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ORIGINAL PAPER

Enzymatic hydrolysis of *Asclepias syriaca* fibers in the presence of ionic liquids

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Abstract Attempts have been made to enhance *Asclepias syriaca* (As) fiber saccharification by cellulase in the presence of ionic liquids. Three ionic liquids (1-butyl-3-methylimidazolium chloride, [BMIM]Cl; 1-ethyl-3-methylimidazolium chloride, [EMIM]Cl; and 1-butyl-4-methylpyridinium chloride, [BMP]Cl) were used. In comparison with conventional cellulose pretreatment processes, the ionic liquids were used under milder conditions corresponding to the optimum activity of cellulase. The structural modification and thermal properties of As fibers were analyzed by DSC, TG, DTG, and FT-IR. As fibers were hydrolyzed by cellulase more easily in aqueous ionic liquid media. For As fibers hydrolyzed in the presence of ionic liquids, saccharification rate during the first 3 h of the hydrolysis reaction was significantly improved, especially when the fibers were hydrolyzed in the presence of [BMP]Cl. FT-IR spectra revealed that As fibers undergo modification during enzymatic hydrolysis in the presence of ionic liquid. The increased rate of hydrolysis was accompanied by an increase in the degree of crystallinity of the As fibers. It was apparent from thermal analysis that the well ordered crystalline phase and the intermolecular regularity of carbohydrates obtained from the As fibers considered in this study were affected by the ionic liquid through changing of the fiber structure.

Keywords Carbohydrates · Enzymes · Ionic liquids · IR spectroscopy · Thermochemistry

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Introduction

Cellulose is a major fraction of plant biomass. It is the most abundant renewable resource in the world, and can be useful in conventional petrochemical refineries in an economy based on renewable resources [1, 2]. In its natural state, cellulose is highly crystalline in structure with individual cellulose polymer chains held together by strong hydrogen bonding and van der Waals forces. The individual cellulose chains are linear condensation polymer molecules made up of anhydroglucose units joined together by β-1,4-glycosidic bonds [3] with degrees of polymerization ranging from 1,000 to 15,000 units. In general, neither water molecules nor catalysts of hydrolysis (for example cellulase enzymes) are able to easily penetrate the crystalline matrix [4].

Enzymatic hydrolysis of cellulose is a heterogenous catalytic process in which the enzymes are adsorbed by cellulose surfaces in order to affect hydrolysis. The process is controlled by two stages: the adsorption of enzymes on cellulose particles, and the formation of enzyme–substrate complexes, both being associated with several enzyme- and substrate-related factors [5]. Enzyme-related factors include inhibition by products (cellobiose and D-glucose), thermal stability, synergism, and adsorption. Substrate-related factors include the presence of hemicelluloses and lignin, cellulose crystallinity, the degree of polymerization, and accessible external and internal surfaces of cellulose. To make cellulose more susceptible to hydrolysis, pretreatment is needed to reduce the crystallinity and increase the porosity of the cellulose [6].

Different pretreatment methods can be used to enhance the digestibility of cellulose materials [7]. Pretreatment methods are generally classified into biological, physical, chemical, and physicochemical, and require extreme

conditions, for example high temperatures and pressures or strong acids or bases making special equipment necessary, or a long residence time. A cost-effective pretreatment strategy to improve cellulose hydrolysis is, therefore, still required.

The application of ionic liquids (ILs) as solvents in carbohydrate chemistry has recently been reviewed [8, 9]. Some ionic liquids, especially those containing the Cl^- anion, dissolve cellulose [10]. Ionic liquids have the ability to dissolve large amounts of cellulose under very mild conditions. The feasibility of recovering nearly 100% of the ILs used, in their initial purity, makes them attractive [11–13]. Recently, cellulose solubilities of up to 39, 25, and 10% (*w*/*w*) have been reported for the ILs 3-methyl-*N*-butylpyridinium chloride [11], 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) [14], and 1-allyl-3-methylimidazolium chloride ([AMIM]Cl) [15], respectively.

Milkweed is an industrial crop. The seed pods are harvested for their floss, which is a good thermal insulator and is being produced commercially as a hypoallergenic fiber filler for comforters and pillows. Milkweed seeds end up as by-products of floss production and have limited applications as plants for landscaping and erosion control. Seeds contain 21% oil and 32% crude protein (dry basis) [16]. Milkweed seed protein has functional properties that may find use as a thickener, protein extender in adhesives, or emulsifier in paints [17].

In addition to the seed hairs, milkweeds produce long, quite strong, but brittle bast fiber in their stems. These fibers can be used as a substitute for wood in pressed panels. The possibility of using *Asclepias syriaca* (As) fibers for paper manufacture has also been reported [18, 19].

In our study, As fibers were used as substrate for enzymatic hydrolysis with cellulase in the presence of the ionic liquids 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl), 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), and 1-butyl-4-methylpyridinium chloride ([BMP]Cl. The extent of hydrolysis was estimated by determination of the soluble sugars released during saccharification. Modifications of the As fibers were studied by FT-IR spectroscopy and thermal investigation by simultaneous TG/TDG/DSC analysis.

Results and discussion

Hydrolysis of As fibers

Studies have revealed that one advantage of dissolving cellulose in an ionic liquid is reduction of the degree of crystallinity and a positive effect on the hydrolysis rates. This hypothesis is based on the fact of increased availability and the amorphous nature of the cellulose chains during enzymatic treatment. Thus, the cellulose would be more amenable to hydrolysis by the enzymes and hence the reaction rate is increased. It has been proved that the structure of cellulose fibers regenerated from used ionic solutions by addition of water is less crystalline than the original untreated cellulose [20].

The efficiency of the increase in As fiber saccharification by cellulase in the presence of ionic liquid was investigated. As shown in Fig. 1a, b, the rate of hydrolysis was improved.

An increased rate of hydrolysis was observed for As fibers treated with [BMIM]Cl, the saccharification yield being greater than that obtained from untreated As fibers after 24 h (Fig. 1a). The rate of enzymatic hydrolysis was significantly increased by treatment of the As fibers with [BMP]Cl, as is apparent from Fig. 1b.

The solid-state structures, including crystalline and non-crystalline structures, have a large effect on carbohydrate hydrolysis kinetics. Thus, it seems that As fibers hydrolyzed in the presence of ionic liquids were less crystalline and adsorbed more cellulase than the reference. Thus, the As fibers were hydrolyzed by cellulase much faster in the presence of ionic liquid. After 120 h of hydrolysis, the extent of hydrolysis of As fibers treated with [BMP]Cl was greater than saccharification conversion achieved using [EMIM]Cl or [BMIM]Cl. Enhancement of the efficiency of As fiber saccharification

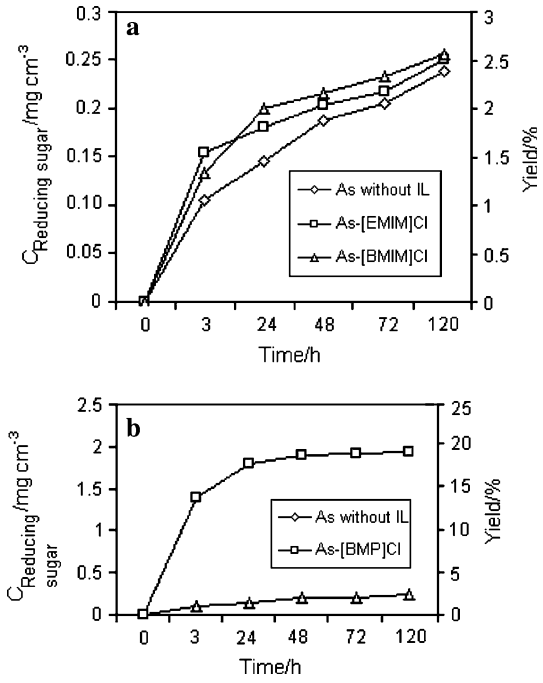

Fig. 1 Effect of ionic liquids on the rate of enzymatic hydrolysis of As fibers. Substrate samples were incubated with cellulase (30 FPU/g substrate) and ionic liquid for 120 h at 40 °C and pH 4.8: **a** [EMIM]Cl and [BMIM]Cl; **b** [BMP]Cl

by cellulase in the presence of the ionic liquids was in the order [BMP]Cl > [BMIM]Cl > [EMIM]Cl.

FT-IR spectroscopy

Figure 2 shows the FT-IR spectra of *Asclepias syriaca* fibers before and after enzymatic hydrolysis for 120 h without and with ionic liquid. The IR spectra in the 800–1,800 cm^{-1} region were used to characterize the nature of As fiber hydrolysis in the presence of ionic liquids. The 1,430, 1,160, and 897 cm^{-1} absorption bands can be used to study the type of crystalline cell and the crystallinity changes, because these bands in the spectrum of crystalline cellulose I differ clearly from those in the spectra of cellulose II and amorphous cellulose. The absorption band at 1,430 cm^{-1} is assigned to the CH_2 symmetric bending in cellulose, being specific to the mixture of crystalline cellulose I and II. The absorption band at 897 cm^{-1} is assigned to C–O–C stretching at the β-(1 → 4)-glycosidic linkage in cellulose I [21].

As shown in Fig. 2a, the absorption band at 1,430 cm^{-1} was strong for untreated As fibers, but weak for treated fibers. An increase in the width of this band would represent the increase in the cellulose I content and the decrease in cellulose II. Furthermore, the most significant and best defined absorption band, that at 897 cm^{-1}, corroborated the presence of crystalline cellulose I. This absorption band was intense for untreated As fibers but weak for those treated with [BMIM]Cl (Fig. 2b).

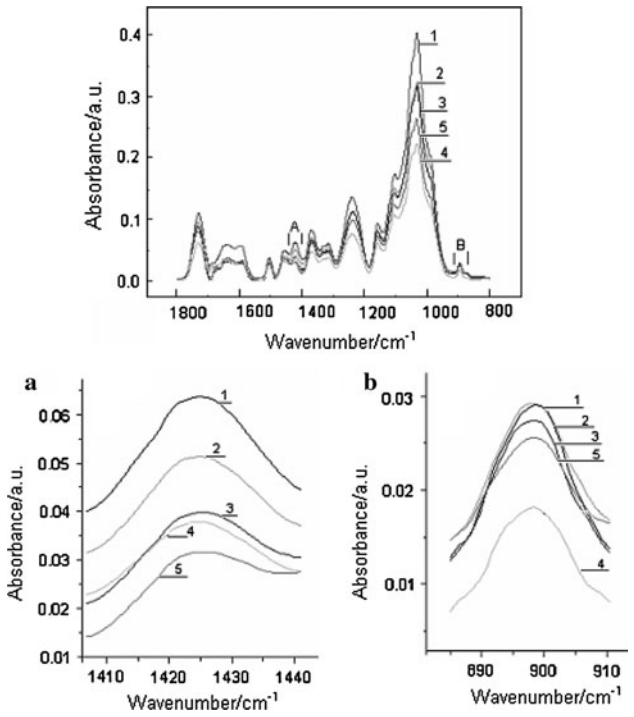

Fig. 2 FT-IR spectra of As before hydrolysis (*1*); and after enzymatic hydrolysis for 120 h in the absence (*2*) and presence of the ionic liquids: [EMIM]Cl (*3*); [BMIM]Cl (*4*); [BMP]Cl (*5*); **a** zoom of the 1,410–1,440 cm^{-1} area, **b** zoom of the 890–910 cm^{-1} area

The spectra shown in Fig. 2 indicate that the untreated As fibers have a cellulose I crystal type. This is in accord with the well-known fact that almost all native celluloses in the higher plants have the crystal structure of cellulose I. The cellulose I structure was transformed into the amorphous or cellulose II structure after As fibers were hydrolyzed in the presence of ionic liquid.

Absorbance at 1,430 and 897 cm^{-1} is quite sensitive to the crystal structure of cellulose in lignocellulosic materials. Thus, the absorbance ratio A_{1430}/A_{897}, which is known as the crystallinity index [22] or the lateral order index (LOI), has been used to reflect the cellulose I fraction in the structure of cellulosic material [23].

The FT-IR spectra show characteristic cellulose peaks around 1,000–1,200 cm^{-1} [24, 25]. The band near 1,160 cm^{-1} is representative of the anti-symmetric bridge stretching of C–O–C groups in cellulose and hemicelluloses, and the band near 1,318 cm^{-1} can be ascribed to CH_2 wagging vibrations in cellulose and hemicelluloses.

The FT-IR results showed that peaks of cellulose materials were lower after enzymatic hydrolysis, especially in the presence of ionic liquids, indicating that some of the cellulose was degraded. The band at 1,635–1,640 cm^{-1}, which has been attributed to the bending vibrations of absorbed water, significantly decreased after enzymatic hydrolysis in the presence of ionic liquids. Another two infrared ratios were calculated:

1. A_{1372}/A_{2900}, which is known as the total crystallinity index (TCI) [21]; and
2. A_{3308}/A_{1330}, known as hydrogen-bond intensity (HBI) [23].

The values obtained, listed in Table 1, are closely related to the crystal system and degree of the intermolecular regularity.

A high index value indicates that the material has high crystallinity and an ordered structure. As shown in Table 1, after enzymatic hydrolysis the LOI decreased for As fibers from 2.41 to 1.72 without ionic liquid, and to 1.14–1.40 in the presence of ionic liquid. The TCI of the samples increased significantly after enzymatic hydrolysis in the presence of ionic liquids (Table 1). This indicates that part of the crystalline structure of cellulose was transformed into the amorphous form in the presence of ionic liquid. As a consequence, the fragmental and porous cellulose materials with amorphous structure could provide more surfaces for enzymes to attack. This is confirmed by the increased enzymatic hydrolysis of the cellulose in the presence of ionic liquid.

Table 1 Crystallinity indices and hydrogen bonding intensity of As fibers before and after enzymatic hydrolysis

Sample	TCI (A_{1372}/A_{2900})	LOI (A_{1430}/A_{897})	HBI (A_{3308}/A_{1330})
As initial, non-hydrolyzed	0.84	2.41	5.35
As/EH without ionic liquid[a]	0.96	1.72	4.38
As/EH in [EMIM]Cl	1.04	1.40	4.10
As/EH in [BMIM]Cl	0.98	1.14	4.73
As/EH in [BMP]Cl	1.06	1.19	3.86

[a] EH means enzymatic hydrolysis after 120 h

LOI and HBI are significantly lower than those calculated before enzymatic hydrolysis. The lowest values were obtained for As fibers after enzymatic hydrolysis in the presence of [BMP]Cl. The values were affected by the presence of ionic liquid in the reaction medium in the order:

TCI: [BMIM]Cl < [EMIM]Cl < [BMP]Cl
LOI: [BMIM]Cl < [BMP]Cl < [EMIM]Cl
HBI: [BMP]Cl < [EMIM]Cl < [BMIM]Cl

Thus, the well ordered crystalline phase and the degree of intermolecular regularity of carbohydrates from the substrate considered in this study were affected by the presence of ILs in the reaction medium through changing of the fiber structure.

Thermal analysis

Thermograms of the As fibers are shown in Fig. 3. The degree of crystallinity of the cellulosic material was shown to affect the shape of the thermograms. The increase of degradation temperature for As fibers hydrolyzed in the presence of ionic liquids is related to the intense crystalline region which undergoes significant changes.

In the DSC curve obtained from dried non-hydrolyzed As fibers heated under nitrogen (Fig. 3a), two endothermic peaks were observed around 220 and 344.9 °C and an exothermic process was registered at 309.8 °C, corresponding to the thermal decomposition of cellulose. The maximum decomposition of non-hydrolyzed As fibers was recorded as 48.35% weight loss at 343.5 °C.

In the DSC curve of As fibers hydrolyzed without ionic liquid (Fig. 3b) an endothermic process occurred at around 371.8 °C. This resulted in approximately 52% weight loss, as shown by the pattern of the TG line. Two endothermic transitions in the DSC curve of As fibers hydrolyzed in the presence of [EMIM]Cl were observed around 220 and 369 °C (Fig. 3c). A weight loss of 50% is recorded at 365.7 °C. In the DSC curve of As fibers hydrolyzed in the presence of [BMIM]Cl (Fig. 3d), two significant endothermic peaks were observed around 265.0 and 372.2 °C. A 47.5% weight loss up to 372 °C is apparent on the TG curve; this effect corresponds to cellulose decomposition. In the DSC curve of As fibers hydrolyzed in the presence of [BMP]Cl (Fig. 3e), the endothermic peak at around 354.0 °C could be assigned to thermal decomposition of cellulose. At 368.2 °C a weight loss of 48% was noted as an effect of an exothermic process. In all cases, the first endothermic effect corresponding to water removal during the thermal decomposition process appeared at around 80 °C.

Thermal analysis of As fibers hydrolyzed in the presence of ionic liquid revealed a decrease in weight loss of 2–4% at the maximum decomposition temperature; this is evidence of increased hydrolysis of As fibers in the presence of ionic liquids compared with fibers hydrolyzed without ionic liquid.

Experimental

Materials

Acetic acid, sodium acetate dihydrate, 3,5-dinitrosalicylic acid (DNS), sodium hydroxide, sodium potassium tartrate (Rochelle salt), phenol, sodium metabisulfite, and ethanol were obtained from Sigma–Aldrich (Germany). Ionic liquids [EMIM]Cl, [BMIM]Cl, and [BMP]Cl (Fig. 4) were obtained from Fluka and were used without further purification. Cellulase from an *Aspergillus niger* strain was supplied by BioChemika, Fluka. The *Asclepias syriaca* (As) fibers were used without preliminary treatment.

Enzymatic hydrolysis

The As fibers were hydrolyzed without or with ionic liquid in vials using a WNB 7-45 water bath. The total vial volume was 5 cm^3 and the As fiber concentration 2 mg/cm^3. An As fibers-to-ionic liquid ratio of 1:15 (*w*/*w*) was used and cellulase addition of 30 FPU/g As fibers was used in the hydrolysis reaction. The mixture was buffered with 50 mM acetic acid–sodium acetate solution, pH 4.8. The hydrolysis reaction was carried out at 40 °C for 120 h. All As fiber samples were hydrolyzed using the same cellulase stock solution. The release of soluble reducing sugars was periodically measured by use of the DNS assay.

Analysis

Chemical analysis of As fibers was performed according to TAPPI (the leading association for the worldwide pulp, paper, packaging and converting industries) methods. The milkweed fibers were composed of 54.9% cellulose, 8.0% hemicelluloses, 19.3% lignin, and 0.9% ash.

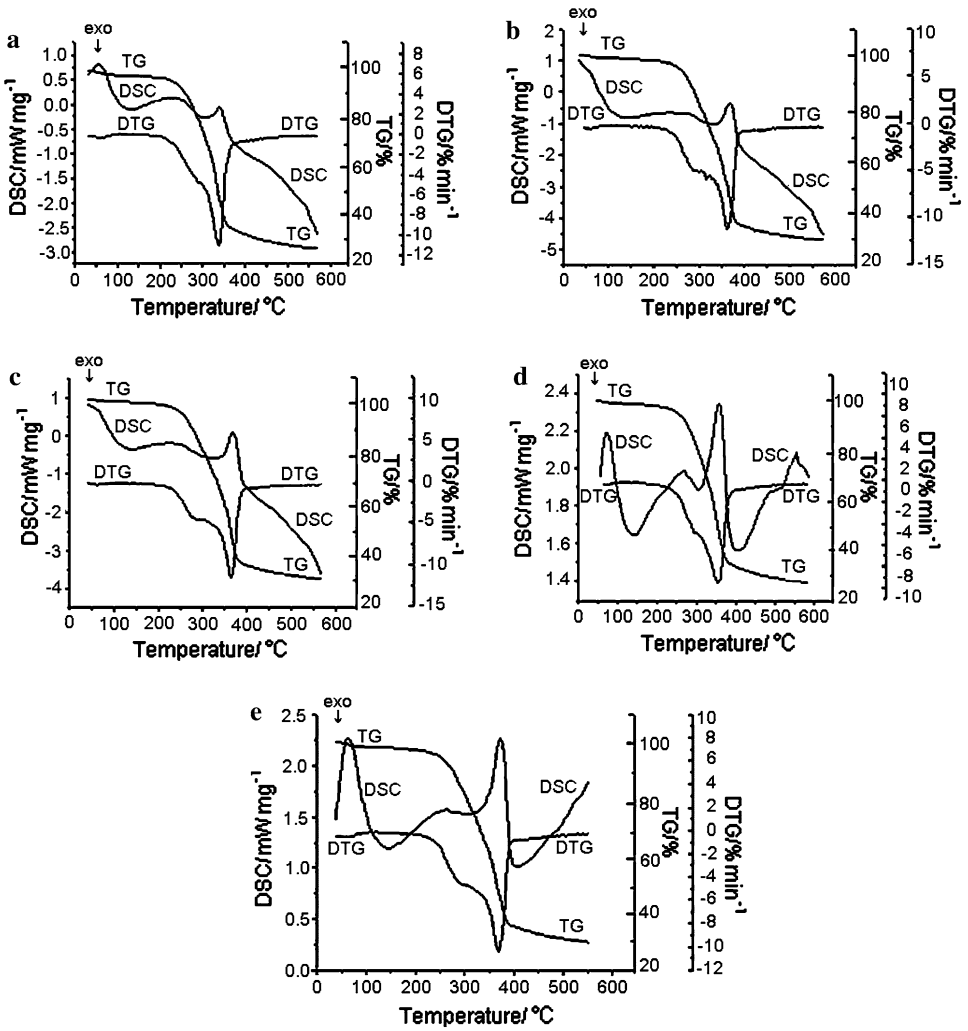

Fig. 3 TG/DTG/DSC curves for non-hydrolyzed As fibers (**a**); for fibers hydrolyzed without ionic liquid (**b**); and for fibers hydrolyzed in the presence of [EMIM]Cl (**c**), [BMIM]Cl (**d**), and [BMP]Cl (**e**)

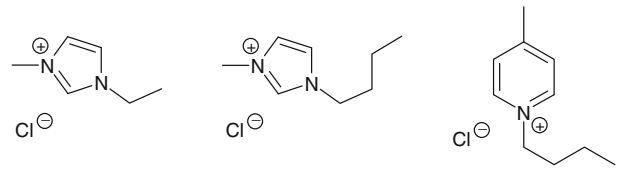

Fig. 4 Structures of the ionic liquids (ILs) used in the hydrolysis reaction

Reducing sugar was measured by the DNS assay [26], with glucose as standard. Each hydrolysate sample was analyzed in triplicate. Cellulase activity was determined by the standard filter paper assay and expressed as filter paper units per gram (FPU) [27]. One FPU is defined as the amount of enzyme that releases 1 μmol glucose equivalents per minute from Whatman No. 1 filter paper.

The color intensity of the hydrolysate samples was measured by use of a Cole-Palmer spectrophotometer at 540 nm. Yield of reducing sugars from treated As fibers was calculated by use of Eq. 1 as follows:

$$\text{Yield}\,(\%) = (\text{reducing sugars weight}/\text{dry substrate weight}) \times 100 \quad (1)$$

Fourier transform infrared spectroscopy (FT-IR) was performed with a Bruker Vertex 70 spectrophotometer. The spectra (4,000–400 cm^{-1}) were recorded with a resolution of 4 cm^{-1} and 64 scans per sample. Samples were prepared by mixing approximately 2.0 mg with 120 mg of spectroscopic grade KBr and pressing to produce 13 mm diameter pellets.

TG/DTG/DSC analysis was performed using a Netzsch STA 449 F1 Jupiter system under nitrogen atmosphere. The STA system combines the DSC and TG methods and accomplishes the measurement of heat flow and mass change under completely identical conditions. Samples (~5 mg) were placed in Al_2O_3 crucibles hermetically closed with lids then heated at 10 °/min from room temperature to 600 °C, with nitrogen as purging gas at a flow

rate of 50 cm^3/min. TG and DSC curves recorded with ±0.5 °C precision were analyzed using Netzsch Proteus analysis software.

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