Biodiversity restoration and conservation of inland water ecosystems for environmental and human well-being

BioReset

BiodivRestore-406

2020 - 2021 Joint Call

Joint COFUND Call on "Conservation and restoration of degraded ecosystems and their biodiversity, including a focus on aquatic systems"

Deliverable 2.2.1

Evaluation of laccase-producing biomaterial based on colonization with *P. ostreatus* with respect to removal efficiency of selected Emerging Contaminants in Wastewater

Lead Beneficiary	Work package	Delivery month
SLU	**2**	**18**

1. Executive Summary

Pollution is threatening the biodiversity of inland waters that are vital to society and the future of the Earth. A major source of this pollution are effluent discharges from wastewater treatment plants (WWTPs). Treatment processes used in WWTPs do not efficiently remove emerging contaminants, such as pharmaceuticals and microplastics, which lead to health hazards to non-target species, including humans. This polluting source limits the conservation and restoration of freshwater systems. At the same, there is a need for strategies for up-scaling restoration solutions and for rapid and simple to use methodologies to assess conservation and restoration progress, i.e. assessment strategies anticipating the success of conservation/remediation measures in suitable timescales, ensuring reliable data comparison over time and space, and guiding intervention measures. Thus, the **BioReset** project proposes to advance treatment processes (chemical, physical, biological and their combination) to promote ecosystem recovery and conservation and to develop assessment strategies.

Deliverable 2.2.1 is intended to develop the use of mycoremediation based on the white-rot fungi *Pleurotus ostreatus* for WP2, within **BioReset**. Additional information regarding communication and academic works within the work package is outlined in this report.

2. Task description

WP2 regards improving the effectiveness and upscaling of WW treatments. Within this, Task 2.2 focuses on bioremediation with white-rot fungi (mycoremediation). This task will focus on: **i)** construction of laccase-producing biomaterials (D.2.2.1) and **ii)** use of this material for safe removal of EC from WW. Low-cost and less used biomasses will be evaluated and developed as laccase-producing biomaterial. This will be obtained through colonization with safe and well-known white-rot fungi (e.g. *Pleurotus ostreatus*). Bioremediation based on fungi is particularly promising in the decontamination of WW from EC because of the nonspecific nature of the ligninolytic enzymatic system, which is able to degrade a wide range of EC. Factors stimulating the release of laccase as well as the time span of enzyme release will be studied. Selected laccase-producing biomaterials will be used for filtration of WWTP effluents and their effect on contaminants (e.g., azithromycin, clarithromycin, sulfamethoxazole, trimethoprim, venlafaxine and its metabolite o-desmethylvenlafaxine, clotrimazole, fluconazole and miconazole) included on the most recent EU Watch List will be evaluated. The analysis of the contaminants will be performed with an established in-house method based on SPE and LC-MS/MS.

3. WP2 - Task 2.2 team members

The Team members in WP2, Task 2.2, are:

Name	Organization	Role
Malin Hultberg	SLU	Task coordinator
Oksana Golovko	SLU	Researcher

4. Developed activities

From preliminary experiments, two different set-ups for immobilized fungi were selected for studying laccase production and EC removal by *P. ostreatus*. In the first set-up, the material based on wood chips were evaluated and sawdust (2-4 mm) based on birch (*Betula* sp.) were selected. In the second set-up, an innovative approach based on fungal-lignin pellets were selected and evaluated.

Use of sawdust substrate for production of ligninolytic enzymes

Monitoring of enzyme activity in sawdust colonized by *P. ostreatus* over time in the bioreactors indicated that the enzymes were washed out very rapidly. Initial enzyme activity in the outlet water was 35-40 U of laccase per liter, but after less than 1 h enzyme activity was below 5 U/L and continued to decrease. No impact of either substrate aeration or nutrient supplementation were observed. In a second experimental set-up, the flow rate was reduced from 1 to 0.3 L/h and the bioreactors were monitored over a longer period (10 days) in order to assess whether it was possible to achieve fungal growth and thereby increase enzyme activity under these conditions. Fungal growth in the bioreactors was observed visually, but enzyme activity in the outgoing water was practically negligible in both treatments.

Impact of sawdust substrate colonized with P. ostreatus on concentrations of selected pharmaceuticals

The impact of the crude enzyme suspensions from colonized sawdust in removing the selected pharmaceuticals was observed to be low. Compared with the autoclaved control without enzyme activity, a significant decrease in concentration was observed for the compound sulfamethoxazole in all treatments. Considering the short exposure time and moderate levels of enzyme activity applied, it seems safe to conclude that for the sulfonamides, treatment based on sawdust colonized by *P. ostreatus* is a realistic opportunity. In the next experiment, attempts were made to increase enzyme activity in the crude enzyme suspensions of *P. ostreatus* to assess whether higher removal efficiency of the pharmaceuticals could be obtained. Compared with the autoclaved control without enzyme activity, a significant decrease was observed for five pharmaceuticals (sulfamethoxazole, trimethoprim, metoprolol, lidocaine, venlafaxine) in the crude enzyme suspension with the highest activity. Our results suggest that with a short exposure time, which can be expected if a treatment process based on ligninolytic enzymes is integrated into a WWTP, enzyme activity is highly relevant for removal efficiency even though the target pharmaceuticals are present in very low concentrations. We therefore decided to explore innovative approaches (described below) to increase the enzyme activity of *P. ostreatus* in wastewater.

Pellets-based method for efficient production of laccase and fungal biomass in wastewater

Commercial mushroom spawn of *Pleurotus ostreatus* was used to develop a white-rot fungal pellet in water. Pellets of an approximate size of 0.3-0.8 cm, composed of the grain spawn and mycelium of *P. ostreatus* growing out from the spawn, developed in the wastewater. The addition of lignin, as an inducer of ligninolytic enzyme activity, to the wastewater initially resulted in an opaque and dark brown suspension. However, over time lignin accumulated in the mycelium resulting in brown pellets composed of mycelium and lignin and transparent water with slight brownification. The accumulation of lignin in the mycelium is an interesting finding. It is beneficial from a water treatment perspective as the small lignin particles to a large extent will be removed from the water while still evidently increasing laccase activity in water. The activity of laccase was significantly higher compared to the control already after 24 h. The addition of lignin had a low impact on the pH of the wastewater, with initial pH of 7.2±0.04 for the synthetic wastewater and 7.3±0.03 for the synthetic wastewater with added lignin.

Impact of pellets-based method on concentration of selected pharmaceuticals

The pellet was tested for removal of 11 different pharmaceuticals in two different nominal concentrations. In both concentrations, there was a significant decrease in the total sum of pharmaceuticals in the water after treatment compared to the autoclaved control which lacked enzymatic activity. When the effect of the treatment was studied for the individual compounds, a very similar pattern was observed in both experiments. Concentrations of the antibiotics azithromycin, erythromycin, sulfamethoxazole and trimethoprim in the water phase were reduced by more than 50% in both experiments when compared to the control. Out of 11 tested compounds, 9 compounds were significantly reduced by the experiment in the lowest nominal concentration and 8 compounds in the experiment with the highest nominal concentration. Thus, for a majority of the added compounds, the concentration in water was decreased after treatment.

No significant differences were observed between the amount of biomass recovered from the treatment (living fungus) and the control (autoclaved fungus). The average amount of biomass recovered from each replicate was 1.2±0.04 g dw, approximately 24 g /L of dw. This corresponds well to the initial addition of 40.0 g of spawn (fresh weight, moisture content ranging between 47-53%) and 5.0 g dw of lignin. Thus, in the studied system, the solid phase composed of spawn, fungal hyphae and lignin, represented only a minor part (2.4% of total weight). In the solid phase, pharmaceuticals were accumulated to a significantly higher degree in the treatment, which was not autoclaved. Thus, it seems like the living hyphae were better at absorbing the pharmaceuticals compared to the dead fungal biomass. As for the water phase, a very similar pattern was observed in both experiments when the effect of the treatment was studied for the individual compounds in the solid phase. Erythromycin, tramadol and metoprolol showed the highest tendency to accumulate in the living pellets while azithromycin, fluconazole and trimethoprim were below detection limit in the experiment with the lowest nominal concentration and only recovered to a low extent in the experiment with the highest nominal concentration. Only sulfamethoxazole was recovered to a higher degree from the autoclaved control and this difference was significant only in the experiment with the lowest nominal concentration.

Total content of individual pharmaceuticals and their sum, including both liquid and solid phase, was calculated for each treatment to determine if reduction of pharmaceuticals in the wastewater treated with the living fungus was due to accumulation of the compounds in its biomass. However, our results suggests that the observed decrease of pharmaceuticals in the wastewater was mainly due to enzymatic degradation. The finding of a similar relative reduction for most of the individual compounds in both experiments suggests that the degrading enzymes were not saturated under the applied experimental conditions.

5. Results

Of the two different set-ups evaluated within task 2.2, it is evident that the pellets approach was more successful in treatment of EC in wastewater. It should be highlighted that the use of filamentous fungi in wastewater treatment has evident benefits considering the harvest of biomass compared to other biological treatments based on microalgae or bacteria. The laccase-producing pellets developed within **BioReset** can easily be harvested by coarse filtration. In addition, the grain spawn, lignin, and the produced mycelium can be considered less harmful from an environmental standpoint and pH was only affected to a low extent. Thus, on-going and future work in task 2.2 will focus on this method to produce laccase-producing biomaterial for mycoremediation.

6. Associated indicators

Publications

1. Hultberg, M., Asp, H., Bergstrand, K.J.B., Golovko, O. (2023). *Production of oyster mushroom (Pleurotus ostreatus) on sawdust supplemented with anaerobic digestate*. Waste Management 155, 1-7.
2. Silva, A.D.M., Sousa, J., Hultberg, M., Figueiredo, S.A., Freitas, O.M., Delerue-Matos, C. (2022). *Fluoxetine Removal from Aqueous Solutions Using a Lignocellulosic Substrate Colonized by the White-Rot Fungus Pleurotus ostreatus*. International Journal of Environmental Research and Public Health 19 (5), 2672.

Communications

1. Golovko, O., Hultberg, M., *Green Bioremediation with White-rot Fungi*, SETAC Europe 33 Annual Meeting, April 30 – May 4, 2023, Dublin, Ireland. (Poster presentation)

Grants awarded

1. The fungal pellets concept was awarded the SLU Alnarp and Sparbanken Scania Innovation Award in 2023.