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Effect of natural deep eutectic solvent molar ratio variation on cowpea shell pretreatment

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Abstract
This research investigated the effect of pretreatment on cowpea shells using lactic acid-choline chloride (L: C) solvent with varying molar ratios (L: C 5:1, L: C 9:1, and L: C 10:1). The chemical composition of the pretreated samples was analysed, and their surface morphology was evaluated using scanning electron microscopy (SEM). In addition, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction were used to study the structural changes in the functional groups and crystallinity of the pretreated cowpea shells. The results obtained showed that the pretreatment process made a significant impact on the chemical composition of the cowpea shells. The solvent of L: C (10: 1) made the highest impact with an increase in the percentage of glucan in the pretreated samples by 33 % and a decrease in acid-insoluble lignin by 11. 5 %. Acid-soluble lignin and xylan remained relatively constant in different molar ratios. SEM analysis revealed surface erosions and biomass deconstruction in the pretreated samples, with the degree of erosion increasing with higher acid molar ratios. FTIR analysis identified distinct changes in the functional groups of pretreated cowpea shells compared to those of untreated shells. Specific peaks at different wavelengths 2936 $cm^{-1,}$ 1034 $cm^{-1,}$ and 1693 cm^{-1} were identified, indicating variations in cellulose, hemicellulose, and lignin content. The pretreatment of samples with varying ratios (L: C 5:1, L: C 9:1, and L: C 10:1) exhibited increased crystalline indices of 78%, 79%, and 81%, respectively, compared to the untreated sample with a crystalline index of 66%. The pretreatment of cowpea shells with lactic acid: choline chloride solvent induced significant changes in their chemical composition, surface morphology, functional group structure, and crystallinity index. These findings contribute to our understanding of the potential applications of pretreated cowpea shells.

Keywords: Cowpea shells, pretreatment, Natural deep eutectic solvent, lactic acid, choline chloride lignin, cellulose, hemicellulose

1. Introduction

The physicochemical properties of biomass, including its chemical composition, crystalline index, accessible surface area, functional groups, lignin content, and morphology, can be modified by pretreatment. Such modifications are essential for reducing the recalcitrant nature of biomass. Pretreatment methods can be categorised as nonbiological, biological, or a combination. Biological processes include the use of microorganisms to treat substrates, and non-biological methods involve the use of physical processes and chemical approaches (such as ionic liquid, deep eutectic solvents (DES), and natural deep eutectic solvents (NADES). However, some of these methods face challenges such as severe reaction conditions, high energy consumption, environmental toxicity, and high costs (Elgharbawy et al., 2016). Therefore, the development of sustainable, cost-effective, and environmentally friendly pretreatment processes is crucial for biorefinery projects.

Ionic liquids (examples of ionic liquids include choline acetate, and 1-Butyl-3-methylimidazolium tetrafluoroborate) also known as green solvents, have emerged as greener alternatives to conventional solvents due to their negligible vapour pressures and chemical/thermal inertness (Greer et al., 2020). The groundbreaking work of Swatloski et al. (2002) demonstrated the application of ionic liquids for the dissolution of cellulose, paving the way for their application in biomass pretreatment. Although biomass pretreatment with ionic liquids has shown effectiveness, concerns remain regarding cost, recyclability, reapplication, and inhibitor generation (Kumar & Sharma, 2017). Furthermore, their environmental toxicity and biodegradation have raised questions (Oskarsson & Wright, 2019). Deep eutectic solvents (DES) on the other hand, constitute a novel family of ionic liquids formed by hydrogen bond interactions involving a combination of two or three affordable and non-hazardous components that possess the ability to self-associate (Smith et al., 2014). DES is formed by combining a hydrogen bond acceptor (HBA) (for example, choline chloride) with a hydrogen bond donor (HBD) example lactic acid capable of forming a complex with each other. DES was discovered by Abbot et al. (2003), during their investigation of the solvent properties of choline chloride/urea mixtures. DES is considered a greener alternative to ionic liquids due to its lower cost, ease of preparation with higher purity, and properties resembling ionic liquids, such as low melting point, thermal stability, adjustable physicochemical properties through component variations, low volatility, high substrate solubility (Yang & Wen, 2015). These advantages have made deep eutectic solvents the focus of biocatalysis, biotechnology, and bioengineering research.

However, as technology advanced Chio et al. (2011), discovered a novel set of ILs and DES called Natural Deep Eutectic Solvents (NADES). Through data analysis, they observed that specific simple molecules, including amino acids, choline, and organic acids are consistently present in significant amounts in microbial, mammalian, and plant cells. They found that combining these primary plant metabolites in specific molar ratios forms a viscous liquid that mimics synthetic ionic liquids and deep eutectic solvents. NADES are preferred over ionic liquids due to their cost effectiveness, ease of synthesis, nontoxicity, biocompatibility, recyclability, reusability, and high biodegradability (Kumar & Sharma, 2017). Additionally, NADES has the advantage of having low or no inhibitory compound formation (Kumar et al., 2016). These key advantages make NADES an attractive choice for biomass pretreatment, and they can be prepared using three different methods. The heating and stirring method: the hydrogen bond donors and acceptors are combined and heated to about 80 ^{0}C while continuously stirring until a clear colour liquid is obtained (Wan & Mun, 2018). The vacuum evaporating method involves mixing the components with a rotatory evaporator at 50 ° C (Dai et al., 2013). In the freeze-drying method: components are mixed in water and freeze-dried to obtain clear liquid (Zhang et al., 2022).

One of the advantages of deep eutectic solvents is their tailorable properties. Different solvents can be formed by combining hydrogen bond donors and acceptors in different molar ratios. It can be applied in different fields such as bio-catalysis, extraction of natural products, biomass pretreatment, clinical therapy and pharmaceutical products (Yang, 2018). The use of NADES has been applied for the pretreatment of different wastes such as rice straw (Kumar et al., 2016), corn cob (Zhang et al., 2016), coffee silverskin (Procentese & Rehmann, 2018), oil palm fronds (New et al., 2019) However, in this study we aim to explore the pretreatment of cowpea shell using NADES. Cowpea (*Vigna unguiculata*) is a legume crop widely cultivated for its edible seeds and consumed legume grain in countries such as Asia, Tropical Africa, South America, select regions of southern Europe, and the United States (Alemu et al., 2019). However, the cowpea plant produces a significant amount of agricultural residue (shells) after harvest which is often improperly disposed of, thereby causing environmental pollution (Koul et al., 2022). According to Ben-Iwo et al (2016) and Simonyan & Fasina, (2013), in the year 2010, 9.77 million tons of cowpea shell residue was generated in Nigeria. A study by Kemausuor et al (2014), showed that cowpea shells residue has the potential of generating 17 Mega Liters of cellulosic ethanol per year. But for a successful bioconversion of cowpea shells, a sustainable and environmentally friendly pretreatment such as the NADES approach is essential.

To determine the best molar ratio for the efficient deconstruction of biomass using NADES, it is important to investigate the impact of varying molar ratios between the hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA). The effect of this variation between choline chloride and lactic acid composition (1:9, 1:5, 1:2, 1:1) has been studied on the pretreatment of rice straw (Kumar et al., 2018). Thi & Lee (2019), also studied the effect of different choline chloride: lactic acid ratios (1: 2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:15) in the pretreatment of oil palm empty fruit bunch (OPEFB). The pretreatment of corncob waste was studied by Zhang et al.,(2016) using choline chloride-lactic acid combined in different molar ratios (1:2, 1:5, 1:10, 1:15), dicarboxylic acid: choline chloride (1:1) and polyalcohol: choline chloride (2:1). Li et. al (2018), varied lactic acid choline chloride molar ratio (5:1, 3:1 and 1:1) to study the pretreatment of rice straw. Another study by Su et al. (2021), studied the effect of varying molar ratios

of Choline chloride: Lactic acid (1:2, 1:4, 1:6, 1:8, 1:10) on the pretreatment of poplar sawdust at different temperatures of 110°C and 130°C. However, to the best of the authors' knowledge, the effect of this variation on cowpea shells pretreated with NADES has not been explored. This study therefore focuses on the effects of the different molar ratios between the hydrogen bond donor (HBD, lactic acid) and hydrogen bond acceptor (HBA, choline chloride) on the chemical composition, morphological characteristics, and structural properties of cowpea (*Vigna unguiculata)* shells.

2.0 Material and methods

2.1 Materials

Cowpea (*Vigna unguiculata)* shells were obtained from Tunga, located at coordinates 9 ° 17 '12.048' N, 6 ° 34' 59.988' E, within the Chanchaga local government area of Minna, Niger State, Nigeria. To protect against pest infestation and microbial growth, the Cowpea shells were carefully stored in airtight bags. Lactic acid (90%) and choline chloride (99%) were purchased from Thermo Scientific (USA), while sulfuric acid (98%), D- (+)-glucose, and D- (+) -Xylose were acquired from Sigma Aldrich Company Ltd, Germany. These chemicals were used in their original form without purification procedures.

2.2 Methods

2.2.1 Raw Material Preparation

The substrate was carefully washed with deionised water and sun-dried for two days before it was milled using a Retsch PM 100 Planetary ball mill (Haan, Germany). The milled sample was then sieved using a laboratory test sieve with a mesh size of 425 µm. Soxhlet extraction technique was used to remove extractives from the sample, using ethanol as the solvent; the extraction process was carried out for 6 hours at 80 ^{0}C. After this, the sample was washed with deionized water and dried at room temperature before storage in airtight containers for subsequent use in experimental procedures.

2.2.2 NADES Preparation and Physical Properties Analysis

Natural deep eutectic solvents (NADESs) were prepared by combining lactic acid (L) and choline chloride (C) in varying molar ratios, 5: 1, 9: 1, and 10:1. The solvents were prepared using the protocol described by Dai et al. (2013). Briefly, the reagents (lactic acid and choline chloride) were carefully measured and added to a tightly capped bottle, followed by incubation in a water bath set at 50 ° C with continuous stirring at 150 rpm for 30 minutes. Once a clear solution was obtained, it was left undisturbed overnight to ensure that phase separation did not occur, which indicates the stability of the solvent. The physical properties (density, conductivity, and viscosity) of the solvents were analysed at 25°C. The solvent densities were measured using the Sigma 700 tensiometer (Biolin Scientific USA). Conductivities were analyzed using a conductivity meter (Jenco instruments, Model: 3020M). The dynamic viscosity values of the solvents were measured using an RV-Viscometer (Model: NDJ-8S; W&J Instrument Co., Ltd. China).

2.2.3 NADES pretreatment

Following the study conducted by Kumar et al. (2016), the pretreatment process was carried out with a solid loading of 5% (w/w) and L: C solvents hydrated with 5% (w/w) water content. According to their research findings, 5 % water dilution (v/v) improved their pretreatment of rice straw. The hydration of the solvents with water has been proven to improve the fluidity of the solvent (Gabriele et al., 2019), hence increasing the mass transfer between the solvent and the biomass (Nolasco et al., 2022).

The pretreatment was carried out at a temperature of 120 °C, as determined by Li, Hou, Lin, Zhang, and Fu (2018), for 5 h based on the preliminary research findings of this work (not published) and (Yu et al., 2013). The experiment was carried out using a Schott-capped bottle placed within a high-temperature bath circulator MaXircu CH-8 model manufactured by Daihan Scientific, Korea. For proper washing of the pretreated substrate the methodology described by Procentese et al. (2015) and Li et al. (2018) was adopted. The slurry obtained after pretreatment was first washed with water to remove some of the solvents and dissolved lignin before subsequently, re-washing with 5 ml of an antisolvent (ethanol) and water. In a sustainable approach, the ethanol used for washing was carefully collected and recovered using a vacuum rotavapor from Büchi Labortechnik, Switzerland, which allowed reuse throughout the entire process. Once the solid residue was recovered, it was subjected to an oven drying process at 35 ° C. The dried solid residue was analysed using the National Renewable Energy Laboratory (NREL) method (Sluiter et al., 2008) to determine the composition of structural carbohydrates (glucan and xylan) and lignin.

2.2.4 Glucan, xylan, and lignin analysis of treated and untreated cowpea shell

This study applied protocols outlined by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008) to determine the structural carbohydrates (glucan and xylan) in the untreated and pretreated cowpea shells. Specifically, 0.3 g of the substrates were mixed with 3 ml of 72% sulfuric acid and incubated for 1 hour at 30 ° C in a water bath. The acid concentration was then adjusted to 4% by adding 84 ml of deionised water, followed by autoclaving at 121 ° C and 1.3 bar for 1 hour using a Hirayama autoclave (HV-110, Japan). The resulting filtrate from the dilute acid hydrolysis was further diluted, and the pH was adjusted using calcium carbonate. Subsequently, the neutralised filtrates were filtered through 0.45 μm nylon syringe filters and filled into HPLC autosampler vials. The composition of glucose and xylose was analysed using an Agilent 1260 Infinity HPLC system equipped with a refractive index detector. The analysis was carried out at 55 ° C, using an Aminex HPX-87C column (300 x 7.8 mm, Bio-Rad Laboratories, Hercules, California, USA) at 60 ° C. The column temperature was maintained at 60 °C, and the mobile phase consisted of 0.005 M H_2SO_4 at a flow rate of 0.6 ml/min. Glucose and xylose values obtained were converted to the glucan and xylan equivalent using equations developed by NREL (Sluiter et al., 2008).

The acid-insoluble lignin content was determined using the residue obtained from the acid hydrolysis step. It was placed in a crucible, dried at 105 ° C for 4 hours and then placed in a muffle furnace at 575 ° C for 4 hours. The weight loss of the acid-insoluble residue after ashing was measured gravimetrically to determine the acid-insoluble lignin content (AIL). The acid-soluble lignin composition in the filtrates was evaluated using the Agilent Technologies Cary 60 UV-vis spectrophotometer. The absorbance at a wavelength of 288 nm was used to calculate the acid-soluble lignin content using the equation developed by NREL (Sluiter et al., 2008). Experiments were performed in duplicate, and the results are presented as a percentage of the substrate on an oven-dried basis.

2.2.5 Scanning electron microscopy (SEM) analysis

Scanning electron microscopy (SEM) was employed to examine the morphological characteristics of both untreated and treated cowpea shells. The investigation was carried out using a Zeiss Gemini Ultra Plus field emission scanning electron microscope (FEG-SEM) (Germany). The imaging was carried out under specific conditions, including an acceleration voltage of 2 kV and 5000, 10,000, and 30,000 magnifications. A carbon coating was applied to the substrate using the Quorum Q150T coating unit to enhance the conductivity and imaging quality of the sample.

2.2.6 Fourier transform infrared (FTIR) analysis.

Fourier transform infrared (FTIR) spectroscopy was carried out using the Perkin Elmer Spectrum 100 apparatus (USA) to identify the various functional groups in the chemical structure. The FTIR spectrum was obtained within a spectral range of 600 to 4000 cm^{-1}.

2.2.7 X-ray diffraction (XRD) analysis

The untreated and pretreated samples were analysed using a PANalytical X'Pert Pro powder diffractometer with an X'Celerator detector, variable divergence, and fixed receiving slits with Fe-filtered Co-Kα radiation (λ=1.789Å). Samples were scanned from $2\theta = 10^0$ to $2\theta = 60^0$ with an increment of 0.008. The crystallinity index (CrI) was calculated using the following peak height method formula (equation 1) by Segal et al. (1959).

$$CrI = \frac{I_{002} - I_{AM}}{I_{002}} \times 100 \qquad (1)$$

Where I_{002} is the maximum intensity of the lattice diffraction for cellulose I, and the minimum diffraction intensity for the amorphous material between the 002 and the 101 peaks (I_{AM}).

3.0 Results and Discussions

3.1 Physical properties of NADES

Data on the physical properties of a solvent is a major prerequisite before it can be considered for use in industrial applications (Haghbakhsh et al., 2019). Table 1 shows the physical properties: densities, viscosities and conductivities of the solvents prepared. These properties are affected by variations in the molar ratio of the hydrogen bond donor and hydrogen bond acceptor (Bušić et al., 2022). From Table 1, can be observed that increasing the hydrogen bond donor molar ratio affects the density, viscosity, and conductivity values of the solvents. From the results presented in Table 1, the density of L: C (5:1) is the lowest (1.187 g/ml) followed by L: C (9: 1) with L: C (10:1) having the highest density value of 1.197 g/ml. The increasing trend in density observed as the hydrogen bond donor molar ratio increases can be attributed to the reduction in available free space for bonding between the hydrogen bond donor and hydrogen bond acceptor (Ijardar et al., 2022). This trend agrees with the research findings

reported by Shafie et. al (2019), where an increase in hydrogen bond donor (citric acid) molar ratio increased the density of the solvent. And agrees with the result obtained by Singh et al. (2018) in which increasing the molar ratio of HBD (glycerol) increased density. The viscosity of solvents determines their suitability as a reaction medium for an industrial process (Ijardar et al., 2022). Based on Table 1, L: C (5:1) is the most viscous (200 mPa/s) while L: C (10:1) has the lowest viscosity at 166.55 mPa/s. This viscosity variation can be attributed to the diluting effect of lactic acid (HBD) on the hydrogen bond between lactic acid and choline chloride. This dilution leads to an increase in the molecular movement within the solvent to improve fluidity (Ijardar et al., 2022). Conductivity is another important physical property that governs the rate at which heat energy passes through a substance (Chen et al., 2021). The conductivity measurements as seen in Table 1, show a progressive increase in value as the lactic acid content of the solvents increases with L: C (10:1) giving the highest conductivity value of 1182.50 micro-siemens. This implies that a decrease in the salt (ChCl) content of the solvent improved the conductivity of the solvents. This observed trend is in line with the research findings of Yan et. al (2017), where thermal conductivity measurements were conducted using choline chloride (salt) and ethylene glycol for solvent preparation.

Table 1: Physical properties of different NADES prepared at 22 ^{0}C.

NADES	Density (g/ml)	Viscosity (mPa/s)	Conductivity (µS)
L: C (5:1)	1.187 ± 0.013	200.20 ± 0.565	151.80 ± 0.14
L: C (9: 1)	1.196 ± 0.010	186.50 ± 1.620	1176.50 ± 2.13
L: C (10:1)	1.197 ±0.013	166.55 ± 0.070	1182.50 ± 2.12

3.2 Glucan, xylan and lignin analysis of treated and untreated cowpea shell

The percentage composition of the untreated cowpea shells was obtained as glucan (21.32 %), xylan (21.46 %), acid-insoluble lignin (28.37 %) and acid-soluble lignin (8.36 %). Another study on cowpea shells harvested from Ijebu-Ode in Ogun state, Nigeria by Abiola-Olagunju, Akinwande and Mako (2020), obtained the following values for hemicellulose (24 %) and an acid detergent lignin (22.55 %) which closely aligns with values reported in this study. However, the cellulose content of 31.82 % obtained from their study is higher and this can be attributed to genotypic differences and their interaction with the local environmental conditions (Guragain et al., 2017). The effect of molar ratio variation between lactic acid (HBD) and choline chloride (HBA) on the pretreatment of cowpea shells is shown in Table 2. From the results obtained the acid-soluble lignin decreased marginally as lactic acid content of the solvent increased. The acid-insoluble lignin decreased significantly achieving 31 %, 38 % and 41 % removal with (L: C) ratios of 5:1, 9:1, and 10:1 respectively. The delignification effect of the NADES on biomass has been associated with the quantity of hydrogen bond donors that make up the solvent (Li et al., 2018) and also the viscosity of the solvent (Zhang et al., 2016). Therefore, the high lignin removal obtained by L: C (10:1) can be associated with its high lactic acid (HBD) ratio composition and its low viscous nature which promotes mass transfer. This result highlights the impact of increasing the acidity of the solvent on the lignin removal and it aligns with the research findings of Kumar et al. (2018) and Li et al. (2018) on the effect of molar ratio of rice straw pretreatment.

The xylan composition as seen in Table 2, remained relatively stable within the range of 21 -20 % even as the acidity of the solvent increased. The stability of xylan from beechwood in L: C (10: 1) was also reported by Lynam et al. (2017). Another report by Kumar et al. (2016), also shows zero solubility of xylan in NADESs studied indicating high stability. However, this phenomenon is not in agreement with the research findings of Li et al., (2018). In their study, rice straw was pretreated at 120 ^{0}C for 3 h using (L: C) ratios of 1:1, 1: 3 and 5:1. The result obtained shows a progressive increase in xylan and lignin removal with a maximum value of 58 and 48 % respectively. It is worth noting that xylan (hemicellulose), is bound to cellulose in lignocellulosic biomass by lignin (Zoghlami & Paës, 2019), therefore xylan removal is dependent on lignin dissolution. This implies that the high lignin removal achieved by Li et al. (2018) enhanced the hemicellulose removal.

The glucan composition of the pretreated cowpea shells increased with the increment of the hydrogen bond donor in the NADES. This resulted in the highest glucan percentage of 54% achieved with L: C (10:1). This trend aligns with previous research conducted by Li et al. (2018) and Kumar et al. (2018). The increased glucan content can be associated with the reduction in lignin content due to an increase in the acidic hydrogen ions in the solvent (Su et al., 2021). However, the increased acidity of the solvent adversely affects the solid recovery percentage. The highest solid recovery (62 %) was obtained when L: C (5: 1) was applied which reduced significantly as acidity increased.

This finding is in line with prior research by Zhang et al. (2016), Shen et al (2019), and Li et al. (2018) on the effect of higher lactic acid molar ratio on solid recovery after pretreatment.

Table 2: Structural composition of the untreated and pretreated cowpea shel

	% Solid Recovered	Glucan (%)	Xylan (%)	Acid Insoluble Lignin (%)	Acid Soluble Lignin (%)
Untreated	100	21.32 ± 0.11	21.46 ± 0.30	28.37 ± 0.03	8.36 ± 0.65
L: C (5: 1)	62 ± 0.11	50.44 ± 0.08	20.03 ± 0.41	19.57 ± 0.1	7.85 ± 0.01
L: C (9:1)	58 ± 0.02	50.42 ± 0.53	19.63 ± 1.05	17.58 ± 0.20	7.93 ± 0.01
L: C (10:1)	42 ± 0.04	54.21 ± 0.23	20.5 ± 1.22	16. 84 ± 1.25	6.70 ± 0.01

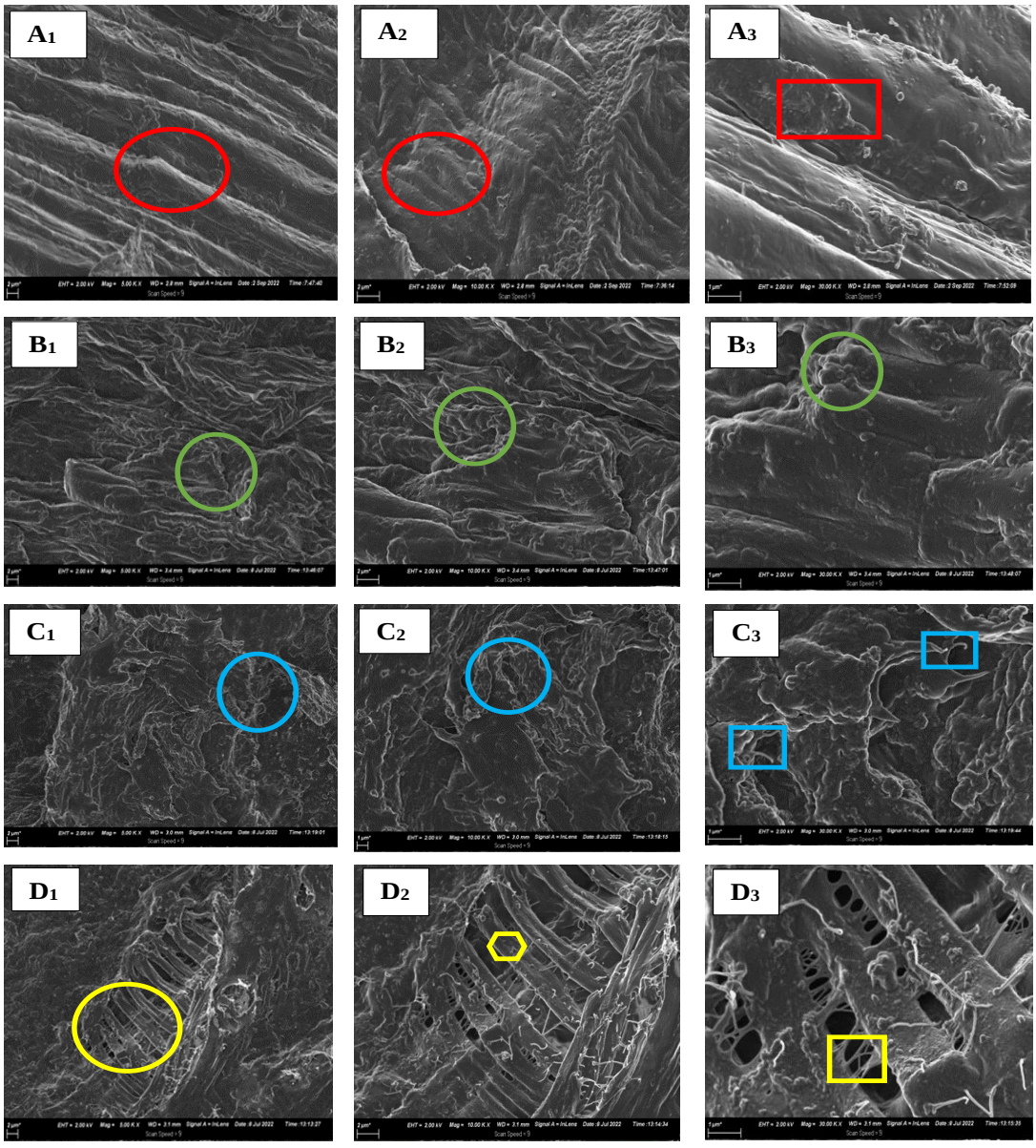

Figure 1: SEM analysis of untreated and pretreated substrate. Where A, B, C, and D denote untreated, L: C (5:1), L: C (9:1), and L: C (10: 1), respectively. The subscripts denote magnification 5000, 10000, and 30000 respectively.

3.3 Scanning electron microscopy (SEM) analysis

SEM is an imaging tool that allows the analysis of surface morphology and examination of microstructure to study surface erosions, deconstruction processes, and the relocalization of components of the cell wall (Karimi & Taherzadeh, 2016). Figure 1 (A- D), presents the surface morphology of the untreated and treated cowpea shell at various magnifications (5000, 10000, and 30000) respectively. Figure A_1 -A_2 shows the ridged surfaces of the cell walls of the untreated cowpea shells (red circles) with minimal disruptions observed in Figure1A_3 (red box). These slight disruptions can be attributed to the size reduction process (ball milling). The smooth surface can be identified as the lignin component of the substrate covering the cellulose rod-like fibres (Anukam & Berghel, 2021). The pretreatment of the cowpea shells with solvents of different molar ratios increased surface erosion, as shown in Figure 1(B-D).

Figure 1(B_1 -B_3), shows the morphological changes of the substrate pretreated with L: C (5:1) under different magnifications. It can be observed that the solvent disrupted the surface morphology (green circles) when compared to the untreated sample. However, the SEM images for L: C (9:1) show an increased eroded surface (Figure 1(C_1-C_2): blue circles). Observing this effect under higher 30,000 magnification it can be seen that the cross-linked branches of fibre were slightly exposed (Figure1C_3, blue box) and these fibres have been identified as the hemicellulose component of biomass Liu & Yu (2021). The degree of erosion intensified (Figure 1D_1, yellow circle). with the L: C (10:1) ratio exposing the cellulose fibre (Figure 1D_2, yellow hexagon), and the hemicellulose cross-linked branches (Figure 1D_3, yellow box). This confirms the chemical composition analysis in Table 2 which shows a higher cellulose (glucan) composition of cowpea shells pretreated with L: C (10:1). These changes in the surface characteristics of the NADESs pretreated cowpea shells when compared to the untreated can be associated with the disruptions made by the solvent on the molecular framework of the raw sample (Zhang et al., 2016).

3.4 Fourier transform infrared (FTIR) spectroscopy analysis.

Figure 2 illustrates the FTIR plots showing a comparison between untreated and pretreated cowpea shells using different molar ratios. In the untreated and L: C (10: 1) samples, prominent peaks were identified at specific wavelengths: 1034 cm^{-1}, 1693 cm^{-1}, 2936 cm^{-1}, and 3353 cm^{-1}. Previous studies (Fonyuy et al., 2023; Lao et al., 2014); have assigned the peaks at 2936 $cm^{-1}$and 3353 cm^{-1} to cellulose, while the peaks at 1034 cm^{-1} and 1693 cm^{-1} are associated with hemicellulose and lignin, respectively indicating that the substrate is rich in structural carbohydrates and lignin. However, comparing variations in the percentage transmittance of light across the samples shows a higher percentage transmittance in the L: C (10:1) pretreated sample at the peaks identified. This shows that enhanced delignification improved light transmittance through the sample.

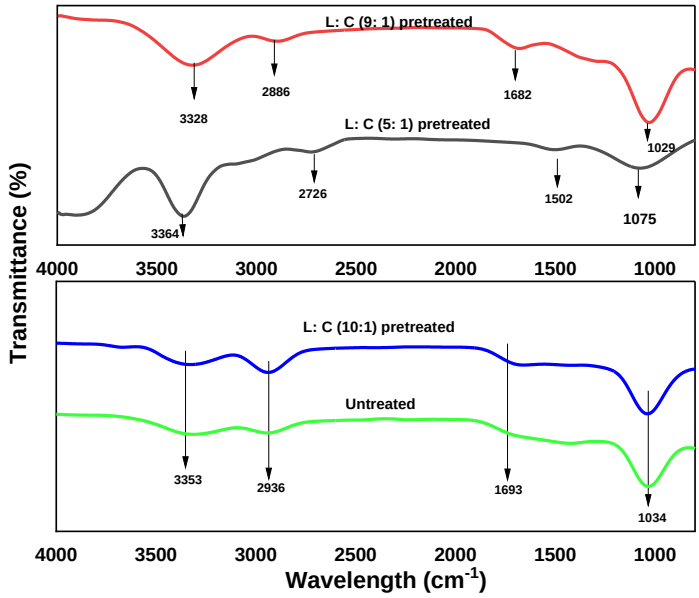

Fig. 2: FTIR analysis of untreated and pretreated substrates

Similarly, in the pretreated cowpea shells in L: C (9:1) molar ratio, the major peaks were identified at 1029, 1682, 2886 and 3328 (cm ^{1}). According, to Olatunji & Madyira (2023), the peak at 1029 cm^{-1} (non-structural CHO) is related to hemicellulose, while the peak at 1682 cm^{-1} corresponds to cellulose (Feng et al., 2018). The peak at 2886 cm^{-1} (-CH stretch) is attributed to lignin (Bi et al., 2018), and the peak at 3328 cm^{-1} is associated with cellulose (Barathi et al., 2013). In L: C (5:1) pretreated cowpea shells, the peak at 1075 cm^{-1} is indicative of β (1-3) glucan (Pérez-Bassart et al., 2023) while the peak at 1502 cm^{-1} suggests the presence of lignin (Song et al., 2019) . The peak at 2726 cm^{-1} falls within the wavelength range of 2830- 2695 representing C-H stretching commonly found in cellulose, hemicellulose, and lignin (Zhuang et al., 2020). Additionally, the peak at 3364 cm^{-1} falls within the range between 3600–3100 cm^{-1} wavelength which is associated with OH stretching of cellulose. The distortion at the peaks for lignin and hemicellulose, when compared to the untreated sample, indicates a reduction in the content of both lignin and hemicellulose due to the pretreatment process.

3.5 XRD (X-ray diffraction)

To investigate the impact of pretreatment on sample crystalline index, both untreated and pretreated samples were examined using XRD analysis. The crystalline index for the untreated sample and samples pretreated with the different variations (L: C 5:1, L: C 9:1, and L: C 10:1) was determined from Figure 3, yielding values of 66%, 78%, 79%, and 81%, respectively. A noticeable increase in crystallinity index emerged when comparing untreated and pretreated samples. This increase is attributed to the reduction in the lignin and xylan composition in the pretreated samples, thereby enhancing the cellulose fraction in the samples (Li et al., 2019; Maibam & Goyal, 2022). A similar trend of increased crystalline index after pretreatment was also reported by Procentese et al. (2015). Additionally, an increase in lactic acid molar ratio correlated with a higher crystalline index in the pretreated samples, signifying increased removal of lignin with increasing pretreatment severity.

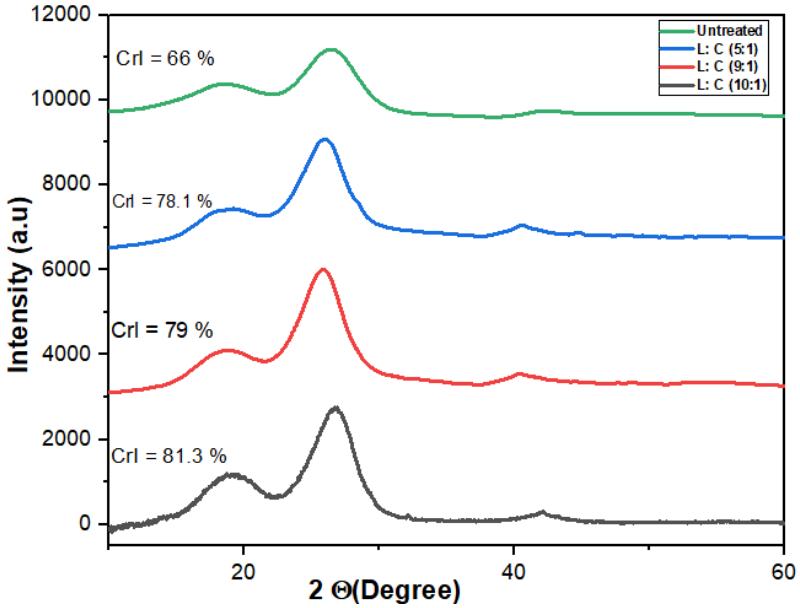

Figure. 3: XRD plots for pretreated and Untreated cowpea shells.

4.0. Conclusion

The pretreatment of cowpea shells with lactic acid: choline chloride solvent with varying molar ratios made a significant impact on the chemical composition, surface morphology, and functional group structure. The percentage of glucan increased with higher lactic acid molar ratios, while the percentage of insoluble lignin decreased. The highest glucan percentage of 54 % was achieved with L: C (10:1). SEM analysis showed surface erosions and biomass deconstruction in the pretreated samples, indicating disruption of the cell wall structure. FTIR analysis showed significant changes in the functional groups of the pretreated cowpea shells, indicating variations in the content of cellulose, hemicellulose, and lignin content. The XRD analysis confirmed the impact made by the pretreatment on the crystallinity index.

These findings highlight the potential of natural deep eutectic solvents for the pretreatment of cowpea shells to facilitate the conversion of this understudied waste to more useful products. Therefore, more research is recommended in other fields to explore the specific properties and performance of these pretreated cowpea shells in different applications such as fermentable sugar production, as well as optimisation of the pretreatment process to enhance their suitability for specific purposes.

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