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Optimizing of Lignin Peroxidase Production by The Suspected Novel Strain of *Phanerochaete chrysosporium ITB Isolate*

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Abstract : *Phanerochaete chrysosporium ITB isolate* that suspected as the novel strain of *P. chrysosporium*, potential as a source of lignin peroxidase (LiP). Several conditions to optimize the production of LiP in submerge batch bioreactor, such as inoculum development, carbon source, temperature, agitation, pH, surfactant (Tween-80) and addition of activity enhancers (veratryl alcohol) were tested in this experiment. The spores of *P. chrysosporium ITB isolate* from optimum inoculum development inoculated in modified Tien Kirk medium to produce LiP. It was shown that the optimum cultivation time to produce optimum inoculum development was two weeks since the highest number of spores produced ($3.53.10^7$ spores/mL) and the viability of the spores was still over 90 %. Highest specific activity of LiP from *P. chrysosporium ITB isolate* was 77.4 ± 13.1 U/mg, achieved at optimized condition: the medium consist of sawdust 1 %, ammonium sulfate 20 mM, tiamin-HCl 0,01 %, veratryl alcohol 300 ppm, Tween-80 0,025 %, basal medium and trace element components were the same as Kirk's medium but in aqua demineralization, cultivated to 1. 10^5 spore/mL, and were grown at 37 ^oC and 50 rpm for five days.The result depict that *P. chrysosporium ITB isolate* can produce high activity of lignin peroxidase in modified Tien Kirk medium.
Keywords: *Phanerochaaete chrysosporium*, ITB isolate, lignin peroxidase.

Introduction

Lignin peroxidase (EC 1.11.1.14) is an extracellular hemeprotein, dependent of H_2O_2, with an usually high redox potential and low optimum pH.LiP does not require mediators to degrade high redox-potencial compounds but it needs hydrogen peroxide to initiate the catalysis[1]. LiP catalyze the H_2O_2-dependent oxidative cleavage of a variety of non-phenolic lignin compounds (diarylpropane), β-O-4 non-phenolic lignin model compounds and a wide range of phenolic compounds (e.g. guaiacol, vanillyl alcohol and catechol)[2]. LiP is also capable to oxidizing a variety of xenobiotic compounds including polycyclic aromatic hydrocarbons, polychlorinated phenols, nitro, aromatics, and azo dyes[3].LiP reported have been used for removal phenolic contaminants, decolorization of synthetic dyes, bio pulping process, and production of bioethanol from lignocellulose biomass[1, 4].It's make the demand of LiP are expected to increase along with the increase in research and applications of the activity of this enzyme for human needs.

The white rot Basidiomycetes *P. chrysosporium* have been the best studied lignin-degrading fungus. Lignin peroxidase (LiP) and manganese peroxidase (MnP) have been the most intensively studied extracellular

enzyme of this fungus[5]. Most of them show higher MnP expression than LiP or almost equal in Kirk's medium such as *P. chrysosporium* ATCC 24725 (BKM-F-1767), *P. chrysosporium* ATCC 34541 and *P. chrysosporium* ME-446[6,7]. *P. chrysosporium ITB isolate* from Microbiology Laboratory of Institut Teknologi Bandung was the suspected novel strain of *P. chrysosporium*[8]has a unique profile. The strain always produce LiP higher than MnP even in a specific medium to produce manganese peroxidase[9], this fact made the strain was potential as a source of LiP.

LiP as a part of ligninolytic system of *P. chrysosporium* was secreted only during secondary metabolism in response to nutrient limitation[10] and in very low amounts[11].The production of LiP has been studied using Kirk's medium. Most of these studies have been carried out using glucose as carbon source. There were only few studies dealing with utilization of agriculture waste as carbon sources including sawdust, rice straw and bagasse. Agriculture waste as a carbon source could be support low-cost LiP production. There are several conditions to optimize the secretion or production of LiP in submerge batch bioreactor, such as inoculum development, composition of nutrient in production medium, culture condition (pH, temperature, agitation), surfactant (Tween-80) and addition of activity enhancers (veratryl alcohol)[7, 10, 12, 13]. In this study, we optimizing several condition to produce LiP from the suspected novel strain of *Phanerochaete chrysosporium ITB Isolate* in modified Kirk's medium, especially, inoculum development to produce highest number and viability of spores, agriculture waste as carbon source, time, temperature, agitation, pH, Tween-80 and veratryl alcohol.

Experimental

Development Inoculum

The suspected novel strain of *Phanerochaete chrysosporium* ITB isolate was obtain from Culture Collection of Microbiology Laboratory of Institut Teknologi Bandung. This strain was purified with monospore technique on potato dextrose agar (Difco). The monospore was cultured on potato dextrose agar slant at 30 ^{o}C to produce spore. Variation of cultivation time was 1, 2, 3 and 4 weeks. After cultivation, the spores were washed from slant agar with 0.02 % Tween-80 by loop, left to stand for five minutes, poured into sterile containers, homogenized by vortex for ten minutes and left to stand for thirty minutes. The mixtures were filtered by sterile glass wool. The number of spores in a spore solution was calculated by Hemocytometer (Assistants) and total plate count (TPC) methods. The spore viability is the ratio of TPC with spore number in hemocytometer. The experiments were carried out with three replications.

Growth of Fungus in Lignin Peroxide Production Medium

Production medium of LiP were modified form Kirk's Medium[14]: basal medium 1 X, glucose 10 %, trace element solution 1 X, tiamine-HCl 0.01%, except that dimethylsuccinate was replace by 20 mM acetate buffer pH 4.5 and ammonium tartrat replace by ammonium sulfate 20 mM. Each medium inoculated with a spore suspension with an initial concentration of 1. 10^6 spores/mL, were grown at 37 °C and 100 rpm. After inoculation, production medium filtered using Whatman no.1. The residue obtained was determined the dry weight of the mycelium and the filtrate was determined extracellular protein levels and activity of LiP.

Optimizing of Lignin Peroxidase Production

Experiments were carried out with experimental according completely randomized design, using one independent variable to determine the effect of each variable on specific activity of LiP. The number of initial spores used in this experiment was 1. 10^5spores/mL. Optimization of the carbon source as much as 1% (w/v) using glucose, sawdust, bagasse and rice straw was observed after 3, 4 and 5 days incubation. Optimization of incubation time (0,1, 2, 3, 4, 5, 6, 7, and 8 days), temperature (30, 37, 45, 50 and 60 °C), shaking (0, 50 and 100 rpm), pH (3.0, 3.5, 4.5 and 5.5 with acetate buffer 20 mM), the concentration of Tween 80 (0, 0.025, 0.05 and 0.1%), and the concentration of veratryl alcohol (0, 100, 300, 500, 700, 1000 ppm) using the optimum conditions of the previous experiments. Each experiments was performed three replications.

Determination of Lignin Peroxidase Activity and Spesific Activity of Lignin Peroxidase

LiP activity was determined by modified ofTien methods[15].As much as 800 µL of alcohol veratril 10 mM was added with 1000 µL of tatrat acid 0.2 M, 1680 mL aquaDM and 200 µL enzyme solution. The mixture

was incubated for 2 minutes, then the reaction was initiated by addition of 320 mL of H_2O_2 5 mM. The absorbance was measured at a wavelength of 310 nm at zero and first minute.LiP activity was calculated according to the following:

$$\text{LiP activity (U/mL)} = \frac{(At-Ao)\text{x Vtotal(mL)}.10^6}{\varepsilon maks \text{ x d x Venzym (mL) x t}}$$

Where ε_{maks} is molar extinction coefficient of veratryl aldehyde (9300 $M^{-1}cm^{-1}$), d is thick of cuvette (cm), and t was one minute

Specific activity of LiP was LiP activity per mg of protein (U/mg protein). The extracellular protein determined according Lowry Methods[16]

Result and Discussion

Optimum Inoculum Development of *P. chrysosporium ITB isolate*

Operationally, isolation of spore suspension from the slant culture was easier and higher in recovery volume than the isolation of spores from solid medium in a Petri dish (data not shown). Determination of the viability and the number of spores in a spore suspension at various times of incubation was an effort to produce high of the LiP production and its reproducibility. The number of spores increased from the first week to the second week but decline sharply in the third week, while the viability start to decline since the second week and decline sharply in the third week (Figure 1). The optimum incubation to produce the spore suspension of *P. chrysosporium ITB isolate* with this method was two weeks with the highest number of spores produced ($3.53.10^7$ spores/mL) and the viability of the spores over 90 %.

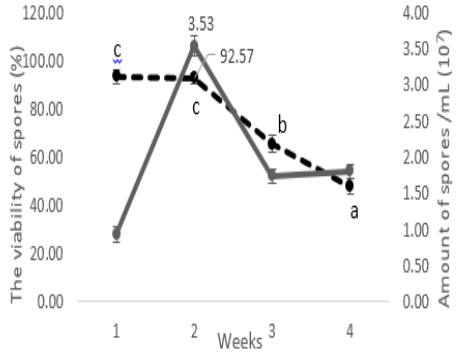

Figure 1. The product of *P. chrysosporium* spores on 4 weeks incubation. The number (—) and viability (- - -) of spores after 1, 2, 3 and 4 weeks of incubation. Data are shown as means ± standard deviation of three independent experiments.

Growth of *P. chrysosporium* in Lignin Peroxide Production Medium

The growth of fungus usually was observed by dry weight of mycelium, but this parameter couldn't be used in media containing material that insoluble in water such agriculture waste as carbon source. The only way is to use a total plate count (TPC) technique. Unfortunely, this technique is takes a long time to be used as a routine analysis. The increasing mass of microbes is directly proportional with other cell component such as DNA, RNA and protein[17]. Using the analogy, we could be observing the growth of fungus by measuring the extracellular protein.This method of course more easily, cheaper and faster than the method of dry weight and TPC, especially for the fungus growth in media containing material that insoluble in water. This experiment was conducted to observe how the growth and production of LiP by *P. chrysosporium ITB isolate* in the modified Kirk's medium. Beside, to determine whether the value of an extracellular protein levels can be used as an alternative parameter to observe the growth of fungi. The results showed that increased levels of extracellular protein profile of *P. chrysosporium ITB isolate* in a modified Kirk's medium similar with the increased profile of dry weight of mycelium (Figure 2A). Pearson correlation between the levels of extracellular

protein and dry weight of mycelium was 0.976. This result confirmed that the parameters of extracellular protein levels can be used to observe the growth of *P. chrysosporium*.

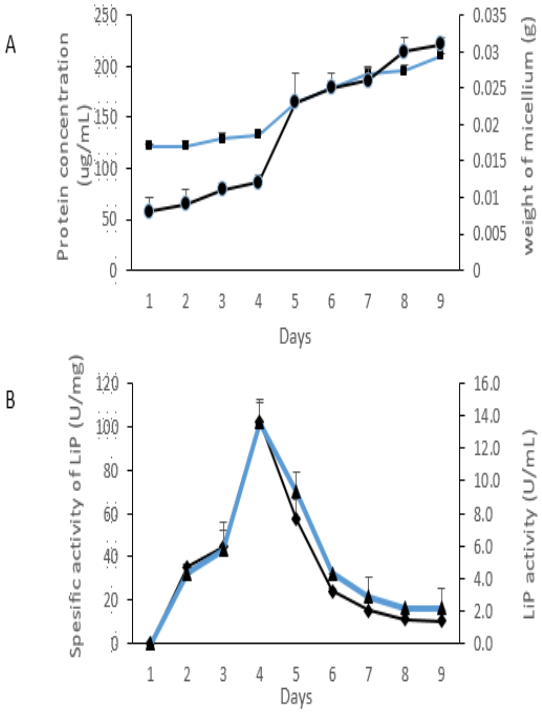

Fig 2. The growth of *P. chrysosporium* in modified Kirk's Medium with glucose as carbon source. Correlation between dry weight of mycelium (●) and protein concentration (■) in A and correlation between activity of LiP (▲) and specific activity of LiP (♦) in B. Data are shown as means ± standard deviation of three independent experiments

Optimum conditions for the production of LiP presented with highest of LiP activity. It's become problem if different conditions of the experiment produce different type and amount of extracellular protein. We can't directly compare among the conditions by LiP activity, specific activity of LiP is more appropriate. The specific activity of LiP is the LiP activity per mg protein. Using this parameter is actually more specific because it directly shows the comparison of LiP with overall extracellular protein that produced. Both curves coincide as shown in Figure 2B and Pearson correlation between the LiP activity and the specific activity of LiP was 0.986.

LiP was secreted only during secondary metabolism in response to nutrient limitation. The profil of growth of *P. chrysosporium*BKM-F-1767in Kirk's medium is as follows that inoculum spores of started the log phase growth on 17-24 h after inoculation, the fungus entered declined 2-3 days after inoculation and on the third day showed the beginning of secondary metabolism and produce the highest LiP at seven days incubation[6]. Different strain of *P. chrysosporium* produce the optimum of LiP in different incubation time. *P. chrrysosporium* (ATCC 20696) reach highest LiP production in Kirk's medium at five days incubation[7]. In this experiment, *P. chrysosporium ITB isolate* reach the highest LiP production in Kirk's modified medium at four days after incubation, but at fifth days its declined sharply (Figure 2B) although dry weight of mycelium and extracellular protein content increase on the fourth day to the fifth day of the most significant and likely to rise up to the seventh day (Figure 2A).This result confirms that the production of LiP by *P.chrysosporium* influenced by the type strains used and *P. chrysosporium ITB isolate* is a different strain with *P.chrysosporium* BKMF -F-1767.

Optimum Carbon Source for Lignin Peroxidase Production by *P. chrysosporium ITB isolate*

We observed extracellular protein, activity and specific activity of LiP on 4, 5 and 6 days incubation to assign optimum carbon source in order to find the low-cost agricultural waste as carbon source observed as shown in Table 1. Biomass of *P. chrysosporium ITB isolate* and microscopy observation performed on the fifty day as shown in Figure 3.

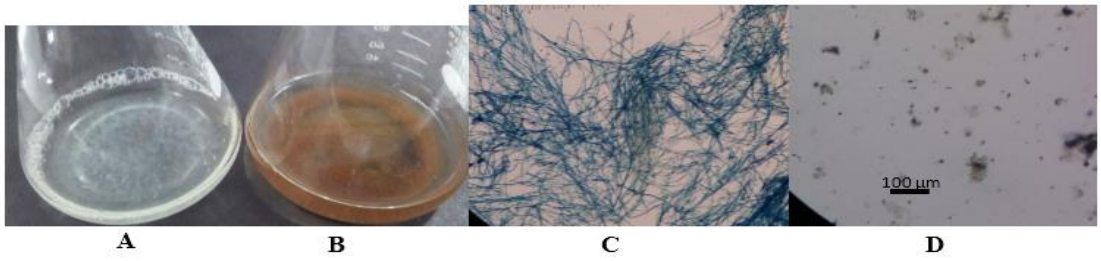

Figure 3. Biomass of *P. chrysosporium* ITB isolate after 5 days of incubation in glucose medium (A), in the sawdust medium (B), microscopic observations using staining and magnification 100X in glucose medium (C) and sawdust medium (D). Hypha of *P. chrysosporium* indicate with blue color.

Table 1.The influence of carbon sources and time of incubation on dry weight, extracellular protein content, activity and specific activity of LiP.

Carbon source	Days	LiP activity (U/mL)	Extracellular protein level (µg/mL)	Specific activity of LiP (U/mg)
Glucose	4	2.90±1.20^{a}	165.92±17.70^{a}	17.60±8.57^{abcd}
	5	5.76±1.27^{a}	161.60±9.50^{a}	26.67±1.63^{d}
	6	2.30±0.00^{a}	159.39±13.5^{a}	13.56±1.10^{bc}
Sawdust	4	3.60±1.21^{a}	130.92±2.20^{a}	26.97±9.06^{abcd}
	5	5.76±1.27^{a}	128.97±4.40^{a}	44.70±10.86^{abcd}
	6	2.20±0.00^{a}	125.50±3.00^{a}	17.17±0.42^{c}
Rice straw	4	2.02±0.00^{a}	276.63±5.30^{b}	7.37±0.35^{a}
	5	2.90±1.21^{a}	278.43±4.20^{b}	10.30±4.50^{abcd}
	6	0.73±1.27^{a}	281.61±7.20^{b}	2.47±4.27^{abc}
Bagasse	4	2.20±0.00^{a}	266.77±4.40^{b}	8.07±0.15^{ab}
	5	2.20±0.00^{a}	277.31±4.30^{b}	7.73±0.12^{ab}
	6	2.20±0.00^{a}	282.60±4.00^{b}	7.60±0.10^{ab}

a, b, c and d mark is a notation as result of statistically analysis by the Brown-Forsythe test continued with Games-Howell at $\alpha = 0.05$. The same notation indicates no significant difference among treatments.

Each of agricultural waste has different chemical composition that will affect the profile of growth and LiP production of *P. chrysosporium*. Statistical analysis by Brown-Forsythe test continued with Games-Howell at $\alpha = 0.05$ (Table 1) showed that LiP activity was not significantly different among treatments. It was confirmed that to determine the optimum conditions of production LiP among carbon source materials should be based on specific activity of LiP value as discussed above. Based on the extracellular protein level, there are two group i.e low extracellular protein level group that produce in the medium of sawdust and glucose and high extracellular protein level group that produce in medium of straw and bagasse. The extracellular proteins produced by sawdust lower than glucose. This is presumably because the growth rate in sawdust medium is slower due to more complex carbon sources than glucose. This expectation has proven through comparison of macroscopic and microscopic observation of hyphae of *P. chrysosporium* in sawdust and glucose media. Threads of hyphae clearly visible in glucose medium, while in the medium of sawdust are not visible (Figure 3 A, B). The hyphae in glucose is longer and clearly visible whereas in the medium of sawdust is short (Figure 3C, D). This result is in line with other research noted that the growth of *P.chrysosporium* in lignin (0.05 %) and glucose (0.2 %) medium slower than in glucose (0.2 %) medium[18].

The *P. chrysosporium* capable to degrade both the lignin and carbohydrate components, but the expression of enzymes depends on the carbon source or wood species. Rice straw and bagasse contain cellulose and hemicellulose component higher than sawdust[19]. *P. chrysosphorium* was cultivated using cellulose as a major carbon source it induced production of different glyco- hydrolyzing (GH) family of proteins but in lignin

medium induced proteins as major production role in lignocellulose degradation[18]. This explains that the levels of extracellular proteins in straw and bagasse medium as in the group of high extracellular protein level.

The highest specific activity of LiP was in sawdust at fifth day incubation, but standard deviation value of the treatment was high and makes this treatment statistically not recommended as the optimum condition. Statistically, the optimum conditions for the production of LiP from *P. chrysosporium ITB isolate* was suggested in glucose medium at fifth day incubation and in sawdust at sixth day incubation. We decided to choose sawdust as optimum carbon source to produce LiP from *P. chrysosporium ITB isolate*. LiP from *Pycnoporus sanguineus* MTCC-137 also has been found in the presence of sawdust on the fifth day after inoculation, compared to coir-dust, bagasse, corn cob and wheat straw[20]. These results also consistent with previous research that the ligninolytic system, including lignin peroxidase (LiP), is activated during secondary metabolism, regulated by the availability of nutrients and indicates that LiP activity level is enhance by the presence of lignin, lignin-related compounds or low-molecular-weight aromatic compound[12].

Optimum incubation time of lignin LiP production of *P. chrysosporium ITB isolate*

The composition of sawdust (as optimum carbon source from agricultural waste) is significantly different from glucose, so it is important to re-evaluate the optimum incubation time to LiP production. Growth profile of *P. chrysosporium* ITB *isolate* and production of LiP in a medium containing sawdust as shown in Figure 4.

Growth profile and LiP production of *P. chrysosporium* ITB *isolate* in a medium containing sawdust as carbon source (Figure 4), slightly different with in the medium containing glucose (Figure 2). Specific activity of LiP at the beginning of cultivation was very high. This is presumably because of their functional groups on the sawdust that is involved in absorbance at 310 nm that occur when LiP activity measurements. Sawdust contain 24 % of lignin[19]. The basic building blocks of lignin can be schematically simply into "C9" units each made up of a phenolic moiety bearing three aliphatic carbons. The aromatic components are moreover differently substituted by methoxy groups, whereas the aliphatic portions are characterized by the variable presence of C=C unsaturation, hydroxyl functionalities, and other less frequent substituents[21]. Unsaturated C=C (conjugated C=C) are known to absorb energy at ultraviolet wavelength (200-400 nm). Lignin consists of large group of aromatic polymers with a characteristic absorption band at 280 nm, a simple lignin content detection method was used to measure the absorbance of medium at 280 nm[22].

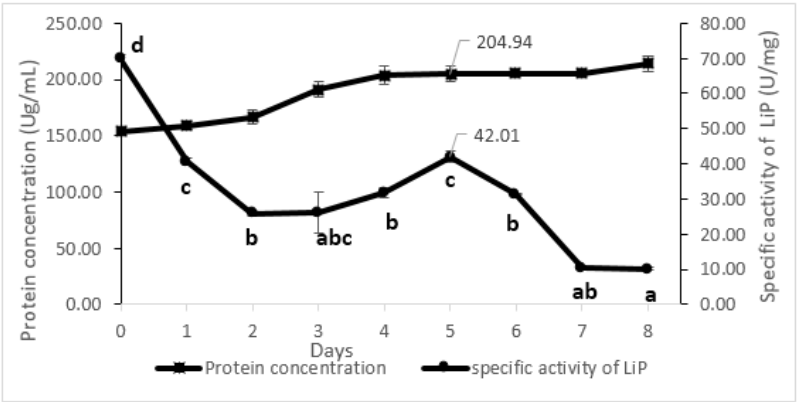

Figure 4. Protein concentration and specific activity of LiP after 1 to 8 days of incubation in Kirk's modified medium with a sawdust as carbon source. a, b, c and d mark is a notation as result of statistically analysis by the Brown-Forsythe test continued with Games-Howell, α = 0.05. The same notation indicates no significant difference among treatments.

The specific activity of LiP decreased at one and two days incubation, allegedly due to degradation of the structure of sawdust as a result of metabolism of *P. chrysosporium*, so functional groups involved in absorbance at 310 nm is reduced. This allegation is relevant to increased levels of extracellular proteins occurred that indicate the occurrence of the growth of this fungus. The increase in the specific activity of LiP occurred since the third day and the highest in fifth day. Specific activity of LiP decrease in the sixth day after

the incubation, while an increase in protein content remains the case until the eighth day. It was suspected because the increasing of protease activity at that days.

Optimum temperature, agitation and pH for LiP production of *P. chrysosporium ITB isolate*

Growing conditions including temperature, agitation and pH affects the number of LiP production from *P. chrysosporium*. Figure 5A showed that the specific activity of LiP increases at temperature from 30 to 37 °C, but decreased at temperature over 37 °C (45 and 50°C), and the optimum temperature LiP production is achieved at 37 °C. Figure 5B showed that agitation until 100 rpm did not significantly effect to the production of the specific activity of LiP ($p>0.05$), but the relatively higher specific activity LiP produced at a speed of 50 rpm. Given *P. chrysosporium ITB isolate* suspected as novel strain, the authors are interested in studying the effect of pH on production the specific activity of LiP. Figure 5C showed that the specific activity of LiP on the medium at pH 3; 3.5 and 4.5 were not differ significantly ($p>0.05$), whereas at pH 5.5 decline sharply.

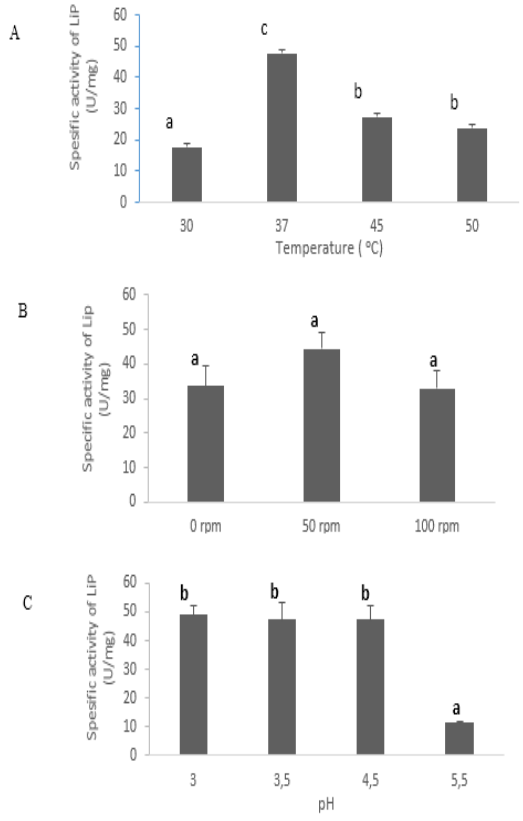

Figure 5. Optimum temperature, agitation and pH for LiP production by *P. chrysosporium ITB isolate* in Kirk's modified medium with a sawdust as carbon source after fifth day of incubation. a, b, and c mark is a notation as result of statistically analysis by the One Way ANOVA test with Tukey at α = 0.05. The same notation indicates no significant difference among treatments.

The optimum temperature is within the optimum temperature range of previous research (30 until39 °C) [6, 7, 13, 23], likewise with the agitation[24]. *P. chrysosporium* is an obligate aerobic fungus that requires oxygen for growth and development. The oxygen dissolve content (DO) in the water is only 8 mg/ L at 20 °C, so the agitation is needed to increase the content of DO in the medium[25], but according due to shaking at high speed (100 and 200 rpm) known to inactivate ligninase[24].But, the optimum pH medium in this result slightly different from Kirk[14]. *P. chrysosporium ITB isolate* can produce LiP were not significantly different either at medium pH of 3.0, 3.5 and 4.5 (Figure 5). Whereas, almost publications on the growth and production peroxidase of *P. chrysosporium* using medium pH of 4.5, referring to the classic study conducted Kirk et al[14]. The study concluded that the optimum culture pH for lignin decomposition was 4 to 4.5, with marked suppression over 5.5

and less than 3.5.Our result contributes to the low cost of LiP production. The adjustment of pH medium with buffer is not necessary again. We can directly dissolve the component of medium with aqua to produce the medium with pH is 3.0.

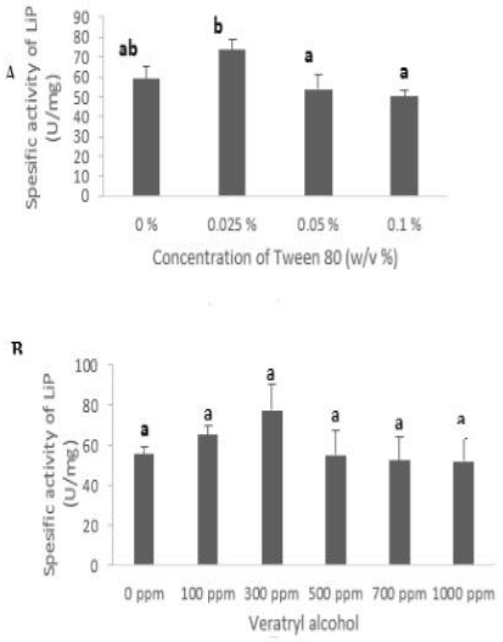

Figure 6. Effect of Tween-80 & veratryl alcohol concentration on LiP production of *P. chrysosporium ITB isolate* in Kirk's modified medium with a sawdust as carbon source for fifth day of incubation at 37 °C, 50 rpm and pH = 3.0. Statistically analysis by the One Way ANOVA test with Tukey at α = 0.05.

Optimum of Tween-80 and veratryl alcohol concentration on LiP production of *P. chrysosporium ITB isolate*

Surfactants addition such as Tween-80 and veratryl alcohol in medium of *P. chrysosporium* reported to increase the production of LiP. The specific activity of LiP increase at the addition of 0.025 % Tween-80, but decreased at the addition of 0.05 and 0.1% as shown in Figure 6A. Figure 6B showed that addition of veratryl alcohol in this study does not significantly affect the increase in specific activity of LiP ($p>0,05$), however LiP specific activity was relatively higher resulting in the addition of 300 ppm.The result showed that optimum of addition of Tween-80 and veratryl alcohol also similar with previous research[7].

Conclusion

Highest specific activity of LiP from *P. chrysosporium ITB isolate* was 77,4 ± 13,1 U/mg, achieve inmodified Kirk's medium contain sawdust 1 % (w/v), ammonium sulfat 20 mM, Tween-80 0,025 %, and veratryl alcohol 300 ppmin aqua demineralized, cultivated to 1. 10^5 spore/mL, and were grown at 37 ^oC and 50 rpm for five days.

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