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ISOLATION OF ENDOPHYTIC FUNGI FROM *DIPTEROCARPUS GRANDIFLORUS* BLANCO AND EVALUATION OF XYLANASE ENZYME ACTIVITY FROM FERMENTATION BROTH

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ABSTRACT

Xylanase enzyme, one example of secondary metabolites generated by the endophytic microbes was an enzyme which degrades Xylan. The current research study was focused on isolation of endophytic fungi from *Dipterocarpus grandiflorus* Blanco that produced xylanase enzyme and evaluation of this enzyme activity. Isolation of endophytic fungi from Twigs of *D. grandiflorus* Blanco were performed via direct seeding inoculation technique utilizing Corn Meal Malt (CMM) medium, followed by purification of these isolates in Potato Dextrose Agar (PDA) media and macroscopic observations to characterize these isolates. The presence of xylanase enzyme was determined using Dinitro Salicylic Acid (DNS) method. Over 12 days our study results yield a total of eight (8) endophytic fungi isolates with six xylanase enzyme producing isolates (Dg II,3 $_{a2}$, Dg II,3 $_{a3}$, Dg II,3 $_{b1}$, Dg II,3 $_{c1}$, Dg II,3 $_{e1}$, Dg II,3 $_{f1}$). Highest extracellular xylanase activity was found in Dg II, 3_{fl} (4.036 U/mL) while the lowest one was 0.426U/mL in isolate Dg II, $3_{bl.}$

INTRODUCTION: In Indonesia, enzymes played important roles (i) in industrial processes such as food industry for preservative bread, (ii) in diagnostic purposes of diseases, (iii) in molecular, biology analysis and chemical reactions and lastly (iv) as active ingredients in detergents. The need of the xylanase enzyme has dramatically increased recently, particularly in pharmaceutical, food and beverages, and paper industries. Surprisingly however, sources of enzymes generated by local industries were unable to satisfactorily meet this requirement and they have to import large quantities of enzyme from overseas, which was very costly (Judoamidjojo *et al*, 1989). Based on this evidence, scientists were encouraged to find other methods of enzyme production that is enzyme generating endophytic microbes.

Endophytic microbes, which can be bacteria, fungi or yeast, live symptomless in various part of the host plant. They produce secondary metabolites that generated oligosaccharide degrading enzymes, growth factors, hormones as well as substances which could potentially act as antimicrobial and antifungal agent (Petrini *et .al*., 1992).

It was well documented that Xylanase enzyme can be generated by endophytic microbes and mostly used by pulp industry. The benefit of using this enzyme was to reduce environmental contaminations due to the use of organic substance, such as chlorine for bleaching purposes (Kumala *et al*., 2008, and Judoamidjojo *et al*, 1989).

In Indonesia, variety of medicinal plants grow in its tropical land, however, not many research and scientific studies were performed to explore the numerous endophytic microbes living within the plants and their enzyme products. The current research studies carried out many investigations on endophytic microbes focusing on the activity of xylanase enzyme produced by endophytic fungi isolated from *Dipterocarpus grandiflorus* Blanco, namely "KERUING" plant. Besides its usage as building material, The Keruing plant was also used as traditional medicine to cure urinary infections by the laymen (Apanah and Turnbull, 2003 and Naiolah. 2000).

METHODS:

Materials: Endophytic fungi isolate were obtained from Bogor.
Culture media: Potato Dextrose Agar (PDA)(*Oxoid*), Yeast extract (*Oxoid).*
Liguid fermentation media: *Potato Dextrose Yeast* (PDY).
Reagents used in the analysis of hydrolysis product were xylan *Dinitro Salicylic Acid* (DNS), calcium carbonate (*Kimia Farma*), Xylosa (*Fluka),* Xylan (*Sigma*), Phosphate buffer pH 8.

Methods: Isolations of endophytic fungi were carried out via surface sterilization and direct seeding technique. Plant colony identifications were done macroscopically by colony characteristic observations such as growth rate, color and colony morphology (Kumala *et al*., 2006). Fresh and healthy *Dipterocarpus grandiflorus* Blanco were sampled from Kebon Raya Bogor in dry season. Part of the plants used in this study is twigs (diameter 0.8 – 10 mm). The plant samples were characterized in herbarium Balitbang, Puslitbang Biology Department of LIPI, Bogor, West Java, Indonesia.

Isolation and screening of endophytic fungi: Endophytic fungi isolations were carried out *via* direct seeding method and surface sterilization methods were adapted from (Petrini *et al*., 1992, Tomita, 1995, and Caroll, 1988), briefly, samples were washed under running water for about 10 min to remove dust and dirt, then they were cut into small pieces and air-dried at room temperature. Twigs were cut into pieces (dimension of 1 cm). Surface sterilization was conducted as followed: specimen were soaked in 76 % ethanol for 1 min, in hypochlorite solution 5.3 % for 5 min and finally in ethanol 76 % for 30 sec. The surface-sterilized pieces of twigs were aseptically sliced length-wisely. Then, they were

immediately placed on the surface of CMN medium which contained chloramphenicol (0.005%w/w). This was followed by incubation at temperature of 27-29^{o}C for 5-7 days. Selection processes were applied to differentiate endophytic fungi from other microbes grown in the culture. The selected endophytic fungi were transferred and cultured in PDA media.

Purification of isolated endophytic fungi: Colony identification was done macroscopically by observing colony characteristics Colonies with same morphology from one isolates were pooled together. The un-identified colonies were separated and grouped according to their morphology status. Purified endophytic fungi were cultured in slant agar. Each of the isolated fungi was cultured in different types of culture media (stock and work cultured). Stock culture was kept in freeze dried while working culture was kept in slant agar (Kumala *et al*., 2006).

Shake fermentation method: To obtain the xylanase enzyme, liquid fermentation using PDY medium, consists of potato dextrose broth 24.0gL^{-1}; yeast extract 2.0gL^{-1} and $CaCO_3$ 5gL^{-1} at pH 6.0 was conducted. Endophytic fungi were incubated in Petri dish, for 7 days. Liquid fermentation was carried out in a 250 mL Erlenmeyer flask, containing 50 mL PDY medium and 5 pieces of the endophytic fungi (the mass of the fungi 1x1 cm), insided an orbital shaker incubator rotating at 150 rpm for 5 days at room temperature.

After that, fermentation was continued in 1L PDY medium for 7 days under the same conditions. Separation of cell mass from supernatant which contain the enzyme was carried out in cold centrifuge (-4 ^{o}C), at 2000 rpm for 20 min. Supernatant which contained the extracellular xylanase enzyme was kept in the study for assays of the enzyme activity.

Xylanase enzyme activity: Dinitro Salicylic Acid (DNS) method was employed to confirm the presence of xylanase enzyme in the isolated endophytic fungi (Kumala *et al*., 2008).

Determination of maximum absorbance wave length (λ_{max}) of Xylosa: Stock solution of xylosa consists of 1 mg/ml was prepared. From this solution take 0.5 ml incubated at 48^{o} C for 10 minutes. Then added 1.0 ml of DNS reagent to the solution, then mixture and heated at 100^{o}C for 5 minutes. After that added distilled water to make up to 50.0 ml final volume. The maximum absorbance was measured.

Standard curve of xylosa: A xylose stock solution (1 mg/mL) was prepared and diluted down to five concentrations (0.1, 0.2, 0.4, 0.6 and 0.8 mg/ml). Reaction mixtures were then prepared using the same method as mentioned above section ("Determination of xylosa maximum absorbance wavelength"). Absorbance of these solution was measured on wavelength (λ) at 371.5 nm.

Xylanase enzyme assay: 1% ($^{b}/_{v}$) Xylan solution (0.5 ml) was mixed with 0.5 ml of enzyme supernatant and incubated at 48^{o}C for 10 minutes. 1.0 ml of DNS-reagent was added, heated in boiling water for 5 minutes. The reaction mixture was then diluted in distilled water (total volume of 50.0 ml) and enzyme activity was measured at λ 371.5 nm. Sample concentration was calculated, by converting the absorbance of sample solution into xylose standard curve. Control study was conducted with the same method as above except for the addition of supernatant after heating in boiling water for 5 minutes. The control solution absorbance was later converted into xylose standard curve to calculate its concentration. Xylanase activity was defined as 1 micromole of xylose release per minute under assay condition and this can be calculated using the following formula:

$$\text{Enzyme activity (UmL}^{-1}) = \frac{([S] - [K]) \times 1000 \times \text{Dilution factor}}{\text{Time of incubation} \times \text{Xylose MW}}$$

[S] - Sample concentration
[K] - Control concentration
Dilution factor - 200 x
Incubation time - 10 minutes.
Xylosa (molecule weight) - 150.13

RESULTS:

TABLE 1: MACROSCOPIC CHARACTERISTICS OF ENDOPHYTIC FUNGI ISOLATE OF *DIPTEROCARPUS GRANDIFLORUS* BLANCO

Isolate code	Colony diameter (cm) at day 7	Morphology and color of colony	Reverse colony
Dg II,3 $_{a1}$	5.0	White, edge of colony undulate	White green- brownish
Dg II,3 $_{a2}$	9.2	White, edge of colony undulates with concentric ring	Yellow- brownish
Dg II,3 $_{a3}$	9.9	White, edge of the colony serratus and with concentric ring	White-brownish
Dg II,3 $_{b1}$	10	White, edge of the colony entire	White-brownish
Dg II,3 $_{c1}$	9.5	White like cotton, edge of colony serratus	White brownish
Dg II,3 $_{c2}$	9.0	White greenish, edge of colonies. Erratus with concentric ring	Green-greenish
Dg II,3 $_{e1}$	9.0	White like cotton. Edge of colony undulates	White greenish
Dg II,3 $_{f1}$	4.7	White like cotton edge of colony serratus	White greenish

Table 1 shows the characteristic macroscopic fungi isolate of *Dipterocarpus grandiflorus* Blanco incubated at room temperature for 7 days. For each fungus, there were differences in shape, circle diameter of the growth fungi and also difference color.

TABLE 2: ACTIVITY OF EXTRACELLULAR ENZYME FROM SHAKE FERMENTATION OF ENDOPHYTIC ISOLATE OF *DIPTEROCARPUS GRANDIFLORUS* BLANCO

Isolate code	Enzym activity
Dg II,3 $_{a1}$	>10
Dg II,3 $_{a2}$	4.036
Dg II,3 $_{a3}$	1.985
Dg II,3 $_{b1}$	0.426
Dg II,3 $_{c1}$	0.533
Dg II,3 $_{c2}$	>10
Dg II,3 $_{e1}$	0.618
Dg II,3 $_{f1}$	2.624

Table 2 summarizes the activity of extracellular enzyme (supernatant) from shaking fermentation. Fungi with the isolate code DgII, 3_{a1} and DgGII, 3_{C2} had no activity.

DISCUSSION: Endophytic fungi isolates require approximately 5-7 days growing at room temperature (27-30^{o}C). For the purpose of this study, the endophytic fungi cultivation was carried out in PDA medium. Endophytic microbe's cultivation needs nutrients and other minerals suitable for endophytic microbe growing in the media, cell synthesis, cell metabolism, energy production and cellular movement. In general, endophytic fungi had a slow growing characteristic, but optimal culture condition could accelerate its growth.

Good fermentation medium should provide all nutrients needed by the microbes for energy production, growth and cell synthesis. Carbon and nitrogen rich compound were the most importance components in fermentation medium since microbes and several fermentation products were mostly composed of carbon and nitrogen elements apart from organic salts and some vitamins and minerals. PDY which contains carbon source deriving from potatoes, dextrose and yeast extract as nitrogen source were used in the fermentation process conducted in the current study.

Liquid fermentation medium in general have many advantages when compared to solid fermentation medium because the composition and concentration of the medium can be determined quite easily and optimal growth condition can be established (Stanbury, and Whitaker, 1987). The type of fermentation performed in this study was flask-shaking fermentation, at room temperature (27-30^{o}C) for 14 days, with shaking speed of 150 rpm. The product was supernatant containing xylanase enzyme, extracellular enzyme and activities of these enzymes were tested.

In the assays of xylanase enzyme activity, incubation at 48^{o}C was conducted to accelerate the enzymatic reaction between xylanase and xylan substrate so that the reaction occurs optimal condition. While incubation at 100^{o}C was done to inactivate the enzyme hence, enzymatic reaction could stop.

From eight *Dipterocarpus grandiflorus* Blanco fungi isolates, six isolates that have extracellular enzyme was obtained. They were (i) isolate Dg I I, 3_{a2} with activity value 4.036 UmL$^{-1.}$; (ii) Isolate DgII, 3_{a3} with activity value 1.985 UmL$^{-1.}$; (iii) Isolate Dg II, 3_{b1} with activity value 0.426 UmL$^{-1.}$; (iv) Isolate Dg II, 3_{c1} with activity value 0.533 UmL$^{-1.}$; (v) Isolate Dg II, 3_{e1} with activity value 0.618 UmL^{-1} and (vi) isolate fungi Dg II, 3_{f1} with activity value 2.694 UmL$^{-1.}$. The highest value was Dg II, 3_{a2} with value 4.036 UmL^{-1} and the lowest value was 0, 426 UmL^{-1}.

Isolate fungi Dg II, 3_{a1}, and Dg II,3_{c2} have no activity. Qureshy AF, *et al.*, (2000) mentioned that xylanase enzyme activities relied on several factors: pH, temperature as well as substrate and enzyme concentrations. The other said that *Aspergillus* sp and *Trichoderma* sp were xylanase enzyme generating endophytic fungi species. Baraznenok *et al*., (1999) It maight be the two fungi that have no activity was not from the Aspergillus or Trichoderma sp.

Fermentation using xylan carbon source as inducer could increase the production of xylanase enzyme. Kumala., *et al*. (2008) in her study demonstrated that xylan fermentation medium generated highest xylanase enzyme activity as observed in endophytic fungi isolate B3-2 (41,21 UmL^{-1}). The current study, however, used PDY liquid medium which does not contain xylan as inducer, this explained the lower activities of the enzyme when compared to that of using xylan in fermentation medium. Thus, it was advisable to use xylan containing fermentation medium to produced high activity xylanase enzyme.

CONCLUSION: Isolate of endophytic from twig of *Dipterocarpus grandiflorus* Blanco yield 8 fungi isolates. Endophytic fungi isolate produced xylanase enzyme activity ranged from 4.036 UmL$^{-1.}$ to 0.426 UmL^{-1}.

Isolate fungi endophytic did not produce xylanase enzyme were Dg II,3_{a1} and isolate fungi Dg II,$3_{c2.}$

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