BIODELIGNIFICATION OF AGRO-INDUSTRIAL WASTE AS THE SUBSTRATE TO PRODUCE LACCASE ENZYME FROM TRAMETES VERSICOLOR

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A B S T R A C T

Agro-industrial waste in Indonesia is a significant problem for the environment. Optimization of agro-industrial waste utilization is an exciting thing to be researched further. This study aims to utilize agro-industrial waste to optimize laccase enzyme production. The agro-industrial waste used is in the form of bagasse, rice straw, and corn stalks. The waste is used as biomass and treated to extract cellulose through bio-delignification. Cellulose serves as a substrate substance in the production of laccase enzymes. Laccase enzymes can be applied as a biocatalyst in the environmental, pharmaceutical, and chemical industries. Cellulose from agroindustry waste was synthesized by heat treatment using steam explosion with the operation conditions 200°C and 10 bar for 10 minutes. The production of laccase enzymes was done using the green technology submerged fermentation method for 32 hours at 25°C. The optimal result in producing laccase enzyme is using substrate from corn stalks and adding nutrients with an activity value of 25.46 U/mL. The reaction kinetics of laccase enzyme and commercial enzyme against 0.1 M substrate were modelled using the Euler method, and the k value was $2x10^{-4}$ min^{-1} (crude laccase) and $1.9x10^{-2}$ min^{-1} (commercial laccase).

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I. INTRODUCTION

Indonesia is an island country with relatively large agricultural land. One problem that is also being eliminated is agro-industrial waste. This agro-industry waste can be an alternative energy source for the textile and chemical industries. Biowaste can be used as a substrate in the production of enzymes. Laccase enzymes are an exciting topic to study because they have potential in chemical products. Laccase enzyme is identified as a fungus but was recently isolated in *Azospirillium lipoferum* bacteria [1]. Laccase production is widely reported to be done using solid-state fermentation. In submerged as opposed to solid-state fermentation, Pleurotus ostreatus exhibited unusual laccase synthesis. Submerged fermentation cultures yielded four laccase isoforms and 13,000 U l^{-1} of laccase, and 5.6 g l−1 of biomass output. On the other hand, cultures cultured in solid-state fermentation produced 4.5 g of biomass l^{-1} and had three laccase isoforms, with a substantially lower laccase activity of 2,430 U l^{-1}. The outcomes demonstrate that *P. ostreatus* functions significantly better in submerged fermentation than in solid-state fermentation [2].

The production of laccase enzymes from *Trametes versicolor* has been shown to have enzyme production potential with a 16-day fermentation process with a laccase yield of 1241.07 U/g of corn stalk substrate. The results show that laccase can be produced explicitly using substrates from agro-industrial waste [2].Agro-industrial waste can be used as biomass because it has specific characteristics in mushroom growth. Cellulose biomass comprises three main components: cellulose, hemicellulose and lignin. Cellulose is the main component in plant cell walls and dominates up to 50% of plant dry weight [4]. Spruce grows in the lowlands of the tropics and can also grow in parts of the subtropics. The main benefit of peppermint is that it is a raw material for making sand sugar. The pumpkin waste, commonly called the bagasse, is a by-product of the pumpkin fluid extraction process from parts of the pumpkin plant stem. Bagasse can be used as a substrate to produce activated carbon [5]. Additionally, one plant produces about 35-40% of the pumpkin's weight [6].

This cellulose compound can be transformed into other products, such as oxalate acid. Oxalate compounds can be used as explosives, colouring, and rayon for laboratory analysis [7]. In the metal industry, oxalate acid is a coating material that protects metals from corrosive and cleaning agents for automotive radiators, metals, and equipment. It contains lignocellulose and has a fibre length of between 1.7 mm and 2 mm with a diameter of about 20 micrometres, so

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it is economically used not only as a fuel energy source. Nevertheless, it can also be used as a raw material for paper, canvas, mushroom, etc. Even peppermint can be used as animal feed and as an alternative energy source, such as bioethanol or biogas [8]

Rice straw is the most considerable agricultural waste and is not fully utilized due to technical and economic factors. In some farmers, rice straw is often used as a mulch when planting palm oil. Only a small proportion of farmers use rice straw as an alternative feed for cattle during the dry season because it is challenging to get greenery. On the other hand, sludge as agricultural waste is often a problem for farmers, so it is often burned to deal with the problem. According to the Central Statistical Authority, the national grain production reached 71.29 million tonnes per year in 2011. While the production of grain straw can reach 12-15 tonnes per hectare per harvest, it varies depending on the location and type of varieties used. Rice straw is known to have a high cellulose content, reaching 39.1% dry weight, 27.5% hemicellulose, and 12.5% lignin [6].

Actinomycetes and other bacteria belonging to other genera are also capable of breaking down extracted lignin [9], [10], [11]. Lignin biodegradation by different microorganisms has been intensively explored, and it has been demonstrated that inoculation with lignin-degrading microbes can expedite the composting process and increase compost quality [9]. The links between fungal humification and lignin breakdown were assessed by Lopez et al. (2006)[9]. Various ligninolytic organism species have unique degradation methods, which may also have distinct effects on the production of humus [12].

The use of wastes from corn stems as a source of cellulose can increase the value of agricultural waste. Corn stems contain 42.6% cellulose, 21.3% hemicellulose and 8.2% lignin, so the high potential of corn stems has a chance of being one of the alternative sources of cellulosis [13]. The agroindustrial waste contains phenolic compounds. The quantity, number, and location of hydroxyl groups within a molecule are all connected to the antioxidant activity of phenolic acids [14], [15]. Cyclic voltammetry is used to measure electrochemical reduction potentials, which provide information about the capacity to reduce chemicals. The results of this method show that processes involving phenols are not always reversible. Phenolic substances have a wide range of actions, such as:

- disrupting the cycle of reactions started by free radicals;
- slowing down or increasing enzyme activity;
- chelating metals like iron and copper, which can prevent their involvement in Fenton reactions that can produce significant quantities of hydroxyl radicals [16].

A dense fermentation system is best suited for fermenting processes for microorganisms requiring little moisture, such as fungi and other microorganisms. The solid substrate is a source of carbon, nitrogen, minerals, and growth-supporting factors used as a growth site for microorganisms. Microorganisms that grow through a solid fermentation system are in growing conditions under their natural habitat; such microorganisms can produce enzymes and metabolism more efficiently than in a liquid fermenting system[3], [6], [8], [17].

Huang et al. (2006) immobilized the laccase enzyme that would be used as a bio-component in the adrenaline sensor. Immobilization was committed against the laccase enzymes produced from *Pynoporus sanguineus* by adsorption on the surface of the nanoparticles CuTAPc-Fe_3O_4. The yield of immobilization obtained was 20%, with residual activity after storage for one month of 85% of the reactivity [18].

Osma et al. (2011) research using *Trametes versicolor* produced a laccase activity of 63.5 U/g. Fermentation was done by solid-state fermentation with wheat decoction as its substrate and resulted in a cheaper production process with a final price of 0.04 cents €/U [19]. The enzyme production was also carried out by other researchers using the microorganism *Trametes versicolor,* obtaining an activity of 272.62 U/g. Fermentation was done by solid-state fermentation with olive leaves as its substrate. The substrate was 1.4-1.6 mm, adding 1% yeast extract (w/w) [20]Adekunle et al. (2017) also observed laccase enzyme production using the microorganism *Trametes versicolor*. The substrate is a corn stem treated with a steam explosion, which has not been treated in any way. Substrates that are not treated produce an activity of 1241.07 U/g, while the substrate treated makes an extensive activity of 2600.33 U/g. The use of explosion steam has been shown to reduce the level of non-cellulose by adding nutrients during fermentation [3].

Based on previous research, the study focuses on producing laccase enzymes using agro-industrial waste varieties of cornstalk, bagasse and rice straw. The agro-industrial waste was given a steam explosion treatment to extract the lignin and cellulose compounds in the delignification process. The resulting biomass is used as a substrate to produce laccase enzymes by submerged fermentation. This research can be used as a basis for optimization in producing laccase enzymes for applications in the health and chemical industry.

II. MATERIALS AND METHODS

Enzyme production was carried out by *Trametes versicolor* fungus, which was obtained from the Indonesia Culture Collection. The substrate was treated to obtain the cellulose through a steam explosion process. All chemicals used in this study were purchased from Sigma Aldrich such as NH_4NO_3, $(NH_4)_2HPO_4$, KH_2PO_4, $MgSO_4.7H_2O$, $CaCO_3$, $FeSO_4.7H_2O$, $CuSO_4.5H_2O$, Fe_3SO_4.

A. Pretreatment of Agricultural Waste

The agricultural wastes used in this study were rice husk, bagasse, and corn stalks. The waste was collected from the Depok area. It is cut into pieces, dried, and smoothed. The explosion was conducted for 10 minutes at 200°C and 10 bar. The treated substrate was collected, grinded and filtered with a 10-micron mesh filter.

B. Crude Laccase Enzyme Production

The laccase enzyme production in this study used a solid-state fermentation method using *Trametes versicolor*

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as a microorganism. The medium was designed and standardized with nutrients at pH 5. The substrate was prepared in solid form at room temperature. Inoculation of fungus is done by spreading 1 ml of the substrate into a mold of *Trametes versicolor*. Laccase enzyme production is allowed to be fermented for 32 hours at 25°C. The enzyme produced was obtained by extraction and separation. The extraction process was done by adding 50 mM sodium acetate buffer with pH 5.0 and stirring it for 60 min at 120 rpm. The mixed solution was centrifuged for 10 min at 4C and 14000 rpm. The clear supernatant is crude laccase enzyme. The activity of the produced enzyme was analyzed using a spectrophotometer UV-VIS at a wavelength of 240 nm. The enzyme activity test used a mixture of 2,2-azinobis-3-Ethylbenzothiazoline-6-Sulfonic Acid *(*ABTS*)* and tartaric acid buffer 0.1% (w/v) pH 3.0. the obtained unit activity indicated the amount of enzyme needed to oxidize 1 µmol of 2,2-azinobis-3-Ethylbenzothiazoline-6-Sulfonic Acid per minute.

C. Kinetic Oxidation of ABTS

The oxidation of ABTS is observed to measure the amount of free radical antioxidant activity that reacted at pH 7.4. the amount of 0.1 M ABTS substrate and the tartaric acid buffer is reacted with the three different concentrations of crude enzyme, i.e., 10 mg/mL, 30 mg/mL, and 50 mg/mL for 3 minutes, then rapidly analyzed with High-Performance Liquid Chromatography with C18 column. The results of oxidation are used for enzymatic kinetic studies.

III. RESULTS AND DISCUSSIONS

The primary purpose of this research is to utilize agro-industrial waste as an alternative to solve the environmental issues in Indonesia. Besides, biomass from agro-industrial waste has massive potential in cellulose production. Three kinds of substrates are used in the waste: corn stalk, bagasse and rice straw. Corn stems obtained after the harvest are generally only discarded or burned. Rice straw waste is usually used as feed for livestock, basins or mansion floors, building materials (floors, walls), and burned as fuel.

The agro-industrial used in this research was estimated to be a substitution of commercial substrate. Moreover, the research includes three prominent steps: biowaste pretreatment, enzyme production, and enzymatic analysis (Figure 1). The biowaste is treated with high pressure and thermal conditions to extract the lignocellulose. The extracted lignocellulose is allowed to react with the solvent through a biodelignification process using an enzyme. The treated substrate is used as a mold for fungi growth by adding nutrients to the substrate. The delignification process involves altering the chemical structure of the lignocellulosic biomass to break the chemical bonds that bind lignin to the other constituents of lignocellulosic materials (cellulose and hemicellulose). This allows lignin to be broken down selectively. Because it is ecologically safe, biological pretreatment is one of the various pretreatments that can be applied [9-11], [21].

Substrate use is given steam explosion treatment and nutrient addition to give greater activity values than a substrate with steam-free treatment. This comparison reinforces information on the nutritional addition of fermentation processes and waste treatment [22]. The substrates used in this solid-state fermentation are corn stalk, bagasse, and rice straw. The type of substrate will influence the growth of *Trametes versicolor* in the fermentation process, which directly affects the quality of the *laccase* produced, i.e., the unit of *laccase* activity. There are two fundamental differences between these two types of substrate. The first difference is the shape of the substrate.

According to Mirsalami (2024), the shape of the substrate in solid-state fermentation will affect oxygen uptake, the growth of fungi, and the fermenting processes in them. The use of solid-state fermentation methods can also improve the nutrients contained in the media. The fermentation process can increase the mineral content. The

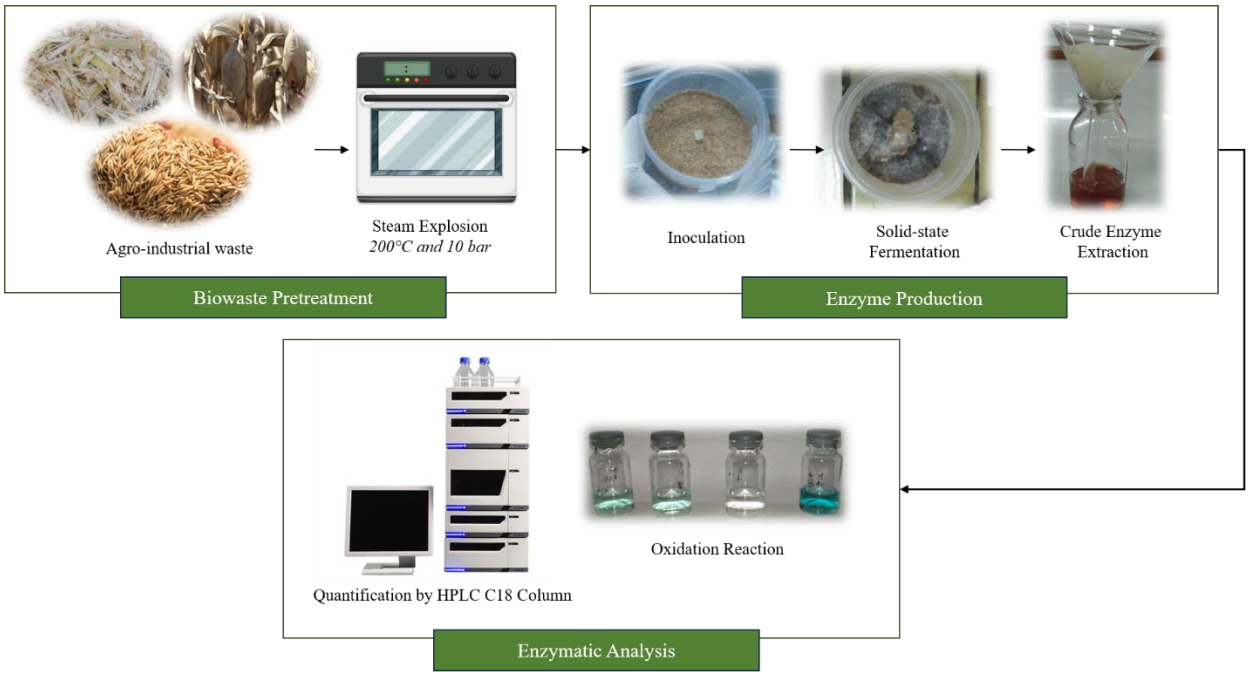

Figure 1 Schematic Overview of the Enzyme Production

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fermentation of laccase with rice husks was carried out by Mirsalami (2024) for 12 hours at 37°C. In contrast, in this study, the process of the third variation of the substrate was performed for 32 hours at 37°C [23]. Previous studies have proved that solid-state fermentation of *Trametes versicolor* using agro-industrial waste showed an activity value of 6.885 U/mL [24]. In this research, we reported an increase in enzymatic activity with a value of 25.46 U/mL. The nutrients added in the fermentation process affected the performance of the enzyme and increased its enzymatic activity. Substance conditions affect how much substrate is used when the enzymes bind to one another.

The phenomenon that occurs when fermentation causes osmosis is that the smaller size of the substrate particle provides a larger surface area to penetrate microorganisms [25]. However, tiny size can lead to substratum accumulation and interfere with respiration and aeration. By contrast, large particles have an area large enough for the efficiency of the aeration process, but the surface location for the Penetration is minimal [26]. The substrates are treated similarly, i.e. dried, smoothed, and then grated to a homogeneous size. So, the substrate size factor is less influential in this experiment.

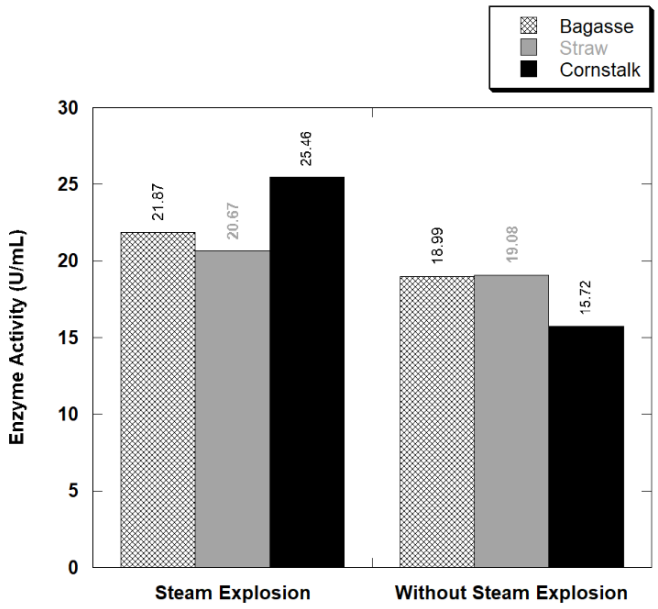

Figure 2 The effect of steam explosion against laccase enzyme production

Based on Figure 2, it can be seen that the difference in the result of each substrate's activity depends on the treatment of steam explosion. Enzyme production with a cornstalk substrate showed the highest enzyme activity at 25.46 U/mL, whereas substrates without explosion treatment only achieved enzyme activity of 15.72 U/ml. Steam exploitation helps to break down the lignocellulose component more effectively. This causes such components to be more easily digested by cane, producing higher activity units at the same fermentation time than samples without steam explosion.

In this study, the fungal strains used were a single strain. Somehow, the formation of laccase in a variety of fungal strains is inhibited by high glucose concentrations. By preventing the induction of laccase and allowing just constitutive enzyme synthesis, an excess of sucrose also decreased laccase production. This issue was resolved by using polymeric substrates, such as cellulose. While nitrogen depletion is a common trigger for fungal laccases[27], it has also been observed that in certain strains, nitrogen does not affect the activity of the enzyme [28]. Some research utilizing a low carbon-to-nitrogen ratio found high laccase activity, while other investigations demonstrated that higher laccase production was produced at a high carbon-to-nitrogen ratio [29-30]. Moreover, laccase was generated sooner when the fungus was grown in nitrogen-rich media than nitrogen-limited media [31-32].

Steaming the biomass enables quick heating without unduly diluting the sugars generated in the process. Because of the organic acids produced by the acetyl groups in hemicelluloses, the steam condenses throughout the process at such high pressures that it penetrates the biomass and starts an autohydrolysis reaction [33]. Glycosidic linkages break as a result, causing hemicellulose to become soluble. Moreover, the liquid that has condensed within the lignocellulosic fibres evaporates once more upon instantaneous relief of pressure, resulting in the mechanical disintegration of the lignocellulosic matrix. The immediate decompression also causes the particle size of the pretreated biomass to decrease[34-35].

As a comparison, the substrate is used without being treated with a steam explosion, and nutrients are added; the activity value is higher than when the substrate is treated with the steam explosion but is not nutrient-free. The difference in current enzymatic activity values between the two types of treatment is not very significant (Figure 2). Bagasse with the steam explosion is 21.87 U/mL, and it shows 18.99 U/mL without steam explosion. The differences in enzyme activity are also influenced by the effect of nutrition on the fermentation process and the lignocellulose content in each substrate. From the picture above, it can generally be seen that the cornstalk substrate produces higher units of activity when compared to other substrates. Corn stems have higher lignocellulose values when compared to the two different types of substrate. This allows the cane to produce enzymes with a better value of activity when compared to other substrates [25]. Enzyme production processes of laccase are heavily affected by the substrates and purity of the enzyme. Laccase enzymes with a purity of 42% have enzyme-specific activity of 420 units/mg protein [17]. Enzyme catalysis, by knowing the kinetic properties of enzymes, is characterized by observing product formation or substrate reduction as a function of incubation time. Enzyme kinetic characterization is performed using the method of substrate reduction performed by previous researchers[36-39]. The enzyme and substrate concentrations were investigated in the oxidation reaction to obtain the k value in each data point.

The tests were carried out by reacting ABTS as a substrate and extracting the enzyme *laccase* character as an enzyme. The experiments were conducted by varying ABTS 5 and 10 mg/mL concentrations. The enzymes used were raw *laccase* extract from the fermentation of the *Trametes versicolor* cane by solid-state fermenting method. The test was carried out by adding 5 mg/mL with the composition of 100% raw extracts of the enzyme laccase. The reaction was carried out over an hour at a 5-minute interval at a

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temperature of 25°C. After incubation, ABTS levels were quantified by measuring absorption to convert data into enzyme concentrations.

TABLE 1

OXIDATION REACTION OF ABTS WITH COMMERCIAL ENZYME

CS = 0.1 M, CE= 5 MG/ML; 10 MG/ML

t (min)	Cs (1) (M)	Cs (2) (M)
0	0.1	0,1
5	0,09837	0,09781
10	0,09603	0,09568
20	0,09278	0,09072
30	0,08753	0,08453
40	0,08304	0,07945
50	0,07934	0,07406
60	0,07474	0,06920

Based on Table 1, it can be seen that there is a decrease in the concentration of ABTS. This indicates that the raw extract of the enzyme laccase has catalyzed an ABTS oxidation reaction. The laccase enzyme acts as a biocatalyst against the substrate throughout the oxidation reaction. ABTS technique has been well performed to identify the characteristics of phenolic compounds in samples. Additionally, a previous study reported an observation against two antioxidant standards, five fruits, and four phenolic compounds, which are examined for their antioxidant capacity and reaction kinetics. Compared to the regular (pH 7.4) version, the modified ABTS technique performed at pH 4.5 with sodium acetate buffer is highly stable and simple to apply to fruit samples. The assay technique, pH, and reaction time all affected the estimated antioxidant capacities. The endpoint analysis of all experiments was made possible by the steady, straightforward reaction kinetics of the traditional antioxidant standards, trolox and ascorbic acid. Gallic acid and quercetin obtained stable endpoints. However, the most complex reaction kinetics and reaction rates among the phenolic compounds under investigation were shown by chlorogenic and caffeic acids, making endpoint analysis impossible [34].

In order to examine the kinetic parameters of the oxidation reaction, the oxidation reaction was done using two kinds of enzyme forms, commercial laccase enzyme and crude laccase enzyme, respectively. Figure 3 describes the kinetic parameters of the substrate oxidation reaction, the data being processed with order 1 reaction modelling, and numerical integration approaches. The oxidation analysis was performed by reacting ABTS as a substrate and a commercial laccase enzyme with an activity unit of 10 U/mg. The experiments were conducted by varying the concentrations of 5 mg and 7 mg extracts of enzymes. The test was carried out by adding 5 mg of commercial laccase extract to the ABTS substrate and the sodium tartrate buffer. The reaction was carried out for an hour with a 5-minute interval at a temperature of 25°C. After incubation, ABTS levels are quantified by measuring absorption to obtain data that will be converted into enzyme concentrations.

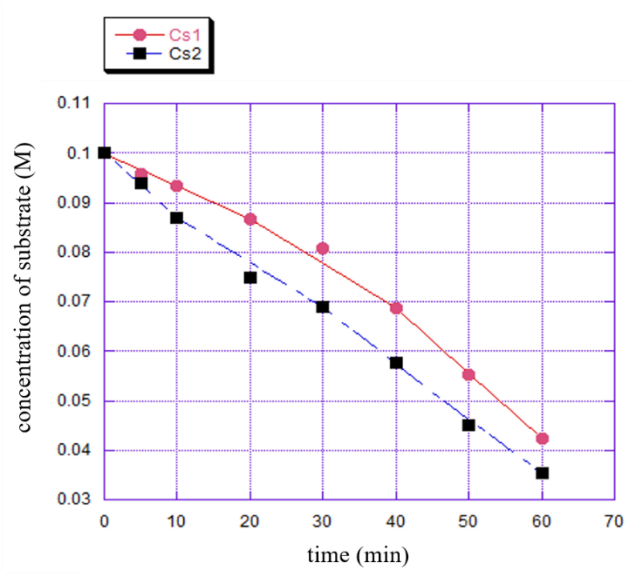

Figure 3 Fitting curve graph of oxidation reaction between ABTS and commercial enzyme

As shown in Figure 3, the modelling results against oxidation data with commercial enzymes show fitting curves that hamper perfectly. Based on the modelling results using the kinetic model of the first order, the reaction coefficients are 0.0065 and 0,0002. The Euler method used in this study for solving ordinary differential equations with first-order numerical shows the relationship between the rate of formation of the linear product and the substrate concentration is such that with the increase of the substratum concentration, the rate is also increased. The rate constant value for crude and commercial enzymes is $2x10^{-4}$ min^{-1} and$1.9x10^{-2}$ min^{-1}. The rate of commercial enzyme is show faster than crude enzyme due to its purity. This value means the rapidity of the enzyme to oxidize the substrate in each minute.

TABLE 2

OXIDATION REACTION OF ABTS WITH CRUDE LACCASE ENZYME CS = 0.1 M, CE= 5 MG/ML; 10 MG/ML

t (min)	Cs (1) (M)	Cs (2) (M)
0	0,01	0,1
5	0,09573	0,09381
10	0,09326	0,08695
20	0,08678	0,07472
30	0,08065	0,06899
40	0,06876	0,05753
50	0,05534	0,04506
60	0,04237	0,03529

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Based on the data obtained in Table 2 above, it can be seen that there is a decrease in the concentration of ABTS. This indicates that the raw extract of the enzyme laccase has catalyzed an ABTS oxidation reaction. The above data is processed with order 1 reaction modelling and numerical integration approaches to obtain the kinetic parameters of the oxidation reaction. The modelling graph can be seen in Figure 3. The decrease in the concentration hypothesized due to the quantity and orientation of the hydroxyl groups at the aromatic ring binding site and the kind of substituent determine antioxidant activity. All of the phenols evaluated in this experiment had three hydroxyl groups, which gave them the strongest antioxidant activity in the ABTS assay. IC50 values for gallic acid are lower than IC50 values for pyrogallol, suggesting that the carboxylic group somewhat enhances this action. Among the hydroxylated phenolic acids, 3,4-dihydroxyphenylacetic acid is the strongest and is the next in line of antioxidants. Research has shown that the kind of spacer that exists between the benzene ring and carboxylic acid affects their activity and that this activity is higher for phenolic acids containing a methylenic group [40-42].

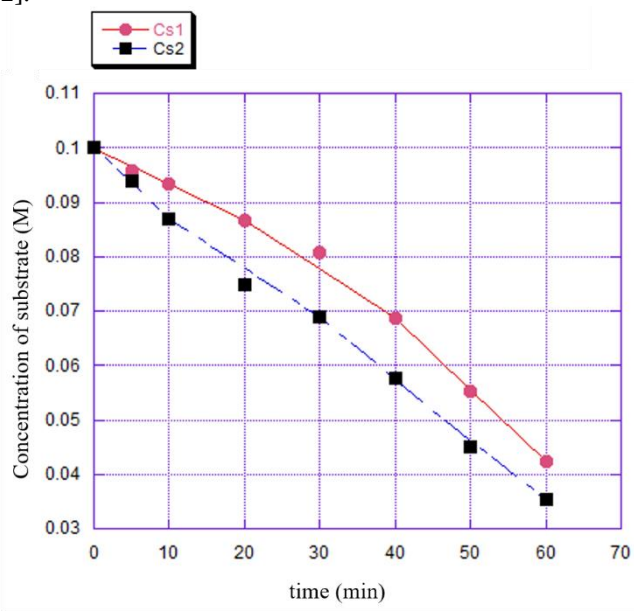

Figure 4 Fitting curve graph of oxidation reaction between ABTS and crude laccase enzyme

The modelling of oxidation reaction data using crude enzyme laccase showed slight shifting changes in Cs1 (Figure 4). Based on modelling using the order 1 kinetic model, the reaction constant is 0.014 with an error of as much as 5.64% and 0.019 with an error of 2.96%. The relationship between the formation rate of the linear product and the substrate concentration is such that with the increase in the substratum concentration, the reaction speed will also increase.

IV. CONCLUSIONS

Laccase enzyme can be produced by solid-state submerged fermentation with agro-industrial waste as substrate. The highest enzyme activity is laccase production from cornstalk as substrate with 25.46 U/mL. The steam explosion affected cellulose extraction in biomass and can be used as a substrate for enzyme production. The kinetic oxidation reaction values are $2x10^{-4}$ min^{-1} and $1.9x10^{-2}$ min^{-1} for crude and commercial laccase, respectively.

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