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Decolorization of reactive azo dyes using a sequential chemical and activated sludge treatment

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Textile wastewater contains high concentrations of organic substances derived from diverse dyes and auxiliary chemicals, some of which are non-biodegradable and/or toxic. Therefore, it is essential that textile wastewater is treated and that these substances are removed before being discharged into the environment. A combination of advanced oxidation processes (AOPs) to obtain partial dye degradation followed by a biological treatment has been suggested as a promising method for cost-effective decolorization of wastewater. The aim of this study was to develop and evaluate a combined method of partial Fenton's oxidation and biological treatment using activated sludge for decolorization of azo dyes, which represent an important group of recalcitrant, toxic textile dyes. Using Reactive Violet 5 (RV5) as a model dye, color removal was significantly higher when the combined Fenton treatment/activated sludge method was used, as opposed to separate application of these treatments. More specifically, pretreatment with Fenton's reagent removed 52.9, 83.9 and 91.3 % of color from a 500 mg l^{-1} RV5 aqueous solution within 60 min when H_2O_2 concentrations of 1.0, 1.5, and 2.0 mM were used, respectively. Subsequent biological treatment was found to significantly enhance the chemical treatment, with microbial decolorization removing 70.2 % of the remaining RV5 concentration, on average. Molecular analysis of the microbial community within the activated sludge revealed that exposure to RV5 shifted the community composition from diverse towards a highly-specialized community harboring taxa with azo dye degrading activity, including *Trichosporon*, *Aspergillus* and *Clostridium* species.

[**Key words:** 454-Pyrosequencing; Activated sludge; Advanced oxidation process; Decolorization; Fenton's oxidation; Reactive Violet 5]

Textile wastewater is typically intensely colored and contains high concentrations of organic substances derived from diverse dyes and auxiliary chemicals (1,2). More than 10,000 different dyes are used in the textile industry, of which approximately 280,000 tons are discharged worldwide every year (3). About 70 % of these dyes are synthetic azo dyes, representing a highly diverse group of dyes characterized by nitrogen to nitrogen double bonds (so-called azo bonds; −N═N−) (4). Many azo dyes and their metabolites are recalcitrant in nature and can be highly toxic to both terrestrial and aquatic life (5,6). Furthermore, disposal of azo dyes into surface water strongly affects not only its aesthetic qualities, but also its transparency, by which photosynthesis in aquatic plants is hampered (7). Therefore, it is essential that these dyes and auxiliary chemicals are removed from textile wastewaters using appropriate and effective methods prior to their discharge into the environment (5,8).

Many approaches have been proposed to remove dyes from textile wastewaters, including chemical coagulation/precipitation, physical adsorption, electrochemical oxidation, chemical oxidation, and biological anaerobic/aerobic degradation and/or conversion (6,9–12). Recently, advanced oxidation processes (AOPs) have been proposed as a promising technique for wastewater treatment as AOPs are able to oxidise a wide range of compounds that are otherwise difficult to degrade (13). Among AOPs, oxidation using Fenton's reagent is an attractive treatment for decolorization and degradation of dyes because it uses effective, easy to handle, non-toxic reagents (i.e., Fe^{2+} and H_2O_2) (14,15). Many studies have been performed regarding the decolorization of dyes using Fenton's oxidation (15,16). Additionally, its capacity to improve organic biodegradability of toxic or non-biodegradable wastewaters has been described (17). Despite the apparent potential AOPs show for the degradation and removal of recalcitrant dyes, disadvantages of these processes include high reagent costs, input of energy and production of iron sludge waste in the Fenton process, which requires management and safe disposal (18).

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To circumvent these limitations, various methods have been investigated, including the use of photo-Fenton techniques (19), electro-Fenton techniques (20), sono-Fenton techniques (21) and Fenton-like oxidation techniques (22). Additionally, a combination of AOPs to obtain partial dye degradation followed by a biological treatment has been shown to have potential to achieve effective decolorization and mineralization of azo dyes (13). Thus far, most studies have focused on the combination of a chemical pretreatment followed by a biological treatment with a specific microbial strain or microbial assemblages such as biofilms which are able to complete the dye's degradation (23–25). Surprisingly, a combination of AOP and activated sludge processes has remained underexplored, despite some studies suggesting that a Fenton treatment combined with a (aerobic) biological treatment could be an interesting option for the treatment of recalcitrant compounds, including dyes (26,27). Furthermore, only little is known about the impact of azo dyes on the microbial populations in activated sludge processes after dye exposure. The main objective of this study was to develop and evaluate a combined method of Fenton's oxidation and a biological treatment using activated sludge to achieve decolorization and enhance mineralization of azo dyes using Reactive Violet 5 (RV 5) as a model dye. To this end, our first goal was to evaluate and optimize Fenton's oxidation process to achieve partial degradation of azo dyes in order to make them easily biodegradable. Secondly, we evaluated the potential of activated sludge to perform dye removal, and finally the overall performance of a combination of both methods was evaluated. In order to identify key microbes in the biological treatment, activated sludge microbial communities were characterized using 454 amplicon pyrosequencing.

MATERIALS AND METHODS

Reagents Experiments were performed using the reactive (mono)azo dye Reactive Violet 5 (RV5; Fig. S1) as model dye. RV5 is widely used in textile and dyeing industries and is typically found in high concentrations over other reactive dyes in dyebath effluents (28). Additionally, it is, for example, also used in antifreeze (29). Both RV5 and all other required chemicals were obtained from Sigma–Aldrich (Saint Louis, MO, USA), unless mentioned otherwise.

Fenton's oxidation Fenton's oxidation was performed in 250 ml Erlenmeyers filled with 200 ml demineralized water containing RV5. Erlenmeyers were incubated at 24°C and continuously stirred (300 rpm) using a Cimarec i Poly 15 stirrer (Thermo Fisher Scientific, Waltham, MA, USA) to prevent oxygen depletion. Experiments were carried out at an initial pH of 5.0, which is known to decrease during the experiment to a pH around 3–4 (30), as was also observed in our experiments. In a first experiment, Fenton's reagent decolorization capacity (i.e., the ability to remove color) was evaluated using different dye concentrations ranging from 100 to 900 mg l^{-1}. For this experiment, a H_2O_2 concentration of 1.5 mM and a Fe^{2+} concentration of 0.15 mM was used as proposed in Lucas et al. (23). In a second experiment, the decolorization capacity of different H_2O_2 concentrations (ranging from 0.0 to 2.5 mM, while maintaining the molar ratio between H_2O_2 and Fe^{2+} at 10:1 (23)) was evaluated for a dye concentration of 500 mg l^{-1}. For all experiments, Fe^{2+}, H_2O_2 and dye solutions were freshly prepared from $FeSO_4.7H_2O$, H_2O_2 and RV5 stock solutions, respectively. The required amounts of Fe^{2+} and H_2O_2 were added simultaneously to the dye solution. Dye decolorization capacity (%) was determined by measuring the solution's absorbance at the start of the experiment and after 1, 2, 3, 4, 5, 7.5, 12.5, 20, 30, 45 and 60 min, and was calculated as:

$$Decolorization\ capacity\ (\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} x100 \qquad (1)$$

Measurements were performed immediately after sampling to avoid further decolorization. Absorbance readings were performed using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at visual maximum peak wavelength (530 nm), and demineralized water without azo dye was used as a blank solution to calibrate the spectrophotometer. All experiments were performed twice.

Microbial decolorization Microbial decolorization was assessed using fresh activated sludge from a well-operating municipal wastewater treatment plant located in Flanders, Belgium. Besides municipal wastewater, the plant also treats septic material from households, but little to no industrial wastewater. Following aeration of 1 h, the sludge was centrifuged and added (10 g l^{-1}) into autoclaved minimal medium (0.5 l), of which 10 ml was added to 90 ml RV5 containing demineralized water. The final test medium consisted of 5.0 g l^{-1} glucose, 1.0 g l^{-1} $(NH_4)_2SO_4$, 1.0 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} yeast extract and 0.1 g l^{-1} $CaCl_2.2H_2O$ (23), and dye concentrations ranged between 100 and 500 mg l^{-1}. The experiment was performed in duplicate. Treatments without sludge were included as a control and showed that no spontaneous color degradation nor dye absorption occurred throughout the duration of the experiment (data not shown). Incubations were carried out at 20°C on an orbital shaker at 150 rpm for a total of 168 h. Samples were gathered periodically after 0, 24, 48, 72, 96, 120 and 168 h of incubation and were centrifuged to remove solid particles prior to absorbance measurement. Absorbance readings and calculations of decolorization capacity were performed as described above.

Sequential Fenton's oxidation and microbial decolorization In a final experiment, the efficacy of a combination of Fenton's oxidation (1.0, 1.5 or 2.0 mM) and activated sludge was evaluated for a dye amount of 500 mg l^{-1}. After 60 min of Fenton's oxidation, 90 ml of the Fenton treated RV5 water was combined with 10 ml of the activated sludge minimal medium solution, followed by incubation on an orbital shaker as described above. As a comparison, treatments with the chemical reagent or the activated sludge alone were included. Samples were gathered at the start of the experiment and 1, 3, 5, 7.5, 12.5, 20, 30, 45 and 60 min after Fenton treatment, and after 24, 48, 72, 96, 120 and 168 h of incubation and were analyzed as described above. The experiment was repeated twice.

Microbial community characterization For a number of treatments described above, molecular microbial community analyses were performed at the end of the experiment (i.e., after 168 h of incubation). Furthermore, analyses were performed on the activated sludge before addition of the sludge to the dye medium. Genomic DNA was extracted from 0.15 g precipitated, homogenized sludge using the Power Soil DNA isolation kit (MoBio Laboratories Inc., Solana Beach, CA, USA) according to the manufacturer's instructions. Subsequently, two 454 pyrosequencing amplicon libraries were built as described previously, including one for bacteria (primers used: S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21) and one for fungi (primers used: ITS86F/ITS4) (Tables S1 and S2) (two PCR replicates per DNA extract) (31,32). Next, amplicon libraries were sequenced, and the sequencing results were analyzed as described previously (32), using the UPARSE v8.0 standard pipeline (http://drive5.com/uparse/). Sequences from both PCR replicates per sample were combined and grouped into operational taxonomic units (OTUs) based on a 3 % sequence dissimilarity cut-off. Due to uneven sequencing depth and correlation between number of sequence reads and number of OTUs per sample (data not shown), the number of sequences was rarefied to 700 sequences per sample for bacteria and 2200 for fungi, while excluding initial global singletons (i.e., OTUs represented by a single sequence in the unrarefied data) (31). Bacterial OTUs were assigned taxonomic identities by using the 'classify.seqs' command in Mothur (v. 1.36.1) (https://www.mothur.org/) and the Silva taxonomy database v. 119 (https://www.arb-silva.de/), which we manually curated to include additional microbes previously observed in activated sludge (using the Midas database, http://www.midasfieldguide.org/). Taxonomic assignments were considered reliable when ≥0.80 score value was found. Fungal OTUs were identified by querying a representative sequence (selected by UPARSE) against GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html), excluding uncultured/environmental entries. Rarefaction curves were generated for each sample using the Vegan package (v. 2.4–1) for R (Fig. S2) (http://www.R-project.org/; https://cran.r-project.org/web/packages/vegan/index.html). Nonmetric multidimensional scaling (NMDS) and Chao1 and Ace coverage calculations were performed using the R-package Vegan (v. 2.4–1) and the 'summary.single' command in Mothur (v. 1.36.1). Sequence data obtained in this study have been deposited in the Sequence Read Archive under BioProject accession PRJNA355983.

RESULTS AND DISCUSSION

Chemical decolorization of Reactive Violet 5 As pollutant concentration is an important parameter in textile wastewater treatment, first Fenton's reagent decolorization capacity was evaluated using different RV5 dye concentrations. The concentrations used in this study were 100, 200, 300, 500, 700 and 900 mg l^{-1}. Further, a H_2O_2 and Fe^{2+} concentration of 1.5 mM and 0.15 mM was used based on previous research (23). After 60 min of Fenton's reaction, dye removal decreased from 98.4 to 60.8 % when increasing the dye concentration from 100 to 900 mg l^{-1} (Fig. 1). Most activity was seen during the first 45 min of the reaction, after which no substantial further decolorization was observed

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