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Determination of the Optimum Conditions for Extracting Polyphenol Oxidase and Laccase Enzymes From *Malva Parviflora* and Their Role in The Decolorization of Some Dyes

Ali Jabbar R. AL-Sa'ady, Majid Hameed Hilal*

Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

The current work was undertaken to obtain the crude extract of PPO and laccase enzyme from the leaves of *Malva parviflora*. All leaves were washed with tap water, then the extraction was performed to acquire the crude extract's enzymes. One gram of *Malva parviflora* leaves was homogenized in various volumes of distilled water (1:10, 1:5, and 1:2 w: v). The results showed that polyphenol oxidase and laccase with a ratio of 1:10 (w:v) gave the highest specific activity of 112.3 with 0.394 U/mg proteins. In addition, *Malva parviflora* leaves were homogenized with several types of buffers with two concentration (0.2 and 0.1 M) for PPO and laccase extraction. These buffers were potassium phosphate (pH 7.0), Tris-HCl (pH 8.0), and sodium acetate (pH 6.0). The results showed that sodium acetate (0.2 M, pH 6) was the best extraction buffer for PPO and laccase with specific activity values of 206.8 and 1.8 U/mg, respectively. Different times for PPO and laccase extraction were determined and the better time was found to be after five minutes, with specific activity values of 251.9 and 1.876 U/mg, respectively, while after 2 and 10 minutes the specific activity values of the enzymes were significantly decreased. Decolorization efficiency of different dyes by the enzyme mixture (PPO and laccase) was determined. It was found that nine dyes (Neutral red, Toluidin blue, Safranin, Crystal violet, Methylene blue, Blood, Phenol red, Henna, and Acridine orange) were altered in their absorbance after incubation with the enzyme mixture for a limited time period.

Keywords: Polyphenol oxidase, Laccase, *Malva parviflora*, Extraction, Decolorization.

تحديد الظروف المثلى لاستخلاص انزيم البوليفينول أوكسيديز واللاكيز من نبات الخباز ودراسة دور هذه الانزيمات في إزالة بعض الأصباغ

علي جبار رشك, ماجد حميد هلال*

قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

تم إجراء العمل الحالي للحصول على المستخلصات الخام لانزيم البوليفينول اوكسيديز وانزيم اللاكيز من أوراق نبات الخباز (*Malva parviflora*). حيث غسلت جميع الأوراق بماء الصنبور ثم استخلصت للحصول على المستخلص الأنزيمي الخام. علق واحد غرام من أوراق الخباز (*Malva parviflora*) في أحجام مختلفة من الماء المقطر وكانت هذه النسب 10:1 ، 5 :1 ، و 2 :1 (وزن:حجم). أظهرت النتائج بأن نسبة الاستخلاص 10:1 (وزن:حجم) كانت افضل نسبة لاستخلاص انزيم البوليفينول اوكسيديز واللاكيز لتعطي

*Email: majid.hameed40@yahoo.com

أعلى فعالية نوعية تقدر 112,3 و 0,394 وحدة/ملغرام بروتين وعلى التوالي، اما عند استخدام النسب الأخرى فقد تم الحصول على فعالية نوعية أقل. الخطوة الثانية هي تعليق أوراق نبات الخباز (*Malva parviflora*) مع عدة أنواع من المحاليل المنظمة (وبتراكيذ 0,2 و 0,1 مولاري) لاستخلاص أنزيم البوليفينول اوكسيديز واللاكبيز. ان هذه المحاليل المنظمة هي فوسفات البوتاسيوم (الأس الهيدروجيني 7)، التريس حامض الهيدروكلوريك (الأس الهيدروجيني 8) ، وخلات الصوديوم (الأس الهيدروجيني 6) . اظهرت النتائج أن محلول خلات الصوديوم (0,2 مولر، وأس هيدروجيني 6) كان أفضل محلول لاستخلاص أنزيم البوليفينول اوكسيديز و اللاكبيز مع فعالية نوعية 206,8 و 1,8 وحدة/ملغرام على التوالي، في حين أعطت المحاليل المنظمة الأخرى ذات التراكيذ والأس الهيدروجيني المختلفة فعالية نوعية أقل. تم تحديد أوقات مختلفة لاستخلاص أنزيم البوليفينول اوكسيديز و اللاكبيز حيث كان أفضل وقت للاستخلاص بعد مرور خمسة دقائق وبفعالية نوعية 251,9 و 1,876 وحدة/ملغرام على التوالي، بينما انخفضت الفعالية النوعية للإنزيمات بعد الدقيقة الثانية و الدقيقة العاشرة. تم تحديد كفاءة إزالة الأصباغ المختلفة بواسطة خليط الإنزيمات (البوليفينول اوكسيديز واللاكبيز). حيث وجد ان تسعة أصباغ (Toluidin blue, Neutral red, Acridine orange,) حيث وجد ان تسعة أصباغ (Toluidin blue, Neutral red, Acridine orange,) تغيير في امتصاصيتها بعد الحضان مع خليط الإنزيمات لفترة زمنية محددة .

Introduction

Polyphenol oxidases (PPOs) (EC 1.10.3.2) are copper-consisting oxidoreductases which catalyze the oxidation and hydroxylation of phenolic compounds in the existence of molecular oxygen. These enzymes are a wide prevalent group of enzymes found in bacteria, plants, animals, and fungi. Nearly 50 % of the tropical fruits are discarded due to quality disorders resulting from the browning by these enzymes. The browning is fundamentally catalyzed by the polyphenol oxidase enzymes. Due to the deleterious effects on food products by enzymatic browning, PPO has been widely studied in different tissues [1]. Polyphenol oxidases are placed fundamentally in the thylakoid membrane of the mitochondria and chloroplasts of plants. Tanning reactions occur after the disarray of tissues rich in phenols, which may cause linking of soluble polyphenol oxidase with a particles fraction [2]. In the presence of atmospheric oxygen and PPO, monophenol is hydroxylated to o-diphenol and the diphenol can be oxidized to o-quinones, which is then subjected to polymerization to result in the dark brown polymers [3]. PPOs possess various applications in industrial processes such as medicine and food. These enzymes were also utilized to tear out phenolics from wastewaters, non-phenolic compounds, and some of pigments and dyes [4]. Laccases are multicopper enzymes that are qualified of oxidizing several aromatic compounds via a mechanism of a radical catalyzed reaction in the existence of oxygen. They are a family of blue and multi-copper oxidases which comprise, beside laccase, plant ascorbate oxidase, the protein ceruloplasmin of the mammalian plasma, and bilirubin oxidase. Laccases contain 4 atoms of copper in the active site, which play a significant role in their catalytic activity. These copper atoms are categorized into 3 types depending on their spectroscopic and functional characteristics; type 1 (blue), type 2 (normal) and type 3 (coupled binuclear) [5]. The first laccase from bacteria was proclaimed from *Azospirillum lipoferum* as a multimeric enzyme [6]. Also, laccase was found in different plants, e.g. *Rhus vernicifera*, *Schinus molle*, *Magnifra indica* (mango), *Pistachia palaestina* and *Pleiogynium timoriense*. Laccase can be found in other fruits besides mango, while some studies mentioned that the laccase enzyme might be present in tomato. Laccases are applied in numerous industrial sectors, such as discoloration of wine, paper processing, chemical production from lignin, and environmental pollutants detoxification [7]. The applications of decolorization of pigments and dyes from the industrial waste of textile by bacterial and fungal laccase is being largely accumulated. This study aimed to determine the optimum conditions for the extraction of PPO and laccase enzymes from *Malva parviflora*, including the type of extraction buffer, extraction time and extraction ratio. The study also aimed at testing the ability of these enzymes to decolorize different dyes.

Materials and Methods

Chemicals

Catechol, o-tolidine, Na-acetate, bovine serum albumin, Tris-HCl and Coomassie brilliant blue were purchased from Sigma Co., while the other chemicals were supplied by BDH Chemicals.

Determination of polyphenol oxidase activity

Catechol was utilized as a substrate to estimate enzyme activity. The reaction solution contained 0.1 ml of the enzyme and 2.9 ml of substrate (0.01 M) in phosphate buffer (0.1M, pH 7.0). A blank sample was represented by three ml of the substrate solution [8]. The oxidation of catechol was determined by measuring the absorbance increase at 420 nm after 3 min using a spectrophotometer. One unit for activity of polyphenol oxidase was defined as the amount of polyphenol oxidase enzyme that accumulates in absorbance of 0.001/min. The enzyme activity was measured according the following equation:

$$\text{Activity of PPO (U.ml}^{-1}\text{)} = [(As_2 \text{ sample} - As_1 \text{ sample}) - (Ab_2 \text{ blank} - Ab_1 \text{ blank})]/(0.001 \times t)$$

As₂sample: is the end absorbance of the sample, **As₁sample:** is the first absorbance of the sample; **Ab₂blank:** is the end absorbance of the control, **Ab₁blank:** is the initial absorbance of the control, and (t) it is the reaction time in minutes [9].

Laccase activity

The activity of laccase enzyme was determined according to the method of Kalral *et al.*[10] through the utilization of o-tolidine as a substrate, while o-tolidine oxidation was estimated via measuring the absorbance accretion at 366 nm by using a spectrophotometer.

Determination of protein concentration

Protein concentration was estimated according to the Bradford method [11].

Extraction ratio

One gram of *Malva parviflora* leaves was homogenized in various volumes of distilled water using a blender for extraction of laccase and PPO enzymes. The extraction proportions were 1:10, 1:5, and 1:2 (w:v). Protein concentration and enzyme activity were also estimated.

Extraction buffer

The leaves of *Malva parviflora* plant were homogenized with various types of solution buffers at a ratio of 1:10 (w:v) for laccase and PPO extraction. These buffers are Tris-HCl at a concentration of 0.2 and 0.1 M (pH 8), potassium phosphate 0.2, and 0.1 M at pH 7, and sodium acetate 0.2, and 0.1 M (pH 6). Distilled water was also used for the extraction of laccase and PPO from the leaves of *Malva parviflora*. In each treatment, the protein and the enzyme activity were estimated [12].

Extraction time

PPO and Laccase enzymes were extracted from *Malva parviflora* at different times (2, 5 and 10 min.), by homogenizing the leaves in 0.2 M of Na-acetate buffer using a blender [9]. The enzyme activity and the protein were also estimated.

Decolorization of some dyes by PPO and Laccase enzyme

Fourteen types of dyes were prepared at a concentration of 25 mg/l. These dyes included nigrosin, phenol red, henna, brilliant green, toluidine blue, neutral red, indigo carmine, crystal violet, safranin, acridine orange, methylene blue, bromophenol blue, and blood. The absorbance of each dye was measured depending of its λ -max. The reaction mixture was prepared by adding 1 ml of the mixed enzymes (PPO and laccase) to 3 ml of the dye solution. The mixture was incubated for 27 min, then the changes in the absorbance for each dye were measured. Control samples were prepared for each dye under the same conditions but without the addition of enzyme. Decolorization efficiency of the mixed enzymes was assessed by monitoring the decrease in absorbance under the maximum wavelength of the dye [13].

Results and discussions

Effects of the extraction ratios on PPO and Laccase activities

Three extraction proportions were tested to determine the better ratio for PPO and laccase extraction from *Malva parviflora* leaves. Figures-(1 and 2) show that the extraction ratio of 1:10 (w:v) was the best ratio for PPO and laccase extraction, with specific activity values of 112.3 and 0.394 U/mg proteins, respectively. In this context, Ridgway and Tucker (1999) [14], found that 1:3 (w: v) was a better proportion for the extraction of PPO from apple, while Yagar, and Sagiroglu [15] found that 1:1 (v: w) was the better proportion for the extraction of PPO from quince.

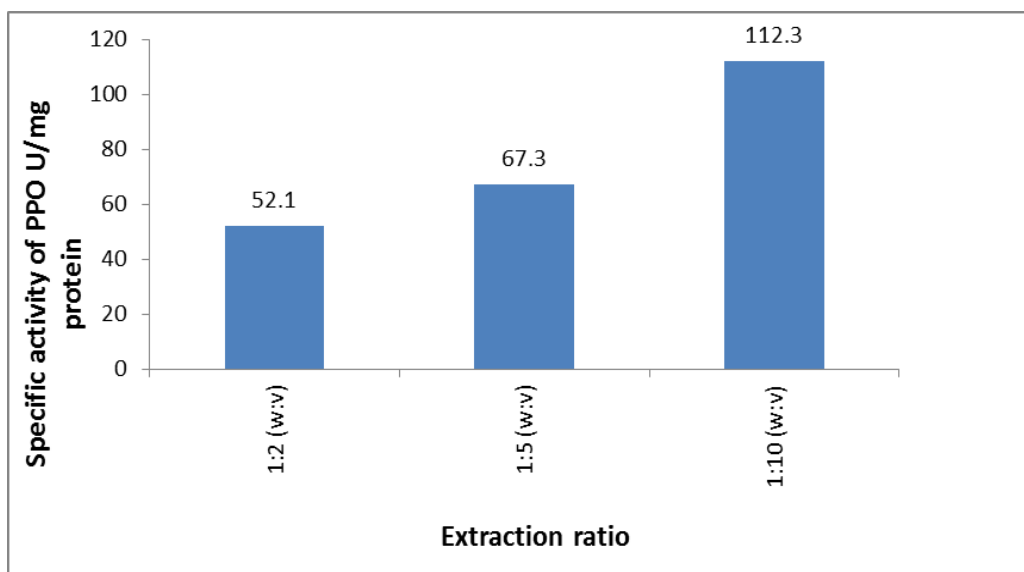


Figure 1-Effects of extraction ratios on PPO activity extraction from *Malva parviflora* leaves.

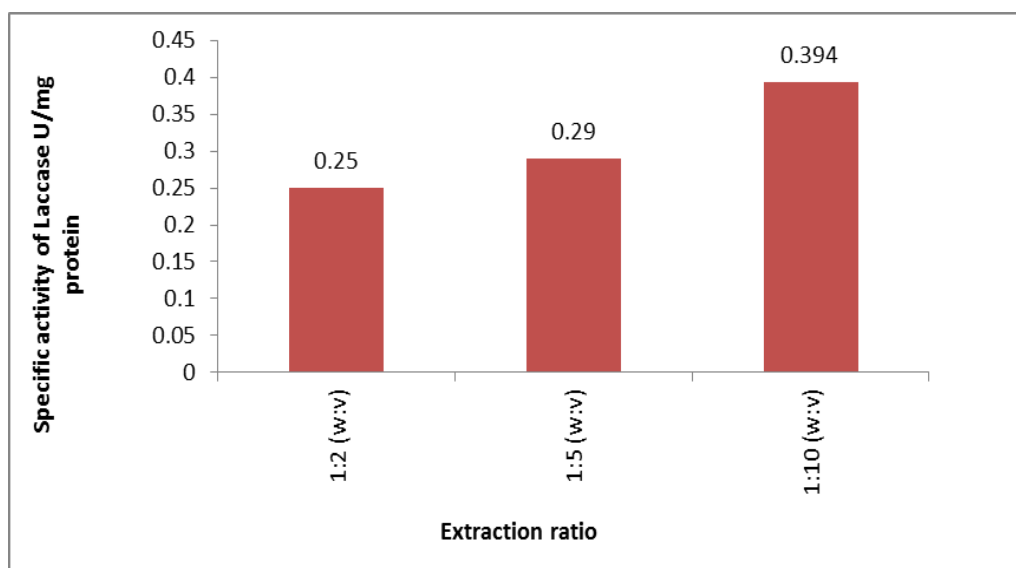


Figure 2-Effects of extraction ratios on Laccase activity extraction from *Malva parviflora* leaves.

Effect of extraction buffer

Specific activities of PPO and laccase enzyme were measured after extraction by using different buffers. The results are illustrated in Figures-(3 and 4) which show that 0.2 M sodium acetate buffer (pH 6) was the best extraction buffer for PPO and laccase extraction, with specific activity values of 206.8 and 1.8 U/mg protein, respectively, while the other buffers with different concentrations exerted low specific activity values. Aziz and AL-Sa'ady (2016) [9] found that potassium phosphate buffer (0.05 M, pH 7) was the best extraction buffer for PPO extraction from banana peel, with a high specific activity (300 U/mg), while other buffers with different pH and concentrations showed low specific activity. However, Aziz, *et.al.*, (2018) [4] reported that the best method for the extraction of PPO from banana peel with a high specific activity was the homogenization and dissolution in 100 ml phosphate buffer (0.05 M, pH 7) that contains 0.5% polyethylene glycol and ascorbic acid (0.01M) using a blender.

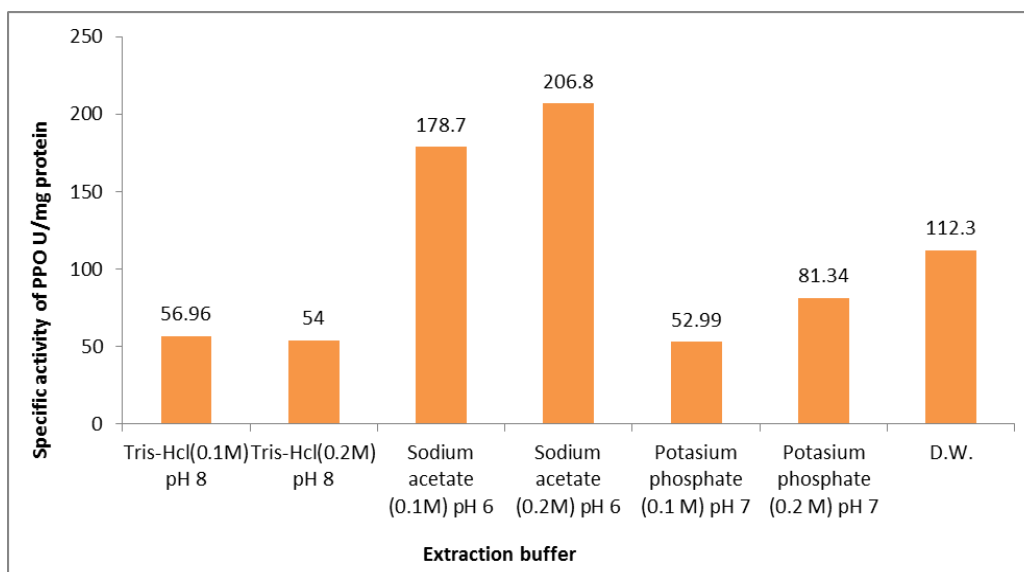


Figure 3-Effects of different extraction buffers on PPO extraction from *Malva parviflora* leaves.

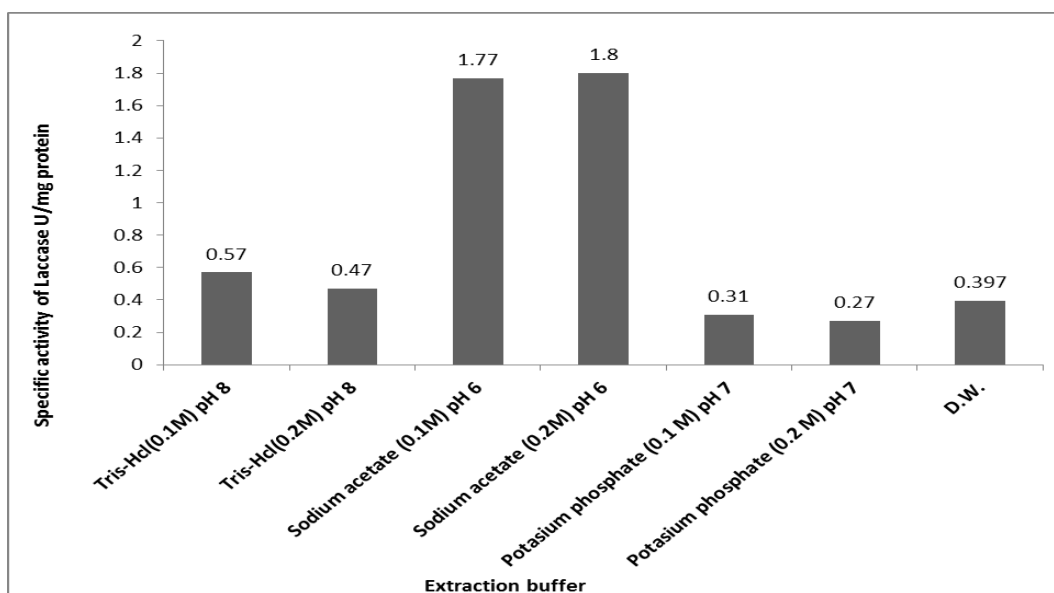


Figure 4-Effects of different extraction buffers on laccase extraction from *Malva parviflora* leaves.

It is worthy to mention that the effects of pH on the stability and enzyme activity may be through the influence on the protein structure of the enzyme via the alkalinity or acidity of the solution, due to the variation in the ionization state of the different amino acid residues [16]. The activity of enzymes is affected by pH in many ways. First, the enzyme stability is influenced by the environmental pH, since intensives acidity or alkalinity lead to the denaturation of the enzyme. Second, each enzyme has an optimum pH for its maximum activity and stability, and the enzyme is stable at certain limits above and under the optimum. Third, pH of the reaction mixture may influence the binding of the enzyme with its substrate. Also, the effects of pH on the solubility of the materials extracted from plant cells was reported [9].

Extraction time

Various extraction times were used for the extraction of PPO and laccase enzyme. The preferable time was shown to be five minutes, with specific activity values of 251.9 and 1.876 U/mg, respectively, while after 10 minutes the specific activities were decreased, possibly because the increase in the blender temperature that impacts the total activity of these enzymes. The diminution in the enzyme activity at high-temperature may result from the shift in the enzyme structure that blocks the active sites, along with enzyme denaturation [17]. Aziz and AL-Sa'ady (2016) [9] found that the better time

for PPO extraction from banana peels was after one minute, with a high specific activity of 981 U/mg. While, Aziz *et.al.* (2018) [4] reported that the best time for PPO extraction from banana peels was 1 minute.

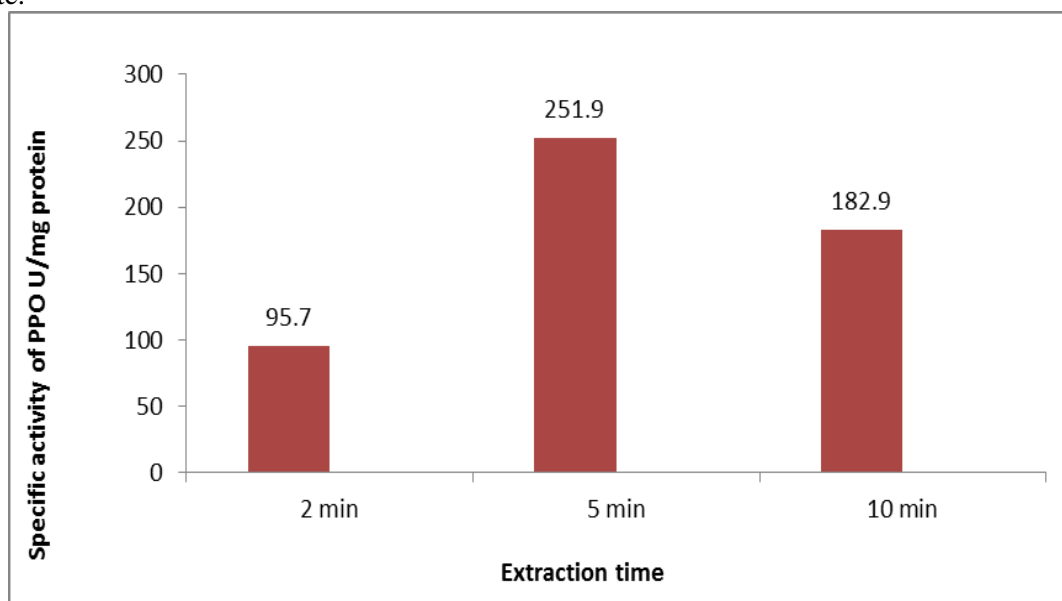


Figure 5-Effects of extraction time on PPO extraction from *Malva parviflora* leaves.

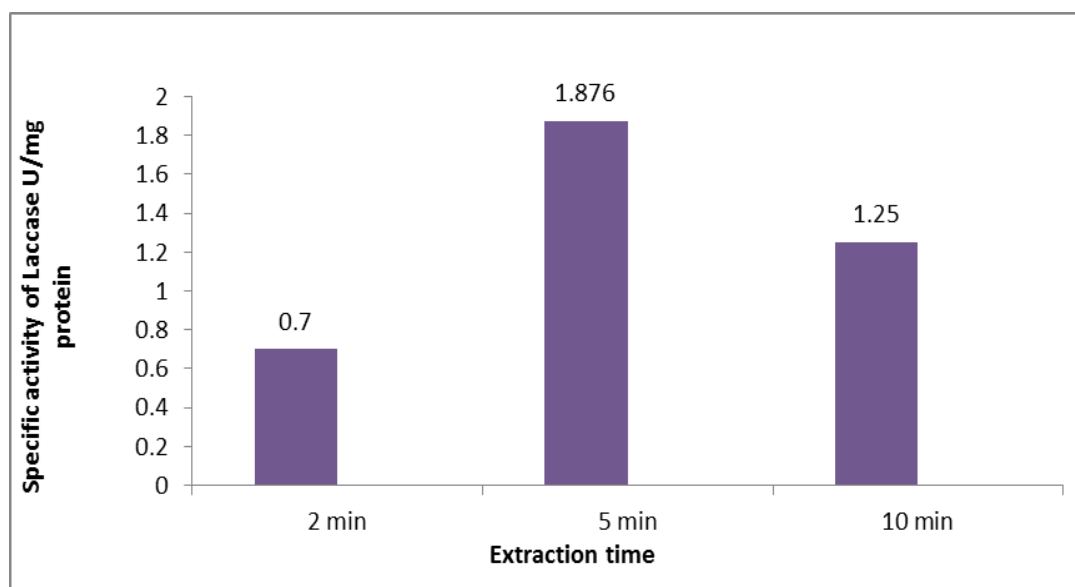


Figure 6-Effects of extraction time on Laccase extraction from *Malva parviflora* leaves.

Dye decolorization

The enzymes mixture was used for the decolorization of different dyes (nigrosin, phenol red, henna, brilliant green, giemsa stain, toluidine blue, neutral red, safranin, acridine orange, bromophenol blue, indigo carmine, methylene blue, crystal violet, and blood) in order to demonstrate their potential in the treatment of dyestuff wastewater. The results showed that only nine dyes (Neutral red, Toluidin blue, Safranin, Crystal violet, Methylene blue, Blood, Phenol red, Henna, and Acridine orange) were reduced in their absorbance, while no change occurred after treatment with other dyes for 27 min (Table-1).

These results agree with the results of AL-Sa'ady *et.al.* [18], who used laccase produced from *Bacillus* sp. in the decolorization of different dyes. Aziz, *et.al.* [4] tested the ability of the immobilized PPO from banana peel to decolorize different types of dyes, including neutral red, acridine, and toluidine. They showed a change in their absorbance values after incubation with the enzyme for a certain time

Table 1-Decolorization of different dyes by enzyme mixture from *Malva parviflora* leaves.

Dye Time	λ - max (nm)	Ab.at zero time	3 min	6 min	9 min	12 min	15 min	18 min	21 min	24 min	27 min
Brilliant green	625	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95	1.95
Bromophenol blue	590	2.43	2.43	2.43	2.43	2.43	2.43	2.43	2.43	2.43	2.43
Giemsa stain	530	1.62	1.62	1.62	1.62	1.62	1.62	1.62	1.62	1.62	1.62
Toluidine blue	630	2.75	2.56	2.46	2.32	2.27	2.12	2.12	2.12	2.11	2.11
Neutral red	545	1.43	1.32	1.25	1.09	0.89	0.76	0.67	0.64	0.62	0.55
Acridine or- ange	495	1.59	1.36	1.18	1.06	0.97	0.86	0.74	0.63	0.546	0.546
Indigo car- mine	495	1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53	1.53
Crystal violet	588	2.43	2.23	2.21	2.08	1.97	1.74	1.52	1.43	1.27	1.07
Safranin	534	1.98	1.87	1.72	1.61	1.45	1.37	1.24	1.24	1.24	1.24
Methylene blue	665	3.27	3.02	2.83	2.64	2.47	2.28	1.98	1.79	1.63	1.63
Nigrosin	580	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48	1.48
Phenol red	570	1.98	1.84	1.73	1.66	1.52	1.39	1.17	1.01	0.95	0.95
Henna	425	2.33	2.21	2.06	1.83	1.65	1.47	1.25	1.06	0.97	0.73
Blood	417	2.77	2.63	2.52	2.39	2.17	1.89	1.57	1.31	1.16	1.03

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