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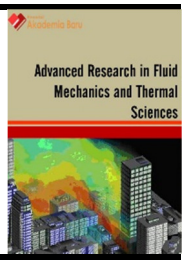

Factors Affecting the in-Situ Hydrolysis of Empty Fruit Bunches in Ionic Liquid Compatible Cellulase System

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ABSTRACT

Utilization of the abundant raw materials in Malaysia as well as surmounting the obstacle of the shortcoming of fossil fuels resources are current concerns of the research. Cellulase development aims to enhance the bioethanol production from lignocellulosic biomass such as empty fruit bunches (EFB) that resists enzymatic hydrolysis due to its complex structure. Hence, ionic liquids (ILs) have been introduced with the ability to disrupt the lignocellulose to be accessible by enzymes and thus it is expected to achieve a significant improvement by conducting both treatment and hydrolysis in one step by leaping cellulose regeneration. Palm kernel cake (PKC) was utilized as the primary media for cellulase production through solid-state bioconversion (SSB) by Trichoderma reesei. Factors that affect the hydrolysis in the ionic liquid-cellulase (IL-E) system was investigated using one-factor at a time (OFAT) and reducing sugar obtained as the response to the study. It was found that the biomass could be loaded up to 35% (w/v) and agitation has influenced the rate of reducing sugar production where increasing the speed was associated with higher concentration. Higher IL concentration in the combined system caused lower enzymatic activity and hence, less sugar was produced. The results obtained can be employed for the one-step process in the conversion of EFB into fermentable sugar.

1. Introduction

It is well known that lignocellulosic biomass is one of the most abundant yet beneficial renewable materials and counts as an alternative source for fossil fuels [1]. Oil palm industries generate enormous amounts of lignocellulosic oil palm biomass (OPB) which includes palm shells, palm fibers, palm kernels, palm trunks, empty fruit bunches (EFB) and palm oil mill effluent (POME). Cellulose, the main component of interest in lignocellulose, is protected by lignin and hemicelluloses that wrap the molecule [2] which raised the difficulties in the enzymatic hydrolysis and requires a preparation step. Therefore, several methods were employed to achieve cellulase-

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accessible substances such as milling [3], hot water treatment [4], steam explosion, acid and alkaline hydrolysis [5, 6], irradiation [7] and microbial treatment [8] followed by subsequent enzymatic hydrolysis.

Although the conventional approaches on acid or alkaline pretreatment have the advantages such as cost reduction, they contribute to environmental problems as well as the high energy consumption [9]. Moreover, cellulose recovery requires a washing step with large amounts of anti-solvents (e.g. water) to prepare for the hydrolysis, which is associated with the high cost and excessive waste generated [10].

ILs were introduced as efficient tools for lignocellulose pretreatment as they could disrupt the hydrogen bonds and expose the lignocellulosic material to the cellulase which enhances the hydrolysis process [11]. The high cost of ILs and the concern over the environment on the disposal of these solvents are also some of the main obstacles hindering the large-scale application in the pretreatment of lignocellulosic biomass. Fortunately, ILs may have an excellent recyclability [12] and can be reused to reduce the cost as well as the impact on the environment. Considering the cost associated with commercial enzymes and ILs, this study focused on obtaining a locally produced cellulase by utilizing an abundant raw materials from palm oil industries, palm kernel cake (PKC), as the media for fungal growth, EFB for the production of reducing sugar as it contains high cellulose (23.7-65.0%)[13].

The use of ILs has been reported in the pretreatment of lignocelluloses for cellulose generation [14], followed by conversion into sugar. For instance, treatment of *Eucalyptus* with 1-butyl-3-methylimidazolium acetate [BMIM]OAc, that was then subjected to enzymatic hydrolysis brought about 13.7% conversion of the glucan into glucose [15]. Additionally, cellulase enzyme stability was also studied by the researchers to seek for a compatible IL-enzyme system, such as in cholinium-based ILs [16, 17]. Some factors have been reported to have an influence on the in-situ hydrolysis. For instance, particle size, water content, biomass loading, enzyme loading, temperature of pretreatment and mixing as well as the IL concentration [9, 18–20].

Consequently, the current study focused on the combination of IL and cellulase in one system (a single reactor) for fermentable sugar production. Few factors were investigated including biomass loading into the system, cellulase loading, agitation, and IL concentration. The investigation aims to have an insight on individual factors on the sugar production from the EFB material that could be converted into the fuels in the future direction. For the elimination of the cellulose generation step and combining both pretreatment and enzymatic hydrolysis as a single reactor process, this study might have a great starting point for further improvement in bioethanol production.

2. Methodology

2.1 Materials

First, Palm kernel cake (PKC) and empty fruit bunches (EFB) were collected from Sime Darby West Mill Plantation in Carey Island, Malaysia. Samples were dried until constant weight obtained, then ground to 1.0-2.0 mm. *Trichoderma reesei* (RUTC30) was purchased from American Type Culture Collection ATCC. Chemicals were obtained from Fisher Scientific, MERCK, and Sigma-Aldrich, Malaysia.

2.2 Production of Cellulase by Solid-State Fermentation (SSF)

The Cellulase was produced locally by solid-state fermentation of palm kernel cake (PKC), rice husk (RH) and empty fruit bunches (EFB) as the primary media. The spores' suspension of Trichoderma

reesei (RUTC30) was inoculated into the three different media and incubated for seven days. Crude enzyme was extracted after mixing the fermented samples with sodium citrate buffer (pH 4.8±0.2) followed by centrifugation [21]. The cellulase activity was assessed using the method reported by Ghose [22].

2.3 Stability of Cellulase in Different Ionic Liquids (ILs)

The cellulase was incubated in different ILs, for certain periods of time, then, the enzyme activity was determined using carboxymethyl cellulose (CMC) as the substrate [23]. The initial activity of *PKC-Cel* was taken as the control (100%) and subsequent readings were calculated as residual activities. The ILs used were choline acetate [Cho]OAc, choline butyrate [Cho]Bu, 1-ethyl-3-methylimidazolium acetate [EMIM]OAc, 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM]DEP, 1,3-dimethyl imidazolium dimethyl phosphate [DMIM]DMP and tetrabutyl phosphonium acetate [TBPH]OAc.

2.4 The Effect of Different Factors on The Compatible System

Classical one-factor-at-a-time (OFAT) was employed to evaluate the optimal levels of the factors that contributed to in the enzymatic hydrolysis in presence of the IL chosen from the stability test. The factors that were investigated were IL concentrations, biomass loading, cellulase loading, and agitation speed. Unless stated otherwise, temperature for the pretreatment was 100°C for one hour at 500 rpm in a thermomixer, followed by cellulase addition at 100 U/g loading and mixing at 45°C for 12 hours and 500 rpm. The hydrolysate was obtained by centrifugation at 10,000 rpm for 10 minutes and the supernatant was analyzed for reducing sugar using the DNS method with glucose standard curve [24]. The analysis of the significance of factors was executed using one-way ANOVA, Tukey's test and 95% confidence in Minitab 17.1.0.

2.4.1 Effect of IL concentration

IL concentration was investigated as one of the main factors that affect the in-situ hydrolysis. The system composes of IL and buffer solution (citrate buffer pH 4.8±0.2) in different ratios: 10, 20, 40, 60, 80 and 100% of the IL to the buffer. The hydrolysis was monitored over a period of 12 hours and the reducing sugar concentration was measured in mg/g EFB using the DNS method.

2.4.2 Effect of biomass loading

The effect of biomass loading was investigated by introducing different amounts of the biomass to the system starting from 5% to 40% biomass relative to the IL volume. The reaction time was fixed to 12 hours and the IL concentration at 10%, cellulase loading at 100 U/g.

2.4.3 Effect of cellulase loading

By fixing the other factors, the influence of cellulase enzyme loading was investigated at different concentrations starting from 20 to 100 units/gram EFB.

2.4.4 Effect of agitation

Agitation was investigated as mixing the reactants is one of the important factors that are involved in any reaction. Agitation ranged from 200 to 600 rpm using a thermomixer with a temperature control. The same amount of the biomass (EFB) was loaded into microcentrifuge tubes and the IL was added into each tube, heated for an hour with mixing, then the *PKC-Cel* (cellulase) was added into each tube at 80 U/g. The hydrolysis was carried on for 12 hours at 45°C, followed by centrifugation and reducing sugar analysis.

3. Results and Discussion

3.1 Cellulase Production

At the first screening, all used substrates have served as a medium for cellulase enzyme production at different activities. The production could be arranged in the following order: PKC>RH>EFB as shown in Figure 1. PKC served as a better substrate in terms of cellulase production as it contains more accessible nutrients compared to EFB and rice husk.

Cellulase activity was calculated using both CMC and filter paper assays. The obtained activity was 24.14±1.82 FPU/ml (123.33±9.12 Unit (U)/ gram dry substrate (gds) and 157.872±1.56 CMC unit/ml (789.386±7.8 U/gds) on the seventh day of the fermentation. The enzyme optimal conditions were determined at 45 °C ad pH 5.0 [21].

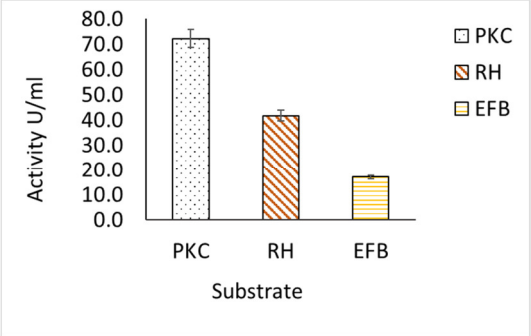

Fig. 1. Production of cellulase from three different substrates: Palm kernel cake (PKC), Rice husk (RH) and Empty fruit bunch (EFB)

3.2 Stability of PKC-Cel in ILs

The effect of a few different ILs on the locally produced PKC-Cel was investigated at different time intervals (Figure 2). In [Cho]OAc, 63.15% of the activity remained after six hours. In contrast, [Cho]Bu has shown stability up to 50% of initial activity after six h. While in [EMIM]OAc, the activity dropped dramatically to 4.7%. Surprisingly, phosphate-based ILs showed different trends. In [EMIM]DEP, *PKC-Cel* maintained 36% of its activity, whereas only 8.16% of the activity was detected in [DMIM]DMP. In [TBPH]OAc, the enzyme exhibited an inhibitory effect where only 20% of the activity was recorded. However, the inhibitory effect in phosphonium-based IL effect was less than imidazolium-based IL. It was found that PKC-Cel had the highest stability in [Cho]OAc (>60%) in the pure IL.

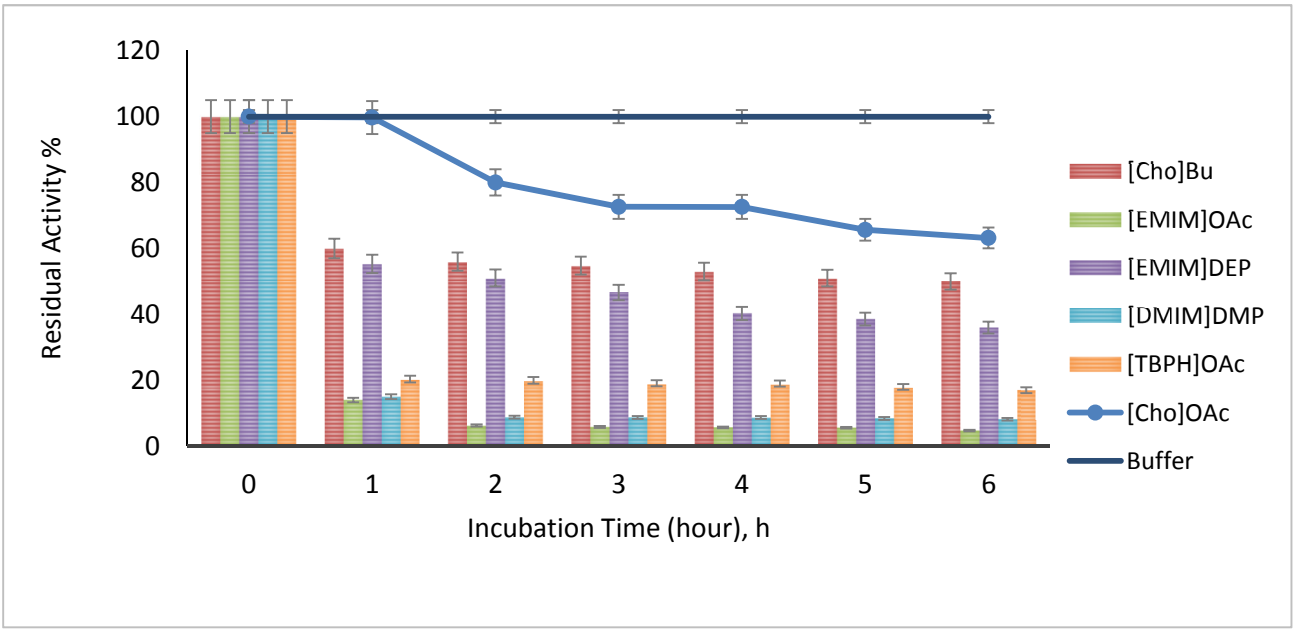

Fig. 2. Stability of PKC-Cel in different ILs. ILs are: [Cho]OAc, choline butyrate [Cho]Bu, 1-ethyl-3-methylimidazolium acetate [EMIM]OAc, 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM]DEP, 1,3-dimethyl imidazolium dimethyl phosphate [DMIM]DMP and tetrabutyl phosphonium acetate [TBPH]OAc

In most cases, it was reported that ILs having hydrophobic nature, less viscosity, kosmotropic anion and chaotropic cation enhance the activity and stability of enzymes. However, the correlation could not be generalized because of many contradictory results [25]. [DMIM]DMP and [EMIM]OAc were both tested in the enzymatic hydrolysis system and showed that ILs concentration above 40% resulted in cellulase deactivation. At 90% (v/v) of [DMIM]DMP, endoglucanase retained roughly 50% of its activity [26]. Similarly, in [EMIM]OAc, cellulase retained 40% of the activity after one hour and less than 1% after four hours [27].

3.3 The Effect of Different Factors on The Compatible System

3.3.1 Effect of IL concentration

It was noteworthy that increasing the IL concentration in relative with the buffer has a slight inhibitory effect on the PKC-Cel as observed from Figure 3. The inhibition could be related to the high salinity of the ILs which interferes with the protein folding and results in inactivation of the enzymes [28]. Therefore, carboxylic acid anions were introduced to lower the melting points and viscosities of the ILs. Moreover, this improves ILs ability to react with the hydroxyl groups in the cellulose and enhance the dissolution power [29]. Consequently, the IL, [Cho]OAc was chosen to run further investigations.

As seen in Figure 3, the reducing sugar released from EFB was produced in elevated concentrations of the [Cho]OAc in the combined system as both enzyme and IL are in contact with the substrate, EFB. In addition, the value of means that do not share a letter are significantly different, using Tukey's method and 95% confidence. In another word, each concentration was categorized in a separate group (A, B, C, D, D, E and F).

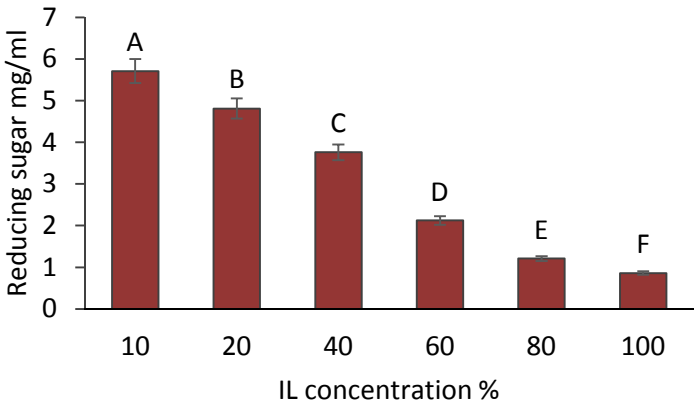

Fig. 3. Effect of IL concentration on the obtained reducing sugar in IL-E hydrolysis system (R^2 = 0.9927)

As obtained from the OFAT analysis, the results showed the significance of the IL concentration as a factor whereas it can facilitate the hydrolysis at a certain limit and inhibit the enzyme at the same time at elevated concentrations. Therefore, a balanced formulation is required in order to achieve the maximum sugar production as the outcome of the enzymatic hydrolysis in the combined system. Nine ILs were tested by Li *et al.,* [30] for activity and enzymatic saccharification of untreated corn cob in the presence of 20% (v/v) of: 1,3-dimethyl imidazolium dimethyl phosphate [MMIM]DMP, [EMIM]DEP, [EMIM]DMP, 1,3-dimethyl imidazolium methylsulfate [MMIM][MeSO4], N,N-dimethyle thanolammonium acetate and lactate [DMEA]OAc, [DMEA]La, [BMIM]Cl, N-butyl-N-methyl pyrrolidinium chloride [BmPy]Cl, and [BMIM][PF_6]. No inhibition of the cellulase was observed in [MMIM]DMP, [DMEA]La, and [DMEA]OAc in 20% (IL/buffer) (v/v). After enzymatic hydrolysis of untreated corn cob for three hours, the RS concentration in IL/buffer system was 1.13, 1.17, and 1.15 g/L, respectively. Based on this data, lignocellulose-dissolving, IL [MMIM]DMP, was a good candidate for pretreatment in view of its good biocompatibility with both lignocellulose solubility and cellulase activity [31]. According to Thomas *et al.,* [32], enzymes were most active in the dimethyl phosphate-ILs, followed by acetate.

3.3.2 Effect of biomass and cellulase loading

Enzyme concentration is a significant factor in any enzymatic reaction. Increasing the enzyme concertation increase the reaction rate in the ideal reaction. However, in the presence of the IL. The enzyme showed a slight deactivation effect. Moreover, additional biomass did not result in higher sugar production from EFB. This might be associated with the IL capacity to attack the hydrogen bonds in the lignocellulose molecules and the possible shape conformational changes of the enzyme active site that might result of the IL which causes the enzyme to be less active, hence receiving a new substrate molecule will be more difficult by time. Effect of biomass loading is presented in Figure 4. It showed the maximum biomass that can be loaded is 35% to achieve 5.54 mg/ml of reducing sugar, whereas further addition did not result in a significant increment of the reducing sugar production as proved by one-way ANOVA, Tukey's test (95% confidence). Biomass loading of 35% and 40% laid in the same group (A), as the recorded difference was insignificant. High loading could reverse the effect of pretreatment that increases the enzymatic accessibility of biomass as higher solid loading results in mixing problems and further causes heat and mass

transfer limitation in the system. Moreover, high loading can also lead to the formation of inhibitory compounds, such as furfural, which inhibits enzymatic hydrolysis [33].

Increasing the biomass loading raises a mixing problem, thus slows down the hydrolysis reaction, and does not contribute to a very high yield. However, there is a maximum limit in which we could reach to achieve both an efficient and balanced conversion. In theory, the solubility of enzyme-substrate might affect the efficiency of the enzymatic hydrolysis, in which a thicker and less soluble form might disrupt the conversion process; making the opposite might result in the otherwise [34, 35]. In two-step hydrolysis of the [EMIM]OAc pretreated biomass, it resulted in sugar yields of 80% for glucose and 50% for xylose at substrate loadings up to 33% (w/w). At 50% (w/w) biomass loading, sugars obtained were 55% and 34% for glucose and xylose, respectively [36] which pointed to the reduction of sugar yields upon higher loading. It was reported that pretreatment of cotton stalks at 30% (w/w) showed that higher biomass loading relates to the incapability of [EMIM]OAc to extract lignin effectively in a multi-step hydrolysis [37].

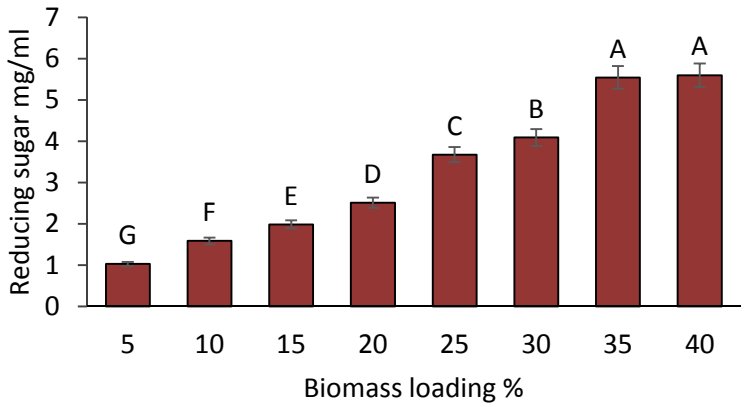

Fig. 4. Effect biomass loading on the obtained reducing sugar in IL-E hydrolysis system (R^2 =0.9958)

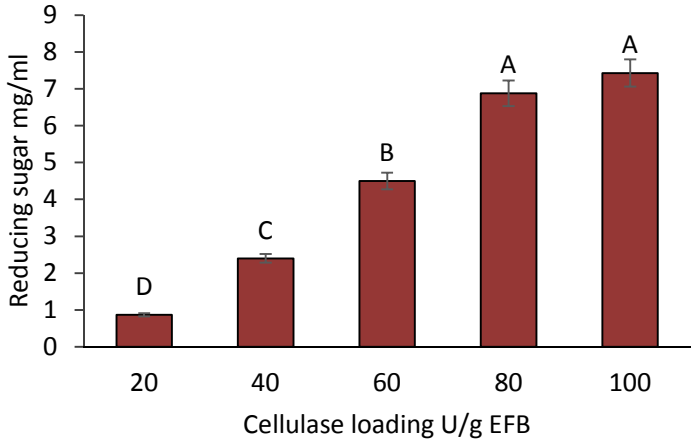

Fig. 5. Effect of cellulase loading on the obtained reducing sugar in IL-E hydrolysis system (R^2 = 0.9898)

On the other hand, increasing the cellulase loading on the fixed amount of the biomass resulted in higher reducing sugar production at 35% loading of the EFB, as illustrated in Figure 5. Different concentrations of the cellulase showed differences in produced sugar and therefore, they were

significant by Tukey's test. As can be seen from Figure 5, the concentration of 80 and 100 U/g of cellulase could be categorized in the same group, 'A', which means increasing the concentration further did not enhance the hydrolysis further. However, more biomass loading will require the addition of enzyme to facilitate the hydrolysis.

3.3.3 Effect of agitation

Mixing is an essential process during the reaction to ensure more contact between the substrate-enzyme [S-E] and the substrate-IL [S-IL] which results in higher productivity. The trend of the obtained sugar is presented in Figure 6.

From the attained results, increasing agitation facilitated the hydrolysis and thus increased the reducing sugar production from the EFB biomass.

The viscosity of IL affects the mixing and mass diffusion during pretreatment. Therefore, a higher biomass concentration under agitation allows more frequent contact and collision between the biomass particles, caused impinging on the biomass surface, inadvertently promoting cellulose dissolution from the matrix. It can be concluded that increasing the loading is another promising approach to improve pretreatment efficiency. 500 rpm seems to be good enough to achieve the desired yield as concluded from the tested range, based on Tukey's test results where both 500 and 600 rpm are grouped under the same letter (A). Thus, they showed no significant difference.

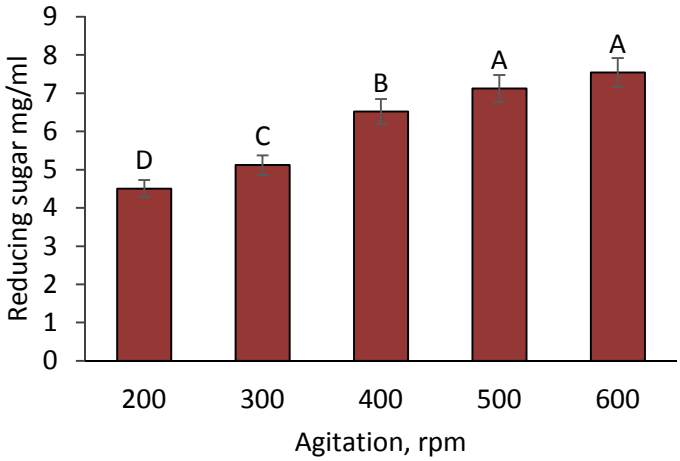

Fig. 6. Effect of agitation on the obtained reducing sugar in IL-E hydrolysis system (R^2 = 0.9898)

4. Conclusions

Based on the findings of the OFAT analysis, the obtained data demonstrated the capability of the IL-E system to facilitate the hydrolysis without the washing step to regenerate the cellulose. Compatibility of PKC-Cel with IL can be further applied in the application of IL-E in bioethanol production.

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