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Fungal Production of Xylanase from Oil Palm Empty Fruit Bunches via Solid State Cultivation

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Abstract*—The abundance of Oil Palm Empty Fruit Bunches (EFB), the lignocellulosic biomass waste from Crude Palm Oil (CPO) industries, offers a potential for its application as raw material for biorefinery. Its xylan content in particular, suggests that EFB can be used as raw material for xylanase production. This article studies xylanase production by fungal Trichoderma viride ITB CC L67, Aspergillus niger ITB CC L51, and Penicillium sp. ITB CC L96 using EFB as the substrate in solid state fermentation. The aims of this research are to evaluate the incubation time to produce the highest xylanase activity, to explore the effect of particle size of EFB on xylanase activity and to determine the optimal operation condition of each significant variable, including temperature and substrate ratio (g EFB in mL liquid medium) using Response Surface Methodology (RSM). The method of Central Composite Design (CCD) is employed to investigate the individual crucial component of the operation condition, which significantly affected the enzyme activity. The incubation time of T. viride, A. niger, and Penicillium sp. were observed to produce xylanase . The result show that the xylanase activity is increase in the early phase of incubation at 0-36 hours, and remained constant afterward. At the time of 36 hours, the activity of xylanase produced by T. viride, A. niger and Penicillium sp. were subsequently 815 U/mL, 624 U/mL and 789 U/mL or equal to 8,146 U/g, 6,243 U/g and 7,892 U/g dry substrate. The particle size of the EFB is affect the growth and hence affects on xylanase production. It shows that the smaller size of EFB resulted in better fungal growth, which was indicated by higher biomass dry weight and higher protein content, as well as higher xylanase activity. The optimum conditions obtained xylanase activity is 740.6 U/mL or 5,095.5 U/g substrate, produced by T.viride ITB CC L.67 at 32.8oC and substrate ratio of 0.63.

Keywords*— Empty Fruit Bunches (EFB); optimization; solid state fermentation; *xylanase; xylose

I. INTRODUCTION

Xylanolytic enzymes or the so-called *xylanases* are highly exploited enzyme in industries, including food industry such as bread making and starch separation, and textile industries, and also in improving nutritional properties of animal feed, and bleaching of cellulose pulp in paper manufacturing [1]. Other than these, *xylanase* also has numerous potential applications in the pretreatment of *lignocellulosic* material in a biorefinery. For example, xylanases are utilized in catalyzing the *endohydrolysis* of 1.4-β-*xylosidic* linkages in *xylan, lignocellulosic* material to produce xylose-monomer. Xylose is the raw material of xylitol production, an alternative sugar which is low in calorie that prevents dental damage.

A wide variety of microorganisms produces xylanase. *Xylanase* produced from bacteria that have been reported include *Streptomyces sp.* [2], and *Bacillus pumilus SV-85S* [3]. The production of *hemicellulase* by these fungi in Solid State cultivation are particularly interesting as they secrete the enzymes into the medium and provide *xylanase* activities that are much higher than those found in yeast and bacteria [4]. Many studies have been conducted on the production of xylanase enzymes from fungi, including those from *Aspergillus niger XY-1* using wheat bran as substrate [5]; *A. niger DFR-5* using wheat bran and soybean cake as substrates [6]; mutagenesis of *A. niger F-19* [7]; *Penicillium including Penicillium sp. AHT-1* using the walnut shell and beech wood as substrates [8]; *P. sclerotiorum* using a variation of lignocellulosic material as substrates [9]; and *P. canescens* using wheat straw as substrate [10]. Xylanase from *Trichoderma sp* has been reported by *Trichoderma reesei* SAF3 using xylan as substrate [11]; *T. reesei* RUT-C30 [12]; *T. reesei QM 9414* using wheat straw as substrate [13]; *T.reesei* [14]; and *Trichoderma sp.SC9* [15]. Relatively few studies have been conducted on xylanase production using EFB substrates via SSF. The increasing number of palm oil production results in more waste, including biomass waste namely oil palm stem, leaf sheath, husk, shell, and empty fruit bunches. Among those, the oil palm empty fruit

bunches (EFB) are produced approximately 20-22% of fresh palm oil fruit bunches [16], mostly from the separation of the oil palm fruits from their bunches. The processing of palm oil biomass waste is therefore necessary to prevent the negative socio-environmental impacts of the palm oil industry.

Nowadays, EFB is mostly returned to the farm as compost or fertilizer. However, considering the composition of EFB which consists of 35-42% cellulose, 25-35% hemicelluloses, and 11-23% lignin [17]–[19]. The biomass waste has a high potential to be used as raw material for various chemical processes following the concept of the *biorefinery*. For example, the cellulosic material could be used as the feedstock of bioethanol, biofuel, or pulp mills. The hemicellulose could also be used to get a variety of valuable products such as xylose and xylitol.

In its use, hemicellulose must first be hydrolyzed into its components which consist of 6 carbon atom sugar (mannose and galactose) and 5 carbon atom sugar (xylose and arabinose). This process could be performed chemically, at high pressure and temperature using acid or alkaline as the catalyst [20] or at ambient condition using biological xylanolytic enzyme as the catalyst [21]. The enzymatic hydrolysis is preferred because the process is conducted at the moderate operating condition. Another advantage is that this process does not need high purity chemicals as raw materials and complex utilities. In most cases, the bioprocess applies waste as raw materials. Since the major factor for broad application is the enzyme cost, approaches that either decrease the medium cost or increase production efficiency should be investigated.

Since EFB has a relatively high content of xylan, EFB is considered to be a prospective raw material for *xylanase* production. This can make *xylanase* to be abundantly available, and therefore has a potential market. However, Inoculum type, moisture, pH, temperature, substrate, and nutritional factors are reported to be important factors in SSF [22], [23], which need to be studied and optimized for *xylanase* production from EFB.

This research aims at examining the production of fungal *xylanase* using EFB as a carbon substrate in solid state fermentation. This article covers the evaluation of incubation time, the particle size of EFB, and the temperature and the solid-liquid ratio (media composition) to *xylanase* production. The optimum condition for *xylanase* cultivation is sought further.

II. Material and Methods

A. Preparation of Raw Material

EFB was collected from Incasi Raya Palm Oil Mill, West Sumatera, Indonesia. The raw materials are washed with clean water then followed by drying using sun and oven at 105°C overnight. Dry EFB was ground and was sieved using Tyler sieve 20 mesh and 60 mesh [24].

B. Fungal Preservation

Three fungal strains were tested during the experiments: *Aspergillus niger ITB CC L.51, Trichoderma viride ITB CC L.67* and *Penicillium sp* ITB CC L.96. *A. niger* were cultivated in potato dextrose agar. The spore of fungal was immobilized on dried rice pellets then made into inoculum by mixing with sterile distilled water to generate a cell concentration reaches of 1×10^6 spores/mL [25].

C. Cultivation Medium Preparation

The nutrient for fungal fermentation was preserved by mixing dried EFB as a main carbon source for the fungal growth with a liquid mineral medium which was adapted from [26]. The mineral medium contained 1.5 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.3 g/L urea, 0.03 g/L$CaCl_2$, and 0.2 g/L $MgSO_4.7H_2O$. The nutrients on the medium before use, sterilized in advance to avoid contamination in an autoclave for 15 minutes at 121°C before use.

D. Solid State Cultivation

1) Cultivation Time Evaluation: the cultivation timing process is intended to determine the time that is capable of producing the highest *xylanase* activity. The experiment was done with the three fungal strains, 5 g OPEFB was added with 10 mL of liquid medium with a substrate ratio of 0.5. The fungal cultivations of 1×10^6 spores/mL were carried out in 300 mL Erlenmeyer at 30°C and were conducted over the of 12-72 hours cultivation time. One shake flask culture was intended to provide a one-time sample. Sampling was done every 12 hours and the obtained samples were used for the analysis of cell concentration (cell dry weight and content of protein) and activity of *xylanase* [27].

2) Evaluation of EFB Particle Size: The evaluation of EFB particle size experiments were conducted at the optimum cultivation time obtained in the preceding section. Particle sizes evaluated were 20 and 60 mesh. Each run was performed in Duplo. Samples were taken at the end of the cultivation and were used for cell concentration and *xylanase* activity analysis.

3) Optimization of Xylanase Production: The *xylanase* production was optimized using the methodology of the response surface, which is the experimental design of the Central Composite Design (CCD), on three variables: fungal strains, cultivation temperature, and solid to the liquid substrate. The fungal strains used were *A. niger* ITBCC L.51, *T. viride* ITBCC L.67, and *Penicillium* sp ITBCC L.96. Cultivation temperature was varied at 28-42°C, with the ratio of substrate evaluated, was in the range of 0.46-0.71 (gr EFB/mL liquid medium) [28]. Each run was performed in two replicates, using the best EFB particle size and for the optimum cultivation time that has been obtained previously.

E. Enzymes Harvesting

At the time of cultivation process is completed, then proceed with the harvesting process in the fermentation system. The harvesting process of the enzyme was performed according to [5]. Enzymes were harvested from the fungal fermentation by mixing four times of volume distilled water to the enzyme extract. Subsequently, the extract was agitated with a sterile stick and then stirred at 100 rpm and room temperature for 1 hour. The extract obtained was then filtrated by vacuum to separate solid and particulates. Further, the extract was separated using centrifugation for 15 minutes at 10,000 rpm at 5°C. The enzyme extract could be used for analysis of activity.

F. Analysis

1) The Composition Lignocellulosic Material: The primary composition of EFB was determined following two standard methods: TAPPI (TAPPI standard T 13m-54 and T 19 - 50) in which the components of EFB were expressed in their polymeric form and NREL Technical Report NREL/TP-510-42623 [29] in which the component of EFB were expressed in their monosaccharide form. The TAPPI analysis was performed in the Laboratory of Chemical and Instruments, Center for Pulp and Paper, Ministry of Industry, Indonesia whereas the NREL analysis was performed in the Laboratory of Microbiology and Bioprocess Technology, ITB, Indonesia.

2) Cell Concentration: Cell concentration was analyzed as dry cell weight and total protein content according to [30]. The samples for cell dry weight analysis were obtained following similar initial procedure from enzyme harvesting until vacuum filtration. Supernatant from vacuum filtration was then filtered again using a weighed filter paper with a pore size of 0.2 µm. The filter and the cake (cell biomass) were afterward oven-dried at 50°C. When constant weight was reached, the samples were put into a desiccator and weighed. The samples for total protein content analysis were prepared following similar initial procedure from enzyme harvesting until vacuum filtration. Supernatant from vacuum filtration was stored at 4^{o}C refrigerator for 24 hours before cell disruption. The cells were disrupted by adding 1 N NaOH with the ratio of 1:1 for 15 minutes at 100^{o}C. Afterward, the protein content was measured by Bradford method [31] using bovine serum albumin as its standard.

3) Xylanase Activity: The enzyme activity was measured by Larchwood xylan (Sigma Co., USA) as substrate according to [5]. In brief, the reactant containing 0.5 mL of 1% (w/v) *Larchwood xylan* (pH 5.0 acetate buffer) mixed with the enzyme as much as 0.5 mL then incubated using a water bath for 15 minutes at 40°C. After incubation was *complete, the reaction was stopped by adding a dinitrosalicylic* (DNS) acid solution of 1.5 mL and boiled for 5 minutes. The reducing sugars resulted were quantified by the *dinitrosalicylic* acid method using D-xylose as standard [32] with a spectrophotometer at 540 nm wavelength. One unit (U) of *xylanase* activity was defined as the number of enzymes needed to produce 1 µmol of xylose per minute under the assay conditions.

Laccase activity of the crude enzyme was determined using the analytical method according to [33], a solution of 1160 µL of 0.4 mM ABTS (2,2'-*azinobis*-di (3 *ethylbenzthiazoline sulphonate*)) in sodium acetate buffer (pH 4.5) was put into a disposable 1.5 mL cuvette. Forty microliters clarified sample was then added into the cuvette and was mixed accordingly. The presence of *laccase* in the sample will convert ABTS into a blue-green color of cation radical. The optical density of the color was determined using spectrophotometer (Genesis 10 UV Visible) at 420 nm at an interval of one minute for five minutes. One International Unit of *laccase* activity is defined as the amount of enzyme that oxidizes 1 µmol of ABTS per minute. *Cellulase* activity analysis was determined according to NREL method Laboratory Analytical Procedure (LAP), NREL/TP-510-42628 [34]. The detection of *glycosidic* bond cleavage by this method involves the parallel and identical treatment of three categories of experimental tubes (assay mixtures, blanks and controls, and glucose standards). The substrate is a 50 mg *Whatman* No. 1 filter paper strip (1.0 x 6.0 cm).

4) Sugar Content by HPLC: The sugar content in the crude enzyme such as xylose and glucose was measured by HPLC using HPX-87H column (*Aminex, Biorad*) using 0.005 M sulfuric acid as the mobile phase at column temperature of 60 °C and refractive index detector (RID) at detector temperature of 40°C according to NREL method Laboratory Analytical Procedure (LAP), NREL/TP-510-42623 [29].

III. Result and Discussions

A. Characterization of EFB

The composition of *lignocellulosic* biomass waste EFB was characterized to confirm whether or not it had the proper and sufficient components to be used as the substrate, for fungal cultivation for *xylanase* production. The observed results and literature value of compositions of EFB are presented in Table 1.

Our analysis showed that EFB consisted of around 22% hemicellulose xylose as the main constituent of the EFB hemicellulose. This number is comparable to previously reported references. For example, [17] measured *xylan* content in EFB to be 24.02%, whereas [19] measured the hemicellulose content of EFB to be 22.03% (Table 1). The relatively high xylose content of EFB suggested that the EFB had the potential to be used for *xylanase* or *xylito*l production.

TABLE I
THE POLYMER AND MONOMER COMPOSITION OF EFB

Polymeric Composition Obtained from TAPPI Analysis				
Composition	**(%-dry weight)**	**Literature Values (%-dry weight)**		
		[17]	**[19]**	**[18]**
Cellulose	43.32	42.85	38.00	31.80
Hemicellulose	23.67	24.10	35.00	22.03
Lignin	22.10	11.70	23.00	17.79
Other	10.91			
Monomeric Composition Obtained from NREL Analysis				
Glucose	35.80			32.98-33.40
Xylose	19.62			19.28-19.60
Arabinose	1.50			1.47-2.37
Manose	-			1.50-1.22
Galactose	-			1.03-1.42

B. Cultivation Time

To evaluate optimal cultivation time to produce xylanase, the time cultivation profile of enzyme activities was conducted for 72 hours and the samples were taken every 12 hours (the observed results are presented in Fig. 1). Each of the *T. viride*, *A.niger*, and *Penicillium sp.* cultivation cell dry weight and protein content is presented in Figure 1a-b, where similar trends were observed. Up to 24 hours, the cells grew slowly and got faster at 24-36 hours. After 36 hours, the cell dry weight and protein content were observed to be more or less constant. These observations indicated that the fungus needed to undergo 24 hours of lag phase before their

exponential growth and they already reached their stationary phase by 36 hours of cultivation. At the same growth condition, the three fungi (*T.viride, A.niger*, and *Penicillium sp*) grew at a similar speed and reached similar biomass weight.

T. viride, *A. niger*, and *Penicillium sp.* were observed to produce xylanase throughout their cultivation time. The xylanase activity was observed to increase in the early phase of cultivation, 0-36 hours, and remained constant afterward (Fig. 1c). *T. viride* and *Penicillium* were observed to produce a similar level of *xylanase* activity, whereas *xylanase* activity produced by *A.niger* was measured to be significantly lower. At the time of 36 hours of cultivation conditions, the average activity of *xylanase* produced by *T. viride, A. niger and Penicillium sp.* were subsequently 815 U/mL, 624 U/mL and 789 U/mL or equal to 8,146 U/g, 6,243 U/g and 7,892 U/g dry substrate. By comparison, the research found that the highest xylanase activity produced by *Neosartorya spinosa* at solid state cultivation using wheat bran as substrate was at 72 hours of cultivation with xylanase activity of 20.6 U/mL (which is lower than result gained from this research) [26].

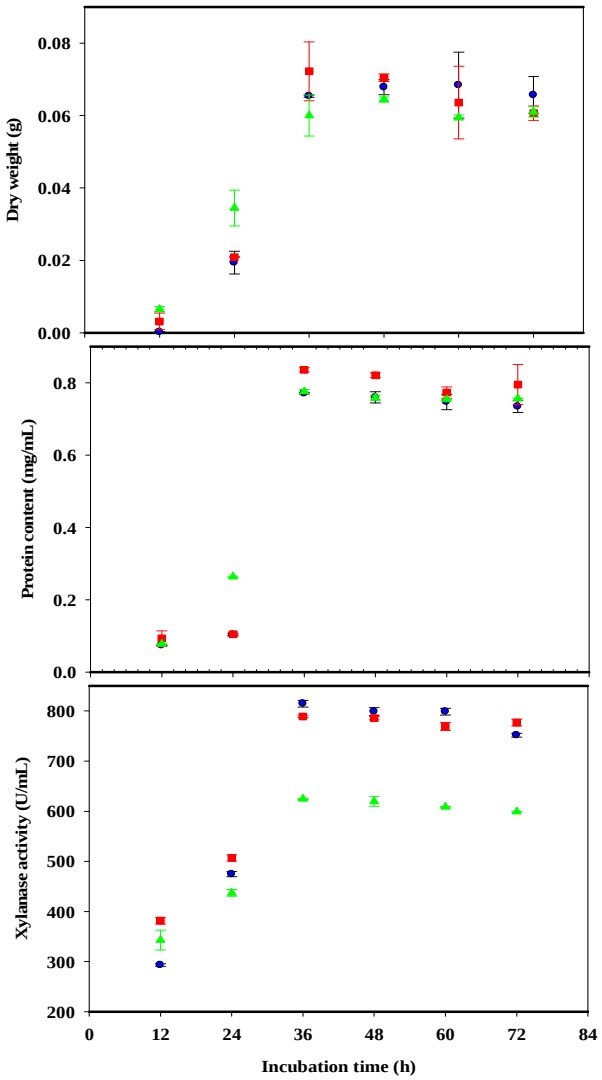


Fig 1. Cell dry weight (1a), protein content (1b) and xylanase activity (1c) for cultivation time evaluating

● *T. viride*, ■ *Penicillium*, ▲ *A. niger*

Other researchers reported that the xylanase produced by *Aspergillus niger* in Solid state cultivation provides the highest activity at 48 hours of cultivation with *xylanase* activity at 14,637 U/g dry substrate [5], which is higher than our result among others due to differences in the initial hemicellulose compound in the substrate. According to [4], *xylanase* enzymes were produced during fungal growth because *xylanase* was required to catalyze the hydrolysis of xylan. This enzyme is produced mainly by microorganisms in order to break down plant cell wall hemicellulose which is included, along with other enzymes that hydrolyze polysaccharides, and also digest *xylan* for growth. Our results showed that the hemicellulose content in the medium from EFB served as a good inducer for the fungi to produce *xylanase*.

Holker and Lenz modeled the fungal growth on a solid substrate as follows: the fungal hyphae would grow using existing nutrition on the surface of solid substrates at first, then fungal mycelia would form and grow, spread over the surface of a solid substrate [35]. Enzymes produced by the mycelia would then diffuse into the solid matrix, and catalyze the degradation of macromolecules into monomers. Monomers will be used as nutrients. Enzymes and other products were released into the solid matrix and fulfilled the space that needs to be extracted for specific purposes at the end of the process. The similar profiles between the fungal growth and produced enzyme activity observed in these experiments validated the model.

Our results showed the decreasing growth and enzyme production after 36 hours of cultivation (Fig. 1). What factors dictated this, however, were not investigated further in the research. The decreasing of *xylanase* activity may occur due to the growth of fungi that are not optimal, lack of moisture, macro and micronutrient deficiencies in the cultivation medium, enzyme inhibition by the end product, or a change in pH during the cultivation process [36].

C. Influence of Particle Size of EFB

The particle size of the substrate may significantly affect the fungal growth on a solid substrate and hence affects *xylanase* production. According to [32], the particle size in solid-state cultivation significantly affected microbial growth, absorption of heat, and mass transfer. The use of smaller particles provides more surface area for adhesion, and thus providing a favorable environment for the heat transfer and exchange of oxygen and carbon dioxide between air and the surface of the solid substrate. However, particles that are too small can cause agglomeration of the substrate which can interfere with microbial respiration, resulting in disruption of cell growth [37].

In this research, the EFB size was varied to 20 mesh and 60 mesh and was used as the substrate for the 36 hours cultivation of *T. Viride* for the production of *xylanase*. Table 2 shows that the smaller size of EFB resulted in better fungal growth, which was indicated by higher biomass dry weight and higher protein content, as well as higher *xylanase* activity.

TABLE II
INFLUENCE OF PARTICLE SIZE

No	Analyze	Dimension	Particle Size of EFB	
			60 mesh	20 mesh
1	Dry weight cell	g	0.065 ± 0.001	0.054 ± 0.003
2	Protein content	mg/mL	0.771 ± 0.002	0.697± 0.030
3	*Xylanase* activity	U/mL	814.659 ± 6.644	793.048± 2.658

The experiments should be continued further with a smaller particle size of EFB, where a decrease in cell growth and enzyme production may be seen, as was suggested by [37]. However, reducing the particle size of EFB was both still technologically challenging and high energy consumption. Therefore, it was not performed in this research.

D. Characterization of Enzyme Extract

The previous report suggested that in addition to producing *xylanase*, the evaluated fungal species (*Trichoderma*, *Aspergillus*, and *Penicillium*) were also producing cellulase enzymes [12]. To check the presence of these enzyme activities, sugar concentration in the solution was measured. The presence of xylose, which is the breakdown product of hemicellulose, would confirm the presence of *xylanase* activity. On the other hand, the presence of glucose, which is the breakdown product of cellulose, would confirm the presence of *cellulase* activity. Table 3 shows the concentration of glucose and xylose from the three fungal cultivations. Both xylose and glucose were measured, which indicated the presence of both *xylanase* and *cellulase* activity. It is interesting to see that the ratio of glucose to xylose in *T.viride* cultivation was much lower than the other, which may lead to the high *xylanase* activity in the produced enzyme.

TABLE III
SUGAR CONTENT AND CHARACTERIZATION OF CRUDE ENZYME PRODUCED IN THE FUNGAL CULTIVATION

Content		Microorganisms		
		T.viride	***A.niger***	***Penicillium sp***
Protein Content (mg/mL)		0.770	0.77	0.84
Glucose Concentration (g/L)		0.05	0.23	0.15
Xylose Concentration (g/L)		0.34	0.29	0.23
Xylanase	U/mL	814.66	624.38	789.29
	U/mg protein	1057.59	805.07	944.45
Cellulase	FPU/mL	24.70	33.60	30.80
	FPU/mg protein	32.08	43.30	36.84
Laccase	U/mL	$1.44x10^{-6}$	$2.70x10^{-6}$	$1.10x10^{-7}$
	U/mg protein	$1.87x10^{-6}$	$3.57x10^{-6}$	$1.32x10^{-7}$

To confirm the results further, specific enzyme assays were performed to test the *xylanase*, *cellulase*, and *laccase* activities in the produced enzyme extract. We observed that the produced enzyme has all—the *xylanase*, *cellulase*, and *laccase* activities (Table 3). *Xylanase* activity was observed to be the main activity measured in the enzyme extract, in the range of 620-815 U/mL. *Cellulase* activity was significantly measured as well, in the range of 25-34 PFU/mL. On the other hand, minimal *laccase* activity was measured in the crude enzyme sample, in the range of $1.10x10^{-7}$-$2.70x10^{-6}$ U/mL. The small laccase activity may be due to the sterilization of EFB before the cultivation that may act as a thermal pretreatment of the *lignocellulosic* material. However, this should be confirmed further by a separate study.

We also measured the protein content in the enzyme extract or crude enzyme, and thereby the enzyme activity could also be expressed as the specific activity or the enzyme activity per gram protein (Table 3). The composition of measured enzyme activities indicated that the produced enzyme had relatively high specificity for hemicellulose or xylan degradation.

E. Optimization of Substrate Ratio and Temperature

The optimization of *xylanase* production was performed following Response Surface Methodology (RSM) on effects of temperature and substrate ratio for each fungi using central composite design (CCD). Detail experiment design along with the resulting enzyme activity is presented in Table 4.

TABLE IV
CCD EXPERIMENTAL DESIGN FOR XYLANASE PRODUCTION

run	F1	F2	Xylanase Activity (U/mL)		
	(°C)	Solid Liquid ratio	***Tricoderma viride ITB L.67***	***Aspergilus nier ITB L.51***	***Penicillium sp ITB L.96***
1	35	0.46	387.6	687.8	343.4
2	35	0.58	786.0	640.8	526.7
3	42	0.58	413.9	601.4	344.4
4	35	0.58	726.3	664.3	530.9
5	30	0.5	708.0	715.5	566.6
6	35	0.58	708.0	656.8	565.2
7	35	0.58	647.9	622.5	545.5
8	40	0.5	732.4	708.0	346.7
9	35	0.58	738.4	683.1	549.7
10	35	0.71	717.9	673.2	368.3
11	30	0.67	699.6	609.8	364.1
12	40	0.67	384.8	614.0	349.1
13	28	0.58	453.8	705.7	322.8
14	35	0.46	385.2	730.1	381.5
15	42	0.58	426.6	648.3	338.7
16	30	0.5	720.7	702.8	545.5
17	40	0.5	736.7	665.7	312.9
18	35	0.71	722.6	608.9	366.5
19	30	0.67	719.3	611.7	365.0
20	40	0.67	404.0	578.8	330.3
21	28	0.58	452.9	720.7	341.6

Within the range of experiments, with a temperature of 30-42°C and solid to liquid ratio in the substrate of 0.46-0.71, *xylanase* activities were measured in the range of 322.8 to 786 U/mL. Parameters of the experiment were calculated. The general ability of the above polynomial and the

accuracy model evaluated by the determination coefficient (R^2) and variant analysis (ANOVA) for the experimental results are presented in Table 5. From multiple regression analysis, we found that regardless of the significance of coefficients, the *xylanase* production could be explained by the quadratic equations as presented in equation 1-3.

$$Y_1 = 415.8T + 15,241.5R - 201.1TR - 4.8T^2 - 6,824.7R^2 - 10,909.5 \quad (1)$$

$$Y_2 = -15.9T - 15.9R + 6.2TR + 0.1T^2 + 947.9R^2 - 1628 \quad (2)$$

$$Y_3 = 192.7 + 7,648.7R + 113.7TR - 3.7T^2 - 10,145R^2 - 4,894.1 \quad (3)$$

Where Y_i is the response value or the produced *xylanase* activity for *Trichoderma*, *Aspergillus*, and *Penicillium* (U/mL). Respectively, T and R represent temperature and substrate ratio. The Student's t-distribution and the corresponding P-value, along with the parameter, are given in Table 5. The parameter coefficient and the corresponding P-value suggested that both temperature and substrate composition have significant effects on the *xylanase* production.

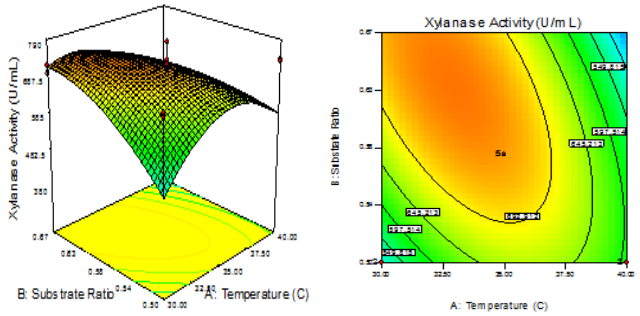
TABLE V
ANALYSIS OF VARIANCE FOR THE RESPONSE

Variable	*T.viride ITB L.67*	*A.niger ITB L.51*	*Penicillium sp ITB L.96*
Model	Quadratic	Quadratic	Quadratic
Source	P-value prob> F		
Model	0.087	< 0.003	<0.0001
T-Temperature	0.195	0.018	0.039
R-Substrate ratio	0.5790	0.0003	0.117
TR	0.090	0.808	0.016
T^2	0.021	0.764	<0.0001
R^2	0.228	0.453	0.0002
R-square	0.44	0.662	0.799

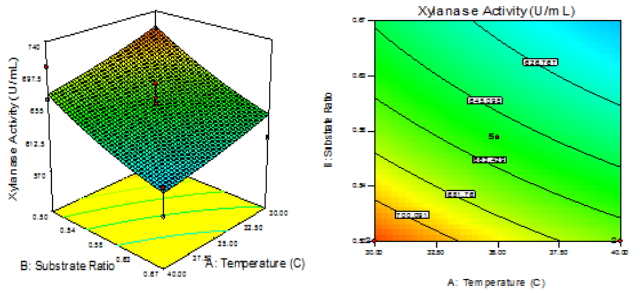
Figure 2 shows that the contour plots and 3D response surfaces graphs were shown a correlation of the temperature and substrate ratio that has the effect of cultivation conditions on *xylanase* activity. Generally, during microbial growth, the solid medium is disintegrated, which may lead to changes in the geometric and physical characteristics of the medium and significantly affect the heat and mass transfer process [23]. Our results confirmed that solid to liquid ratio significantly affected the growth of fungi and the production of *xylanase*. Solid to liquid ratio in the substrate up to 0.58 increases *xylanase* activity. However, higher solid to liquid ratio in the substrate of 0.58-0.67 decreases *xylanase* activity. At high solid to liquid ratio, the system was very viscous, thereby may inhibit heat and mass transfer. Besides, the temperature was also an essential factor because each strain has its optimum temperature for growth [38].

Specific for each fungus, significant factors influencing the xylanase activity of *A. niger* are temperature and substrate ratio (Table 4). At 35°C, *xylanase* activity decreased with increasing ratio of up to 0.67. This suggests that *A. niger* prefers a somewhat moist medium (more water) because it can facilitate spores growth and cause a higher production of *xylanase* activity.

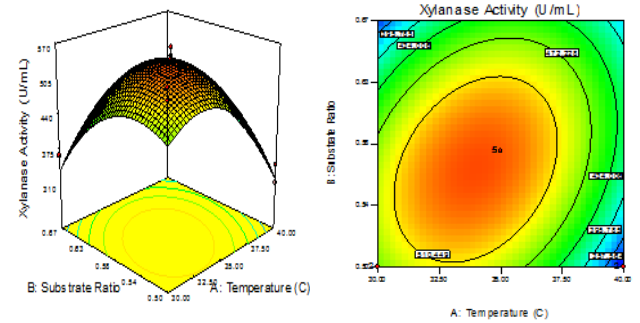
Xylanase activity of *T. viride* increased along with the increasing temperature of up to 35°C but then decreased until the temperature reached 42°C. Results of all three types of fungal *xylanase* activity in optimum condition can be seen in Tables 5 and 6. Table 6 shows that the highest enzyme activity produced by *T. viride* was at 32.8°C, with substrate ratio of 0.63 and 740.6 U/mL or 5,095.5 U/g substrate. A validation experiment was performed at the optimal condition of *T.viride* and consistently produced *xylanase* activity of 753 U/mL or 5,180.6 U/g dry substrate.



a. Trichoderma viride ITB L.67



b. Aspergillus niger ITB L.51



c. Penicillium sp ITB L.96

Fig. 2 The Interaction Between Temperature and Substrate Ratio using Response Surface for Xylanase Production by Fungal:

TABLE VI
OPTIMIZED RESULT FOR THE RESPONSE OF *XYLANASE* PRODUCTION

Fungi	°C	Substrate Ratio	*Xylanase* Activity		Validation of *Xylanase* Activity
			U/mL	U/g substrate	
T.viride ITB L.67	32.8	0.63	740.6	5,095.3	753 U/mL (5,180.6 U/g)
A.niger ITB	30	0.50	713.6	4,909.6	

L.51					
Penicillium sp	34	0.57	548.7	3,775.1	

Meanwhile, at a substrate ratio of 0.58, *xylanase* activity declined with the increase of temperature of up to 40°C. This is consistent with the optimum living condition of *A. niger*, which is about 28-30°C [5], [39]. *Xylanase* activity of *Penicillium sp* increased along with the increase of temperature up to 35°C but then decreased until the temperature reached 40°C. This is consistent with some cultivation by several researchers in the genus *Penicillium* at a 30-35°C temperature as *P. sclerotiorum* [40] and *P. canescens* [10].

F. Comparison with Other Producing Fungi

Lakshmi et al. performed the production of *xylanase* in solid substrate fermentation using EFB as a substrate using *A. Tereus* and *A. fumigatus*. The cultivation was performed for 60 hours at 30°C, giving *xylanase* activity of respectively 13,370 U/g dry substrate and 12,898 U/g dry substrate [36]. The reported *xylanase* activity was much higher than the obtained results of this research, despite using the same EFB substrate. Lakshmi's experiment used additional carbon source, xylose, and fructose, in the medium that might improve the fungal growth and thus provide more biocatalysts for the enzyme production [36]. However, providing pure sugar also means extra cost.

Prado et al. reported the *xylanase* production in solid-state fermentation conditions using *Neosartorya spinosa* at 35°C, pH 5, solid to liquid ratio of 0.5 for 72 hours, utilizing various *lignocellulosic* materials, namely wheat bran, rice bran, cassava, corn cobs, maize straw, and sugarcane bagasse. The produced *xylanase* activities were in the range of 0.5-15.1 U/mL [26], or significantly lower than the obtained results of this research.

Other researchers conducted a study on the production of *xylanase* from wheat bran using *A.niger* XY -1 at 28°C and reported the produced *xylanase* activity of 14,637 U/g dry substrate [5]. Other produced *xylanase* using *Penicillium sclerotiorum* at 30°C gave xylanase activity at 6-7 U/mL [40]. Kar et al. reported on the production of fungal xylanase utilizing *T. reseei* SAF3 which produced optimal xylanase activity of 3.42 U/mL at 48 hours of cultivation [11].

Overall, the obtained results showed that producing xylanase enzyme from OPEFB by solid state cultivation of *T.viride, A.niger*, or *Penicillium sp* gave a reasonably high xylanase activity at comparable operation condition such as temperature and solid and liquid ratio in particular. This indicates that fungal cultivation at EFB, the *T.viride* in particular, was a possible process for xylanase production.

IV. Conclusions

We have studied the fungal production of xylanase mainly regarding the evaluation of cultivation time, particle size and optimization of fermentation conditions including temperature and substrate ratios. The results confirmed that EFB contains sufficient hemicellulose and xylan to be used as a substrate for xylanase production using solid-state fungal cultivation. Furthermore, the size of the used EFB fiber affected the fungal growth and the activity of produced xylanase. Every three tested fungal strains, *A.niger* ITB CC L.51, *T.viride* ITB CC L.67, and *Penicillium sp.* ITB CC L.96 produced enzymes with *xylanase* activity as well as *cellulase* and *laccase*. From the analysis of fungal growth profile, we found that *xylanase* was produced at a growth cell phase with optimal production when the fungal was cultivated until the end of its exponential growth phase. In optimizing the produced xylanase activity, we found that both temperature and solid to liquid ratio in the substrate affected the produced *xylanase* activity. *T.viride* ITB CC L.67 was suggested as the best *xylanase* producer when cultivated at 32.8°C with substrate solid to liquid ratio of 0.63.

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