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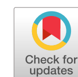
Agricultural waste as a source of peroxidase for wastewater treatment: Insight in kinetics and process parameters optimization for anthraquinone dye removal



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A B S T R A C T

The number of different technologies available for colored wastewater treatment shows the vastness of the issue. Finding the most feasible one, yet taking into account the cost, the efficiency and eco-friendliness is like searching needle in a haystack. Here, we have taken the eco-friendliness to a level above, using one bio-waste material for treatment of another waste. Potato peel and soybean hull are agroindustrial waste that is at the same time abundant source of potent enzymes such as peroxidases that can be used in oxidation reactions. In this study, the anthraquinone dye Acid Violet 109 is used as a model for simulation of colored wastewater and for oxidation by peroxidase from two bio-waste sources: potato peel and soybean hull. The operational conditions were optimized and a kinetic study was performed in order to obtain detailed information about the behavior of the enzymes when faced with extreme substrate concentration. Under the optimal conditions, $72.78 \pm 3.13\%$ and $66.12 \pm 2.51\%$ biodegradation was achieved with potato peel peroxidase, and soybean hull, respectively. As most of the industrial wastewater treatments require higher temperatures, both soybean hull and potato peel peroxidase were subjected to and proved to be able to operate at significantly higher temperatures up to 70°C.

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1. Introduction

The industrial expansion has contributed to economic development; nonetheless it is the ground cause of environmental pollution. Cumulative water pollution affects climate change, extinction of species, poor quality of living etc. It is a global issue that affects, in a great manner, the environmental welfare.

Textile dyeing process as a source of water pollution is energy and water consuming process while 5%–40% of the dyes used are released in the wastewater (da Silva et al., 2010a). Dyes' persistence to light and temperature together with their complex aromatic structure makes them difficult for degradation by conventional wastewater treatment. Dyes in wastewater are aggravating photosynthesis owing to the fact that they are disabling the adsorption of sunrays in surface water and thus shifting the ecosystem balance. Releasing of colored effluents into rivers and oceans has a negative effect on the surrounding flora and fauna. Suppression of growth, development and reproduction of aquatic biota are

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the main consequences caused by these pollutants if present in water. Not only are colored effluents susceptible to bioaccumulation, but they also possess carcinogenic and mutagenic properties that compromise the human health and the environment (Mathur et al., 2005). Colored constituents of textile effluents may be azo, anthraquinone, nitro, methane and other dyes. Second major dye class are anthraquinone dyes that are mostly used for wool, polyamide and silk dyeing. Even though anthraquinone dyes have lower color intensity and narrow range of different hues, their application is popular because of their color fastness and brilliance. Feasible optical properties and thermal stability add up to their frequent use. Nonetheless, their toxicological profiles state that most of anthraquinone dyes are mutagenic, carcinogenic and allergenic (Environmental Control, Inc., 1981). These unfavorable properties have led to the development of different colored effluents' treatments classified as physical, chemical and biological. Trending method of treatment is advanced oxidation process due to its fast reaction rates and small quantity of reactants. On the other hand, it is still cost-ineffective process that has to be specifically optimized for particular effluent (Krishnan et al., 2017). Biological methods where plenty of microorganisms can be used, are considered to be the most efficient for wastewater treatment (Busca et al., 2008). While their main advantage is the low operational cost of the process, there are few parameters such as dye concentration, pH and temperature of the effluent that significantly affect the decolorization process (Cheremisinoff, 2002). In addition, the entire microorganism-catalyzed process is controlled and dependent on microbial growth. Enzymatic wastewater treatment takes over the microbial methods on the grounds of shorter treatment duration, the absence of lag-phase, ability of decolorizing effluents with higher dye concentration, simplicity of process management and reduction in sludge volume (Karam and Nicell, 1997).

Peroxidase is an oxidoreductase that uses hydrogen peroxide as an electron acceptor for catalysis of oxidative reactions. In order to abet the process eco-friendliness and design it in accordance to principles of circular economy, the application of peroxidase isolated from bio-waste material is highlighted. Peroxidases originating from the plant material have strong potential utility for bioremediation of different pollutants from wastewaters. Horseradish peroxidase is the most studied enzyme derived from plant material, which is used as a very effective biocatalyst in the treatment of various recalcitrant pollutants (i.e. dyes, phenols, etc.) present in wastewater (Shen et al., 2019; Ahirwar et al., 2017; Wagner and Nicell, 2005; Ely et al., 2017; Šekuljica et al., 2016). Šekuljica et al. (2015) observed the oxidation of two anthraquinone dyes using commercial horseradish peroxidase, while Silva et al. (2012) extracted peroxidase from turnip for decolorization of a reactive dye. Different plants have been selected as a source of enzymes that are later used for decolorization of textile effluents (Sarkar et al., 2017; Arabaci and Usluoglu, 2014; Bettin et al., 2019; Villegas et al., 2018). The use of enzymes from waste materials that have not undergone any purification process is the goal of the researcher when it comes to wastewater treatment. Also, the minced parts of the waste material as a source of peroxidases are seen as natural immobilized enzyme systems that are much easier to manipulate and the efficiency is slightly different compared to the crude extract or purified stock solution of the target enzyme. Potato pulp use, as well as use of soybean flakes, soybeans, and bananas in the treatment of contaminated wastewater is in the ascending phase of research Kurnik et al. (2015), Dahiru et al. (2018), Jadhav et al. (2013), Chagas et al. (2015), Wright and Nicell (1999). Treating the industrial solid waste as a material for extraction of enzymes, and using the extract from what once was waste, for treatment of another waste fits perfectly with the design of sustainable development. According to our knowledge, the available literature for potato peel use is limited to the removal of azo dyes by adsorption (Hoseinzadeh et al., 2014). The enzyme itself has not been used for dye degradation. There are studies dealing with azo dyes removal (Villegas et al., 2018) and benzidines (Altahir et al., 2015) using soybean hull peroxidase, but not with anthraquinone dyes' removal.

The aim of this study is to evaluate of peroxidase isolation from waste plant material: potato peel and soybean hull. Afterwards, the peroxidase isolated from these sources were applied in decolorization reactions, where anthraquinone dye was used as a model. The enzyme efficiency in the observed reaction was brought to the highest level by varying the decolorization parameters: contact time, pH, enzyme, hydrogen peroxide and dye concentration. Taking into account the conditions prevailing in the polluted wastewater, such as high temperatures, the isolated peroxidases were examined from the aspect of temperature optimum and compared with each other. In addition, differences in the efficiency of isolated peroxidases are directly related to the kinetic characteristics of the given enzymes obtained by inspecting the initial kinetics and modeling the experimental data by the corresponding kinetics of bisubstrate reactions.

2. Materials and methods

2.1. Materials and reagents

Soybean hull and potato peel were obtained from Sojaprotein and Chips Way factories. The anthraquinone dye C.I. Acid Violet 109 was purchased from DCC colorants (Ningbo, 315199 China). Hydrogen peroxide and pirogallol were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals and solvents were of the highest commercial grades purchased from Merck (Darmstadt, Germany) and Lach-Ner (Bratislava, Czech Republic).

2.2. Peroxidase extraction from plant material and preparation of crude enzyme source

Extraction procedures were carried out according different methods available in the literature with slight modifications. The potato peel was soaked in buffer solution (phosphate buffer, 100 mM, pH 6) 1:4, homogenized and left overnight at

4 °C. Next, the extract was filtered through gauze, heated at 65 °C in a 3 min, and cooled in an ice bath. The crude enzyme extract was centrifuged at 10^4 rpm, 15 min. The supernatant was collected for enzyme activity determination and stored at −18 °C (Idesa and Getachew, 2018).

The peroxidase extraction from soybean hull was performed as follows: the hull were homogenized for 10 min with distilled water 1:4. Then, the extract was filtered through gauze, heated at 65 °C in a 3 min, in order to inactivate catalase, and cooled in an ice bath. The crude enzyme extract was centrifuged (Heraeus™ Fresco™ 17 Microcentrifuge, Thermo Scientific, Waltham, USA) at 10^4 rpm 15 min. The supernatant was collected for enzyme activity determination and stored at −18 °C (Ghaemmaghami and Alemzadeh, 2010; Parsiavash et al., 2015).

2.3. Peroxidase activity assay

The peroxidase activity was determined spectrophotometrically using pyrogallol as a standard substrate. The absorbance increase at 420 nm is noted for a solution of 0.013 M pyrogallol, 3% v/v hydrogen peroxide and 10 μl of enzyme solution. One unit of activity is defined as the amount of peroxidase that will form 1 mg of purpurogallin from pyrogallol under the assay conditions.

2.4. Biodegradation assays of AV 109 dye

The process parameters that affect the degree of peroxidase-catalyzed dye biodegradation are: pH of the reaction mixture, dye concentration, hydrogen peroxide concentration, enzyme concentration and temperature. All experiments were performed in a batch reactor in a 100 ml beaker with constant stirring (Thermo Scientific Cimarec™ Multipoint 6 Magnetic stirrer, Waltham, United States), 150 rpm. Optimal pH value was determined by monitoring the biodegradation rate within pH 3–9. Citrate buffer was used for pH 3.0, acetate buffer for pH 4–5, phosphate buffer for pH 6–8, and Tris-buffer for pH 9, all of them with concentration of 50 mM. Enzyme concentration influence on the couple of enzymes was varied from 0.1–1 U in order to find the optimal one for every enzyme and the biodegradation was monitored during 50 and 30 min for potato peel and soybean hull peroxidase, respectively. Under the optimal pH and enzyme concentration, the hydrogen peroxide and dye concentration effect on the biodegradation was examined. Two consecutive set of experiments were carried out: in the first ones, dye concentration was varied from 10–100 mg/L, and in the second ones, H_2O_2 concentration was varied from 0.1–1 mM, at room temperature. The efficiency and temperature influence on the biodegradation catalyzed by potato peel and soybean hull peroxidase was monitored at different temperatures: 25, 38, 50, 60 and 70 °C. The reaction mixture was placed in water baths (Memmert, Schwabach, Germany) and heated at the above mentioned temperatures under pre-determined optimal process parameters. All the reactions were carried with constant stirring and the biodegradation was followed spectrophotometrically (UV/Vis Ultrospec 3300 Pro, Amersham Bioscience, UK) and calculated as:

$$BIODEGRADATION\ (\%) = 100\left(\frac{A_0 - A_t}{A_0}\right) \tag{1}$$

where A_0 is the initial absorbance and A_t is the absorbance of medium after biodegradation at the λ_{max}(nm) of AV 109 dye, 590 nm. All experiments were performed in triplicate and relative standard deviations of triplicate measurements were less than 5%.

2.5. Storage stability of peroxidase from potato peel and soybean hull

The storage stability of peroxidase originating from potato peel and soybean hull was determined by measuring the biodegradation % during storage of crude enzymatic extract at 4 °C for 5 weeks as previously described in 2.4.

2.6. Kinetic study of AV 109 dye biodegradation

The initial rates of AV 109 oxidation process catalyzed by potato peel and soybean husk peroxidase were studied in the presence of hydrogen peroxide by measuring the change in absorbance at 590 nm. The reaction was performed at 25 °C in 50 mM acetate buffer, pH 4.0. Measurements were conducted with different concentrations of reducing substrate, AV 109 dye at a fixed hydrogen peroxide concentration and *vice versa*. Based on the kinetic cycles, the initial velocities were determined and the dependences of the initial velocities and the initial concentrations of the substrates (initial velocity *vs.* initial substrate concentration) were fitted by an appropriate mathematical model of bisubstrate reactions.

3. Results and disscusion

3.1. Process parameters optimization of AV 109 biodegradation

In this paper, enzyme was extracted from bio-waste material so as to use it for degradation of anthraquinone dye from textile wastewater. Crude enzyme extracts from potato peel and soybean hull were collected and their peroxidase activity

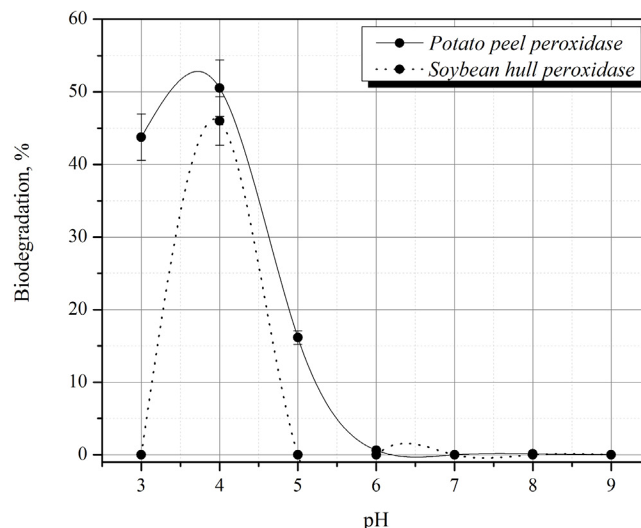

Fig. 1. The influence of the initial pH on AV109 decolorization at room temperature by peroxidase from potato peel and soybean hull, (reaction conditions: dye concentration 30 mg/L, H_2O_2 concentration 0.1 mM, enzyme concentration 0.2 U).

was determined as previously described. Highest enzyme activity expressed the soybean hull crude extract with 220 IU/mL, followed by potato peel with 1 IU/mL. The soybean hull peroxidase that was used by Chagas et al. (2015) showed activity of 31 U/mL, while the potato peel peroxidase characterized by Bharadwaj (Bharadwaj, 2019) showed 0.432 U/mL activity. Generally, in each enzymatically catalyzed biodegradation the key factors are pH, temperature, characteristics of the substrate, mixing, incubation time, as well as the concentration of dye and the enzyme as well as the addition of the mediator. Therefore, the optimum pH value of AV 109 biodegradation catalyzed by peroxidases from each bio-waste material was determined by dissolving the dye in different buffer solutions ranging pH 3–9. Under different pH conditions dye molecules change their electrical charge, resulting in easier dye removal depending on the method used (Mahmoud et al., 2007). Apart from this, enzymes also require specific pH, in which their interaction with the substrate is at highest level. The obtained results on the effect of pH on the biodegradation of AV 109 dye catalyzed by potato peel and soybean hull peroxidase are shown in Fig. 1.

It is clearly shown in Fig. 1 that the optimum pH at which the enzymes were tested, reached maximum activity at 4. The pH value or range where the enzyme can express its highest activity may have vast variation which is strongly related to the dye's ionization forms. Main variables that have significant role in determining the optimal pH are the enzymatic residues in the active site and the type of dyes (Al-Ansari et al., 2009). Altahir et al. showed this pH co-dependency on two acid azo dyes, decolorizing them with soybean peroxidase. Although the same enzyme is used, the pH optimum was drastically different for the both dyes, which demonstrates the relevance of the pH parameter (Altahir et al., 2020b). Peroxidase from potato peel showed activity in the range pH 3–5, which is not the case with peroxidase from soybean hull. Soybean hull peroxidase was active only at pH 4, with a decolorization rate of 46.00 ± 3.34%. Possible explanation can be that in soybean hull only one peroxidase isoenzyme can be found (Al-Ansari et al., 2011), while in potato peel are present more peroxidase isoforms (Bernards et al., 1990). This can be the reason why soybean hull peroxidase shows activity only at pH 4. On the one hand, it is more simple to characterize and operate a process where only one isoenzyme is present. On the other hand, when more isoenzymes are present, the process is more insusceptible to changes. While investigating the pH optimum, the reaction time was assessed. The data regarding time course of AV 109 dye degradation under the different pH are not shown, however optimal contact time 50 and 30 min for the biodegradation of AV 109 dye catalyzed by peroxidase from potato peel and soybean hull, respectively was adopted. The degree of biodegradation achieved in the potato peel and soybean hull peroxidase catalyzed reactions were 50.53 ± 3.88 and 46.00 ± 3.34%, respectively of the initial AV 109 dye concentration introduced into the system.

3.2. Effect of enzyme concentration on decolorization rate

Cost is the biggest obstacle, but finding new sources and new advances in biotechnology make enzymatic catalysis on an industrial scale a feasible alternative. Enzymes from various wastes can be obtained in the form of extracts by simple concentration methods and are often more active than purified enzyme preparations. The use of enzymes obtained from agricultural waste material emphasizes the cost-effectiveness of the biodegradation process of synthetic dyes. In order to examine the initial enzyme concentration influence on the biodegradation, the enzyme concentration was varied from 0.1 U to 1 U. The results are shown in Fig. 2. As for peroxidase from potato peel, the level of dye removal increases with

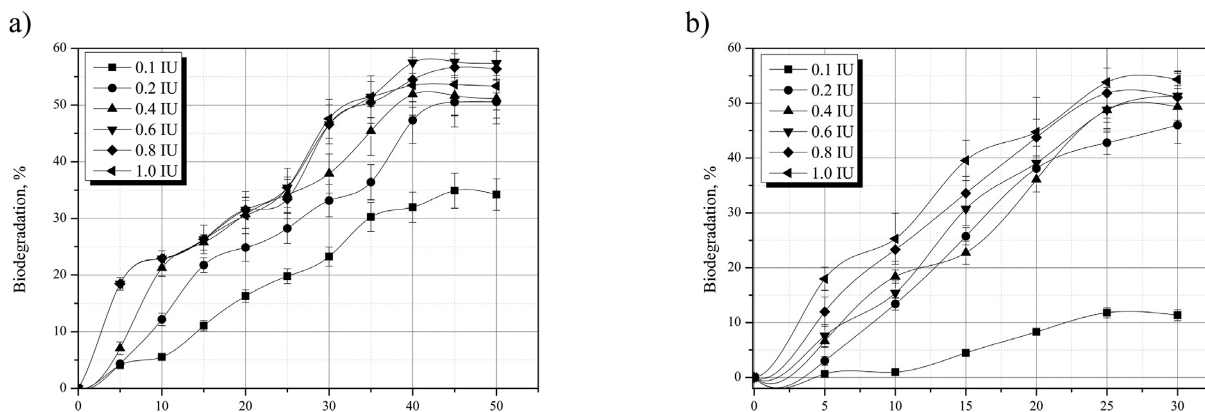

Fig. 2. The biodegradation rate of Acid Violet 109 catalyzed with (a) potato peel and (b) soybean hull peroxidase, (reaction conditions for both enzymes: dye concentration 30 mg/L, H_2O_2 concentration 0.1 mM, temperature 25 °C, pH 4, contact time: 50 min for potato peel peroxidase, 30 min for soybean hull peroxidase).

the increase of enzyme concentration up to a certain point where the enzyme concentration practically has no impact on the decolorization rate. The highest biodegradation rate was obtained at the initial potato peel peroxidase activity of 0.6 IU, 57.35 ± 2.12%. A further increase above 0.6 IU did not have a significant effect on the biodegradation of the tested dye. This can be due to the fact that higher enzyme concentration requires more H_2O_2. In the case of peroxidase from soybean hull, the decolorization rate is directly proportional to the enzyme concentration. With 1U enzyme, dye removal of 54.32 ± 1.11% was achieved. Chiong et al. used crude peroxidase extract from luffa and soybean for methyl orange dye removal (Chiong et al., 2006). Their finding for the luffa peroxidase concentration optimization can be related to the results obtained for potato peel and soybean hull peroxidase. Potato peel peroxidase uses hydrogen peroxide as an electron acceptor in order to catalyze the oxidation reaction. If only the enzyme concentration is increased, but not the H_2O_2 concentration, a probable scenario is where all H_2O_2 molecules will be used, yet not all enzyme molecules will be active due to the lack of H_2O_2. Same goes with soybean hull peroxidase, only the H_2O_2 demand for this enzyme may be lower, and at the inspected enzyme concentration range enough H_2O_2 molecules are present to activate the enzyme.

3.3. Effect of H_2O_2 and dye concentration on decolorization rate

Peroxidase uses hydrogen peroxide as an activator so it is an important parameter for optimization. Low H_2O_2 concentration can limit the reaction, but excessive amount can have inhibitory effect. The influence of H_2O_2 was assessed by varying its concentration from 0.1–1 mM for each enzyme. Decrease in the decolorization rate, caused by H_2O_2, can be observed at peroxidase from each agroindustrial waste. The optimal H_2O_2 concentration was 0.01 mM for both, potato peel and soybean hull peroxidase where the biodegradation rate of 57.35 ± 2.12 and 54.32 ± 1.11% respectively, was recorded. Chiong et al. managed to remove 81.5% of methyl orange dye with soybean hull peroxidase using 2 mM H_2O_2 (Chiong et al., 2006), which is 20 times higher concentration of hydrogen peroxide than the one needed for biodegradation of AV109 with soybean hull peroxidase, with slightly higher percentage of decolorization. Hidalgo et al. observed the oxidation of various Domalan dyes which composition is a combination of anthraquinone and azoic acid-dispersed dyes using peroxidase from lentil stubble (Hidalgo et al., 2011). In the case of Green domalan dye, optimal H_2O_2 concentration was 0.3 mM, while concentration above 1 mM had inhibitory effect. The same out-turn happens with potato peel peroxidase and soybean hull peroxidase in AV109 biodegradation. Taking in consideration that H_2O_2 must be handled with caution, and adding its cost-ineffectiveness, lower H_2O_2 concentrations are favored (see Fig. 3).

To examine the effect of dye concentration, it was varied from 10–100 mg/L and the results are shown in Fig. 4. The optimal dye concentration for peroxidase from soybean hull was 10 mg/L with 60.39 ± 2.49% of decolorization. At dye concentration of 100 mg/L, the decolorization rate dropped for 12.06 ± 1.19% and 38.13 ± 2.11% for soybean hull and potato peel peroxidase, respectively. In the case of peroxidase from potato peel, the highest decolorization rate was 65.11 ± 1.59% at substrate concentration of 40 mg/L. It is important to note that at 40 mg/L dye concentration soybean hull peroxidase managed to achieve 34.11 ± 3.33% dye removal. It is not its optimal substrate concentration, but the decolorization rate is slightly lower than in the case of the other enzyme. There are also references in the literature with commercial enzymes that are similar or quite different from the results obtained in this study. Altahir et al. used commercial crude dry solid soybean peroxidase for decolorization of two 0.5 mM azo dyes, Acid Black 2 (AB2) and Acid Orange 7 (AO7). Decolorization rate of 95% was achieved after 3 h with 1.2 U/mL enzyme activity for both dyes using 1.5 mM and 1.25 mM hydrogen peroxide for AO7 and AB2, respectively (Altahir et al., 2020a). Another group of authors, Marchis et al. Studied the oxidative degradation of Remazol Turqouise Blue G133 by commercial soybean peroxidase (Marchis et al., 2011). After 4 h, 96% dye degradation was achieved with 2.06×10^{-4} mM enzyme, 200 mg/L

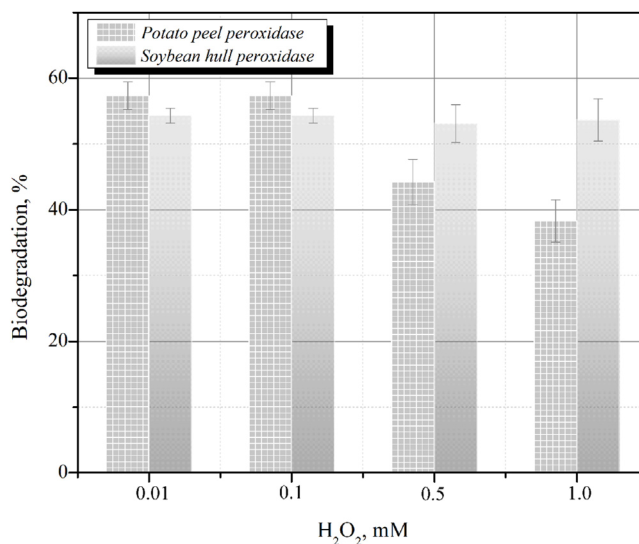

Fig. 3. The influence of hydrogen peroxide concentration on biodegradation rate of AV 109 catalyzed by potato peel and soybean hull peroxidase, (reaction conditions for both enzymes: dye concentration 30 mg/L, temperature 25 °C, pH 4; contact time: 50 min for potato peel peroxidase, 30 min for soybean hull peroxidase; enzyme concentration: 0.6 U for potato peel peroxidase, 1U for soybean hull peroxidase).

dye concentration and 0.1 mM hydrogen peroxide. Furthermore, Da Silva et al. investigated the enzymatic degradation of several dyes: Drimarene Blue X-3LR (DMBLR), Drimarene Blue X-BLN (DMBBLN), Drimarene Rubinol X-3LR (DMR), and Drimarene Blue CL-R (RBBR) by horseradish peroxidase (da Silva et al., 2010b). With 3.5 U/mL enzyme activity, 0.55 mM hydrogen peroxide and 120 mg/L dye concentration, they managed to degrade 77%–99% of the mentioned dyes. Two dyes: DMBLR and RBBR were biodegraded after only 5 min, while the others for 1h. Šekuljica et al. optimized the decolorization of two anthraquinone dyes: Acid Blue 225 (AB225) and Acid Violet 109 (AV109) using commercial horseradish peroxidase. With 0.15 IU/mL they achieved 94.7% and 89.36% decolorization of AV109 and AB225, respectively. The AV109 decolorization was done in 15 min at pH 4, 30 mg/L dye concentration and 0.4 mM hydrogen peroxide. As for AB225, the reaction time was 32 min, pH 5, 30 mg/L dye concentration and 0.04 mM hydrogen peroxide (Šekuljica et al., 2015). It can be concluded that commercially available enzymes are able to withstand higher dye concentration and in some cases the biodegradation is a lot faster. However, the biodegradation capability of the enzyme depends on the dye structure which is closely related to the reaction time (da Silva et al., 2010b). According to our knowledge, commercial enzyme preparation from potato is not available, nor published researches with commercial potato peroxidase, so comparison is not possible.

All experiments regarding process parameters optimization for AV109 dye removal catalyzed by peroxidase from potato peel and soybean hull were performed in a dye solution prepared in buffer with distilled water. However, these conditions are far from the real system, hence the efficiency of both enzymes was tested under the optimal conditions, only in artificially prepared wastewater of a complex composition. In order to elucidate the effect of wastewater on the enzymatic biodegradation, synthetic wastewater was prepared according to Yaseen and Scholz with AV 109 concentration of 40 mg/L and 10 mg/L for potato peel peroxidase and soybean hull peroxidase, respectively (Yaseen and Scholz, 2018). Other parameters such as enzyme and hydrogen peroxide concentration that were previously optimized in experiments with purified water were taken as such. The results given in Supplementary Material showed that synthetic wastewater has a negative effect on the biodegradation rate when soybean peroxidase is used. Decrease in decolorization for 20% was observed. However, the biodegradation rate of synthetic wastewater by potato peel peroxidase did not have any effect. The decolorization remained unchanged. The results lead to an interesting conclusion that soybean is greatly affected by the other constituents of the wastewater. Possible negative influence can come from the metal ions that can act as inhibitors, or that the benzoyl group could compete with the substrate dye molecules and cause inhibition. The peroxidase from potato peel appears to be more stable than the soybean hull peroxidase, and is not affected by the presence of different compounds in the synthetic wastewater. Nonetheless, soybean peroxidase was found to be active at only one pH (pH 4) indicating the existence of only one enzyme isoform. While in the case of peroxidase from potato peel it has been confirmed that it is active in the pH range 3–5 therefore, the sustained activity in artificial wastewater can be attributed to one or more of the existing forms of isoenzymes.

3.4. Temperature optimum

The optimal temperature of peroxidase was examined by monitoring the decolorization rate at different temperatures: 25–70 °C. The results are given in Fig. 5. Peroxidase from potato peel achieved 72.78 ± 3.13% dye removal at 50 °C while at

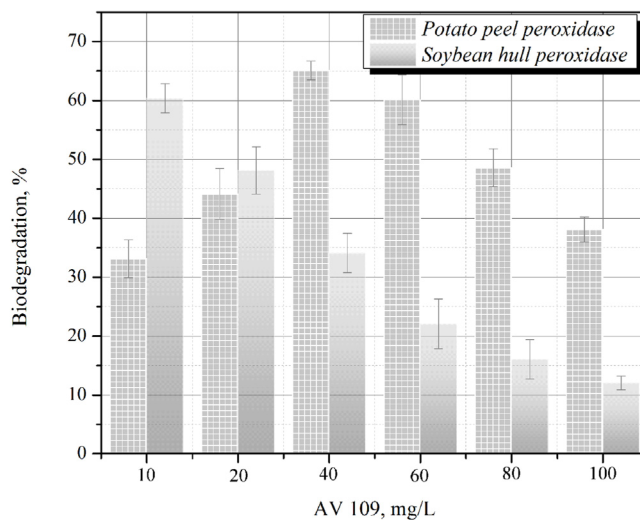

Fig. 4. The effect of the dye concentration on the dye removal catalyzed by potato peel and soybean hull peroxidase, (reaction conditions: temperature 25 °C and pH 4 for both enzymes; contact time: 50 min for potato peel peroxidase, 30 min for soybean hull peroxidase, H_2O_2 0.01 mM; enzyme concentration: 0.6 U for potato peel peroxidase, 1U for soybean hull peroxidase).

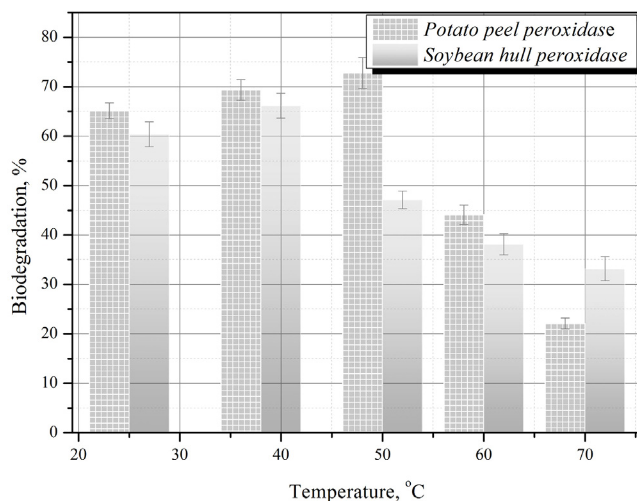

Fig. 5. The influence of temperature on the AV 109 biodegradation catalyzed with peroxidase from potato peel (reaction conditions: pH 4; contact time: 50 min; enzyme concentration: 0.6 U; 0.01 mM H_2O_2; 40 mg/L dye concentration) and peroxidase from soybean hull (reaction conditions: pH 4; contact time: 30 min; enzyme concentration 1U; 0.01 mM H_2O_2; 10 mg/L dye concentration).

60 °C the decolorization rate decreased to 44.12 ± 1.98%. Soybean hull peroxidase was stable at all temperatures, showing the highest decolorization rate of 66.12 ± 2.51% at 38 °C. Among the vigorous operational conditions required for effluents' treatment is high temperature. This parameter can reach value up to 65 °C, so treatments effective at high temperatures are of interest (Dey and Islam, 2015; dos Santos et al., 2007; Zaharia et al., 2012). At 60 and 70 °C, 38.13 ± 2.12 and 33.17 ± 2.45%, respectively dye was removed, leading to a conclusion that if higher temperature is needed for any process where peroxidase is the working enzyme of choice, the recommendation is to use peroxidase from soybean hull. Chagas et al. also concluded that at 50 °C and 60 °C, the soybean hull peroxidase retains its activity (Chagas et al., 2015).

The optimal parameters for AV109 biodegradation are given in summary in Table 1.

3.5. Storage stability of peroxidase from potato peel and soybean hull

Industrial application requires extremely stable and efficient enzymes. Accordingly, we examined the stability of peroxidases originating from potato peel and soybean hull stored for 5 weeks at 4 °C and their biodegradation potential of model anthraquinone dye was measured. The obtained results are given in Fig. 6. As presented in Fig. 6 the potato peel

Table 1
Optimal process parameters for maximum AV109 biodegradation.

Process parameters	*Potato peel peroxidase*	*Soybean hull peroxidase*
pH	4	4
Time, min	50	30
Enzyme dose, IU	0.6	1.0
H_2O_2, mM	0.01	0.01
AV109, mg/L	40	10
Temperature, °C	50	38
BIODEGRADATION, %	72.78 ± 3.13	66.12 ± 2.51

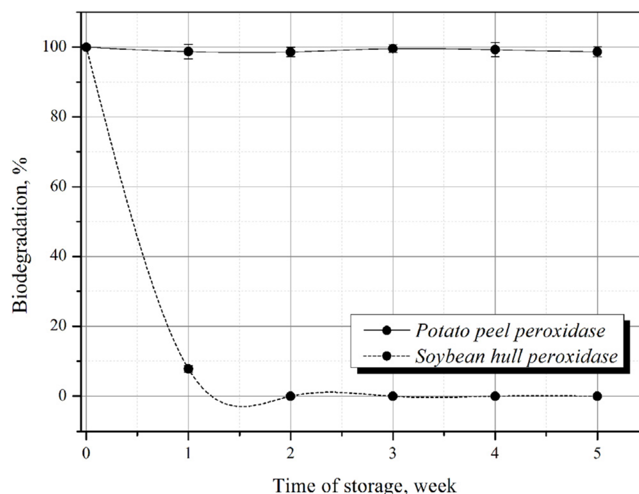

Fig. 6. Storage stability of potato peel and soybean hull peroxidase.

peroxidase activity was almost changeless during 5 weeks of storage at 4 °C. Only a slight decrease in activity, which is reflected in the reduction of biodegradation by 2% was observed after storage under the given conditions, indicating that the enzyme in this form has the potential for use in large-scale processes, i.e. at the industrial level. It is apparent from Fig. 6 that soybean hull peroxidase behaves completely differently during storage. Namely, a sharp decline in activity is recorded after 1 week of storage, which is confirmed by the finding that a biodegradation rate of 7.81 ± 1.09% was achieved, while after a 2 weeks this enzyme completely loses activity. These observations clearly indicate that potato peel peroxidase is remarkably stable on storage than soybean hull peroxidase.

3.6. Kinetic model fitting

The transition from the laboratory level of peroxidase application in the treatment of colored wastewater to the industrial level is possible only if the kinetics of the observed reaction are fully elucidated. Although data on the initial kinetics of biodegradation of synthetic dyes are scarce in the literature, it has been suggested by the Šekuljica and co-workers that the peroxidase catalyzed AV 109 dye biodegradation comports in the form of bisubstrate Ping-Pong Bi–Bi reactions (Šekuljica et al., 2015). Accordingly, the initial kinetics of the degradation reaction were examined and the experimental data obtained were modeled with the corresponding mathematical models shown by Eqs. (2)–(4). Peroxidase catalyzes oxidation reactions using H_2O_2 as an electron acceptor. As it involves two substrates, it abides the Ping-Pong Bi–Bi mechanism. Briefly, the peroxidase binds H_2O_2 first so that the enzyme is transformed to its oxidized form, and then it can be reduced by the second substrate, in this case the dye, which is at the same time being oxidized. Due to the consecutive binding of the substrates it is referred to as an ordered mechanism. When the enzyme is oxidized by H_2O_2, an intermediate is formed (EI, compound I) with H_2O release. EI oxidizes the second substrate and shifts to another intermediate state — EII, compound II. EII is reduced to its primary state E by second substrate molecule (Yaseen and Scholz, 2018).

Mathematically, Ping-Pong mechanism without inhibition can be inscribed:

$$v_0 = \frac{\vartheta_{max}\,[H_2O_2]_0\,[Dye]_0}{K_m^{Dye}\,[H_2O_2]_0 + K_m^{H_2O_2}\,[Dye]_0 + [H_2O_2]_0\,[Dye]_0} \quad (2)$$

Table 2
Kinetic parameters attained by modeling of experimental data with Ping-Pong Bi–Bi mechanism using equations describing hydrogen peroxide and dye inhibition model.

H_2O_2 inhibition					
	v_{max}	K_m^{Dye}	$K_m^{H_2O_2}$	$K_I^{H_2O_2}$	R^2
Potato peel peroxidase	1.326	2.553×10^{-6}	7.52×10^{-5}	0.03095	0.9918
Soybean hull peroxidase	0.9881	0.2531	0.2276	/	0.9845
Dye inhibition					
	v_{max}	K_m^{Dye}	$K_m^{H_2O_2}$	K_I^{Dye}	R^2
Potato peel peroxidase	2.573	2.365×10^{-4}	3.652×10^{-5}	1.034×10^{-5}	0.9901
Soybean hull peroxidase	4.311	3.223×10^{-4}	3.691×10^{-6}	5.488×10^{-6}	0.9881

Ping-Pong mechanism with H_2O_2 inhibition:

$$v_0 = \frac{\vartheta max\,[H_2O_2]_0\,[Dye]_0}{K_m^{Dye}\,[H_2O_2]_0\left(1+\frac{[H_2O_2]_0}{K_I^{H_2O_2}}\right)+K_m^{H_2O_2}\,[Dye]_0+[H_2O_2]_0\,[Dye]_0} \tag{3}$$

and Ping-Pong mechanism with dye inhibition:

$$v_0 = \frac{\vartheta_{max}\,[H_2O_2]_0\,[Dye]_0}{K_m^{Dye}\,[H_2O_2]_0+K_m^{H_2O_2}\,[Dye]_0\left(1+\frac{[Dye]_0}{K_I^{Dye}}\right)+[H_2O_2]_0\,[Dye]_0} \tag{4}$$

where v_0-initial rate of the reaction, v_{max}- maximum rate of the reaction, $[H_2O_2]_0$- initial hydrogen peroxide concentration, $[Dye]_0$- initial dye concentration,
$K_m^{H_2O_2}$–Michaelis constant for H_2O_2,
K_m^{Dye}-Michaelis constant for dye,
$K_I^{H_2O_2}$ and
K_I^{Dye} inhibition constants for hydrogen peroxide and dye, respectively.

The kinetic assay was conducted by varying the H_2O_2 concentration and keeping the other parameters constant with the obtained optimal values, and by varying the dye concentration in another set of experiments. Fig. 6a clearly shows the completely different effect of hydrogen peroxide on peroxidase from potato peel and soybean hull. Specifically, the experimental values of the influence of hydrogen peroxide on the biodegradation of AV 109 dye catalyzed by peroxidase from the potato peel show good agreement with the kinetic model of Ping-Pong Bi–Bi bi reaction with substrate inhibition (Table 2). In addition, model without inhibition proved to be the best ($R^2 = 0.9845$) when it comes to the effect of hydrogen peroxide on the peroxidase from soybean hull in the observed reaction. The foregoing is best confirmed by the values of the Michaelis and inhibition constants obtained from the assumed mathematical model. Namely, the Michaelis constant for the potato peel and soybean hull peroxidase were 7.52×10^{-5} and 0.2276 mM, respectively indicating a significantly higher affinity of soybean hull peroxidase towards hydrogen than potato peel peroxidase. The lower affinity of potato peel peroxidase is explained by inhibition presence, which is confirmed by the value of the inhibition constant given in Table 2, 0.03905 mM. Apparently, soybean hull peroxidase compared to potato peel peroxidase is more liable to H_2O_2 concentration. Unlike hydrogen peroxide, the dye is shown to be a potent inhibitor of both, potato peel and soybean hull peroxidase. Notwithstanding the dye is an inhibitor of both peroxidases, the strength of inhibition is not equal as evidenced by the values of inhibition constants. Particularly, the dye inhibition constants for potato peel and soybean hull peroxidase were found to be 1.034×10^{-5} and 5.488×10^{-6} mM, respectively. Thus, it was confirmed that potato peel peroxidase had a higher affinity for AV 109 dye (optimized dye concentration was 40 mg/L) than soybean hull peroxidase (optimized dye concentration was 10 mg/L). If we compare the results with the available literature data, it can be seen that the tested peroxidases are more sensitive to the effect of AV109 than the commercial horseradish peroxidase (Šekuljica et al., 2015). However, this is not the case when hydrogen peroxide inhibition is at stake. Soybean hull peroxidase has been shown to be more stable than commercial horseradish peroxidase in terms of the influence of hydrogen peroxide. On the other hand, in the AV 109 biodegradation catalyzed with potato peel peroxidase, increased concentrations of both substrates the dye and hydrogen peroxide lead to the formation of non-productive or dead-end complex (see Fig. 7).

4. Conclusion

The experimental data from this study can be of a great use when designing a wastewater treatment process. The chosen sources of peroxidase add up to the eco-friendliness of the process from the start, as bio-waste from one industry can further be used in another manufactory for their waste treatment. Depending on the available operating means

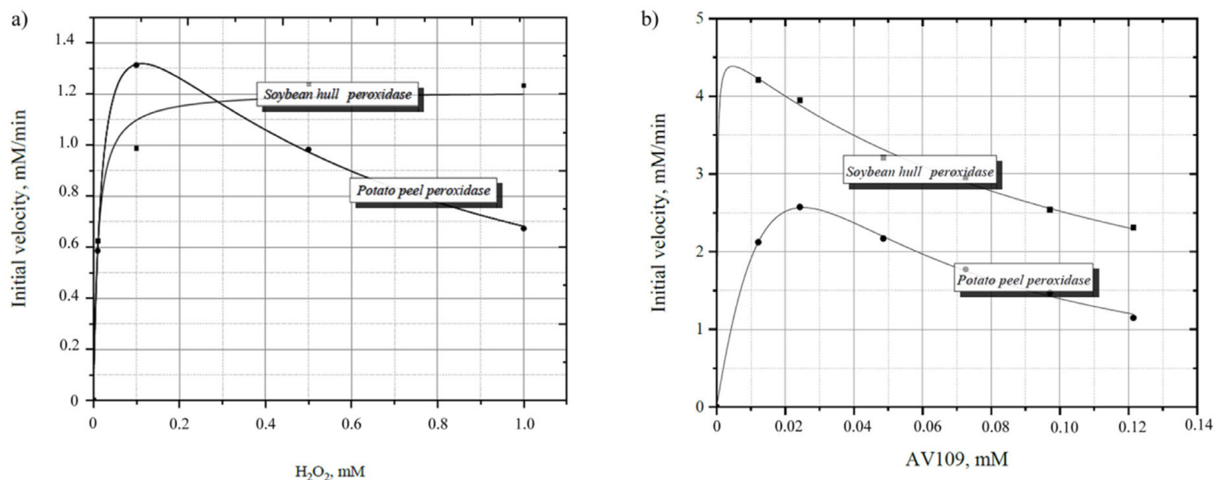

Fig. 7. (**a**) The influence of hydrogen peroxide concentration on the initial rate of the reaction under the constant dye concentration for potato peel peroxidase 0.0485 mM and soybean hull peroxidase 0.0121 mM. The curves are fitted according the experimental data and the kinetic model for inhibition with hydrogen peroxide and (**b**) The influence of dye concentration on the initial rate of the reaction under the constant hydrogen peroxide concentration, 0.01 mM for both soybean hull and potato peel peroxidase.

and material goods, different enzyme source can be selected, according to the performances needed. When potato peel peroxidase was used 92% decolorization was achieved. On the other hand, soybean hull peroxidase removed 88% of dye. Given the toxicity of concentrated H_2O_2, it is crucial to note that optimal H_2O_2 concentration for potato peel is five times more than the one needed for soybean hull peroxidase. The soybean hull peroxidase showed the highest temperature resistance. When it comes to dye concentration, soybean hull will be the least affected with the dye load. Potato peel peroxidase was susceptible to increase of dye concentration, but this peroxidase showed least variations in all parameters examined. On the whole, every enzyme source has its own benefits, but further studies should lead to more efficient biodegradation and maximal utilization of such sources.

CRediT authorship contribution statement

Milica Svetozarević: Investigation, Writing - original draft, Formal analysis. **Nataša Šekuljica:** Conceptualization, Methodology, Writing - review & editing. **Zorica Knežević-Jugović:** Supervision. **Dušan Mijin:** Supervision, Writing - review & editing.

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.2020.101289.

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