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# Deep eutectic solvents compatible *Aspergillus niger* cellulase and its utility for *in situ* pre-treatment and saccharification of wheat straw

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ABSTRACT

Deep eutectic solvents (DES) represent an emerging class of ionic liquids with many novel properties and wide industrial applications. Very recently, they are being used as natural solvent systems for lignocellulosic biomass pre-treatment. The present study deals with the stability of *Aspergillus* niger cellulase in three types of DES namely reline (choline chloride and urea), glyceline (choline chloride and glycerol) and ethaline (choline chloride and ethylene glycol) at high concentrations (70%, v/v). The tertiary structural changes in the presence of varying concentrations of DES were monitored by intrinsic fluorescence indicating the maintenance of active site of cellulase in the presence of DES. Further, the applicability of DES stable cellulase was checked for the *in situ* pre-treatment and saccharification of wheat straw. The maximum reducing sugars (~16mg/ml) were generated under the optimised conditions by using ethaline as the chosen DES. Thus, the pre-treatment of wheat straw using DES compatible cellulase in a one-pot process represents an economical feasible approach in the utilisation of lignocellulosic biomass for the generation of biofuels.

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## 1.Introduction

The generation of a green biorefinery addresses the utilisation of agro industrial wastes for the production of biofuels as a replacement of the conventional methods of employing petroleum reserves and other non renewable sources [Budzianowski, 2016]. This cost effective approach seems viable from both academic and industrial point of view, keeping in mind the availability and abundance of the substrates and the need to meet the growing energy demand [Jung et al., 2013; Gao & Rehmann, 2014]. The lignocellulosic biomass (composed of cellulose, hemicellulose and lignin) has immense potential to be used for the generation of biofuels, platform chemicals and various other valuable by-products. However,the recalcitrance of the lignocellulosic biomass often poses problems for its optimal utilisation. Hence, pre-treatment of biomass becomes a necessity in order to remove the lignin-hemicellulose layer, reduce cellulose crystallinity and to increase its accessibility to hydrolases for efficient saccharification [Khare et al., 2015].

Several pre-treatment methods which have been successfully employed in the past are acid, alkali, hotwater, lime, ammonia fibre expansion (AFEX), microwave and steam explosion [Menon & Rao, 2012]. However, some of these conventional pre-treatment methods have led to the generation of adverse conditions and by-products which negatively influenced the sugar yields as well as the down stream processing of biomass. In recent years, ionic liquids (ILs) have been increasingly used for biomass pre-treatment [Zhu et al., 2006]. ILs are defined as salts which remain liquid at room temperature and are composed of an anion and cation portion. These possess certain favourable properties such as negligible vapour pressure, low viscosity, thermal stability, tuneability etc. which makes them highly efficient solvent systems for pre-treatment [Ghandi,2014]. The commonly used ILs which have been employed for biomass saccharification include [EMIM][OAc],[BMIM][MeSO4], [BMIM] [Cl], [MMIM] [DMP]etc. [Qing et al., 2014; Xia et al., 2014; Sadaf et al., 2016; Grewal et al. 2017]. In spite of their superior cellulose solubilising ability and inertness, the utilisation of ILs for scale up processes remains a major bottleneck. The shortcomings of ILs are due to (i) high production costs of ILs (ii) complex recycling procedures and (iii) toxicity towards major saccharifying enzymes [Shi et al., 2013]. Due to latter, the conventional pre-treatment processes advocate the washing of IL spre-treated biomass with anti-solvents like water or ethanol for the complete removal of ILs. The hydrolytic enzymes (cellulases, hemicellulases) are then added for biomass saccharification.

In order to overcome the above drawbacks, an emerging class of ILs termed as "Deep Eutectic Solvents (DES)" have emerged as viable alternatives. DES are composed of quarternary ammonium or phosphonium salts with hydrogen bond donors such as carboxylic acids, sugars, polyols, sugar alcohols, amides etc. [Smith et al., 2014]. The

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various inexpensive DES components commonly used are lactic acid, acetic acid, formic acid, choline chloride, urea, betaine etc. [Sheldon, 2016]. The easy availability and inexpensive nature of the individual components as well as the biodegradability and biocompatibility factors have made DES attractive replacement solvents for conventional ILs in many industrial applications [Zhao & Baker, 2013]. The commonly used DES include, glyceline (choline chloride: glycerol), reline (choline chloride: urea), maline (choline chloride: malonic acid) [Abbott et al., 2011; Zhang et al., 2012]. Considering the biocompatibility of DES towards hydrolytic enzymes, *in situ* pre-treatment and saccharification of lignocellulosic biomass can be carried out in a cost effective manner. The DES stable lignocellulolytic enzymes have the advantage to be put together along with pre-treated biomass and catalyse simultaneous biomass hydrolysis. Procentese et al. [2015] showed the pre-treatment of corn cob using choline chloride based DES leading to the production of 41 g of reducing sugars per 100g substrate. In another study, lactic acid based DES was used for the pre-treatment of rice straw resulting in the production of 333 mg/g reducing sugars [Kumar et al., 2016].

On similar lines, the current work aims to study the compatibility of various DES with cellulase from *Aspergillus niger.* The cellulase was found to be stable in all the screened DES upto 70% (v/v) concentrations. The enzyme was further utilised in the simultaneous saccharification of DES pre-treated wheat straw, which is an abundant under-utilised agricultural waste [Sarkar et al., 2012].

The work encompasses the following (i) stability of *A. niger* cellulase in DES (ii) applicability of this cellulase in the *in situ* pre-treatment and saccharification of wheat straw (iii) studying the structural and morpho-chemical changes occurring in the enzyme and substrate respectively in presence of DES.

## 2. Materials and Methods

### *2.1 Materials*

Cellulase *(Aspergillus niger),* choline chloride and carboxymethyl (CM)-cellulose were sourced from Sigma Chemicals (St. Louis, USA). Urea, ethylene glycol and glycerol were purchased from Fisher Scientific (Waltham, Massachusetts, USA). Ethaline, a mixture of choline chloride and ethylene glycol (1:2 mole ratio), reline, a mixture of choline chloride and urea (1:2 mole ratio) and glyceline, a mixture of choline chloride and glycerol (1:2 mole ratio) were purchased from Scionix Ltd (London, U.K). All other chemicals used were of analytical grade. The biomass sample i.e. wheat straw was obtained locally and sieved to 2 mm particle size. The same lot stored at 4 °C was used throughout the study. The chemical composition of wheat straw (% w/w) was 41.13 ± 0.5 cellulose, 35.7 ± 0.3 hemicellulose, and 13.47 ± 0.5 lignin.

### *2.2 Stability of cellulase in deep eutectic solvents*

The stability of *Aspergillus niger* cellulase (5U/ml) in different DES i.e. glyceline, ethaline and reline was measured by incubating cellulase dissolved in sodium acetate buffer (0.05 M, pH 5.0) with different concentrations (10-70 %(v/v) of DES at 37 °C. The appropriate aliquots were taken at different time intervals and endoglucanase activity was measured spectrophotometrically according to Ghose [1987]. The reaction mixture consisted of 0.5ml CM-cellulose substrate (1% w/v in 0.05M sodium acetate, pH 5.0) and 0.5ml of appropriate enzyme aliquot. The reaction mixture was incubated at 50 °C for 30 min and the amount of reducing sugars released were determined by the dinitrosalicyclic acid (DNS) method using glucose as standard [Miller et al., 1960].

One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute under standard assay conditions. The cellulase enzyme incubated in assay buffer without DES was run as control and its activity was considered as 100 %. The stability of cellulase in the individual components of DES was assessed by following the same protocol and the endoglucanase activity was determined under standard assay conditions as described above.

### *2.3 Intrinsic fluorescence of cellulase in deep eutectic solvents*

The cellulase enzyme incubated with varying concentrations of different DES i.e. glyceline, ethaline and reline for 1h at 37°C was used for fluorescence measurements. The intrinsic fluorescence spectra were recorded on a Perkin Elmer spectrofluorometer using 1 cm path length quartz cuvettes. The excitation wavelength was kept at 295 nm and emission spectra were recorded between 310 and 490 nm. The protein concentration was kept at 50.0 ug/ml and respective blanks containing only buffer and DES were also run simultaneously. The data was recorded in triplicate and averaged.

### *2.4 Pre-treatment and saccharification of wheat straw using deep eutectic solvents*

Wheat straw was pre-treated with different DES (ethaline, glyceline and reline) respectively and then hydrolysed enzymatically without removing DES from the mixture. For the pre-treatment step, 10% (w/w) slurry of wheat straw in respective DBS was prepared. The slurry was heated in an oil bath at 100 °C for 3h with constant stirring. A control was also taken where the biomass was pre-treated with sodium acetate buffer (0.05 M, pH 5.0) instead of DBS. The mixtures were cooled to room temperature. For the subsequent step of enzymatic hydrolysis, the biomass/DES mixture was diluted with sodium acetate buffer (0.05 M, pH 5.0) to obtain 50 % (w/v) of DES. The enzymatic saccharification was initiated by adding cellulase solution (50U/g biomass) and incubating the reaction mixture at 37 °C at 150rpm. The aliquots were taken out at regular time intervals and reducing sugars were estimated by DNS method [Miller et al., 1960].

### *2.5 Optimisation of pre-treatment conditions for biomass hydrolysis*

The effect of temperature and biomass loading during pre-treatment of wheat straw was determined by varying one parameter at a time. The wheat straw was pre-treated at various temperatures between 30-160°C and biomass loading was varied from 5-20 % (w/w). All the other pre-treatment and subsequent saccharification conditions were kept constant as described previously. The amount of reducing sugars released from pre-treated wheat straw after saccharification by cellulase was estimated by DNS method [Miller et al., I960].

### *2.6 Structural changes in wheat straw due to deep eutectic solvents pre-treatment and saccharification*

The ethaline pre-treated wheat straw samples were subjected to scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR) to elucidate the structural changes. To record the surface morphological features of wheat straw before and after pre-treatment, the samples were dehydrated and coated with gold before examining under scanning electron microscope (ZEISS Model EVO 50, UK, NT Delhi facility, New Delhi, India). For FT-IR, the dehydrated samples were mixed with the spectroscopic grade KBr and processed according to the method of Bodirlau & Teaca [2009] using Agilent Technologies Gary 600 Series FTIR Spectrometer. Each spectrum was an average of 32 scans with a spectral resolution of 4 $cm^{-1}$. The scanning range was from 500 to 4000 $cm^{-1}$.

All the experiments were done in triplicate and data shown are the average ± standard deviation from triplicate runs.

## 3. Results and Discussion

### *3.1 The stability of A. niger cellulase in deep eutectic solvents*

Cellulases are important enzymes involved in the hydrolysis of the most abundant polymer present on the earth, cellulose. The ionic liquids (IL) stable cellulases have been in demand for their efficient usage *in situ* biomass saccharification [Grewal et al. 2017]. ILs stable cellulases have been reported to be produced by *Aspergillus fumigatus, Halorhabdus utahensis, Penicillium janthinellum, Pyrococcus horikoshii* and *Thermatoga maritime* [Zhang et al., 2011; Bose et al., 2012; Trivedi et al., 2013]. However, more potent IL stable cellulases with high activities are still required for scale up processes. The reports on the stability of cellulases in DES are very scarce and hence require more research. In the present study, stability of *A. niger* cellulase was evaluated in three different deep eutectic solvents (DES) *viz.* reline, maline and glyceline upto 70% (v/v) concentrations for 72h (Fig.1). The cellulase maintained ~70% residual activity in 70% (v/v) glyceline and ethaline while retaining 50% activity in the presence of reline. Intrigued by these results, the effect of individual components of DES on cellulase activity were also observed. The results suggested (data not shown) that cellulase activity in presence of DES was affected majorly by the hydrogen bond donors like urea, glycerol and ethylene glycol rather than the salt choline chloride. The reports on the effect of individual components of DES are contradictory. A recent study stated that both the hydrogen bond donors, glycerol, urea and salt, choline chloride were individually responsible for inhibiting catalase enzyme [Harifi-Mood et al., 2017]. On the other hand, another study observed that choline chloride salt alone is not toxic [Juneidi et al., 2016].

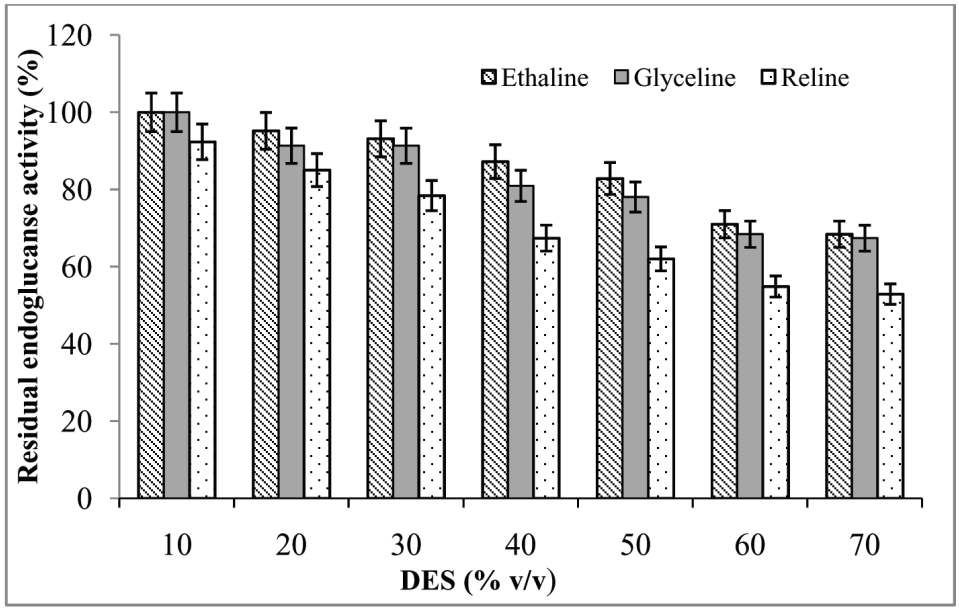


**Fig.1 Stability of *A. niger* ercellulase in deep eutectic solvents**
*A. niger* cellulase (5U/ml) was incubated with different types of DES at varying (10-70%, v/v) concentrations for 72h at 37°C followed by estimation of the residual endoglucanase activity.

### *3.2 Intrinsic fluorescence of A. niger cellulase in the presence of deep eutectic solvents*

To evaluate the tertiary structural changes if any, that might be occurring in the interaction of *A. niger* cellulase with DES, intrinsic fluorescence was performed in the presence of varying concentrations of reline, glyceline and ethaline. A general trend of the decrease in fluorescence intensity was seen as a function of DES concentration (Fig.2). However the decrease was more prominent in the case of reline and glyceline indicating structural perturbations of cellulase. This might be due to the gradual unfolding of the protein assisted with fluorescence quenching (Fig 2A and B). In the case of ethaline, no significant change in the fluorescence intensity was observed suggesting the maintenance of tertiary structure of the protein in this DES (Fig 2C). Deep eutectic solvents are known to affect the secondary and tertiary structure of proteins [Xu et al., 2017]. ChCI/Urea and ChCI/Gly have been observed to cause a red shift in the fluorescence emission spectra of lysozyme [Esquembre et al., 2013]. A recent study conducted on bovine liver catalase showed the fluorescence quenching of protein in the presence of 100 mM glyceline and reline respectively [Harifi-Mood et al., 2017]. The results indicated that ethaline maintains the structure as well as the activity of the cellulase whereas reline and glyceline disturbed the tertiary structure of the cellulase while maintaining the active site of the protein.

### *3.3 Applicability of A. niger cellulase in biomass saccharification*

To evaluate the effectiveness of the DES compatible cellulase system for the hydrolysis of biomass, wheat straw was used as the lignocellulosic substrate. The results showed that the cellulase saccharification of DES pre-treated wheat straw was significantly improved compared to the wheat straw pre-treated with buffer (Fig.3). The maximum reducing sugars were generated within 48h for all the DES, after which the levels became constant. The amount of reducing sugars released were 11 mg/ml, 12 mg/ml, 8 mg/ml in glyceline, ethaline and reline respectively. Out of all the DES, ethaline was further chosen for optimisation of pre-treatment conditions for maximum sugar generation. Recent studies have shown the efficiency of DES in biomass saccharification. Glyceline has been used in the pre-treatment of corn cob with a glucose yield of 96% (Zhang et al., 2016). A eutectic mixture of choline chloride and formate has also been used for the pre-treatment of corn stover with a yield of 99% [Xu et al., 2016]. The use of natural deep eutectic solvents (NADES) containing lactic acid and choline chloride for the pre-treatment of rice straw resulted in generation of 333mg reducing sugars per g rice straw [Kumar et al., 2016].

### *3.4 Effect of temperature and biomass loading on ethaline pre-treatment of wheat straw*

The effect of temperature on wheat straw pre-treatment was observed at temperatures ranging from 30-160 °C. The results showed that on increasing the pre-treatment temperature from 30 °C to 100 °C, the amount of reducing sugars were maximum at 70°C as compared to the control indicating the utility of the process at lower temperatures. The lower energy consumption makes this one-pot bio-process cost effective. A high sugar recovery (almost 80%) at comparatively lower temperatures was considered advantageous in a recent study [Procentese et al., 2015]. The optimisation of biomass (wheat straw) loading showed that the amount of reducing sugars increased with the increase in biomass loading from 10 to 20 % (w/w). The maximum reducing sugars were obtained at 48h with 20 % (w/w) biomass loading. This can be due to the presence of higher amount of substrate availability to the enzyme. A high biomass loading is desirable, so that ILs are less consumed thus making the whole process economically feasible [Ninomiya et al., 2015]. The effective IL pre-treatment with biomass loadings as high as 50% has been reported [Cruz et al., 2013]. Another study, observed the generation of high amount of sugars, 55% glucose and 30% xylose respectively at 50% biomass loading during IL pre-treatment [Wu et al., 2011]. The final optimised curve after ethaline and buffer pre-treatment is shown in Fig.4 representing maximum reducing sugar yield of 16mg/ml.

### *3.5 Morpho-chemical characterisation of wheat straw*

SEM and FTIR were carried out to study the structural changes that occur in the wheat straw after DES pre-treatment and cellulase hydrolysis. The morphology of untreated wheat straw seems to be rigid and regular with a smooth surface (Fig. 5(i)A). On the other hand, the ethaline pre-treated samples were seen to be partially disrupted (Fig. 5(i)B). This was probably due to the removal of lignin and hemicellulose during pre-treatment. Fig. 5(i)C shows a porous and broken surface which might be possibly due to the action of cellulase on the ethaline pre-treated wheat straw. These results support the effectiveness of ethaline pre-treatment with subsequent enzymatic hydrolysis. To further substantiate the changes occurring in wheat straw after ethaline pre-treatment FTIR analysis was carried out. The peak at 1243 $cm^{-1}$ which represents ether bonds between lignin and carbohydrates was seen to reduce in intensity (Fig. 5(ii). This might be due to the removal of lignin wrapped around cellulose fibres [Qing et al., 2014]. The peak at 1732 $cm^{-1}$ which is due to C=0 stretch present in ester bonds between lignin and carbohydrates decreased attributing to the removal of lignin. The absorption intensity of the peak

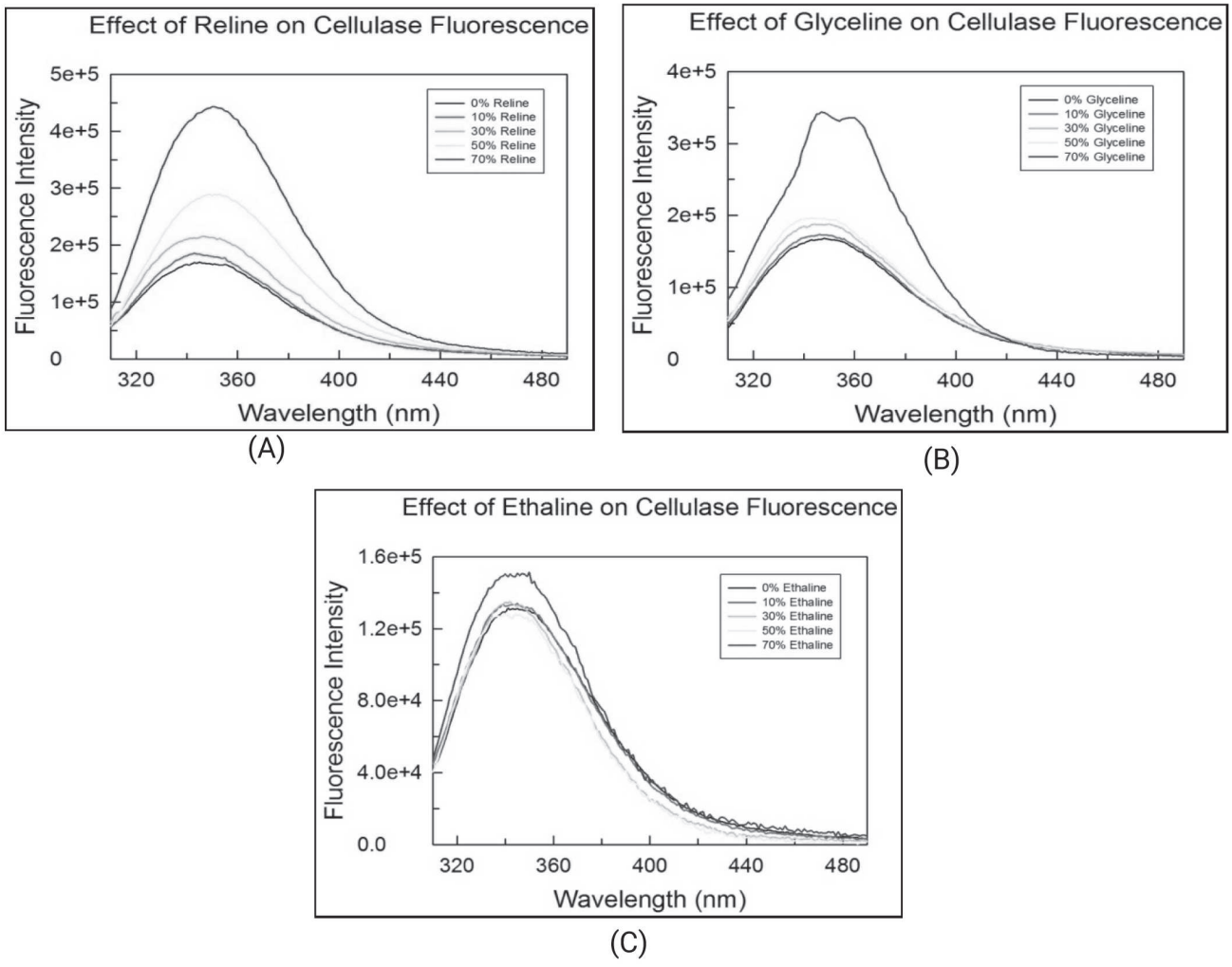


**Fig.2 Intrinsic fluorescence intensity of *A. Niger* rcellulase in the presence of deep eutectic solvents**
*A. niger* cellulase (50ug/ml) was incubated in 0-70% (v/v) concentrations DES at 37 °C for 1h. Sample aliquots were withdrawn and subjected to fluorescence spectroscopy. Fluorescence intensity in the presence of (a) reline (b) glyceline (c) ethaline

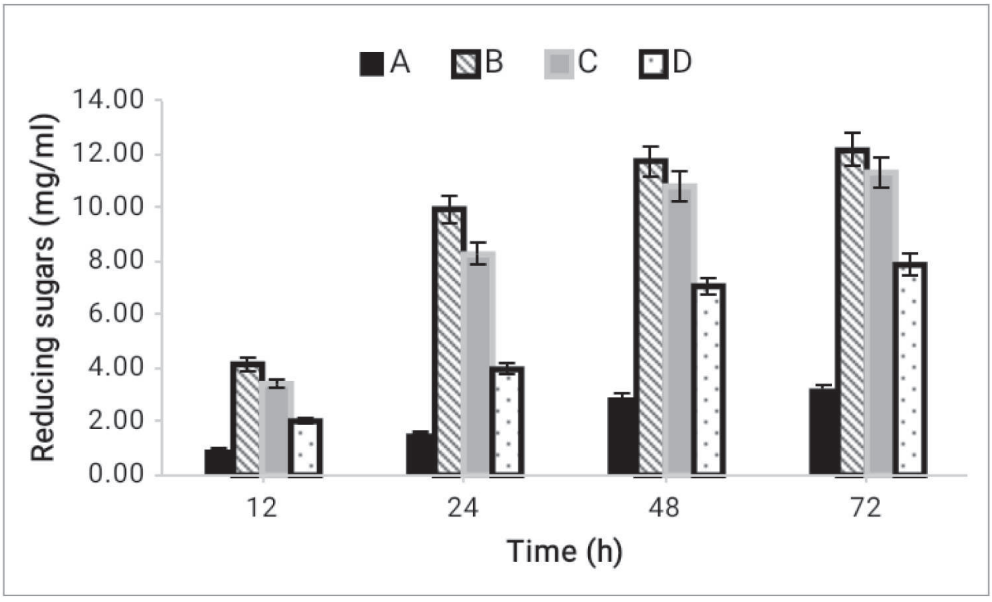


**Fig.3 Application of *A. niger* cellulase in the *in situ* pre-treatment and saccharification of wheat straw**
Wheat straw (0.5g) was pre-treated with ethaline, glyceline and reline (1ml) for 3h at 100°C, followed by addition of cellulase (50U/g wheat straw) for 72h at 37°C. The reducing sugars generated after these pre-treatments were estimated by DNS method.
(A) control (pre-treated with buffer, 50mM sodium acetate, pH 5.0) (B) ethaline pre-treatment (C) glyceline pre-treatment (D) reline pre-treatment

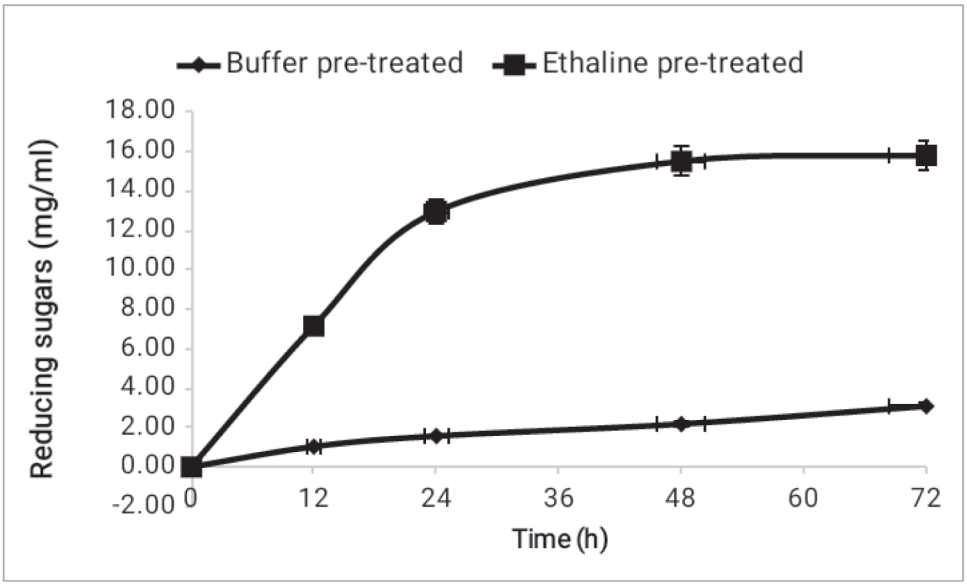


**Fig.4 The effect of optimised pre-treatment conditions on saccharification of wheat straw**
Wheat straw (0.5g) was pre-treated with (i) 1ml ethaline at 70 °C for 2h, followed by the addition of cellulase (50U/g wheat straw) (ii) 1ml buffer (50mM sodium acetate, pH 5.0) for 2h, followed by the addition of cellulase (50U/g wheat straw). The reducing sugars generated were estimated by DNS method.

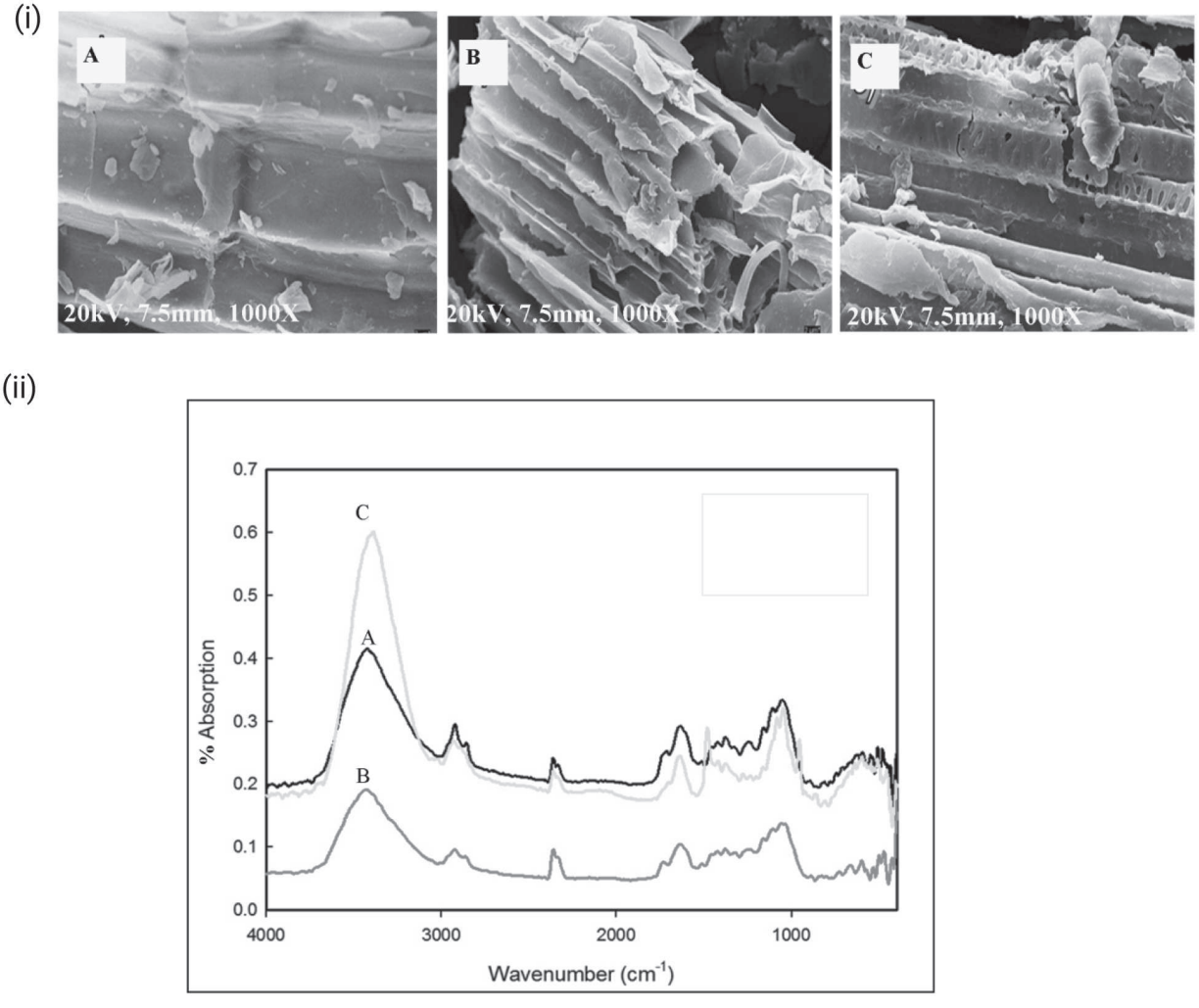


**Fig.5 SEM and FTIR of wheat straw**
The wheat straw was processed and viewed under (i) SEM with 1000x magnification (A) untreated wheat straw (B) ethaline pre-treated (C) etja;pome and cellulase treated ii) FTIR of wheat straw (A) untreated (B) Buffer pre-treated (C) ehaline pre-treated

at 1633 $cm^{-1}$ which represents stretching of acetyl groups of hemicellulose also decreased suggesting the removal of the hemicellulose sheath [Shafiei et al., 2013]. Also, there was an increase in the peak intensity of O-H stretching at 3500 $cm^{-1}$ which can be attributed towards an increase in number of O-H groups due to the cleavage of ether linkages.

Hence, the results of SEM and FTIR confirmed the structural deconstruction changes occurring in wheat straw after pre-treatment with ethaline which led to better accessibility by cellulase for efficient hydrolysis in the subsequent saccharification step.

## 4. Conclusions

The *A. niger* cellulase was found to be stable at high concentrations of DES (70% v/v) *viz.* ethaline, reline and glyceline. The tertiary structural changes occurring in the cellulase in presence of three different DES were monitored by intrinsic fluorescence spectroscopy. The stability of cellulase in DES was exploited for *in situ* pre-treatment and saccharification of wheat straw. The morpho-chemical changes occurring in the wheat straw before and after ethaline pre-treatment were confirmed by FTIR and SEM suggesting the dissolution and deconstruction of biomass and facilitating the efficacy of one-pot bioprocess. The maximum reducing sugars (16mg/ml) were generated after ethaline pre-treatment at 70°C for 3h at 20% (w/w) biomass loading. This process conducted at relatively lower temperatures shows less energy consumption and better utility of DES pre-treatment for biomass derived applications.

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