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STRUCTURAL ALTERATION OF OIL PALM EMPTY FRUIT BUNCH BY PERACETIC ACID COMBINED WITH ULTRASONIC PRETREATMENT FOR XYLANASE PRODUCTION BY *Neosartorya* sp.

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ABSTRACT

The excess of oil palm empty fruit bunches (OPEFB) has caused significant ecological issues. Combined peracetic acid (PAA) and ultrasonication were used for OPEFB pretreatment. PAA solutions of varying strengths and duration of ultrasonication at a frequency of 37 kHz at 60°C were examined for OPEFB pretreatment. At 10% PAA for 60 min, the highest levels of cellulose (52.0 ± 0.2%), hemicellulose (39.5 ± 0.2%), and decreased lignin (11.3 ± 0.4%) were achieved. In the ideal pretreatment condition, the highest glucose levels (28 mg g^{-1}) and xylose (19 mg g^{-1}) was observed. Under a scanning electron microscope (SEM), a significant structural change in OPEFB before and after pretreatment was discovered. FTIR of OPEFB fibre had an absorption peak at 1727 cm^{-1}, which relates to the hemicellulose's C=O stretching. From the XRD studies, both crystalline and semi-crystalline zones may be seen in the diffractogram curves of OPEFB cellulose. Neosartorya *sp. BCC 18065 produced more xylanase (92 ± 0.03 IU g^{-1}) on pretreated OPEFB compared to untreated OPEFB (61 ± 0.01 IU g^{-1}). With more xylanase enzymes present during fermentation, xylose production was enhanced. The results demonstrate that structural changes in OPEFB can be changed by PAA-assisted ultrasonication pretreatment, which also improves enzymatic hydrolysis and saccharification.*

Keywords: characterisation, OPEFB, peracetic acid, ultrasonication, xylanase.

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INTRODUCTION

Up to 85% (or around 49.56 million tonnes) of the world's palm oil was produced in Indonesia and Malaysia in 2016, making them the two biggest producers in the world (Nurika *et al*., 2023). The oil palm empty fruit bunch (OPEFB) which is frequently discarded and incinerated at the palm oil mill, can be used as fertiliser at the plantation. Therefore, the OPEFB must be converted to protect the environment (reduce greenhouse gas emissions) and secure the future of the oil palm industry. The country would certainly benefit from the conversion of this waste into useful products. More and more people are becoming interested in regulating OPEFB, especially in industrial conversion. By utilising all fractions derived from the OPEFB as the raw material, it is expected that OPEFB valorisation to be maximised and thus, reduces the residues in the process of producing fuels and various chemical products of commercial interest (Rame, 2018; Tahir *et al*., 2022).

The majority of palm solid residues are lignocellulosic components, which have different compositions of lignin, cellulose and hemicellulose (Farhan *et al*., 2021). Currently, lignocellulosic substrates promote alternative feedstocks for the synthesis of bioethanol (Selvakumar *et al*., 2023). Typically, a substrate with a good proportion

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of cellulose and hemicellulose is utilised for the enzymatic synthesis of ethanol. OPEFB is suggested as an alternative feedstock for ethanol production, which indicates that the majority of its primary components are fermentable sugars, including xylose and glucose (Mardawati *et al*., 2022).

Cellulose is generally present as a linear polymer of glucose and hemicelluloses, which are branched copolymers of many sugars (*i.e.*, uronic acid, arabinose, mannose, galactose, and xylose), and lignin being the aromatic heterogeneous polymer of three monolignans units (*p*-coumaryl, sinapyl, and coniferyl) (Zhu and Pan, 2022). It is possible to hydrolyse cellulose and hemicelluloses to produce sugar. Due to the recalcitrant character of lignocellulosic biomass, an effective pretreatment step is required to maximise the yield of fermentable sugar and the intensity of enzymatic hydrolysis. Pretreatment is consequently required to delignify and promote the breakdown of the lignocellulosic moiety (Tran *et al*., 2019). Pretreatment involves modifying the biomass so that hemicelluloses and cellulose may be hydrolysed (enzymatically) more quickly, with higher sugar yields. Certain pretreatment conditions are required to eliminate lignin through sugar retention.

There are many pretreatment procedures, involving physical, chemical, and biological treatments which have been used to change the structural and chemical structure of the lignocellulosic substrate and to increase the yield of fermentable sugars (Kucharska *et al*., 2018). The steam pretreatment which employs a high-temperature-pressure reactor, is a viable pretreatment strategy to improve the enzymatic saccharification of OPEFB. Small-scale batch operating equipment, such as laboratory flasks, were mainly used in the research on the auto-hydrolysis process of biomass for the generation of bioethanol (Sari *et al*., 2021). The oxidative pretreatments involve various mechanisms, depending on the situation. The options for pretreatment include the utilisation of ozone or oxygen, moist oxidation, and the application of oxidising solvents like hydrogen peroxide (H_2O_2) and peracetic acid (PAA) (Bussemaker and Zhang, 2013). The hydroxonium ions produced by strong oxidising agents like PAA target the electron sites of lignin, and thus have been described as selective and effective as delignification agents (Jia *et al*., 2015). In the presence of a sulfuric acid catalyst, PAA can be made by combining H_2O_2 with acetic acid. By removing as little lignin as possible, this PAA pretreatment process effectively de-lignifies biomass, thereby improving the probability that the cellulose fraction will be converted (Gill *et al*., 2021).

The ultrasonic solution treatment is an innovative emerging technique which has the potential to replace pretreatment technology. By dividing the intermolecular connection, sonication uses ultrasonic radiation to agitate the particles and facilitate the disintegration of molecules. There is a chance to successfully replace traditional hydrolysis techniques with ultrasonication (Wong *et al*., 2009). However, lignocellulosic biomass sonication depends on several parameters, including ultrasonic intensity, frequency, power, medium used, temperature, time and solid loading (Luo *et al*., 2014). Aqueous mediums are subjected to ultrasonic treatment to cause cavitation, which generates conditions of high temperature, pressure, and powerful shear forces. Lignin is released after the breaking of lignin-carbohydrate linkages that occur as a result of ultrasound exposure (Subhedar and Gogate, 2014). Studies by Subhedar *et al.* (2015) and Olughu *et al.* (2021), demonstrated the impact of ultrasonication on the destruction of lignocellulosic substrates, however, there is not much current information existing on the effectiveness of PAA-assisted ultrasonication on OPEFB. Due to this, we predicted that using PAA and ultrasonication together, it might significantly improve the enzymatic saccharification of OPEFB fibre residues.

The main enzymes which are responsible for destroying the xylosidic linkages in the biomass xylan structure are known as xylanases. Microbial xylanases exhibit a range of substrate specificities and biochemical characteristics, thus making them appropriate for use in a variety of biotechnological and industrial applications (Bhardwaj *et al*., 2019). *Neosartorya* spp. is a member of the *Aspergillaceae* family and is known as an ascomycete. They have special heat-resistance features that enable them to survive in extreme temperatures. *Neosartorya glabra* is used for the production of exo-polygalacturonase (Desagiacomo *et al*., 2021). The genus *Neosartorya fischeri* produces β-Glucosidase enzyme, which has a broad substrate specificity and shows glucanase, cellobiase, xylanase, and glucosidase activities (Yang *et al*., 2014).

Utilising inexpensive substrate is beneficial economically from an industrial perspective for synthesising enzymes. The ability of PAA-aided ultrasonication to pretreat the OPEFB and increase enzyme production was the primary focus of the current study. The effects of pretreatment-induced structural and compositional changes to OPEFB on solid substrate fermentation (SSF) production of value-added bioproducts including xylanase and sugars were investigated. This is the first study to report the use of the fungus *Neosartorya* sp. BCC 18065 to produce xylanase and sugar on pretreated OPEFB.

MATERIALS AND METHODS

OPEFB Substrate

OPEFB was collected from the palm oil mill industry (Trang Province, Thailand). The collected OPEFB was then continually cleaned with purified water to eliminate any debris, dust, or other unwanted contaminants, and then dried in an oven for 24 hr at 70°C-80°C to get a constant dry weight. The necessary particle size of 1 to 2 mm was achieved by using a hammer mill to crush the dried OPEFB fibre, which was then processed through a sieve shaker (18 mm meshes) before being kept in sealed plastic bags at ambient temperature until used in the experiment. For the proximate analysis, a fresh OPEFB substrate was also created.

Peracetic Acid-assisted Ultrasonication Pretreatment of Biomass

In three separate 250 mL Erlenmeyer flasks, 5 g of dried OPEFB substrate and 100 mL of PAA were combined at three different concentrations (w/v): 5%, 10%, and 15%. After thoroughly mixing the contents, the flasks were submerged in an ultrasonic bath (Germany's Elma E series) and sonicated for various lengths of time: 40, 60, and 80 min at 37 kHz frequency with an ultrasonic power of 200-250W at 60°C. For the control treatment, no sonication is required. The produced slurry residue was then divided into solid and liquid parts, once the experiment finished. The solid filtrate was oven-dried for 24 hr at 60°C-70°C and then kept for future research, following a distilled water rinse. Exactly 20 mL of 1% v/v H_2SO_4 was mixed with 1 g of solid substrate when its pH was around 7.0. After that, they undergo autoclave hydrolysis for 1 hr at 121°C. Whatman No. 1 filter paper was used to filter the hydrolysed samples after the reaction finished. The quantitative and qualitative characteristics of reducing sugars were ascertained by the obtained supernatant.

Assay Methods

The components of the OPEFB substrates (cellulose, hemicelluloses and lignin) were evaluated, and moisture and ash content were also analysed according to the AOAC (1990) methods. The hydrolysate's total soluble sugars (TS) were measured using the simple, rapid phenol-sulfuric acid method (Dubois *et al*., 1956). The 3, 5-dinitro salicylic acid (DNS) method (Miller, 1959) was employed to quantify the concentration of reducing sugars. By using the stated method (Khabarov *et al*., 2006), the amounts of xylose and arabinose were determined.

FTIR Spectrum of OPEFB

The surface functional groups of both untreated and pretreated OPEFB were recognised by Fourier transform infrared (FT-IR) spectroscopy (Perkin Elmer Spectrometer, England). Before the examination, tests were blended in with potassium bromide (KBr), and the resultant combination was squeezed into a pellet. The IR spectrum was gathered with a resolution value set at 4 cm^{-1} across a range of 500-4000 cm^{-1}.

Scanning Electron Microscopy (SEM) of OPEFB

Utilising SEM and imaging, morphological and structural alterations in pretreated and untreated OPEFB were found. First, double-sided carbon tape was used to attach the solid samples to the aluminium sample stabs. After that, a picture was captured using an acceleration voltage of 10 kV.

X-ray Diffraction of OPEFB

An X-ray diffractometer (XRD, PHILIPS, X'Pert MPD) was used with a 40 kV applied voltage and a 30 mA current to observe the complex crystalline structure of OPEFB both before and after pretreatment. With a step size of 0.03° in the range of 2 = 5°-50° and the Equation (1) presented below (Segal *et al*., 1959) as a reference, the crystallinity index (CrI) was calculated from the samples.

$$\mathrm{CrI}\ (\%) = \frac{I_{002} - I_{18.0°}}{I_{002}} \times 100 \qquad (1)$$

where I_{002} denotes the maximum diffraction intensity at the 002 lattice, and $I_{18.0°}$ denotes the maximum diffraction intensity at 18.0°, 2θ°.

Xylanase Producing Fungus Strain and Inoculum Preparation

Neosartorya sp. BCC 18065 was purchased from the National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathum Thani, Thailand. Stock cultures of the organism were preserved on Potato Dextrose Agar (PDA) Slant at 40°C.

A 250 mL clean Erlenmeyer flask was utilised to make a 100 mL broth that included (% w/v): xylan (Birchwood) (0.5), KCl (0.01), Peptone (0.09), $(NH_4)_2HPO_4$ (0.04), and $MgSO_4.7H_2O$ (0.01). The pH was then adjusted to 7.0, and the flask was then sterilised at 121°C for 15 min. To get the mycelial suspension as inoculum, *Neosartorya* sp. BCC 18065 spore suspension was then injected and incubated in a continuous rotating shaker (at 120 rpm) at 33°C-340°C for 72 hr.

Solid Substrate Fermentation (SSF)

Separate SSF tests employing untreated and pretreated OPEFB were conducted. Along with the 5 g of OPEFB substrate, the 500 mL conical flasks contained a basal fermentation medium made up of the following ingredients: (g L^{-1}): $MgSO_4.7H_2O$ 0.6; $(NH_4)_2SO_4$ 1.4; KH_2PO_4 1.6; $CaCl_2.2H_2O$ 0.4; urea 0.2; yeast extract 0.2; proteose peptone 0.25; together with a mineral salt solution with traces of elements [$MnSO_4.7H_2O$, 4.60; $CoCl_2.2H_2O$, 1.50; $ZnSO_4.7H_2O$, 3.34; $FeSO_4.7H_2O$, 3 mg L^{-1} (pH 7.0)]. The fermentation medium's moisture percentage was retained between 70% and 72% using a basal medium. They were autoclaved at 121°C for 15 min, after uniformly mixing the contents of the Erlenmeyer flasks. The flasks were then allowed to reach room temperature. Following this, flasks were inoculated with a 5 mL *Neosartorya* sp. BCC 18065 spore suspension (1×10^8 spores mL^{-1}) and cultured at ambient temperature for 6-7 days under aseptic conditions. To eliminate the fungal biomass, the broth was centrifuged after being collected under the laminar airflow hood chamber during fermentation. The collected supernatant was then analysed for xylanase and estimate xylose sugars.

Assay of Xylanase

The xylanase activity was calculated following Bailey *et al*. (1992). For this assay, 1.8 mL of a 1.0% (w/v) Azo-xylan (birch wood) solution was made in 50 mM sodium citrate buffer (pH 5.3) with 0.2 mL of enzyme dilution (in 50 mM sodium citrate buffer, pH 5.3) and allowed to incubate for 5 min at 50°C. The DNS method which required mixing 3 mL of DNS reagent and incubating the mixture for 5 min at 95°C, was employed to calculate the quantity of released reducing sugars. The end reaction was stopped by conditioning it with cold water. The solution's 540 nm wavelength was then measured with a UV-visible spectrophotometer. The quantity of enzyme that generates 1 mol of xylose equivalent every min is considered one international unit of enzyme activity.

RESULTS AND DISCUSSION

PAA/Ultrasonic Pretreatment of OPEFB

Pretreatment is a vital step in converting lignocellulose into fermentable sugars. The purpose of the pretreatment is to separate the lignin and disrupt the lignocellulose's structure, and in this study, it is typically carried out using PAA and ultrasonication. *Table 1* displays the chemical and proximate composition of untreated OPEFB. The primary constituents of untreated OPEFB are cellulose (32.6 ± 0.4%), hemicelluloses (28.4 ± 0.2%), and lignin (25.0 ± 0.4%). This reveals that cellulose and hemicellulose together make up around 50% of the dry weight of OPEFB. A pretreatment was applied to increase the cellulose and hemicellulose yields from untreated OPEFB biomass.

TABLE 1. CHEMICAL COMPOSITION OF UNTREATED OPEFB

No.	Components	%
1	Moisture	10.0 ± 0.2
2	Ash	1.5 ± 0.3
3	Cellulose	32.6 ± 0.4
4	Hemicellulose	28.4 ± 0.2
5	Lignin	25.0 ± 0.4
	Sugars	**mg g^{-1}**
6	Total sugars	20.2 ± 0.1
7	Reducing sugars	18.4 ± 0.3
8	Glucose	10.8 ± 0.1
9	Xylose	7.4 ± 0.2
10	Arabinose	3.6 ± 0.8

Note: Except moisture in OPEFB, all the remaining composition of substrate was analysed based on dry weight basis (%). Values are mean of two replicates.

The PAA concentrations of around 5%, 10%, and 15% (w/v) were examined against three different ultrasonication durations (40, 60, and 80 min) at 60°C to establish the most efficient ratio of PAA concentration and ultrasonication duration for OPEFB biomass hydrolysis. The composition of the pretreated OPEFB was determined under various circumstances and shown in *Figure 1*. It indicates that pretreatment makes lignin soluble, increasing the quantity of cellulose and hemicellulose.

The ideal pretreatment conditions for achieving the highest yield were observed to be 10% PAA with a 60 min ultrasonication period at 60°C. The findings showed that both cellulose (52.0 ± 0.2%) and hemicellulose (39.5 ± 0.2%) were increased along with a decrease in lignin (11.3 ± 0.4%). Delignification is a crucial indicator of an efficient pretreatment process because lignin prevents the enzymatic breakdown of carbohydrates. The proportion of lignin removed during the pretreatment under various circumstances ranged from 5.5%-54.8% (*Figure 1*). This demonstrates that PAA might respond selectively to lignin without significantly degrading cellulose and that it can help separate cellulose from other components. This present result was compared with Palamae *et al*. (2014), who found that acid-insoluble lignin, hemicellulose, and cellulose made up around 16%, 36%, and 47% respectively, in the pretreated

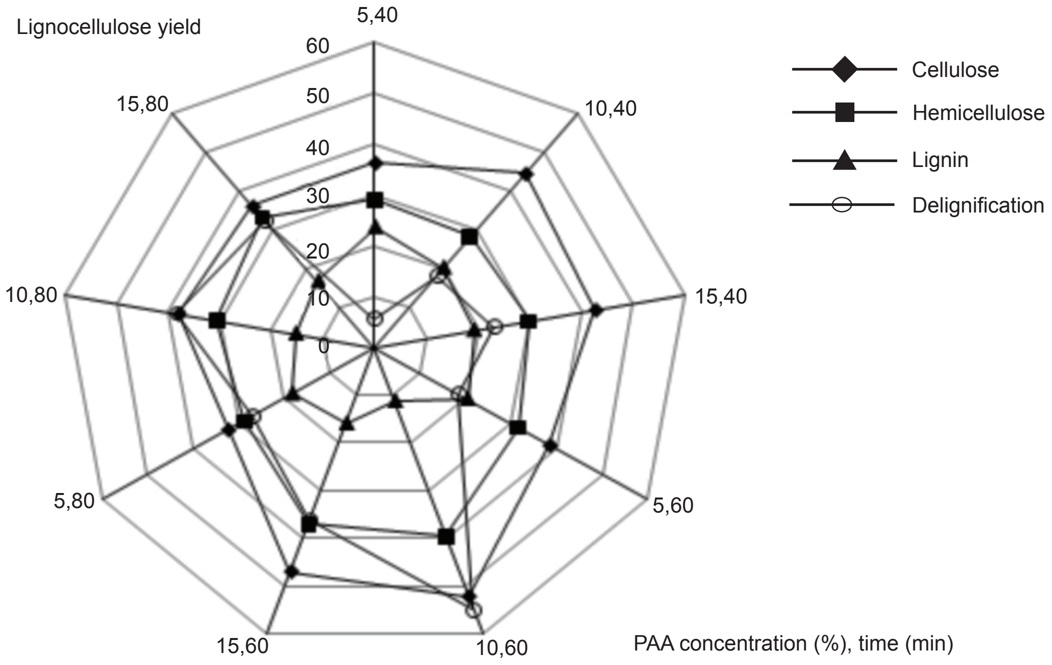

Figure 1. PAA assisted ultrasonication pretreatment of lignocellulose yield.

EFB fibre under optimal circumstances (20 mL PAA per g of OPEFB, 35°C, reaction time for 9 hr). The majority of the hemicellulose was retained, whereas around 53% of the lignin was removed. The extended pretreatment duration (80 min), however, was the cause of the lower cellulose and hemicellulose yields. To successfully delignify and increase the carbohydrate content of OPEFB, a shorter pretreatment period (40 min) with a lower PAA concentration (5%) is insufficient. The sonication time has the biggest impact on the pretreatment that is obtained. As the duration of sonication increases, the amount of delignification and sugar released from the biomass also increases, but the sonication which lasts longer than a specific period of time, has no further advantage.

Switchgrass (*Panicum virgatum* L.) was subjected to ultrasonic irradiation, and Olughu *et al*. (2021) studied the molecular, microstructural, and delignification alterations that occurred. The percentage of delignification, according to their research, varied between 1.86% and 20.11%. The results demonstrated that sonication time, followed by acoustic power, has the greatest influence on ultrasonic delignification. Switchgrass undergoes chemical and structural changes as a result of ultrasonic irradiation. The improvement in delignification was made possible by the hydrodynamic shear forces brought on by sonication. The variation in lignin percentage between pretreated and untreated EFB suggests that lignin was degraded during combined pretreatment. The extent of delignification increases from 16.00% to 44.00% with a change in temperature from 313 K to 373 K, with 353 K being the optimum temperature, above which an increase in temperature has only a minimal impact on delignification, as reported by Subhedar *et al*. (2015) in their study of delignification of waste newspaper using ultrasound. Different types of biomass must have different optimum temperatures; hence, it is critical to investigate how temperature influences EFB for a specific ultrasonic reactor configuration. Moreover, studies show that ultrasonication may shorten pretreatment periods and be beneficial for the extraction of cellulose and hemicellulose from lignocellulosic material. Chemical pretreatment is used to delignify lignocelluloses, and this recovery may be mainly due to the reduction in lignin. Based on the difference in lignin percentage between pretreated and untreated EFB, the lignin was eliminated during combined pretreatment.

Additionally, the pretreatment may produce a higher percentage of glucan and xylan in the pretreated OPEFB. *Figure 2* shows the yields of various sugars during the PAA/ultrasonic pretreatment of the OPEFB substrate. The findings of this investigation suggested that the PAA/ultrasonic pretreatment might significantly increase the yield of fermentable sugar. The pretreated OPEFB had a much higher quantity of sugar than the untreated OPEFB. Research by Patil *et al*. (2019) showed that when compared to traditional methods used for sawdust, the ultrasonic-assisted operations resulted in effective delignification as well as a higher yield of reducing

Figure 2. Effect of PAA assisted ultrasonication on different sugar yields.

sugars in a treatment time of 3 hr at 50°C. Overall, it was found that compared to traditional treatment methods, ultrasonic-assisted delignification and enzymatic hydrolysis produced around 1.5 times more lignin removal and 1.6 times more reducing sugar yield, respectively. The findings of the present experiment suggest that pretreated OPEFB produced more glucose than untreated OPEFB. PAA pretreatment greatly decreased lignification rates in the OPEFB, especially when it was combined with ultrasonication. The pretreatment, however, was completed using 10% PAA and a 60 min ultrasonication time when the temperature was low (60°C).

To avoid losing the reaction mixture due to substantial heating, experiments were not carried out over 60°C. The yield of reducing sugars in the hydrolysis process may be negatively impacted by the degradation of polysaccharides, particularly cellulose, which can occur when temperatures are much higher than normal (Subhedar and Gogate, 2014). This mild pretreatment condition facilitates the delignification process by preserving cellulose and hemicellulose in the final OPEFB substrate and promoting cellulolysis and xylanolysis. Under ideal pretreatment conditions, pretreatment with PAA improves the high-yield recovery of carbohydrate (Glucose) content in the solid phase and reduces carbohydrate loss (Kundu *et al*., 2021). After pretreatment, the hardwood and softwood recovered 90%-95% of their glucan content. The recovery of all sugars except glucan declined as response time increased.

Based on the findings depicted in *Table 1*, the original, untreated OPEFB substrate had low quantities of both total and additional sugars. At 10% PAA and 60 min of ultrasonication, the pretreated OPEFB produced higher concentrations of reducing sugar (42.0 mg g^{-1}), glucose (28.0 mg g^{-1}), xylose (19.0 mg g^{-1}), and arabinose (7.8 mg g^{-1}). The yield of sugar was extremely low at both short retention (40 min) and high retention (80 min) times. The yield of the released sugar, however, decreased as the concentration of acid increased (15%). The formation of furfural, hydroxyl methyl furfural, and acetic acid in more challenging conditions appears to be the reason for the drop in sugar yields. When these by-products were produced during the pretreatment, there may be less cellulose in the solid residue, which would reduce the yield of released sugars. Increasing pretreatment time and temperature would result in a decrease in the output of xylose and arabinose since hemicelluloses are frequently more easily degraded than cellulose. Furfural production was linked to a greater xylose content than glucose in hydrolysates.

Based on these findings, pretreatment duration and PAA concentration significantly influenced sugar yields. Sugar yields decrease as the pretreatment time exceeds the optimal point (80 min). The present study is compared with that of Song *et al*. (2015), who used three levels of ultrasound power (0%, 30%, and 70%) to prepare poplar wood biomass, and they discovered that the ultrasound-assisted 15 min dilute acid pretreatment will increase the sugar production by roughly 10% to 40%. They also reported that the longer periods at relatively low power densities of around 0.5 W mL^{-1} showed minimal impact and increased ultrasonic power, and time during ultrasonic treatment, all which had substantial effects on hydrolysis efficiency.

FTIR Spectrum of OPEFB

OPEFB's structural modification was successfully achieved via PAA/ultrasonic treatment. The hydroxonium ion or H_3O^+, which PAA generates in acidic media attacks the electron sites of lignin and causes electrophilic substitution with ring hydroxylation, oxidative ring-opening, oxidative demethylation, cleavage of β-aryl ether bonds, displacement of side chains, and epoxidation of olefin structure (Zhao *et al*., 2017).

The FTIR spectra of untreated and pretreated OPEFB are shown in *Figure 3*. In both the untreated and pretreated OPEFB, they displayed strong peaks at 3100-3400 and 1000-1100 cm^{-1}. The large absorption band in the spectral region of 3100-3400 cm^{-1} is a result of the particular hydrogen-bonded -OH stretching vibration of cellulose, which appeared in the FTIR spectra of both untreated and pretreated OPEFB (Senusi *et al*., 2020). In addition, the lignin's C-O-C stretching vibrations are represented by the peak at 1000-1100 cm^{-1}. The OPEFB fibre also had an absorption peak at 1727 cm^{-1}, which relates to the hemicellulose's C=O stretching (Ghali *et al*., 2012). These peaks, which were seen in both the pretreated and untreated OPEFB, suggest that hemicellulose was still available after pretreatment. A single absorption peak at 1319 cm^{-1}, which represented the C-H in-plane bending of cellulose (Shi and Li, 2012), was only found in the pretreated OPEFB. The untreated OPEFB missed this peak. It appears that a portion of the lignin was solubilised during the PAA pretreatment procedure and may have been lost. Chemical analysis of the (*Figure 1*) sample clearly shows that lignin has been solubilised, whereas cellulose and hemicellulose have been maintained in the pretreated OPEFB. After the pretreatment, the vibrational peak of about 2900 cm^{-1}, which is connected to the C-H stretching vibration in cellulose and hemicelluloses was reduced, suggesting partial lignin removal and hemicelluloses (Shang *et al*., 2020). The peak at 1645 cm^{-1} in the pretreated OPEFB may be caused by the adsorbed water's bending mode when it interacts with the cellulose fibre (Inamuddin *et al*., 2019).

The pretreated OPEFB sample did, however, show enhanced intensities in the ranges of 3400, 1200 and 1500 cm^{-1}, as well as 600-850 cm^{-1}. These findings, however, suggested that the OPEFB substrate has a variety of absorption bonds that are often observed at 1030, 1239, 1457 and 1727 cm^{-1}. Both untreated and pretreated OPEFB exhibited

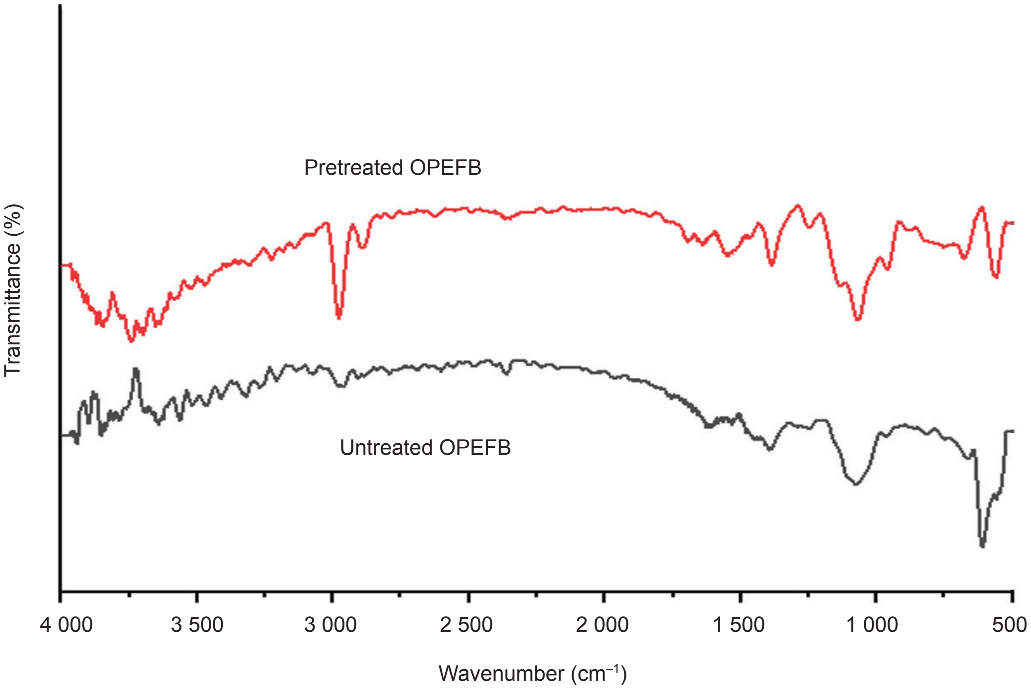

Figure 3. FTIR spectra of untreated and pretreated OPEFB.

hemicellulose assignment at 1727.00 cm^{-1} (C=O conjugates in xylans). However, a well-defined peak with high intensity at 1030.00 cm^{-1} was discovered and gradually disappeared as PAA concentration increased (Pandey and Pitman, 2003). This peak is also attributed to guaiacyl moieties. As the temperature rises, the peak of about 1239.00 cm^{-1} is the first to start decreasing in size, and data indicates that this band has a mixed origin. According to Acquah *et al*. (2016), it is created via syringyl nuclei in lignin and C=O linkages in hemicelluloses. All spectra had the same peak at 1457.00 cm^{-1}, which is connected to the asymmetric bending of the CH_3 and methoxy ($-OCH_3$) groups found in lignin, demonstrating the retention of lignin in the matrix (Labbe *et al*., 2005).

The absorption spectral bands in the 1200.00-800.00 cm^{-1} region may indicate the type of polysaccharide present. According to Labbe *et al*. (2005), typical arabinoxylan peaks have regions of between 1152.00 and 995.00 cm^{-1}, due to the vibrational stretching and bending of C-O, C-C, and C-OH. The degree of branching at the O-2 and/or O-3 sites is strongly correlated with the absorption intensity in this region (Inamuddin *et al*., 2019). Furthermore, all hemicellulosic fractions have a high absorbance at 896.00 cm^{-1}, which is relevant to either the C-1 group frequency or the ring frequency, indicating the occurrence of β-glycosidic connections between xylose units in the hemicelluloses (Revanappa *et al*., 2010). The spectral band identified in the current study was in the region of 898.05 cm^{-1} in both untreated and pretreated OPEFB.

SEM of OPEFB

To further examine the effectiveness of pretreatment on the OPEFB surface, SEM analysis was also performed. SEM images of both untreated and pretreated OPEFB are shown in *Figure 4a* and *4b*. The untreated OPEFB surface had a rough, hard appearance and was covered with silica bodies (*Figure 4a*). The structure is compact and densely arranged, and there are no holes or pores observed on the OPEFB surface. Due to the presence of high levels of lignin and hemicellulose, the structure looks highly compact. Hence, little cellulose and hemicellulose were accessed by enzymes. The most well-known mineral seen on the exterior of woody plants is often silica bodies (silicon dioxide), which are created when soil minerals invade and solidify in sedimentary cavities between and inside the cell wall during plant growth (Yoon and Kim, 2008). As can be observed, the surface shape of the untreated fibre is the main physical barrier preventing enzymes from accessing the surface, cellulose, and hemicellulose hydrolysis for the creation of sugar. Pretreatment is, therefore, necessary to remove these silica solids and destroy the resistant fibre surface in order to permit the entrance of an enzyme. The surface morphology of OPEFB after 60 min of 10% PAA-assisted ultrasonication is displayed in *Figure 4b*. It was found that the pretreatment effectively removed the silica bodies deposited on the outside of the fibre strands by leaving unfilled sedimentary cavities. The degradation of the lignocellulosic structure was increased by pretreatment. The compact structure of the OPEFB was significantly damaged as a result of the removal of the binding polymers. Intra- and intermolecular linkages are present in lignocellulosic biomass components, which contribute to the compact structure of the biomass. SEM images demonstrate the breakdown of these structures, which is consistent with the decomposition of the chemical constituents of the treated OPEFB. The processing caused the lignin covering and fibre surface to become disturbed. Surface disturbance might make cellulose and hemicellulose more accessible to microorganisms and enzymatic processes for future bioconversion. Additionally, it was shown that pretreatment reduced the fiber bundle size, which could expand the area accessible for enzyme entry and improve enzyme accessibility. The creation of holes and cracks along the internal components of the OPEFB suggested that PAA-assisted ultrasonication had been effectively utilised to alter the structure of the OPEFB.

According to Mesa *et al*. (2011), the pretreatment disintegrated the lignin and lignin-carbohydrate connections, leaving behind thick residues that were primarily composed of cellulose and hemicellulose. Another physical barrier preventing enzyme action on both cellulose and hemicelluloses is the coating of lignin on the fibre surface. Delignification is therefore an important stage in the pretreatment process to remove this physical barrier to enzymatic saccharification.

X-ray Diffraction (XRD) of OPEFB

XRD experiments were conducted to ascertain the degree to which raw OPEFB and chemically treated fibers are crystalline. The findings of the XRD examination of untreated and pretreated OPEFB are shown in *Figure 5*. Untreated OPEFB cellulose has a maximum cellulose I content of roughly 28.10%, whereas pretreatment lowers the cellulose I concentration to around 25.23%. However, after 60 min of hydrolysis, it was determined that the fiber crystallinity index had somewhat decreased, possibly as a result of excessive PAA exposure. After 60 min of sulphuric acid hydrolysis, the pretreated OPEFB had a CrI of about 54%, which was lower than the untreated OPEFB data, which had a CrI of 55% (Fahma *et al*., 2010). The CrI values of the regenerated cellulose-rich solids were reduced, making them more amenable to

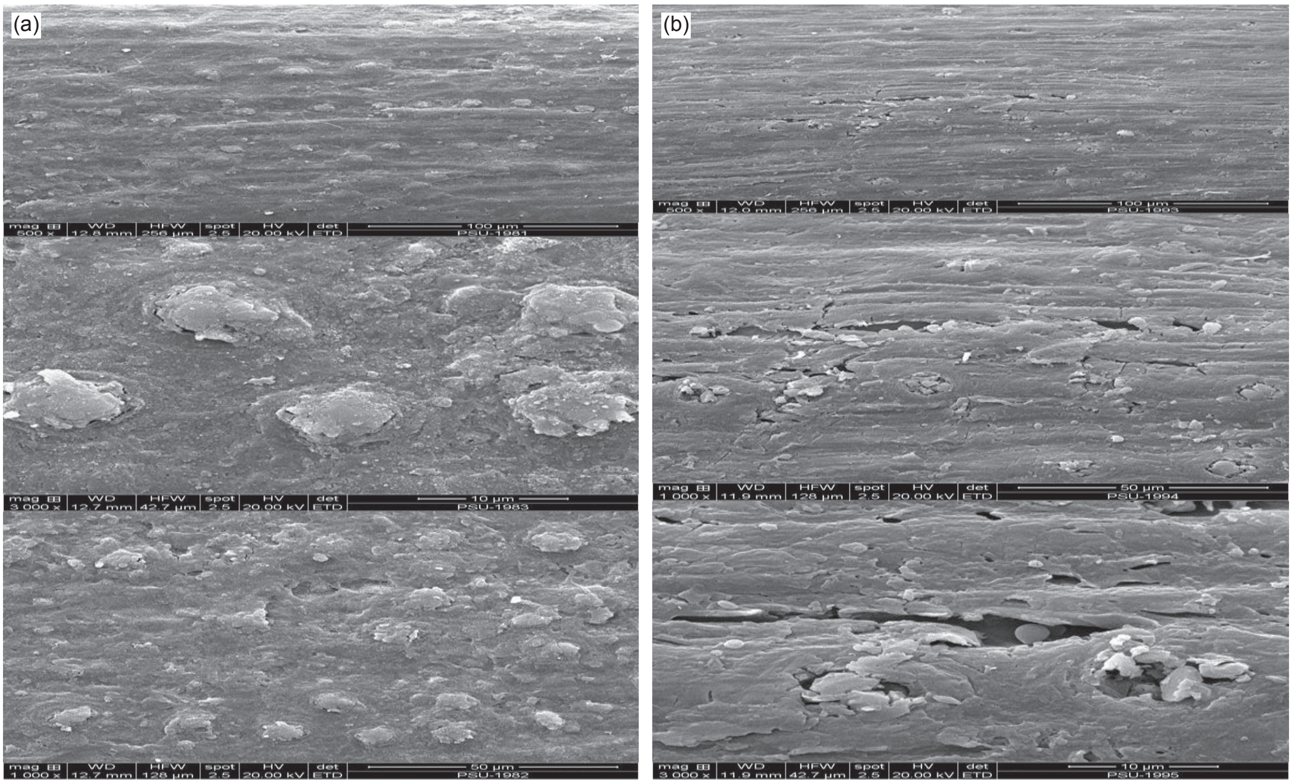

Figure 4. (a) Scanning electron microscope (SEM) images of untreated OPEFB at 500x, 1000x and 3000x magnification. (b) Scanning electron microscope (SEM) images of OPEFB treated at 600°C for 60 min (10% PAA), at 500x, 1000x and 3000x magnification.

enzymatic hydrolysis. The materials' CrI was not considerably impacted by pretreatment, but a more significant decrease in CrI was seen for straw, which explains the reduced recalcitrance of straw cellulose. A correlation exists between the stiffness of the cellulose structure and crystallinity, which can lead to fibres with higher tensile strengths. Both crystalline and semi-crystalline zones may be seen in the diffractogram curves of OPEFB cellulose in *Figure 4a* and *4b*. The existance of a visible peak on the shoulder at two diffraction angles between 22.0° and 25.4° shows that this type of behaviour is caused by the presence of a minimal amount of cellulose II. During the ultrasonication pretreatment procedure, the potential energy of the expanded bubble is converted into the kinetic energy of a liquid jet that passes through the interior of the bubble, penetrates the opposing bubble, and strikes the surface molecule of the cellulose. Continuous physical pressures contribute to particle breakage and initiate the process of cellulose polymers being cut into shorter chains of cellulose. The combined PAA treatment improved the cellulose yield. Comparable to untreated OPEFB, pretreated OPEFB has a lower crystallinity index. This could be because the non-selective processes like ultrasonication, can remove both amorphous and crystalline celluloses.

Xylanase Production in SSF

The goal of the current work was to produce xylanase enzyme using *Neosartorya* sp., a fungus, from OPEFB, which had been made with PAA-assisted ultrasonication and left untreated. It was discovered that OPEFB prepared with 10% PAA/ultrasonic for 60 min produced more enzymes (92 ± 0.02 IU/g) than untreated OPEFB (61 ± 0.01 IU/g) (*Figure 6*). The enzyme activity was higher on the fifth day, and as fermentation continued, the enzyme activity on both OPEFB substrates continued to decline. The lack of hemicellulose (xylan) and the depletion of the medium's micro- and macronutrients may be the causes of the decrease in enzyme synthesis.

Additionally, *Figure 7* displays the yield of xylose sugar during enzymatic hydrolysis. Compared to the pretreated substrate (46 ± 0.02 mg g^{-1}), less xylose was generated from the untreated OPEFB (34 ± 0.03 mg g^{-1}). On the fifth day, the xylose output reached its maximum; subsequently, it declined, possibly because of the consumption of sugar for fungal development. The results of this study showed that OPEFB pretreatment seemed to promote the synthesis of xylanase, which was associated with a higher level of xylose. The hemicellulose in the pretreated OPEFB was predicted to be broken down more

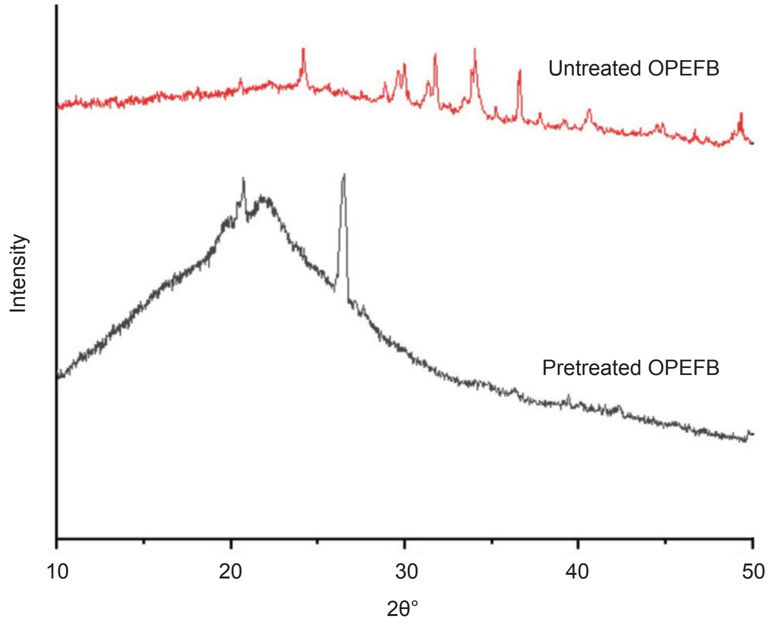

Figure 5. X-ray diffraction of untreated and pretreated OPEFB.

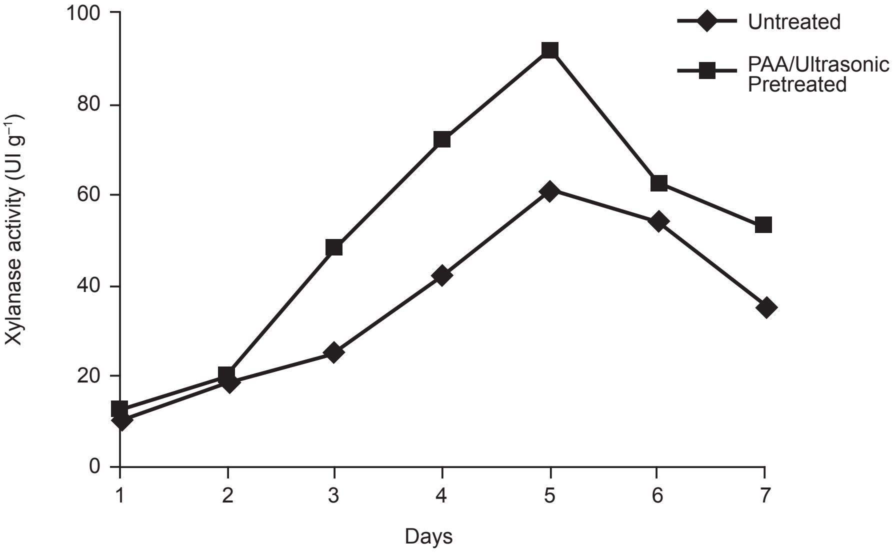

Figure 6. Xylanase enzyme activity (IU/g) on untreated and PAA/Ultrasonic pretreated OPEFB.

effectively, and theoretically, more xylose would be produced with higher xylanase activity. It has been demonstrated that pretreatment efficiently delignifies OPEFB substrates by lowering cellulose crystallinity and loosening the lignin structure, thereby increasing the substrate's porosity. The results of this study reveal that the fungus ability to produce xylanase may vary depending on the substrate used. As a result, xylanase production is enhanced when the substrate has more readily available hemicellulosic components. The results of this study were compared to those of Alves-Prado *et al*. (2010), whom reported that *Neosartorya spinosa* was an effective producer of xylanase when cultured on SSF using wheat bran and corncobs after a 72 hr fermentation.

CONCLUSION

According to the current findings, PAA-assisted ultrasonication is appropriate for the generation of fermentable sugars with holo-cellulose (cellulose and hemicellulose). Additionally, the pretreatment increases the quantity of lignin removed from the treated substrate. The elimination of lignin is indicated by an FTIR result, and XRD examination supported the reduction in cellulose crystallinity,

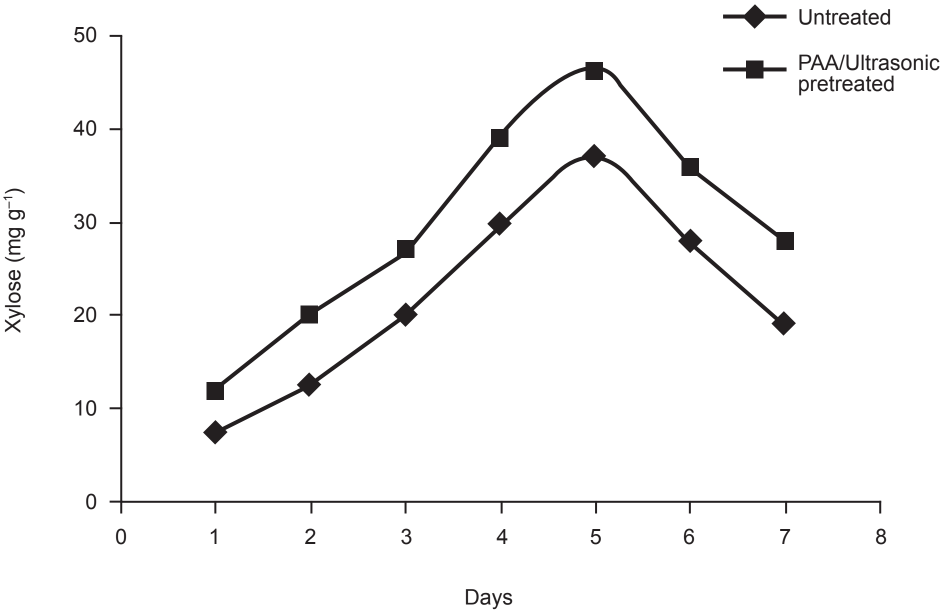

Figure 7. Yield of xylose sugar during enzymatic hydrolysis on untreated and PAA/Ultrasonic pretreated OPEFB.

which is crucial for the yields of fermentable sugar in pretreated OPEFB. As opposed to alternative pretreatment techniques, the current method is appealing since it is low-energy, low-cost, and economically feasible because the procedure is done at room temperature. Additional research will concentrate on statistical optimisation techniques (RSM, Taguchi, *etc.*) to increase enzyme production from a fungus strain.

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