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Use of Purified Laccase from Prickly Lettuce (*Lactuca serriola* L.) In Removal of Phenolic Compound from Some Foods

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Abstract: **Laccase from Prickly lettuce leaves was purified through three sequential steps using $(NH_4)_2SO_4$, ion-exchange and gel filtration chromatography. At the end of the process, enzyme was 17.04 fold purification and yield of 76.66% with a specific activity 141.996 U/mg. The molecular weight was 69.37 kDa as estimated by gel filtration chromatography (Sephacryl S-200). The visible absorption spectrum showed one peak at 618 nm that is typical for type I Cu (II). The optimum pH activity was 6 and enzyme was stable between 4-7, while temperature activity was 40 and enzyme was stable at 60°C for 15 min. Carbohydrate content of purified laccase was 12% as determined by phenol sulfuric acid, and enzyme was 100% inhibited by 10mM of Sodium azide, l-cysteine, dithiothreitol and EDTA and 98, 96, 94, 91, 93 and 82% by SDS, Hg^{+2}, Al^{+2}, Ba^{+2}, Ni^{+2} and Mn^{+2} respectively at same concentration, while inhibition at 1Mm was 45, 39, 62, 59, 51, 53, 48, 55 and 41% respectively, and the Km and Vmax values was 53.68 µM and 641.2 µM\min respectively. The treatment of apple juice with 1, 5, 10, 20 u\mg of laccase led to removed 11.35, 44.72, 88.03 and 89.21 % of phenol compounds respectively, while the treated with 10 u\mg of laccase for 10, 20, 30, 40, 50, 60 min led to removed 28.42, 60.93, 88.03, 89.37, 90.01 and 90.04% of phenol compounds respectively.**

Keywords: **Laccase, Prickly lettuce leaves, *Lactuca serriola* L., Purification, Phenolic compound, Apple juice.**

1. INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing enzyme, also known as multi copper blue oxidases, belong to the oxidoreductase group of enzymes that catalyzes the oxidation of a wide variety of organic and inorganic compounds by coupling it to the reduction of oxygen to water like phenol, aniline, 4-hydroxybenoic acid and 2, 2'-azino-bis-(3-ethyl benzothiazoline-6-sulphinic acid) (ABTS) [1][2]. Laccase is generally found in higher plants and fungi but recently it was found in some bacteria, these enzymes contain 15-30% carbohydrate and have a molecule mass of 60-90 kDa [3]. Plant laccases have been reported to show higher molecular mass than fungal laccases which may be due to a higher extent of glycosylation than fungal ones, The glycosylation has been reported to play a role in copper retention, thermal stability and activity of the enzyme [1]. In plants, laccase plays a role in lignifications whereas in fungi it has been implicated in delignification, sporulation, pigment production, fruiting body formation, and plant pathogenesis [3].

Several methods were used to purified enzyme from different sources such as protein precipitation by ammonium sulphate, ion exchange and gel filtration chromatography, so, thermo stable alkaline laccase from papaya (*Carica papaya*) was purified by using affinity chromatography [1], and the purification protocol of laccase from edible mushroom *Clitocybe maxima* included use ammonium sulfate saturation, ion-exchange chromatography on DEAE-cellulose, SP-Sepharose, and Q-Sepharose, and gel filtration by fast protein liquid chromatography on Superdex 75 [4], also laccase

from *Fomitiporia mediterranea* was purified through concentrated crude extract by tangential ultra filtration, precipitation by ammonium sulphate saturation, ion-exchange chromatography on DEAE-cellulose and gel filtration by Superdex 200 column chromatography [5], While, laccase from a newly isolated *Trichoderma harzianum* WL1 was purified by ultra filtration, Sephadex G-100 column chromatography and Concanavalin-A affinity chromatography [6], and the purification of laccase from white rot fungus *Pleurotus sajorcaju* was carried out using ammonium sulfate, DEAE-cellulose and Sephadex G-100 column chromatography [7].

Laccase is considered to be a potentially important industrial enzyme. It can be applied extensively in many fields [8], laccase play an important role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant, removal of endocrine disruptors, pulp delignification, pesticide or insecticide degradation, organic synthesis, waste detoxification, textile dye transformation, biosensor and other applications [3][9].

Polyphenols are phytochemicals, including a large variety of structures from small molecules, polyphenols are important components of food possessing, anti-inflammatory, antioxidative, chemopreventive and neuroprotective activities, the estimated intake from vegetables and fruits of the dietary polyphenols is approximately 1 g/day, but, During the oxidative metabolization of these compounds, they give rise to toxic molecules like the superoxide anion ($.O^{-2}$) which may produce DNA damages, simultaneously, processing in air presence the food containing phenols, dark polymers are obtained as oxidation products, that fact leading to change of food quality, like browning color that happened by enzymatic reaction, therefore different treatments for avoiding such transformations have been suggested, among these a free or immobilized laccase treatments [10][11]. So, the present study aimed to purification and characterization of laccase from leaves of *Lactuca serriola* L. that available in Iraq as a wild plant as an economic source and use enzyme to removal phenol compound from Apple fresh juices.

2. MATERIALS AND METHODS

2.1 Plant material:

Fresh green Prickly lettuce leaves were collected form the University of Baghdad campus land in May 2015. The leaves were washed in water to remove dirt and dust and dried to use it as a source to enzyme extraction.

2.2 Enzyme assay and protein estimation:

The laccase activity was determined using ABTS as the substrate [12]. The assay mixture contained 5mM ABTS, a 100mM sodium acetate buffer (pH 5.0), and 100µl aliquots of an appropriately diluted enzyme solution. The oxidation of ABTS was monitored spectrophotometrically by measuring the increase in the A420 (ε=36,000$M^{-1}cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1µmole of ABTS per minute at 25°C. The protein concentration was estimated using a Bradford assay [13] with bovine serum albumin as the standard and 595nm absorbency.

2.3 Enzyme extraction from Prickly lettuce:

crude extract was prepared by homogenizing 20g Prickly lettuce leaves in 60ml Tris-HCl buffer 100mM, pH 7.5 that containing 0.1% (w/v) polyvinylpyrrolidone (PVP) and 7mM mercaptoethanol by using blender. The homogenate was centrifuged at 5000rpm for 30min at 4°C to obtained supernatant [1].

2.4 Purification of laccase:

Laccase was purified according to the method of [5] with partially modified as follows. Crude extract 200ml was brought to 40-80% saturation $(NH_4)_2SO_4$ and centrifuged at 5000rpm for 30min. The resulting precipitate was dissolved in distilled water and dialyzed against distilled water overnight at 4°C and concentrated by polyethylene glycol (PEG 20000) to 10ml which loaded on a DEAE-Cellulose column (1.5×30cm). Sodium phosphate buffer 0.05M, pH 5 was used as calibration and washing purification step. The elution step Carried out by gradient NaCl 0-1M in the same buffer. Fractions separated at a flow rate of 36ml\hour were monitored by a UV-Spectrophotometer at 280nm and determination

of enzyme activity (U\ ml). The active fractions were collected and loaded on a Sephacryl S-200 column (1.5×60cm) which equilibrated with sodium phosphate buffer 0.05M, pH 7 containing 0.15M NaCl at a flow rate of 18ml\hour. Active fractions were pooled, dialyzed against distilled water overnight at 4°C and lyophilized by Freeze dryer.

2.5 Molecular weight estimation:

The molecular weight of the enzyme was estimated by gel filtration chromatography on a Sephacryl S-200 column [12]. The molecular weight markers used were lactoferrin 80kDa, bovine serum albumin 67kDa, ovalbumin 43kDa and trypsin 23kDa.

2.6 Spectroscopic studies:

The absorption spectrum was determined at room temperature between 400 and 600nm on a visible spectrophotometer [5].

2.7 Carbohydrate content:

Carbohydrate content of purified laccase was determined by phenol sulfuric acid (Dubois et al., 1956).

2.8 Characterization of laccase:

The effect of optimum pH on laccase activity and stability was determined within a pH range of 2-8 (pH 2-4; 50mM citrate-phosphate buffer, pH 4.5-6; 50mM sodium-acetate buffer, pH 6.5-8; 50mM Tris-HCl buffer) using ABTS as the substrate, optimum pH for stability was determined after laccase incubation with buffer for 15min. Optimum temperature for activity of the purified laccase was examined over a temperature range of 30-80°C, at the optimum pH value and with ABTS as the substrate. Optimum temperature for stability was determined after laccase incubation within limited temperature for 15min. The effects of various inhibitors and metal ions on purified enzyme activity were determined using assay mixtures 2ml, comprised of appropriately diluted enzyme, 50mM sodium acetate buffer pH 4.8, and 0.1mM ABTS, with various concentrations of inhibitors or 5mM metal ions. Laccase activity in the absence of inhibitors or metal ions was defined as 100%. Km and Vmax values of the pure laccase were determined by measurement of enzyme activity with various concentrations 0.01-0.1mM of ABTS as the substrates. Kinetic constants were calculated by the Michaelis-Menten method [15].

2.9 Application:

2.9.1 Apple juice preparation:

Apple juice was prepared after removing the seeds by using a blender and centrifugation at 3000rpm for 10min to remove the precipitate.

2.9.2 Determination of total phenolic compounds:

Phenolic compounds concentration before and after treated with laccase was determined by using Folin-Ciocalteau method, and gallic acid (3,4,5-trihydroxybenzoic acid) was used to prepare calibration curves with concentration between 10-100mg\ml, all samples were measured by absorbance at 765nm [16].

2.9.3 Effect of laccase activity and time on the removal of phenolic:

The effect of laccase activity on the removal of phenolic compounds was studied by add 1ml of enzyme solution 1, 5, 10 and 20U/mg in citrate buffer 20mM pH 5 to the 1000ml apple juice and incubation at 35°C and 50rpm for 30min. The reaction mixture was then analyzed for remained phenolic concentration (%) as described above. The effect of time was studied by add 10U/mg to the apple juice and incubation for 10, 20, 30, 40, 50 and 60min at the same experiment conditions [17].

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3. RESULTS

3.1 Purification of the enzyme:

Laccase from Prickly lettuce leaves was purified through three sequential steps using $(NH_4)_2SO_4$, ion-exchange (Figure 1) and gel filtration (Figure 2) chromatography. At the end of the process, enzyme was 17.04 fold purification and yield of 76.66% with a specific activity of 141.996 U/mg (Table 1).

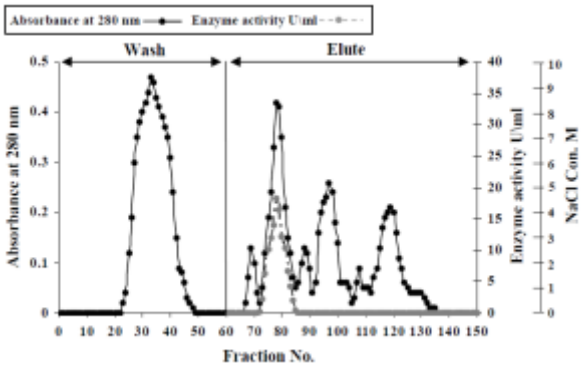

Figure 1: purification of Laccase by DEAE-Cellulose column (1.5×30cm). Sodium phosphate buffer 0.05M, pH 5 was used as calibration and washing purification step. The elution step Carried out by gradient NaCl 0-1M in the same buffer. Fractions separated at a flow rate of 36ml\hour.

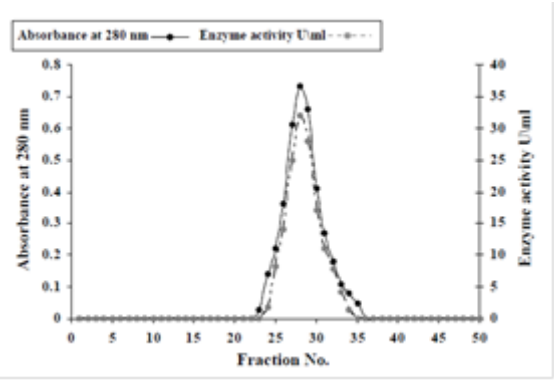

Figure 2: purification of Laccase by Sephacryl S-200 column (1.5×60cm). Sodium phosphate buffer 0.05M, pH 7 containing 0.15M NaCl was used as calibration and elution at a flow rate of 18ml\hour.

Table 1: Purification steps of laccase from Prickly lettuce (*Lactuca serriola* L.) leaves.

Step	Volume (ml)	Protein (mg\ml)	Total protein (mg)	Enzyme activity (U\ml)	Specific activity (U\mg)	Total activity (U)	Fold purification	Yield (%)
Crude extract	200	0.72	144	6	8.33	1200	1	100
Precipitation 40-80% $(NH_4)_2So_4$	10	1.37	13.7	103.34	75.43	1033.4	9.06	86.17
DEAE-Cellulose	33	0.29	10.44	29.58	102	976.14	12.24	81.36
Sephacryl S-200	27	0.24	9.36	34.07	141.96	919.89	17.04	76.66

3.2 Molecular weight estimated:

The molecular weight of purified laccase was 69.37kDa as estimated by from gel filtration chromatography Sephacryl S-200 with standard proteins (Figure 3).

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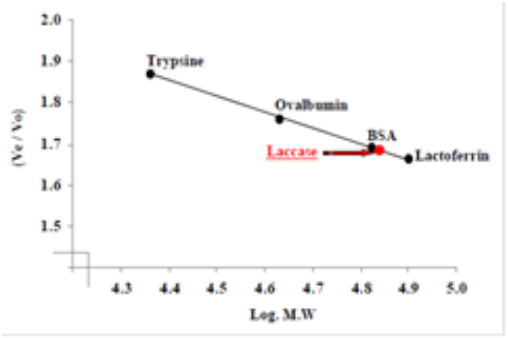

Figure 3: Molecular weight estimated of purified laccase by gel filtration chromatography Sephacryl S-200 with standard proteins

3.3 Spectroscopic studies:

The visible absorption spectrum of the purified lactase showed one peak at 618nm that is typical for type I Cu (II) (Figure 4)

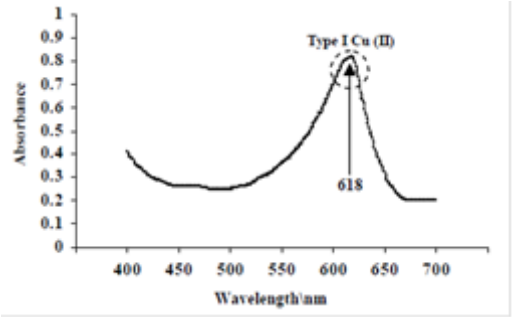

Figure 4: visible absorption spectrum of the purified laccase from Prickly lettuce (*Lactuca serriola* L.) leaves to detect type of Cu

3.4 Carbohydrate:

Carbohydrate content of purified laccase was 12% as determined by phenol sulfuric acid.

3.5 Characterization of laccase:

3.5.1 Effect of pH activity and stability:

The optimum pH activity for the laccase was 6 and enzyme was stable between 4-7 and its loss about 70 and 55% from original activity at pH 2 and 8 respectively (Figure 5).

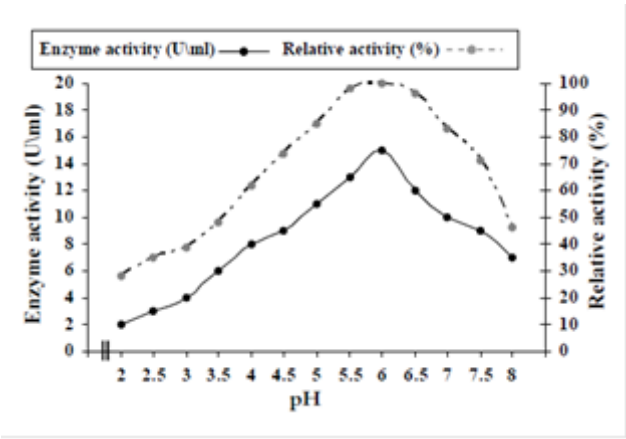

Figure 5: Effect of optimum pH activity and Stability of laccase from Prickly lettuce (*Lactuca serriola* L.) leaves.

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3.5.2 Effect of Temperature Activity and Stability:

Temperature activity of purified laccase was 40 and enzyme was stable at 60°C for 15min. and it loss more than 95% of its original activity at 80°C for same time (Figure 6).

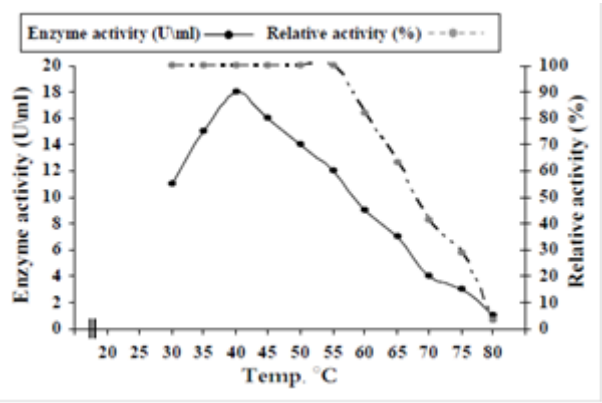

Figure 6: Effect of optimum temperature activity and Stability of laccase from Prickly lettuce (*Lactuca serriola* L.) leaves.

3.5.3 Effects of various inhibitors and metal ions:

The enzyme was 100% inhibited by 10mM of Sodium azide, l-cysteine, dithiothreitol and EDTA and 98, 96, 94, 91, 93 and 82% by SDS, Hg^{+2}, Al^{+2}, Ba^{+2}, Ni^{+2} and Mn^{+2} respectively at same concentration, while inhibition at 1Mm was 45, 39, 62, 59, 51, 53, 48, 55 and 41% respectively (Table 2).

Table 2: Effects of various inhibitors and metal ions in activity of laccase from Prickly lettuce (*Lactuca serriola* L.) leaves

No.	inhibitors	Inhibition (%)	
		1 mM	10 mM
1	Sodium azide	45	100
2	l-cysteine	39	100
3	dithiothreitol	62	100
4	EDTA	59	100
5	SDS	51	98
6	Hg^{+2}	53	96
7	Al^{+2}	51	94
8	Ba^{+2}	48	91
9	Ni^{+2}	55	93
10	Mn^{+2}	41	82

3.5.4 Km and Vmax constant:

The results in (Figure 7) showed that Km and Vmax values were 53.68µM and 641.2µM\min respectively for purified laccase from Prickly lettuce (*Lactuca serriola* L.) leaves.

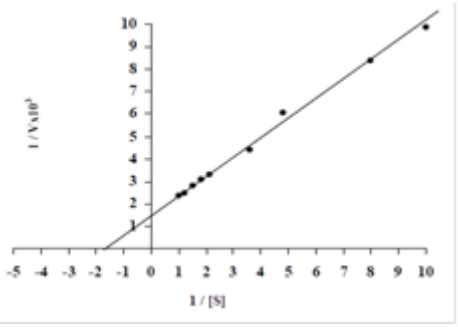

Figure 7: Km and Vmax values were 53.68 µM and 641.2 µM\min respectively for purified laccase from Prickly lettuce (*Lactuca serriola* L.) leaves.

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3.6 Application:

3.6.1 Effect of laccase activity and time on the removal of phenolic compounds from apple juice:

The treatment of apple juice with 1, 5, 10, 20U\mg of laccase led to removed 11.35, 44.72, 88.03 and 89.21% of phenol compounds respectively (Figure 8), while the treated with 10U\mg of laccase for 10, 20, 30, 40, 50, 60min led to removed 28.42, 60.93, 88.03, 89.37, 90.01 and 90.04% of phenol compounds respectively (Figure 9).

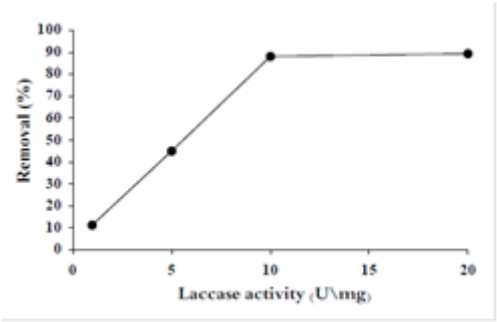

Figure 8: Effect of laccase activity on the removal of phenolic compounds from apple juice by purified laccase from Prickly lettuce (*Lactuca serriola* L.) leaves

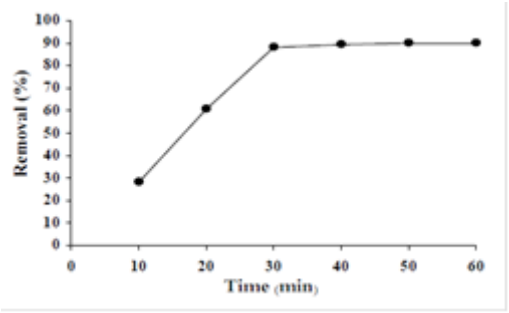

Figure 9: Effect of time on the removal of phenolic compounds from apple juice by purified laccase from Prickly lettuce (*Lactuca serriola* L.) leaves.

4. DISCUSSION

4.1 Purification of the enzyme:

Many researches were referring to purified laccase from different sources by many techniques, but all them were aimed to getting of enzyme in higher purity. For example, Murugesan *et al.* (2006) [7] was purify of laccase from white rot fungus *Pleurotus sajorcaju* by using three steps including ammonium sulfate 70%, DEAE-cellulose, and Sephadex G-100 and got the enzyme in 10.3 fold and 53% yield. While, Ben Younes *et al.* (2007) [18] was able to purify of laccase from the white rot fungus *Perenniporia tephropora* by precipitate with $(NH_4)_2SO_4$ 80% saturation, Bio gel P 100, Mono Q, Mono Q HR and Superdex 200 and got enzyme with 4.17 fold, 8% yield and 180 U/mg specific activity. Also, Sadhasivam *et al.* (2008) [6] could to purified laccase from a newly isolated *Trichoderma harzianum* WL1 by ultra filtration, Sephadex G-100 and Concanavalin-A affinity chromatography with 151.7 purification fold and 0.39% yield. As well, Abou-Mansour *et al.* (2009) [5] notice that the purification of laccase from *Fomitiporia mediterranea* by using $(NH_4)_2SO_4$, DEAE and Superdex chromatography lead to got enzyme with 10 fold purification, 0.5% yield. While, Tapwal *et al.* (2014) [2] was purify enzyme from *Alternaria alternate* by treated with ammonium sulfate saturation of 80% and gel filtration with 4.02 fold purification and 169 U/mg specific activity. Also, Jaiswal *et al.* (2015) [1] refer to Purification of a thermo stable alkaline laccase from papaya (*Carica papaya*) by Con-A affinity chromatography and getting 1376.70 fold and 61.5% yield with specific activity 41.30 U/mg.

4.2 Molecular weight estimated:

Almost, the molecular weight of laccase was estimated by gel filtration chromatography and SDS-PAGE method, so that maybe lead to give different value of enzyme as well as the difference in the source from which is purified enzyme from

it, so, in this regard, Ullrich, *et al.* (2005) [19] used SDS-PAGE and got a single protein bond with a molecular weight of 66kDa for purified laccase from the medicinal mushroom *Agaricus blazei*. Also, Ben Younes, *et al.* (2007) [18] refer that the molecular weight of purified laccase from the white rot fungus *Perenniporia tephropora* was 65kDa as estimated from gel filtration chromatography and 63kDa as determined from SDS-PAGE. While, Mi and Park, (2008) [12] estimated molecular weight of laccase from Basidiomycete *Fomitella fraxinea* by used SDS-PAGE and Sephacryl S-200, the molecular weight of enzyme was 47 and 47.6kDa respectively, while Abou-Mansour, *et al.* (2009) [5] found that the molecular weight of lactase from *Fomitiporia mediterranea* was 60.8kDa when estimated by Superdex chromatography and SDS-PAGE, As well, Zhanga, *et al.* (2010) [4] getting a monomeric protein bond with a molecular weight of 62kDa as estimated by SDS-PAGE for purified laccase from the edible mushroom *Clitocybe maxima*. Also Tapwal, *et al.* (2014**)** [2] used SDS-PAGE and got a single protein with a molecular weight of 45kDa for purified Laccase from *Alternaria alternate*. While, Lee, *et al.* (2012) [15] was purified laccase from yeast *Yarrowia lipolytica* and he found that molecular weight it 67kDa when estimated by SDS-PAGE and Sephacryl S-300. Moreover, Jaiswal, *et al.* (2015) [1] refer that most of the plant laccases reported so far was found to be monomeric having subunit molecular within the range of 60-100kDa.

4.3 Spectroscopic studies:

In the holoenzyme form, most laccases have four copper atoms per monomer, these copper atoms are classified in three groups using UV/visible spectroscopy, the type I copper (T1) is responsible for the intense blue color of the enzymes and has a strong electronic absorption around 600nm, the type II copper (T2) is colorless and The study this type of is bound to three nitrogen atoms, the type 3 copper (T3) consists of a pair of copper atoms that give a weak absorbance near the UV spectrum, the T2 and T3 copper sites are close together and form a trinuclear centre in which binding dioxygen and four-electron reduction to water occur [10]. Therefore, many studies were referring to this case. For instance, the purified laccase from the white rot fungus *Perenniporia tephropora* showed a spectrum with two absorption maxima at 280 and 614nm [18]. But, the spectrum of laccase from a newly isolated *Trichoderma harzianum* WL1 had a peak at 608 and 325nm suggesting the presence of types I and III Cu centers [6]. While, the absorption spectrum of laccase from *Fomitiporia mediterranea* showed three peaks at 280, 340 and 620nm, the peak at 620nm is typical for type I Cu (II), and the peak at 340nm suggests the presence of the type III binuclear Cu (II) pair [5].

4.4 Carbohydrate:

Therefore laccase was considered and identified as a glycoprotein and many studies suggest that [6][15]. For instance Madhavia and Lele (2009) [10] indicate that Laccase is a glycosylated monomer or homodimer protein generally having fewer saccharide compounds 10-25% in fungi and bacteria than in the plant enzymes and noted that the carbohydrate compound contains monosaccharides such as hexoamines, glucose, mannose, galactose, fucose, and arabinose, and Glycosylation in laccase is responsible for secretion, proteolytic susceptibility, activity, copper retention, and thermal stability.

4.5 Characterization of laccase:

4.5.1 Effect of pH activity and stability:

Many studies have referred to different value for optimum pH of laccase that purified from many sources. It was 5.5 from the medicinal mushroom *Agaricus blazei* [19]. 5.0 of laccase produced by a white rot fungus *Pleurotus sajorcaju* [7]. 3.0 For the purified laccase from *Pycnoporus sanguineus* [20]. 5.0 And 4.0 of laccase from the white rot fungus *Perenniporia tephropora* were for ABTS and 2,6-DMP, respectively and enzyme was more stable at alkaline values of pH 8.0 than at acidic values of pH [18]. 4.5 From a newly isolated *Trichoderma harzianum* WL1 [6]. Between 6.8-7.4 for laccase from *Rhus vernicifera* [10], and 3-5 with optimum activity at pH 4.5 for enzyme from *Alternaria alternate* [2]. On the other hand, it was 8.0 and 6 with catechol and ABTS, respectively for thermo stable alkaline laccase from papaya (*Carica papaya*) [1].

The differences in optimum pH between fungal and plant laccase it may be caused by the variance in physiological functions, the acidic conditions of fungal growth may be lead to produce low pH optimum of enzyme that responsible of mechanism for removing toxic phenols from growth medium under natural conditions, while, plant laccase that have main role in synthetic process such as lignin formation, being intracellular have their pH optimum nearer to the physiological range, In addition [10].

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4.5.2 Effect of Temperature Activity and Stability:

The optimum temperature values of laccase can vary considerably dependence on the source of the enzyme, so, the differences in optimum temperature for activity and stability of laccases from different sources might be due to the variance in the number of disulphide bonds in the enzyme structure [1]. On this basis, many studies have pointed to this subject, for example, the optimum temperature of laccase from white rot fungus *Pleurotus sajorcaju* was 40°C [7]. Laccase from the white rot fungus *Perenniporia tephropora* was very stable at low temperatures, but it was inactivated rapidly at temperatures above 50°C, and completely inactivated within 2hour at 70°C [1]. While it was 70°C from Basidiomycete *Fomitella fraxinea* for ABTS oxidation and enzyme remained stable up to 40°C [12]. Also, it was 60°C from the edible mushroom *Clitocybe maxima* [4]. The purified laccase from *Alternaria alternate* has a broad temperature sensitive 35-70°C and the optimum temperature for it was observed at 65°C [2]. Moreover, the activity of laccase from papaya (*Carica papaya*) was increased up to 70°C and after wards decreased rapidly [1].

4.5.3 Effects of various inhibitors and metal ions:

In general, laccases respond similarly to several inhibitors of enzyme activity. Therefore many chemicals and metal ions were used in this field to find out its effects on the enzyme such as laccase from *Pycnoporus sanguineus* which strongly inhibited activity by sodium azide, l-cysteine, and dithiothreitol [20]. Also Sadhasivam, *et al.* (2008) [6] found that enzyme from newly isolated *Trichoderma harzianum* WL1 was completely inhibited activity by sodium azide at the concentration of 20µM and several metal cations, especially Hg^{+2}. As laccase produced by a white rot fungus *Pleurotus sajorcaju* which completely inhibited activity by sodium azide [7]. While effect of metal ions on the laccase activity from Basidiomycete *Fomitella fraxinea* was tested using ABTS as the substrate, the enzyme was 57, 43, 51 and 52% inhibited by 1 mM of Ni^{+2}, Al^{+2}, Mn^{+2} and Ba^{+2} respectively, slightly stimulated by 1mM of K^{+} and Ca^{+2}, and essentially unaffected by 1mM of Cu^{+2} where the laccase was completely inhibited by 0.5mM sodium azide, l-cysteine, DTT, EDTA, DMSO, and SDS [12]. As well the effect of various effectors namely, metal ions, reducing agent (DTT), chelating agent (EDTA) and detergent (SDS) on thermo stable alkaline laccase from papaya (*Carica papaya*) activity were studied by Jaiswal, *et al.* (2015) [1] who noted that Mn^{+2}, Cd^{+2}, Ca^{+2} and Na^{+} activated laccase in 0.1-10mM concentration, while enzyme inhibited at concentrations beyond 0.1mM with complete inhibition inactivity at 10mM SDS and EDTA also inhibited laccase activity.

The inhibition of laccase activity by EDTA might be due to the metal chelating of Cu in the catalytic site of the enzyme. However, the change in the conformation of the protein structure might be the cause of the inhibition of activity by SDS, also the small anions such as halides (excluding iodide), azide, cyanide, and hydroxide bind to the type 2 and type 3 copper, resulting in an interruption of the internal electron transfer and activity inhibition [10].

4.5.4 Km and Vmax constant:

The study of Km and Vmax value is consider important on of characteristic studies, because it provide some parameter of enzyme, substrate and the affinity between them, so different methods were used to study this parameter depending on the type of substrate and varieties in concentration [21]. Such as Ullrich, *et al.* (2005) [19] who noted that kinetic constants were determined (K_m, k_{cat}) for laccase from the medicinal mushroom *Agaricus blazei* were 63 µM, 21 s^{-1} for ABTS, 4 µM, 5 s^{-1} for syringaldazine, 1,026 µM, 15 s^{-1} for DMP and 4307 µM, 159 s^{-1} for guaiacol, and Sadhasivam, *et al.* (2008) [6] who found that purified laccase from a newly isolated *Trichoderma harzianum* WL1 showed K_m values of 180 and 60 µM, respectively, and V_{max} values of 3.95 and 1.42 U mg^{-1} protein, respectively, for the substrates ABTS and guaiacol. The apparent Km and Vmax values of laccase from Basidiomycete *Fomitella fraxinea* for ABTS and 2,6-DMP were determined from a Lineweaver-Burk plot, where the Km values were 270 and 426 µM, respectively, and the Vmax values were 876 and 433.3 µM/min, respectively [12]. Also, the Km of laccase from *Fomitiporia mediterranea* was 25 mM for 2,6-dimethoxyphenol (DMP) (2.5-250 mM) as substrate for laccase [5]. Moreover, the Km and Vmax values of laccase from papaya (*Carica papaya*) were found to be 0.04, 1.48 and 11.33mM, and 0.04, 0.15 and 0.46M.min^{-1}.ml^{-1} for ABTS, catechol and hydroquinone respectively [1].

4.6 Application:

4.6.1 Effect of laccase activity and time on the removal of phenolic compounds from apple juice:

Phenolic compound that found in foods and juices can be lead to change in color that It is well known that browning reaction by effect of oxidoreductase enzymes and some chemical reactions, so various pre and post treatments are

available to avoid this case of fruit juices like used gelatin, bentonite, silica gel and others materials to increase stability of these products. Many enzymatic treatments have been used for fruit juice stabilization, and laccase is one among them. Some results reported that laccase treatment increased the susceptibility of apple juice browning during storage [10].

Same results was observed in study of Okazaki, *et al.* (2002) [22] which indicated that increasing of laccase concentration (originated from *Coriolus versicolor*) from 0 to 50µg/ml enhanced BPA removal to 100%. Chakroun, *et al.* (2010) [23] has used purified laccase from *Trichoderma atroviride* 0.3U/ml for elimination of phenolic pollutants including 2,4-dichlorophenoxyacetic acid, 4-chlorophenol, *o*-cresol or and catechol led to 21, 28, 100 and 100% removal of pollutants respectively after 24 h incubation. Also Lee, *et al.* (2012) [15] was found that Purified laccase from yeast *Yarrowia lipolytica* removed 30-52% of the phenolic compounds in rice straw Prehydrolysate. According to Asadgol, *et al.* (2014) [17] the purified laccase from in *Paraconiothyrium variabile* 5U/ml could remove 80 and 59.7% of phenol and bisphenol A respectively after 30 minutes of treatment of phenolic pollutants. Moreover, Kushwah, *et al.* (2014) [8] indicate that purified laccase from *Agaricus bisporous* could be used for the detection of phenolic compounds in waste water samples, because he obtained the signals from the waste samples were found to be very similar with the standard phenol solutions having the same concentration.

5. CONCLUSION

Results of this study was showed the possibility of laccase purification from Prickly lettuce (*Lactuca serriola* L.) uncomplicated steps and get the enzyme in high activity and good yield, as well as the use of enzyme to removal of phenolic compound from some foods.

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