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Biodegradation of clotrimazole and modification of cell properties after metabolic stress and upon addition of saponins

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ABSTRACT

Azole fungicides constitute an extensive group of potential emerging pollutants which can be found in natural environment. This study focuses on the biodegradation of clotrimazole and the characterization of cell surface properties of microorganisms capable of degradation of this compound. The influence of long-term contact of bacteria with clotrimazole and the impact of the addition of *Saponaria officinalis* extract on cell surface modification was also checked. The biodegradation of clotrimazole did not exceed 70%. The presence of plant extract increased biodegradation of fungicide. The cells metabolic activity after one-month exposure to clotrimazole was the highest for each tested strain. Moreover, metabolic stress led to a strong modification of cell surface properties. The results are promising for determining the impact of clotrimazole on environmental microorganisms.

1. Introduction

Azole derivatives belong to the group of antifungal agents that have a broad spectrum of activity. They are widely used in agriculture as active ingredients of biocides as well as pharmaceuticals in human and veterinary medicine or personal care products (Chen and Ying, 2015; García-Valcárcel et al., 2016; Kahle et al., 2008; Peng et al., 2012). One of the most important compounds in the group of azole fungicides is clotrimazole, 1-[(2-chlorophenyl)(diphenyl)methyl] − 1H-imidazole. This medicament is effective in the treatment of various fungal infections, especially dermatological and gynecological ones. Due to the serious side effects and ability to self-induced biotransformation in the liver, clotrimazole is always used topically (Mutschler et al., 2014). The active ingredients of topically administered drugs are being absorbed by the skin only in 5–10% which means that 90–95% of medicament may be rinsed out from the skin and subsequently enter wastewater (Peng et al., 2012). Except for the presence in households and hospital wastewaters, clotrimazole may enter the environment as improperly disposed household or manufacturing waste (Burkina et al., 2015; Chen and Ying, 2015).

The presence of clotrimazole in various environmental compartments has been documented in a number of reports. Kahle et al. (2008) determined the amounts of azole fungicides in various municipal activated sludges. In their study clotrimazole was detected at concentrations ranging between 12 and 78 ng L^{-1}. Moreover, Chen et al. (2013) investigated the occurrence of three azole biocides in biosolid-amended soils. Clotrimazole turned out to be present at a concentration equal to 492 ng g^{-1} of the biosolid sample. Furthermore, Lacey et al. (2012) established levels of clotrimazole in wastewaters from 0.5 to 8.65 μg L^{-1}.

Undesirable presence of clotrimazole in the environment may affect endocrine system of aqueous and soil organisms or have negative impact on indigenous environmental microorganisms (Chen and Ying, 2015; Peng et al., 2012). In addition, it has been shown that agricultural use of azole fungicides results in water contamination which in turn leads to azole resistance in human pathogen *Aspergillus fumigatus* (Berger et al., 2017; Hollomon, 2017; Jeanvoine et al., 2017; Verweij et al., 2009). This saprophytic mould is commonly present in the environment and can cause various diseases such as invasive aspergillosis, chronic aspergillosis or allergic syndromes (Jeanvoine et al., 2017).

The ubiquity of pharmaceuticals in various environmental compartments, such as soil, groundwater, surface water or wastewater, is a serious environmental concern (Almeida et al., 2013; Favier et al., 2015; Hörsing et al., 2011; Lacey et al., 2012; Popa Ungureanu et al., 2016; Tiwari et al., 2017). Hence, it is very important to remove them from the environment. Environmentally friendly and promising removal technique is bioremediation which is based on the ability of microorganisms to transform chemical substances into simpler, less toxic or non-toxic compounds (Azubuike et al., 2016; Srivastava et al., 2014). The factors affecting bioremediation include among others microbial features and properties of degraded compound (Boopathy, 2000; Ren et al., 2018; Srivastava et al., 2014). It has been proven that

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the prolonged contact of microorganisms with toxic compounds, being the non-specific carbon source, can change their properties and be the factor enhancing biodegradation (Pacholak et al., 2017; Popa Ungureanu et al., 2016; Smułek et al., 2015). Another element affecting biodegradation is the presence of surface active agents in microbial culture – they increase the bioavailability of the degraded compounds to the microbial cell and influence its surface properties (Elliot et al., 2011; Sun et al., 2018; Wang et al., 2017). It should be noted that surface properties of bacterial cells which often correspond to their biodegradation ability can be easily measured including such parameters as hydrophobicity of cell surface, permeability of bacterial membrane and zeta potential of microbial suspension (Hori et al., 2009; Obuekwe et al., 2009; Sadowska et al., 2014; Smułek et al., 2015).

As clotrimazole is the emerging organic pollutant showing the negative impact on non-targeted organisms as well as indigenous environmental microorganisms, the overriding aim of the study was to estimate changes in microbial cells (cell surface hydrophobicity, inner membrane permeability and zeta potential) induced by prolonged contact of microorganisms with clotrimazole. Experiments were performed with the use of bacterial cells before long-term contact and after one and three months of their exposure to clotrimazole. Moreover, the ability of three bacterial species, isolated from activated sludge to biodegradation of clotrimazole was checked. Another objective of this work was the evaluation of clotrimazole toxicity towards selected bacterial strains.

Besides, in order to determine the influence of natural surfactants coming from *Saponaria officinalis* on clotrimazole biodegradation and cell surface properties modification, in parallel the experiments with the addition of saponin-rich plant extract were performed.

2. Materials and methods

2.1. Microorganisms

Microorganisms used in this study were isolated from activated sludge that was collected aseptically from the municipal sewage treatment plant. The samples collected containing microorganisms were diluted with sterile mineral salt medium (composition [$g\,L^{-1}$]: Na_2HPO_4 2.79, KH_2PO_4 1.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4{\cdot}H_2O$ 0.2, $Ca(NO_3)_2{\cdot}7H_2O$ 0.01, ammonium iron(III) citrate 0.01, H_3BO_3 0.0003, $CoCl_2{\cdot}6H_2O$ 0.0002, $ZnSO_4{\cdot}7H_2O$ 0.0001, $Na_2MoO_4{\cdot}2H_2O$ 0.00003, $MnCl_2{\cdot}4H_2O$ 0.00003, $NiCl_2{\cdot}6H_2O$ 0.00002, $CuCl_2{\cdot}2H_2O$ 0.0001) with the addition of sterilized glucose and clotrimazole solutions. Afterwards, the samples were incubated at 30 °C with shaking at 120 rpm. Every 7 days, the microorganisms were transferred to a fresh culture medium. In the final isolation stage the cultures contained clotrimazole as the sole source of carbon and energy. After 10 weeks of cultivation, the bacteria were isolated and identified biochemically and genetically with the use of Vitek 2® system and 16 S rRNA gene amplification, respectively. Microbial species used in the experiments were: *Aeromonas* sp. AS1, *Alcaligens faecalis* and *Aeromonas* sp. PA3.

2.2. Growing conditions

In order to evaluate the impact of long-term contact of microorganisms with clotrimazole on bacterial cells, the selected bacteria were incubated at 30 °C with shaking at 120 rpm in liquid cultures in the presence of fungicide as the sole source of carbon for 3 months. Every 7 days they were subcultivated under sterile conditions in order to provide fresh nutrients. Each culture contained 45 mL mineral salt medium, 0.2 mL of clotrimazole solution (the final concentration of clotrimazole in the culture was 0.1 mg L^{-1}) and 5 mL previous culture. In this paper cell lines subjected to metabolic stress are described as "stressed bacterial strain".

2.3. Chemicals

For preparing all media and aqueous solutions the deionised and ultrapurified Mili-Q water were used. Salts used to the preparation of MSM (mineral salts medium) were purchased from Avantor Performance Materials Poland S.A. Other fine chemicals, e.g. clotrimazole, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, used in experiments were of the highest purity grade (98% or greater). They were purchased from Sigma-Aldrich. The natural surface active agent used in the experiments was *Saponaria officinalis* extract. The extract contained non-ionic surfactants – saponins which were obtained using the Soxhlet extractor with methanol used as an extraction solvent (Smułek et al., 2017). The extraction yield was equal to 10% and the presence of saponins in the extract obtained was confirmed by measuring the lyophilized extract with FTIR spectrometer and analyzing it using LC–MS/MS system with a low resolution mass spectrometer. The critical micelle concentration of the extract was 1.00 g L^{-1}.

2.4. Biodegradation assay and LC-MS/MS analysis

For evaluation of the biodegradation of clotrimazole by selected bacterial strain, the cells cultures were prepared in sterile 250 mL ShotDuran® laboratory glass bottles. Each bottle contained 45 mL mineral salt medium, 5 mL inoculum and previously filtered methanol solution of clotrimazole. The final concentration of fungicide in each sample was 0.1 mg L^{-1}. Simultaneously, analogous cultures with the addition of *Saponaria officinalis* extract were cultivated. The concentration of surfactants in the systems tested was chosen according to their CMC (critical micelle concentration). Abiotic control samples (without the presence of microorganisms) were also carried out. All the tests were incubated on a rotary shaker (agitation rate 120 rpm) at 30 °C for 36 days. Every few days samples were taken in order to analyze the amount of clotrimazole present in the culture.

The concentration of residual clotrimazole in bacterial cultures was determined with the use of LC-MS/MS analysis. The analysis was performed using the UltiMate 3000 chromatograph from Dionex (Sunnyvale, CA, USA) coupled with the API 4000 QTRAP mass spectrometer from Applied Biosystems/MSD Sciex (Foster City, CA, USA). Clotrimazole was determined on the Gemini-NX C18 (100 mm × 2.0 mm I.D.; 3 μm) column from Phenomenex (Torrance, CA, USA) thermostated at 35 °C, onto which 5 μL of samples were injected. A gradient elution at 0.3 mL min^{-1} flow-rate of mobile phase was used. The gradient started from 20% of $5{\cdot}10^{-3}$ mol L^{-1} ammonium acetate in water and 80% of methanol and changed to 90% of methanol in 3 min. Then, methanol was increased to 100% in 1 min and the chromatographic run was finished 4 min after the injection. There were 3 min of mobile phase equilibration before the next run. The eluate from the column was directed to the mass spectrometer through the ESI source working in the positive ionization mode. Nitrogen was used in both the source and mass spectrometer. The following parameters of the ESI source and mass spectrometer were used: curtain gas pressure 20 psi, nebulization gas pressure 45 psi, auxiliary gas pressure 45 psi, source temperature 300 °C, ESI voltage 4500 V, decluttering potential 60 V, collision gas at medium pressure. The multiple reaction monitoring was used with 200 ms dwell time. The quantitative transition was performed from 277.2 *m/z* to 165.2 *m/z* at 29 V collision energy and the confirmatory transition – from 277.2 *m/z* to 242.2 *m/z* at 28 V collision energy.

2.5. Microbial adhesion to hydrocarbons

In order to evaluate the cell surface hydrophobicity of tested strains, the method based on microbial adhesion to hydrocarbons was used (Górna et al., 2011). The modification of hydrophobicity was measured for each bacterial species before metabolic stress and after a month and