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AN INVESTIGATION BASED ON PHARMACEUTICAL COMPOUND REMOVAL IN MULTIPLE CULTURES OF *STREPTOMYCES* MIUG 4.89 AND ACTIVATED SLUDGE

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Key words*: pharmaceutically active compounds, clofibric acid, activated sludge, bioremediation*

INTRODUCTION

The potential negative effect of pharmaceutical compounds to aquatic and terrestrial wildlife makes interest on the study of their existence, activity and removal from waste water (Yan, Q. et al., 2014). Organic micropollutants such as pharmaceuticals enter the water cycle either as the original compound or in a metabolized form at low loads resulting in relatively low concentrations. Their existence in aquatic system depends on their physicochemical characteristics, particularly on their hydro-solubility, stability and half-life of the molecules (Dirany, A., et al. 2011).

Many processes can take place in compounds in the aquatic environment, like photochemical degradation, dilution and transportation, sorption onto solid matrices, within the aquatic system with potential absorption in biological species (Mceneff, G., et al. 2015, Karungamye, Petro Novert, 2020).

Previous studies have shown that pharmaceutical active compounds (PhACs) show a remarkably high persistence and are not eliminated efficiently during the wastewater treatment plants (Tauxe-Wuersch, A., et al. 2005, Semrany, S.,et al., 2012; Salgado, R., et al., 2012).

Among them, clofibric acid (CLA) is one of this of pharmaceutical contaminants substances reported like drug metabolite, who is detected in the aquatic ecosystems. However, several studies reported it presence wastewaters, in surface waters (Ternes T.A., 1998; Andreozzi R., et al., 2003)and also in drinking waterafter treatment process (Heberer T.,2002; Boyd G.R.,et al., 2003)in concentration levels ranging from ngL^{-1} to $\mu g\ L^{-1}$. To date, a few reports about the potential of different species belonging to the genus *Streptomyces* for bio-accumulate or to degrade a wide variety of xenobiotic compounds have been published. More recently, onestrain belonging to the genus *Streptomyces,* named MIUG 4.89, was previously selected by Popa Ungureanu et al. 2016 for its ability to degrade clofibric acid (up to35%).

In this paper, the present study evaluates the ability of selected strains to remove CLA and their adaptability in multiple cultures with activated sludge.

MATERIALS AND METHODS

Microorganism and chemicals

The strain *Streptomyces* MIUG 4.89, used in this work was provided from the Cultures Collection of the Bioaliment Research Center, Faculty of Food Science and Engineering of ''Dunarea de Jos'' University of Galati, Romania, with acronym MIUG. The isolated bacteria was maintained by cultivation on Gauze agar medium and maintained at 4 °C, as well as sub-cultured at regular intervals for the experiments.

Activated sewage sludge sampleused in this work was collected from aeration tank of the MunicipalSewage Treatment Plant of Galati.

All the chemicals reagents, CLA, methanol (HPLC grade), acetic acid and nutrient media were provided from Sigma–Aldrich (St. Louis, MO, USA).

Bioremediation Experiments

Inoculum preparation has been made in Gause's medium, in faverto promote increased a biomass growth. The used medium contained the following: starch, 20g, K_2HPO_4, 0.50 g; $MgSO_4$•7H2O, 0.50 g; KNO_3, 1.0 g; $FeSO_4$•7 H2O, 0.01; NaCl, 0.5 g; per litre of distilled water. All media were adjusted to a final pH of 7.2 and then sterilised in an autoclave for 15 min at 120 ^{0}C. The flasks were incubated at 25 °C for 72 h on a rotary shaker (SI-300R Incubator Shaker Jeio Tech, Korea) at 150 rpm.

The dynamics of culture growth was established by measuring the optical density of the cultures medium at 600 nm using JASCO UV/VIS spectrophotometer (Japan). After strain development by cultivation in liquid Gauze culture medium, the active biomass was separated by centrifugation at 10000 rpm for 10-15 minutes.

The pellet was washed twice with ultrapure water. The washed cells suspension was then placed in a pre-weighed aluminium pan and dried in a hot air oven at 100 °C for 24 h until constant weight. The

dried filters were weighed after being allowed them to cool in a desiccator for 30 minutes to bring them to room temperature.The amount of dry biomass in the culture broth was inferred from the following, Equation (1):

$$\text{Dry biomass concentration (g/L)} = \frac{(W_1 - W_2)}{V}, (1)$$

Where W_2 is the weight of the filter with biomass obtained after drying (g), W_1 is the weight of the corresponding empty filter (g), and V is the volume of sample (L).

Activated sludge sample was washed four times with tapwater 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. The reactor was continuously ventilated and maintained at room temperature. Once a month, 200 µL of organic nutrient was added in order to diversify the carbon sources in the environment.

Sludge samples were taken from the reactor and then separated by centrifugation (4000 rpm, for 5 minutes) and washed at least three times using tap water to remove residual chemicals from the maintenance medium. The washed activated sludge was then used in biosorption and biodegradation experiments.

Clofibric acid biodegradation by activated sludge

For the biodegradation experimentsa basal liquid medium (Hopwood, 1967) was used. It contains the following compounds (g $L^{-1}$of distilled water): L-aspragine, 0.50; glucose, 0.50; K_2HPO_4, 0.50, $MgSO_4 \cdot 7H_2O$, 0.20; $FeSO_4 \cdot 7\ H_2O$, 0.01supplemented with 0.2 mg L^{-1} CLA. Activated sludge was added in a concentration of 20% dry substance. The flaks were maintained 25ºC, 150 rpm, for 10 days and at constant stirring rate (150 rpm).

Evaluation of the capacity of clofibric acid removes in multiple cultures of Streptomyces MIUG 4.89 and activated sludge

All biodegradation tests were performed in the same basal liquid medium. Also, in this study, it was aimed to establish the optimal conditions for achieving the biostimulation of the biodegradation of pharmaceutical compound, in multiple cultures (*Streptomycetes* - activated sludge), in submerged cultivation conditions (aerobic and facultative anaerobes), for 10 days, when the biomass dried ratio was varied - *Streptomyces* inoculum: activated sludge at values of 1:1 and 1:2.

All assays were performed in triplicate. However, controls groups included abiotic and heat killed organisms were also carried out in order to study the biosorption of the target molecule and the biotransformation ability of selected strains.Samples were periodically taken from the shake flasks to monitor biomass yield production (expressed as dry weight) and residual pollutant content.

Analytical methods

The crude culture supernatants, after biomass separation by centrifugation at 10000 rpm for 10 minutes, were analyzed for quantification of residual CLA content. Supernatants were filtered using a 0.2 µm pore size syringe filters (PALL Life Science, New York, USA) and then transferred to HPLC vials for subsequent HPLC analysis.

The degradation rate was determined with the High Performance Liquid Chromatography (HPLC, Agilent 1200 Series, Santa Clara, CA, USA) technique, equipped with a columnC18 column (150 mm x 4.6 mm, with particle size 5µm) and aphotodiode array detector (PDA).

The mobile phase consisted of methanol, ultrapure water and 0.1% acetic acid (70:30, v/v, flow-rate 1.0 mL/min^{-1} and the sample volume was 50 µl). The detection wavelength was set at 270 nm. Analysis was carriedout in isocratic mode.The retention time was 2.4 minutes and the instrumental quantification limit (LOQ) for CLF was < 0.2 mg L^{-1}.

RESULTS AND DISCUSSION

Biodegradation of CLA with clofibric acid biodegradation by activated sludge

In our work, it was examined the ability of the activated sludge to remove studied target compound, in a concentration of 0.2 mg L^{-1}, by cultivated in aerobe submerged conditions in the liquid Hopwoodbasal medium. After 5 days of incubation, the CLA biotransformation yield was 20% (Fig. 1) and the concentration of dry biomass remained constant this time. Also, Kosjel et al.2007, reported 55% removal of clofibric acid using sludge starter culture active.Many methods have been proposed to remove CLA from the wastewater. However, biodegradation are the one important method of elimination of clofibricacid from wastewater.

Clofibric acid does contain a chlorophenolic group, but its resistance to biodegradation is in fact due to the steric hindrance from a single extra methyl group (Evangelista, S., et al., 2010;). Also, the nature of aliphatic side chain of compound influence their persistence in the aquatic environment, affecting their susceptibility to biological elimination. (Winkler, M., et al 2001; Tran, N. H., et al. 2009; Li, X.; Xu, T., et al. 2008; Wang, Q. et al. 2007).

After 10 days of submerged cultivation CLF from liquid medium was degraded up to 30% when the dry biomass ratio *Streptomyces* MIUG 4.89: activated sludge was 1:1 (fig. 2).

It was observed that the CLA biotransformation yield is higher than the one obtained under the same conditions with activated sludge biomass that showing the role with many cultures approaches for the removal of this

compound. The total removal of CLA was not obtained in the both situation.

Arias et al., 2003, obtain some similar results whenused a mixed culture: *T. versicolor* ATCC 42530- activated sludge that was exposed to a BPA compound.

The effect of delayed addition of starter cultures on the biodegradation efficiency of clofibric acid was also investigated.

The starter culture *Streptomyces* MIUG 4.89 was inoculated after five days of the process with activated sludge, and at the same time the ratio between the two cultures was varied.

From Fig. 3, it can be observed that CLA removal yield until reaching a valued of 58.5 %, when the wet biomass ratio Streptomyces MIUG 4.89: activated sludge was also 1:1.

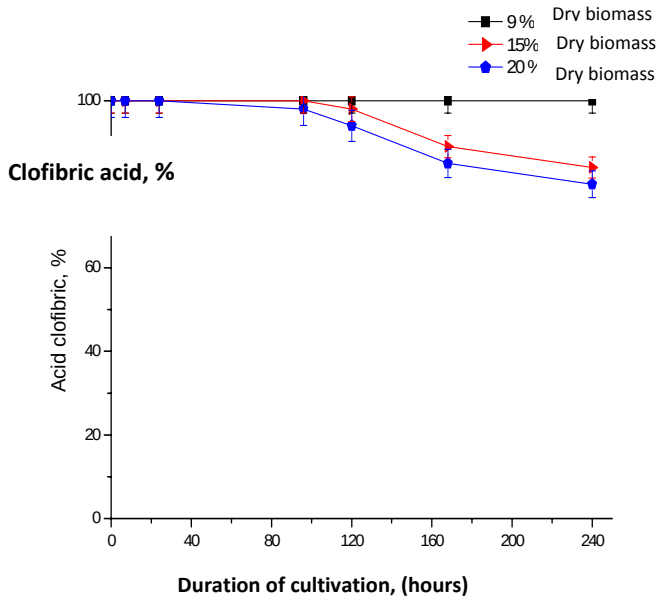

Fig.1. Clofibric acid degradation by activated sludge at various concentrations, in minimal medium supplemented with 0.2 mg L^{-1} CLA

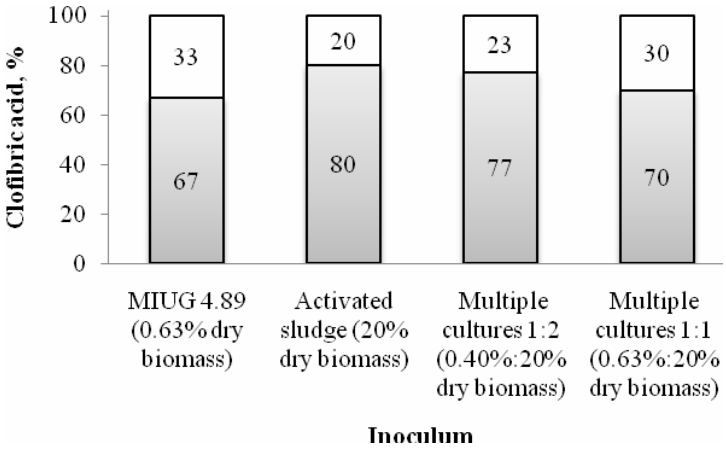

Fig. 2. The biodegradation capacity of clofibric acid in multiple cultures of *Streptomyces* MIUG 4.89: activated sludge by varying the ratio between cultures (□ percentage of target molecule removal, ■ percentage of non-degraded target molecule)

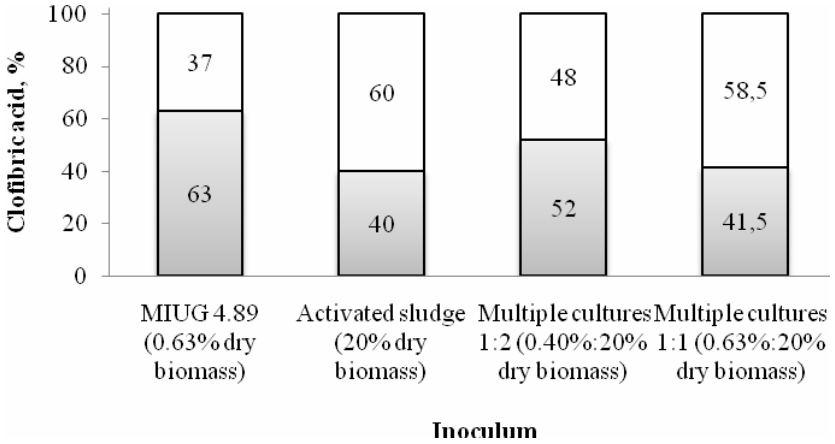

Fig. 3. The ability of multiple cultures to CLA removal, by inoculation delayed at 5 day intervals; (□ percentage of target molecule removal, ■ percentage of non-degraded target molecule)

The results obtained in our work with multiple cultures can be a promising alternative for the efficient elimination of clofibric acid (Winkler, M., et al 2001; Tran, N. H., et al. 2009; Li, X.; Xu, T., et al. 2008; Wang, Q. et al. 2007).

Also, biotic and abiotic tests with biomass inactivated by autoclavingwere carried out under similar conditions. The obtained results of biosorption tests indicated that this phenomenon is negligible (less than 3%).

CONCLUSIONS

This work demonstrates the possibility to use of activated sludge from the Municipal Sewage Treatment Plant of Galati for clofibric acid removal in simple cultivation controlled conditions.

The activated sludge were able to biotransform the pharmaceutical compound in percentage to 20%, during cultivation in a minimal basal medium, in aerobe conditions in submerged system in presence of 0.2 g L^{-1} CLA.

The efficiency of biotransformation of clofibric acid increases when it usedmultiple cultures *Streptomyces*: activated sludge, in a 1:1 ratio. However, under these conditions, the multiple cultures showed a low degradation yield; a maximum elimination yield was only of 58.5%, when the selected strain *Streptomyces* MIUG 4.89 was inoculated staggered, in concentration of 0.63% dry substance, after five days from the start of the process active.

To complete this work, more studies should be carried out to evaluate the degradation product of clofibric acid and its impact to the environment.

ABSTRACT

The consumption of pharmaceuticals has increased worldwide and new compounds are continually being introduced in the market. Studies on the occurrence of pharmaceuticals show that the widely used pharmaceuticals acid clofibric are present in relevant concentrations in the environment. This molecule is recognized as highly resistant to biodegradation (WWTP biomass or bacteria) and has high persistence in the environment. Hence, to date, the elimination of this molecule continues to be a subject of interest. This study employs a viable alternative for micropollutant removal.

The aim of the present study was to investigate the potential of selected strain to degrade CLA and their adaptability in multiple cultures with activated sludge. Additionally, the possibility of biostimulation of the activity of microorganisms from the consortium by optimizing their functionality in the biodegradation process of pharmaceutical compound was also examined. The results showed a removal efficiency of CLA of 58.5% when the wet biomass ratio *Streptomyces* MIUG 4.89: activated sludge was 1:1. This work represents the first study of the ability of multiple cultures *Streptomyces* MIUG 4.89 and activated sludge to remove this refractory compound.

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