Improved biofilm carriers for fungal exploitation in wastewater treatment

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Improved biofilm carriers for fungal exploitation in wastewater treatment

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Abstract. In Moving Bed Biofilm Reactors (MBBR) the biofilm grows protected within small plastic carriers (known as biofilm carriers), which are designed with high internal surface area. The biological wastewater treatment process consists of adding biofilm carriers in aerated or anoxic tanks to support biofilm attachment and growth. Some of the authors conceived, designed and realised an improved carrier (new shape and material) for fungal biofilm development. The improved biofilm carriers were tested in laboratory conditions and good results were obtained. This new biofilm carrier will be used to treat cellulosic (tannery and papermill) wastewaters and is grafted with cellulose fibers for the self-sustainability of the fungal biomass. This will facilitate substrate adhesion, due to biochemical capabilities of the selected strains, which involve secretion of extracellular enzymes, that can break down substrate by combined action of several degradative processes, such as demethylation, oxidative cleavage of the propane side chain, cleavage of ether bonds between monomers etc.

1. Introduction

For wastewater treatment different technologies and processes are known worldwide. The biological treatment technologies based on different consortia of microorganisms are the most common processes applied especially for the removal of the organic compounds. The effectiveness of the activated sludge process was demonstrated for the municipal wastewaters [1], but for the industrial effluents these biological treatment processes are not sufficient due to the fact that these polluted waters contain a significant concentration of other elements that are not reduced by common bacteria. In the case of the industrial wastewater treatments, other advanced technologies (ex. tertiary treatment processes mainly based on chemical-physical technologies) are applied.

In this context, the authors aim to develop a tertiary wastewater treatment technology dedicated to the biodegradation of some specific recalcitrant compounds, like cellulose, tannins or absorbable organic halides (AOXs), that are mainly found in papermill, tannery, textile etc. wastewaters. The cellulosic compounds are very difficult to be removed from the effluent by using only conventional biological treatments based on the catabolic capacity of bacteria (activated sludge).

The authors propose a new treatment technology based on fungi-functionalized Moving Bed Biofilm Reactor (MBBR). Inside the conventional MBBRs the consortia of microorganisms form biofilms, that attaches to free solid-phase carriers also known as biomedia or biofilm carriers [2]. The biofilm carriers are often realised from plastic materials that have a close-to-water density. High-density polyethylene (HDPE) or polyvinyl alcohol (PVA) are the most common materials from which the biomedia is made [3].

The biofilm developed on the carriers transform or accumulate pollutants. Common applications for MBBRs include nitrification, oxidation (organic carbon, iron- and sulfur-oxidation) and removal of heavy metals. The toxic chemicals can be also reduced when the biofilm carriers are taken out from the system and the biofilm is removed by washing.

During the last years, the MBBR technology for wastewater treatment has been intensively studied and developed [4] - [5], but not applied in combination with fungi (ex. White Root Fungi WRF) for wastewater treatment.

The technology proposed by the authors will lead to validation of an innovative MBBR designed for new bioactive fungal tools. The central element of this new technology is based on an innovative fungal carrier containing cellulose. Cellulose is the nutrient source for the proliferation of WRF. The fungi metabolism is used by the authors, in the depletion of AOX, tannins and cellulose from the polluted waters. Currently, the removal of AOXs from wastewater is based on anaerobic dehalogenation processes. Fungi were already described as capable to deplete AOX from kraft pulp bleaching effluents at lab scale [6]. The authors propose an innovative MBBR that will perform in a single step the removal of AOXs that conventionally requires the combination of two processes (both aerobic and anaerobic) when only bacteria are involved. Also, fungi were described as capable of biotransformation of tannins in wastewater [7]. Tannins are known for growth inhibition of microorganisms. Despite the antimicrobial properties of tannins [8], fungi are resistant to tannins and can utilise them as carbon source. For these reasons, the authors propose fungi utilizations in MBBRs for AOX, tannin and cellulose removal.

The species of microorganisms in the white rot categories have been successfully used in the bioremediation of effluents from the textile industry [9],[10], which are capable of producing various isolation forms of extracellular oxidases, including laccase, lignin peroxidase (LiP) and Mn peroxidase, enzymes involved in the degradation of lignin and lignocellulosic substrates, their lignylolytic system being directly involved in the degradation of various xenobiotic compounds, including textile dyes [11]. Their bioremediation activity is based on the production of non-specific extracellular enzymes capable of degrading recalcitrant lignin polymers under the action of specific enzymes such as lignin peroxidase (ligninase, LiP, EC 1.11.1.14), manganese-dependent peroxidase (MnP, EC, 1.11.1.13), laccaza (benzendiol: oxygen oxidoreductase, EC 1.10.3.2.) and peroxidase (VP, EC 1.11.1.16), similar mechanisms being involved in the complex degradation of synthetic dyes [12],[13]. The main disadvantage of microbial enzyme bioremediation is their high cost and thermal denaturation in contact with wastewater specific to the textile industry, but these inconveniences can be easily eliminated due to the renewable nature of these enzymes in cell cultures.

2. Material and methods

2.1. Fungal strains

One of the fungal strains used in the present study, *Bjerkandera adusta MUT 2295*, was obtained from Mycotheca Universitatis Taurinensis (MUT) and preserved at 4°C on Malt Extract Agar (MEA). This strain has been previously used in studies aimed at treating landfill leachate, pharmaceutical and tannery wastewater [14-16].

Also, the efficiency of *Cerioporus squamosus* strain (White Rot Fungi) was tested, towards COD removal from a natural wastewater sample, for biodegradative screening of the strain, which will be used in future experiments for augmentation of HDPE carriers. *Cerioporus squamosus* is a basidiomycete bracket fungus, with a widespread distribution, being found in North America,

Australia, Asia, and Europe. The strain was previously grown as a starter culture, in Czapek-Dox nutritive broth (7 days at 28ºC). Also, the experiment was carried out in the Czapek-Dox nutrient broth, which was dissolved in wastewater originating from textile finishing processes, in 50 mL Erlenmeyer flasks (one as control sample and one for strain sample). After preparation, media was autoclaved at 121°C for 15 min., and after cooling, the strain flask was inoculated with 5 mL of the initial starter culture of *Cerioporus squamosus*. Both samples (control and strain) were incubated for 4 days at 28ºC. After incubation, the contents were filtered on a 20 μm membrane and analysed.

2.2. COD reduction method

The determination of COD was made according to the international standard SR ISO 6060 (1989), "Determination of the chemical oxygen demand". The level at which the determined experimental results are close to the theoretical values depends primarily on the analytical water load and the degree of oxidation, the COD analysis strongly depending on the composition of the analysed water. According to the standard, many organic compounds are oxidized in a proportion of 90% - 100%, and if the samples contain a large amount of such compounds (urban effluent), the COD value is a good approximation of the theoretical consumption of oxygen. For the refluxing boiling step, a COD thermoreactor was used: ECO6, Velp Scientifica, with setting temperature up to 200°C. All reagents used to determine the COD content were of analytical quality:

1. Sulfuric acid: ρ = 1,84 g/mL; c(H2SO4) = 4 mol/L;
2. Silver sulfate (Ag_2SO_4);
3. Potassium dichromate (reference standard solution): c(K2Cr2O7) = 0.040 mol/L;
4. Iron (II) and ammonium sulfate titrate solution: c[$(NH_4)2Fe(SO_4)2 \times 6H_20$] = 0.12 mol/L;
5. Ferroin (indicator solution).

The COD content, expressed in milligrams of oxygen per liter, was calculated according to the following formula: COD (mg/L) = [800 x C x (V_1-V_2)]/V_0

where: C = the concentration of the amount of iron (II) and ammonium sulphate solution; V_0 = sample volume to be analysed before dilution (if performed), in milliliters; V_1 = the volume of iron (II) and ammonium sulphate solution used to titrate the blank sample, in milliliters; V_2 = the volume of iron (II) and ammonium sulphate solution used to titrate the sample to be analyzed, in milliliters; 8000 = the molar mass of ½ O_2, in milligrams per liter.

2.3. Carriers

Wheel-shape carriers were provided by DFR Systems SRL and made by HDPE, cellulose and talcum. In particular, 12 types of carriers were tested including big (internal diameter: 2.2 cm), as can be observed in Figure 1, and small ones (internal diameter: 1.2 cm). Due to the addition of talcum and cellulose, the initial shape of the carriers suffered some modifications (Biofilm carriers from Figure 1 compared with the carriers in Figure 2 – first row).

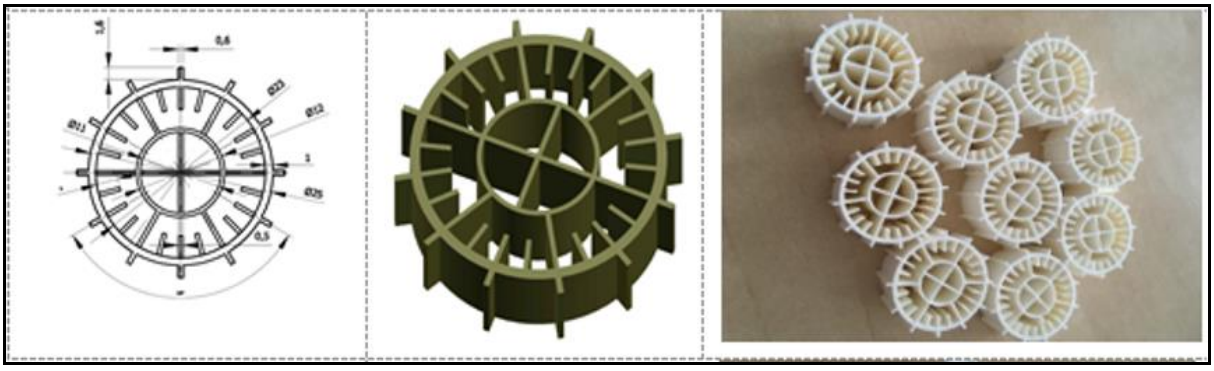

Figure 1. Biofilm carriers with bigger internal diameter (2D and 3D designing, carriers realized with 3D printer).

For each group (big and small), 4 composition were employed, characterized by different percentages of cellulose and HDPE. Among small carriers, two types were tested: regular or with additional spokes (Figure 2). A summary of the main features of the carriers tested is represented in Table 1.

Table 1. Size and composition of different carriers.

Color	Composition	Size	Spokes	Abbreviation
White	100% HDPE	Big		BW
		Small	Regular	SW
			With Spokes	SSW
Yellow	3% cellulose, 5% talcum and 92% HDPE	Big		BY
		Small	Regular	SY
			With Spokes	SSY
Red	5% cellulose, 5% talcum and 90% HDPE	Big		BR
		Small	Regular	SR
			With Spokes	SSR
Brown	7% cellulose, 5% talcum and 88% HDPE	Big		BB
		Small	Regular	SB
			With Spokes	SSB

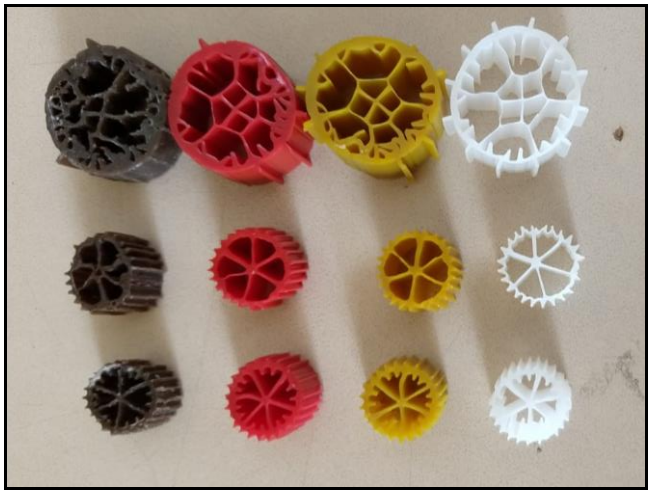

Figure 2. From left to right, first line: BB, BR, BY and BW. Second line: SB, SR, SY and SW. Third line: SSB, SSR, SSY and SSW.

2.4. Fungal attachment to carriers

Fungal (Bjerkandera adusta MUT 2295) attachment to carriers was performed with a modified protocol from Bardi et al., 2017. After the pre-cultivation on MEA, *B.adusta* was homogenized under sterile conditions in 9.0 g/L NaCl, and inoculated into 500 mL flasks containing glucose and yeast extract liquid medium (GLY = 5.0 g/L glucose; 1.9 g/L yeast extract, 250 mL/flask) and HDPE-cellulose carriers, previously sterilized through autoclave. Homogenate was added to all flasks using 10 mL/flask. In particular, 6 big carriers were added to each flask, while in case of small carriers, 24 pieces were located into each flask. Each condition was repeated twice for a total of 24 flasks, which were incubated in agitation (150 rpm) for one week at room temperature, to allow fungal adhesion to carriers. During the incubation, the progress of the attachment was monitored daily and, at the end of the test, 1 mL was withdrawn from each flask culture for enzymatic activity measurement.

3. Results and discussions

Out of 12 types of carriers, Bjerkandera adusta MUT 2295 attachment could be observed in two types. In particular, a partial attachment could be seen in BR already after three days of incubation (Figure 3). However, from that day upon the end of the experiment (7 days) fungal attachment on the carrier did not proceed any further. Fungal attachment on such carriers interested approximately 20% of carrier surface. It is worth noting that such attachment could be observed only in two out of 6 carriers/flask in both flasks in which BR carriers were added. Also, in the case of BB carriers, a partial attachment could be observed after 3 days of incubation, which, at that time, interested approximately 30% of carriers surface and that could be observed in all the BB carriers (in both flasks). At the end of the experiment, all the BB carriers were fully occupied by fungal biomass (Figure 4).

Figure 3. Flasks with BR carriers after one week of incubation.

Figure 4. Flasks BB carriers after one week of incubation.

No attachment could be observed in all the other carriers, suggesting that small carriers were not suitable for *B. adusta*, with or without additional spokes. Similar results have been reported by Spina et al. 2012 [17]. Indeed, these authors did not observe biomass attachment when inoculating *B. adusta* MUT 2295 in agitated conditions on circular industrial carriers with similar shape and size [17]. From the results achieved, it is reasonable to expect that the internal structure of big carriers, with small internal chambers, favored fungal adhesion and growth. In addition, it seems reasonable to hypothesize that higher concentration of cellulose could have enhanced fungal attachment on carriers. Indeed, partial and complete attachment occurred in red (BR) and brown carriers (BB), respectively, which contained the highest cellulose percentages. The positive effect of cellulose in fungal attachment could be explained as a consequence of *B. adusta* cellulolytic properties [18]. Considering that full immobilization occurred in 7% cellulose carriers and only partial adhesion occurred in 5% ones, it is reasonable to consider such concentration as a threshold for *B. adusta* attachment, which was achieved in the same timing (7 days) of fungal attachment on commonly employed PUFs [15], [19-21].

Regarding the *Cerioporus squamosus* strain attachemt to the biofim carriers, good results were obtained (Figure 5 - 8). The best results were obtained for SSB biofilm carriers (Figure 8).

Figure 5. SSW with *C. squamosus*

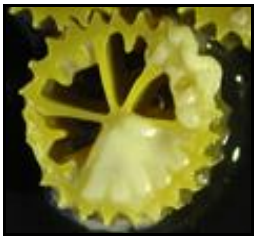

Figure 6. SSY with *C. squamosus*

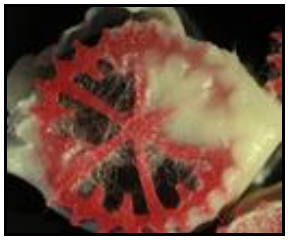

Figure 7. SSR with *C. squamosus*

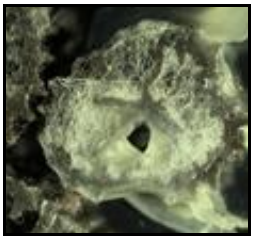

Figure 8. SSB with *C. squamosus*.

COD analysis is a general indicator of water quality, measuring the dissolved oxygen depletion capacity in samples contaminated with organic matter. More specifically, the analysis determines the equivalent oxygen quantity required for the chemical oxidation of organic compounds in water. Often, COD analysis is used to estimate BOD (Biochemical Oxygen Demand) values, with strong correlations between these two.

For *Cerioporus squamosus* the two samples (control and strain) were processed according to ISO 6060 standard, each of which was subjected to acid digestion for 2h at 150ºC (Figure 9 and 10). Since the nutrient media has high glucose content (30 g/L), which can significantly influence the COD value, a blank containing 3% glucose-added residual water was prepared (used as control).

Figure 9. Acid digestion of samples in ECO6 thermoreactor - c*ontrol sample.*

Figure 10. Acid digestion of samples in ECO6 thermoreactor - *Strain sample.*

The zero sample (V1 in the formula) was prepared and processed from distilled water, this being used as standard water. Furthermore, it was subjected to acid digestion, as well as the samples, and the titration value with iron (II) and ammonium sulphate was used in the COD calculation of each sample. The result of COD value and reduction rate of strain sample was of 32428 mg/L (41.93%), when compared to the controls sample which contained residual glucose water to mimic the composition of the samples, with COD value of 55849 mg/L.

4. Conclusions

Experiments to reduce chemical oxygen demand made on unprocessed wastewater sample revealed significant reduction by *Cerioporus squamosus* strain (41.93%), after only 4 days, which makes this particular strain a perfect candidate for reduction of certain pollutants from industrial wastewaters.

Regarding *B. adusta* MUT 2295, as a future perspective, the same experiment could be performed with other fungi, commonly used in bioremediation processes, to verify whether cellulose concentration and carriers' size could influence their biomass attachment.

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