Biodegradation of Erythromycin with Environmental Microorganism *Pseudomonas aeruginosa* 3011

M. Šabić,[a,*] L. Čižmek,[a] M. Vuković Domanovac,[a] and E. Meštrović[b]

[a]Department of Industrial Ecology, Faculty of Chemical Engineering and Technology, University of Zagreb, Marulićev trg 19, 10000 Zagreb, Croatia

[b]PLIVA HRVATSKA d.o.o., Prilaz baruna Filipovića 25, 10000 Zagreb, Croatia

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If pharmaceuticals occur in the environment, they can cause potential problems due to possible accumulation in the ecosystems. Biodegradation transforms xenobiotics using microorganisms rather than chemicals that create their own disposal problems. Biodegradation can make use of genetically modified microorganisms, which raises ethical and ecological issues, or it can make use of microorganisms that occur in the environment. The aim of this work was to study the biodegradation of the pharmaceutical erythromycin by environmental microorganisms. Initial concentration of erythromycin ranged from 0.02 mg dm^{-3} to 2000 mg dm^{-3}, while initial concentration of *Pseudomonas aeruginosa* 3011 was $7.05 \cdot 10^8$ CFU cm^{-3}. Inhibition concentration 50 % was 124.2 mg dm^{-3}. The results from the antimicrobial susceptibility experiment showed that *P. aeruginosa* 3011 is resistant to erythromycin. Average biodegradation efficiency was 33.43 %. These results suggest that pharmaceuticals in waste can be efficiently biodegraded through selection of appropriate, naturally occurring microorganisms, and optimization of degradation conditions.

Key words:
biodegradation, erythromycin, *Pseudomonas aeruginosa* 3011, batch reactor

Introduction

Pharmaceuticals include more than 4000 molecules with different physicochemical and biological properties and distinct modes of biochemical action.[1] They belong to the group of emerging contaminants in the environment and are the most relevant substances for the possible impact on aquatic ecosystems due to their frequent use and physicochemical properties. With more advanced analytical methods, residues of pharmaceuticals are found in concentrations up to μg dm^{-3} in surface water[2] in developed countries, and up to mg dm^{-3} in developing countries[3]. Release sources of pharmaceuticals in the environment are, for example, households, hospitals, livestock farms, pharmaceutical production, wastewater treatment plants or improper disposal of out-of-date medicines.[4] Most of these discharges have not been regulated yet, and the environmental fate of pharmaceuticals is not well understood.[5,6] Therefore, there is a growing interest for the disposal of such residues that could have potential negative effects on ecosystems.[7] Antibiotics, among all pharmaceuticals, are extensively used in treatment of diseases, and as such, their antimicrobial residues can have potentially adverse effects on humans.[8] In the last decades, antibiotic compounds have been considered as ''emerging contaminants'' due to their relatively high consumption and pseudo-persistence in the environment.[6,9]

Conventional wastewater treatment approaches are usually ineffective at degrading pharmaceutical contaminants like antibiotics, because most such contaminants show poor solubility in water.[4] Therefore, different research groups have been focusing on other degradation processes, such as photochemical transformations and photocatalytic decompositions in order to increase removal efficiency.[10] Recently, a lot of research has been focused on the area of microbial biodegradation or biotransformation of antibiotics[11–13], because biological treatment is a preferred option in terms of economic costs and ecological footprint. The ability of microorganisms to metabolize complex chemicals and large amounts of organic compounds represent one of the most important processes.[14,15] Most of the studies on biodegradation of pharmaceuticals investigated the removal of these compounds by mixed cultures of microorganisms.[13,16] However, it is easier to isolate the effects and behavior of individual strains, which could facilitate process optimization. Many bacteria

*Corresponding author: msabic@fkit.hr

have developed enzymatic pathways not only to deactivate antibiotics (which usually involves one or bond cleavages, but not complete breakdown of the antibiotics), but also to metabolize the antibiotics (involving their complete breakdown/transformation).[17,18]

The aim of this research was to study the susceptibility of environmental microorganisms to the macrolide antibiotic erythromycin. The main interest was to investigate the possibility of biodegradation by *Pseudomonas aeruginosa* 3011. This would contribute to the development of simpler and more efficient processes for the removal of pharmaceuticals in the environment.

Materials and methods

Antimicrobial susceptibility test

Prior to the biodegradation experiments, the antimicrobial susceptibility tests[19] on antibiotic erythromycin were performed with bacteria *Pseudomonas aeruginosa* 3011, *Bacillus subtilis* 3020, *Streptomyces rimosus* R7, and mold *Aspergillus niger* 405. All microorganisms are stored on agar slopes in the refrigerator at +4 °C at the Faculty of Chemical Engineering and Technology, Zagreb. For the cultivation of the mentioned microorganisms, nutrient agar (Biolife, Italy) and malt extract agar (Biolife, Italy) were prepared. The microorganisms were cultivated by inoculating the nutrient agar plates with *Pseudomonas aeruginosa* 3011 and *Bacillus subtilis* 3020, and incubated at 37 °C for 24 hours, while *Streptomyces rimosus* R7 and *Aspergillus niger* 405 were cultivated on malt extract agar plates and incubated at 28 °C for 3 days. The antimicrobial susceptibility tests were conducted with the selected environmental microorganisms. The concentration of erythromycin in the antimicrobial susceptibility tests ranged from 0.02 to 2000 mg dm^{-3}. The inoculated agar plates with discs on surface were incubated in the same conditions as previously indicated for each microorganism.

Biodegradation experiments

Three experiments, Exp. 1, Exp. 2 and Exp. 3, were conducted. In Exp. 1, the biodegradation of erythromycin by *B. subtilis* 3020 was studied. The other two experiments studied the biodegradation of erythromycin by *P. aeruginosa* 3011. Suspensions of bacterial cultures *P. aeruginosa* 3011 and *B. subtilis* 3020 were prepared, and their optical density was determined spectrophotometrically at λ = 600 nm of 0.7 and 1.5, respectively (Hach, model DR/2400, USA). The colony-forming unit was determined at the beginning of the experiment using the decimal dilutions method. The colony-forming units of *B. subtilis* 3020 were $2.2 \cdot 10^7$ CFU cm^{-3} in Exp. 1, while for the *P. aeruginosa* 3011 they were $8.6 \cdot 10^8$ CFU cm^{-3} in Exp. 2, and $5.5 \cdot 10^8$ in Exp. 3.

The substrate used in the biodegradation experiments was a macrolide antibiotic, erythromycin (PLIVA Hrvatska d.o.o.). Erythromycin was dissolved in water at the temperature of 28 °C[20]. Working solutions were prepared in the following concentrations: γ_{01} = 0.02 mg dm^{-3}, γ_{02} = 0.2 mg dm^{-3}, γ_{03} = 2 mg dm^{-3}, γ_{04} = 20 mg dm^{-3}, γ_{05} = 50 mg dm^{-3}, γ_{06} = 80 mg dm^{-3}, γ_{07} = 100 mg dm^{-3}, γ_{08} = 150 mg dm^{-3}, γ_{09} = 200 mg dm^{-3}, γ_{10} = 2000 mg dm^{-3}. The initial erythromycin concentrations are marked as S1-S10. Concentration of erythromycin before and after the experiments was monitored by ultra performance liquid chromatography (Waters H-Class, USA), at λ = 210 nm, using column Waters BEH C-18 100×21 mm. The mobile phase, pumped at 0.61 cm^3 min^{-1}, consisted of 12.7 mmol dm^{-3} anhydrous disodium hydrogen phosphate dissolved in water, pH 8.9 (A) and 25 % methanol in acetonitrile (B) with gradient elution. The initial conditions of biodegradation in Exp. 1 – Exp. 3 with an initial number of colony-forming units, CFU, and the initial concentration of the substrate S1-S10 are shown in Table 1. In the first two experiments, concentrations of erythromycin were used according to the logarithmic scale, from the lowest S_{01} = 0.02 mg dm^{-3}, up to the largest, S_{10} = 2000 mg dm^{-3}. In Exp. 3, the concentration interval was reduced from S_{04} = 20 mg dm^{-3} to S_{09} = 200 mg dm^{-3}.

Biodegradation experiments were conducted in Erlenmeyer flasks (100 cm^3) in an incubator shaker

Table 1 – *Initial concentrations of biomass and erythromycin for Exp. 1 – Exp. 3*

Experiment #	CFU (cells cm^{-3})	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
1	$4.3 \cdot 10^7$	+	+	+	+	–	–	–	–	+	+
2	$1.1 \cdot 10^{10}$	+	+	+	+	–	–	–	–	+	+
3	$4.3 \cdot 10^8$	–	–	–	+	+	+	+	+	+	–

"+" – analyzed concentrations

"–" – not analyzed concentrations

(Unimax 1010, Heidolph) at 28 °C and 160 rpm for 64 hours in Exp. 1, and 24 hours in Exp. 2 and Exp. 3. To remove the possibility of photodegradation of erythromycin, the experiments were performed in the dark.[21]

Erlenmeyer flasks contained 1 cm^3 of the suspension of microorganisms, 4 cm^3 of phosphate buffer (0.85 g KH_2PO_4, 2.18 g K_2HPO_4 and 3.34 g $Na_2HPO4 \cdot 7H_2O$ in 100 cm^3 distilled water, pH = 7.36), 21 cm^3 of BOD dilution water, 10 cm^3 of nutrient solution (0.8 g nutrient broth and 0.6 g sodium acetate dissolved in 100 cm^3 distilled water)[22], and 4 cm^3 of the prepared test solutions of erythromycin with initial concentrations S1-S10. BOD dilution water was prepared by adding 1 cm^3 of each of the following solutions to 1 dm^3 distilled water: magnesium sulfate solution (2.3 g $MgSO_4 \cdot 7H_2O$ in 100 cm^3 distilled water), calcium chloride solution (2.8 g $CaCl_2$ in 100 cm^3 distilled water), ferric chloride solution (25 mg $FeCl_3 \cdot 6H_2O$ in 100 cm^3 distilled water) and ammonium chloride solution (170 mg NH_4Cl in 100 cm^3 distilled water). The control solution was prepared with 25 cm^3 of BOD dilution water without the test solution of erythromycin, and it was used to correct for sample turbidity, color or precipitation in test solutions.[22] All test concentrations were prepared in duplicate.

The pH-value of solutions that were used in the biodegradation experiment and the final mixture solution were tested prior to implementation in all experiments. The pH ranged from 6.5 to 7.3, and the final pH of the mixture solution was about 7.5. The pH value is very important for conducting this experiment because erythromycin[23] is inactive at pH values lower than 3, while the optimal pH value for the growth of microorganisms is 6–9.[24]

During the experiments, the samples were monitored for changes in optical density. Samples were Gram stained after 16 and 24 hours, and microscopic analysis was conducted under light microscope (Olympus BX50, Olympus Optical Co Ltd., Japan) equipped with a microphotography system (Olympus DP 10 camera). Samples were filtered and dried at the end of the experiments to determine the concentration of biomass and residual concentrations of erythromycin. In addition, the colony-forming unit was determined at the end of the experiment.

Results and discussion

Antimicrobial susceptibility testing

Before setting up the biodegradation experiment, antimicrobial susceptibility tests were conducted with selected environmental microorganisms. The results of the preformed experiments are shown in Table 2.

Table 2 – *Results of antimicrobial susceptibility testing on erythromycin*

S_0 (mg dm^{-3})	Zone diameter (mm)			
	S. rimosus R7	*B. subtilis* 3020	*A. niger* 405	*P. aeruginosa* 3011
0.02	/	no zone	/	no zone
0.2	/	no zone	/	no zone
2	/	8 ; – ; –	/	no zone
20	/	10 ; 17 ; –	/	/
50	9 ; – ; –	14 ; 20 ; 27	no zone	no zone
100	10 ; – ; –	17 ; 22 ; 30	no zone	no zone
200	12 ; 15 ; –	19 ; 23 ; 31	no zone	no zone
2000	/	21 ; 27 ; –	/	12 ; 20 ; –

/ – test was not conducted

The results show that the most susceptible microorganism to erythromycin was *Bacillus subtilis* 3020. This is because erythromycin is effective against Gram-positive bacteria.[25] The bacteria *Streptomyces rimosus* R7 had intermediate resistance to the tested antibiotic, even though it is a Gram-positive bacteria as *B. subtilis* 3020. This is because it belongs to the genus of Streptomyces, which have the ability of producing antibiotics and probably have resistance genes for the same one.[26] The bacteria *Pseudomonas aeruginosa* 3011 and mold *Aspergillus niger* 405 were resistant to a wide range of examined concentrations of erythromycin. Enzyme erythromycin esterase was successfully isolated from *Pseudomonas* sp., which increased the resistance of the said microorganism on the tested pharmaceutical.[27] It is possible that the bacteria *Pseudomonas aeruginosa* 3011 has the same enzyme because it belongs to the same genus of bacteria, and therefore does not show susceptibility to erythromycin.

Biodegradation process

The results from Exp.1 shown in Fig. 1, indicate that the lower concentration of substrate is less toxic, respectively, the sample is similar to the control. This means that the bacterial culture had grown at lower concentrations of erythromycin. The toxic concentration which prevented growth of *B. subtilis* 3020 in the first 24 hours was 2 mg dm^{-3}. For the concentration of erythromycin of 0.02 mg dm^{-3} the sample was 90.17 % similar to the control, while for the concentration of erythromycin of 0.2 mg dm^{-3} the absorbance of the sample was greater than for the control solution. Therefore, it can be concluded that there was no inhibition in the assay, respectively, bacteria *B. subtilis* 3020 showed resistance to concentrations of erythromycin lower than 2 mg dm^{-3}. In addition, from Fig. 1 it is evident

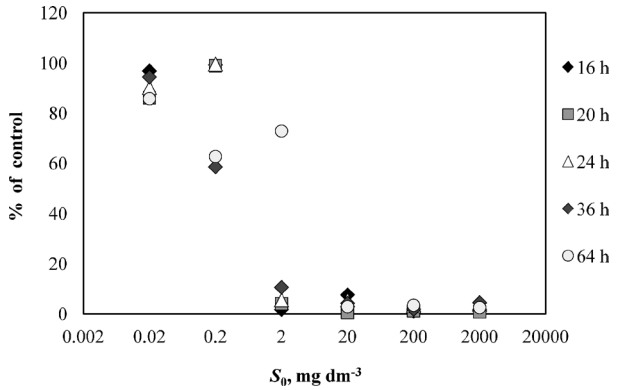

Fig. 1 – *Change in the percentage of the controls and the concentration of substrates S1–S4, S9 and S10 in the process of biodegradation of erythromycin with B. subtilis 3020 in Exp. 1*

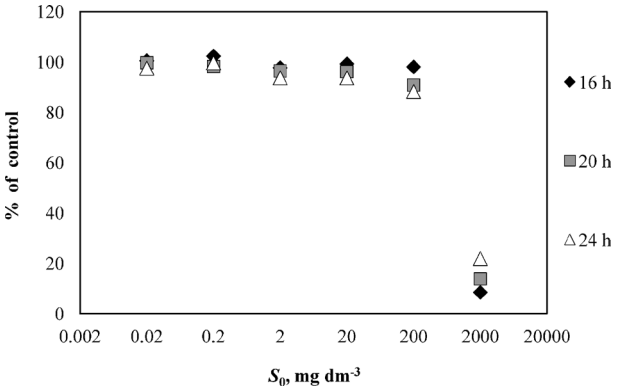

Fig. 2 – *Change in the percentage of the controls and the concentration of substrates S1–S4, S9 and S10 in the process of biodegradation of erythromycin with P. aeruginosa 3011 in Exp. 2*

that, in the 36th hour, absorbance of the sample with a concentration of erythromycin of 2 mg dm^{-3} began to grow, and that in the 64th hour the sample was 72.92 % similar to the control. This indicates the adjustment of the bacterial culture *B. subtilis* 3020 on substrate, erythromycin. In this experiment, erythromycin showed inhibitory activity and not bactericidal.[28] The results also showed that there was a drop percentage of control in the 36th and 64th hour of the sample with the concentration of the substrate of 0.2 mg dm^{-3}. Absorbance values dropped 1.7 times in the 36th hour, and 1.6 times in the 64th hour. This means that there was a decay of biomass because of the disappearance of nutrients or due to the toxic influence of the substrate. Based on the results, the biodegradation time in further experiments was defined as 24 hours.

In Exp. 2, after 16 hours, the production of biomass started in the experimental solutions in a form of brown fibers. Production of biomass was expressed in control samples and in samples with a lower concentration of substrate (0.02–20 mg dm^{-3}), whereas in the samples with a substrate concentration of 200 and 2000 mg dm^{-3}, biomass accumulation occurred only around the 24th hour. This was probably caused by the high concentration of substrate, i.e. it was necessary for the biomass to adjust to the conditions in the reactor so that it could have begun to grow rapidly. Also, in the course of this experiment, the green-yellow pigment pyocyanin appeared in the control samples after 24 hours, which is characteristic for the bacteria *P. aeruginosa*. In the other samples, the pigment did not appear as a result of the presence of a substrate, which probably acted as an inhibitor to the production of the pigment.[29]

The results shown in Fig. 2 indicate that the samples with lower concentration are more similar to control solutions. In this experiment, all tested concentrations of erythromycin showed no inhibitory activity, except S10, 2000 mg dm^{-3}, where there was a complete inhibition of growth of bacterial culture. The sample was consistent with control of 22.0 % after 24 hours, while for other concentration samples this value was 90–100 %, which is 4.5 times higher absorbance. The results show that in the 16th hour, production of biomass reached a maximum, and then at 20th and 24th hour, the values slightly declined. The exception was the sample with the concentration of substrate S10, 2000 mg dm^{-3} where the rise in the percentage of control over time was present from 8.6 % to 22.0 %, indicating the adaptation of bacterial culture to adverse effects.[30]

In Exp. 2, the resistance of the tested microorganism was observed according to almost all tested concentrations of substrate. IC_{50}, 50 % inhibition concentration, is a concentration of substance, which inhibits the growth of bacterial cells by 50 %.[22] According to the results above, IC_{50} value for erythromycin was 124.2 mg dm^{-3}.

The Exp. 3 was conducted for interval of concentrations from S4 to S9. The results of Exp. 3 are shown in Fig. 3. It can be seen that none of the tested concentrations of erythromycin had an inhibitory effect on the bacterial culture *P. aeruginosa* 3011. The results show that the values in the 16th hour were more than

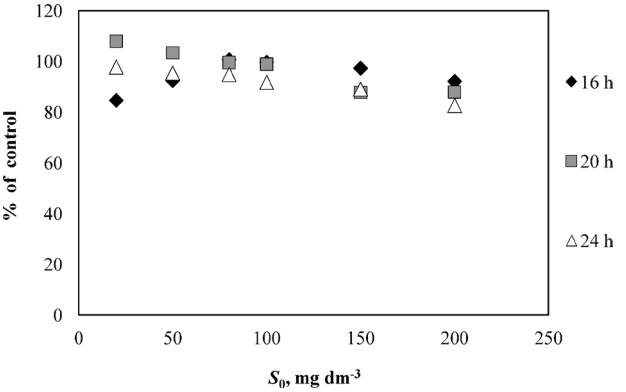

Fig. 3 – *Change in the percentage of the controls and the concentration of substrates S4–S9 in the process of biodegradation of erythromycin with P. aeruginosa 3011 in Exp. 3*

100 % for the lower concentrations, and after 24 hours the values decreased. This indicates the possibility of biodegradation, as the bacteria, after adjusting to the present conditions and after utilization of simple nutrient sources, started to degrade the complex organic molecules, in this case erythromycin.[30,31] After the bacteria had utilized all the nutrients, the biomass began to decrease, which is evident after 24 hours. In addition, it is shown that the biomass concentration increased in all samples, regardless of the concentration of substrate, which suggests possible adaptation of the bacterial culture to the conditions in the reactor.

The effectiveness of the biodegradation process was observed by determination of the concentration of erythromycin at the beginning and end of Exp. 3. From the results shown in Fig. 4, it is evident that the percentages of biodegradation for all tested concentrations of substrate in the solutions were similar. This indicates that the selection of the bacterial culture *Pseudomonas aeruginosa* 3011 for the experiment of possible erythromycin biodegradation, was good because in only 24 hours, the pure bacterial culture was able to biodegrade 33.43±5.7 % of the complex organic molecule.[12]

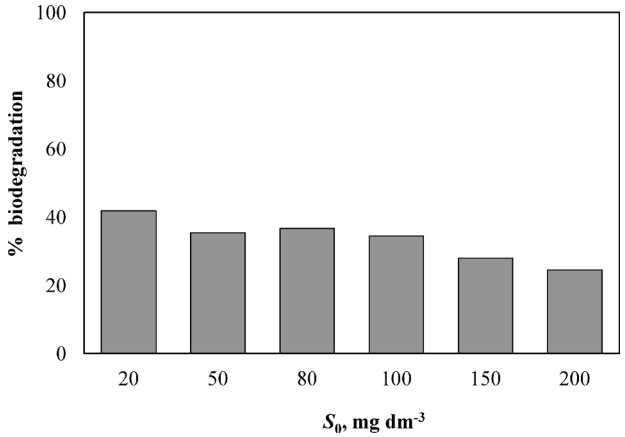

Fig. 4 – *Efficiency of biodegradation process of erythromycin in Exp. 3*

Microbiological analysis

To find out how many viable bacterial cells were present in a sample, the colony-forming units were determined.[24] The results for Exp. 1 – Exp. 3 are shown in Table 3. From the results, it can be seen that the average number of viable bacterial cells of *Bacillus subtilis* 3020 for the tested concentrations of erythromycin was $8.9 \cdot 10^6$ CFU cm^{-3}. The number of viable *Pseudomonas aeruginosa* 3011 cells in Exp. 2 decreased to $7.1 \cdot 10^7$ CFU cm^{-3}, while in Exp. 3 the number of viable bacteria cells maintained in all tested erythromycin concentrations. The results indicate that Gram-positive bacteria are more susceptible to erythromycin.[25]

Although the results shown in Figs. 1–3 indicate that biomass concentration during biodegradation did not differ from the control solutions, differences in the number of living cells are visible even at the lowest concentrations of erythromycin. Thus, at concentrations of substrate S1, 0.02 mg dm^{-3} in Exp. 1 and Exp. 2, 10 times less bacteria had survived than in the control solution. These results show that there was a specific inhibition of cell growth present in the experiments, probably at the beginning when the bacterial cultures had not yet adapted to the new conditions.[32]

Table 3 – *Values of the total number of viable cells for different initial concentrations of erythromycin*

S_0 (mg dm^{-3})	CFU (cells cm^{-3})		
	Exp. 1	Exp. 2	Exp. 3
0 (control)	$4.3 \cdot 10^7$	$1.1 \cdot 10^{10}$	$4.3 \cdot 10^8$
0.02	$3.5 \cdot 10^6$	$2.8 \cdot 10^9$	/
0.2	$4.4 \cdot 10^6$	/	/
2	$5.4 \cdot 10^5$	/	/
20	$1.8 \cdot 10^5$	$5.2 \cdot 10^9$	$3.2 \cdot 10^8$
100	/	/	$2.7 \cdot 10^8$
150	/	/	$2.4 \cdot 10^8$
200	/	$4.2 \cdot 10^9$	$2.8 \cdot 10^8$
2000	$3.1 \cdot 10^5$	$7.1 \cdot 10^7$	/

/ – analysis was not conducted

Microorganisms use organic substances as a source of nutrients and energy.[24] Microscopic analysis consisted of a visual examination and determination of the morphological characteristics of the cells. Microphotographs of bacterial culture *Bacillus subtilis* 3020 used in Exp. 1, and stained according to Gram, are shown in Fig. 5. It can be seen that, with increasing the initial concentration of erythromycin in the experiments, bacterial cells morphologically changed, respectively, the bacterial cells became elongated and the chains of bacteria broke. Erythromycin binds to 50S subunit of ribosomal RNA and in that way causes inhibition of the synthesis of proteins.[33] It is possible that erythromycin causes damage to the cytoplasmic membrane and compromises cell wall synthesis, leading to loss of cytoplasmic material[34], which can result in change of cell structure.

A microphotograph of control solutions with bacterial culture *Pseudomonas aeruginosa* 3011 is given in Fig. 6a. It is evident that the cells are small and that it is a Gram-negative bacterium.[12] The changes in the size of bacterial cells occurred with the increase in concentrations of the substrate. The sample with the lowest concentration of S1, 0.02 mg dm^{-3} was smaller and fitted the pattern of the control solution. The higher the concentration of erythromycin in the sample, the larger were the bacterial cells. We as-

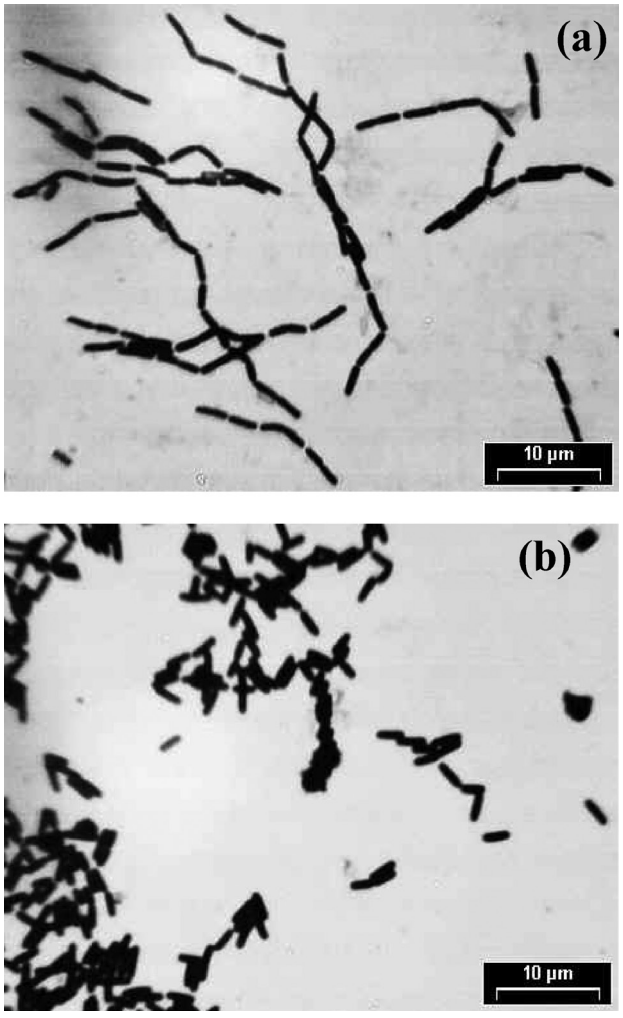

Fig. 5 – *Microphotographs of bacterial culture B. subtilis 3020 taken in the 24th hour of biodegradation of erythromycin for a) control solution, and b) S3 in Exp. 1., M = 1000×*

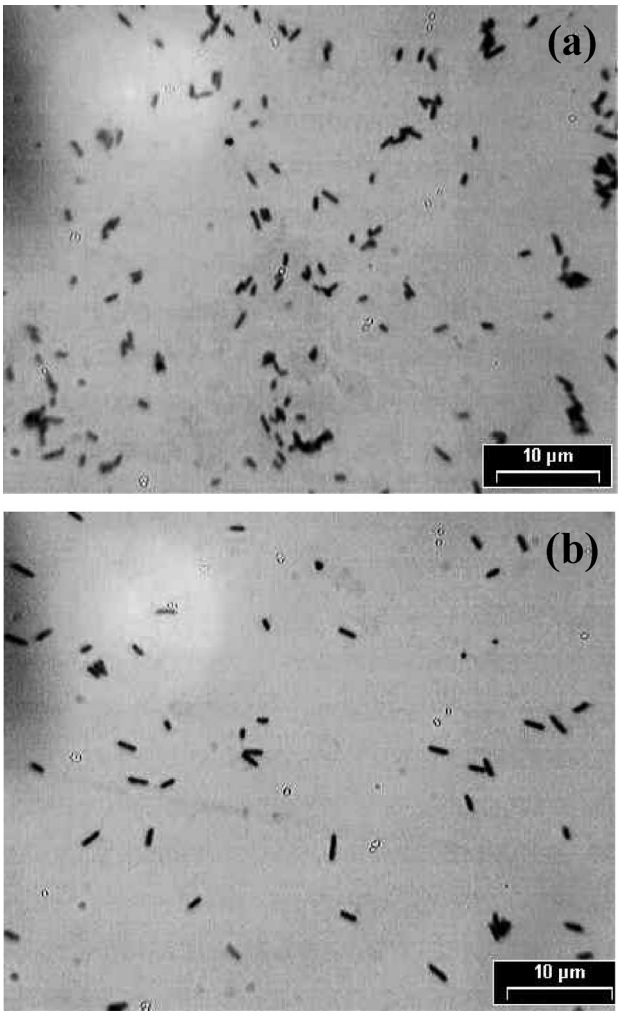

Fig. 6 – *Microphotographs of bacterial culture P. aeruginosa 3011 for: a) control solution, b) S7, in Exp. 3 taken in the 24th hour of biodegradation of erythromycin, M = 1000×*

sume that this may be due to the adjustment of the bacterial culture *P. aeruginosa* 3011 to the new conditions, where the nutrient is a hardly degradable substance. The morphology of living cells varied with the concentration of the substrate, i.e. the higher the concentration of erythromycin, the larger the cells were, but the value of CFU of the bacterial culture *P. aeruginosa* 3011 was lower.

Conclusion

In order to prevent potential problems in the ecosystems, pharmaceuticals have to be removed in an economically effective but eco-friendly way, such as biodegradation. Removal efficiency can be improved by inoculating naturally occurring microorganisms into contaminated sites. The obtained results have shown biodegradation efficiency of erythromycin by different environmental microorganisms. During antimicrobial susceptibility tests and biodegradation experiments, *Pseudomonas aeruginosa* 3011 showed high resistance to various concentrations of erythromycin. The average biodegradation of erythromycin for Exp. 3 was 33.43 % after 24 hours. The highest removal efficiency of erythromycin obtained was 44.1 % for the substrate concentration S4. The concentration of erythromycin affects the morphology of *P. aeruginosa* 3011, i.e. by increasing the concentration of erythromycin, the concentration of biomass reduced and the cells grew bigger. Biodegradation of erythromycin by bacteria *P. aeruginosa* 3011 shows good results, and can serve as a basis for further research in the area of biological treatment of waste streams.

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