Effect of the ionic liquid [bmim]Cl and high pressure on the activity of cellulase

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The effect of the ionic liquid 1-butyl-3-methylimidazolium chloride ([bmim]Cl) and of high pressure on the activity of cellulase from *Aspergillus niger* were studied separately and in combination. The enzyme activity decreased with increasing concentrations of [bmim]Cl, reaching 50% the value in aqueous buffer with 20% [bmim]Cl. However, when the enzyme is held in 10% [bmim]Cl and is then assayed in 1% [bmim]Cl, it showed only 8% reduction of activity. These results can be explained by the fact that the activity of the enzyme in [bmim]Cl is linearly correlated with the decrease of the thermodynamic water activity (a_w). Under pressure the enzyme activity varied from less 60% (at 200MPa) to equal (at 400 MPa), compared to atmospheric pressure. In 10% [bmim]Cl under pressure, cellulase activity is improved compared to atmospheric pressure, varying from equal (at 600 MPa) to 1.7-fold higher (at 100 MPa). This opens the possibility to improve cellulase activity in ionic liquids, and possibly of other enzymes, by carrying out the reaction under pressure.

Introduction

The limited reserves of fossil fuels have increased attention to the use of renewable biomaterials for energy and production of chemicals. However, production of biofuels and chemicals from edible agricultural feedstocks raises problematic questions, since it competes for agricultural land needed for food and feed production.[1] In the last few years, research has focused on the possible feasible use of cellulose, the most abundant renewable bioresource, for the production of fermentable sugars that can be converted to fuels such as ethanol or chemicals such as lactic acid.[1-3] For this possibility to become viable, a limiting and crucial step is the hydrolysis of cellulose to fermentable sugars,[4] due to the protection of the sugar molecules against chemical and enzymatic hydrolysis, by the tight packing of cellulose chains in microfibrils and cellulose's poor solubility in aqueous solutions.

In the last few years ionic liquids (ILs) have emerged as a new class of solvents for biocatalysis, either as single or co-solvents and have attracted increasing attention for research.[5,6] More recently it was found that some ILs can dissolve cellulose,[2,4,7-10] opening up interesting possibilities for the use of these solvents to render cellulose more accessible to enzymatic hydrolysis. The IL 1-butyl-3-methylimidazolium chloride, [bmim]Cl, is indicated in the literature as one of the ILs with a better capacity to dissolve cellulose.[4,10] This has led to several recent publications concerning enzymatic hydrolysis of cellulose after pretreatment with ILs[11,12] or directly (*in situ*) in mixtures of aqueous buffer/ILs.[3,13] However, in the latter case, activity of cellulase has been shown to be lower than in aqueous solutions for several ILs.

The Earth is predominantly a high-pressure environment, with 62% of the total biosphere being characterized by pressures greater than 100 bar (10 MPa).[14] However, information on the effects of pressure on biomolecules is still relatively scarce,[15] being a field of increasing interest. Firstly used in the fields of chemistry and physics, the use of the so called high (hydrostatic) pressure (HP) is now an established method for cold pasteurization of foods, using pressures of about 500 MPa (~5000 atm).[16] HP is also considered a green technology, since it uses water as a compression media and is energetically efficient. In the area of enzymology HP has the potential to change enzymes' activity and selectivity.[17-19] Also, enzymatic catalytic reactions can be carried out under HP in different solvent media as for instance in organic solvents.[20] The effect of HP on an enzymatic reaction rate is governed by the activation volume (*Va*) of the reaction, defined as the overall molar volume difference between the transition state and ground state of the enzyme–substrate complex.[21] If *Va* is positive (negative), the reaction rate is decreased (increased) by pressure, while if it is zero there is no effect.

The present work was initiated with the objective to study the effect of the ionic liquid, [bmim]Cl, and of high hydrostatic pressure up to 675 MPa, used separately and in combination, on activity of cellulase, using carboxymethylcellulose as substrate. Although there are already some publications on the effect of pressure on enzymes' activity in IL, using supercritical carbon dioxide,[22-24] the pressure level used in these cases was around 10 MPa, much below those used in this work (50–675 MPa). As the authors are aware, this is the first study carried out concerning the effect of HP on the activity of an enzyme in the presence of an ionic liquid at these pressure levels. This approach

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can open interesting possibilities by conjugating the peculiar solvent properties of ILs and the effect of HP on enzymatic reactions.

Results and discussion

Effect of [bmim]Cl

In Fig. 1 it can be seen that in 10% [bmim]Cl the activity of cellulase and yield of reducing sugars is lower compared to buffer and the difference increases with the reaction time, reaching less 55% of the reducing sugars formed at 50 min in buffer. Turner *et al.*,[3] also found that activity of cellulase was lower in 0.5 to 1% [bmim]Cl using cellulose azure as substrate. These results may be due to the IL causing enzyme inactivation.

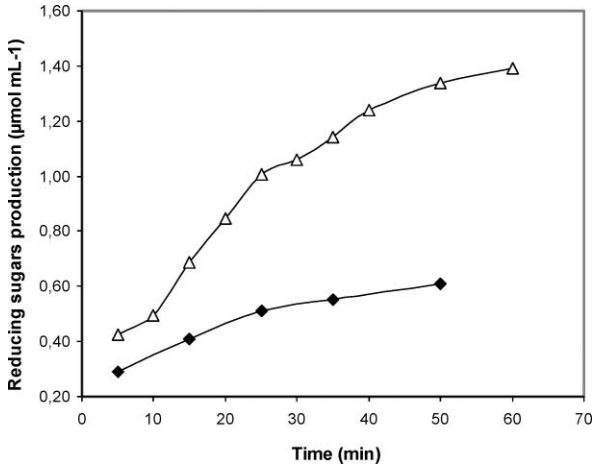

Fig. 1 Reducing sugars production by cellulase in buffer (△) and in 10% [bmim]Cl (♦).

To test for possible inactivation of cellulase in [bmim]Cl, experiments were run in which the enzyme was held in 10% [bmim]Cl for different periods of time, and afterwards an aliquot was taken and added to a solution of 1% CMC in buffer to quantify the activity. This procedure resulted in dilution of [bmim]Cl to 1% in the reaction media.

Fig. 2 shows that there is a linear correlation between cellulase activity and the previous residence time of the enzyme in 10% [bmim]Cl. For 20 min of residence of the enzyme in 10% [bmim]Cl, when activity is measured in 1% [bmim]Cl, there is only 8% reduction of cellulase activity. This value is much lower than the difference verified in Fig. 1, indicating that the decrease of activity verified in 10% of [bmim]Cl is mostly reversible. Similar results were obtained when horseradish peroxidase was transferred from several ILs at different concentrations to buffer with 0.5-2% of the ILs.[25]

Cellulase activity decreases with increasing concentrations of [bmim]Cl, showing about 50% of the activity in buffer in 20% [bmim]Cl for 20 min reaction time (Fig. 3). The reduction of the thermodynamic water activity (a_w) has been shown to be the determinant key parameter for the activity of enzymes in organic solvents, by controlling the hydration level of enzymes.[26] Since [bmim]Cl is a salt and salts decrease the water activity, the a_w value of the different [bmim]Cl solutions was determined using a Rotronic Hygrosckop DV-2 hygrometer (Bassersdorf, Switzerland). The results showed a linear dependence of a_w with [bmim]Cl concentration ($y = -0.0936x + 95.2$, $R^2 = 0.958$). Cellulase activity shows a linear correlation with a_w (Fig. 4, $y = 0.67x - 0.61$; $R^2 = 0.960$). α-Chymotripsin activity in imidazolium-based ionic liquids was shown to be influenced by the hydration level, in a way similar to that in organic solvents.[27] The effect of a_w on cellulase activity explains the results obtained after transference of the enzyme from 10% to 1% [bmim]Cl. Recovery of activity is due to the fact that the enzyme activity in 1% [bmim]Cl is assayed in a higher a_w condition.

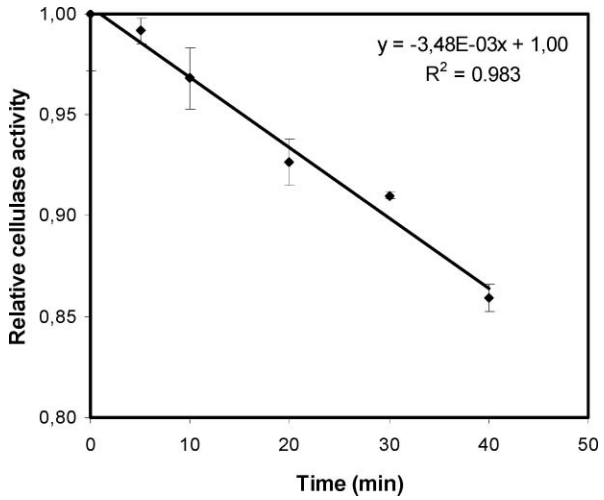

Fig. 2 Relative cellulase activity measured in 1% [bmim]Cl, as a function of the previous residence time of the enzyme in a 10% of [bmim]Cl solution.

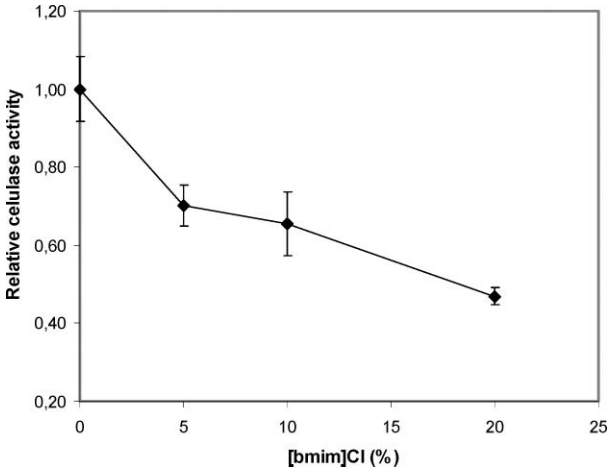

Fig. 3 Relative cellulase activity *vs.* concentration of [bmim]Cl.

Effect of high pressure

To verify whether possible effects of high pressure on cellulase activity were due to changes on CMC and not on the hydrolysis reaction, CMC was subjected to pressure at 300 and 500 MPa at 30 °C for 15 min. Cellulase activity was quantified at atmospheric pressure (during 5, 10, 20 and 40 min) using this high pressure processed CMC as substrate and no effects were observed (results not shown). Also, to verify whether the pressure treatments used could inactivate cellulase, the enzyme

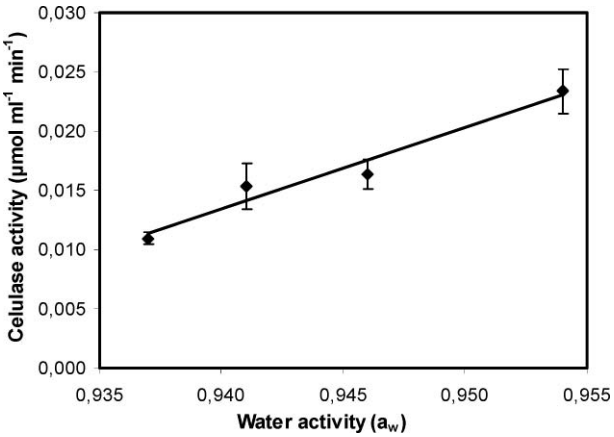

Fig. 4 Cellulase activity *vs.* a_w values of the [bmim]Cl solutions used.

was processed at 400 and 600 MPa at 30 °C for 15 min and no change of activity was verified at atmospheric pressure (results not shown).

Fig. 5 shows that cellulase activity under pressure first decreases (from 50 to 200 MPa), then increases (from 200 to 400 MPa), and then decreases again (from 400 to 675 MPa). At 400 MPa cellulase activity is not affected by pressure, while for the other pressures it varies from 60–80% the value at atmospheric pressure. These results show that cellulase is active under pressure and so has the potential to be used in these conditions. Murao *et al.*[28] reported that while activity of several cellulases at 37 °C was enhanced by pressure (in the range of 200 to 400 MPa), some other cellulases showed lower activity under pressure. For cellulase from *Aspergillus niger* (the same used in the present work) these authors only indicated that the best result was obtained at 400 MPa (115% the activity at atmospheric pressure). However, no activation volumes were reported to mathematically describe the effect of pressure on cellulase activity.

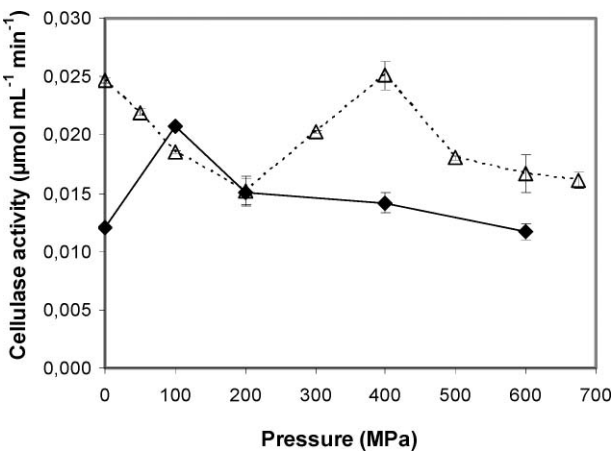

Fig. 5 Effect of high pressure on cellulase activity in buffer (△) and in 10% [bmim]Cl (♦) (the experiments at 300 and 500 MPa without IL and at 400 and 600 MPa with IL, were repeated in a second run and these results are the average of four replicates).

The activation volumes obtained in this work are presented in Table 1, showing that between 0.1 to 200 MPa the activity of the enzyme is inhibited by pressure (Va = 6.17 cm^3 mol^{-1}), while the opposite is verified between 200–400 MPa (Va = −6.33 cm^3 mol^{-1}). Between 500 and 675, the reaction is slightly inhibited by pressure (Va = 1.68 cm^3 mol^{-1}).

Table 1 Activation volumes (*Va*) obtained for activity of cellulase under pressure in buffer and in 10% [bmim]Cl

Pressure range (MPa)	Activation volume/cm^3 mol^{-1}
Buffer	
0.1–200	6.17 (R^2 = 0.990)
200–400	−6.33 (R^2 = 0.991)
500–675	1.68 (R^2 = 0.992)
10% [bmim]Cl	
200–600	1.58 (R^2 = 0.915)

Cellulase activity under pressure in the presence of 10% [bmim]Cl (Fig. 5) was not affected at 600 MPa and was higher at the other pressures studied (respectively, 1.7-, 1.3-, and 1.2-fold at 100, 200, and 400 MPa). At 100 MPa with 10% [bmim]Cl the cellulase activity reaches 85% the value in aqueous buffer at atmospheric pressure.

The activation volume determined for cellulase activity with bmim[Cl], from 200 to 600 MPa was 1.58 cm^3 mol^{-1}, meaning that is this pressure range the reaction is slightly inhibited by pressure.

Overall, while in aqueous buffer the activity under pressure varies from equal to lower the value at atmospheric pressure, in 10% [bmim]Cl it varies from equal (at 600 MPa) to higher (1.7-fold at 100 MPa). This opens good possibilities to improve cellulase activity in ILs using high pressure, as well as other enzymes. The reasons for such an effect of HP in cellulase activity in presence of [bmim]Cl are not known and should be studied in further work.

Conclusions

Cellulase activity in 5–20% [bmim]Cl solutions is lower (30 to 50%) compared to buffer and correlates linearly with the decrease of the thermodynamic water activity (a_w) caused by [bmim]Cl. When cellulase is held in 10% [bmim]Cl and then assayed in 1% [bmim]Cl, activity recovery occurs, the activity being higher, this fact being explained by the higher a_w of 1% [bmim]Cl solution. Under pressure, cellulase activity in buffer varies from 60% to 100% (at 400 MPa) the value at atmospheric pressure. In 10% [bmim]Cl cellulase activity under pressure varies from equal to 1.7-fold higher (at 100 MPa) the value at atmospheric pressure. These results open the possibility to improve cellulase activity, and possibly of other enzymes in ILs, by carrying out the reaction under pressure.

Materials and methods

Lyophilized cellulase (E.C. # 3.2.1.4) from *Aspergillus niger* was purchased from Fluka (St. Louis, MO) and used dissolved in 0.1 M acetate buffer, pH 5.0. Sodium carboxymethylcellulose (CMC) and [bmim]Cl were purchased from Sigma (St. Louis, MO) and Fluka (St. Louis, MO), respectively. Sodium acetate and acetic acid were purchased from Fluka (St. Louis, MO) and used as 0.1 M acetate buffer, pH 5.0. All the other reagents were of analytical grade.

Quantification of cellulase activity

Cellulase activity was determined at 30 °C by the spectrophotometric quantification of the reducing sugars produced, using the 3,5-dinitrosalicylic acid (DNS) method,[29] as glucose equivalents, by means of a standard curve of glucose ($R^2 = 0.996$). Previous experiments confirmed a linear relation between activity and enzyme concentration and reaction time. A solution of 1% CMC was used as a substrate, which was dissolved under continuous stirring, in the acetate buffer solution at room temperature during 45 min. To 450 µL of substrate solution, 35 µL of buffer were added and the reaction started by the addition of 15 µL of enzyme solution (2.5 mg mL^{-1}). After 20 min the reaction was stopped by the addition of 1.0 mL DNS, followed by boiling during 5 min, cooling, addition of 1.0 mL water and measurement of absorbency at 540 nm using a Shimadzu, UV/VIS 1240 spectrophotometer (Kyoto, Japan). In the case of activity of cellulase in [bmim]Cl, the substrate solution consisted of 1% cellulose dissolved in mixtures of buffer and 5–20% [bmim]Cl. Previous experiments indicated that there was no effect caused by the presence of [bmim]Cl on the quantification of reducing sugars.

Cellulase activity under high pressure

Activity of cellulase under high pressure was carried out using a hydrostatic press from Unipress Equipment, Model U33 (Warsaw, Poland), with a pressure vessel of 100 mL (35 mm diameter and 100 mm height), surrounded by an external jacket, connected to a thermostatic bath to control the temperature. Prior to the experiments the temperature of the pressure vessel was equilibrated at 30 °C. The enzymatic reaction was initiated at atmospheric pressure as described above, and immediately after two 400 µl eppendorf tubes were filled with the reaction mixture and capped. The two tubes were then packaged on a heat sealed plastic bag, without air, that was introduced in the pressure vessel and pressure build up was initiated. After the desired pressure had been achieved, the enzymatic reaction continued for 10 min under pressure and then decompression took place. The bag was taken out of the pressure vessel and transferred to the water bath at 30 °C to complete the 20 min reaction time, after which the reaction was stopped and the activity quantified at atmospheric pressure.

Activation volume calculation

The effect of pressure on the velocity of the enzyme catalysed reaction is described by the activation volume (*Va*) calculated by the Eyring equation:

$$\ln(V) = \ln(A) - \frac{PVa}{RT} \quad (1)$$

Where P is the pressure (MPa); v is the activity of the enzyme (µmol min^{-1} mL^{-1}); *Va* is the activation volume (cm^3 mol^{-1}); T is the absolute *T*/K, A is a constant, and R (8.314 cm^3 MPa^{-1} mol^{-1} K^{-1}) is the universal gas constant. The activation volumes were calculate by linear regression analysis.

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