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Ligninase Profiling and Optimization of Laccase Production from Indigenous Wood Rot Fungus (WRF) $KLUM_2$ in Kirk Medium-*Alkali Lignin Kayu Jati* (MK-ALKJ)

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Abstrak

The production of ligninase by wood rot fungus (WRF) is determined by carbon source and growth condition. The goal of this study is to determine the ligninase profile produced by WRF $KLUM_2$ in Kirk Medium using teak wood alkaline lignin as a carbon source known as *Kirk Medium-Alkali lignin Kayu Jati* (MK-ALKJ), optimization of dominant ligninase production in the MK-ALKJ compared to the one that is produced in the Kirk's medium with glucose as a carbon source (MK-Glucose). This research was conducted in an experimental laboratory consisting of: (1) spore suspension preparation, (2) ligninase profiling at various growth times, (3) ligninase profiling at various temperature variations, (4) optimization of laccase production including pH and the amount of nitrogen source. Growth was identified based on the specific activity of lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. The results showed that relatively the three types of ligninase, namely LiP, MnP, and laccase, were produced in the same amount by the wood rotting fungus isolates $KLUM_2$ in MK-ALJK. All three were produced with the highest yield of respectively 55.65; 52.48; 57.64 U/mg. Laccase as the dominant ligninase can be optimized to reach 83.52 U/mg by inoculating 2.10^7 spore cells in MK-ALKJ in 37 °C, pH = 3.5, and a nitrogen source of 20mM $(NH_4)_2SO_4$ for 6 days. Therefore, it can be concluded that the ligninase activity of indigenous WRF $KLUM_2$ in MK-ALJK medium is higher than in the MK-Glucose.

Keywords: Kirk-Medium, $KLUM_2$ isolate, ligninase, wood rot fungus.

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1. INTRODUCTION

Ligninase enzyme is a typical enzyme produced by the wood rot fungi (WRF) group (Deshmukh & Sao, 2015). Ligninase is divided into two groups, heme peroxidase and phenol oxidase. Heme peroxidase consists of lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and versatile peroxidase (EC 1.11.1.16). Phenol oxidase only consists of laccase (EC1.10.3.2) (Hamid, 2013). The medium that is commonly used for the growth and production of ligninase is Kirk's medium (Urek *et al.*, 2007; Acevedo *et al.*, 2011; Susanti *et al.*, 2016). The components in Kirk's medium are carbon sources; ammonium sulfate (source of nitrogen); thiamine-HCl (precursor of B vitamins necessary for fungus growth); veratryl alcohol (ligninase inducer); KH_2PO_4 (buffers); $MgSO_4.7H_20$, $CaCl_2$, NaCl, $FeSO_4$, $CuSO_4.5H_2O$, $CoCl_2$, and $ZnSO_4$ (micro minerals), nitriloacetate; and $ALK(SO_4)_2$ (trace minerals) (Susanti *et al.*, 2016).

According to Dekker *et al.*, (2001), the ligninase enzyme is a secondary metabolite produce in the iodophase or stationary phase by the WRF in a medium with a limited carbon and nitrogen sources. Therefore, the use of complex carbon sources is expected to accelerate the iodophase conditions, hence ligninase activity can be produced faster and higher. Complex carbon sources such as lignin extract are known to produce ligninase with higher activity than glucose and glycerol carbon sources (Hu *et al.*, 2016; 14 Kenkebashvili *et al.*, 2012). *Genoderma applanatum* in Indulin AT carbon source media (lignin extract) produced higher

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ligninase (LiP 7.512 U/mg; MnP 13.133 U/mg; laccase 0.977 U/mg), compared to acacia sawdust carbon source (LiP 5.420 U/mg; MnP 13.705 U/mg; laccase 0.906 U/mg), Ball Milled Cellulose (LiP 7.512 U/mg; MnP 1.751 U/mg; laccase 0.356 U/mg) and glucose (LiP 4.512 U/mg; MnP 0,275 U/mg; laccase 0.064 U/mg) (Artiningsih, 2009).

Ligninase production by wood-rotting fungi is also influenced by growth conditions such as temperature and pH. WRF isolates classified as mesophyll fungi can live in a temperature range of 15-40 °C (Pham *et al.*, 2019). The optimum pH for the production of heme peroxidase is at pH of 4.5-5.5 (Acevedo *et al.*, 2011), and for the production of phenol oxidase (laccase) is between pH of 3-7 (Hamid *et al.*, 2013).

Indigenous WRF $KLUM_2$ isolated from the weathered cocoa husk of Sepawon plantation, Kediri, East Java has the potential as a source of ligninase since it can reduce methylene blue in selective media (Delila, 2016). Arinta (2017) showed that WRF $KLUM_2$ in Kirk's medium with a glucose carbon source (MK-Glucose) produced a LiP ligninase profile (60.67 U/mg) much higher than MnP (46.56 U/mg) and laccase (23.38 U/mg). In this study, we are going to observe the ligninase profile of WRF $KLUM_2$ grown in Kirk medium using teak wood alkaline lignin as a carbon source known as *Kirk Medium-Alkali lignin Kayu Jati* (MK-ALKJ). Observations were made at various growth times and temperatures. The production optimization was carried out towards the dominant ligninase type which included the optimization at various pH and the amount of $(NH_4)_2SO_4$.

2. MATERIALS AND METHODS

Materials and tools

The materials used in this study were the indigenous WRF $KLUM_2$ of a collection group of Dr. Evi Susanti at the Biochemistry Research Laboratory, State University of Malang, which is continuously subcultured in PDA (Potato Dextrose Agar) medium. Materials with a grade of p.a. are: Potato Dextrose Agar (PDA), hydrogen peroxide (H_2O_2), $MnSO_4.7H_20$, $CaCl_2$, NaCl, $FeSO_4$, $CoCl_2$, $ZnSO_4$, $AlK(SO_4)_2$, H_2SO_4, HCl, guaiacol, tartaric acid, citric acid, veratryl alcohol, Folin-Ciocalteu reagent, sodium citrate, citric acid, nitriloacetate, Tween-80, and ammonium sulfate. The technical grade materials are: distilled water, lysorin, detergent, teak powder, rubbing alcohol and 70% alcohol. Wood rot fungi local isolate KLUM2 is a collection of the Biochemistry Laboratory, Department of Chemistry, State University of Malang.

The equipment used in this study were test tubes, Erlenmeyer flask, watch glass, spatula, stirring rod, beaker, volume pipette, dropper pipette, measuring cup, glass funnel, filter paper, sieve, milipore, ose wire, enten wire, plastic wrap, spirit lap, lighter, tissue, inoculum bottle, tip, micropipette, microtube, hot plate and stirrer, centrifuge (80-1 Table Top Low speed), oven (Memmert U10 Oven), incubator (Memmert BM400), autoclave (Tommy SX vertical chamber), vortex (Dragon Lab MX-5), refrigerator, analytical balance (Precisa XT 120 A), and Visible spectrophotometer (Spectrophotometer B-ONE, Vis 50 DA).

Spore Suspension Preparation

All stages of spore suspension preparation were conducted aseptically. A pure isolate of indigenous WRF $KLUM_2$ was inoculated on Potato Dextrose Agar (PDA) media and incubated for 14 days. The spores formed were resuspended using a 0.02% Tween-80 solution with the help of an ose needle, and vortexed for 10 minutes, and then left idle for 30 minutes. The mixture was filtered using sterile cotton in a sieve to obtain a spore suspension. The number of initial spores in each production was set at 2.10×10^7 cells. Determination of the volume of the inoculated suspension with an OD value of OD_{660} = 0.41 is equivalent to a cell density of 22.6×10^5 cells/mL, the volume of spore suspension required for each experiment is calculated based on equation (2) which is a derivative from equation (1) :

$$\Sigma \text{ cell} = \frac{OD_{660\ measured}}{OD_{660\ standar}}\ x\ stand.\ cell\ density\left(\frac{cell}{ml}\right).vol(ml) \quad (1)$$

$$\text{Vol.(mL)} = \frac{2{,}10 \times 10^7 \text{x OD 660 standar}}{\text{cell density x OD660 measured}} \ldots\ldots\ldots\ldots\ldots (2)$$

Kirk Medium-Alkali lignin Kayu Jati (MK-ALKJ) Preparation

Teak wood powder was added with 1% sulfuric acid and heated at 80 °C for 20 minutes, until the solution becomes cloudy brown. 4% sodium hydroxide solution then added and boiled for 30 minutes until the

solution turns from brown to dark brown and smells pungent. The mixture was autoclaved at 121 °C with the pressure of 15 psi. The results of the autoclave were filtered using filter paper and the residue obtained was neutralized until it reached pH 7 with hot distilled water. The alkaline lignin residue further baked at 70 °C to obtain dry product.

The alkaline lignin medium was made using a modified method of Susanti et al. (2016). The composition of the production media per L consists of: 10 g Alkali lignin teak wood; 1 X basal medium; 1 X trace element solution; 0.5 g $NH_4(SO_4)_2$, 0.3 g Tween-80; 640 ml acetate buffer pH 4.5 0.2 M; 0.5 g veratryl alcohol; and 0.001 g thiamine-HCl. Trace element solution 1 X per L contains: 3 g $MgSO_4.7H_2O$; 5 g NaCl; 0.1 g $FeSO_4.7H_2O$; 0.1 g $CoCl_2$; 0.1 g $ZnSO_4.7H_2O$; 0.1 g $CUSO_4.5H_2O$; 10 mg $AlK(SO_4)_2.12H_2O$ and 1 g nitriloacetate, and 1 X per L basal medium contains: 0.2 g KH_2PO_4: 0.05 g $MgSO_4.7H_2O$; and 0.01 g CaCl .

Ligninase Profiling of WRF KLUM$_2$ in MK-ALKJ

Ligninase profiling was conducted in 2.10 x 10^7 spore cells of WRF $KLUM_2$ inoculated into 20 mL of MK-ALKJ with pH=4.5, incubated for 12 days. The fermented products on days 0, 2, 4, 6, 8, 10, and 12 were centrifuged at 1000 rpm for 15 minutes. The supernatant obtained was crude extract of ligninase, tested for protein content and activity of ligninase enzymes including LiP, MnP, and Laccase activites. The second observation was carried out based on the time of ligninase production at various temperature of 27, 30, 37, and 45 °C. Each treatment was conducted in triplicate.

Assay of Protein Concentration

A total of 0.5 mL protein standard solution containing 0 (blank), 25, 50, 75, 100 g/mL of bovine serum albumin (BSA) protein and the sample was put in a dry and clean test tube with the addition of 2.5 mL of Biuret solution, briefly vortexed. It was left idle at room temperature for 10 minutes and 0.25 mL of Folin-Ciocalteu 1 N was added then vortexed and left incubated for 20 minutes. The absorbance was measured at a wavelength of 750 nm. The obtained absorbance data used to create protein standard curve. Determination of sample protein concentration was determined by interpolating sample absorbance value into the protein standard curve.

LiP Activity Test

LiP activity test conducted by taking 0.8 mL of a 10 mM veratryl alcohol solution placed in a test tube with the addition of 1 mL of 0.2M tartaric acid solution, 1.5 mL of crude extract of the enzyme, and 0.5 mL of 50 mM H_2O_2 solution. The absorbance was measured at a wavelength of 310 nm, at minutes 0 and 1 after the addition of H_2O_2. Based on the absorbance value obtained, the activity of the LiP enzyme was determined using the equation (3), the value of the excitation coefficient molarity (ε_{max}) is 90300 $M^{-1}cm^{-1}$.

$$Enzyme\ Activity\left(\frac{U}{ml}\right) = \frac{(At-A0)\times V_{tot}\times 10^6}{\varepsilon_{max}\times d\times V_{\ enzyme}\times t} \ldots.(3)$$

A_t : Absorbance at minute 1
A_0 : Absorbance at minute 0
ε_{max} : substrate molar absoprtivity
d : thickness of cuvette (cm)
t : time (minutes)
V_{total} : total volume ingredients (ml)
V_{enzyme} : enzyme filtrate volume (ml)

MnP Activity Test

A total of 0.5 mL acetate buffer solution pH 5.5 was added with 0.8 mL of 1 mM guaiacol solution and 1 mL of 20 mM citric acid solution. 0.5 mL of 50 mM H_2O_2, 1.5 mL of 0.1 mM $MnSO_4$ solution and 0.2 mL of crude extract of the enzyme then added. The mixture left idle for 15 minutes. The MnP activity test was determined based on the amount of guaiacol that was oxidized, which was then measured at a maximum wavelength of 465 nm at minute 0 and minute 1. The absorbance value obtained is to determine the activity of the MnP enzyme using equation (3), with a molar excitation coefficient value (ε_{max}) of 12100 $M^{-1}cm^{-1}$.

Laccase Activity Test

Laccase activity was measured based on its ability to oxidize guaiacol. Oxidized guaiacol was measured spectrophotometrically at a wavelength of 470 nm with a molar excitation coefficient of 6740 M^{-1} cm^{-1} (Praveen *et al.*, 2010). The step of the laccase

activity test was 0.8 mL of 10 mM guaiacol solution which was added with 0.8 mL of pH 5 0.2 M acetate buffer and 0.2 mL of extra crude enzyme, then measured at a wavelength of 470 nm at minute 0 and 1. The absorbance value obtained is to determine the activity of the Laccase enzyme using equation (3). With a molar excitation coefficient (ε_{max}) of 6740 $M^{-1}cm^{-1}$.

Optimization of the Production of the Dominant Ligninase Enzyme KLUM2 Isolate

Optimization of the production of the dominant ligninase enzyme KLUM2 isolate includes pH and the amount of nitrogen source. The variation of pH used refers to the work by Susanti *et al.*, (2016), which are 3; 3.5; 4/5; 5; and 5.5 using 0.2 M acetate buffer. The nitrogen source used was $(NH_4)_2SO_4$ with 5; 10; 20; and 40 mM variations. Each independent variable is observed by referring to the previous subchapter, by adjusting the observed independent variables. The dependent variable is the protein content and the activity of the dominant ligninase enzyme.

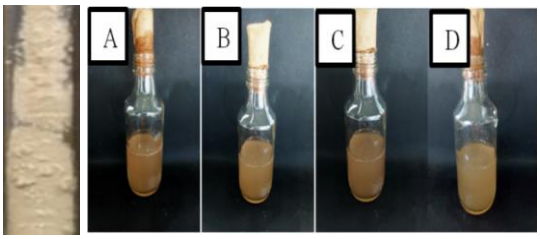
3. RESULTS AND DISCUSSIONS

Inoculum Preparation and Spore Suspension of WRF $KLUM_2$

The WRF $KLUM_2$ is a collection of the research group of Dr. Evi Susanti at the Biochemistry Research Laboratory, State University of Malang, which is continuously subcultured in potato dextrose agar (PDA) medium. At the spore preparation stage, the cultures were incubated at 37 °C for 14 days and the inoculum was obtained in the form of a brownishs pore suspension (Figure 1a). This result is similar with the results of Delila's work (2017). The spore suspension on first until third repetitions was cloudy brown, while on the fourth repetition, it was lighter (Figure 1b). This is comparable to the OD value shown in Table 1, which indicated that the lighter the brown color, the lower the OD value.

Table 1. OD_{660} WFH $KLUM_2$ Spore Suspension

Experiment	$0D_{660}$
1	1.252
2	1.242
3	1.305
4	1.167



(a) (b)

Figure 1. (a) Culture of WRF $KLUM_2$ in PDA slant which was incubated at 37 °C on the 14th day and (b) WFH $KLUM_2$ Spore Suspension Experiment 1 (A), Experiment 2 (B), Experiment 3 (C), Experiment 4 (D**).**

Growth of WRF $KLUM_2$

The color and turbidity of MK-ALKJ which had been inoculated with WRF $KLUM_2$ spore suspension for 12 days had changed. On day 0, MK-ALKJ was clear. Entering day 2 and 3, it became slightly cloudy and became cloudier with increasing growth time. This is in line with Arinta (2017) that stated the growth of $KLUM_2$ wood rot fungi isolate spores in PDB (Potato Dextrose Broth) medium becoming reddish brown after 6 days. The opinion was confirmed by the results of measurements of protein levels carried out on days 0, 2, 4, 6, and 8. Protein levels on day 0 were 67.22 g/mL, increased to 77.08 g/mL on day 2. A gradual increase occurred until day 8 to 81.11 g/mL (Figure 2).

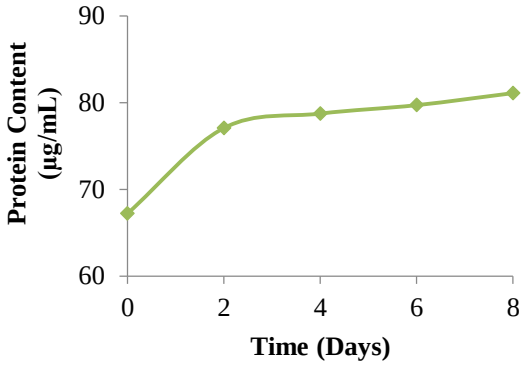


Figure 2. Growth curve of WRF $KLUM_2$ in MK-ALKJ at 37 °C, pH 4.5 and $(NH_4)_2SO_4$ 20 mM

The increase in protein levels showed that the spore suspension of the $KLUM_2$ wood rot fungi isolate experienced growth as mentioned from Susanti *et al.*, (2016) which stated that an increase in protein content indicated an increase in cell components,

which was directly proportional to the increase in the mass of a microorganism and was a feature of microbial growth. Susanti *et al.*, (2016) also showed that the increase in extracellular protein levels of *P. chrysosporium* ITB isolate was proportional to the increase in dry weight of the mycelium.

The growth of WRF $KLUM_2$ in MK-ALKJ from day 2 to 8 tends to be gentle. Contrasting growth in the Kirk's medium with a glucose carbon source (Arinta, 2017), which shows a sharp increase (Figure 3.). This condition is similar with Susanti *et al.*, (2016). The growth of *P. chrysosporium* ITB isolate using sawdust carbon sources is gentler than in glucose carbon sources medium. This is because glucose can immediately become energy source for the formation of primary metabolite components such as cell biomass constituents so that the increase in protein levels is higher and faster, whilst the carbon source in MK-ALKJ is harder to digest, forcing the wood rot fungi to produce ligninase to degrade the carbon source as an energy source for its growth. As a result, the increase in protein during growth is relatively sloping (Artiningsih, 2006; and Susanti *et al.*, 2016).

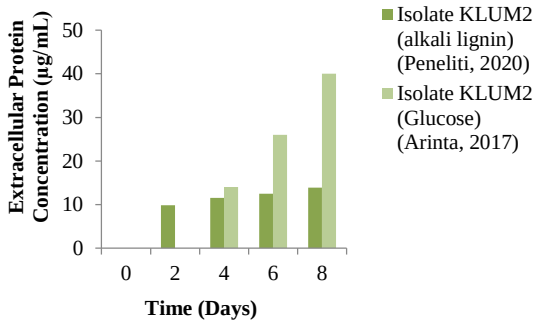


Figure 3. Comparison of extracellular protein concentration of WRF $KLUM_2$ increment cultured in different carbon sources.

Ligninase Profile of WRF $KLUM_2$ in MK-ALKJ at Various Growth Times

The results of the study in Figure 4 show that on day 0 the specific activity value was measured. Whereas on day 0 there should have been no activity at all, this is presumably due to the presence of interfering compounds in the growth medium components, which also absorb during the measurement of enzyme activity. This opinion is supported by a decrease in the value of specific activites on the 2nd day. The decrease occurs because the components of the medium are changed and used to produce energy and cell growth. Furthermore, there was an increase from the eigth to the twelfth day. These results were similar with the ligninase profile Kirk's medium with glucose carbon source which showed ligninase activity by the $KLUM_2$. Isolate began to increase and showed the highest activity on day 6 (Arinta, 2017). The lower ligninase until the sixth day was assumed because the cells are adapting to the medium, then after the nutrients in the medium are reduced, the production of ligninase will also decrease (Subagyo *et al.*, 2014).

The ligninase profile on the sixth day shows that the three enzymes of LiP, MnP, and Laccase that were produced were relatively equal. This result is different from Arinta (2017) which showed that LiP was more dominant than MnP and Laccase in MK-glucose. This proves that the type of carbon source affects the ligninase profile of the WRF $KLUM_2$. Cells do not need to immediately produce ligninase in MK-glucose. On the other hand, although the growth of the WRF in MK-ALKJ is slow, lignin alkaline as the only carbon sources in the medium will trigger ligninase production to convert alkaline lignin into a carbon source that can be converted into energy for growth.

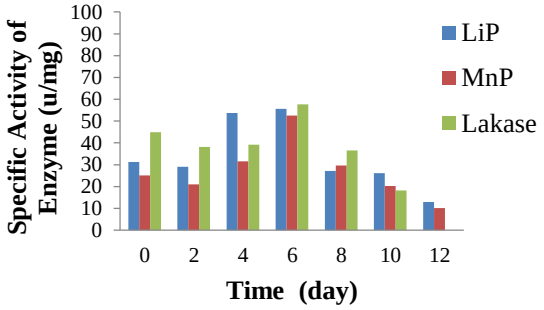


Figure 4. Specific activity of ligninase produced by WRF $KLUM_2$ at pH 4.5, 37 °C, $(NH_4)_2SO_4$ 20 mM at various growth times.

Ligninase Enzyme Profile of WRF KLUM2 at Various Growth Temperatures

The production of ligninase at various temperatures was conducted at the optimum time of ligninase production which is shown in Figure 5 for the sixth day. In general, the temperature gave the same effect on the production of LiP, MnP and Laccase from the $KLUM_2$ isolate, which increased from 27 to the optimum temperature at 37 °C, then decreased at 45 °C and showed consistency

that laccase was the dominant ligninase produced in MK-ALKJ. This fits with Susanti's (2016) statement that stated mesophyll fungus generally growth at temperatures between 25-40 °C which have enzymes in the form of thermolabile proteins that have been denatured at temperatures above 40 °C.

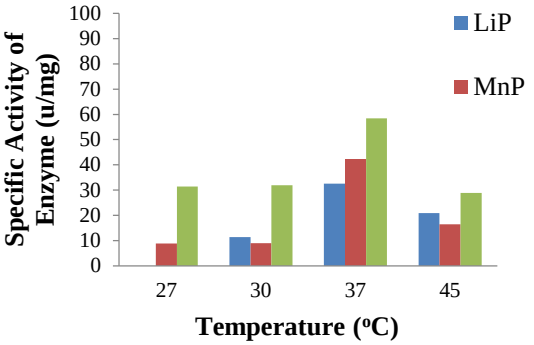


Figure 5. Specific activity of Ligninase produced by WRF $KLUM_2$ in MK-ALKJ on day 6 at pH 4,5, $(NH_4)_2SO_4$ 20 mM at various temperatures.

pH Optimization for Dominant Ligninase Production by Wood Rot Fungi Isolate $KLUM_2$ in MK-ALKJ

The results showed that pH affected laccase production (Figure 6). Laccase production increased to pH 3.5 (63.32 U/mg), then decreased to 50% at pH 4.5. Thus, laccase production at the optimum condition was obtained at pH 3.5. This study still corresponds with Hamid *et al.*, (2013) which stated that the optimum conditions for enzyme production for the phenol oxidase (laccase) group ranged from pH 3-7. Neto *et al.*, (2009) showed that the production of Laccase by *L. crinitus* which is a Basidiomycetes group was optimum at pH 3.5.

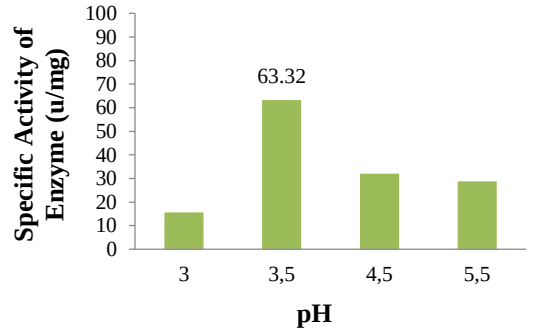


Figure 6. Specific Activity of Laccase Enzyme produced by WRF $KLUM_2$ in MK-ALJK, at 37 °C, $(NH_4)_2SO_4$ 20 mM, at various pH levels.

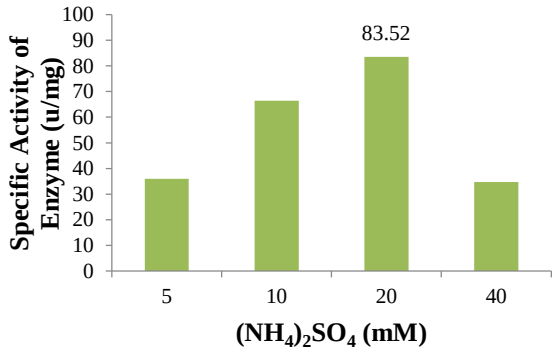
Other studies such as Manimozhi et al (2012) showed that the pH value of 5.5 is the optimum value for Laccase production from *Agaricus heterocystis*. Kumar, (2016) reported that the optimum pH for Laccase production from *Aspergillus Flavus* was at pH 7. This proves that the optimum pH for Laccase production varied depending on the fungus strain.

Optimization of $(NH_4)_2SO_4$ levels for Dominant Ligninase Production by the indigenous Wood Rot Fungi $KLUM_2$ Isolate in Kirk-Alkali Lignin Medium

The optimization of $(NH_4)_2SO_4$ levels was conducted after knowing the optimum pH level for the production of dominant ligninase (laccase) $KLUM_2$ isolate. The results showed that the levels of $(NH_4)_2SO_4$ affected the production of laccase. $(NH_4)_2SO_4$ has a role as a nitrogen source for the growth of WRF $KLUM_2$ and induces ligninase production. Figure 7 shows low laccase activity at 5 mM $(NH_4)_2SO_4$ levels, because at that amount the nitrogen source is still low. Thus, the formation of ligninase is also low. Laccase activity began to increase at 10 mM levels, then experienced a maximum increase at 20 mM levels of 83.52 U/mg, and decreased drastically at 40 mM levels. The production of ligninase requires an adequate nitrogen source to induce and supply ligninase precursors. The excess number of nitrogen sources resulted in insufficient or even excessive energy supply, thus triggering the $KLUM_2$ isolate to be slow and not induced to produce ligninase enzymes. Mantovani *et al.*, (2007) argue that a lack or excess of nitrogen content can inhibit the growth of a fungi. However, Mikiashvili et al (2006) stated that in secondary metabolism such as laccase production, nutritional requirements differ depending on the culture and strain conditions of the fungus, laccase activity can increase in medium with limited nitrogen or vice versa. Hence, it can be concluded that the optimum condition of laccase production is 83.52 U/mg by wood rot fungi isolate $KLUM_2$ in MK-ALKJ is at pH 3.5 and nitrogen content of 20 mM, at 37 °C for 6 days.

Laccase produced by the $KLUM_2$ isolate in MK-ALJK on the sixth day with an incubation temperature of 37 °C, pH 4.5, and levels of $(NH_4)_2SO_4$ at 20 mM, showed a higher specific activity value (83.52 U/mg),

compared by Arinta's work (2017) on the production of laccase by the same fungi in MK-glucose which is 23.28 U/mg. These results prove that the carbon source affects the amount of ligninase produced. Glucose carbon source is a simple carbon source that can directly enter cells and is used for cell growth so that cells do not need to immediately produce ligninase. On the other hand, alkaline lignin is a complex carbon source similar to the natural substrate of wood rot fungi. This causes slow growth but triggers the production of ligninase to convert alkaline lignin into a carbon source that can be converted into energy for growth.



Gambar 7. Specific Activity of Laccase Enzyme Produced by WRF $KLUM_2$ in MK-ALJK on Day 6, with temperature at 37 °C, pH 3,5 at various concentration of $(NH_4)_2SO_4$.

4. CONCLUSIONS

Relatively, the three types of ligninase, which are LiP, MnP, and laccase, were produced in the same amount by the wood rot fungi isolates $KLUM_2$ in MK-ALKJ. All three enzymes were produced with the highest yield of 55.65, 52.48 and 57.64 U/mg, respectively. Laccase as the dominant ligninase can be optimized to reach 83.52 U/mg by means of 2.10^7 spore cells inoculated in MK-ALKJ at 37 °C, pH 3.5, and a nitrogen source of 20mM $(NH_4)_2SO_4$ for 6 days. Therefore, it can be concluded that the ligninase activity of indigenous WRF $KLUM_2$ in MK-ALJK medium is higher than in the MK-Glucose.

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