



Chemical and structural changes of pretreated empty fruit bunch (EFB) in ionic liquid-cellulase compatible system for fermentability to bioethanol

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Abstract

The pretreatment of empty fruit bunch (EFB) was conducted using an integrated system of IL and cellulases (IL-E), with simultaneous fermentation in one vessel. The cellulase mixture (*PKC-Cel*) was derived from *Trichoderma reesei* by solid-state fermentation. Choline acetate [Cho]OAc was utilized for the pretreatment due to its biocompatibility and biodegradability. The treated EFB and its hydrolysate were characterized by the Fourier transform infrared spectroscopy, scanning electron microscopy, and chemical analysis. The results showed that there were significant structural changes in EFB after the treatment in IL-E system. The sugar yield after enzymatic hydrolysis by the *PKC-Cel* was increased from 0.058 g/g of EFB in the crude sample (untreated) to 0.283 and 0.62 ± 06 g/g in IL-E system after 24 and 48 h of treatment, respectively. The EFB hydrolysate showed the eligibility for ethanol production without any supplements where ethanol yield was 0.275 g ethanol/g EFB in the presence of the IL, while lower yield obtained without IL-pretreatment. Moreover, it was demonstrated that furfural and phenolic compounds were not at the level of suppressing the fermentation process.

Keywords Structure · Pretreatment · Cellulase · Ionic liquid · Hydrolysis · Lignocellulose

Abbreviations

ILs	Ionic liquids
IL-E	Ionic liquid-cellulase system
EFB	Empty fruit bunch
<i>PKC-Cel</i>	Cellulase produced on palm kernel cake
PKC	Palm kernel cake
[EMIM]OAc	(1-Ethyl-3-methylimidazolium acetate)
[Cho]OAc	Choline acetate

[Cho]Bu	Choline butanoate
[EMIM]DEP	(1-Ethyl-3-methylimidazolium diethyl phosphate)
[EMIM]Me(H)PO ₂	(1-Ethyl-3-methylimidazolium methylphosphonate)

Introduction

Significant amounts of lignocellulosic oil palm biomass are generated from palm oil industries. A hectare of oil palm plantation can yield about 21.6 tons of biomass per year. Fronds and empty fruit bunch (EFB) are amounted to 50.3 and 20.4%, respectively (Chew and Bhatia 2008), with the most noteworthy rate of cellulose displayed in EFB. The EFB is an abundant agro-industrial waste with less monetary value which can cause a fundamental issue for the environment. It is disposed into landfills, utilized as a part of soil treatment or burned. Given the relatively high content of carbohydrate in EFB, it counts as a cheap source of ethanol and lignocellulosic derivatives, for instance, glucose, xylose, mannose, and animal feed (Ishola and Taherzadeh 2014).

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Empty fruit bunch characteristics might vary from one source to another due to several variables such as the age, size, growth phase, geographic area, soil condition, and climate effects associated with the oil palm tree, as well as the testing methods applied to the resulted EFB. It comprises of 65% cellulose, 29.2% lignin, 28.8% hemicellulose, and up to 3.7% extractive substances (Abdullah and Sulaiman 2013). Due to its compositions, this agro-industrial substance serves as an alternative resource for biofuels production and may be utilized for sugar production and bioconversion to different types of products. The cellulose itself is widely known as one of the four prime components of lignocellulosic materials which is protected by lignin and hemicelluloses (Laureano-Perez et al. 2005). For an enzymatic hydrolysis process to take place towards the cellulose, the difficulty arises as the substance needs to be uncovered through a pretreatment process for the enzyme to make a contact. In other words, without the assistance of a pretreatment process, the main encounter of EFB saccharification is the content of hemicellulose and lignin (Bari et al. 2009). Thus, it is a prominent fact that one of the key steps for an effective use of biomass in the conversion process to ethanol is the pretreatment of lignocellulose (Hahn-Hägerdal et al. 2006).

Several approaches have been applied to convert the complex carbohydrates content of EFB. Some of them include the treatment of the material with sulfuric acid (Harun and Danquah 2011) and the treatment with sodium hydroxide and steam (Choi et al. 2013), followed by enzymatic hydrolysis. However, these acid and alkali treatments could have resulted in the release of furfural and hydroxymethylfurfural which contribute to the toxicity and inhibition of microbial growth.

Ionic liquids (ILs) have been introduced as another effective tool for lignocellulose pretreatment since they enable hydrogen bonds disruption and facilitate the molecule exposure to the cellulase for the hydrolysis process to take place (Mai et al. 2014). However, there is a possible downside where ILs might cause losses in cellulases activities. This issue has been addressed and coordinated with the focus of investigation towards delivering IL-cellulase compatibility that can withstand the effects of specific ILs. Some ILs are selective solvents and can produce fewer byproducts which offer more enzyme stability and normal microorganism growth (Elgharbawy et al. 2018; Reddy 2015). These were evident when inspecting the saccharification of yellow poplar biomass with 15% [EMIM]OAc (1-ethyl-3-methylimidazolium acetate) ionic liquid. Many researchers have been interested in studying the cellulases stability to detect the compatibility of the related enzyme when it is mixed with ILs in an IL-enzyme system, such as cholinium-based ILs (Ninomiya et al. 2015b). One possible interesting method is the single-step hydrolysis, or also known as one-pot hydrolysis, where the IL pretreatment

and lignocellulosic biomasses are combined in a single vessel for the bioconversion into ethanol. The elimination of the IL recovery process in this method may lead to a simple, in-situ conversion of the lignocellulose and would be beneficial in designing an integrated system for the production of ethanol. Moreover, the inhibitory effect that may arise can be minimized by controlling the temperature and the concentration during the biological process (Chong et al. 2013).

Most of the recent studies have demonstrated that the sugar production from lignocellulose underwent a pretreatment step followed by the cellulose regeneration through a washing process, with enzymatic hydrolysis followed after. The generated hydrolysate shall then undergo fermentation to produce ethanol if applicable. For instance, bagasse (Ninomiya et al. 2015c) and rice straw (Pooornejad et al. 2014) have been used for that purpose. Other recent investigations have demonstrated the one-step processing of lignocellulose in IL using cotton cellulose (Auxenfans et al. 2012) and sawdust (Auxenfans et al. 2017) using [EMIM]DEP (1-ethyl-3-methylimidazolium diethyl phosphate), [EMIM]OAc, and [EMIM]Me(H)PO₂ (1-ethyl-3-methylimidazolium methylphosphonate) ionic liquids, respectively. Choline acetate and ultrasonication were used for the pretreatment of bagasse incompatible enzymatic hydrolysis with 72% saccharification yield (Ninomiya et al. 2015a).

This study utilized EFB in a single reactor, with a focus on the integration of IL and cellulase (from waste byproduct). The cellulase enzyme itself was locally produced on the agro-industrial waste, palm kernel cake (PKC) without any other nutrients supplements and named as the PKC-*Cel*. In addition, comprehensive analyses were executed to assess the suitability of the hydrolysate for fermentation, before and after the hydrolysis process. It is predicted that this approach would offer a clear knowledge on the impact of IL-enzyme interactive system on the direct procedure of lignocellulose transformation into sugar and ethanol.

Materials and methods

Raw materials

The basic raw materials used in this research are palm kernel cake (PKC) and empty fruit bunch (EFB) from palm oil mill plantation. The samples were provided by Sime Darby Plantation, Carey Island Malaysia in a single batch of 25 kg for each and was consumed throughout the span of the study. After collection, samples were oven dried at 60 °C until a consistent weight was achieved, then ground to the desired size of less than 0.5 mm, and subsequently stored in air-tight containers for future use.

Production of PKC-Cel

Locally produced cellulase was extracted as a product of the fermentation of PKC as previously described in our report (Elgharbawy et al. 2016a). The cellulase enzyme was called 'PKC-Cel' in reference to the main medium that was used for production. PKC-Cel was produced through solid-state fermentation of palm kernel cake as the basal medium. The fermentation was conducted by inoculating samples with the spores' suspension of *Trichoderma reesei* (RUTC30) for 7 days. Crude enzyme was extracted with sodium citrate buffer (pH 4.8 ± 0.2) followed by centrifugation, and micro- and ultra-filtration to remove the cell debris and to confirm the range of the molecular weight protein obtained. The retentate containing the enzyme was collected and tested for enzyme activity. The activity was measured using filter paper assay described by Ghose (1987) and Endo- β -1,4-D-glucanase assay using carboxymethyl cellulose (CMC) (Salvador et al. 2010).

Enzymatic hydrolysis of EFB

In a glass vial, the EFB (1.875 g) was weighted, IL (5 g) was added, mixed until homogenized, and heated at 75 °C for 60 min and cooled before the buffer solution was added to achieve 10% IL-buffer solution (v/v). After that, the ultra-filtered (UF) cellulase was loaded (45 FPU/g of the EFB). After 48 h of the enzymatic reaction, the reducing sugar concentration was assessed using DNS assay. Steps and procedures were comprehended in the previous publication (Elgharbawy et al. 2016b).

Characterization of the native and treated EFB for IL-E evaluation

The chemical constituents of the EFB were determined following the standard outlined in the NREL test methods with some modifications based on a recent publication (Ninomiya et al. 2015a).

Structural carbohydrates and sugar determination

The determination of lignin and hemicellulose was made by an amount of 0.1 g of the sample mixed with 2 mL of a 72% (v/v) H_2SO_4 aqueous solution for 2 h at room temperature, then diluted with 75 mL of water, and autoclaved at 121 °C for 15 min. The acid-diluted hydrolysate was filtered. The solid remainder was used for the assessment of the acid-insoluble lignin (AIL) by measuring the weight after drying at 100 °C for 12 h. The amount of acid-soluble lignin (ASL) was determined from the ultraviolet (UV)

absorbance of the filtrate at 205 nm (absorption coefficient of 110 L/g/cm).

The sum of the acid-insoluble and acid-soluble lignin was expressed as the total lignin amount (Ninomiya et al. 2015c). The amount of hemicellulose was calculated from the xylose content as stated in Eq. (1):

$$\text{Hemicellulose (g/g biomass)} = \text{xylose concentration g/L} \times \frac{\text{volume of sample}}{\text{weight of dry EFB}} \times \text{Correction factor} \left(\frac{132}{150} \right). \quad (1)$$

The ratios are obtained from the corresponding polymeric sugars (xylan and arabinan) and calculated from the concentration of the monomeric sugars, using anhydrous correction factors of 0.88 (or 132/150) for xylose and arabinose (Nieves et al. 2016).

Cellulose content in the native and treated EFB was determined by anthrone method using microcrystalline cellulose for standards (Updegraff 1969). The phenol-sulfuric acid assay was employed to measure the carbohydrate content of the biomass (Dubois et al. 1956).

Total soluble sugar was measured by dissolving 100 mg of the EFB sample in 100 mL of distilled water; the total free sugar existing in the sample was extracted and filtered through Whatman No.1 filter paper. The analysis of the obtained filtrate was performed by applying phenol-sulfuric method. The total reducing sugar (TRS) of glucose, galactose, mannose, and arabinose were determined in total as described by Salvador et al. (2010).

Monosaccharides and oligosaccharides were determined by high-performance liquid chromatography (HPLC) using COSMOSIL SUGAR-D column (4.6 mm I.D. \times 250 mm), US, which consists of high-purity porous spherical silica gel. The stationary phase consists of secondary and tertiary amine. Glucose, xylose, mannose, arabinose, and galactose were all determined in hydrolyzed samples by running along standard sugar solution from each sugar. Samples were prepared for the analysis by filtering 1.0 mL of each sample into a clear glass HPLC vial using a 0.2 μ m nylon syringe filter. Four different concentrations of each sugar were prepared using deionized water as the solvent. The standard dilutions were then transferred into the HPLC vials using the syringe filter. HPLC was employed on Waters 600 system (Hampton, US) with a refractive index detector (RID) equipped with acetonitrile as the mobile phase at the ratio of 70:30, 75:25, and 80:20 in deionized water with fixed flow rate of 1.0 mL/min and 30 °C temperature. A membrane filter was utilized to filter the mobile phase to avoid column blockage.

Acetic acid was quantified using Agilent 1200 HPLC system (California, USA) with a refractive index detector (RID) equipped with REZEX ROA column (Phenomenex, USA). Sulfuric acid (5 mmol/L) was used as the mobile

phase at a fixed flow rate of 0.6 mL/min with isocratic elution at ambient temperature. The sample injection volume was 20 μ L. Standard curves were generated using different concentrations of acetic acid. A guard column with similar packing was used to increase the longevity of the main column. The analysis results were kindly provided by UKM, Institute of Systems Biology (INBIOSIS), Bangi, Selangor, Malaysia.

Morphological structural changes

Screening electron microscopy (SEM) was used for native (untreated) and treated samples characterization. All treated samples were dried using freeze dryer (LABCONCO) and stored at -80 °C. The dried samples of both treated and untreated were used for observation using scanning electronic microscope (SEM) of Hitachi SU1510 SEM at, 500 \times , 1000 \times , and 5000 \times magnification at back-scatter detector (BSE) mode. The analysis was performed at Quasi-S Sdn. Bhd, UKM-MTDC Smart Technology Center, Bangi, Malaysia.

Fourier transform infrared spectroscopy (FTIR)

The native (raw) EFB, IL-treated EFB, and the hydrolysate were frozen dried for 72 h, and then analyzed by FTIR within the range of 4000–500 cm^{-1} with 16 scans at a resolution of 4 cm^{-1} . Spectral outputs were recorded in the absorbance mode as a function of wavenumber.

Swelling capacity

As demonstrated by Noori and Karimi (2016), swelling capacity of the native and pretreated EFB was measured by adding 0.1 g of the dry sample into a bag of nonwoven material and then immersed in distilled water for 1 h. The difference in weight of the dry material (W_1) and the swollen material (W_2) was recorded, and the swelling capacity was calculated as shown in Eq. (2):

$$\text{Swelling capacity} = \frac{W_2 - W_1}{W_1}. \quad (2)$$

Enzyme adsorption and desorption

Samples (0.05 g) of the untreated or pretreated substrate and 400 FPU/g cellulase were mixed with citrate buffer (5.0 mL, 50 mM pH 4.8) in 15-mL-centrifuge tubes. All tubes were incubated at 4 °C for 2 h and mixed at 100 rpm. The content was then centrifuged at 4000 rpm and 4 °C for 15 min, and the protein content of the unabsorbed cellulase was measured in the supernatants using Bradford assay. For enzyme

desorption estimation, the prepared samples were diluted with 5 mL citrate buffer at 4 °C and incubated in the shaker for two more hours at 4 °C, centrifuged and the protein content was determined. Equation (3) was used to quantify the average percentages of cellulase desorption:

$$\text{Cellulase desorption (\%)} = \left(\frac{C - D}{C} \right) \times 100, \quad (3)$$

where C and D are the average amounts of cellulase adsorbed on the pretreated solids (mg/g) over 2 h and the unabsorbed protein (mg/g) after desorption over 2 h, respectively (Noori and Karimi 2016).

Crystallinity index

To investigate the effect of pretreatment on the EFB structure, the absorbance obtained from FTIR analysis was analyzed. Absorption band of 1430 cm^{-1} is the corresponding assignment of cellulose I which is resistant to hydrolysis, whereas band 896 cm^{-1} represents cellulose II which is readily hydrolyzed (Kljun et al. 2011).

Determination of total nitrogen (TN) and total phosphorus (TP)

HACH Method 10071 (Test 'N Tube Vials) was used to determine the total nitrogen content in the samples obtained after hydrolysis. HACH Method 10127 (Test 'N Tube Vials) using Molybdovanadate reagent was used for assessing the phosphorus in the samples.

Determination of minerals

The contents of Sodium (Na), potassium (K), calcium (Ca), and copper (Cu), were determined using in-house Method No. STP/Chem/A13 Microwave Digestion-AAS as referred by UNIPEQ-MTDC Technology Center, Universiti Kebangsaan Malaysia (Ref: ULUKM/1787/16). Iron (Fe), zinc (Zn), and manganese (Mn) were determined using atomic absorption spectrophotometry (AAS). All samples were prepared in 0.2% nitric acid including standards.

Determination of total phenolic compounds (TPC)

Folin–Ciocalteu method (Wolfe et al. 2003) was applied for TPC determination in the samples at 760 nm using Multiskan™ GO Microplate Spectrophotometer. The absorbance was referred to a standard curve of prepared gallic

acid solutions and expressed as gallic acid equivalents in milligrams per 100 g of sample. Assays were performed in triplicate for each sample.

Determination of furfural

Furfural content was determined using phenylhydrazine solution method and absorbance was recorded at 446 nm (Zarei 2009). The same steps were tracked for the standard preparations except that the sample solution was replaced with different concentrations of furfural solution (0.5, 1, 1.5, and 2.0 g/L).

Fermentation to ethanol

An Erlenmeyer flask (250 mL) was used for fermentation in which 100 mL of the hydrolysate was autoclaved. After it had cooled down, it was inoculated with 2.5 mL of the prepared inoculum (*Saccharomyces cerevisiae*), plugged with a cotton cap, appropriately sealed, and incubated at 30 °C and 150 rpm. After 72 h, the obtained broth was centrifuged (8000 rpm, 15 min) and the ethanol concentration was assessed using chromic acid method (Caputi et al. 1968) and gas chromatography coupled mass spectrometry (GC–MS). The GC–MS used was Agilent 7890A GC, and 5975C MS with CTC-PAL autosampler equipped with headspace sampling module (Agilent Technologies, Wokingham, UK), DB-WAX (122-7032) capillary column, and Helium gas as a carrier at 25 cm/s measured at 150 °C. Sugar consumption was estimated using DNS assay over 84 h. The recovery of ILs from ethanol was conducted by distillation.

Results and discussion

Characterization of the native (untreated) and treated substrate to evaluate the effect of IL-E

Pretreatment and saccharification of EFB

The 24 h-hydrolysis in IL was performed to evaluate which IL could yield the highest sugar content as presented in Table 1. The EFB samples were exposed to a pretreatment in [Cho]OAc and [Cho]Bu ionic liquids, which later subjected to hydrolysis. The conversion differences between pretreated and native EFB indicated that the IL pretreatment disrupted the structure and improved the accessibility of the enzyme to the substrate.

The findings from this study demonstrated that [Cho]OAc and [Cho]Bu were perfectly compatible with *PKC-Cel* which was shown by the lignin diminishment along the treatment. Enhancement of the saccharification was similarly observed with the ILs' presence in the system. With the aid of [Cho]OAc, 0.14 ± 0.05 g of glucose was obtained from 1 gram of EFB. The yield was slightly lower when [Cho]Bu was used. Despite of the high ratio on the utilization of IL against the biomass in this laboratory-scale study, the possibility of recycling and immobilizing the IL during the process, as well as the implementation of one-vessel reaction, may help the commercialization of this method in the future. The results obtained are comparable with the studies conducted on biomass hydrolysis without ILs. Enzymatic hydrolysis of pretreated biomasses of weeds has been conducted with mechanical agitation and sonication under constant solid loading. It was shown that sonication accelerated the hydrolysis rate of weed biomass. However, it took 10 h of treatment

Table 1 EFB-pretreated and hydrolyzed sample analysis in two IL-compatible system

Entry	Acid-soluble lignin % (w/w)	Acid-insoluble lignin % (w/w)	Total lignin % (w/w)	Cellulose content % (w/w)
Content after pretreatment with IL compared with native EFB ^a				
Native EFB	1.14 ± 0.1	22.5 ± 0.5	23.6 ± 0.6	2.1 ± 1.01
[Cho]OAc	0.91 ± 0.05	15.9 ± 0.3	16.8 ± 0.35	32.6 ± 1.5
[Cho]Bu	1.10 ± 0.05	17.0 ± 0.5	18.1 ± 0.55	26.2 ± 0.54
Entry	Converted hemicellulose % (w/w)	Residual hemicellulose content % (w/w)	Total reducing sugar (g/L)	Glucose (g/L)
Content after hydrolysis in the presence of IL ^b				
Native EFB	0.6 ± 0.00	23.1 ± 0.87	10.0 ± 0.1	0.12 ± 0.1
[Cho]OAc	21.4 ± 0.2	10.8 ± 1.67	31.7 ± 0.1	16.6 ± 0.5
[Cho]Bu	15.7 ± 0.2	12.3 ± 2.05	30.1 ± 0.1	15.1 ± 0.1

^aConditions: 60 min pretreatment, 75 °C

^bConditions: 10% IL, 50 °C, 45 FPU/g EFB, 24 h enzymatic hydrolysis

to obtain 35–40 g/L of reducing sugar (Borah et al. 2016). The biomass of *P. hysterothorus* pretreated with sulfuric acid and delignified was subjected to enzymatic hydrolysis and yielded around 400 mg reducing sugar/g biomass. The results obtained in the current study (600 mg/g EFB) were higher considering the shorter period and enzyme consumption in both cases (Singh et al. 2015).

It can be seen that dispatching the lignin and hemicellulose partially improved the saccharification. The anions in ILs serve as hydrogen bond acceptors, as they interact with the hydroxyl groups in cellulose and weaken its structure. At the same time, the cations interact with lignin through hydrogen bonds as well as π - π interaction (Asakawa et al. 2015). These interactions between the IL components and the lignocellulose are assumed to reduce the inhibitory effect towards PKC-Cel. To support this hypothesis, it was reviewed by Zhao (2016) that insufficient water to hydrate kosmotropic or borderline anions (such as acetate, lactate, and chloride) of ILs bearing high H-bond basicity tends to interact strongly with enzymes causing their inactivation. Consequently, the enzyme stabilizing/activating kosmotropic anions (such as OAc^- and Cl^-) in diluted aqueous solutions become enzyme-inactivating agents in ILs with low (less than 1%) water contents. Perhaps, this could explain the withstanding of *PKC-Cel* in the IL-enzyme solution throughout the hydrolysis.

The highest sugar production was recorded with [Cho]OAc in the previous research by the authors (Elgharbawy et al. 2016a). As acquired from the analysis, the specimen treated with [Cho]OAc contained 24, 22.5, and 0.09 g/L of glucose, xylose, and arabinose individually. On the other hand, the native EFB hydrolysis released 0.12, 0.50, and 0.13 g/L of glucose, xylose, and arabinose respectively (applying the same conditions without IL pretreatment; 50 °C, 45 FPU/g EFB, and 24 h enzymatic hydrolysis). As seen from the sugar analysis, it points out that there are several types of reducing sugars in the hydrolysate, such as galactose, glucose, glyceraldehyde, fructose, or ribose.

The anions from ILs and hydrogen particles of the hydroxyl in the cellulose chain form hydrogen bonds to interact. These bonds interfered with the H-bonding of the cellulose, so that the hydrolytic rate increases and yields more sugar. Apart from that, the crystallinity and the degree of polymerization are diminishing by IL treatment which weakens the biomass structure (Yang and Fang 2015).

Above and beyond, an improvement in the hydrolysis of cellulose was realized to be achieved by the effect of the IL which was three times larger than the native samples due to the enlargement of the pores size of the EFB and lignin disruption (Sun et al. 2013). During and after the treatment, the structure of cellulose was exposed to polymorphic changes. The alterations in the structure facilitated the EFB enzymatic hydrolysis. Moreover, cellulose I is the crystalline form that

is hydrolysis-resistant. Transformation of cellulose I to other forms such as cellulose II or amorphous structures could improve their vulnerability to enzymatic hydrolysis (Samayam et al. 2011).

Changes in cellulose, hemicellulose, and lignin

Ionic liquids' ability to disturb the structure of the lignocellulosic biomass was demonstrated. The ILs' pretreatment has an impact on the hydrolysis, affecting the sugar production and the glucose. The content of the cellulose measured has varied. The native EFB cellulose that available for hydrolysis was determined at $2.1 \pm 1.0\%$ (w/w), and the content increased as the ILs were applied. [Cho]OAc offers the most remarkable cellulose content (available for hydrolysis after 24 h), $32.6 \pm 1.5\%$ (w/w), Table 1. Without pretreatment with ILs, the changes of the hemicellulose appeared null.

Removal of lignin boosts the biomass hydrolysis and the pretreatment with ILs conveys an imposing and crucial task. The lignin removal provides smoothness of the hydrolysis phase. The total lignin content from the native EFB was recorded at $23.6 \pm 0.6\%$ (w/w) with more reduction detected with [Cho]OAc at $16.81 \pm 0.35\%$ (w/w).

Total sugar for the native hydrolyzed EFB was 10.02 ± 0.1 g/L, while 31.7 ± 0.1 g/L was achieved in [Cho]OAc. In a comparable example, the elevated recognizable glucose concentration after hydrolysis was 16.57 ± 0.5 g/L with [Cho]OAc.

Scanning electron microscopy (SEM) for morphological structural changes

The structure and morphology were analyzed using SEM. Micrographs were produced for the EFB native sample and the ones treated with [Cho]OAc and [Cho]Bu (Figs. 1, 2). The structure of both samples in ILs gave comparable outcomes, but analysis of the pretreated samples had slight differences. With both ILs, the distorted surface was shown at first glance compared with the native EFB which seemed to be unbending and robust. The examination of the IL-pretreated samples after an hour revealed the cellulose strands. Figure 1 shows comparable images of the EFB with [Cho]Bu and PKC-Cel which are similar to the images obtained with [Cho]OAc. Both [Cho]OAc and [Cho]Bu demonstrated clear dissimilarities in the morphology of native EFB, after IL pretreatment and after IL-cellulase integration. It was expected to observe differences as a consequence of the lignin disruption and the cellulose crystallinity reduction which interrupts the tissue system. However, this was seen from the FTIR analysis as well as the chemical analysis of the biomass. Figures 1b and 2b are demonstrating the stretching and disruption of the surface in comparison with the native EFB. The exposure became clear with the

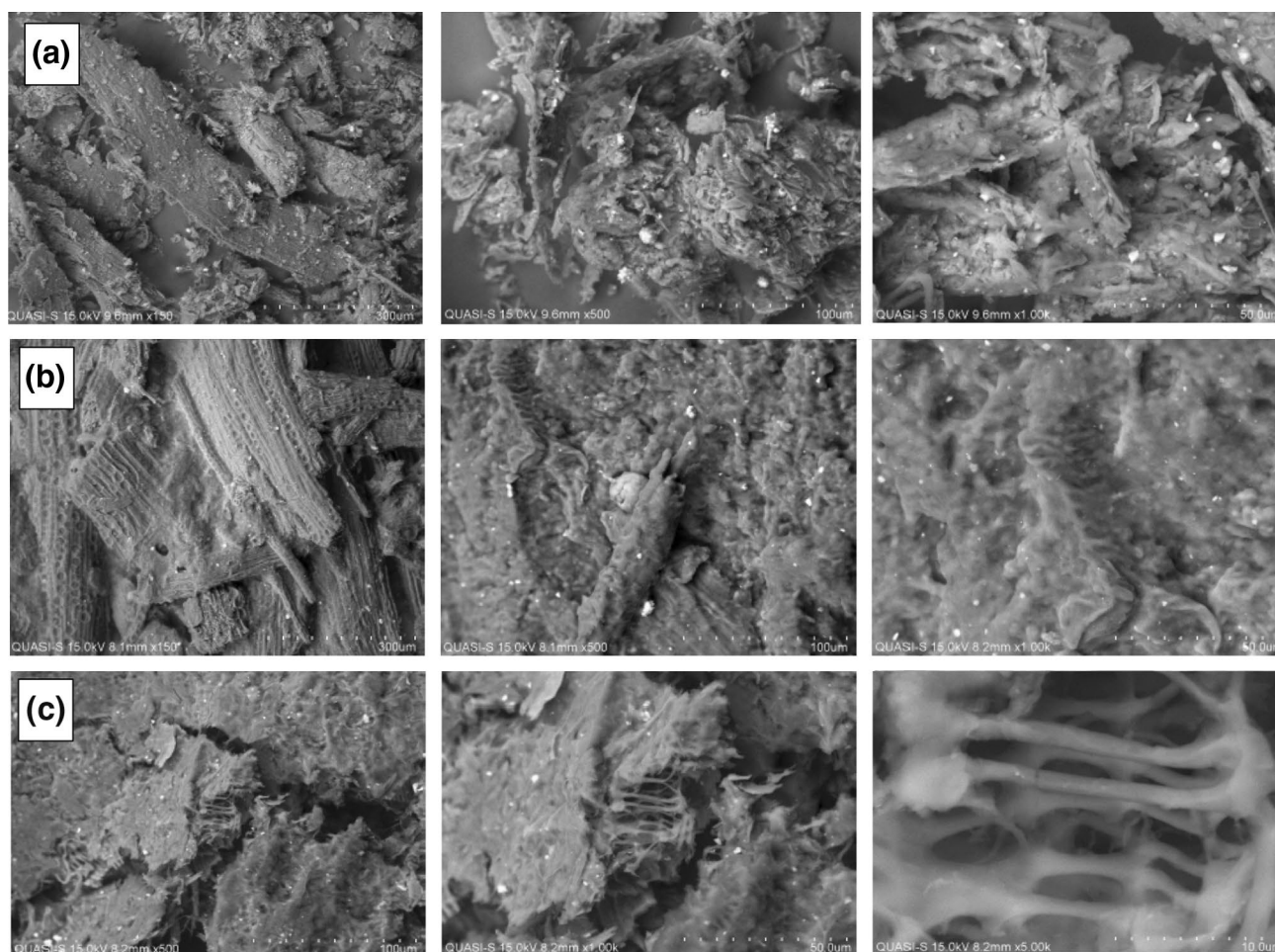


Fig. 1 SEM images of (a) native EFB (untreated empty fruit bunch) 150, 500 and 1000 \times , (b) EFB treated with [Cho]Bu, 150, 500 and 1000 \times (c) EFB treated with [Cho]Bu and PKC-Cel (cellulase), 500,

1000 and 5000 \times The images were obtained after one hour of the incubation period in IL and in E-IL system, (BSE) mode

integrated system. The surface was rough and solid in the native EFB but started being destroyed slowly in the presence of the IL.

Morphology and characterization of the surface changes showed that EFB-pretreated samples had a compacted structure with immaterial pores. The pretreated EFB had more open structures (Figs. 1c, 2c). The pretreatment with IL and PKC-Cel in one system made a smooth and swollen structure, and resulted in alterations of the surface and resulting in the exposure of the cellulose fibers. In general, it can be noted that lignocelluloses with less crystalline structures are more proper for enzymatic hydrolysis which is shown in the following sections.

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of native, IL-pretreated, and hydrolyzed samples in [Cho]OAc and [Cho]Bu are shown in Fig. 3.

Consistent with a study led by Abdul et al. (2016) on the treatment of oil palm EFB with AFEX, lignin, and hemicellulose structures were expected to be at 1800–900 cm^{-1} . The absorbance bands demonstrated prominently less intensity around 1505–1557 cm^{-1} in [Cho]OAc-pretreated samples. A similar result was denoted in [Cho]Bu at 1503–1556 cm^{-1} , as outlined in Table A.1 (supplementary data). The changes in the peaks were anticipated with the C=C vibration of the lignin aromatic ring. [Cho]OAc pretreatment and [Cho]Bu contributed to the lignin disruption of EFB. As observed, the absorbance expanded at around 896 and 1430 cm^{-1} compared with the native EFB which demonstrates the presence of cellulose (Nomanbhay et al. 2013).

Changes were also noted at 1720–1740 cm^{-1} in the EFB sample treated with [Cho]OAc and a similar range in [Cho]Bu was recorded compared to native EFB. The reduction in the peak intensity was recognized with a C–O–C extending hemicellulose band vibration and a C–H bonding of

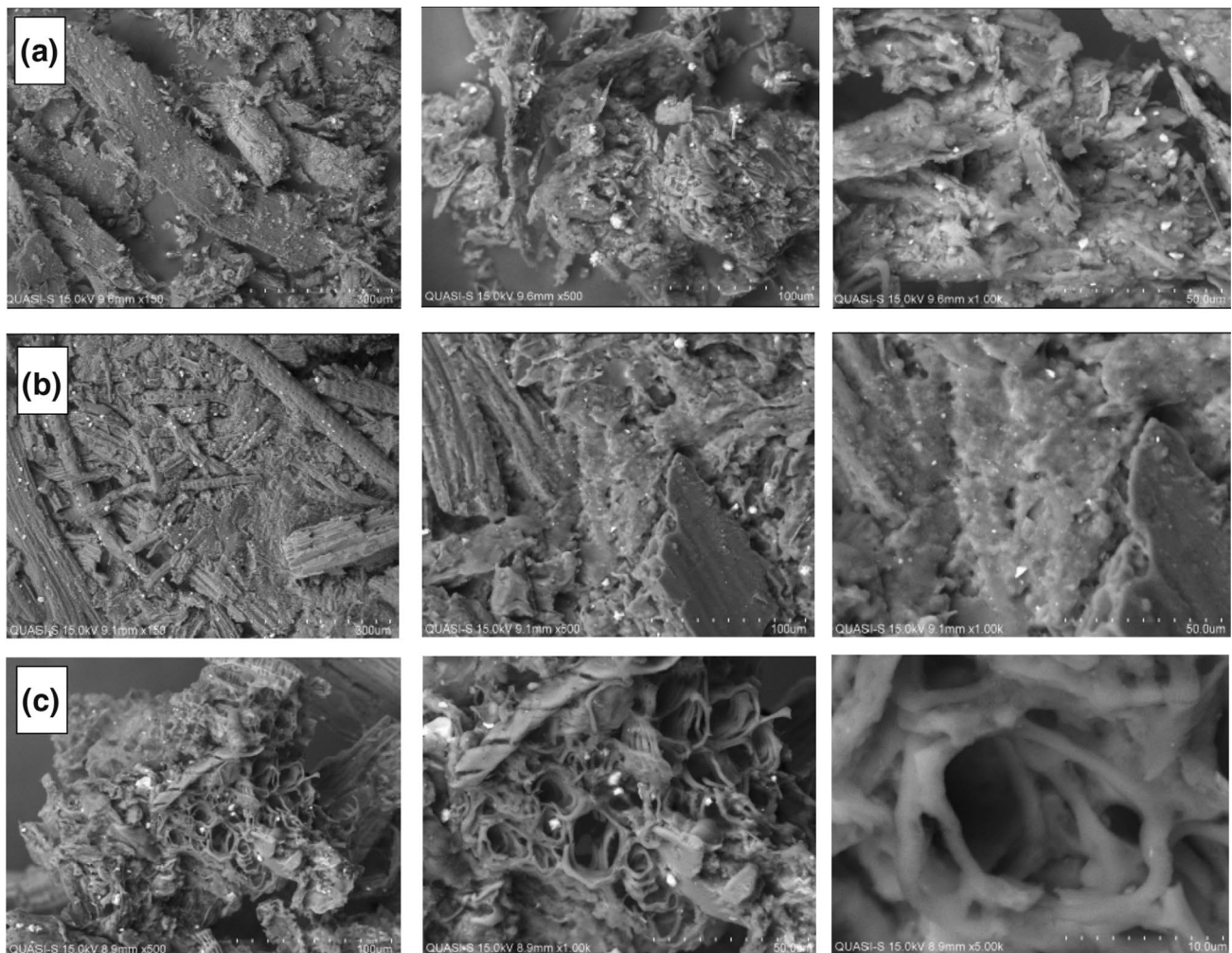


Fig. 2 SEM images of (a) native EFB (untreated empty fruit bunch) 150, 500 and 1000 \times , (b) EFB treated with [Cho]OAc (150, 500 and 1000 \times) (c) EFB treated with [Cho]OAc and PKC-Cel (cellulase),

(500, 1000 and 5000 \times). The images were obtained after one hour of the incubation period in IL and in E-IL system. (BSE) mode

cellulose. The results are supported by Abdul et al. (2016) who specified that the absorbance at 1738 cm^{-1} of EFB is related to carboxylic and carbonyl bonds which are found in hemicellulose. After [Cho]OAc and [Cho]Bu pretreatment were integrated with *PKC-Cel*, reducing sugar peaks appeared in the spectra ranging from 2947 to 3633 and 1707 to 1584 cm^{-1} . At 896 cm^{-1} , the band points towards cellulose II which is more acquiescent to hydrolysis. Cellulose I was reduced, while cellulose II appeared at 3751 cm^{-1} .

Chemical analysis of the treated-hydrolyzed EFB

Beside FTIR and SEM analysis, chemical analysis was also employed to acquire the information of the sample performance after the hydrolysis. The hydrolysate obtained contains carbon and nitrogen sources and minerals as demonstrated in Table 2.

Total phosphorus was 11.8 mg/g of EFB which contributes to 0.98% of the EFB total weight. The nitrogen content assessed in this study was 0.3 g/L . Metal ions of Zn^{2+} , Mg^{2+} , and Mn^{2+} have been determined at 0.183 , 0.103 , and 0.171 mg/L , respectively.

It is notable that the ability of yeast to form ethanol depends on several factors: for example, microbial strains and nutrients. The essential supplements in the media are carbon and nitrogen. Nitrogen is vital for yeast development and affects the ethanol generation and also the ethanol tolerance. Apart from carbon and nitrogen, micronutrients are also essential for cell growth and the fermentation process. Baharuddin et al. (2011) have estimated the cellulose at 50.3% of the EFB dry weight, lignin at 18% , whereas hemicellulose was 26.1% . Total nitrogen, phosphorus, and potassium were determined at 1 , 0.6 , and 2.3% , respectively, of the dry weight of the EFB.

Fig. 3 FTIR spectra of **a** native (untreated) EFB at the absorbance bands of 500–4000 cm^{-1} , **b** [Cho]Bu-pretreated EFB in enzymatic system (E-IL), **c** [Cho]OAc-pretreated EFB in enzymatic system (E-IL)

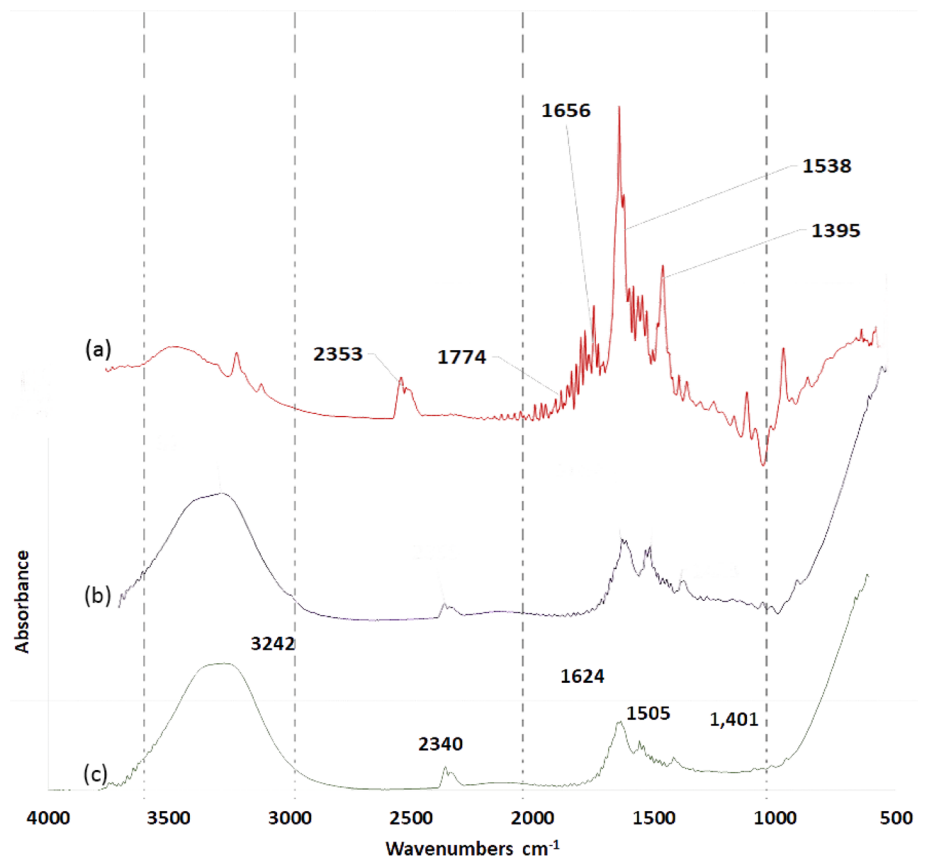


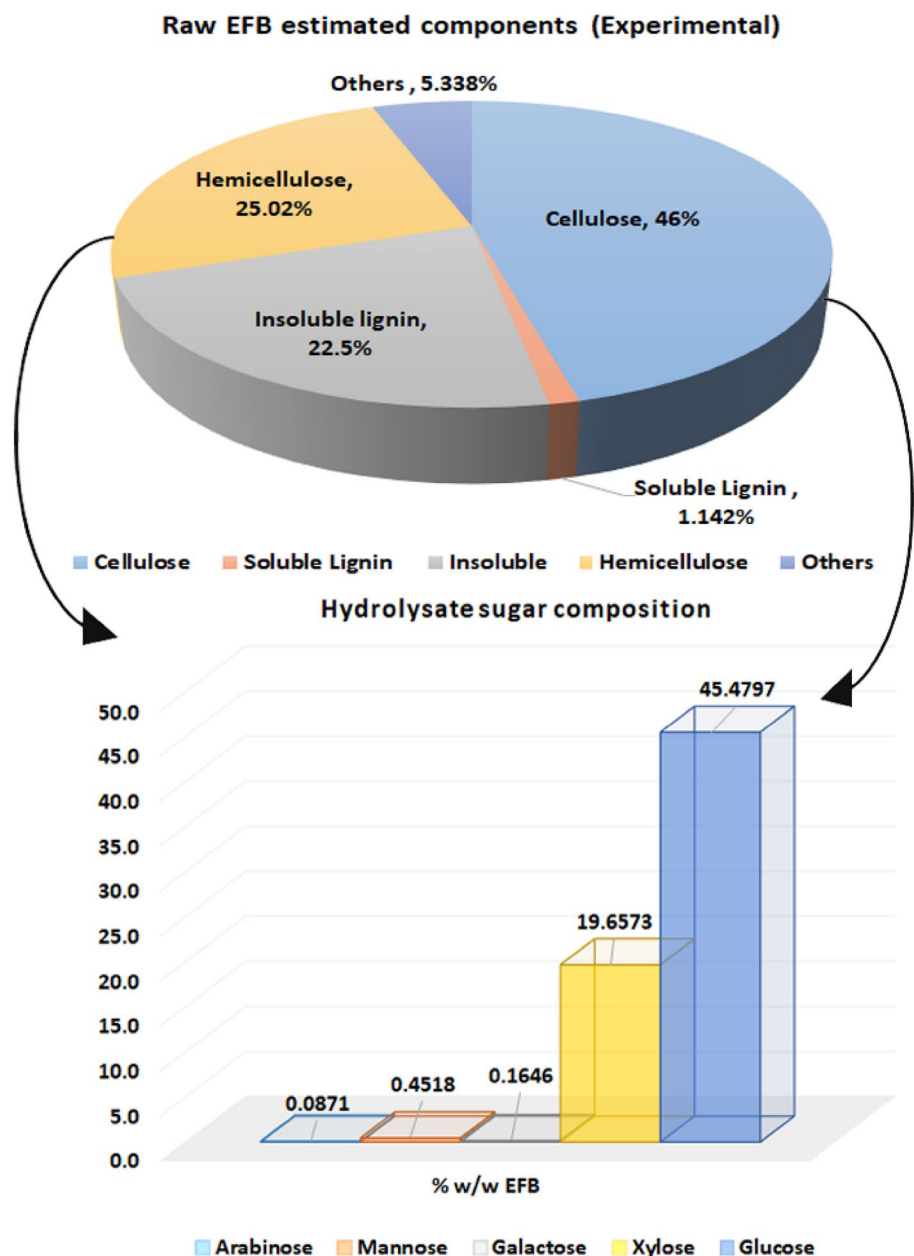
Table 2 Analysis of the hydrolysate obtained after [Cho]OAc-cellulase hydrolysis

Entry	Value/unit	% (w/w) EFB
Minerals composition of the hydrolysate		
Calcium, Ca	0.6 ± 0.05 mg/g	0.06
Sodium, Na	$1.6.70 \pm 0.05$ mg/g	0.14
Potassium, K	3.2 ± 0.5 mg/g	0.32
Copper, Cu	0.02 ± 0.007 mg/g	1.5×10^{-3}
Total phosphorus, TP	11.8 mg/L	0.98
Iron, Fe	0.3 ± 0.01 ppm	1.40×10^{-4}
Zinc, Zn	0.2 ± 0.01 ppm	1.30×10^{-4}
Manganese, Mn	0.2 ± 0.01 ppm	1.24×10^{-4}
Magnesium, Mg	0.1 ± 0.01 ppm	1.70×10^{-5}
Chemical composition of the hydrolysate		
Protein	236.7 ± 2.75 $\mu\text{g/mL}$	1.27
Total soluble Sugars, TOS	4.7 ± 0.7 mg/mL	0.47
Total reducing sugar, TRS	79.0 ± 1.65 g/L	61.9
Total nitrogen, TN	300.0 ± 6.06 mg/L	2.12
Total phenolic compounds, TPC	0.4 ± 0.05 mg/g	0.04
Acetic acid	0.6 ± 0.05 g/L	0.03
Furfural	2.0 ± 0.5 g/L	0.11

The ideal amounts of supplements for the ethanol from sweet sorghum juice were reported as Zn^{2+} 0.01 g/L, Mg^{2+} 0.05 g/L, and Mn^{2+} 0.04 g/L to achieve 120 g/L of the ethanol

(Deesuth et al. 2012). The results obtained seem to be consistent with the published data. The vital trace elements for yeast growth and ethanol production are Zn^{2+} , Mg^{2+} , and

Fig. 4 Detailed analysis of sugar components for raw EFB and its hydrolysate obtained after treatment in the IL-E system. Pretreatment time and temperature: 60 min and 75 °C, hydrolysis time: 48 h, IL concentration 10%, PKC-Cel loading 45 FPU/g EFB, and temperature 50 °C



Mn^{2+} . Zinc influences both cell growth and the metabolism of yeast cells. The ethanol production and resistance were both improved by Zn^{2+} -supplemented culture. Magnesium is vital for cell growth and enzymatic activities of yeast cells and roles as an enzyme cofactor. It positively affects ethanol production as it reduces the proton, so anion could penetrate the plasma layer by interrelating with the phospholipids layer, with stabilizes the bilayer. This supports the improvement of yeast tolerance to ethanol. The metal ion Mn^{2+} is a vital factor in *S. cerevisiae* metabolism as it is a component of some proteins that build enzymes associated with the fermentation such as pyruvate carboxylase (Deesuth et al. 2012).

Compounds that inhibit the microorganisms can be formed during the pretreatment and hydrolysis; for example, acetic acid, compounds derived from carbohydrates, and byproducts from lignin, including furfural and phenolic acids. Upon solubilization and hydrolysis of hemicellulose, acetic acid can be released into the medium. The degradation of xylose under acidic conditions results in furfural formation and can reach moderately high concentrations in severe pretreatment conditions of lignocelluloses (Palmqvist et al. 1999).

Acetic acid (0.62 ± 0.05 g/L), and furfural (2.1 ± 0.5 g/L) fall in the recognized level that did not restrain ethanol production as demonstrated in relevant studies (Ylivero et al.

Fig. 5 Detailed biomass balance of the EFB pretreatment and hydrolysis in E-IL system. The process starts with 1.875 g of the EFB

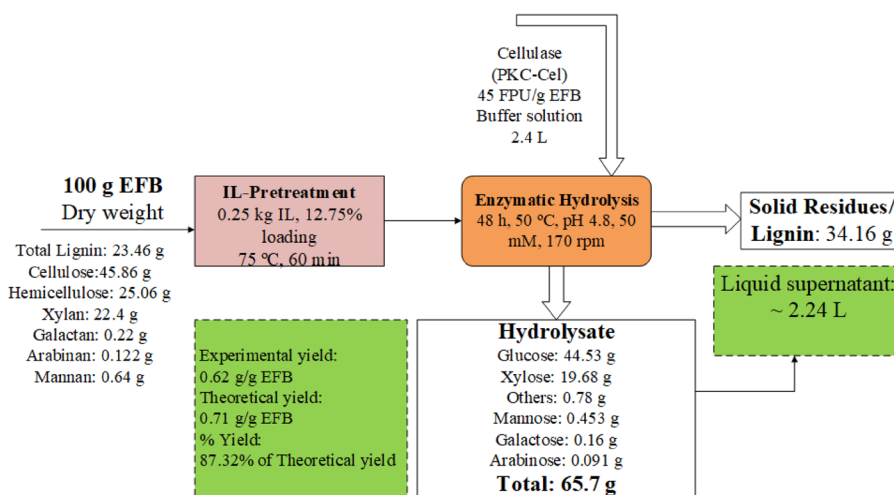


Table 3 Analysis of the treated and treated hydrolyzed EFB

Entry	Crystallinity index (CrI)	Swelling capacity (g/g)
Native EFB	2.1 ± 0.55	0.4 ± 0.32
Treated EFB, [Cho]Bu	1.3 ± 0.45	1.7 ± 0.44
Treated EFB, [Cho]OAc	1.6 ± 0.65	1.8 ± 0.56
Treated EFB, [Cho]Bu + E	0.6 ± 0.05	NA ^a
Treated EFB, [Cho]OAc + E	0.6 ± 0.05	NA ^a

^aNot applicable: as the mixture, in this case, was not in solid form due to performing one-step pretreatment and hydrolysis
Crystallinity index = A1430/A896

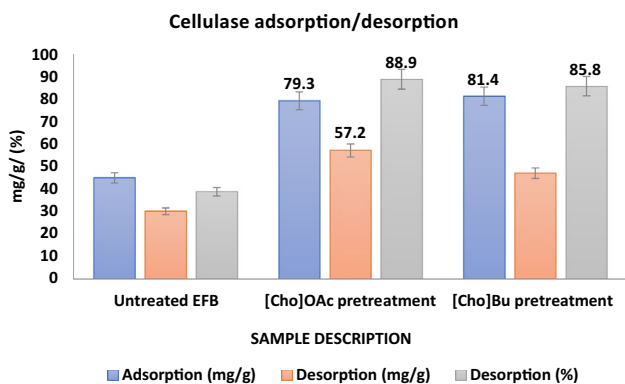


Fig. 6 Cellulase adsorption and desorption on EFB treated with [Cho]OAc and [Cho]Bu. Data include treated samples and raw samples

2013). To achieve an acceptable range that does not result in metabolism inhibition, the maximum concentration of the acetic acid was 3.0 g/L (Palmqvist and Hahn-Hägerdal

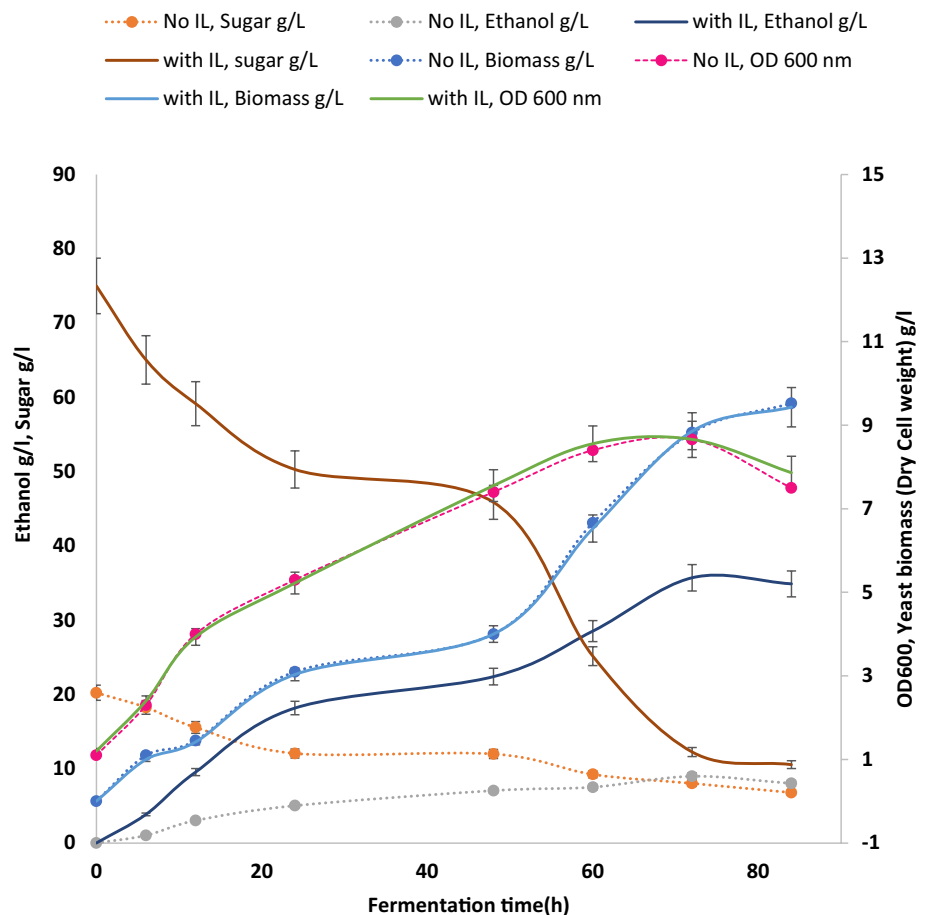
2000). Furfural reduction may occur to form its corresponding alcohol, which has a less inhibitory effect, assumed to be catalyzed by alcohol dehydrogenase (Dabirmanesh et al. 2012). A high concentration of cells in the presence of furfurals may lead to a rapid bioconversion by yeast cells as it reduces the impact of inhibition. *Saccharomyces cerevisiae* could endure high furfural levels at a high concentration of the cells, without significant changes in the ethanol generation. In the continuous culture containing 17.0 g/L of furfural, the yeast effectively released ethanol (Ylivero et al. 2013).

The content of total phenolic compounds (0.36 ± 0.05 mg/g) did not fall in the range of fermentation suppression. Through the hydrolysis, free amino nitrogen could substitute the external nitrogen source. The investigation demonstrated that total protein (TP) and nitrogen (TN) were 0.237 g/L (1.27% w/w) and 0.3 g/L (2.12% w/w), respectively. Despite having a low level in the process, there are yet similar to the data reported on EFB, which showed that around 2.3 ± 0.1% of the dry weight was protein (Kim and Kim 2015).

Sugar component analysis of monosaccharides in the hydrolysate acquired was assessed using HPLC, and the obtained data are shown in Fig. 4. It can be seen that the major constituent in the mixture was glucose (45.47%), followed by xylose (19.65%). Other components of arabinose (0.087%), mannose (0.45%), and galactose (0.16%) were the minor sugars found in the hydrolysate. In this term, galactose and arabinose contributed to the smallest concentrations achieved.

Empty fruit bunch (EFB) was analyzed for its carbohydrate content and revealed 36.6 ± 0.6% (w/w) glucan, 24.2 ± 0.5% (w/w) XMG (xylan, mannan, and galactan), 1.2 ± 0.1% (w/w) arabinan, and 22.0 ± 0.4% (w/w) insoluble lignin. The residual percentage was conveyed as extractives

Fig. 7 Yeast growth, sugar consumption, and ethanol production during fermentation of IL-EFB hydrolysate and raw EFB samples for bioethanol production



and crude protein (Kim et al. 2015). The results obtained are consistent with the recent data reported in other studies.

In the current study, most of the cellulose was converted into glucose (99%). Based on the analysis, the enzymatic process demonstrated hydrolysis effectiveness. The conversion percentage of hemicellulose to xylose was 78.8% while the residual ended up as arabinose, galactose, and mannose. Unconverted sugars or monosaccharides that were not detected by the HPLC might have contributed to the total sugar obtained.

The presence of xylose, galactose, mannose, and arabinose in the mixture was consistent with the reported composition of the EFB. The obtained constituents were compared with the initial EFB used, as presented in Fig. 5. Majority of the EFB was converted to glucose and xylose with sugar yield of 1.23 g in the hydrolysate. The experimental yield was 87.3% of the theoretical yield with only 0.5% error in terms of cellulose conversion into glucose.

Swelling capacity and crystallinity index (CrI)

Swelled structure and lower cellulose crystallinity could be obtained by pretreatment at low temperatures, while pretreatment at high temperatures devastated the biomass and created deep pores (Noori and Karimi 2016).

As indicated by the crystallinity list (CrI) shown in Table 3, the capacity of [Cho]Bu treatment to reduce the crystallinity of cellulose was higher than that of [Cho]OAc, through consolidation of both IL and PKC-Cel in one system decreased the CrI value. IL could break down the hydrogen bonds of the cellulosic structure and alter cellulose I to cellulose II that can be hydrolyzed by enzymes. It is reported that [Cho]Bu has higher standard enthalpy and higher thermal expansion compared to [Cho]OAc. This shows more molecular energy relative to [Cho]OAc (Muhammad et al. 2012).

Swelling of cellulose is a consequence of the IL entering the amorphous region of the cellulosic substance, which enhanced the portability of the cellulose chains, weakened the hydrogen bonds, and diminished the crystalline parts. The higher the swelling of the EFB, the more accessibility is provided which allows the hydrolysis to take place (Table 3).

During IL-pretreatment, cations enter and move through the small pores in the cellulose enhancing the polyionic character of the pretreated lignocellulose. The cation remains in the structure as a counter charge to COO^- and increases the swelling of the material (Reddy 2015). The major mechanism for the enzymatic hydrolysis enhancement by IL at the moderate temperature is the reduction of the crystallinity by cellulosic dissolution (Xu et al. 2017). At elevated temperatures, the main impact of IL on lignocelluloses is the destruction of the structure, resulting in lignin and hemicellulose decomposition (Soudham et al. 2015).

The highest adsorption of cellulase was observed in EFB with [Cho]Bu (81.4 ± 3.3) followed by [Cho]OAc (79.3 ± 2.8) (Fig. 6). Pretreatment with IL can soften and extract the compounds off from EFB. As seen in the treated samples, the rate of cellulase desorption expanded in the IL-E contrasted with the crude ones. The IL pretreatment improved cellulase desorption by expanding the surface area and diminishing the lignin content, which leads to an effective hydrolysis. One of the likely reasons for cellulase deactivation is the ability of lignin to adsorb the cellulase irreversibly. Therefore, hydrolysis in the presence of IL can lessen the lignin content as lignin can bound to IL, while cellulose is being exposed which promotes the cellulase desorption and enhances the hydrolysis (Zhao 2016).

Fermentation of hydrolysate to ethanol

The concentration of total sugar was found to be 70–75 g/L (0.62 ± 0.06 g/g EFB) after 48 h of the hydrolysis. The hydrolysate was used for fermentation media of ethanol production. Figure 7 shows the profile of the sugar and ethanol concentration during the 84 h of the fermentation. Over time, the ethanol synthesis increases as the cell concentration increased. After 72 h, the ethanol production reached its maximum. It can also be observed from the graph that as the incubation period increased, the optical density corresponding to growth also increased. It was then declined after the 72 h' period. The rapid reduction in reducing sugar concentration was observed after 48 h, whereas, at the time, ethanol started to increase considerably. The productivity of the ethanol was estimated at $0.4178 \text{ g/L}^{-1}\text{h}^{-1}$. In the current study, the yield of ethanol was 0.275 g for each gram of EFB. The ethanol concentration was 34.58 g/L after 72 h without additional supplements.

The observed ethanol yield was 0.127 g ethanol/g EFB in case of the raw EFB whilst the IL-E system generated 0.275 g ethanol/g EFB; 87.94% of the theoretical yield. The results obtained in the current study were compared with the reported data in different pretreatment conditions.

The ionic liquid [EMIM]OAc was used for Eucalyptus pretreatment which later was subjected to hydrolysis

with Novo-Celluclast and produced 0.172 g ethanol per each gram of the biomass (Lienqueo et al. 2016). The data obtained demonstrated the potential of the EFB hydrolysate to be converted into ethanol without supplementation. The fermentation in the presence of IL did not suppress the growth of the yeast cells. It was reported that cholinium is a vitamin B-complex and widely used as food additive, the cholinium-based ionic liquids are generally looked upon as environmentally harmless and, thus, acknowledged as non-toxic (Ossowicz et al. 2016).

Conclusion

Pretreatment of EFB in an integrated system of ionic liquid (choline acetate) and PKC-Cel (cellulase) (IL-E) yielded 87.3% of the theoretical glucose yield. In [Cho]OAc-enzyme system, 99% of the cellulose was converted to glucose after 48 h in IL-E system. The EFB hydrolysate was assessed as a potential media for ethanol production. Furthermore, the presence of IL in the fermentation medium did not possess toxic effect on the growth of the yeast cells as the IL used is derived from vitamin B-complex.

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Compliance with ethical standards

Conflict of interest We declare no commercial or financial conflict of interest underlying the current project.

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