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KONTINUALNI SISTEM ZA OBEZBOJAVANJE OTPADNIH VODA. PRIMENA UMREŽENE PEROKSIDAZE IZ POLJOPRIVREDNOG OTPADA U UKLANJANJU BOJE

FLOW IN THE TUBE: CROSSLINKING OF PEROXIDASE FROM AGRICULTURAL WASTE FOR DYE REMOVAL IN MICROTUBULAR REACTOR

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Poljoprivredni otpad predstavlja lako dostupan prirodni izvor ugljenika, koji se može koristiti za dobijanje proizvoda sa dodatom vrednošću. Poljoprivredni otpad se može koristiti kao sirovina za proizvodnju električne energije, biogorivo, biogas i proizvodnju biođubriva. Dalja primena poljoprivrednog otpada može biti kao supstrat za fermentaciju u čvrstom stanju (eng. solid state fermentation), za proizvodnju antibiotika, enzima i fitohemikalija. Izolovanje enzima iz otpadnog materijala kao sirovi ekstrakt doprinosi konceptu održivosti i značajno smanjuje cenu enzima. Enzim od interesa u ovom radu je peroksidaza izolovana iz sojinih ljuspica. Sirova peroksidaza iz sojinih ljuspica umrežena je oksidovanim pektinom na unutrašnje zidove PTFE cevnog mikroreaktor, i ispitivan je uticaj koncentracije umreživača na aktivnost enzima i efikasnost umrežavanja, kao i uticaj protoka reagensa na aktivnost enzima i efikasnost umrežavanja. Nakon toga, vršeno je ispitivanje uticaja prečnika cevnog mikroreaktora na efikasnost umrežavanja i aktivnost peroksidaze: 0,5 i 0,8 mm. Nakon imobilizacije ispitana je mogućnost uklanjanja antrahinonske boje umreženom peroksidazom u cevnom mikroreaktoru. U ovom radu je takođe je ispitivana mogućnost ponovne upotrebe imobilisanog enzima.

Ključne reči: *mikroreaktor; peroksidaza iz otpadnog materijala; čistija proizvodnja; C.I. Acid Violet 109; imobilizacija*

Agro-industrial waste has gained special attention as an abundatly available natural carbon source, which can be used for value-added products such as generating power, biofuel, biogas and biofertilizers production. Further application of agro-industrial waste may be as a substrate for solid state fermentation for production of antibiotics, enzymes, and phytochemicals. Isolation of enzymes from waste material as crude extracts contributes to the concept of sustainability and lowers their cost significantly. The enzyme of interest in this study was peroxidase isolated from soybean hull. Furthermore, the crude peroxidase from soybean hull was crosslinked by oxidized pectin onto the inner walls of PTFE microtubular reactor. The influence of the crosslinker concentration on the enzyme activity and crosslinking efficiency was evaluated, together with the effect of the reagents flow rate on the enzyme activity and crosslinking efficiency. Two different microtubular diameters were examined for the crude peroxidase crosslinking: 0.5 and 0.8 mm. After the immobilization, the possibility of anthraquinone dye removal by crosslinked peroxidase in the microtubular reactor was examined. The reusability of the immobilized enzyme was also evaluated in this study.

Key words: *microreactor; peroxidase from waste material; cleaner production; C.I. Acid Violet 109; immobilization*

1 Introduction

Waste material from the food industry or from any industry that processes agricultural materials is commonly used for feedstock or disposed on landfills. The improper disposal of waste materials rich in carbohydrates, proteins and bioactive compounds, such as burning or dumping on an un-

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planned landfill, may lead to adverse impact on the environment. The untreated waste contributes to increasing greenhouse gas emissions, which means that it has direct influence on climate change [1], [2]. Furthermore, it leads to economic loss because valuable nutrients are not being used. The agro-industrial waste can be used for value-added products such as generating power, biofuel, biogas and biofertilizers production [3]–[6]. Further application of agro-industrial waste may be as a substrate for solid state fermentation for production of antibiotics, enzymes, and phytochemicals [7], [8]. Isolation of enzymes from waste material as crude extracts contributes to the concept of sustainability and lowers the costs significantly. Moreover, the application of continuous flow contributes to automatization of processes and increases process productivity. The use of microreactors has several advantages over batch reactors: high surface-to volume ratio; better mixing and mass transfer; laminar flow; process intensification and cleaner production which contributes to supporting the circular economy concept. Another characteristic of microtubular reactors is the possibility of numbering-up, where the scale-up is possible with increasing the number of same reactors, so the process efficiency remains constant on lab and industrial scale [9]. The immobilization of enzymes contributes to their reusability. In this study, peroxidase was isolated from soybean hull. The crude peroxidase from soybean hull was crosslinked by oxidized pectin onto the inner walls of PTFE microtubular reactor. The influence of the crosslinker concentration on the enzyme activity and crosslinking efficiency was evaluated, together with the effect of the reagents flow rate on the enzyme activity and crosslinking efficiency. Two different microtubular diameters (0.5 and 0.8 mm) were examined for the crude peroxidase crosslinking. After the immobilization, the removal of the dye C.I. Acid Violet 109 by crosslinked peroxidase in the microtubular reactor was examined. The reusability of the immobilized enzyme was also evaluated in this study.

2 Materials and Methods

2.1 Materials

Soybean seeds were obtained from the local groceries; C.I. Acid Violet 109 from DCC colorants (Ningbo); pyrogallol, monobasic potassium phosphate anhydrous, dibasic potassium phosphate trihydrate, sodium periodate from Sigma Aldrich, hydrogen peroxide 35% and sodium bicarbonate from Zorka Šabac; citric acid from Alkaloid; and trisodium citrate dihydrate from Alkaloid. Apple pectin was purchased from Sigma-Aldrich. All reagents were of analytical grade.

2.2 Isolation of peroxidase from soybean hull

The isolation of peroxidase from soybean hull was done as previously described [10]. Briefly, wet mass of 30g soybean hull was soaked in 120 mL distilled water, and homogenized. The mixture was left for extraction overnight at 4 ºC. Next, filtration through gauze was performed. The filtrate was heated at 65 ºC for 3 min, then cooled to room temperature. The mixture was centrifuged, and the supernatant was stored at 4 ºC.

2.3 Periodate oxidation of pectin from apple

The oxidation of pectin was done by periodate oxidation described by Ivanovska et al. with slight alterations [11]. Pectin from apple, 2g was added in Erlenmayer flask together with 20% ethanol (20 % *v/v*). After stirring the mixture, 0.5 M sodium periodate was added. The reaction was performed at 60 °C, 2h in dark conditions. The oxidized pectin was precipitated with cold 2-propanol, vacuum filtrated and then freeze-dried.

2.4 Imobilization of soybean hull peroxidase in microtubular reactor

The soybean hull peroxidase was immobilized by oxidized pectin as cross-linker in the microreactor which length was 1 m and diameter 0.5 mm. The flow rate of the peroxidase solution was 0.0008 mL/min, while the flow rate of the 0.5 % cross-linker was 0.001 mL/min. The residence time for the cross-linking reaction was 108.8 min. Next, 50 mM citric buffer solution pH 4 was pumped through the microreactor to clear away the peroxidase that is not immobilized.

2.5 Activity determination of cross-linked peroxidase in microtubular reactor

The determination of the immobilized soybean hull peroxidase activity was performed by pyrogallol as a standard substrate [12]. Briefly, pyrogallol was added to a 100 mM phosphate buffer pH 7, resulting in final concentration of 13 mM. The substrate solution inlet flow rate was 0.1 mL/min, the same as the hydrogen peroxide flow rate. The absorbance was measured at 420 nm. One unit of activity (μmol/min) is defined as the amount of peroxidase that will form 1 mg of purpurogallin from pyrogallol in 1 min under the assay conditions.

2.6 Influence of the cross-linker concentration on the enzyme activity and immobilization efficiency

The effect of the cross-linker concentration was assessed by varying the concentration of oxidized pectin in the range 0.05 - 1%. The flow rate of the cross-linker solution was 0.001 mL/min, while the peroxidase flow rate was 0.008 mL/min. The immobilization efficiency was measured as the difference in the mass of protein in the initial solution and the mass of protein in the outlet solution, divided by the protein mass in the initial solution.

2.7 Influence of the flow rate and the reactor's diameter on the enzyme activity and immobilization efficiency

The influence of the reactants flow rate was examined by changing the rate of the inlet flow as follows: F1 constisting of peroxidase flow rate of 0.0004 mL/min and cross-linker flow rate of 0.0005 mL/min; F2 consisting peroxidase flow rate of 0.001 mL/min and cross-linker flow rate of 0.0008 mL/min; and F3 consisting of peroxidase flow rate of 0.0008 mL/min and cross-linker flow rate of 0.001 mL/min. The same flow rates were examined in a microreactor with 0.5 mm and 0.8 mm diameter.

2.8 Operational stability of cross-linked peroxidase in microtubular reactor

The cross-linked soybean hull peroxidase in microtubular reactor was tested for the decolorization of the anthraquinone dye C.I. Acid Violet 109 under optimal conditions that were determined in previous research studies. After one cycle of decolorization, 50 mM citric buffer solution pH 4 was pumped through the microreactor to clear away residues. The cycles were rerun until a significant change in the decolorization was noticed. The absorbance for the AV109 decolorization was measured at 590 nm.

3 Results and discussion

In this study, cross-linking of crude peroxidase in a microtubular reactor was performed and the decolorization of anthraquinone dye AV109 was validated. Peroxidase was isolated from agricultural waste such as soybean hull. In this way waste valorization was achieved. The cross-linking of peroxidase was carried out by oxidized pectin. The activity of the immobilized peroxidase was measured using pyrogallol as a standard substrate and was 21.31 μmol/min.

3.1 The influence of the cross-linker concentration on the enzyme activity and immobilization efficiency

The influence of the cross-linker concentration on the enzyme activity and immobilization efficiency is given in Fig. 1. As it can be seen from the figure, the increase of the cross-linker concentration has a positive effect on the immobilization efficiency, where it increases from 14.9 % for 0.05 % (w/v) cross-linker, to 26.6 % efficiency for 1 % (w/v) solution of cross-linker. As for the activity of the enzyme, it drops with the increase of cross-linker concentration. Lioret et al. came across similar finding when they studied the immobilization of laccase in a continuous flow microreactor [13].

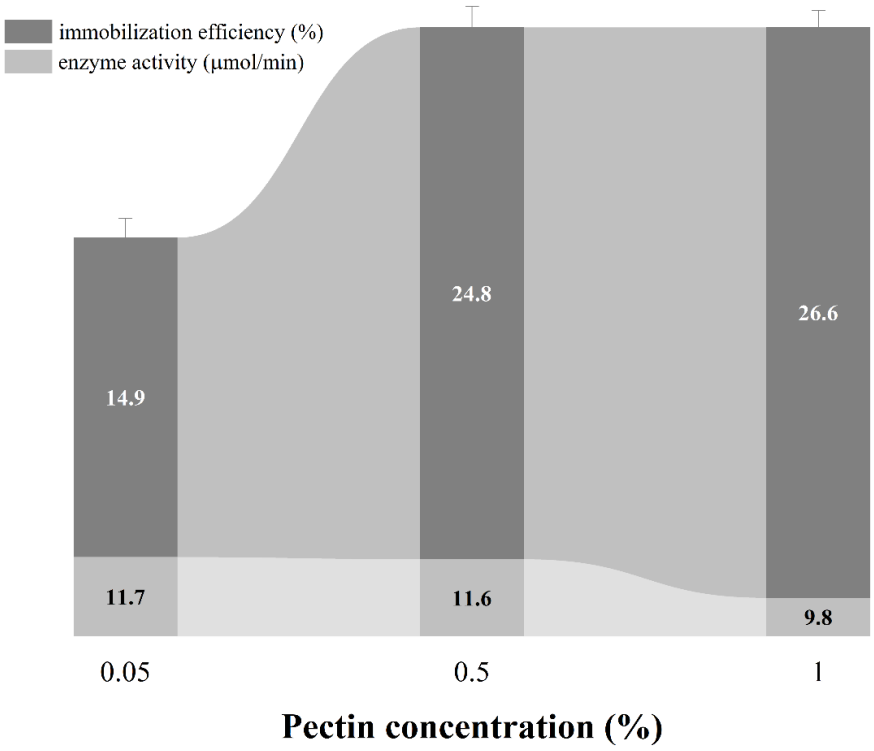

Fig 1. The influence of the cross-linker concentration on the enzyme activity and immobilization efficiency. Conditions: cross-linker flow rate 0.001 mL/min; enzyme flow rate 0.0008 mL/min; enzyme activity 60 U/mL; temperature 25 ºC; length of the reactor 1m; diameter 0.5 mm

3.2 The influence of the flow rate and the reactor's diameter on the enzyme activity and immobilization efficiency

The influence of the flow rate on the enzyme activity and immobilization efficiency in a microreactor with 0.5 mm diamaeter is given in Fig 2. The highest enzyme activity was measured at the flow rate F3: peroxidase flow rate of 0.0008 mL/min and cross-linker flow rate of 0.001 mL/min. It can be concluded that the immobilization efficiency is the highest when the cross-linker flow rate is higher than the flow rate of the enzyme. This indicates the importance of the ratio between the cross-linker and the soybean hull peroxidase.

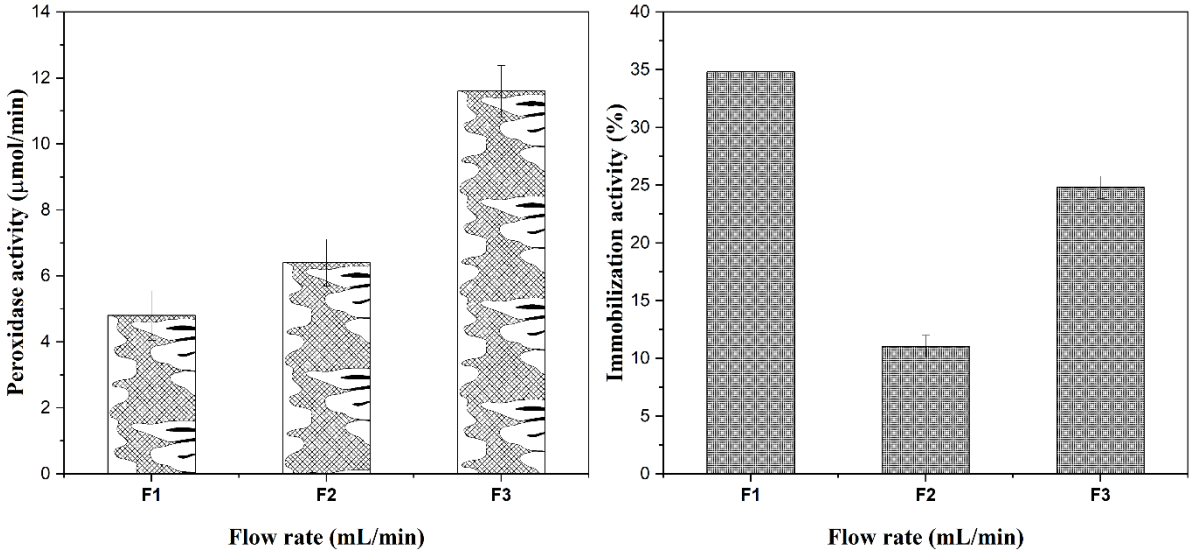

Fig 2. The influence of the flow rate on: a) the enzyme activity and b) immobilization efficiency. Conditions: enzyme activity 60 U/mL; cross-linker concentration 0.05 % (w/v), temperature 25 ºC, length of the reactor 1m; diameter 0.5 mm

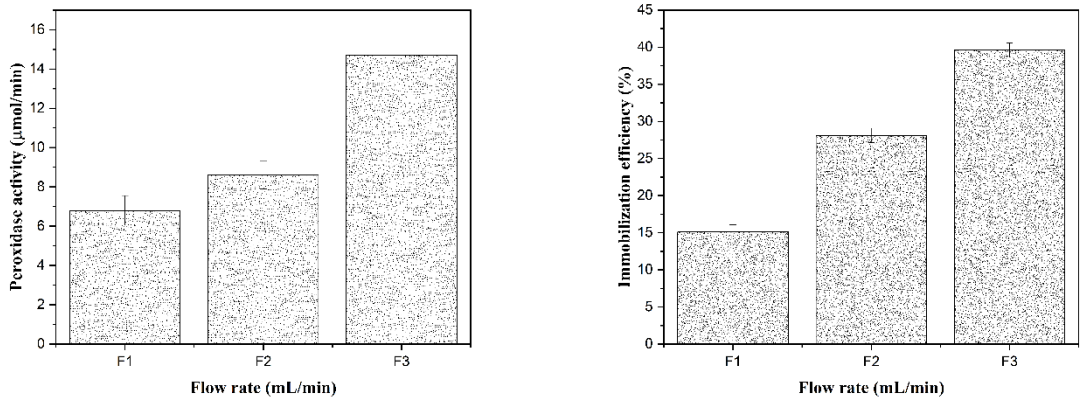

Fig 3. The influence of the flow rate on: a) the enzyme activity and b) immobilization efficiency. Conditions cross-linker concentration 0.05 % (w/v), temperature 25 ºC, length of the reactor 0.390 m; diameter 0.5 mm

The influence of the flow rate on the enzyme activity and immobilization efficiency in a microreactor with 0.8 mm diameter is given in Fig 3. As it can be seen from Fig. 3, the enzyme showed to be most active when the immobilization was performed at F3: peroxidase flow rate of 0.0008 mL/min and cross-linker flow rate of 0.001 mL/min. The reactor's diameter had no significant influence on the enzyme activity. The immobilization efficiency was the highest when the immobilization was carried out at F3, and the lowest at F1: peroxidase flow rate of 0.0004 mL/min and cross-linker flow rate of 0.0005 mL/min. It can be concluded that with decrease of the flow rate, the immobilization efficiency also decreases.

3.3 Operational stability of cross-linked peroxidase in microtubular reactor

The operational stability of cross-linked soybean hull peroxidase in a microtubular reactor used for decolorization of AV109 is given in Fig. 4. The decolorization reaction was performed at pH 4, dye concentration 10 mg/L, hydrogen peroxide 0.2 mM and enzyme activity 11,7 μmol/min. The cross-linked peroxidase retained 80% of its activity after 6 consecutive cycles. After 10 cycles, the activity dropped to 65% of the initial value. Kalsoom et al. worked on immobilization of soybean peroxidase in polyacrylamide gel. The peroxidase retained 60% of its activity after 6 cycles of diazo dye degradation [14].

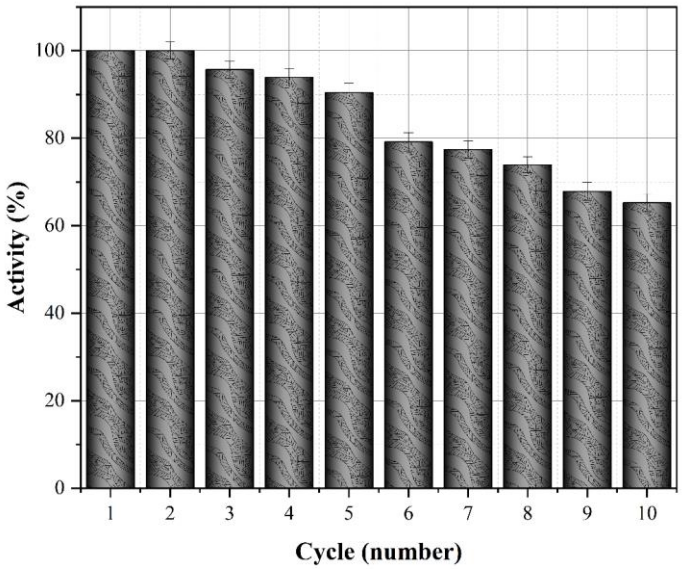

Fig 4. Opertional stability of immobilized soybean hull peroxidase in microtubular reactor. Conditions: pH 4; enzyme activity 11,7 μmol/min;, temperature 25 ºC, hydrogen peroxide concentration 0.2 mM; dye concentration 10 mg/L

4 Conclusion

In this study, immobilization of peroxidase from waste material in continuous flow microtubular reactor was performed. The enzyme was cross-linked with oxidized pectin. The influence of the cross-linker concentration was examined, together with the reactor's diameter and the flow rate of the reactants. After optimization of the immobilization conditions, the operational stability of the immobilized peroxidase in the microreactor in the decolorization reaction of AV109 was validated. After 10 consecutive cycles, the soybean peroxidase retained 65 % of its activity.

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