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NEWLY STRATEGY FOR BIODEGRADATION OF PHARMACEUTICALS IN CONTROLLLED BIOTECHNOLOGICAL CONDITIONS

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The presence of emerging contaminants in the environment, such as pharmaceuticals, is a growing global concern. Studies on the occurrence of pharmaceuticals show that the widely used pharmaceuticals containing the diclofenac and acid clofibric are present in significant concentrations in the environment. *In situ* bioremediation techniques, based on augmentation strategy, have received the most attention, because it is friendly to environment and implies relatively low cost. This study employs an alternative solution for the emerging pollutants removal from the wastewaters. Thus, in order to enhance the biodegradation of recalcitrant xenobiotic pollutants, the possibility of biostimulation of the activity of multiple microbial consortia (selected fungal strain and activate sludge) was investigated. The obtained results showed a high yield of removal of target molecules (clorfibric acid and diclofenac) when the biomass ratio of a selected fungal strain of *Trametes pubescens*: activated sludge was 1:1. The idea for use consortia of selected and wild microorganisms in pharmaceuticals biodegradation is a valuable one which can be applied with efficiency in *in situ* bioremediation processes of polluted waters.

Keywords: bioremediation, clofibric acid, diclofenac, *Trametes pubescens* activated sludge, emergent organic pollutants

INTRODUCTION

The presence of micropollutants such as pharmaceuticals (PhACs), industrial chemicals, personal care products and many other chemical compounds in the aquatic environment have become a significant problem worldwide[1]. Their existence in aquatic system depends on their physicochemical characteristics, particularly on their hydro-solubility, stability, and half-life of the molecules[2]. Hence, these compounds have been found in wastewaters and in surface waters, as they are currently not completely eliminated in wastewater treatment plants (WWTPs). A group that has recently received a lot of attention due to its persistent occurrence in different water sources is that of analgesic, lipid regulators and non-steroidal anti-inflammatory drugs[3,4].

Clofibric acid (CLA) is the main pharmacologically active compound in pharmaceutical products used for controlling blood lipid content. Their concentration levels in surface water and effluent from sewage treatment plants (STPs) have been shown to lay in the nanograms per liter (L) to micrograms per L range[5,6].

Diclofenac (DFC), a phenylacetic acid derivative classified as a non-steroidal anti-inflammatory drug (NSAID), due to wide prevalence is considered as one of the most important contaminants of emerging concern[7,8].

Among PhACs, clofibric acid (CLA) and diclofenac (DCF) are commonly detected in aquatic environments due to their incomplete removal during wastewater treatment processes. Physicochemical methods lead to the best CLA and DCF degradation yields (> 90%), but the inherent drawbacks is the tendency of the formation of secondary toxic by-products[9].

Considering the compounds CLA and DFC with the property of low biodegradation rate, bioconcentration and bioaccumulation in aquatic organisms, inevitably they would pose unpredicted effects on the biology which are exposed to them for long term[10].

Bioremediation is an eco-safe and economically justified alternative for harsh chemical treatments being based on the individual microbial strain able to detoxify the particular compound or microbial consortia with more complex metabolic properties.

Among fungi, white rot species show high efficiency in degradation of a wide range of xenobiotic compounds, this ability being of great interest for the development of environmentally friendly biotechnological processes to be applied in food production, medical application and bioremediation purposes[11].

Only few studies showed the ability of white-rot fungi like as *Trametes versicolor, Irpex lacteus, Ganoderma lucidum* and *Phanerochaete chrysosporium*, to degrade carbamazepine, ibuprofen, diclofenac and clofibric acid. Some studies reported the removal of CLA, in concentration of 10 mg L^{-1} in a high percentage close to 97%[12]. Among others, one strain *Trametes pubescens* was recently selected for its ability to degrade CLA and DCL (up to 30%) during cultivation in submerged system under aerobic conditions at an initial CLA and DCL concentration of 15 mg L^{-1} .[13] More recently, it was evaluated the maximum removal yield of CLA (58.5%) when the wet biomass ratio of selected strain *Streptomyces MIUG 4.89*: activated sludge was 1:1[14] .

Commonly water treatment processes were not designed for the micropollutants removal, although some reports have demonstrated that some types of pharmaceuticals can be eliminated by conventional wastewater treatment system, such as activated sludge. The application of various microorganisms presented in sludge sample is widely used for wastewater treatment. Complex microbial communities in activated sludge are highly responsible for the removal of available nitrogen and carbon[10].

In this framework, the present study evaluates the ability of selected strain of *Trametes pubescens* and their adaptability in multiple cultures with activated sludge to biodegradation of CLA and DCF in biotechnological controlled conditions.

MATERIALS AND METHODS

Microorganism and chemicals

The white-rot fungal strain *Trametes pubescens* was provided from the Cultures Collection of the Faculty of Biology, *Alexandru Ioan Cuza* University of Iasi. The strain was maintained by subculturing on nutrient agar plates at 4°C. Subcultures were routinely made every 30 days.

Activated sludge sample used in this work was collected from aeration tank of the Municipal Sewage Treatment Plant of Galati.

All the chemicals reagents, CLA, DCF, acetonitrile, methanol (HPLC grade) and ingredients for culture media formulation were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Inoculum preparation

A mycelial suspension of white-rot fungal was obtained by inoculation of three plugs (6 mm in diameter) of agar plugs, from the growing zone of fungal on plates, in 150 mL of malt extract medium which was shaken (135 rpm) at 25^0C for 4–5 days. Pellets formed were washed with sterile deionized water and then the dense mycelial mass was aseptically blended with a homogenizer, to obtain homogeny mycelial inoculum. This mycelium was then aseptically inoculated in basal liquid medium target compounds and this procedure was repeated each time for fungal inoculum.

Activated sludge sample was washed four times with tap water to remove the remains of chemical compounds. Then it was inoculated in a glass reactor with a capacity of 8 L, with the following compounds (g L^{-1}): peptone 0.64; NH_4Cl 15.2; K_2HPO_4 0.11; CH_3COONa 140, pH 6.0, for activation before use for biodegradation process.

For use in biodegradation process the both activated inoculum (*Trametes pubescens :* activated sludge) were mixed in ration 1:1 and 1:2.

Biodegradation experiments

The biodegradation experiments were performed by submerged cultivation in basal sterile liquid medium (MM) containing (g L^{-1}): glucose 5, yeast extract 5, peptone 20, $MnSO_4·H_2O$, 0.50; $CuSO_4·5H_2O$, 0.50, supplemented with 15mgL^{-1} target compounds (CLA and DCF), pH 5.5. The cultivation took places on orbital shaker SI-300R Incubator Shaker (Jeio Tech, Korea) at constant stirring rate (135 rpm) and temperature of 25ºC, for 14 days.

All biodegradation tests were performed in the same MM liquid medium. For each experiment, 10 mL of fungal inoculum (OD_{600} =1.3) was added. Also, in this study, it was aimed to establish the optimal conditions for achieving the biostimulation of the biodegradation of pharmaceutical compounds in multiple cultures (*Trametes pubescens* – activated sludge), in submerged cultivation conditions (aerobic and facultative anaerobes), for 14 days, when the biomass dried ratio was varied – fungal inoculum: activated sludge at values of 1:1 and 1:2.

Biosorption assays with heat killed biomass inactivated by autoclaving were performed under similar conditions to those described for biodegradation test in order to evaluate possible adsorption of the target molecules on the cell biomass.

Analytical Methods

Samples were aseptically taken at regular intervals and investigated for biomass concentrations (dry weight) and the residual pollutants content.

For determining l dry weight of biomass, the cultures were vacuum filtered over preweighed glass-fiber filtred (Whatman, Barcelona, Spain). The filters containing the mycelial mass were dried at 100^0 C to constant weight.

Residual concentrations of CLA and DCF were determined using a high performance liquid chromatography (HPLC, Agilent 1200 Series, Santa Clara, CA, USA) system equipped with a photodiode array operating at a wavelength of 230 nm for to quantify the residual CLA and 278 nm for DCF, respectively. Chromatographic separation was achieved on a C18 column (150 mm x 4.6 mm, with particle size 5μm) and at 40 °C. A mobile phase consisting of a mixture of methanol/ultrapure water acidified with 0.1% (v/v) acetic acid (50:50, v/v) were used for clofibric acid. Also, diclofenac separation was performed under isocratic conditions whit 70/30 acetonitrile/ ultrapure water acidified with 0.1% (v/v) acetic acid. The flow rate was 1.0 mL/min^{-1} and the injection volume was 50 μL.

Statistical analysis

All assays were performed in duplicate and the data presented represented the mean values of these replicates. Each dataset was analysed with ANOVA on Statistica 13 (Statsoft, USA) software. The results where $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Biodegradation of target molecules by activated sludge

In this study, it was investigated the ability of the activated sludge to biotransform of the studied pharmaceutical compounds, in a concentration of 15 mg L^{-1}, by cultivated in aerobe submerged controlled biotechnological conditions in the liquid basal medium (MM) As shown in Fig. 1, emergent organic pollutants were almost completely degraded by the activated sludge, after 10 days of submerged cultivation. These observations are in agreement with those reported for carbamazepine when exposed of multiple cultures (*Trametes versicolor* ATCC 42530 – activated sludge)[15]. It was observed that this removal may be influenced by various factors such: temperature can affect the microbial activity in sludge and the maximum removal of target molecules was seen at low temperatures. Also, acidic pH eliminating drugs was considered very favorable where the hydrophobicity plays a very important role.

In all the experiments, only 10% of target molecules were removed due to adsorption in the biomass as observed from the difference in CLA and DCF concentrations between the heat-killed controls and the uninoculated ones. Similar results have been previously reported for the same molecules[16,17]. Moreover, several studies reported that CLA sorption to sludge, sediments and suspended matter is not considered to be an important contribution to the elimination of this molecule from surface and waste water[18,19].

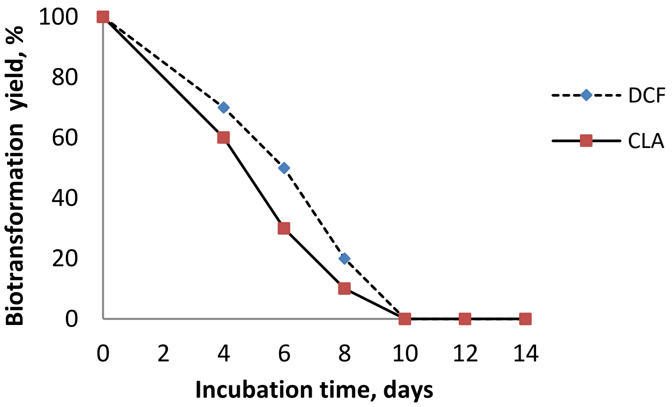

Figure 1. Biotransformation yield of pharmaceutical compounds by activated sludge.

Evaluation of the biodegradation capacity of refractory pharmaceuticals molecules using multiple cultures

The aim was to establish the optimal conditions for achieving the biostimulation of the biodegradation of pharmaceutical compounds in multiple cultures (fungal culture and activated sludge), under submerged conditions by cultivation (aerobic), for 14 days, when the dry biomass ratio was varied to values of 1:1 and 1:2.

In addition, during after 6 days of incubation time, a progressive augmentation of target molecules removal yield can be observed were the activated sludge and the fungal culture are inoculated simultaneously in a ratio of dry biomass fungal culture: activated sludge of 1:1 (Fig. 2 and Fig. 3). These observations are in agreement with those reported previously by Dereszewska *et al.*, for DFC when exposed to multiple cultures[20].

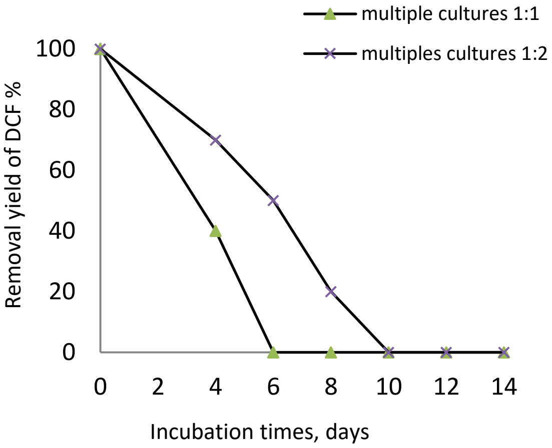

Figure 2. Removal yields of DFC by the multiple cultures cultivated in aerobe submerged conditions in the liquid MM medium, (after 14 days at 25ºC and 135 rpm).

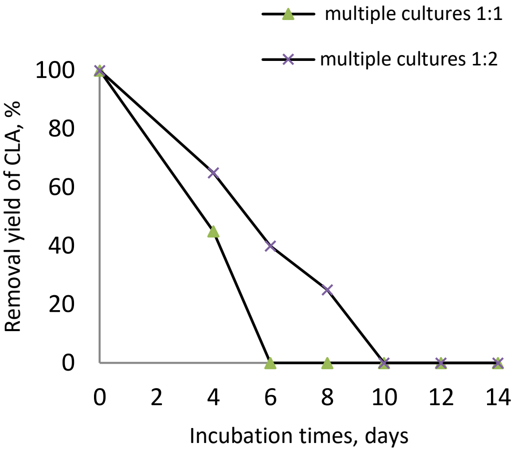

Figure 3. Removal yields of CLA by the multiple cultures cultivated in aerobe submerged conditions in the liquid MM medium, (after 14 days at 25ºC and 135 rpm).

This yield of biodegradation is higher than the one obtained under same conditions but with the separate single inoculum (fungal or activate sludge), suggesting the positive role of the used microbial consortia strategy for the removal of these recalcitrant pharmaceutical molecules. Also, this superior biotransformation yield of the emergent organic pollutants explains the adaptability of the selected fungal strain of *Trametes pubescens* in mutiple consortia with the wild microorganisms which improving the efficiency of the biodegradation process.

CONCLUSIONS

This is an original study on the degradation of CLA and DCF using microbial consortia of selected and wild microorganisms. The results confirm the approaches for use of selected fungal strain and activated sludge from the Municipal Sewage Treatment Plant of Galati for the elimination of pharmaceuticals residual in waters in a controlled biotechnological process of biodegradation. In addition, the microbial consortia diversity and counts have a significant impact on the removal of the target molecules. The reached maximum elimination yield of both studied recalcitrant compounds was after 6 days of incubation when the combined inoculum ratio was: a 1:1.

The proposed treatment could be interesting from the applicability point of view, as the use of these composting-like technologies is widely extended in WWTPs. However, to complete this work, more studies should be carried out to identification of the degradation metabolites and its impact to the environment.

REFERENCES

1. Luo, Y., Guo, W., N.G.O., Huu, H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S. & Wang, X.C. *A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Science of the Total Environment*, **2014**, 473–474, pp. 619–641.
2. Dirany, A., Efremova Aaron, S., Oturan, N., Sirés, I., Oturan, M. A., & Aaron, J. J. *Study of the Toxicity of Sulfamethoxazole and Its Degradation Products in Water by a Bioluminescence Method during Application of the*

Electro-Fenton Treatment. Analytical and Bioanalytical Chemistry, **2011**, 400(2), 353–360.

3. Boix, C., Ibáñez, M., Sancho, J.v., Parsons, J.R., Voogt, P.d., Hernández, F. *Biotransformation of Pharmaceuticals in Surface Water and during Waste Water Treatment: Identification and Occurrence of Transformation Products*. Journal of Hazard Materials, **2016,** 302,175–187.
4. Chonova, T., Keck, F., Labanowski, J., Montuelle, B. *Science of the Total Environment Separate Treatment of Hospital and Urban Wastewaters: A Real Scale Comparison of Efluents and Their Effect on Microbial Communities*. Science of Total Environmental, **2016**, 542, 965–975.
5. Sacher, F., Lange, F. T., Brauch, H. J., & Blankenhorn, I. *Pharmaceuticals in ground waters: analytical methods and results of a monitoring program in Baden-Württemberg, Germany. Journal of Chromatography* A, *2001*, 938, 199–210.
6. Salgado, R., Noronha, J. P., Oehmen, A., Carvalho, G.,& Reis, M.A. M. *Analysis of 65 pharmaceuticals and personal care products in 5 wastewater treatment plants in Portugal using a simplified analytical methodology. Water Science and Technology,* **2010,** 62, 2862–2871.
7. Kamal, M.A.H.M., Iimura, N., Nabekura, T., Kitagawa, S. *Enhanced skin permeation of diclofenac by ion-pair formation and further enhancement by microemulsion. Chemical and Pharmaceutical Bulletin*, 2007, 55 (3), 368–371.
8. Kruglova, A., Ahlgren, P., Korhonen, N., Rantanen, P., Mikola, A., Vahala, R. *Biodegradation of ibuprofen, diclofenac and carbamazepine in nitrifying activated sludge under 12 C temperature conditions. Science of Total Environmental***, 2014**,499, 394–401.
9. Jones, O. A. H., Voulvoulis, N., & Lester, J. N. *The occurrence and removal of selected pharmaceutical compounds in a sewage treatment works utilizing activated sludge treatment. Environmental Pollution*, 2007, 145, 738–744.
10. Lee, J., Ji, K., Kho, Y.l., Kim P., Choi, K. *Chronic exposure to diclofenac on two freshwater cladocerans and Japanese medaka. Ecotoxicology and Environmental Safety***, 2011**, 74 (5), 1216.
11. Oldenkamp, R., Beusen, A.H., Huijbregts, M.A. *Aquatic risks from human pharmaceuticals—modelling temporal trends of carbamazepine and ciprofloxacin at the global scale. Environmental Research Letters*, **2019**, 14, 034003.
12. 12. Marco-Urrea, E., Perez-Trujillo, M., Blanquez, M., Vicent, T., Caminal, G. *Biodegradation of the analgesic naproxen by Trametes versicolor and identification of intermediates using HPLC-DAD-MS and NMR*. Bioresource Technology, **2010**, 101, 2159–2166.
13. Ungureanu, C., Bahrim G., Favier L., Tănase, C. *Increase of the Trametes versicolor efficiency in the bioremediation process for diclofenac biodegradation in aquatic environment. The Annals of the University Dunărea de Jos of GalaţiFascicle VI –Food Technology,* **2015**, 39(1), 25-35.
14. 15. Rodríguez-Rodríguez, C., Jelić, A., Alcina Pereira, M., Sousa, D., Petrović, M., Alves, M., Barcelo, D., Caminal, G., Teresa Vicent, T. *Bioaugmentation of Sewage Sludge with Trametes versicolor in SolidPhase Biopiles Produces Degradation of Pharmaceuticals and Affects Microbial Communities. Environmental Science & Technology,* **2012,** 46, 12012−12020.
15. 16. Salgado, R., Oehmen, A., Carvalho, G., Noronha, J. P., & Reis, M. A. M. *Biodegradation of clofibric acid and identification of its metabolites.* Journal of Hazardous Materials, **2012**, 241-242, 182–189.
16. 17. Yan, Q., Gao, X., Huang, L., Gan, X. M., Zhang, Y. X., Chen, Y. P., Peng, X. Y., & Guo, J. S. *Occurrence and fate of pharmaceutically active compounds in the largest municipal wastewater treatment plant in Southwest China: Mass balance analysis and consumption back-calculated model,* Chemosphere, **2014**, 99, 160–170.
17. 18 Fent, K.,Weston, A. A.,&Caminada, D. *Ecotoxicology of human pharmaceuticals.* Aquatic Toxicology, **2006**, 76, 122–159.
18. 19. Kosjek, T., Heath, E., Pérez, S., Petrović, M., & Barceló, D. *Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole - time-of-flight mass spectrometry.* Journal of Hydrology, 2009, 372, 109–117.
19. 20. Dereszewska A. and Stanislaw Cytawa S. *Effect of Diclofenac Concentration on Activated Sludge Conditions in a Biological Wastewater Treatment Plant,* Water, **2023**, 15,1838.