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Delignification of Halophyte *Atriplex crassifolia* by Green Recyclable Deep Eutectic Solvents for Enhanced Production of Biogas

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Abstract: Deep eutectic solvents (DESs) have upgraded the practices of valorizing lignocellulosic feedstock by lessening biomass recalcitrance through delignification in precise and economical manner. In this study, the influence of a series of deep eutectic mixtures was evaluated on the halophyte *Atriplex crassifolia* for achieving elevated biogas production. Initially, the biomass was pretreated via several DESs with varying hydrogen bond donors (HBDs) including carboxylic acids, amine/amide, and polyols/glycols. DES composed of choline chloride (ChCl) and lactic acid (LA) evidenced as the most effective solvent in achieving high lignin removal rates and was further optimized by evaluating the parameters of molar ratio of DES components, solid-to-liquid ratio, and solvent addition. A maximum delignification value of 89.5% was achieved by 15% diluted ChCl: LA (1:2) DES at a biomass loading of 1:15. The solubilization rate of diluted ChCl: LA was also raised up to 38%. FT-IR analysis revealed significant lignin elimination from ChCl: LA pretreated substrates. Moreover, $\geq$88% of ChCl: LA DES was recovered after up to three pretreatment cycles, retaining $\geq$85% delignification efficiency. Fresh DES-pretreated *Atriplex crassifolia* recorded 32.2 mL/g of biogas production yield due to increased cellulosic content. The findings validated *Atriplex crassifolia* as an efficient feedstock for biogas production and confirmed the affectivity of ChCl: LA pretreatment in eliminating the lignin barrier, ultimately making cellulosic sugars readily biodegradable and highly accessible for anaerobic microorganisms.

Keywords: deep eutectic solvents; pretreatment; choline chloride; lactic acid; delignification; *Atriplex crassifolia*; biogas

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1. Introduction

Ever-increasing energy demands and a shortage of non-renewable energy means have outstretched the need for sustainable energy resources. Currently, an immense share (i.e., $\geq$84%) of global energy consumption is sustained through fossil fuels such as coal, oil, petroleum etc. [1,2]. Due to these concerns, researchers have shifted the focus toward expanding renewable energy reserves. Given the core issue of global warming, renewable biofuels have been reckoned as the chief backers of energy for restricting the consumption of fossil fuels [3,4]. Biofuels, comprising biogas, biodiesel, bioethanol etc., seem favorable alternatives for traditional fossil fuels [5]. Among biofuels, biogas is a promising green energy source. Reviewing the past 10 years of research, ~90% rise occurred in the biogas industry. In 2017, 70% of the total world's biogas was generated in Europe (64TWh), emphasizing the significance of biogas production from natural renewable green resources [6]. Lignocellulosic biomass (LCB) is a renewable and copious carbon-based feedstock on Earth and is considered as a promising primary source for materializing the notion of bioenergy [7]. On the contrary, agricultural produce is facing intense constraints because of the decline in arable lands due to soil salinization and shortage of freshwater. Approximately 43% of the world's surface is occupied by arid and semi-arid lands, whereas 98% of water is saline. Accordingly, ~20% of irrigated agricultural lands are salt-affected, and it is the cause of an economic downturn of USD 27.3 billion every year [8], compelling the need to exploit salt-tolerant halophytes for generating renewable energy.

Halophytes are an upright source of low-cost LCB [9] as they can be cultivated on degraded saline lands. So far, various plants have been inspected for generating biogas, including maize, grass, sunflower, clover etc. [10–13]. However, minimal work has been conducted on exploring halophytes in this respect. Several halophytic plant species assessed for this purpose include *Halodule uninervis*, *Halophila stipulacea*, *Salicornia sinus-persica*, *Arundo donax*, *Halophila ovalis*, and *Salicornia bigelovii* [14]. Genus Atriplex (commonly named saltbush and orache) is a widely distributed halophyte consisting of 250 species. It belongs to the family Amaranthaceae and subfamily Chenopodioideae (plants of the world online). Among Atriplex halophytes, *Atriplex saggitata* (Borkh) has been reported for its usage as a feedstock for generating biogas [15].

Though LCB is a promising feedstock for generating bioenergy, its highly complex structure is an impediment in achieving this goal. Pretreatment is a chief step to break this recalcitrant barrier, unpacking the cellulosic sugars to make them readily accessible for anaerobes. Effective pretreatment destroys the LCB's supramolecular structure, eventually improving biogas yield. Approximately 40% of the total process cost is constituted by the pretreatment step alone [16]. Although conventional pretreatment methods (acid, alkali, biological, chemical) lessen the biomass recalcitrance to a certain extent [17], these strategies have multiple limitations as well, such as equipment corrosion, increased operating expense, and costly chemical recovery strategies [18]. Hence, green, sustainable, and mild pretreatment approaches are increasingly emphasized. DESs appear as favored solvents for pretreatment purposes. DESs are a group of sustainable organic solvents and can be employed as effective alternatives to ionic liquids. They exhibit low melting points, low toxicity, high purity, and can be easily prepared. Furthermore, they are not flammable, less volatile, biodegradable, and exhibit high air and thermal stability as well [19]. The delignification mechanisms in DES systems are interrelated with the capacity of electronegative halogen anion (Cl^-) from ChCl to form hydrogen bonding with hydroxy groups present in lignin, followed by aromatic compound dissolution [20]. In case of dissolution of cellulose, the preliminary intermolecular hydrogen bonds present in cellulose (β-(1,4)-glycosidic) are broken down by DES, leading to the generation of new hydrogen bonds between hydroxyl groups in polysaccharide and DES [21,22]. The H-bond basicity of DES weakens the intra- or intermolecular hydrogen bonds in cellulose, demonstrating the solvent's ability to act as an HBA. Additionally, it has also been evaluated that anions with the capability to be a HBA such as OAc^-, $(MeO)_2PO_2^-$, Cl^-, $HCOO^-$, imidazole, or morpholine are outstanding candidates to form DESs [23].

In comparison to traditional solvents, DESs have high compatibility for both enzymes and microorganisms [24]. In addition to all these positive traits, one of the chief attributes is their easy recyclability and reuse, thus making the process of biogas production cost-efficient. Researches have evidenced $\geq$90% of DES recovery yield [25,26]. All these aspects of DESs make them highly suitable to utilize as pretreatment solvents for biogas production. Studies have revealed the optimistic effects of utilizing DESs such as ammonium thiocyanate-urea [27] and choline chloride-oxalic acid [28] on enhancing biogas yield.

As already mentioned, this research work, as far as we know, will be the first study that utilizes a halophyte, i.e., *Atriplex crassifolia*, for biogas generation. Moreover, as DES-based pretreatment is quite a new approach, in-depth analysis of these eutectic solvents has not been extensively performed. Very few studies are available that provide a detailed survey of utilizing and optimizing DES as a pretreatment solvent. For these reasons, in the present work, three types of DES, carboxylic acids, polyols/glycols, and amine/amides, have been evaluated for pretreating a halophyte to attain increased cumulative biogas production. Additionally, process optimization has been carried out by electing some under-researched parameters, such as type of HBD, the molar ratio of DES constituents, solid-to-liquid (S/L) ratio, and effect of diluted DES (water/solvent addition in DES) for achieving increased delignification values. This study further holds a comprehensive explanation of the recyclability and reusability of the eutectic solvent, highlighting the potential of recovered DES mediated pretreatment on biogas production, thus making the process economically feasible.

2. Materials and Methods

2.1. Substrate and Anaerobic Sludge/Inoculum

Atriplex crassifolia was obtained from the Government College University, Lahore, Pakistan. The halophyte was initially dried and later cut into small bits (1–2 cm). These pieces were ground into fine powder (particle size: 1.19 mm) and preserved at room temperature in tightly closed zipper bags. The anaerobic sludge was taken biweekly from the wastewater treatment plant of Water and Sanitation Agency (WASA), Lahore. The sludge was reactivated at 37 °C for ~5 days before inoculation. Anaerobic sludge was degassed by pre-incubating it at the same operating temperature as of inoculum source to prevent the production of background gas while limiting the incidence of residual biodegradable organic content during testing [29,30]. The characterization of both anaerobic sludge and the substrate was provided in Table 1.

Table 1. Characterization of pre-hydrolyzed biomass and inoculum (anaerobic sludge).

Characterizing Factors	*Atriplex crassifolia*	Anaerobic Sludge
Cellulose (%)	37.5 ± 1.3	-
Hemicellulose (%)	32.5 ± 1.1	-
Lignin (%)	19.2 ± 0.4	-
pH	5.5 ± 0.6	7.9 ± 0.1
TS (g/L)	52.4 ± 1.5	24.3 ± 0.9
VS (g/L)	41.8 ± 1.2	14.6 ± 0.2
tCOD (g O_2/L)	103 ± 3.5	28.5 ± 1.1

2.2. Preparation of Deep Eutectic Solvents

DESs were prepared by dissolving choline chloride (i.e., HBA) with varied types of HBD at a 1:2 molar ratio (Table 2). All the eutectic solvents were heated at 80 °C under constant vigorous stirring at 500 rpm until a homogenous and transparent liquid was formed. All prepared solvents were cooled and dried to avoid moisture absorption and later stored at room temperature in sealed flasks [27,31].

Table 2. Several hydrogen bond donors combined with choline chloride to make DESs.

Carboxylic Acids	Polyols/Glycols	Amines/Amides
Acetic acid (AA)	Ethylene glycol (EG)	Urea (U)
Oxalic acid (OA)	Glycerol (GLY)	Formamide (FM)
Lactic acid (LA)	Xylitol (X)	Acetamide (Ac)
Citric acid (CA)	1,2-Propanediol (1,2-PDO)	Monoethanolamine (MEA)
Levulinic acid (LVA)	1,3-Propanediol (1,3-PDO)	Diethanolamine (DEA)

2.3. Pretreatment Optimization of Atriplex crassifolia

The choline chloride-based DESs were initially screened by varying the types of HBD. For this, 5 g of substrate was dissolved in the 50 mL DES. The solution was then incubated for 4 h at 120 °C. The DES that showed the highest delignification efficiency was further optimized by selecting physicochemical parameters including molar ratio of DES constituents, solid-to-liquid ratio, and effect of solvent addition as independent variables. Pretreated *A. crassifolia* was filtered and rinsed with ethanol/water in order to remove the remaining DES constituents, and washing step was continued until the pH become neutral, evincing the thorough elimination of DES components from the feedstock. Later on, washed biomass samples were dried at 70 °C for 3 h. The dried substrates were stored in sterilized zip-lock polythene bags prior to use [27,32]. The biomass residue recovery attained after the pretreatment and SR% of DES on samples were calculated by the formulas provided below [32,33].

Equations used to calculate (a) percentage recovery of biomass after DES mediated pretreatment and (b) solubilization rate (SR%) of ChCl-LA based DES on halophyte *Atriplex crassifolia.*

$$\text{Pretreated biomass solid recovery } (\%) = \frac{W_{SF}}{W_{RAW}} \times 100 \tag{1}$$

$$\text{Solubilzation rate } (\text{SR } \%) = \frac{m_0 - m_1}{m_0} \times 100 \tag{2}$$

where, W_{SF} = Weight of solid fraction obtained after pretreatment; W_{RAW} = Weight of raw substrate; m_0 = Oven-dried weight of raw sample; m_1 = Oven-dried weight of the pretreated residues

2.4. Biogas Production via Anaerobic Digestion

Biogas was generated by the process of AD in batch fermentation experiments. The nutrient medium was formed by dissolving (g/L) $NiCl_2.6H_2O$ (0.00005 g), $CaCl_2.2H_2O$ (0.075 g), Na_2SeO_3 (0.00005 g), $CoCl_2.6H_2O$ (0.0005 g), K_2HPO_4 (0.35 g), $CuCl_2$ (0.00003 g), KH_2PO_4 (0.27 g), $MgCl.6H_2O$ (0.1 g), $FeCl_2.4H_2O$ (0.2 g), $NaNO_4$ (0.00001 g), H_3BO_3 (0.00005 g), NH_4Cl (0.53 g), $ZnCl_3$ (0.00005 g), and $MnCl_2$ (0.0005 g) in 50 mL distilled water [34]. The inoculum-to-substrate ratio (ISR) was 2:1. A total of 10 mL of inoculum was added in a flask containing 5 g of pretreated biomass. The final volume was raised to 200 mL by adding mineral medium and distilled water [35]. To develop anaerobic conditions, the nitrogen gas (99% pure) was flushed into the headspace of the reactor for two minutes. The set-up was incubated at 35 ± 1 °C with pH adjusted at 7. The gas released during AD was passed through 0.5 N NaOH to capture CO_2, while the residual gas was assumed to be biogas [29]. The inoculum & raw substrate and inoculum alone were also digested as controls to compare the extent of biogas production from DES-pretreated halophyte. For AD process, the retention time was 24 days and the biogas production was measured daily. The pH of all set-ups was checked at the end of the digestion process to ensure the absence of alkaline solution saturation [36]. The biogas quantification (mL) was performed by the water displacement method.

2.5. Recovery and Reuse of Deep Eutectic Solvents

For recovering DES, water was added to the pretreatment liquor after solid–liquid separation, and later the solution was kept overnight at 4 °C to precipitate the lignin. The DES solution was then centrifuged (10,000× *g*) for 10 min to separate the precipitated lignin. After lignin removal, DES was recovered from the DES–water mixture through vacuum distillation. The residual water was evaporated by setting the rotatory vacuum evaporator (Heidolph Rotary Evaporator, Laborota 4002) at 80 °C. The recovered DES reagent and water were further utilized for the next pretreatment and lignin precipitation cycles, respectively [37,38]. The DES recovery yield was calculated by the following formula:

$$\text{Y}_{DES} \text{ recovered } (\%) = \frac{V_{DES} \text{ recovered } (\text{mL})}{V_{DES} \text{ used for pretreatment } (\text{mL})} \times 100 \tag{3}$$

All the recovered DES solutions were again utilized for pretreatment and termed in accordance with the relevant number of recovered generations; for instance, DES-1, -2, -3, -4, and -5 were recycled in first five generations, respectively [37]. The recovered DES yield, pretreatment efficiency, and extent of biogas production were determined for each regenerated DES in the same manner as described earlier.

2.6. Analytical Methods

The lignin content of *Atriplex crassifolia* and the delignification extent after DES-mediated pretreatment were determined based on standard procedures acquired from the National Renewable Energy Laboratory [39]. The cellulose content on a dry matter (DM) basis was estimated by the method reported by Gopal and Ranjhan et al. [40]. Acid detergent fibre (ADF) and neutral detergent fibre (NDF) were calculated to determine hemicellulose (NDF-ADF) content of the halophyte as reported by Van Soest and Robertson et al. [41]. COD was measured by an open-reflux method (Method 5220B). Volatile solids (VS) and total solids (TS) of both inoculum and substrate were determined gravimetrically (VS; Method 2540E, TS; Method 2540B) as described by APHA et al. [42]. Fourier-transform infrared spectroscopy (FT-IR) of DES-pretreated and raw biomass was also conducted (wave number; 4000 cm^{-1} to 500 cm^{-1}) for studying the structural alterations in pretreated substrate. The experimental setup for lab scale anaerobic digester was acquired from Admasu et al. [43] with slight modifications. The biogas production (mL) was measured by the amount of water displaced. The pH was detected via pH meter (adjusted by 1% H_2SO_4 or NaOH).

2.7. Statistical Analysis

All the provided experiments were carried out in replicates (repeated thrice). Statistical data analysis was performed via SPSS Modeler 16.00 (IBM Analytic, New York, NY, USA). The representation of all experimental outcomes was performed by MS Excel. The Y-error bars interpreted the standard deviation (±SD) of all replicates.

3. Results and Discussion

3.1. Effect of Hydrogen Bond Donors Type

Varied HBDs were mixed with ChCl (HBA), as described earlier. Among all, ChCl: LA-based DES displayed the highest delignification rate of 69.5%. The SR% of ChCl: LA i.e., 25.8% on halophyte was also relatively more significant compared to other DESs. The cellulose yield in carboxylic acid DESs varied from 43.2% to 58.4%. Carboxylic acids showed the trend of LA > LVA > AA > OA > CA as shown in Table 3. The results also inferred that among carboxylic acid HBDs, monocarboxylic acids exhibited higher delignification capacity when compared with di- or tri-carboxylic acids. Otherwise stated, the influence of the length of alkyl chain on delignification was interrelated to the extent of carboxyl groups. Consequently, monocarboxylic acids showed greater lignin solubility as they exhibited shorter alkyl chains, while the trend was certainly opposite for di- and tri-carboxylic acids [44]. The results agreed well with those obtained by Hou et al. [45] and Tan et al. [32], who reported low delignification potential of binary carboxylic acid-based DESs in pretreating rice straw and oil palm empty fruit bunch (OPEFB), respectively.

In polyol/glycol-based DESs, ChCl: 1,3-PDO showed a higher lignin removal rate of 62.3% due to strong hydrophobicity. Among diol-based DESs, longer-chain alkyl diols were more effective than shorter-chain glycols. The results were similar to those obtained by Hou et al. [46], who reported that the lignin solubility values of cholinium ILs rose with the increase in alkanoate anion chain length. As the hydrophilicity of HBDs increased, the delignification capacity decreased. It was because more hydroxyl groups in HBD caused higher lignin retention in the biomass. Moreover, extra hydroxyl (-OH) groups also brought extensive hydrogen bonding, which made DES highly viscous and stable. Hence, more energy was needed to lose substrate bonded structures to facilitate its interaction with the solute [19]. For this reason, among all polyol/glycol-based DESs, xylitol showed the lowest potential for lignin removal. The finding agreed well with that of Hou et al. [45], who demonstrated lowest delignification potential of xylitol as a HBD. Among amine/amide DESs, ethanolamine-based eutectic solvents outperformed acetamide, formamide, and urea due to their stronger basicity compared to amides. DEA displayed slightly less delignification values than MEA as the additional hydroxyl-ethyl group at DEA intensified steric hindrance in the DES, thus reducing the fractionation efficiency [47].

Table 3. Characterization of *Atriplex crassifolia* after pretreatment from various deep eutectic solvents.

Type of DESs	% Cellulose	% Hemicellulose	% Lignin	% Delignification
Carboxylic acid-based DES				
ChCl: LVA	54.6 ± 1.3	25.6 ± 0.5	6.5 ± 0.3	66.1 ± 2.1
ChCl: LA	58.4 ± 1.7	21.6 ± 0.3	5.9 ± 0.2	69.3 ± 2.4
ChCl: AA	50.3 ± 1.5	27.3 ± 0.7	7.8 ± 0.8	59.4 ± 1.8
ChCl: OA	47.5 ± 1.4	29.4 ± 0.9	9.1 ± 0.7	52.6 ± 1.6
ChCl: CA	43.2 ± 0.9	30.5 ± 1.4	9.7 ± 1.1	49.5 ± 1.5
Polyol/glycol-based DES				
ChCl: EG	49.3 ± 1.0	28.4 ± 1.1	8.2 ± 1.0	57.3 ± 0.9
ChCl: G	42.5 ± 1.2	29.5 ± 0.7	9.7 ± 0.9	49.5 ± 1.3
ChCl: X	38.1 ± 0.8	31.4 ± 1.3	11.4 ± 1.1	40.6 ± 0.7
ChCl: 1,2-PDO	50.3 ± 1.1	27.3 ± 0.8	7.2 ± 0.5	62.3 ± 2.3
ChCl: 1,3-PDO	52.2 ± 2.1	25.3 ± 0.5	7.6 ± 0.3	60.4 ± 2.2
Amine/amide-based DES				
ChCl: U	41.2 ± 0.7	29.3 ± 1.0	9.8 ± 1.0	48.9 ± 0.6
ChCl: FM	45.3 ± 0.6	26.7 ± 1.1	8.0 ± 0.6	57.8 ± 1.1
ChCl: Ac	86.4 ± 2.0	30.8 ± 1.2	11.6 ± 0.9	39.6 ± 1.2
ChCl: MEA	76.2 ± 1.4	23.7 ± 0.1	6.6 ± 0.4	65.6 ± 1.9
ChCl: DEA	78.2 ± 1.9	25.4 ± 0.6	7.4 ± 0.8	61.5 ± 1.7

Proton-catalyzed bond cleavage reactions were primary mechanisms to effectively separate lignin molecules from biomass. Therefore, acidic DESs having carboxylic acids as HBDs displayed high delignification values in pretreatment compared to amine/amide or polyol/glycol mixtures. It was because active protons from carboxylic acids facilitate the proton-catalyzed bond cleavage reactions in LCB-like glyosidic bonds, lignin–carbohydrate links, and ether bonds. Carboxylic acid DESs were also less viscous, which increased their mobility and penetration into biomass [48]. Moreover, HBDs that exhibited low pKa values significantly influenced the DES acidity as highly acidic DESs eliminated considerable lignin and hemicellulose amounts. Predominantly, the greater the HBD's polarity, the higher the acidity of the DES, eventually stimulating elevated delignification efficiencies [19]. Therefore, carboxylic acids-based eutectic mixtures showed higher lignin removal potential than all other HBD types. Oh et al. [49] and Thi and Lee [50] also reported that acidic DESs were highly efficient solvents for attaining high lignin removal ratios than hydroxyl and amine/amide-based eutectic solvents. As ChCl: LA showed best performance as a pretreatment solvent, it was selected for further optimization studies to achieve high biogas production rates.

3.2. Effect of Molar Ratio of DES Constituents

The halophyte was pretreated by ChCl: LA DES at varied molar ratios of 1:1, 1:1.5, 1:2, 1.5:1, and 2:1. The results indicated that with the increment in LA ratio, the lignin removal rate also increased. The highest delignification value of 73.4% was attained at a molar ratio of 1:2 with a cellulose retention rate of 62.4%, while ChCl: LA at a molar ratio of 2:1 was ineffective for the fractionation of halophyte as only 41.3% of lignin was eliminated, and the SR% was also lowered to 17% (Figure 1). The results agreed well with the work of Li et al. [51], which showed increased lignin and hemicellulose removal with the increase in molar ratio of LA to ChCl. In general, the DES acidity had an essential role in performing the catalytic cleavage of the linkage present between hemicellulose and lignin molecules. The more acidic the DES was, the larger the lignin removal values were. Hence, ChCl: LA

DES at 1:2 molar ratio was selected as the best DES to augment biomass digestibility under mild pretreatment conditions.

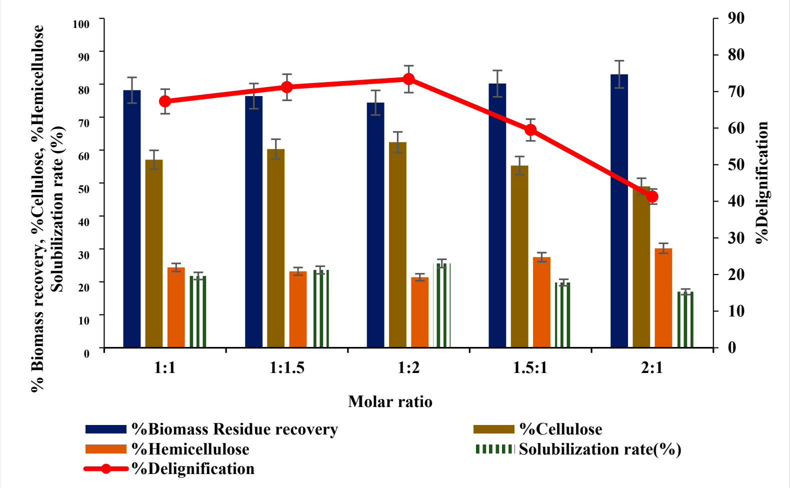


Figure 1. Effect of different molar ratios of ChCl: LA on delignification of *Atriplex crassifolia*.

3.3. Effect of Solid-to-Liquid Ratio

The amount of solvent had a significant influence on the final fractionation; therefore, the effect of various biomass loadings (1:5, 1:10, 1:15, 1:20, and 1:25) was also evaluated, as illustrated in Figure 2. When the S/L ratio increased from 1:5 (*w*/*v*) to 1:15 (*w*/*v*), the delignification values elevated to 82.4%. The hemicellulose value also lowered to 13.3%, while the cellulose content enriched up to 73.4%. In the presence of excessively low S/L ratio, DES could not thoroughly saturate the biomass sample, and hemicellulose and lignin content could not be eliminated. With the increase in S/L ratio, the cellulose values elevated, reached maximum, and started to decline with further increase. The results complied well with the findings reported by Sai and Lee [52], who pretreated empty fruit bunches with ChCl: LA DES and stated that extremely high S/L values would cause excessive biomass and cellulose degradation. The optimal S/L ratio of 1:15 was selected for further experimentation as it was suitable for disallowing biomass degradation and led to a higher cellulosic yield.

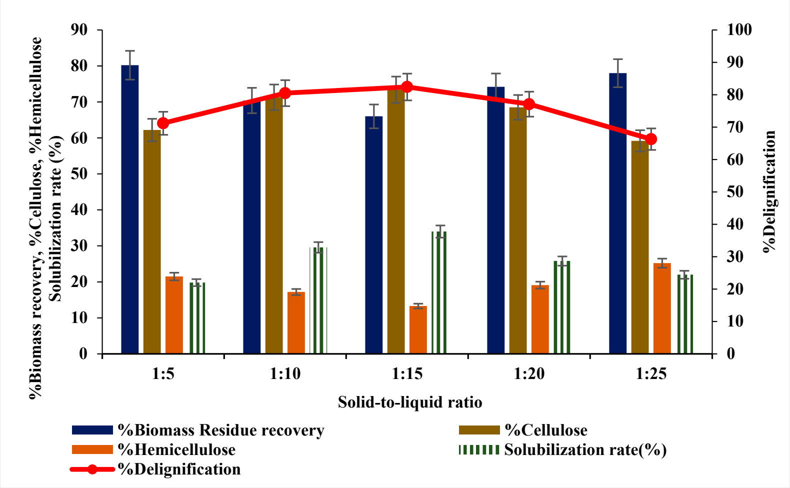


Figure 2. Effect of the S/L ratio on ChCl: LA-mediated pretreatment of *Atriplex crassifolia*.

3.4. Effect of Water Addition

Viscous nature of DES is a major restraint in achieving effective pretreatment yield as it decreases its mass transfer during pretreatment. Elevated reaction temperature may reduce the DES viscosity, but negatively affect biomass residue recovery. Another way to lessen the DES viscosity is to dilute it by adding water. To evaluate the influence of diluted ChCl: LA pretreatment on lignin dissolution, the water content varied from 5% to 25% with an increment of 5% while maintaining the reaction conditions at 120 °C for 4 h. The lignin elimination values elevated with the increase in water content, and the highest delignification was achieved at 15% water addition. The SR% was also enhanced to 38%, ensuring effective mass transfer of ChCl: LA DES on halophyte *Atriplex crassifolia* (Figure 3). The cellulose enrichment of 79.3% was also achieved at this concentration. However, with the further rise in water content, cellulosic yield and lignin extraction values started to decline. DESs are viscous solvents and their viscosity drops with the upsurge in water content. Compared to DES mixtures, water is less viscous and exhibits low density values. Therefore, the presence of water lowered the overall viscosity of ChCl: LA DES. Subsequently, a high lignin removal rate at 15% water addition might be the result of increased mass transfer of DES reagent owing to its low viscosity, which eventually enhanced the accessibility and digestibility of the halophyte [38]. These verdicts were in line with the conclusions acquired from Hou et al. [53] and Yiin et al. [54], stating increased delignification values by diluted malic acid-based DESs. According to New et al. [55], the increase in water content in DES also engendered a negative effect on lignin removal as his work validated decreased delignification rateswhen ChCl: U was momentously diluted. In the present study, the delignification values declined when the water content increased above 15%. The limiting factor, in this case, was the low concentration of ChCl: LA DES as the solvent was significantly diluted by distilled water. Soares et al. [56] also uncovered the potential of aqueous ChCl: LA solution to eliminate excessive lignin content from *Eucalyptus globulus* wood due to its hydrotropic behavior that ultimately enhanced the lignin dissolution.

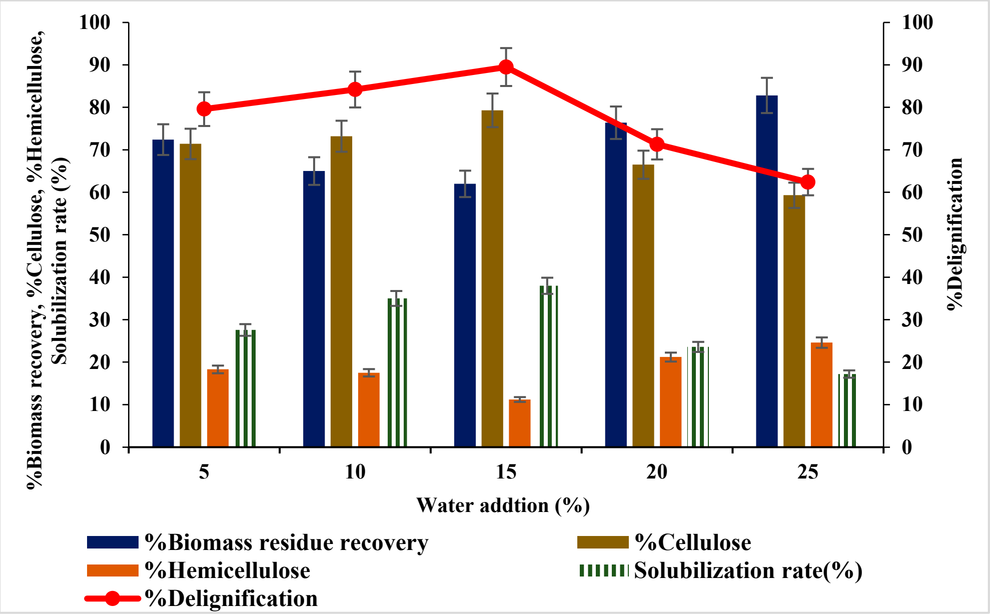


Figure 3. Effect of diluted ChCl: LA DES on delignification of *Atriplex crassifolia*.

3.5. FTIR Analysis of ChCl: LA Treated and Untreated Substrate

The FT-IR of DES-pretreated and raw substrate was performed for determining the structural variations in *A. crassifolia* after fully optimizing diluted ChCl: LA-based pretreatment. The peak intensities considerably diverged in both samples and the FT-IR spectrum presented noteworthy variations in functional groups of diluted ChCl: LA treated *A. crassifolia* in comparison to the raw biomass. ChCl: LA-treated sample showed significant lignin elimination and dissolution of lignin–cellulose bond. The peak 3415 cm^{-1} deviated to 3436 cm^{-1}, specifying free hydroxyl (-OH) groups. This occurred due to the elimination of amorphous non-cellulosic materials from the halophytic sample. An insignificant deviation at peak 2900 cm^{-1} implied the rupture in methyl and methylene groups in the cellulose molecules. The alteration in C=O stretching at peak 1715 cm^{-1} indicated the rupture of ether linkage present between hemicellulose and lignin. Furthermore, in contrast with untreated substrate, a decline in the intensities of aromatic skeletal peaks was observed at 1511 cm^{-1}, 1301 cm^{-1}, and 1243 cm^{-1}, confirming the considerable removal of lignin content [33,57] as depicted in Figure 4.

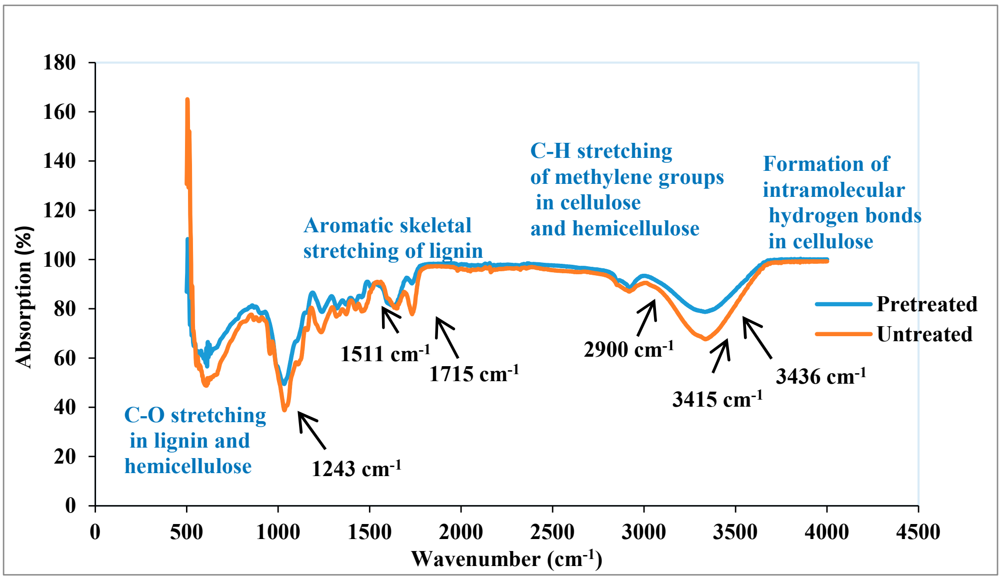
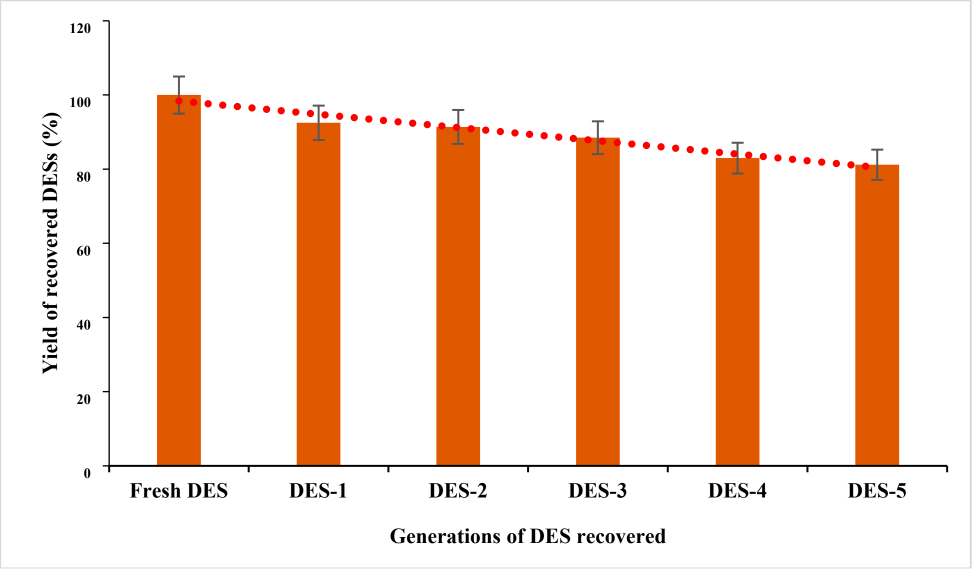


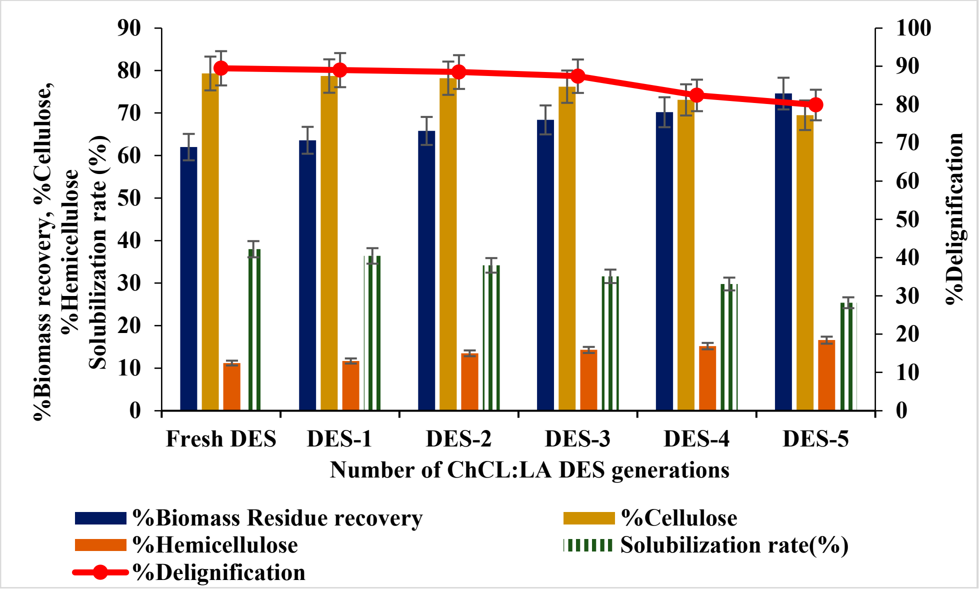
Figure 4. FT-IR spectrum of ChCl: LA pretreated and raw *Atriplex crassifolia*.

3.6. Recovery and Reuse of ChCl: LA DES

On account of the ability to dissolve fibers, freshly recovered DES and DES-5 showed varied recovery potentials. The first three generations of DES provided recovery yields of 92.5%, 91.4%, and 88.5%, respectively (Figure 5a). The results directed that ≥80% of DES could be effectively recycled for up to five generations, suggesting high recovery rates. However, the viscosity of the ChCl: LA decreased with each cycle because of the existence of residual moisture. Recovered ChCl: LA DES was again reutilized for pretreatment; ≥85% lignin was eliminated and the SR% of ChCl: LA DES on *Atriplex crassifolia* was also recorded as ≥30.0% after three pretreatment cycles, as shown in Figure 5b. All the DESs provided high cellulose retention values as well. The slight alterations in SR% and pretreatment capacities confirmed the utilization of regenerated DES as effective solvent to make the pretreatment process profitable and cost-effective. Wang et al. [37] also reported DES recovery via vacuum distillation and concluded that recovered DES maintained the pretreatment efficiency.



(**a**)

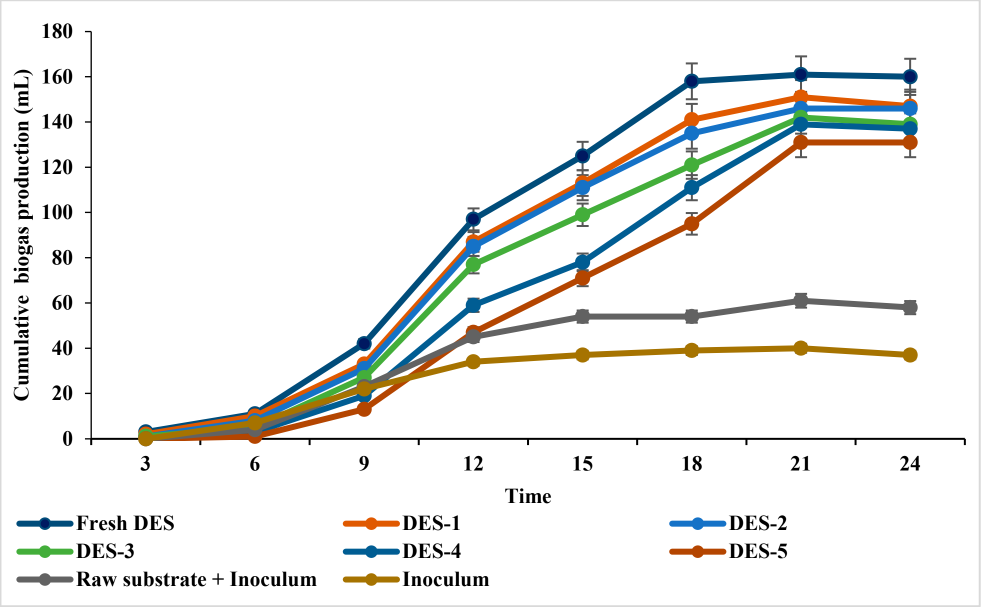


(**b**)

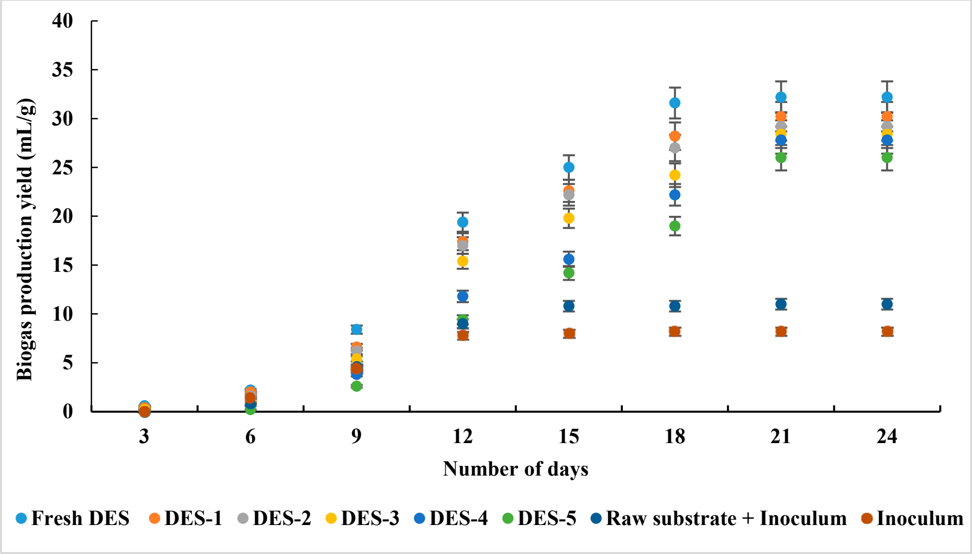
Figure 5. (**a**) Recovery yield (%) of ChCl: LA DES up to five pretreatment cycles. (**b**) Characterization of *Atriplex crassifolia* after pretreating the substrate from regenerated ChCl: LA DESs.

3.7. Biogas Potential of ChCl: LA Pretreated Substrate

All the ChCl: LA (fresh + recovered) pretreated halophyte samples were subjected to biogas production through AD process. The gradual rise in biogas production was observed until 9 days of incubation, and the peak values were obtained after 21 days. The fresh DES and DES-1 pretreated biomass samples provided maximum biogas production values of 161 mL and 151 mL with biogas production rates of 0.32 mL/h and 0.29 mL/h, respectively (Figure 6a). The inoculum alone produced only 40 mL of biogas while the raw substrate generated an extremely meager biogas yield of 12.2 mL/g, as illustrated in Figure 6b, owing to its highly recalcitrant structure towards anaerobes. The highest cumulative biogas production yield was noted from fresh ChCl: LA pretreated *Atriplex crassifolia*, i.e., 32.2 mL/g. Moreover, all DES-pretreated substrates provided production yields of ≥26 mL/g. These results indicated the efficacy of ChCl: LA pretreatment in begetting high biogas production values as ChCl: LA pretreatment lessened the biomass recalcitrance by breaking LCB complex structure and eliminating high lignin amounts. Yu et al. [58] also informed increased biogas yields after pretreating substrate with DESs prior to anaerobic fermentation.



(**a**)



(**b**)

Figure 6. (**a**) Cumulative biogas production (mL) from fresh and recycled ChCl: LA DES-pretreated halophyte samples. (**b**) Biogas production yield (mL/g) of fresh and recovered ChCl: LA DES-pretreated *Atriplex crassifolia* samples.

4. Conclusions

Among all types of DES, carboxylic acid-based DESs showed relatively better performance due to their acidic and less viscous nature. Among them, ChCl: LA displayed highest delignification values. Under optimized conditions, approximately 89.5% of lignin elimination was achieved, and cellulose content was enriched to 79.3%. Furthermore, the DES also displayed high recovery potential as ≥88% of ChCl: LA was recovered after three cycles of pretreatment, while the delignification potential of the recovered DES was also retained. ChCl: LA-based delignification of halophyte *Atriplex crassifolia* proved highly effective in increasing biogas production yields as well. All the DES-pretreated substrates yielded high cumulative biogas production values in comparison to the raw substrate because biogas yield increased by 37.9% for fresh ChCl: LA treated substrate due to its increased content of cellulosic sugars, eventually suggesting the efficacy of ChCl: LA based pretreatment for attaining enhanced biogas production values.

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