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Utilising ionic liquids for the *in situ* swelling of Avicel towards enhanced enzymatic saccharification.

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In this study, choline carboxylate ionic liquids were explored as solvents to enhance the hydrolysis of cellulose by enzymes. We found that [Cho][Pn] was the most effective IL, enhancing the activity of the enzyme and thus the production of glucose. Interestingly in [Cho][Pn] a significant portion of glucose was subsequently converted to fructose.

Ethanol is the largest biofuel in production and is derived from first generation feedstock such as sugar and starch crops.[1, 2] Significant progress towards the conversion of lignocellulosic biomass into ethanol is underway however remains a challenge for biorefineries.[3] The use of biomass in biorefineries has the advantage of not being a food source such is the case with sugar cane and corn which may impact the longer term sustainability. Unlike sugar and starch crops, carbohydrates from biomass are more difficult to hydrolyse and as a result, currently yields less product. Two main methods are currently employed, for the hydrolysis of biomass into fermentable sugars. The first and older method uses acids as catalysts, while the second uses enzymes. While enzymes offer more potential, the use of enzymes for the hydrolysis of lignocellulose materials is limited by two major factors; the thermal instability of the enzymes and the need for pre-treatment. Pre-treatment of lignocellulosic materials is often necessary to improve the accessibility of the enzymes to the hydrolysable elements of the lignocellulose material.[2]

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In recent decades, ionic liquids (ILs), which are salts consisting entirely of ions, has gained significant attention due to their unique and tunable chemical and physical properties.[4-7] Once considered a niche and scientifically interesting solvents, due to their versatility, ILs are now finding use in a broad range of fields as well as use in industry as environmentally friendly solvents.[8] The ability of a number of ILs to facilitate the solubility of difficult to dissolve compounds such as cellulose and other biopolymers has offered new opportunities for biomass processing.[9-14] Additionally, ILs have been shown to stabilise biomolecules including enzymes.[15, 16] As such, attempts have been made to combine the pre-treatment and enzyme saccharification process however, when using high concentrations of imidazolium ILs, a lower enzyme activity (when compared to buffered solutions) or complete denaturing of the enzyme has been reported.[17, 18] Some improvements in glucose production using the imidazolium ILs has been achieved employing a multi-step process whereby after the pre-treatment step conducted at elevated temperatures in high IL concentration, the temperature is reduced and buffer is added to significantly reducing the IL concentration before adding the enzyme.[19, 20] Here we explore the dissolution capability of several choline carboxylate ILs as previous reports on some of the choline based ILs showed biocompatibility.[21] Indeed choline dihydrogen phosphate has been shown to stabilise proteins very effectively.[16] In addition choline acetate [Cho][OAc] has been used as an effective solvent for pre-treatment of lignocellulosic biomass.[13, 22] We report improved glucose production utilising a high concentration of the IL - choline propionate [Cho][Pn] which was most likely linked back to both the ability of this IL to stabilise the enzyme. Additionally [Cho][Pn] swells the Avicel, improving the accessibility of the

enzyme to the amorphous regions of the Avicel. We found that in the [Cho][Pn] solution, glucose was converted to fructose even under the mild conditions used here. The ability of the choline carboxylate ILs utilised in this study, to dissolve cellulose was investigated (full experimental given in supplementary information). Avicel (PH-101) was added to the pre-heated IL at 80 °C for choline propionate [Cho][Pn] and choline hexanoate [Cho][Hex] and the mixture was stirred and heated for 1 hour. For [Cho][OAc] the temperature was increased to 90 °C due to the higher melting point of the IL. Visually, it could be seen that dissolution in [Cho][Hex] and [Cho][OAc] had not occurred however in [Cho][Pn], Avicel appeared dissolved. To determine if dissolution had indeed occurred, the samples were subjected to polarised optical microscopy (POM). From the images in Figure 1, it can be confirmed that dissolution occurred only in [Cho][Pn].

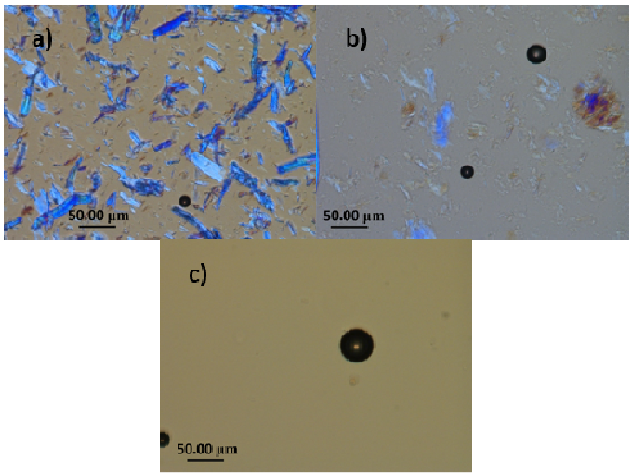

Figure 1: POM images of 5 wt% Avicel after 60 mins at 80° C in a) [Cho][Pn] b) [Cho][Hex] and c) in [Cho][OAc] at 90 °C (due to m.p)

Although the other choline ILs were unable to completely dissolve cellulose, from the POM images it appears that some dissolution has occurred in the [Cho][Hex] and complete dissolution will occur if given enough time while [Cho][OAc] was largely ineffective as has been previously reported [13,22].

Next, the enzyme activity as a function of IL concentration for the various choline ILs was tested. Figure 2 shows the DNS results, the maximum enzyme activity was found to occur at pH 5 and this point denotes 1 in Figure 2. The pH of the IL:water solutions was measured to be pH 7 as such point 0% on Figure 2 is a buffered pH 7 solution. It can be seen that as the concentration of [Cho][Hex] and [Cho][Pn] increased, the measured enzyme activity also increased. A similar trend was not observed for [Cho][OAc] with the relative enzyme activity remaining constant at all [Cho][OAc] concentrations investigated here. We also measured the relative activity of the enzyme in a solution containing 20 wt% 1-butyl-3-methylimidazolium acetate [Bmim][OAc] a well-known cellulose dissolving IL. The enzyme activity in [Bmim][OAc] was lower when compared to the choline ILs used here and increasing the amount of [Bmim][OAc] resulted in zero activity. Included on Figure 2 is the DNS result for 90 wt% [Cho][Pn]: 10 wt% water at a higher temperature of 80 °C. At this temperature and IL concentration, the highest enzyme activity was measured. This was very promising, suggesting that the enzyme remained active at this elevated temperature.

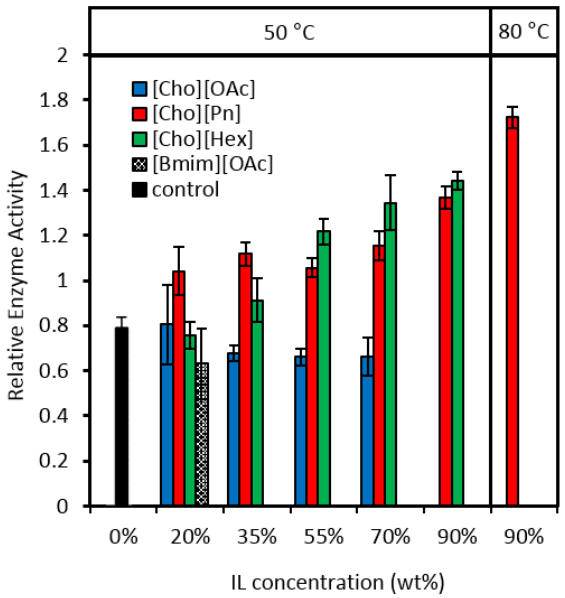

Figure 2: DNS results for the amount of glucose produced upon addition of cellulase (6 U/mL) and an incubation time of 20 mins, sample identification in figure legend. Enzyme activity is represented relative to incubation in sodium acetate buffer (1 denotes sugars produced in NaOAc buffer (pH 5))

The increase in sugars produced by the [Cho][Hex] and [Cho][Pn] as a function of IL concentration maybe linked to improved accessibility for the enzyme to the amorphous regions of the Avicel. At 50° C as more [Cho][Hex] or [Cho][Pn] was added, the Avicel substrate became increasingly swollen, see Figure 3. Swelling of the Avicel substrate was also observed for [Bmim][OAc] however as has been previously reported, this IL is known to denature the enzyme.[17] Little to no swelling of the Avicel substrate was observed for [Cho][OAc].

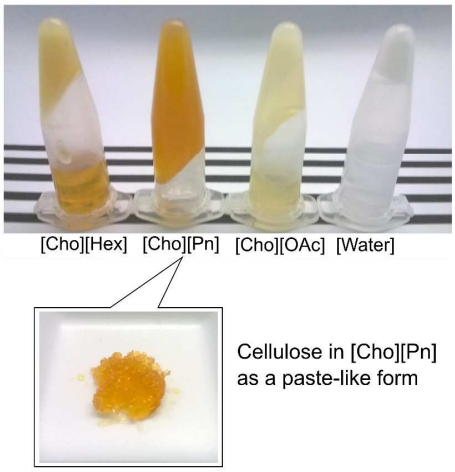

Figure 3: Avicel in 90 wt% IL: 10 wt% water after heating at 50 °C for 20 minutes.

It can be seen that for the [Cho][Pn] case, the Avicel is swollen forming a gel. This ability to swell the Avicel likely improves the enzymes accessibility enhancing hydrolysis.

While the DNS method is recommended by IUPAC commission on biotechnology for measuring standard cellulose activity, the DNS method is not sugar specific and can often led to an overestimation.[23] Therefore to validate the results, we used HPLC to quantify the amount of glucose produced by the enzyme. Figure 4 shows the amount of glucose produced, as measured by HPLC, for various [Cho][Pn] : water concentrations at 50 °C for 20, 40 and 60 minute incubation times: solid lines. The DNS results from Figure 2 showed an increase in sugars produced as the [Cho][Pn] content increased. However, it can be seen that for all [Cho][Pn] concentrations the amount of glucose produced at 50 °C was significantly lower than the buffer solution. Additionally, little difference in glucose produced was measured as a function of [Cho][Pn] concentration. We therefore increased the incubation temperature to 80 °C and measured the glucose produced by a solution containing 90 wt% [Cho][Pn]: 10 wt% water as well as 90 wt% [Cho][Hex]: 10 wt% water at 20, 40 and 60 minute incubations: Figure 4 dotted lines. Increasing the incubation temperature results in a different trend. At 80 °C, the solution containing 90 wt% [Cho][Pn]: 10 wt% water produced significantly more glucose after 20 minutes when compared with the sodium acetate buffer solution. The solution containing 90 wt% [Cho][Hex]: 10 wt% water also produced a higher glucose amount, 15 % higher, after 20 minutes when compared to the sodium acetate buffer. For both IL solutions, the maximum amount of glucose produced occurred within the first 20 minutes. Increasing the incubation time resulted in a decrease in the amount of glucose detected. This was the opposite of what was expected and what was observed in the sodium acetate buffer. It was unlikely that enzyme denaturation was the cause of the reduced glucose rather the more likely scenario was glucose degradation or isomerisation. The absolute amount of glucose produced was low ~14% as the enzyme used in this study is the cellulase variant endo-β-glucanase, specific to hydrolysing amorphous regions of cellulose through random cleavage of β-glucosidic bonds and is inactive to crystalline regions. We selected this enzyme to demonstrate that improvements in the amount of glucose produced are linked to improved accessibility for the enzyme due to the Avicel being swollen by the [Cho][Pn]. It is also plausible that the [Cho][Pn] is disrupting the crystalline regions and increasing the amount of amorphous regions. The Avicel substrate used here is ~83% crystalline as received. Regenerated material from [Cho][Pn] shows a reduction in the degree of crystallinity of the Avicel, however when comparing this regenerated Avicel to regenerated Avicel from [Cho][OAc], larger differences as measured by FTIR were observed in the hydroxyl region 3700-3000 cm^{-1}. The broadening of this peak indicates lower crystallinity, 1106 cm^{-1} (γ(CO), CO stretching), and 1058 cm^{-1} (γ_{as}(ring), anti-symmetric in-phase ring stretching) with the addition of a peak at 1412 cm^{-1} ($\delta_s(CH_2)$, CH_2 symmetric bending) for the [Cho][OAc] regenerated Avicel suggesting [Cho][OAc] was more effective at reducing the crystallinity of Avicel (spectra shown in supplementary information).[24, 25]

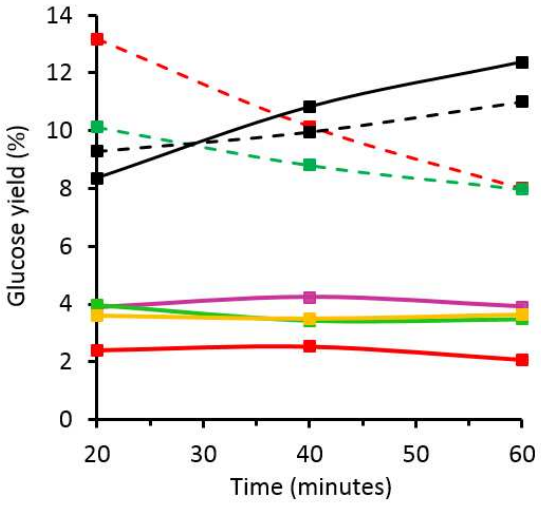

Figure 4: Percentage of glucose detected using HPLC where solid lines represent incubation at 50 ℃, dashed lines represent

incubation at 80 ℃. Black is sodium acetate buffer, red is 90 wt% [Cho][Pn]: 10 wt% water, green is 90 wt% [Cho][Hex]: 10 wt% water, purple 70 wt% [Cho][Pn]: 30 wt% water and yellow 40 wt% [Cho][Pn]: 60 wt% water.

To investigate the decrease in the glucose concentration as a function of time, we monitored the glucose concentration in 90% [Cho][Pn]: 10 wt% water in the absence of Avicel and enzymes at 80 °C. Indeed, again we observed a decrease in the amount of glucose as a function of time. Coupled to the decrease in the amount of glucose, there was an increase in the amount of fructose, as shown in Figure 5.

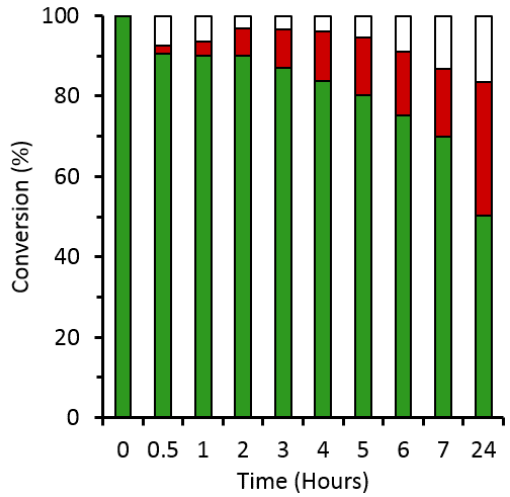

Figure 5: Conversion of glucose to fructose as a function of time in 90 wt% [Cho][Pn]: 10 wt% water solution at 80 ℃. Where green represents amount of glucose and red represents amount of fructose

The base catalysed conversion of D-glucose to D-fructose is well known in carbohydrate chemistry and can be described by the Lobry de Bruyn–Alberda van Ekenstein transformation. Generally associated with the conversion of D- glucose to D-fructose is D-mannose and while additional compounds were present in the HPLC chromatogram, they were not identified as D-mannose, suggesting some other intermediate compounds were formed here.

Conclusions

We report on the use of the biocompatible ILs for the enzymatic saccharfication of Avicel. Of the choline ILs tested, [Cho][Pn] was found to be the most effective, with improved hydrolysis as a function of increased [Cho][Pn] concentration. Increasing the hydrolysis temperature from 50 °C to 80 °C also saw a further enhancement in the amount of glucose produced. The solution containing 90 wt% [Cho][Pn] was found to perform the best. The key to the improved glucose production is likely due to improved enzyme accessibility as we showed that [Cho][Pn] could effectively swell the Avicel. We also found that in the 90 wt% [Cho][Pn]: 10 wt% water solution, at 80 °C the glucose was converted to fructose.

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