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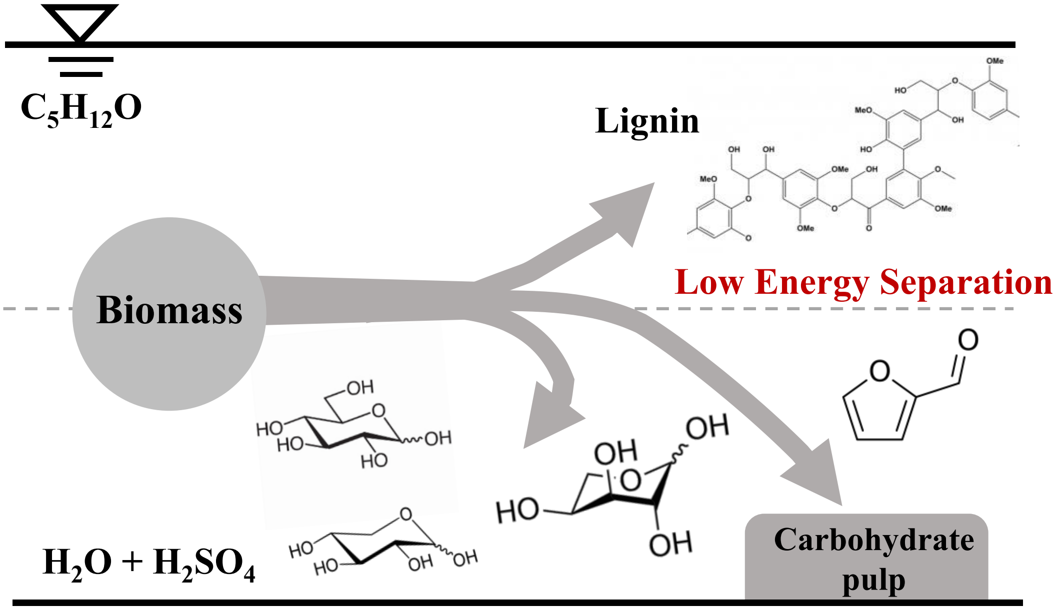

Highlights

- Three pretreatment scenarios were compared for *Acacia Confusa* wood biorefinery;
- Biphasic pretreated substrate resulted 92.2% digestibility and 82% SSF yield;
- Preserved 42.47% of original β-O-4 linkages in fractionated lignin,
- More than 50% of wood carbon was recovered as ethanol and reactive lignin;
- Saved 47.2% of energy to produce net positive energy output of 1.77 MJ/ kg of wood.

Biphasic Pretreatment for Energy and Carbon Efficient Conversion of Lignocellulose into Bioenergy and Reactive Lignin

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ABSTRACT

The valorization of lignocellulosic biomass depends on the effective fractionation of its major components to overcome its natural recalcitrance. Pretreatment overcomes biomass recalcitrance to facilitate effective fractionation of its components and to make substrate accessible for different chemicals and enzymes for efficient conversion to the fuels or building block chemicals. However, pretreatment is the most energy-intensive process which determines the efficiency of downstream processing for integrated biorefinery. Here, we reported biphasic pentanol-water pretreatment to facilitate an energy-efficient fractionation of major lignocellulose components. To compare, three different pretreatment scenarios *i.e.,* dilute acid (S-I), ethanol (S-II), and biphasic pentanol-water (S-III) were investigated to evaluate the energy and carbon footprint of *Acacia Confusa* wood biorefinery to produce benchmark fuel (bioethanol) and reactive lignin. Results showed that S-III pretreatment yielded 70.27% lignin dissolution in the organic phase while leaving cellulose as an insoluble solid residue. Simultaneous saccharification and fermentation resulted in 92.2% cellulose digestibility of pretreated substrate and 82% fermentation yield of released sugar in the hydrolysate. S-III pretreatment also preserved 42.47% of reactive β-aryl ether (β-O-4) linkages which is 31.12% higher than S-II pretreatment. Carbon balance showed that S-III process recovered 50.33% wood carbon in the product streams as ethanol and reactive lignin, which is higher than S-I (17.10%) and S-II (49.59%) approaches. Finally, the overall energy balance showed S-III pretreatment yielded a net positive energy of 1.77 MJ/kg wood which was much higher than the S-I (-0.14 MJ/kg) and S-II (-1.94MJ/kg) process. It was due to the physical separation of dissolved sugars and lignin which reduced about 47.23% of the total energy consumption compared to S-II biorefinery indicating the feasibility of S-III pretreatment.

The short version of the paper was presented at ICAE2020, Dec 1-10, 2020. This paper is a substantial extension of the short version of the conference paper.

Keywords: Energy footprint, Carbon balance, Bioethanol, Biphasic pretreatment, Pentanol-water, Reactive lignin.

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1. Introduction

Lignocellulose biorefinery is expected to be an alternative to petroleum refinery for sustainable production of biofuel and chemicals [1]. Cellulose, hemicellulose, and lignin are the major components of lignocellulosic biomass which can be used as the building-block chemicals for many other chemicals [2, 3]. For example, cellulose and hemicellulose sugars can be used to produce bioethanol, biobutanol, furfural, lactic acid, GVL etc. Lignin can be utilized to produce polyphenol, polyurethanes, phenol resin, carbon fibers, and other valuable aromatic chemicals [4]. However, lignocellulosic biomass possesses strong recalcitrance to biodegradation due to the complex structure of cell wall components *i.e.* cellulose, hemicellulose, and lignin [5]. The network structures and strong interaction between hemicellulose and lignin limit the cellulose accessibility due to the strong interaction between hemicellulose and lignin. Therefore, pre-treatment is the most critical step in the biorefinery which could fractionate lignocellulose components by altering the structure of cell wall components and enhances the accessibility of cellulose to different chemicals and enzymes for efficient conversion [6]. Therefore, biorefinery efficiency largely depends on the effective pretreatment of lignocellulose and it is the most energy-intensive process in bioconversion [7].

Over the decades, numerous studies have been devoted to effective fractionation to maximize the efficiency of the biomass valorization process [8, 9]. Liquid hot water, dilute acid, dilute alkali, organosolv, ionic liquid, steam explosion, ammonia fibre expansion pretreatment are the most common pretreatment methods for lignocellulose [10, 11]. Among these methods, dilute acid (DA) pretreatment is a cost-effective method to remove biomass recalcitrance to produce energy and chemicals [12]. However, DA pretreatment encounters low digestibility of substrate and low-value residual lignin due to severe condensation during pretreatment [13]. With the benefit of lignin recyclability, organosolv among other chemical

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methods is getting more attention in lignocellulosic biorefinery [14]. In organosolv pretreatment, lignocellulose is fractionated by dissolving hemicellulose and lignin in the water-organosolv mixture leaving cellulose as an insoluble pulp which has high digestibility [15]. To implement a sustainable lignocellulosic biorefinery, not only fractionation of major components is important, the energy and carbon efficiency of the fractionation process must be considered. After organosolv pretreatment, the separation of lignin from the spent liquor and the recovery of organic solvent usually complicates the overall process. Lignin and hemicellulose are commonly fractionated through the use of large amount of water as an antisolvent [14]. The addition of water significantly increases the energy consumption in distillation to concentrate dissolved sugars in the liquor and organic solvent recovery [13, 16].

Lignin-first biorefinery has been receiving increasing interest recently as this holistic biorefinery concept focuses on reducing later lignin depolymerization/modification steps during the fractionation process and broadens the application of lignin [17-19]. Organosolv pretreatment with water and immiscible organic solvent facilitates the physical separation of biomass components in three different phases. Hydrolyzed hemicellulose and lignin are dissolved in the aqueous and organic phases respectively while cellulose fraction remained as a solid residue [20]. A previous study using one-pot catalytic n-butanol/water biphasic system was investigated to convert eucalyptus sawdust into a cellulose pulp, hemicellulose-derived polyols, and lignin-derived phenolics [21]. The valorization of hemicellulose and lignin was achieved at 200°C temperature in presence of Ru/C catalyst with hydrogen pressure. The phase separation at lower temperature offers a facile strategy to separate lignin-derived chemicals in butanol and polyols in water. Another example is an acid-catalyzed water/n-butanol biphasic system which promoted the separation of biomass components in three different phases of which lignin can be recovered and cellulose residues have 96% enzyme digestibility [22].

Wang et al., reported a one-pot mild fractionation technique for poplar wood using a phenol/water system to increase the processibility of wood components [23]. The study reported about 90% lignin removal with 73% cellulose retained in the solid substrate under the optimized condition at 120℃.

Although biphasic organosolv pretreatment can ease the separation of lignin and hydrolyzed hemicelluloses after the pretreatment, it still faces the challenge from the mass transfer limitation caused by the biphasic solvent system during pretreatment [20]. The partial solubility of butanol and phenol (8-10%) in water [24] at room temperature showed that a significant portion of solvent will be wasted during separation. Also, the aqueous phase may contain inhibitors such as phenol which may need additional extraction before biorefinery application. Moreover, the lignin integrity and modification after pretreatment would highly affect its further application. To address these issues, in this study, an integration of lignin-first fractionation and biphasic organosolv pretreatment has been proposed which can realize the effective fractionation of lignin with high energy and carbon efficiencies to produce bioethanol and reactive lignin from the whole biomass.

Herein, we designed and investigated an integrated biorefinery using pentanol-water biphasic pretreatment of acacia wood that simultaneously produces reactive lignin as well as a carbohydrate pulp which is amenable to bioethanol production. Pentanol is almost immiscible (1.7-2%) with water which can facilitate more efficient solvent recovery to physically separate hemicellulose and lignin fractions. Most importantly, the proposed biorefinery process including pretreatment, fractionation/distillation, saccharification, and fermentation (SSF) were fully studied to calculate energy footprints for bioethanol production and reactive lignin recovery. The calculation has also identified the energy benefits in the fractionation process by comparing with homogenous organosolv pretreatment (ethanol pretreatment) and dilute acid pretreatment. The advantageous effect of pentanol-water pretreatment on lignin quality/reactivity was also assessed by quantifying the amount of different linkages and molecular weight of the fractionated lignin. Finally, carbon efficiency was quantified to determine the potential of mitigating greenhouse gas (GHG) emissions of biorefinery conversion.

2. Methodology

2.1. Materials and apparatus

The *Acacia Confusa* wood was used as biomass feedstock throughout the study which was collected from the local supplier. The wood sample was stored in the cool room for further experiments. All the chemicals and pretreatment solvents used in the study for analytical procedures were of reagent grades. Commercial cellulase (CellicCtec-2) was a generous gift from the Novozymes Investment Co. Ltd. (Beijing, China). The structural sugars were quantified by the high-performance liquid chromatography coupled with refractive index detector and Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA). The structure of lignin was assessed by two-dimensional heteronuclear single quantum coherence (2D HSQC) NMR, gel permeation chromatography (GPC), and infrared (IR) spectroscopy.

2.2. Optimization of pentanol-water solvent system

The pretreatment of *Acacia Confusa* wood was carried out in a metallic cylinder digester manufactured by Yudian, China. The digesters were sealed tightly, mounted on a 15L rotating cylinder. About 50 gms of dry biomass were cooked in each digester with various combinations of temperature, solid to liquid ratio, solvent, and catalyst compositions to optimize pentanol-water biphasic solvent (**Table 1**). For comparison and optimization, one parameter was varied while keeping all other parameters constant.

Table 1. Pretreatment conditions for optimization of pentanol-water biphasic solvent system for *Acacia Confusa* wood residues.

Sample	**Pretreatment conditions**			
	(%) Pentanol	**(mM) H_2SO_4**	**℃ Temp.**	**Liquid/ solid**
Untreated	-	-	-	-
$P_{00}C_{45}T_{170}S_7$	0	45	170	7
$P_{10}C_{45}T_{170}S_7$	10	45	170	7
$P_{20}C_{45}T_{170}S_7$	20	45	170	7
$P_{35}C_{45}T_{170}S_7$	35	45	170	7
$P_{50}C_{45}T_{170}S_7$	50	45	170	7
$P_{65}C_{45}T_{170}S_7$	65	45	170	7
$P_{80}C_{45}T_{170}S_7$	80	45	170	7
$P_{95}C_{45}T_{170}S_7$	95	45	170	7
$P_{65}C_{00}T_{170}S_7$	65	0	170	7
$P_{65}C_{15}T_{170}S_7$	65	15	170	7
$P_{65}C_{30}T_{170}S_7$	65	30	170	7
$P_{65}C_{45}T_{170}S_7$	65	45	170	7
$P_{65}C_{60}T_{170}S_7$	65	60	170	7
$P_{65}C_{85}T_{170}S_7$	65	85	170	7
$P_{65}C_{100}T_{170}S_7$	65	100	170	7
$P_{65}C_{120}T_{170}S_7$	65	120	170	7
$P_{65}C_{45}T_{170}S_5$	65	45	170	5
$P_{65}C_{45}T_{170}S_9$	65	45	170	9
$P_{65}C_{45}T_{140}S_7$	65	45	140	7
$P_{65}C_{45}T_{200}S_7$	65	45	200	7

The pretreated samples were abbreviated as $P_iC_jT_kS_l$; where P_i was the pentanol composition; C_j was the catalyst concentration; T_k was the temperature, and S_l was the liquid to solid ratio. The values of the respective parameters were shown in the subscription.

2.3. Pretreatment design

To compare pentanol-water with conventional solvents, three different biorefinery scenarios were investigated to produce building-block sugars/basic fuels and reactive lignin for value-added chemicals from *Acacia Confusa* wood residues. The major process units and products obtained from all three approaches were demonstrated in **Fig.1**.

Scenario-I (S-I): Dilute acid was used to fractionate lignocellulose sugars due to the strong hydrolyzing effect of acid in the aqueous medium. Wood chips were mixed and heated at 170°C for 30 minutes in a stainless-steel reactor with a solid-liquid ratio of 1:3. Carbohydrate sugars were hydrolysed and fermented to produce bioethanol while lignin was obtained as a solid residue after SSF of pretreated substrates.

Scenario-II (S-II): Ethanosolv pretreatment fractionates lignocellulose components and provides an option to recycle fractionated lignin. The solvent was prepared by mixing 65% ethanol and 45 mM H_2SO_4 as catalyst. Pretreatment was carried out at 170°C for 60 minutes with a solid-liquid ratio of 1:7. Lignin was precipitated from the pretreatment liquor using water as anti-solvent. Liquor was distilled to recover solvent (ethanol) and dissolved sugars for further use. Carbohydrate pulp and liquor sugars were subjected to saccharification and fermentation to produce bioethanol.

Scenario-III (S-III): Pentanol-water biphasic pretreatment was carried out at optimized conditions (65% pentanol, 170°C, 45mM H_2SO_4, and 1:7 solid-liquid ratio), aiming energy-efficient fractionation of lignocellulose. The pretreatment liquor was physically separated to aqueous (dissolved sugars) and organic fractions (containing lignin) by a separatory funnel while leaving cellulose as a solid residue. The aqueous phase was used for fermentation after neutralization by KOH using BLBIO-2020 model pH controller. The organic phase containing lignin can be directly utilized to produce lignin foam or lignin monomer for high-value applications [20].

[Please insert Fig.1 here]

2.4. Chemical composition analysis

Chemical composition analysis was conducted before and after pretreatment to determine the true value of the waste-derived biomass. The analytical procedure of structural carbohydrates and lignin had been well-documented by NREL [25]. Before analysis, wood samples were air-dried and ground into a small powder to ensure maximum reagent penetration. A part of the sample was dried in an oven at 105 °C for at least 12 hours to be constant weight. The moisture content was determined by calculating the loss in weight of the wood sample. The sample was mixed with 72% H_2SO_4 and hydrolyzed at 30°C temperature in a water bath for 60 mins. The solution was diluted to 3.97 % H_2SO_4 (w/w) and autoclaved at 121°C for another 60 mins for complete hydrolysis. The autoclaved masses were filtered to separate lignin residue and filtrate containing cellulose and hemicellulose sugars. An aliquot was used to determine the structural sugars while lignin was gravimetrically determined from the dried mass of the residue.

2.5. Simultaneous Saccharification and fermentation (SSF)

The SSF of pretreated substrates was carried out in a 250 mL Erlenmeyer flask with a total working volume of 100 mL at 5% (w/v) substrate loading. The pH of the mixture was adjusted at 5.5-6.2 by auto addition of KOH using BLBIO-2020 model pH controller. The enzyme

Cellic-Ctec2 at 15 FPU/g-cellulose and precultured yeast at 5 g/L were added to the mixture for hydrolysis and fermentation. The yeasts were precultured in yeast extract peptone dextrose (YPD) medium (2% (w/v) glucose, 1% (w/v) yeast extract, and 2% (w/v) peptone extract) at 37℃ for 9 hrs. The SSF experiments were conducted for 72 hrs and aliquots were collected at 3, 6, 9, 24, 48, and 72 hrs to determine saccharification and fermentation efficiencies.

2.6. Characterization of lignin

Lignin structure and its transformation during pretreatment were determined by two-dimensional heteronuclear single quantum coherence (2D HSQC) nuclear magnetic resonance (NMR)[26]. 2D HSQC NMR analysis also gives quantitative information of different linkages in lignin samples. For the test, around 50 milligrams of lignin sample were dissolved in deuterated DMSO. 2D HSQC spectra were recorded using a JEOL ECZR 5000 MHz spectrometer equipped with a 5 mm ROYAL probe. For the 1H dimension, the number of complex points was 1024 with a spectral width of 11 ppm and recycle delay of 1.5s. In the 13C dimension, the number of transients was 64, and time increments 256 with 1.5 s interscan delay. The peaks from syringyl (G) and guaiacyl (G) aromatic units of lignin were used as the internal reference. The quantitative values were determined by comparing the integral volume of HSQC correlations [26]. JEOL's Delta 5.2.1 software was used to determine the volume integrals of HSQC correlations.

Infrared (IR) spectra of lignin samples were recorded using a Perkin Elmer Fourier-transform infrared (FTIR) spectrometer with a diamond crystal attenuated total reflectance accessory (ATR-FTIR). Powdered lignin samples were placed on top of a diamond crystal and exposed to an infrared beam in the region between 4000 and 400 cm^{-1} with 12 scans and 4 cm^{-1} resolution. The infrared beam penetrated and reflected off the surface of the sample through the diamond crystal in the form of an evanescent wave. The room temperature and relative humidity were around 26 °C and 35% respectively.

The number-average (Mn) molecular weights of all lignin samples were determined by gel permeation chromatography (GPC, Agilent 1200, USA)[26]. Approximately 10 milligrams of lignin samples were dissolved in 2 mL 0.11 M LiCl solution in N, N-dimethylacetamide and filtered mixture through 0.45 mm filter. An aliquot of 10μL for each sample was injected for GPC analysis. The column temperature was maintained at 80℃ and eluted with the mobile phase N, N-dimethylacetamide at the rate of 1 ml/min. The analysis was carried out by the HPLC equipped with Agilent PLgel MIXED-B column and refractive index detector.

2.7. Energy balance

The energy consumption of the pretreatment process was determined by the enthalpy change of related components of the total system using the following equation-

$$E_H = \frac{1}{\eta}\left[\int_{T_0}^{T_t}\left(\sum_{i=1}^{n} m_i^{T_j} \cdot Cp_i^{T_j}\right)dT + \sum_{i=1}^{n} m_i^{T_k} \cdot L_i^{T_k}\right] \qquad (1)$$

where E_H is the change of enthalpy or total energy consumption, (kJ); η is the heat transfer efficiency; T_0 is the room temperature (°C); T_t is the pretreatment temperature (°C); m is the total mass including substrates and reagents (kg); C_t is the heat capacity of the component (kJ/kg/°C); L is the latent heat of the component (kJ/kg); T_j is the specific temperature of selected component (°C); T_k is the boiling point of the selected component (°C); subscripted 'i' denotes the type of component and n is the total number of components. The energy consumption was calculated based on the complete heat transfer while neglecting the interactions between the components of substrates and reagents. It was also considered that 50% of the consumed heat was recovered during the pretreatment process.

The consumption for solvent recovery in the distillation process (E_{Dstl}) was calculated using the equilibrium curve of the solvent-water system [27] as follows:

$$E_{Dstl} = L \cdot e_{steam} = R_c \cdot V \cdot e_{steam} \qquad (2)$$

where, *L and* V are liquid and vapor flow rates (kg-mole/h); e_{steam} is the energy density of the heating stream (kJ/kg); and R_c is the specific constant of L/V ratios [27].

2.8. Carbon efficiency of the process:

The carbon balance of wood biorefinery determines the potentials of mitigating greenhouse gas (GHG) emissions of the process. The carbon balance was evaluated based on mass flow of the biorefinery process and molecular formula of the components. The carbon efficiency of the process was calculated based on the amount of carbon recovery in the products.

3. Results and discussion

The pretreatment of biomass reduces inherent lignocellulose recalcitrance which ultimately increases the enzymatic digestibility of cellulose residue [28]. The ultimate objective of our pretreatment design was to remove lignocellulose recalcitrance by dissolving hemicellulose and lignin in the pretreatment liquor while leaving the maximum amount of cellulose as a solid residue. Three scenarios, namely DA (S-I), ethanol (S-II), and pentanol-water (S-III) pretreatments were performed to compare and demonstrate effective fractionation of lignocellulosic.

3.1. Optimization of pentanol-water solvent system

The efficiency of the proposed process was determined in terms of hemicellulose removal, lignin removal, cellulose recovery, and enzyme digestibility of the pretreated substrates. The results were shown in **Fig 2(a)** to **Fig. 2(d)**. Hemicellulose removal increased with the pretreatment severity which included acid concentration (pH), cooking temperature, and solid-liquid ratios used in the pretreatment. All these parameters increased the hydrolysability of hemicellulose linkages during pretreatment. Besides, the higher acid concentration reduced the pretreatment time and temperature for effective/ complete removal of hemicellulose. The high acid concentration increased the availability of protons (H_3O^+) in the system which was mainly responsible for the cleavage of all lignocellulose linkages [13]. The complete hemicellulose removal was obtained at 60 mM acid concentration in the pretreatment liquor for cooking 60 minutes at 170℃. There was a slight increase (92.63% to 92.98%) of hemicellulose removal when the temperature was increased from 170 ℃ to 200 ℃ at 45 mM acid concentration. On the other hand, the increase of pentanol composition reduced the hemicellulose removal due to the reduced mobility of (H_3O^+) in concentrated pentanol solution. The hemicellulose removal reduced from 93.92% to 59.15% as the pentanol composition increased from 10% to 95% in the liquor.

[Please insert Fig.2 here]

Lignin removal showed a similar response as hemicellulose removal and the lignin solubility increased with the pentanol composition up to 65% which enhanced the hydrophobic interaction between lignin and solvent. However, lignin removal started decreasing after 65% since lignin dissolution was highly dependent on hemicellulose dissolution. At the very high acid concentration ($\geq$ 85 mM acid) or pretreatment severity, lignin removal from the solid pulp was reduced due to lignin condensation reaction. The formation of condensed C=C bond in the lignin reduced its dissolution from the solid pulp [5]. The maximum lignin removal was obtained at 170 °C and 45 mM of acid concentration which was about 70.30% of the original lignin which was comparatively lower than some of the reported studies [14, 23]. Maybe, it was due to the limited mass transfer between biomass lignin and pentanol phase. However, this study facilitated a much easier separation of lignin with the higher amount of original β-O-4 linkages which was discussed later in the lignin characterization section.

The solid recovery was reduced due to the removal of hemicellulose and lignin in the pretreatment process as shown in **Fig 2(a)** to **Fig. 2(d)**. The solid residues obtained after pretreatment and fractionation mainly contained cellulose which was about 72.10% of the total solid. Biorefinery efficiency largely depends on the cellulose recovery as a readily usable solid residue which can be used to produce building-block sugars or bioethanol. The cellulose recovery decreased with increasing acid doses and cooking temperature in the pretreatment. The higher cooking temperature and acid doses increased the severity of pretreatment that was responsible for higher cellulose degradation. For example, the cellulose recovery reduced from 90.56% to 69.62% as the acid doses increased from 15 mM to 120 mM at the same cooking temperature (170°C). At same acid dose and solid-liquid ratio, the cellulose recovery also reduced from 92.36% to 83.83 % when the cooking temperature was increased from 140 °C to 200°C. However, the effects of solvent composition and solid-liquid ratios were minimal on the cellulose recovery.

Therefore, the optimum conditions were found as 65% pentanol, 45 mM acid concentration, and 170 °C temperature with 1:7 solid-liquid ratio. The optimized pretreatment conditions resulted in 92% hemicellulose and 70.27% lignin solubilization in the solvent system while resulting in an insoluble fraction with 88.49% cellulose recovery.

3.2. Pretreatment efficiency of different solvents

The compositions of pretreated wood residues and hydrolysates were illustrated in **Fig.3,** where the composition of pretreated substrates was expressed in terms of raw substrate and solid yield. S-I pretreatment removed 98.47% hemicellulose from substrate while leaving all the lignin in the solid cellulosic residues. For organosolv pretreatment, S-II and S-III, the solid yield was significantly (53.74% and 52.08% of its initial weight respectively) reduced due to the removal of easily hydrolysable hemicellulose and lignin. S-II pretreatment removed 90.19% hemicellulose and 74.01% lignin from *Acacia Confusa* wood residue. S-III approach resulted similar performance on hemicellulose (89.49%) and lignin (70.27%) removal from raw wood. In addition, approximately 90% of the original cellulose was recovered in solid residue which suggested that S-III pretreatment was not too harsh to hydrolyze cellulose.

[Please insert Fig.3 here]

There was a trade-off between substrate-related factors such as hemicellulose and lignin removal from pretreated substrates in pretreatment [28]. Pentanol served as a solvent for hydrophobic lignin in the fractionation process while dilute acid cleaved inter-unit ester and ether linkages of hemicellulose and lignin. The use of mineral acid significantly increases H_3O^+ ion in pentanosolv liquor and subsequently increases the fractionation efficiency of the solvent system. The hydrolyzing effect of the acid catalyst accelerated effective hydrolysis of hemicellulose to its sugars and dissolved in the aqueous phase. The effective hydrolysis of hemicellulose, in turn, makes lignin removal easier due to the framework structure of hemicellulose and lignin.

Dissolved hemicellulose and cellulose were recovered in liquor as building block sugars and the sugar yields in the liquor were determined. S-III pretreatment recovered 4.02% and 13.81% of raw biomass as cellulose and hemicellulose, respectively. Approximately, 1.70%, 1.93%, and 1.28% of raw biomass were degraded to hydroxymethylfurfural (HMF), furfural, and acetic acid, respectively in the pretreatment liquor. S-II pretreated liquor yielded similar amount of carbohydrate sugars and by-products in the liquor.

S-I pretreatment produced relatively higher amount of HMF (2.95%), furfural (3.80%), and acetic acid (2.28%) while reducing hemicellulose sugar recovery (12.65%) in the pretreatment liquor. The degradation of sugar into fermentation inhibitors also increased as the acid concentration increased. It was due to the strong hydrolyzing effect of acid during pretreatment which led to degradations of hemicellulose sugars to fermentation inhibitory products (Delbecq et al., 2018).

3.3. Simultaneous saccharification and Fermentation

Effective pretreatment facilitates the separation of cellulosic fractions that are readily digestible during enzymatic hydrolysis. High cellulose digestibility is a prerequisite for high sugar/ethanol yield in biorefinery conversion. SSF is the most promising and time-saving process for saccharification and fermentation in one reactor. To investigate the effect of three different pretreatment scenarios on the digestibility and fermentation, SSF was carried out in fermentation broth with yeast at 5% solid loading and 15 FPU/gm of enzyme loading.

[Please insert Fig.4 here]

Fig.4 showed the ethanol concentration, fermentation yield and digestibility of solid residues obtained from S-I, S-II, and S-III pretreatment. S-II and S-III exhibited increased digestibility and glucose yields compared to S-I pretreated sample. The related calculations from leftover sugars and ethanol showed 47.12, 88.74, and 92.14% cellulose digestibility of S-

I, S-II, and S-III the pretreated substrates, respectively. It is well-known that a higher removal of lignin usually leads to a higher enzymatic hydrolysis rates and higher glucose yields for both S-II and S-III. Hydrophobic interaction between enzyme and condensed residual lignin significantly reduced digestibility (sugar release) of S-I pretreated substrate [29]. The released sugars resulted in approximately 76.0, 82.4, and 82.0% fermentation yields for S-I, S-II, and S-III, respectively. A similar ethanol conversion (82.68%) was obtained from hemicellulose sugars dissolved in the hydrolysates.

3.4. Characterizations of Lignin

Fractionated lignins were analyzed by 2D HSQC NMR spectra and compared with the benchmark ball-milled lignin (BML) . The NMR spectra were analyzed in terms of major lignin sub-units (S and G units), inter-unit linkages (β-O-4, β-5, and β-β), and lignin-carbohydrate complex (ester and benzyl ether linkages). The aromatic units (S and G units) were used as the internal standards for quantitative determination. The quantitative analysis was carried out using the integral value of α-position of the linkages at the same contour level [26].

Hardwood naturally contains about 46–75% syringyl units (S-lignin) and 25–50% guaicyl lignin (G-lignin) [3]. G-lignin contains more reactive sites and can be easily reacted with some other groups to form condensed structure. This conversion depends on the pretreatment conditions and solvent used for fractionation. S-I fractionated lignin contained the lowest amount of G-lignin and the highest amount of S-lignin indicating severe condensation during pretreatment [30]. On the hand, the highest amount of G-lignin in S-III fractionated lignin supported lignin stabilization and protection during biphasic pretreatment **Fig.5(a)**.

The lignin depolymerization starts from the cleavage of β-O-4 linkage which is regarded as the main pathways for lignocellulose fractionation. However, the functionality of fractionated lignin is directly proportional to the amount of β-O-4 linkages persevered in the fractionated lignin, for example, monolignol production. Therefore, in the lignin-first biorefinery, the prime concern is the fractionation of lignocellulose components while keeping the maximum amount of beneficiary β-O-4 linkages of lignin. In high severity condition (low pH), pretreatment induces the formation of detrimental -C=C- linkages (condensed lignin) which ultimately reduces the amount of beneficiary β-O-4 linkages in the fractionated lignin. Therefore, in S-I pretreatment, no β-O-4 linkage was detected in the fractionated residual lignin **Fig.5(a).** Severe condensation reaction of lignin was enhanced in the aqueous medium by a strong hydrolyzing effect. On the other hand, S-II pretreatment retained 7.05% β-O-4 linkages in the fractionated lignin while S-III pretreatment retained three-fold higher β-O-4 linkages in the fractionated lignin, i.e. 26.37% β-O-4 linkages. In other words, S-III pretreatment can preserve the lignin structure with approximately 42.47% β-O-4 linkages by comparing with BML (62.08%). It is believed that pentoxy stabilization and high solubility of lignin in pentanol phase can effectively separate lignin from the high acid severity environment. The phase separation can help to protect reactive linkages of lignin from vulnerable aqueous acidic environment and preserve a higher proportion of the most important β-O-4 linkages. In addition, the lowest amount of condensed β-5, and β-β linkages also supported the phenomena of preserving high proportion of reactive lignin S-III fractionation.

[Please insert Fig.5 here]

All fractionated lignins showed similar IR spectrum with a distinctive signal at 1029 cm^{-1} as shown in **Fig.5(b)**. It was attributed by the complex deformation of C–C, C=C bending, and C–O–H groups. For S-I, the intense peak at 1029 cm−1 may be due to excessive formation of condensed structure (C–C and C=C) in fractionated lignin. In ATR-FTIR spectra of all lignin samples, absorption bands found at 1546, 1505, and 1459 cm^{-1} corresponding to the vibrations of the phenyl alkane aromatic ring. The wide absorption band at 3382 cm^{-1} attributed to both the aromatic and aliphatic OH groups, while the absorption bands for C–H vibration of the –CH_2 and –CH_3 groups appeared at 2933, 2885, and 1459 cm^{-1} respectively [31]. The band at 1694 cm^{-1} may be due to non-conjugated carbonyl linkages while the conjugated carbonyl groups with aromatic rings appeared at two different positions at 1630 and 1694 cm^{-1}, which were probably due to p-coumaric acid and ferulic acid group in the lignin macro-molecules. The characteristic band of the syringyl ring appeared at 1392 cm^{-1}, while the stretching vibrations of C−O and C−C in the syringyl ring were assigned at 1122 cm^{-1} [32]. The presence of p-hydroxyphenyl groups appeared as an absorption band at 815 cm^{-1}. The vibration bands of C–C, C–O, and C=O stretching could be merged at 1225 cm^{-1}. A complex vibration band relating to the C–H, C–O, and C–C deformation was assigned at 1029 cm^{-1} [31, 33].

Molecular weight distribution of fractionated lignins were demonstrated with respect to retention time of GPC column **(Fig.5(c))**. The number average molecular weight of S-III lignin (7593 Da) was slightly higher than the S-II fractionated lignins (6380 Da). Lignin molecular weight is directly proportional to the amount of β-O-4 linkages in the lignin [26]. This supported the positive effect of S-III pretreatment to stabilize lignin during fractionation to obtain high lignin integrity. Although dilute acid (S-I) cleaved almost all β-O-4 linkages of fractionated, condensation and repolymerization resulted high molecular weight (5775 Da).

3.5. Carbon efficiency of the process.

The flow of carbon was demonstrated in **Fig.6** for each pretreatment approach for acacia wood biorefinery. The desired carbon output streams were ethanol and fractionated lignin with reactive functional groups/linkages. Sugar degradation products such as HMF, furfural, and acetic acid appeared as byproduct streams.

In S-I pretreatment, only 17.10% of wood carbon was converted to bioethanol while recovering 40.96% carbon as condensed residual lignin. Carbon remained in residual lignin can only be used for low-value applications due to severe condensation during pretreatment. In addition, only 24.15% carbon can be obtained in the form of reducible sugars due to lower digestibility (47.12%) of dilute acid pretreated substrate. A total of 8.73% of wood carbon was converted to CO_2 which was a metabolic product during fermentation. The amount CO_2 formation was directly proportional to ethanol production from reducible sugars. A small portion of wood carbon was converted to fermentation inhibitors such as HMF (3.85%), furfural (5.42%), and acetic acid (2.08%).

[Please insert Fig.6 here]

Carbon efficiency was significantly increased due to higher amount of bioethanol (27.84%) and reactive lignin (21.75%) in product stream of S-II. As compared to S-I, an additional 10.74% carbon was obtained in the product stream as bioethanol and 21.75% carbon as reactive lignin which can be converted to value-added aromatic chemicals. Besides, the higher digestibility (21.75%) of the pretreated pulp produced a higher carbon conversion (49.60%) in acacia wood biorefinery. Similar carbon efficiency was obtained in S-III pretreatment, the proposed S-III pretreatment preserved 49.29% of carbohydrate carbon to ethanol. However, fractionated lignin in S-III showed higher structural integrity and functionality due to higher proportion of reactive functional groups/linkages compared with the lignin recovered from S-II. In addition, S-III pretreatment is advantageous in fractionation and energy consumption which were discussed in the next sections. Considering pretreated pulp and fractionated lignin as the products, approximately 70% carbon efficiency was achieved for acacia wood biorefinery by S-III process which is comparable to the recent report (77.6%) in Science. [34].

3.6. Energy balance

The overall energy footprint was calculated for different process units to convert 1 kg of dry wood to bioethanol as shown **Fig.7**. Total energy consumption for size reduction (chipping & milling) was similar for S-II, and S-III (0.36 MJ), while S-I consumed higher energy (0.54 MJ) due to intensive milling after pretreatment. Pretreatment is the most energy-intensive process which consumed 32.90-60.14% of the total energy required for the conversion process. The energy required in pretreatment is directly proportional to solvent volume and pretreatment temperature. Again, solvent volume depends on the mechanism of pretreatment and solubility of targeted lignocellulose components/solutes [29]. Dilute acid (S-I) pretreatment only dissolves readily hydrolysable hemicellulose and therefore, requires less amount (1:3) of solvent/water [35]. On the hand, organosolv requires relatively higher solvent volume (1:7); since it aims to dissolve both lignin and hemicellulose in the liquor while leaving cellulose [36, 37]. Therefore, the energy consumption of S-I (1.72 MJ) was much lower than S-II (2.47 MJ) and S-III (2.39 MJ) pretreatments. Energy consumption difference between S-II and S-III was due to difference in specific heats of ethanol and pentanol. The higher energy consumption of S-II and S-III pretreatment was compensated by high energy yield in product. The product energy of S-I, S-II, and S-III pretreatment approaches were 3.261 MJ, 5.59 MJ, and 5.74 MJ, respectively.

[Please insert Fig.7 here]

For lignin first integrated biorefinery (S-II and S-III), fractionation of lignocellulose components, products, and solvent recovery are major challenges which consumes the major portion of total energy required [13]. For S-II (conventional organosolv) pretreatment, dissolved sugars and lignin fractionated the addition of water (three volume) as anti-solvents to the pretreatment liquor. The addition of anti-solvent increases the total volume of pretreatment liquor which in turn consumes a large extent of energy for solvent recovery and enrichment of liquors. Thus, S-II process consumed 4.69 MJ energy for fractionations and solvent recovery. S-III pretreatment fractionated lignin and hemicellulose sugars with a high distribution coefficient between pentanol and water phase, respectively. The physical separation of aqueous and organic phases provided energy-saving fractionation while maintaining the almost complete fractionation of hemicellulose sugars and lignin in the respective phases. S-III process reduced about 47.23% of the total energy consumed than S-II, due to no energy requirement in the liquor enrichment and solvent recovery. The organic phase containing lignin can be directly used to produce lignin foam and high-value lignin aromatic chemicals to strengthen the concept of integrated biorefinery. Thus, S-III process yielded 1.77 MJ of net positive energy from one kg of *Acacia Confusa* wood. It should be noted that the calculation was based on the results of laboratory experiments to evaluate the potential of full-scale biorefinery. Certain errors may be expected at large scale due to different rates of mass and heat transfer in different reactors.

4. Conclusion

The proposed pentanol-water biphasic (S-III) pretreatment can be a budding candidate for pretreatment of lignocellulose in the biorefinery, which saved 47.23% of the total energy consumption compared to conventional S-II process by reducing hemicellulose and lignin fractionation. S-III process yielded a net positive energy 1.77 MJ/kg of wood with 50.33% carbon recovery in product stream as ethanol and reactive lignin. It resulted in approximately 70.27% lignin removal and 92.14% enzyme digestibility of substrates. S-III pretreatment preserved up to 42.47% of original β-O-4 linkages due to the physical separation of lignin from a vulnerable acidic environment. Thus, the use of pentanol as a pretreatment solvent is possible, thereby can achieve the objectives of high energy output and saccharification yield in the biorefinery. The productivity of both lignin and carbohydrates are both close to all state-of-the-art techniques and may be further increased. However, the successfulness of the biphasic (S-III) process will rely on effective solvent recovery, lignin removal, and recovery on large scale, which need to be further optimized.

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Figure Captions

Fig.1. Demonstration of major unit operations and unit process for (S-I) dilute acid, (S-II) ethanol pretreatment, and (S-II) pentanol-water biphasic pretreatment system.

Fig.2. Process optimization for pentanol-water biphasic solvent using pretreatment efficiency for varying (a) solvent composition, (b) liquid to solid ratio, (c) catalyst concentration, and (d) pretreatment temperature.

Fig.3. Chemical compositions of pretreated *Acacia Confusa* wood residues and pretreatment liquor. The data are represented as gram per 100 gm of raw biomass used for pretreatment.

Fig.4. SSF yield in terms of (a) ethanol concentration, (b) fermentation yield, and (c) digestibility for S-I, S-II, and S-III pretreated substrate using *Saccharomyces Cerevisiae* yeast to produce ethanol.

Fig.5. Characterization of fractionated lignin in terms of (a) semi quantitative values of major sub-units and linkages (b) infrared responses and (a) molecular weight distribution.

Fig.6. Carbon flow diagram of S-I, S-II, and S-III for biorefinery conversion of *Acacia Confusa* wood residues. The values were expressed as the percentage of carbon based on the carbon content of the entire wood.

Fig.7. Comparative energy footprints for bioethanol production from *Acacia Confusa* wood biomass for S-I, S-II, and S-III.

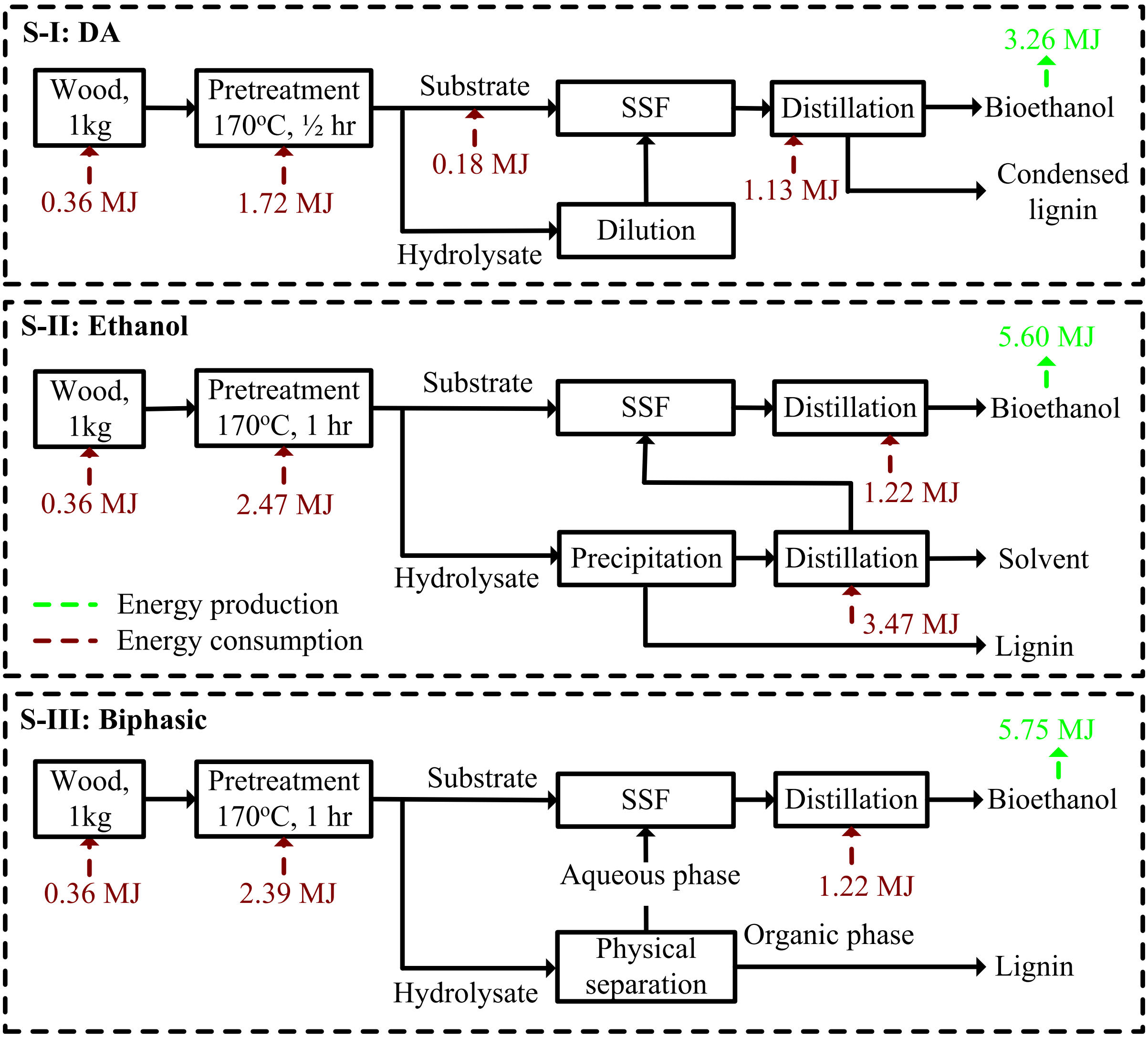

S-I: DA
Wood, 1kg
Pretreatment 170°C, ½ hr
Substrate
SSF
Distillation
Bioethanol
3.26 MJ
0.36 MJ
1.72 MJ
0.18 MJ
1.13 MJ
Dilution
Hydrolysate
Condensed lignin
S-II: Ethanol
Wood, 1kg
Pretreatment 170°C, 1 hr
Substrate
SSF
Distillation
Bioethanol
5.60 MJ
0.36 MJ
2.47 MJ
1.22 MJ
Hydrolysate
Precipitation
Distillation
Solvent
3.47 MJ
Lignin
Energy production
Energy consumption
S-III: Biphasic
Wood, 1kg
Pretreatment 170°C, 1 hr
Substrate
SSF
Distillation
Bioethanol
5.75 MJ
0.36 MJ
2.39 MJ
Aqueous phase
1.22 MJ
Organic phase
Physical separation
Lignin
Hydrolysate

Fig.1

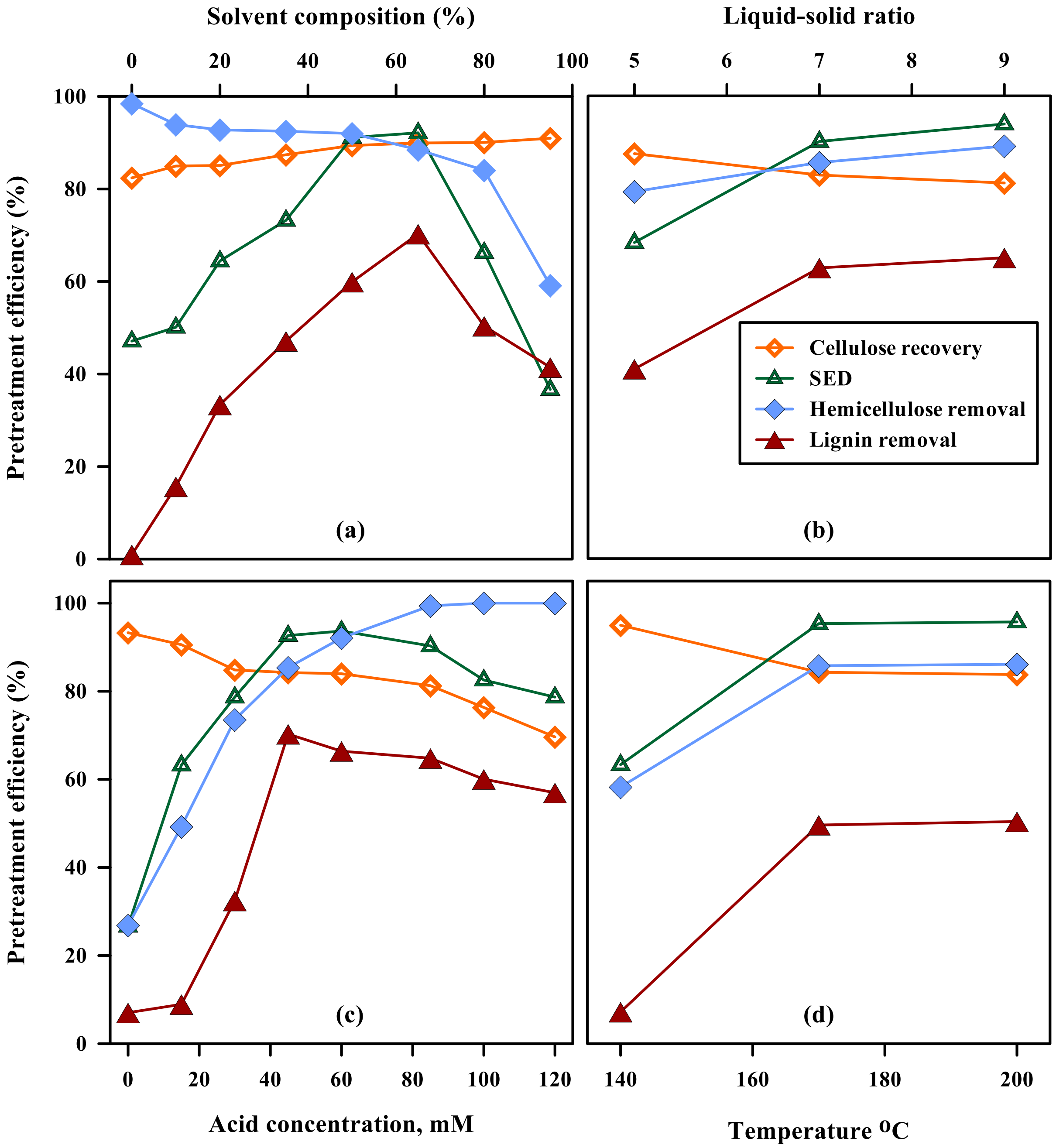
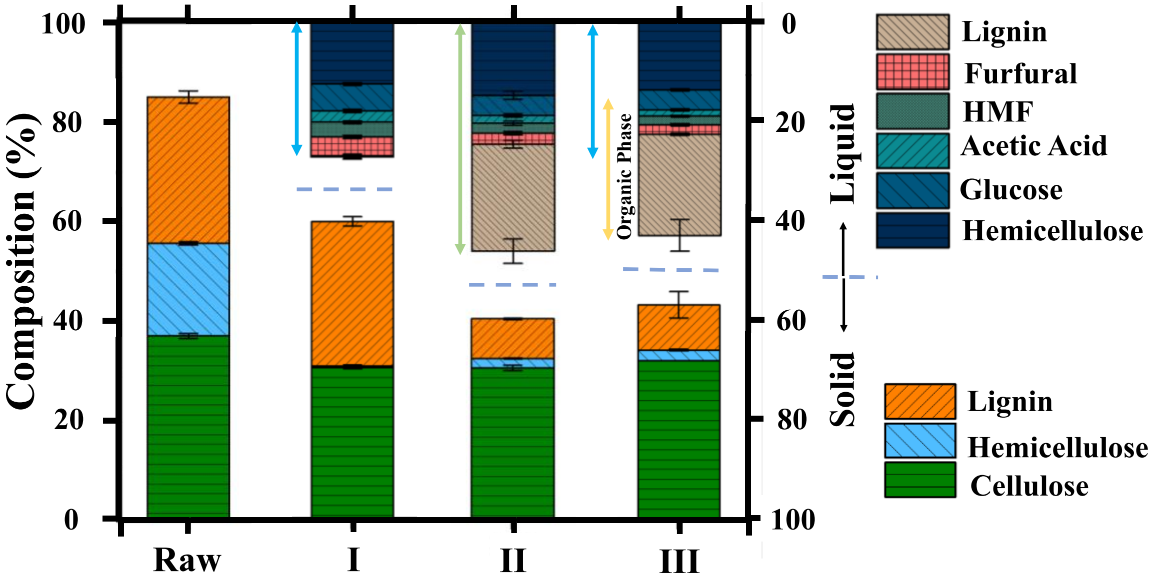

Solvent composition (%)
0
20
40
60
80
100
Liquid-solid ratio
5
6
7
8
9
Pretreatment efficiency (%)
0
20
40
60
80
100
(a)
(b)
Cellulose recovery
SED
Hemicellulose removal
Lignin removal
Pretreatment efficiency (%)
0
20
40
60
80
100
(c)
(d)
0
20
40
60
80
100
120
Acid concentration, mM
140
160
180
200
Temperature ^{0}C

Fig.2



Composition (%)
100
80
60
40
20
0
Raw
I
II
III
0
20
40
60
80
100
Liquid
Solid
Organic Phase
Lignin
Furfural
HMF
Acetic Acid
Glucose
Hemicellulose
Lignin
Hemicellulose
Cellulose

Fig.3

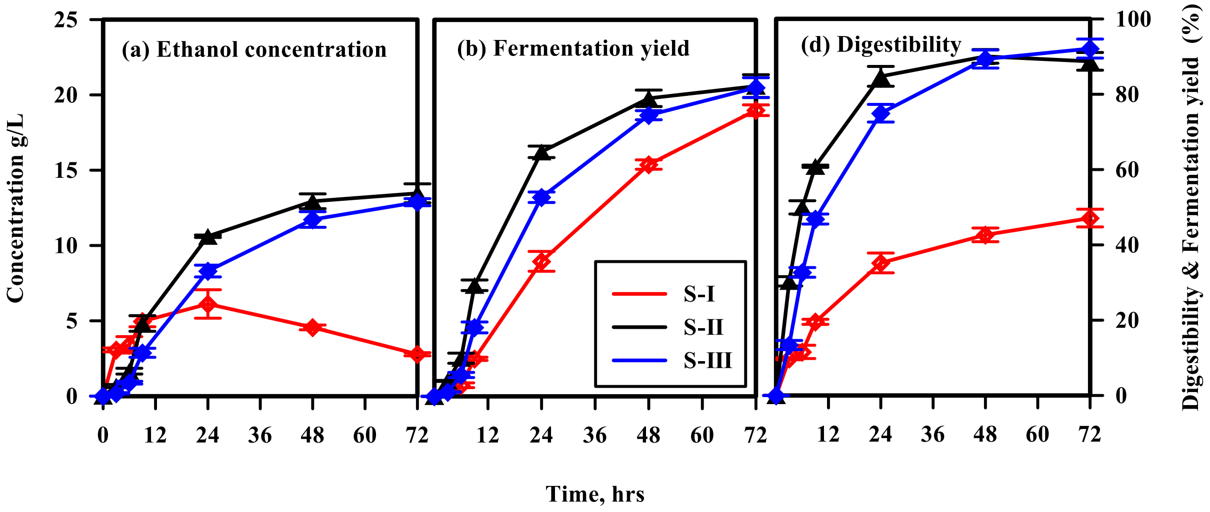

(a) Ethanol concentration
(b) Fermentation yield
(d) Digestibility
Concentration g/L
Digestibility & Fermentation yield (%)
Time, hrs
S-I
S-II
S-III
0
5
10
15
20
25
0
20
40
60
80
100
0
12
24
36
48
60
72

Fig.4

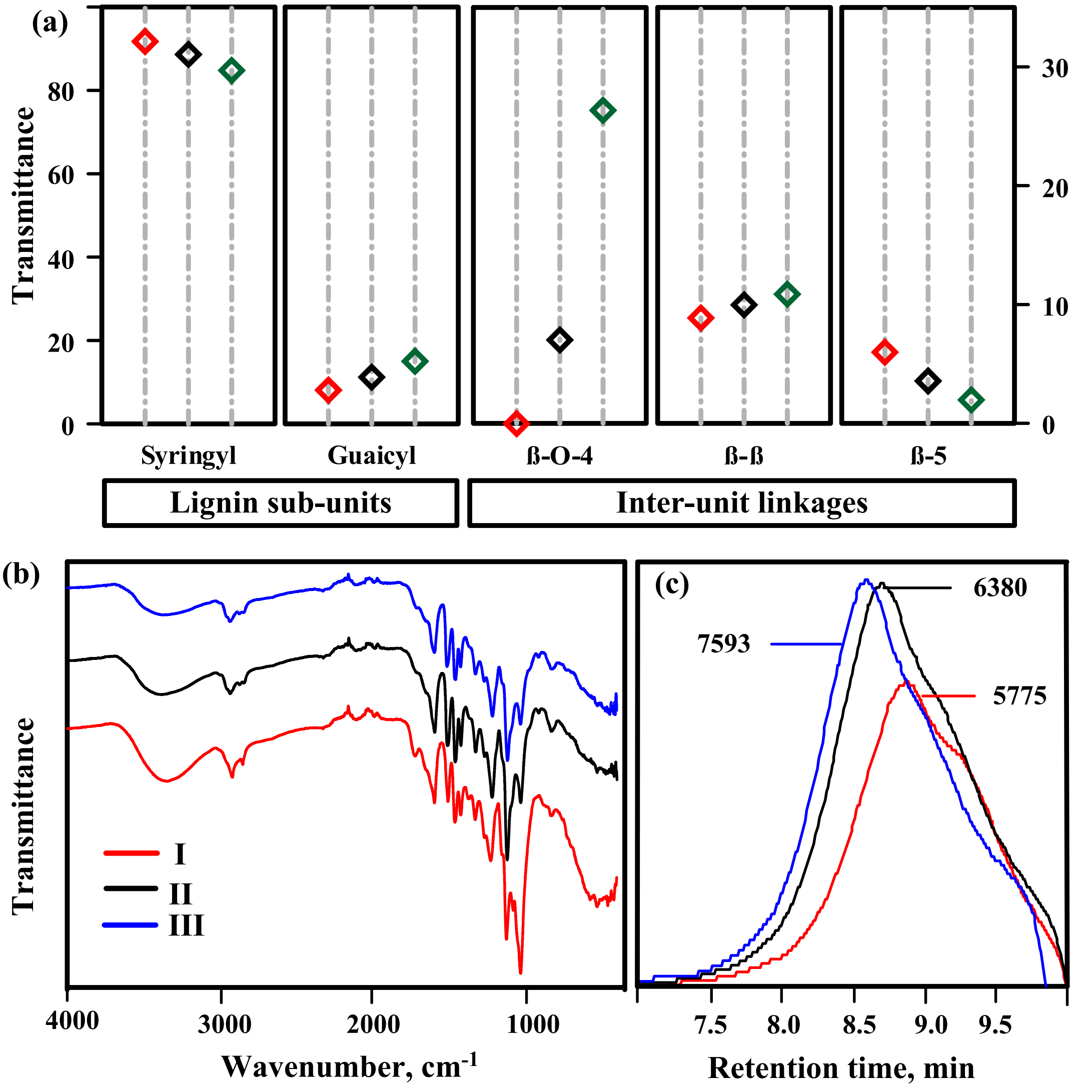

(a)
Transmittance
0
20
40
60
80
0
10
20
30
Syringyl
Guaicyl
ß-O-4
ß-ß
ß-5
Lignin sub-units
Inter-unit linkages
(b)
Transmittance
I
II
III
4000
3000
2000
1000
Wavenumber, cm^{-1}
(c)
6380
7593
5775
7.5
8.0
8.5
9.0
9.5
Retention time, min

Fig.5

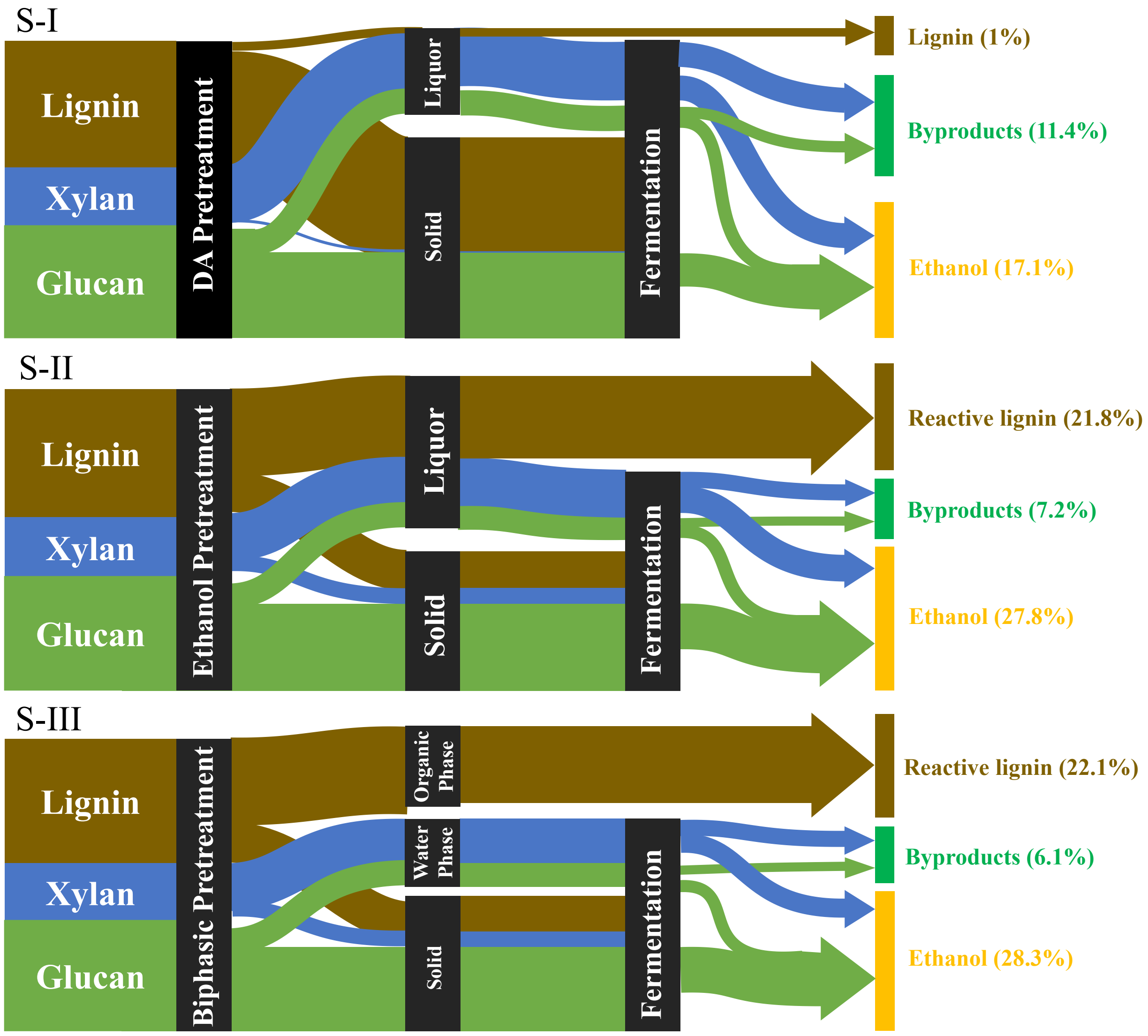

S-I
Lignin
Xylan
Glucan
DA Pretreatment
Liquor
Solid
Fermentation
Lignin (1%)
Byproducts (11.4%)
Ethanol (17.1%)
S-II
Lignin
Xylan
Glucan
Ethanol Pretreatment
Liquor
Solid
Fermentation
Reactive lignin (21.8%)
Byproducts (7.2%)
Ethanol (27.8%)
S-III
Lignin
Xylan
Glucan
Biphasic Pretreatment
Organic Phase
Water Phase
Solid
Fermentation
Reactive lignin (22.1%)
Byproducts (6.1%)
Ethanol (28.3%)

Fig.6

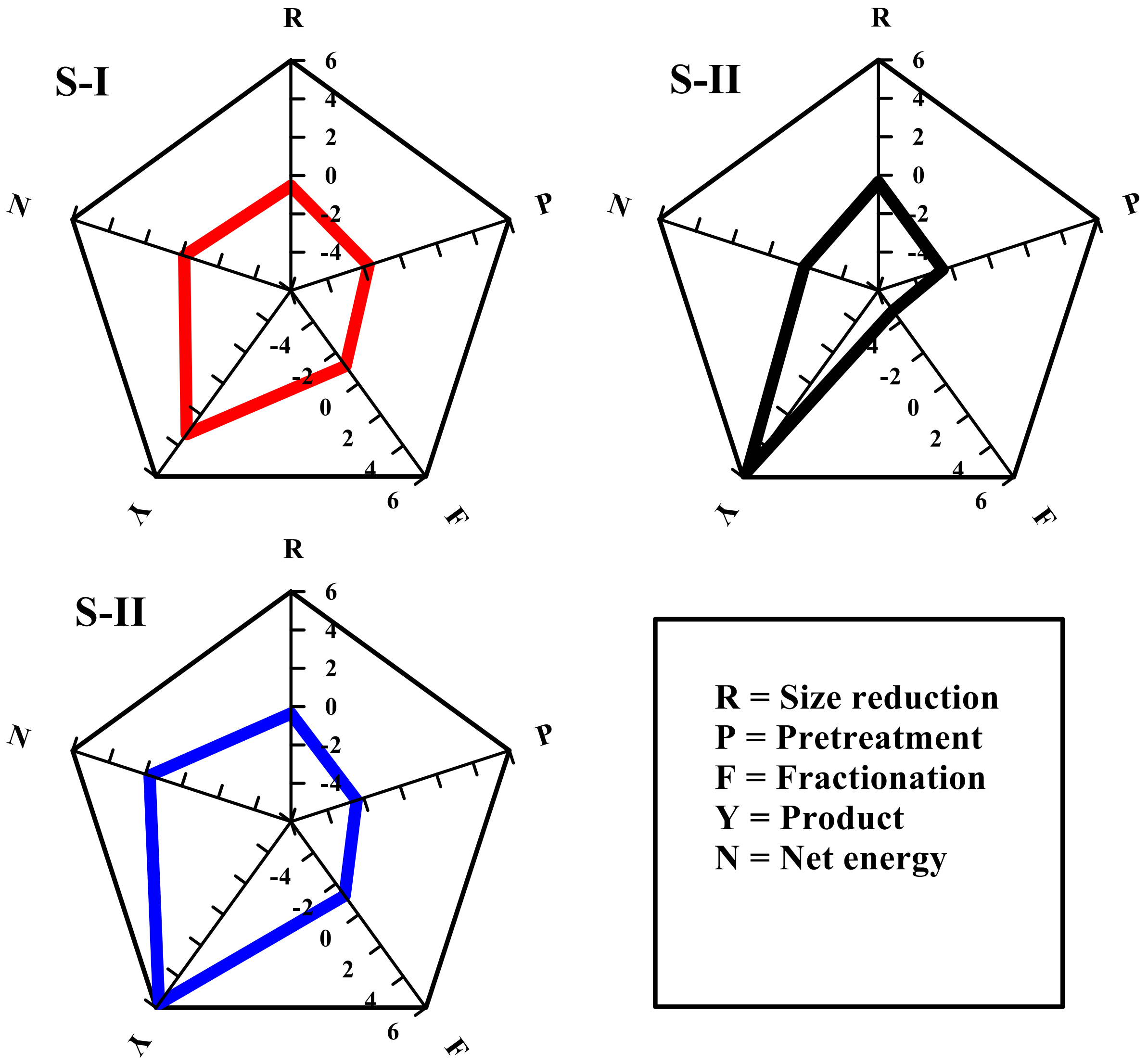

S-I
S-II
S-II
R
P
F
Y
N
6
4
2
0
-2
-4
R = Size reduction
P = Pretreatment
F = Fractionation
Y = Product
N = Net energy

Fig.7

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: