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Microbial Removal of Sulfamethoxazole by Pure Bacterial Cultures Isolated from the Environment

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Abstract

Pharmaceuticals are an important group of environmental micropollutants which have recently attracted much attention of the international scientific community due to their global dissemination, and because of their ability to stimulate specific biological responses at very low concentrations. The fate of many of these pharmaceuticals in the environment has not been thoroughly investigated. Sulfamethoxazole (SMX) is one of the pharmaceuticals that display significant bioactivity, it is considered to be a persistent pollutant. The biodegradation of this compound by enriched bacteria isolated from activated sludge and household compost has been studied. Nine bacterial strains were tested for their ability to grow in minimal mineral salt medium (MMSM) in presence of 6 mg/l of SMX as a sole source of carbon and energy. The results revealed that all the strains are able to grow and resist SMX. After a screening on a liquid medium, S4 and S2 strains identified as *Pseudomonas* sp. and *Arthrobacter nicotianae*, respectively, were selected for their particular ability to resist this micropollutant. The removal rates achieved by these strains after 48h of incubation in MMSM at 26°C were 34.27% and 32.95%, respectively (initial SMX concentration was 3 mg/l), as quantified using HPTLC, where adsorption and abiotic removal mechanisms were shown to be absent, showing that SMX is certainly biodegradable.

1. Introduction

The incidence and fate of pharmaceutical compounds (PhaCs) in the environment has increasingly attracted researcher's interest during the last decade. This type of contaminants is considered to be emerging pollutant due to their recalcitrant nature and their sublethal or chronic effect on the non-target organisms [1-3]. PhACs are primarily released through wastewater treatment plant (WWTP) discharges [4]. However, these plants are not currently designed for complete removal of PhACs [5]. Extensive WWTP sampling campaigns have been explored in several countries where various WWTP treatment technologies have been applied and extensive information are available on PhAC influent and effluent concentrations. Reported concentrations values were typically in the order of μg/l or lower than ng/l [6].

Antibiotics are among the most used pharmaceuticals in human and veterinary medicine. They are used as growth promoters or for therapeutic purposes. They have been detected in the influent and effluent of sewage treatment plants and surface water in the range of ng/l and μg/l. The main problem related to their presence in the environment is the appearance of resistant bacteria [7-8]. This problem is now considered as a major public health concern due to the increased occurrence of associated clinical infections [9]. Sulfamethoxazole (SMX), an important broad-spectrum antibacterial with superior stability, is complex nitrogen containing heterocyclic compound with a molecular formula of $C_{10}H_{11}N_3O_3S$. It is currently prescribed to treat urinary tract infections and it is also used in veterinary practices, aquaculture and livestock breeding both for treating diseases and as a growth promoting agent [8]. An increased number of studies have reported the detection of SMX in

groundwater, in effluents of WWTP and in rivers. According to Xu et al., SMX was detected in the Haihe River at concentration of 50-370 ng/l which is higher than those of other types of antibiotics [9]. This antibiotic mainly enters wastewater via human excretion as unmodified SMX (13–5%) or as its human transformation products N-acetyl-SMX (43–55%) or N-SMX-glucuronide (10–13%) [10]. The removal of pharmaceuticals in the environment is essential to prevent environmental contamination and possible adverse effects [11], this removal may occur through various mechanisms, including biodegradation, abiotic transformation and sorption to biomass or suspended solids. In general, sorption process and biodegradation are considered to be the most important removal mechanisms [12].

The capacity of various microorganisms to biodegrade persistent organic pollutants has been widely studied during the last decades to deal with the extensive contamination of the environment; the attention has been focused on bacteria and filamentous fungi [13]. SMX has previously been considered by many researchers to be persistent to biodegradation [14], furthermore, in the conventional wastewater treatment plants, SMX biodegradation have been measured to range from 0% to 90% [7]. Because of its low adsorption, its reduction is mainly due to microbial activity [10].

At lab scale, there have been conflicting results regarding the biodegradation of SMX, they vary from significant removal in activated sludge [7], to minimal biodegradation [15]. To understand the consequences of pharmaceuticals pollution on the environment, it is imperative to know the fate of these drugs and this includes knowing whether they are biodegradable, to what extent, and whether there are any metabolites that are created during the biodegradation. This study deals with the evaluation of the capacity of an acclimated culture of aerobic bacteria isolated from activated sludge and household compost to degrade SMX in a defined liquid medium.

2. Experimental

2.1. Collection and enrichment of samples

In order to obtain efficient degraders of sulfamethoxazole (SMX), two samples were collected. Activated sludge from waste water treatment plant of RABTA (Jijel-Algeria) and household compost from a region of KAOUS (Jijel-Algeria), the samples were enriched with 5mg of Primasol® drug (200 mg SMX and 40 mg trimethoprim) twice a month and incubated at room temperature, mixed and moistened every 7 days [16].

2.2. Bacterial cultures and chemicals

To isolate the dominant bacteria present in the two samples (activated sludge and household compost), serial dilutions were carried out and spread on Mineral Minimal Salt Medium agar (MMSM) (FeSO$_4$·7H$_2$O (0.013 g/l), CaCl$_2$·2H$_2$O (0.013 g/l), MgSO$_4$·7H$_2$O (0.25 g/l), Na$_2$HPO$_4$ (7.5 g/l), KH$_2$PO$_4$ (5 g/l), NH$_4$NO$_3$ (5 g/l), yeast extract (0.05 g/l), Agar (15 g/l), pH 7.0) supplemented with 6 mg/l of SMX [17]. The plates were aerobically incubated at 37°C for 48 h. Different morphologies of colonies were chosen and isolated by repeated streak culturing on nutrient agar. SMX (Polypharma, purity ≥ 99.0%) was kindly provided from the Laboratoire National de Controle du Medicament (Algeria). The stock solution was prepared in sterile distilled water and methanol (50:50% v/v) and introduced in the MMSM when necessary. All solvents used as chromatographic eluents in the experiment (ethyl acetate and methanol) were of chromatography grade, and they were purchased from SIGMA ALDRICH and FLUKA.

2.3. Screening of SMX degraders

It is a preliminary test allowing us to choose the most efficient bacterial strains able to grow in MMSM containing SMX (6 mg/l) as sole source of carbon and energy. Ten ml of MMSM were inoculated with the isolated strains. The samples were incubated in a rotary shaker at 26°C under agitation for 4 days. Microbial growth was monitored by a UV-Visible spectrophotometer (Amercham) at 600 nm every 24 h (the test was performed in duplicate) [16]. Thus the most tolerant isolate was finally characterized on the basis of 16S rDNA technique. Amplification of the 16S rDNA gene from the genome was done by the polymerase chain reaction (PCR) using specific primers, PCR products were sequenced and the obtained sequences were then analyzed using nucleotide Blast search data base and have been deposited in the GenBank sequence database.

2.4. *Effect of different concentrations of SMX on bacterial growth*

In order to determine the maximal concentration of SMX that is supported by the selected bacteria and did not have a negative effect on their optimal growth, different concentrations of SMX were evaluated in duplicate. A series of tube containing 9 ml of MMSM medium and different concentrations of SMX (5 mg/l-1000 mg/l) were inoculated with 1ml of an overnight culture of the selected strain (S2 and S4). Inoculated MMSM medium without SMX was used as control. The samples were incubated in a rotary shaker incubator at 26°C (150 rpm) for 48 h and the bacterial growth was monitored by UV-Visible spectrophotometer at 600nm [2].

2.5. *Biodegradation test*

The biodegradability of SMX was investigated using the Closed Bottle Test (OECD 301C) [18]. It was performed in 250 ml Erlenmeyer flasks containing 100 ml of MMSM inoculated with 5% of an overnight culture of selected strain S4 and S2. The overnight culture was centrifuged; the resulting pellet was washed two times by distilled water and added to the flasks. SMX was added into MMSM as a sole source of carbon and energy for growth, with a final concentration of 3 mg/l. For evaluation of the abiotic hydrolysis and the adsorption of SMX on biomass, control experiments were conducted following the same procedure using the mineral medium, without microorganisms and with 5% of autoclaved bacterial cells, respectively. The flasks were covered with aluminum foil to prevent photo-degradation and incubated in a rotary shaker at 26°C at 150 rpm for 48 h. During growth experiments, the culture medium was sampled at regular time intervals and the cell growth was monitored by optical density measurement at 600 nm. To estimate drug concentration, 2 ml of the culture medium was taken and centrifuged at (6000 rpm) for 10 min to separate biomass then the supernatant was filtered through a Millipore filter (0.22 μm) and stored at -20°C until analysis.

2.6. *Chromatography analysis*

A filtered supernatant sample of 1.5 ml was evaporated to dryness under nitrogen vacuum at 60°C. The residues were dissolved in 0.5 ml methanol and analyzed by the High Performance Thin Layer Chromatography (HPTLC) on 20 cm×20 cm HPTLC plates coated with silica gel 60F254 (Merck). Samples and standard solutions (20 μl) were applied to the plates as 5 mm bands, using a CAMAG (Muttenz, Switzerland) ATS 4 automatic sample-application device equipped with a 100μl syringe and N2 flow. The development was performed in a CAMAG glass twin-trough chamber, with ethyl acetate and methanol (75:15%, v/v) as mobile phase to a distance of 8 cm from the lower edge of the plate [19]. *In situ* densitometric scanning at 290 nm was performed with a CAMAG TLC Scanner 3, in absorbance mode, using the deuterium light source, and equipped with win CATS 1.4.2 software. Under these conditions SMX was detected at an R_f of 0.26. Degradation of SMX in time-course degradation experiments was assessed by comparing its concentration in the heat-killed control flasks with that in the experimental flasks. Heat-killed controls consisted of autoclaved cultures (121°C for 30 min) which were setup under identical conditions to those of the experimental cultures. The amount of adsorbed SMX was determined from the difference in SMX concentration between uninoculated and heat-killed control. Peak Area of SMX was used to estimate the percentage of degradation using the following Eqs (1) [20]. EI (%) is the SMX removal rate (%). C_0 is the peak area of SMX in the beginning of experiment and C_i in the end.

$$EI\ (\%) = \frac{C_i - C_0}{C_0}(100) \qquad \text{Eqs (1)}$$

3. Results and discussion

3.1. *Screening of SMX degraders*

Different colonies were obtained and 9 pure strains were repeatedly screened by streak culturing. The ability of these isolates to grow on MMSM medium containing 3 mg/l of SMX as sole source of carbon and energy was tested in this experiment. Table 1 illustrates the growth of the isolates on MMSM in presence of SMX at 26°C during 96 h. Results indicated that, for most strains, cell growth started in the first hours of incubation and increased gradually with time, it reached its maximal level after 48 h of incubation except for S7 where the cell growth started after 24 h of incubation. After 72 h, a decrease in cell growth was observed for most strains only

for strain S4. The fact that bacterial isolates were able to grow in MMSM containing SMX as sole source of carbon and energy, gives evidence that, these isolates could have the ability to use this pharmaceutical as a substrate. According to the literature, biodegradation of xenobiotics often leads to a lag phase more or less important [21]. As seen in table1; the rapid growth of some isolates may be explained by a higher acclimation of the microbes that had been enriched under similar conditions. On the other hand, for the isolates displaying a slow growth after 24 h, another possible explanation may be relevant. Madigan et al. suggested that a lag time is observed when cells are transferred from a rich culture medium to a poor one. When the substrate in the medium is changed some enzymes are induced to metabolize the new substrate (it is the time required for adaptation to the new substrate) [22]. A remarkable decrease in bacterial growth observed after 48 h of incubation can be attributed probably to a possible toxic effect of the transformation products, as found for the metabolites of several xenobiotics [23] or to a depletion of substrate. Based on the obtained results the isolates S4 and S2 had the most important ability to grow on MMSM containing SMX as sole source of carbon and energy and they have been selected to be further investigated. These strains (S4 and S2) were identified by their 16S RNA sequence, and had the highest similarity (97.27% and 99.38%) with *Pseudomonas* sp. and *Arthrobacter nicotianae*, respectively. They were deposed in GenBank database under the following accession numbers: KJ740439, KJ740438.

Table 1: Growth (OD_{600nm}) of the isolates on MMSM containing SMX (3 mg/l) as sole source of carbon during 96 h

Strains	Origin	0 h	24 h	48 h	72 h	96 h
S1	Household compost	0.231	0.431	1.08	0.751	0.406
S2	Household compost	0.206	0.881	1.09	1.061	0.819
S3	Household compost	0.201	0. 697	0.860	0.571	0.443
S4	Activated sludge	0.298	0.782	0.879	1.05	1.128
S5	Activated sludge	0.259	0.426	1.171	0.768	0. 760
S6	Household compost	0.228	0.976	0.86	0.811	0.764
S7	Activated sludge	0.5	0.12	1.04	1,02	0,690
S8	Household compost	0.223	0.250	1.10	1.08	0.778
S9	Household compost	0.269	0.850	0.973	0.633	0.496

3.2. Effect of different concentrations of SMX on bacterial growth

This experiment was performed to determine the SMX concentration that did not have an effect on the optimal growth of S2 and S4 strains. Comparing the cell growth in the control and the experimental tubes (Figure 1), it can be observed that the growth of S2 and S4 increased gradually with the increase of SMX concentration, it reached its maximal level at a concentration of 15mg/l and 35mg/l, respectively (0.995 for S2 and 0.971 for S4). This increase revealed that the antibiotic (SMX) may favor the growth of resistant bacteria [9]. A slow decrease in bacterial growth was observed at SMX concentration up to 125 mg/l for S2 and 200 mg/l for S4. This reduction of growth may be explained by the toxic effect of high concentrations of SMX. According to the obtained results, it can be concluded that SMX affects the optimal growth of tested bacteria only at very high concentrations.

In this context, García-Galán et al. reported that the SMX is non-toxic to most microorganisms tested, including different bacterial strains as *Vibrio fischeri*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [24]. Another study carried out by Xu et al. showed that *Bacillus* strains were more resistant to different concentrations of SMX [9]. On the other hand, Ferrari et al. showed that the algae (*Pseudokirchneriella subcapitata* and *Synechococcus leopoliensis*) are sensitive to SMX at low concentrations (0.090 µg /land 0.0059 µg/l, respectively); it showed a high toxicity on both species [25]. Tolerance of both strains to high concentrations of the antibiotic, enables them to be strong candidates for the treatment of drugs contaminating the environment, which offers a green alternative technology to incineration and other physico-chemical treatments.
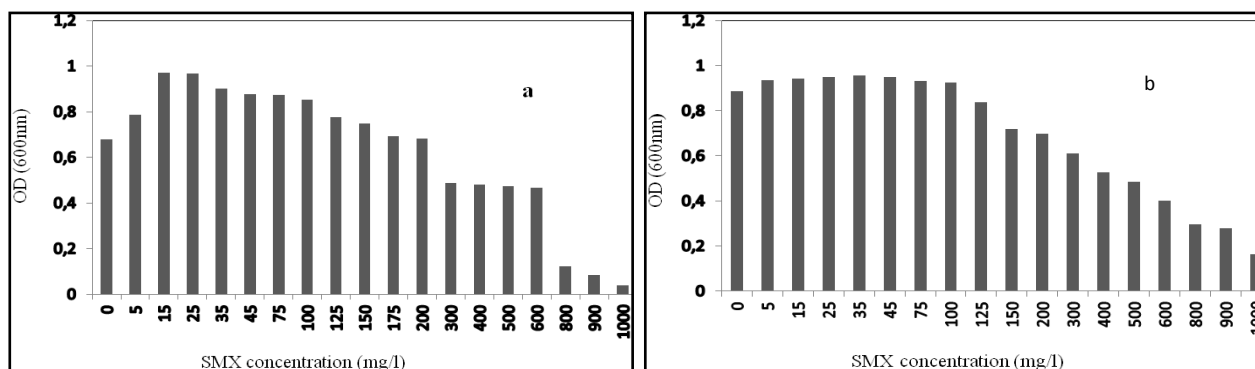
Figure 1: Effect of different concentrations of SMX on the growth of the strain S2 (a) and S4 (b) in MMSM medium after 48 h of incubation at 26°C under shaking.

3.3. *Biodegradation study*

The rate of SMX biodegradation was studied in 250 ml Erlenmeyer flasks during 48 h and the percentage of removal was calculated after measuring the residual SMX by HPTLC. Representative chromatogram depicting the R_f of SMX in the above mentioned solvent system is shown in (Figure 2).
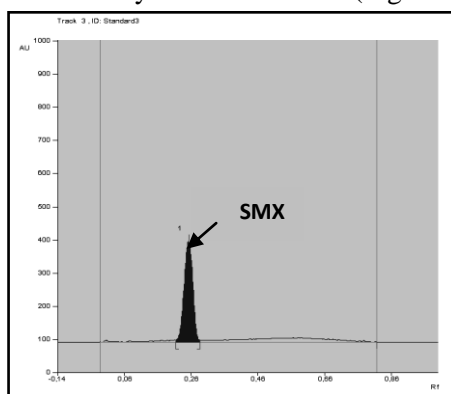
Figure 2: A chromatogram of SMX in the mobile phase ethyl acetate and methanol (75:15%, v/v) (R_f=0.26)

The obtained results showed the absence of SMX elimination in both abiotic and adsorption flasks. During the same period of time, the elimination rate of SMX in microbiologically active samples (in presence of S2 and S4) was in order of 34.27% and 32.95%, respectively; the results are presented in (Figure 3). Both strains were able to grow on MMSM containing SMX as sole source of carbon and energy with a maximal growth obtained after 48h of incubation, consequently, the cell growth of the two strains seems to be in line with the degradation process. Data from literature on the environmental fate of SMX demonstrated that in addition to biodegradation process, abiotic degradation (photolysis, volatilization and hydrolysis) and sorption may also be involved in the depletion of SMX from the environment [3]; however, the abiotic process was excluded in our experiment because no reduction in SMX concentration was recorded. The adsorption of SMX to biomass was found to be absent, these results give evidence that this compound is not able to adsorb on the bacterial surface. Our results were in agreement with those obtained by Yu et al. who found that SMX is weakly adsorbed to the biomolecules (microorganisms) [26], in addition Rodarte-Morales et al. observed that SMX is unable to adsorb on *Phanerochaete chrysosporium* mycelium [2]. The absence of degradation of SMX in the abiotic and adsorption samples, confirmed that microbial activity of the two strains *Pseudomonas* sp and *Arthrobacter nicotianae* is highly involved in SMX elimination. SMX degradation is also observed in other *Proteobacteria*, such, *Brevundimonas* sp., *Variovorax* sp., and *Ralstonia* sp., and *Actinobacteria*, such as *Microbacterium* sp. and *Rhodococcus* sp. [27]. Our results were in strong agreement with those reported on the degradation of SMX by *Rhodococcus rhodochrous* with an elimination rate of 29% [28]. Similarly, Le-Minh et al. observed that SMX is biodegradable with a high level by the micro-biota of the activated sludge [29]. In another study, five

strains capable of degrading ^{14}C-labeled SMX to $^{14}CO_2$ were isolated from a membrane bioreactor acclimatized to SMX, carbamazepine, and diclofenac. Two of these strains are belonging to the phylum *Actinobacteria*, while three were members of the *Proteobacteria* [30].
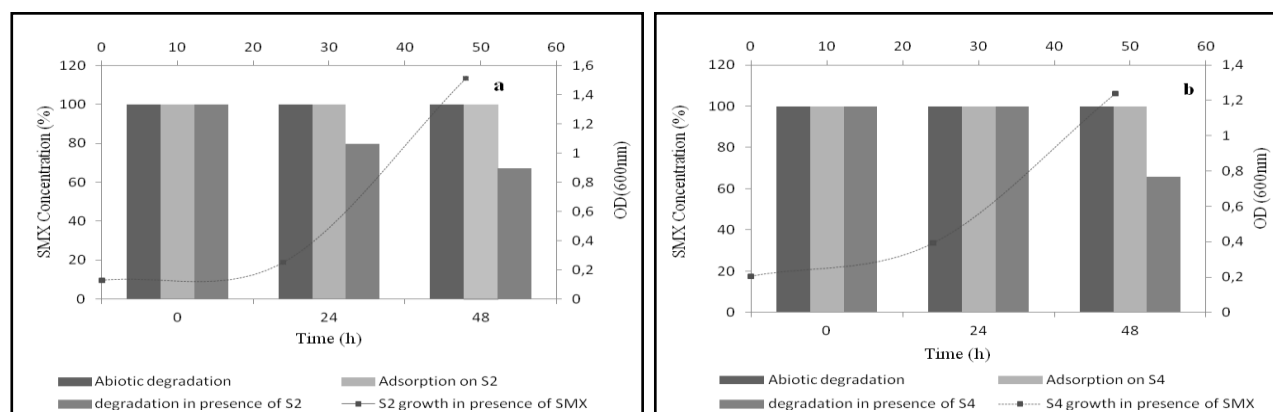
Figure 3: Elimination rate of SMX in presence of (a) S2 and (b) S4 strains and in abiotic and adsorption samples in MMSM after 48 h of incubation

Besides bacteria, there are other microorganisms which can degrade pharmaceutical compounds such as fungi. Rodarte-Morales et al. demonstrated that *P. chrysosporium* can degrade completely SMX after an incubation period of 4 days [2]. Also, a versatile peroxidase from the ligninolytic fungus *Bjerkandera adusta* showed SMX breakdown activity [27]. On the other hand, our results were in contrast with the classification of Joss et al. which considered SMX as a non-biodegradable substance and/or difficult to biodegrade [15].

Much less is known about the microbial degradation of sulfonamides, for these reasons informations on genes and enzymatic mechanisms involved are still limited [27]. Ricken et al. described the molecular mechanism used by *Mycobacterium* sp. strain BR1 for the breakdown of SMX [31]. They reported that in this bacterium, a NADH dependent type I ipso-hydroxylation results in the SMX fragmentation. Using the same mechanism, *Microbacterium* sp. strain BR1 is able to degrade other sulfonamides, as long as the sulfonyl bonded heteroatom-containing moieties, such as (3-amino-5-methylisoxazole), can act as moderate leaving groups. Furthermore, the strain BR1 showed a similar pattern of sulfonamide breakdown, degrading all the sulfonamides with an amine hetero-aromatic moiety, but not those containing poor leaving groups such as urea (glimepiride, glibenclamide) or NH_2 of the sulfanilamide group (furosemide) [27]. Larcher and Yargeau reported that the microbial degradation of SMX and formation of metabolites are due to the enzymatic machinery displayed by these bacteria. For example, arylamine N-acetyltransferase is produced by *P. aeruginosa* and *Rhodococcus* species and these enzymes showed high specificity for aromatic amines and can use SMX as substrate [7].

Furthermore, several studies reported the ability of the genus *Pseudomonas* to degrade pharmaceutical compounds, in this context, Larcher and Yargeau found that *P. aeruginosa* can biodegrade SMX in MMSM medium [7]. Evangelista et al. found that *P. putida*, and *P. fluorescens*, growing on glucose in a medium containing one of three chlorophenoxy acids at a concentration of 0.1 g/L (clofibric acid, (R)-2-(4-chloro-2-methylphenoxy) propionic acid (mecoprop or MCPP) and 4-chloro-2-methylphenoxyacetic acid (MCPA)) degraded these compounds to varying degrees; from non measurable to almost complete removal [17].

Conclusion

The obtained results indicated that both strains (S2 and S4) degrade SMX with considerable efficiency, which suggest their use as a mixed culture for bioremediation of pharmaceutical effluents, but further studies are recommended to elucidate the sulfonamide degradation pathway by these microorganisms. According to our knowledge the biodegradation of SMX by the pure bacterial strain of *Arthrobacter nicotianae* has not been described before.

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