BIODEGRADATION OF IBUPROFEN AND METFORMIN BY BACTERIA ISOLATED FROM CHIHUAHUA, MEXICO

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Abstract. Metformin is one of the medications used to treat non-insulin-dependent diabetes mellitus, and ibuprofen is one of the most used non-steroidal anti-inflammatory drugs worldwide. The use of these pharmaceuticals has currently generated contamination of rivers and soil since they are highly stable compounds. Therefore, the objective of this work was to evaluate the biodegradation of ibuprofen and metformin by bacteria of the genus *Bacillus*, *Paenibacillus*, and *Pseudomonas* isolated from sediments of bodies of water in the state of Chihuahua. Regarding the removal of ibuprofen, the *Bacillus* strain showed the highest removal values of 52.53% at 480 h, while *Pseudomonas* and *Paenibacillus* reached 42.93% and 44.8%, respectively. On the other hand, 99.99% of the removal of metformin was observed by the *Pseudomonas* strain at 480 h; *Bacillus* and *Paenibacillus* strains showed a removal of 97.79% and 92.36%, respectively. Thus, it is concluded that the use of the bacterial strains isolated in this study is a promising alternative for the treatment of pharmaceutical products that currently have environmental risks.
Keywords: *bioremediation, environmental health, Bacillus, Paenibacillus, Pseudomonas*

Introduction

Pharmaceutically active compounds, a certain category of micropollutants also included in the category of emerging contaminants, are reported in wastewater due to the development of human and commercial activities. Among emerging contaminants, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), metformin, hormones, pesticides, surfactants, personal care products, industrial additives, and a wide variety of chemical compounds have been reported as ethinylestradiol and diethylstilbestrol are capable of disrupting endocrine functions at low concentrations (García-Gómez et al., 2011; Nava-Carrillo et al., 2008). In this sense, the use of drugs worldwide has increased in recent decades as a consequence of the different diseases developed by human beings. Zamora-Vélez et al. (2021) conclude that the use of drugs increased significantly due to the SARS-CoV-2 virus pandemic, causing a health and environmental crisis. At a global level, different pharmaceutical compounds have been reported present in surface waters, marine waters, and as traces in waters used for human consumption (García-Gómez et al., 2011; Tejada et al., 2014; Fatehifar and Borghei, 2018; Meléndez-Marmolejo et al., 2020; Prampano and Duarte, 2020; Zamora-Vélez et al., 2021). In addition to the fact that pharmaceutical products are designed to be polar and stable compounds, which means that they are incompletely metabolized by humans and excreted into the sewage system, the few research reported conducted on

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the elimination of these contaminants has not allowed the design of water treatment systems that carry out their adequate elimination. Also, the repercussions due to ignorance and lack of environmental legislation when controlling discharges into bodies of water are a concern for health and safety in the environment (Tisler and Zwiener, 2019; Sanabria and Montagut; 2023).

Ibuprofen is an NSAID that has been reported as the third most consumed drug in the world, between 8.9% and 14% is excreted unchanged (Murdoch and Hay, 2015). Since a large amount of ibuprofen has the potential to enter the environment, its presence has been detected in irrigation, drinking, and wastewater (Siemens et al., 2008; Xu et al., 2009; Estrada-Arriaga et al., 2016). Metformin hydrochloride is a medication synthesized in the 1970s for the treatment of patients with non-insulin-dependent diabetes mellitus since it is an anti-hyperglycemic agent. This compound is a high contaminant in different aquatic ecosystems, such as rivers and/or soils depending on the place where they are discharged. This contamination is the origin of new diseases, according to the individual resorption properties in the intestine, up to 90% of the unchanged drug (metformin) is eliminated with urine within 12 h, while the rest is excreted with feces (Mijaylova et al., 2016; Arango-Granados et al., 2019).

Several investigations with biological methods have been carried out for the elimination of emerging contaminants, among which microbial remediation stands out. This is a technology in which microbial metabolism is used under specific conditions to rapidly decompose contaminants, targeting their degradation to final components, such as water and CO_2. In this sense, organisms such as fungi, bacteria, or microbial consortia turn out to be efficient in the removal of contaminants, such as pharmaceutical products (Gavrilescu et al., 2015; Estrada-Arriaga et al., 2016; Fortunato et al., 2016; Navrozidou et al., 2019; Tisler and Zwiener, 2019; Vokovi´c et al., 2019; Wu et al., 2020). For example, bacteria of the genus *Bacillus* and *Pseudomonas* have been reported in the removal of emerging pharmaceutical contaminants, presenting removal values that range between 23% and 100% of ibuprofen and metformin, respectively; therefore, they reduce the possibility of drugs entering into aquatic systems (Fortunato et al., 2016; Joshi et al., 2018; Vokovi´c et al., 2019; Prampano and Duarte, 2020). Thus, the objective of this study is to evaluate the biodegradation of ibuprofen and metformin by bacteria of the genus *Bacillus*, *Paenibacillus*, and *Pseudomonas* isolated from sediments of water bodies in the state of Chihuahua, Mexico.

Materials and methods

Microbial strains and growth conditions

The bacterial strains *Bacillus freudenreichii* and *Paenibacillus illinoisensis* were reported by Soto-Padilla et al. (2022). The *Pseudomonas* strain (B215003) was isolated from sediment collected in Conchos River (29°32′41.59” N 104°28′35.66” W) located in the state of Chihuahua, Mexico. The bacterial strains were grown in nutrient broth (DIFCO) at 37°C for 24 h at 200 rpm (222DS-LABNET) to be used as inoculum (Soto-Padilla et al., 2022).

Morphological and phylogenetic characterization of the studied bacteria

To carry out the morphological characterization of the studied bacteria, Gram staining and scanning electron microscopy (SEM) (Hitachi SU5000) were performed.

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To do this, a 48-h cell culture in nutrient broth (DIFCO) was carried out and centrifuged at 6000 rpm for 10 min, the pellet was washed and fixed with 2% formaldehyde, and the drying was carried out by dehydration using acetone and ethyl alcohol (Cruz-Soto et al., 2023). For phylogenetic identification, it was analyzed at the Genomic Biotechnology Center of the National Polytechnic Institute in Reynosa, Tamaulipas, Mexico. PCR amplification of 1500 bp from the 16S rRNA gene was performed using the universal primers: 27F(5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'TACGGYTACCTTGTTACGACTT-3'). The amplification program used was 30 cycles: denaturation at 95°C for 30", alignment at 54°C for 30", and elongation at 72°C for 1 min. The sequences obtained were subjected to bioinformatic analysis with the programs: Finch TV, CLUSTALX 2.1, SEAVIEW, and MEGA 4.0 (Soto-Padilla et al., 2022). The National Center for Biotechnology Information (NCBI) was consulted for sequence comparison. Phylogenetic trees were constructed using the Neighbor-joining method; and the Tamura-Nei model of distance analysis.

Bacterial resistance to ibuprofen and metformin

To carry out the minimum growth inhibitory concentration of bacteria *Bacillus freudenreichii*, *Paenibacillus illinoisensis* and *Pseudomonas* sp. 10% of pre-inoculum volume: volume ratio (v/v) was taken at an optical density of 0.6-0.8 nm at a wavelength of 600 nm (Lambda 25), and inoculated in nutrient broth (DIFCO) previously supplemented with the concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150 or 200 mg/L of ibuprofen [2-(4-isobutyl phenyl)propionic acid] (SIGMA), and in the same way for metformin (metformin hydrochloride) (SIGMA). Then, the inoculated culture medium were subsequently incubated at 37°C for 48 h at 200 rpm (222DS-LABNET). Bacterial growth was evaluated by UV-Vis spectrometry at a wavelength of 600 nm (Lambda 25) (Hernández et al., 2016; Marchlewicz et al., 2017).

Removal kinetics of ibuprofen and metformin

To carry out the kinetics of removal of 2-(4-isobutyl phenyl) propionic acid (ibuprofen) and metformin hydrochloride by bacteria *Bacillus freudenreichii*, *Paenibacillus illinoisensis* and *Pseudomonas* sp., 10% of pre-inoculum of each bacteria was taken in volume: volume ratio (v/v) at an optical density of 0.6-0.8 nm at a wavelength of 600 nm, and inoculated in nutrient broth previously supplemented with 100 mg/L of ibuprofen [2-(4-isobutylphenyl) propionic acid] or metformin (metformin hydrochloride), aliquots of the culture media were taken at various time intervals (0, 48, 240 and 480 h), and centrifuged at 6000 rpm for 15 min (Hernández et al., 2016; Cruz-Soto et al., 2023). Solid-phase extraction of the drugs was carried out using column HyperSep C8 (Thermo Scientific) in vacuum extraction, the extracted sample of the drugs was preserved at 4°C in methanol (Sunaric et al., 2013). Standard curves were made to determine the concentration of the drugs at concentrations of 10 to 100 mg/L, the samples were analyzed by UV-Vis spectroscopy at 220 nm and 236 nm for ibuprofen and metformin, respectively (Mijaylova et al., 2016; Marchlewicz et al., 2017). All experiments were conducted in triplicate and controls were used for ibuprofen and metformin. It was complemented with a Fourier Transform Infrared (FTIR) spectroscopy analysis (Fahim et al., 2014; Dasgupta, 2017).

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Results

The morphological traits of the studied strain are shown in *Figure 1*, which indicated the bacterial cells have a bacillary form, and the Gram stain was negative, average size of 1.0 μm. In addition, the phylogenetic relationship confirmed the taxonomy of the studied strain B215003, as *Pseudomonas* sp. (*Fig. 2*).

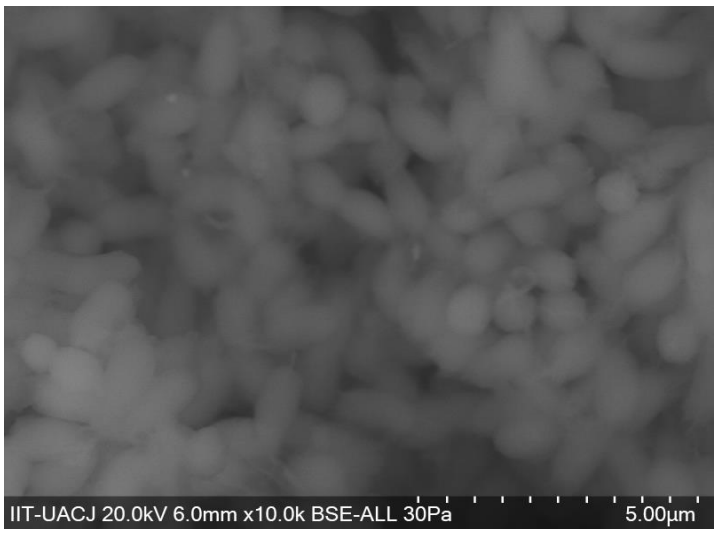

Figure 1. *Bacillary bacterial cells of the genus Pseudomonas sp. isolated from sediments of the Conchos River*

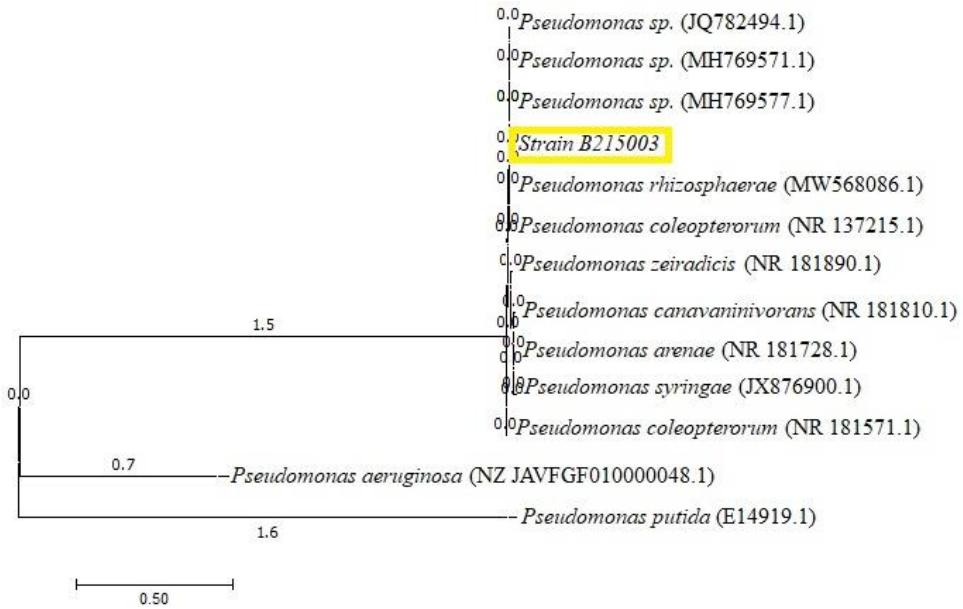

Figure 2. *Phylogenetic analysis of Pseudomonas sp. isolated from sediments of the Conchos River whit software MEGA 4.0*

Regarding to the resistance presented by the studied bacteria to different exposed concentrations of drugs, it can be identified that *Pseudomonas* sp. was inhibited by ibuprofen, due to a concentration of 20 mg/L inhibited its growth by approximately

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75%. On the other hand, *Bacillus freudenreichii* and *Paenibacillus illinoisensis* inhibited their growth by approximate 25% at 50 mg/L, and at 200 mg/L, respectively, compared to the control (*Fig. 3*).

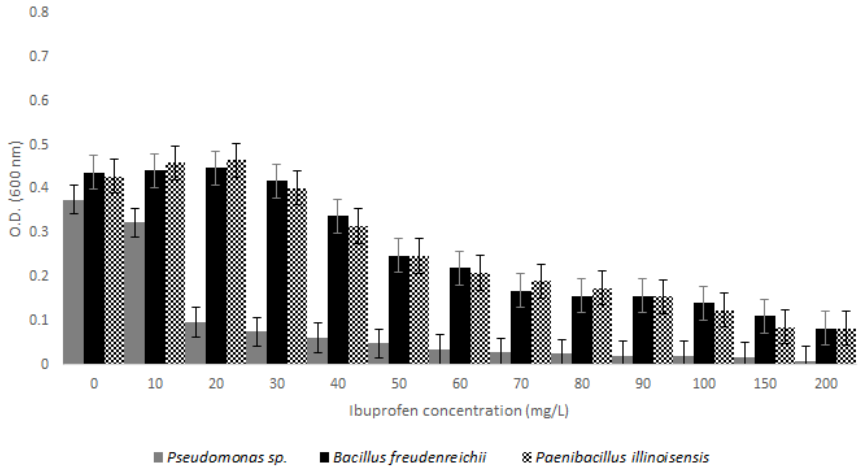

Figure 3. *Minimum inhibitory concentrations of ibuprofen for Pseudomonas sp., Bacillus freudenreichii and Paenibacillus illinoisensis*

Likewise, *Pseudomonas* sp. shows an inhibition of around 50% compared to the control at 10 mg/L of metformin. Similarly, up to the highest evaluated concentration of 200 mg/L, *Bacillus freudenreichii* shows that its cell growth is not affected by the presence of metformin. On the contrary, at a concentration of 50 mg/L the cell growth exceeds the cell development of the control by approximately 75%. The growth of the bacteria of the genus *Paenibacillus illinoisensis* appears similar in the absence and presence of all evaluated concentrations of metformin (*Fig. 4*).

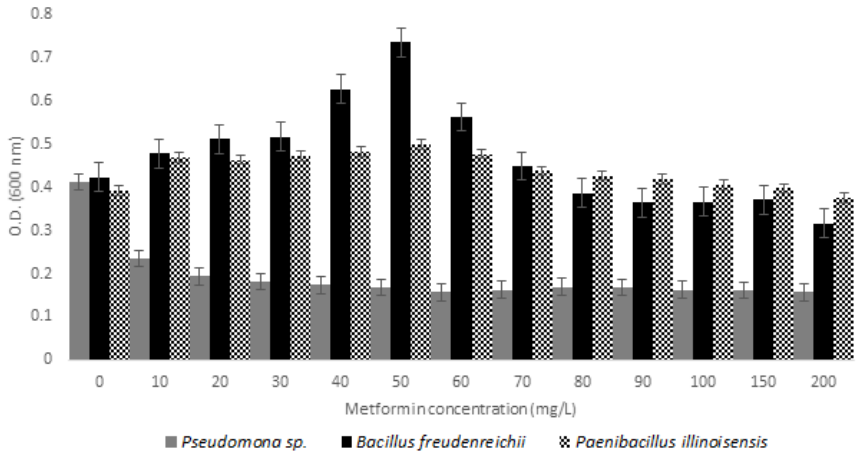

Figure 4. *Minimum inhibitory concentrations of metformin for Pseudomonas sp., Bacillus freudenreichii and Paenibacillus illinoisensis*

When evaluating the removal of ibuprofen by the studied bacterial strains, maximum removal percentages of 42.9%, 44.8% and 52.5% were obtained after 480 h by *Pseudomonas* sp., *Paenibacillus illinoisensis* and *Bacillus freudenreichii*, respectively

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(*Fig. 5*). It can be seen that *Pseudomonas* sp. and *Paenibacillus illinoisensis* already had a removal percentage greater than 30% after 48 h, while *Bacillus freudenreichii* did not show removal of ibuprofen at the same time.

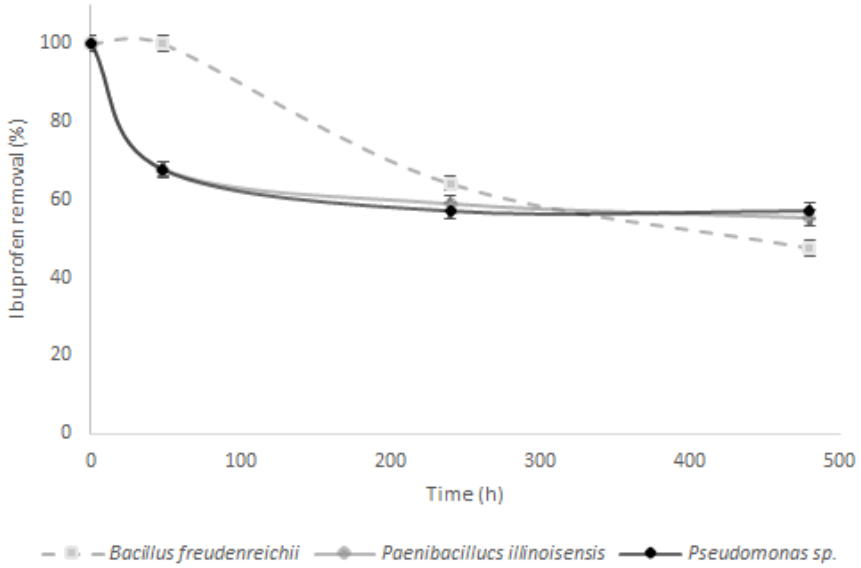

Figure 5. *Ibuprofen removal percentage curve at 480 h of Pseudomonas sp., Bacillus freudenreichii and Paenibacillus illinoisensis*

In *Figure 6*, we observe that in the first 48 h the strains studied present a significant removal of metformin, where *Bacillus freudenreichii* presented 85.1%. *Paenibacillus illinoisensis* showed 87.5% and *Pseudomonas* sp., presented 83.7%. However, the *Pseudomonas* sp. presented the highest percentage of removal after 480 h, showing 99.99%, while at this same time, *Bacillus freudenreichii* and *Paenibacillus illinoisensis* presented a metformin removal of 97.79% and 92.36%, respectively.

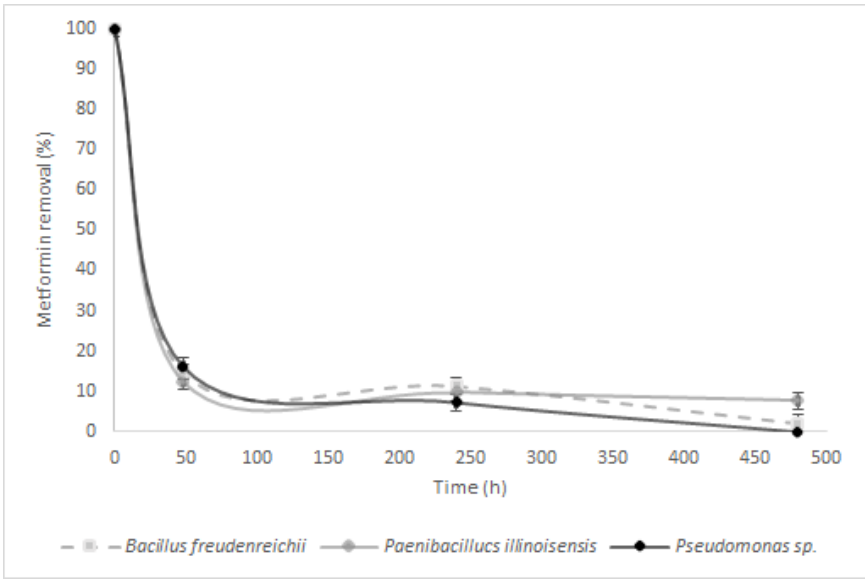

Figure 6. *Percentage removal of metformin at 480 h by Pseudomonas sp., Bacillus freudenreichii and Paenibacillus illinoisensis*

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When analyzing the FTIR spectrograms of both ibuprofen and the products obtained after its degradation by the studied strains, we observe that there is very little variation. In the spectrogram of ibuprofen, four main functional groups can be distinguished: 1) OH group at a wavelength of 2956; 2) OH group at a wavelength of 2832; 3) C-C at a wavelength of 1627; and 4) COO- at a wavelength of 1381 (*Fig. 7a*). Later, upon exposure of ibuprofen to the bacterial strains, the spectrograms show a slight decrease in the signal of the C-C functional group at a wavelength of 1627 (*Fig. 7b, c, d*).

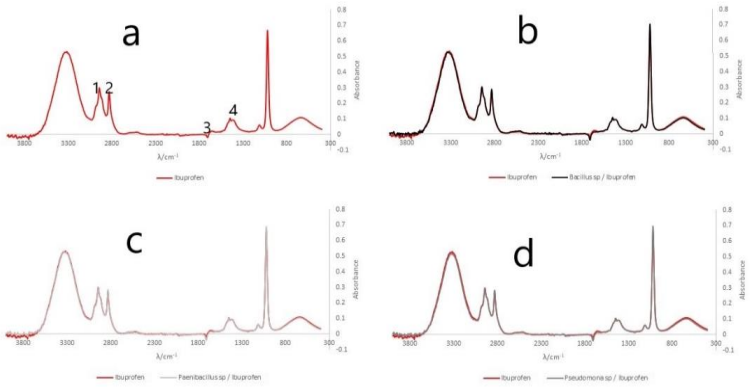

Figure 7. *FTIR spectrograms of ibuprofen. a) nutritional broth with ibuprofen; b) nutrient broth with ibuprofen after growth with Bacillus freudenreichii; c) nutrient broth with ibuprofen after growth with Paenibacillus illinoisensis; and d) nutrient broth with ibuprofen after growth with Pseudomonas sp.*

Figure 8a shows the spectrogram of metformin hydrochloride by FTIR, highlighting 1) Primary stretching of the characteristic N-H functional group with the metformin molecule between the range of 3370-3292 wavelength; 2) Secondary stretching of the N-H functional group at a wavelength of 2972; and 3) Stretching of the C-N group between 1632-1568 wavelength. In *Figure 8b, c, d*, a decrease in the signal of the N-H functional group of the primary stretching and a decrease until the elimination of the signal at the wavelength between 1632-1568 corresponding to the subsequent C-N stretching can be observed to the exposure of metformin with each of the bacterial strains.

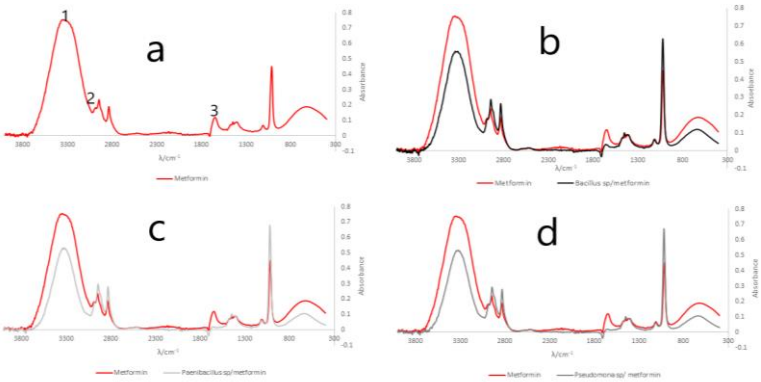

Figure 8. *FTIR spectrograms of metformin. (a) Nutrient broth with metformin; (b) nutrient broth with metformin after growth with Bacillus freudenreichii; (c) nutrient broth with metformin after growth with Paenibacillus illinoisensis; and (d) nutrient broth with metformin after growth with Pseudomonas sp.*

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Discussion

The bioremediation process of pharmaceutical products has been reported applying phytoremediation, as well as microbial removal (fungi and bacteria); within the various pharmaceutical compounds, the removal of antibiotics, hormones, and NSAIDs, has mainly been identified (Aissaoui et al., 2017; Del Alamo et al., 2017; Joshi et al., 2018; Singh and Ummalyma, 2020). Bittencourt et al. (2016), Quintana et al. (2005), and Tisler and Zwiener (2019) have reported the application of bacteria from primary sludge for the removal of hormones and pharmaceutical products, demonstrating that various genera of bacteria can be applied in bioremediation processes of emerging contaminants. Soto-Padilla et al. (2022) reports the capacity of bacterial strains of the *Bacillus* and *Paenibacillus* genera isolated in bodies of water in the state of Chihuahua, with the ability to remove lead and arsenic, concluding that the bacterial strains *Bacillus freudenreichii* and *Paenibacillus illinoisensis* can be applied in processes for removing emerging contaminants. Marchlewicz et al. (2017), report the degradation of ibuprofen using *Bacillus thuringiensis*. In the same way, Prampano and Duarte (2020), have used bacterial strains of *Bacillus* and *Pseudomonas*, and demonstrated the capacity of these microorganisms to use NSAIDs as the only source of energy and be able to reduce their presence in water systems. In *Figure 1,* we can identify the traits reported for species of the genus *Pseudomonas*, agreeing with what was reported as a Gram-negative, aerobic bacilli and grow at temperatures that vary from 18°C to 42°C (Bustos et al., 2009). The phylogenetic relationship relates the bacteria studied to species of the genus *Pseudomonas*, which are applied in the removal of emerging contaminants (Prampano and Duarte, 2020). The phylogenetic tree in which the similarity relationships shows that it belongs to the genus *Pseudomonas,* but a species is not assigned, because 100% similarity occurs with species sp. (*Fig. 2*). The results obtained in the evaluation of minimal inhibitory growth (*Fig. 3*) coincide with the results presented by Prampano and Duarte (2020) and Marchlewicz et al. (2017), who demonstrate that the presence of ibuprofen in bacterial strains of the genus *Bacillus* and *Pseudomonas* does not affect their growth, since bacterial growth occurs at 200 mg/L. Regarding the effect on the bacterial strains evaluated in the presence of metformin (*Fig. 4*), no research is reported that includes these evaluations, they mainly focus on the detection of metformin biodegradation products (Gurav and Bathia, 2018; Tisler and Zwiener, 2019). When comparing the results obtained in the removal of ibuprofen, it is observed that the percentages shown in our results (*Fig. 5*) are within the ranges of ibuprofen removal carried out by other researchers, ranging from 37.33% to 95.9% removal using strains of the genus *Bacillus* and *Pseudomonas*, as well as microbial consortia (Marchlewicz et al., 2017; Fatehifar et al., 2018; Prampano and Duarte, 2020; Khan et al., 2022). Zwinner et al. (2002) and Quintana et al. (2005) observed the degradation of ibuprofen-forming metabolites during degradation in bioreactors. These authors suggest that the first reaction of ibuprofen utilization is the transformation of the aliphatic chain of ibuprofen under oxic conditions. Mijaylova et al. (2016) showed the degradation of metformin by evaluating with a bacterial consortium, in the aerobic phase 71% of it was removed in 576 h, in respect to the results obtained in the experiment, the percentages of removal are higher than the reported, since the strain of *Bacillus freudenreichii* removed 97.79%, *Paenibacillus illinoisensis* removed 92.36% and the strain *Pseudomonas* sp. removed 99.995% of metformin hydrochloride in 480 h. Tisler and Zwiener (2019) show the removal of metformin in a treatment plant using activated sludge which results in the degradation in an aerobic system of 99% of 5 mg/L of

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metformin in 7 days. Fourier transform infrared spectroscopy (FTIR), in recent years, has proven to be a simple, rapid, and ecological method for the quantification of many active components in pharmaceutical samples, since in most cases no prior preparation is required. of the sample. The functional groups identified in the IFTR spectrograms of ibuprofen and metformin show characteristic groups reported by Dasgupta (2017) and Fahim et al. (2014) respectively, when comparing each of the drugs, the decrease in certain functional groups can be visualized, which are directly related to the removal of said drug due to the presence of bacterial strains.

Conclusion

According to the results obtained and the analyses carried out, it can be concluded that the *Bacillus freudenreichii* strain may be applied in ibuprofen biodegradation processes, and in the case of metformin biodegradation, the *Bacillus freudenreichii*, *Paenibacillus illinoisensis*, and *Pseudomonas* sp. strains may be applied.

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