International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Penicillin as a Carbon and Energy Source for Microorganisms from Crude - Oil Contaminated Soil A Novel Approach to Bioremediation

Leonela Herrera[1], Maximiliano Eduardo Gutierrez[2], Maite Soledad Baztan[3], Graciela Natalia Pucci[4]

Microbiology - Biochemistry Department - CEIMA - Universidad Nacional de la Patagonia San Juan Bosco – Argentina
Email: *puccigraciela[at]gmail.com*

Abstract: ***Antibiotics, including penicillin, are considered emerging pollutants due to their continuous input and persistence in the environment even at low concentrations. Thus, several techniques for the degradation and mineralization of antibiotics have been evaluated to reduce their environmental impact. The objective of this work was to search for bacterial consortia from crude oil - contaminated soils, capable of using penicillin as a carbon and energy source and/or secreting enzymatic compounds to neutralize this antibiotic. The degradation of penicillin was evaluated using the closed - bottle technique, in which the bottle contained penicillin, the bacterial inoculum, and a mineral medium at a concentration of 220 ppm. The bacteria were counted, the degradation of penicillin was determined by optical density at 245 nm, and FT - IR spectroscopy was performed. At 14 days, FT - IR spectrum analyses of penicillin showed the disappearance of peaks at 1673 cm^{-1} and 1106 cm^{-1}. These results indicate that some bacteria from the oil - contaminated soil studied had developed the ability to degrade penicillin (by secreting* extended *spectrum β - lactamases (ESBL) and metallo - β - lactamases (MBL)), whereas others had developed resistance to penicillin. The bacterial communities used penicillin at a rate of 54.34%. Eleven strains were isolated and identified as Pseudomonas.***

Keywords: penicillin, degradation, bacteria

1. Introduction

Antibiotics are the second most common group of drugs present in wastewater worldwide due to failures in industrial waste treatments and their irrational use in humans and animals (Poutou - Piñales et al., 2022). In addition, antibiotics are currently considered emerging pollutants due to their continuous input and persistence in the environment even at low concentrations. Thus, several techniques have been evaluated for the degradation and mineralization of antibiotics to reduce their environmental impact (Poutou - Piñales et al., 2022; Homem et al., 2011; Abdurahman et al., 2021). These techniques include physical ones, such as sedimentation and filtration, chemical oxidation, UV ultrasound and ions, and biological ones, such as the use of microorganisms and their enzymes (Liu et al., 2021; Pan et al., 2016; Cycon et al., 2019; Wu et al., 2021; Calcio et al., 2021).

Regarding the latter, some bacteria, such as *Bacillus* sp., *Klebsiella* sp., *Shewanella* sp., *Sphingobacterium* sp., and *Stenotrophomonas maltophilia*, have been shown to be able to degrade tetracyclines (Ahmad et al., 2021), whereas others such as micrococci and *Stenotrophomonas maltophilia* have been shown to be able to degrade ciprofloxacin (Gutierrez et al., 2022). In *Stenotrophomonas maltophilia*, the degradation routes so far identified include reactions such as denitromethylation, decarbonylation, and deamination (Ahmad et al., 2021). Some of the bacteria mentioned above are found in hydrocarbon - contaminated soils of Argentine Patagonia and have become adapted to using recalcitrant carbon resources (Acuña & Pucci 2023).

Alexy et al. (2004) studied the degradation of different antibiotics such as amoxicillin, benzylpenicillin, ceftriaxone, cefuroxime, chlortetracycline, clindamycin, erythromycin, gentamicin sulfate, imipenem, ofloxacin, and sulfamethoxazole by the closed - bottle technique, in which the bottle contained a bacterial inoculum, the antibiotic to be tested, a mineral medium. Results showed that less than 60% of these antibiotics were biodegraded in 28 days. This technique is inexpensive to study antibiotic degradation because it has a bias towards the growth of Gram negative bacteria, which are highly resistant to antibiotics.

Little is known about the impact of antibiotics on the growth from microbial populations of oil - contaminated soils. The aim of this study was to investigate oil - degrading microbial populations to find a bacterial community able to use penicillin G as a carbon and energy source, in order to better understand the behavior of microbial populations able to degrade penicillin G.

2. Material and methods

Study of the ability of the bacterial community to biodegrade penicillin by the closed bottle test. To search for bacterial consortia able to biodegrade penicillin, the bacterial community was obtained from hydrocarbon - contaminated soils from Argentine Patagonia and then inoculated into a mineral salt medium containing 20 mg L^{-1} penicillin G as the sole carbon source. This was achieved through ten successive subcultures before incubation at 28°C on a rotary shaker. The ability of the bacterial community to biodegrade penicillin was measured using the closed - bottle technique, by monitoring CO_2 accumulation using NaOH to capture it. To this end, 5 mL of the bacterial community with a turbidity of 1 McFarland was poured into a brown bottle with 95 mL of mineral salt medium (K_2HPO_4 0.5 g; KH_2PO_3 0.5 g; $MgSO_4$ 0.2 g; $(NH_4)NO_3$ 1 g; yeast extract 0.025 g; pluripeptone 0.025 g) and without other hydrocarbons or nutrients. NaOH was titrated using HCl 0.1N (Bartha et al., 1979).

Enumeration and isolation of aerobic bacteria
Culturable bacteria from the sample were counted using the standard plate dilution method. A serial dilution of physiological sterile water (pH 7.2) was vortexed for 1 min at low speed. Aliquots of 100 µL of 10^{-1} to 10^{-6} dilutions were grown on trypticase soy broth agar (TSBA), which consisted of trypticase soy broth (30 g/L) and granulated agar (15 g/L).

FT - IR spectroscopy
Samples were then analyzed on a Varian equipment, operated in the spectral window from 400 to 4000 waves/cm with 32 scans/sample with a resolution of 4 waves/cm, a scanning speed of 10 kHz and a DTGS detector. Three spectra were sequentially acquired for each sample. These spectra were processed and analyzed with the aid of the Varian resolution software. The region from 700 to 900 waves/cm was used for bacterial fingerprinting.

Optical density
The degradation of penicillin G by the bacterial community was determined by optical density (OD) at 245 nm for 14 days.

Search for β - lactamases
Next, to determine whether the bacterial community studied was able to secrete enzymes able to neutralize penicillin, β - lactamases were searched for Extended - spectrum β - lactamases (ESBL) were detected by using the double - disc synergy method (Lezameta et al., 2010), following the Jarlier method (Committee of the French Society for Microbiology). In this test, plates with Müeller Hinton culture medium were inoculated with the bacterial strains, with a turbidity of 0.5 McFarland. An amoxicillin/clavulanic acid disk was placed in the center of a petri dish, and ceftazidime, ceftriaxone and aztreonam disks were placed around it at a 20 - mm center - to - center distance from the former. The dish was read after 24 - 48 hours of incubation at 28°C.

To search for metallo - β - lactamases (MBL) able to produce carbapenemases (Sánchez et al., 2008), the plates were inoculated according to the recommendations of the Clinical and Laboratory Standards Institute for the disc diffusion test. In the same way, as in the previous test, plates with Müeller Hinton culture medium were used and inoculated with a strain suspension with a turbidity of 0.5 McFarland. Then, discs containing ethylenediaminetetraacetic acid (EDTA) and commercial discs containing ceftazidime, imipenem, and meropenem were placed at a 15 - mm center - to - center distance from the former. The dish was read after 24 - 48 hours of incubation at 28°C.

Strain identification
To identify the bacterial strains, acid extraction was performed on 40 mg of bacteria obtained from previous three successive subcultures on TSBA plates, starting with saponification using methanol: sodium hydroxide: water (150 mL: 45 g: 150 mL), followed by methylation with 6N hydrochloric acid and methanol (325 mL: 275 mL), and then extraction with n - hexane - methyl tert - butyl ether (1: 1) and washing with sodium hydroxide - water (10.8 g - 900 mL), according to the procedure of the identification system (MIDI Newark, DE, USA - www.midi - inc. com). Fatty acids were determined as methyl esters by gas chromatography according to the Sherlock - MIDI procedure.

3. Results and Discussion

In the present study, we investigated the biodegradation of penicillin G by a bacterial community from oil - contaminated soils by using the closed bottle technique (Fig 1). Penicillins belong to the β - lactam class of antibiotics and contain a β - lactam ring fused with a thiazolidine ring. In agreement with that found by Al - Ahmad et al. (1999), the biodegradation in the closed bottle within the period studied (14 days) increased only for penicillin. This indicates that the bacterial community of this work can break the ß - lactam ring in 14 days. This also indicates that the bacteria used had not adapted to the test compound (i. e. penicillin). The compounds containing a ß - lactam ring could have been chemically degraded by hydrolytic ring opening without oxygen consumption. In our case, penicillin biodegradation was followed by CO_2 accumulation and monitored by FT - IR. However, the hydrolysis products were not readily biodegradable and bacterial activity was still present. The absorbance at 245 nm allowed monitoring the presence of chemical groups of penicillin by the spectrophotometer. The biodegradation or use of this antibiotic produces mineralization (Poppo Trujillo, 2009), which can be used to assess this process (Martínez Pérez, 2007). Similar results have been observed in previous studies on the degradation of ceftazidime, demonstrating that the bacterial consortia from oil - contaminated sites can also adapt to other complex compounds (Gutiérrez et al., 2022). Other authors have shown that, under certain conditions, antibiotics are biodegradable in water (Drillia et al., 2005; Gartiser et al., 2007) and in the soil (Reis et al., 2020). This result may depend on the adsorption, *i. e.*, low bioavailability, of the test compounds.

The results of the present study were evaluated through FT - IR spectroscopy analysis. This analysis showed the following absorption bands (Fig.2) and a change in the structure of penicillin. On day 14, the band at 3165 cm^{-1} showed a decrease compared to that observed on days 1 and 7. This band represents the O - H bond of the functional group –COOH (carboxylic acid). At 3040 cm^{-1}, the intensity of the characteristic band of the C - H bond of the aromatic ring also decreased. At 1765 cm^{-1}, the characteristic band of the stretching of the C=O (ketone) bond present in the β - lactam ring, as well as the band at 1338 cm^{-1}, which represents the C - N bond on the β - lactam ring, also showed a decrease in their intensity. The absorption band at 1673 cm^{-1}, which corresponds to the deformation of the N - H bond of the amide functional group of the penicillin structure, also decreased in intensity. Another band that showed a decrease in its intensity was the band at 953 cm^{-1}, which represents the C - OH stretching of the –COOH (carboxylic acid) functional group. The absorption band that disappeared was the one that appeared at 1106 cm^{-1}, which represents the bending of the C - CO - N bond of the C=O (ketone) functional group of the β - lactam ring. The disappearance of this absorption band suggests that the antibiotic was hydrolyzed due to the presence of β - lactamases, which were found in some of the bacteria isolated. This evidences that β - lactamases act by breaking the amide bond of the β - lactam ring, previously

bound to the carboxyl group (β - lactam antibiotics, sf.). Reis et al. (2020) reported the degradation of penicillin through hydrolysis and photolysis measured by organic matter but stated that the degradation occurs mainly by the hydrolysis of the β - lactam ring, which generates degradation products that are often found at higher concentrations than the original form. According to Cirilo et al. (2013), that product would be penicilloic acid.

Antibiotics present in the environment are biodegraded by microorganisms and enzymes. In the present study, the bacterial consortium studied used penicillin as a carbon and energy source, generating a degradation curve monitored at 245 nm and a growth curve observed by CO_2 accumulation (Fig.1). This growth corresponded to an increase from $2.89x10^7$ colony - forming units (cfu) /mL to $2.71x10^8$ cfu/mL, where 11 bacterial domains of Gram - negative ESBL - and MBL - positive bacteria were isolated (Table 1). Microorganisms can produce antibiotic - degrading enzymes, such as β - lactamases, which can cleave the β - lactam rings of different toxins, such as cyanotoxins, and antibiotics, such as penicillins and cephalosporins. Based on the substrate specificity of β - lactamases, they can be roughly divided into three categories: penicillinases, cephalosporinases, and oxime - type cephalosporinases (Singh et al., 2023). Penicillin enzymes easily break down penicillin antibiotics, while cephalosporin enzymes have higher activity in breaking down cephalosporin antibiotics. Cephalosporin oxime enzymes can degrade both penicillin and cephalosporin, with particular efficiency against cephalosporin oxime. Enzyme systems can be constructed for effective antibiotic degradation (Liu et al., 2021); these are often immobilized on Fe_3O_4 magnetic nanoparticles (Gao et al., 2017b) to facilitate β - lactamase immobilization for penicillin degradation.

ESBL were detected in seven of the eleven strains isolated, and seven of them showed ability to produce metallo - β - carbapenemases (Table 1). These bacterial strains, which hydrolyze penicillin G through β - lactam or carbapenemase enzymes, are resistant to this antibiotic but can use it as a source of carbon and energy when associated with other bacteria. The study of the bacteria isolated showed that several were able to produce β - lactamases, which coincides with the biodegradation of cephalosporins in lake sediments and soils (Reis et al., 2020).

Further studies on the biodegradation of antibiotics should be focused on specific factors of bacterial communities that have been identified to be necessary for the biodegradation of anthropogenic molecules, and on the optimization of the factors already identified as necessary for the biodegradation of antibiotics to prevent antibiotic resistance.

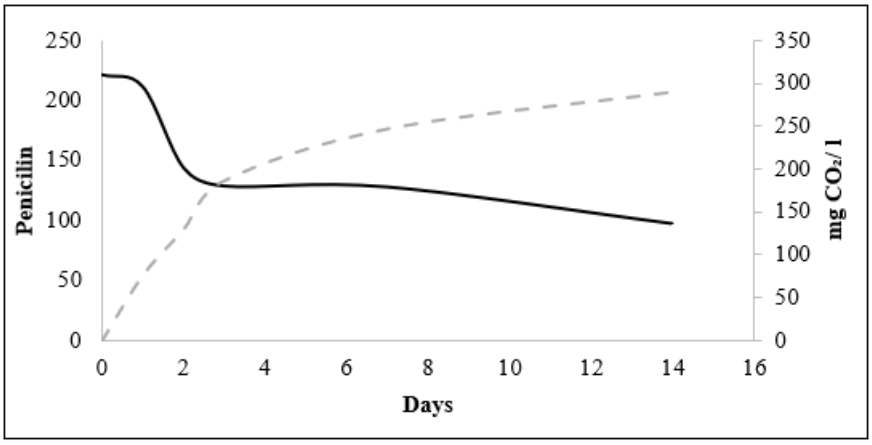

Figure 1: Closed - bottle test by CO_2 accumulation and optical density at 245 nm

Table 1: Bacteria isolated from the oil - contaminated soil community studied and ESBL and MBL enzyms.

Bacterial strains isolated	ESBL	MBL	ID
1 - PG1	(+)	(+)	*Pseudomonas aeruginosa 0.884*
2 - PG2	(+)	(+)	*Bacillus sp 0.520*
3 - PG3	(+)	(-)	*Cellulomonas femi 0.694*
4 - PG4	(-)	(-)	*Virgibacillus pantothenticus 0.785*
5 - PG5	(-)	(+)	*Cellulomonas fimi 0.686*
6 - P15	(-)	(-)	*Pseudomonas aeruginosa 0.817*
7 - P1	(+)	(+)	*Pseudomonas aeruginosa 0.838*
8 - P2	(+)	(+)	*Pseudomonas aeruginosa 0.736*
9 - P3	(+)	(-)	*Pseudomonas aeruginosa 0.863*
10 - P4	ESBL (+)	+	*Pseudomonas aeruginosa 0.935*
11 - P5	ESBL (-)	+	*Pseudomonas aeruginosa 0.940*

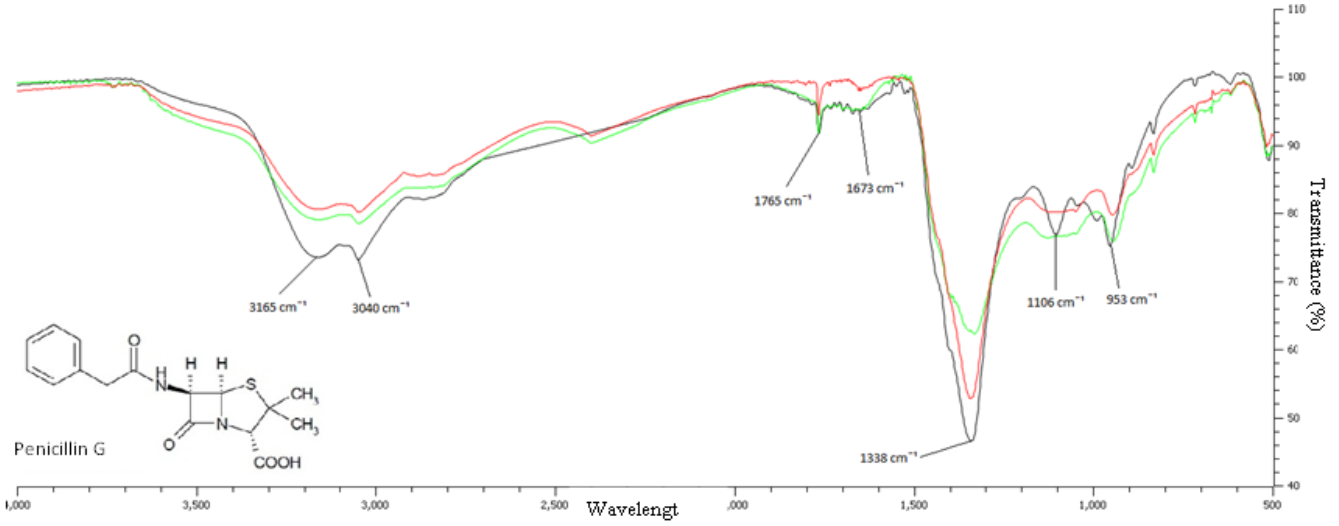

Figure 2: FT - IR analysis of penicillin. The FT - IR spectrum of penicillin G showed absorption bands at 3, 388 cm^{-1} (O–H stretching), 3, 272 cm^{-1} (C–H stretching), 3, 004 cm^{-1} (C–H stretching), 2, 101 cm^{-1} (C≡C stretching), 1, 701 cm^{-1} (C = O group), 1, 644 cm^{-1} (C = C), 1, 419 cm^{-1} (C–H stretching), 1, 360 cm^{-1} (C–H bending), 1, 232 cm^{-1} (C–O stretching), and 1, 093 cm^{-1} (O–H bending). Only the functional groups at 1, 232 cm^{-1} (C–O stretching) and 1, 419 cm^{-1} (C–H stretching).

References

[1] Abdurahman, M. H., Abdullah, A. Z. and Shoparwe, N. F. (2021). A comprehensive review on sonocatalytic, photocatalytic, and sonophotocatalytic processes for the degradation of antibiotics in water: Synergistic mechanism and degradation pathway. *Chem. Eng. J.*413, 127412.

[2] Acuña, A. y Pucci, G. N. (2023). Desarrollo de una comunidad bacteriana y de cepas aisladas a expensas de utilización de Claritromicina como fuente de carbono y energía. Re*vista Ingeniería y Sanitaria Ambiental.*147: 42 - 47.

[3] Ahmad, F., Zhu, D. and Sun, J. (2021). Environmental fate of tetracycline antibiotics: Degradation pathway mechanisms, challenges, and perspectives. *Environ. Sci. Eur.*33, 64.

[4] Al - Ahmad, A., Daschner, F. D. and Kümmerer, K. (1999). Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of wastewater bacteria. *Archives of environmental contamination and toxicology*, *37*, 158 - 163.

[5] Alexy, R., Kumpel, T. and Kummerer, K. (2004). Assessment of degradation of 18 antibiotics in the Closed Bottle Test. *Chemosphere*.57, 505–512.

[6] Bartha, R. (1979). Effect of environmental parameters on the biodegradation of oil sludge. *Applied and Environmental Microbiology*.37 (4): 729 - 239. Doi. org/0099 - 2240/79/04 - 0729/11$02.00/0.

[7] Calcio Gaudino, E., Canova, E., Liu, P., Wu, Z. and Cravotto, G. (2021). Degradation of Antibiotics in Wastewater: New Advances in Cavitational Treatments. *Molecules*, 26, 617.

[8] Cirilo, A., LLombart, C. y Tamargo, J. (2013). Introducción a la química terapéutica (2° edición). Diaz de Santos. Barcelona.

[9] Cycon, M., Mrozik, A. and Piotrowska - Seget, Z. (2019). Antibiotics in the Soil Environment - Degradation and Their Impact on Microbial Activity and Diversity. *Front. Microbiol.*10, 338.

[10] Drillia, P., Dokianakis, S. N., Fountoulakis, M. S., Kornaros, M., Stamatelatou, K. and Lyberatos, G. (2005). On the occasional biodegradation of pharmaceuticals in the activated sludge process: The example of the antibiotic sulfamethoxazole. *J. Hazard. Mater.*122 (3): 259 - 265.

[11] Gao, X. J., Fan, X. J., Chen, X. P., &amp, Ge, Z. Q. (2018). Immobilized β - lactamase on Fe 3 O 4 magnetic nanoparticles for degradation of β - lactam antibiotics in wastewater. International *Journal of Environmental Science and Technology*, 15, 2203 - 2212

[12] Gartiser, S., Elke, U., Radka, A. and Kümmerer, K. (2007). Ultimate biodegradation and elimination of antibiotics in inherent tests. Chemosphere, 67 (3): 604 - 613.

[13] Gutiérrez, M. E., Acuña, A. J. y Pucci G. N. (2022). Utilización de ciprofloxacina como única fuente de carbono.7mo. Encuentro de investigadores de la Patagonia Austral.24, 25 y 26 de octubre. Puerto San Julián.

[14] Homem, V. and Santos, L. (2011). Degradation and removal methods of antibiotics from aqueous matrices. *A review. J. Env. Manag.*92, 2304–2347.

[15] Lezameta, L., Gonzáles, E., y Tamariz, J. (2010). Comparación de cuatro métodos fenotípicos para la detección de betalactamasas de espectro extendido. *Revista Peruana de Medicina Experimental y Salud Pública.* Vol.27. http: //www.scielo. org. pe/scielo. php?script=sci_arttext&amp, pid=S1726 - 46342010000300006.

[16] Liu, P., Wu, Z., Abramova, A. V. and Cravotto, G. (2021). Sonochemical processes for the degradation of antibiotics in aqueous solutions: *A review. Ultrason. Sonochem.*74, 105566.

[17] Martínez Pérez, M. D. L. Á. (2007). Diseño de una prueba de biodegradabilidad anóxica de compuestos orgánicos en medio líquido. Universidad autónoma del Estado de Hidalgo. Instituto de Ciencias Básicas e Ingeniería. Centro de investigaciones químicas.

[18] Pan, M. and Chu, L. M. (2016). Adsorption and degradation of five selected antibiotics in agricultural soil. *Sci. Total Environ.*545–546, 48–56.
[19] Poppo Trujillo, D. (2009). Aplicación del test de generación de CO_2 por Head Space en el estudio de la biodegradación aerobia del Diazinon en lodos activados.
[20] Poutou - Piñales, R., Pedroza - Rodríguez, A. M. and Quevedo - Hidalgo, B. E. (2022). Degradation of Antibiotics. *Scholarly Community Encyclopedia*.
[21] Reis, A. C., Kolvenbach, B. A., Nunes, O. C. and Corvini, P. F. (2020). Biodegradation of antibiotics: the new resistance determinants–part II. *New biotechnology*, 54: 13 - 27.
[22] Sánchez, D., Marcano, D., Spadola, E., León, L., Payares, D., Ugarte, C., Salgado, N., Maggi, G., Guevara, A., Torres, S., Rodríguez, J., Flores, A., Tarazona, B. (2008). Metaloenzimas tipo VIM detectadas en aislamientos clínicos en Pseudomonas aeruginosa en cuatro hospitales de Venezuela. *Revista del Instituto Nacional de Higiene Rafael Rangel*. Vol.39. https: //ve. scielo. org/scielo. php?script=sci_arttext&amp, pid=S0798 - 04772008000200004.
[23] Singh, A., Chaurasia, D., Khan, N., Singh, E., &amp, Bhargava, P. C. (2023). Efficient mitigation of emerging antibiotics residues from water matrix: Integrated approaches and sustainable technologies. *Environmental Pollution*, 121552.
[24] Wu, S. and Hu, Y. H. (2021). A comprehensive review on catalysts for electrocatalytic and photoelectrocatalytic degradation of antibiotics. *Chem. Eng. J.*409, 127739.