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Integrated Production of Xylitol, Ethanol, and Enzymes from Oil Palm Empty Fruit Bunch through Bioprocessing as an Application of the Biorefinery Concept

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Citation: Mardawati, E.; Nawawi, M.I.S.; Caroline, V.; Imanisa, T.W.; Amanda, P.; Mahardika, M.; Masruchin, N.; Fitriana, H.N.; Rachmadona, N.; Lani, M.N. Integrated Production of Xylitol, Ethanol, and Enzymes from Oil Palm Empty Fruit Bunch through Bioprocessing as an Application of the Biorefinery Concept. *Fermentation* **2023**, *9*, 882. https://doi.org/10.3390/fermentation9100882

Academic Editor: Emmanuel Atta-Obeng

Received: 21 August 2023
Revised: 20 September 2023
Accepted: 22 September 2023
Published: 29 September 2023

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Abstract: Oil palm empty fruit bunch (OPEFB), an abundant source of lignocellulosic biomass waste, is rich in hemicellulose and is converted into xylose for xylitol production. The remaining cellulose-rich residue can be efficiently hydrolyzed into glucose, which serves as a substrate for bioethanol and enzymes. This process aligns with an integrated biorefinery model aimed at optimizing the utilization of OPEFB. This study optimizes a two-stage enzymatic hydrolysis fermentation for OPEFB conversion into value-added products. Using a 4% NaOH pretreatment, lignin was degraded while preserving hemicellulose and cellulose. This hydrolysis yielded 12.27 g/L of xylose and 36.86 g/L of glucose. Ethanol production, using varied fermentation media, achieved maximum concentrations of 0.043 g/L for xylitol and 21.35 g/L for ethanol, with substrate-to-product yields of 0.005 g/g and 0.374 g/g, respectively. Furthermore, enzyme production by *Aspergillus niger* was assessed on multiple parameters, recording a peak cellulase activity of 55.16 ± 20.24 U/mL and enzyme weight of 42.748 kDa. The OPEFB substrate yielded the highest protein content of 0.00942 ± 0.00010 mg/mL. These findings demonstrate the feasibility and efficiency of the two-stage enzymatic hydrolysis strategy in facilitating integrated biorefinery processes for efficient and sustainable OPEFB utilization.

Keywords: oil palm empty fruit bunch; biorefinery; lignocellulose; two-stage enzymatic hydrolysis

1. Introduction

The cultivation of oil palm serves as a pivotal economic catalyst within the Indonesian context, making substantial contributions to the national economy by serving as a leading plantation commodity and a significant generator of foreign exchange. As attested by Alkusma et al. [1], the relentless expansion of plantations and the burgeoning oil palm industry correlates with a heightened generation of waste materials, notably including substantial quantities of oil palm empty fruit bunches (OPEFB)—a form of solid waste. As reported by Yanti and Hutasuhut [2], in the year 2018, up to 6.7 million tons of OPEFB were disposed of, raising environmental challenges due to pollution and landfill congestion.

OPEFB is classified as lignocellulosic biomass waste, representing a reservoir of substantial untapped potential. Its constitution includes cellulose (ranging from 24% to 65%), hemicellulose (comprising 21% to 34%), and lignin (constituting 14% to 31%) [3]. Particularly noteworthy is its significant hemicellulose content, a complex polysaccharide polymer that includes glucose, mannose, arabinose, and xylose units, thereby conferring structural support to the cellular walls. Importantly, up to 82% of the hemicellulose in OPEFB can be converted into xylose, thereby serving as a pivotal precursor for xylitol production [4].

Xylitol is a sugar alcohol used in several sectors, including the food, pharmaceutical, and health industries. It is commonly used in the manufacturing of confectioneries, chewing gum, carbonated beverages, and oral hygiene items. In a study conducted by Muryanto et al. [5], the residue obtained after the extraction of xylitol, rich in cellulose, has the potential for conversion into bioethanol, a kind of liquid fuel energy. Additionally, residues of xylitol and ethanol production may contain partially degraded lignin, as well as incompletely converted hemicellulose and cellulose, serving as substrates for enzymatic reactions. Enzymes like cellulase, xylanase, and laccase are often derived from the metabolic processes of microorganisms [6].

In the pretreatment phase, the primary objective is to eliminate lignin, reduce cellulose crystallinity, and convert OPEFB into pulp to enhance the feasibility of hydrolysis and fermentation. This is achieved by subjecting the polysaccharide crystals to a heating process at a temperature of 120 °C [7]. During hydrolysis, hemicellulose is degraded, facilitating the extraction of fibers, and polysaccharide chains are broken down into monosaccharides [8]. The hydrolysate is then used in the fermentation phase, where xylitol is produced by the yeast *Meyerozyma caribbica*. This particular yeast strain is recognized for its resilience to inhibitory substances, making it an ecologically sustainable and cost-effective choice for this process. The achievement of effective xylitol bioconversion through fermentation is contingent upon many parameters, including temperature, pH, aeration conditions, substrate concentration, and the existence of other molecules apart from xylose, such as glucose. In addition, the xylose solution undergoes a conversion process, resulting in the formation of the xylitol solution [9]. The adequate preparation of the medium is crucial for achieving good fermentation of xylitol. The use of different compositions of the medium has a significant impact on the resulting products.

On the other hand, the solid waste produced during the process of xylitol manufacture from OPEFB is significant due to its substantial cellulose content, making it a promising feedstock for bioethanol production within a biorefinery framework. Enzymatic hydrolysis can degrade cellulose polymers into glucose, enabling further processing of residual solid waste. Compared to acid hydrolysis, enzymatic hydrolysis generates more glucose, resulting in more significant bioethanol production [10].

While previous studies have mostly focused on bioethanol production, this study adopts a novel perspective by examining bioethanol generation using varying quantities of cost-effective and readily available fermentation media [11]. The objective is to maximize the extraction of ethanol from a cost-effective substrate. Based on the findings of Mardawati et al. [4], it is imperative that the materials used in the process satisfy the fundamental nutritional demands necessary for the proliferation of microorganisms. These needs include key components such as carbon, nitrogen, non-metallic constituents (specifically sulfur and phosphorus), metallic elements including calcium, zinc, sodium, potassium, copper, manganese, magnesium, and iron, as well as vitamins, water, and energy.

This study employs a two-stage enzymatic hydrolysis technique, focusing on using OPEFB waste for bioethanol synthesis and xylitol production. Both steps of hydrolysis require the use of hemicellulase enzymes to catalyze the breakdown of hemicellulose into xylose, serving as a substrate for xylitol fermentation. The cellulose-rich xylitol residue is subjected to further hydrolysis to produce glucose hydrolysate that may be used for ethanol production. The resulting solid waste from the process of hydrolysate separation is used as a substrate for lignocellulosic enzyme synthesis. This study demonstrates the

use of the OPEFB biorefinery for the combined synthesis of xylitol and bioethanol, with subsequent fermentation using different medium compositions.

Enzyme synthesis can be accomplished through fermentation by microbial organisms, often bacteria or fungi [12]. It has been shown that fungi exhibit a greater hydrolysis rate in comparison to yeast and bacteria. They are capable of producing a diverse range of hydrolytic and oxidative enzymes that have the ability to break down the components of lignocellulosic materials. The selection of *Aspergillus niger*, a filamentous fungus known for its production of cellulase, xylanase, and laccase enzymes, was based on its favorable performance and cost-effectiveness compared to other choices [13]. The development of fungi is influenced by several parameters, including substrate concentration, nutrient supply, aeration, pH, temperature, incubation conditions, moisture content, and fermentation duration [14].

Microorganisms play an active role in fermentation and naturally reproduce in environments conducive to their development [15]. The present study used the submerged fermentation technique to produce the three enzymes. The methodology used a liquid substrate, with the addition and replacement of nutrients conducted in a continuous manner within the submerged fermentation medium. This environment is particularly appropriate for microorganisms, including fungi, that prefer elevated levels of moisture [16].

The primary aim of this research was to evaluate the use of a fermentation medium in conjunction with a two-stage enzymatic hydrolysis procedure for the production of xylitol and bioethanol. Furthermore, an additional objective for this research was to assess the enzymatic activity of cellulase, xylanase, and laccase enzymes produced by *Aspergillus niger* during cultivation on an integrated OPEFB substrate. The enzyme evaluation was conducted by taking into account key variables, including activity, protein content, specific activity, and enzyme mass.

2. Materials and Methods

2.1. Materials Preparation and Source

All components of the OPEFB parts were cleaned under running water and subjected to drying at 60 °C in an oven for a period of 24 h. The dried material was subsequently fragmented into finer particles using a disc mill and sorted via a sieve with a mesh size ranging from 60 to 80 mesh or 0.25 to 0.177 mm size. The prepared materials were stored in air-tight bags at ambient temperature until they were used.

Enzymatic hydrolysis was performed using the Cellic HTec2 and Cellic CTec2 enzymes sourced from Novozymes in Copenhagen, Denmark. The activity of these enzymes was 75 IU/mL and 130 FPU/mL, respectively. In this study, *Meyerozyma caribbica* Y67 from the Indonesia Culture Collection (InaCC)-BRIN, Indonesia, was used to ferment xylitol. While *Candida* sp. Unpad CC Y26 was used to make ethanol derived from environmentally exposed OPEFB. Additional chemicals were purchased from Sigma Aldrich in St. Louis, Missouri, and Merck in Singapore. All chemicals were of laboratory grade and used immediately upon receipt. All solutions were prepared with 18.2 MΩ cm Milli-Q water (Millipore, St. Louis, MO, USA).

2.2. Pretreatment of Oil Palm Empty Fruit Bunches

A preliminary study was undertaken to identify the optimal pretreatment method for OPEFB. The experimental investigation involved the evaluation of different pretreatment methods, specifically acid and alkaline catalysts, with variations in the duration of the pretreatment process for each catalyst. The OPEFB flour was dissolved in either 4% NaOH or 4% H_2SO_4 solutions after being weighed on an analytical scale. Referring to the study by Sari et al. [17], the materials were mixed in an Erlenmeyer flask using a solid-to-liquid ratio of 1:20 (b/v). This means that 20 g of OPEFB flour was added to 400 mL of solution. The mixture was processed in an autoclave at 121 °C for periods of 15, 30, and 60 min. The optimal pretreatment methods would be selected based on lignin reduction, hemicellulose, and cellulose retention, as well as process duration.

2.3. Enzymatic Hydrolysis of Pretreated Oil Palm Empty Fruit Bunches Using Cellic Htech Enzyme

The pretreated OPEFB powder was sterilized by immersing 10 g in 100 mL of pH 5 acetate buffer in an Erlenmeyer flask and autoclaving it for 15 min at 121 °C. Hydrolysis was carried out in a shaker incubator (N-Biotek, Seoul, South Korea) at 60 °C and 150 rpm for 96 h using 38.5 I.U./g of biomass Cellic HTec. Following hydrolysis, the liquid fraction was separated through centrifugation at 6000 rpm for 20 min. Sampling was performed every 24 h. The solid residue was oven-dried at 105 °C oven for 24 h. The dried solid residue was employed as a substrate for the synthesis of ethanol.

Equations for calculating theoretical xylose and glucose concentrations (g/L) in the feedstocks are defined in Equations (1) and (2), respectively, as follows:

Theoretical xylose concentration (g/L):

$$\frac{\text{Mass of lignocellulose (g)} \times \text{hemicellulose content in linocellulose} \times 0.88}{\text{working volume}} \quad (1)$$

Theoretical glucose concentration (g/L):

$$\frac{\text{Mass of lignocellulose (g)} \times \text{hemicellulose content in linocellulose} \times 0.9}{\text{working volume}} \quad (2)$$

2.4. Xylitol Fermentation of Oil Palm Empty Fruit Bunches Hydrolysate by M. caribbica

Inoculum preparation started by sterilizing 50 mL of potato dextrose broth (PDB) at 121 °C in an autoclave for 15 min. Following sterilization, the sterile medium solution was inoculated with a single loopful of *Meyerozyma caribbica* cells and incubated at 30 °C for 48 h, with a shaking speed of 200 rpm in a shaker incubator. Subsequently, 5% of this cultivated inoculum was mixed with 200 mL of fresh PDB medium and incubated again in a shaker incubator at 30 °C for 48 h at 200 rpm. The mixture was then centrifuged at 6000 rpm for 12 min to separate the cell pellet from the medium. The cell pellet was subsequently homogenized in 50 mL of the desired fermentation medium.

This study examined three different xylitol fermentation media. The first medium, labeled Medium A, has been employed in prior research for xylitol fermentation, enriched with a variety of minerals and vitamins [18]. The composition of this medium was 9.438 g/L $(NH_4)_2SO_4$, 2.5 g/L KH_2PO_4, 0.05 g/L $CaCl_2{\cdot}2H_2O$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L citric acid, 0.035 g/L $FeSO_4{\cdot}7H_2O$, 0.0092 g/L $MnSO_4{\cdot}7H_2O$, 0.011 g/L $ZnSO_4{\cdot}7H_2O$, 0.001 g/L $CuSO_4{\cdot}7H_2O$, 0.002 g/L $CoCl_2{\cdot}6H_2O$, 0.0013 g/L $Na_2CoO_4{\cdot}2H_2O$, 0.002 g/L H_3BO_3, 0.0035 g/L KI, 0.0005 g/L $Al_2(SO_4)_3$, 0.1 g/L myo-inositol, 0.02 g/L calcium-pantothenate, 0.005 g/L thiamine hydrochloride, 0.005 g/L pyridoxal hydrochloride, 0.005 g/L nicotine acid, 0.001 g/L aminobenzoic acid, and 0.0001 g/L d-biotin. The second medium, labeled Medium B, was formulated with 5 g/L yeast extract, 2 g/L KH_2PO_4, 2 g/L $(NH_4)_2HPO_4$, and 0.3 g/L $MgSO_4{\cdot}7H_2O$. The third medium, referred to as Medium C, is an AB-mix, which is a commercial hydroponic fertilizer that contains macroelements, such as N, Ca, K, Mg, S, and P, as well as microelements, including Fe, Mn, Cu, B, Zn, and Mo.

2.5. Ethanol Production Using Semi-Simultaneous Saccharification and Fermentation (Semi-SSF) Method

The Semi-SSF technique was used to produce bioethanol from OPEFB residue. A 250 mL Erlenmeyer flask with a working volume of 100 mL was used. The OPEFB residue and cellulase enzyme concentrations in the total citrate buffer solution at pH 5 were 10% (*w*/*v*) and 65 FPU/gram biomass, respectively. Before enzyme addition, both biomass and buffer were sterilized. The saccharification process was conducted in the incubator shaker at 50 °C for 96 h at a shaking speed of 130 rpm.

For inoculum preparation, 50 mL of potato dextrose broth (PDB) medium was sterilized and mixed with one loopful of *Candida* sp. Unpad CC Y26 cells. The inoculum solution was incubated at 30 °C and 200 rpm for 48 h in a shaker incubator. Then, 5% of the prepared

inoculum was mixed with 200 mL of fresh PDB medium and incubated under the same conditions. The cell pellet was separated from the media by centrifuging the inoculum at 6000 rpm for 12 min.

After 96 h of the hydrolysis period, neither filtration nor enzyme inactivation was carried out on the hydrolysate. A cell pellet of *Candida* sp. Unpad CC Y26 and a two-fold concentrated fermentation medium were then added to the hydrolysis Erlenmeyer flask. Fermentation was conducted at 30 °C, 150 rpm for an additional 96 h in an incubator shaker.

This section encompasses an investigation of three distinct types of xylitol fermentation media. The first medium (Medium 1) compositions were 1.8 g/L yeast extract, 0.9 g/L $(NH_4)_2HPO_4$, and 0.6 g/L $(NH_2)_2CO$. The second medium (Medium 2) compositions were 4 g/L peptone, 3.6 g/L yeast extract, 2 g/L KH_2PO_4, 1 g/L $MgSO_4{\cdot}7H_2O$, and 3 g/L $(NH_4)_2SO_4$. The third medium (Medium 3) compositions were 5 g/L peptone, 3 g/L yeast extract, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.11 g/L $CaCl_2$, and 0.05 g/L $ZnSO_4$.

2.6. Extracellular Enzymes Production by Aspergillus niger Unpad CC C42

The study investigated variations of OPEFB substrates, including untreated and mechanically treated OPEFB, xylitol production residue, and ethanol fermentation residue. For enzyme production, 2.4 g of PDB and 100 mL of distilled water were homogenized using a magnetic stirrer. A concentration of 2.5 g of OPEFB biomass at a concentration of 2.5% was added and sterilized in an autoclave at 121 °C for 15 min. To enhance extracellular enzyme production, 1% Tween 80 was added to the fermentation flask. Fungal spore density was measured using a hemocytometer according to Avin's procedure [19]. Aseptically, a spore density of 6.8×10^7 cells/mL was inoculated into the fermentation flasks and incubated at 30 °C and 200 rpm for 9 days using an incubator shaker. Sampling was performed every three days, with the samples centrifuged at 6000 rpm for 20 min to separate the fungal biomass and extracellular enzymes in the supernatant. Subsequently, the supernatant was subjected to various tests to evaluate the enzymatic activity, protein concentration, specific activity, and molecular weight of xylanase, cellulase, and laccase enzymes.

2.7. Analysis Method

The lignocellulose composition was determined using the Van Soest method, as described by Hindrichsen et al. [20]. This method enables the simultaneous quantification of cellulose, hemicellulose, and lignin concentrations in the biomass. For analyzing the hydrolysis and fermentation samples, high-performance liquid chromatography (HPLC) was conducted using a Waters 1515 pump, a 2707 autosampler, and UV and refractive index (RI) detectors with an Aminex HPX-87H column (Milford, MA, USA). The mobile phase used was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min. The growth of *M. caribbica* was monitored by measuring optical density at 600 nm and converted to dry cell weight (DCW) using a calibration curve. Meanwhile, to evaluate the growth of *Candida* sp. Unpad CC Y26 cells, the total plate count (TPC) method was employed due to the precipitation of yeast cells during fermentation, and the calibration curve was also used to convert colony-forming units (CFUs) to dry cell weight (DCW). The growth of *Aspergillus niger* cells can be measured based on their spore density using a hemocytometer. A scanning electron microscope (JEOL, JSM-6330F; Tokyo, Japan) was used to examine the surface of the OPEFB before and after various treatments.

2.8. Enzyme Activity Test

Enzyme activities of cellulase and xylanase were measured using the Jampala et al. [21] method. Each test tube contained 0.5 mL of crude enzyme and 1 mL of 0.05 M citrate buffer pH 4.8. For cellulase and xylanase assays, 0.5 mL of 1% carboxymethyl cellulose (CMC) and 1% xylan were used as substrates, respectively. After 30 min incubation in a 50 °C water bath, 2 mL of DNS reagent was added, followed by heating in a 90 °C water bath for 10 min, then chilled. The color change was seen, and the absorbance was measured at 540 nm using a spectrophotometer. Enzyme activities were calculated based on standard

glucose (y = 92.347x − 0.0633, R^2 = 0.9973) and xylose (y = 40.118x + 0.444, R^2 = 0.995) equations. The glucose and xylose standard curve (0–10 g/L) for cellulase activity from Adney and Baker [22] was utilized.

The laccase activity was spectrophotometrically determined as per the method of Yuliana et al. [23]. A mixture of 60 µL of 0.5 M acetate buffer at pH 5, 20 µL of 1 mM 2.2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and 50 µL of the crude enzyme was incubated in a microplate at 37 °C for 30 min, with absorbance measured at 420 nm. Enzyme units were defined as the laccase activity that oxidized 1 µmol of ABTS per minute.

Specific activity refers to the number of enzyme activity units per milligram of protein under optimum conditions. This is commonly used to indicate the amount of enzyme involved in a specific enzymatic reaction. To determine the specific activity of xylanase, the enzyme activity units and xylanase protein concentration were measured [24]. One unit of xylanase activity was defined as the amount of xylanase activity that produced 1 mg of xylose under optimum conditions. A higher specific activity value indicated a better ability of the enzyme to utilize the substrate.

2.9. Protein Content

The Bicinchoninic Acid (BCA) protein assay kit was used for protein quantification, following the Thermoscientific protocol [25]. The protein testing was performed by adding 200 µL of BCA Working Reagent to each column of the microplate samples to be tested. The BCA Working Reagent was prepared by mixing BCA Reagent B with A in a ratio of 1:50. Subsequently, a total of 200 µL of BCA Working Reagent was added to each well containing 25 µL of the enzyme sample. After shaking for 30 s and incubating at 37 °C for 30 min, the absorbance was measured at 562 nm using a spectrophotometer. Protein concentration was derived from a BSA (0–200 g/L) standard curve (y = 0.0008x + 0.0064 (R^2 = 0.946).

2.10. Enzyme Molecular Weight

SDS-PAGE was performed using the Laemmli (1970) method with 15% separating and 4% stacking gels. After mixing 15 µL of the sample with 10 µL of loading dye, the mixture was heated at 95 °C for 10 min. Five microliters of samples were loaded into each well. Electrophoresis was carried out using Mini Protean Tetra Cell units (Bio-Rad Laboratories, Inc., Richmond, CA, USA) for approximately 60 min at 110 V. Proteins were stained with Simply Blue and destained with Aquades after separation. A broad-range molecular weight standard marker (10–245 kDa) was used to determine the molecular weight (MW) of the enzymes.

2.11. Statistical Analysis

Student *t*-test was used to determine the statistical significance of all measurements. The data were presented as mean ± standard deviation. A *p*-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Preliminary Study for Oil Palm Empty Fruit Bunches Pretreatment

Pretreatment serves as a crucial step that breaks down the cell wall bonds, making the hemicellulose and cellulose more accessible to enzymatic action [26]. The effective pretreatment results in the optimal recovery of valuable hemicellulose derivative products while minimizing the production of undesirable degradation compounds [27]. Moreover, the pretreatment should enhance the susceptibility of cellulose-containing solids to enzymatic hydrolysis, reduce waste generation, and lower energy consumption [28]. Table 1 presents the lignocellulose content profile of OPEFB before and after the pretreatment stages. Utilizing a 4% NaOH solution as an alkaline catalyst led to a more significant reduction in lignin content over time compared to a 4% H_2SO_4 catalyst. However, longer pretreatment durations resulted in considerable hemicellulose loss, which is undesirable since hemicellulose serves as a raw material for xylitol production. Therefore, the optimal method for

pretreatment involved a 15 min application of a 4% NaOH solution, owing to its high hemicellulose content and low lignin content. These findings align with previous research [28], indicating that the use of an alkaline catalyst triggers saponification in lignocellulose, leading to its subsequent modification. This modification alters the lignin structure, resulting in a significant reduction in lignin content within the solid residue. In contrast, using an acid catalyst primarily decomposes hemicellulose and dissolves lignin, resulting in the dissolution of a substantial amount of hemicellulose and an acid-soluble lignin.

Table 1. Comparison of the lignocellulose content of OPEFB in the initial condition and after pretreatment.

Lignocellulose Component	% Dry Weight						
	Raw OPEFB	OPEFB after Pretreatment					
		A15	A30	A60	B15	B30	B60
Neutral detergent fiber (NDF)	79.7	77.8	75.6	72.8	76.0	75.0	71.2
Acid detergent fiber (ADF)	56.3	58.4	58.4	54.8	52.8	54.8	51.8
Hemicellulose	23.4	19.4	17.2	18.0	23.2	20.2	19.4
Lignin	19.1	16.6	14.6	13.6	9.5	8.6	7.6
Cellulose	36.0	41.0	43.0	40.6	42.9	45.4	43.0
Ash	1.2	0.8	0.8	0.6	0.4	0.8	1.2

The pretreatment variations consisted of A15 (4% H_2SO_4 for 15 min), A30 (4% H_2SO_4 for 30 min), A60 (4% H_2SO_4 for 60 min), B15 (4% NaOH for 15 min), B30 (4% NaOH for 30 min), and B60 (4% NaOH for 60 min).

Figure 1a illustrates the surface of untreated OPEFB, which remains coated with lignin. This dense and resilient lignin structure in OPEFB acts as a barrier to the enzymatic hydrolysis process, inhibiting the effective conversion of hemicellulose and cellulose into monosaccharides. In contrast, Figure 1b presents the morphological changes in OPEFB fibers after pretreatment, revealing a significant alteration in the lignin structure. This treatment effectively softens and irregularizes the lignin layer, a finding that aligns with the findings from Soontornchaiboon et al. [29]. They suggest that alkaline pretreatment enhances the texture of lignocellulose, resulting in the formation of larger and more abundant cavities. As seen in Figure 1b, after pretreatment, the OPEFB surface appears more open, and its outer texture becomes smoother and fragmented. These structural changes enable enzymes to effectively hydrolyze hemicellulose into xylose and cellulose into glucose, as the enzymes can now more easily access the internal substrates. Such transformations in surface morphology are crucial for optimizing the bioconversion process.

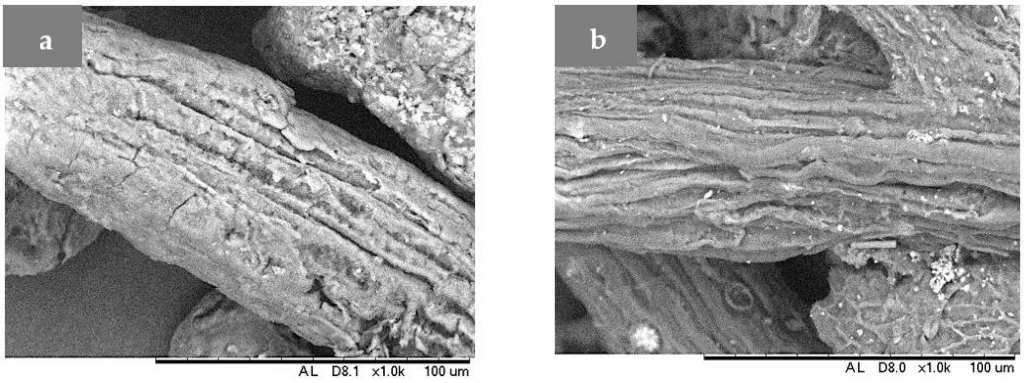

Figure 1. Morphology of untreated OPEFB (**a**) and pretreated OPEFB (**b**).

3.2. Enzymatic Hydrolysis of Oil Palm Empty Fruit Bunches

Enzymatic hydrolysis serves as a pivotal stage in the production of both xylitol and ethanol. Enzymatic hydrolysis has a favorable energy utilization profile, as it can be conducted at lower temperatures and has the potential to generate up to 70% sugar content.

However, it is essential to acknowledge that the reaction rates for this process tend to be relatively slow [30,31].

In this study, the xylitol production was initiated with an alkaline pretreatment of OPEFB feedstock, followed by enzymatic hydrolysis. The chosen pretreatment strategy has demonstrated significant efficacy in achieving elevated concentrations of xylose and glucose [32,33]. Furthermore, this method enhances delignification, increases the porosity of OPEFB, and improves enzyme accessibility during the subsequent enzymatic hydrolysis stage [4]. The decline in lignin content after pretreatment OPEFB is shown in Table 2 revealing a reduction from an initial 19.1% in raw OPEFB to 9.6% in the pretreated material.

Table 2. Oil Palm Empty Fruit Bunches lignocellulosic content for hydrolysis feedstocks.

No	Composition	Dry Weight (%)		
		Raw Material	Pretreated OPEFB	Xylanase-Hydrolyzed OPEFB
1	NDF	79.7	75.6	79.9
2	ADF	56.3	52.6	72.4
3	Hemicellulose	23.4	23.0	7.5
4	Cellulose	36.0	42.6	62
5	Lignin	19.1	9.6	8.9

The first enzymatic hydrolysis of OPEFB involved a blend of xylanase enzymes, specifically Cellic Htech, due to its capacity to effectively hydrolyze xylan, which constitutes the primary component of hemicellulose [31]. After 96 h of hydrolysis (Table 3), the total amount of xylose concentration was found to be 12.27 g/L, corresponding to 60.62% of the theoretical xylose yield from the feedstock. The effectiveness of hemicellulose hydrolysis is demonstrated by a noticeable reduction in hemicellulose content after the hydrolysis procedure, from an initial 23% down to 7.5%, as presented in Table 2. In addition, the hydrolysis process has yielded a glucose concentration of 31.73 g/L, which represents 83.9% of the theoretical glucose in the feedstock. The high glucose concentration resulting from the first hydrolysis indicates that pretreatment with a 4% NaOH solution effectively removes a substantial portion of the lignin and thereby enhances the accessibility of enzymes to cellulose fibers. The outcomes of the initial hydrolysis exhibit differences when compared to the findings of a prior investigation [34] that employed identical feedstock and xylanase. The low glucose concentration (2.75 g/L) in this study was attributed to the use of autohydrolysis as the pretreatment method. In the subsequent enzymatic hydrolysis, the solid residue obtained from the initial hydrolysis of OPEFB was subjected to treatment with cellulase, specifically using Cellic Ctech. The objective of this hydrolysis process is to produce a high concentration of glucose to further facilitate ethanol production. According to the data presented in Table 3, it is clear that the second hydrolysis process resulted in a glucose concentration of 36.86 g/L. This concentration corresponds to about 66% of the theoretical glucose in the current feedstock. Similar to the initial hydrolysis, the glucose concentration generated during the subsequent hydrolysis was seen to be higher compared to the findings reported by Mardawati (2022) [34]. Mardawati achieved a glucose concentration of 22.37 g/L, utilizing identical feedstock (solid residue of xylanase hydrolysis) and enzyme concentration and type. The primary distinguishing factor between the current study and prior studies is the chosen pretreatment method, which significantly improves the substrate's accessibility to the enzymes.

Table 3. The concentration of xylose and glucose in the Oil Palm Empty Fruit Bunches hydrolysate.

Sample	Type of Enzyme	Xylose		Glucose	
		Concentration (g/L)	**Theoretical Xylose in the Feed Stock (%)**	**Concentration (g/L)**	**Theoretical Xylose in the Feed Stock (%)**
Hydrolysate I	Cellic Htech	12.27	60.62	31.73	83.9
Hydrolysate II	Cellic Ctech	0	0	35.86	66

3.3. Xylitol Fermentation Using Meyerozyma caribbica

During the fermentation process, the growth of *M. caribbica* cells was closely monitored in three distinct types of media: Medium A, enriched with minerals and vitamins; Medium B, containing yeast extract and an additional nitrogen source; and Medium C, a cost-effective alternative fermentation medium composed of commercial hydroponic plant fertilizer. Various factors can influence microbial cell growth, including medium composition, nutrient availability, temperature, pH, and oxygen levels [35]. The growth patterns of *M. caribbica* cells in these three fermentations media are illustrated in Figure 2.

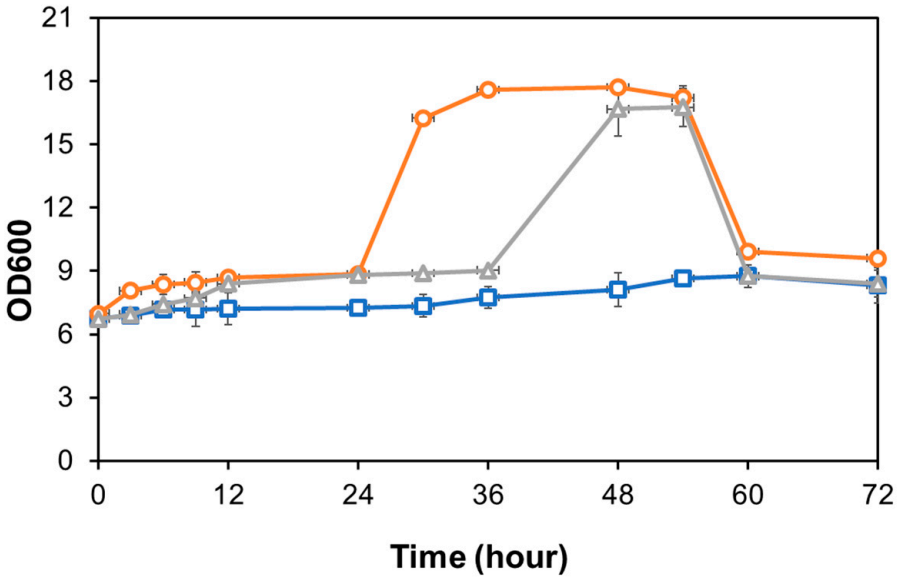

Figure 2. *M. caribbica* cell growth during xylitol production using three distinct media. Medium A (blue, rectangle), Medium B (orange, circle), and Medium C (gray, triangle). The data shown are the average of two replicate studies, and the error bars show the standard deviations.

The growth of *M. caribbica* cells in Medium A showed a relatively low rate, exhibiting only a 30% increase from its initial value after 60 h of fermentation. However, despite the limited cell growth, the fermentation process was highly efficient in producing xylitol, with a yield of 0.005 g/g, and ethanol, with a remarkable yield of 0.32 g/g, which was the highest yield observed among all the tested samples (Table 4). Analyzing the medium's composition showed that Medium A is devoid of complex compounds like yeast extract or peptone that are typically used to promote cell growth. Thus, the carbon source for cellular metabolism is solely derived from hydrolysate. It becomes evident that Medium A was intentionally formulated to favor fermentation over cell growth, particularly in anaerobic conditions [36,37].

Table 4. Substrate composition and the fermentation metabolite products (t = 54 and t = 72).

Parameter	Exponential End (t = 54)			Fermentation End (t = 72)		
	Medium A	Medium B	Medium C	Medium A	Medium B	Medium C
Initial Xylose Concentration (g/L)	12.265	12.265	12.265	12.2650	12.2650	12.2650
Final Xylose Concentration (g/L)	5.839	5.919	3.233	3.6970	4.5250	2.9780
Xylose Utilization (%)	52.393	51.741	73.640	69.8573	63.1064	75.7195
Initial Glucose Concentration (g/L)	31.726	31.726	31.726	31.7260	31.7260	31.7260
Final Glucose Concentration (g/L)	11.888	1.9060	19.436	7.0770	0.259	17.9160
Glucose Utilization (%)	62.529	93.992	38.738	77.6934	99.184	43.5290
Xylitol Concentration (g/L)	0.041	0.000	0.043	0.0400	0.0000	0.0360
Xylitol Yield from Xylose Consumption (Yp/s) (g/g)	0.005	0.000	0.005	0.0047	0.0000	0.0039
Xylitol Yield from Cell Count (Yp/x) (g/g)	0.022	0	0.0042	0.0253	0.0000	0.0215
Ethanol Concentration (g/L)	6.326	5.397	0.504	7.206	5.1370	0.000
Ethanol Yield from Glucose Consumption (Yp/s) (g/g)	0.3189	0.199	0.0330	0.2923	0.163	0.0000
Ethanol Yield from Biomass Formation (Yp/x) (g/g)	3.3383	0.5809	0.0400	4.5608	3.2513	0.0000
Biomass Concentration (g/L)	8.645	17.20	16.75	8.325	9.61	8.395
Biomass Yield from Xylose Consumption (Yx/s xil) (g/g)	0.295	1.6105	1.1216	0.1844	0.3398	0.1804
Biomass Yield from Glucose Consumption (Y x/s glu) (g/g)	0.0955	0.3248	0.8242	0.0641	0.0530	0.1144
Specific Growth Rate (μ) (1/h)	0.0049	0.0208	0.0144	0.0049	0.0208	0.0144

The cellular growth of *M. Caribbica* in Medium B can be characterized by four distinct phases. These phases comprise the adaptation phase (0–24 h), the logarithmic phase (24–36 h), the stationary phase (36–54 h), and ultimately, the death phase (54–72 h). Unlike Medium A, Medium B contains yeast extract as an additional carbon source. Yeast extract, being a complex compound, has the capacity to stimulate cell proliferation significantly, leading to a cell growth rate 2.3 times higher than the initial value at 30 h into the fermentation. The utilization of Medium B proves to be highly conducive to ethanol production by *M. caribbica*, resulting in a yield of 0.2 g/g after 54 h of fermentation (Table 4). However, it should be noted that xylitol production was not observed in this medium.

Similar to Medium B, the growth pattern in Medium C can also be classified into four distinct phases. These phases encompass the adaptation phase (0–36 h), the logarithmic phase (36–48 h), the stationary phase (48–54 h), and finally, the death phase (54–72 h). Interestingly, the adaptation phase in Medium C is relatively extended in duration, while the logarithmic and stationary phases are relatively shorter compared to Medium B. Medium C is rich in various essential components, including calcium nitrate, iron (Fe), potassium nitrate, KH_2PO_4, $(NH_4)_2PO_4$, KNO_3, $MgSO_4$, $MnSO_4$, $CuSO_4$, $ZnSO_4$, boric acid, and Mo [38]. Medium C, which is a rich source of N, P, and K as well as sugar from the hydrolysate, is able to increase cell growth by 2.5 times compared to the initial value, but the resulting fermentation products are low, namely xylitol with a yield of 0.005 g/g and ethanol with a yield of 0.033 g/g (Table 4).

Among the various media tested, medium A was the most effective in terms of production of fermentation products despite the relatively low level of cell growth. This finding highlights a promising avenue for maximizing the conversion of sugars in the hydrolysate into product synthesis, prioritizing it over cellular growth. Additionally, the growth of *M. caribbica* cells in Medium C, which is expected to be used as a cost-effective alternative to the fermentation medium, exhibited a satisfactory growth rate efficiency (μ) of 0.0144. Nevertheless, further optimization is required to improve product yields in this medium.

A notable finding from the investigation of three different media compositions was the absence of substantial xylitol production exceeding 5 g/L, despite the presence of approxi-

mately 12.65 g/L xylose derived from hydrolysate and a xylose utilization rate exceeding 50% in all cases. Previous research has shown that, for effective xylitol production, the xylose concentration in the fermentation medium should be higher than that of glucose. This is primarily due to the inhibitory effect of high glucose content on the activity of xylose reductase, a key enzyme for xylitol production [39]. As a result, the metabolite pathways in the cells are diverted from xylitol to ethanol production.

3.4. Ethanol Production Using Semi-SSF Method by Candida sp. Unpad CC Y26

During the ethanol fermentation process, the growth of *Candida* sp. Unpad CC Y26 cells was closely monitored in three distinct complex media. Medium 1 contains a small amount of yeast extract and two types of inorganic nitrogen sources. Medium 2 is rich in yeast extract and peptone and includes additional inorganic nitrogen sources and minerals. Medium C, on the other hand, contains high levels of yeast extract, peptone, and minerals. The growth patterns of *Candida* sp. Unpad CC Y26 cells in these three-fermentation media are depicted in Figure 3.

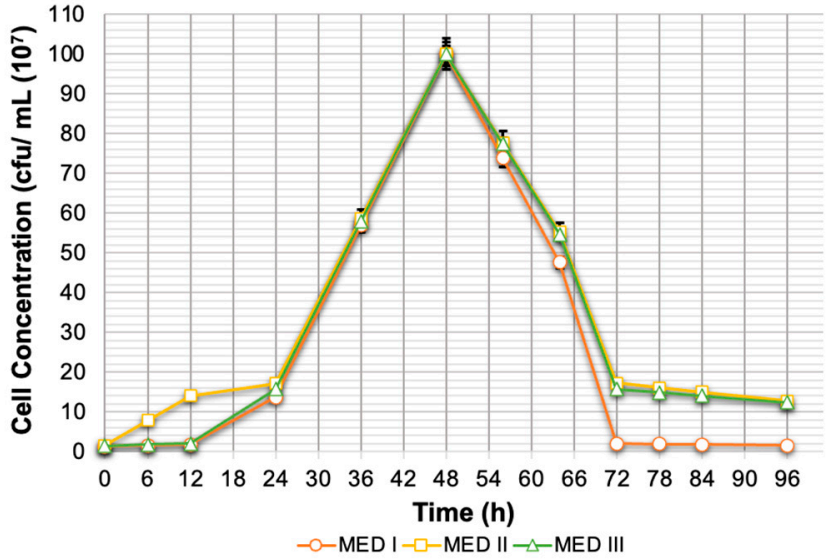

Figure 3. The growth phase of isolate yeast Y26 cells.

In general, the cell growth patterns across the different media showed similarities, with the exception of Medium 1. This medium is characterized by its lower nutrient concentrations, resulting in a corresponding decrease in cell count during the final 24 h of fermentation. On the other hand, Medium 2, with its more complex nutrient composition, did not display an initial adaptation phase. In Mediums 1 and 3, the cellular adaptation phase began at hour 0 and continued until the 24th hour. Subsequently, a logarithmic growth phase was observed, starting at hour 0 for Medium 2 and from the 24th to the 48th hour for Mediums 1 and 3. Following this growth phase, there was a notable decline in viability, known as the accelerated death phase, which occurred between the 48th and 72nd hours. The significant reduction in cellular count observed at 48 h can be attributed to the low glucose concentration at that specific time. Notably, at the 48th hour, Medium 2 exhibited a substantial concentration of ethanol (21.35 g/L), with an ethanol yield of 0.626 g/g. In contrast, negligible ethanol production was observed in Mediums 1 and 3, as shown in Table 5.

Table 5. Substrate composition and the fermentation metabolite products (t = 48 and t = 96).

Parameter	t = 48			t = 96		
	Medium 1	**Medium 2**	**Medium 3**	**Medium 1**	**Medium 2**	**Medium 3**
Initial Glucose Concentration (g/L)	36.86	36.86	36.86	36.86	36.86	36.86
Final Glucose Concentration (g/L)	2.62	2.80	3.04	0.50	1.66	1.50
Glucose Utilization (%)	92.88	92.40	91.73	98.64	95.48	95.92
Ethanol Concentration (g/L)	0.03	21.35	0.04	0.02	1.32	0.04
Ethanol Yield from Glucose Consumption (Yp/s) (g/g)	0.0008	0.6260	0.0011	0.0006	0.0374	0.0012
Ethanol Yield from biomass (Yp/x) (g/g)	0.003	2.476	0.004	1.00	1.375	0.042
Biomass Yield from Glucose Consumption (Yx/s glu) (g/g)	0.253	0.253	0.255	0.001	0.028	0.027
Initial Biomass Concentration (g/L)	0.11	0.14	0.13	0.11	0.14	0.13
Final Biomass Concentration (g/L)	8.76	8.76	8.76	0.13	1.11	1.08
Specific Growth Rate (μ/hour)	0.07918	0.14828	0.07958	0.07918	0.14828	0.07958

The variations in ethanol concentration can be attributed to a range of multiple factors, including, but not limited to, glucose consumption for microbial growth and the duration of the fermentation process. As fermentation proceeds, the available nutrients in the substrate become progressively depleted, and yeast may readily consume ethanol, leading to a decrease in ethanol concentration within the fermentation broth. In the case of Medium 2, our investigation determined that the most favorable results were achieved with a fermentation time of 48 h, which corresponds to the peak of the logarithmic growth phase. During this period, ethanol concentration significantly dropped to 0.034 g/L at 72 h but rebounded to as much as 1.316 g/L at 96 h. It is worth noting that Pant et al. conducted a similar study using *Candida sojae* JCM 1644 and lignocellulosic hydrolysate as sources of sugar. In their research, the majority of ethanol production occurred within a 24 h fermentation period, demonstrating a volume productivity of 41.92 g/L and a product yield of 0.28 g/g. However, it should be emphasized that ethanol yield generally decreases as fermentation time extends [39].

The role of nutrients in the fermentation medium was crucial in enhancing the growth of microorganisms and, consequently, increasing the concentration of bioethanol produced. Nitrogen, in particular, served as an essential nutrient source for microorganism growth by facilitating the formation of nucleic and amino acids. Adequate nutrient availability provided the microbes with ample energy to convert glucose into ethanol [40]. Alminderej et al. demonstrated that incorporating an inorganic nitrogen source, such as ammonium chloride, resulted in a significant 106% increase in ethanol productivity [41]. Furthermore, the supplementation of calcium sources, like $CaCl_2$, led to a notable enhancement of 31.3% in ethanol productivity. Thus, in our investigation, Medium 2, which was supplied with more inorganic nitrogen sources in the form of ammonium sulfate, exhibited significantly higher ethanol production compared to Medium 3, which contained additional calcium. It is important to note that both mediums were supplemented with yeast extract and peptone.

3.5. Extracellular Enzymes Production by Aspergillus niger Unpad CC C42 Using Various Oil Palm Empty Fruit Bunches Residue

The primary objective of this study is to evaluate the suitability of four different feedstock types as substrates for the production of cellulase, xylanase, and laccase enzymes within the context of the OPEFB waste biorefinery. The four types of OPEFB feedstocks tested are untreated OPEFB, mechanically treated OPEFB, OPEFB subjected to xylanase hydrolysis, and ethanol fermentation solid residue. Table 6 displays the characteristics of the lignocellulosic composition of the four feedstocks. The variations in porosity and the quantity of cellulose, hemicellulose, and lignin in each type of OPEFB that was examined are expected to influence the activity of extracellular enzymes, specifically cellulase, xylanase,

and laccase, which are produced by the fungus *Aspergillus niger*. The experiment was conducted over a period of 9 days, with sampling intervals set at three days, in order to ascertain the duration required to achieve the desired activity level of each extracellular enzyme produced by different types of OPEFB.

Table 6. Oil Palm Empty Fruit Bunches feedstocks lignocellulosic content.

No	Composition	% Dry Weight			
		Raw Material	Mechanically Treated OPEFB	Xylanase-Hydrolyzed OPEFB	Ethanol Fermentation Solid Residue
1	NDF	79.7	73.5	79.9	66.9
2	ADF	56.3	56.9	72.4	59.7
3	Hemicellulose	23.4	16.6	7.5	7.2
4	Cellulose	36.0	37.3	62	38.7
5	Lignin	19.1	7.6	8.9	19.8

The growth pattern of *Aspergillus niger* over a nine-day span on various types of OPEFB showed a significant similarity, with the highest growth achieved on untreated OPEFB, which amounts to 1.282 × 10^7 cells/mL. This similarity can be attributed to the substantial and intact presence of the lignocellulosic components in this particular sample. Figure 4 illustrates the logarithmic growth phase within a time frame of 0 to 144 h. During this phase, cellular growth and division occur at an optimal rate, facilitated by the ample supply of nutrients. As a result, this favorable condition leads to a substantial escalation in cellular activity. During the initial stage of this phase, a significant quantity of extracellular enzymes is synthesized with the purpose of hydrolyzing lignocellulosic fibers. Subsequently, the phase of mortality spans a duration of 144 to 216 h, as evidenced by the observed decline in cellular population. In this particular stage, there is a decline in food availability, leading to the generation of compounds that possess potential toxicity that inhibit cellular proliferation. Consequently, the growth rate of *A. niger* experiences a reduction [42].

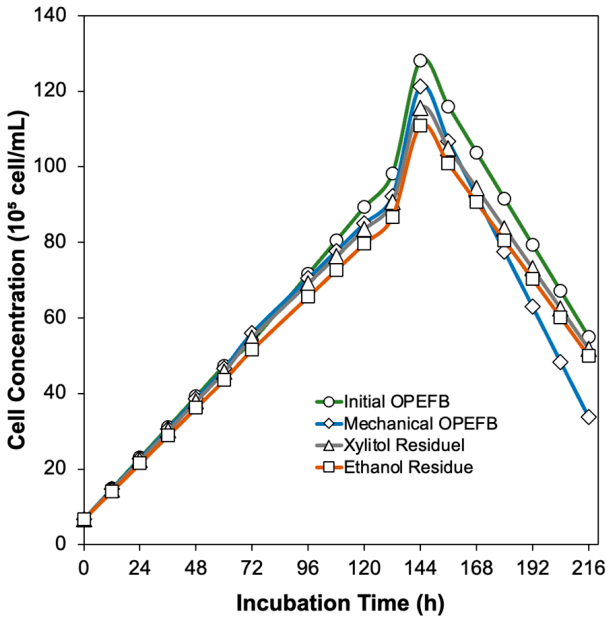

Figure 4. Cell growth of *A. niger* in different substrate types.

According to the data presented in Table 7, it is evident that the ethanol fermentation solid residue of OPEFB exhibited the highest cellulase enzyme activity and specific activity. This peak activity was observed following a nine-day incubation period and resulted

in a cellulase enzyme activity of 55.16 ± 20.24 U/mL. This observation demonstrates a positive correlation between the duration of incubation and the level of cellulase activity generated. Cellulase is an exoenzyme synthesized by *A. niger* for the purpose of catalyzing the hydrolysis of cellulose present in the OPEFB, resulting in the production of glucose for their carbon source. Both the cellulose content and the porosity of the OPEFB significantly influence the activity and synthesis of the cellulase enzyme [43]. It can be inferred that the cellulase exhibits the highest specific activity when applied to the OPEFB substrate residue derived from ethanol fermentation. This can be attributed to the extensive treatment undergone by OPEFB involving alkali, enzymes, and microorganisms. As a result, this form of OPEFB is expected to possess greater porosity, facilitating enhanced accessibility of cellulase to the desired cellulose target region.

Table 7. Cellulase, xylanase, and laccase activity.

Substrate Type	Incubation Time (Days)	Cellulase Activity (U/mL)	Xylanase Activity (U/mL)	Laccase Activity (U/mL)
Raw OPEFB	3	30.66 ± 6.68	24.46 ± 7.00	0.0000148 ± 0.0000035
	6	34.98 ± 1.14	38.19 ± 0.26	0.0000234 ± 0.0000070
	9	39.49 ± 6.86	39.96 ± 0.04	0.0000139 ± 0.0000044
Mechanically treated OPEFB	3	30.39 ± 2.88	22.92 ± 7.52	0.0000127 ± 0.0000019
	6	35.78 ± 16.67	34.30 ± 6.56	0.0000102 ± 0.0000013
	9	39.51 ± 45.89	35.61 ± 6.20	0.0000108 ± 0.0000031
Xylanase-hydrolyzed OPEFB	3	20.98 ± 4.04	21.69 ± 9.10	0.0000099 ± 0.0000086
	6	25.11 ± 3.14	29.47 ± 7.65	0.0000143 ± 0.0000035
	9	37.60 ± 5.97	38.41 ± 1.47	0.0000150 ± 0.0000045
Ethanol fermentation solid residue OPEFB	3	30.93 ± 0.89	32.53 ± 0.60	0.0000147 ± 0.0000174
	6	42.14 ± 5.02	36.42 ± 0.83	0.0000143 ± 0.0000032
	9	55.16 ± 20.24	36.60 ± 0.89	0.0000052 ± 0.0000036

Hemicellulose and lignin are the outermost components of lignocellulose. Various treatments, including physical, chemical, and biological treatments, prioritize the reduction of these two components by targeting them initially. As a result, the concentrations of both hemicellulose and lignin decrease when the treatment is given. Based on Table 7, the highest xylanase activity of 39.9590 ± 0.0378 U/mL and the highest laccase activity of 0.0000234 ± 0.0000070 U/mL were obtained from the raw OPEFB samples. These samples, which retain significant amounts of hemicellulose and lignin, create an optimal environment for *A.niger* to achieve maximal enzymatic activity. Specifically, the raw samples exhibited the highest activity levels in the production of xylanase and laccase when compared to other OPEFB substrates.

Figure 5 shows the SDS-PAGE profile of crude enzymes produced from different kinds of OPEFB. Due to the fact that the sample is in its initial enzymatic state, the resultant protein bands exhibit considerable heterogeneity and display a smeared appearance. Kiribayeva et al. reported that the molecular weight of xylanase in microorganisms ranged from 8 to 145 kDa [44]. Das et al. (2011) conducted a study wherein SDS-PAGE analysis was employed to identify the presence of three protein bands. These bands corresponded to exo-1,4-glucanase, endo-1,4-glucanase, and 1,4-glucosidase, with molecular weights of around 25, 30, and 75 kDa, respectively [45]. According to Vantamuri and Kaliwal, laccase has three isoenzymes with molecular weights of 15, 35, and 67 kDa, respectively [46]. Several protein bands, between 30 and 45 kDa, around 20 kDa, and around 100 kDa, can be observed, which are indicative of the presence of cellulase, xylanase, and laccase enzymes.

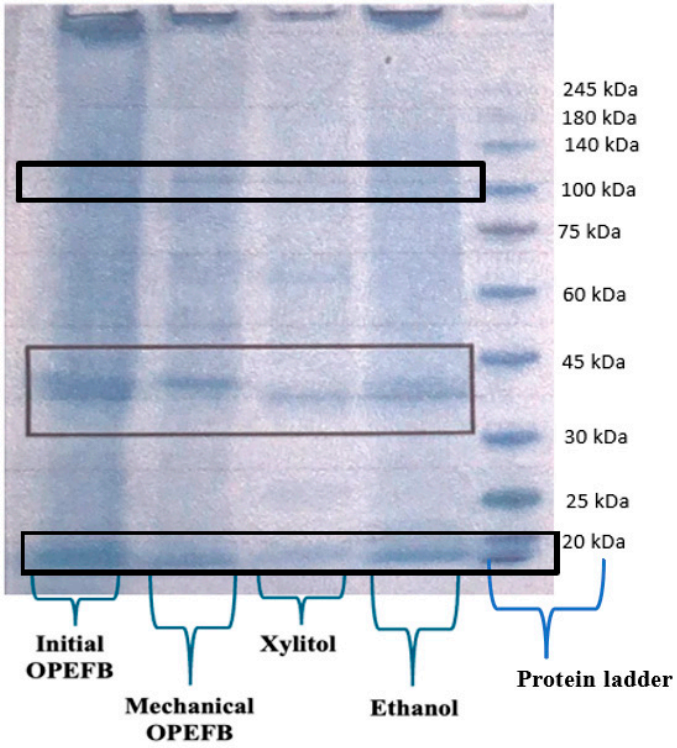

Figure 5. SDS-PAGE profile of crude enzyme produced from different kinds of OPEFB.

4. Conclusions

Our study emphasizes the promise of a two-step enzymatic hydrolysis process for integrated ethanol and xylitol production using OPEFB as a substrate. We successfully increased cellulose content to 62.0% and produced glucose at concentrations suitable for bioethanol production. The preserved hemicellulose content offers prospects for xylitol production, although the yield of xylitol was limited. Substantial ethanol production and effective enzyme generation were achieved from ethanol residue. These findings highlight the potential for integrated xylitol, enzyme, and bioethanol production in future research efforts. Key challenges to be addressed include optimizing xylitol yield and scalability. The commercial viability of this approach will depend on cost-effectiveness at scale and market demand for these products. Further research and development are needed to fully exploit the benefits of this innovative approach.

Author Contributions: Conceptualization, E.M., N.M., H.N.F. and M.M.; methodology, E.M., N.M. and H.N.F.; software, M.I.S.N., V.C. and T.W.I.; validation, E.M., H.N.F. and M.N.L.; formal analysis, P.A., N.M., H.N.F., N.R. and M.M.; investigation, M.I.S.N., V.C. and T.W.I.; resources, E.M. and H.N.F.; data curation, P.A., M.M., N.M. and N.R.; writing—original draft preparation, M.I.S.N., V.C., T.W.I. and N.R.; writing—review and editing, E.M., N.R. and M.N.L.; visualization, N.R.; supervision, E.M., N.M., H.N.F. and M.N.L.; project administration, E.M.; funding acquisition, E.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by RKDU Universitas Padjadjaran with grant number 1549/UN6.3.1/PT.00/2023, and the APC was funded by the Directorate of Research and Community Engagement Universitas Padjadjaran.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors are grateful to the University of Padjadjaran, the National Research and Innovation Agency, as well as the Higher Education, Research, and Technology for the generous funding and provision of essential facilities. The authors would like to thank PT. Condong Garut, Indonesia, who provided OPEFB samples for this study.

Conflicts of Interest: The authors declare no conflict of interest.

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