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RESEARCH ARTICLE

Screening of Antibiotics Biodegradability from Wastewater

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ABSTRACT

Objective: One of the sources of environment antibiotics contamination is wastewater treatment plants (WWTPs), there by constituting a global public health risk. This present study aimed to investigate the biodegradability of antibiotics and antiseptics and highlights the biodegradation of Ciprofloxacin as a sole carbon source by a bacterium isolated from the sludge "El Kouwaer," WWTP located in Mascara.

Materials and Methods: In the present study, biodegradability of some antibiotics and antiseptic were tested at 50 mg/l concentration through active sludge microorganisms by Manometric Respirometry Method (OECD 301F). Further analysis of 16S rRNA gene sequencing used to identify MK4 strain isolated from the sludge. Furthermore, ATR-FTIR spectroscopy was conducted in order to identify its biodegradation in the presence of different carbon sources and LCMS/MS spectrometry were used to identify the metabolite degradation.

Results: Our Results revealed that four antibiotics tested were readily biodegradable (60%) as Ciprofloxacin, Doxycycline, Amoxicillin, Ampicillin, and Penicillin. Conversely, other was not readily biodegradable, such as Azithromycin (36.11%), Cephalexin (36.20%), and Metronidazole (33.33%). Meanwhile, the remaining antibiotics under examination were degraded, with Sulfamethoxazole (25.75%), Clarithromycin (25.36%), and Nifuroxazide (16.33%) recording degradation. Ciprofloxacin was chosen to represent the most biodegraded antibiotic. Based on 16S rRNA gene, MK4 strain was related to *Klebsiella oxytoca* (99.99%). ATR-FTIR revealed that the strain *K. oxytoca* MK4 caused changes in the structure of the Ciprofloxacin, in the presence of various sources of carbon, with varying effects on bacterial growth and biodegradation.

Conclusion: In this study, the identified strain *K. oxytoca* MK4 facilitated the degradation of Ciprofloxacin.

Keywords: Wastewater, antibiotic, antiseptics, biodegradability, ATR-FTIR spectroscopy, LCMS/MS metabolites.

INTRODUCTION

Antibiotics represent a widely-used pharmaceutical product, used for treating humans.[1] The metabolism of antibiotics in the human body is slight and they are excreted through urine and feces. Then, they are transmitted to sewage treatment plants where there is no effective removal by conventional biological treatments, and they reach the aquatic environment as emerging and persistent micro-pollutants processes.[2] Similar to penicillins, some antibiotics have rapid degradation, while others, such as Ciprofloxacin (CIP), tylosin, and tetracyclines, exhibit different degradation rates or behaviors. CIP and oxolinic acid are known to be relatively persistent in water.[3] Thus, it seems that wastewater treatment plants are major reservoirs of antibiotic-resistant bacteria which are transmitted into aquatic environments.[4] The European Water Framework Directive included many antibiotic groups in the "watch-list".[5] The challenge is to remove antibiotics from wastewater treatment plants (WWTPs), as documented in multiple studies.[6] As a result, significant parts of these substances tend to accumulate in active sludge[7], particularly in systems with anaerobic digestion where they have a lack treatment.[8] Among the commonly used antibiotics, amoxicillin, trimethoprim, and CIP are frequently detected at high levels in both soil and water environments.[9] Studies and data have indicated that biodegradation[10] is among the range of methods that have been proven effective in eliminating various types of antibiotics in WWTPs.[10] The removal of antibiotics by microbial biodegradation in sewage sludge and aquatic environ-

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ments was reported to be an important elimination process.[11] During sludge treatment, between 0-40% of CIP by biodegradation was found.[12,13] Unfortunately, a significant quantity of CIP remained present in the digested sludge found in wastewater treatment plants, indicating its persistence.[14] The microbial community structure involved in the degradation of CIP has not been reported yet.[3,15]

The biodegradability test is a screening method used to evaluate the biodegradability or mineralization potential of chemicals based on predefined criteria.[16] The focus of this work was to examine the biodegradability of some antibiotic compounds to predict their fate in biological systems treating wastewater using the OECD 310 guideline. The assessment of the biodegradability of specific antibiotics and antiseptics was conducted. In 28-day manometric respirometry tests, the percentages of biodegradation of the targeted compound (CIP) were computed. CIP was selected as a representative of flouroquinolones due to its extensive usage as an antibiotic in most regions across the globe. The main goals of the present study were to isolate and identify the predominant indigenous bacterial cultures from effluent wastewater, to evaluate their ability to biodegrade CIP in the presence of various carbon sources, and to assess their impact on bacterial growth and biodegradation. Additionally, the degradation pathways were analyzed by utilizing the Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) technique to determine structural changes. Additionally, the objective of the present study is to identify the metabolites or transformation products that are generated during the biodegradation process.

MATERIALS AND METHODS

Sample Collection

The wastewater treatment plant "El Kouwaer", the subject of the current study, is located 3.2 km from the Mascara department and 361 km from Algiers in the Kouwaer area (35°23'02.6"N 0°08'36.4" E). Two kinds of samples (wastewater influent and effluent) were taken every month from June 2019 to May 2020. Also, for the biodegradability test, a total of nine samples of activated sludge were collected from the aeration tank of a WWTP. During the same day, the samples collected were transported to the laboratory using sterile bottles at 4°C and analyzed.[17]

Preparation of Activated Sludge Samples

Samples of activated sludge underwent some preparations such as keeping them to settle for 1 h until the supernatant was drawn off. Then, the activated sludge samples were subjected to a series of steps, including washing once, centrifugation, and resuspension in Mineral salt medium MMSM.[18]

Physico-Chemical Analysis of Wastewater Samples

Physico-chemical parameters of wastewater samples were measured for pH, temperatures (air and water temperatures) and dissolved oxygen, and also, pollution indicators: Biological oxygen demand (BOD_5) and Chemical oxygen demand (COD).[19,20] The BOD_5/COD ratio was calculated. Results were compared with those obtained in Algeria.[19]

Biodegradability Evaluation

Manometric Respirometry Test

A Manometric respirometry test (MRT, OECD 301F) was performed using 300 ml of sterile mineral medium[18] containing each of the tested compounds (10 antibiotics and 01 antiseptic) and inoculated with 0.5 ml of inoculum (activated sludge) and 20 drops per liter of nitrification inhibitor N- *allylthiourea* used to selectively inhibit nitritation and nitratation bacteria. The mixtures formed were kept in sterile BOD bottles at 30°C±1 during 28 days under constant agitation 150 tr/min.[18] Anutomatic analyzer (System OxiTop® OC100, Germany) was used daily to measure the aerobic biodegradation.[21]

BOD_{28} Determination

BOD after 28 days was calculated according to OECD (1992) guidelines. Mean replicate values for each batch were calculated daily from collected data (equation 1).

$$\text{BOD} = \frac{\text{O2 mg/L substance absorption} - \text{O2 mg/l blanc absorption}}{\text{mg/L test substance}}$$

mg/l..... (1)

Percentage of Biodegradation

Percentage of biodegradation of 10 antibiotics and 01 antiseptic was determined by measuring the quantity of oxygen consumed by the inoculums used. This value was adjusted by deducting the oxygen consumption of the blank, and expressed as percentage of the theoretical oxygen demand (ThOD) as shown in equation 2.[18]

$$\text{Biodegradation (\%)} = \left[\frac{DBO}{ThOD}\right] x100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where: BOD: Biochemical Oxygen Demand of the compound (mg/l) after 14 days or 28 days. ThOD: Theoretical Oxygen Demand required to completely oxidize the compound (mg/L)

Theoretical Oxygen Demand (ThOD) was calculated at the end of the experiment according to equation 3 (OECD, 1992).

$$\text{ThOD}=\frac{16\left[2c+0.5(h-cl-3n)+3s+\left(\frac{5}{2}\right)p+0.5na-\ \right]mg/mg}{\text{MW}} \quad \ldots\ldots\ldots (3)$$

In terms of biodegradation of the test compounds, OECD (18) stipulates that for a compound to be classified as having passed for ready biodegradability, it should have attained 60% of ThOD, within a period of 10 days after oxygen consumption reached 10%.

Further, CIP was selected to represent the most widely used and mostly biodegraded. Thus, it was chosen for the isolation and identification of predominant indigenous bacterial cultures from effluent wastewater able to degrade CIP.

Ciprofloxacin Degradion Bacteria

Isolation

The process used for acclimating and enriching microorganisms was the same as described in the studies of Liyanage & Manage.[22,23] In an Erlenmeyer flask, a mixture was prepared containing 50 ml of sample (activated sludge) and CIP at a final concentration of 60 mg/l. Then, the volume was finalized with sterile water up to 100 ml, and incubated at 28 °C±1/14 days under agitation at 100 rpm. After that, resistant bacteria were inoculated in a Luira Broh medium containing 60 mg/l of CIP to isolate, and the medium was incubated at 28°C/3 days.[22,23] The enriched culture was plated onto Mineral salt medium agar plates supplemented with 20 mg/l of CIP. The isolated colonies that grew on the plates were purified and assessed for their capability to degrade CIP. Among them, a specific strain named MK4 exhibited the ability to degrade CIP, and was chosen for subsequent investigation.

Molecular Identification of Ciprofloxacin Resistance Bacteria

The Bacterial Genome DNA Extraction Kit (Nucleospin de Macherey-Nagelen) was used to extract Genomic DNA. The 16S rRNA amplified fragment of approximately 1465 bases using two primers 27 F/1492 R; forward (5'AGAGTTTGATCCTGGCTCAG-3') and reverse (5'TACGGYTACCTTGTTACGACTT-3').[24] DNA sequences were analyzed with the Basic Local Alignment Search Tool at the National Center for Biotechnology Information website (NCBI, http: //www. ncbinlm. nih. go v/).[25] The phylogenetic tree was constructed using the MEGA 11 software.[26]

The optimal tree is shown using the Neighbor-Joining method inferred the evolutionary history.[27] The bootstrap test, with a specified number of replicates (in this case, 100), is used to assess the robustness of the phylogenetic tree.[28] Using the Maximum Composite Likelihood method, the evolutionary distances were computed.[29] All positions with less than 5% site coverage were eliminated, i.e., fewer than 95% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1467 positions in the final dataset.

Ciproflaxin Biodegradation Test

In order to test effect ciproflaxin concentration on biodegradation, resistant bacteria were transferred to 5 ml of Luira Broh broth supplemented with CIP at different concentrations (5 mg/l, 2.5 mg/l, 1.25 mg/l, 0.625 mg/l, 0.312 mg/l, 0.15mg/l) and incubated at 28°C/24 h. Then, centrifugation was performed and turbidity was adjusted to (OD=0.35) at a wavelength of 590 nm.[23] 150 µl of bacterial suspension was inoculated into microplate and incubated at 28°C. Absorbance was measured by ELISA microplate at 595 nm during 0, 24 and 48 experimental hours.[23]

Effect of Organic Substrate on Growth and Biodegradability

In order to determine best organic substrate affecting the most biodegraded antibiotic by strain MK4 biodegradability of the antibiotic at a concentration of 5 mg/ml exposed to different carbone sources (glucose, sodium acetate, and sucrose) was realized in erlenmeyer flasks containing 50 ml of MMSM medium and inoculated with bacterial suspension (3.0%), and incubated at 30 °C. The effect of sodium acetate was used as control. Bacterial growth and biodegrability were measured at the different specific time points (0, 24, 48 h).[30]

ATR- FTIR Analysis

After 14 days of incubation period, ATR- FTIR spectral was used to analyze the structural properties of degradation product in each sample.[31] The sample containing CIP, strain MK4 and carbone sources (glucose, sodium acetate and sucrose) prepared as described previously was analyzed by ATR-FTIR technique. A scanning range from 400 to 4000 nm was performed using the ATR-FTIR instrument (Cary 63). The obtained analytical spectrum was compared to a library of reference spectra to identify the specific functional groups present in the samples.

Potential Intermediates of Ciprofloxacin Biodegradation

The approach described by author Pan et al.[32] was used for the samples collected from the biodegradation experiment. Ultra-performance liquid chromatography tandem mass spectrometry with a MS QQQ Mass spectrometer was utilized to analyze the intermediate metabolites from CIP biodegradation. Prior to conducting chemical analysis, liquid samples underwent filtration using a 0.22-μm nylon membrane. The LC/MS/MS analysis was performed using 6400 Series Triple Quadrupole with an electrospray ionization source employed for the identification of CIP and its biodegradation intermediates. A Column Comp

(G1316A) C18 column (150 mm×2.1 mm, 3 μm) was utilized, and the mobile phase consisted of solvent A (ultrapure water with acetonitrile). Samples were injected with a volume of 50 μl, and the column oven temperature was maintained 0.8°C.

Statistical Analysis

In this study, the significant difference ($P<0.05$) in the CIP biodegradation and growth bacteria was determined using analysis of variance (ANOVA) to check the significant difference ($P<0.05$) in the CIP biodegradation under the conditions of different nutrients.

RESULTS

Physico-Chemical Analysis of Wastewater Samples

Physical parameters including temperature (air and water)$_2$, pH, and oxygen dissolved of the influent and effluent wastewater samples were recorded (Figure 1).

Temperature

Results of analysis conducted on water samples showed that wastewater temperature was close to ambient temperature representing significant values ranging between (T=8.79°C) and (T=17°C). However, unregistered air temperature values varied from (T=13°C) and (T=23°C).

pH

A range pH value of influent water samples was between 7.5 and 10.22. Likewise, pH of enfluent average 7.91 and 10.25. Those measures were close to neutral or alcalin pH.

Oxygen

Monthly change of oxygen in wastewater (influent and effluent) showed the presence of significant amounts, varying between 0.30 mg/l to 4.19 mg/l. There was a trend between atmospheric temperature and microbial oxygen uptake; experiments received in summer showed highest value uptake of 4.19 mg/l, however, the lowest value of 2.98 mg/l correspond to February 2020, which was the winter season.

Pollution Indicators

Chemical Oxygen Demand

COD values unregistered of influent wastewater ranged from 175 mg O_2/l to 1480 mg O_2/l (Figure 2). On the other hand, COD of effluent wastewater recorded were much lower; they varied between 56 mg O2/l and 368 mg O_2/l

Biological Oxygen Demand

BOD_5 values of influent samples varied between 150 mg/l to 900 mg/l (Figure 2). Whereas, reduction in BOD_5 content of effluent wastewater was observed ranging between 5 mg O_2/l and 65 mg O_2/l.

BOD_5/COD Ratio

To characterize the nature of the effluent, the ratio of BOD_5/COD was used (Figure 3). COD and BOD_5 are parametres to quantification of the biodegradable or non-biodegradable polluting load of the inoculum. The ratio BOD_5/COD used as an index of biodegradability in water, as it gives an initial estimate of the biodegradability of the material organic from our effluents. The BOD_5/COD ratio value was between 2 and 3 for the samples taken, which indicates that it is moderately loaded with biodegradable organic matter. But, the BOD_5/COD ratio of some sample was less than 2, which indicates that the effluent this time is lightly loaded with inorganic matter.

Biodegradability Evaluation

BOD_{28} Determination

All substances tested showed varied BOD values during 28 days (Figure 4). BOD_{28} values for different antibiotic compounds varied from the lowest 3.1 mg/L for nifuroxazide mg/l, whereas, the highest registered value 186.34 mg/l for CIP before 10 days, followed by penicillin and doxycillin after 14 days. The BOD_{28} values for the negative control (inoculum in mineral medium) recorded was between 1.24 mg/l to 6.2 mg/l. The BOD_{28} value for the positive control batch (sodium acetate in mineral medium) unregistered was between 39.06 mg/l to 57.66 mg/l.

Percentage of Biodegradation

Percentage of biodegradation revealed that some antibiotic compounds were highly biodegraded within 14 days of incubation. Then, biodegradation increased after 28 days. While, for other antibiotic substances, the highest biodegradation was achieved within 28 days (Figure 5). All the experiments demonstrated the percentage degradation of the reference compound (Sodium acetate) at a concentration of 50 mg/l, ranging from 82.05% within 14 days to as high as 189.75% within 28 days. These results indicate that the reference compound achieved ready biodegradation after 28 days. Out of following 11 antibiotics, three were considered as readily biodegradable (biodegradability above the threshold of 60%) including ciprofoloxacin (138.33%), doxycillin (66.66%), and penicillin (54.23%) at 14 days. Other antibiotics, such as cephalexin (36.20%), azithromycin (36.11%), metronidazol (33.33%), (25.75%) clarithromycin (25.36%), and nifruxazid (16.36%) were moderate or low-degradable.

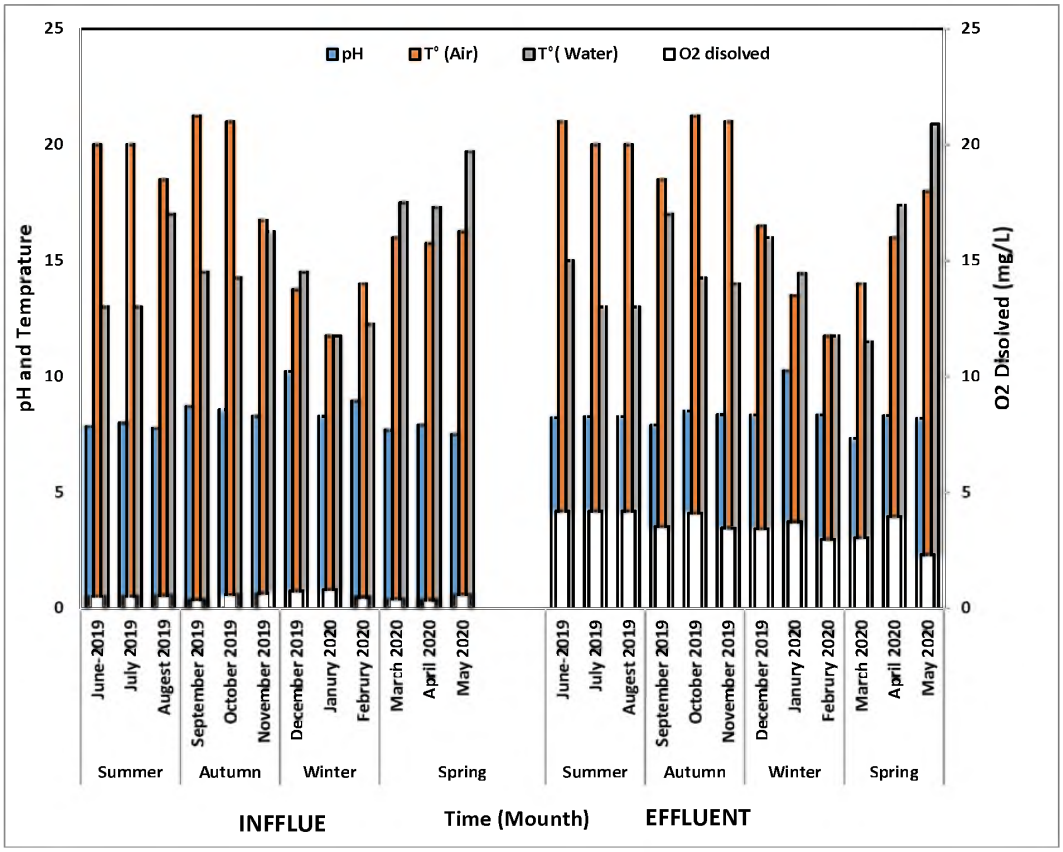

Figure 1. Physico-chemical parameters of wastewater samples.

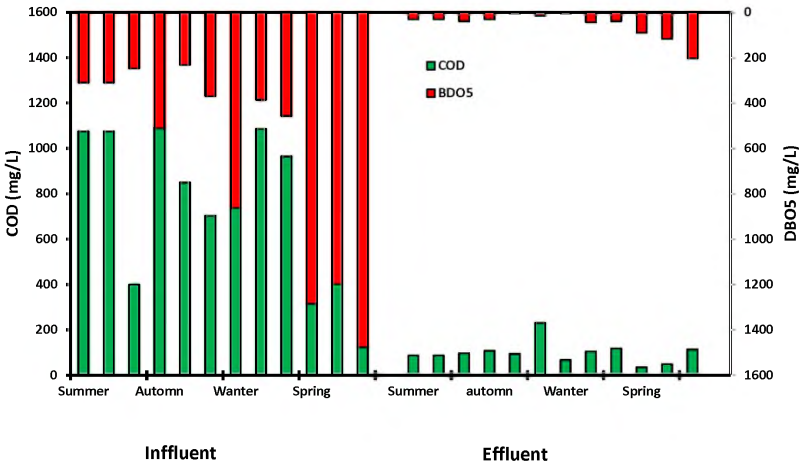

Figure 2. Pollution parameters of wastewater samples.

Ciprofloxacin Degrading Bacteria

Identification

Morphological analysis revealed that the MK4 strain (MK474159.1) was Gram-negative and rod shaped (Figures 6B and C), forming on a GN medium incubated at 30°C/24 h. Phylogenetic analysis of 16S rRNA gene sequence indicated that it belongs to the *Klebsiella oxytoca* species. The MK4 strain showed a high level of homology (99% of similarity) at 85% with the *K. oxytoca* HE650838 and *K. oxytoca* AY150697

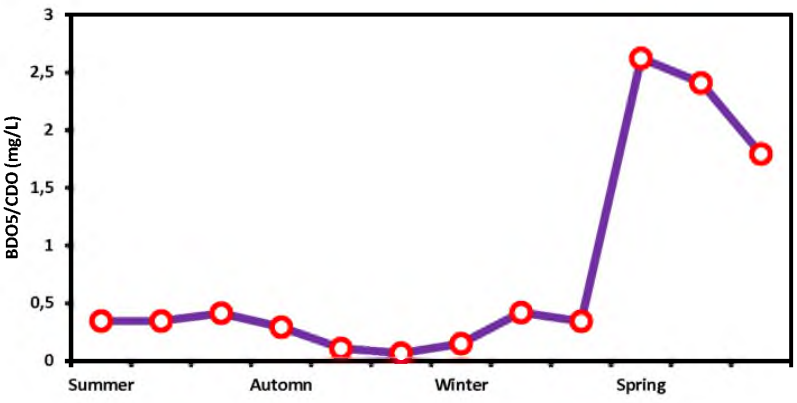

Figure 3. BOD_5/COD ratio of wastewater samples.

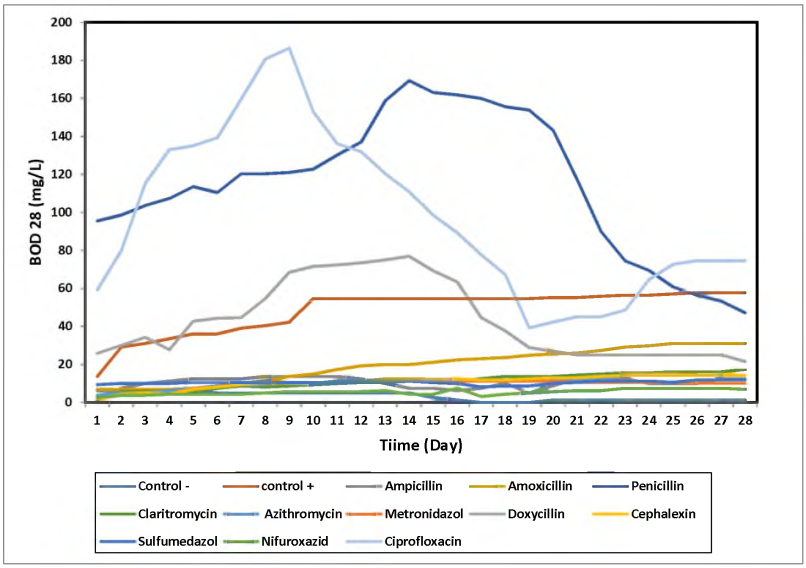

Figure 4. BOD_{28} of antibiotic compounds analysis.

strains. A detailed phylogenetic analysis of the strain's placement is illustrated in Figure 6A.

Effect of Ciproflaxin Concentration on Biodegradation

In this part of study, the MK4 strain was adopted as a model bacterium to assess the biodegradation of CIP. Figure 7 illustrates the growth curves of the MK4 strain in various concentrations of CIP. In the control group (without CIP), the growth curve displayed a decline due to the absence of carbon and energy sources in the medium. Growth of the MK4 strain in different concentrations of CIP showed that maximal growth was observed within 24 h; then, growth decrease at the end of 48 h when the CIP concentration was less than C3=1.25 mg/l. Higher than C4=0.625 mg/l, the maximal bacterial growth was observed at 24 h, then decreased at the end of incubation at 48 h. Growth of strain MK4 in the control group, showed no variation at 24 and 48 h (Figure 8). The highest growth of the MK4 strain was observed at concentrations C5, C4 and C6 during 24 h, showing optical densities of 3.711; 3.302; 2.967, respectively.

Effect of Organic Substrate on Growth and Biodegradability

In order to determine the best organic substrate for CIP degradation by MK4 strain, different carbon sources were tested on bacterial growth. Growth of the MK4 strain was significantly increased in the presence of the three carbon sources tested including glucose sodium acetate, and sucrose (Figure 9). The highest bacterial growth was observed for sucrose (OD=0.5248) at 24 h, then decreased at 48 h, rep-

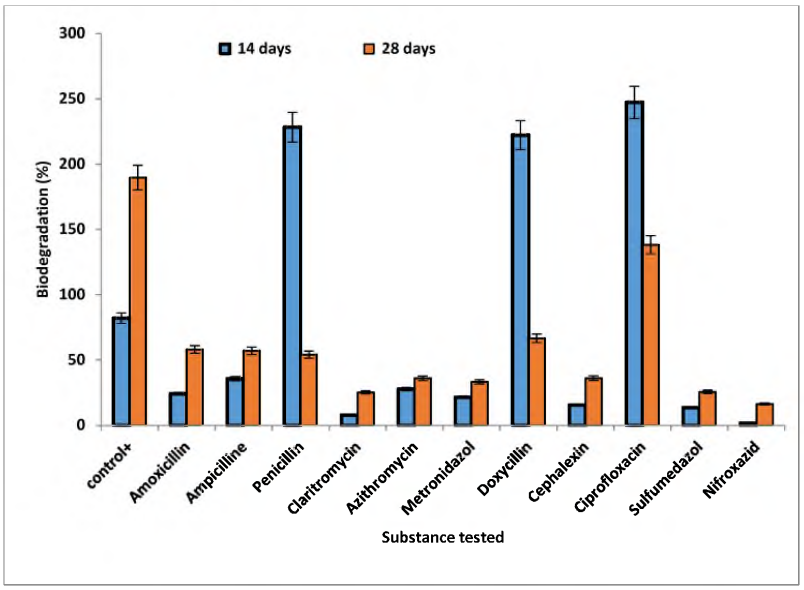

Figure 5. Percentage of biodegradation (%) of antibiotic substances.

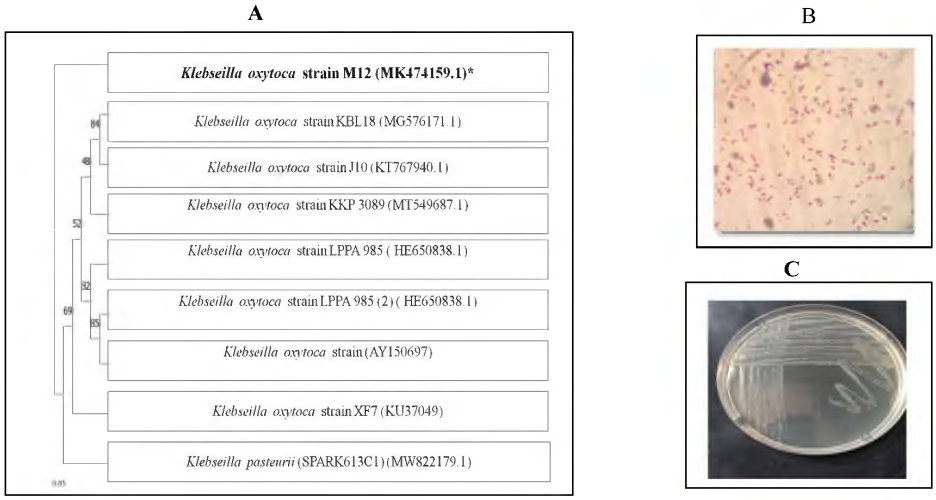

Figure 6. Macroscopic, microscopic and phylogenetic analysis by neighbor-joining method based on 16S rRNA gene sequence of MK4 strain and related strains (A: phylogenetic tree MK4; B: Microscopic characteristics of MK4 strain; C: Macroscopic characteristics of MK4 strain).

resenting OD=0.162. For glucose and acetate, respectively, growth increased from OD=0.2961 and OD= 0.207 at 24, to OD=0.2869and OD=0.3907 at 48 h. In these conditions, comparable growth of the MK4 strain was observed in the cultures that were fed with CIP and carbone sources, with no significant difference ($p>0.05$) between them.

ATR-FTIR Analysis

All spectra of (CIP+Glu) at T=0 h, T=24 h, and T=48 h in (Figure 9) showed the same peaks indicating the presence of same function. Function O-H is visible approximately at 311 cm^{-1}, 2729 cm^{-1}, 2509 cm^{-1} and a peak at 2346 cm^{-1} is characteristic of the C≡N nitrile group. Additionally, a peak at 1704 cm^{-1} is characteristic of the C=O ketone group, indicating a change of the pyridazine and quinolone rings.

Infrared spectra of CIP samples revealed different functions of substances produced in the presence of various carbone sources: glucose (GLU), sucrose (sac) and sodium acetate(acetate), at T=0, T=24 h, T=48 h (Figure 9). Comparison result of samples (CIP+acetate) at T0 and (CIP+sac) at T0, (CIP+ sac) at T24h (Figure 9) with standard spectrum revealed same peaks at 3267 cm^{-1}, indicating the presence of (O-H) hydroxyl in carboxyl group, and vibrations absorption at 1634

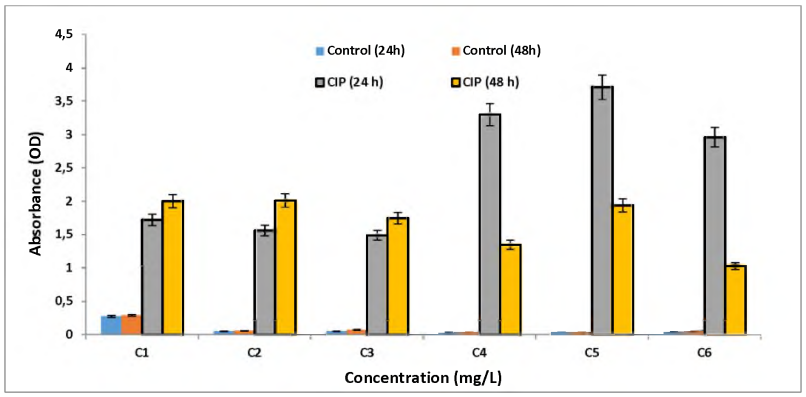

Figure 7. Impact of ciprofloxacin concentration on growth of the MK4 strain.

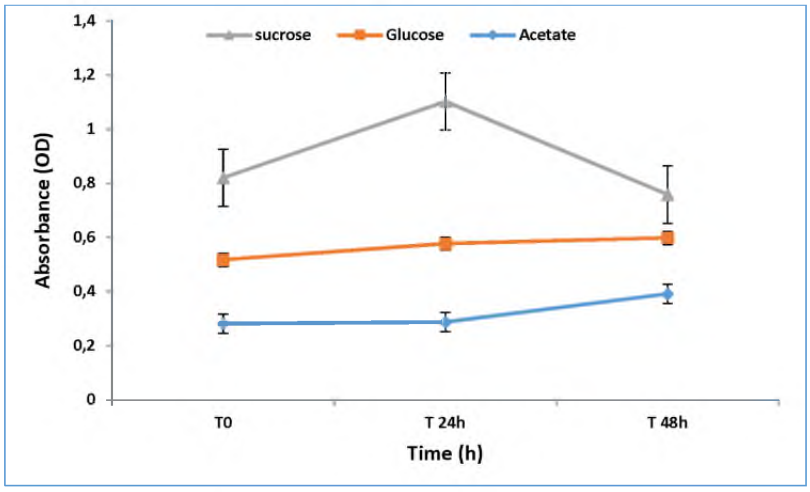

Figure 8. Impact of ciprofloxacin concentration on growth of the MK4 strain.

cm^{-1} and 2111 cm^{-1} were attributed to C=C function while peaks were 1075 cm^{-1} and 1184 cm^{-1} are characteristic of the C-F stretching function. Results analysis of (CIP+ acetate) at T24 and T48h, (Figure 9 a), showed that all peaks disappear while other peaks at 2921 cm^{-1}, 1396 cm^{-1} and 1075 cm^{-1} were detected. Peaks of the spectrum (CIP+ sac) at 48 h in the (Figure 9 b) revealed the presence of band at 2172 cm $^{-1}$, indicating the stretching of $-H$ in alkynes, while a peak at 1615 cm^{-1} is indicative of symmetric stretching of C-C=C in alkanes, which is a characteristic feature of this functional group.

All spectra of (CIP+Glu) at T=0 h, T=24 h, and T=48 h in (Figure 9c) showed the same peaks indicating the presence of same function. Function O-H is visible approximately at 3112 cm^{-1}, 2729 cm^{-1}, 2509 cm^{-1} and a peak at 2346 cm^{-1} is characteristic of the C≡N nitrile group. Additionally, a peak at 1704 cm^{-1} is characteristic of the C=O ketone group, indicating a change of the pyridazine and quinolone rings.

Taken together, current findings suggest that the MK4 strain resulted in the decomposition and conversion of the aforementioned groups or components of CIP. At T=0 h and T=24 h, the ATR-FTIR spectra obtained with sodium acetate and sucrose had some degree of similarity, indicating the presence of the same function groups. After T=48 h, the ATR-FTIR spectra of the three carbone sources (sucrose, glucose and sodium acetate) were different, meaning the presence of variable functions.

Potential Intermediates of Ciprofloxacin Biodegradation

After 14 days of acclimatization of the sample added microbiota, LC/MS/MS analysis was employed to identify the biodegradation products of CIP. The major intermediate compound was produced by the MK4 strain during the degradation, which had been cultivated with CIP for 14 days. In contrast, the absence of any peaks in the negative control (without microorganisms) corresponds to the absence of these particular metabolites. The suggested configurations of CIP intermediates

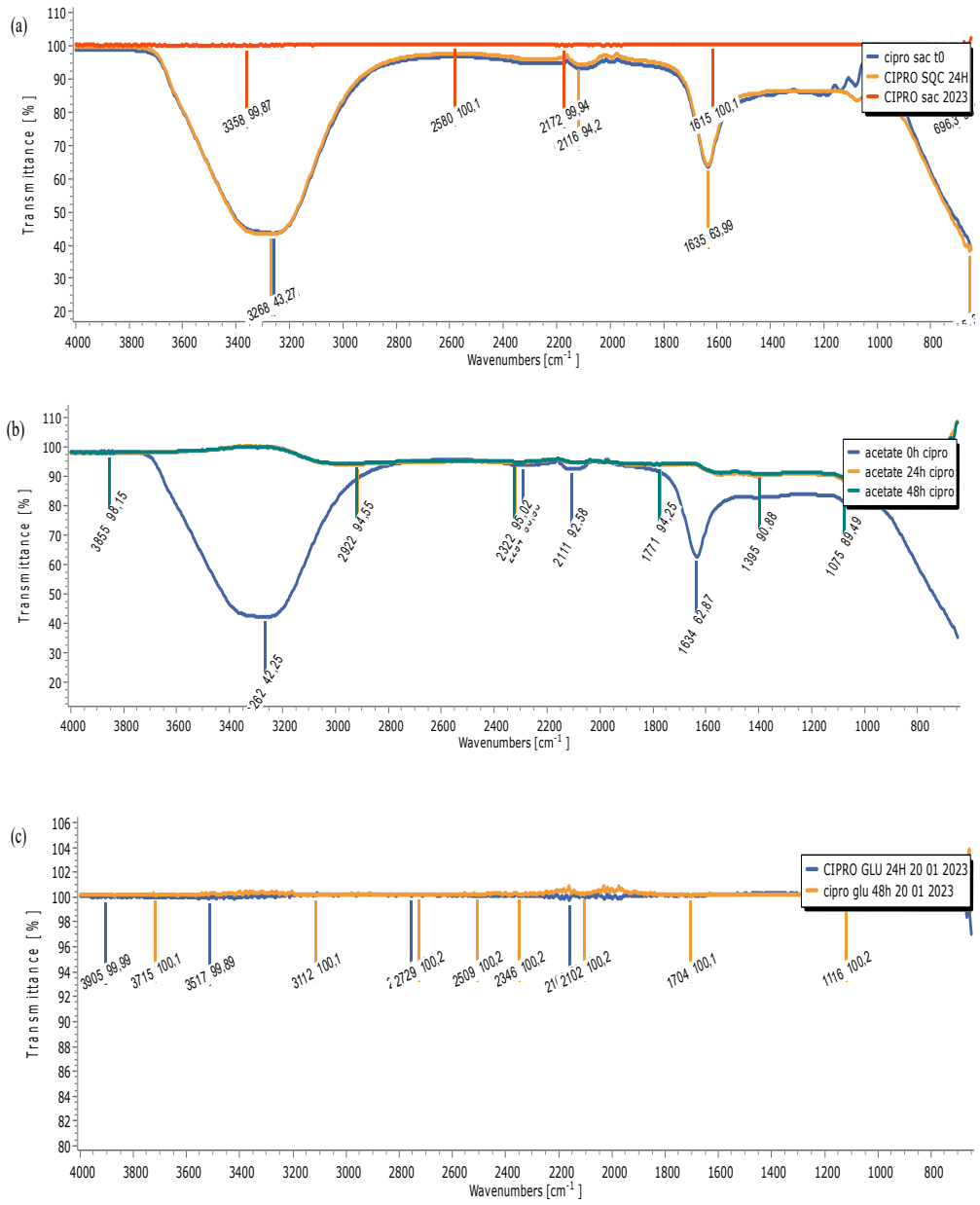

Figure 9. ATR –FTIR analysis of CIP degradation (a) in presence of sodium acetate; (b) in presence of sucrose; (c) in presence of glucose.

were derived from the analysis of the overall ion chromatogram and information previously documented in publications by Girardi et al.[33] Additionally, the ChemBioDraw Ultra software was employed in this process.

Figure 10 displays the protonated molecules [M-H] - of the intermediate metabolites. In order to confirm the structures more accurately, the protonated molecules of the major intermediate metabolites were chosen for production analysis. Based on the identified products, four potential degradation pathways were proposed, as illustrated in Figure 10. Initially, the elimination of the piperazine through desethylation of the piperazine substituent and hydroxylation from CIP led to the formation of the first intermediate metabolite, named CIP-A1, which can be identified as C13H10FNO3, and it exhibits (280.8 m/z) mass-of-charge. Through the subsequent elimination of the amino group (-NH_2) from the benzene ring of CIP-A1, an intermediate metabolite was generated, named CIP-A2, at a mass-to-charge of 193.9 m/z, and it is identified as C10H9NO3. Additionally, at a mass-to-charge ratio at 162.9 m/z, another metabolite was formed and derived from CIP-A1, named (CIP-B), which is C7H7FN2O2. Furthermore, the introduction of an OH group onto the benzene ring of CIP resulted in the creation of CIP-C, which was identified as C17H19FN3O4 and displays a mass-to-charge ratio at 342.9 m/z.

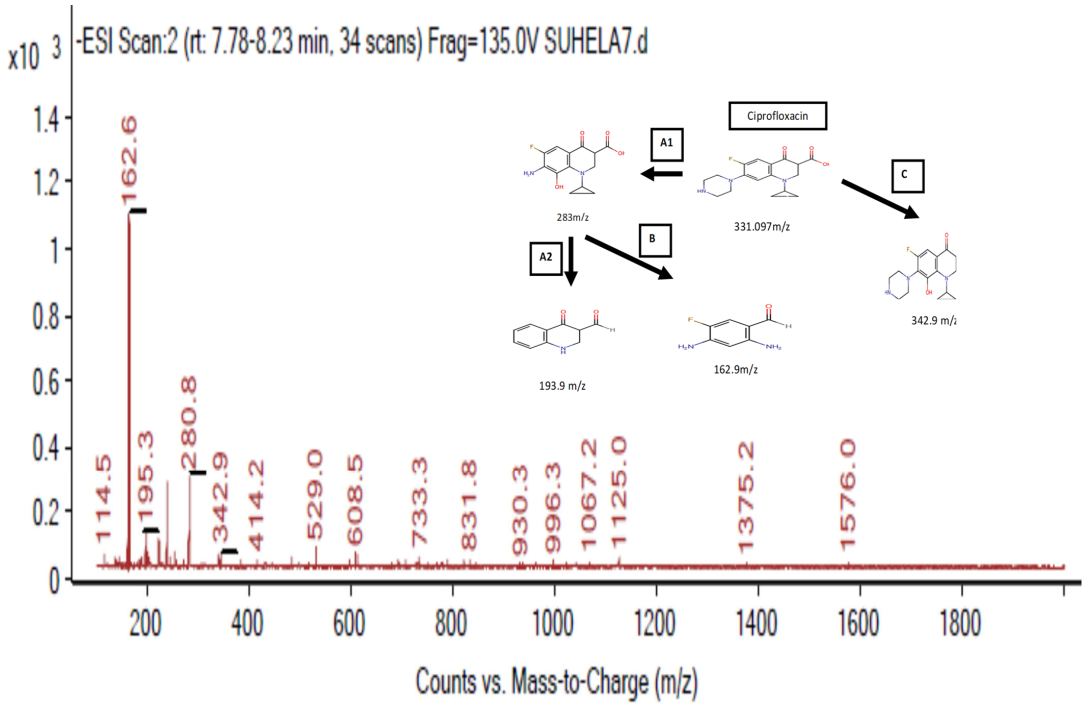

Figure 10. Proposed chemical structure of intermediate products of CIP by LC-MS/MS.

DISCUSSION

Assessing the biodegradability of some antiseptic and antibiotic compounds in wastewater to predict their fate in biological systems seems of great importance as an ecofriendly elimination of environment pollution. Current findings revealed that air and water temperatures of WWTP varied between 8.79 to 23°C. The variation of temperature depends on time of sampling, and the different processing stages of WWTP. Temperature directly influences the development of microorganisms present in wastewater. It is one of the most important ecological factors for sludge function and the purification activity by microorganisms.[34]

Neutral to basic pH of wastewater samples: influent (7.5-10.22), and effluent (7.91-10.25), can be explained by the presence of chemicals of the El Kouyer station. In accordance with the FAO[35], the pH levels fall within the typical range of 6.5 to 8.4. Many authors mentioned that wastewater pH depends on the stage of treatment.[36]

Monthly change of oxygen in wastewater (influent and effluent) of the El Kouyer station varied between 0.30 mg/l to 4.19 mg/l. This change is due to the differences in oxygen consummation, limiting of microbial respiration, and is attributed to the seasonal temperature variations.[37,38]

The current study indicated that COD of wastewater was reduced from: influent samples (175 mg O_2/l to 1480 mg O_2/l) to effluent samples (56 mg O_2/l and 368 mg O_2/l). They were obtained with treated water in compliance with Algerian discharge standards (120 mg O_2/l).[19]

The decrease of BOD_5 values of influent samples (150 mg/l-900 mg/l) to effluent wastewater (5 mg O_2/l -65 mg O_2/l) indicates that WWTP eliminates the maximum rate of biodegradable organic pollution. Also, high BOD5 levels, implying the effect of microbial oxygen demand, cause hypoxia conditions for the aquatic flora.[39]

In order to characterize the nature of the effluent, the ratio of BOD_5/COD was calculated. According to Bechac et al.[40], the ratio in question remains within the normal range of 1 to 3. Abdouni et al.[41] stated that this ratio depends on the nature and origin of sewage which can be domestic or industrial. It is associated with the growth of abundant bacteria and a decrease in the oxygen ratio after the oxygen is consumed by the microorganisms.[42] The observed differences in BOD_5 values can be due to the variations in seasonal temperature changes which may affect microbial respiration.[37,38] Another possible reason for the relatively low blank BOD_5 could be an insufficient amount of inoculum, as suggested by Kümmerer et al.[43] and Seyfried et al.[44] These studies mentioned that the high inoculum had a positive effect on biodegradation experiments, leading to a reduction in the lag phase and an enhanced representation of competent degraders.

Several antibiotics were tested for their biodegradability in our study. These were classified as readily biodegradable to persistent or recalcitrant in the environment by ECHA.[45] In

a comparison of our results with the guidelines OECD[18] and the studies of Painter[36] and O'Malley[46], the tested reference compound sodium acetate exhibited rapid and complete degradation with a percentage of 82.05% via MRT within 14 days. The biodegradation of the reference compound must be rapid (14 days) and reach at least 60% of degradation. Guziałowska-Tic and Tic[47] reported 83.33% degradation of sodium acetate using MRT on the 14th day at a concentration of 100 mg/L. Bergheim et al.[48] and Piętka-Ottlik et al.[49] demonstrated 78% biodegradation of sodium acetate within 14 days but at a concentration of 30 mg/l.

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The process of antibiotic compounds was found to be low for most of them.[50] Al-Ahmad et al.[51] tested the biodegradation ability of penicillin G, CIP, cefotiam, meropenem, and sulfamethoxazole using the closed bottle test. They found that only penicillin G was biodegradable to some degree, with approximately 27% being removed after 28 days, or increase to 35% in 40 days. However, none of the others were readily biodegradable. ß-lactam antibiotics were recorded to be highly biodegradable in several studies.[15,52] On the other hand, macrolides seem to be more recalcitrant for biodegradation.[53] While some antibiotics, such as penicillins, have been reported to degrade easily, antibiotics such as CIP, tetracyclines and tylosin have been reported to be more recalcitrant and cause more dispersal in the environment at higher concentrations with their persistence and accumulation in the environment.[54] Li et al.[55] tested 16 antibiotics using both OECD 302 B and OECD 301 B tests, and they found that only benzyl penicillin (penicillin G) was completely mineralized. Kummerer et al.[43] tested the biodegradability of CIP, ofloxacin, and metronidazole, and found that none of them were biodegraded. The biodegradation assessments of trimethoprim and sulfamethoxazol conducted by Bertelkamp et al.[56] demonstrated that trimethoprim was a biodegradable molecule, but SMX, with zero or very low biodegradability coefficients, was more persistent. According to our results, it was determined that Sulfamethoxazole was degradable (25.75%). Although several researchers have documented varying rates of removal for tetracycline, and some studies indicate that tetracyclines are not biodegradable, there is a lack of documented evidence regarding their mineralization.[57,58] Gartiser et al.[59] studied the degradation of 17 antibiotics from different antibiotic groups as cephalosporins: (Ceftriaxone, Cefuroxime); B-lactams (Penicillins: Amoxicillin, Benzylpenicillin); macrolides (Clarithromycin, Erythromycin);tetracyclines (Tetracycline, Chlortetracycline) macrolides (Clarithromycin, Erythromycin); quinolones (Ofloxacin); carbapenem (Imipenem); lincosamides (Clindamycin); nitroimidazole (Metronidazole); aminoglycosides (Gentamicin); polyeneantimycotics (Nystatin); sulphonamides (Sulfamethoxazole, Trimethoprim); glycopeptides (Vancomycin, Monensin) by inherent biodegradability test combined with the Zahn–Wellens test in the Closed Bottle Test.[18] By result, all these substances are classified as "readily biodegradable", also in WWTPs and thus have no accumulation in the aquatic bodies.[60] This variation in % of degradation of antibiotics compounds in the present results may be due to some factors such as the nature and source of the inoculum (active sludge or second effluent), and the viability of the inoculum is one of the conditions test. Physical-chemical factors such as pH and temperature can limit or at least slow down microbial activity, and affect the removal efficiency of antibiotics when optimum requirements are not attained.[61] Because of the relation between the atmospheric seasonal temperature and microbial oxygen uptake, the microbial respiration can be limited.[37,38] According to the guideline[18], when the substance tested exhibits a biodegradation rate of less than 60%, the pH value should remain between pH=6 to pH=8.5. So, it is crucial to maintain pH neutrality as it greatly influences the activity of microbial inoculum.

CIP is a third generation antibiotic of the fluoroquinolone family with a large antibacterial activity.[62] Based on molecular identification with morphological characteristics the CIP degrading bacterial strain was identified as *K. oxytoca.* Chen et al.[63] found that although the strains were present in all water samples, large numbers of them were present in WWTP and activated sludge. Additionally, *K. oxytoca* epidemics are frequently linked to strains that have enzymes which inactivate the antibiotic activity such as carbapenems and extended-spectrum beta-lactamases.[64–67] The resistance mechanisms of *K. oxytoca* may be due to the over-expression of gene coding of the beta-lactamase enzyme or to the presence of multiple copies of beta-lactamase genes. Both of these mechanisms lead to the over-production of beta-lactamase enzymes.[68,69] In addition, some studies have noted that the genomes of several *K. oxytoca* isolates are resistant to other wide antibiotics families, such as tetracycline and fluoroquinolones.[70] Several studies highlighted the potential of *K. oxytoca* as a promising candidate for the biodegradation of antibiotics in wastewater treatment processes. Wang et al.[71] reported enhanced degradation of norfloxacin with a rate of 90% within 72 h. Also, a previous study of Wang[15] found that the *K. oxytoca* HKE-10 strain can degrade CIP at a range of concentration between 0.8 mg/L and 100 mg/L

in 5 days. In the present study, the use of glucose and sodium acetate stimulated bacterial growth and CIP degradation. The *K. oxytoca* MK4 growth increased rapidly in presence of glucose and sodium acetate compared to sucrose. This might suggest that co-metabolism plays a crucial role in eliminating the antibacterial activities of CIP. Thus, those organic substrates were considered the best sources for CIP degradation as they resulted in rapid bacterial growth and an elimination rate of CIP after 24 h incubation. Co-substrates play a dynamic and significant role in the degradation reaction of emerging contaminants. Moreover, previous studies have demonstrated that the presence of easier-to-use substrates, such as sodium acetate and glucose, can enhance the degradation rate of antibiotics.[72,73] In the current study, the presence of another source of carbon and energy favored the bacterial growth and elimination of the CIP, and compared to the negative control. It is well known that an adequate supply of a simple source of carbon and energy stimulates bacterial growth and its co-metabolism. On the other hand, in the presence of a complex carbon source (sucrose), the bacteria may take a long lag phase duration to adapt.

In our study, the ATR-FTIR spectrophotometer provides a distinct and clear view of antibiotic degradation by the *K. oxytoca* MK4 strain in presence of the different sources of carbon. The significative reduction in vibration that corresponds to the piperazine ring, as shown, indicates that various locations on the piperazine ring may have undergone oxidation, and potentially formed carboxy groups. In addition, the displacement of the corresponding bands in the degraded sample, as compared to those in the standard, can be attributed to the existence of metabolites. Based on the results reported by Guo et al.[74], it can be inferred that the cleavage of the piperazinyl ring can lead to the formation of transformation products. The appearance of two new stretching absorption peaks of C-F (1075 cm^{-1}, 1185 cm^{-1}, 1116 cm^{-1}) respectively, and other news peaks corresponding to CC, C=O and CN groups, suggests that the CIP antibiotic has undergone changes as a result of bacterial degradation.[75,76] The vibrations observed at (1472 cm^{-1}, 1628 cm^{-1}) correspond to the bonds in the benzene ring as well as those in the pyrazine ring. These vibrations were still detectable in the spectrum of *M. luteus* and *B. subtilis* even after the incubation of 14 days as reported by Tan et al.[75] and Yan et al.[76] The spectra for *L. gesseri, Enterobacter* sp., and *Bacillus* sp. bacteria closely resembled the degradation products of CIP recorded in previous studies.[77,78] However, it was observed that the products of the quinolone ring structure remained detectable even after the incubation period; this suggests that the piperazinyl ring cleavage may be responsible for the elimination of the antibacterial activity of CIP. Further research may be required to fully comprehend the chemical structure and characteristics of these compounds. Several studies have indicated that the degradation of CIP involves at least two distinct parts of the CIP molecule; the piperazinyl substituents and quinolone moiety.[79,80] These two components can undergo separate degradation processes during biodegradation and can be subject to biotransformation or degradation by microbial enzymes. The same finding in the study of Wang[15] showed that the *K. oxytoca* HKE-10 strain is capable of efficiently degrading CIP through amide bond hydrolyzed, followed by quinolone degradation via oxidation and ring opening. The degradation of CIP was confirmed by ATR-FITR, which revealed structural changes in both the piperazine ring and the quinolone moiety of the molecule.

The results indicate that the presence of glucose and sodium acetate stimulated bacterial growth and CIP degradation for the first 24 h, compared to sucrose where the bacterium started growing and exhibited a high capability to degrade CIP after 48 h. Pan et al.[32] tested the influence of sodium acetate as a co-substrate and observed approximately 60% of the antibiotic elimination after 5 days. They reported that sodium acetate triggered the bacterial growth and improved the breakdown of CIP. Nguyen et al.[81] recorded degradation of CIP at a level of 80-90% by adsorption on sludge during the treatment of wastewater whose substance is stable. Moreover, the adsorption process is the step that helps the activated sludge system to degrade about 50-% of CIP by anaerobic biodegradation.[82,83] These findings suggest that *K. oxytoca* strain could be a promising candidate for the bioremediation of CIP-contaminated environments, including wastewater treatment plants and polluted soil.

In our study, CIP changed to four products mainly by degradation and hydroxylation of the piperazinyl substitute. Thus, the transformation process of CIP was observed to occur in two regions of the molecule: the piperazinic portion and the quinolone portion by two different reactions: (A) decomposition of piperazinyl substituent; and (B) hydroxylation. The study conducted by Liu et al.[12] discovered that the biological oxidation process carried out by a mixed culture specifically targeted the piperazine ring of CIP. However, the antibiotic quinolone component of CIP remained unchanged and unaffected by the transformation process.

During our study, we observed the biodegradation of CIP and its metabolite CIP-A1. The metabolite CIP-A1 exhibited a protonated molecule signal at m/z 283, which had been previously identified during the biotransformation of CIP using the fungi *Pestalotiopsis guepini*.[78] In addition, we observed the elimination of the amino group (-NH_2) from the benzene ring of CIP-A1, resulting in the formation of CIP-A2, with a molecular weight of 193. This finding aligns with the research conducted by Xiaobin et al.[84] Additionally, we observed the formation of CIP-C, with a molecular weight of 348, which is consistent with previous reports by Alexandrino.[13] In the fact, the FQ such as CIP transformed in natural environments by a consortia of non-characterized microorganisms but not by a single microorganism. For instance, Feng et al.[85] used a consortium isolated from activated sludge which has been used in many CIP biotransformations. Furthermore, the same

biodegradation products of CIP identified as CIP-A and CIP-C were found as the bacterial community responsible for the degradation of CIP by the strains belonging to different genera such as *Stenotrophomonas, Phenylobacterium, Pseudoxanthomonas, Dysgonomonas, Ferruginibacter*, and *Leucobacter* in the study reported by Liao.10 The same metabolite product (CIP-C) in our findings was reported in the study of Wetzstein et al.[86], showing the biodegradation of CIP by the *Gloeophyllum* striatum, the brown rot fungus. In addition, the metabolite CIP-A is a result of CIP biodegradation by certain fungi.[77,87,88]

The study of Jia et al.[89] investigated the degradation of CIP using a mixture of bacteria, specifically anaerobic sulfate-reducing bacteria such as *Desulfobacter* sp., which were isolated from a sludge system. These bacterial strains were found to produce metabolites known as CIP-A and CIP-C in our study. The formation of these metabolites occurred through a desethylation reaction of the piperazine substituent and the subsequent hydroxylation processes. However, some studies reported the biodegradation of CIP by one strain, as reported by Jung et al.[90] They reported the biotransformation of CIP by an *Escherichia coli* strain which is highly resistant to CIP and isolated from a municipal WWTP. This strain is able to degrade CIP and inactivate certain FQs via acetylating the piperazine ring by the aminoglycoside acetyltransferase enzyme coding in its genome. Also, Pan et al.[32] reported that CIP can transformed into seven products, and five of them (M2, M3, M4, M7, M16) were also demonstrated in other studies, by the thermophilic bacterium *Thermus* sp. isolated from acclimated sludge.

CONCLUSION

The biodegradability of antibiotic and antiseptic drugs in wastewater has been thoroughly investigated. The results of this study indicate that some antibiotics tested did not meet the expected levels for biodegradation established according to the OECD 301F guidelines. Therefore, it can be concluded that while certain antibiotic drugs are assumed to be readily biodegradable compounds, some of the tested compounds degrade by less than 20%, rendering them non-biodegradable and potentially persistent. This, in turn, serves as an indicator of the chemicals being potentially persistent. It is noteworthy that, as in the case of ecotoxicity, the biodegradability assessment must be conducted through several independent test runs to take into account the variability in the microbial inoculum. An alternative approach could be the analysis of the taxonomic composition of the inoculum, which can help identify significant variations within the microbial community and identify the microorganisms that are potentially responsible for the transformation process. In this study, a bacterial strain was isolated and identified as *K. oxytoca* MK4, which facilitated the degradation of CIP and the analysis of its transformation products. The ATR-FTIR spectrums used in the analysis showed changes in antibacterial active sites of the antibiotic tested CIP (piperazine ring and quinolone part) and confirmed the degradation of CIP by the bacteria isolated. As such, this bacterial strain is a potential candidate for introduction into wastewater effluents to remove CIP in effluent water before discharging into the environment. This research also demonstrated that indigenous bacterial strains enhance biodegradation when added to active sludge. From these results, the gene encoding the CIP degradation by the dominant isolates of active sludge should be investigated via metagenomics, transcriptomics and proteomics.

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Availability of data and materials

The 16S rRNA gene sequence data of the ciprofloxacin degrading *Klebseilla oxytoca* MK4 deposited to National Centre of Biotechnology Information (NCBI) GenBank with accession number MK474159.1.

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