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Biorefinery design with combined deacetylation and microwave pretreatment for enhanced production of polyhydroxyalkanoates and efficient carbon utilization from lignocellulose

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ABSTRACT

Lignocellulosic biomass has emerged as a sustainable and economically viable feedstock for polyhydroxyalkanoate (PHA) production. However, it has seldom been used as part of an integrated process for effective carbon utilization. An innovative combined deacetylation and microwave pretreatment strategy (DEA-MW) and a wild-type *Cupriavidus necator* strain with the potential to utilize a lignocellulose-based carbon source and PHA biosynthesis were selected to set up the module needed to enable a new sustainable platform for biomass pretreatment and waste carbon valorization. DEA-MW pretreatment was introduced to mitigate the concentration of inhibitory acetic acid and phenolic compounds in the biomass hydrolysate. This pretreatment enhanced deacetylation and delignification, resulting in 95.50 % deacetylation and 45.30 % delignification. When DEA-MW pretreated rice husk biomass was coupled with microbial fermentation, *C. necator* promoted the fermentability of the rice husk hydrolysate. With a 3 g/L of levulinic acid from hydrolysis, as the sole carbon source, we achieved a concentration of PHA 440 mg/L with a content of 65 %. We also successfully recovered 72.22 % of lignin using a combined DEA-MW pretreatment. A detailed structural analysis showed that β-O-4, β–β, and β-5 linkages had been preserved in the recovered lignin. This study has shown that DEA-MW pretreatment plays a role in promoting biomass conversion and enhancing PHA production and opens up new possibilities for efficient and cost-effective lignocellulose-based biorefinery processes.

1. Introduction

In light of recent concerns regarding the rapid depletion of fossil resources and environmental pollution caused by the excessive use of fossil-based products, intensive research is now being undertaken to develop biorefinery technologies, that can produce value-added products from sustainable and carbon–neutral lignocellulosic biomass [1–3]. Of the various emerging bio-based products, polyhydroxyalkanoate (PHA) has garnered increasing attention because of its sustainable features such as biodegradability at ambient conditions and biocompatibility meaning that is a green alternative to fossil-based plastic [4–6].

So far, more than 150 monomer units of PHA have been identified [7]. Of these, poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) have been subjected to the most intense study [8]. However, the stiffness and crystalline characteristics of PHB limit its broader applications. The introduction of a 3-hydroxyvalerate (3HV) monomer unit into the side chain of the PHB polymer can lead to a PHBV copolymer that is more elastic, flexible, and easily processable than PHB [8,9]. Therefore, PHBV has potential applications in polymer industries as well as in biomedical fields. It has been biosynthesized by various prokaryotic microorganisms as intracellular carbon and energy storage materials [10]. PHA metabolism has long been studied by the model bacterium *Cupriavidus necator,* formerly known as *Ralstonia eutropha* [11], it was also known as *Wautersia eutropha* [12]. *C. necator* is particularly efficient at accumulating PHA, which accounts for up to 90 % of its cell dry weight. It grows rapidly and is safe for both the environment and humans [13].

The main problem preventing their immediate industrial application is that PHAs are more expensive to produce than fossil-based plastics. To overcome this, research is focusing on how best to use renewable and low-cost lignocellulose material [14,15]. However, the intrinsic heterogeneity of cellulose-hemicellulose-lignin in lignocellulose makes it recalcitrant to chemical and enzymatic deconstruction [16]. Therefore, a pretreatment step is often required to break the recalcitrant structure

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of biomass and improve the biomass conversion of cellulose and hemicellulose contents into fermentable sugars for downstream applications [17].

Of the pretreatments available, microwave-assisted hydrothermal pretreatment is being widely studied because it has a high biomass conversion efficiency and a short reaction time [18,19]. As previously reported, a hemicellulose fraction solubilized by an acid-catalyzed pretreatment improves the access of cellulose to hydrolysis [20]. However, a previous study also showed that severe microwave pretreatment conditions are always required for the hydrolysis of cellulose, specifically when raw biomass is used as feedstock [21,22]. The severity of these pretreatment conditions may degrade the hemicellulose-based sugars and lignin and form toxic byproducts such as acetic acid, vallinic acid, *para*-coumaric acid, syringaldehyde, which have been reported to impede the production of microbial PHA from lignocellulose [13,23].

The addition of a deacetylation step with an alkaline solution prior to the microwave-assisted hydrolysis step is recommended to eliminate the acetyl groups from the backbone of the xylan [24,25]. Deacetylation improves the efficiency of both biomass hydrolysis and the subsequent fermentation processes [25–27]. The reasons for improving biomass hydrolysis and subsequent fermentation processes previously investigated for bioethanol production from biomass [28,29]. It shows that i) deacetylation mitigates the degradation of hemicellulose and the formation of inhibitors, resulting in reduced inhibition in the subsequent downstream processes [28,30]; ii) deacetylation leads to partial delignification and the removal of silica, which plays an important role in enhancing the specific surface area of biomass and creating substantial nanoscale porosity to biomass, thus promoting efficient cellulose hydrolysis [31]; iii) deacetylation suppresses the formation of acetic acid during the hydrolysis step, hence mitigating acetic acid inhibition thus improve rate fermentation processes [32]. Considering these advantages of microwave heating and deacetylation, we can anticipate that the combined deacetylation-microwave pretreatment of lignocellulosic biomass will significantly reduce the formation of inhibitory compounds in the biomass hydrolysates and improve its fermentability for PHA production. This approach is extremely limited to PHA production from biomass.

Moreover, the deacetylation pretreatment with an alkaline solution ionizes phenols and solubilizes the low molecular weight of lignin in the deacetylation liquor [31,33]. Recently, lignin extraction and valorization have gained more recognition as contributing to sustainable and economically viable biorefinery processes [34]. The "lignin-first" pretreatment approach has been devised to extract lignin and improve sugar yield [35]. Simultaneously, the extraction of valorizable lignin that does not compromise the efficiency of carbohydrate extraction not only improves the economic viability of lignocellulose-based biorefining but also minimizes the waste generated by the biorefinery framework [36]. Using pretreatment to boost PHA production while extracting valuable lignin is challenging because it often leads to the formation of uncondensed lignin [37]. Moya et al. [28] reported the use of alkaline deacetylation pretreatment before the acid hydrolysis of biomass, which not only removes a great deal of the lignin fraction but also removes such inhibitors as furan, phenolics, and, particularly, acetic acid from acidic biomass hydrolysate. Consequently, this approach enhances the fermentability of biomass hydrolysates, although it is important to note that it does not recover the lignin. In a related study, Chu and coworkers [29] have recently reported that deacetylation and steam explosion pretreatment in the presence of sodium carbonate and sodium sulfite enhance cellulose hydrolysis and its microbial conversion efficiency. However, deacetylation pretreatment removed 45 % of lignin from biomass but the structural characteristics of lignin are still unclear [17]. Interestingly, Wu et al. [31] reported an intriguing observation that lower-temperature alkaline pretreatment of biomass extracts the partial fraction of lignin, retaining up to 40 % of its native β-O-4 linkages. This significant finding suggested that solubilized lignin fragments undergo limited changes during the alkaline pretreatment process. On the other hand, microwave-assisted processes have been considered efficient for extracting lignin from biomass [38]. Microwave techniques proved to be a promising alternative to conventional heating used in the extraction of lignin [39]. The time required to complete of reaction is significantly reduced as compared to conventional heating. The advantage of the use of a microwave-assisted process is the excitation of polar molecules and ions, which directly transform this energy into heat via dipole–dipole rotation and friction in the matrix [40]. Therefore, combining deacetylation with microwave heating is a promising choice for the pretreatment of the biomass. However, it is noteworthy that, to the best of our knowledge, the extraction of lignin and subsequent PHA production from biomass is limited.

Based on the above considerations, the present study aims to design a biorefining process with combined deacetylation and microwave pretreatment followed by microbial fermentation to boost the production of PHAs from rice husks (RHs). RHs are sustainable, low-cost, and abundantly available in Catalonia, Spain. To achieve a circular economy, this agricultural waste should be recycled and used to reduce the heavy dependency on fossil fuels. This research investigates the effect of DEA-MW on biomass composition, structural characteristics, and chemical hydrolysis, and its role in mitigating the formation of microbial growth inhibitors. This step is crucial to improve the efficiency of subsequent microbial fermentation processes. Moreover, the lignin isolated and recovered by DEA-MW pretreatment was characterized by using ATR-FTIR (attenuated total reflectance Fourier transform infrared spectroscopy) and 2-D HSQC NMR (two-dimensional heteronuclear single quantum correlation), demonstrating that extracted lignin valorization potential was significant.

2. Material and methods

2.1. Raw biomass and chemicals

In this research, rice husk was used as a representative lignocellulosic carbon substrate. Rice husk (RH) biomass was obtained from the Cambra Arrossera del Montsià, Catalonia, Spain, and oven-dried at 105 °C to achieve a moisture content below 5 %. The raw RH was milled and then sieved through a 400 μ mesh for pretreatment and fermentation purposes. Sodium hydroxide (NaOH), and sodium sulfite (Na_2SO_3) were purchased from Sigma Aldrich. Sulfuric acid was obtained from Fisher Chemical.

2.2. Biomass Compositional analysis

The composition of RH was analyzed using the method described by Sluiter et al. [41], as presented in Table 1 (see supporting information). In brief, 0.3 g of oven-dried RH biomass was treated with 3 mL of 72 % w/w of sulfuric acid in glass test tubes. This mixture was then placed in a water bath at 30 °C ± 2 °C for 60 min and stirred every 10 min. After 60 min of the hydrolysis reaction, the tubes were taken out of the water bath. The mixture was then transferred to autoclave bottles, and the acid concentration was diluted to 4 % by adding 84 mL of deionized water. The autoclave bottles were placed in an autoclave rack and autoclaved at 121 °C ± 2 °C for 60 min. Once the autoclave cycle had finished, the mixture was allowed to cool to room temperature, after which the suspensions of solid residue and liquid hydrolysates were separated via vacuum filtration. The solid residue recovered was used to determine the acid-insoluble lignin by the gravimetric method. The liquid hydrolysate samples were neutralized with barium carbonate ($BaCO_3$) and analyzed using High-Performance Liquid Chromatography (HPLC) equipped with an Aminex HPX-87H column. The eluent was an aqueous solution of sulfuric acid (5 mM H_2SO_4) and the flow rate was 0.6 mL/min at a column temperature of 80 °C. Glucose, xylose, galactose, arabinose, and acetyl content (determined as acetic acid), furfural, and 5-hydroxymethylfurfural (HMF) in the biomass hydrolysate were analyzed on a

Table 1
Biochemical composition, carbohydrate sugar recovery, acetyl groups, and lignin removal of untreated RH and deacetylated RH.

Treatment	Glucan content %	Xylan content %	Lignin content %	Acetyl content %	Solid yield %	Glucan recovery %	Xylan recovery %	Lignin removal %	Acetyl removal %
Raw RH	34.51 ±0.44	16.46 ±0.37	25.18 ±0.24	2.55 ±0.06	–	–	–	–	–
[a]DEA	48.32 ±0.31	18.74 ±0.12	23.29 ±0.16	0.20 ±0.07	71.10	99.55 ±0.60	82.25 ±0.20	34.39 ±0.91	94.39 ±0.04
[b]DEA-MW	53.23 ±0.26	15.96 ±0.07	21.04 ±0.13	0.18 ±0.06	64.66	99.73 ±0.24	63.70 ±0.70	45.30 ±0.71	95.50 ±0.18

Data are presented as the average value ± standard deviation. [a,b] Glucan and xylan recovery, and the removal of lignin and acetyl groups from DEA-pretreated RH and DEA-MW pretreated RH, were calculated based on the pretreated solid yield obtained after deacetylation

refractive index detector (RID). The sample peaks were identified and quantified by comparing the retention times and plotting the calibration of the curve using analytical standards glucose, xylose, arabinose, galactose, acetic acid, furfural, and HMF.

2.3. Biorefinery design

We designed biorefinery configurations that incorporate combined deacetylation-microwave pretreatment, dilute acid hydrolysis, and microbial fermentation. Our aim was to improve the biomass fractionation and the microbial utilization efficiency of the RH biomass (Fig. 1**)**.

2.3.1. Deacetylation and biomass hydrolysis

2.3.1.1. Deacetylation with conventional heating (DEA). The alkaline deacetylation process was carried out as outlined by Wu et al. [31]. Briefly, oven-dried rice husks RH (10 g) were treated by impregnating them with a solution containing 4.8 % w/w of sodium hydroxide (NaOH) and 3 % w/w sodium sulfite (Na_2SO_3). The liquid-to-biomass ratio in the impregnation solution was maintained at 12:1 (g to mL), with a composition of 4.8 % w NaOH and 3 % w Na2SO3. The mixture was then heated at 80 °C in the oil bath for 180 min. Subsequently, the mixture was allowed to cool, and the solid was separated by vacuum filtration. The liquid fraction (deacetylation liquor), was stored for the lignin recovery. The deacetylated RH solid was then washed with deionized water until the pH of the washing liquid reached 7. Finally, the solid was dried in an oven to reduce its moisture content. Compositional changes in the deacetylated RH biomass were determined by using the NREL method as described above.

2.3.1.2. Deacetylation with microwave (DEA-MW) mediated deacetylation and delignification. The alkaline deacetylation was performed in a microwave Milestone Synthwave Single Chamber Reactor. In typical experiments, 10 g of oven-dried RH biomass was added to a PTEF vessel containing the solution of sodium hydroxide (NaOH, 4.8 %w/w) and sodium sulfite (Na_2SO_3, 3.0 % w/w), with a RH biomass-to-liquid ratio of 1:12 (g to mL). The agitation was kept constant at 600 rpm throughout the experiment and the mixture was heated at 80 °C for 90 min at a microwave power of 400 W. Then, the reaction mixture was allowed to cool, and the deacetylated RH solid fraction was separated from the liquid fraction by vacuum filtration. The black liquid fraction was stored at 4 °C to recover the lignin. The deacetylated RH solid was washed with deionized water until the pH of the washing reached 7 and then oven-dried. Compositional changes in the DEA-MW pretreated RH biomass were determined by using the NREL method as described above. The pretreated solid yield and degree of deacetylation were calculated as

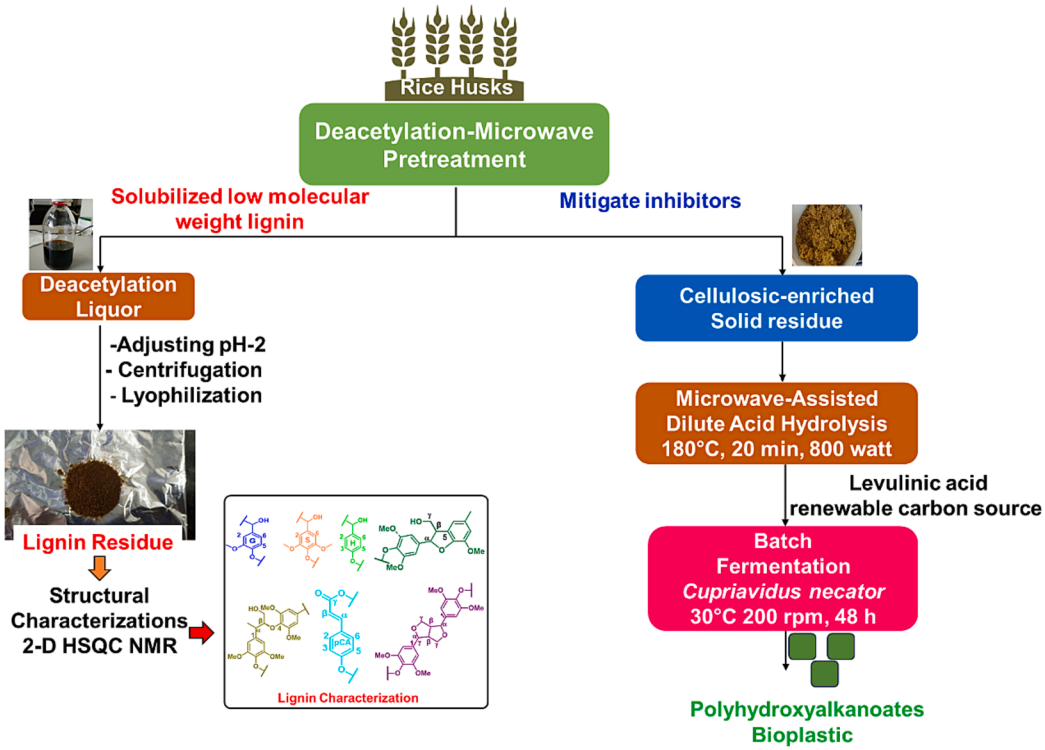


Fig. 1. Biorefinery design for the production of PHA biopolymer from RH biomass via deacetylation-microwave pretreatment and microbial fermentation and concurrent extraction and characterization of lignin.

follows.

$$\%\ \text{Pretreatment solid yield} = \frac{\text{Weight of solid after deacetylation}}{\text{Weight of solid before deacetylation}} * 100 \quad (1)$$

$$\%\ \text{Degree of deacetylation} = 1 - \frac{[\text{acetyl groups in the deacetylated RH (g)}]}{[\text{acetyl groups in the raw RH (g)}]} * 100 \quad (2)$$

2.3.1.3. Microwave-assisted hydrolysis. In the typical experiments, 0.3 g of deacetylated RH biomass was loaded in the glass reactors containing 10 mL of 0.5 M sulfuric acid solution. The glass reactors containing RH in 0.5 M sulfuric acid solution were fitted with loose PTFE caps to ensure pressure balance. The reaction chamber was filled with 250 mL of deionized water. The chamber was pre-pressurized with nitrogen at 30 bar to prevent the mixture from boiling. The mixture was stirred at 600 rpm throughout the experiment. The experiment was carried out at 140 °C, 160 °C, 180 °C, and 210 °C for 20 min of reaction time and used a microwave power of 1200 W. After the reaction had finished, the suspensions were centrifuged to separate the liquid hydrolysates and solid residue. An aliquot of liquid hydrolysates was filtered through nylon 0.45 μm and analyzed by HPLC to determine the yield of levulinic acid, furfural, acetic acid, 5-hydroxymethylfurfural (5-HMF), glucose, and xylose following equation (3).

$$\%\ \text{Yield of water} - \text{soluble products} = \frac{C*V}{\text{Weight of raw RH biomass}} * 100 \quad (3)$$

where, C is the concentration (mg/mL) measured by HPLC of glucose, xylose, acetic acid, levulinic acid, formic acid, furfural, and HMF and V is sample volume in mL.

2.3.2. Microbial fermentation

Cupriavidus necator DSM 545 strain, obtained from Leibniz Institute DSMZ, Germany, was used for PHA production with levulinic acid-enriched RH hydrolysate as the sole carbon source. The strain was maintained by monthly subculture on agar plates with DSMZ medium 1 (5 g/L peptone, 3 g/L beef extract). For seed culture, a single colony from an agar plate was aseptically transferred into 10 mL of DSMZ medium 1 solution and grown at 30 °C, 200 rpm for 24 h [10]. After cell growth, cells were centrifuged in a Benchtop centrifuge (Thermo Fisher Scientific) at 9000 rcf * g for 10 min, then collected and the pellet was washed with phosphate buffer. Subsequently, the washed cell pellet was transferred into 100 mL of modified mineral medium [42] containing 3g/L levulinic acid from the hydrolysis process. This concentration was achieved by diluting the original RH hydrolysate with the mineral medium. Subsequently, cultivation was conducted in 500 mL of the conical flask at pH-7.0, 30 °C, and 200 rpm for 48 h. The C/N ratio was adjusted to 30 by varying the concentration of $(NH_4)_2SO_4$ as described by Jaremko and Yu [11]. The modified mineral medium contained the following elements (g/L): (3.0) KH_2PO_4, (5.86) $Na_2HPO_4{\cdot}2H_2O$, (2.0) $(NH_4)_2SO_4$, (0.4) $MgSO_4{\cdot}7H_2O$, (0.02) $CaCl_2{\cdot}2H_2O$, (0.12) $MnCl_4{\cdot}4H_2O$, (0.12) $(NH_4)_5[Fe(C_6H_4O_7)]$ and 1 mL of trace elements solution as described in [42].

2.3.3. Cell dry weight (CDW)

The cell growth during the batch culture study was determined using cell dry weight. Specifically, 5 mL samples were withdrawn at 12, 24, 36, 48, 60, and 72 h, and these samples were centrifuged at 9000 rpm for 10 min at 4 °C to separate cell pellets from the supernatant. The separated cell pellets were then oven-dried until a constant weight was achieved. The PHA content within bacterial cells was calculated by determining the ratio of PHA concentration to cell dry weight as described by Kavitha S et al. [43].

2.3.4. PHA quantification

The PHA was quantified by methanolysis of cell pellets as described by Mohan et al. [13] and and Braunegg et al.[44]. Briefly, 5 % w sulfuric acid and 30 % w methanol in chloroform (total volume 3 mL) with 0.5 g/L of benzoic as an internal standard were added to cell pellets, and the mixture was then incubated at 105 °C for 180 min. After the methanolysis reaction had finished, 1 mL of 1 M NaCl solution was added, and the mixture was vortexed for 40 s. The chloroform layer at the bottom was transferred into the clean bottle containing approximately 30 mg of sodium sulfate to remove traces of the aqueous layer. Samples were filtered via a 0.22 μm nylon filter, identified using a gas chromatography-mass detector (GC–MS), and quantified using a gas-chromatography-flame ionization detector (GC-FID). The GC analysis was performed using an Agilent 6890 equipped with a capillary column, 30 m x 0.25 mm i.d., 0.25 μm. The GC conditions were as follows: 10 °C /min ramp from 40 to 80 °C and temperature increased in 25 °C steps from 80 to 250 °C, and was held at 250 °C for 3 min. Nitrogen was the carrier gas with a flow rate of 30 mL/ min. Analytes were identified and quantified by GC–MS and GC-FID at a temperature of 275 °C with a hydrogen flow of 30 mL/min and an airflow rate of 350 mL/min. Polymer contents were determined by constructing a calibration curve with standards methyl(R)-3-hydroxybutyrate (3HB) (Sigma Aldrich, purity-99 %) and methyl(R)-3-hydroxyvalerate (3HV) (Sigma Aldrich, purity- $\geq$ 98 %). The amount of PHA accumulated by *C. necator* was calculated as follows.

$$\text{PHA content in \% w} = \frac{[\text{PHA}]}{\text{CDW}} * 100 \quad (4)$$

where the [PHA]-concentration is expressed in mg/L by GC and CDW-cell dry weight in mg/L.

2.3.5. Extraction of PHA polyester

Chloroform was used to extract the PHA biopolymer from the cell pellets. A total of 20 mL of chloroform was mixed with 0.5 g of lyophilized cell pellets and then the mixture was warmed to 45 °C for 3 h. The resulting suspension was filtered through a 0.22 μm syringe filter to remove cell debris. The filtrate was poured into the ice-cold methanol to precipitate PHA biopolyester. The precipitated PHA was redissolved in chloroform, which was then evaporated on the rotary vapor and dried to obtain the PHA polyester at 40 °C.

2.3.6. Lignin extraction and recovery

Lignin was isolated and recovered from deacetylated liquor by decreasing its pH to 2 with an HCl solution as described by Wu et al. [31]. When the pH of the deacetylation liquor was decreased, lignin precipitated. The precipitated lignin was washed with deionized water several times and lyophilized to a dry solid. The removal and recovery of lignin were calculated as follows.v

$$\%\ \text{Lignin removal} = 1 - \frac{[\text{Lignin in pretreated solid residue}*\text{pretreatment solid yield}]}{[\text{Lignin in raw RH biomass}]} * 100 \quad (5)$$

$$\%\ \text{Lignin recovery} = \frac{\text{Mass of lignin in pretreated solid}}{\text{Lignin in raw RH biomass}} * 100 \quad (6)$$

2.3.7. Analytical methods

2.3.7.1. ATR FTIR (Attenuated total internal reflectance-Fourier transform infrared) analysis. The chemical groups that changed after deacetylation were examined using attenuated total internal reflectance-Fourier transform infrared (ATR-FTIR, JASCO). The samples were measured by averaging 32 scans from 500 to 4000 cm^{-1} at a spectral resolution of 1 cm^{-1}.

2.3.7.2. XRD (X-ray diffraction) analysis. The XRD patterns of untreated and deacetylated RH were obtained from the Brucker D8-Discover diffractometer scanning from 5 to 30° at a rate of 3° per minute. The crystalline index (CI) was calculated using the Segal method (equation (7).

$$\%\ CI = (I_{020} - I_{am})/I_{020} \quad (7)$$

where, I_{020} is peak intensity at a 2θ angle close to 22°, which represents the crystalline part of cellulose in biomass, and I_{am} is peak intensity at a 2θ angle close to 16, which represents the semicrystalline part of cellulose in biomass.

2.3.7.3. ESEM (Environmental scanning Electron Microscopy) and EDX (Energy dispersive X-ray) analysis. To investigate morphologies and microstructural changes in pretreated RH samples, ESEM analysis was conducted as described in [45]. In addition, EDX was coupled with ESEM to verify changes in elements (C, O, S, Si) present after deacetylation.

2.3.7.4. 2D HSQC NMR (Heteronuclear Single-Quantum Correlation) analysis. The lignin obtained from DEA-MW pretreatment was analyzed by 2D HSQC NMR as previously described in [31,45,46]. In detail, an isolated lignin sample of 50 mg was dissolved in 0.6 mL of DMSO-d_6 solvent and analyzed on a Bruker Avance 400 MHz. MestReNOVAx64 software was used to process 2D-HSQC NMR spectra. The integral values for the $S_{2/6}$, G, and H units were calculated as described in [47].

2.3.7.5. ^1H NMR (Proton nuclear magnetic Resonance) analysis. The structural characterization of PHA extracted from microbial biomass was conducted using ^1H NMR (Bruker Avance 400 MHz). For this analysis, 5 mg of PHA sample was dissolved in 1 mL of $CDCl_3$ solvent and TMS (Tetramethylsilane) was used as the reference standard. The ^1H NMR spectra were processed with MestReNOVAx64 software.

3. Results and discussion

The fundamental challenge presented by the bioconversion of biorefinery-based waste to PHA is that most current biomass pretreatments and hydrolysis processes generate toxic microbial growth inhibitors and solid lignin waste within the biorefinery framework. These issues prevent lignocellulosic materials from efficiently producing PHAs and effectively utilizing carbon. Previous studies have shown that deacetylation before biomass hydrolysis is critical to improving bioconversion, as it removes inhibitory compounds [24,29]. Deacetylation also solubilizes the lignin fraction, the attractive properties of which – low molecular weight, a higher percentage of β-O-4, β–β, and β-5 linkages, and hydrophilic properties – make it a promising aromatic carbon source for the production of renewable products via chemical or biological pathways [36,48]. Based on this understanding, we combined deacetylation and microwave pretreatment (DEA-MW) and used it to mitigate the formation of growth inhibitory compounds, the fractionation of lignin, and the fermentation of rice husk hydrolysate. DEA-MW pretreatment is introduced into the current biorefining process to enable PHA production and optimize carbon efficiency so that the

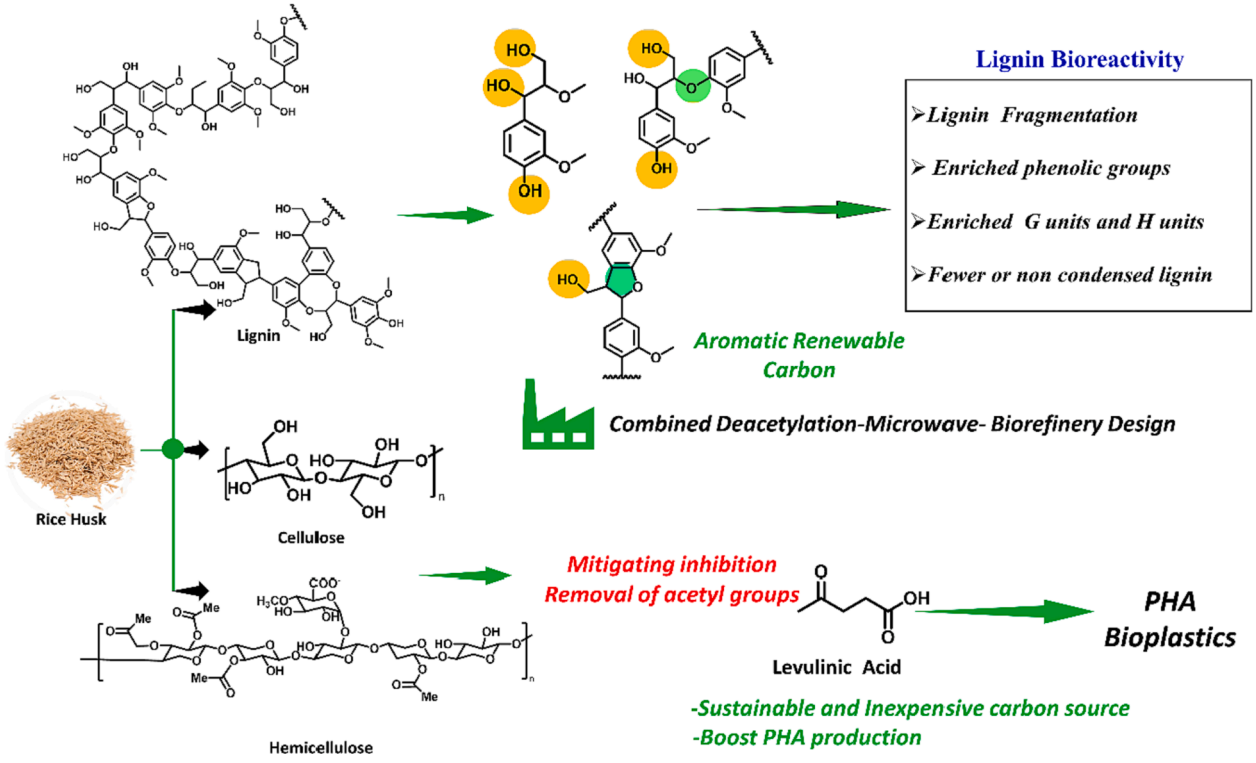


Fig. 2. Proposed biorefinery design using deacetylation-microwave pretreatment to improve PHA production and the efficiency of the carbon utilization of rice husk.

lignocellulosic biorefinery framework is more efficient (Fig. 2). Considering these features, the DEA-MW pretreatment employed a fermentation process and was compared with the deacetylation by conventional heating in terms of the mitigation of the formation of inhibitory compounds, lignin fractionation, and PHA yield.

3.1. Effect of combined deacetylation-microwave pretreatment on solid yield and biomass compositions

Previous literature reports have shown that deacetylation enhances microbial fermentability of biomass hydrolysates [24,25,29], with temperature, time, and alkali concentration all these are influencing the extent of the deacetylation. Specifically, when the deacetylation of biomass was performed at room temperature, it required a longer residence time and a higher concentration of alkali. On the other hand, at higher temperatures (80 °C-100 °C), a lower concentration of alkali and shorter residence time was employed. Chen and coworker. [49] demonstrated that increasing the deacetylation temperature from 60 °C to 80 °C, resulted in the increased extraction of lignin up to 23 %. This observation could be attributed to the hydrolysis of lignin-carbohydrates complex (LCC) linkages and the ionization of phenolic groups in lignin fragments. As NaOH-Na_2SO_3-based deacetylation has been successfully employed on agriculture waste by Wu et al. [31], we aim to compare this method using microwave heating, microwave-assisted alkali pretreatment had previously demonstrated its efficacy in selectively removing acetyl groups and efficiently extracting lignin from biomass [39,50,51]. At conventional heating, the deacetylation of raw RH biomass was carried out using a solution of NaOH (4.8 %w/w)+ (3 % Na_2SO_3) at 80 °C, 3 h, resulting in the removal of 94.39 % of acetyl groups, solubilization of 20 % of carbohydrates, and 34.39 % of lignin (Table 2). On the other hand, microwave-assisted deacetylation using NaOH (4.8 %w/w) + (3 % Na_2SO_3), which could retain the majority of glucan (99.77 %) in water-insoluble fraction, removed of 95.50 % of acetyl groups, and 45.50 % lignin within water-soluble fraction in just 90 min (Table 2).

Previously, Singh et al. [52], reported that lignin extraction increased to 82 % through microwave-assisted alkaline pretreatment under the following conditions: microwave power 900 W, 15 % NaOH, Temperature-121 °C, Time 1 min. A significant increase in the extraction of lignin is much higher than conventional hydrothermal-assisting heating. The potential of microwave heating to extract higher lignin due to the change of heterogenous structure of lignocellulose and then improve the reactivity. Thus, most of the hemicellulose fractions are promptly degraded during the microwave-assisted process, which is beneficial for the efficient delignification of biomass [51]. Jin et al. [53], conducted pretreatment of biomass using microwave-water, microwave-NaOH, and microwave-$Ca(OH)_2$. Their results of microwave-assisted pretreatment revealed that microwave-assisted alkali pretreatment led to enhancement in cellulose content. Dávila et al.[50] optimized microwave-assisted alkaline process to fractionate simultaneously cellulose and lignin from vine shoots biomass. Their results of microwave-assisted alkali pretreatment revealed that at optimum pretreatment conditions (150 °C, 6 % W NaOH, and 30 min, microwave power- 400 W) they could achieved to extract 57 % w of cellulose, and 21.84 % w of lignin from biomass. To complement to those work, we optimized the microwave-assisted alkaline pretreatment of RH biomass at mild conditions (4.8 %w/w NaOH + 3 %w/w Na_2SO_3, 80 °C, microwave power-400 W). Our result at this optimum condition have advantages of the high amount of removal of acetyl groups (95.50 %) recovery of glucose (99.75 %) and lignin (72.22 %). On top of that we analyzed extracted lignin fraction on 2D HSQC NMR to elucidate the structural characteristics of lignin fraction, which shows that β-O-4, β–β, and β-5 linkages had been preserved in the recovered lignin. Importantly, our proposed biomass pretreatment (DEA-MW) allows for the extraction of lignin without compromising subsequent carbohydrates-based PHA production. This results address the dilemma faced by current biorefineries to decide in between carbohydrates -first or lignin-first approach, demonstrating the efficiency of our proposed DEA-MW pretreatment in the context of biomass valorization and effective carbon utilization.

Both DEA and DEA-MW pretreatments led to a similar amount of deacetylation, but these pretreatments differed in the extent of lignin removal (Table 2). These deacetylation processes – deacetylation and delignification – were then assessed using ATR-FTIR, XRD, ESEM and elemental analysis and also to see how they improved a microbial PHA-based fermentation.

Table 2
The effect of microwave temperature on glucan to levulinic acid (LA) conversion (reaction time of 20 min).

Temperature (°C)	Heating type	Glucose (g/L)	LA (g/L)	% Yield per gram of RH	
				Glucose %	LA%
120	microwave	2.65 ± 0.15	0.30 ± 0.65	8.83	0.99
160	microwave	5.97 ± 0.11	3.54 ± 0.48	19.9	11.8
180	microwave	0.32 ± 0.21	6.32 ± 0.08	1.06	21.06
180	[b]oil bath	4.57 ± 0.21	2.52 ± 0.21	15.23	8.4
210	microwave	0.19 ± 0.02	5.63 ± 0.05	0.63	18.76

[a]Yield of LA(%) $= \frac{\text{Conc LA}\left(\frac{g}{l}\right)*V\,(L)}{\text{Wt of RH}\,(g)}*100$, RH is 0.3 g of DEA-MW pretreated sample, 0.5 M H_2SO_4, volume is 10 mL (0.01*L*), and microwave power is 1200 W. Data is presented as the average value ± standard deviation. [b] Conventional oil bath heating for 8 h.

3.2. Characteristics of the deacetylated biomass

3.2.1. ATR-FTIR analysis

ATR-FTIR analysis was carried out to gain some basic information about DEA and DEA-MW pretreatments and their effect on deacetylation. Fig. 3 displays FITR spectra showing the structural characteristics of raw RH, DEA-pretreated RH, and DEA-MW pretreated RH samples. The changes in structural characteristics can be observed in FITR spectra, either through the disappearance of bands or the reduction of the content of functional groups. The absorption band at 1720 cm^{-1} corresponds to the carbonyl group (-C = O) stretching and at 1358 cm^{-1} and 1260 cm^{-1}attributed to phenol hydroxyl (Ar-OH) stretching and methoxy group (-O-CH_3), which disappears after deacetylation pretreatment. This is likely due to the addition of Na_2SO_3 (3 % w/w) during the alkaline pretreatment, which enhanced the elimination of lignin and acetyl groups while preserving most of the glucan content in DEA-pretreated RH and DEA-MW-pretreated RH (Table 2). The nucleophilic addition of $SO3^{-2}$ facilitates the solubilization of a large amount of lignin fragmentation as lignin in biomass becomes more ionizable and hydrophilic [31]. However, DEA-MW pretreatment showed a more significant decrease in peaks at 1360 cm^{-1} (corresponding to O-CH_3) and 1245 cm^{-1} (corresponding to C-O stretching of the acetyl groups present in hemicellulose and lignin) in DEA-MW-pretreated RH sample compared to DEA-pretreated RH. This observation suggests that DEA-MW pretreatment efficiently extracts the lignin as compared to deacetylation performed using conventional heating (Table 2). This is likely due to the microwave heating and alkali agents NaOH + Na_2SO_3 creating the synergetic effect. The microwave-assisted process offers a rapid and efficient means of applying heat to biomass, inducing transformative changes in its recalcitrant and heterogeneous structure. This heightened reactivity is further through reaction with NaOH + Na_2SO_3. Specifically, these alkali agents contribute to the rendering of lignin more hydrophilic and ionizable. Consequently, this modification

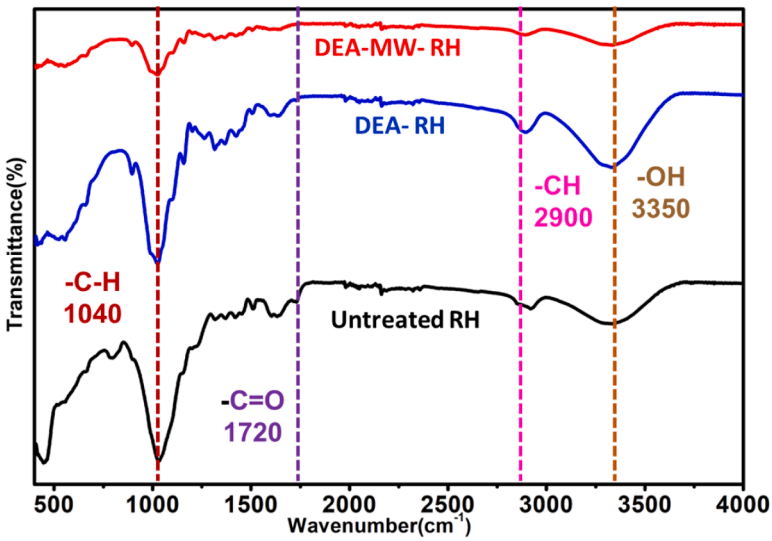


Fig. 3. ATR-FTIR spectra of untreated RH and pretreated RH after deacetylation.

facilitates more efficient dissolution of lignin. The enhanced dissolution of lignin exposed a large amount of xylan, which could be subsequently deacetylated by added hydroxide ions (OH^-) The same finding was noted in previous studies conducted by Sun et al. [38], Dávila et al. [50], and Janker-Obermeier [54]. The absorption bands at 3350 cm^{-1} and 2900 cm^{-1} in Fig. 3 indicate the - OH stretching and C-H stretching, reducing in DEA pretreated and DEA-MW pretreated samples compared to that in untreated samples. Upon comparing the DEA-pretreated RH sample and DEA-MW-pretreated RH, DEA-MW-pretreated RH indicated a more significant decrease –OH stretching and C-H stretching. This observation attributed to hydrogen bonds partially broken methyl and methylene groups of cellulose deconstructed, resulting in improved accessibility of cellulose for subsequent hydrolysis process. The peak appears at 1040 cm-1 in DEA- pretreated sample and DEA-MW pretreated samples confirming cellulose content retained after deacetylation pretreatment (Table 2).

3.2.2. *Elucidating crystalline, morphological, and elemental changes after deacetylation*

As we proposed above, the deacetylation pretreatment might also alter the structure of the cellulose in biomass, making it more amenable for downstream applications. Thus, the crystalline, morphological, and elemental changes that occurred during the deacetylation were assessed by X-ray Diffractometer (XRD), Environmental Scanning Electron Microscopy (ESEM), and Energy-dispersive X-ray Spectroscopy (EDX). The XRD spectra and ESEM images are shown in Fig. 4**a,b.** The XRD patterns and crystalline index (CrI) of untreated RH after deacetylation are shown in Fig. 4**a.** It is noteworthy that CrI was slightly lower and sharpness was observed in the amorphous region in the deacetylated samples than in the untreated RH samples, which could be attributed to the removal of acetyl groups disrupting of the regular structure of the cellulose.

It ranged between 31 % and 25 % for untreated RH, DEA-pretreated RH, and DEA-MW pretreated RH. This observation indicated that cellulose crystallinity and macromolecular structure were still retained after deacetylation, which is consistent with the FTIR and biocompositional analysis mentioned above (Table 2).

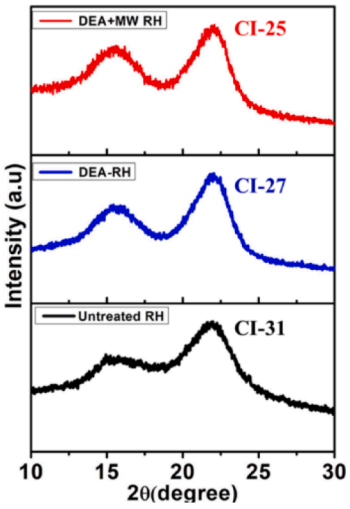
As shown in Fig. 4**b.** the untreated RH substrate displayed a regular, tough structure with a smooth and dense surface. However, after deacetylation pretreatments, there were holes and cracks on the surface of the biomass. The alkaline deacetylation pretreatment with 4.8 % NaOH readily ionized phenol groups of lignin in biomass and the addition of 3 % Na_2SO_3 incorporated the sulfonic group into the lignin molecules, which enhanced the hydrophilicity and solubility of lignin and led to the extraction of more lignin [29]. The sulfur content (Fig. 4**b**) in the deacetylated RH substrates is increased, which confirms the nucleophilic attack of SO_3^{-2} on the lignin molecules [55]. Moreover, the alkaline deacetylation pretreatment exhibited the potential to solubilize the silica in the biomass. The elemental analysis presented in (Fig. 4**b**) shows that silica content in deacetylated samples is significantly lower than in untreated RH. The partial removal of lignin and silica via deacetylation from RH biomass generates cracks and nanoscale porosity on the surface of the biomass, (Fig. 4**b),** thus improving the accessibility of cellulose for hydrolysis [31].

3.3. *Microwave-assisted dilute acid hydrolysis*

Untreated RH, DEA-pretreated RH, and DEA-MW pretreated RH samples were hydrolyzed to produce levulinic acid using microwave-assisted dilute acid treatment (Fig. 5**).** In Table 3 we systematically optimized the reaction conditions. The effect of temperature on the microwave-assisted hydrolysis of cellulosic biomass to levulinic acid was assessed. By varying the temperature initially from 120 °C to 210 °C for 20 min, the concentration of glucose decreased as the temperature increased because it was converted into levulinic acid, hence increasing the yield. The highest yield of 21.06 % of levulinic acid per gram of RH substrate was achieved at optimum conditions of 180 °C, 20 min, with 6.32 g/L of levulinic acid. At a higher temperature than the optimum 180 °C, the levulinic acid degrades and the yield of levulinic acid is lower (Table 3**).** This observation is in line with the findings of previous studies [56,57]. When the same sample was heated in an oil bath at the same temperature for 8 h, the yield was 8.4 % levulinic acid. When conventional heating is used, the reaction time is always longer and the yield is always lower than when microwave heating is used [57].

Fig. 5**.** shows that levulinic acid (carbon source) and other inhibitors in hydrolysates after microwave-assisted dilute acid hydrolysis of raw RH DEA-pretreated RH, and DEA-MW pretreated RH. It is noteworthy that the deacetylation suppressed the formation of acetic acid in DEA and DEA-MW-pretreated RH hydrolysate, while raw RH hydrolysates yielded 2.18 % acetic acid (0.67 g/L acetic acid). In addition, total furan and phenolic concentrations in DEA and DEA-MW-pretreated RH hydrolysates were significantly reduced, as mentioned above, so the deacetylation effectively partially solubilizes the xylose and lignin contents.

(a)



(b)

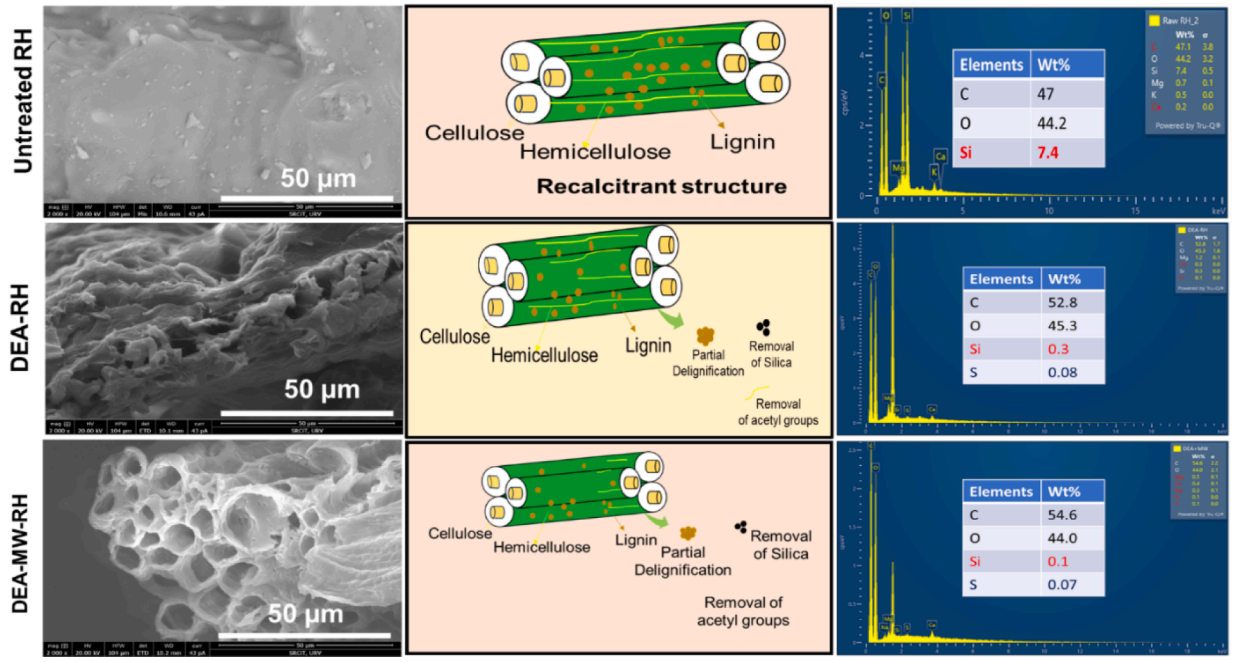


Fig. 4. A) XRD spectra, b) ESEM and EDX analysis of untreated RH and pretreated RH biomass after deacetylation.

3.4. PHA production using RH hydrolysates

The hydrolysate solutions obtained from untreated RH and DEA and DEA-MW pretreated RH were evaluated for PHA fermentation. The acidic hydrolysate solutions were neutralized with KOH solution (2 M) before being used for microbial fermentation. In the initial experiment, we investigated the growth of *C. necator* on the untreated RH hydrolysate but, unfortunately, we observed no bacterial growth and no biosynthesis of PHA. This observation can likely be attributed to the presence of such inhibitory compounds as acetic acid (0.67 g/L) and unidentified phenolics in the untreated RH hydrolysate. Acetic acid can diffuse into the cell where it dissociates into acetate ions and protons. Since the pKa of the acetic acid is significantly lower than the intracellular pH, the excess proton is actively expelled from the cell to maintain the membrane potential. This process consumes more energy, which may adversely affect growth [23]. Phenolic compounds also inhibit the growth of bacteria, since they partition into cell membranes and lose their structural integrity, which impairs their function as selective barriers and enzyme matrices [58]. Marudkla et al. [23] reported that a concentration of acetic acid greater than 0.5 g/L inhibited the growth of *C. necator*. Furthermore, Mohan et al [13] studied the role of various potential inhibitors on microbial activity and revealed that a

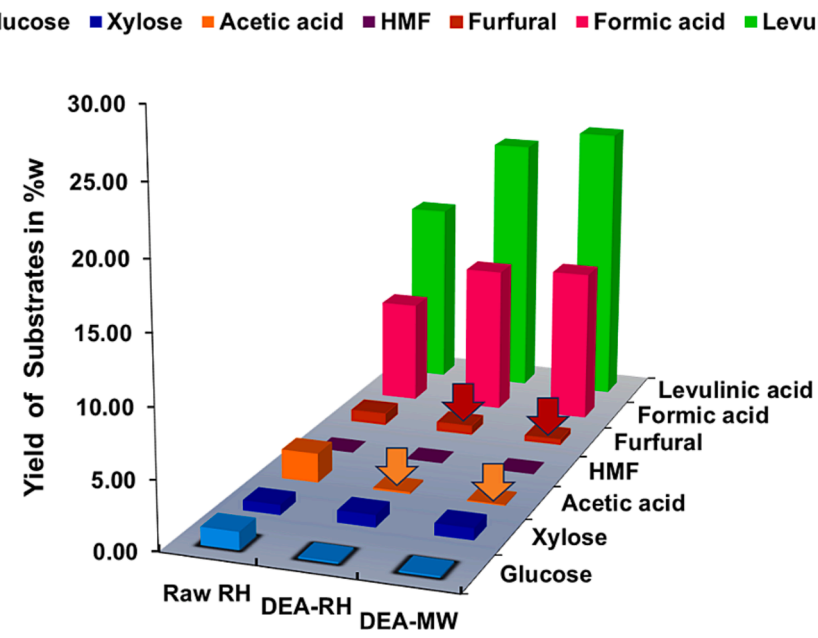


Fig. 5. Microwave-assisted hydrolysis of raw RH, DEA-pretreated RH, and DEA-MW pretreated RH. MW conditions: 0.5 M H_2SO_4, 3 % RH loading, at 180 °C and 1200 W.

concentration of phenolics above 2.5 mM and formic acid above 2 g/L inhibits the growth of *C. necator*. Notably, hydrolysate solutions derived from raw RH were found to contain acetic acid and phenolics that surpassed the inhibitory threshold level mentioned above. As a result, this might lead to the death of *C. necator* cells and halt PHA production. To mitigate the inhibition of acetic acid and phenolic monomers, we investigated pretreatments with deacetylation by conventional heating (DEA) and deacetylation by microwave heating (DE-MW). Based on the concentration of formic acid in the pretreated hydrolysate solution, fermentation media were prepared with 3 g/L of levulinic acid, which ensured that the concentration of formic acid was below the inhibitory level. Compared to untreated RH hydrolysates, the pretreated hydrolysates were able to support bacterial growth and PHA biosynthesis to a much greater extent. This improvement was mainly due to the removal of microbial growth inhibitory compounds such as acetic acid and lignin during the deacetylation pretreatment. Please refer to the supporting information for the HPLC chromatogram, which illustrates the utilization of levulinic acid by *C. necator*. The cell dry mass (CDM), titer of PHA mg/L, and PHA yield mg/g of levulinic acid are shown in (Fig. 6. **a, b, c, d and e).** Recently there are some studies have been conducted using alkali-assisted emerging techniques such as ultrasound, enzymes, microwave for PHA production. we compared our results with these studies (see Table 4 in supporting information).

As illustrated in Fig. 6**a,** both the growth of bacteria and the PHA content were higher on the hydrolysate obtained from DEA-MW pretreated RH than from DEA-pretreated RH. This indicates that the DEA-MW pretreatment is more efficient at removing inhibitory compounds and improves CDW (cell dry weight, microbial growth) and PHA production. As shown in Fig. 6a in DEA-MW pretreated hydrolysates, biomass formation was observed after 8 h of lag phase, increased significantly after 12 h, and reached its maximum at 48 h. This level was maintained until the 72 h fermentation (CDW-630 mg/L). The levulinic acid concentration was consistently reduced and was completely consumed in 48 h (see supporting information for HPLC chromatogram). Notably, the PHA titer (Fig. 6b) started to increase after 12 h. This observation suggests that the nitrogen source might be completely depleted before 12 h, while levulinic acid remained sufficient in the fermentation medium (see supporting information for HPLC chromatogram of levulinic acid utilization). In response to nitrogen-limiting conditions, *C.necator* ceased its growth and engaged its metabolic activities in intracellular accumulation of PHA biopolyester Fig. 6b [59,60].

As shown in Fig. 6**b,** the PHA concentration significantly increased after 24 h and continued to increase until 48 h. The decrease in PHA production after 48 h of fermentation time might be attributed to the consumption of accumulated PHA by bacteria [61]. The maximum PHA concentration of 440 mg/L; and 65 % of PHA content was achieved using DEA-MW pretreated RH hydrolysate solution (Fig. 6**e).**

Producing PHAs from lignocellulosic hydrolysate remains challenging due to its low fermentability [13]. To overcome this challenge, strategies such as detoxification[13], the use of genetically modified strains [61,62], and high cell density cultivation [6] were used to produce PHAs from the hydrolysates of lignocellulose. Mohan et al. [13] investigated the possibility of fermenting raw pine wood hydrolysate by ion exchange purification followed by activated carbon. They reported a PHA content of 64.07 % and a yield of 0.43 g/g using 4 g/L of detoxified pine wood hydrolysate. Recently, Khomlaem et al. [6] used cell retention culture to achieve a high PHA concentration from corn cob hydrolysates. They reported a PHA concentration of 57.6 g/L (56.5 % of CDW). However, the PHA concentration in this study is 440 mg/L (65 % PHA content). Therefore, more investigation needs to be done to optimize PHA production using native biomass as a carbon source.

3.4.1. Characteristics of PHA extracted from DEA-MW pretreated RH

3.4.1.1. GC–MS and GC-FID analysis. GC–MS analysis of the hydroxy acid distribution of PHA from DEA-MW pretreated RH indicated that PHA consisted mainly of 3-hydroxybutyric acid (3HB, *m/z*-118.06) and 3-hydroxyvaleric acid (3HV, *m/z*-132.08). To quantify these 3HB and 3HV monomer compositions in PHA, GC-FID analysis was performed, revealing that PHA contained a 42.24 % mole fraction of 3HB monomer and a 63.34 % mole fraction of 3HV monomer. PHBV was determined to be the copolymer of PHA consisting mainly of 3HB (57.76 % mole) and 3HV (57.76 % mole). Please refer to the supporting information for GC–MS and GC-FID chromatograms of PHA extracted from DEA-MW pretreated RH.

Previously, Koller et al. [63] conducted a study investigating the impact of varying concentrations of 3HV-related precursors on PHA

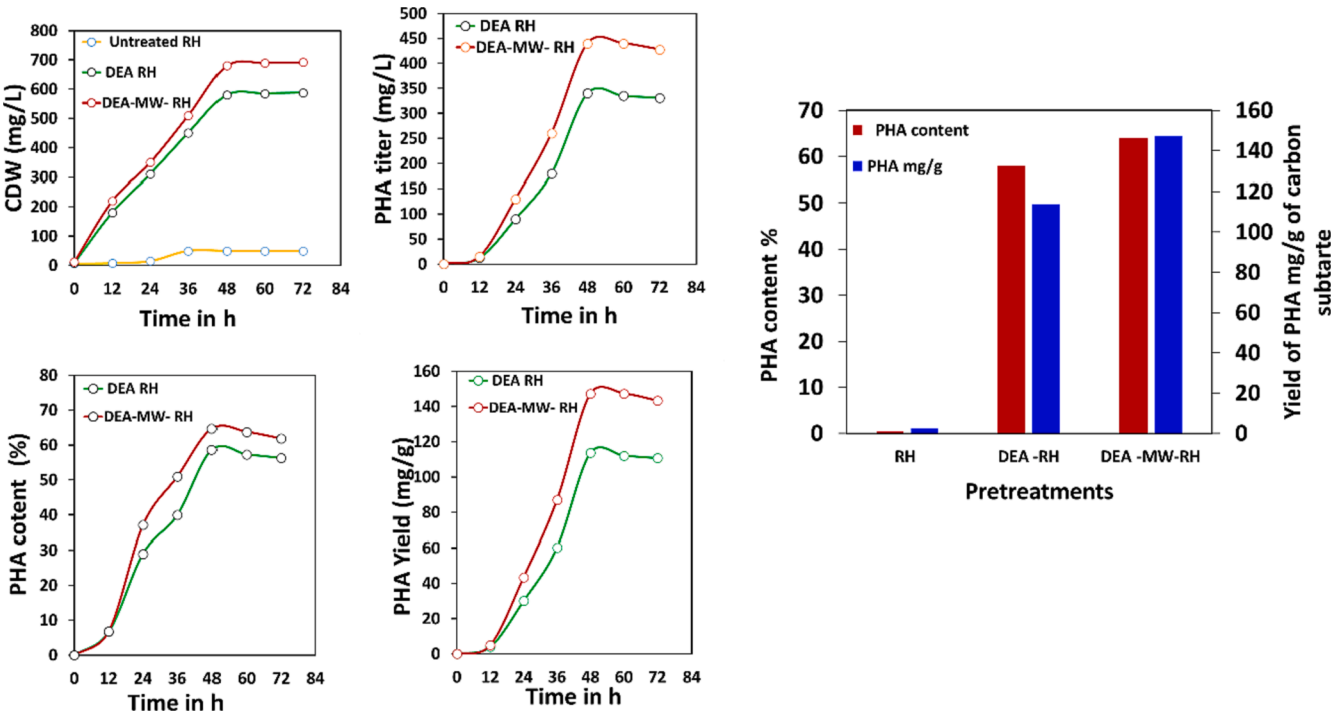


Fig. 6. Effect of untreated RH, and DEA and DEA-MW pretreated RH hydrolysate solutions on a) the growth of bacteria (cell dry weight mg/L). b) PHA titer mg/L. c) PHA content (%) and d) PHA yield (mg/g). e) comparison of PHA yield and content obtained from DEA-RH and DEA-MW-RH at optimal 48 h.

produced by *Hydrogenophaga pseudoflava* using hydrolyzed whey permeate and additional 3HV-related precursors. The authors observed a notable enhancement in HV fraction, increasing it from 18 % to 50 % when levulinic acid concentrations were raised from 0.5 g/L to 1 g/L. This observation suggests that concentrations of 3HV and 3 HV content in PHA directly correlated to the provided HV precursor concentration; the more precursor added, the higher the achieved 3HV concentration, resulting in higher 3HV content in PHA. In another study, Novackova et al. [64] explored evolved *C.necator* strains for their PHBV production capabilities through submerged cultivation in a mineral medium by supplying 3.5 g/L of levulinic acid. The authors reported the PHA content of 55.4 % and 20.6 % of HV fraction in PHA. Further, our results have the advantage of producing higher 3HV (57.76 % mole) content from RH hydrolysates. The higher mole percentage of the 3HV fraction in PHA can be attributed to the higher concentration of levulinic acid. Notably, we increased the concentration of levulinic acid above 3 g/L, and the growth of bacteria was adversely affected.

3.4.1.2. NMR analysis. In the ^{1}H NMR spectra (Fig. 7), the peak signal at 0.87 ppm corresponds to –CH_3 groups in the 3HV monomer, while the doublet –CH_3 protons at 1.20 ppm attributed to the methyl group in the 3HB monomer. Moreover, a peak triplet at 1.56 ppm is a characteristic of the methylene group in the HV monomers and a multiplet proton splitting pattern appears at 2.50 ppm, which is attributed to –CH_2, the side chain of the 3HV monomer, and the methylene proton (close to the carbonyl group) of the 3HB monomer. The peaks that appear at 5.20 ppm are characteristic of the –CH group in the 3HB monomer, while the peak at 5.08 ppm is characteristic of the –CH group in the 3HV monomer. The outcome of the NMR study coincides with that of the study by Koller et al. [63] and Bossu et al. [65] It has been confirmed, then, that

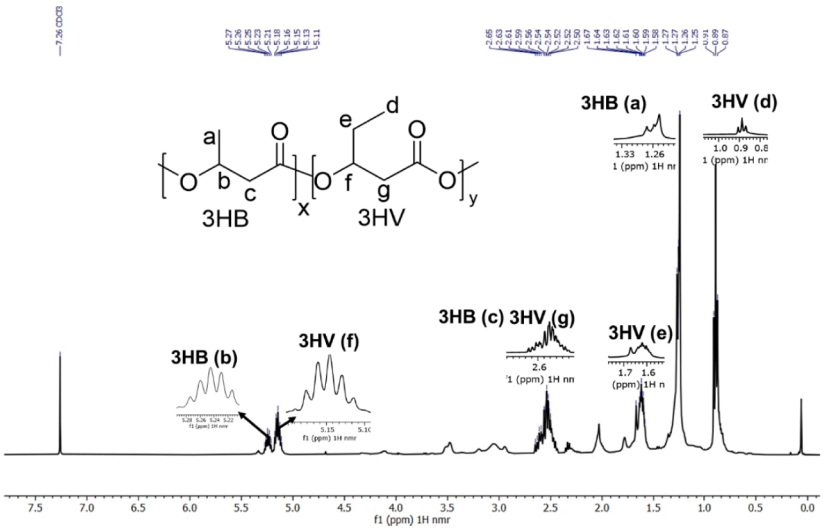


Fig. 7. ^{1}H NMR spectra of PHA accumulated by *C. necator* DSM 545.

the PHA obtained is the chemical characteristic of PHBV polyester.

3.4.2. Characterization of lignin

3.4.2.1. ATR -FTIR spectra. The ATR-FTIR spectra of DEA-pretreated RH and DEA-MW pretreated RH are presented in Fig. 8**.** The absorption band at 3300 cm^{-1} is attributed to –OH vibration stretching in phenol and aliphatic structures, while the peak at 2900 cm^{-1} is characteristic of C-H asymmetric stretching in methyl and methylene groups. It should be pointed out that these peaks were observed to be weak in untreated RH. The absorption band in the deacetylated samples appeared at 1640 cm^{-1} indicating aromatic skeletal vibration (C-C stretching) and -C = O stretching of lignin groups. It shows that the carbon at position 5 in the G unit might react with the aryl propane in the side ring of the lignin [66]. This observation implies that deacetylation extracts lignin with several G units (guaiacyl units). This hypothetical conclusion finds support in a small shoulder that appeared at 1250 cm^{-1}, attributed to the G unit [67]. Furthermore, an absorption band appeared at 1160 cm^{-1} which is characteristic of the S unit (syringyl unit). The sharp absorption band in deacetylated samples at 1040 cm^{-1} signifies modification in the vibrational characteristics of the aromatic ring within the G unit. This alteration is indicative of the structural deformation of G units in the aromatic ring [38]. The variation in peak intensities corresponding to S and G units illustrates that the process is effective at isolating and recovering lignin.

3.4.2.2. 2D HSQC NMR analysis. To further elucidate structural characteristics and interlinkages in the lignin sample isolated from combined deacetylation-microwave pretreatment, we conducted a 2D HSQC NMR analysis. The predominant sub-units and interlinkages elucidated from 2D HSQC spectra are depicted in Fig. 9**c**. It was found that alkaline solubilized lignin retained 78.05 % of its native β-O-4 bond (as shown in Fig. 9**a and b**). This result was comparable to the quantity of β-O-4 linkages found in milled wood lignin isolated from RH [68]. In addition, other linkages such as β-5 and β- β were found to be 4.20 % and 17.70 %, respectively. The S/G ratio which represents the proportion of syringyl units and guaiacyl units in the lignin sample has an S/G ratio value of 0.15, close to the original lignin [66]. This observation strongly suggested that the lignin fraction underwent minimal changes during alkaline solubilization in line with similar studies that isolated lignin through methods that used deep eutectic solvent (DES), mechanocatalysis (MC), and microwave-assisted processes, all under mild conditions [38,47,69]. Our HSQC analysis identified the signals corresponding to p-hydroxybenzoate (PB), and p-coumarate (pCA) in deacetylated RH lignin, which had also been previously reported [45]. This observation further supports the notion that carboxylic acids are prone to ionization under reduced pH conditions, enabling the removal of lower molecular weight lignin.

Table 3
Relative composition of syringyl (S units), guaiacyl (G units), and *para*-hydroxyphenyl (H units), S/G unit, end groups, and β-O-4, β–β, and β-5 interlinkages in isolated lignin by deacetylation-microwave pretreatment as estimated by HSQC NMR.

DEA-MW RH	Integral Volumes	%
Lignin Recovery	–	72.22
[a]Lignin Sub Units	S Unit-152.57	17.44
	G Unit-514.87	58.85
	H Unit- 207.31	23.69
S/G ratio	–	0.15
[b]End Groups	p-coumarate (p-CA)- 95.3	10.89
	p-hydroxy benzoate (PB)- 72.67	8.30
[c]Interlinkages	β-O-4–71.96	78.05
	β–β-16.32	17.70
	β-5–3.91	4.20

[a] Content % expressed based on fraction of S + G-H units, [b] Content % End groups as fraction of S + G + H units, and [C] Content % expressed fraction of β-O-4, β–β, and β-5 interlinkages. The detailed equations for the calculation of S, G, and H units are given in supporting information.

The HSQC spectrum (Fig. 9**b**) also shows that the hemicellulose fraction in RH biomass undergoes solubilization during the deacetylation pretreatment. Predominantly, xylan fractions are mainly solubilized, primarily due to their connection to lignin through the lignin-carbohydrate complex (LCC) [70–72]. It should be pointed out that lignin isolated from RH via combined DEA-MW exhibits characteristics such as low molecular weight, the perseverance of inherent linkages such as β-O-4, β- β, and β-5, and favourable hydrophilic properties. Neither were any condensed sub-unit structures observed in the lignin isolated via DEA-MW pretreatment. These properties make it an

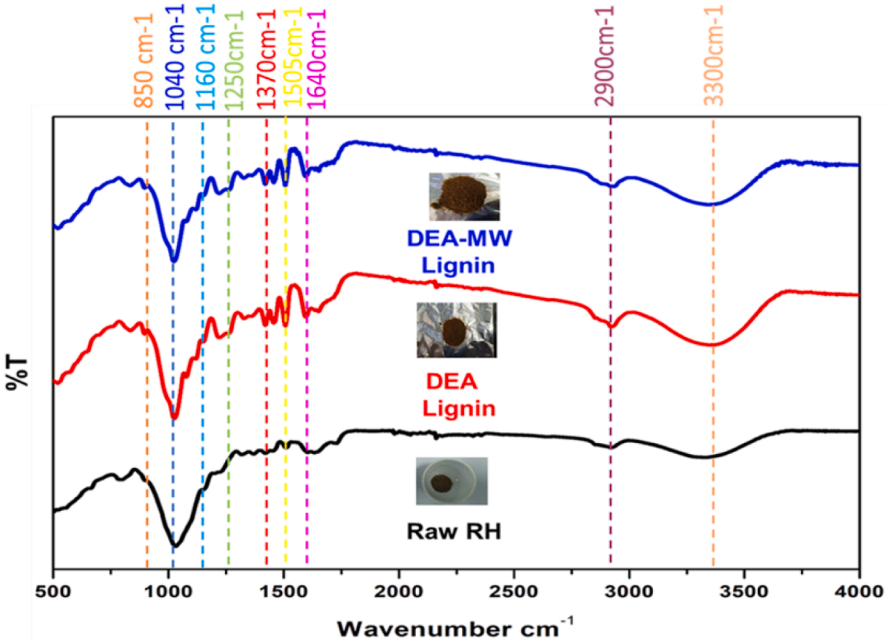


Fig. 8. ATR-FTIR analysis of lignin in raw RH, DEA-RH, and DEA-MW RH.

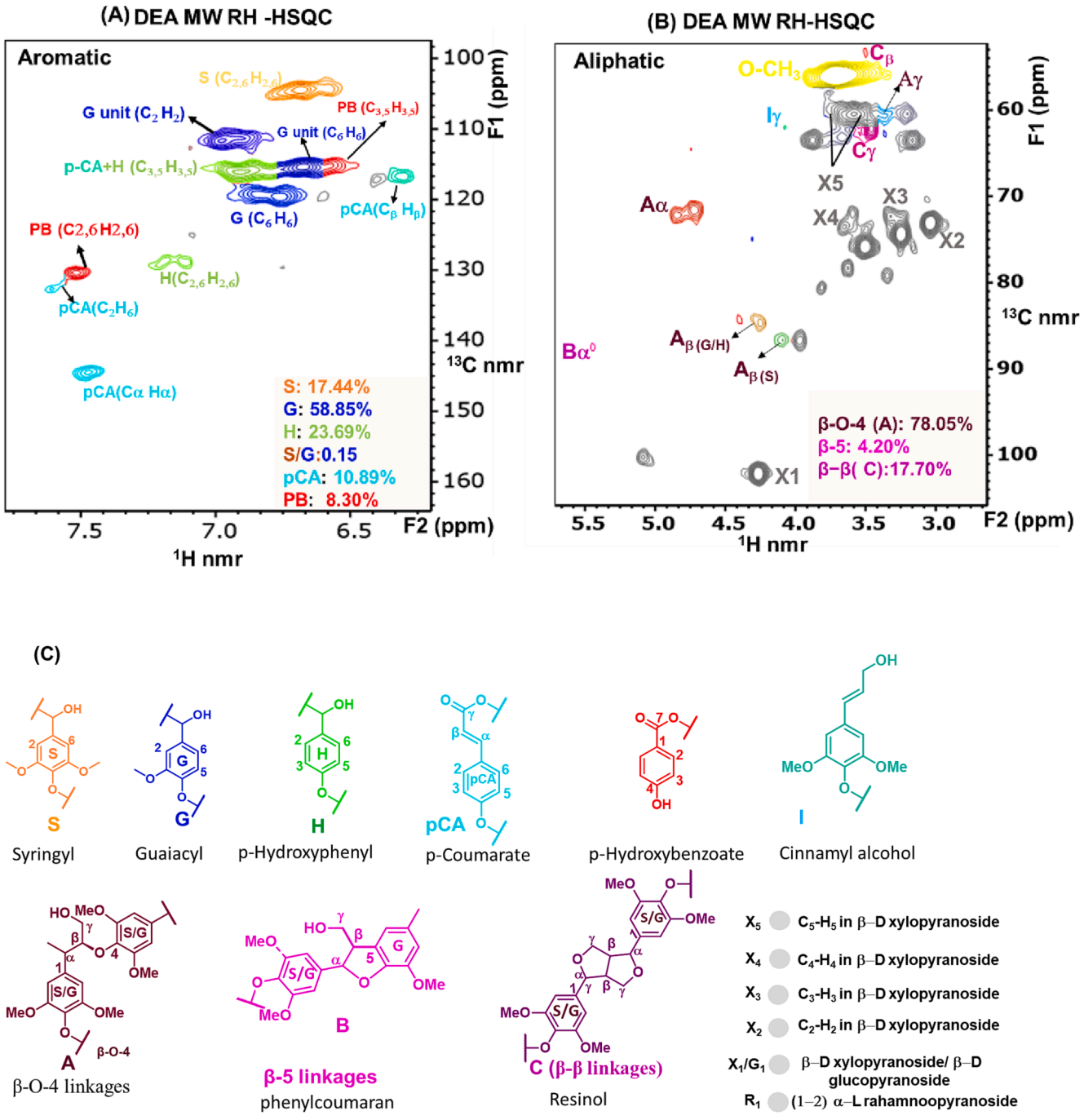


Fig. 9. 2D HSQC NMR of the lignin isolated from the deacetylation-microwave treated RH sample, where a) aromatic region of DEA-MW pretreated RH, b) aliphatic region of DEA-MW pretreated RH, and c) chemical structures of the lignin subunits and interlinkages.

attractive candidate for the production of bio-based products using chemical [35,48,73,74] and biological methods [36,75–77], thus enhancing the sustainability of the lignocellulosic biorefinery. It should be noted that lignin isolated from RH via combined DEA-MW exhibits characteristics such as low molecular weight, the presence of inherent linkages such as β-O-4, β- β, and β-5, and favorable hydrophilic properties. These properties make it an attractive aromatic carbon source for the production of bio-based products using chemical [35,48,73,74] and biological methods [36,75–77]. The structural characteristics of lignin isolated from DEA-MW pretreatment are shown in Table 5. The cross signals between the ^{13}C and the ^{1}H NMR of each counter line in the HSQC spectrum have been assigned (see supporting information, Table 5).

4. Conclusions

In summary, this study explored a combined DEA-MW approach for the production of bio-based biodegradable and biocompatible PHA bioplastics using agricultural waste rice husks as feedstock. The combined DEA-MW pretreatment efficiently curbs the formation of inhibitors such as acetic acid and phenolics, improves biomass fermentability, and boosts the yield of PHA. DEA-MW pretreated RH generated the maximum PHA concentration, content, and yield (440 mg/L, 65 %, and 147 mg/g, respectively). Furthermore, the isolation of lignin via combined deacetylation-microwave pretreatment opens an additional avenue for using all the components of RH. We recovered 72.22 % lignin with well-preserved native β-O-4, β–β, and β-5 linkages

and DEA-MW pretreated RH still contained non-condensed sub-units. This holistic approach not only contributes to improving PHA production but also emphasizes the importance of maximizing resource utilization and minimizing waste generation in bioplastic production, in harmony with the principle of the circular economy. In essence, the combined deacetylation-microwave pretreatment followed by dilute acid hydrolysis and microbial fermentation showcase promising synergetic biorefinery processes for sustainable and cost-effective PHA production. However, the next step of the investigation should address the scaling-up of the process to allow for techno-economic analysis and environmental assessment.

CRediT authorship contribution statement

Vaibhav Vilas Andhalkar: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Daniel Montané:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Data curation. **Francesc Medina:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Magda Constantí:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.149754.

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