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Development of choline-based deep eutectic solvents for efficient concentrating of hemicelluloses in oil palm empty fruit bunches

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Abstract−Lignocelluloses complexity has led to poor dissolution efficiency in solvents. This study was conducted to concentrate hemicellulose content in oil palm empty fruit bunches (EFB) using green solvent, choline chloride (ChCl)-based deep eutectic solvent (DES). Results showed that ChCl : formic acid (FA) is the most effective among the DES in concentrating hemicellulose content in treated EFB and exhibits the highest dissolution power of lignin. The toxicity test showed that all the synthesized DES had negligible effect against *Escherichia coli* and *Salmonella typhimurium*. Nevertheless, all of them possessed comparable cell proliferation to their individual counterparts, ChCl, glycerol, lactic acid (LA) and FA, which implied that these DES could be used in the intended industries.

Keywords: Oil Palm Biomass, Formic Acid, Glycerol, Lactic Acid, Toxicity

INTRODUCTION

A wide spectrum of technologies has emerged to produce or extract bio-sourced components. These components have the potential to alter modern life by providing a sustainable future, as non-renewable resources from mother nature will be replaced by renewable biomass [1]. Li et al. discovered that a porous composite made from egg-shell yolk and NiO/C can be used as lithium-ion battery anode material [2]. Lignocellolusic biodiesel has been produced successfully by using an integration system consisting of solar-biopower and fungi [3]. Galliano et al. [4] studied the experimental factors that affected aqueous dye-sensitized solar cell performance in order to produce photovoltaic device without any toxicity. The same research group discovered that the efficiency of aqueous solar cells can be enhanced by the treatment of $TiCl_4$ [5]. In general, bio-sourced products are primarily biopower [4,5], transportation fuels [3,6], biogas [7], natural bio-products [8], bioplastics [9], biocomposites [10] and biomonomers [11].

In Malaysia, 5.81 million hectares of land are utilized for oil palm cultivation [12]. There is a massive reservoir of resources from the crop as the oil palm generates only 10% of oil, with the remaining 90% as oil palm lignocellulosic biomass [13]. The enormous amount of this biomass is present in solid form as empty fruit bunches (EFB), palm kernel shell (PKS), mesocarp fiber (MF), oil palm frond (OPF) and oil palm trunk (OPT) [13]. EFB, one of the main by-products, however, has limited applications compared to other oil palm biomass. In general, lignocellulosic biomass, as the name implies, is primarily composed of three polymeric constituents: cellulose, hemicellulose and lignin. These components could be transformed into many value-added products, including biodiesel [14], biogas [15], syngas [16], chemicals [17], polymers [18], composites and monomeric sugars [19]. However, lignocellulosic biomass is not susceptible to dissolution and degradation, attributed to cross linkages of lignin with celluloses and hemicelluloses forming complex matrix. Hydrolysis of lignocellulosic biomass is required to degrade its backbone, making polysaccharides more exposed to the next course of reaction. This is termed pre-treatment and it can be accomplished *via* chemical or enzymatic reaction; the latter is more environmentally friendly due to its milder operating conditions, higher selectivity, lower energy costs and higher sugar yields [20]. Literature data show that hydrolysis rate of lignocellulosic biomass can be enhanced by disrupting the present lignin, reducing cellulose crystallinity and degree of polymerization. Such pre-treatment reduces the recalcitrance structure of lignocellulosic biomass, leading to better accessibility of cellulosic surface area and substrate porosity [14].

Pre-treatment of lignocellulosic biomass, be it *via* biological, physical, chemical or physiochemical, could result in some changes of biomass. While physical changes occur during pre-treatment involving grinding [21], steaming and steam extrusion [22], thermal-mechanical extrusion [22] or ultrasonication [23], chemical changes take place when biomass is pretreated with acids [24], alkali [25], organic solvents [26], ionic liquids [27] and oxidants [28]. Physico-chemical pretreatment uses steam [29], supercritical CO_2 explosion [30] and ammonia fiber explosion [31]. A more preferred pretreatment in recent years has been a biological method involving microbial communities [32]. Nevertheless, a number of challenges remain in these types of pre-treatment, primarily associated with low feasibility due to energy-intensive processes requiring high temperature and pressure, as well as expensive chemical reagents. Therefore, it is crucial to find alternative methods that can prevail over these limitations.

Hemicellulose is widely abundant, though cellulose is the major

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chemical composition, in most of the plant biomass. The extracted raw materials, glucose, xylose, galactose, arabinose, mannose, rhamnose, xylan and furfural [33] from hemicellulose *via* catalysis reactions are primarily used in various industries such as pharmaceutical, oil refining, plastics, food as well as biochemicals. Hemicellulose is relatively unstable thermally and chemically as compared to lignin and cellulose [34]. Conventional pretreatment methods developed so far have focused on disrupting the intramolecular bonds of lignin and hemicellulose in order to increase accessibility of cellulose for subsequent hydrolysis. There are, however, limited studies performed on increasing the yield of hemicellulose, which is one of the crucial economic factors in technological development. In recent years, deep eutectic solvent (DES) has emerged as a promising green solvent for a wide range of applications, including metal electrodeposition [35], metal electropolishing [36], metal extraction [37], gas adsorption [38], biotransformation [39], organocatalysis [40] and biomass transformation [41].

DES is widely known as an advanced generation ionic liquid in view of its similar physicochemical characteristics: powerful solvent capacity, low volatility and high electrochemical conductivity [42]. DES can be synthesized easily by mixing two or more inexpensive high melting point components under mild heat. The resulting melting point is usually significantly lower than the individual components as the components involved self-associate between hydrogen bond donor (HBD) and acceptor (HBA), and behaves like a single phase [43]. As a green solvent, DES is non-flammable, biocompatible and biodegradable, also is easily available and economical due to its tunable physicochemical properties. The production cost of DES is low and it is easy to be prepared without any further purification step. Different types of DESs can be designed for the intended applications due to flexibility in adjusting their functionalities. Although DES has been aggressively exploited this decade, and the associated individual components toxicity well-documented, knowledge level and representative data of pre-treating oil palm biomass using DES is still lacking and limited.

Glycerol (Gly), lactic acid (LA) and formic acid (FA) (Fig. 1) are HBD widely used in pharmaceutical and food industries [44,45] due to their natural characteristics, biodegradability, non-toxicity and high solubility in water. Choline chloride (ChCl), an organic quaternary ammonia salt as an electron acceptor (HBA) (Fig. 1), is commonly used as a supplement in animal feed formulation. Previous studies indicated that ChCl-based supplements prevent fat accumulation in liver and abdominal area of Japanese quails [46] and increase milk production in Etawah Grade goats [47]. The European Food Safety Authority (EFSA) reported that ChCl does not pose an adverse effect on the environment despite its high level reported in animal feed [48].

Fig. 2. Oil palm empty fruit bunches.

Therefore, our study aimed to extract hemicellulose from EFB using ChCl-based DES: ChCl : Gly, ChCl : LA and ChCl : FA. The conversion efficiency of the DES employed was calculated. The toxicity of the DES against three bacterial strains, *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*, was also investigated.

MATERIALS AND METHODS

EFB was collected from a palm oil mill (Fig. 2). ChCl was purchased from Sigma-Aldrich, USA, while Gly, FA and LA were purchased from a local chemical supplier, R&M Chemicals, based in Malaysia. Bacterial strain of *E. coli* ATCC 25922 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany, whereas *S. typhimurium* ATCC 14028 and *S. aureus* ATCC 29213 were obtained from Taylor's University Lakeside Campus, Malaysia.

1. Synthesis of DES

ChCl and Gly were used to produce DES for the pre-treatment of extractive-free EFB. Two different DES concentrations were prepared based on 1 : 1.5 and 1 : 3 molar ratio of ChCl : Gly. The mixture was stirred and heated to 80 °C for 2 h until a clear solution was formed. The same procedure was repeated for other DES, ChCl : LA and ChCl : FA with a same molar ratio. All the synthesized DES were kept in a desiccator prior to pretreatment experiments.

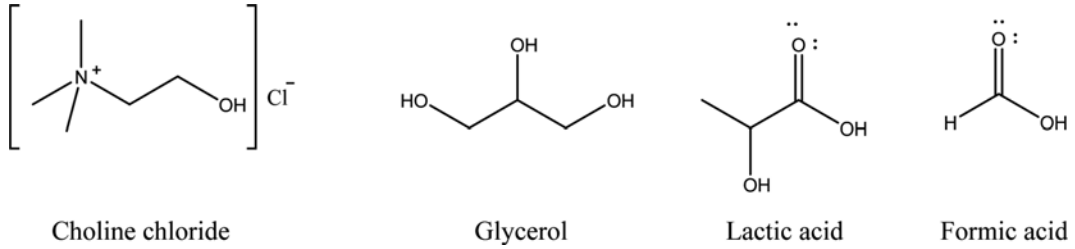

Fig. 1. Chemical structures of choline chloride, glycerol, lactic acid and formic acid.

The viscosity of the DES was measured using a viscometer (Anton Paar DV-2 P). The test was carried out in triplicate for each sample.

2. Extractive-free EFB

Approximately 50 g of air-dried and sieved EFB with particle size of 212 μm were subjected to a Soxhlet apparatus using *n*-hexane for 2 h. The extracted hexane was then evaporated to dryness under vacuum to obtain a mass of extractives, while extractive-free EFB was dried at 60 °C in an oven until constant weight. The percentage of extractives was calculated using Eq. (1):

$$\%\ \text{of extractive} = \frac{w_0 - w_f}{w_0} \times 100\% \quad (1)$$

where w_0 and w_f indicate the initial and final weight of EFB.

3. Pre-treatment of EFB

A ratio of 1 : 19 w/w extractive-free EFB : DES was heated at 100 °C with vigorous stirring for 2 h. The mixture was then filtered and rinsed with distilled water to ensure no residual DES remained on the pre-treated EFB. The pre-treated EFB was dried at 60 °C in an oven to a constant weight. The test was carried out in triplicate for each sample.

4. Characterization of Untreated and Pre-treated EFB

4-1. Lignin Content

Lignin content of EFB was determined according to the three methods as described in Kirk et al. [49], TAPPI T222 and TAPPI UM 250 Test Methods with some modifications. An amount of 0.5 g extractive-free EFB was added into 5 mL of sulfuric acid (H_2SO_4, 72%) and stirred at room temperature for 1 h. The mixture was made up to 145 mL with distilled water, autoclaved at 120 °C for 1 h, and then filtered. The filtrate representing acid soluble lignin (ASL) content of the EFB was measured by a UV-Vis spectrophotometer (Thermo Scientific GENESYS 20) and the absorbance recorded at 325 nm. The solution with 4% of H_2SO_4 was served as blank. The filtered residue, acid insoluble lignin (AISL), was rinsed with hot water and oven-dried at 60 °C to a constant weight. Percentages of AISL and ash were calculated according to Eq. (2), while percentage of ASL was based on Eq. (3). Ash content of the EFB was determined *via* thermogravimetric analysis (TGA). The test was carried out in triplicate for each sample.

$$\%\ \text{of acid insoluble lignin (AISL)} = \frac{\text{Mass of residue}}{\text{Initial mass of EFB}} \times 100\% \quad (2)$$

$$\%\ \text{of acid soluble lignin (ASL)} = \frac{\frac{A}{a \times b} \times df \times V \times \frac{L}{1000\ ml} \times 100}{\frac{W \times \%\ T_{final}}{100}} \quad (3)$$

where A is the difference of absorbance value between blank and treated extractive-free EFB at 325 nm, a is an absorptivity value, 110 L $g^{-1}cm^{-1}$ and b is cell path length, 1 cm. V is the total volume of H_2SO_4 (72%) and distilled water. W is the initial weight of EFB in grams. df represents dilution factor and % T_{final} is the percentage of total solid content of EFB.

4-2. Hemicellulose Content

Hemicellulose content of biomass was determined according to Lin et al. [50] with slight modifications. One gram of EFB was added into 10 mL of 0.5 M NaOH solution and heated at 80 °C for 3.5 h. The mixture was filtered and rinsed with distilled water. The solid residue obtained was dried at 60 °C in an oven until a constant weight. The percentage of hemicellulose content in EFB was calculated based on Eq. (4). The test was carried out in triplicate for each sample.

$$\%\ \text{of hemicellulose} = \frac{w_o - w_f}{w_o} \times 100\% \quad (4)$$

where w_o represents the initial mass of EFB and w_f represents the final mass of EFB.

4-3. Cellulose Content

The cellulose content of EFB was determined using Eq. (5) based on Li et al. [51].

$$\text{Cellulose content (\%)} = 100\% - (\%\ \text{of lignin} + \%\ \text{of hemicellulose} + \%\ \text{of extractives} + \%\ \text{of ash}) \quad (5)$$

5. Toxicological Test

The percentage of cell proliferation for all the synthesized DESs and the starting materials against three bacterial strains was determined using microbroth dilution method based on international standard methodology M7-A6 [52] with some modifications. All bacterial strains were cultured in nutrient broth aerobically at 33-37 °C. The medium used for the experiment was Mueller Hilton broth (MHB). In brief, 75 μL of bacterial inoculum was plated into each well, followed by adding 75 μL of sample at the concentration of 1 mg/mL. The cultured inoculum served as a positive control, while the fresh MHB was a blank. The plate was then incubated at 33-37 °C for 19 h. The plate was measured at an absorbance of 600 nm using a SPECTROstar Nano microplate reader (BMG Labtech). The percentage of cell proliferation was calculated based on Eq. (6). The test was carried out in triplicate for each sample.

$$\%\ \text{cell proliferation} = (S - B)/(C - B) * 100 \quad (6)$$

where S is the average of absorbance of sample, B is the average of absorbance of blank and C is the average of absorbance of positive control.

6. Statistical Analysis

All data obtained were performed statistically using GraphPad Prism 7 and presented as mean±standard deviations. The standard deviation represents the reproducibility of the experiments in this study. The data were first analyzed by one-way analysis of variance (ANOVA), followed by Sidak's Multiple Comparison Test. Results were considered to be statistically significant when the *p* value was <0.05.

RESULTS AND DISCUSSION

The percentages of lignin, hemicellulose and cellulose present in the untreated (raw) and pre-treated EFB are presented in Figs. 3, 4 and 5, respectively, and summarized in Table 1. All the experiments were carried out in triplicate and the experiments are reproducible as all the standard deviations were found to be less than 5%. Significant differences were observed among lignin, hemicellulose and cellulose contents between the untreated and pre-treated EFB, except for cellulose content of ChCl : Gly (1 : 3) treatment group. Notably, all the synthesized DES possessed good solubility for lig-

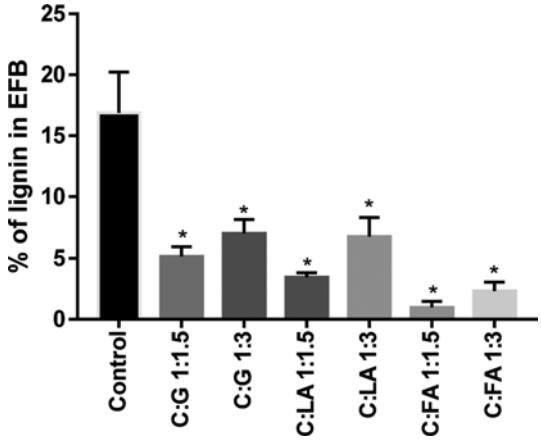

Fig. 3. Percentage of lignin in empty fruit bunches. Note: Control represents untreated EFB, C : G represents ChCl : Gly, C : LA represents ChCl : LA, C : FA represents ChCl : FA and "*" indicates p<0.05 as compared to that of untreated EFB.

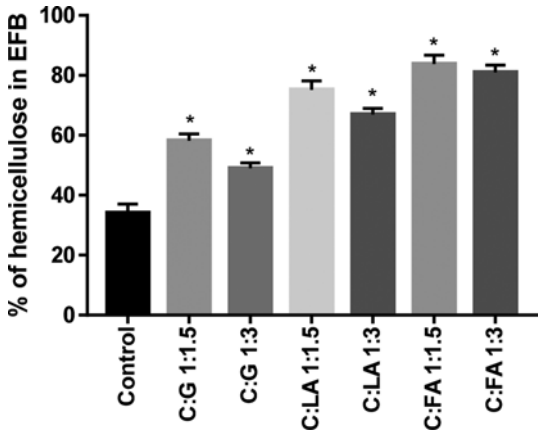

Fig. 4. Percentage of hemicellulose in empty fruit bunches. Note: Control represents untreated EFB, C : G represents ChCl : Gly, C : LA represents ChCl : LA, C : FA represents ChCl : FA and "*" indicates p<0.05 as compared to that of untreated EFB.

nin and cellulose. An average 74.61% of lignin and 55.34% of cellulose could be extracted by DES where the percentage of cellulose could be increased to 63.91 if excluding ChCl : Gly (1 : 3) treatment group. The results are in good agreement with the published data where the DES employed ChCl : Gly [53], ChCl : FA [43] and ChCl : LA [43,54] are able to disrupt biomass recalcitrance and

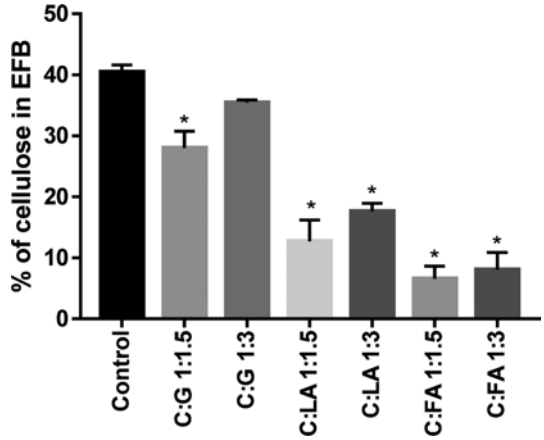

Fig. 5. Percentage of cellulose in empty fruit bunches. Note: Control represents untreated EFB, C : G represents ChCl : Gly, C : LA represents ChCl : LA, C : FA represents ChCl : FA and "*" indicates p<0.05 as compared to that of untreated EFB.

expose the surface area. In addition, the results are also in line with the data previously reported by Mikel et al. [55]. The findings imply that the addition of FA to the catalytic conversion reaction promoted the depolymerization of lignin by converting lignocellulosic biomass into bio-oil, attributable to its capability in cleaving strong intermolecular bonds in lignin.

Meanwhile, statistically significant differences were observed for hemicellulose and cellulose content of the pre-treated EFB among the ChCl : Gly groups. The same goes to hemicellulose content of the pre-treated EFB among the ChCl : LA groups. On the other hand, different molar ratio of ChCl : FA did not alter the content of hemicellulose and cellulose in the pre-treated EFB significantly. Although there was no significant difference in lignin content of the pre-treated EFB for each DES at two different molar ratios, the results in Table 1 showed that higher proportion of HBD gave higher percentage of lignin and cellulose, and lower percentage of hemicellulose of the pre-treated EFB. Thus, higher concentration of HBD prompted to remove hemicellulose more efficiently compared to lignin and cellulose.

Kumar et al. [54] showed that ChCl : LA (1 : 5) successfully removed 57.8% of lignin from rice straw after 12 h of incubation at 60 °C. The same DES (1 : 9 and 1 : 10), as reported by Jablonsky et al. [56] showed lower removal of 14.6% and 29.1% of lignin, respectively, at higher concentration of LA from wheat straw even though longer (24 h) incubation time was used. Comparing those

Table 1. Percentage of lignin, hemicellulose and cellulose in untreated and pre-treated empty fruit bunches

	Untreated EFB	Pre-treated EFB using DESs (in molar ratio)					
		ChCl : Gly		ChCl : FA		ChCl : LA	
		1 : 1.5	1 : 3	1 : 1.5	1 : 3	1 : 1.5	1 : 3
Lignin	16.89±3.33	5.16±0.80*	7.04±1.10*	0.98±0.50*	2.32±0.74*	3.45±0.37*	6.78±1.52*
Hemicellulose	34.03±3.03	58.24±2.20*	48.92±1.88*	83.82±2.86*	81.00±2.34*	75.22±2.92*	66.99±1.94*
Cellulose	40.47±1.14*	28.00±2.79*	35.43±0.45	6.60±2.06*	8.07±2.81*	12.72±3.48*	17.63±1.30*

Note: "*" indicates $p<0.05$ as compared to that of untreated EFB

with our data (58.3-94.2% lignin removal at molar ratios of 1 : 1.5 and 1 : 3 at 100 °C for 2 h), it was found that high proportion of HBD in DES and a prolonged reaction time not only had not promoted lignin degradation, but *vice versa* with a reduced percentage removal, indicating that ChCl plays a role in either concentrating hemicellulose or removing lignin, but the mechanism is still ambiguous. The results are in good agreement with previously reported data by Zhang et al. [57]. Zhang et al. [58] revealed that molar ratio of DES plays an important role in altering the DES physicochemical properties for biomass pre-treatment, such as viscosity, freezing point, conductivity and pH. It was acknowledged that high freezing point or viscosity of DES [58] is undesirable as it is not cost effective and there will be higher intramolecular interaction amongst the hydrogen bonds, resulting in less free space within DES, hence, lower mobility for intermolecular interaction. As a result, the efficiency of DES in degrading the targeted chemical compositions such as lignin, cellulose and hemicellulose is reduced. All the synthesized DES are liquid at room temperature, implying that their freezing points are lower than room temperature (25-27 °C). In addition, the viscosity of the synthesized DES with the molar ratios of 1 : 1.5 and 1 : 3 at 20 °C was 450.40±0.17 cP and 334.30±0.10 cP for ChCl : Gly; 41.10±0.10 cP and 4.93±0.21 cP for ChCl : FA; 302.50±0.10 cP and 165.57±4.83 cP for ChCl : LA, respectively. The result is in good agreement with the literature data [58] where the higher the viscosity of DES, the lower the dissolution power if compared among the three DES groups. However, this is not applicable if compared within the group as other factors need to be taken into account.

Our findings showed that the acid-type DES, ChCl : FA and ChCl : LA are more effective in disrupting lignin linkages dominated by aromatic bonds and cellulose crystallinity associated with β-(1,4)-glycosidic linear bonds. Interestingly, the abundant β-(1,3)-glycosidic bonds of hemicellulose remained untouched, implying that there were restricted spaces for hydrolysis and/or degradation of hemicellulose to occur significantly during DES pre-treatment. It indicated that the acid-type DES are highly specific and behave selectively towards lignocellulosic components. The results obtained did not correspond well with the findings of Kumar et al. [54] reporting ChCl : LA has no significant dissolution effect on cellulose [54]. Among the synthesized DES with the percentages of 94.2% and 86.2% for molar ratios of 1 : 1.5 and 1 : 3, respectively (Table 1), ChCl : FA had the highest dissolution power on lignin. The treatment group showed remarkably high hemicellulose content compared to the other two treatment groups. Structurally, Gly, FA and LA are alcohol-based solvents with different chain length of alcohol in the order of Gly (C3)>LA (C2)>FA (C1). Although Gly presents multiple hydrogen bond donor sites (-OH) and is more advantageous than FA and LA (Fig. 1), theoretically, the longer the hydrocarbon site chain, the poorer the hydrogen bonding capacity and the lower in solubilizing lignin. FA, being the simplest carboxylic acid, is the most versatile in mobilizing its electron lone pairs, hence able to attack and breakdown not just the ether linkages between lignin, but others like covalent, hydrogen bonds as well as van der Waals force linking the celluloses during EFB pre-treatment at mild condition. This would result in better solubility of lignin and cellulose. On contrary, the side chain of LA (~$CH(OH)CH_3$) is relatively larger compared to that of FA (~H) (Fig. 1). Hence, it has much restricted electron donating power, in depolymerizing the two chemical compositions. Therefore, a DES with a simpler ligand (in this case FA) attached to it has better capability to compete with the hydrogen bond network exhibited in the lignocellulosic matrix, leading to higher dissolution power in forming intermolecular hydrogen bonds with the two chemical compositions, hence facilitating the dissociation of lignin and cellulose. Lynam et al. [43] reported that ChCl : FA (1 : 2) dissolved 14% of lignin and <1% of cellulose while ChCl : LA (1 : 10) dissolved 13% of lignin and <3% of cellulose from the mixture of isolated biomass components at 60 °C. The lignin and cellulose removal efficiencies are much lower compared to those treatments at 100 °C. Thus, Zhang et al. [57] reported that the optimal pre-treatment temperature and time of corncob using ChCl : LA (1 : 2) were 90 °C and 24 h in terms of lignin recovery and glucose yield. In short, the higher the electron donating capacity of ligand in the order of FA>LA>Gly, the better the dissolution power of DES in order of ChCl : FA> ChCl : LA>ChCl : Gly.

An increase in temperature and time during pretreatment often leads to the production of undesirable by-products including enzyme and fermentation inhibitors [59], high energy consumption as well as low enzymatic hydrolysis efficiency [57]. Therefore, varying molar ratio, types of DES, acid strength, optimal temperature and time

Table 2. Percentage of cell proliferation of starting materials and all the synthesized DES against three bacterial strains at a concentration of 1 mg/mL

	E. coli	*S. typhimurium*	*S. aureus*
Choline chloride (ChCl)	100	98.19±1.27	65.89±1.65
Glycerol (Gly)	100	100	51.09±4.82
Formic acid (FA)	96.69±2.61	100	35.74±4.17
Lactic acid (LA)	100	100	41.61±4.42
ChCl : Gly 1 : 1.5	100	100	54.92±2.72
ChCl : Gly 1 : 3	100	100	53.49±3.14
ChCl : FA 1 : 1.5	100	97.78±1.71	47.65±2.84
ChCl : FA 1 : 3	100	98.55±1.88	44.75±4.95
ChCl : LA 1 : 1.5	100	96.29±2.30	52.45±3.47
ChCl : LA 1 : 3	100	100	50.73±2.63

are all equally crucial factors in finding an optimum condition of pretreatment to ensure sufficient interaction between DES (ligand) and the targeted lignocellulosic components before biomass disruption occurring. The study indeed indicated that EFB could be easily and rapidly delignified without much hassle using acid-type DES.

The results on toxicological test indicated that all starting materials, ChCl, Gly, LA, FA and the six synthesized DES, were benign to both the Gram-negative bacterial strains, *E. coli* and *S. typhimurium*, with more than 95% cell proliferation at the concentration of 1 mg/mL (Table 2). Conversely, they exhibited toxicity against *S. aureus* at the same concentration. High tolerance of *E. coli* and *S. typhimurium* was due to the strong outer membrane of the Gram-negative bacterial strains where it is made up of lipopolysaccharide and protein. It formed a formidable barrier which restricted the attack of DES from penetrating into the bacterial cell envelopes [60]. On the other hand, no barrier was established for *S. aureus* as its cell wall consists solely a thick peptidoglycan layer, therefore, more vulnerable to DES.

In summary, there is no significant difference observed among all starting materials and DES against *E. coli* and *S. typhimurium*. In contrast, all samples showed some degree of toxicity towards *S. aureus* with the lowest cell proliferation observed for both the employed ligands or organic acids, LA and FA. This implies that the ecotoxicity of the synthesized DES is very much dependent on the type of ligand and very little on the proportion used, which eventually is strain specific.

CONCLUSION

Different types of DES and the associated molar ratios of ligands possess different dissolution power towards lignin and cellulose. ChCl : FA was the most effective among all the synthesized DES in concentrating the hemicellulose content in EFB at mild condition, implying that the ChCl : FA-treated EFB could be used to produce hemicellulose-derived platform chemicals, which are primarily used in various industries such as pharmaceutical, oil refining, plastics, food as well as biochemicals. The toxicity of all the synthesized DES on the Gram-negative strains, *E. coli* and *S. typhimurium*, was negligible at 1 mg/mL. Even though the DES showed some toxicity on *S. aureus*, the level was lower than that of individual ligand used, indicating that these DES could be used in the intended industries.

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