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Biodegradation of synthetic dyes by free and cross-linked peroxidase in microfluidic reactor

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ABSTRACT

This paper aims to reduce the gap between intensified innovative process designs and the application of enzyme technologies at an industrial scale that often take full advantage of microreactor systems. Soybean and potato processing waste was used as the source of peroxidase in both the free and immobilized form for the biodegradation of the Acid Violet 109 anthraquinone dye (AV109) in a PTFE (polytetrafluoroethylene) microreactor which is 0.5 mm in diameter and 6 m in length. High biodegradation efficiencies of 95 and 76% were achieved by soybean and potato peroxidase respectively after 3 min of residence in the microreactor, with a noticeable lack of hydrogen peroxide inhibition. The reactor's design, with regard to its diameter and length, significantly affected the biodegradation efficiency. The polymer membrane formation by the cross-linking approach with oxidized pectin and soybean or potato peel peroxidase on the inner PTFE wall was SEM confirmed. The cross-linking efficiency with oxidized pectin of both peroxidases is inversely correlated with the activity, hence the highest recorded activity of immobilized peroxidase was 11 and 19 μmol/min of soybean and potato peroxidase, respectively, at an initial pectin concentration of 0.05%. A promising reusability for AV109 biodegradation in the microreactor with immobilized soybean and potato peroxidase was confirmed, since the immobilized peroxidases retained 65 and 35% of the initial activity after the tenth cycle, respectively. The LC–MS and GC–MS techniques confirmed the biodegradation of the AV 109 dye, and the intermediates with a lower molecular weight were separated and identified.

1. Introduction

Microfluidic systems based on microchannels which are on a micron- or submicron scale for microflow manipulation are implemented in an array of enzyme catalyzed processes (Zhang et al., 2021). The continuous flow delivered by microfluidic reactors provides exquisite benefits: a high surface-to volume ratio; better mixing and mass transfer; laminar

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and highly ordered flow; process intensification and cleaner production. In addition, by carefully considering the reaction kinetics and flow dynamics, it is possible to easily increase the scale of the process towards an industrial level by multiplying the number of the same reactors in the series (Šalić and Zelić, 2018).

The enzyme-driven bioremediation of wastewater is a high-performance specific process which is easily manipulated. The enzyme-based treatment of colored wastewater enables the production of an effluent where the dye concentration is significantly below the predicted no-effect concentration (PNEC) level and is mostly performed in large-scale reactors (Darwesh et al., 2019; Li et al., 2020). However, there is a growing concern in the implementation of the continuous flow microreactor technology, making this process more efficient and cost-effective (Cao et al., 2021). A novel approach in the biodegradation of synthetic dyes founded on the 4R's principle (Reduce, Reuse, Recycle and Restore) represents the application of crude extracts of peroxidases obtained by one-step extraction from low-cost waste biological materials (soybean, cabbage, potato, watermelon, lemon, banana peel) tailored to microfluidic systems (Mathkor et al., 2019). The application of microfluidic enzyme reactors at a large scale is limited by enzyme stability, underlying the necessity for enzymatic process engineering. Immobilized microfluidic enzyme reactors have a potential for the removal of synthetic dyes due to the possibility to separate reaction derivatives from the enzymes in a continuous flow. Furthermore, the substrates are continuously supplied to the system, avoiding or mitigating enzyme inhibition by products or excess substrates. Although small, the microchannels of the reactor are a very suitable space for enzyme immobilization. So far, data on the successful application of various immobilization techniques in microchannels can be found in literature (Zhu et al., 2020; Honda et al., 2005; Lloret et al., 2013; Costas et al., 2008; Matto and Husain, 2006).

The current study was carried out to expand the potential application of peroxidases originating from agro-industrial waste by their use in free and immobilized forms within fully automated microreactor systems in order to address the limitations associated with the high cost and low stability of these peroxidase-based biodegradation approaches. In this regard, the possibility of microfluidic systems for the biodegradation of synthetic dyes in a continuous flow reactor catalyzed by peroxidase from soybean hull and potato peel was investigated. The output and the intrinsic system specific parameters were optimized in order to enhance biodegradation efficiency. Hence, the main enzymatic reaction parameters in the continuous flow were thoroughly optimized: initial enzyme, hydrogen peroxide and AV 109 dye concentrations. Additionally, the influence of the design, more precisely the diameter and length of the microreactor, on the efficiency of the enzyme in the biodegradation reaction of AV 109 dye was carefully considered. With a particular focus on the development of immobilized peroxidase microreactors, an investigation of the initial concentration of cross-linking agent, oxidized pectin, on both soybean and potato peroxidase immobilization was performed. The immobilized system was characterized in terms of biodegradation capacity along with operational stabilities. The last objective of the current study is the analysis of AV 109 dye biodegradation products. The soybean and potato peroxidase catalyzed biodegradation of AV 109 dye was investigated in detail by examining the transformation products generated during degradation. The determination of biodegradation products was performed by LC–MS and GC–MS analysis and herein the transformation products and possible mechanism of this model of anthraquinone dye degradation catalyzed by soybean and potato peroxidase are reported.

2. Materials and methods

2.1. Materials and reagents

Soybean hulls and potato peels were obtained from Sojaprotein (Serbia) and Chips Way factories (Serbia); C.I. Acid Violet 109 from DCC colorants (Ningbo, 315199 China); pyrogallol, monobasic potassium phosphate anhydrous (KH_2PO4), dibasic potassium phosphate trihydrate ($K_2HPO_4 \times 3\ H_2O$) and TRIS from Merck (USA); hydrogen peroxide 35% from Carlo Erba; sodium acetate from Alkaloid (N. Macedonia); and glacial acetic acid from Lach:ner (Czech Republic). Apple pectin (Mw – 30000–100000, degree of esterification 70%–75%) was purchased from Sigma-Aldrich (St. Louis, USA). All of the reagents were of analytical grade.

2.2. Soybean hull and potato peel recovery from low-cost agro-industrial waste

Peroxidase from soybean hull and potato peel waste was recovered as previously described (Svetozarević et al., 2021). In brief, potato peels and soybean hulls were incubated in a phosphate buffer (100 mM, pH 6) and distilled water, respectively, overnight and then filtered through gauze, incubated at 65 °C for 3 min, cooled in an ice bath and centrifuged at 10^4 rpm for 15 min in order to obtain a crude enzyme extract. The crude enzyme extract was stored at −18 °C until later use.

2.3. Peroxidase activity assay

Soybean hull and potato peel peroxidase activity was measured at 420 nm with a spectrophotometer (UV/Vis Ultrospec 3300 Pro, Amersham Bioscience, UK) using pyrogallol as a standard substrate (Šekuljica et al., 2016). In brief, the enzymatic activity was monitored during 3 min in the mixture consisting of: 1 ml of 0.013 M pyrogallol, 0.01 ml of 3% *v/v* hydrogen peroxide and 0.01 ml of enzyme solution (T = 25 °C). One unit of activity (μmol/min) is defined as the amount of peroxidase that catalyzes the conversion of 1 μmol of pyrogallol per minute.

2.4. Biodegradation assays of AV 109 dye in PTFE microreactor catalyzed by soybean hull and potato peel peroxidase

The process parameters were optimized by changing the concentrations of the enzyme in the flow, hydrogen peroxide and AV109 at pH 4. The biodegradation was monitored until there was no significant change in the absorbance, which was confirmed to be the residence time. The residence time in the reactor was varied by changing the flow (ml/min) of the reactants through the microreactor. Further optimization was performed by changing the length and diameter of the reactor. All experiments were done at room temperature. The experiments were conducted in a microreactor system consisting of three plunger pump units (LC-20AD XR, Shimadzu USA Manufacturing Inc) and two micromixers. The microreactor tube was made from PTFE by VICI Jour. The output parameters — the flow rate of the reagents was kept in a constant ratio 1:1:1, while the reactant flows were varied. For the investigation of the enzyme concentration's influence on the biodegradation rate, the enzyme concentration in the flow was varied from 0.05–0.6 IU/mL for soybean hull peroxidase (SHP), and 0.2–1.0 IU/ml for potato peel peroxidase, at a constant $[H_2O_2]/[AV109]$ concentration flow ratio of 5.5. In the same experiments, the residence time was obtained. The hydrogen peroxide concentration effect was investigated by varying its concentration in the flow from 0.1–1.0 mM at a constant enzyme concentration in the flow of 0.8 and 0.2 IU/ml; for potato peel peroxidase and soybean peroxidase, respectively, and a constant dye concentration in the flow of 30 mg/l for both enzymes. The dye concentration was optimized by varying the initial concentration of AV109 in the flow from 30–360 and 90–450 mg/l for soybean peroxidase and potato peel peroxidase, respectively, at a constant enzyme concentration in the flow of 0.8 and 0.2 IU/ml, and hydrogen peroxide concentration in the flow of 1.0 and 0.2 mM, for potato peel peroxidase and soybean hull peroxidase, respectively. After optimizing the concentration of the enzyme, the dye and hydrogen-peroxide in the flow, the length of the reactor was varied. The biodegradation reaction was optimized in terms of length using a microreactor with a diameter of 0.5 mm and varying lengths of 2.5, 6 and 15 m; while in the microreactor of an 0.8 mm diameter, the examined lengths were 1, 2.3 and 6 m. The biodegradation rate was monitored spectrophotometrically, at 590 nm. It is significant to mention that the initial dye concentration in the flow differs from the actual dye concentration in the flow in the microreactor where the biodegradation takes place. Because there are three inlet flows, the actual dye concentration in the flow in the microreactor is three times lower. In all calculations, the actual concentration in the flow in the microreactor was taken into consideration.

The biodegradation rate was calculated using the equation:

$$\textit{Biodegradation efficiency}, (\%) = 100\left(\frac{C_0 - C_t}{C_0}\right) \tag{1}$$

where C_0 is the initial dye concentration and C_t is the residual dye concentration after biodegradation calculated using the calibration curve for AV 109 dye $y = 0.013 \times C$. All experiments were performed in triplicate and relative standard deviations of triplicate measurements were less than 5%.

2.5. Cross-linking of soybean hull and potato peel peroxidase in microchannel reactor using oxidized pectin

2.5.1. Pectin cross-linker preparation by periodate oxidation

The pectin cross-linker was prepared as previously described with slight modifications (Talekar et al., 2014). 2 g of apple pectin was dissolved in 20% *(v/v)* ethanol, homogenized at room temperature followed by the addition of 0.5 M sodium metaperiodate (3 ml) and the pH was adjusted to 3.5 using dilute hydrochloric acid and sodium bicarbonate solutions. The mixture was incubated at 60 °C for 2 h with constant shaking, 150 rpm (KS 4000 i control, IKA, Staufen, Germany) in dark. After 2 h, the oxidized pectin was recovered using an excess of cold isopropanol, vacuum filtered and dried using a laboratory freeze dryer (Martin Chris Beta 2–8, LSC basic, Germany). Pectin oxidation was confirmed by FTIR analysis.

2.5.2. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared (FT-IR) spectra were recorded in transmission mode, using a Nicolet™ iS™ 10 FT-IR Spectrometer (Thermo Fisher Scientific) with Smart iTR™ Attenuated Total Reflectance (ATR) sampling accessories. The ATR-FTIR spectra were recorded in the 500–4000 cm^{-1} range with 20 scans per spectrum.

2.5.3. SEM

A scanning electron microscope (Tescan FE-SEM Mira 3 XMU) operated at 5 keV was employed to characterize the morphology of the tubes prior to and after the immobilization procedure. Prior to SEM analysis, all samples were coated with a thin gold layer by using a sputter coater (Polaron SC503, Fisons Instruments, United Kingdom).

2.6. Cross-linking of peroxidase on the inner wall PTFE tubes with oxidized pectin

For the cross-linking of peroxidase onto the inner wall of PTFE with oxidized pectin as a cross-linker, a microreactor with 1 m length and 0.5 mm diameter was used. A 0.05% aqueous solution of oxidized pectin was pumped into the PTFE tubes at a flow of 0.001 ml/min, while the crude enzyme extract (0.25 mg/ml of soybean peroxidase and potato peroxidase, used separately) was introduced at a flow rate of 0.0008 ml/min. After residence of 108.8 min, the outlet solution, which

contains the unbound enzyme, was collected and subjected to a total protein concentration determination by modified Lowry assay (Hartreee, 1972). The amount of cross-linked peroxidase was expressed as the difference in the mass of protein in the initial solution and the mass of protein in the outlet solution. Thus, the peroxidase pectin cross-linking efficiency (CLE) in the microreactor was calculated as follows:

$$CLE\ (\%) = \left(\frac{m_{RT=0} - m_{RT=108.8}}{m_{RT=0}} \right) \times 100 \quad (2)$$

Where, $m_{RT=0}$ is the mass (mg) of peroxidase in the enzyme stock used for the immobilization and the $m_{RT=108.8}$ is the mass (mg) of protein remained in the outlet solution after the immobilization, i.e. 108.8 min. The enzyme activity was measured using pyrogallol as a substrate after the flushing of the immobilized microreactor with an acetic buffer 0.05 M, pH 4. Briefly, a 13 mM solution of pyrogallol in a phosphate buffer (0.1 M, pH 7) was pumped at a flow of 0.1 ml/min along with 0.97 mM H_2O_2 at 0.1 ml/min (T = 25 °C). After 1 min of residence, the absorbance was measured at 420 nm. One unit of activity (μmol/min) is defined as the amount of peroxidase that catalyzes the conversion of 1 μmol of pyrogallol per minute.

2.7. Operational stability of peroxidase immobilized in microreactor

The peroxidases from soybean hulls and potato peels cross-linked in the PTFE microreactor (0.5 mm diameter, 1 m length) were applied in the removal of the AV 109 dye under the previously established reaction parameters for dye degradation catalyzed by free peroxidases. After one cycle of biodegradation, the microreactor was flushed with an acetic buffer 0.05 M, pH 4 and fresh dye and hydrogen peroxide solutions were injected. The cycles were repeated until a substantial decrease in the activity of the immobilized enzymes was noticed.

2.8. Determination of biodegradation products by LC-ms and GC-ms analysis

For the characterization of the biodegradation products, LC–MS and GC–MS analysis was performed. A Thermo Scientific Accela/TSQ Quantum Access MAX triple quadrupole HPLC–MS system with the mobile phase (A: H_2O 0.1% HCOOH, 5 mM $HCOONH_4$ and B: MeOH 0.1% HCOOH, 5 mM $HCOONH_4$) at the flow rate of 0.3 cm^3/min was used. The following gradient program was used for the analysis: 100% A for 0.5 min, then gradient ramp to 70% B at 7 min, 100% B at 9 min and hold. The instrumental parameter was as follows: full scan 50–1000 m/z, HESI, negative ionization, spray voltage, of 3700 V, Sheath and aux gas of 55 and 15, respectively. Vaporizer and capillary temperature of 270 °C and 300 °C, respectively. The sample injection volume was 0.01 ml, while the separation was done on a Thermo Scientific Accucore aQ, 100 × 2.1 mm, 2.6 μm column. The data were analyzed by Thermo Xcalibur 2.2 software. Semivolatile organics produced from AV109 biodegradation were measured by GC–MS, using a Varian CP-3800 GC with Saturn 2200 MS and CP-8200cx autosampler (Varian Inc., Palo Alto, CA, USA). This system was equipped with a 1079 split/splitless injector port set to 280 °C. The initial column oven temperature was set to 60 °C, ramped at 8 °C min^{-1} rate to 300 °C, and held for 10 min. The constant carrier gas flow rate was 1.0 ml min^{-1}. To identify the AV109 biodegradation compounds, 1 μL of the solution sample was analyzed on a FactorFour (VF-5 ms) capillary column (30 m × 0.25 mm ID; film thickness 0.25 μm). MS operated in the electron ionization mode at 70 eV, using the full scan analysis in the mass range from 45 to 450 *m/z*.

2.9. Determination of the chemical oxygen demand (COD)

In order to assess the quality and toxicity of the dyed solution after the enzymatic treatment, COD analysis is performed. The analysis is performed by the adapted US EPA dichromate method (410.4). When the enzymatic reaction is over, samples are collected. Blank samples contain dye, hydrogen peroxide and thermally inactivated enzyme with their optimal concentration. After the samples are prepared, 0.2 ml of each are pipetted to a reaction vial for high range COD (HI93754C-0*), at a 45-degree angle. The vials are sealed, mixed and refluxed by COD reactor, 2 h, 150 °C. After the digestion, vials are cooled to about 120 °C, mixed once again and cooled to room temperature. Next, the COD is measured by Multiparameter Photometer HI83314 and the COD high range method.

3. Results and discussion

3.1. Process parameters optimization of AV109 biodegradation in continuous flow

In this study, the investigation of anthraquinone dye AV109 biodegradation in the microreactor was performed. The enzyme used for the dye oxidation was isolated from low-cost agro-industrial materials as a crude enzyme extract. Potato peels and soybean hulls were used as sources of peroxidase. According to our previous research, the optimal pH for the removal of the AV 109 dye catalyzed by free soybean hull and potato peel peroxidases in batch reactor conditions for both enzymes was pH 4 (Svetozarević et al., 2021).

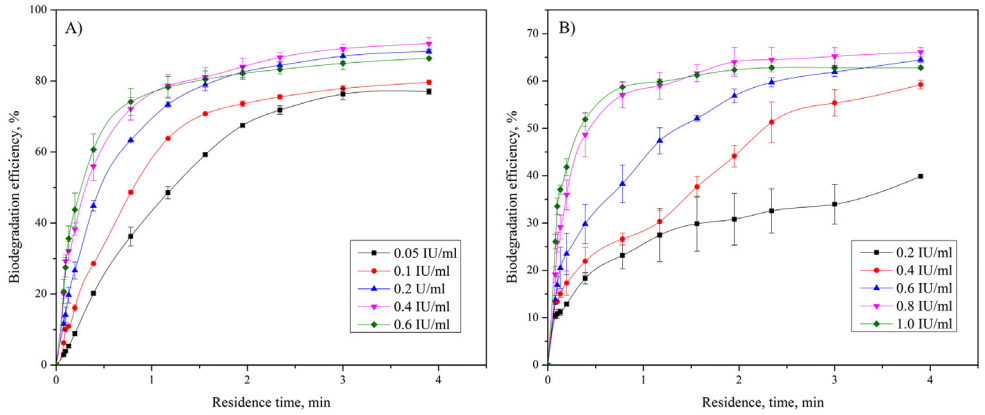


Fig. 1. Enzyme concentration optimization for AV109 biodegradation in continuous flow by: (A) soybean hull peroxidase, (B) potato peel peroxidase. Reaction conditions: 0.2 mM hydrogen peroxide; 30 mg/l dye, pH 4, T = 25 °C, $L_{reactor} = 6$ m, $d_{reactor} = 0.5$ mm for both enzymes.

Although low-cost agro-industrial waste materials were used as the enzymes' source, the enzyme concentration is a vital parameter in terms of process efficiency and cost-effectiveness. The one-step extraction method contributes to a simple, robust and easy handling method. The influence of the enzyme concentration on the biodegradation was examined by varying the initial enzyme concentration in the flow from 0.05 to 0.6 IU/ml for soybean peroxidase, and from 0.2 to 1.0 IU/ml for potato peroxidase. The efficiency of both peroxidases in the removal of the AV 109 dye performed in a continuous flow are shown in Fig. 1.

It can be clearly seen from Fig. 1(A) that the soybean hull peroxidase has a higher potential for dye degradation at a lower enzyme activity. With only 0.2 IU/ml of soybean peroxidase an 88.39% of biodegradation was achieved, while only 64.52% of biodegradation was achieved with 0.8 IU/ml of the potato peroxidase in the flow (Fig. 1 (B)). The biodegradation stopped changing significantly after 3 min, thus the residence time of 3 min was adopted for further experiments. By comparing the obtained results with the results of the biodegradation in a batch reactor, it can be concluded that the continuous system has the potential for application in the biodegradation of synthetic dyes (Svetozarević et al., 2021). Namely, batch biodegradation using 1 IU of soybean peroxidase reached 54.32% of dye removal, while in the continuous system, a biodegradation of 87.80% was achieved using 5-fold lower enzyme dose, 0.2 IU. It can be assumed that better mixing and mass transfer in the continuous system increased the efficiency of peroxidase in the observed reaction. Biodegradation in the batch reactor lasted 30 min while 3 min in the continuous system was enough to reduce the dye concentration from 30 to 3.85 mg/l.

Soybean and potato peroxidase use hydrogen peroxide as an electron acceptor, reducing the hydrogen peroxide to water, while the oxidized state of the enzyme catalyzes the oxidation of the substrate — in this case AV109. The hydrogen peroxide concentration can affect greatly the biodegradation efficiency, so its optimization is of great importance. In order to compare the biodegradation in the continuous system with the previously developed process in the batch system, the influence of initial hydrogen concentrations of 0.1, 0.2 and 1.0 mM were selected to examine the effect on the biodegradation catalyzed by both the soybean and potato peroxidases in the continuous system (Fig. 2). The increase of peroxidase efficiency in correlation to the increase in initial hydrogen peroxide concentration can be observed from Fig. 2(A). Namely, the sharp increase in biodegradation efficiency catalyzed by soybean peroxidase from 66.15 to 88.03% is established. Similarly, the biodegradation efficiency of potato peroxidase increased from 43.77 to 72.66% as the initial peroxide concentration increased (Fig. 2B). However, the most important observation in this experimental series is that there is no peroxide inhibition in both enzymatic reactions, which is not the case in a batch reactor. In the batch reactor, a pronounced inhibitory effect of hydrogen peroxide on potato peel peroxidase efficiency was observed (Svetozarević et al., 2021). Annotatively, it is possible to circumvent the main industrial application limitation of enzymatic dye degradation by using microreactors. The improvement of this dye removal technique is the absence of enzyme inactivation caused by excess hydrogen peroxide.

One of the main issues that sets back the enzymatic treatments of colored wastewater is a high dye load that can have inhibitory effect on the enzyme. The actual dye concentration flow was varied 10–120 mg/l for soybean hull peroxidase and 10–150 mg/l for potato peel peroxidase. The results are shown in Fig. 3.

From Fig. 3(A) can be concluded that the dye concentration and the biodegradation efficiency are inversely correlated. The higher the dye concentration is, the lower the biodegradation efficiency. Obviously, the dye has a strong inhibitory effect on biodegradation efficiency. The optimal dye concentration for soybean hull peroxidase was 10 mg/l where 95.13% biodegradation efficiency was achieved. The same result on the influence of the initial dye concentration on the efficiency of soybean peroxidase is presented in the literature, more precisely; high initial dye concentrations have a strong inhibitory effect on this enzyme. As for potato peel peroxidase, quite the opposite results were obtained, Fig. 3(B). This enzyme has proven to be active at high dye concentrations, up to 150 mg/l. It is the optimal AV109 concentration,

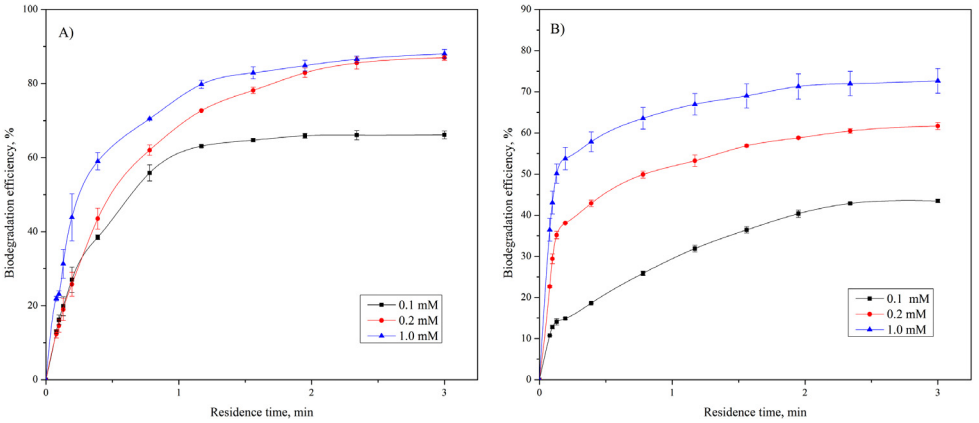


Fig. 2. Hydrogen peroxide concentration optimization of AV109 biodegradation in continuous flow by: (A) soybean hull peroxidase, (B) potato peel peroxidase. Reaction conditions: 0.2 IU/ml for soybean hull peroxidase and 0.8 IU/ml for potato peel peroxidase; 30 mg/l dye, pH 4, T = 25 °C, $L_{reactor} = 6$ m, $d_{reactor} = 0.5$ mm for both enzymes.

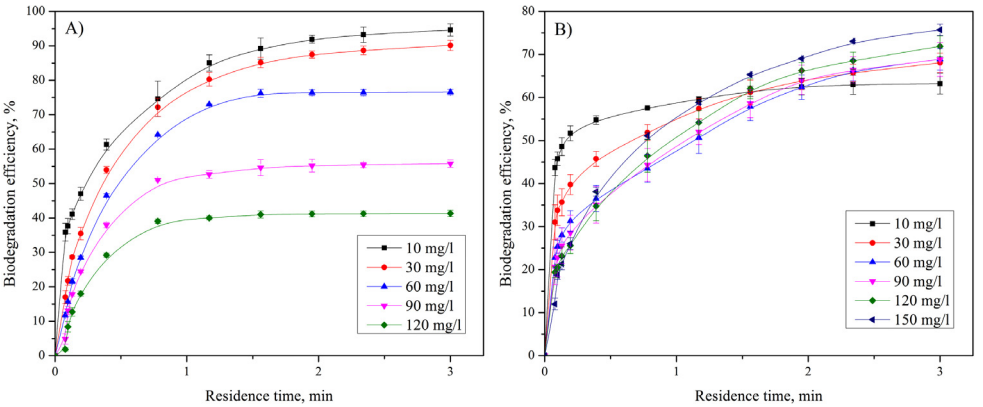


Fig. 3. (A) Dye concentration optimization of AV109 biodegradation in continuous flow by: (a) soybean hull peroxidase, (B) potato peel peroxidase. Reaction conditions: 0.2 IU/ml enzyme concentration, 0.2 mM hydrogen peroxide for soybean hull peroxidase and 0.8 IU/ml enzyme concentration, 1 mM hydrogen peroxide for potato peel peroxidase; pH 4, T = 25 °C, $L_{reactor} = 6$ m, $d_{reactor} = 0.5$ mm for both enzymes.

with 75.71% biodegradation efficiency. This result is in complete contrast to the behavior of potato peroxidase in batch conditions. The dye concentration did not show any inhibitory effect on the potato peel peroxidase in a continuous flow, revealing a high potential for the commercial application of this potato peroxidase based removal of anthraquinone dyes. A considerable potential of potato peel/pulp in the biodegradation of organic pollutants has been reported in the literature. Peroxidase originating from sweet potato has been successfully implemented in the batch process of methyl orange biodegradation. This peroxidase removed 91.4% of the initial dye concentration with 102.5 μmol/l of H_2O_2 and 81.2 IU/ml of the enzyme was documented (Luan et al., 2017). Furthermore, the potato pulp peroxidase showed a phenol removal efficiency of over 95% for optimized phenol concentrations (Kurnik et al., 2015). Thus, with the efficient biodegradation of an extremely high AV 109 dye load, the process intensification property of microreactors was confirmed.

The microreactor's geometry plays a significant role when the performance of microreactor is the focus of attention. Among geometric parameters of substantial importance are its length and diameter. Herein, we have examined the influence of the length and diameter of the PTFE microreactor on the efficiency of peroxidase biodegradation of the AV 109 dye under the previously optimized process conditions. The influence of the reactor diameter and length was examined under previously optimized residence time conditions. The results are given in Fig. 4.

As it can be seen from Fig. 4(A), reactors with a diameter of 0.5 mm and 0.8 mm were used for optimization. Reactors with varying lengths of 2.5, 6 and 15 m were examined at an 0.5 mm diameter, and 1, 2.5 and 6 m lengths at an 0.8 mm diameter. The biodegradation efficiency varies, 91.03–95.48% for both diameters in the reaction catalyzed by soybean peroxidase. Although there is no large deviation in biodegradation efficiency, it is evident that a more efficient process is in a reactor with a smaller diameter, more precisely 0.5 mm. The influence of the reactor's length on the biodegradation efficiency is apparent when high concentration of the dye is employed, in particular when the potato peroxidase catalyzes biodegradation, Fig. 4(B). The optimal length of the reactor has proven to be 6 m, with a 76.95% biodegradation efficiency.

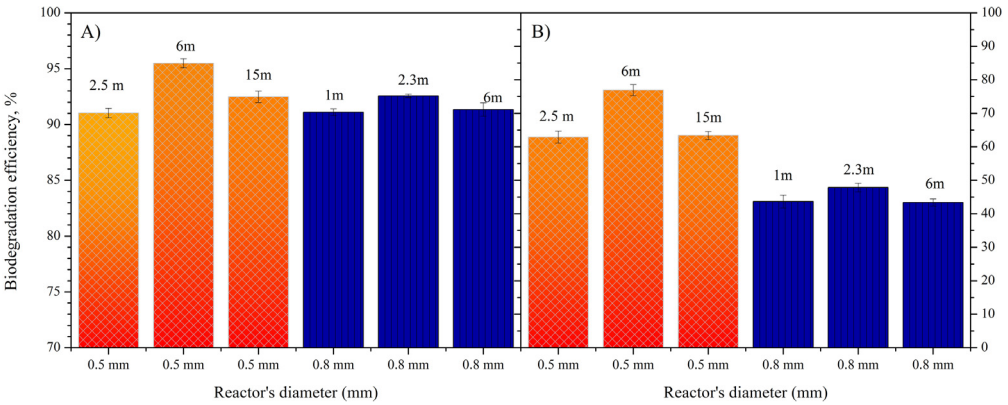


Fig. 4. Reactor's length optimization of AV109 biodegradation in continuous flow by: (A) soybean hull peroxidase, (B) potato peel peroxidase. Reaction conditions: 0.2 IU/ml enzyme concentration, 0.2 mM hydrogen peroxide, 10 mg/l dye for soybean hull peroxidase and 0.8 IU/ml enzyme concentration, 1 mM hydrogen peroxide, 150 mg/l for potato peel peroxidase; pH 4, T = 25 °C $L_{reactor} = 6$ m, $d_{reactor} = 0.5$ mm for both enzymes.

Shorter or longer lengths than 6 m showed a decrease in decolorization. This leads to a conclusion that even though the reactor's length is greater, that does not necessarily lead to a more efficient process. In this case, the reactor's diameter has a more significant role in biodegradation efficiency, as proven by the reactors of different lengths and 0.8 mm diameter, where 43.38–47.82% biodegradation was achieved. Similar findings were acquired by Onal et al. in their study of unsaturated aldehydes' hydrogenation (Gupta et al., 2013). With an increase of the microreactor's diameter from 0.5 to 1 mm, the reaction rate dropped.

3.2. Immobilization of soybean and potato peroxidase in continuous flow and application in AV 109 dye biodegradation

Efficient processing implies the application of immobilized enzymes that are persistent forms of native ones with higher stability and enhanced reusability over repeated reaction cycles maintaining a satisfying catalytic activity. The results shown above confirm that it is possible to develop a fully automated process for the biodegradation of synthetic dyes with a free enzyme in the microreactor systems. Microreactor systems have attractive and favorable properties for reactions catalyzed by immobilized enzymes such as prompt mass and heat transfer along with a large specific surface area. With the idea of developing an environmentally friendly solution for the biodegradation of synthetic dyes, the process of peroxidase immobilization in a microreactor is also planned. The immobilization plan considered the formation of polymer enzyme membranes by the cross-linking technique on the inner wall of PTFE tubes. Apart from the glutaraldehyde and paraformaldehyde, the recent trend in enzyme cross-linking involves the use of natural polymers in an oxidized form (Honda et al., 2005). Therefore, apple pectin was successfully oxidized by the periodatic method available in the literature and the oxidation success was confirmed by FT–IR analysis (Fig. S1).

The main differences between FT–IR spectra of virgin and oxidized pectins is the appearance of a new peak at 1729.91 cm^{-1} corresponding to dialdehyde peaks in periodate oxidized polysaccharides, such as apple pectin. Furthermore, the appearance of a peak at 1617.57 cm^{-1} is related with free COO^{-} groups in oxidized pectin. In addition, the typical broad band at 3200–3550 cm^{-1} assigned to stretching vibration of O-H groups, appears with lower intensity in the FT-IR spectrum of oxidized pectin. It is well known that the vicinal diols in pectin are cleaved by periodate oxidation, to form dialdehyde derivatives. In oxidized pectin, fewer alcohol groups are present than in virgin pectin, as the FT-IR analysis confirms. These findings coincide completely with previous literature reports (Gupta et al., 2013). Later, the cross-linking efficiency of oxidized pectin was examined in peroxidase immobilization. The immobilization procedure was quite simple and consisted of injecting a solution of peroxidase (flow rate of 0.0008 ml/min) and oxidized pectin (flow rate of 0.001 ml/min) in a concentric laminar flow with the aim of forming a polymer layer of cross-linked enzyme on the inner wall of the PTFE tube. The effect of different initial cross-linker concentrations and the initial enzyme concentration on the immobilization efficiency and activity of peroxidase immobilized in PTFE tubes was studied and the results are given in Fig. 5.

It is evident that the quantity of the cross-linker has a major impact on the characteristics of cross-linked soybean peroxidase (Fig. 5(A)). Namely, with the increase in the initial concentration of oxidized pectin beyond 0.05%, the amount of bound enzymes in the polymer membrane further increases and has a negative impact on peroxidase activity. Thus, at the lowest initial concentration of oxidized pectin, the highest activity of immobilized peroxidase was obtained, which is very often attributed to the formation of multiple layers within the membrane and by steric disturbances caused by densely distributed enzymes inside the membrane. Even though scarce, there are some data regarding the microreactor design in terms of oxidoreductase immobilization. Thus, for example, a microreactor system of laccase immobilized in PTFE tubes with a diameter of 0.5 mm and a length of 13 cm (26 μl total volume of microreactor) was designed by

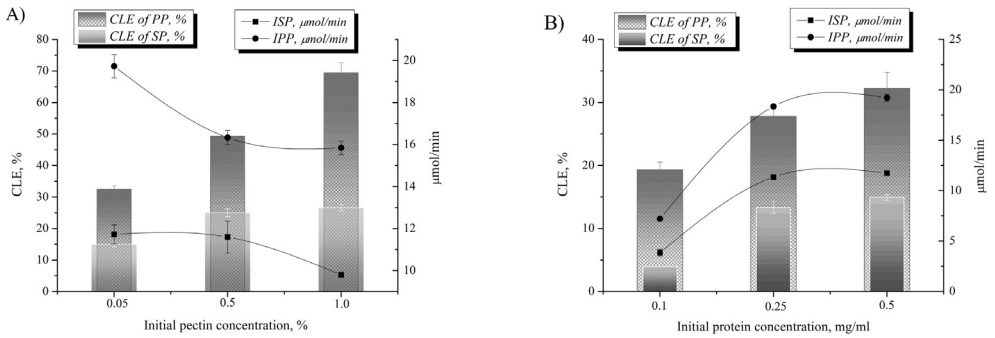


Fig. 5. (A) The initial pectin concentration influence on the cross-linking efficiency (CLE, %) and the activity of immobilized soybean and potato peroxidase (μmol/min); Reaction conditions: concentration of soybean and potato peroxidase in the flow: 0.5 mg/ml (B) The initial protein concentration influence on the cross-linking efficiency (CLE, %) and the activity of immobilized soybean and potato peroxidase (μmol/min); Reaction conditions: concentration of pectin in the flow 0.05%.

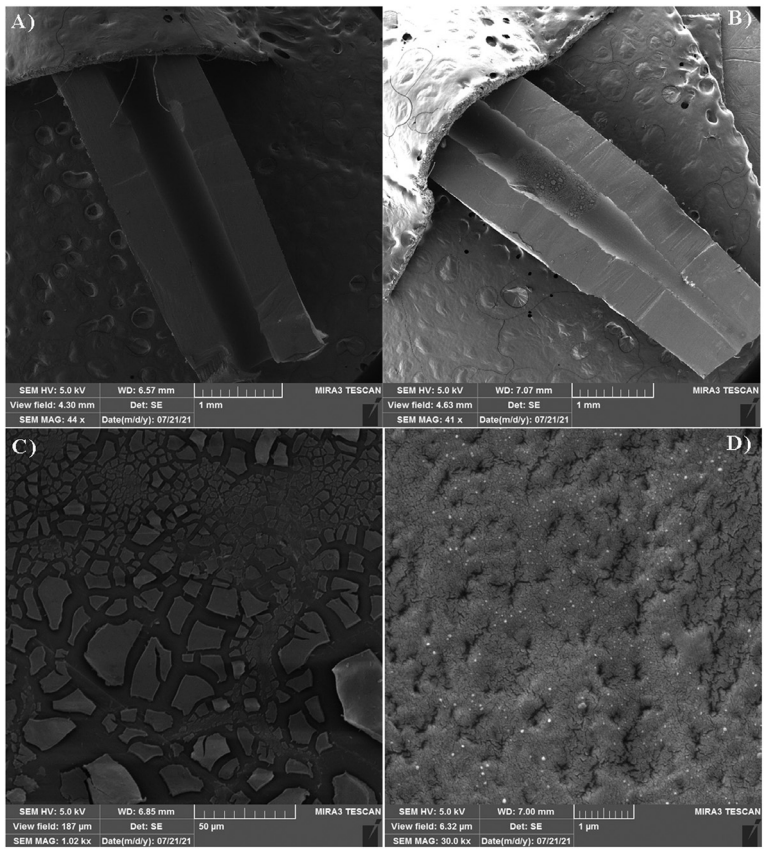


Fig. 6. SEM images of PTFE tubes (A – 44× magnification) and peroxidase polymer membrane on the inner PTFE tube (B – 40× magnification, C – 1000× magnification, D – 30000× magnification).

the cross-linking technique with a mixture of glutaraldehyde and paraformaldehyde with the addition of coupling agent, poly(L)-lysine. Similar conclusions about the effect of the cross-linking agent were drawn in this study, for instance, a progressive increase in the concentration of the cross-linking agent affects the reduction of the activity of immobilized laccase (Lloret et al., 2013). The influence of the initial cross-linker concentration on the potato peroxidase cross-linking in the PTFE tube is also shown in Fig. 5(A). Apparently, the potato peroxidase cross-linking in PTFE tube was even more efficient under similar initial cross-linker concentrations. A sharp increase in initial cross-linker concentration from 0.05 to 1% resulted in a remarkable immobilization efficiency increase from 32%–69%, meanwhile the relative activity

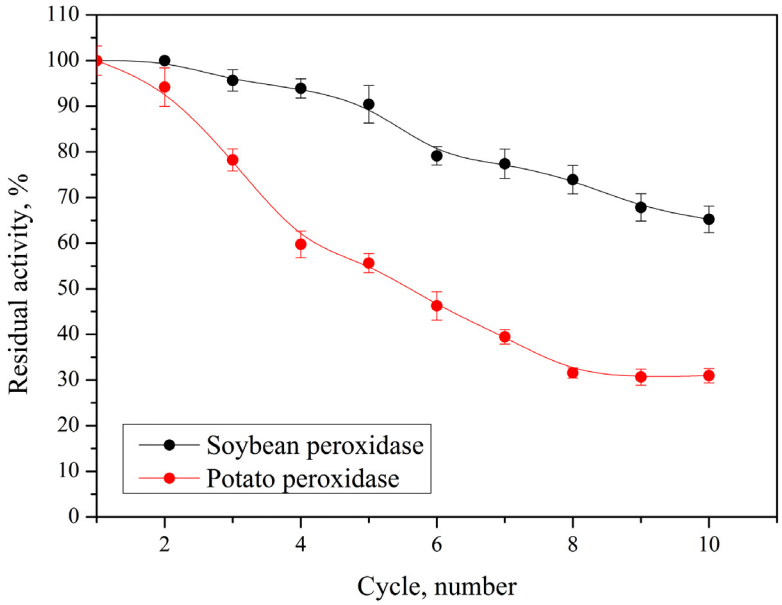


Fig. 7. Operational stability of peroxidase immobilized in microreactor. Reaction conditions for soybean peroxidase: 10 mg/l AV 109 dye, 0.2 mM H_2O_2; for potato peroxidase: 150 mg/ml AV 109 dye and 1 mM H_2O_2.

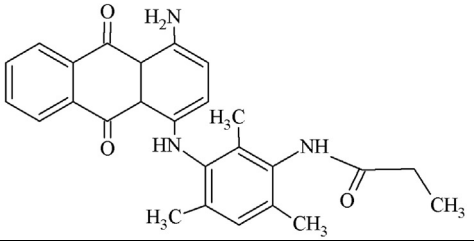
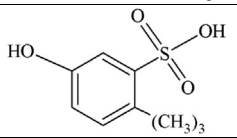
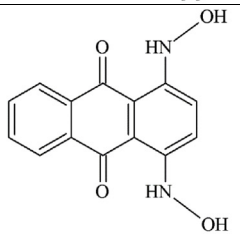
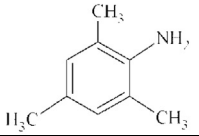
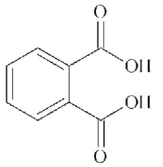
of immobilized potato peroxidase has remained almost unchanged, at 18.35 μmol/min. It is quite clear that the cross-linking agent has exactly the same effect on the immobilization efficiency and the activity of both the soybean and potato peroxidase. Further immobilization procedures were directed towards determining the initial protein concentration's influence on the cross-linking efficiency and the activity of the immobilized soybean and potato peroxidases. According to the results displayed in Fig. 5(B) one can conclude that at the initial enzyme concentration of 0.25 mg/ml the highest activity of cross-linked soybean peroxidase is recorded which is 11 μmol/min and remains unchanged with further increases in the initial enzyme's concentration up to 0.5 mg/ml. The same effect of the initial enzyme's concentration on the immobilization efficiency and the activity of potato peroxidase can be observed in Fig. 5(B). Namely, with an increase in the initial enzyme concentration the increase in cross-linking efficiency was recorded along with increase in the activity of immobilized potato peroxidase.

The verification of the accomplished peroxidase immobilization in PTFE tubes is also given in Fig. 6. Fig. 6 shows the surface morphology of PTFE tubes prior to and after the peroxidase immobilization by cross-linking technique using oxidized pectin. It is absolutely obvious that the peroxidase immobilization changed the surface morphology of PTFE tubes in terms of surface smoothness. Namely, before the immobilization, the PTFE surface was smooth without any signs of unevenness or cracks on the surface. However, with the formation of the polymer membrane consisting of cross-linker and the enzyme, the PTFE surface acquired a rougher texture, with many apparent aggregates of heterogeneous distribution. These observations are in line with previously reported SEM images of peroxidase immobilization along with pectinase immobilization *via* polyaldehyde kefiran on a glass bead (Hosseini et al., 2020; Marchisa et al., 2012).

Consequently, for the design of the microreactor with immobilized potato peel peroxidases, the concentration of pectin in the flow was 0.05% and the potato peroxidase concentration in the flow was 0.5 mg/ml. Also, for the design of the microreactor with immobilized soybean hull peroxidases, the concentration of pectin in the flow was 0.05% and the soybean hull peroxidase concentration in the flow was 0.5 mg/ml. These microreactor designs were examined in the following experiments. The microreactor with immobilized soybean and potato peroxidases were examined with the continuous removal of the AV 109 dye carried out at optimum operating conditions with free soybean and potato peroxidases in terms of biodegradation efficiency and stability and the results are given in Fig. 7.

One of the rationales for the immobilization of the enzyme is the possibility of reuse, which facilitates the separation of the enzyme from the reaction mixture, thus reducing the cost of the process, which further enables enzyme application in real life. Fig. 7 shows the operational stability of both soybean and potato peroxidases cross-linked in the PTFE microreactor, verified through ten consecutive cycles. As it can be seen, the soybean peroxidase retained up to 80% biodegradation efficiency of the AV109 dye in six consecutive cycles. Afterwards, the biodegradation efficiency slightly declines and reaches 65% after the tenth cycle. The operational stability of potato peroxidase is slightly lower. For instance, the efficiency of potato peroxidase in each subsequent cycle decreases approximately the same amount, about 10% and at the end of the tenth cycle the efficiency of biodegradation is approximately 35%. The reduction in the activity of immobilized enzymes during the operational stability test is mainly explained by the effect of leaching the enzyme from the carrier. Indeed, it is likely that lysine residues from unwanted proteins in the extract were the ones that participated in

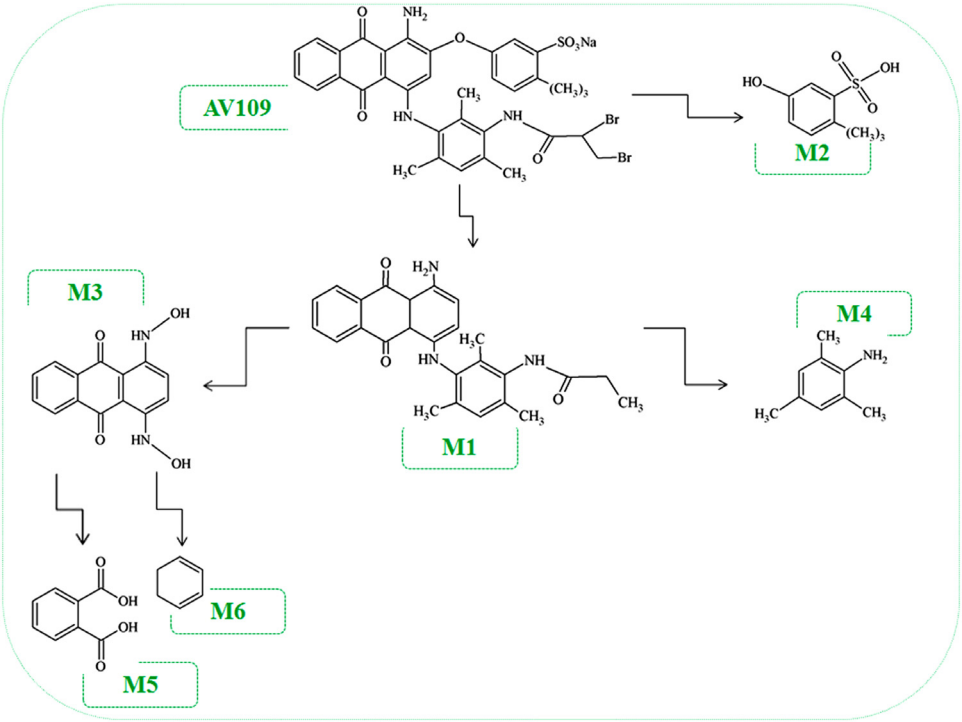
Table 1
The byproducts of AV 109 dye biodegradation identified by LC–MS and GC–MS analysis.

Molecular formula	*m/z*	Proposed structure
$C_{26}H_{27}N_3O_3$	428.84	
$C_6H_5O_4S$	254.92	
$C_{14}H_{10}N_2O_4$	293.93	
$C_9H_{11}NO_2$	135.85	
$C_8H_6O_4$	165.18	
C_6H_8	80.00	

the cross-linking and that the target peroxidase enzyme was trapped in the space. Accordingly, it is quite certain that the enzyme will be washed out of the microreactor after a certain number of cycles. Additionally, during each new reaction cycle, the microenvironment around the immobilized enzyme changes, which can cause a disturbance in the structural conformation of the enzyme and a result in a slight decrease in activity (Morsi et al., 2021). In particular, this phenomenon may explain the lower operational stability of immobilized potato peroxidase. A high dye load of 150 mg/l in each biodegradation cycle catalyzed by immobilized potato peroxidase can induce particular structural changes in the enzyme molecule, which significantly reduces its efficiency. Commercial soybean peroxidase immobilized in polyacrylamide matrix was used in six cycles of Trypan Blue degradation, wherein the entrapped soybean peroxidase removed 60% of the dye after the sixth cycle (Kalsoom et al., 2013). The soybean peroxidase from agro-industrial waste immobilized in chitosan beads using glutaraldehyde as cross-linking agent was applied for phenol removal. The immobilized enzyme showed a potential of 50% efficiency in the oxidation of caffeic acid after 4 consecutive cycles (Chagas et al., 2015). A comparison of the operational stability in this study with available literature data reveals the feasibility of an application of microreactor technology in biodegradation processes.

3.3. The biodegradation mechanism in terms of enzymatic analysis and byproduct detection

Apart from the efficiency appertaining to dye removal, of particular importance is the environmental safety of the output stream that remains after the applied biodegradation procedure. Indeed, the only way to make an assumption about the safety and quality of treated colored water is to elucidate the mechanism of enzymatically catalyzed biodegradation of



Scheme 1. The proposed biodegradation pathway of AV 109 dye catalyzed by peroxidase (potato, soybean).

anthraquinone dye AV 109. Therefore, in an attempt to comprehend the possible route of anthraquinone dye biodegradation, the byproducts were collected at different residence times and analyzed *via* LC–MS and GC–MS techniques. According to the mass spectra and *m/z* values the molecular weights and structural information of six biodegradation byproducts are given in Table 1.

The LC–MS analysis of byproducts showed the mass spectra of 6 intermediates (Fig. S2–S9). Apparently, AV 109 dye biodegradation starts with the formation of **(M1)** N-(3-((4-amino-9,10-dioxo-9,10-dihydroanthracen-1-yl)amino)-2,4,6-trimethylphenyl)propionamide (*m/z* of 428.8; retention time 11.9 min) and **(M2)** 2-(tert-butyl)-5-hydroxybenzenesulfonic acid on the retention time of 9.73 min, corresponding to a *m/z* 254.92. Later, (**M1)** is decomposed to **(M3)** 1,4-bis(hydroxyamino)anthracene-9,10-dione (retention time 9.00 min with a *m/z* 293.93) and **(M4)** 2,4,6-trimethylaniline with a *m/z* 135.85 at retention time of 5.47 min. The quinone ring cleavage of **M3** results in the formation of benzene-1,2-dicarboxylic acid **(M5)** at a retention time 13.29 min with a *m/z* 165.18 and cyclohexa-1,3-diene **(M6)** with a *m/z* of 80 at retention time of 10.95 min was confirmed by GC–MS. The benzene-1,2-dicarboxylic acid (phthalic acid) formation during peroxidase catalyzed biodegradation of dyes with an anthraquinone chromophoric group is in line with available literature data. A similar result was obtained after the identification of Acid Blue 129 biodegradation catalyzed by laccase and Reactive Blue 5 biodegradation catalyzed by DyP from *Thanatephorus cucumeris* Dec 1 (Sugano et al., 2009; Alam et al., 2021). Aside from the biodegradation of anthraquinone dyes catalyzed by class II peroxidases, phthalic acid also appears as a product of the anthraquinone ring opening during the biodegradation of anthraquinone dyes catalyzed by class III peroxidases, plant peroxidases, more precisely horseradish peroxidase (Bilal et al., 2019). Additionally, a sulfate ion was identified at a retention time of 0.75 min with a *m/z* of 218.74, and Br^- at a retention time of 0.85 min with a *m/z* of 103.87 and 202.78. Low weight biodegradation products were reported by L. Liu et al. who investigated the degradation of anthraquinone dye Alizarin Red by chloroperoxidase (Liu et al., 2014). Herein it was confirmed that the activity of peroxidases from agro-industrial waste resulted in the fragmentation of the AV 109 dye into several smaller byproducts. Moreover, the disappearance of the dye is directly related to the disruption of the chromophore structure. In accordance with the identified biodegradation products of the AV 109 dye catalyzed by peroxidases originating from soybean and potato processing industries the biodegradation pathway is given in Scheme 1.

3.4. COD

After examining the efficiency of biodegradation and determining the degradation products, it is necessary to check the toxicity of the resulting solution. Accordingly, the values of chemical oxygen demand in the dye sample before biodegradation and after biodegradation with both, peroxidase from soybean hulls and potatoes were measured. The

results clearly indicate an improvement in water quality after the treatment, more precisely a reduction in chemical oxygen demand. Namely, in the sample before biodegradation catalyzed by peroxidase from potato the COD value of 1852 mg O_2/l was measured, while the COD value in the sample after the treatment was reduced to 362 O_2/l. Furthermore, in the sample before biodegradation catalyzed by soybean hulls peroxidase the COD value of 653 mg O_2/l was measured, while the COD value in the sample after the treatment was reduced to 92 mg O_2/l. Apparently, this enzymatic process in the microreactor can be considered for use in the treatment of colored wastewater because effluents with reduced toxicity are obtained.

4. Conclusion

In this study, the biodegradation of anthraquinone dye in continuous flow by peroxidase was performed. The low-cost agro-industrial materials used as a source of the enzyme, together with the simple one-step extraction elevates the potential use of enzymes at an industrial scale. Furthermore, the implementation of microreactors for dye biodegradation proved to be a feasible technology which provided a high biodegradation efficiency at a short residence time. Soybean hull peroxidase achieved a biodegradation efficiency of 95% at a residence time of 3 min, while the biodegradation efficiency of 76% was completed in 3 min of residence time with potato peel peroxidase. Moreover, the smaller reactor's diameter contributed to better mixing and an enhanced contact among reagents. Successful cross-linking of soybean hulls and potato peel peroxidase with oxidized pectin on the inner wall of the microchannel was achieved and confirmed via SEM analysis. After 10 cycles of reuse soybean peroxidase retained 65% of its initial activity, while potato peroxidase retained only 35%. With the LC– MS and GC–MS analysis 6 biodegradation products were identified, giving an increase in the biodegradability of the dye. Together with the high operational stability of the immobilized enzyme microreactor, this is a promising design of a greener and cleaner process for the degradation of synthetic dyes with great potential for scaling up.

CRediT authorship contribution statement

Milica Svetozarević: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Nataša Šekuljica:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision. **Antonije Onjia:** Methodology. **Nemanja Barać:** Investigation. **Marina Mihajlović:** Validation, Formal analysis. **Zorica Knežević-Jugović:** Writing – review & editing, Supervision. **Dušan Mijin:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2022.102373.

References

Alam, R., Ardiati, F.C., Solihat, N.N., Alam, M.B., Lee, S.H., Yanto, D.H.Y., Watanabe, T., Kim, S., 2021. Biodegradation and metabolic pathway of anthraquinone dyes by Trametes hirsuta D7 immobilized in light expanded clay aggregate and cytotoxicity assessment. J. Hazard. Mater. 405, 124176. http://dx.doi.org/10.1016/j.jhazmat.2020.124176.

Bilal, M., Rasheed, T., Zhao, Y., Iqbal, H.M.N., 2019. Agarose-Chitosan hydrogel-immobilized horseradish peroxidase with sustainable bio-catalytic and dye degradation properties. Inter. J. Biol. Macromol. 124, 742–749. http://dx.doi.org/10.1016/j.ijbiomac.2018.11.220.

Cao, Q., Sang, L., Tu, J., Xiao, Y., Liu, N., Wu, L., Zhang, J., 2021. Rapid degradation of refractory organic pollutants by contionus ozonation in a micropacked bed reactor. Chemosphere 270, 128621. http://dx.doi.org/10.1016/j.chemosphere.2020.128621.

Chagas, P.M.B., Torres, J.A., Silva, M.C., Corrêa, A.D., 2015. Immobilized soybean hull peroxidase for the oxidation of phenolic compounds in coffee processing wastewater. Int. J. Biol. Macromol. 81, 568–575. http://dx.doi.org/10.1016/j.ijbiomac.2015.08.061.

Costas, L., Bosio, V.E., Pandey, A., Castro, G.R., 2008. Effects of organic solvents on immobilized lipase in pectin microspheres. Appl. Biochem. Biotechnol. 151, 578–586. http://dx.doi.org/10.1007/s12010-008-8233-0.

Darwesh, O., Ali, S., Matter, I., Elsamahy, T., Mahmoud, Y., 2019. Enzymes immobilization onto magnetic nanoparticles to improve industrial and environmental applications. Methods Enzymol. 630, 481–502.

Gupta, B., Tummalapalli, M., Sarwar Alam, M., 2013. Functionalization of pectin by periodate oxidation. Carbohydr. Polymers 98, 1160–1165. http://dx.doi.org/10.1016/j.carbpol.2013.06.069.

Hartreee, H.F., 1972. Determination of protein: A modification of the lowry method that gives a linear photometric response. Anal. Biochem. 48, 422–427. http://dx.doi.org/10.1016/0003-2697(72)90094-2.
Honda, T., Miyazaki, M., Nakamura, H., Maeda, H., 2005. Immobilization of enzymes on a microchanel surface through cross-linking polimerization. Chem. Commun. 40, 5062–5064. http://dx.doi.org/10.1039/b510605b.
Hosseini, S.S., Khodaiyan, F., Mousavi, S.M.E., Azimi, S.Z., Gharaghani, M., 2020. Immobilization of pectinase on the glass bead using polyaldehyde kefiran as a new safe cross-linker and its effect on the activity and kinetic parameters. Food Chem. 309, 125777. http://dx.doi.org/10.1016/j.foodchem.2019.125777.
Kalsoom, U., Ashraf, S.S., Meetani, M.A., Rauf, M.A., Bhatti, H.N., 2013. Mechanistic study of a diazo dye degradation by soybean peroxidase. Chem. Cent. J. 7, 93. http://dx.doi.org/10.1186/1752-153x-7-93.
Kurnik, K., Treder, K., Skorupa-Klaput, M., Tretyn, A., Tyburski, J., 2015. Removal of phenol from synthetic and industrial wastewater by potato pulp peroxidases. Water Air Soil Pollut. 226, 254. http://dx.doi.org/10.1007/s11270-015-2517-0.
Li, H., Yuan, Z., Shang, X., Shang, H., Liu, Darwesh O Li C Fang J, 2020. Application of gradient acid fractionation protocol to improve decolorization technology by lignin-based adsorbent. Int. J. Biol. Macromol. 172, 10–18. http://dx.doi.org/10.1016/j.ijbiomac.2020.12.206.
Liu, L., Zhang, J., Tan, Y., Jiang, Y., Hu, M., Li, S., Zhai, Q., 2014. Rapid decolorization of anthraquinone and triphenylmethane dye using chloroperoxidase: Catalytic mechanism, analysis of products and degradation route. Chem. Eng. J. 244, 9–18. http://dx.doi.org/10.1016/j.cej.2014.01.063.
Lloret, L., Eibes, G., Moreira, M.T., Feijoo, G., Lema, J.M., Miyazaki, M., 2013. Improving the catalytic performance of laccase using a novel continous-flow microreactor. Chem. Eng. J. 223, 497–506. http://dx.doi.org/10.1016/j.cej.2013.03.018.
Luan, J., Zhuang, Y., Shen, Y., 2017. The decolorization and removal of methyl orange with sweet potato peroxidase as biocatalyst and the toxicological assessment of its degradation products. J. Biotech. 256S, S44–S116. http://dx.doi.org/10.1016/j.jbiotec.2017.06.975.
Marchisa, T., Gerrato, G., Magnacca, G., Crocellà, V., Laurenti, E., 2012. Immobilization of soybean peroxidase on aminopropyl glass beads: Structural and kinetic studies. Biochem. Eng. J. 67, 28–34. http://dx.doi.org/10.1016/j.bej.2012.05.002.
Mathkor, T.H., Hasan, H.R., Daham, Z.M., 2019. Screening and extraction of peroxidase enzyme from diffeent plant wastes. Biochem. Cell. Arch. 19, 2739–2746. http://dx.doi.org/10.35124/bca.2019.19.S1.2739.
Matto, M., Husain, Q., 2006. Entrapment of porous and stable concanavalin A-peroxidase complex into hybrid calcium alginate-pectin gel. J. Chem. Technol. Biotechnol. 81, 1316–1323. http://dx.doi.org/10.1002/jctb.1540.
Morsi, R., Al-Maqd, K.A., Bilal, M., Iqbal, H.M.N., Khaleel, A., Shah, I., Ashraf, S.S., 2021. Immobilized soybean peroxidase hybrid biocatalysts for efficient degradation of various emerging pollutants. Biomolecules 11, 904. http://dx.doi.org/10.3390/biom11060904.
Sugano, Y., Matsushima, Y., Tsuchiya, K., Aoki, H., Hirai, M., Shoda, M., 2009. Degradation pathway of an anthraquinone dye catalyzed by a unique peroxidase DyP from Thanatephorus cucumeris Dec 1. Biodegradation 20, 433–440. http://dx.doi.org/10.1007/s10532-008-9234-y.
Svetozarević, M., Šekuljica, N., Knežević-Jugović, D., 2021. Agricultural waste as a source of peroxidase for wastewater treatment: Insight in kinetics and process parameters optimization for anthraquinone dye removal. Environ. Technol. Innov. 21, 101289. http://dx.doi.org/10.1016/j.eti.2020.101289.
Talekar, S., Nadar, S., Joshi, A., Joshi, G., 2014. Pectin cross-linked enzyme aggregates (pectin-CLEAs) of glucoamylase. RSC Adv. 4, 59444–59453. http://dx.doi.org/10.1039/C4RA09552A.
Šalić, A., Zelić, B., 2018. Synergy of microtechnology and biotechnology: Microreactors as an effective tool for biotransformation processes. Food. Technol. Biotechnol. 56, 464–479. http://dx.doi.org/10.17113/ftb.56.04.18.5673.
Šekuljica, N., Prlainović, N., Jakovetić, S., Grbavčić, S., Ognjanović, N., Knežević-Jugović, Z., Mijin, D., 2016. Removal of anthraquinone dye by cross-linked enzyme aggregates from fresh horseradish extract. Clean - Soil Air Water 44, 891–900. http://dx.doi.org/10.1002/clen.201500766.
Zhang, H., Bai, Y., Zhu, N., Xu, J., 2021. Microfluidic reactor with immobilized enzyme- from construction to applications: A review. Chin. J. Chem. Eng. 30, 136–145. http://dx.doi.org/10.1016/j.cjche.2020.12.011.
Zhu, Y., Chen, Q., Shao, L., Jia, Y., Zhang, X., 2020. Microfluidic immobilized enzyme reactors for contionus biocatalysis. React. Chem. Eng. 5, 9–32. http://dx.doi.org/10.1039/C9RE00217K.