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Cellulosic ethanol production from green solvent-pretreated rice straw

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ABSTRACT

Cellulosic ethanol production from green solvent (GS) -pretreated rice straw and the effects of green solvents on cellulase enzyme cellic ctec2 and cellobiose-fermenting yeast strain *Clavispora* NRRL Y-50464 was comprehensively investigated. Using choline chloride/glycerol (CC-GLY) treated rice straw, maximum reducing sugars of 226.7 g/L was obtained with a saccharification efficiency of 87.1% at 20% solids loading and 12 FPU cellic ctec2. An ethanol production of 36.7 g/L was observed from 8% of glucose within 36 h with a conversion efficiency of 90.1%. Incubation of cellulase in green solvents (CC-GLY, choline chloride/1,2-propane diol (CC-PD), choline chloride/ethylene glycol (CC-EG)) at high concentrations (up to 30%, *v/v*) had no inhibitory effect on cellic ctec2. Moreover, CC-GLY and CC-PD at 10% (*v/v*) did not affect the growth rate, sugar consumption and ethanol production from *Clavispora* NRRL Y-50464, while 10% (*v/v*) CC-EG repressed and delayed the cell growth of the microbe. While, strain Y-50464 was highly sensitive in other green reagents that were evaluated in this study. The green solvent-pretreatment efficiently removed lignin from the ricestraw and improved fermentation efficiency significantly. Green solvents with pH < 3.0 inhibited of cellic ctec2 enzyme activity and growth of *Clavispora* strain NRRL Y-50464. When pH of the green reagents was neutralized or adjusted to pH 5.0, normal enzyme activity and cell growth was observed. Moreover, with selective green reagents *i.e.* CC-GLY, CC-PD, and CC-EG, cellulosic ethanol production can be accomplished using cellic ctec2 and strain NRRL Y-50464.

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 *n*atural *d*eep *e*utectic *s*olvents (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 to 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 other 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 heterogenic 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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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 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 cellulosic 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 presized 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 1
List of green solvents tested in this study.

S. No.	Group	Green solvents		Molar ratio	Legend
		Component 1	Component 2		
1	Acidic	Choline chloride	Malic acid	1:1	CC-MA
2		Choline chloride	Citric acid	1:1	CC-CA
3		Choline chloride	Tartaric acid	1:1	CC-TA
4		Choline chloride	Lactic acid	1:5; 1:9	CC-LA
5		Choline chloride	Oxalic acid	1:1	CC-OA
6	Neutral	Choline chloride	Malonic acid	1:1; 1:2	CC-MAL
7		Choline chloride	Ethyleneglycol	1:1	CC-EG
8		Choline chloride	1,2-propane diol	1:1	CC-PD
9		Choline chloride	Urea	1:1	CC-UR
10		Choline chloride	Glycerol	1:1	CC-GLY

2.3. Enzyme stability studies in green solvents

The stability of Cellic Ctec2 enzyme in green solvents was measured by incubating the enzyme solution in different concentrations (0, 0.5, 2.5, 5.0, 7.5, 10, 15%, v/v) of green solvents in citrate buffer (50 mM, pH 5.2) at 37 °C for 48 h. Total cellulase activity (FPase) 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 stabile 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