Cellulosic ethanol production from green solvent-pretreated rice straw

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1. Introduction

Recently, new types of ‘green solvents’ were identified and recognized as potential solvents for a wide range of biological and non-biological applications (Paiva et al., 2014; Dai et al., 2013). These solvents were termed as natural deep eutectic solvents (NADES) and are made up of low-cost, naturally available chemical compounds with high melting points. Compared to the conventional organic solvents these solvents have boosted advantages in diverse areas of applications. Besides having physico-chemical properties similar to chemically synthesized ionic liquids (ILs), NADES or termed as green solvents are entirely composed of low-cost natural components, primarily plant-based metabolites, which are biodegradable, non-toxic and eco-friendly (Paiva et al., 2014). These solvents are low-melting and low-transition temperature solvents that are convenient for process of biomass pretreatment at or near ambient temperatures. Furthermore, green solvents are biocompatible solvents with infinite possibility to form wide-liquid ranges and specific substrate solubility properties. The most common recently developed green reagents comprises of binary solvents where choline chloride (CC) is mixed in different molar ratios with several hydrogen bond donors, such as malonic acid, citric acid, and glycerol (Dai et al., 2013). In addition, preparation of green reagents is a simple process, and these catalysts could be easily recovered and recycled, thus may significantly reduce the overall-cost of the process. Utilizing such green solvents for biomass pretreatment is one of the key areas which need to be addressed in developing an economically viable cost-effective method for production of liquid biofuels.

Global dependence on the rapidly depleting finite petroleum reserves in the fast growing industrialization era and the population growth has forced us to explore alternative renewable resources for sustaining life on earth. Lignocellulosic biomass is the most abundant renewable reserve and promising bioresource enriched in cellulosic and hemicellulosic fractions, which can be efficiently hydrolyzed and routed towards production of liquid fuels. However, these biomass materials are highly recalcitrant to microbial or enzymatic breakdown due to intercellular heterogeneous complex matrix formation with lignin moieties (Van Dyk and Pletschke, 2012). Thus biomass pretreatment became an essential step in order to overcome the recalcitrance towards high yield bioconversion to fermentable sugars. Among numerous

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http://dx.doi.org/10.1016/j.bcab.2016.04.008
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pretreatment methods using physical, chemical and biological treatments, dilute acid, ammonia fiber expansion, ammonia recycled percolation and mild-alkali pretreatment treatments appeared suitable for commercial scale applications (Kumar et al., 2009; Wyman et al., 2013; Karimi et al., 2013). These methods are effective to breakdown lignocellulosic materials, but suffer high cost of capital expenditure (CAPEX) and operational expenditure (OPEX) (Gonzalez et al., 2011). In addition, the recyclability and reuse of the catalysts are a cost-intensive process and not sustainable.

Recently, deep eutectic solvents (DES) have been reported as attractive reagents in strengthening enzyme stability and enhancing enzyme activity of lipase and laccases (Huang et al., 2013; Choi et al., 2011). The NADES pretreatment does not require extensive amounts of water for pre-washing the pretreated biomass residues, and reduces deleterious effects of acid or alkali on cellulase activity during sugars production. Thus employing NADES pretreatment, the overall production costs could be significantly decreased compared to the dilute acid or mild-alkali pretreatment methods.

Rice straw was selected in the current investigation due to its abundance availability in surplus amounts, especially in the Asian countries. Foreseeing the potential use of this renewable biomass and its bioconversion into liquid fuels, voluminous research studies were reported from several decades, employing a wide range of pretreatment methods. However, majority of the pretreatment methods suffers with certain drawbacks viz., poor enzymatic saccharification, environmentally hazardous, non-biodegradable, non-renewable, multi step down-stream processing, solvent recovery, recycling, and high capital and operational expenditures. Here, we have prepared few green reagents including NADES reagents and low transition temperature mixtures (LTTMs) and their bioconversion into liquid fuels, voluminous research studies were reported from several decades, employing a wide range of pretreatment methods. However, majority of the pretreatment methods suffers with certain drawbacks viz., poor enzymatic saccharification, environmentally hazardous, non-biodegradable, non-renewable, multi step down-stream processing, solvent recovery, recycling, and high capital and operational expenditures. Here, we have prepared few green reagents including NADES reagents and low transition temperature mixtures (LTTMs) and studied their effect on commercially available cellulase Cellic Ctec2 and a β-glucosidase producing yeast strain Clavispora NRRL Y-50464. Besides these, we have also evaluated the cellulose ethanol production from selective green-reagent pretreated rice straw.

2. Methods

2.1. Lignocellulosic biomass

Rice straw was used as the lignocellulosic biomass residue for pretreatment and enzymatic hydrolysis. Rice straw was obtained from local agricultural fields in Anand district, Gujarat. The lignocellulosic biomass was extensively washed with water before pretreatment and then dried in sunlight for 3–4 d until the moisture content reached < 5% (w/w). The dried residue was then cut into small pieces (2–10 mm) using a hammer mill. The pre-sized biomass was directly used without any further processing.

2.2. Preparation of green solvents

Two component reaction mixtures employing choline chloride (CC) based green solvents were prepared for the current investigation. The hydrogen bond donors tested in this study included malonic acid (MAL), malic acid (MA), 1,2-propanediol (PD), citric acid (CA), tartaric acid (TA), glycerol (GLY), ethylene glycol (EG), lactic acid (LA), urea (UR) and oxalic acid (OA). These reagents were added separately in different molar ratios to choline chloride in screw capped conical glass bottles. The mixtures were then incubated in shaking water bath for 2 h to 12 h between 40 °C and 80 °C at constant 100 rpm until a clear liquid solution was formed following the protocols described previously by us (Kumar et al., 2015). Total 10 different types of choline chloride based green solvents were prepared (Table 1).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Group</th>
<th>Green solvents</th>
<th>Molar ratio</th>
<th>Legend</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acidic</td>
<td>Choline chloride, Malic acid</td>
<td>1:1</td>
<td>CC-MA</td>
</tr>
<tr>
<td>2</td>
<td>Choline chloride</td>
<td>Citric acid</td>
<td>1:1</td>
<td>CC-CA</td>
</tr>
<tr>
<td>3</td>
<td>Choline chloride, Tartaric acid</td>
<td>1:1</td>
<td>CC-TA</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Choline chloride</td>
<td>Lactic acid</td>
<td>1:5; 1:9</td>
<td>CC-LA</td>
</tr>
<tr>
<td>5</td>
<td>Choline chloride</td>
<td>Oxalic acid</td>
<td>1:1</td>
<td>CC-OA</td>
</tr>
<tr>
<td>6</td>
<td>Neutral</td>
<td>Choline chloride, Malonic acid</td>
<td>1:1; 1:2</td>
<td>CC-MAL</td>
</tr>
<tr>
<td>7</td>
<td>Choline chloride</td>
<td>Ethylene glycol</td>
<td>1:1</td>
<td>CC-EG</td>
</tr>
<tr>
<td>8</td>
<td>Choline chloride</td>
<td>1,2-propanediol</td>
<td>1:1</td>
<td>CC-PD</td>
</tr>
<tr>
<td>9</td>
<td>Choline chloride</td>
<td>Urea</td>
<td>1:1</td>
<td>CC-UR</td>
</tr>
<tr>
<td>10</td>
<td>Choline chloride</td>
<td>Glycerol</td>
<td>1:1</td>
<td>CC-GLY</td>
</tr>
</tbody>
</table>

2.3. Enzyme stability studies in green solvents

The stability of Cellic Ctec2 enzyme in green solution was measured by incubating the enzyme solution in different concentrations (0, 0.5, 2.5, 5.0, 10, 15%, v/v) of green solvents in citrate buffer (50 mM, pH 5.2) at 37 °C for 48 h. Total cellulase activity (FPhase) was measured by standard filter paper assay method following the protocol published by T.K. Ghose (1987). One unit of enzyme activity is defined as the amount of enzyme required to release 1 μM of reducing sugar per min under the assay conditions. Control experiments voiding either enzyme or the additives were also performed to nullify background absorbance. Mean values from triplicate experiments were calculated and presented.

2.4. Growth of Clavispora NRRL Y-50464

Clavispora NRRL Y-50464 obtained from USDA-ARS Patent Culture Collection was prepared in YPG medium as previously described by us (Liu et al., 2012; Chapla et al., 2015). Briefly, different green solvents were added separately into the growth media containing 5% glucose as carbon source and then the growth profile including glucose consumption and ethanol production was evaluated. Samples were taken at regular intervals and measured the absorbance at 600 nm for microbial growth. HPLC method (Schimadzu LC 2010C, Schimadzu Co., Kyoto, Japan) using HPX-87h aminex ion exclusion column (Biorad, Hercules, CA) was used to measure glucose and ethanol concentrations (Chapla et al., 2015).

2.5. Rice straw pre-treatment and enzymatic hydrolysis

Among the prepared green solvents CC-GLY, CC-MAL and CC-LA were used for biomass pretreatment. Since cellic ctec2 was not stable in the some of the green reagents (as shown in detail in Section 3), experiments on biomass pretreatment and enzymatic hydrolysis were performed in CC-GLY reagent. The detailed procedures for biomass pretreatment and enzymatic hydrolysis were recently described by us (Kumar et al., 2015). In brief, the green reagents were added separately to the biomass residue at 5% and 10% solids loading in a 250 ml screw capped conical flask and was incubated at different temperatures (60 °C to 121 °C) and for different time periods (30 min to 12 h). After pretreatment, the reaction mixture was diluted 6- to 7-fold to precipitate lignin in distilled water following a recently described protocol by us (Kumar et al., 2015). In order to separate the lignin extract, the liquid
fraction was separated from the residual pretreated biomass by glass fiber filtration using Millipore vacuum pump (Millipore Inc., USA). The lignin extract was then precipitated by simple dilution method by adding ice cold water (~ 6–7 fold) into the liquid fraction. The precipitated lignin was separated by membrane filtration and then dried until the moisture content was < 5% (w/w).

The solid residue was separated by membrane filtration and subjected to drying at 55 °C until the moisture content reached < 5% (w/w). The moisture content was measured by Infrared moisture analyzer (Sartorius MA100, Germany). Enzymatic hydrolysis was performed at 10%, 15% and 20% of solids loading using 9, 12 and 15 FPU of Cellic Ctec2 enzyme, respectively. The biomass saccharification was conducted in a horizontal free falling reactor (Bangalore Genei, India) at 50 °C with a constant agitation speed of 16 rpm for 48 h. Samples were collected at regular intervals and then the reducing sugars concentration was measured using standard DNSA method (Dashtban et al., 2010) and simultaneously the cellobiose, glucose and xylose contents were also quantified using HPLC.

2.6. Ethanol fermentation

Ethanol production from the reducing sugars was carried out using Clavispora NRRL Y-50464 in submerged fermentation conditions. The glucose concentration in the reducing sugars was estimated and adjusted to 5% or 8% by dilution with deionized water. A 10 x YP+ media was added to the contents and sterilized at 121 °C for 20 min as previously described (Liu et al., 2012; Chapla et al., 2015). An overnight grown culture with an absorbance of 2.58 at 600 nm was taken and the cells were separated by centrifugation and then resuspended in fresh 1x YP+ media. A 3% (w/w) inoculum was added to the fermentation media and incubated at 37 °C for 72 h. Samples were withdrawn at regular intervals and measured the ethanol production and glucose consumption using HPLC as mentioned above.

2.7. Effect of additives

To evaluate the effect of additives on Clavispora NRRL Y-50464 growth different concentrations of additives were added in the YPG media. The additives tested were sodium benzoate (0.005 – 0.1%), sodium azide (0.01 – 0.1%) and ethanol (1 – 10%), respectively. The growth profile, glucose consumption and ethanol production from these tested conditions was measured as described above.

2.8. Analysis of lignin extracts

Quantitative and qualitative analysis of the lignin extract was analyzed using UV–visible UV-1700 series spectrophotometer (Shimadzu Scientific Instruments, Singapore) following a protocol previously described (Kumar et al., 2015). Briefly, the lignin extract was diluted in the respective green solvents and absorbance was recorded in the range between 200 and 800 nm with 1 nm spectral resolution. All samples were referenced to pure green solvents. The extent of delignification was estimated on the basis of the lignin extracted into the green solvent solution and was also measured by UV–vis spectroscopy following the NREL LAP-004 protocol (Ehrman, 1996) and by standard chemical compositional analysis described by Goering and Van Soest (1970). All the analytical and biochemical experiments were carried out in triplicates and the mean values were presented.

2.9. Analysis of pretreated biomass

Morphological characteristics of the pretreated biomass were studied using scanning electron microscopy (SEM). Since the samples were non-conductive, gold coating of the samples were used in Scanning Electron Microscope (Carl Zeiss EVO 50 XVP) after mounting on aluminium stubs fixed with double-coated carbon tape for 90 s. The vacuum chamber was maintained at current of 1 mA and 30 kV voltage and then analyzed using the following setting conditions: 20 kV EHT, 50 μm aperture, and 1000 X magnification. Besides morphological characteristics, crystallinity index ratios of the pretreated biomass were studied using x-ray diffraction (XRD). The crystallinity percentage of untreated and green solvent pretreated rice straw biomass was determined using an X-ray diffractometer (XRD 6000 Shimadzu). Further, crystallinity index ratio and FTIR analysis of the pretreated biomass and lignin extract were performed as described previously (Kumar et al., 2015).

2.10. Physicochemical characterization of green solvents

Since there were no extensive reports on the physicochemical properties of some of the green solvents that were prepared in this current study, we have studied the conductivity and viscosity properties. In our previous study, we have reported that addition of water to the NADES reagent increased the total lignin extract from pretreated rice straw biomass (Kumar et al., 2015). To understand and correlate the changes in the conductivity and viscosity profiles, here we have studied these two critical parameters. Conductivity test was measured at ambient temperature (27 °C) using a Eutech PCD650 conductivity instrument (Thermo Fisher Scientific Inc., USA) equipped with pH and temperature measurement facility. Viscosity test was measured using Redwood Viscometer (Model no: CS504, make: Haryana Co., India) at atmospheric pressure at 30 °C, respectively. The Viscometer instrument is equipped with hammer finished stainless steel with electrical heating arrangement, silver plated oil cup with precision stainless steel jet, cup cover, ball valve and thermometer-clip. A 100 ml sample was placed in a sample holder surrounded by a temperature regulated water jacket. Kinematic viscosities were calculated based on the principle of falling time by the effect of gravity. All the analytical and biochemical experiments were carried out in triplicates and the mean values were presented.

3. Results and discussion

3.1. Physicochemical properties of green solvents

Recently we have reported few physicochemical properties for selected NADES reagents (Kumar et al., 2015). In this report, we have presented the research work on preparation and characterization of few more green solvents (Fig. S1). The conductivity profile of the green solvents varied considerably among each other. Addition of water to the green solvents showed significant variability in solvent conductivity. The conductivity of the green solvents may be correlated with their individual chemical components. The plausible reason attributed to the ionic form of the choline chloride and partial ionization of the hydrogen bond donors (acids/sugars/diols). Francisco et al. (2013a, 2013b) reported extensively on the physico-chemical properties and the effect of water and ethanol on few selected choline chloride based NADES reagents. CC-EG solvent had the highest conductivity (7.659 mS/cm), while CC-CA showed lowest conductivity (0.081 mS/cm) (Table 2). The detailed conductivity profile of the acidic and neutral green solvents with increasing water content is shown in Fig. 1. Recently, Dai et al. (2015) evaluated the viscosity and conductivity of 16 different NADES reagents. The conductivity profile of the green solvents tested in this study was closely correlated
Table 2
Conductivity (mS/cm) of the tested green solvents at ambient temperature.

<table>
<thead>
<tr>
<th>Compositions/legend/molar ratio</th>
<th>Conductivity (mS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline chloride:Malic acid (CC-MA)</td>
<td>0.197</td>
</tr>
<tr>
<td>Choline chloride:Citric acid (CC-CA)</td>
<td>0.081</td>
</tr>
<tr>
<td>Choline chloride:Tartaric acid (CC-TA)</td>
<td>ND</td>
</tr>
<tr>
<td>Choline chloride:Lactic acid (CC-LA)</td>
<td>2.271</td>
</tr>
<tr>
<td>Choline chloride:Oxalic acid (CC-OA)</td>
<td>5.702</td>
</tr>
<tr>
<td>Choline chloride:Malonic acid (CC-MAL)</td>
<td>0.708</td>
</tr>
<tr>
<td>Choline chloride:Ethylene glycol (CC-EG)</td>
<td>7.659</td>
</tr>
<tr>
<td>Choline chloride:1,2-Propane diol (CC-PD)</td>
<td>3.03</td>
</tr>
<tr>
<td>Choline chloride:Urea (CC-UR)</td>
<td>3.51</td>
</tr>
<tr>
<td>Choline chloride:Glycerol (CC-GLY)</td>
<td>1.909</td>
</tr>
<tr>
<td>Deionized water</td>
<td>0.00210</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.000</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>5.70</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>50.8</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>11.8</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>0.00107</td>
</tr>
<tr>
<td>1,2-Propane diol</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* The molar ratios of all the tested green solvents were 1:1, except CC-LA, where 1:5 was used.

Fig. 1. Effect of water addition on changes in conductivity profile of (A) acidic and (B) neutral green solvents at ambient temperature.

Table 3
Compositional analysis of untreated and pretreated rice straw.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Residual straw (g)</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Holocellulose (%)</th>
<th>Lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>200</td>
<td>40.0 ± 1.8</td>
<td>28.0 ± 2.0</td>
<td>68.0 ± 19</td>
<td>11.0 ± 1.8</td>
</tr>
<tr>
<td>CC-GYL pretreated</td>
<td>191</td>
<td>43.1 ± 1.1</td>
<td>28.0 ± 0.6</td>
<td>68.1 ± 13</td>
<td>6.9 ± 0.3</td>
</tr>
<tr>
<td>CC-MAL pretreated</td>
<td>193</td>
<td>44.3 ± 1.6</td>
<td>26.2 ± 0.8</td>
<td>70.2 ± 19</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>CC-LA pretreated</td>
<td>190</td>
<td>46.0 ± 0.9</td>
<td>24.6 ± 1.3</td>
<td>70.0 ± 12</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>Alkali treated*</td>
<td>144</td>
<td>47.2 ± 1.2</td>
<td>19.2 ± 1.6</td>
<td>66.4 ± 13</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>Acid treated*</td>
<td>120</td>
<td>37.5 ± 1.9</td>
<td>0.6 ± 0.02</td>
<td>38.1 ± 0.8</td>
<td>8.1 ± 0.2</td>
</tr>
</tbody>
</table>

* Data was obtained from our recent research experiments reported by us earlier (Kumar and Parikh 2015).

3.2. Pretreatment of rice straw biomass

Green solvent pretreated rice straw showed significantly higher level of lignin removal, among which, CC-LA treatment displayed the highest level of lignin removal of 57.2 ± 3.6% followed by CC-MAL and CC-GLY with reduced lignin of 51.8 ± 2.3% and 37.2 ± 5.1%, respectively. However, after CC-LA treatment, a slight loss in hemicellulose content in the biomass was observed. But, the CC-MAL and CC-GLY solvent pretreatments did not affect the cellulose and hemicellulose contents (Table 3). These results clearly indicated the specificity and selectivity of the green solvents towards the lignin solubility. We recently observed the solubility of purified cellulose, xylan and lignin in selected NADES reagents (Kumar et al., 2015). Choline chloride was reported to stabilize the cellulose system by forming strong hydrogen bond interactions, thus highly unlikely to dissolve the cellulose (Abbot et al., 2006). Our XRD analysis of CC-GLY showed that no solubilization of crystalline and amorphous regions after pretreatment was observed (Fig. S3). The percentage crystallinity [CrI (%)] was slightly reduced from 33.48% to 31.85%. This difference was found mainly due to the increase in the amorphous cellulose content while the intensity of crystalline cellulose remained at nearly the same level. Besides, XRD data demonstrated that there was a clearly detectable structural deformation of the crystalline and amorphous regions of the treated rice straw. Such cellulose decomposition would increase the availability of amorphous cellulose for rapid enzymatic hydrolysis. The cellulose and hemicellulose content in the pretreated biomass after CC-GLY treatments was 43.1 ± 1.1% and 28.0 ± 0.6%, respectively, and for CC-MAL 44.3 ± 1.6% and 26.2 ± 0.8%, respectively. These results support the hypothesis on the non-dissolution property of choline chloride on cellulose and hemicelluloses components. Moreover, SEM analysis of CC-GLY and CC-MAL pretreated rice straw did not show any major deconstruction in the structural organization of the cellulosic matrix (Fig. 2). But green solvent pretreatment clearly removed the surface lignin from the biomass exposing the internal cellulosic and hemicellulosic fractions that could be easily accessible towards enzymatic hydrolysis.

UV-visible spectroscopy studies of the lignin extract showed absorbance maxima at 280 nm typical for aromatic ring compounds (Fig. 3a). On the basis of final pH of the green solvents, these were categorized into two groups: acidic (pH 2.0-2.6) and with their study, where initially the conductivity was increased with water addition and thereafter decreased reaching a peak value. CC-OA had the highest conductivity (96.75 mS/cm) with 70% water dilution. Dai et al. (2015) also reported that increase in conductivity of NADES reagent was mainly due to the decrease in the viscosity of the solvent. The viscosity of the green solvents was suddenly dropped after addition of 20% (v/v) water and thereafter no significant change was detected. Maximum viscosity was observed with CC-MA, followed by CC-MAL (Fig. S2). The detailed viscosity measurement of CC-LA with water addition was reported recently (Kumar et al., 2015).
neutral (pH 6.0–pH 7.0), respectively. Acidic green solvents include choline chloride/malic acid (CC-MA), choline chloride/citric acid (CC-CA), choline chloride/tartaric acid (CC-TA), choline chloride/lactic acid (CC-LA) and choline chloride/oxalic acid (CC-OA). While neutral green solvents include choline chloride/malonic acid (CC-MAL), choline chloride/1,2-propane diol (CC-PD), choline chloride/glycerol (CC-GLY), choline chloride/ethylene glycol (CC-EG) and choline chloride/urea (CC-UR). Interestingly, lignin extracted from acidic green solvents had shown a single prominent peak at 280 nm, while with neutral green solvents an additional shoulder around 305–315 nm was observed. It is known that p-coumaryl alcohol had a characteristic absorption maximum at 280 nm and branched chain aromatic ring structure components such as coniferyl alcohol and sinapyl alcohol absorbs at higher wavelengths around 300–315 nm (Vivekanand et al., 2014). Supporting the UV results, FTIR analysis also confirmed the purity of the lignin extract after green solvent pretreatment (Fig. 3b). Details regarding the peaks detected in FTIR analyses that are characteristic to the lignin were described in our recent report (Kumar et al., 2015). These results indicated that the lignin extract was of high purity with no contamination from cellulose or hemicellulose fractions.

3.3. Enzymatic hydrolysis and ethanol production

Enzymatic saccharification of the green solvent pretreated rice straw produced maximum reducing sugars of 226.7 g/L at 20% solids loading and 12 FPU per gm of Cellic Ctec2 in 48 h incubation. Based on the theoretical glucose yield conversion from the pretreated rice straw, the saccharification efficiencies were calculated. Highest saccharification efficiency was found to be 87.1% with 10% solids loading and marginally decreased to 81.6% with 20% solids loading. While with mild-alkali pretreated rice straw, the maximum saccharification efficiency of 74.3% was achieved at 10% solids loading and thereafter significantly reduced (Fig. 4). While with untreated biomass, the saccharification efficiency was only 56.6% with maximum reducing sugars of 66 g/L was obtained with 10% solids loading. These data further supports our recent observation on increasing reducing sugars with green solvent pretreatment at high solids loading as compared to the mild-alkali pretreatment (Kumar and Parikh, 2015). Fermentation was performed using the so formed reducing sugars. Compared to glucose the total concentrations of cellulbiose and xylose in the reducing sugars from green solvent pretreated rice straw were found to be significantly low (Fig. S4). The measured concentration of cellobiose and xylose in reducing sugars from green solvent pretreated rice straw at 10% solids loading and 9 FPU enzyme were 3.2 g/L and 11.4 g/L, respectively. Initially the reducing sugar solution was diluted with sterile distilled water to 5% and 8% glucose concentration (from HPLC measurements), sterilized and fermented to ethanol using the Clavispora NRRL Y-50464 yeast strain. Although there was no significant difference in the rate of glucose consumption was observed with both reducing sugars containing 5% glucose and pure glucose. However, the maximum ethanol produced was 22.6 g/L in 24 h compared to 19.7 g/L in 18 h. While with 8% glucose, maximum ethanol yield was increased to 36.7 g/L at a delayed time of 36 h, while reducing sugars at similar concentrations of glucose was completely utilized (Fig. 5). The ethanol conversion efficiencies were estimated and found to be ~89.5 ± 0.51% with both 5% and 8% glucose concentrations. Recently, we have shown ethanol production from pure cellulose reached 40.44 g/L in a bottled SSF after 72 h (Liu and Cotta, 2015). These results suggest the potential of...
Clavispora NRRL Y-50464 as industrial yeast for lower-cost cellulosic ethanol production.

3.4. Effect of green solvents on activity of cellulase Cellic Ctec2

Recently we have reported enzymatic saccharification of mild-alkali pretreated rice straw using Celluclast 1.5 L and Novozyme 188 where maximum reducing sugars of 0.854 g/g were produced within 48 h at 10% solids loading with a saccharification efficiency of 96.5% (Kumar and Parikh, 2015). Also, we have recently reported use of NADES reagent for rice straw biomass pretreatment and high purity lignin extraction (Kumar et al., 2015). In continuation to the NADES pretreatment, here we have further evaluated the effect of green solvents along with other NADES reagents on the enzyme activity of a commercial Cellic Ctec2 cellulase enzyme. The enzyme was incubated separately in respective green solvents and the residual enzyme activities were analyzed intermittently for 48 h. The cellulase activity was slightly decreased in CC-MAL, CC-GLY, CC-PD and CC-UR solvents when compared to the control without green solvents (Fig. 6). While, nearly complete loss of cellulase activity was observed within 24 h in CC-MA, CC-TA, CC-LA and CC-OA, respectively. Interestingly, incubation of cellic ctec2 in high concentration (30% (v/v)) of green solvents at room temperature, the cellulase activity was increased from 104 FPU/ml to 141 FPU/ml after 48 h and whereas in CC-EG the activity was increased from 108 FPU/ml to 160 FPU/ml after 24 h (Fig. 6). Very recently, Gunny et al. (2015) also reported the cellulase enzyme, Carezyme 1000 L stability in 1:2 M ratio of CC-GLY, CC-EG and CC-MAL solvents. They have shown that at 30% NADES reagents, the cellulase activities were marginally decreased in CC-GLY and CC-EG after 48 h. Our results were also comparable with Gunny et al. report showing good stability in GLY and EG solvents without any loss in enzyme activity (Gunny et al., 2015). But, unlike Carezyme 1000 L enzyme, Cellic Ctec2 was found to be highly stable in 15% CC-MAL solvent (Fig. 6). The most plausible reason for cellic ctec2 stability at higher concentrations of green solvents could be due to lower molar ratio of hydrogen bond donors. For example, the cationic effect of malonic acid is comparatively lower at 1:1 when compared to 1:2 ratio. Besides, the other reason could be the neutral pH nature of CC-MAL reagent. It is known that cellic ctec2 is highly active and stable at and around pH 5.5. This might be an important factor for high stability of cellic ctec2 in neutral green solvents. Beside cellulases, other enzyme activities such as laccases were also found to be stable in 50% CC-MAL solvent (Choi et al., 2011). Along with this, the high stability of cellic ctec2 in CC-GLY and CC-EG were mainly due to their protein stabilizing properties. It is known that glycerol and diols reagents are high-quality enzyme stabilizers (Gu and Jerome, 2013).

3.5. Effect of green solvents on Clavispora NRRL Y-50464

No significant changes in the growth pattern of Clavispora NRRL Y-50464 was observed when grown in lower concentrations (< 10%) of CC-GLY, CC-EG and CC-PD solvents, except 10% CC-EG where a delayed growth with prolonged logarithmic phase was observed (Fig. 7a). Beyond 10% concentration, irrespective of the green solvent the growth of microbe was severely hampered. In
Fig. 6. Total cellulase (FPase) activity of Cellic Ctec2 in the presence of different natural deep eutectic solvents at various concentrations after 24 and 48 h incubation period at 37 °C. (A) choline chloride/malic acid; (B) choline chloride/citric acid; (C) choline chloride/tartaric acid; (D) choline chloride/lactic acid; (E) choline chloride/oxalic acid; (F) choline chloride/malonic acid; (G) choline chloride/ethylene glycol; (H) choline chloride/1,2-propane diol; (I) choline chloride/urea; and (J) choline chloride/glycerol.
acidic green solvents no detectable growth was observed even at very low concentrations (Fig. 7d). This could be mostly due to the strong acidic nature of the solvents that strongly affected the pH of the growth medium. In order to overcome such external effects, the growth medium was adjusted to pH 6.8 with different buffering agents such as NaOH, Tris base, HEPES, MOPS and phosphate buffers, but still no detectable growth was observed. This was mainly due to the high molar concentrations (5–7 M) of buffering chemicals that were added to adjust the pH to 6.0, which showed a profound negative effect on the microbial growth. Similarly, the rate of glucose consumption and ethanol production were also affected in these green solvents (Fig. 7b and c). No difference in rate of glucose consumption was observed with 5% and 10% CC-GLY and 5% CC-EG; where the glucose was completely consumed within 18 h (Fig. 7b). While with 5% CC-PD the glucose was consumed in 21 h. In 10% CC-EG and 10% CC-PD nearly 16 g/L of residual glucose was detected in the culture medium after 24 h (Fig. 7b). Similarly, lowest ethanol was produced in 10% CC-EG and 10% CC-PD, where maximum ethanol reached up to 7.5 g/L in 24 h unlike in other green solvents the maximum ethanol produced was 19.7 g/L within 24 h (Fig. 7c). The quantitative analysis of glucose consumption and their ethanol production in these solvents were clearly demonstrated by HPLC where decrease in glucose peak and increase in ethanol peaks were detected (Fig. S5). Sitepu et al. (2014) reported resistance of Clavispora spp in IL solvents, where Clavispora lusitaniae UCDFST 81–467.1 and Clavispora opuntiae UCDFST 81–502.1 were found tolerate 5% 1-ethyl-3-methylimidazolium acetate.

3.6. Effect of antimicrobial agents on Clavispora NRRL Y-50464

The effect of anti-microbial agents on growth of Clavispora

Fig. 7. Effect of neutral green solvents on Clavispora NRRL Y-50464. (A) Growth curve, (B) glucose consumed, (C) ethanol produced and (D) Microbial growth curve in acidic green solvents.
NRRL Y-50464, in different concentrations (0.005–0.1%) of sodium azide and sodium benzoate was studied. Interestingly, sodium benzoate had not shown any significant effect on the microbial growth. However, the cell biomass was marginally decreased with increasing concentration of sodium benzoate (Fig. S6a). Concomitantly, the rate of glucose consumption and ethanol production were also affected with the growth (Fig. S6b and S6c). The rate of glucose consumption in sodium benzoate was found to be comparable with the neutral green solvents, except in 0.1% sodium benzoate where nearly 6.0 g/L of residual glucose was detected after 24 h incubation. Maximum ethanol yield of 17.4 g/L was obtained up to 0.05% sodium benzoate, thereafter increasing the concentration of sodium benzoate to 0.1% the ethanol yield was marginally decreased and reached 14.3 g/L in 24 h. Whereas, addition of sodium azide in the growth medium had shown a strong negative effect on NRRL Y-50464 growth and no detectable growth was observed even at very low concentrations (0.005%) of sodium azide (Fig. S7). In general sodium benzoate does not kill the microorganism but it stops the microbes to grow and is most effective in acidic conditions (pH < 4.0). It is mainly added in foods and beverages as a preservative for long term storage. Whereas, sodium azide is a strong antimicrobial agent and known to inhibit the growth of a wide range of yeasts, gram negative and gram positive bacteria (Lichstein and Snyder, 1944). In this study we observed that Clavispora NRRL Y-50464 was resistant against sodium benzoate while susceptible to sodium azide. Further, in simultaneous saccharification and fermentation (SSF) experiments 0.02% sodium benzoate was supplemented into the reaction mixture to avoid external bacterial contamination.

3.7. Ethanol tolerance of Clavispora NRRL Y-50464

Addition of ethanol in the growth culture media had shown negative effect on the growth rate of NRRL Y-50464. A prolonged stationary growth phase was observed with addition of 1–4% ethanol and thereafter no detectable growth was observed (Fig. S8). Although the Clavispora was found to tolerate 4% ethanol, however, the growth was very less with prolonged logarithmic phase and delayed doubling time. Higher ethanol concentrations (5% and above) resulted not only decreased the efficiency of substrate utilization, but also resulted in lower ethanol yields. The ethanol tolerance of Clavispora spp. was not reported till date. This is the first time we have showed the growth profile of Clavispora NRRL Y-50464 with external supplementation of ethanol. Further microbial studies on adaptation of Clavispora NRRL Y-50464 in higher ethanol concentrations are needed to understand insights of different biochemical pathways.

3.8. Process flow on cellulosic ethanol production from green solvent pretreated rice straw

An integrated zero-waste technology on cellulosic ethanol production was proposed in this research (Fig. S9). The entire process includes three main stages (a) green solvent synthesis and lignocellulosic biomass pretreatment (b) lignin and ethanol production (c) solvent recovery and reuse. The detailed process conditions were described in the above sections. In brief, stage 1 includes lignocellulosic biomass was pretreated with freshly prepared green solvents of selective molar ratio and different solids loadings until a homogenous mixture was formed. The liquid fraction of homogenate contained lignin extract and green solvents. In stage 2, the liquid fraction was separated by mechanical squeezing and thorough washing with distilled water until a clear extract was obtained. The homogenate enriched in cellulose and hemicelluloses were air dried. While the lignin extract present in the liquid fraction was precipitated by distilled water. The residual solid fraction was further subjected to enzymatic hydrolysis and ethanol fermentation. In the final stage, the green solvents were recovered from the water by simple distillation process and the recovered green solvents were reused in the next cycle of biomass pretreatment. In this process entire process no waste or by-product was generated (Fig. S8). The cost economics, mass balance and energy and exergy evaluation studies of the proposed process will further clearly demonstrate the techno-economic feasibility of the integrated technology.

4. Conclusion

Acidic green solvents inhibit the cellulase activity and growth of Clavispora NRRL Y-50464 while neutral green solvents had shown no significant effect. Enzymatic hydrolysis of green solvent pretreated biomass produced similar amounts of reducing sugar yields compared to mild-alkali pretreatment and the reducing sugars were efficiently fermented into ethanol in submerged fermentation conditions. Our study demonstrated use of NADES agents and other green solvents for biomass pretreatment towards developing a potential ‘green technology’ in cellulosic ethanol production.

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 Divyanshu Arya, Arya Patel and Ayesha Patel for helping in conducting SEM, FTIR and conductivity experiments. The authors are thankful to Novozymes Inc Ltd for providing Cellic Ctec2. The research work is financially supported by Indian Council of Agricultural Research (ICAR), under All India Co-ordinated Research Project (AICRP) –EAAI and CRP Programs, through the project numbers (VVN/RES/DRET-LBT/2014/3 and VVN/CRP/2015/6) Govt. of India.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jbcb.2016.04.008.

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