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Tolerance to cytostatic drugs bleomycin and vincristine by white rot fungi

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Abstract: Cytostatic drugs have become one of the greatest environmental hazards. They exhibit toxic, carcinogenic, mutagenic and teratogenic effects on flora and fauna, including people. They are poorly eliminated in conventional wastewater treatment plants and their mixtures could possess higher ecotoxicity than individual drugs. Fungi are organisms with enormous potential for biodegradation of a variety of toxic chemical pollutants. The aim of this work was to estimate tolerance of five fungal strains to selected anticancer drugs, which will be useful to determine the potential for their possible use in cytostatics removal and may be significant in the context of wastewater treatment application. Test was conducted on *Fomes fomentarius* (CB13), *Hypholoma fasciculare* (CB15), *Phyllotopsis nidulans* (CB14), *Pleurotus ostreatus* (BWPH) and *Trametes versicolor* (CB8) and the chosen drugs were bleomycin and vincristine. Their ability to grow in the presence of selected cytostatics was evaluated in cultures conducted on two solid media which differed in the richness of nutrient compounds. Fungal strains tolerance was expressed as a half maximal effective concentration.

Results showed that fungi display better tolerance to high cytostatics' concentrations in the medium rich in carbon source. Regardless of the medium used, the differences in growth ability were lower for bleomycin (the tolerance was higher). The greatest tolerance for bleomycin was shown by *Pleurotus ostreatus*. Results suggest that more efficient elimination of bleomycin would be possible to obtain, strain BWPH seems to be the best fungal candidate for this drug degradation assay and, probably, in wastewater treatment application tests in a longer perspective.

Introduction

Thousands of drugs are used globally and therefore pharmaceuticals are becoming ubiquitous in the environment (Balcerzak and Rezka 2014; Jie et al. 2017). They have been identified all over the world in the seas, lakes, rivers, groundwater, soil and lake sediments (Balcerzak and Rezka 2014). Pharmaceuticals constitute a potential risk group of multiclass chemicals which exhibit negative effects on micro- and macroflora and fauna as well as on human health (Olicón-Hernández et al. 2017). Considering the growing number of people suffering from cancer, cytostatic drugs became significant environmental risk factors, which results from their toxic, carcinogenic, mutagenic and teratogenic effects (Balcerzak and Rezka 2014, Castellet-Rovira et al. 2018). In addition, their mixtures in real samples could possess greater toxicological effect as compared to the individual drugs (Ferrando-Climent et al. 2015). Cytostatics are particularly critical micropollutants due to their poor elimination in conventional wastewater treatment plants, either by biological conventional activated sludge treatments or by advanced technologies studied, such as membrane bioreactors, electrolysis and advanced oxidation processes (Castellet-Rovira et al. 2018, Ferrando-Climent et al. 2015). Even though the development of removal technologies for these compounds is not keeping pace with the swift increase in their use, many attempts have been made to solve this problem. Wastewater fungal treatment seems to be a promising alternative. Fungi are versatile and robust organisms with enormous potential for bioremediation and biodegradation of a variety of toxic chemical pollutants (Asgher et al. 2008). They have a high tolerance to toxic substances in the environment and a variety of strategies to resist these compounds. These strategies include both non-enzymatic processes, such as bioadsorption and biomineralization (bio-precipitation), as well as biotransformation (which includes hydroxylation, oxidation, sulfoxidation, and dealkylation reactions) and biodegradation mediated by extracellular and intracellular enzymatic systems (Asgher et al. 2008, Olicón-Hernández et al. 2017). White-rot fungi (WRF), which are mostly basidiomycetes, had come into the area of interest of industrial and environmental microbiology (Asgher et al. 2008, Prakash 2007). These fungi have successfully been

utilized in degradation of environmental pollutants, such as polyaromatic compounds, pesticides, dyes, heavy metals, and polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), trinitrotoluene (TNT) and a range of other toxic pollutants, such as cyanides, azide, carbon tetrachloride and pentachlorophenol (Gadd 2001, Olicón-Hernández et al. 2017, Prakash 2007, Przystaś et al. 2010, Singh et al. 2010). Ligninolytic enzymes play the most important role in the degradation of persistent organic pollutants, mostly due to their low substrate specificity. Besides causing the transformation of lignin particles, they also degrade and detoxify xenobiotic compounds of aromatic structure (Bending et al. 2002, Gadd 2001, Kües 2015, Lee et al. 2014, Przystaś 2016, Singh et al. 2010). It is worth to notice that PAHs and dyes not only have complex structure (multiple aromatic rings combined with a variety of functional groups) but also are recalcitrant, toxic, mutagenic and cancerogenic, just like cytostatic drugs (Gadd 2001, Przystaś 2016, Singh et al. 2010). This makes the biological transformation of anticancer drugs using WRF a promising alternative to standard WWTP. The use of their oxidoreductase enzymes could be proposed as cost – effective, efficient and environmentally friendly solution for municipal, industrial or hospital water treatment technologies (Asgher et al. 2008, Castellet-Rovira et al. 2018, Naghdi et al. 2018, Olicón-Hernández et al. 2017).

There is lack of knowledge about the fungal candidates for pharmaceutical degradation screening by testing their growth ability. Thus, the aim of this work was to evaluate the tolerance of five fungal strains to selected anticancer drugs, namely bleomycin and vincristine, which will be useful to determine the potential for their possible use in cytostatics removal. *Trametes versicolor* and *Pleurotus ostreatus* represent the most commonly used fungal species in the biodegradation of Pharmaceutically Active Compounds (PhACs) but also other less studied strains with potential for bioremediation were included. Our results may be significant in the context of application of investigated fungal strains in wastewater treatment.

Materials and methods

Test compounds

Standards for chemical analysis of Bleomycin sulfate (CAS: 9041-93-4) and Vincristine sulfate (CAS: 2068-78-2) were supplied by Chemat (Gdańsk, Poland).

Test solutions preparation

Concentrated stock solutions (2500 mg/L) of each chemical were prepared in deionized water and stored in the dark at 4°C. The test solutions were prepared immediately before tests by mixing the appropriate volumes of the stock solutions with test media.

Fungi isolation

Pure cultures of selected microorganisms were isolated by spore and tissue method from fruit bodies and propagated. These were: *Fomes fomentarius* (CB13), *Hypholoma fasciculare* (CB15), *Phyllotopsis nidulans* (CB14), *Pleurotus ostreatus* (BWPH) and *Trametes versicolor* (CB8). The strains were collected in the Culture and Recreation Park and Bolesław Chrobry Park in Gliwice, Southern Poland.

Fungi species identification

Fungi species were pre-identified using morphological analysis which consisted of macro- and microscopic observations. Identification of strains has been confirmed using molecular methods. Genomic DNA was extracted using a modified CTAB (hexadecyltrimethylammonium bromide) method according to Gálvez et al. (2017). PCR (Polymerase Chain Reaction) was done using DreamTaq Green DNA polymerase (Thermo Scientific, Espoo, Finland). The composition of each reaction mixture and PCR conditions were described earlier by Kozłowska et al. (2018). Species identification was based on the Internal Transcribed Spacer ribosomal DNA sequence analysis using two primers: ITS4 – 5'– TCCTCCGCTTATTGATATGC -3' and ITS5 – 5'– GGAAGTAAAAGTCGTAACAAGG –3' (White et al. 1990).

For sequence analysis, PCR-amplified DNA fragments were purified with exonuclease I (Thermo Scientific) and shrimp alkaline phosphatase (Thermo Scientific). DNA fragments were labeled using forward primer and the BigDyeTerminator 3.1 kit (Applied Biosystems, Foster City, CA, USA). Conditions of amplicon purification and labeling were described earlier by Gorczyca et al. (2018).

Sequence reading was performed using Applied Biosystems equipment. Sequences were compared to the NCBI GenBank-deposited sequences using BLASTn algorithm.

All isolated species were deposited in the Fungal Strain Collection of The Biotechnology Centre, The Silesian University of Technology, Gliwice, Poland.

Toxicity tests

Strains' ability to grow in the presence of selected anticancer drugs was tested by placing 8 mm disc of fungal culture on solid media: MEA (Malt Extract Agar, BTL) which is rich in carbon and nitrogen, and MSB (Mineral Salt Broth) containing 10g/L glucose, 0.2 g/L ammonium tartrate, 10 mg/L thiamin, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 g/L $CaCl_2$, with the addition of different concentrations of chosen cytostatics, and incubating for six or twelve (in the case of strains CB14 and CB15) days at 26°C. The cytostatics were tested three-five times for each organism and each replicate covered a range of at least five different concentrations of the drugs (arranged in a geometric series with factor 2). The highest tested concentration was 100 mg/L and the lowest was 0.01 mg/L. Fungal tolerance was measured by establishing the EC_{50} (effective concentration at which 50% of the tested effect is reached), with reference to control without a drug. Concentrations causing 50% growth inhibition were calculated by logarithmic-probit analysis method with defining 95% confidence. Calculations were performed with Microsoft Excel software.

Statistical analyses

Shapiro-Wilk test was used to test the data normality, and then, based on the statistic results, the Kruskal-Wallis rank sum test was used to examine the significance of the differences between the growth medium and examined drug within the tests for individual fungus. The p values <0.05 were considered statistically significant. Dunn's test was conducted as a post-hoc pairwise multiple comparison test to discern which of the pairs have significant differences. Dunn's test was further adjusted by the Holm method.

In addition, to examine whether there are significant differences between the media for all fungi within one chosen drug, Mann-Whitney *U* test and Student's *t*-test were performed for bleomycin and vincristine, respectively. Statistical analyses of the obtained results were performed with Statistica 12.0 Software (StatSoft) and MS Excel.

Results and discussion

Molecular identification

Five fungal strains used in this study were identified based on the analysis of the ITS1-ITS2 sequence. The DNA fragments were amplified using PCR with ITS4-ITS5 primers and sequenced. The complete sequences of these products (ca. 600 bp) were compared to the reference ITS sequences deposited in the GenBank Database (Table 1).

Fungal strains tolerance to cytostatics

In the present study, the ability of five white-rot fungi strains to grow in the presence of two anti-neoplastic drugs, bleomycin and vincristine, was evaluated. Growth inhibition tests' results are shown in Figures 1 and 2.

The results were also analyzed to note whether there were statistically significant differences for a given fungus between its growth in the company of different drugs and on different media, what is presented in Table 2. In addition, in Table 3 it is shown between which groups are those differences.

Table 1. Identification of fungal strains on the basis of the ITS1-ITS2 sequence, and respective reference GenBank ITS sequences

Symbol	Identified fungal species	Sequence identity
CB13	*Fomes fomentarius*	99% identity to the *Fomes fomentarius*, acc. numbers: MG719678.1; MG719676.1; KM433840.1
CB15	*Hypholoma fasciculare*	100% identity to the *Hypholoma fasciculare*, acc. numbers: KY950514.1; KY950442.1; KU836538.1
CB14	*Phyllotopsis nidulans*	99% identity to the *Phyllotopsis nidulans*, acc. numbers: GQ142019.1; DQ404382.1
BWPH	*Pleurotus ostreatus*	99% identity to the *Pleurotus ostreatus*, acc. numbers: LN877895.1; JQ316531.1; EU622256.1
CB8	*Trametes versicolor*	98% identity to the *Trametes versicolor*, acc. numbers: KR673701.1; MG231888.1; HM016848.1

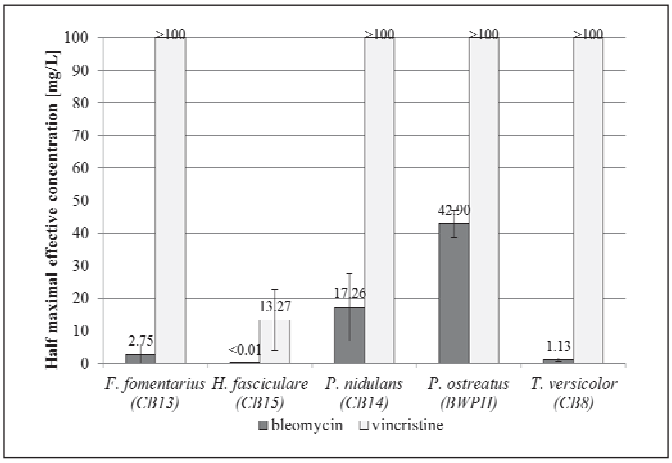

Fig. 1. Fungal strains tolerance to bleomycin and vincristine on MEA medium expressed as a half maximal effective concentration

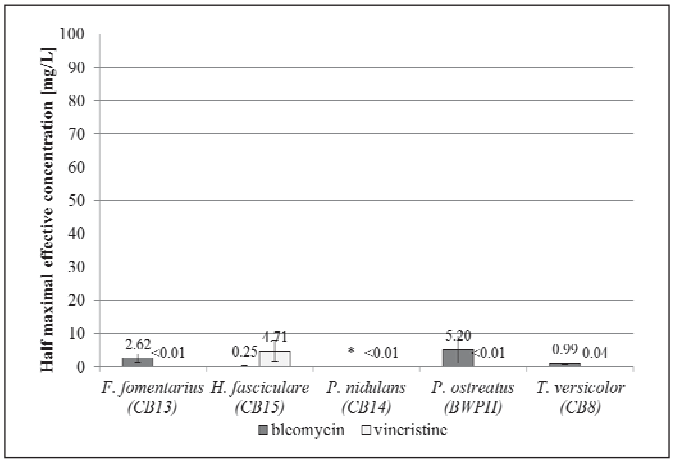

Fig. 2. Fungal strains' tolerance to bleomycin and vincristine on MSB medium, expressed as a half maximal effective concentration; * low growth at blind sample, which leads to measure inaccuracy

Table 2. Statistical analysis of significance of the differences between individual fungus growth in the company of different drugs and on different medias using Kruskal-Wallis rank sum test (results with $p<0.05$ were considered statistically significant)

	F. fomentarius (CB13)	*H. fasciculare* (CB15)	*P. nidulans* (CB14)	*P. ostreatus* (BWPH)	*T. versicolor* (CB8)
Kruskal-Wallis chi-squared statistic:	11.411783	16.342901	11.307692	15.535814	11.942857
Degrees of freedom:	3	3	2	3	3
p-value:	0.009695	0.000964	0.003504	0.001412	0.007581

Table 3. Results of post – hoc Dunn test, further adjusted by the Holm – Bonferrony method (results with $p<0.05$ were considered statistically significant)

	F. fomentarius (CB13)			*H. fasciculare* (CB15)			*P. nidulans* (CB14)			*P. ostreatus* (BWPH)			*T. versicolor* (CB8)		
	MEA BLM	MEA VCN	MSB BLM	MEA BLM	MEA VCN	MSB BLM	MEA BLM	MEA VCN	MSB BLM	MEA BLM	MEA VCN	MSB BLM	MEA BLM	MEA VCN	MSB BLM
MEA VCN	0.550	–	–	0.005	–	–	0.271	–	–	0.137	–	–	0.302	–	–
MSB BLM	0.704	0.828	–	0.356	0.158	–	–	–	–	0.509	0.509	–	0.829	0.297	–
MSB VCN	0.016	0.003	0.006	0.003	0.872	0.143	0.003	0.055	–	0.001	0.310	0.028	0.302	0.003	0.302

Presented tolerance test is a presumptive study to screen fungi for their potential abilities to degrade chosen cytostatic drugs. The ability to grow in the presence of anti-cancer compounds may indicate that the strain may be capable to conduct biotransformation processes. The main mechanism in the degradation of the PhACs and aromatic compounds by basidiomycota is due to synergistic effects of extracellular and non-specific nature of the multi-enzyme system, comprising mainly of lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), versatile peroxidase (VP) and laccase, along with other accessory enzymes, including intracellular cytochrome P450 monooxigenases and nitroreductases (Asgher et al. 2008, Castellet-Rovira et al. 2018, Cerniglia 1997, Lee et al. 2014, Naghdi et al. 2018, Olicón-Hernández et al. 2017). Two of the tested species (*Pleurotus ostreatus* and *Trametes versicolor*) are regarded as model organisms in basic and applied laccase research (Jie et al. 2017). *Fomes fomentarius* is another well-known laccase-producing basidiomycete (Jie et al. 2017, Ramesh and Pattar 2009). These enzymes are the main family of important applications in bioremediation, but pharmaceutical's removal is usually obtained by a combination of laccase with other enzymes, such as versatile peroxidase (which has a wider removal spectrum) and glucose oxidase or tyrosinase (Jie et al. 2017). Besides laccases, *Pleurotus* sp. possess another lignin degrading enzymes, i.e. MnPs, aryl alcohol, glyoxal and pyranose oxidases, with extraordinary oxidation potential, while *T. versicolor* has different peroxidases, such as VPs, MnPs or LiPs, which are capable of mineralizing a variety of recalcitrant aromatic compounds (Asgher et al. 2008, Castellet-Rovira et al. 2018, Singh et al. 2010). Thus, it can be expected that these fungi will show the highest growth potential in the presence of the tested drugs and the degradation ability of investigated substances. Indeed, strain BWPH (*P. ostreatus*) appeared as the fungal strain that may be used as a remedy for both tested drugs in the case of studies conducted on MEA medium, because it has the greatest ability to grow in high concentrations of both cytostatics.

All tested fungal species were able to degrade aromatic dyes and mono-aromatic pesticides (Bending et al. 2002, Jayasinghe et al. 2008, Nozaki et al. 2008, Prakash 2007, Ramesh and Pattar 2009). In addition, *T. versicolor* and *P. ostreatus* have already been shown to have a special capacity to remove a wide amount of recalcitrant PhACs including β-blockers, antibiotics, anti-inflammatory and psychiatric drugs, achieving even their mineralization, but extremely little is known about their usefulness in cytostatics' elimination (Castellet-Rovira et al. 2018, Ferrando-Climent et al. 2015, Prakash 2007). According to our knowledge, only a few studies on cytostatic drugs removal using WRF have existed until now. Ferrando-Climent et al. (2015) performed a study on the elimination of anticancer drugs (ciprofloxacin, cyclophosphamide, ifosfamide, methotrexate, azathioprine, etoposide, docetaxel, paclitaxel, vincristine and tamoxifen) using an alternative biological treatment based on the fungus *T. versicolor*. Studies were performed for 8 days in a 10 L fluidized bed bioreactor inoculated with this fungus and were set up in order to evaluate the elimination of cytostatics from real hospital wastewater. Most of them were removed in 48 to 100% by combined sorption-biodegradation processes. This fungal treatment was not efficient only in eliminating cyclophosphamide and ifosfamide, even under optimal growth conditions. Interestingly, the elimination of cyclophosphamide and ifosfamide was also tested by Castellet-Rovira et al. (2018) with six different ligninolytic fungi: *Trametes versicolor*, *Ganoderma lucidum*, *Irpex lacteus*, *Stropharia rugosoannulata*, *Gymnopilus luteofolius* and *Agrocybe erebia*. The removal rate of 20% or more was achieved by *S. rugosoannulata* and *G. lucidum* and the biodegradation rate of over 25% was obtained by *I. lacteus* and surprisingly *T. versicolor*, although their efficiency might have been caused by non-biological mechanisms. The same outcome about those two drugs' elimination results from Haroune et al. (2014) research conducted on *T. hirsute* (< 40% cytostatics removal). Those two studies indicate that even though *T. versicolor* tolerance is not the highest, these strains should not be omitted while considering fungi with the ability to biodegradate bleomycin and vincristine, especially that Ferrando-Climent et al. (2015) already confirmed that the species are able to eliminate the vinca alkaloid.

The differences in nutrient content between Malt Extract Agar and Mineral Salt Broth should force fungi to use selected anti-cancer drugs as a carbon source in the second medium. In the case of bleomycin, the tolerance of fungal strains is similar on both tested media, what is confirmed by the lack of statistical differences in the growth ability (p=0.322; U-value=878). There were also no statistically significant differences examined by Kruskal Wallis-Dunn test in MEA vs MSB comparison set with this drug (Tab. 3). Obtained results indicate that *Pleurotus ostreatus* tolerates the presence of this drug at best (Fig. 1 and 2). Bleomycin was the most toxic to *Hypholoma fasciculare* which, possibly, does not produce enzymes needed for its degradation. *Trametes versicolor*

displayed EC_{50} at the level of 1, which is surprisingly low when the wide use of this strain for degradation of dyes, pesticides and pharmaceuticals is considered. On the other hand, *Trametes versicolor*, together with *Fomes fomentarius*, are the strains with almost no differences in tolerance to bleomycin between two media tested. That indicates that the lack of nutrients does not affect their ability to grow dramatically, which can suggest that they produce enzymes needed for bleomycin degradation and then use the products as a carbon source. *Phyllotopsis nidulans* showed impaired growth even for blind sample on MSB with approximately 1.8 cm diameter of colony growth, which made it impossible to determine the EC_{50}.

Results obtained in the test with vincristine were unexpected. In all tested strains except CB15, there were no differences in growth between blind sample (control without a drug) and the highest concentration with cytostatic used (100 mg/L) on MEA. In contrast, three of four strains were not able to grow at the lowest tested concentration (0.01 mg/L) on MSB. This could suggest the existence of a mechanism blocking the access of toxic substances to the entire mycelium and selective use of the substrates from the medium. The reason of extreme differences in fungal tolerance to vincristine, depending on the tested medium, was possibly similar to those presented by Cerniglia (1997). The author claimed that in the case of polycyclic aromatic hydrocarbons, fungi do not utilize them as the sole source of carbon and energy. Therefore, to allow fungi to metabolize them, the medium should be supplemented with an additional carbon source which does not negate the fact that some WRF have the ability to cleave the aromatic rings and mineralize PAHs. This may also be valid for vincristine and tested fungal strains. On the other hand, studies on elimination of trace organic contaminants (TrOC) by *T. versicolor* showed that the removal of hydrophilic ones, with log D<3, was negligible (Nguyen et al. 2014). Vincristine K_{OW} is equal 2.82 and according to Popowicz and Koszelnik (2015) it is approximately equal to log D. This indicates that sorption process of vincristine as hydrophilic compound would be low, though the degradation by intracellular enzymes would not take place. Notably, effective degradation of PhACs by fungal cultures is not only caused by higher hydrophobicity but electron-donating groups, such as amine and hydroxyl, which also leads to drugs' elimination (Naghdi et al. 2018). Such groups do have both tested compounds: bleomycin and vincristine. In addition, fungi are able to produce biosurfactants. These diverse amphiphilic surface-active compounds interact between phases of different polarities, reduce interfacial tension and increase interactions between molecules. These compounds with hydrophilic and hydrophobic portions are well-known tools used in bioremediation. Here, both tested drugs contain aromatic structures, thus, it could be possible that biosurfactants will improve their mobility and increase their bioavailability, as it has previously been observed for PAHs (Olicón-Hernández et al. 2017).

P. nidulans (CB14) and *H. fasciculare* (CB15) incubation times were twice longer than those of the remaining strains, but it is not the reason of better *H. faciculare* vincristine tolerance on MSB. According to Szałek et al. (2013) this cytostatic is stable for at least 31 days at room temperature (15–25°C). This means incubating temperature was not the factor affecting the durability and toxicity of the drug. Measuring accuracy may be more likely reason, due to the very limited growth of this fungus on MSB, such as the strain CB14, which, consequently, strongly influenced the results observed.

In general, the tested fungi showed greater ability to grow in the presence of bleomycin regardless of culture medium composition. In the long term, the potential to depredate this drug by tested fungal strains should be analyzed. Nevertheless, the removal performance does not only depend on fungal species, their secreted enzymes, and molecular structure of target compounds, but also on factors such as temperature, pH or aeration, and those parameters need also to be investigated (Naghdi et al. 2018). The existence of hydroxylation, oxidation, sulfoxidation, formylation, deamination, dealkylation and dehalogenation mechanisms involved in the removing processes is of high importance, even if they do not always result in desirable or complete mineralization of the compounds. They may lead to the formation of intermediate transformation products or just the sorption of parent compounds by the biomass (Castellet-Rovira et al. 2018, Ferrando-Climent et al. 2015, Jie et al. 2017, Olicón-Hernández et al. 2017). In addition, detoxification does not necessarily occur, since by-products of the process can be occasionally more recalcitrant or even more toxic than the parent compound (Ferrando-Climent et al. 2015, Naghdi et al. 2018). All those issues need to be taken into account when mycoremediation is considered as a promising alternative, which can replace or supplement cytostatic drugs removal and treatment processes used at present.

Conclusions

The objective of the present study was to assess the tolerance of five fungal strains and, in longer perspective, their capacity to degrade recalcitrant cytostatic drugs: bleomycin and vincristine. To accomplish this goal EC_{50} values were evaluated on two solid media differing in the content of nutrients. Results showed that fungi display better tolerance to high cytostatics' concentrations in the medium rich in carbon source. Regardless of the medium used, the differences in growth ability were lower for bleomycin (the tolerance was higher). Possibly, better elimination rates would be possible to obtain with this drug. The *Pleurotus ostreatus* (BWPH) strain seems to be a promising fungal candidate to further study the bleomycin degradation and, probably, in wastewater treatment application tests in the long term.

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