

Lignocellulosic Agroresidual Waste Materials to Liquid Biofuels – A Green Technology Process

Aesha Patel, Ekta Shah, Shaishav Sharma, Gaurav Dixit, Adepu Kiran Kumar*

Bioconversion Technology Division, Sardar Patel Renewable Energy Research Institute, Post box No. 2, Vallabh Vidyanagar – 388120, Anand, Gujarat, India

Email: kiranbio@gmail.com,

Abstract

Lignocellulosic biomass is the most abundant biomass available in the world. The recalcitrant cell wall of lignocellulosic biomass makes pretreatment a necessary action. Efficient pretreatment results in production of maximum sugars from enzymatic hydrolysis and minimize their loss. Various solvents have been developed for the pretreatment of lignocellulosic biomass viz. ionic liquids, DES and natural deep eutectic solvents (NADES). Recently, research on NADES is considered to be a new subject in chemistry and is rapidly gaining interest. Besides environmental friendly, employing NADES in advancement of the existing biorefinery seems to be a promising approach for clean fractionation or pretreatment of the lignocellulosic biomass residues. Production of cellulosic ethanol was carried out by lactic acid and choline chloride (LA-CC) mixture based NADES pretreated ricestraw through separate saccharification and fermentation process. Also, comprehensive evaluation on solvent recovery and its reusability, extraction of high purity lignin and xylan, and reducing sugars production were performed. Pretreatment at 5% and 10% solid loadings using LA-CC mixture at molar ratio of 9:1 and subsequent enzymatic saccharification at 10% and 25% biomass loadings produced maximum glucose yields of 38.1 ± 1.2 g/L and 90.2 ± 2.3 g/L with glucan conversion efficiency of 40.3 % and 31.4 %, respectively. Further fermentation of produced reducing sugars with *Clavispora NRRL Y-50464* at 9 % glucose concentration produced maximum ethanol yield of 37.1 g/L in 36 h with 80.8% conversion efficiency. Besides, NADES pretreatment of ricestraw solubilised 72-75 % of biomass bound lignin from which nearly 86 – 90 % lignin was recovered from the liquid spent wash extract. Moreover, nearly 99% of the treated NADES reagent was recovered and reused in three consecutive biomass pretreatment cycles with no loss in both pretreatment efficiency and production of reducing sugar yields. This clearly indicates a promising integrated process of green technology for cellulosic ethanol production from ricestraw.

Keywords

NADES; recovery and reuse; biorefinery; lignin; cellulosic ethanol

1. Introduction

Recently, lignocellulosic biomass, which mainly consists of cellulose, hemicellulose, and lignin has received increased attention as a promising renewable and carbon-neutral feedstock for production of biofuels and chemicals due to its high availability, and low cost [1]. For example, cellulose and hemicellulose can be hydrolyzed to monosaccharides, followed by biotransformation into various biofuels such as ethanol, H₂, and biodiesel [2].

Among the recently developed green solvents, choline chloride and lactic acid based NADES mixture is one of the most extensively studied bionic reagents due to their potential applications [3,4]. Since NADES possess drastically lower melting temperatures, chemical reactions can be performed with these reagents at or near ambient conditions, especially in extraction of thermolabile compounds from biological and chemical sources [5]. Unlike conventional dilute acids, NADES pretreatment does not generate inhibitory by-products such as acetic acid, furfurals and HMF that hinder enzymatic catalytic activity and hence no additional detoxification step was required [6,7]. Although, molecular understanding of several NADES reagents are still in nascent stage, comprehensive evaluation of the physico-chemical properties of choline chloride + lactic acid based NADES mixtures were studied to a greater extent [8].

Along with the clean and green nature, employing NADES in advancement of the existing biorefinery seems to be a promising approach for clean fractionation or pretreatment of the lignocellulosic biomass residues. This new process valorises the abundantly available biomass or agro-residual waste materials into a variety of materials, biochemicals and biofuels, respectively. Although several conventional

methods including ionic liquids have been reviewed for the pretreatment of lignocellulosic biomass [9,10], but existing pretreatment methods still lack in obtaining high yields and purity of the desired products. In addition, lignocellulosic biomass pretreated with harsh conventional organic solvents undergoes non-specific degradation and generates undesirable by-products that are not suitable for subsequent processes.

The current study evaluates different integrated processes for rice straw delignification, recovery of high purity lignin and xylan, enzymatic hydrolysis and production of cellulosic ethanol using CC-LA (9:1) based NADES reagent. Besides, comprehensive evaluation on recovery of the treated NADES reagent and its reusability efficiency for consecutive biomass pretreatment cycles was detailed.

2. Materials and Methods

2.1. Raw Materials

Rice straw was selected as the lignocellulosic biomass residue for pretreatment and enzymatic hydrolysis. Experiments related to the preparation of biomass for pretreatment, synthesis of NADES reagents, and *Clavispora* NRRL Y-50464 yeast culture media composition and growth conditions were reported earlier by us [11,12]. Enzymatic hydrolysis was performed using Cellic Ctec2, a kind gift from Novozymes Inc, USA. *Clavispora* NRRL Y-50464 was a kind gift from USDA-ARS, USA. All the chemicals used in biomass pretreatment, enzymatic hydrolysis and yeast fermentation were of analytical grade quality and are of highest purity, except for HPLC studies, where HPLC grade chemicals were used.

2.2. NADES pretreatment and enzymatic hydrolysis

Four different conditions of biomass pretreatment and enzymatic hydrolysis were tested for process evaluation of cellulosic ethanol production. The tested conditions were Set-I: pretreatment (Pt) at 5% solids loading (SL) and enzymatic hydrolysis (EH) at 10% SL; Set-II: Pt at 5% SL and EH at 25% SL; Set-III: Pt at 10% SL and EH at 10% SL; and Set-IV: Pt at 10% SL and EH at 25% SL, respectively. All the biomass pretreatments were carried out using lactic acid + choline chloride (LA-CC) at a molar ratio of 9:1 at 1 atm pressure and 121°C for 15 min. The pretreated biomass was then washed repeatedly with distilled water until the pretreatment solvent was completely removed from the biomass and no detectable solvent

was found in the flow through which was confirmed by HPLC analysis [13]. Cellic ctec2 was used for conversion of pretreated biomass to reducing sugars. Enzymatic saccharification was performed at an enzyme loading of 9 FPU per g biomass which corresponds to 62 mg/g biomass or 100 mg/g glucan. Optimum conditions of temperature (50°C) and pH (pH 5.2) as determined for Novozymes Cellic ctec2 were maintained during the enzymatic hydrolysis. Samples were withdrawn at regular time intervals and reducing sugars concentration was measured by standard DNSA method [14] and simultaneously the glucose and xylose contents were quantified using HPLC [13,11]. Alongside, enzymatic hydrolysis of untreated and mild-alkali pretreated rice straw was carried out under similar test conditions and compared with the NADES pretreated rice straw.

2.3. Cellulosic ethanol production

Ethanol was produced from reducing sugars in submerged fermentation process using *Clavispora* NRRL Y-50464. Except 6% (w/v) inoculum and 10% glucose, all other fermentation conditions were maintained similar to our earlier studies [11]. Control experiments were carried out using 9 % pure dextrose. Samples were withdrawn at regular time intervals and consumption of glucose and production of ethanol was analysed using HPLC.

2.4. Lignin extraction and recovery

The pretreated biomass was washed with water and the liquid fractions obtained were collected, pooled and incubated at room temperature for lignin precipitation. The lignin precipitate was separated from the solvent mixture using membrane filtration method (0.9µm glass fiber filters). The pellet was then washed thoroughly with water to remove residual NADES reagent and further dried at 55°C until the moisture content reached < 1 %.

2.5. Recovery and reuse of NADES reagent

After lignin separation, NADES reagent was separated from the residual liquid fraction using rotary vacuum evaporation at 60°C. Both the recovered NADES reagent and water were reused for next cycle of biomass pretreatment and lignin precipitation respectively. The water content in the recovered NADES reagent was measured by automatic Karl Fisher Titrator (KAFI+) (LabIndia, India). The recovery percentage of NADES reagent was calculated based on the quantification of choline chloride and lactic acid in the recovered NADES reagent using HPLC. Simultaneously, the recovered

NADES reagent after lignin separation were pooled with the liquid fractions of fermentation process and the process was evaluated for an integrated technology for biomass pretreatment, lignin extraction and recovery, and solvent recovery and reuse.

2.6. Recovery of xylan

The xylan fractions obtained from the biomass pretreatment was precipitated using anti-solvent phenomenon following the protocol described by Nakamura *et al.*, [15]. Acetonitrile was added to the liquid extract of NADES mixture in 1:10 ratio and vortexed for 30 min at 1400 rpm incubated at room temperature for 30 min to 1 h for xylan precipitation. The precipitate was separated by

2.7. Analytical methods

Delignification efficiency was estimated based on the amount of lignin extracted into the NADES solution. Lignin recovery was calculated using the total dry weight of lignin powder per gram of biomass and represented in gm% of lignin.

A graphical representation of each individual process steps involved in biomass pretreatment, enzymatic hydrolysis and ethanol fermentation combined with down-stream processing for the solvent recovery and reuse, extraction and recovery of value added products viz. lignin and xylan are detailed in Fig. 1.

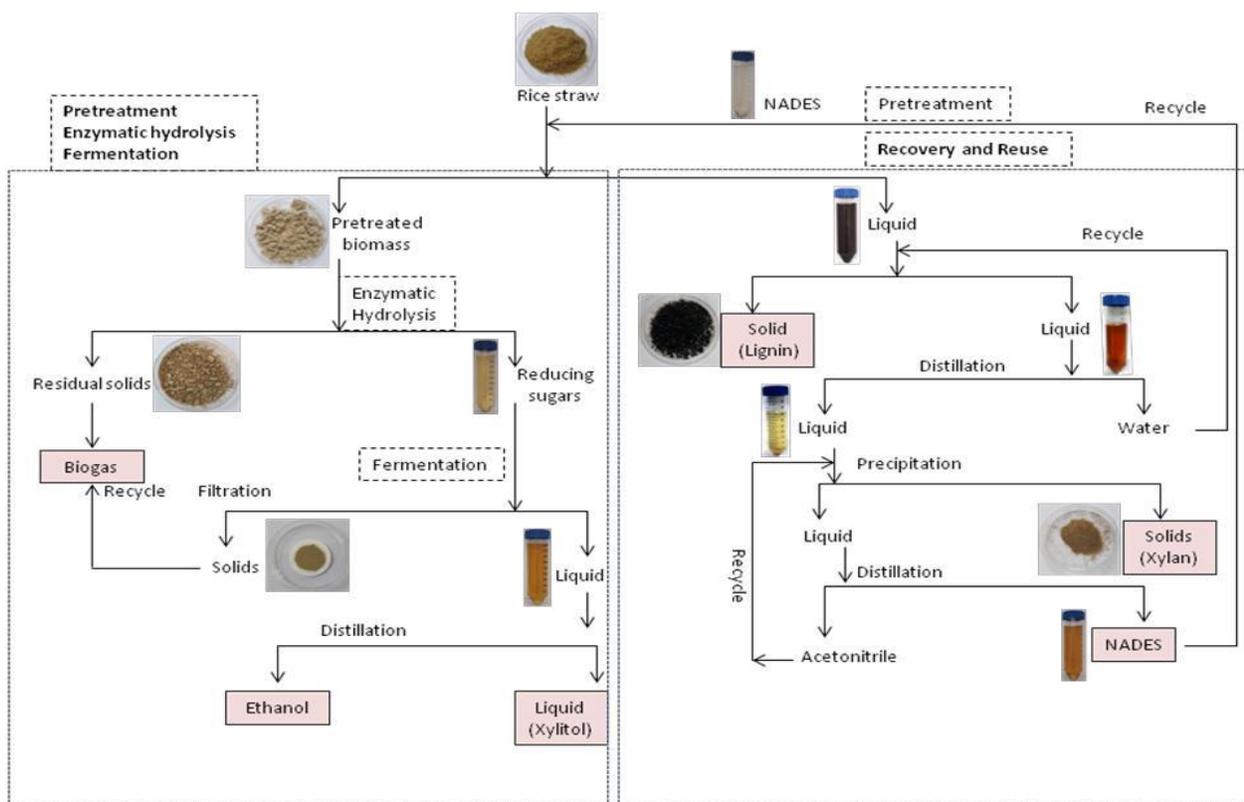


Figure 1. Graphical representation of individual steps of integrated biorefinery process for rice straw pretreatment, bioethanol production, recovery and reuse of NADES reagent

centrifugation at 1500 rpm for 30 min and was washed twice with acetonitrile and solvent was evaporated. The temperature maintained during recovery process of water and acetonitrile was 40°C and 60°C, respectively. Quantification of recovered solvents (%) was performed using volumetric methods.

3. Results

3.1. NADES deconstruction of rice straw

NADES pretreatment of ricestraw at 5 % (Set-I and Set-II) and at 10 % of solids loading (Set III and Set-IV) resulted in biomass losses of 35.66% and 38.24%, respectively. The residual glucan, xylan, lignin, ash and silica contents in the NADES pretreated biomass of Set-I was 62.13%, 9.9%, 4.3%, 10.2% and 9.1%; Set-II was 63.10%, 8.2%, 4.1%, 10.42% and 8.9%; Set-III was 68.16%, 5.6%, 3.9%, 10.16% and 9.6%; and Set-IV was 68.45%, 5.8%, 3.5%, 10.37% and 9.4%, respectively (Table 1). Compared to untreated RS (40±1.8% cellulose, 28±2.0% hemicelluloses and 10±0.9% lignin), NADES pretreatment resulted in 55-70% more cellulose availability for enzymatic conversion to reducing sugars.

3.2. Lignin and xylan recovery

Due to high lignin insolubility in water, lignin started to precipitate as soon as the washed liquid fractions (3-7-fold water content) were pooled and complete precipitation of lignin at the bottom of the container was observed within 1 h. The primary reason behind lignin precipitation with addition of water was due to the change in the physico-chemical nature of the reaction mixture which favoured lignin insolubility [16]. The % lignin in the NADES pretreated liquid extract was 62.2% (Set-I), 63.5% (Set-II), 71.9% (Set-III) and 72.3% (Set-IV), respectively (Table 1). Maximum lignin solubility of 72% from 10% SL pretreated biomass was achieved. Nearly, 86% of the solubilised lignin obtained was recovered (on dry weight basis) in powdered form. Addition of acetonitrile to the lignin-free NADES extract resulted in >99% precipitation of soluble xylan fraction.

Table 1. NADES pretreatment of rice straw under different process conditions

3.3. Recovery of NADES reagent

Pretreatment process	Set – I	Set – II	Set – III	Set – IV
Solids loading (%)	5.0	5.0	10	10
Biomass (kg)	1.0	1.0	1.0	1.0
NADES content (kg)	15.47	15.47	7.33	7.33
Biomass recovery (%)	65.36	65.99	62.11	62.85
Glucan (%)	62.13	63.10	68.16	68.45
Xylan (%)	9.9	8.2	5.6	5.8
Lignin (%)	4.3	4.1	3.9	3.5
Ash (%)	10.21	10.42	10.16	10.37
Silica (%)	9.1	8.9	9.6	9.4
Lignin extracted (%)	62.2	63.5	71.9	72.3

Nearly 99% of the NADES reagent was recovered from the xylan-free liquid extract. The moisture content in the recovered NADES reagent was measured and found to be 1±0.3%. Since, no significant losses were ascertained from the NADES recovery process, the solvent was reused consecutively in three cycles of biomass pretreatment without additional solvent. No significant difference in biomass recovery, solubility efficiency of cellulose, hemicellulose and lignin was observed after each cycle of pretreatment with the recovered NADES reagent (Table 2)

3.4. Enzymatic saccharification of NADES pretreated RS

Saccharification efficiency was found to increase with increase in the solids loading during enzymatic hydrolysis *i.e.* Set-II and Set-IV produced more sugars than Set-I and Set-III processes (Table 3). The reducing sugars were increased from 69.8 g/L (70.8% saccharification efficiency) to a maximum of 170.7 g/L (51.9% saccharification efficiency) when the solids loading were increased from 10% to 25%, respectively (Fig. 2). No significant difference in production of reducing sugars was observed when 5% and 10% pretreatments were used in enzymatic hydrolysis. However, at similar biomass loading in enzymatic hydrolysis, mild-alkali pretreated rice straw from 10% pretreated (Set-II and Set-IV) material produced 171.7 g/L and 169.3 g/L reducing sugars which was nearly 2-fold higher compared to 5% pretreated (Set-I and Set-III) material (69.8 g/L and 65.5 g/L reducing sugars). In both the conditions, reducing sugars produced with NADES treatment were higher than untreated rice straw.

3.5. Cellulosic ethanol production

Fermentation of reducing sugars at 9% glucose concentration with 6% (*w/v*) *Clavispora* NRRL Y-50464 cells yielded maximum ethanol production of 36.7 g/L within 36 h with a conversion efficiency of 79.9% (Fig. 3). Control experiments with pure dextrose fermentation at 9 % (*w/v*) produced maximum ethanol (30.8 g/L) within 5 h, and nearly complete consumption of glucose was observed. Addition of 6% (*w/v*) of Y-50464 inoculum radically reduced the period for pure glucose fermentation. At similar initial glucose concentration, fermentation of untreated, mild-alkali pretreated RS produced maximum ethanol yields of 31.6 g/L in 27 h and 33.2 g/L in 25 h, respectively. Thereafter, marginal

decrease in ethanol production was observed with the untreated and mild-alkali treated RS sugars.

Table 2. Recovery of NADES reagent, lignin and water from different cycles of biomass pretreatment

Solids Loading	No. of cycles	NADES (kg)			Lignin (g %)			Water (L)		
		Initial	Final	% recovery	Initial	Final	% recovery	Initial	Final	% recovery
5%	1	1.55	1.53	99	11	6.6	60	12.94	12.79	98.8
	2	1.53	1.52	99	11	6.8	62	10.82	10.68	98.8
	3	1.52	1.50	99	11	6.4	58	9.69	9.56	98.8
10%	1	0.73	0.73	99	11	7.0	63	5.97	5.90	98.8
	2	0.73	0.72	99	11	6.9	63	5.23	5.16	98.8
	3	0.72	0.71	99	11	7.0	63	4.86	4.80	98.8

Table 3. Enzymatic hydrolysis of NADES pretreated rice straw in different process conditions

Saccharification process	Set – I	Set – II	Set – III	Set – IV
Solids loading (%)	10	25	10	25
Biomass (g)	50	125	50	125
Cellic ctec2 (g)	3.1	7.75	3.1	7.75
Sugar yield (g/L)	69.8	171.7	65.5	169.3
Glucose (%)	37.2	92.1	38.6	90.3
Xylose (%)	13.1	30.0	13.2	37.0
Saccharification efficiency (%)	70.8	51.9	66.4	51.7

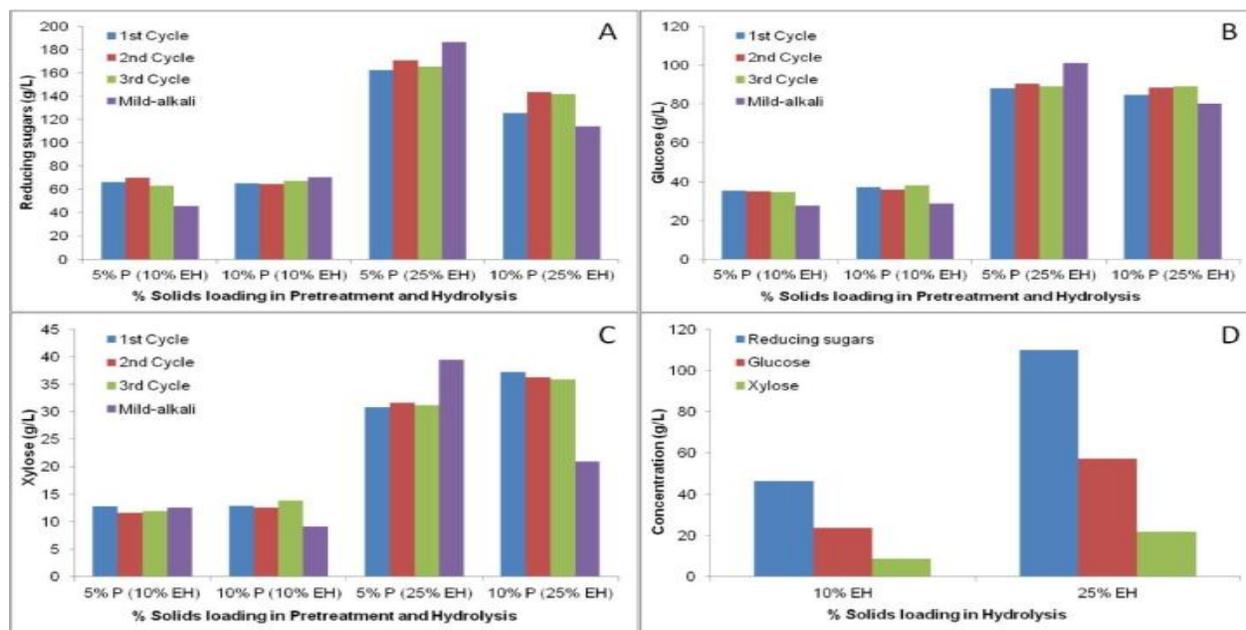


Figure 2. Enzymatic hydrolysis from pretreated rice straw. The figure clearly depicts the concentration (g/L) of reducing sugars (A), glucose (B), xylose (C) from NADES and mild-alkali treated rice straw. Concentration of reducing sugars, glucose and xylose from untreated biomass is shown in D. NADES reagent was recovered, evaluated for three consecutive pretreatment cycles (1st, 2nd and 3rd cycles) and compared with mild-alkali and untreated biomass residue

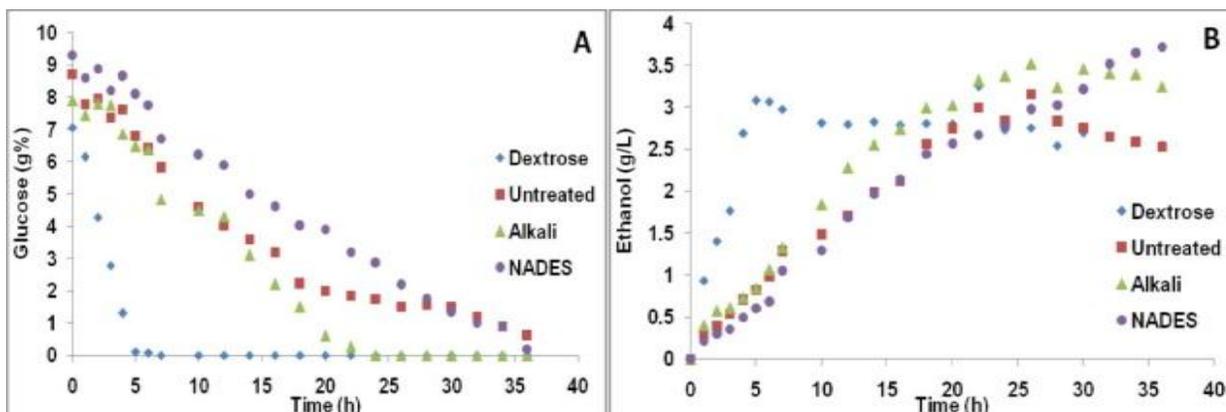


Figure 3. Rate of glucose consumption (A) and ethanol production using *Clavispora* NRRL-Y-50464

4. Conclusion

The current study demonstrates applicability of CC-LA mixture as a potential NADES reagent for efficient solubilisation and recovery of high purity lignin (86 – 90 %) and high purity xylan (99%) from lignocellulosic agro-residue, rice straw. Also, the NADES solvent could be effectively recovered (99%) and recycled (3-times and above) for consecutive biomass pretreatments without losing its efficiency. Further, higher solids loading tested in both biomass pretreatment (10%) and enzymatic hydrolysis (25%) processes generate concentrated sugars (171.7 g/L) which may be directly fermented to ethanol with higher conversion efficiencies (79.9%). Moreover, employing higher solids loading *i.e.* Set-IV in this integrated process significantly reduced input of NADES in biomass pretreatment, required during recovery processes could be reduced significantly. water and acetonitrile during lignin and xylan precipitation, respectively. Hence, the energy required during recovery processes could be reduced significantly.

5. Acknowledgments

The authors are thankful to the Director, Sardar Patel Renewable Energy Research Institute, Gujarat, India, for support of this research. The authors are thankful to Dr. Lewis Liu, United States Department of

Agriculture, Agriculture Research Service for helpful technical discussions. The research work is financially supported by Indian Council of Agricultural Research (ICAR), under All India Co-ordinated Research Project (AICRP) –EAAI program, Govt. of India with Grant Number VVN/CRP/2015/3 and Department of Biotechnology (DBT), Govt. of India with Grant Number BT/PR12368/PDB/26/431/2014.

6. References

- [i] Phitsuwan.P., Sakka, K., Ratanakhanokchai, K., 2013. Improvement of lignocellulosic biomass in planta: a review of feedstocks, biomass recalcitrance, and strategic manipulation of ideal plants designed for ethanol production and processability. *Biomass Bioenergy* 58, 390–405.
- [ii] Gomez, L.D., Steele-King, C.G., McQueen-Mason, S.J., 2008. Sustainable liquid biofuels from biomass: the writings on the walls. *New Phytol.* 178, 473–485.
- [iii] Yiin, C. L., Quitain, A.T., Yusup, S., Sasaki, M., Uemura, Y., and Kida, T. (2016). Characterization of natural low transition temperature mixtures (LTTMs): Green solvents for biomass delignification. *Bioresource Technology.* (199), 258-264.



- [iv] Alvarez-Vasco, C., Ma, R., Quintero, M., Guo, M., Geleynse, S., Ramasamy, K. K., Wolcott, M., and Zhang, X. (2016). Unique low-molecular-weight lignin with high purity extracted from wood by deep eutectic solvents (DES): a source of lignin for valorization. *Green Chemistry*. (18), 5133-5141.
- [v] Gu, T., Zhang, M., Tan, T., Chen, J., Li, Z., and Zhang, Q., (2014). Deep eutectic solvents as novel extraction media for phenolic compounds from model oil. *Chemical Communications*. (50), 11749-11752.
- [vi] Dai, Y., Spronsen, J. V., Witkamp, G. J., Verpoorte, R., and Choi, Y. H. (2013), Natural deep eutectic solvents as new potential media for green technology. *Analytical Chimica and Acta*. (766), 61-68.
- [vii] Paiva, A., Craveiro, R., Aroso, I., Martins, M., Reis, R. L., and Duarte, A. R. C. (2014). Natural deep eutectic solvents – Solvents for the 21st century. *ACS Sustainable Chemistry and Engineering*. (2) 1063–1071.
- [viii] Francisco, M., Bruinhorst, A. V., and Kroon, M. C. (2012). New natural and renewable low transition temperature mixtures (LTTMs): screening as solvents for lignocellulosic biomass processing. *Green Chemistry*. (14) 2153-2157.
- [ix] Jorgensen, H., Kristensen, J. B., Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels Bioproducts and Biorefining*. (1), 119–134.
- [x] Mora-Pale, M., Meli, L., Doherty, T. V., Linhardt, R. J., and Dordick, J. S. (2011). Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass. *Biotechnology and Bioengineering*. (108), 1229-1245.
- [xi] Kumar, A. K., Parikh, B. S., Shah, E., Liu, L. Z., and Cotta, M. A. (2016). Cellulosic ethanol production from green solvent-pretreated rice straw. *Biocatalysis and Agricultural Biotechnology*. (7), 14-23.
- [xii] Chapla, D., Parikh, B. S., Liu, L. Z., Cotta, M. A., and Kumar, A. K. (2015), Enhanced cellulosic ethanol production from mild-alkali pretreated rice straw in SSF using *Clavispora* NRRL Y-50464. *Journal of Biobased Materials and Bioenergy*. (9), 1-8.
- [xiii] Kumar, A. K., Parikh, B. S., and Pravakar, M. (2015). Natural deep eutectic solvent mediated pretreatment of rice straw: Bioanalytical characterization of lignin extract and enzymatic hydrolysis of pretreated biomass residue. *Environmental Science and Pollution Research*. (23) 9265-9275.
- [xiv] Dashtban, M., Maki, M., Leung, K. T., Mao, C., and Qin, W. (2010). Cellulase activities in biomass conversion: measurement methods and comparison. *Critical Reviews in Biotechnology*. (30), 302–309.
- [xv] Nakamura, A., Myafuji, H., Saka, S., Mori, M., and Takahashi, H. (2010). Recovery of cellulose and xylan liquefied in ionic liquids by precipitation in anti-solvents. *Holzforchung*. (64), 77-79.
- [xvi] Fernando, E. F., Vallejos, E. M., and Area, M. C. (2010). Lignin recovery from spent liquors from ethanol water fractionation of sugar cane bagasse. *Cellulose Chemistry and Technology*. (44), 311-318.