strains. Therefore, these three bacteria are the best candidates to be added to wastewater to remove CPX before it enters the natural environment.

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