Amoxicillin Biodegradation with *Bacillus subtilis* and *Pseudomonas aeruginosa*: Characterization of Relevant Degradation Products

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The widespread use of antibiotics can result in the release of a large number of residues into the environment. In order to reduce the exposure risk, considerable research has been carried out in the field of antibiotic residues. We aim to explore the possibilities of antibiotic amoxicillin biodegradation in laboratory scale by adding microbiological cultures, *Pseudomonas aeruginosa* (Gram-negative) and *Bacillus subtilis* (Gram-positive), which are a type of bacteria that are commonly found in the environment, such as in soil and water. Apart from the degradation itself, special attention was paid to the identification of antibiotic residues. The biodegradation process was investigated, revealing a 99.2 % degradation efficiency at amoxicillin concentration of 0.2 mg mL^{-1}, and four degradation products were identified. For reaction monitoring and identification of degradation products, UHPLC and LC-MS analyses were performed. Furthermore, to provide additional evidence, the products were independently synthesized and then compared spectrometrically with the obtained biodegradation products. These results could provide new aspects to the behavior of amoxicillin, and pave the way for further monitoring and studies of its residues in the environment.

Keywords

antibiotic residues, environment contamination, amoxicillin, biodegradation, biodegradation products

Introduction

Antibiotics are one of the main medicinal discoveries of the last century and are used today for the treatment of a large number of bacterial infections, not only in humans but also in livestock production.[1–3] Uncontrolled and improper antibiotic consumption has a significant influence on bacterial communities. As they are exposed to high levels of antibiotic residues, they can react in many different ways, including the development of resistance.[4,5] Thus, antibiotics often lose efficiency in the treatment of diseases and infections that were curable previously, so there is an increasing need for the introduction and use of new antibiotics with a wide spectrum. Regardless of their origin, antibiotics eventually end up in the soil and aquatic environment through wastewater, sludge, and manure used in agriculture. Finally, they may appear in the exposed parts of the environment or even in food,[6,7] thus exposing humans and livestock to selectively resistant microorganisms.[1,8] Many aquatic organisms are harmed by residual antibiotics, which is why some antibiotics are under severe observation programs, for example, amoxicillin and ciprofloxacin.[9]

There are various methods of attempting to reduce and control the concentration of residual antibiotics in the environment, aiming primarily to reduce development of resistance among the bacterial species. The biological activity of antibiotics in different matrices in nature depends on how well they are suited or rejected by environmental conditions.[10–12] So far, a number of mechanisms of antibiotic degradation in nature have been revealed. The mechanisms can be biotic, such as biodegradation by bacteria and fungi, and abiotic, which include hydrolytic, photolytic, oxidation or reduction actions. The mechanism depends on the physical or chemical properties of the substance and local con-

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ditions in the environment that is in the focus of observation.[1] The transformation of the substance itself is generally faster in aerobic conditions than in those anaerobic, and higher temperatures favor the degradation of antibiotics.[13] Unfortunately, the degradation products can also affect the microbiological community itself, and these effects do not have to be the same as those of the "mother" component.

One of the most common groups of antibiotics are beta-lactam antibiotics, which include amoxicillin. Due to the beta-lactam ring's tendency to hydrolysize, beta-lactam antibiotics are rarely found in the environment in their original form. So far, a number of successful studies on the biodegradation of antibiotics, including beta-lactam antibiotics, have been performed, but a scant amount of information is provided on the decomposition products and their impact on the environment, which may be present in a larger proportion than the drug itself.[14]

Baghapour *et al.*[15] have carried out bacterial biodegradation of amoxicillin with a submerged biological aerated filter in an aquatic environment. The maximum efficiency of amoxicillin removal was 50.7 %; increasing the initial amoxicillin concentration resulted in a more effective removal.[15]

Yang *et al.*[16] have examined the degradation of amoxicillin, tetracycline, and sulfonamide antibiotics in wastewater sludge with bacteria that degrade antibiotics under aerobic and anaerobic conditions. Four bacterial strains were used for antibiotic degradation: SF1 (*Pseudomonas* sp.), A12 (*Pseudomonas* sp.), B (*Bacillus* sp.), and SANA (*Clostridium* sp.). Strains SF1 and A12 showed the highest degradation capacity under aerobic conditions, and strains B and SANA showed the highest efficiency under anaerobic conditions. The highest degree of decomposition occurred with a combination of two strains. The anaerobic biodegradation required a longer time to achieve the same overall efficiency. The efficiency of amoxicillin biodegradation with strains SF1 and A12 in aerobic conditions was 93.4 % after 8 days, and with strains B and SANA in anaerobic conditions it was 93.6 % after 15 days.[16]

In this work, the biodegradation of amoxicillin was studied in laboratory conditions using environmental bacterial cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*, focusing on the resulting degradation products.

Materials and methods

Chemicals

Amoxicillin solutions (Amox) were prepared in laboratory by dissolving amoxicillin ($C_{16}H_{19}N_3O_5S$, Sigma Aldrich, Saint Louis, USA) in ultra-pure water (LC grade) in concentrations of $\gamma_1 = 0.2$ mg mL^{-1} and $\gamma_2 = 0.5$ mg mL^{-1}.

Bacterial cultures of *Bacillus subtilis* (3020) and *Pseudomonas aeruginosa* (3011) from the Microorganism Collection of the University of Zagreb, Faculty of Chemical Engineering and Technology, Department of Industrial Ecology were used for biodegradation. Nutrient agar (Biolife TS-401810) was a nutrient medium used for growing bacterial cultures. *P. aeruginosa* is designated Pa, and the designation for *B. subtilis* is Bs.

Biodegradation experiment

Biodegradation experiments were performed in 1000-mL Erlenmeyer flasks on a thermostatic rotary shaker (Heidolph Unimax 1010, Heidolph Instruments GmbH & Co., Germany) at $T = 25\pm1$ °C and 150 rpm for 7 days. To eliminate the influence of photodegradation, the biodegradation experiment was performed in the dark. Two experiments were performed for each bacterial culture with initial amoxicillin concentrations of 0.2 and 0.5 mg mL^{-1}. The optical density of the prepared bacterial suspension was 0.13 A.U., at λ = 600 nm (Hach, DR/2400, Loveland, Colorado, USA). Each flask contained 490 mL of amoxicillin and 10 mL of bacterial cell suspension. Samples were collected on days 0, 0.5, 1, 3, 5, and 7, filtered through a 0.45-µm filter (Millipore Millex-HV, Hydrophilic PVDF), and stored at 4 °C until analysis.

Analytical methods

UHPLC analysis

Solutions for LC analysis were freshly prepared mixtures of LC-grade chemicals, methanol (J.T. Baker) and formic acid (Sigma-Aldrich). Aquatic solutions were prepared from UHPLC-grade laboratory water, filtered through a Milli Q Advantage 10 water purification system by Merck Millipore. Analysis was performed by an UHPLC Agilent 1290 Infinity II LC system with a quaternary pump and diode array detector (Santa Clara, CA, USA). Biodegradation was monitored sequentially using the UHPLC method with gradient elution and reversed phase column Phenomenex Gemini C18. The dimensions of the column were 100 x 4.6 mm with a particle size of 5 µm. It was a C18 column with trimethylsilyl chloride (TMS) endcapping and with a fully porous organo-silica solid support. Chromatographic separation was undertaken using mobile phase comprised of 0.1 % formic acid (aquatic solution) as eluent A, and methanol with 0.1 % formic acid as eluent B. Flow rate throughout the analysis was 0.8 mL min^{-1} and injection volume was 15 µL. Run time of one injection was 15 min-

utes in total; the run started with 90 % of aquatic eluent, and in the minutes from 10–12 the organic eluent increased from 10 to 40 %, and then dropped to 10 % until minute 14.

UHPLC-MS analysis

The degradation products were identified by LC-MS method. The focus was on scanning the masses of the main peaks detected by UHPLC analysis, whose intensity changed mostly through the sampling time. The analysis was performed using a similar UHPLC Agilent instrument, an UHPLC 1290 Infinity II LC system with a quaternary pump and diode array detector (Santa Clara, CA, USA), but coupled to an Agilent 6495C triple quadrupole system with an AJS ESI ion source (Santa Clara, CA, USA). The method on coupled instruments was performed on the same reversed phase column, Phenomenex Gemini C18, and under the same flow and gradient conditions. The chemicals used for the preparation of solutions were LC-MS-grade water for chromatography, commercially available from Merck, formic acid from Sigma-Aldrich, and LC-MS-grade methanol from Merck. The injection volume was 15 μL, and the needle was washed through the flush port with methanol. The mass spectra were collected under the source parameters listed in Table 1. These parameters are specific for Agilent 6495C triple quadrupole system with an AJS ESI ion source, adjusted for analysis described in this work.

Table 1 – *Source parameters for mass spectrometry (Agilent 6495C)*

Parameter	Value
Gas temp. (°C)	250
Gas flow (L min^{-1})	15
Nebulizer (psi)	20
Sheath Gas Heater	400
Sheath Gas Flow	12
Capillary (V)	3000
V charging	1500

All the UHPLC-MS analyses were performed and processed with Data Acquisition and Qualitative Analysis package included in Agilent MassHunter Workstation Software.

Results and discussion

Amoxicillin biodegradation

The best biodegradation results were obtained with Bs-1 at amoxicillin concentration of 0.2 mg mL^{-1} (Fig. 1). After 30 minutes, the relative area of amoxicillin dropped to 55.3 %, and after one day of biodegradation process to 6.3 %. After 7 days, amoxicillin was degraded almost completely with the relative area in the reaction solution of 0.8 %. Comparatively, biodegradation with Pa-1 in 0.2 mg mL^{-1} solutions was significantly slower; the concentration of amoxicillin after 30 minutes was 94.3 %, and after 7 days 55.7 % of the original area (Fig. 2). Similar results were obtained in 0.5 mg mL^{-1} amoxicillin solutions with Bs-2 and Pa-2, where the area of amoxicillin achieved after 7 days was 52.0 % with Bs, and 65.5 % with Pa, respectively.

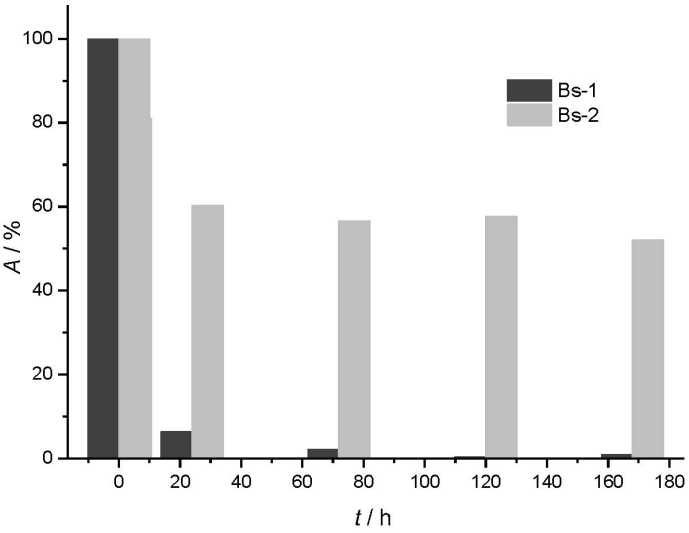


Fig. 1 – *Relative area of amoxicillin peak with Bacillus subtilis (Bs); Bs-1 (γ_1 = 0.2 mg mL^{-1}) and Bs-2 (γ_2 = 0.5 mg mL^{-1})*

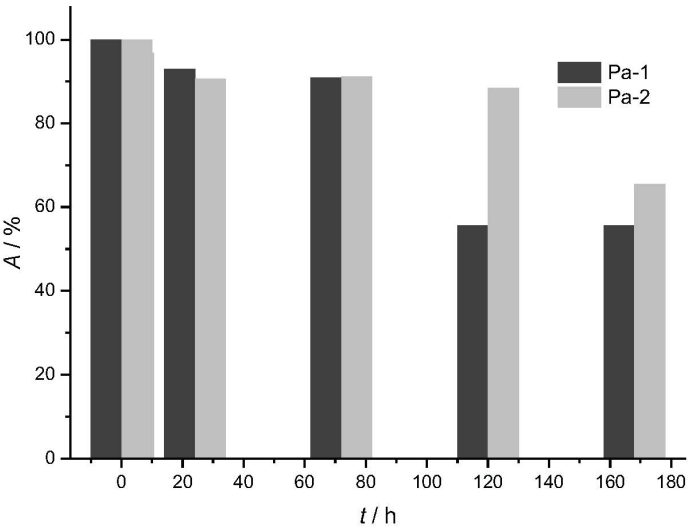
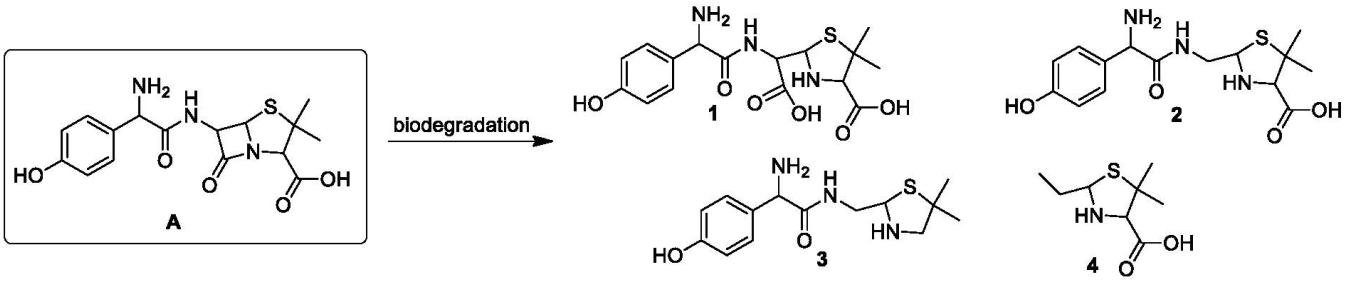


Fig. 2 – *Relative area of amoxicillin with Pseudomonas aeruginosa (Pa); Pa-1 (γ_1 = 0.2 mg mL^{-1}) and Pa-2 (γ_2 = 0.5 mg mL^{-1})*

Identification of biodegradation products

In parallel with the biodegradation of amoxicillin, a certain number of degradation products were also observed, depending on the reaction condi-



Scheme 1 – *Biodegradation of amoxicillin*

tions. In the case of degradation with Bs, degradation products **1**–**4** were observed (Scheme 1), while degradation with Pa gave products **1** and **2**. Relative area of the products, through 7 days, are summarized in Tables 2 and 3.

As expected, the highest proportion of degradation products was observed in the case of biodegradation with Bs, concentration of 0.2 mg mL^{-1} (Table 2), where the fastest degradation of amoxicillin occured. Product **1**, formed by the opening of the beta-lactam ring of amoxicillin, was identified as the main degradation product, which is expected for beta-lactam antibiotics.[14] Decarboxylation of product **1** led to product **2**, observed in similar area, respectively, while secondary decarboxylation gave product **3**, parallel with the formation of compound **4**, as side products. Biodegradation with Bs of higher concentrations (0.5 mg mL^{-1}), in accordance with the degradation of amoxicillin, indicates significantly lower proportions of degradation products. Pa biodegradation at both concentrations (Table 3) indicates the presence of two products, ring-opening compound **1** as the main degradation product and decarboxylated product **2** in traces. The influence of concentration on the proportion of products was not observed.

Table 2 – *Relative area (%) of biodegradation products with Pseudomonas aeruginosa (Pa)*

t/h	Pa (γ = 0.2 mg mL^{-1})		Pa (γ = 0.5 mg mL^{-1})	
	1	2	1	2
0	0	0	0	0
0.5	2.6	0.2	2.5	0.3
24	3.4	0.6	4.5	0.3
72	5.1	0.8	5.3	0.5
120	17.2	1.2	7.5	0.6
168	16.8	0.6	17.3	0.8

Table 3 – *Relative area (%) of biodegradation products with Bacillus subtilis (Bs)*

t/h	Bs (γ = 0.2 mg mL^{-1})				Bs (γ = 0.5 mg mL^{-1})			
	1	2	3	4	1	2	3	4
0	0	0	0	0	0	0	0	0
0.5	36.2	1.3	0	0.3	14.0	0.7	0.3	0.1
24	64.0	7.5	1.2	1.5	23.9	5.7	0.6	0.7
72	44.8	25.8	2.1	4.6	18.5	11.2	1.0	1.9
120	43.2	35.3	3.9	3.9	11.4	13.3	2.1	3.5
168	22.2	32.1	4.5	10.3	9.9	13.8	2.4	3.9

For now, there are no known data on the toxicity of the formed products, since the focus of this research was to carry out biodegradation and characterize the degradation products in the mentioned concentrations. Therefore, part of the future aspects would include a more detailed analysis of the environmental impact of the obtained products.

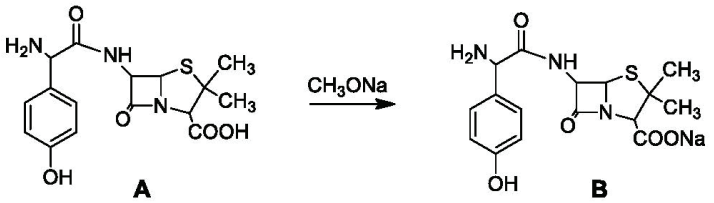
Preparation of products 1 and 2

As part of the additional confirmation of the structures, the main degradation products **1** and **2** were prepared by independent synthesis, in order to analyze the products on UHPLC and compare the masses of the synthesized compounds with the masses of the identified degradation products of amoxicillin. The synthesis was carried out from the sodium salt B (Scheme 2), previously prepared according to known procedure, from amoxicillin and sodium methoxide.[17]

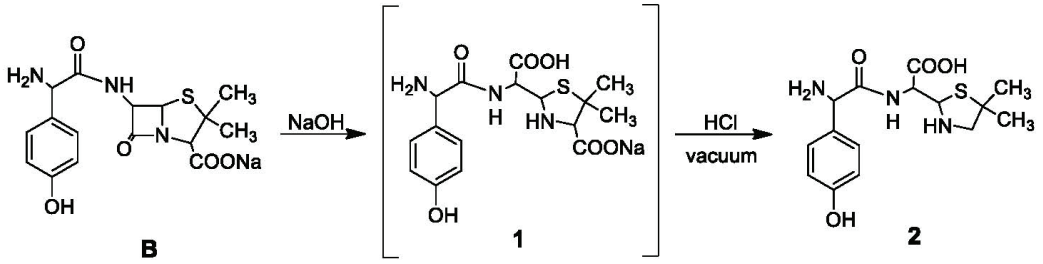
The next step refers to the hydrolysis of the beta-lactam ring to product **1** (Scheme 3), followed by decarboxylation in the presence of hydrochloric acid, and the formation of product **2** according to Garag.[18] The presence of products was confirmed by LC-MS method, whose masses entirely coincide with the masses of degradation products.

Conclusions

Biodegradation of the beta-lactam antibiotic amoxicillin proved to be very successful in laboratory conditions using *Bacillus subtilis* microbial culture, whereby after 7 days, almost all amoxicillin was removed from the solution. The primary process was the expected hydrolysis of the beta-lactam



Scheme 2 – *Synthesis of the amoxicillin sodium salt* ***B***



Scheme 3 – *Synthesis of products* ***1*** *and* ***2***

ring, followed by decarboxylation. These results, obtained on small simple molecules, can be an excellent guide to future aspects of simple and effective removal of complex structures, and identification of residual compounds with a significant impact on the environment.

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