BIODIVERSITAS
Volume 26, Number 5, May 2025
Pages: 2117-2128

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d260510

Fungal diversity for enhanced sugar production comparing rotting and non-rotting fungi in pretreatment of empty palm oil bunches

INA MONICA FIRSTY[1], RATNA SETYANINGSIH[1], SRI SUGIWATI[2], ATANU KUMAR DAS[3], RONI MARYANA[2,♥]

[1]Graduate Program of Bioscience, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

[2]Research Center for Chemistry, National Research and Innovation Agency. Jl. Puspitek Serpong Gate, KST BJ Habibie, Banten 15314, Indonesia. Tel.: +62-21-756-0929, Fax.: +62-21-756-0929. ♥email: roni.maryana@brin.go.id

[3]Department of Pulp, Paper and Packaging, MoRe Research Örnsköldsvik AB, RISE Research Institutes of Sweden. Hörneborgsvägen 10, SE-892 50, Domsjö, Örnsköldsvik, Sweden

Manuscript received: 22 August 2024. Revision accepted: 30 April 2025.

Abstract. *Firsty IM, Setyaningsih R, Sugiwati S, Das AK, Maryana R. 2025. Fungal diversity for enhanced sugar production comparing rotting and non-rotting fungi in pretreatment of empty palm oil bunches. Biodiversitas 26: 2117-2129.* Empty Palm Oil Bunch (EPOB) is an abundant lignocellulosic biomass in Indonesia with significant potential for conversion into valuable products. This study explores various types of fungi in enhancing sugar production from EPOB. This study compared white-rot fungi (*Trametes versicolor* and *Pycnoporus cinnabarinus*) with non-white-rot fungi (*Aspergillus niger*, *Aspergillus terreus*, *Trichoderma harzianum*, and *Trichoderma viride*) to see the effect on lignin and cellulose content, as well as enzyme activities. Using Response Surface Methodology (RSM), this study optimized temperature, pH, and substrate concentration. Among the *fungi*, *A. niger* showed the best results for lignin removal (7.58 ± 1.5%) and cellulose increase (33.56 ± 0.00%). *P. cinnabarinus* had the highest laccase and MnP activities (9.26 and 123.81 U/mL), while *A. niger* produced the highest LiP activity (3276.8 U/mL). X-Ray Diffraction (XRD) analysis revealed a reduction in crystallinity in the pretreated samples. The best pretreatment conditions with *A. niger* were found to be 30°C, pH 5, and 7 g of EPOB, achieving the highest lignin removal (14.21 ± 0.50%). *A. niger* also led to the highest cellulose content (53.08%) at 25°C. Enzyme hydrolysis of *A. niger*-pretreated EPOB produced 1.09 g (36.30%) of reducing sugar after 48 h at 50°C. This study highlights how leveraging fungal diversity can optimize biomass pretreatment processes.

Keywords: Cellulose, fungi, lignin, pretreatment, sugar

Abbreviations: EPOB: Empty Palm Oil Bunch; RSM: Response Surface Methodology; LiP: Lignin Peroxidase; MnP: Manganese Peroxidase; DNS: 3,5-Dinitro Salicylic Acid

INTRODUCTION

Oil palm is one of the most efficient oil-producing plants in terms of cost. Production of oil palm generates various types of waste, including empty palm oil bunches (25%), fibers (14%), shell waste (7%), liquid waste and palm sludge (Bolong et al. 2016). Empty Palm Oil Bunches (EPOB), a major Indonesian commodity and abundant biomass, exhibit a recalcitrant lignocellulosic composition: 9.4 ± 0.2% ASL, 23.0 ± 0.6% AIL, 53.8 ± 0.3% holocellulose (40.8 ± 0.7% cellulose, 13.1 ± 0.3% hemicellulose), 7.2 ± 0.4% ash, and 3.9 ± 0.5% extractives (Simanullang et al. 2023). Lignocellulosic biomass is the largest source of carbon worldwide (Behera and Ray 2016; Cortes-Tolalpa et al. 2017) and is recognized as a valuable carbohydrate source for biofuel production (Mustafa et al. 2017). EPOB is a potentially biomass for producing monomeric sugars, glucose and xylose (Solihat et al. 2017; Rizal et al. 2018). Breakdown of lignocellulose complexes can produce lignin, cellulose, and hemicellulose. Lignin isolation with optimization has been carried out by Hidayati et al. (2023) with good results. The isolated lignin can be used as a functional material. Purified cellulose can also be used as a valuable product. Anwar et al. (2024) have studied the synthesis and characterization of cellulose acetate from sugar palm stem. Conversion of carboxymethylcellulose obtained from EPOB cellulose has been carried out by Yimlamai et al. (2021). EPOB must undergo several processes to release glucose, the key component that can be transformed into a variety of valuable products. One promising approach is biomass bioconversion, where EPOB is used as a substrate for bioethanol production. Under optimized conditions, this process not only helps reduce environmental pollution and production costs but also boosts renewable energy sources (Fasiku and Wakil 2022).

Pretreatment is a crucial first stage that aims to remove compounds that probably inhibit the hydrolysis rate, one of which is by partly or completely degrade lignin (Sjulander and Kikas 2022) of the biomass so that cellulose can easily bind to enzymes for hydrolysis. Pretreatment of biomass involves many types of method; physical methods for size reduction (ball milling, ultrasonication, steam, pyrolysis, gamma irradiation), chemical delignification using acids, and biological methods with microorganisms or enzymes. Biological pretreatment is a green approach due to its mild conditions and the absence of inhibitory byproducts or

hazardous waste (Ummalyma et al. 2019). However, this method has some limitations, including a lengthy fungal growth period on lignocellulosic media and the fact that its ligninolytic activity depends on the compatibility between the substrate and the fungus used (Yadav and Vivekanand 2021).

Many fungi act as saprobes, deriving nutrients from various types of dead organic matter. Among these saprobes, fungal species that thrive on wood or in contaminated environments have developed efficient mechanisms for producing degrading compounds, such as ligninolytic enzymes. Fungi are increasingly used for biomass pretreatment due to their ability to degrade complex organic materials. Among them, rot fungi—adapted to woody substrates—are particularly notable for their diverse enzymatic capabilities. They fall into three main groups: white-rot, brown-rot, and soft-rot fungi, each with distinct mechanisms for lignin degradation. Key enzymes involved include laccase, Lignin Peroxidase (LiP), and Manganese Peroxidase (MnP) (Weng et al. 2021).

While previous studies have investigated white-rot fungi such as *Pycnoporus cinnabarinus* and *Trametes versicolor* (Sahu and Pramanik 2015), and brown-rot fungi like *Trichoderma viride* and *Trichoderma harzianum* (Safari et al. 2022; Zhang et al. 2023) and *Aspergillus terreus*, there is limited research on the comparative effectiveness of diverse fungi for pretreatment. This study focuses on *Aspergillus niger*, evaluating their lignin degradation capabilities and enzyme activity using a novel pretreatment approach with a 14-day fungal suspension. Response Surface Methodology (RSM) is employed to optimize conditions, including temperature, pH, and substrate concentration.

MATERIALS AND METHODS

Sample collection

EPOB obtained from Sulawesi, Indonesia, was dried in the sun to reduce moisture content to under 10%. The dried EPOB was then ground and screened to achieve a particle size of 40 mesh. The six fungal isolates were obtained from the Indonesian Culture Collection (InaCC), Research Center for Biology, Indonesian Institute of Science, Cibinong, Bogor. They were *Trametes versicolor* InaCC F200, *Aspergillus terreus* InaCC F30, *Pycnoporus cinnabarinus* InaCC F780, *Trichoderma viride* InaCC F241, *Trichoderma harzianum* InaCC F87, *Aspergillus niger* InaCC F539. Potato Dextrose Agar (PDA) was used as a growth medium for fungi. The fungal isolates were grown under conditions of 30°C and pH 6 for 7 days.

Fungal pretreatment of EPOB

EPOB 40 mesh, approximately 5 g in weight-that had been sterilized using an autoclave at 121°C for 20 min-added with distilled water in a 1:2 ratio, was placed in an erlenmeyer flask. Fungal isolates, with an incubation time of 7 days, were dissolved in 10 mL of distilled water. The fungal spores were measured using a haemocytometer. Fungal suspension about 1 mL was then aseptically poured onto the EPOB, and then incubated at 28°C for 14 days with a pH of 6 (Sugiwati et al. 2020). The fungal treatment was conducted with variations in temperature (25, 30, and 35°C), pH (4, 5, and 6), and substrate concentration (5, 7, and 9 g) in the pretreatment process (Figure 1).

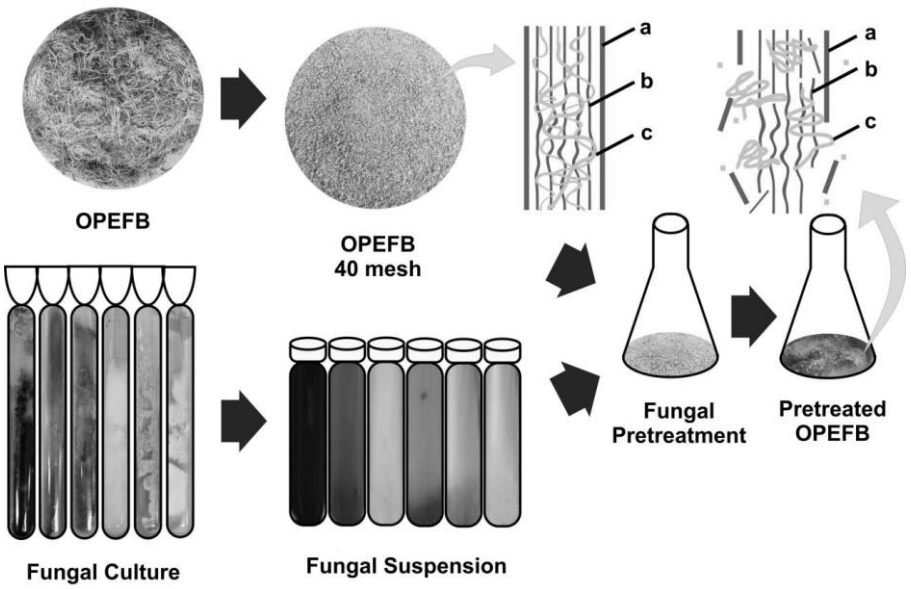

Figure 1. Scheme of fungal treatment using fungal suspension. a: Lignin, b: Cellulose, c: Hemicellulose

Determination of lignin and cellulose content

The analysis was conducted to determine the content of lignin, cellulose, and hemicellulose in EPOB before and after pretreatment. Referring to Sluiter et al. (2008), 0.3 g of 40 mesh TKKS, were added with 3 mL of 72% H_2SO_4, then incubated for 2 hours at 30°C, and homogenized with a vortex regularly. The sample was then diluted to 87 mL, then vacuum autoclaved, and filtration was carried out, using a 0.45 µm cellulose acetate membrane. The filtrate was collected in a test tube and then used to calculate dissolved lignin and sugar content.

Lignin dissolved content was measured by spectrophotometry at a wavelength of 205 nm. Non-dissolved lignin content was calculated with the ash content obtained from the filtrated biomass sediment.

$$\%\ \text{Acid Soluble Lignin (ASL)} = \frac{\left(\frac{\text{UV abs} \times \text{fp}}{110}\right) \times \frac{87}{1000}}{\text{Oven Dry Weight (ODW)}} \times 100\%$$

$$\%\ \text{Acid Insoluble Lignin (AIL)} = \frac{\text{Weight} - \text{ash}}{\text{Oven Dry Weight (ODW)}} \times 100\%$$

$$\%\ \text{Lignin (ASL)} = \%\ \text{ASL} + \%\text{AIL}$$

The neutralized filtrate was filtered using a syringe filter, and then injected into the High Performance Liquid Chromatography-Refractive Index Detector (HPLC-RI) for measurement of cellulose and hemicellulose content:

$$\text{CS} = \frac{\text{HS}}{\text{H}_{\text{std}}} \times \text{C}_{\text{std}}\text{G}$$

$$\%\ \text{Sugar} = \frac{\text{CGS}}{\text{ODW} \times \frac{100}{87}} \times 100\%$$

$$\%\frac{\text{Cellulose}}{\text{Hemicellulose}} = \%\ \text{Sugar} \times \text{AC}$$

CGS is the concentration of glucose or xylose detected by High Performance Liquid Chromatography (HPLC). H_s is the area of the sample detected by HPLC, H_{STD} is the area of the standard detected by HPLC. C_{STDG} is the concentration of glucose and xylose standard, ODW is the oven dry weight, and AC is the anhydro correction, which is 0.9 for glucose, and 0.88 for xylose.

Ligninase activity

Laccase activity

Laccase activity measurement method refers to Kalra et al. (2013), where 1 mL of crude fungal enzyme extract was mixed with 3 mL sodium acetate buffer (pH 5). The mixture was then added with 1 mL of 2 mM guaiacol (2-methoxyphenol), then incubated for 15 minutes at room temperature. After that, the absorbance was measured with a spectrophotometer at 450 nm. At the same time, a blank solution was prepared using 1 mL of distilled water as a sample, which reacted similarly to the enzyme sample. The amount of guaiacol reduction was calculated using the Lambert-Beer equation, with a molar absorbance of guaiacol of 12,100/M/cm.

Manganese Peroxidase (MnP) activity

MnP enzyme activity was determined by measuring the oxidation of guaiacol as a substrate at 465 nm with an extinction coefficient of 465 = 12100 M/cm (Wunch et al. 1997). A total of 0.25 mL of 0.5 M phosphate citrate buffer (pH 5.5), 0.5 mL of 4 mM guaiacol, 0.25 mL of 50 mM $MnSO_4$, 0.25 mL of crude extract of the fungal enzyme, and 0.25 mL of 50 mM H_2O_2 were mixed. The absorbance of the solution was measured at incubation times 0 and 30 minutes. A blank was made without adding $MnSO_4$ and with the addition of distilled water.

Lignin Peroxidase (LiP) activity

The method refers to del Pilar Castillo et al. (1997) with modifications. LiP activity was determined based on the oxidation reaction of veratryl alcohol to veratryl aldehyde. Crude extract of fungal enzyme about 0.2 mL; 0.05 mL of 5 mM H_2O_2; 0.1 mL of 8 mM veratryl alcohol; 0.2 mL of 0.05 M tartrate buffer pH 3, and 0.45 mL of distilled water were put into a cuvette and shaken. The absorbance was read at 310 nm at time intervals of 0 and 30 minutes. The extinction coefficient of veratryl alcohol is 9.300 M/cm.

Measurement of ligninolytic enzyme activity:

$$\text{EA} = \frac{\text{A} \times \text{V}}{\text{t} \times \text{e} \times \text{v}}$$

Where:

EA : Enzyme Activity (U/mL)
A : Absorbance as a spectrophotometry result
V : Total mixture volume in a reaction (mL)
v : Volume of crude extract enzyme in a reaction
t : Incubation time (s)
e : Extinction coefficient

***Cellulase* activity**

Carboxymethylcellulase (CMCase) activity

CMCase activity determination is based on the reducing sugar content produced by the degradation of carboxymethylcellulose (CROMOLINE) at 2% (p/v). Referring to Dos Santos et al. (2012), the reaction assay was performed by mixing 0.5 mL of 50 mM sodium citrate buffer pH 4.8 with 0.5 mL of crude extract of the fungal enzyme, and 0.5 mL of CMC (2% by volume) as a substrate into a test tube. The reaction control used 0.5 mL of the buffer solution and 0.5 mL of the crude extract of the fungal enzyme. Blank was prepared with 0.5 mL of 3,5-Dinitro Salicylic Acid (DNS) and 0.5 mL of buffer. Samples were incubated at 50°C for 10 minutes. The reaction was stopped by adding 0.5 mL of DNS and incubated in boiling water for 5 minutes. A 6.5 mL of distilled water was then added for absorbance measurement at 540 nm.

$$\text{Enzyme activity}\left(\frac{\text{U}}{\text{mL}}\right) = \frac{(\textit{Glucose conc. of sample} - \textit{Glucose conc. of control}) \times \textit{Dilution rate} \times 1000}{\text{Incubation time} \times \text{Glucose MW} \times \text{Vol. of enzyme}}$$

Filter Paper Cellulase (FPAse) activity

FPase activity consists of a mixture of endoglucanase and exoglucanase resulting from the degradation of Whatman No. 1 filter paper, which measures 1.0 cm × 6.0 cm (Ghose

1987). Referring to Adney and Baker (2008), the enzyme assay was carried out by mixing Whatman no.1 filter paper strip (1 × 6 cm) with 1 mL of 0.05 M Na-citrate buffer pH 4.8, and 0.5 diluted crude extract of fungal enzyme (with glucose concentration more than 2 mg and less than 2 mg). The enzyme control solution was prepared by mixing 1 mL of buffer and 0.5 mL of crude extract of fungal enzyme. Substrate control was prepared by mixing 1.5 mL of buffer with 1 strip of filter paper. Blank was prepared using 1.5 mL of buffer. Standard solution was prepared by making glucose stock solution with dilutions (2 mg/mL, 3.3 mg/mL, 5 mg/mL, 6.7 mg/mL). Blank, control, and standard solutions were incubated with the enzyme assay at 50°C for 60 min. The reaction was stopped by adding 3 mL of DNS and then incubated in boiling water for 5 minutes. Distilled water was added for dilution. The absorbance was measured at 540 nm. Calculate Filter Paper Unit (FPU):

$$\text{Filter paper activity} = \frac{0.37}{(\text{Enzyme})\ \text{releasing 2.0 mg glucose}} \frac{\text{units}}{\text{mL}}$$

Analysis of Cellulose Crystallinity Index (CCI)

The powdered sample was prepared for analysis using X-Ray Diffraction (XRD). CCI was calculated from the resulting XRD pattern, with the following equation (Shankar et al. 2022).

$$CCI = \frac{Imax - Imin}{Imax}\ x\ 100\%$$

Where:

I_{max} : Diffraction intensity at 2θ = 22.37° corresponding to crystalline region

I_{min} : Diffraction intensity at 2θ = 9.87° corresponding to amorphous region

Enzymatic hydrolysis

Following the method of Maryana et al. (2022) with a few modifications, 15% EPOB (solid loading) of the total volume of 20 mL was used. A total of 3.00 g of the substrate was placed in Erlenmeyer flask. Citrate buffer solution about 15.68 mL of 0.05 M pH 4.8 was added. Surfactant tween 20 about 0.223 mL was added, followed by the addition of 2 types of enzymes, 0.860 mL cellulase and 0.171 mL β-glucosidase (5:1). The mixture was incubated for 3 × 24 hours using a shaker incubator at 50°C, 100 rpm. Sampling was taken every 12 hours, and the reducing sugar was measured using DNS method.

Measurement of reducing sugar

A sample volume of 0.5 mL hydrolyzed EPOB (every 12 hours sampling) was mixed with 0.5 mL of DNS reagent. The mixture was then incubated in boiling water for 5 minutes. After that, the mixture was cooled at room temperature, and then 1.5 mL distilled water was added. The absorbance was measured spectrophotometrically at 540 nm. A standard curve was prepared by dilution of standard glucose solution (100-800 ppm) (Johnston 2002).

Data analysis

Lignin, lignin removal, and cellulose after fungal pretreatment were processed with RSM using Statistical Package for the Social Sciences (SPSS). Analysis of Variance (ANOVA) to obtain a linear regression function, which was further used for graph visualization with MathCad to obtain an optimum response surface and response contour. XRD patterns were obtained and processed with Origin. The software was used to determine and visualize the optimal conditions of temperature, pH, and substrate in the pretreatment process of EPOB by fungal isolate suspension.

RESULTS AND DISCUSSION

EPOB contains a lignocellulose complex. High lignin content in biomass can inhibit the access of cellulases to cellulose, thus reducing the hydrolysis rate. Various types of fungi have their own ability to degrade lignin. The differences in the enzymes produced lead to different abilities. This study was conducted using 6 types of fungi from the white rot (*Pycnoporus cinnabarinus* InaCC F780 and *Trametes versicolor* InaCC F200) and non-white rot (*Aspergillus terreus* InaCC F30, *Trichoderma viride* InaCC F241, *Trichoderma harzianum* InaCC F87, and *Aspergillus niger* InaCC F539) groups.

Chemical composition of untreated and fungal treated EPOB

Untreated EPOB contained 32.27% cellulose and 6.65% hemicellulose. The lignin content of EPOB was 36.41%. The increase in cellulose content is due to cellulose that is exposed after lignin removal (Shirkavand et al. 2017). The pretreatment with the six types of fungi resulted in varying reductions in lignin content, ranging from 1.02% to 7.58% (Table 1). This variability highlights the diverse abilities of the fungi in lignin removal and their impact on cellulose and hemicellulose content, which has implications for the potential bioethanol yield from EPOB, and can be observed by its morphology (Figure 2).

Ligninase activity

The degradation of lignocellulosic biomass leads to a loss of lignin and an increase in sugars (cellulose and hemicellulose), which is caused by the activity of ligninolytic enzymes. Numerous fungal species generate Lignin-modifying Enzymes (LEs) and have intricate enzyme systems for lignin degradation, such as Laccase (LCC), Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), and Versatile peroxidase (Vp) (Asemoloye et al. 2020; Wang et al. 2020). Rot fungi species have different abilities to degrade these complexes. All six fungi produced laccase, an enzyme known for its role in breaking down lignin (Figure 3). However, the production levels varied, with *P. cinnabarinus* demonstrating the highest activity for MnP among the fungi tested. This suggests that *P. cinnabarinus* is particularly effective in utilizing MnP for lignin degradation. On the other hand, *A. niger* showed the highest activity for LiP, indicating its superior capability in lignin breakdown compared to the other fungi, which showed relatively low LiP activity. This variation in enzyme production highlights the diverse enzymatic profiles of the fungi, underscoring the importance of choosing the right fungal species for effective pretreatment of lignocellulosic biomass.

Table 1. Lignin, cellulose, and hemicellulose content of before and after fungal pretreatment of EPOB

Isolate	Lignin content (%)	Lignin removal (%)	Cellulose (%)	Hemicellulose (%)
Untreated	36.41	-	32.27 ± 0.00	6.65 ± 0.00
InaCC F780 *Pycnoporus cinnabarinus*	34.23 ± 0.5	5.99 ± 2.8	42.03 ± 0.00	15.21 ± 0.00
InaCC F200 *Trametes versicolor*	35.61 ± 1.1	2.2 ± 0.0	42.84 ± 0.00	15.24 ± 0.00
InaCC F30 *Aspergillus terreus*	35.11 ± 0.9	3.58 ± 2.5	44.31 ± 0.00	17.92 ± 0.00
InaCC F241 *Trichoderma viride*	35.65 ± 0.1	2.08 ± 0.3	47.38 ± 0.00	18.72 ± 0.00
InaCC F87 *Trichoderma harzianum*	36.04 ± 0.3	1.02 ± 0.9	43.57 ± 0.00	15.30 ± 0.00
InaCC F539 *Aspergillus niger*	33.65 ± 0.6	7.58 ± 1.5[1]	48.57 ± 0.00	8.81 ± 0.00

Note: [1]Isolate selected for optimization pretreatment

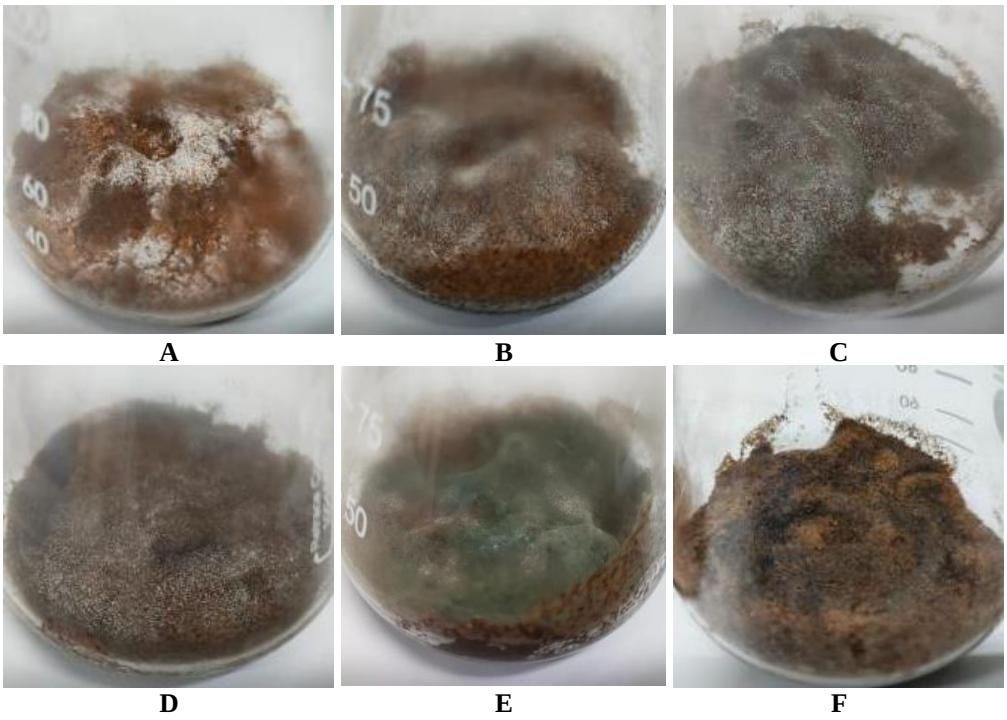

Figure 2. Fungal pretreatment of EPOB for 14 days, on 7 g substrate, at 30°C; A. InaCC F780 *Pycnoporus cinnabarinus*; B. InaCC F200 *Trametes versicolor*; C. InaCC F30 *Aspergillus terreus*; D. InaCC F241 *Trichoderma viride*; E. InaCC F87 *Trichoderma harzianum*; F. InaCC F539 *Aspergillus niger*

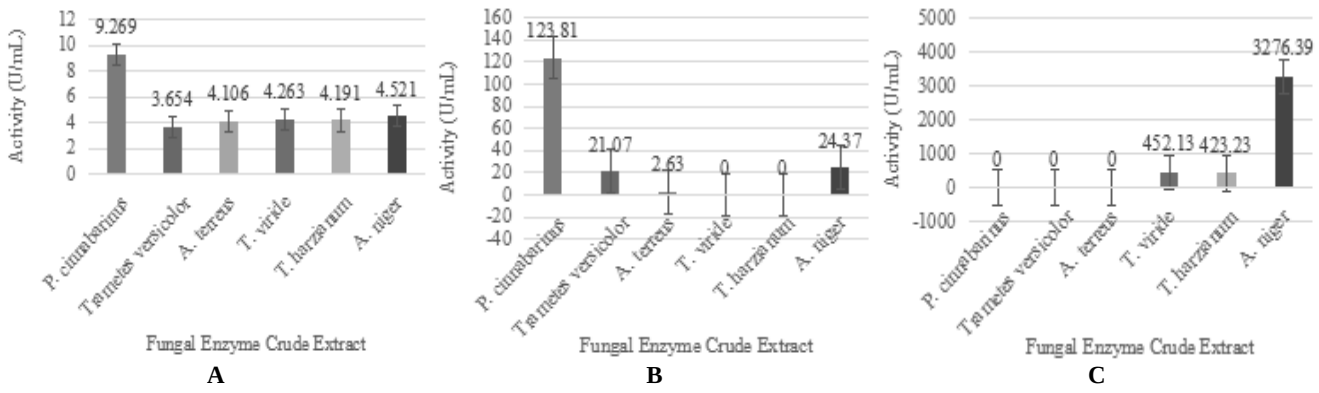

Figure 3. Ligninase enzyme activity: A. Laccase activity; B. MnP activity; C. LiP activity produced by fungal enzyme crude extract

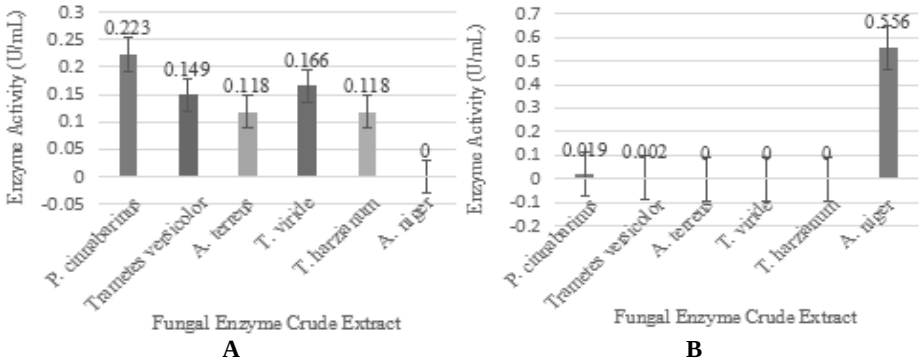

Figure 4. *Cellulase* activity of fungal *enzyme* crude extract: A. CMCase activity; B. FPase activity

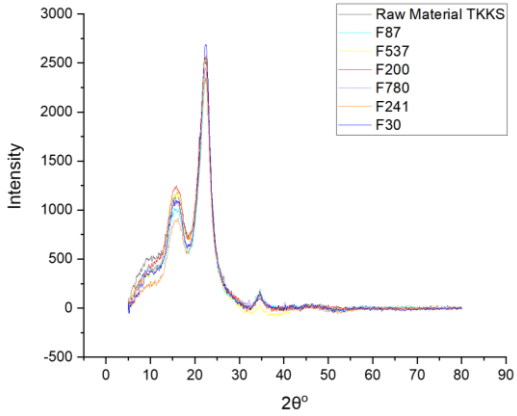

Figure 5. XRD spectra of control and fungal pretreated EPOB

Cellulase activity

One of the challenges in biological pretreatment is that microorganisms also digest cellulose for their growth needs, so the cellulose content that should increase after pretreatment decreases. All fungi, except *A. niger*, produced CMCase, which is crucial for breaking down carboxymethyl cellulose into simpler sugars. However, *A. niger* stands out by producing the highest level of FPase, an enzyme essential for breaking down the more complex cellulose structures found in filter paper. In contrast, the other five fungi exhibited very low FPase activity (Figure 4). This disparity in enzyme production highlights the diverse enzymatic profiles among the fungi, underscoring the specialized roles each fungus plays in cellulase activity and the potential for targeted applications in biomass degradation.

Cellulose Crystallinity Index (CCI)

In the pretreatment process, fungi can act through various mechanisms, such as altering the crystalline structure of cellulose. XRD analysis was utilized to explore the CCI of the untreated sample and the pretreated sample using the six fungi. The CCI of the untreated sample was 49.93%. Among the six fungi, *T. harzianum* managed to decrease crystallinity by 3.84% (the highest). *P. cinnabarinus* and *T. versicolor* were also capable of reducing the CCI of EPOB (Table 2). This study also showed the spectra of untreated and pretreated EPOB (Figure 5).

Table 2. CCI of sample pretreated using six different fungi

Sample	CCI (%)
Untreated	49.93
InaCC F780 *Pycnoporus cinnabarinus*	49.51
InaCC F200 *Trametes versicolor*	48.48
InaCC F30 *Aspergillus terreus*	52.08
InaCC F241 *Trichoderma viride*	56.46
InaCC F87 *Trichoderma harzianum*	48.01
InaCC F539 *Aspergillus niger*	53.90

Fungal pretreatment optimization on lignin removal and cellulose response

One fungal isolate with the highest ability to degrade lignin was selected for further pretreatment optimization. Besides its ability to degrade lignin, *A. niger* can also increase cellulose content (Table 3). *A. niger* is also capable of producing high LiP activity. Optimization of pretreatment conditions was conducted to obtain optimal conditions for the fungi to achieve the highest lignin degradation and an increase in cellulose and hemicellulose content from pretreatment conditions. As a result, 3D plot surface is made of optimization result in lignin removal response (Figure 6) and cellulose response (Figure 7).

The results showed an increase in cellulose of up to 20.71%. A decrease in lignin causes an increase in cellulose content. However, this was not shown in all experiments in this study. The highest cellulose yield (optimal) was obtained in experiments R7 and R13, with optimal conditions of a temperature of 25°C temperature, while pH and the amount of substrate had no significant effect. Experiments R1, R2, R3, R4, R5, R9, and R10 showed an increase in lignin removal followed by increasing cellulose content, which means that the main material to be processed into yield is also being increased. Meanwhile, the decrease in lignin content followed by decreasing cellulose can be caused by fungi, which also digest cellulose for their growth.

Enzymatic hydrolysis

The saccharification process was carried out for 48 hours, with sampling every 12 h, and the optimal results were obtained at the 48th hour with 1.09 g of reducing sugar (Figure 8), which means 36.30% of reducing sugar present in 3 g of EPOB that has been pretreated and hydrolyzed. Based on the mass balance calculation of 7 g of initial EPOB biomass, the resulting reducing sugar amounted to 1.788 g.

Table 3. RSM table of pretreatment conditions using *A. niger* on EPOB towards lignin, lignin removal and cellulose response

Runs	Conditions			Lignin (%)	Lignin removal (%)	Cellulose (%)
	Temp. (°C)	pH	Substrate cons. (g)			
Untreated	30	6	5	36.41	-	32.37 ± 0.00
R1	30	5	7	31.24 ± 0.00	14.21 ± 0.50	51.11 ± 0.00
R2	35	5	7	35.33 ± 0.00	4.72 ± 0.00	46.29 ± 0.00
R3	35	4	9	33.74 ± 0.00	2.96 ± 2.44	46.45 ± 0.00
R4	30	5	9	31.58 ± 0.00	13.26 ± 0.55	52.43 ± 0.00
R5	25	4	5	34.18 ± 0.00	6.12 ± 0.61	51.4 ± 0.00
R6	30	5	7	32.32 ± 0.00	10.16 ± 2.88	45.59 ± 0.00
R7	25	5	7	33.98 ± 0.00	6.68 ± 0.00	52.81 ± 0.00
R8	35	6	9	35.05 ± 0.00	3.73 ± 0.00	48.26 ± 0.00
R9	25	4	9	35.72 ± 0.00	1.90 ± 1.71	48.87 ± 0.00
R10	30	5	7	33.46 ± 0.00	8.10 ± 0.70	51.19 ± 0.00
R11	30	5	7	32.92 ± 0.00	9.58 ± 2.67	47.23 ± 0.00
R12	30	4	7	34.52 ± 0.00	5.20 ± 1.27	48.79 ± 0.00
R13	25	6	9	35.51 ± 0.00	2.46 ± 0.00	53.08 ± 0.00
R14	30	5	7	33.12 ± 0.00	9.03 ± 0.70	50.81 ± 0.00

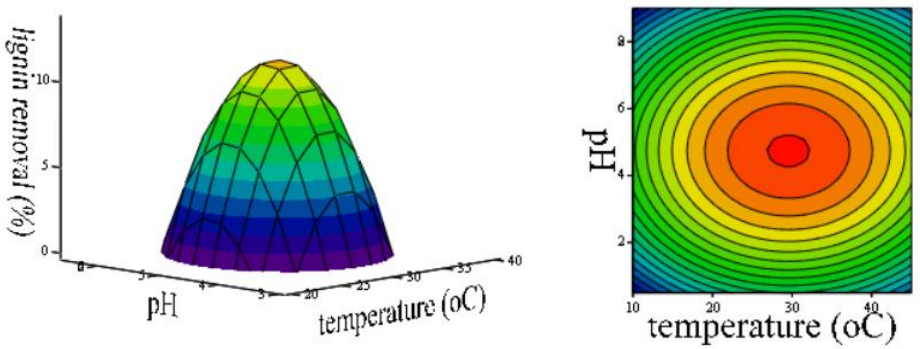

Figure 6. 3D plot of surface response to lignin removal response on 7 g substrate with temperature and pH variation

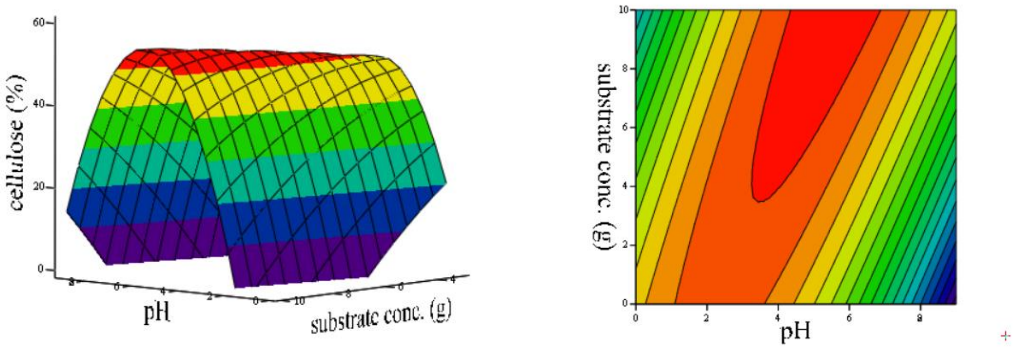

Figure 7. 3D plot of surface response to cellulose response on 25°C with pH and variation of substrate concentration

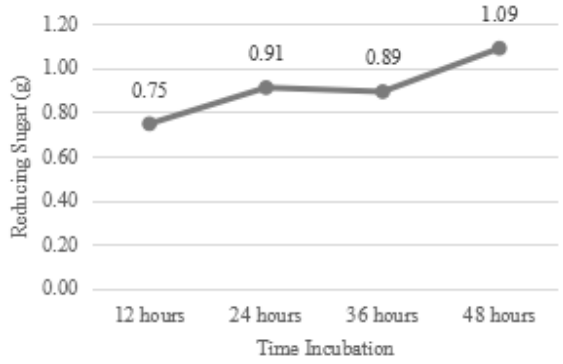

Figure 8. Reducing sugar content of hydrolyzed EPOB by cellulase

Discussion

Fungi, with their remarkable diversity and enzymatic capabilities, were employed in the pretreatment process as a critical preliminary step in enhancing sugar production. The effectiveness of fungal pretreatment of Empty Fruit Bunches (EFB) is highly influenced by factors such as the secretion of ligninolytic enzymes, the moisture content of the EFB, the incubation duration, and the type of fungal species used (Janusz et al. 2017; Suksong et al. 2020). Due to their versatile enzymatic capabilities (Payne et al. 2015), fungi are employed in a range of applications, including the decomposition of EPOB (Tahir et al. 2019; Rasyid et al. 2020; Kusumaningtyas et al. 2022), biodecolorization processes (Nurhayat et al. 2022), and the degradation of petroleum hydrocarbons (Novianty et al. 2021).

The primary benefit of biological such as fungal delignification lies in its safe and eco-friendly nature, as it avoids the use of chemicals, acids, alkalis, or any reactive substances, thereby minimizing waste generation and lowering processing costs downstream, minimal production of inhibitors and byproducts that do not interfere with the hydrolysis process, enhances the specificity of enzyme reactions, preventing substrate loss or toxic byproducts that may result from chemical modifications. Fungal pretreatment can reduce lignin derivatives (such as vanillin, 4-hydroxybenzaldehyde, and salicylic acid) in the pretreated liquor, which in turn helps minimize inhibition of hydrolase activity (Hidayatullah et al. 2020).

As shown in Table 1, six fungi are quite effective for the pretreatment process due to their ability in degrading lignin and increasing cellulose and hemicellulose content. This ability is attributed to the presence of ligninolytic enzymes, which selectively break down lignin without degrading cellulose into its monomers. All six fungi were effective in reducing the lignin content of EPOB and increasing the cellulose and hemicellulose levels. *P. cinnabarinus* as a basidiomycete fungus, a genus of bracket fungi that produces annual fruiting bodies, bright vermilion in color and console-shaped, on decaying wood (Téllez-Téllez et al. 2016). *P. cinnabarinus* have been studied (Mohtashami et al. 2023) for its diverse bioactivities, which are naturally occurring (Schinagl et al. 2023) and shown to have the ability in degrading lignin (Sahuand and Pramanik 2017; Cerutti et al. 2021), depolymerization of lignin (Gil et al. 2023) due to its ligninolytic enzyme. All strains of *P. cinnabarinus* exhibited strong tolerance to temperatures up to 42°C, with optimal growth occurring within the temperature range of 35-37°C (Schinagl et al. 2023). *T. versicolor* was found to remove 8.86% of *lignin* in EPB biomass (Kamcharoen et al. 2014). Safari et al. (2022) investigated the use of *T. viride* in biomass pretreatment for bioethanol production; however, the extent of lignin removal was not quantified in their study. *T. harzianum* has been shown to reduce lignin content in various biomasses, with the extent of reduction varying depending on several factors (Sijinamanoj et al. 2021). This process is mediated by the production of ligninolytic enzymes, as demonstrated by Zhang et al. (2023). Among them, *A. niger* exhibited the highest lignin removal, alongside an increase in cellulose content compared to the raw material. *A. niger* is known as a cellulolytic fungus capable of degrading cellulose, yet until now, no research has focused on its ability to reduce lignin content in lignocellulosic biomass during pretreatment.

Fungal growth in the pretreatment process is characterized by the spread of hyphae on the surface of the substrate in the Erlenmeyer tube. Mycelium began to be visible with the characteristics of each fungus, on the 5th day. The fungal growth is seen in the Figure 2 shows the appropriate environmental factors. Environmental factors greatly affect the growth of fungi. Among other things, the pH of the substrate is crucial. In their original environment, fungi grow at an optimum pH of 5-6. Substrate moisture also affects the growth of fungi. The moisture of the water causes the hyphae to spread on the surface of the substrate. The abundance of water in the surrounding environment can maintain water content in fungal cells and play a role in nutrient transport. The environmental temperature range for fungal growth is 30-40°C and optimal at 20-30°C. Mycelia density and color change in pretreated substrates are good indicators for the estimation of fungal growth. Fungi at the optimal incubation time are at their highest growth, and able to fully adapt to the environment, and efficiently catabolize the carbon source contained (Manan and Webb 2016).

Microbial degradation and conversion of lignin involve a complex enzymatic system comprising various enzymes and their intermediate and final products. These enzymes collaborate synergistically to break down lignin into smaller molecules with different chemical properties. The diverse types of C-C linkages in the phenylpropane units contribute to the resistance of lignin to degradation. Fungi growing on wood or other biomass break down lignocellulosic materials through a coordinated enzymatic system involving both hydrolytic and oxidative enzymes (Chan et al. 2020). Lignin degradation by fungi is closely associated with the activity of specific enzymes, as illustrated in Figure 3. The enzymes laccase and microbial lignin peroxidase such as MnP and LiP, play key roles in this process (Biko et al. 2020). Lignin degradation and enzyme activity are influenced by various factors. As demonstrated in the study by Pazla et al. (2020), supplementing with calcium and minerals enhances the activity of ligninase enzymes and the lignin-degrading capacity of white-rot fungi. This led to the lowest crude fiber content, the highest

ligninase activity, a reduction in lignin levels, and an increase in crude protein content.

In this study, *P. cinnabarinus* can produce high lignin removal due to its high laccase and MnP activities despite lacking LiP activity. Several gene models for laccases and AA2 peroxidases, such as MnP, LiP, and VP, were found in this species, and were used for enzymatic treatment for lignin modification (Marlic-Garajova et al. 2023). *P. cinnabarinus* was identified as a highly efficient fungal strain for producing lignocellulolytic enzymes involved in the delignification process, outperforming other white rot fungi. Additionally, the research showed that solid-state fermentation yielded higher delignification efficiency compared to submerged fermentation (Sahu and Pramanik 2015). The recently identified aryl-alcohol:oxidoreductase from *P. cinnabarinus* was found to share homology with AAO. This enzyme effectively reduced radical intermediates, such as guaiacol and sinapic acid, which are produced by laccase (Mathieu et al. 2016). Pinheiro et al. (2020) found that *T. versicolor* is capable of producing laccase, with production levels varying depending on the microorganism, the culture medium, and the fermentation conditions. High laccase gene expression has been explored in various applications (Ottoni et al. 2016). Their study highlighted the environmental and industrial significance of achieving high laccase production from *T. versicolor* by utilizing vinasse and cotton gin waste as carbon sources. Ranimol et al. (2018) and Zhang et al. (2023) demonstrated that *T. harizanum* could produce laccase. They performed induction treatments on *T. harzianum* to enhance its lignocellulolytic enzyme production. They found that acid-treated substrates effectively induced the production of cellobiohydrolase, whereas substrates treated with alkaline solutions or sodium chlorite specifically induced β-xylosidase. Additionally, the expression of the laccase gene from *A. terreus* has been studied for its potential use in biodegradation of raw oil (Alharbi et al. 2022). In contrast, *A. niger,* which achieves the highest lignin removal, exhibits relatively low laccase and MnP activities but compensates with very high LiP activity. Different types of fungi contribute to lignin and cellulose degradation in distinct ways. Some white rot fungi degrade lignin and cellulose simultaneously by producing extracellular oxidative enzymes and hydrolytic enzymes. White-rot fungi possess versatile extracellular enzymes that generate H_2O_2, which are crucial in the lignin degradation process carried out by class II peroxidases, such as Aryl-Alcohol Oxidase (AAO) and Glyoxal Oxidase (GLOX) (Daou et al. 2016). The expression of these H_2O_2-generating enzymes varies depending on the fungal species, the type of wood substrate, and physiological conditions. Different H_2O_2-generating enzymes are involved at various stages of fungal decay (Presley et al. 2018). Brown rot fungi can degrade polysaccharides (cellulose and hemicellulose), as well as modify lignin. This fungi produce a limited array of enzymes for lignocellulose depolymerization. Notably, they lack certain cellulose-degrading enzymes, such as cellobiohydrolases (CBHs), and do not possess lignin-modifying heme peroxidases. This leads to a significant loss of wood strength due to the depolymerization of cellulose and hemicellulose, while lignin undergoes only minor modifications. As a result of this type of decay, the biomass shrinks and shows a brown discoloration of the biomass due to oxidation, and becomes small, coarse cubical pieces. Soft-rot fungi are capable of secreting cellulases but are unable to degrade lignin, which usually results in an oxidized brownish color on the biomass, like brown-rot fungi.

One of the major obstacles faced by cellulase enzymes is their limited access to the bulk of cellulose buried within the highly organized and tightly packed fibrillar architecture of cellulose microfibrils. Figure 4 shows that the fungi are still able to produce cellulolytic enzymes, while still increasing the cellulose content. *P. cinnabarinus* is a group of white rot fungi that have shown to have the highest CMCase activity. This shows that *P. cinnabarinus* can produce cellulase and digest cellulose in TKKS. *T. versicolor*, *A. terreus*, *T. viride*, and *T. harzianum* produce CMCase activity. *T. versicolor* is a white rot fungus that is reported to be able to secrete endoglucanase (Manavalan et al. 2015), and this is proven by the presence of CMCase activity in *T. versicolor*. *A. niger* is one of the fungi that have been widely studied regarding its cellulase activity, however, it did not show in this research.

The presence of enzyme activity was demonstrated by enzyme extracts from fungi, but they were not able to hydrolyze the cellulose in EPOB. Cellulose serves as a natural reinforcing agent, with hemicelluloses and lignin acting as a matrix, providing high mechanical cohesion to maintain its structure. Cellulose is a homopolymer made up of β-D-glucopyranose ($C_6H_{12}O_6$) units, linked together by β-(1-4)-glycosidic bonds. Each unit, containing three equatorial hydroxyl groups and adopting a chair conformation, twists by 180° in relation to its neighboring units. When two β-D-glucopyranose units combine, they form cellobiose ($C_{12}H_{22}O_{11}$), the monomeric unit of cellulose (Suhas et al. 2016). Cellulases are divided into 3 (three) main groups, namely endoglucanase, exoglucanase, and beta-glucosidase. Endoglucanases catalyze random bonds in cellulose chains by releasing cellobiose or cellooligosaccharides Figure 4 shows that the six fungi have endoglucanase activity, proven by CMCase activity. This shows that *P. cinnabarinus* (white-rot fungi) produced cellulase and digest cellulose well. CMCase is also produced by *T. versicolor* (Manavalan et al. 2015), *A. terreus*, *T. viride*, *T. harzianum* (brown and soft-rot fungi). In contrast, *A. niger* produced the highest FPase activity compared to the other five fungi. This can happen if *A. niger* produces exoglucanase and beta-glucosidase quite high. The cellulase activity of fungi can also be utilized for enzymatic hydrolysis so that in the future, pretreatment not only degrades lignin but also converts cellulose into glucose, which can be directly used in the fermentation process. This utilization will save the cost of enzyme consumption. However, further research is needed to determine the best conditions for this process.

Exoglucanase can disrupt the crystal structure of cellulose and convert long molecular chains into soluble short molecular fragments. Since the crystalline form of cellulose is very difficult to disrupt, exoglucanase activity is the decisive factor for hydrolyzing cellulose (Chen 2014). The slight decrease in the intensity peak after fungal

pretreatment indicates the simultaneous dissolution of lignocellulosic during fungal pretreatment. Reduction of cellulose crystallinity provides higher rates of bioconversion. However, some case studies mention that high digestibility is associated with an increase in crystallinity. This proves that crystallinity is a characteristic that greatly influences digestibility, but not the only one. Other factors are accessible surface area, porosity, lignin and hemicellulose content, and particle size. The ability of fungi to reduce cellulose crystallinity in EPOB is due to the enzyme activity produced. Based on the result, *T. harzianum* was able to reduce crystallinity by 3.84% (the highest). *P. cinnabarinus* and *T. versicolor* were also able to reduce the CCI of EPOB, as shown in Table 2. This indicates that fungal pretreatment not only reduces lignin but also breaks the inter- and intra-hydrogen bonds of cellulose fibrils and destroys the crystalline structure, which is shown in the peak decrease (Figure 5). This will make it more accessible for the substrate to be accessed by enzymes during hydrolysis (Karimi et al. 2016). Meanwhile, *A. terreus, T. viride*, and *A. niger* increased the CCI.

The results (Table 3) showed that the highest lignin degradation during the 14-day incubation was obtained under conditions of 30°C, pH 5 of EPOB, in R1, R4, and R6 with a percentage of lignin degradation of 14.21 ± 0.50%, 13.26 ± 0.55%, and 10.16 ± 2.88%. The decrease in lignin content caused by fungi is due to the ligninase enzyme produced. This ability follows the optimal temperature of 30°C for the growth of *A. niger* (Astoreca et al. 2007), which is also shown in Figure 6, the lower and the higher temperature, the color change proves that the further away from the optimal conditions (red-colored area), which has the highest lignin removal. Optimization data with lignin removal response was processed using SPSS ANOVA to model the effect of temperature (X_1), pH (X_2), and substrate concentration (X_3) variables on lignin removal response (Y_1). Temperature, pH, and substrate concentration simultaneously influenced lignin removal ($F<0.05$ and $t<0.05$). The convex response surface indicates a well-defined optimal variable (Nyakuma et al. 2020). Each addition of levels/concentration results in higher lignin removal, until it reaches the optimum and will decrease with the addition of levels afterward. The lower quantity of substrate in the flask has great advantages in efficient gas exchange and heat transfer. Thus, providing a greater supply of oxygen for mold growth, and easier removal of carbon dioxide and the heat generated (Manan and Webb 2016).

The results of optimization with RSM produced cellulose response data which then modeled the effect of temperature (X_1), pH (X_2), and substrate concentration (X_3) variables on cellulose response (Y_2). The results of SPSS ANOVA analysis showed that there was a significant effect ($F<0.05$) of temperature, pH, substrate concentration on cellulose. These variables also simultaneously affect the response ($t<0.05$). Figure 7 shows the results of optimum conditions in increasing cellulose content obtained at 25°C, which is represented by the red-colored area. Sulyman et al. (2020) stated that *A. niger* produced the highest cellulase at a temperature of 40°C, which means at 25°C, the increase in cellulose occurs because there is no cellulose digestion process, leading to the high levels detected. Meanwhile, pH variation and substrate concentration did not significantly affect the cellulose content. The curve shows that pretreatment at 25°C exposed cellulose optimally at a pH around 5, and 7 g of EPOB. The convex response surface indicates a well-defined optimal variable (Nyakuma et al. 2020). Each addition of levels/concentrations produces higher cellulose levels, until it reaches the the optimum and will decrease with the addition of levels afterward.

Saccharification was monitored over 48 hours with samples taken every 12 hours. The best results were achieved at 48 hours, producing 1.09 g of reducing sugar, as shown in Figure 8, which means 36.30% of reducing sugar present in 3 g of EPOB that has been pretreated and hydrolyzed. Maryana et al. (2022) mentioned that enzymatic hydrolysis with lignocellulosic substrates is optimum with cellulase (Ctec 2 Sigma Aldrich (Novozyme)) and β-glucosidase (Htec 2 Sigma Aldrich (Novozyme)) enzyme concentrations of 20 FPU/g. Cellulose hydrolysis by cellulase enzymes is optimum at a temperature of 45-50°C and a pH of 4.8. The correct combination of enzymes, temperature, and pH significantly impacts the sugar yield. Sample R4 produced high cellulose of 52.43 ± 0.00% and lignin removal of 13.26 ± 0.00%. Therefore, this sample was selected for enzymatic hydrolysis. The high cellulose content will be converted to glucose with high levels as well. Cellulose is a polymer composed of thousands of D-glucose units with β-(1-4)-glycosidic bonds. Hemicellulose is a highly branched heteropolymer containing sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose, L-fucose), pentoses (D-xylose, L-arabinose), and uronic acid (D-glucuronic acid) with various bond structures, namely α- or β-(1-2, 1-3, 1-4, 1-6). enzymatic hydrolysis uses enzymes from the cellulase and hemicellulase groups that can degrade polysaccharides (cellulose and hemicellulose) into fermentation-ready sugars. The specific structure of cellulose favors the arrangement of polymer chains into a tight, crystalline structure that is insoluble in water and resistant to depolymerization, while hemicellulose is more easily hydrolyzed due to its amorphous structure. Although cellulose is a homopolymer, its degradation requires at least three enzymatic activities: endoglucanases randomly hydrolyze the internal β-(1-4)-glycosidic bonds in cellulose chains, cellobiohydrolases cleave cellobiose units from the chain ends and β-glucosidases convert cellobiose to glucose. These enzymes work synergistically to degrade cellulose by creating new accessible access sites and reducing product inhibition. The optimal conditions for most cellulases are a temperature of 50°C (Ezeilo et al. 2020) and pH values ranging from 4-5.

In conclusion, the pretreatment of lignocellulosic biomass like EPOB using six fungi-*P. cinnabarinus, T. versicolor* (white rot), *A. terreus*, *T. viride*, *T. harzianum*, and *A. niger* (brown/soft rot)-proved effective for enhancing sugar production by degrading lignin and increasing cellulose/ hemicellulose content. *A. niger* InaCC F539 showed the highest lignin removal and cellulose enhancement. Key enzymes like Laccase, LiP, MnP, and cellulases drove lignin degradation and cellulose hydrolysis. Optimal conditions for lignin degradation were 30°C and pH 5,

while cellulose increase peaked at 25°C. This study highlights the potential of fungi, particularly *A. niger*, for biomass pretreatment. Future research should explore scaling up, fungal consortia synergies, genetic engineering for enhanced enzyme activity, and the economic and environmental sustainability of fungal-based methods to advance industrial applications in biofuels and biorefineries.

ACKNOWLEDGEMENTS

The authors would like to thank the *Riset dan Inovasi untuk Indonesia Maju* (RIIM) gelombang VI, National Research and Innovation Agency-LPDP, and the Bioscience Department, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia for supporting this research.

REFERENCES

Adney B, Baker J. 2008. Measurement of cellulase activities. National Renewable Energy Laboratory (NREL). Laboratory Analytical Procedure (LAP). https://www.nrel.gov/docs/gen/fy08/42628.pdf. NREL/TP-510-42628.

Alharbi NK, Alzaban MI, Albarakaty FM, Abd El-Aziz ARM, AlRokban AH, Mahmoud MA. 2022. Transcriptome profiling reveals differential gene expression of laccase genes in *Aspergillus terreus* KC462061 during biodegradation of crude oil. Biology 11 (4): 564. DOI: 10.3390/biology11040564.

Anwar M, Suwanto A, Wahono SK, Prasetyo DJ, Sugiharto SP, Maryana R, Setiyoko A. 2024. Synthesis and characterization of cellulose acetate from fiber waste of sugar palm stem (*Arenga pinnata* sp). AIP Conf Proc 2957: 060024. DOI: 10.1063/5.0183967.

Asemoloye MD, Tosi S, Daccò C, Wang X, Xu S, Marchisio MA, Gao W, Jonathan SG, Pecoraro L. 2020. Hydrocarbon degradation and enzyme activities of *Aspergillus oryzae* and *Mucor irregularis* isolated from Nigerian crude oil-polluted sites. Microorganisms 8 (12): 1912. DOI: 10.3390/microorganisms8121912.

Astoreca A, Magnoli C, Ramirez ML, Combina M, Dalcero A. 2007. Water activity and temperature effects on growth of *Aspergillus niger, A. awamori* and *A. carbonarius* isolated from different substrates in Argentina. Intl J Food Microbiol 119 (3): 314-318. DOI: 10.1016/j.ijfoodmicro.2007.08.027.

Behera SS, Ray RC. 2016. Solid-state fermentation for production of microbial cellulases: Recent advances and improvement strategies. Intl J Biol Macromol 86: 656-669. DOI: 10.1016/j.ijbiomac.2015.10.090.

Biko ODV, Viljoen-Bloom M, van Zyl WH. 2020. Microbial lignin peroxidases: Applications, production challenges and future perspectives. Enzyme Microb Technol 141: 109669. DOI: 10.1016/j.enzmictec.2020.109669.

Bolong N, Saad I, Makinda J, Yaser AZ, Abdullah MH, Ismail AF. 2016. Influence of Oil Palm Empty Fruit Bunch (OPEFB) agro-waste properties as filtration medium to improve urban stormwater. J Teknologi 78 (8): 39-46. DOI: 10.11113/jt.v78.5357.

Cerutti G, Gugole E, Montemiglio LC et al. 2021. Crystal structure and functional characterization of an oligosaccharide dehydrogenase from *Pycnoporus cinnabarinus* provides insights into fungal breakdown of lignocellulose. Biotechnol Biofuels 14 (1): 161. DOI: 10.1186/s13068-021-02003-y.

Chan JC, Paice M, Zhang X. 2020. Enzymatic oxidation of lignin: Challenges and barriers toward practical applications. Chem Cat Chem 12 (2): 401-425. DOI: 10.1002/cctc.201901480.

Chen H. 2014. Chemical composition and structure of natural lignocellulose. In: Biotechnology of Lignocellulose. Springer, Dordrecht. DOI: 10.1007/978-94-007-6898-7_2.

Cortes-Tolalpa L, Salles JF, van Elsas JD. 2017. Bacterial synergism in lignocellulose biomass degradation-complementary roles of degraders as influenced by complexity of the carbon source. Front Microbiol 8: 1628. DOI: 10.3389/fmicb.2017.01628.

Daou M, Piumi F, Cullen D, Record E, Faulds CB. 2016. Heterologous production and characterization of two glyoxal oxidases from *Pycnoporus cinnabarinus*. Appl Environ Microbiol 82 (16): 4867-4875. DOI: 10.1128/aem.00304-16.

del Pilar Castillo M, Ander P, Stenstrom J. 1997. Lignin and manganese peroxidase activity in extracts from straw solid substrate fermentations. Biotechnol Tech 11: 701-706. DOI: 10.1023/A:1018423829918.

Dos Santos TC, Gomes DPP, Bonomo RCF, Franco M. 2012. Optimisation of solid state fermentation of potato peel for the production of cellulolytic enzymes. Food Chem 133 (4): 1299-1304. DOI: 10.1016/j.foodchem.2011.11.115.

Ezeilo UR, Wahab RA, Mahat NA. 2020. Optimization studies on cellulase and xylanase production by *Rhizopus oryzae* UC2 using raw oil palm frond leaves as substrate under solid state fermentation. Renew Energy 156: 1301-1312. DOI: 10.1016/j.renene.2019.11.149.

Fasiku SA, Wakil SM. 2022. Screening of factors responsible for conversion of maize straw into bioethanol. J Microbiol Biotechnol Food Sci 12 (2): e5901. DOI: 10.55251/jmbfs.5901.

Ghose TK. 1987. Measurement of cellulase activities. Pure Appl Chem 59 (2): 257-268. DOI: 10.1351/pac198759020257.

Gil RG, Brambila OMG, Bedrán HV, Martínez JCG, Luna JAC, Brambila MMG. 2023. Depolymerization of lignin by extracellular activity of *Pycnoporus cinnabarinus*, to obtain cellulose. Intl J Chem React Eng 21 (4): 445-460. DOI: 10.1515/ijcre-2022-0037.

Hidayati S, Subeki S, Harahap MPM, Hadi S. 2023. Utilization of lignin monomer isolation from Black liquor of empty palm oil bunches as a prebiotic. Drvna Industrija 74: 53-60. DOI: 10.5552/drvind.2023.0015.

Hidayatullah IM, Setiadi T, Kresnowati MTAP, Boopathy R. 2020. Xylanase inhibition by the derivatives of lignocellulosic material. Bioresour Technol 300: 122740. DOI: 10.1016/j.biortech.2020.122740.

Janusz G, Pawlik A, Sulej J, Świderska-Burek U, Jarosz-Wilkołazka A, Paszczyński A. 2017. Lignin degradation: Microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol Rev 41 (6): 941-962. DOI: 10.1093/femsre/fux049.

Johnston D. 2002. Methodologies for assaying the hydrolysis of cellulose by cellulases. In: Handbook of Food Enzymology. CRC Press, Florida.

Kalra K, Chaucan R, Shavez M, Sachdeva S. 2013. Isolation of laccase producing *Trichoderma* spp. and effect of pH and temperature on its activity. Intl J ChemTech Res 5 (5): 2229-2235.

Kamcharoen A, Champreda V, Eurwilaichitr L, Boonsawang P. 2014. Screening and optimization of parameters affecting fungal pretreatment of oil palm Empty Fruit Bunch (EFB) by experimental design. Intl J Energy Environ Eng 5: 303-312. DOI: 10.1007/s40095-014-0136-y.

Karimi M, Habibi-Rezaei M, Rezaei K, Moosavi-Movahedi AA, Kokini J. 2016. Immobilization of inulinase from *Aspergillus niger* on octadecyl substituted nanoporous silica: Inulin hydrolysis in a continuous mode operation. Biocatal Agric Biotechnol 7: 174-180. DOI: 10.1016/j.bcab.2016.06.001.

Kusumaningtyas P, Candra KP, Sahid A. 2022. Changes in microbial community structure and diversity during decomposition of oil palm empty fruit bunches at different decomposition sites in humid tropical oil palm plantation. Agric Nat Resour 56 (3): 511-524. DOI: 10.34044/j.anres.2022.56.3.08.

Manan MA, Webb C. 2016. Extracted substrate colour as an indicator of fungal growth in solid state fermentation. Malays J Microbiol 12 (6): 445-449. DOI: 10.21161/mjm.s00716.

Manavalan T, Manavalan A, Heese K. 2015. Characterization of lignocellulolytic enzymes from white-rot fungi. Curr Microbiol 70 (4): 485-498. DOI: 10.1007/s00284-014-0743-0.

Maryana R, Bardant TB, Santi EL, Triwahyuni E, Muryanto, Oktaviani, Dahnum D. 2022. Bioethanol production process with SHF method from EFBs pulp and its microcrystalline. IOP Conf Ser: Earth Environ Sci 1108: 012021. DOI: 10.1088/1755-1315/1108/1/012021.

Mathieu Y, Piumi F, Valli R, Aramburu JC, Ferreira P, Faulds CB, Record E. 2016. Activities of secreted aryl alcohol quinone oxidoreductases from *Pycnoporus cinnabarinus* provide insights into fungal degradation of plant biomass. Appl Environ Microb 82 (8): 2411-2423. DOI: 10.1128/AEM.03761-15.

Mohtashami M, Yazdi MET, Gholampour-Faroji N, Ghasemi A, Haddad-Mashadrizeh A. 2023. Thermostability and functionality evaluation of laccases from *Pleurotus ostreatus* and *Pycnoporus cinnabarinus*: An In-silico assay. Biochemical properties of laccases from fungi in silico method. Iran J Pharm Sci 19 (2): 88-98. DOI: 10.22037/ijps.v19i2.42246.

Mustafa AM, Poulsen TG, Xia Y, Sheng K. 2017. Combinations of fungal and milling pretreatments for enhancing rice straw biogas production

during solid-state anaerobic digestion. Bioresour Technol 224: 174-182. DOI: 10.1016/j.biortech.2016.11.028.

Novianty R, Saryono, Awaluddin A, Pratiwi NW, Hidayah A, Juliantari E. 2021. The diversity of fungi consortium isolated from polluted soil for degrading petroleum hydrocarbon. Biodiversitas 22 (11): 5077-5084. DOI: 10.13057/biodiv/d221145.

Nurhayat OD, Ardiati FC, Ramadhan KP, Anita SH, Okano H, Watanabe T, Yanto DHY. 2022. Bioprospecting three newly isolated white-rot fungi from Berbak-Sembilang National Park, Indonesia for biodecolorization of anthraquinone and azo dyes. Biodiversitas 23 (2): 613-623. DOI: 10.13057/biodiv/d230201.

Nyakuma BB, Wong SL, Faizal HM, Hambali HU, Oladokun O, Abdullah TAT. 2020. Carbon dioxide torrefaction of oil palm empty fruit bunches pellets: Characterisation and optimisation by response surface methodology. Biomass Conv Bioref 12: 5881-5900. DOI: 10.1007/s13399-020-01071-8.

Ottoni C, Simões MF, Fernandes S, Santos CR, Lima N. 2016. High laccase expression by *Trametes versicolor* in a simulated textile effluent with different carbon sources and pHs. Intl J Environ Res Public Health 13 (8): 778. DOI: 10.3390/ijerph13080778.

Payne CM, Knott BC, Mayes HB, Hansson H, Himmel ME, Sandgren M, Ståhlberg J, Beckham GT. 2015. Fungal cellulases. Chem Rev 115 (3): 1308-1448. DOI: 10.1021/cr500351c.

Pazla R, Jamarun N, Agustin F, Zain M, Arief A, Cahyani NO. 2020. Effects of supplementation with phosphorus, calcium and manganese during oil palm frond fermentation by *Phanerochaete chrysosporium* on ligninase enzyme activity. Biodiversitas 21 (5): 1833-1838. DOI: 10.13057/biodiv/d210509.

Pinheiro VE, Michelin M, Vici AC, de Almeida PZ, de Moraes Polizeli MdLT. 2020. *Trametes versicolor* laccase production using agricultural wastes: A comparative study in Erlenmeyer flasks, bioreactor and tray. Bioprocess Biosyst Eng 43 (3): 507-514. DOI: 10.1007/s00449-019-02245-z.

Presley GN, Panisko E, Purvine SO, Schilling JS. 2018. Coupling secretomics with enzyme activities to compare the temporal processes of wood metabolism among white and brown rot fungi. Appl Environ Microbiol 84 (16): e00159-18. DOI: 10.1128/AEM.00159-18.

Ranimol G, Venugopal T, Gopalakrishnan S, Sunkar S. 2018. Production of laccase from *Trichoderma harzianum* and its application in dye decolourisation. Biocatal Agric Biotechnol 16: 400-404. DOI: 10.1016/j.bcab.2018.09.003.

Rasyid B, Ala A, Kuswinanti T, Sapareng S. 2020. Exploring functional fungi on organic matter decomposition of oil palm empty bunches as bio-resource in land remediation. Biodiversitas 21 (9): 4247-4252. DOI: 10.13057/biodiv/d210941.

Rizal NFAA, Ibrahim MF, Zakaria MR, Abd-Aziz S, Yee PL, Hassan MA. 2018. Pre-treatment of oil palm biomass for fermentable sugars production. Molecules 23: 1381. DOI: 10.3390/molecules23061381.

Safari WF, Syafaat MS. 2022. Effect of pretreatment and composition of *Trichoderma viride* and *Zymomonas mobilis* consortium on bioethanol production from leaf litter. Indones J Biotechnol Biodivers 6 (2): 52-60. DOI: 10.47007/ijobb.v6i2.134. [Indonesian]

Sahu S, Pramanik K. 2015. Delignification of cotton gin waste and its optimization by using white rot fungus *Pycnoporus cinnabarinus*. J Environ Biol 36 (3): 661-667.

Sahuand S, Pramanik K. 2017. Evaluating fungal mixed culture for pretreatment of cotton gin waste to bioethanol by enzymatic hydrolysis and fermentation using co-culture. Pol J Environ Stud 26 (3): 1215-1223. DOI: 10.15244/pjoes/64641.

Schinagl CW, Siewert B, Hammerle F, Spes G, Peintner U, Schlierenzauer M, Vrabl P. 2023. Growth, morphology, and formation of cinnabarin in *Pycnoporus cinnabarinus* in relation to different irradiation spectra. Photochem Photobiol Sci 22 (12): 2861-2875. DOI: 10.1007/s43630-023-00493-3.

Shankar A, Saini S, Sharma KK. 2022. Fungal-integrated second-generation lignocellulosic biorefinery: Utilization of agricultural biomass for co-production of lignocellulolytic enzymes, mushroom, fungal polysaccharides, and bioethanol. Biomass Conv Bioref 14: 1117-1131. DOI: 10.1007/s13399-022-02969-1.

Shirkavand E, Baroutian S, Gapes DJ, Young BR. 2017. Pretreatment of radiata pine using two white rot fungal strains *Stereum hirsutum* and *Trametes versicolor*. Energy Convers Manag 142: 13-19. DOI: 10.1016/j.enconman.2017.03.021.

Sijinamanoj V, Muthukumar T, Muthuraja R, Rayappan K, Karmegam N, Saminathan K, Govarthanan M, Kathireswari P. 2021. Ligninolytic valorization of agricultural residues by *Aspergillus nomius* and *Trichoderma harzianum* isolated from gut and comb of *Odontotermes obesus* (Termitidae). Chemosphere 284: 131384. DOI: 10.1016/j.chemosphere.131384.

Simanullang S, Ermawar RA, Nawawi DS, Hermiati E. 2023. Optimization of oil palm empty fruit bunch pretreatment with microwave heating and aluminum salt catalysts for enhancing its enzymatic hydrolysis. Biomass Conv Bioref 14: 19239-19255. DOI: 10.1007/s13399-023-04169-x.

Sjulander, N, Kikas T. 2022 Two-step pretreatment of lignocellulosic biomass for high-sugar recovery from the structural plant polymers cellulose and hemicellulose. Energies 15 (23): 8898. DOI: 10.3390/en15238898.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. 2008. Determination of structural carbohydrate and lignin in biomass. National Renewable Energy Laboratory (NREL): Laboratory Analytical Procedure (LAP).

Solihat NN, Sari FP, Risanto L, Anita SH, Fitria F, Fatriasari W, Hermiati E. 2017. Disruption of oil palm empty fruit bunches by microwave-assisted oxalic acid pretreatment. J Math Fund Sci 49 (3): 244-257. DOI: 10.5614/j.math.fund.sci.2017.49.3.3.

Sugiwati S, Hanafi M, Lioe H, Suhartono M. 2020. Effect of growth conditions on β-glucosidase production by local isolate of *Aspergillus niger* using rice bran substrate. Biodiversitas 21 (9): 4058-4066. DOI: 10.13057/biodiv/d210917.

Suhas, Gupta VK, Carrott PJM, Singh R, Chaudhary M, Kushwaha S. 2016. Cellulose: A review as natural, modified and activated carbon adsorbent. Bioresour Technol 216: 1066-1076. DOI: 10.1016/j.biortech.2016.05.106.

Suksong W, Wongfaed N, Sangsri B, Kongjan P, Prasertsan P, Podmirseg SM, Insam H, O-Thong S. 2020. Enhanced solid-state biomethanisation of oil palm empty fruit bunches following fungal pretreatment. Ind Crops Prod 145: 112099. DOI: 10.1016/j.indcrop.2020.112099.

Sulyman AO, Igunnu A, Malomo SO. 2020. Isolation, purification and characterization of cellulase produced by *Aspergillus niger* cultured on *Arachis hypogaea* shells. Heliyon 6 (12): e05668. DOI: 10.1016/j.heliyon.2020.e05668.

Tahir AA, Barnoh NFM, Yusof N, Said NNM, Utsumi M, Yen AM, Hashim H, Noor MJMM, Akhir FNM, Mohamad SE, Sugiura N, Othman N, Zakaria Z, Hara H. 2019. Microbial diversity in decaying Oil Palm Empty Fruit Bunches (OPEFB) and isolation of lignin-degrading bacteria from a tropical environment. Microbes Environ 34 (2): 161-168. DOI: 10.1264/jsme2.ME18117.

Téllez-Téllez M, Villegas E, Rodríguez A, Acosta-Urdapilleta ML, O'Donovan A, Díaz-Godínez G. 2016. Mycosphere essay 11: Fungi of *Pycnoporus*: Morphological and molecular identifcation, worldwide distribution and biotechnological potential. Mycosphere 7 (10): 1500-1525. DOI: 10.5943/mycosphere/si/3b/3.

Ummalyma SB, Supriya RD, Sindhu R, Binod P, Nair RB, Pandey A, Gnansounou E. 2019. Biological pretreatment of lignocellulosic biomass-Current trends and future perspectives. In: Basile A, Dalena (eds). Second and Third Generation of Feedstocks: The Evolution of Biofuels. Elsevier, Amsterdam. DOI: 10.1016/B978-0-12-815162-4.00007-0.

Wang H, Huang L, Li Y, Ma J, Wang S, Zhang Y, Ge X, Wang N, Lu F, Liu Y. 2020. Characterization and application of a novel laccase derived from *Bacillus amyloliquefaciens*. Intl J Biol Macromol 150: 982-990. DOI: 10.1016/j.ijbiomac.2019.11.117.

Weng C, Peng X, Han Y. 2021. Depolymerization and conversion of lignin to value-added bioproducts by microbial and enzymatic catalysis. Biotechnol Biofuels 14 (1): 84. DOI: 10.1186/s13068-021-01934-w.

Wunch KG, Feibelman T, Bennett JW. 1997. Screening for fungi capable of removing benzo[a]pyrene in culture. Appl Microbiol Biotechnol 47: 620-624. DOI: 10.1007/s002530050984.

Yadav M, Vivekanand V. 2021. Combined fungal and bacterial pretreatment of wheat and pearl millet straw for biogas production - A study from batch to continous stirred tank reactors. Bioresour Technol 321: 124523. DOI: 10.1016/j.biortech.2020.124523.

Yimlamai B, Choorit W, Chisti Y, Prasertsan P. 2021. Cellulose from oil palm empty fruit bunch fiber and its conversion to carboxymethylcellulose. J Chem Technol Biotechnol 96 (6): 1656-1666. DOI: 10.1002/jctb.6689.

Zhang Y, Wang R, Liu L, Wang E, Yang J, Yuan H. 2023. Distinct lignocellulolytic enzymes produced by *Trichoderma harzianum* in response to different pretreated substrates. Bioresour Technol 378: 128990. DOI: 10.1016/j.biortech.2023.128990.