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Dicloxacillin Degradation with Free-Living Bacteria

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Abstract Environmental problems such as bacterial resistance have been generated by indiscriminate use of antibiotics, because free-living bacteria have a great ability to adapt to unfavorable environmental, since they develop several defense mechanisms such as genetic resistance, in response of being in contact with this contaminant present in its environment, which is why they acquire special importance due to its biodegradation capacity. In this study, the biodegradation of 40 μg/mL of dicloxacillin with free-living bacteria was evaluated. Wastewater samples were collected from a hospital, from which five resistant strains were isolated, characterized, and identified: *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and two different strains of *Escherichia coli*, being the last one, the most resistant according to the determination of the minimum inhibitory concentration, so degradation was carried out with this strain and in bacterial consortium. Biodegradation was quantified by high-resolution liquid chromatography (HPLC) and the results showed that both the consortium and *Pseudomonas aeruginosa* are highly efficient because the degradation of the antibiotic was 100%, in a time of 3.5 h and 52 h, respectively, and even metabolize the degradation products.

Keywords Biodegradation · Dicloxacillin · *Pseudomonas aeruginosa* · Bacterium · Bacterial consortium

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1 Introduction

Today, there is a great awareness about environmental problems caused by different chemical compounds such as antibiotics, which have provided expectations to deal with several bacterial infections; however, these are governed by criteria of biological evolution and immediately show resistance. An example of this is dicloxacillin (Fig. 1), a beta-lactam antibiotic, derived from penicillin, used for the treatment of infections caused by staphylococci resistant to benzyl-penicillins.

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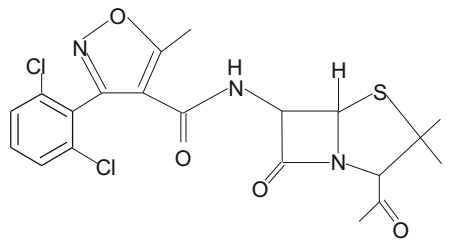

Fig. 1 Chemical structure of dicloxacillin (Colina-Márquez and Castilla-Caballero 2013)

Bioavailability is 50–85%, distributed in the liver, kidneys, synovial fluid, pleural fluid, bone, and bile; the plasma protein binding is 94.4%, while 60% of a dose administered is excreted through urine in its original chemical form (Castle 2007).

Part of the structure and its intermediaries remain intact when passing through conventional wastewater treatment plants, due to its considerable solubility in water and low biodegradability. This represents a threat to the natural bodies of water that receive these compounds after this process (Colina-Márquez and Castilla-Caballero 2013).

As a result, antibiotics have been detected in lakes, rivers, groundwater, and even in drinking water (Binh et al. 2018). Natural attenuation and conventional treatment processes are not efficient to eliminate these antibacterial since they bioaccumulate in macroinvertebrates (Rodriguez-Narvaez et al. 2017), causing several problems being the most important, microbial resistance. Bacteria from different environments have the possibility of exchanging antibiotic resistance genes (Karkman et al. 2017). This represents a risk of proliferation of antibiotic-resistant microorganisms and the creation of multi-resistant phenotypes (Lamba and Ahammad 2017). As such, antibiotics and their effects on the environment have become an important issue in environmental science (Yang et al. 2018).

However, this resistance generated by native microorganisms can be used as a bio-corrective measure for the decontamination of the environment.

Therefore, the bioremediation process is a promising alternative because it uses the ability of natural or genetically modified microorganisms to recover contaminated sites and protect the environment (Sharma et al. 2018).

Among the main advantages of bioremediation are efficiency, low cost, and environmental impact, compared to conventional techniques.

In addition to the above, other methods for the degradation of dicloxacillin have been reported as shown in Table 1, where physicochemical methods stand out.

However, the results have not been satisfactory, since the elimination percentages are low, and reporting the formation of by-products, while when using the fungus *Leptosphaerulina* sp., the percentage of biodegradation was 100%.

Based on the above, free-living bacteria became the ideal candidates for the bioremediation of their environment since they are very resistant due to their ability to survive in an open environment where there is competition for nutrients, as well as adverse environmental factors, in contrast, in laboratory conditions, are more sensitive. For this reason, the present paper aims to test the degradation of dicloxacillin with bacteria isolated from water waste from a hospital.

2 Methodology

2.1 Sampling and Bacterial Characterization

The wastewater sample was collected based on Standard Methods for the Examination of Water and Wastewater (McCrady 2008) from the affluent of a hospital in Mexico in 1-L sterile flask. It was transported to the laboratory

Table 1 Methods for the elimination and degradation of dicloxacillin

Method	Concentration	Degradation %	Reference
Sonochemical degradation	6.4 μM	30.00	Villegas-Guzman et al. (2015a)
Solar heterogeneous photocatalysis	25 μg/L	45.82	Campo and Cohen Rodriguez (2012)
Photocatalytic mineralization	25 mg/L	45.00	Colina-Márquez and Castilla-Caballero (2013)
Photocatalysis with TiO_2	0.213 mM	95.00	Villegas-Guzman et al. (2015b)
Biodegradation with fungus *Leptosphaerulina* sp.	19,000 μg/L	~ 100.00	Copete-Pertuz et al. (2018)

in a cooler at 4 °C. Phenotypically different bacterial colonies were isolated and purified by repeated subculture.

Then, a Gram stain was performed in order to determine the dye affinity and the microscopic morphology of each one. In addition, to include a scanning electron microscopy (SEM) study of each bacterium, for this, it was necessary to fix the bacteria with 2% formaldehyde, for 48 h, then wash with 0.85% saline solution and gradually dehydrated with ethanol (10–100%). The samples were critical point drying, then fixed in the sample holder, and coated with gold. For it is analysis, a scanning electron microscope SU1510 Hitachi at 10 kV was used.

2.1.1 PCR Analysis

The extraction of DNA was realized with Animal and Fungi DNA preparation Kit Jena Bioscience, as it is shown in the following.

2.1.2 Cell Lysis

Bacterial strains were incubated in nutrient broth for 24 h at 37 °C, and then centrifugated at 4000 rpm for 10 min. The samples in the cell pellet were resuspended in 300-μL cell lysis solution to the tissue, homogenized and were added 1.5-μL proteinase K solution to the lysate and mixed by inverting several times, and then incubated at 55 °C for 1 h. The samples were homogenized and centrifugated at 15,000*g* for 1 min; the supernatant was discarded.

2.1.3 Protein Precipitation

The samples were resuspended in 300-μL cell lysis solution, 100 μL of protein precipitation solution was added to the cell lysate, then the solution was mixed well by using a vortex for 20 s and centrifuging at 15,000*g* for 3 min.

2.1.4 DNA Precipitation

The supernatant was transferred to a clean 1.5-mL microtube containing 300 μL of isopropanol > 99%; then, the sample was mixed by inverting gently, let it precipitate.

Centrifugation was carried out at 15,000*g* for 1 min; then, when the DNA was visible, the supernatant was discarded and drained. After that, each sample was dried in a vacuum centrifuge and finally added 50 μL of DNA hydration solution to each dried DNA pellet.

2.1.5 PCR Reactions

PCR reactions were performed in 15 μL containing 1 μL DNA, 3 μL buffer, 10.5 μL H_2O, 0.2 μL primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and 0.2 μL primer 1492R (5′-TACGGTTACCTTGT TACGACTT-3′) of MyTaq PCR kit Master Mix (Bioline). PCRs were realized with a Veriti 96-Well Thermal Cycler under the following conditions: 2 min at 95 °C for initial denaturation, 35 cycles consisting in denaturation for 1 min at 95 °C, annealing for 1 min at 58 °C, and elongation at 72 °C for 2 min, followed by a final 5-min extension step at 72 °C.

2.1.6 PCR Amplifications

Samples were analyzed by gel electrophoresis on 1.5% agarose gels containing 1× TAE and 1× Gel Red, in a horizontal electrophoresis system (Thermo Electron Corporation Power Supply EC250-90) for 30 min at 100 V.

The molecular size marker HyperLadder 100 bp DNA Ladder was used and the agarose gel was revealed with a UVP Photo documenter, 2UV benchtop with Multidoc-IT System.

In addition to the above, fluorescence of the most resistant strain was determined by confocal microscopy TCS SPE/CTR 4000 (Leica), on a slide with and without Gram staining.

2.2 Determination of the Minimum Inhibitory Concentration

To select the bacteria capable of surviving in the presence of dicloxacillin, the minimum inhibitory concentration (MIC) was determined, following the guidelines established in the CLSI (M100 *Performance Standards for Antimicrobial Susceptibility Testing An informational supplement for global application developed through the Clinical and Laboratory Standards Institute consensus process. 27th Edition* 2017). Serial dilutions were made in half starting from dicloxacillin stock of 10,000 μg/mL, in Mueller Hinton broth (MH); then, 50 μL was placed in the wells of the microplate of each solution and inoculated with 50 μL of the bacterial suspension adjusted to 0.5 MacFarland nephelometer (1.5×10^8 UFC mL^{-1}). To

ensure accuracy and reproducibility, two controls were included: one positive (MH broth to which the bacterial suspension was added) and one negative control (MH broth without dicloxacillin and without bacterial suspension). Antimicrobial dilutions between 5000 and 7.8 μg/mL were used.

The MIC was interpreted as the concentration of the antimicrobial, contained in the well of the microplate that inhibited the visible growth of the bacteria, for which it was necessary to compare each well with the positive and negative controls.

2.3 Evaluation of the Degradation Ability

There were prepared two control blanks and two biological degradation models for evaluating the degradation ability.

2.3.1 Control Blanks

Blank 1 (antibiotic stability) consisted of 200 mL of Mueller Hinton broth and 40 μg/mL of dicloxacillin purity 90.4% B.H. mark Fersinsa. The mixture was distributed in sterile tubes and is placed in water baths with slow helical agitation, in temperature 30 °C. The behavior of the antibiotic was monitored by high-resolution liquid chromatography (HPLC).

Blank 2 (bacterial growth in Mueller Hinton broth) consisted of 200 mL of Mueller Hinton broth and 28 mL of bacterial suspension in 0.85% saline solution, the cell density of inoculate fluid was adjusted to 0.5 McFarland nephelometer (1.5×10^8 UFC mL^{-1}). Note: this blank was prepared for more resistant strain and consortium.

2.3.2 Biological Models

Biological model 1 (degradation with more resistant strain): there were used 200 mL of Mueller Hinton broth, 40 μg/mL of dicloxacillin, and 28 mL of a more resistant strain suspension (bacterium in 0.85% saline solution); cell density of inoculate fluid was adjusted to 0.5 McFarland nephelometer (1.5×10^8 UFC mL^{-1}). The combination was distributed in sterile tubes and is placed in water baths with slow helical agitation, in temperature 30 °C. Biodegradation was quantified by HPLC.

Biological model 2 (degradation with bacterial consortium): there were used 200 mL of Mueller Hinton broth, 40 μg/mL of dicloxacillin, and 28 mL of suspension bacterial consortium (9.6 mL of more resistant strain in 0.85% saline solutions adjusted to 0.5 McFarland nephelometer and 4.6 mL of every remaining strain in 0.85% saline solution was adjusted to 0.5 McFarland nephelometer).

Note: The cell density of inoculating fluid (28 mL of consortium suspension) was adjusted to 0.5 McFarland nephelometer (1.5×10^8 UFC/mL).

In order to know the behavior of the bacterial growth with respect to the concentration of antibiotic during the degradation, additional sampling was necessary, which was realized in the following way:

Degradation with the more resistant strain took 1 mL of the sample and realized serial dilution until 1×10^{-7}. Next, the dilutions 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} were sowed for emptying in plaque and duplicate in agar Mueller Hinton. Sampling was realized every 2 h.

Degradation with consortium is the same procedure that degradation with the more resistant strain. In this case, the sampling was realized every hour.

2.4 Quantification by High-Resolution Liquid Chromatography

For quantification, a chromatography Waters 1515 with UV-Vis detector, Agilent, Eclipse XDB-C18, 5 μm, 4.6 × 250 mm column was used. Mobile phase was methanol HPLC grade; water Milli-Q grade with 0.1% formic acid (EMSURE, lot K47929664619, purity 98–100%) in proportion 60/40 *v*/*v*. The quantification was realized at a wavelength of 220 nm, injection volume: 20 μL of sample. The reading was realized by 5 h every 15 min and then every 4 h by 72 h.

3 Results and Discussions

3.1 Sampling and Bacterial Characterization

Five phenotypically different strains were isolated, which were labeled as C1, C2, C3, C4, and C5.

Gram stains showed that all bacteria are Gram negative bacilli; in Fig. 2, the micrographs confirm that, also, they were measured (length and diameter) by quadruplicate and the average of the measurement was reported. The C1 measures $1.77 \pm 0.26 \times 0.44 \pm 0.04$ μm, C2–$1.42 \pm 0.21 \times 0.5305 \pm 0.05$ μm, C3–$1.54 \pm 0.35 \times 0.52 \pm 0.04$ μm, C4–$1.49 \pm 0.49 \times 0.57 \pm 0.01$ μm, y C5–$1.86 \pm 0.11 \times 0.55 \pm 0.03$ μm, the bacilli of C5

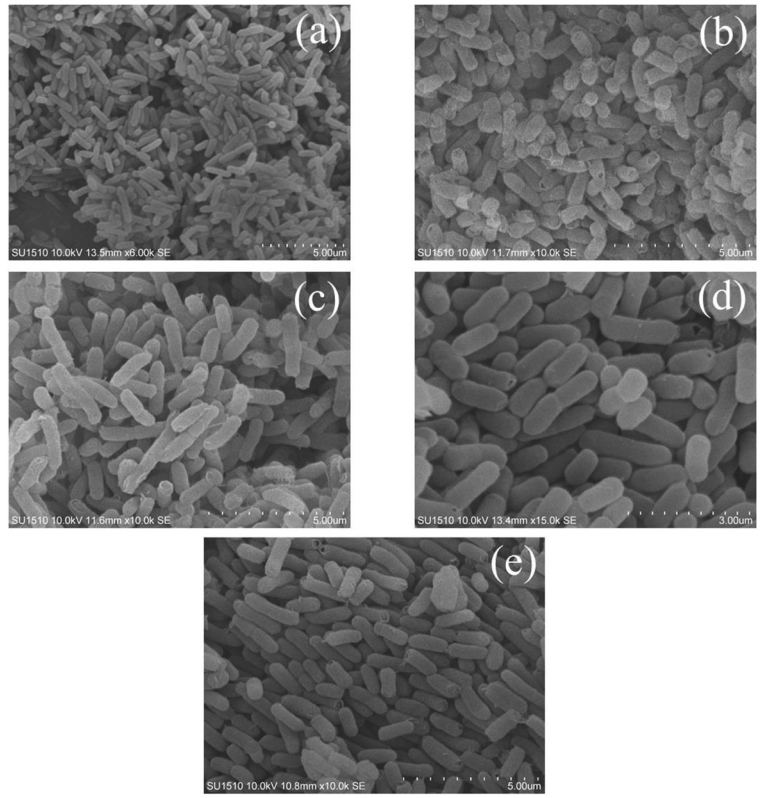

Fig. 2 MEB studies **a** C1, **b** C2, **c** C3, **d** C4, **e** C5

demonstrates to be the largest, followed by C1,C3, C4, and C2, respectively.

Figure 3 shows the gel electrophoresis results in which it can be observed that the five strains have a well-defined amplification band at 1465 pb approximately.

In reference to PCR identification, DNA sequencing analysis was performed with the Geneious prime 2019 program, the database consulted was Gen Bank of the National Center for Biotechnology Information (NCBI). The results of the gender identification of each bacterium are given in Table 2.

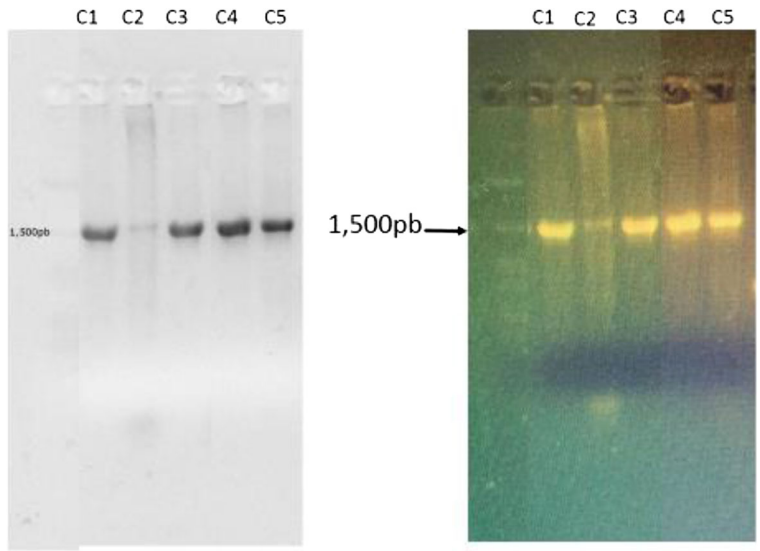

Fig. 3 PCR 1.5% agarose gel electrophoresis

Table 2 PCR bacteria identification

Strain	PCR identification
C1	99.5% *Pseudomonas aeruginosa* sp.
C2	99.9% *Citrobacter freundii*
C3	99.5% *Klebsiella pneumoniae*
C4	99.3% *Escherichia coli*
C5	99.9% *Escherichia coli*

Additionally, the fluorescence of *Pseudomonas aeruginosa* was determined by confocal microscopy. After fixing the sample on a slide, it did not show fluorescence on its own. Therefore, secondary fluorescence was determined to Gram staining, showing fluorescence in green tone in Fig. 4 a and red in Fig. 4 b, while in blue and gray, it reflects only the background of the staining.

In Fig. 4 c, the superimposed image of the green and gray tones is observed, and the gray tone corresponds to the background of the image, d the four images are superimposed, and in this case, it is better appreciated than the blue tone corresponds to the background.

3.2 Determination of the Minimum Inhibitory Concentration

The five strains are considered resistant since they were not inhibited by the recommended concentrations according to the CLSI protocol.

Figure 5 shows the MIC of all the strains under study. *Pseudomonas aeruginosa* is the most resistant to dicloxacillin at a cocentration of 4525 μg/mL, followed by *Escherichia coli* at 4000 μg/mL, *Escherichia coli* at 2450 μg/mL, *Citrobacter freundii* at 1950 μg/mL, and finally *Klebsiella pneumoniae* at 1550 μg/mL. For biodegradation, it was decided to use the consortium and *Pseudomonas aeruginosa* alone due to its metabolic capacities and adaptation to different environments. In addition, it is being widely used for biodegradation.

3.3 Evaluation of Degradation Ability

Figure 6 shows the behavior of the blank with respect to the degradation of dicloxacillin at 30 °C with *Pseudomonas aeruginosa* and the microbial consortium. This degradation was realized for duplicate and the average values and standard deviation are reported in the graphic.

The degradation with *Pseudomonas aeruginosa* was carried out in 52 h while with the bacterial consortium in 3.75 h. This showed the efficiency of the consortium. Also, it was observed that the behavior of the blank of dicloxacillin is stable during the first 25 h; after this, it shows an effect that can be caused by the temperature.

Figure 7 a shows the chromatograms of the behavior of the degradation of dicloxacillin with *Pseudomonas aeruginosa*; at time 0, the presence of two peaks is observed, the first in the range of 2.0 to 4.0 min, and the second in a retention time of 14.5 min, which correspond to the signal of the culture medium and

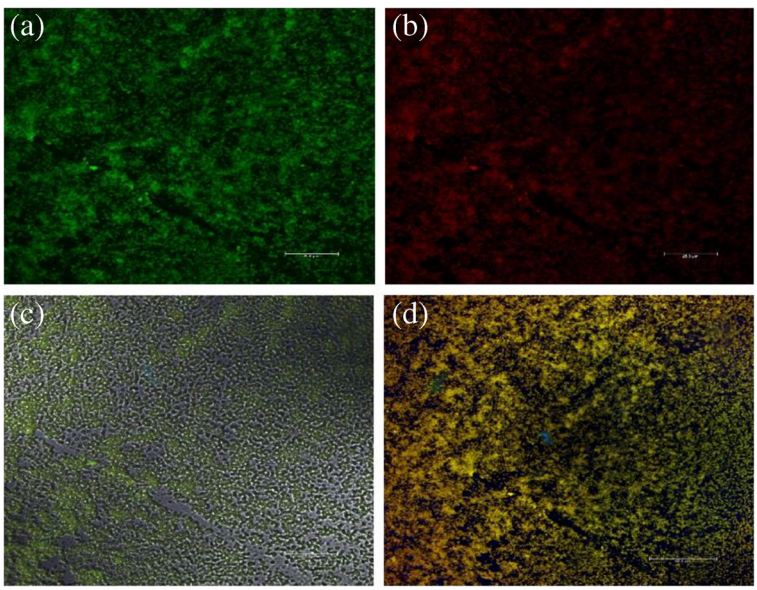

Fig. 4 Confocal microscopy of *Pseudomonas aeruginosa* Gram staining, TCS SPE/CTR 4000 (Leica) equipment **a** fluorescence in green tone, **b** fluorescence in red tone, **c** fluorescence in green and gray, **d** images of the four superimposed fluorescence

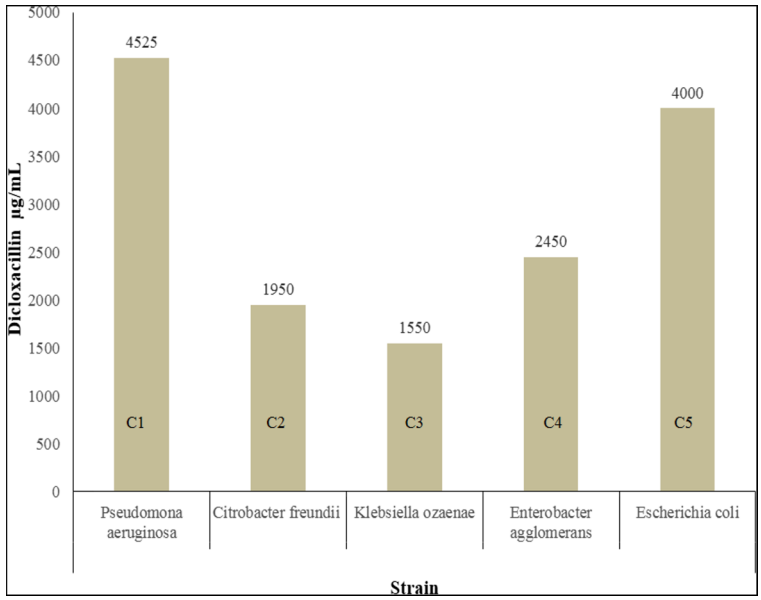

Fig. 5 MIC of dicloxacillin in the five strains isolated

dicloxacillin, respectively. At 12 h, the antibiotic signal decreases considerably and two metabolites are formed, which increase their area while the antibiotic signal decreases as seen from 12 to 48 h. Furthermore, at 52 h, the peak corresponding to dicloxacillin is no longer present and only the two initial metabolites are appreciated. At 72 h, the decrease of the peak corresponding to the culture medium is observed, because the bacteria use it as a source of nutrients, and the two initial metabolites are no longer visible, but the formation of five peaks. When comparing this chromatogram with that of Fig. 8 b which corresponds to blank 2 (*Pseudomonas aeruginosa* in the culture medium), four agree with the metabolism chromatogram of Fig. 8 b with retention times 6.2, 7.1, 7.9, and 9.5. However, the peak area with a retention time of 9.5 min increased, which indicates that it is due to the biodegradation of the dicloxacillin metabolites, and the peak with a retention time of 10.5 min corresponds only to the metabolism of the antibiotic.

The trend of degradation monitoring shows that these metabolites could disappear if more time is left in the presence of *Pseudomonas aeruginosa.*

With respect to the chromatograms of dicloxacillin degradation with bacterial consortium (Fig. 8), it is observed that at zero time, there is the presence of the same peaks shown in Fig. 7; these correspond to the culture medium and dicloxacillin, respectively; after 3 h, the peak of the antibiotic begins to decrease and the presence of nine metabolites is observed, of which 8 are persistent at 3.5 h, while the signal corresponding to

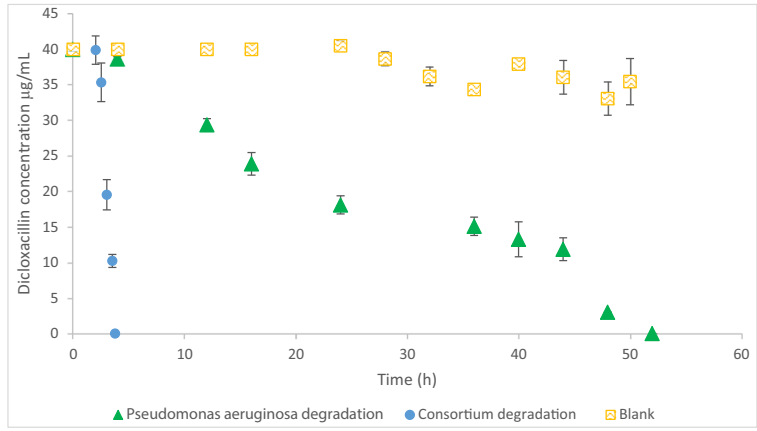

Fig. 6 Behavior of the blank with respect to the dicloxacillin degradation with *Pseudomonas aeruginosa* and bacterial consortium

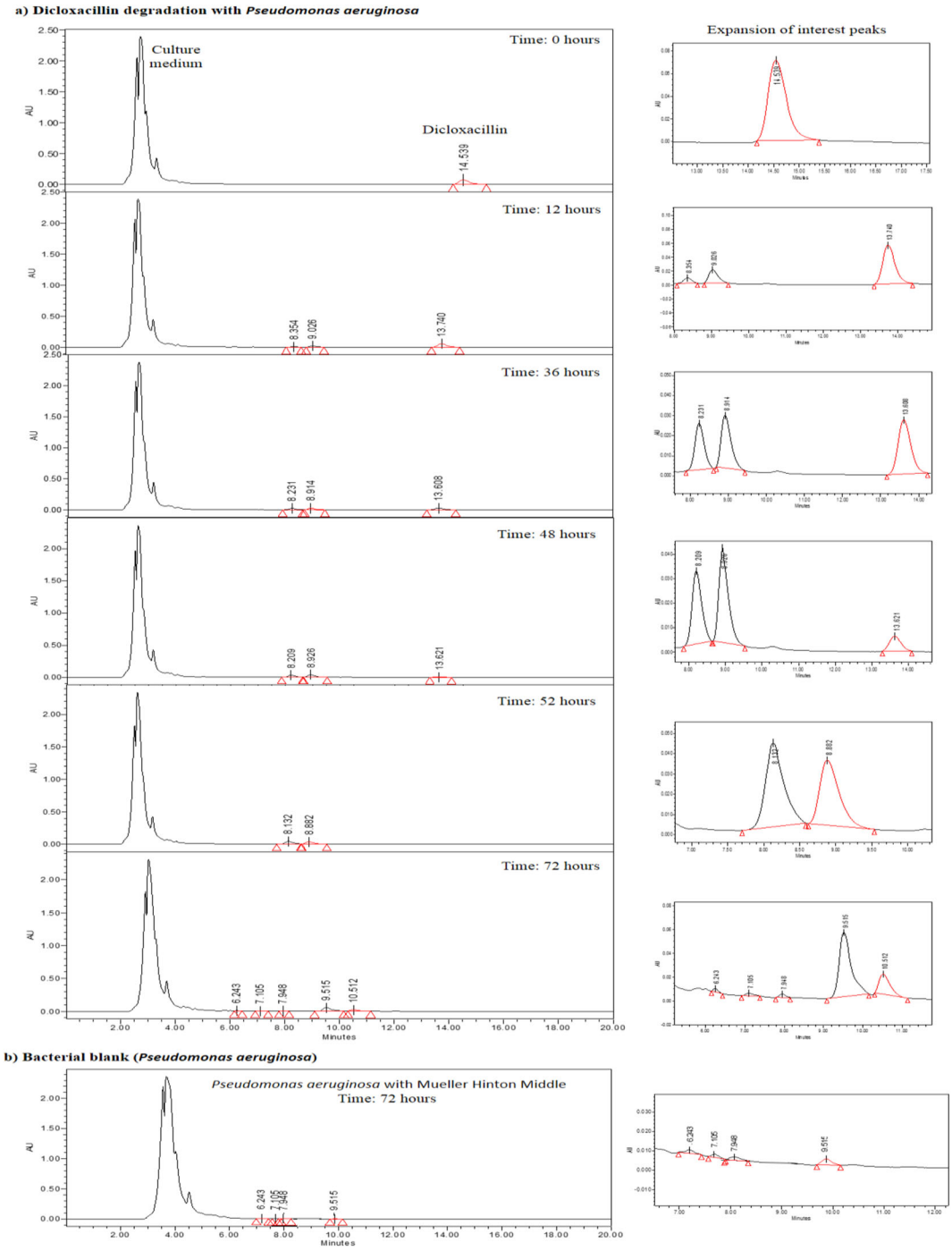

Fig. 7 Chromatograms of **a** behavior of the dicloxacillin degradation with *Pseudomonas aeruginosa* at 30 °C and **b** blank of *Pseudomonas aeruginosa* in culture medium at 30 °C

dicloxacillin has decreased considerably; until at 3.7 h, the signal of the antibiotic is not observed leaving only two metabolites, which after 12 h lead to the formation of five metabolites. After 72 h, only the decrease in the signal of the culture medium and the formation of two peaks are observed; when these peaks are compared with those of the chromatogram of Fig. 9 b which corresponds to blank 2 (bacterial consortium in MH

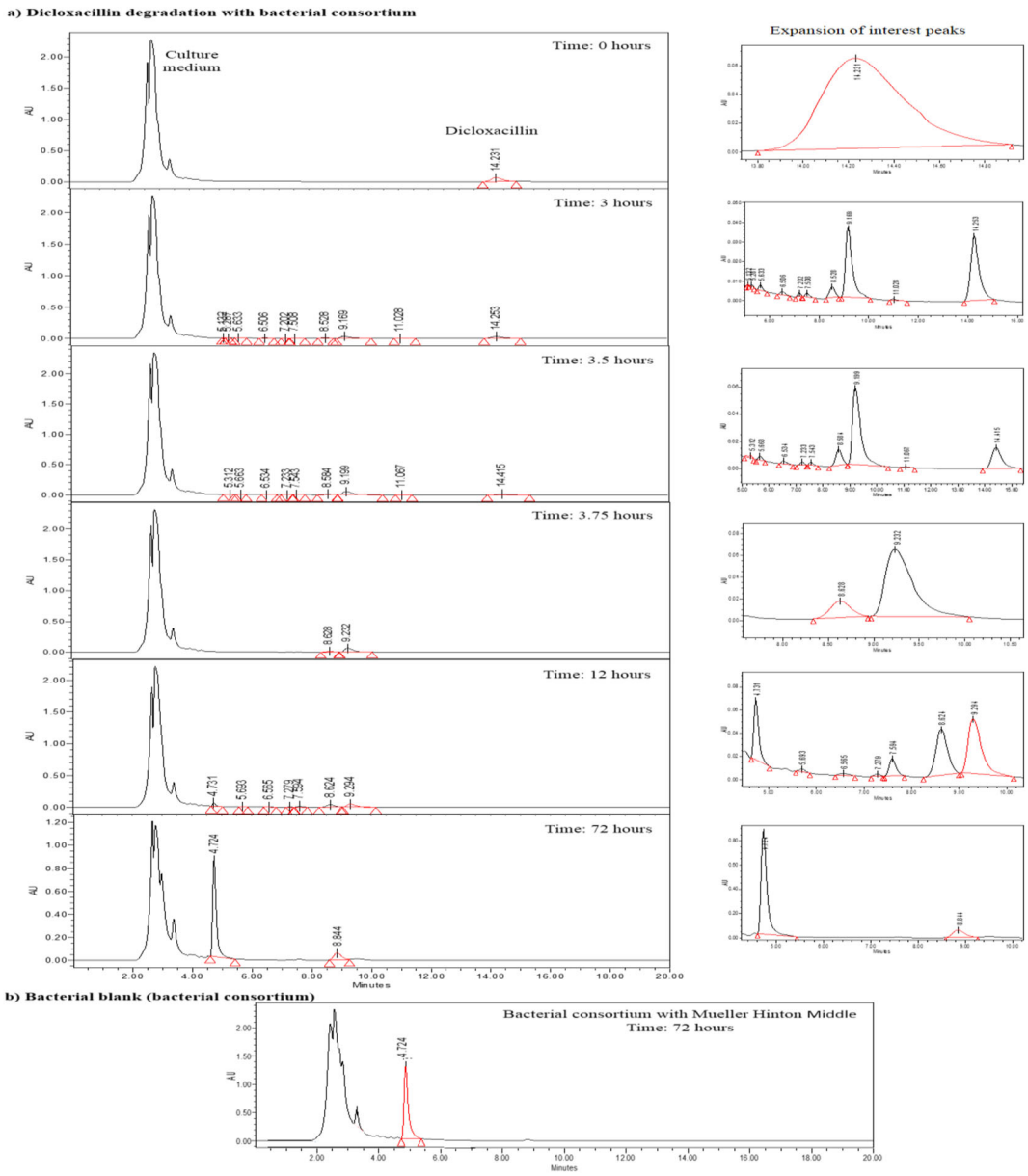

Fig. 8 Chromatograms of **a** behavior of the dicloxacillin degradation with bacterial consortium at 30 °C and **b** bacterial consortium blank in culture medium at 30 °C

medium), it is observed that the peak with retention time of 8.8 is due to the metabolism of dicloxacillin, while the other peak with retention time of 4.7 is a consequence of the metabolism of the bacterial consortium in the culture medium, evidenced by the blank of bacteria with culture medium.

Based on the above, it is determined that the degradation of dicloxacillin is 100% for *Pseudomonas aeruginosa* and in consortium.

When comparing this degradation behavior with Table 1, it is observed that it is higher than that reported with the methods of photocatalysis and sonochemical degradation. In the case of photocatalysis with TiO_2, although 95% mineralization was achieved in 480 min of treatment and the antibiotic was eliminated after 120 min, antimicrobial activity was still detected. This suggests that some byproducts of degradation could also be considered a risk, in terms of the proliferation of

antibiotic-resistant bacteria (Villegas-Guzman et al. 2015b). This indicates that this biodegradation proposal is better than the previous ones since, in addition to eliminating the drug, the bacteria also act on the metabolites as observed in the chromatograms of Figs. 7 and 8.

With respect to biodegradation with *Leptosphaerulina* sp. (Copete-Pertuz et al. 2018), in 8 days, the fungus eliminated the antibiotic, while the proposed method eliminates it in a matter of hours and even metabolites are almost eliminated in 72 h, what makes the use of bacteria more profitable for biodegradation.

Figure 9 shows the degradation of dicloxacillin with respect to the growth of *Pseudomonas aeruginosa*; in this graph, the initial concentration of biomass does not change much in the first 4 h and neither is the decrease in the antibiotic evident; this is because the bacteria are adapting to the environment. However, after 4 h, the exponential phase of these begins, but from 12 to 16 h, the growth is not exponential and the concentration of antibiotics decreases; this could be due to the fact that *P. aeruginosa* possesses an amp C gene inducible, which encodes the β-lactamase hydrolytic enzyme. This enzyme is capable of breaking the amide bond of the β-lactam ring, which leads to the inactivation of β-lactam antibiotics (Pang et al. 2018) as a result of which metabolites are formed as shown in Fig. 9 where it can be seen that precisely at 12 h, there are two metabolites that can be the cause of the bacterial stopping their exponential growth, affecting the enzymatic activity.

Given that the enzymes act as specific biocatalysts involved in a chemical reaction that takes place within the cell, during reaction, they bind effectively to the substrate, forming a transient complex. However, there are environmental factors that modify enzymatic activity such as pH and temperature. In addition to metabolic factors such as the concentration of enzyme or substrate (Arsalan and Younus 2018), since the temperature was kept constant and the amount of substrate is the limiting reagent, it is most likely that the pH was the cause of this stagnation in the exponential growth and this began again when the conditions were favorable.

Also, it can be observed that these curves intersect at 16 h, which is when approximately 50% of the antibiotic has been degraded. This information is very important since it indicates that to degrade 22.5 μg/mL of dicloxacillin is necessary 11.9 log UFC/mL of *Pseudomonas aeruginosa*; this allows to predict the behavior of biodegradation when changes are made in the concentration of the analyte or inoculum (UFC/87 mL).

On the other hand, in Fig. 10, dicloxacillin degradation is observed with respect to the bacterial consortium growth; in this graph, it is seen that the bacterial adaptation phase occurs in the first 2 h while the exponential phase begins after 2 h. Between 3 and 4 h, there is slight stagnation in the growth of the biomass; this was caused by a modification in the enzymatic activity, which does not affect much the exponential growth. An intersection of curves is also observed at 2.6 h, which indicates that 7.7 log UFC/mL of consortium is necessary for degrading 22 μg/mL of dicloxacillin.

With respect to the amount of degraded dicloxacillin, physicochemical methods report from 0.213 mM for photocatalysis with TiO_2 to 25 mg/L in catalytic mineralization, while this work degrades a considerably larger amount (40 mg/L).

3.4 Biodegradation products

During biodegradation with *Pseudomonas aeruginosa*, the presence of seven metabolites was observed, while with the consortium, 10 metabolites were formed of which two

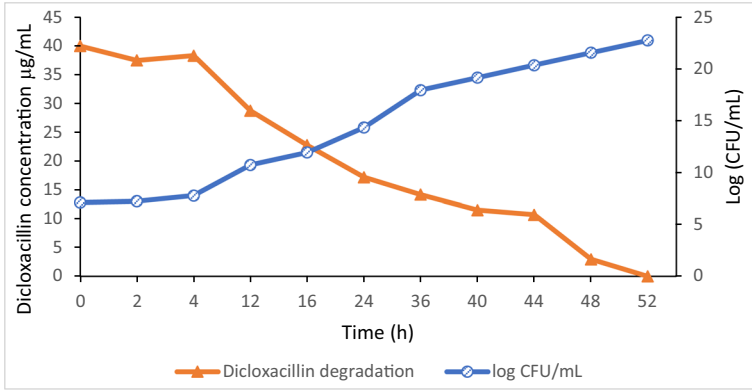

Fig. 9 Dicloxacillin degradation with respect to the *Pseudomonas aeruginosa* growth. CFU: colony forming units

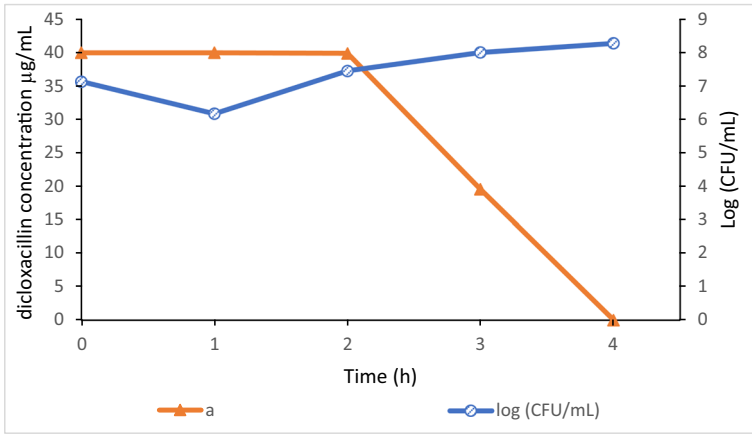

Fig. 10 Dicloxacillin degradation with respect to the bacterial consortium growth. CFU: colony forming units

are persistent in both cases (Figs. 7 and 8). Considering that all penicillins usually consist of two servings:

1. 6-aminopenyllilanic acid, resulting from the binding of a thiazolidine ring and a beta lactam ring
2. A side chain, attached to carbon 6 of the lactam ring (Malgor-Valsecia 1928)

These portions of the dicloxacillin molecule are shown in Fig. 11 marked blue and purple, respectively.

In addition, the breakage of the beta-lactam ring is known to give as the result dicloxacilloic acid, as a result of enzymatic hydrolysis of penicillins (Malgor-Valsecia 1928). And because the bacteria used in this work possess enzymes, one of the persistent and majority products may be dicloxacilloic acid, which when losing the carboxyl group gives way to the formation of dicloxilloic acid. This could be the second persistent metabolite.

In the case of the degradation of penicillin that is the basis of dicloxacillin, it is known that the main

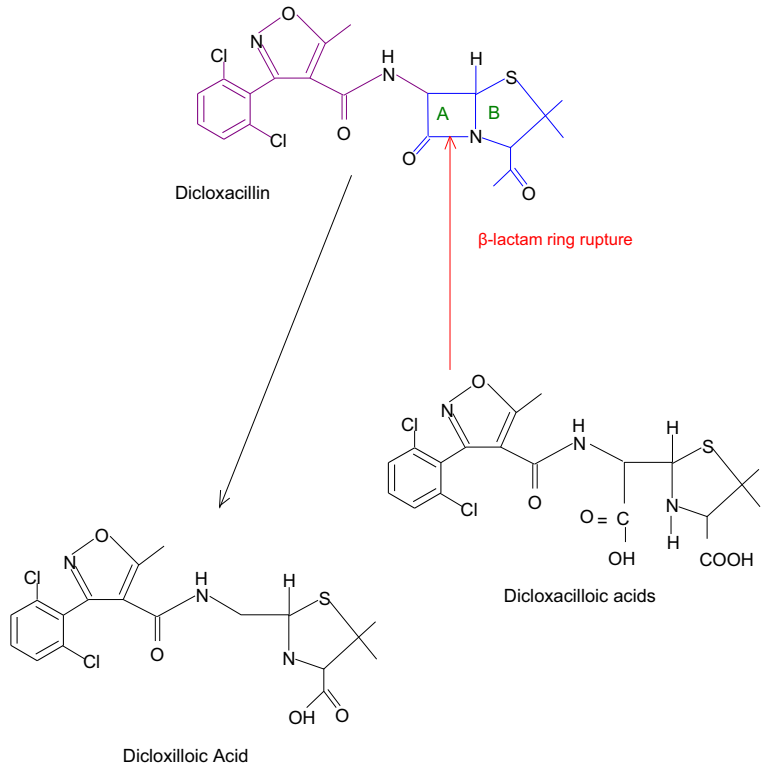

Fig. 11 Dicloxacillin degradation route proposed. **a** Beta lactam ring. **b** Thiazolidine ring

degradation products are penicilloic acid and penilloic acid, as reported by Li et al. (2008).

4 Conclusions

After analyzing and contrasting the results obtained with the work of the other researchers, it was concluded that dicloxacillin degradation using *Pseudomonas aeruginosa* and bacterial consortium as free-living bacterial is 100% efficient. When comparing this biological method with physicochemical methods, it was found to be more efficient because *Pseudomonas aeruginosa* eliminated dicloxacillin in 52 h and at 72 h 71.4% of metabolites, whereas the bacterial consortium eliminated dicloxacillin in 3.5 h and in 72 h 90% of the metabolites. On the other hand, the use of free-living bacteria is more efficient than fungi for biodegradation processes due to their speed of reproduction.

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Compliance with ethical standards

Conflict of Interests The authors declare that they have no conflict of interest.

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