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Effect of Laccase Oxidation Pre-treatment on Coffee (*Coffea arabica*) Bean Processing Waste for Composting Substrate

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Abstract. Laccase oxidation pre-treatment on coffee pulp and husk in this study was expected to improve the quality of compost by removing phenol compounds which can be toxic for microorganisms. The purpose of this study was to determine the effect of pre-treatment of laccase oxidation on coffee bean processing waste (*Coffea arabica*) consisted pulp and husk by determining the total phenol, reducing sugar, organic materials and compost quality. Optimization of laccase production was carried out on 5 types of fermentation medium. All parameters except organic materials were measured spectrophotometrically. The results showed that the highest laccase activity (1081.2 U/ml) was achieved by E medium fermentation which contained 0.45 g of rice husk and 0.1 g glucose as carbon source, some of minerals and without the addition of sawdust and $CuSO_4$. The lowest total phenol and reducing sugar were achieved coffee pulp and husk with laccase oxidation and ABTS pre-treatment as 0.007 ± 0.07 mg/ml and 3.29 ± 0.19 ppm respectively. It can be concluded that indeed pre-treatment of laccase oxidation did not affect the levels of organic matters. All experiment group of pre-treatment showed higher of Total Plate Counts (TPC) comparing to the control. However, the initial oxidation treatment did not have an effect on the levels of N, P, K, C, and the C / N ratio of compost produced when compared to controls. The results of this study could contribute to the process of making compost with the best quality that is environmentally friendly.

INTRODUCTION

Coffee bean processing waste is one of abundant agro-industrial wastes along with the coffee production development program in Indonesia. Every 2 tons of processed coffee will produce about 1 ton of coffee bean processing waste. At the processing stage, more than 2 million tons of coffee bean processing waste are produced in the form of pulp and husk each year [1]. Coffee bean processing waste contains several organic and inorganic materials which are potential to be substrates in several bioprocesses [2] such as bioethanol, biogas, and particle board manufacturing [3]. However, the most common of its waste utilizing is for composting raw materials [4].

High total phenolic compounds, such as chlorogenic acid, epicatechin 21.6%, catechins 2.2%, routine 2.1%, protective acid 1.6%, proantocyanidin 1.8%, and ferric acid 1%, cause coffee bean processing waste become toxic to microorganisms [2][4]. Total phenol 0.05% can be toxic (antimicrobial) to microorganisms because those compounds interact with bacterial cells through the adsorption process and cause protein coagulation and bacterial cell membrane lysis [6]. This matter is able to inhibit the growth of microorganisms may play a role in composting process [7]. Therefore, pre-treatment is needed to reduce total phenol before the waste is used. Laccase oxidation pre-treatment on coffee bean processing wastes is one of methods to reduce total phenol which is considered more environmentally and require lower energy compared to the physicochemical process [5].

Laccase (EC 1.10.3.2) is a multi-copper oxidoreductase enzyme that catalyzes the oxidation process of various types of organic and non-organic compounds including monophenol, diphenol, polyphenol, amino phenol, methoxy

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phenol, and aromatic amine compounds into phenoxy radicals and quinons through electron reactions reducing oxygen to water [8][5]. Laccase oxidation pre-treatment on coffee bean processing wastes in this study is expected to improve the quality of compost by reducing the toxic compounds contained within it.

MATERIALS AND METHODS

Production and Characterization of *Trametes versicolor* Laccase

The optimization of *Trametes versicolor* laccase production was carried out in 5 types of fermentation medium (medium A, B, C, D and E) which are the results of previous research [9][10] fermentation medium modification. Variation of fermentation medium composition showed in Table 1

TABLE 1. The Composition of Laccase Medium Fermentation

Materials	Concentration (g/100ml)				
	Medium A	**Medium B**	**Medium C**	**Medium D**	**Medium E**
Rice husk	0.9	0.9	0.45	0.45	0.45
Yeast Extract	0.3	0.3	0.15	0.15	0.15
Glucose	1	1	0.1	0.1	0.1
Ammonium chloride	0.5	0.5	0.5	0.5	0.5
KH_2PO_4	0.2	0.2	0.2	0.2	0.2
$MgSO_4.7H_2O$	0.05	0.05	0.05	0.05	0.05
$CaCl_2.2H_2O$	0.01	0.01	0.01	0.01	0.01
KCl	0.05	0.05	0.05	0.05	0.05
$CuSO_4$	0.05	0.01	0.05	0.01	-
Sawdust	0.45	0.45	-	-	-

Fungal cultures of *Trametes versicolor* from the PDA medium were taken as much as 1 x 1 cm and inoculated into 100 ml of the fermentation medium. Incubation of fungal cultures was carried out at room temperature (130 rpm). Laccase isolation was carried out on the 3rd day, 4th day and 5th day [11] After incubation, fungal culture was filtered by using Whatman paper no. 1. The filtrate obtained was a crude extract of laccase and characterized furthermore based on laccase activity, total protein, and isoelectric point [12][13][14][15].

Pre-treatment using Laccase Oxidation on Coffee Bean Processing Waste

Pre-treatment laccase oxidation on coffee bean processing waste was carried out to remove phenolic compounds in the coffee skin [16]. Each treatment used 10 g of chopped coffee bean processing waste given four different kinds of treatment. Laccase activity added to the reaction is 12 U / ml. 10 g of coffee bean processing waste and 7.6 ml of laccase were put into a 1000 ml Erlenmeyer flask containing 500 ml of 10 mM citrate buffer pH 5 [10]. The mixtures were incubated at 150 rpm, 40 ^oC [5] for 24 hours [17]. The same treatment was performed on other samples by adding 5 µl ABTS into the mixture. While other treatments used hydrogen peroxide as the oxidizer by adding 7.6 ml H_2O_2 0.345 M [18]. Furthermore, total phenol analysis of coffee bean processing waste was measured spectrophotometrically (λ=760 nm) by using Follin Ciocalteu reagent based on the ability of reaction between phenolic compound and oxidizers. The absorbances obtained were compared with the standard gallic acid curve [19]. Reducing sugar level were measured spectrophotometrically (λ=540 nm) by using *Dinitrosalicylic Acid* reagent. The absorbances obtained were compared with the standard glucose curve.

Composting and Analysis of Compost Quality

The composting process was carried out aerobically [20] by following the composition ratio according to [21]. Briefly, as musch as 6 µl of EM4 (SLP) solution was added to 300 µl of water and 6 µl molasses then mixed with 6 g of lacace pre-treatment coffee bean processing waste. The composting process was carried out for 14 days because composting takes the fastest time around 2 weeks at room temperature [22]. Compost was covered by plastic [20]. The activity of microorganisms in the composting process generated heat so the temperature of compost should be

maintained around 40-50^{0}C to prevent fertilizer damage [23] by opening the lid and turning over the dough. Controlling the temperature was done every day until the compost color changed into blackish brown. Each ripe compost had brownish black characteristics, reduced 30-40% of the biomass, and smelled like soil [23], temperature around 30ºC, humidity 40-60% [24]. Then, compost was dried overnight. Analysis of compost quality was done by measuring the C / N ratio, nitrogen, phosphorus, and potassium [23].

Total Plate Count (TPC) of Compost

Control of microbial growth that play role in the composting process was carried out during the time series of composting process every 24 hours. Total Plate Count on Nutrient agar medium was done by making serial dilutions (10^{-1}, 10^{-2}, 10^{-4},10^{-6}, 10^{-8}, 10^{-10}). Compost was taken and dissolved in sterile aquades. 1 ml of compost solution was taked and put into 9 ml of sterile water in a test tube and homogenized. This stage was a 10^{-1} dilution. Next step, 1 ml of the solution from 10^{-1} dilution was taken and put into another 9 ml of sterile water. The solution was homoginized. This stage was considered as 10^{-2} dilution. This treatment was repeated with the same steps until dilution 10^{-12}. Each dilution series was piped as much as 0.1 ml and poured into Nutrient agar and spreaded by using drigalsky. Inoculated medium was incubated at room temperature (27 to 28 ºC). After 48 hours incubation, colonies on the medium were calculated to get the value of total colonies in Colony Forming unit (CFU) [25].

RESULTS AND DISCUSSION

Optimization of Fermentation Medium and Laccase Characterization

Optimization of Laccase production in this study was carried out by modifying the fermentation medium based on the type of composition (composition A, B, C, D and E). The composition of the fermentation medium D and E showed the existence of laccase activity as shown in Fig. 1, while the composition of the fermentation medium A, B, and C did not show any laccase activity until the end of the incubation period.

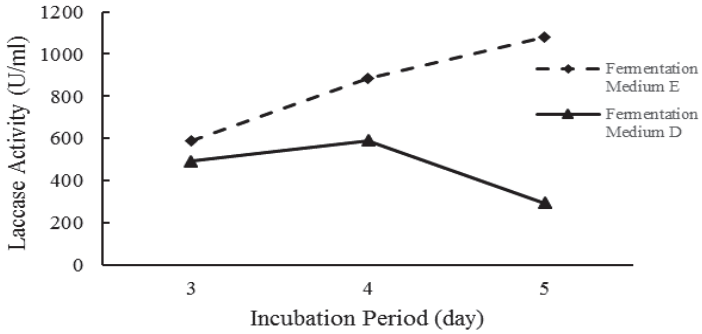

FIGURE 1. Laccase activity on several variations of the incubation period of the fermentation medium.

Based on Fig.1 known that the highest laccase activity was achieved on the 5th day incubation of 1081.2 U / ml from E fermentation medium, while D fermentation medium had the highest activity on the 4th day incubation of 590.1 U / ml. This indicated that E fermentation medium had more stable and higher activity than D fermentation medium until the end of incubation. The low activity on D fermentation medium might be due to the influence of $CuSO_4$ concentration. Cu metal in $CuSO_4$ could induce transcription in laccase formation [26] but excessive addition of Cu metal could reduce the laccase activity [27]. A and B Fermentation medium did not show any laccase activity due to the addition of excess glucose which inhibited the production of lignolytic enzymes including laccase. Glucose plays major role in the formation of cell biomass rather than the production of secondary metabolites [28] because laccase encoding gene can be expressed in mediums with low nitrogen and carbon conditions [28]. In C fermentation medium, the absence of laccase activity was assumed due to the addition of excessive concentrations of $CuSO_4$, so that it was toxic to microorganisms [26].

Laccase with the highest activity of 1081.2 U / ml was further characterized based on the total protein and isoelectric point. The results of total protein analysis were 0.03 mg / ml and the isoelectric point was pH 3. This was

supported by research [8] which demonstrated that one of the laccase characteristic produced from the fungi is having an isoelectric point between pH 3 and pH 7.

Effects of Laccase Oxidation on Coffee Bean Processing Waste

The effect of laccase oxidation pre-treatment on coffee bean processing waste was observed through total phenol levels, reducing sugar and percentage of organic matter analysis (Table 2.).

TABLE 2. Total phenol levels, reducing sugar and percentage of organic matter

Treatment	Total phenol (mg/ml)	Reducing Sugar (ppm)	Organic materials (%)
Control	0.040 ± 0.11	10.11 ± 0.28	51.54
H_2O_2	0.009 ± 0.00	3.31 ± 0.17	55.01
Laccase	0.008 ± 0.07	3.45 ± 0.08	56.10
Laccase + ABTS	0.007 ± 0.07	3.29 ± 0.19	53.73

Based on Table 2. known that total phenol in coffee bean processing waste after treated by laccase oxidation was lower than control. This matter showed that laccase was able to oxidize phenolic compounds within the coffee bean processing wastes such as flavan-3-ol, hydroxynamic acid, flavonol, anthocyanidins, catechins, epicatechins, routine, tannins, and ferulic acids [29] into phenoxy radicals and ortho radicals. -quinone through the reaction of the acceptance of electrons by oxygen into water molecules [30]. Orthoquinone compounds and phenoxy radicals formed were highly reactive and could react with proteins to form complex compounds involving the amino acid lysine [30]. Free phenoxy radicals formed carried out further enzymatic oxidation reactions or non-enzymatic reactions such as hydration, disproportionation and polymerization with other compounds. The lowest phenol content was 0.007 ± 0.07 mg / ml in the treatment of laccase with ABTS. The addition of ABTS played role as mediator in oxidation reactions that increased and expanded oxidation activity by laccase [31].

The results of reducing sugar analysis on coffee bean processing waste treated by Laccase oxidation also showed lower values than controls. The lowest reducing sugar was 3.29 ± 0.19 ppm which was obtained in the coffee bean processing waste treated by laccase oxidation and ABTS. This was caused by the oxidation of reducing sugars by ABTS which had been oxidized to Laccase. The addition of ABTS as mediator of the oxidation acted as an electron carrier and oxidizes non-phenolic substrates which could not be oxidized directly by Laccase because of its higher redox potential [32]. Meanwhile, reducing sugars such as glucose and fructose in coffee bean processing wastes had aldehyde and ketone groups which were able to reduce oxidizing compounds [33]. According to [34], Laccase-ABTS was able to catalyze the oxidation reaction of reducing sugars functional groups, thereby causing reducing sugar to decrease. The reduction of reducing sugars within coffee bean processing waste treated by laccase oxidation and H_2O_2 might also be caused by oxidation reactions were aided by natural mediators in coffee bean processing waste. According to [32], phenolic compounds could also play role as mediator of non-phenolic compounds oxidation reaction by laccase such as 3-Hydroxyanthranilic acid (3-HAA), 4-hydroxybenzoic acid, and phenol red compounds. Natural mediators could also be derived from lignin monomer phenolic compounds, namely p-hydroxycinnamic acid and p-coumaric acid. The results of organic material analysis showed that percentage of organic material did not differ greatly in all types of oxidation pre-treatment. This showed that all types of oxidation pre-treatment used in this study did not have a large effect on the organic materials content of coffee bean processing waste. The difference of coffee bean processing solution color observed in Figure 2.

(a) (b)

FIGURE 2. Color differences when coffee skin waste samples are dissolved in water. (a) control; (b) oxidation pre-treatment.

The color of coffee bean processing waste solution after pre-treatment was looked clearer than the control. This was probably due to the oxidation of aromatic compounds from the pigments [35] such as anthocyanins [36]. Oxidation reaction by laccase could degrade pigment compounds in plants [37] by oxidizing phenolic compounds to produce phenoxy radicals which can cause aromatic ring division of coffee skin colorants [35]. Anthocyanins are classified as pigments called flavonoid compounds [38]. Flavonoid compounds include polar compounds which can dissolve in polar solvents such as water, so that when the coffee skin waste is dissolved in water, the anthocyanin compound will dissolve easily [39]. The dark yellow color in the control solution shows the high levels of anthocyanin compounds dissolved in it, while the coffee skin waste solution with oxidation pre-treatment shows a clearer and pale color which indicates low levels of anthocyanin which dissolves in it.

Application of Substrates Resulting from Oxidation Pre-treatment of N, P, K, C Compost

The results of Total Plate Count (TPC) during the composting process of coffee bean processing waste using an EM4 activator are shown in Fig. 3. The three types of oxidation pre-treatment at composting, except for controls that did not undergo oxidation pre-treatment showed a pattern of TPC values that were almost the same and more stable. Each treatment showed an increase in TPC value at the beginning of composting. TPC values for the 1st and 2nd day composting incubation ranged from $5.5x10^8$ to $2.95x10^{10}$, respectively, indicating that microorganisms used the substrate as an energy source and C source.

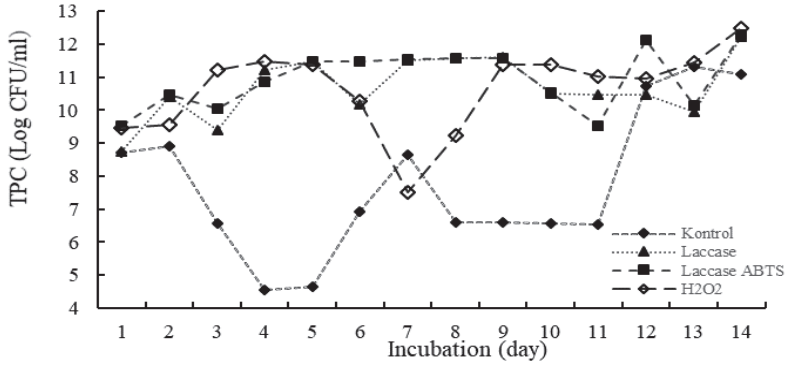

FIGURE 3. TPC of compost

Based on Fig. 3. known that the total number of microbes in the control (compost without oxidation pre-treatment) tends to be lower and fluctuating than all composts during the composting period. This was becaused high total concentration of phenols in coffee bean processing waste which was used for composting. So that it could inhibit the growth of microorganisms in the composting process. Phenol compounds could interact with bacterial cells through an adsorption process involving hydrogen bonds. High total phenol caused protein coagulation and bacterial cell membranes to undergo lysis [40].

The value of TPC in control and coffee bean processing waste treated by H_2O_2 oxidation decreased on days 3 to 7, namely $3.5\ x10^4$ and $3.2x10^7$. In compost with laccase oxidation pre-treatment, TPC value decreased on the 3rd day to reach $2.47x10^9$. The decrease of TPC value in each treatment was likely due to an increase in temperature in the compost due to the decomposition of organic matter by the microorganisms within it [41] as well as to kill pathogenic microorganisms and weeds contained in compost [42]. Furthermore, the TPC value in all compost has increased again until the end of the composting period, which ranges from 10^{10} to 10^{12} CFU / ml. Microorganisms in compost would remodel cellulose and hemicellulose remaining from the previous process into simpler sugars until the decomposed material decreases [43].

The level of compost maturity was observed through color change, texture and aroma. At the end of the composting period, compost appeared blackish color, smelled like soil and weathered wood, and the texture of the compost was finer than the compost base material. The color of compost formed could be seen in Fig. 4.

Based on Fig. 4. showed that control had lighter brown color compared to compost with oxidation pre-treatment. This occurred because the number of microorganisms played role in the composting process was lower than the other three treatments. So that, there was no complete decomposition of organic matter in the control. The difference

in color formed was caused by differences in the number of humus compounds formed. Humus is an aggregate complexes that are not soluble in water, are not scented and are dark brown to black in color originating from the process of decomposition of organic matter [44].

(a) (b)

FIGURE 4. Color of Coffee bean processing Waste Compost. (a) control; (b) compost coffee bean processing waste by oxidation pre-treatment.

The decomposition process affects compost color due to decomposition of organic material contained in compost and produces water vapor and CO_2. So that it took the form of crumbs [45]. When the decomposition process was occured, there was pigment degradation (such as chlorophyll which was very fast and easily decomposed) in coffee bean processing waste. The decomposition process also changed pigment, cellulose, lignin, pectin, starch, humic acid, protein, glucan, tannin compounds and other compounds into a humus compound [46] which was dark blackish brown. At the beginning of composting process, compost had a scent liked the basic ingredient of coffee bean processing waste. During the composting process, coffee bean processing waste were decomposed and the organic materials were utilized by microorganisms. This decay process causes compost that has scented soil or wood that has rotted [47] and forms a complex of humus compounds [46].

TABLE 3. Analysis of Compost Quality

Treatment	Parameter				
	C (%)	N (%)	C/N ratio	P (%)	K (%)
Control	21.58 ± 0.00	1.29	16.73	4.55	0.82
H_2O_2	17.92 ±0.03	1.31	13.68	7.47	0.26
Laccase	17.47± 0.00	1.23	14.20	9.07	0.14
Laccase + ABTS	19.77± 0.00	1.28	15.44	9.84	0.23

Based on Table 3. known that the value of C, N and C / N ratio in all types of samples met the quality requirements of compost from organic waste SNI 19-7030-2004. The lowest C / N ratio was obtained in compost with H2O2 oxidation pre-treatment which was 13.68 and followed by compost with laccase oxidation pre-treatment and laccase + ABTS of 14.20 and 15.44. The low value of the C / N ratio indicated that there was an activity of degradation of organic matter by microorganisms. The level of compost maturity was measured by decreasing the value of the C / N ratio until it approaches the value of the C / N ratio of the soil. C / N ratio values of soil range between 10-12. Organic material which has a C / N ratio value close to the value of the soil C / N ratio, the organic material could be directly used by plants [25][48].

Phosphorus (P) levels in all types of compost had met the standards set by SNI 19-7030-2004 at a minimum of 0.1%. This showed that the inorganic phosphate mineralization process took place well during the composting period. During the composting process, microorganisms would decompose organic phosphate compounds in coffee bean processing waste into inorganic phosphate available to plants with the help of the enzyme phosphatase [49].

Potassium (K) content in all types of compost samples met SNI 19-7030-2004, which was 0.2% except for compost with laccase oxidation pre-treatment of 0.14%. This could be due to number of K solvent bacteria decreased in compost with oxidation pre-treatment because it plays role in the natural potassium cycle. Some microbial species are able to provide potassium in a form that plants can directly use. Microbes produce organic acids that can help release potassium which is bound to potassium-carrying minerals [50].

Based on the analysis of compost quality in Table 3. showed that the results of C, N, P, K and C / N ratios analysis did not differ greatly in all types of compost oxidation and pre-treatment control. This showed that all types of oxidation pre-treatment used in this study did not have major effect on the levels of N, P, K, C and the resulting

compost C / N ratio. This was likely due to the ability of the compost activator in the form of EM4 which was added to accelerate the composting process that occurs in all treatments including control treatments. EM4 activators contain more than 80% of the population of lactic and yeast acid bacteria and a small portion of photosynthetic bacteria, N-fixing bacteria and actinomycetes that can accelerate the decomposition process in the composting process [51]. In addition, according to [52], making coffee bean processing waste compost using an EM4 activator had been able to have a significant effect on improving soil chemical properties in the form of increasing levels of C, N, P, K and was able to increase the number of leaves up to 24.96%, tube diameter 29.59%, production per plot of 50% in the provision of 90g coffee plant compost / plant [53].

CONCLUSION

The composition of E medium fermentation (without the addition of $CuSO_4$ and sawdust) had highest laccase activity of 1081.2 U / ml with. The lowest total phenol levels and reducing sugars were achieved by coffee bean processing waste with laccase oxidation and ABTS pre-treatment 0.007 ± 0.07 mg/ml and 3.29 ± 0.19 ppm respectively. All compost produced has complied with organic waste compost standard according to SNI 19-7030-2004. Pre-treatment of laccase oxidation did not affect the levels of organic matters. All experiments group of pre-treatment showed higher of Total Plate Counts (TPC) comparing to the control. However, oxidation pretreatment did not have an effect on the levels of N, P, K, C, and the C / N ratio of compost produced when compared to controls. For further plans, microorganism growth trends during the composting process that has been achieved could be a reference for optimization in the next research. The best compost from optimization results will be applied to plants for determining the direct effect of compost with laccase oxidation pre-treatment. On the other hand, this study had the disadvantage of differences in the numbers shown by the results of this study was very small because the sample used in small quantities. Therefore for the next research, the number of samples needs to be increased in order the difference of the result study could be clearly seen.

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