Ligninolytic Enzymes Produced by *Gliomastix* sp. in an Organic Waste Medium

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Abstract— **Ligninolytic enzymes are extracelluler enzymes for cutting lignin or lignocelluloses, a complex carbohydrate, to be a more simple carbohydrate. The enzymes have a commercial value for many kinds of industrial purposes, ex. pulp and paper industry. *Gliomastix* sp. was one reported fungi that produced those enzymes. Since organic waste was a cheap and potential organic source for microbial growth, this study was aimed to figure out whether *Gliomastix* sp. T3.7 able to produce ligninolytic enzyme on several chosen organic waste medium, and to detect the effect of temperature and pH on its enzymatic activity. The enzymatic activity was measured using spectrofotometer UV-Vis. The result showed that *Gliomastix* sp. T3.7 was able to produce laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) that belonged to ligninolytic enzymes. Each enzyme showed a different need of temperature and pH condition for each particular optimum activity. On a sugarcane baggase waste medium, *Gliomastix* sp. T3.7 reached an optimum laccase activity of 1,8 U/ml at 35^{0}C and on pH 6, while MnP activity (1 U/ml) at 25^{0}C and on pH 4. The activity of LiP was optimum of 8,1 U/ml on a corn cobs waste medium at 35^{0}C and on pH 5.**

Keywords— **Gliomastix sp, organic waste medium, ligninolytic enzyme.**

Abstrak—**Enzim ligninolitik adalah enzim ekstraseluler untuk memotong lignin atau lignoselulosa, karbohidrat kompleks, menjadi karbohidrat yang lebih sederhana. Enzim memiliki nilai komersial untuk berbagai jenis keperluan industri, ex. industri pulp dan kertas. Gliomastix sp. adalah salah satu melaporkan jamur yang menghasilkan enzim yang. Sejak sampah organik adalah sumber organik murah dan potensi pertumbuhan mikroba, penelitian ini bertujuan untuk mengetahui apakah Gliomastix sp. T3.7 mampu menghasilkan enzim ligninolitik pada beberapa media sampah organik yang dipilih, dan untuk mendeteksi pengaruh suhu dan pH terhadap aktivitas enzimatik nya. Aktivitas enzimatik diukur menggunakan spektrofotometer UV-Vis. Hasil penelitian menunjukkan bahwa Gliomastix sp. T3.7 mampu menghasilkan lakase, mangan peroksidase (MNP) dan lignin peroksidase (LiP) milik enzim ligninolitik. Setiap enzim menunjukkan kebutuhan yang berbeda dari suhu dan kondisi pH untuk setiap kegiatan yang optimal tertentu. Pada media limbah tebu ampas, Gliomastix sp. T3.7 mencapai aktivitas lakase optimum 1,8 U / ml pada 350C dan pH 6, sementara aktivitas MNP (1 U / ml) pada 250C dan pH 4. Kegiatan LIP adalah optimum 8,1 U / ml pada medium limbah tongkol jagung di 350C dan pH 5.**

Kata Kunci—***Metode Numerik, Campuran Konveksi, Ruang Didinginkan, Perpindahan Panas.***

I. INTRODUCTION

Lignocelluloses are the most abundant renewable organic matter on earth and they contribute to the majority of the agroindustrial residues over the world. The utilization of lignocelluloses for ligninolytic enzyme production has been studied extensively. Production of laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) from a wide variety of agro-industrial residues has been reported. Most of the works on ligninolytic enzyme production from agro-industrial residues deal with fungi [1].

Lignocelluloses are featured by biomass recalcitrance in nature where hemicellulose and cellulose are densely packed by layers of lignin [2]. Lignin, like cellulose and hemicelluloses, is a major component of plant materials and the most abundant form of aromatic carbon in the biosphere. As a complex aromatic macromolecule, it provides strength and rigidity to cell walls and tissues of all vascular plants by acting as a glue between the polysaccharide filaments and fibres. In addition, lignin is involved in water transport in plants and forms a barrier against microbial destruction by protecting the readily assimilable polysaccharides. From the chemical point of view, lignin is a heterogeneous, optically inactive polymer consisting of phenylpropanoid interunits, which are linked by several covalent bonds (e.g. aryl-ether, arylaryl, carbon-carbon bonds) [3-5]. The polymer arises from laccase and/or peroxidase initiated polymerization of phenolic precursors via the radical coupling of their corresponding phenoxy radicals [5-6] . Lignin cannot be degraded by hydrolytic enzymes as most other natural polymers (cellulose, starch, proteins, etc.) because of its bond types and heterogeneity.

Lignin biodegradation is fundamental to potential applications for pulp and paper industry. That why a rapid understanding of how to degrade lignin, may provide a challenge of biotechnology applications in pulp and paper manufacturer. Research continues on the lignin degrading enzyme system involved; its nature, production, mechanism of action, and molecular genetics [7-11].

The main extracellular lignin degrading enzymes are laccase, MnP, and LiP. Laccase is a copper-containing polyphenol oxidase. It catalyses the reduction of four-electron oxygen to water and this is typically accompanied by the oxidation of a phenolic substrate to fenoxil radical. Though the laccase produced by *Trametes vesicolor* is able to oxidize nonphenolic substrates provided with 2,2'azinobis (3-ethylbenzthiazoline-6-sulfonate). The molecular weight of laccase is between 60-80 kDa and its isoelectric point is between 3 and 4. Manganese peroxidase is an enzyme

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containing Fe which oxidize phenol to phenoxy radicals by oxidizing $Mn1^+$ to $Mn3^+$ using H_2O_2 as oxidant. $Mn3^+$ was then chelated using oxalic acid or malic that spontaneous oxidize phenolic lignin. The molecular weight of MnP is 40-50 kDa and the isoelectric point is between 3 to 4 though neutral MnP is also found. Lignin peroxidase could also oxidize non phenolic aromatic compound [12].

In the present study, we reported that fungi Gliomastix sp. T3.7 produced laccase, LiP and MnP on organic lignocellulosic waste (rice straw, corn cobs, sugarcane bagasse and sawdust) as medium. Each particular enzymes showed different enzymatic activity over temperature and pH.

II. Method

A. Fungus Preparation

Gliomastix sp. T3.7 was from lab collection of Microbiology and Biotechnology laboratory, ITS Surabaya. The fungus was recultured on PDA medium at 28^0 C for 5 days until the fungus was overgrowth. The fungus culture was then cut in plugs with 10 mm diameter and picked up from PDA medium. Each fungus plugs was further treated as below.

B. Ligninolytic Enzyme Production

Organic wastes were rice straw, corncobs and sawdust from an agriculture area in Ponorogo and sugarcane baggase from Kenjeran, Surabaya. They were cut in to very small pieces of about 40 mesh size. The particular organic waste powder was then dried out at 75^0C for 2 days until it reached a constant dry weight.

A production medium consisted of 5 gr each organic waste powders and 25 ml distilled water were put into 250 ml Erlenmeyer flask and autoclaved at 121^0C, 1.5 atm pressure for about 15 minutes. Before autoclaving, pH of each production medium was adjusted with a serial pH value from 4 to 6. Afterward each adjusted pH medium of each particular organic waste powder was then inoculated with 2 fungus plugs and incubated for 5 days. The incubations were performed in a different temperature ranges from 25^0 to 35^0C.

C. Enzyme Extraction

The produced ligninolytic enzymes were harvested in a crude method. A neutral phosphate buffer (pH 7) was used for extracting the crude enzyme with ratio production culture:buffer was 1:2 (w/v). After pouring the buffer into the production culture, the culture was homogenized with a gentle hand shaking and centrifuged at 5,000 rpm for 20 min on $0\text{-}4^0$ C. The centrifugation was repeated until clean filtrate was obtained.

LiP (1.11.1.14) activity was determined by monitoring of λ310 nm change. Lignin peroxidase activity was determined spectrophotometrically according to [13]. The reaction mixture contained 0.5 M sodium acetate (pH 3.0), 8 mM veratryl alcohol, 0.2 ml enzyme filtrate and 0.45 ml aquades, in a final volume of 1 ml. The reaction was starting by adding 5 mM H2O2 [1 and 14]. One unit (U) was defined as 1 nmol of veratryl alcohol oxidized in 1 minute and the activities were reported as U/ml.

MnP (1.11.1.13) activities was determined by monitoring the oxidation of guaiacol spectrophotometrically at λ 465 nm [15]. The reaction mixture contained 50 mM sodium Na-lactate (pH 4,5), 4 mM Guaiacol, 0.2 ml enzyme filtrate, 1 mM $MnSO_4$ and 0.3 ml aquades, in a final volume of 1 ml. One unit of activity was defined as 1 nmol/l of Mn2+ oxidized per minute. Lignocellulolytic enzyme activity (U) was defined as the amount of enzyme needed to oxidize 1 µM of substrate per minute.

III. Result and Discussion

Activity of ligninolytic enzymes was measured after 5 days inoculation. For the full growth and adaptation onto a solid substrate, the fungus requires this time. Since the fungus mycelium has grown over the organic lignocellulosic waste, it caused nitrogen depletion on medium and changed the metabolic system into a ligninolytic mechanism. Nitrogen depletion was a trigger for initiating a ligninolytic system [11 and 16]. This study was using fungus *Gliomastix* sp. T3.7 as our previous work [17] clearly showed that it was able to degrade lignin by performing a clear zone with a diameter of 1.4 cm. It was greater than other fungi collection in the Microbiology and Biotechnology Laboratory, ITS.

The effect of pH on ligninolytic enzymes activity was shown in Table 1-3. The highest activity of Laccase and MnP was about 1.8 U/ml and 1 U/ml on a sugarcane bagasse waste, respectively, while LiP activity was highest 8,088 U/ml in corn cobs waste. For further optimization studies on bioprocess parameters, sugarcane bagasse and corn cobs were taken as the best organic lignocellulosic waste for maximum ligninolytic enzymes activity of *Gliomastix* sp. T3.7.

Bagasse and and its extract also can serve as efficient inducers of laccase and MnP activies in *Pleurotus ostreatus* [18]. Although the identification of possible inducers compounds in the bagasse or its extract was not undertaken, the presence of several compounds such as p-hydroxybenzoic acid, transcinamic acid, transferulic acid, syringaldehyde, vanilin has been reported in bagasse [18 and 19].

Sugarcane bagasse proved to be the best laccase inducer of *Phlebia radiata* among the various supplements added to different media [20]. Sugarcane bagasse has been reported to be a better substrate for laccase production by *Flavodon flavus* also [21].

Many fungi secrete higher amount of ligninolytic enzymes in the medium in presence of inducers. Many chemical inducers like xylidine, veratryl alcohol, vanilic acid, veratraldehyde, 3,4-dimethoxycinnamic acid, and 3,4,5-trimethoxy cinnamic acid have been used for induction of ligninolytic enzymes of white rot fungi and the others. However, most of these substances are harmful or expensive. Recently investigations have focused on the use of natural substrates and agricultural wastes for achieving this objective because of their easy accessibility and low cost [18].

The effect of pH on activity of ligninolytic enzymes was shown in Table 1-3. The activity of Laccase was highest (1,8 U/ml) at pH 5, as MnP was highest (0,9 U/ml) at pH 4 and LiP was at pH 6 with activity of 8,1 U/ml. For further optimization studies on bioprocess parameters, pH value of 4 to 6 were taken as optimum

for maximum ligninolytic enzymes activity of *Gliomastix* sp. T3.7.

The intact enzyme contains both of positive and negative charged group at any given pH. Such ionizable groups are often apparently part of the active site. A pH variation in medium may resulted a change of ionic forms of the active sites and activity of the enzymes, and hence the reactions rate. Changes in pH may also alter the three-dimensional shape of the enzymes [22-25]. For these reasons, enzymes are very active over a certain pH range [25].

The effect of temperature on ligninolytic enzymes activity was shown in Table 1-3. The highest activity of Laccase was 1.8 U/ml at 35^0 C. MnP was 1 U/ml at 25^0 C and LiP was 8.1 U/ml at 35^0C. For further optimization studies on bioprocess parameters, temperature of 25 and 35^0 C was taken as optimum temperature for ligninolytic enzymes activity of *Gliomastix* sp. T3.7. For many enzymes, denaturations of proteins begin to occur at 45^0 to 50^0 C temperatures. Sensitivity of a protein to denature at elevated temperatures can be varying widely with medium pH, and the influence of various temperature-pH combinations may differ tremendously from enzyme to enzyme [22-25].

Each fungus has unique optimum, minimum and maximum growth temperature; below the optimum growth depresses the rate of metabolism of cells, as well as above the optimal decreases the growth and even death may occur. At higher temperatures (beyond 40^0C), a death rate exceeds the growth rate, which causes a net decrease in the concentration of viable fungal cells as well as enzyme activities.

Lignin degradation is a complicated process. The enzymes system for lignolysis of specific culture depended on the strain, substrate, and environment conditions. Laccase might plays the central role in the lignolysis for one strain or culture, while for others, MnP or LiP, even laccase-MnP or laccase-MnP-LiP might be responsible for the same process [26]. *Gliomastix* sp. T3.7 was a potential microorganism which produced ligninolityc enzyme such as laccase, LiP an MnP. This study was needed to be investigated further until to reach the main goal of a using *Gliomastix* sp.for industrial pulp and paper as extracellular ligninolytic enzymes source.

CONCLUSION

a. *Gliomastix* sp. T3.7 has potential as a ligninolityc enzymes producing fungus, such as laccase, LiP an MnP.
b. Sugarcane bagasse waste was the best inducers for laccase and MnP, whereas corncob was for LiP.
c. Temperature of 25 and 35^0 C and pH value of 4 to 6 were optimum conditions for ligninolytic enzymes activity produced by *Gliomastix* sp. T3.7.

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TABLE 1.
LACCASE ENZYME ACTIVITY ON VARIETY PH, TEMPERATURE AND ORGANIC LIGNOCELLULOSIC WASTE

	Laccase enzyme activity (U/ml)		
Temperature (^{0}C)	pH 4	pH 5	pH 6
		Rice straw	
25	0,734 ^{c}	0,049 ^{a}	0,485 ^{bc}
30	1,403 ^{d}	ND	0,204 ^{a}
35	0,113 ^{a}	0,09 ^{a}	1,449 ^{e}
		Corncobs	
25	0,369 ^{ab}	0,296 ^{ab}	0,35 ^{ab}
30	0,186 ^{a}	0,283 ^{ab}	1,069 ^{d}
35	0,001 ^{a}	0,002 ^{a}	0,448 ^{b}
		Sugarcane bagasse	
25	0,551 ^{bc}	ND	1,723 ^{e}
30	1,117 ^{d}	0,269 ^{ab}	0,468 ^{bc}
35	1,736 ^{e}	1,765 ^{e} *	0,863 ^{c}
		Sawdust	
25	0,221 ^{ab}	0,589 ^{bc}	0,279 ^{ab}
30	1,053 ^{d}	1,456 ^{e}	0,621 ^{bc}
35	0,981 ^{d}	0,718 ^{c}	0,349 ^{ab}

ND : Not Detected. The number followed with the same letter on one group showed insignificantly different with Duncan's test (P<0,05)

TABLE 2.
LIP ENZYME ACTIVITY ON VARIETY PH, TEMPERATURE AND ORGANIC LIGNOCELLULOSIC WASTE

	LiP enzyme activity (U/ml)		
Temperature (^{0}C)	pH 4	pH 5	pH 6
		Ricestraw	
25	ND	3,405	0,556
30	ND	4,253	1,416
35	0,018	4,498	0,735
		Corncobs	
25	0,036	0,233	0,102
30	1,022	0,048	0,066
35	0,018	0,006	8,088*
		Sugarcane bagasse	
25	0,066	0,081	ND
30	0,185	0,269	0,081
35	0,179	0,09	0,723
		Sawdust	

25	0,096	0,197	0,281
30	0,173	0,257	0,281
35	0,245	0,185	0,293

ND : Not Detected.

TABLE 3.
MNP ENZYME ACTIVITY ON VARIETY PH, TEMPERATURE AND ORGANIC LIGNOCELLULOSIC WASTE

MnP enzyme activity (U/ml)			
Temperature (^{0}C)	pH 4	pH 5	pH 6
		Rice straw	
25	ND	ND	0,028 ^{a}
30	ND	0,199 ^{ab}	0,032 ^{a}
35	ND	0,138 ^{ab}	0,657 ^{d}
		Corn cobs	
25	ND	0,032 ^{a}	0,859 ^{d}
30	ND	ND	0,009 ^{a}
35	ND	ND	ND
		Sugarcane baggase	
25	0,964 ^{e*}	0,764 ^{d}	0,464 ^{c}
30	0,147 ^{ab}	0,399 ^{c}	0,055 ^{ab}
35	0,014 ^{a}	0,225 ^{ab}	0,652 ^{d}
		Sawdust	
25	ND	ND	0,073 ^{ab}
30	0,11 ^{ab}	0,523 ^{c}	0,023 ^{a}
35	0,285 ^{bc}	0,005 ^{a}	0,046 ^{ab}

ND : Not Detected. The number followed with the same letter on one group showed insignificantly different with Duncan’s test (P<0,05)