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Pretreatment of Rice Straw using Deep Eutectic Solvent and Saccharification of Pretreated Residue by Crude Cellulase Enzyme

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A B S T R A C T

The present study demonstrated the pretreatment of rice straw using deep eutectic solvent (DES), choline chloride:urea and its comparison with acid and alkaline pretreatment. At a solid loading of 10%, choline chloride (ChCl):urea (1:2) was very effective in lignin extraction from rice straw at 45 °C. It is showed that nearly 40% of lignin was separated in a single step. However, enzymatic hydrolysis of pretreated rice straw with crude cellulase enzyme produced from *C.cinerea*RM-1 showed the reducing sugar yield of 385±8 mg/g with a saccharification efficiency of 31.5±2.5 % in 48 h at 10 % solids loading.

Introduction

With the increasing world's population, energy consumption has increased many folds over the last century. Fossil fuel has been the major source of energy that formed from fossils over millions of years within the earth. Fossil fuels are considered as nonrenewable and depleting continuously with a predicted estimation from the 25 billion barrels to approximately 5 billion barrels till 2050 (Campbell and Laherrere, 1998). On the other hand, a large amount of agro-industrial wastes and crop residues are generated all over the globe and creates many environmental problems if not utilized properly. Mainly three categories of lignocellulosic biomass; woody biomass, agricultural residues, and energy crops are produced. Among the agricultural residues, rice straw is generated in abundance (667.6 million tons) in Asian countries every year and would serve as a great potential feedstock for biofuel production. After harvesting the rice, large amount of rice straw residues i.e., 40-50 cm of loose stubble and 50-60 cm of standing straw are usually left over the field and it is still burnt in the fields causing severe environmental and health issues (Liu *et al.*, 2011; Binod*et al.*, 2010). It is estimated that approximately 205 billion liters of bioethanol can potentially be produced annually by using rice straw (Belal, 2013). Rice straw consists of mainly cellulose (32–47 %), hemicellulose (19–27 %), and lignin (5–24 %).

Therefore, considering the above problems, efforts have been concentrated in exploring the alternative energy sources such as biofuels (biodiesel and bioethanol) by utilizing lignocellulosic biomass. The most important biomass processing challenge is the pretreatment for production of biofuels and it should be simple, environmental friendly and economically feasible (Ravindran *et al.,* 2018). Pretreatment is required for loosen the complex structure of lignocellulosic biomass. Moreover, lignocellulosic biomass requires suitable pretreatment method for solubilization, separation and conversion of its cellulose and hemicellulose components into fermentable sugars (Sun *et al.,* 2016). In addition, a successful pretreatment process increased the yields of hydrolysis, reduces the product degradation and reduces the formation of inhibitory byproducts. A list of pretreatment methods have been developed for different lignocellulosic biomass over the past few decades but till now, there is no single pretreatment method available that could suit to all types of biomass. The most commonly used fundamental types of pretreatment technologies for lignocellulosic biomass include usage of common organic solvents. These technologies have many disadvantages such as low yield, high processing cost and create health and environmental issues (Anwar *et al.,* 2014; Alvira *et al.,* 2010). Therefore, a continuous search is constantly on-going towards more novel, cheaper and efficient green technologies over the past Decade, for solving these challenges (Liu *et al.,* 2014; Dai *et al.,* 2013a; Capolupo and Faraco, 2016). Recently, the “Green Chemistry” concept has emerged as a possible solution to the challenge of using nontoxic and environmental friendly materials for efficient utilization of lignocellulosic biomass. Ionic liquids (ILs) and Deep Eutatic Solvents (DES) are currently gaining importance as an alternative to conventional pretreatment technologies of biomass. ILs possesses attractive properties due to their higher thermal and chemical stability, negligible vapor pressure and non-flammability nature (Wahlström and Suurnäkki, 2015; Wu *et al.,* 2014). However, the use of ILs in the pretreatment is environmentally friendly but it is a cost-intensive process; this limits its use in biorefinery (Hou *et al.,* 2013). Recently, similar to the ILs, DES attracts the attention as a potential green and designer solvent with several chemical and biological applications (Dai *et al.,* 2013a, b; Choi *et al.,* 2011; Huang *et al.,* 2013). According to Paiva *et al.,* (2014), the yield of the DES preparation process may be considered 100 % as no chemical reaction takes place in its production and total waste production is zero.DES is a mixture of a hydrogen-bond acceptor and donor; in most cases, a quaternary ammonium halide salt act as a hydrogen-bond acceptor and amino acid, urea, amine, carboxylic acid or carbohydrate etc. act as a hydrogen-bond donor (Francisco *et al.,* 2012; Zhang *et al.,* 2012). DES has been used extensively in the recent past years in the pretreatment of lignocellulosic biomass for achieving high hydrolysis and fermentation yields. In the current investigation, the pretreatment of rice straw through acid, alkali and DES (ChCl/Urea) and enzymatic hydrolysis for fermentable sugar production were reported.

Materials and Methods

Microorganism and enzyme production

The microorganism used in this study for cellulase enzyme production, was isolated from decomposing wood samples and identified as *C. cinerea*RM-1 NFCCI-3086 by

National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India. Growth conditions of *C. cinerea* RM-1 for cellulase enzyme production were set as described by Poonam (2015).

Raw material and chemicals

Rice straw was procured from the local rice field and washed 3-4 times in distilled water, dried and powdered using laboratory grinder, dried again and stored in polythene begs. This rice straw was used as lignocellulosic biomass for pretreatment studies. Carboxymethyl cellulose (CMC), birchwoodxylan, glucose, xylose, and arabinose were purchased from Sigma. All other chemicals and reagents were of analytical grade.

DES preparation and rice straw pretreatment

DES reagent was prepared in capped bottle by mixing ChCl/Urea at a molar ratio of 2:1 and incubated in incubation shaker at 100 rpm and 70 °C until a clear liquid solution was obtained (Dai *et al.*, 2013a, Francisco *et al.*, 2013b). Various pretreatment experiments including, 2% H_2SO_4, 2% NaOH and DES solvent (ChCl/Urea) were carried out in screw capped conical flasks at 10% solid loadings unless mentioned. Briefly, 10g of rice straw was mixed with 2% H_2SO_4, 2% NaOH and DES solvent in a solid/liquid ratio of 1:10 separately, and subjected to steam treatment at 121 °C and 15 psi pressure for 30 min. Following this, the samples were washed with distilled water for three times. Control and pretreated rice straw samples were analyzed for cellulose, hemicellulose and lignin content.

Lignin separation and recovery of DES

DES was separated from lignin by adding distilled water until the turbid solution obtained. The solution was incubated at 5 °C for 3h. After incubation, the mixture was centrifuged at 10000×g for 15 min, and the pellet was washed with distilled water three times and air-dried to obtain lignin powder. DES was recovered from water solution by incubation in vacuum rotary evaporator at 60 °C. The recovered DES and pure water may be reused in the next biomass pretreatment and lignin precipitation cycle.

Solubility evaluation test of cellulose, xylan, and lignin in DES

Pure cellulose, xylan, and lignin were used for Specific solubility test in DES reagents. All three components were dissolved in 10 ml ChCl/Urea at a molar ratio of 2:1 and 3:1 separately in 10% concentration and incubated at 60 °C for 12h. All samples were filtered through fiber glass filters and dried at 60 °C. The solubility (%) of the NADES reagent was determined by calculating the weight of dried components.

Enzymatic hydrolysis of pretreated rice straw

Enzymatic scarification experiments were carried out at 10% solid loading and cellulase enzyme dose of 10 IU per g in a total reaction volume of 10-ml with citrate buffer in 50-ml sealed bottles. The prepared mixtures were then incubated at 45 °C and15 rpm for 24 and 48h.

The reducing sugars were measured by DNS method (Miller, 1959).The Control experiments were carried out separately either by avoiding cellulose enzyme or the pretreated substrate.

Analytical methods

The cellulase activity (CMCase) was determined using CMC as the substrate following the protocol published by Mandels

M. (1975). The compositional analysis of raw and pretreated straw was done for determining the cellulose, hemicellulose, and lignin content (Sluiter *et al.,* 2008).

Results and Discussion

Pretreatment of rice straw

Table 1 represents the effects of different solvents i.e., dilute acid, mild alkali and DES on delignification of rice straw. Acid treatment of rice straw has very high detrimental effect on hemicellulose content and it decreased from 24.6% to 1.2% while the cellulose and lignin content are not affected to much extent. After alkali pretreatment, the overall cellulose content increased from 35.5% to 43.8% in pretreated rice straw while hemicellulose and lignin content decreased from 24.6% to 18.5% and from 14.5% to 11.56%, respectively. DES pretreatment of rice straw resulted in decrease in lignin content from 14.5% to 8.7%, while cellulose content was increased from 35.5% to 45.8% and hemicellulose content decreased from 24.6% to 21.5%. Thus, it is observed that lignin content was decreased about 40% without affecting the hemicellulose very much after DES pretreatment; thus the overall amount of cellulose was enhanced. Generally, acid pretreated biomass showed significant loss in hemicellulose content (Hendriks and Zeeman,2009). While, DES pretreatment showed no severe effect on hemicellulose and cellulose content of biomass. This trend may found because choline chloride stabilizes the cellulose by making hydrogen bond with it; thus, dissolution of cellulose and hemicellulose is inhibited (Abbott *et al.*, 2006). The cellulose content enhancement after pretreatment may be due to the alteration in biomass structure and crystalline cellulose which might have increased the overall cellulose availability (Kumar and Parikh, 2015). Kandanelli *et al.*, (2018) reported the removal of about 50% lignin from lignocellulosic biomass by using *n*-butanol assisted DES (ChCl: OA) at solid loading of 15 % (*w/v*)at 120°C for 60 min. Kumar *et al.*, (2016) reported that approximately 58% lignin was removed from rice straw after pretreatment with NADES.

Table 2 represents the solubilization studies of pure cellulose, hemicellulose and lignin in DES solution. The pure cellulose and hemicellulose showed no solubilization in DES and remained untouched while lignin showed high solubility (78%); this proves the specificity of DES towards lignin solubilization. While, the % solubility of lignin when present in biomass was found to be comparatively lower than the pure lignin in DES. This could be due to the cross-linking architecture of biomass and strong binding of lignin to cellulose and hemicellulose which poses the lignin to extract. These finding were in close agreement with Kroon *et al.*, (2014), who reported that NADES showed very high selectivity for separation of lignin from a mixture of lignin and cellulose and that lignin solubility values varied with different combinations of NADES reagents.

Saccharification studies

Table 3 represents the enzymatic hydrolysis results of rice straw pretreated with DES at 10% solids loading and 10 IU/g of crude cellulase enzyme produced by *C. cinerea*. The hydrolysis experiments were performed at 45 °C and15 rpm.

The reducing sugars were measured after 24 and 48 h. The results showed the maximum saccharification efficiency of 31.5±2.5 % with reducing sugar yields of 385±8 mg/g after 48 h. Our results were in line with Kumar *et al.*, (2015) who reported that enzymatic hydrolysis of rice straw showed reducing sugars yield of 333±11 mg g^{-1} and saccharification efficiency of 36.0±3.2 % in 24 h at 10 % solids loading.

Table.1 Compositional analysis of raw and pretreated rice straw

S. No.	Treatment	Cellulose%	Hemicellulose%	Lignin%
1.	Untreated rice straw	35.5±1.2	24.6±1.2	14.5±1.1
2.	Acid treated rice straw	34.2±2.2	1.2±0.8	13.6±0.9
3.	Alkali treated rice straw	43.8±3.4	18.52±1.1	11.56±0.9
4.	DES treated rice straw	45.8±2.7	21.5±1.7	8.7±0.4

Table.2 Solubility analysis of pure cellulose, xylan, and lignin in (ChCl/Urea)

DES reagent	% Solubility (10% (w/v) substrate)		
	Cellulose	**Xylan**	**Lignin**
ChCl/Urea (2:1)	0.0	0.0	75.1±6
ChCl/Urea (3:1)	0.0	0.0	73.5±5.5

Table.3 Enzymatic saccharification of DES pretreated rice straw biomass

Time of Saccharification	Reducing sugars (mg/g)	Saccharification efficiency (%)
24h	282±10	20±1.2
48h	385±8	31.5±2.5

Here, we have revealed a green pretreatment process for biomass using deep eutectic solvent (ChCl/Urea) as a potential extraction media for delignification from rice straw and compared with acid and alkali treatment.

A high-quality lignin was extracted from biomass and was separated from cellulose and hemicellulose in a single step by simple precipitation method.

The Results showed that approximately 40% lignin were removed from rice straw using DES treatment. Following delignification, the residual rice straw was subjected to enzymatic hydrolysis and a plenty amount of fermentable sugars (385±8 mg/g) were produced with a saccharification efficiency of 36.0±3.2 %.After pretreatment with DES, degradation products such as furfural and hydroxyl methyl furfural are not formed; therefore detoxification step is not required which is a key step after acid pretreatment.

Therefore, DES pretreatment decreases the overall cost of process by reducing the post-process steps and these green solvents may absolutely be used as the next-generation reagents for sustainable development.

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