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Inhibitory effect of phenolic acid on *Ganoderma boninense* enzyme as an approach on *Ganoderma* infection

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Abstract. *Ganoderma boninense,* the causal of basal stem rot (BSR) has already caused a major loss of revenue in Indonesia oil palm industry. Many methods were used to control *G. boninense* attacks. Phenolic compounds could be accumulated when *G. boninense* attacks oil palm plants and these compounds exhibit anti-*Ganoderma* activity. The aim of this research was to reveal the inhibitory capacity of phenolic acid against ligninolytic enzyme activity of *G. boninense*. Darkish-red colour around the colony of *Ganoderma* on lignin selective medium was observed. Extraction of crude enzymes resulted in 628.49 U/mL Laccase enzymes, 360.42 U/mL MnP enzymes, and 632.62 U/mL LiP enzyme from three replicates. We indicate that the protein concentration of the sample between 30-60 ppm according to standard series. By purified the enzyme three fractionation were obtained consist of fraction 5 which had 1254.4; 15.5; 548.39 U/mL, fraction 6 had 1394.21; 294.77; 141.58 U/mL, and fraction 7 had 196.53; 20.66 and 161.29 U/mL for the activity of laccase, MnP and LiP enzyme respectively. The addition of inhibitor resulted in the decrease of ligninolytic enzyme activity. Both syringic and caffeic acid showed a significant inhibition effect to *Ganoderma*.

1. Introduction

Basal stem rot (BSR) disease caused by *Ganoderma boninense* has already brought major loss of revenue in Indonesian oil palm industry. Currently, *G. boninense* can be found in almost in all oil palm plantations in Indonesia, although the incidence of the disease varies. The disease progresses rapidly not only in the mineral soil but also in peat lands. The incidence of *Ganoderma* disease reported most on nutrient-poor soils. *G. boninense* substrates in oil palm plantations are very abundant and alternative host disease is very broad [1]. Many methods are used to control *G. boninense* attacks, including clean and clearing the field method, crop rotation, eradication of infected plants, the usage of fungicides and biological control, and disease resistance improvement [2].

Chong *et al*. [3] reported that phenolic compounds accumulated when *G. boninense* attacked oil palm plants and these compounds exhibited anti-*G. boninense* activity. A group of phenolic acid has been reported as an enzyme inhibitor. Srivastava *et al* found that 10 phenolic acids from plants inhibited mycelium growth and its ligninolytic and pectinolytic enzymes activities [4]. Chong *et al* showed that phenolic acid especially syringic acid could inhibit the mycelium growth of *G. boninense in vitro* at a certain concentration [3]. *G. boninense* enzyme activities from corresponds to lignin and all other major structural cell wall polymers were detected together with their effect on host wall composition during *G. boninense* infection [5]. There is a high possibility that the phenolic compounds could interrupt *G. boninense* enzymes via competitive inhibitor mechanisms. Meanwhile, Widiastuti *et*

al [6] funded by the State Ministry of Research and Technology are developing organic compound formula as bio-fungicide to *G. boninense*. In the same year, Eris *et al* [1] funded by IBRIEC itself is working on the induction of phenolic acid in oil palm by incorporating chitosan into the planting medium in the nursery stage. For supporting the research that mentioned later, information on the inhibitory effect of phenolic acid to *G. boninense* ligninolytic enzyme is needed. Therefore, the aim of this research is to see whether phenolic acid could inhibit ligninolytic enzyme activity of *G. boninense* or not.

2. Materials and Methods

2.1 Solid state fermentation

An unproductive 15-year-old oil palm tree was cut off, shredded and ground to obtain its powder form. Oil palm powder was then weighed to 20 grams, stored in jam bottle, and autoclaved. Several pieces of *G. boninense* inoculum in PDA were inoculated into the substrate (oil palm powder in the jam bottle). In addition, 2 mL of sterile water was added in order to make the condition moistly favourable for the fungus growth. The incubation period was 30 days.

2.2 Crude ligninolytic enzymes extraction

After incubated for 30 days, 20 mL of 0.2 M pH 7 phosphate buffers were mixed to the inoculated oil palm powder and the mixture was ground using mortar. Crude Ligninolytic enzymes were extracted by filtering the liquids resulted from grinding using Whatman paper No. 41.

2.3 Ligninolytic enzyme activities

Laccase activity was measured by spectrophotometry, as ABTS oxidation at 420 nm ($^{TM}_{420}$ = 36,000 M^{-1} cm^{-1}). The reactive mixture contained per 1 mL: 0.6 mL acetate buffer 0.5 M pH 5, 0.1 mL ABTS solution 1 mM and 0.3 mL enzyme filtrate.

Manganese peroxidase activity was also measured by spectrophotometry and the amount resulted from detraction of enzyme activity measured with and without $MnSO_4$ at 465 nm ($^{TM}_{465}$ = 121,000 M^{-1} cm^{-1}). The first reactive mixture contained 0.1 mL sodium lactate buffer 50 mM pH 5, 0.1 mL guaiacol 4mM, 0.2 mL $MnSO_4$ 1 mM, 0.1 mL H_2O_2 1 mM, 0.3 mL distilled water and 0.2 mL enzyme filtrate. The second reactive mixture was the same as the first one but without addition $MnSO_4$ thus to make the volume 1 mL, distilled water volume was added to its volume becomes 0.5 mL.

Lignin peroxidase activity was evaluated by UV spectrometry of the veratryl aldehyde produced ($^{TM}_{310}$ = 9300 M^{-1} cm^{-1}) during veratryl alcohol oxidation at 310 nm. The reactive mixture contained 0.2 mL acetate buffer 0.05 M pH 5, 0.1 mL veratryl alcohol 8 mM, 0.05 mL H_2O_2 1 mM, 0.45 mL distilled water, and 0.2 mL enzyme filtrate.

The absorbance of all enzyme assays was read at 0 and 30 minutes. For all enzymes under evaluation, one activity unit was defined as the amount of enzyme necessary to oxidize 1 nmol of substrate per minute. The specific activity was expressed as units per milligram of protein.

2.4 Addition of a hypothesized inhibitor

Syringic acid and caffeic acid solution were made which each of both concentrations were 0, 25, 50, and 100 ppm. These substances were examined to see whether they have an inhibition effect or not by adding 1 mL of each concentration of both substances to the enzyme reaction mixture and subsequently, the enzyme activities were checked using the aforementioned methods.

2.5 Purification method

Crude enzymes were purified using a gel permeation chromatography method. Sephadex G-100 and phosphate buffer were used as stationary and mobile phase, respectively. Ten grams of Sephadex G-100 was suspended with 300 mL phosphate buffer 0.1 M pH 6. Column packaging was prepared through stoppage of the column in the inside using cotton that drabble with the eluent to be used. The homogenized stationary phase was slowly poured against the column's wall using a pipette and

allowed to stable until it was separated into two phases. The column flow rate was arranged and calculated (drop/min) so that the flow was moderate. Enzyme extract was then added until it reached the boundary of the stationary phase and the eluent was slowly added through the column's wall using a pipette, consecutively. Every 10 mL of liquid dripping from the column was collected for measurement of the total amount of protein using spectrophotometer at 280 nm. After the enzyme was purified, ligninolytic enzymes activities were measured once again with and without the hypothesized inhibitors.

3. Results and Discussion

3.1. The activity of Ganoderma boninense *enzyme*

Palm trunk powder extracted with 0.2 M phosphate buffer pH 7. Extraction was done with pH 7 phosphate buffer to maintain the pH of the protein that requires a certain pH range and prevents damage to the structure of the protein. Meanwhile, according to Bannerman *et al*. in Wulaningtyas [7], ligninolytic enzymes were extracted with phosphate buffer pH 7 because white root fungi (WRF) generally live at neutral pH. Otherwise, the structure of the protein will be denatured. Extraction was carried out to obtain crude enzyme that would be used in the enzyme activity assay.

Ligninolytic enzymes were on of the extracellular enzymes because of their role in the degradation process. So, it can be detected by using crude enzyme obtained from the supernatant (**Figure 1**). The main function of the extracellular enzyme is necessary to carry out nutrients around it, allowing the nutrients to enter the cell and so it can be absorbed directly by fungal hyphae [7].

WRF is a fungus that is capable of destroying all the major constituent components of wood (cellulose, hemicellulose, and lignin), thus allowing to reduce the content of xylem and phloem as the main constituent of wood logs and cause to become brittle. The process is aided by the enzyme ligninolytic which include laccase and peroxidase enzyme (MnP and LiP) [8]. *Ganoderma boninense* enzyme activity in this study was much higher compared with WRF *Omphalina* sp. and *P. ostreatus* with an average value of laccase, MnP and LiP consecutive 682.49 U / mL, 360.42 U / mL, and 632.62 U / mL. The difference in result might be caused by the different of likelihood, the difference of isolates and substrate used as well.

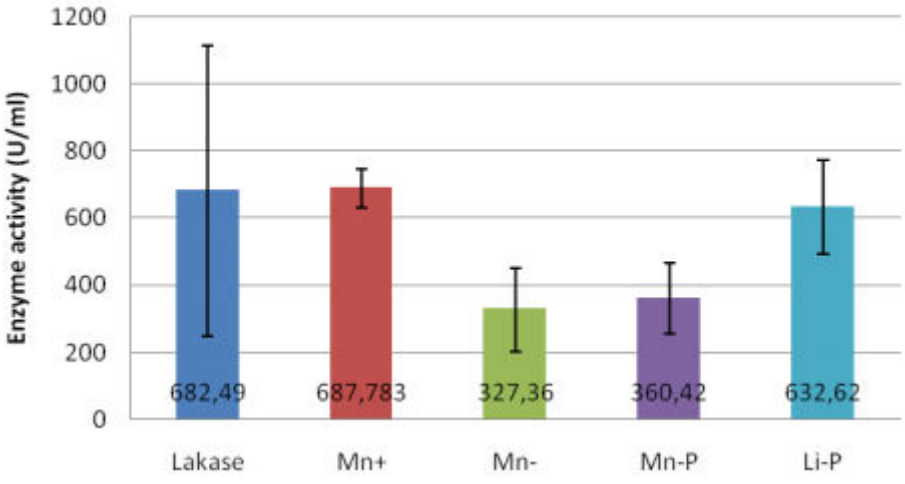

Figure 1. The ligninolytic enzyme activity of *Ganoderma boninense.*

3.2. Purification of an enzyme of Ganoderma boninense

Manganese peroxidase is an enzyme containing heme and is generally considered to be essential for lignin degradation in vivo. Manganese peroxidase catalytic cycle similar to lignin peroxidase, with the addition of Mn^{2+}, were oxidized to Mn^{3+} during the reaction. One unit of MnP proportional to 1 nmol of product produced per minute. Differences MnP considerable value between sample code 11, 17,

and 25, probably due to the rate of growth of mycelium different in each bottle an hour so that the resulting value of different activities.

Lignin peroxidase is also a heme-containing peroxidase. Not all JPP produce lignin peroxidase. But on JPP *G. boninense* produce all the enzymes lignolytic even with a fairly high value when compared with other JPP. The highest yield was obtained from the sample code number 25 in the amount of 974.91 U / mL followed by sample code numbers 17 and 11 of 462.37 U / mL and 460.57 U / mL. The enzyme activity was measured at minute 0 and 30, the difference in absorbance at the time shall be included in the formula for calculating enzyme activity. Enzyme activity graph the relationship between the sample code with the activity of the enzyme produced. Graph enzyme activity is presented in **Figure 1**.

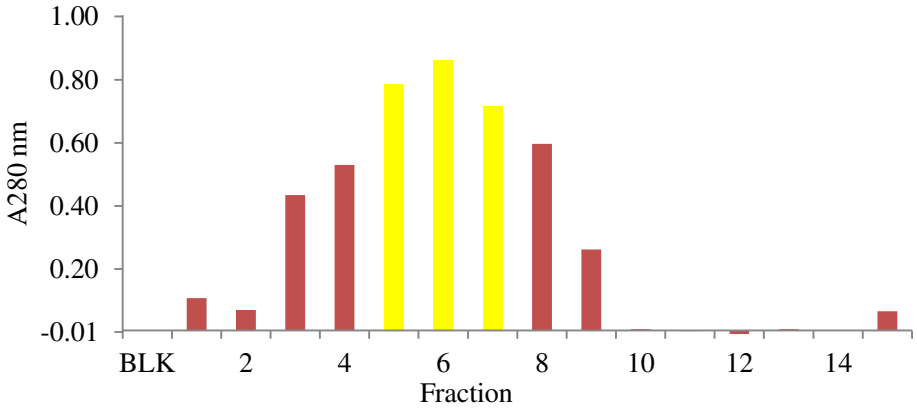

Figure 2. The absorbance of the eluate from gel permeation chromatography for partial purification.

To separate each ligninolytic enzyme, gel chromatography was conducted whereas the separation based on the different MW. We collected 15 fractions of eluate which were fraction 5,6 and 7 had the highest protein concentration (**Figure 2**). Based on of the MW, theoretically laccase which has the highest MW (50-110 kDa) will be collected first, then MnP (46 kDa) and LiP (38-46 kDa) [11]. From the three fraction, we analyzed the enzyme activity. Fraction 5 had 1254.4; 15.5 and 548.39 U/mL for laccase, MnP and LiP respectively. Meanwhile fraction 6 had 1394.21; 294.77; and 141.58 U/mL, and fraction 7 had 196.53; 20.66 and 161.29 U/mL for laccase, MnP and LiP respectively. Aslam and Asgher also found Laccase and MnP enzyme in the same fraction [9].

Because of the wide range of laccase MW, this enzyme was still found in later fraction be in conformity with the smaller one (MnP and LiP). It was too difficult to separate MnP and LiP because of the similar MW. *Ganoderma* has the highest activity of Laccase among the other lignolytic enzyme. Laccase is the oxidase enzyme which caused the injured area and discolours of the plant [10]. Fungal laccase plays a role in pigment formation in spores, detoxification of phenol compound produced during lignin degradation and acts synergistically with peroxidases and another enzyme in the breakdown of lignin [11]. Laccase can oxide a range of compounds such as diphenol, aryl diamine and aminophenol [12]. It assumed virulence of *G. boninense* is also determine with its high laccase activity.

3.3. Addition of Inhibitor

Effect of the inhibitor on ligninolytic enzymes needs to be investigated in vitro as possible control chemicals of *Ganoderma* on oil palm. Syringic and Caffeic acid have a similar structure with a substrate of the ligninolytic enzyme. Lignin consists of phenyl propanoic (p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) unit). The addition of inhibitor brought the decrease of laccase activity.

Syringic acid gives higher inhibition effect than caffeic acid. It could be caused by palm oil as woody angiosperms (hardwoods) consists of S that was the predominant phenolic component (important because lignin is a polyphenol), which comprised 65.6–68.5% of the total phenolic monomers in the oxidation mixtures. The increase of inhibitor brought the high inhibitory effect. Syringic has OH group that could assume has the ability to trap hydrogen peroxide derived hydroxyl radicals (OH•) and may be useful for inhibiting lignin peroxidase enzymes e.g. manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase as found in benzoate [13].

Laccase oxidizes various phenolic compounds including lignin through phenoxy radical formation of phenoxy ring. Laccase using O_2 as oxidant and can oxidize phenolic compounds on a non-specific condition. Laccase enzyme activity obtained from the experiment were 188 U / mL, 115.05 U / mL, and 1743.98 U / mL for sample 11, 17, and 25 respectively (**Figure 3**). Sample 25 produced the highest laccase enzyme activity, possibly due JPP inoculated grow well so that the enzyme can degrade lignin in it.

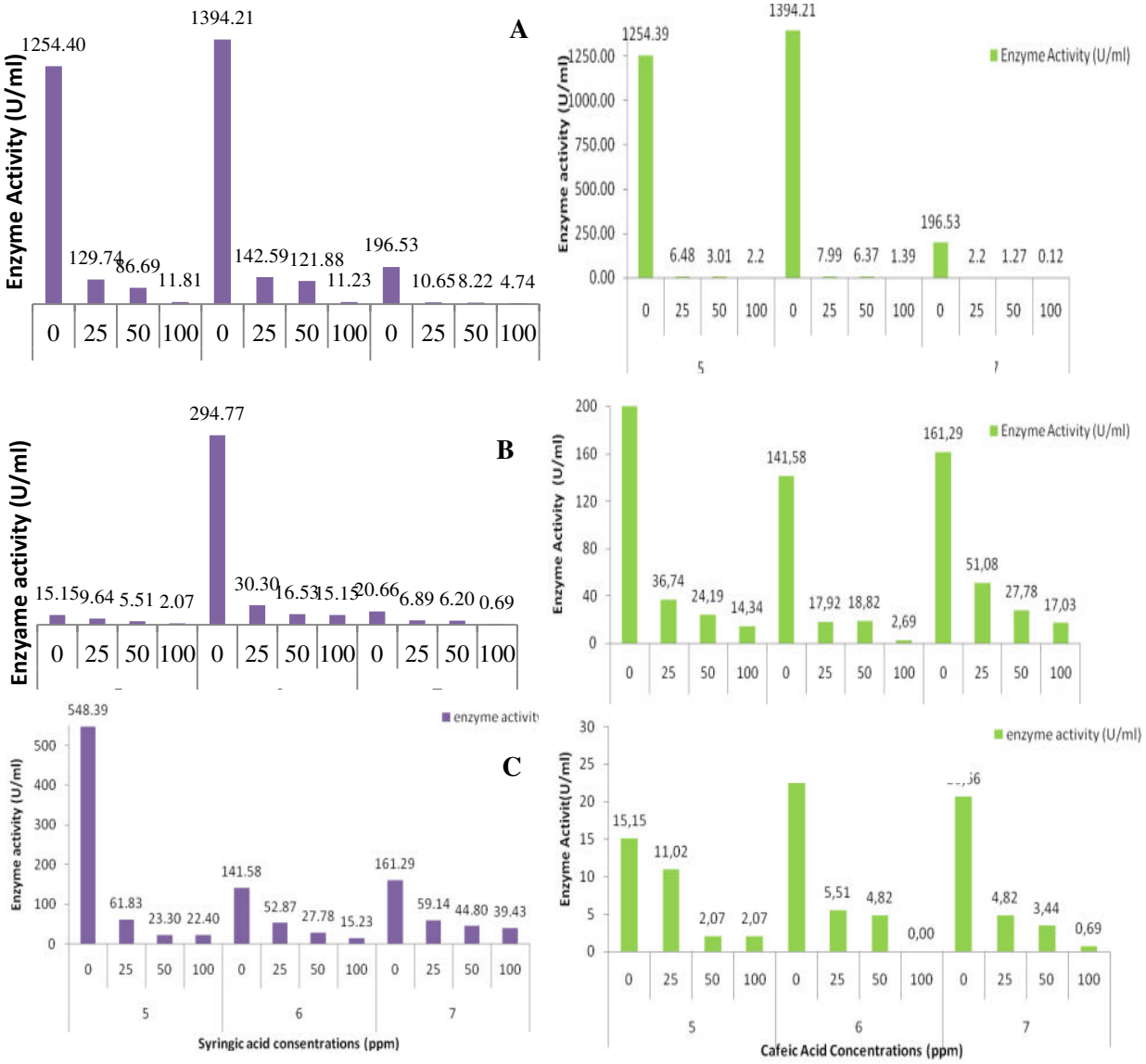

Figure 3. Changes in Laccase (A), MnP (B) and LiP (C) enzyme activity after several concentration additions of Syringic and Cafeic acid.

4. Conclusions

Extraction of crude enzymes resulted in 628.49 U/mL Laccase enzyme, 360.42 U/mL MnP enzyme, and 632.62 U/mL LiP enzyme from three replicates. We indicate that the protein concentration of the sample between 30-60 ppm according to standard series. By purified the enzyme three fractionation were obtained consist of fraction 5 which had 1254.4; 15.5; 548.39 U/mL, fraction 6 had 1394.21; 294.77; 141.58 U/mL, and fraction 7 had 196.53; 20.66 and 161.29 U/mL for the activity of laccase, MnP and LiP enzyme respectively. The addition of inhibitor resulted in the decrease of ligninolytic enzyme activity.

5. References

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