Cross-linking the peroxidase: from potato peel valorization to colored effluents treatment

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Abstract. Despite the obvious benefits from mass production such as an increased productivity, lower product cost and rapid evolution, nowadays we are dealing with energy consumption issues and pollution. The generated waste poses a threat to the environment, so new green techniques are developed constantly. Waste valorization is one of the trending concepts that is a part of sustainability strategies. Potato exploitation due to mass production of chips, hash browns, frozen food and starch leads to a formation of high waste load. There are several available ways for potato peel valorization: as a biofertilizer, as a substrate for microbial growth, as an adsorbent, for extraction of antioxidants and for extraction of enzymes. The enzyme peroxidase is abundant in potato peel. This enzyme uses hydrogen peroxide as an activator and can be readily used for oxidation of different compounds - pollutants. In this study, peroxidase was isolated from potato peel and immobilized as cross-linked enzyme aggregates. Pectin was used as a green cross-linker. The immobilized potato peel peroxidase was used for degradation of a textile anthraquinone dye Lanaset Violet B. Under the optimal process parameters: pH 3, 0.4 mM hydrogen peroxide, 0.8 μmol/min CLEA peroxidase, 10 mg/L dye and 70 min, 85.71±1.45 % dye degradation was achieved. The operational stability, as a key parameter for immobilized enzyme systems, was also examined. After 4 cycles CLEA peroxidase kept 31.57±1.79 % of its biodegradation efficiency.

1. Introduction

The rapid increase in waste production has led to development of numerous waste management solutions. Circular economy is dedicated to extension of products' life cycle, reducing waste to minimum. With product's repair, reuse, recycle whenever it is possible, further value of products is created [1]. Another way of adding value to a product is use of industrial waste. It is estimated that potato peel, as a waste from food industry such as chips industry, hash browns, frozen food, is generated as much as 70-140 000 tons worldwide [2]. Until recently, potato waste material was deposited on landfills with considerable negative impact on the environment. It was also used as animal feed with low added value. Today, potato peel can be used as a biofertilizer, as a substrate for microbial growth, as an adsorbent, for extraction of antioxidants and/or enzymes [3], [4]. Enzymes, as biocatalysts are replacing the conventional chemical catalysts due to their eco-friendliness, and the ability to catalyze reactions under mild conditions [5]. However, the time-consuming downstream process of enzyme production and the cost of high purity enzymes make them not so readily to use. One way of circumventing these drawbacks is extraction of enzymes from alternative sources or from waste material. Furthermore, application of enzymes in the form of crude extract, without purification enriches

the process's sustainability. Further amplification of enzyme use is immobilization. The immobilization of choice is cross-linking enzyme aggregates. In that way, the reuse of enzymes is secured, fitting in circular economy principles. Preparation of oxidized pectin is necessary for introduction of dialdehyde groups which in turn react with amino groups from the enzyme forming covalent bond via Schiff-bases [6]. Oxidized pectin is recognized as a safe reagent for the environment, replacing the toxic glutaraldehyde as an enzyme cross-linker [7]. The isolation of peroxidase from waste material, the immobilization with eco-friendly cross-linker and it use for colored water treatment contributes to the overall process sustainability.

In this study, peroxidase was isolated from waste material – potato peel as a crude extract. The peroxidase was precipitated with ammonium-sulphate and cross-linked with oxidized pectin. The oxidation of pectin was performed by sodium-periodate. After the immobilization procedure and examining the influence of the oxidized pectin concentration on enzyme activity, the CLEA peroxidase was used for decolorization of textile dye Lanaset Violet B. The effect of enzyme activity on the decolorization rate was assessed. The effect of dye load on enzyme activity was also examined.

2. Materials and Methods

2.1. Materials

Potato peels were obtained from the local groceries; Lanaset Violet B from DCC colorants (Ningbo), pyrogallol, monobasic potassium phosphate anhydrous, dibasic potassium phosphate trihydrate, hydrogen peroxide 35% from Carlo Erba; sodium citrate from Alkaloid; and citric acid from Lach:ner. Apple pectin and sodium periodate were purchased from Sigma-Aldrich. All the reagents were of analytical grade.

2.2. Methods

2.2.1. Enzyme extraction. The extraction of peroxidase from plant material was performed as previously described [8]. The potato peels were minced and soaked in distilled water. After 24 h at 4 ºC the extraction mixture was filtered, and the filtrate was subjected to heating up to 65 ºC for 3 min. Next the mixture was cooled to room temperature and centrifuged 10 min at 10 000 RPM. The supernatant was collected and kept in a refrigerator.

2.2.2. Pectin oxidation. 2% *(w/v)* Apple pectin solution in ethanol (20%) was prepared and 0.5 M Na-periodate acid was added [9]. The pH was adjusted to 3.5. The solution was shaken for 2 h at 60 ºC in a dark, sealed Erlenmeyer to avoid autooxidation by light or air. The oxidized pectin was precipitated by excess cold 2-propanol. Afterwards it was freeze dried.

2.2.3. Enzyme activity assay. The peroxidase activity was determined by colorimetric reaction [10]. Pyrogallol was used as a substrate. The change in the absorbance was measured at 420 nm for 3 minutes every 30 seconds. The mixture contains 0.013 M pyrogallol dissolved in phosphate buffer 0.1 M pH 7.0, 3% *v/v* hydrogen peroxide and predefined mass of immobilized enzyme. One unit of activity (μmol/min) is defined as the amount of peroxidase that will form 1 μg of purpurogallin from pyrogallol under the assay conditions. The activity of the cross-linked peroxidase is calculated by the following equation (1):

$$CLEA\ peroxidase\ activity\left(\frac{\mu mol/min}{g}\right) = \frac{\Delta A \cdot V_t}{\Delta t \cdot \varepsilon \cdot m_{CLEA\ peroxidase}} \quad (1)$$

Where:
$\frac{\Delta A}{\Delta t}\left(\frac{1}{min}\right)$ initial reaction rate,
$V_t(mL)$ – total volume of the reaction mixture

$\varepsilon\left(\frac{mmol}{L}\right)$- molar extintion coeficient of purpurogaline,
$m_{CLEA\ peroxidase}$ = mass of cross-linked peroxidase for activity determination

2.2.4. Determination of dialdehyde groups by DNPH method [11]. For the preparation of dinitrophenylhydrazine, 40 mg of it was dissolved in sulfuric acid. Then, 3 mL ethanol was added with constant stirring. After complete homogenization, distilled water was added up to 10 mL. Then, to the 10 mL of DNPH reagent, 0.1 mL 0.3% (*w/v*) pectin solution was added. After 1 h of stirring at room temperature, the absorbance was measured at 357 nm, which corresponds to the amount of the unreacted DNPH. Standard curve was constructed for calculation of aldehyde group concentration, in the range of 2-10 mg/mL DNPH. The content of aldehyde groups was calculated by the following equation:

$$aldehyde\ group\ concentration\left(\frac{mmol}{g}\right): \frac{reacted\ DNP \Big/ 198.14\frac{mmol}{mL}}{0.3\cdot10^{-4}\frac{g}{mL}} \quad (2)$$

Where:
198.14 – molar mass of DNPH (mg/mmol)
$0.3 \cdot 10^{-4}$ – pectin concentration (g/mL)

2.2.5. Determination of protein content by modified Lowry method [12]. Briefly, to a 0.2 mL sample, 0.18 mL reagent A was added (reagent A consists of sodium-tartrate sodium-carbonate and 1M NaOH). The mixture was vortexed and kept in a water bath for 10 min at 50 °C. Afterwards, the mixture was cooled to room temperature and 0.02 mL reagent B was added (reagent B consists of sodium tartrate, copper sulphate and 1M NaOH). The mixture was vortexed and kept at room temperature for 10 min. Next, 0.6 mL reagent C was added (diluted Folin-Ciocalteu reagent) and the mixture was kept for 10 min at 50 °C in a water bath. After the cooling of the mixture to room temperature, the absorbance at 650 nm was measured. Bovine serum albumin in the concentration range 0.1-0.5 mg/mL was used for calibration curve build up.

2.2.6. The effect of pectin concentration on the enzyme activity. The first step of preparing cross-linked enzyme aggregates is precipitation. The peroxidase was precipitated with ammonium-sulphate. Then, oxidized pectin with different concentrations (0.1 – 2 % *w/v*) was added to the precipitated peroxidase. The immobilization lasted 24 h with constant stirring at 4 °C.

2.2.7. The effect of enzyme activity on the decolorization rate. Dye was dissolved in citrate buffer pH 3. Hydrogen peroxide was added in concentration of 0.1 mM, and then CLEA potato peroxidase was also added, varying its activity 0.1 – 1 μmol/min. The change in absorbance was monitored at 590 nm.

2.2.8. The effect of dye load on enzyme activity. The influence of the dye load was examined by preparing dye solutions with different concentration 0.012 mM – 0.121 mM. Next, hydrogen peroxide was added, and then CLEA peroxidase. The reaction was catalysed at room temperature with constant stirring.

3. Results and discussion

3.1. *Pectin oxidation and aldehyde groups' content determination*.
In search of ecological alternative for cross-linker, variety of natural polymers are modified: pectin, chitosan, poly-lysine, dextran etc [9]. The oxidation of pectin was performed by sodium-periodate to obtain dialdehyde groups by vicinal diols cleavage. The oxidized pectin is shown in Fig. 1. The content

of aldehyde groups by DNPH method was determined in two samples: native pectin and oxidized pectin. The content of aldehyde groups in the native pectin amounts for 0.0253 mmol/g, while in the sample of oxidized pectin, the value of aldehyde groups content is 0.0468 mmol/g. The big difference between these two values is an indicator of a successful oxidation. Similar results were obtained by Gupta et al. in their experiments of pectin functionalization [11].

Figure 1. Oxidized pectin before lyophilisation

3.2. The effect of pectin concentration on the enzyme activity

Oxidized pectin has dialdehyde groups that bind covalently to the amino groups from peroxidase forming Schiff bases. On the one hand, insufficient cross-linker concentration could lead to a lower immobilization efficacy. On the other hand, higher pectin concentration could have a negative impact on the enzyme activity. High cross-linker concentration means numerous covalent bonds between the enzyme and pectin, which enhances the enzyme rigidity and lowers its flexibility. Moreover, steric hindrance can happen, and there is great possibility for interaction of the enzyme's active centre amino groups with dialdehyde groups of the crosslinker, which influences directly on the enzyme activity. The peroxidase activity and activity recovery were examined in correlation with pectin concentration. The results are shown in Figure 2. It can be noted that with increase of pectin concentration, the peroxidase activity decreases from 15.18 μmol/min/g when 0.1 % oxidized pectin was used, to 4.49 % μmol/min/g for 0.5 % oxidized pectin. Further increase of pectin concentration (2 %) leads to a more significant

activity decrease - 1.06 µmol/min/g. Similar conclusions were drawn by Kumar et al. [13], where they used glutaraldehyde for laccase cross-linking.

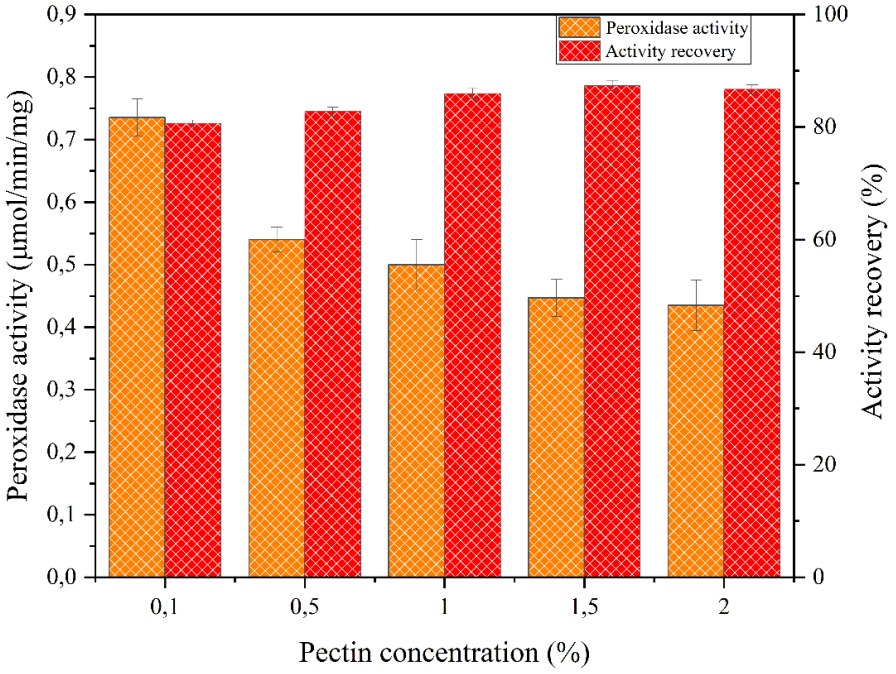

Figure 2. The effect of pectin concentration on potato peel peroxidase activity and activity recovery

3.3. The effect of enzyme activity on the decolorization rate

The enzyme activity was varied in the range 0.1 – 1 µmol/min. Dye was dissolved in citrate buffer solution pH 3. Hydrogen peroxide was added and afterwards CLEA potato peel peroxidase. The results are shown in Fig. 3. With increase of enzyme activity up to 0.8 µmol/min, the decolorization

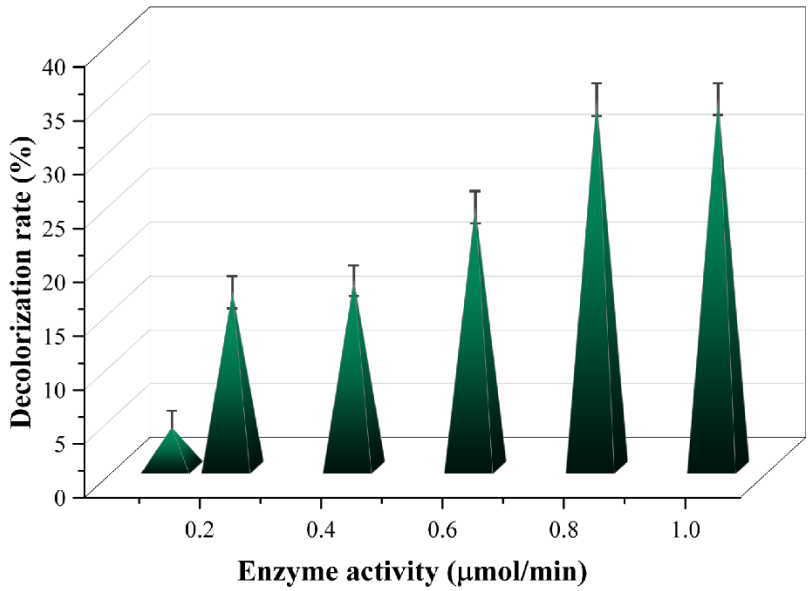

Figure 3. The effect of enzyme activity on the decolorization rate.

rate increased directly proportional. Difference between the decolorization rate when the enzyme activity was 0.8 and 1 μmol/min is insignificant 34.17 and 34.24 %, respectively. So, activity of 0.8 μmol/min/g of potato peel peroxidase was adopted for further experiments.

3.4. *The effect of dye load on enzyme activity*.

For assessment of the dye load influence on the enzyme activity, 6 different dye solutions were prepared in the range 0.012 – 0.12 mM. results are sown in Fig. 4. It can be concluded from the Figure 4 that the highest decolorization rate – 82.2 % was achieved with dye load of 0.012 mM. Further increase of dye load up to 0.12 mM leads to decolorization decrease to 13.1 %. When free potato peel peroxidase was used for the same dye decolorization, 65.11 % was achieved with dye load of 0.048 mM. These findings indicate the presence of limitations in the substrate diffusion, as well as piling up of intermediates, leading to a slower reaction rate.

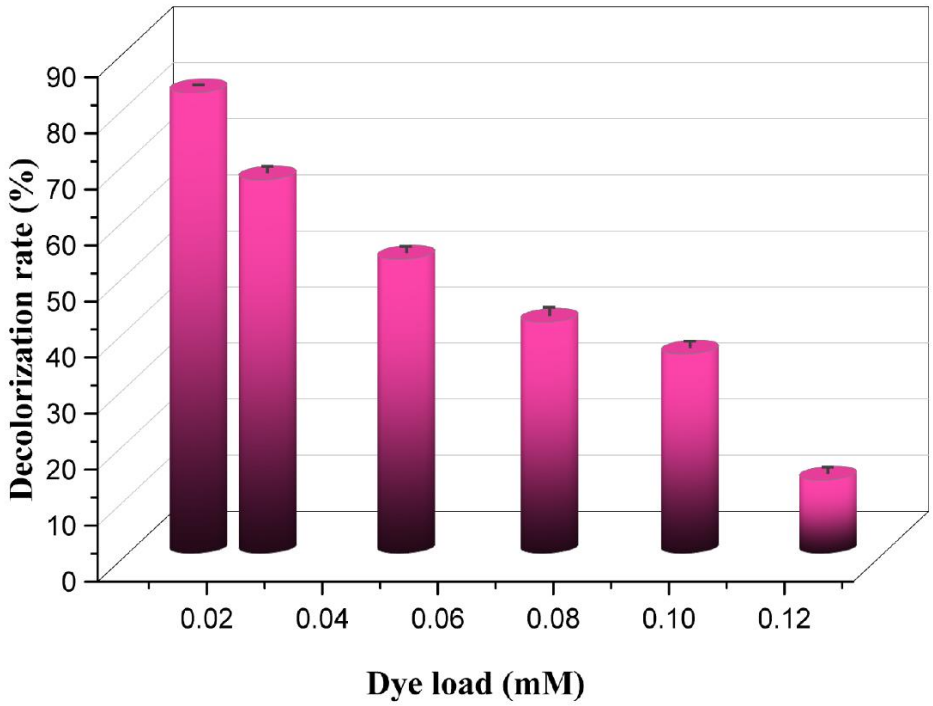

Figure 4. The effect of dye load on enzyme activity.

4. Conclusion

In this study potato peel was used for extraction of peroxidase, enhancing the value of potato waste material. Peroxidase was immobilized as cross-linked enzyme aggregates and used for decolorization of textile dye. Ammonium-sulphate was used for precipitation of enzyme molecules, and oxidized pectin for cross-linking of peroxidase. With oxidized pectin concentration of 0.1 % peroxidase recovery activity was the highest. At pH 3 and CLEA peroxidase activity of 0.8 μmol/min, 82.2 % dye decolorization was achieved. Adding value to potato waste material as a source of peroxidase and the use of the same enzyme for textile dye decolorization presents a potent sustainable concept for cleaner technology of coloured effluent treatment.

5. References

[1] Wang X *et al* 2022 *J. Haz. Mat*. **423** 127023
[2] Sampaio S L *et al* 2020 *Trends Food Sci Technol.* **103** 118–129
[3] Scharf R, Wang R, Maycock J, Ho P, Chen S and Orfila C 2020 *Waste and Biomass Valorization* **11** 2123–2128
[4] Chohan N, Aruwajoye G, Sewsynker-Sukai Y and Gueguim Kana E 2020 *Renew. Energy* **146** 1031–1040
[5] Wahart A, Staniland J, Miller G and Cosgrove S 2022 *R. Soc. Open Sci.* **9** 211572
[6] Yamaguchi H, Kiyota Y and Miyazaki M 2018 *Catalysts* **8** 8050174
[7] Ehrmann A 2021 *Polymers (Basel)* **13**1 3121973
[8] Svetozarević M, Šekuljica N, Knežević-Jugović Z and Mijin D *2020 Maced. J. Chem. Chem. Eng* **39** 196–206
[9] Talekar S, Nadar S, Joshi A and Joshi G 2014 *RSC Adv.* **4** 59444–59453
[10] Šekuljica N *et al* 2016 *CLEAN – Soil Air Water* **44** 891–900
[11] Gupta B, Tummalapalli M, Deopura B and Alam 2013 *Carbohydr. Polym* **98** 1160–1165,
[12] Hartree E 1972 *Anal. Biochem* **48** 422–427
[13] Kumar V, Kumar M, Thiruvenkadaravi K, Baskaralingam P, Kumar P and Sivanesan S 2012 *Bioresour. Technol.* **119** 28–34

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