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Effect of Laccase Oxidation on Phenol Content and Antioxidant Capacity of Roasted Coffee

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Abstract. The endogenous enzyme in fresh coffee beans mostly is from the class of oxidoreductase. However, those enzymes have low effectiveness in phenolic compound oxidation. The fermentation of conventional coffee processing requires a longer time. The ability of laccase could shorten the time of fermentation. Therefore, need exogenous enzyme such as laccase. Laccase is an enzyme that able to degrade lignin. In this study laccase from *Trametes versicolor* is effective for phenol oxidation and able to reduce bitterness in coffee beans. The bitterness of coffee beans is caused mostly by phenolic compounds in coffee such as chlorogenic acid, caffeic acid, and ferulic acid. This study aims to determine laccase oxidation in coffee beans quality by measuring phenol content and antioxidant capacity. The results showed that the lowest total phenol (0.73 ± 0.103 mg/ml) was achieved by laccase oxidation using ABTS as a mediator with antioxidant capacity level as $91.58 \pm 0.001\%$. The benefit of this research is making the best quality of coffee beans with high antioxidant capacity and low phenol content so that it does not require added sweeteners or sugar that can interfere with health.

INTRODUCTION

Coffee is a type of beverage that comes from processing coffee beans (*Coffea canephora* var. Robusta) [1]. Coffee bean processing includes sorting, pulping, fermentation, washing, coffee drying, hulling, roasting, and pulverizing [2]. Fermentation is one of the primary processing that determines the quality of coffee brewing [3]. Traditional fermentation of coffee beans takes longer around 48-72 hours, but by adding exogenous enzyme fermentation becomes faster around 24 hours. The fermentation process breaks down complex compounds into simple compounds by an endogenous enzyme. Endogenous enzyme degrades the remaining mucilage layer on the surface of the skin and decreases the concentration of polyphenol compounds [4]. Polyphenolic compounds are precursors of bitter taste consisting of caffeic acid, chlorogenic acid, and ferulic acid [5].

Endogenous enzyme as an oxidizer of phenol compounds in coffee beans has low effectiveness. Therefore, an exogenous enzyme is added, namely, laccase to increase the effectiveness in oxidizing phenol compounds which cause a bitter taste in the final brewing of coffee [6]. Laccase (EC 1.10.3.2) is a ligninolytic enzyme that able to degrade lignin produced from *Trametes versicolor* fungi [7][8]. Laccase is a multi-copper oxidoreductase enzyme that oxidizes polyphenols to quinones, by removing one hydroxyl electron in phenol compounds to form p-quinone and phenoxy radicals [9][10]. Quinone is an electrophilic molecule that can polymerize with amino acids, simple sugars and aldehydes to form melanoidin which acts as an antioxidant in roasted coffee beans [11][12].

The addition of laccase as an exogenous enzyme is to increase the effectiveness of phenol compounds oxidation in coffee beans, then it could reduce the coffee bitterness. Bitterness taste of coffee beans is caused by phenolic compounds in coffee beans. To determine the most effective phenol oxidation, we use laccase in coffee beans

fermentation process [13]. Laccase oxidation pretreatment on coffee beans in this study is expected to improve the quality of coffee beans roasted.

MATERIALS AND METHODS

Trametes versicolor Fungi

Fungi used for laccase production is *Trametes versicolor* which is a collection from the Mycology Laboratory, Department of Biology, Institut Teknologi Sepuluh Nopember (ITS) Surabaya.

Trametes versicolor Subculture Medium Formulation

The medium used for the *Trametes versicolor* subculture is PDA (Potato Dextrose Agar) solid medium. The PDA medium is taken as much as 39 grams and dissolved in 1 litre of distilled water using a magnetic stirrer until it is homogeneous. The homogeneous medium was sterilized at 121 ° C and 1.5 atm (15 minutes). The medium was poured into 10 ml Petri dishes [14].

Fermentation Medium Formulation

The medium used for enzyme production is a modified fermentation medium of Nyanhongo et al., (2002) method [13]; Fukushima and Kirk (1995)[15]. Table 1 shows the composition of the fermentation medium.

TABLE 1. Composition of Laccase Fermentation Medium

Ingredients	Variation of mass (g)
Rice husk	0.45
Yeast extract	0.15
Glukosa	0.1
Ammonium chloride	0.5
KH ₂ PO ₄	0.2
MgSO ₄ .7H ₂ O	0.05
CaCl ₂ .2H ₂ O	0.01
KCl	0.05

The fermentation medium was dissolved in 100 ml of distilled water and conditioned at pH 5 using a pH meter. The medium was sterilized at 121 ° C (1 atm) for 15 minutes [13][15].

The subculture of *Trametes versicolor* Fungi

The subculture of *Trametes versicolor* isolates by regrowing *Trametes versicolor* on PDA at 28 ° C for 12 days [13][16]. After 12 days *Tremetes versicolor* was inoculated on a 100 ml fermentation medium and incubated for 3 days at room temperature (130 rpm) [17].

Laccase Production and Isolation

Enzyme production was carried out using a modified fermentation medium from the methods of Nyanhongo et al., (2002)[13]. Fungal cultures from the PDA medium were taken as much as 1x1 cm and inoculated in 100 ml of the fermentation medium. Laccase isolation was carried out on the 3rd day [18]. After incubation, the mushroom culture was filtered by using Whatman paper No. 1. The filtrate obtained was used as a crude extract of laccase to test laccase activity, total protein, and isoelectric point [19][20].

Laccase Activity Assay

Laccase activity assay was carried out by inserting 1 ml of 10mM buffer citrate solution pH 4.5, 10 µl crude laccase and 300 µl ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate)) as a substrate [21]. Laccase activity was measured spectrophotometrically ($\lambda=436\text{nm}$) by using ABTS standard curve for calculated [19].

Total Protein Assay

Total protein assay was carried out by making a Bradford reagent by dissolving 10 mg of Coomassie Brilliant Blue G-250 in 5 ml of 95% ethanol, then adding 10 ml of 85% phosphoric acid and distilled until 100 ml. The protein test was carried out by inserting 0.1 ml of crude laccase in a test tube, then adding 5 ml of Bradford reagent. Total protein was measured spectrophotometrically ($\lambda=595\text{nm}$) [22]. The blank solution used was 0.1 ml of distilled water as an enzyme replacement. The absorbance results are then compared with the standard BSA (Bovine Serum Albumin) curve. A standard solution is prepared by dissolving 10 mg of BSA in 50 ml of distilled water. The standard solution was arranged from 0.1 to 1 mg/ml. The value obtained is graphed with the equation $Y = Ax + b$, Y is the absorbance value and x is the value of protein concentration

Isoelectric Point Assay

The isoelectric point assay is carried out by preparing 6 test tubes. Laccase 1 ml was put into each test tube, then a phosphate buffer was added to arrange a pH of 3, 4, 5, 6, 7, and 8, respectively. Furthermore, the test tube was homogeneous with vortex and recorded turbidity. The time taken for the solution to settle was recorded. The formation of the fastest turbidity deposits or most deposits is the pH isoelectric point. The isoelectric point is reached when the sum of the positive and negative charges of the protein is equal so that the precipitation occurs at that pH [23].

Pretreatment of Coffee Beans by Laccase

Pretreatment of coffee beans was done by adding a crude extract of laccase with 12 U / ml activity, and mediators in the form of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) 0.1 mM treated to fermented Robusta coffee beans and without their fermentation. In this research, there are two types of coffee beans that is fermented coffee beans and unfermented coffee beans. Both were treated with laccase and ABTS or laccase mediator system (LMS).

TABLE 2. Laccase Pretreatment in Coffee Beans

Composition	Sample					
	S1	S2	S3	S4	S5	S6
Coffee beans (g)	10	10	10	10	10	10
Crude enzyme <i>laccase</i> (ml)	-	7.6	7.6	-	7.6	7.6
ABTS	-	-	5µl	-	-	5µl

Information :

S1: Sample 1 (fermented coffee beans)

S2: Sample 2 (fermented coffee beans added with Laccase)

S3: Sample 3 (fermented coffee beans added with Laccase and ABTS)

S4: Sample 4 (unfermented coffee beans)

S5: Sample 5 (unfermented laccase coffee beans added)

S6: Sample 6 (laccase and ABTS coffee beans without fermentation added)

After pretreatment, coffee beans were washed using clean water, then dried for 24 hours at a temperature 55°C. Coffee beans were tempered for 24 hours for drops the temperature and is not damaged when occurred hulling process. Roasting was performed at a temperature of 180-250°C for 26 minutes [2].

Total Phenol Determination

Measurement of total phenol in this study serves to determine changes in coffee beans total phenol in each treatment. The principle of this method is the oxidation of phenol compounds in an alkaline atmosphere by Folin-Ciocalteu reagents which produce a blue solution [24]. Coffee extraction was carried out based on Musatto et al., (2011)[25]. Coffee powder samples were prepared to be extracted, then weighed using an analytical balance of 1 gram and dissolved with 40 ml of 60% methanol. The sample is heated for 90 minutes in a water bath. Then the supernatant obtained from centrifugation is filtered using filter paper.

Furthermore, measurement of total phenol levels was carried out based on Kwak et al., (2016) [26] and modified. 1 ml of coffee extract was taken and then added 1 ml of Folin Ciocalteu reagent and homogenized and incubated for 10 minutes. After 10 minutes, 1 ml Na_2CO_3 was added. The mixture is stored at room temperature for 45 minutes. Blank solutions were prepared by replacing reagents with aquades. After incubation, the absorbance value of the sample was measured by a spectrophotometer at a wavelength of 750 nm. Total phenol is calculated based on the standard gallic acid curve. Preparation of a standard gallic acid curve was preceded by making a standard solution of 1 mg/ml by weighing 10 mg of gallic acid and then dissolving it in 10 ml of 60% methanol. Furthermore, standard solutions made various kinds of 0.005; 0.01; 0.015; and 0.02 concentrations. Total phenol was measured spectrophotometrically ($\lambda=750\text{nm}$) by using a 60% methanol blank as a substitute for gallic acid.

Measurement of Antioxidant Capacity

Coffee extraction was carried out based on Musatto et al., (2011) [25]. Sample of coffee powder weighed until 1 g then dissolved with 40 ml of 60% methanol and heated for 90 minutes in water-bath. Then the extract was centrifuged (2.500 rpm) for 20 minutes. Resulting in the coffee extract is filtered using filter paper. Furthermore, the determination of antioxidant capacity was carried out based on Shimamura et al., (2014) [27] and modified. The coffee extract was taken as much as 300 μl then 900 μl of DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was added and homogenized using vortex. Then the solution was incubated for 30 minutes and measured at $\lambda = 517 \text{ nm}$. 300 μl of 60% methanol plus 900 μl DPPH are as a blank solution. The absorbance results have analyzed the percentage of antioxidant activity using the equation as proposed by Ridho, et al [27]:

$$\text{Percentage of Antioxidant Capacity} : \frac{\text{DPPH absorbance} - \text{absorbance of the sample}}{\text{DPPH absorbance}} \times 100\% \quad \dots\dots\dots(1)$$

RESULTS AND DISCUSSION

Effects of Laccase Oxidation on Phenol Content and Antioxidant Capacity of Roasted Coffee

Colour of oxidized roasted coffee by several oxidants in this experiment can be seen in Fig 1. The colour of oxidized roasted coffee powder by laccase showed brighter colour compared to the other experiment group. This is due to the bleaching process by laccase to the aromatic compounds as constituents of colour pigments [16][28]. Laccase is able to do bio-bleaching process by oxidizing phenolic compounds through producing phenoxy radicals that are unstable and will further damage the aromatic rings of coloured compounds in coffee beans [28].

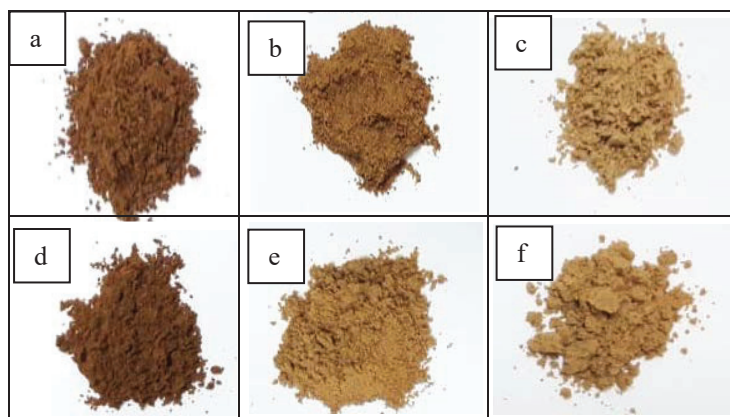


FIGURE 1. Colour of roasted robusta coffee beans after being ground, with various pretreatments a) fermented b) fermented, laccase c) fermented, laccase + ABTS (LMS) d) without fermentation, laccase e) without fermentation, laccase + ABTS (LMS).

The effect of laccase oxidation on robusta coffee beans was observed through the analysis of total phenol and antioxidant capacity (Table 3). At list in Table 3, it can be seen that the roasted robusta coffee beans which are oxidized through the laccase pretreatment have a total phenol concentration which tends to decrease compared to the control. This shows that Laccase is able to oxidize phenolic compounds in coffee such as caffeic acid, chlorogenic acid and ferulic acid into p-quinone and phenoxy radicals [5]. These compounds are unstable compounds that will experience delocalisation to form electrophilic quinones that are highly reactive and capable of polymerizing with other compounds [10][29].

The total phenol concentration will be smaller if the oxidation reaction is added with ABTS. This can be seen in Table 3 with the lowest total phenol value achieved in the laccase + ABTS pretreatment (0.73 ± 0.103 mg/ml). The presence of ABTS in oxidation reactions has the function of increasing the oxidation potential which causes aromatic rings in phenolic compounds to turn into aliphatic [30].

TABLE 3. Total Phenol Results and Antioxidant Capacity of Robusta Roasted Coffee Beans

Pretreatment	Total Phenol (mg/ml)		Antioxidant Capacity (%inhibition)	
	Fermentation	Without Fermentation	Fermentation	Without Fermentation
Control	2.43 ± 0.158	3.11 ± 0.320	86.96 ± 0.005	87.88 ± 0.004
Laccase	1.16 ± 0.054	1.26 ± 0.055	91.42 ± 0.001	90.83 ± 0.004
Laccase + ABTS	0.73 ± 0.103	0.74 ± 0.085	91.58 ± 0.001	91.54 ± 0.0026

In Table 3 it can be seen that the concentration of total phenol does not correlate with the antioxidant capacity of roasted coffee beans. This can be explained that in roasted coffee antioxidant activity is caused by the presence of melanoidin compounds which are the result of a reaction between oxidized phenols and amino acid compounds, simple sugars and aldehydes [11][12]. The antioxidant capacity of roasted coffee could increase with the presence of laccase in the pretreatment process. The addition of ABTS could increase the oxidation potential of the laccase-ABTS system so that it could produce more aliphatic compounds from the oxidation of phenols to form melanoidin [12].

CONCLUSIONS

Laccase was able to ferment robusta coffee beans with the lowest total phenol content of 0.73 ± 0.103 mg/ml achieved in fermented robusta beans with laccase and ABTS pretreatment while the highest antioxidant activity was reached $91.58\% \pm 0.001$. The weakness of previous research is that it does not discuss the effect of the laccase enzyme on the antioxidant capacity of coffee beans. Future plan of this research is will be conducted by organoleptic tests on robusta coffee beans that have been treated using laccase and ABTS.

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