STUDY ON BIOSYNTHESIS OF LACCASE ENZYME FROM FUNGI AND APPLICATION OF HYDROLYSIS OF TEA LEAVES TO OBTAIN BIOACTIVE POLYPHENOLS

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ABSTRACT

Laccase (EC 1.10.3.2) is a poly copper oxidase found in plants, fungi, and bacteria. In this study, 21 fungal strains were screened for laccase activity, strain *Clitopilus* sp. BV8 showed the highest activity at 7,943.1 U/L. Fungal strains were fermented for 7 days on medium containing the following components: $MgSO_4$ (0.5 g/L); KH_2PO_4 (1.5 g/L); glucose (5 g/L); pH 6.0, tomato (2%), urea (2 g/L) and cultured at 25°C under the condition of continuous shaking at 200 rpm, the highest laccase activity was 65 U/mL. Crude laccase enzyme was concentrated from the culture solution with optimum temperature and pH at 40°C and pH 5.0, respectively. Total polyphenol content released and antioxidant activity from green tea leaf extract supported by laccase enzyme were also evaluated. The results showed that in optimal conditions for hydrolysis of tea leaves in 18 hours, the enzyme/substrate ratio was determined to be 5.0 U/gds at pH 5 at 40°C. Total polyphenol content and antioxidant activity reached the highest values of 136.15 mgGAE/g and 78.05%, respectively.

Keywords: *Tea leaves, polyphenol, antioxidant activity, laccase, oxidative enzymes.*

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1. INTRODUCTION

Laccases, which are versatile blue polyvalent oxidases with the capacity to oxidize a diverse range of both aromatic and non-aromatic compounds, are frequently studied in fungi due to their superior redox potential compared to that of bacteria and plants [1]. Numerous filamentous fungi isolate with elevated laccase biosynthesis have been identified, including, such as *Trametes versicolor* [2], *Streptomyces cyaneus* [3], *Melanocarpus albomyces* [4], *Trametes modesta* [5]. Many white rot fungi, in particular, are engaged in lignin metabolism. Laccase is produced by *Pycnoporus cinnabarinus* as a lignin-degrading enzyme, while laccase is produced by *Pycnoporus sanguineus* as a phenol oxidase. Laccase is a polyphenol oxidase whose active site is rich in copper. Laccase is able to oxidize a variety of structures that do not contain phenolic groups and polyphenolic compounds by utilizing small molecules as intermediate electron carriers [6]. Most people are interested in the polyphenol chemicals found in tea leaves. Polyphenols are substances that are found naturally in plants and are very powerful antioxidants. Tea leaves contain polyphenols that are significantly distinct from those in other plants. Catechin compounds make up the majority of the primary ingredients. Green tea catechins, which have potent antioxidant properties, have been employed extensively in functional meals up till now. Free radicals may be eliminated by natural antioxidants like polyphenol compounds, which is an extremely efficient way to stop oxidation [7]. The body is protected by polyphenols against several ailments brought on by free radicals. They include aromatic rings with one, two, three, or more hydroxyl groups (OH) immediately linked to the benzene ring in their

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molecules. The physicochemical attributes or biological activity vary depending on the amount and placement of the -OH groups within the chemical structure. Polyphenols in the cell wall of green tea leaves are often locked together by hydrogen bonds and der Waals bonds, making them difficult to dissolve in water and absorb [8]. To separate these polyphenols, synergistic oxidizing enzymes such as laccase and lignin peroxidase must be used to break these bonds and liberate the polyphenols into smaller, more water-soluble molecules. When oxidizing enzyme preparation is introduced to hydrolyze green tea leaves, it acts on the polyphenols by converting them to enzyme intermediates such as reactive radics (which alter by adding or withdrawing an electron). These intermediates then act on other polyphenols to disrupt their bonds. Other extraction and purification processes were used to get the polyphenols emitted by this network. The enzyme-assisted extraction approach refers to the use of enzyme laccase from the *C. prunulus* BV18 fungus to increase the effectiveness of polyphenol extraction in green tea leaves [9, 10]. The enzyme precisely breaks down the structural components enclosing the plant cells, allowing the solvent and extract to come into touch more easily. Methods of extracting bioactive substances from tea leaves require cell and membrane disruption, and enzymes such as laccase can help strengthen them [11]. Extraction of bioactive compounds from tea leaves. To add originality to the study of deriving biologically active substances from plants, we conducted a study on the application of laccase enzyme to the degradation of green tea leaves in order to obtain bioactive polyphenols.

2. MATERIALS AND METHODS

2.1. Materials

Ba Vi National Park (Ha Noi, Vietnam) was the source of the isolation and purification of 21 studied fungal isolates. They are kept in the laboratory, HaUI Institute of Technology, Hanoi University of Industry. Tea leaves (*Camellia sinensis*) were obtained from Thai Nguyen province (Vietnam). Samples were dried to constant weight at 60°C, ground into powder and stored in desiccant bags, and kept at a cool and dry place.

2.2. Enzyme assay

Laccase activity was determined by the ABTS oxidation method (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) The reaction solution consisted of 50 µl enzyme solution, 100 µl sodium acetate buffer (100 mM, pH). 5), 50 µl ABTS (10 mM) Samples were incubated at 40°C for 30 minutes, spectrophotometrically measured at 420 nm [12].

2.3. Determination of suitable culture conditions for laccase

Effect of carbon and nitrogen sources

The initial basal medium includes $MgSO_4$ (0.5 g/L); KH_2PO_4 (1.5 g/L); glucose (5 g/L); yeast extract (2 g/L); pH 6.5; and supplement (2%) of different substrate sources: Straw, corn, potato, tomato, and soybean. Meanwhile, yeast extract nitrogen for fungal growth from basic culture medium was substituted and other sources tested including (2 g/L): KNO_3; $(NH_4)_2SO_4$; peptones; $NaNO_3$; and urea. Fungal strains are fermented at 30°C, from 1 to 9 days, shaking continuously at 200 rpm. Laccase activity was determined from fungal culture.

Effect of temperature and pH

Fungal strains were cultured on a basic culture medium (pH 6.5) supplemented with substrate (2%) and a suitable nitrogen source. The optimum temperature and pH of the laccase were determined by measuring the enzyme activity according to the method described with the temperature varying from 20 to 40°C and the pH from 3.0 to 8.0.

2.4. Characterization of enzymes

The crude enzyme solution obtained after liquid fermentation was further precipitated with two types of solvents. The aliquot was precipitated with ethanol solvent and ammonium sulfate inorganic salt with concentrations ranging from 40 to 70%, and then centrifugation (10 min, 4000 rpm) collected protein residue. The fractions obtained after centrifugation, protein residues will be

washed and remixed with 100 mM sodium acetate buffer (pH 5.0), continue to centrifuge to collect protein residues, and repeat 2 - 3 times. The effect of pH on laccase activity was tested with pH from 4.0 to 8.0 in 100 mM sodium acetate buffer (pH 4.0 - 5.5) and 100 mM sodium phosphate (pH 6.0 - 8.0). The optimum temperature was determined at 30 to 60°C on ABTS substrate in sodium acetate buffer (100 mM) at pH 5.5.

2.5. Determining suitable conditions for extracting tea leaves by laccase

Tea leaves (5.0 gram per reaction) were milled for 5 minutes to powder, added water (1 : 5, w/v) and sonicated at 40^0C for 10 min. And then, the samples were incubated with laccase enzyme (10 U/gds) from *Clitopilus* sp. BV8 at 40°C and monitored at 2, 18, 21, 24, 42 and 48 hours. Moreover, the effects of different laccase activity [U/gds, U of enzyme laccase per gram dried tea leaves (substrate)] were investigated, including 1.0, 2.0, 3.0, 4.0, and 5.0 U/dgs (laccase) at 40°C, pH 5.0 during 18 hours. The reaction was carried out in 100 mM MOPS buffer (pH 5.0), and for comparison purposes, a control with heat - inactivated enzyme (95°C for 30 min) was used. The hydrolyzate solution was filtered with Whatman filter paper No.41 and centrifuged for 30 minutes at 10,000 rpm. The supernatant extract was collected and dried at 50°C. The obtained samples were analyzed for their total polyphenol content and antioxidant activity.

2.6. Determination of total polyphenol content

Polyphenols in the extract were determined by colorimetry using the Folin - Ciocalteu reagent. This reagent contains a phosphor - tungstenic acid oxidizing agent, during reduction, phenol hydroxy groups are easily oxidized, this oxidizing agent produces a blue color with maximum absorbance at 765 nm. The polyphenol content in the sample is proportional to the sample strength and is calculated as gallic acid. The reaction mixture consists of 1 ml of sample to be quantified, 6 ml of distilled water, and shaken well. Then add 0.5 ml Folin-Ciocalteu reagent 10%, shake well, and let stand. After 5 minutes, add 1.5 ml Na_2HCO_3 20%, shake well. Add distilled water to a volume of 10 ml. Keep test tubes in the dark for 2 hours then measure the absorbance of all samples on a prepared spectrophotometer at 765 nm [13].

2.7. Determination of antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of an extract sample was determined using the method described by Brand-Williams *et al.* (1995) [14]. The sample of desiccated extract was dissolved in DMSO (100%). DPPH was combined with 96% ethanol. The absorbance of a sample was measured at 515 nm using an ultraviolet spectrophotometer. The free radical scavenging capacity (SC, %) of the extract was calculated as follows [14]:

$$SC\ (\%) = \left[100 - \frac{Abs(test) - Abs\ (blank)}{Abs\ (negative)} \times 100\right] \pm \sigma$$

The standard deviation (σ) is calculated by Duncan's formula as follows:

$$\sigma = \sqrt{\frac{(\sum x_i - \bar{x})^2}{n-1}}$$

3. RESULTS AND DISCUSSION

3.1. Screening for fungi with high Laccase activity

The ability of 21 fungal isolates to biosynthesize Lac enzyme was measured by their ability to oxidize ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, Sigma). The results of determining the laccase activity of 21 fungal strains after centrifugation to remove biomass and impurities are presented in table 1.

The screening findings for laccase activity indicated that, 8 of the total 21 strains exhibited activity, equivalent to 38% with activity from 40.6 to 7,943.1 U/L. In which, some strains showed high activity such as *Polystitus* sp. BV5 (454.7 U/L) and *Nemania* sp. BV17 (564.2 U/L). In particular, the strain *Clitopilus* sp. BV8 (7,943.1 U/L) exhibited the highest activity.

Related to this research direction, Thuy *et al.* (2015) isolated and characterized the morphological characteristics of 5 mold strains (BN1, BN2-1, BN2-2, DA3-1 and BV1) with active

laccase from 1,480 to 2,472 U/L. Which, strain BV1 belongs to the genus *Meruliporia* sp. and is the strain with the highest enzyme biosynthesis ability reaching 2,472 U/L and the highest ratio of enzyme activity to dry weight after 5 days of culture (E/M) of 54.04 U/mg [15]. According to Mai *et al.* (2012)[16], *Trametes versicolor* fungus used glucose substrate as an inducer, and the highest enzyme activity was obtained at 4,250 U/L after 7 days of culture at shaking speed 200 v/p, pH 6.0 [17]. Thus, in this study, the strain *Clitopilus* sp. BV8 showed high activity compared with some remaining strains to be studied further.

Table 1. Laccase enzyme activity of fungal strains

TT	Fungal strains	Laccase (U/L)
1	*Auricularia* sp. BV1	137.5 ± 0.2
2	*Ganoderma* sp. BV2	0
3	*Trametes* sp. BV3	0
4	*Bisporella* sp. BV4	0
5	*Polystitus* sp. BV5	454.7 ± 0.5
6	*Lecanicillium* sp. BV6	0
7	*Fomitopsis* sp. BV7	0
8	*Clitopilus* sp. BV8	7,943.1 ± 0.4
9	*Tyromyces* sp. BV9	232.6 ± 0.4
10	*Mycena* sp. BV10	0
11	*Xylaria* sp. BV11	0
12	*Trametes* sp. BV12	0
13	*Ganoderma* sp. BV13	40.6 ± 0.2
14	*Hexagonia* sp. BV14	0
15	*Tyromyces* sp. BV15	0
16	*Coriolus* sp. BV16	126.3 ± 0.1
17	*Nemania* sp. BV17	564.2 ± 0.3
18	*Nigrospora* sp. BV18	252.1 ± 0.6
19	*Inonotus* sp. BV19	0
20	*Psathyrella* sp. BV20	0
21	*Mycena* sp. BV21	0

3.2. Optimal conditions for the production of laccase by the fungus *Clitopilus* sp. BV8

Effect of carbon and nitrogen sources:

The strain *Clitopilus* sp. BV8 was cultured on a medium supplemented with carbon and nitrogen sources from 0 to 9 days of fermentation at 30°C, pH 6.5. The ability to produce laccase enzymes was significantly affected by different carbon sources. The highest enzyme activity was 46 U/mL in the medium supplemented with tomato substrate and the lowest was 18 U/mL in the medium supplemented with corn substrate after 7 days (Figure 1A).

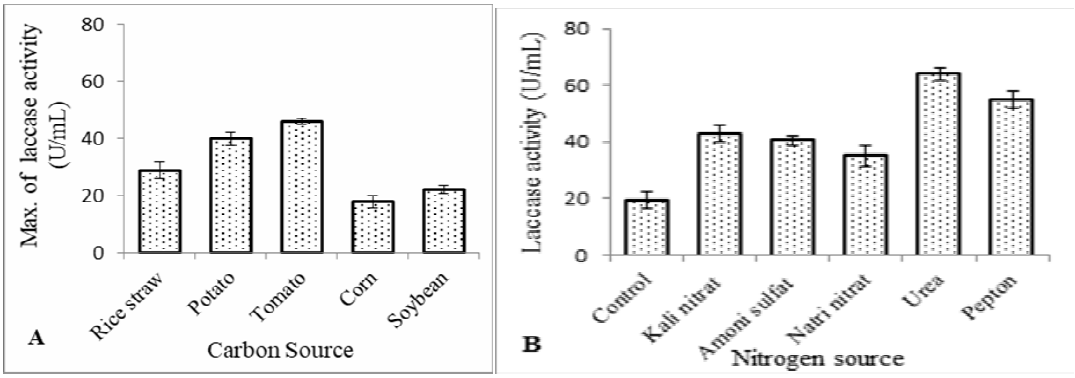

Figure 1. Effect of carbon source (A) and nitrogen source (B) on laccase biosynthesis by *Clitopilus* sp. BV8

The results of figure 1B showed the biosynthesis of laccase was higher than that of the control samples on the media containing nitrogen sources. When a nitrogen source was added to the medium, the laccase activity was between 1.8 and 3.4 times higher than in the control sample. The nitrogen source from urea that biosynthesized the enzyme was highest at 64 U/mL and the lowest from sodium nitrate was 35 U/ml after 9 days of fermentation. Lam and Chien (2013) demonstrated the influence of carbon sources on the growth and development of fungal strains. The fungus *Trametes maxima* CPB30 was surveyed on 8 carbon sources and found that the laccase activity in the medium containing galactose was the lowest at 18 U/ml. In cultures containing other carbon sources, the fungus grew quite well, however, PDA medium was suitable for laccase biosynthesis (579 U/mL) [17]. According to Mai *et al.* (2012), the nitrogen source used 3 g peptone and 1.5 g peptone combined with 1.5 g $(NH7_4)_2SO_4$ for high enzyme activity from 101 U/mL to 120.5 U/ml. However, when there is a combination of inorganic nitrogen and organic nitrogen sources, which can change the pH of the medium, the synthesis of laccase enzyme is higher. The addition of nitrogen source affects the culture medium, and adjusts the pH of the culture medium to facilitate the synthesis of laccase enzyme [16]. Thus, carbon sources from tomatoes and nitrogen from urea increased the biosynthesis of laccase after 7 days of fermentation of *Clitopilus* sp. BV8.

3.3. Effect of temperature and pH

The strain *Clitopilus* sp. BV8 produced the highest laccase enzyme at 25°C (69 U/mL) and the lowest at 40°C (16 U/mL). When the temperature increases from 20 to 25°C, the ability of the fungus to produce enzymes also increases. However, when the temperature increased above 30°C, the fungus did not grow well, so the enzyme activity decreased (16 U/mL) (Figure 2A).

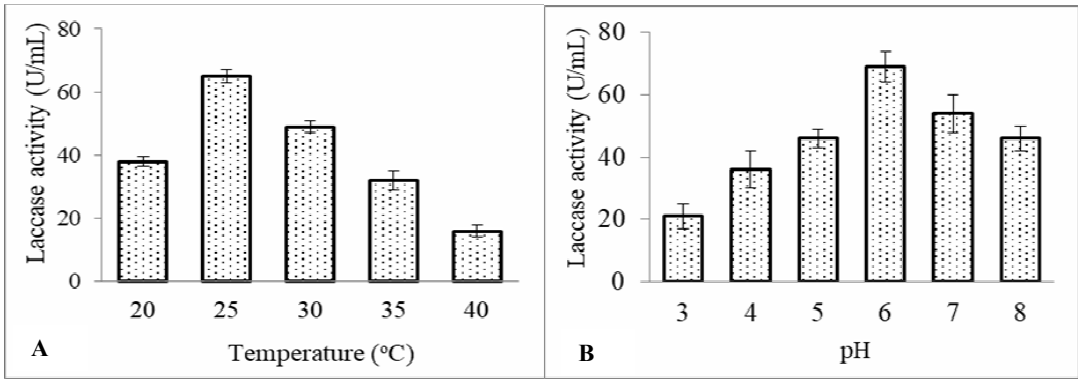

Figure 2. Effect of temperature (A) and pH (B) on laccase biosynthesis by *Clitopilus* sp. BV8

The strain *Clitopilus* sp. BV8 can grow and develop in the pH range from 5.0 to 8.0. The highest laccase activity reached 69.4 U/mL at pH 6.0 and decreased slightly when the pH increased to 7.0 and 8.0. At pH 3, the laccase biosynthetic activity from the fungal strain reached the lowest value of 21.1 U/mL after 9 days. Each fungal species has an appropriate pH range for the best growth and enzyme biosynthesis (Figure 2B). Some studies related to this direction such as Hang *et al.* (2017) studied strain FBV60 (*Pycnoporus* sp. FBV60.) isolated in Ba Vi. Based on morphology and gene sequence, the studied fungus is classified into the phylum Basidiomycetes, genus *Pycnoporus*. The results showed that the fungal strain grew and developed well in the pH range from 4.0 to 9.0, but at pH 6.0, the highest laccase activity was synthesized at 20.47 U/L after 8 days of culture. At pH 3.0 and 10.0, the fungus almost did not grow, the biomass was low and did not produce laccase [18].

3.4. Characterization of laccase from *Clitopilus* sp. BV8 (*Clito*Lac)

The crude enzyme solution obtained by liquid fermentation was further precipitated with various solvents, including ethanol and the inorganic salt ammonium sulfate (40, 50, 55, 60, 65, and 70%). Precipitation was carried out at 18°C, and centrifuged (10 min, 4,000 rpm) to collect protein residue. The fractions obtained after centrifugation and solvent evaporation were reconstituted with 100 mM sodium acetate buffer (pH 5.0) to determine enzyme activity. The results are shown in table 2.

Table 2. Precipitation of crude laccase by different methods

Precipitation method	Concentration (%)						
	40	50	55	60	65	70	Laccase activity (U/mL)
Ethanol	43 ± 0.1	56 ± 0.18	98 ± 0.11	67 ± 0.27	59 ± 0.2	40 ± 0.31	
Amoni sulfate	114 ± 0.15	126 ± 0.25	108 ± 0.28	84 ± 0.25	79 ± 0.14	67 ± 0.32	

The results of table 2 showed that the laccase enzyme activity in the ethanol fractions was low compared to the ammonium sulfate fractions. At the ethanol precipitation fractions, with a solvent concentration of 55%, the highest enzyme activity was 98 U/mL, when the concentration was increased above 55%, the enzyme activity decreased to 40 U/mL at 70%. The highest laccase activity was determined in the ammonium sulfate fractions with a solvent concentration of 50% and the enzyme activity was 126 U/mL. When gradually increasing the solvent concentration, the enzyme activity tended to decrease slightly and reached the lowest value at 70% with enzyme activity of 67 U/mL. The enzymatic activity of ammonium sulfate precipitation was 1.28 times higher than that of ethanol precipitation. Therefore, the method of enzyme recovery by ammonium sulfate inorganic salt is chosen because this method has less effect on laccase activity compared with polar solvent ethanol.

The pH value of laccase was determined between 3.0 and 8.0. The results showed that the laccase enzyme activity obtained from the strain *Clitopilus* sp. BV8 reached a relatively high value in the pH range from 5.0 to 6.0 and reached the highest value of 103 U/mL at pH 5.0. When the pH increased to 6, the enzyme activity decreased slightly by 6 U/mL compared to the enzyme activity at pH 5. After the pH continued to increase, the enzyme activity started to decrease sharply and only reached 55 U/ml at pH 8 (Figure 3A).

Optimal temperature has a great influence on the activity and rate of the reaction, each enzyme only works at a suitable temperature limit. The optimum temperature of the reaction between the enzyme laccase obtained from *Clitopilus* sp. BV8 with ABTS substrate was carried out in the temperature range from 30 to 60°C. The results

showed that the enzyme activity increased gradually from 100 U/ml at 30°C to the highest enzyme activity at 40°C reaching a value of 124 U/mL, and started to decrease gradually after 40°C. After increasing the temperature, the enzyme activity gradually decreased to 17 U/ml at 60°C (Figure 3B).

Thus, the research results showed that laccase biosynthesized from the fungus *Clitopilus* sp. BV8 has an optimum temperature of 40°C and pH 5.

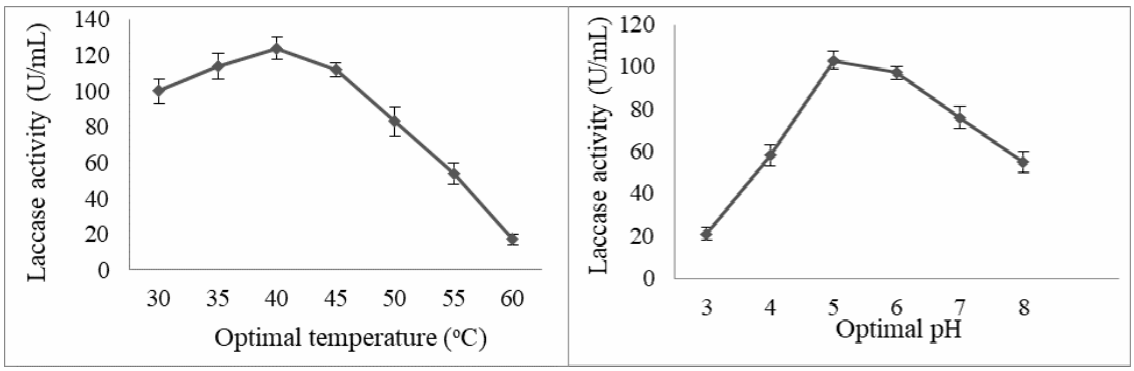

Figure 3. Optimal temperature (A) and pH (B) of *Clito*Lac

3.5. Effect of *Clito*Lac-assisted extraction time on total polyphenol content

The hydrolysis time strongly affects the extraction process of green tea leaves. The longer the extraction time, the higher the efficiency. However, up to a certain point, the extension of extraction time will not be effective. The results of the investigation of the combined hydrolysis time between ultrasound and enzyme on the extraction yield and the main compounds in the extract are shown in figure 4.

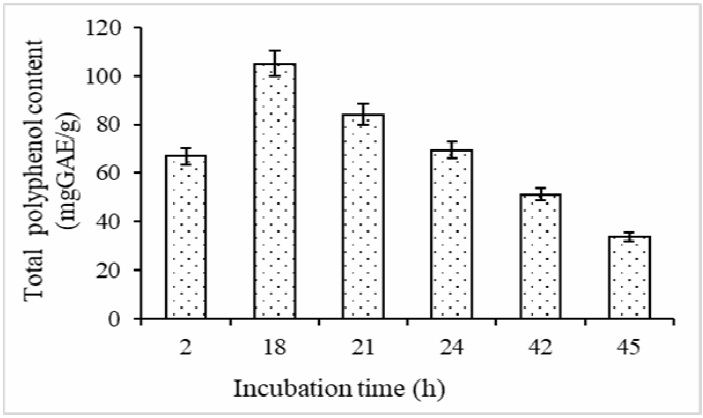

Figure 4. Effect of time on total polyphenols content of tea leaf extract

When increasing the hydrolysis time from 2 hours to 18 hours, the polyphenol content increased rapidly from 66.85 mgGAE/g to 105.04 mgGAE/g and 18 hours is the extraction time to obtain the highest concentration. Continuing to increase the extraction time to 21 hours, the polyphenol content began to decrease, reaching a value of 84.07 mgGAE/g. At 24 hours, the polyphenol content reached 69.49 mgGAE/g. When increasing the time to 42 hours and 45 hours, the polyphenol content decreased sharply reaching 51.29 mgGAE/g and 33.79 mgGAE/g, respectively. It was found that the polyphenol content reached the lowest value at 45 hours and had a high difference compared with the extraction time of 18 hours, reaching a value of 71.25 mgGAE/g. The above results are similar to the study of Thuy *et al.* (2020)[19] on soursop

leaves. If the extraction time is short, the amount of biologically active substances are not completely extracted, but if the time is too long, the active ingredients will be oxidized, and the quantity of the active ingredients will decrease. The survey results showed that the polyphenol content reached the highest level of 94.541 mgGAE/g in the extraction time of 30 minutes. However, quite a lot of reduction between 30 - 45 minutes of extraction [19]. In another study by Quyen *et al.* (2016) on soil gourd leaves. When increasing the extraction time from 10 to 40 minutes, the content increased rapidly from 17.16 to 34.80 mgGAE/g extract and 40 minutes was the time to obtain the highest polyphenol content. However, if the extraction time was increased to 50 and 60 minutes, the polyphenol content increased only slightly and there was almost no difference [20].

3.6. Effect of *Clito*Lac-assisted extraction time on antioxidant activity

The study results show that, the correlation between total polyphenols content for the antioxidant capacity of tea leaf extract. The DPPH free radical technique is frequently used to test antioxidant activity in a very short amount of time when compared to other methods. Several chemical techniques have been investigated to show the strong scavenging potential of tea leaf extract dried extracts. These technologies, however, are not inexpensive and produce a huge number of pollutants. The hydrolysis product of tea leaf extract produced by enzyme-assisted extraction was tested for free radical scavenging activity using DPPH as a free radical in this research [21].

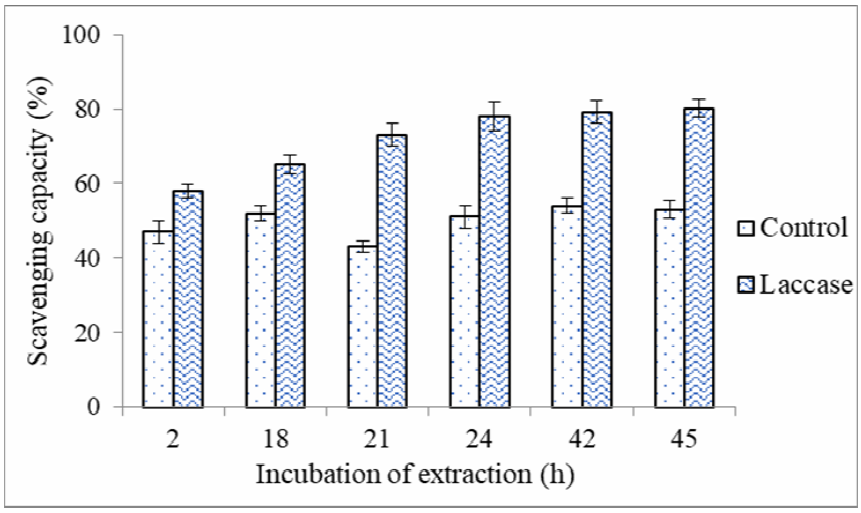

Figure 5. Effect of incubation of extraction on scavenging capacity: (▨) Enzyme-assisted extraction (10U/gds) and (□) inactivated enzyme (control)

Figure 5 shows that, the capacity of dried extract from tea leaf extract for scavenging capacity of free radical. A suitable for total polyphenols content, the hydrolysis products obtained after treatment with laccase showed that, the highest scavenging capacity at 24 hours of incubation (78.23%). A negligible difference was observed with hydrolysis products obtained from 24 hours to 42 hours with scavenging capacity from 79.13 to 80.21%, which were higher than the control (inactivated enzyme) with scavenging capacity from 47.76 to 53.54%.

3.7. Effect of *Clito*Lac enzyme activity on total polyphenol content

The results obtained in figure 3.10 show that enzyme activity has a great influence on polyphenol content. When enzyme activity increased from 1 U/gds to 2 U/gds, the polyphenol content increased sharply from 46.71 mgGAE/g to 90.74 mgGAE/g. However, enzyme activity increased from 3 to 5 U/gds, the polyphenol content increased slightly from 115.32 mgGAE/g to 136.15 mgGAE/g.

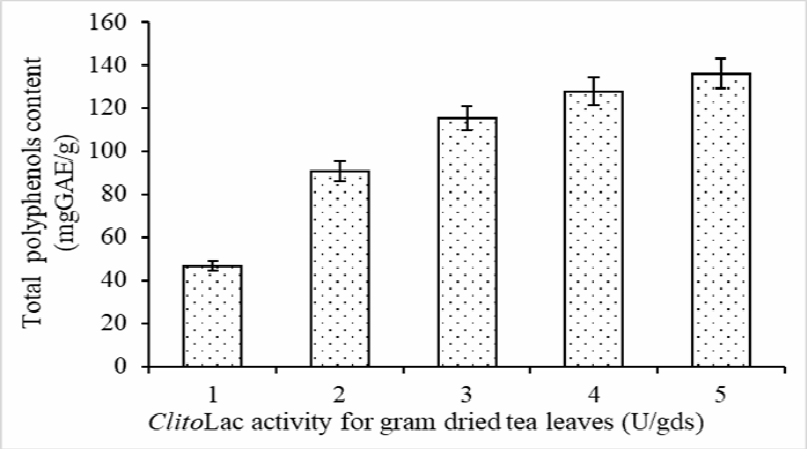

Figure 6. Effect of *Clito*Lac enzyme activity on total polyphenols content of tea leaf extract

It was found that the polyphenol content at the highest value (5.0 U/gds) and the lowest value (1.0 U/gds) had a big difference. This can be explained because when the substrate and enzyme content increase, the reaction rate increases. However, when the enzyme concentration was saturated with the substrate concentration, the reaction rate remained unchanged or increased with increasing enzyme content. So, using laccase enzyme with increasing activity significantly improved the release of products. For that reason, laccase catalyzes the hydrolysis of glycosidic bonds of two or more carbohydrates. Therefore, this enzyme plays a role in the degradation of lignocellulose in plant cell walls. When cells are broken down, it is released from the components of the cell, including proteins, which are known to be complex with polyphenol compounds. Thus, laccase enzyme at 3-5 U/gds was suitable under the above condition. In a study by Dung and Huong (2019) on custard-apple shells using celluclast enzyme. The results showed that, when increasing the enzyme ratio from 0.5% to 1.5%, the polyphenol content increased from 35.89 mgGAE/g to 47.66 mgGAE/g. However, when increasing the enzyme rate to 2.5%, the polyphenol content decreased to 42.65 mg GAE/g [22]. In another study by Buu *et al.* (2018) on the extract of black garlic, the content of polyphenol compounds increased with the increase of cellulase enzyme concentration from 0% to 0.06%, respectively. The value of obtained polyphenol compounds increased from 6.52 mgGAE/g to 11.05 mgGAE/g. However, when the cellulase concentration increased to 0.1%, the obtained polyphenol compounds remained unchanged at 11.11 mg GAE/g [23].

3.8. Effect of *Clito*Lac activity on antioxidant activity

The study results show that, the correlation between polyphenol compounds for the antioxidant capacity of tea leaf extract.

As shown in table 3, the effect of different *Clito*Lac activity (from 1.0 to 5.0 U/gds) was investigated. The antioxidant capacity of the initial dried extract (control sample) was 53.67%. Enzyme treatment had a significant effect on the antioxidant capacity of the samples at 1.0 U/gds compared with the control samples (65.56% and 53.67%, respectively). However, as the results, in the rank of *Clito*Lac activity from 2.0 to 3.0 U/gds a significant change in the liberation of products was observed to be 70.35 to 73.29% for scavenging capacity. The scavenging capacity at 5.0 U is much higher than the control (78.05 and 53.67%, respectively). At 4.0 U/gds, leading to significant changes in the products. However, at higher *Clito*Lac (e.g. up to 4.0 U/gds), the reaction products wouldn't be increased significantly. The highest scavenging capacity using *Clito*Lac (5.0 U/gds) in the enzyme-assisted extraction process was up to 78.05% at 50°C, pH 5.0 during 24 hours incubation. In a study by Buu *et al.* (2018) on

subjects studying the extract of black garlic, the results showed that the DPPH free radical scavenging ability increased when the enzyme concentration increased from 0% to 0.1%, the ability to DPPH free radicals increased from 22.75% to 61.08%. However, there was no difference in DPPH free radical scavenging ability obtained at 0.06% enzyme concentrations; 0.08% and 0.1% [23]. Thus, compared with control extraction, the antioxidant yields were higher in enzyme-assisted extraction system.

Table 3. Effect of different *Clito*Lac activities on antioxidant activity

Enzyme activity (*Clito*Lac, U/gds)	Scavenging capacity (%) obtained by enzyme-assisted extraction (40°C, pH 5.0, 24 hours)	
	*Clito*Lac (test sample)	The inactivated enzyme (control sample)
0	-	53.67 ± 0.12
1	65.56 ± 0.12	-
2	70.35 ± 0.10	-
3	73.29 ± 0.18	-
4	77.79 ± 0.17	-
5	78.05 ± 0.15	-

4. CONCLUSION

In this study, 21 fungal strains were screened for laccase activity, strain *Clitopilus* SP. BV8 showed the highest activity at 7,943.1 U/L. Fungal strains were fermented for 7 days on medium containing the following components: $MgSO_4$ (0.5 g/L); KH_2PO_4 (1.5 g/L); glucose (5 g/L); pH 6.0, tomato (2%), urea (2 g/L) and cultured at 25°C under the condition of continuous shaking at 200 rpm, the highest laccase activity was 65 U/mL. Crude laccase enzyme was concentrated from the culture solution with optimum temperature and pH at 40°C and pH 5.0, respectively. Total polyphenol content released and antioxidant activity from green tea leaf extract supported by laccase enzyme were also evaluated. The results showed that in optimal conditions for hydrolysis of tea leaves in 18 hours, the enzyme/substrate ratio was determined to be 5.0 U/gds at pH 5 at 40°C. Total polyphenol content and antioxidant activity reached the highest values of 136.15 mgGAE/g and 78.05%, respectively.

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