Enzymatic membrane reactor for full saccharification of ionic liquid-pretreated microcrystalline cellulose

Pedro Lozano, Berenice Bernal, Antonio G. Jara, Marie-Pierre Belleville

PII: S0960-8524(13)01654-4
DOI: http://dx.doi.org/10.1016/j.biortech.2013.10.067
Reference: BITE 12570

To appear in: *Bioresource Technology*

Received Date: 9 September 2013
Revised Date: 16 October 2013
Accepted Date: 18 October 2013

Please cite this article as: Lozano, P., Bernal, B., Jara, A.G., Belleville, M-P., Enzymatic membrane reactor for full saccharification of ionic liquid-pretreated microcrystalline cellulose, *Bioresource Technology* (2013), doi: http://dx.doi.org/10.1016/j.biortech.2013.10.067

Title:

ENZYMATIC MEMBRANE REACTOR FOR FULL SACCHARIFICATION OF IONIC LIQUID-PRETREATED MICROCRYSTALLINE CELLULOSE

Authors:

Pedro Lozano[1]*, Berenice Bernal[1], Antonio G. Jara[1] and Marie-Pierre Belleville[2]

Affiliations:

[1] Departamento de Bioquímica y Biología Molecular B e Inmunología. Facultad de Química, Universidad de Murcia, P.O. Box 4021, E-30100 Murcia, Spain.

Fax: +34-868.88.41.48; Phone: +34-868.88.73.92; Email: plozanor@um.es

[2] IEM (Institut Européen des Membranes), UMR 5635 (CNRS-ENSCM-UM2), Université Montpellier 2, Place E. Bataillon, F- 34095 Montpellier, France.

Corresponding author:

Pedro Lozano

Departamento de Bioquímica y Biología Molecular B e Inmunología. Facultad de Química, Universidad de Murcia, P.O. Box 4021, E-30100 Murcia, Spain. Fax: +34-868.88.41.48; Phone: +34-868.88.73.92; Email: plozanor@um.es

Highlights

- Regenerated cellulose permitted full enzymatic hydrolysis in membrane reactors
- Membrane reactors with cellulase and cellobiase provided clear glucose solutions
- Polymeric or ceramic membranes provided constant profiles in glucose production

Abstract

Ultrafiltration reactors based on polymeric or ceramic membranes were shown to be suitable catalytic systems for fast enzymatic saccharification of cellulose, allowing the full recovery and reuse of enzymes. By pre-treating cellulose with the IL 1-butyl-3-methylimidazolium chloride, the suitability of this substrate for enzymatic saccharification in a reactor based on polymeric ultrafiltration membranes was demonstrated, leading to 95% cellulose hydrolysis in 4 hours at 50 ºC. The filtration process gave a clear glucose solution (up to 113 mM) at constant permeate flow (24.7 L h^{-1} m^{-2}), allowing the enzyme to be reused for 9 operation cycles under semi-continuous operation, without any loss of enzyme activity. Under continuous operation mode and using ceramic ultrafiltration membranes at different residence times, the enzymatic reactor showed constant profiles in both the permeate flow rate and the glucose concentration, demonstrating the excellent suitability of the proposed approach for the saccharification of cellulose.

Keywords: Cellulose Saccharification, Membrane Reactor, Cellulase, Ionic Liquids, Biofuels.

1. Introduction

The use of non-edible lignocellulosic biomass for producing second generation bioethanol through clean and sustainable approaches, where the hydrolysis of cellulose into fermentable sugars is key step, is one of the greatest challenges on the research and industrial agenda. However, despite the fact that cellulose contains a small amount of relatively easily accessible amorphous regions with few lateral interactions between the polysaccharide chains, they mainly consist of crystalline domains that are supported by multivalent inter- and intramolecular hydrogen bonds, involving a high recalcitrance to its depolymerization in glucose units (Bommarius *et al.,* 2008; Mizuno *et al.,* 2012; Brandt *et al.,* 2013; Hamada *et al*., 2013).

The most commonly used approaches to carry out cellulose hydrolysis are chemical (using dilute and concentrated acids) and enzymatic hydrolysis. The enzymatic hydrolysis of cellulose has several advantages over acidic hydrolysis because of the high specificity of the biocatalyts used to break the β(1 → 4) glycosidic bonds, avoiding the undesired transformation of glucose into furfurals, which act as inhibiting by-products in the subsequent fermentation step for producing bioethanol. The full depolymerization of cellulose to its glucose units can be carried out by the cellulase complex, involving the synergistic action of endo-1,4-β-D-glucanases (EGs, EC 3.2.1.4), exo-1,4-β-D-glucanases or cellobiohydrolases (CBHs, EC 3.2.1.91), and beta-glucosidases (EC 3.2.1.21) (Rosgaard *et al*., 2007; Lehmann *et al*., 2012). The EGs cleave glycosidic bonds, preferentially in amorphous cellulose regions, to generate reactive ends for CBHs, which act progressively to degrade cellulose from either the reducing (CBH I) or non-reducing (CBH II) ends, to generate mainly cellobiose. At high concentrations, cellobiose inhibits CBH activity, and the presence of beta-glucosidase (cellobiase) to convert cellobiose into glucose is necessary for optimal cellulose saccharification (Singhania *et al.,* 2013). However, major obstacles to the practical realization of the full potential of enzymatic

hydrolysis include the high cost of enzymes, as well as the slow reaction rate due to the recalcitrant character of this polymeric substrate (Hamada *et al*., 2013). For this reason, any hydrolytic approach for cellulose saccharification requires pretreatment of the cellulosic materials to increase their susceptibility to hydrolysis, the effectiveness of this process being considered as a key factor in the overall efficiency (Zhao *et al.,* 2009; Galbe and Zacchi*,* 2012, Mizuno *et al*., 2012; Ohira *et al*., 2012; Uju *et al*., 2013). The discovery made by Rogers's group, concerning the ability of some ionic liquids (ILs) *e.g*. 1-butyl-3-methylimidazolium chloride ([Bmim] [Cl]), etc. to dissolve cellulose (Swatloski *et al*., 2002), has opened up new opportunities for the valorization of large amounts of waste cellulose-containing materials because ILs are non-volatile, have low melting points and high thermal stability (Ohira *et al*., 2012; Zhao *et al.*, 2012; Brandt *et al.,* 2013). However, it has been widely reported how these ILs that are excellent for dissolving cellulose, (*e.g*. [Bmim][Cl], etc.), producing fast enzyme deactivation as a result of protein unfolding (Turner *et al*., 2003; Salvador *et al*., 2010; Lozano *et al*., 2011). In this context, the re-precipitation of cellulose from IL solutions into polar molecular solvents (*e.g*. water, ethanol, etc.) is the most popular cellulose pretreatment approach, because it involves full disruption of the crystalline structure of this polysaccharide (Lindmand *et al.*, 2010), which improves its subsequent enzymatic hydrolysis in buffered media (Dadi *et al*., 2006; Shill *et al*., 2011; Husson *et al*., Mizuno *et al*., 2012; 2011, Uju *et al*., 2013). In this context, the full recovery and reuse of ILs used for cellulose pretreatment has been proposed as the key for preserving the green integrity of the overall process of cellulose saccharification (Shill *et al*., 2011; Lozano *et al*., 2012; Brandt *et al.,* 2013). Furthermore, since the final destination of the hydrolyzed cellulose solutions is fermentation by yeast to produce bioethanol, the presence of residual IL is undesirable for its effect on the viability of yeast cells (Ouellet *et al.,* 2011, Hong *et al., 2012*). In the same way, although enzymes are environmentally friendly, non-toxic and non-corrosive catalysts, their recovery and/or reuse

is to be encouraged for all processes liable to scaling-up in order to reduce the technological costs (Franssen *et al.,* 2013).

Recently, we proposed a sustainable cyclic process for preparing amorphous cellulose from homogeneous cellulose solutions in the IL 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) through antisolvent precipitation with equimolar water–ethanol mixtures under ultrasounds at 60ºC (Lozano *et al.*, 2012). By means of this approach, the IL is fully recovered (up to 99.7%) and can be successfully reused in further cellulose dissolution/precipitation cyclic processes. Furthermore, the regenerated amorphous cellulose is an excellent substrate for enzymatic hydrolysis, permitting full hydrolysis that provides a clear glucose solution, suitable for growing *Saccharomyces cerevisiae* aerobically without further purification.

On the other hand, enzymatic membrane reactors are one of the most popular approaches in the field of biotechnology for enzyme reuse, particularly when combined with downstream processing (Mori *et al*., 2005; Andric *et al*., 2010; Franssen *et al*., 2013). Ultrafiltration membrane reactors allow carrying out biocatalytic continuous processes by using enzymes in free form, which it is advantageous for degradation of polymeric substrates (*e.g.* pectin, etc.) with respect the use of immobilized biocatalyst, where the accessibility of the substrates to the enzyme is restricted. Furthermore, by the appropriate selection of pore size/cut-off of the membrane, the complete rejection of the enzyme is ensured and the resulting biocatalytic system can be reused as long as the activity remains high. In addition, the continuous extraction of products from the medium reduces any inhibitory effects on biocatalyst improving reaction rate and product yields (Rios *et al.*, 2007). In this context, the enzymatic hydrolysis of water-soluble polysaccharides, such as pectins (Lozano *et al*., 1987) and starch (Paolucci-Jeanjean *et al*., 2001), has been successfully carried out using ultrafiltration membrane reactors, in which the membrane acts as a barrier for the enzyme, but is permeable for the produced monosaccharides.

Ultrafiltration membrane reactors have also been reported for many biorefinery applications (Abels *et al*., 2013), including the enzymatic hydrolysis of cellulose as aqueous suspensions (Gan *et al.,* 2002; Belafi-Bako *et al*., 2006). In all cases, the enzymatic saccharification resulted in low conversion yield (up to 50 % hydrolysis), because of the recalcitrant character of the substrate, although this was improved to nearly 90% hydrolysis when a water-soluble cellulose derivative (*e.g.* CM-cellulose) was used as substrate (Liu *et al.,* 2011).

This paper describes for the first time the application of membrane reactor technology to the enzymatic hydrolysis of IL-pretreated cellulose, in order to provide a fast and efficient approach for the full saccharification of cellulose permitting full recovery and reuse of the enzymes. In this context, two configurations of enzymatic membrane reactors, based on polymeric or ceramic ultrafiltration membranes, were tested under both semi-continuous and the continuous operation modes. Furthermore, recognized operational parameters that reveal membrane reactor efficiency, including as the enzyme stability during reuse as well as the permeate flux profile, were also studied.

2. Material and Methods

2.1 Materials

Cellulase from *Trichoderma reesei* (Celluclast 1.5L®, EC 3.2.1.4), and cellobiase from *Aspergillius niger* (Novozyme 188®, beta-1,4-glucosidase, EC 3.2.1.21) were a gift from Novozymes S.A (Spain). Polyethersulfone (PES) membrane (Vivaflow50) cassettes were obtained from Sartorius (Spain). Tubular ceramic membranes with three channel geometry were obtained from Tami Industries (France). Microcrystalline cellulose (20 μm powder) and other chemicals were purchased from Sigma-Aldrich-Fluka (Spain). The IL 1-

butyl-3-methylimidazolium chloride, [Bmim][Cl], (99% purity) was obtained from IoLiTec GmbH (Germany).

Prior to use, enzyme preparations were ultrafiltered to eliminate all the low possible molecular weight additives, as follows: 25 mL of Celluclast or Novozym 188 were diluted in 225 mL of 50 mM citrate buffer pH 4.8, and the resulting solutions were concentrated 10-fold by ultrafiltration at 8ºC, using a Vivaflow 50 (Sartorious) system equipped with PES membranes (10 kDa. cut-off). For each enzyme, the process was repeated three times, leading to a cellulase (0.16 U mg^{-1} prot., 154.9 mg prot. mL^{-1}), or cellobiase (1.33 U mg^{-1} prot., 93.6 mg prot. mL^{-1}) solutions, respectively, which were used for cellulose hydrolysis.

2.2 Preparation of regenerated cellulose (RC)

Microcrystalline cellulose (10g) was added to a 1-L Erlenmeyer flask containing 100 g melted [Bmim][Cl] at 115ºC, and the mixture was incubated with mechanical stirring for 1 h at the same temperature, which gave a clear, colourless and viscous cellulose solution. This solution was then cooled to 60ºC in a glycerol thermostatic bath, and the amorphous cellulose was regenerated by adding 500 mL (approx. 5-fold IL-cellulose volume) of an equimolar (23.5/76.5, v/v) water/ethanol solution pre-heated at 60ºC. The resulting suspension of regenerated cellulose (RC) was vigorously stirred for 15 min. The RC gel was recovered by filtration through a nylon membrane (0.1 mm mesh), then washed twice with 500 mL of equimolar water/ethanol solution applying 150 W ultrasounds (Ultrasons, Selecta, Spain) for 15 min. Finally, the RC was washed two-two times with ultrapure water (MilliQ-Millipore System) and mechanical stirring for 15 min (Lozano *et al*., 2012), resulting in a white gel-like solid of RC with a 85% (w/w) moisture content, as measured by weight loss of RC after drying in an oven for 14 h at 105ºC.

2.3. Membrane reactor for enzymatic saccharification of RC under semi-continuous operation mode.

As can be seen in Figure 1, the reactor system consisted of a stirred tank (100-mL total capacity) connected through a peristaltic pump with an ultrafiltration Vivaflow-50 unit based on PES membranes (50 cm^2 overall surface, 10 or 5 kDa cut-off). Under semicontinuous operation mode, both the biocatalytic and the filtration steps were carried out separately. Firstly, the reactor tank was loaded with a 2% (w/w) RC suspension (60 mL) in 50 mM citrate buffer pH 4.8, and then maintained under magnetic stirring for 30 min at 50ºC until a homogeneous suspension was observed. The biocatalytic step was started by adding both cellulase (1.84 mL, 38 U g^{-1} cellulose) and cellobiase (1.24 mL, 128 U g^{-1} cellulose) glycohydrolases. The solution was maintained under magnetic stirring for 4 h at 50ºC, and without recirculation. Then, the filtration step was started by applying a 120 mL/min recirculation flow rate through the ultrafiltration units at 1.2 bar back-pressure, obtaining a fully clear permeate product at a flow rate controlled by an automatic balance. At regular time intervals, 65 µL-aliquots were taken from either the reactor tank or the permeate flow, and suspended in 10 mM bicarbonate buffer, pH 9.8 (1.035 mL) to stop the reaction, then centrifuged at 13,000 rpm for 5 min. The resulting clear phase was used to quantify glucose and cellobiose by HPLC, and the total reducing sugars were quantified by the dinitrosalicylic acid (DNS) method. All experiments were carried out in duplicate.

2.4. Membrane reactor for enzymatic saccharification of RC under continuous operation mode.

The reactor system consisted of a stirred tank (600-mL total capacity) connected through a peristaltic pump with the ultrafiltration module, containing a 3-channel tubular ceramic membrane (23 cm length, 3.5 mm hydraulic diameter, 76 cm^2 filtration surface area, 5 kDa cut-off) (see Figure 2). Firstly, the reactor tank was loaded with 200 or 500 mL of the RC

suspension (0.8, 1.2, 1,6 or 2 % w/v, respectively) in 50 mM citrate buffer pH 4.8, before being maintained under magnetic stirring for 30 min at 50ºC until a homogeneous suspension was observed. The enzymatic saccharification step was started by adding both cellulase (38 U g^{-1} cellulose) and cellobiase (1.24 mL, 128 U g^{-1} cellulose), and the solution was maintained under stirring for 1.5 or 2 h at 50ºC and without recirculation to obtain an almost clear reaction mixture unable to block the filtration unit. Then, the filtration step was started by applying a 215 mL min^{-1} recirculation flow rate through the ultrafiltration module and at 0.5 bar back-pressure, obtaining a fully clear permeate product at a flow rate controlled by an automatic balance, while the reactor tank was continuously fed with fresh RC solution at the same flow. At regular time intervals, 65 µL-aliquots were taken from the permeate flow, and suspended in 10 mM bicarbonate buffer, pH 9.8 (1.035 mL), and the resulting mixture was used to quantify the total reducing sugars by the dinitrosalicylic acid (DNS) method. All experiments were carried out in duplicate.

2.5 Enzyme retention capacity of ultrafiltration membranes

The enzyme retention capacity of both polymeric (10 or 5 kDa cut-off) and ceramic (5 kDa cut-off) membranes was determined by ultrafiltration assays. For each membrane case, 200 mL of either 4.76 mg mL^{-1} (0.76 U mL^{-1}) cellulase or 1.93 mg mL^{-1} (2.56 U mL^{-1}) cellobiase solution in 50 mM citrate buffer pH 4.8 were ultrafiltrated in the corresponding membrane reactor for 2 h at 120 mL min^{-1} flow rate, 0.5 bar transmembrane pressure and 50ºC. Then, samples from the retentate and permeate fractions, respectively, were withdrawn for quantification of the enzyme activity. For each enzyme, the enzyme retention capacity of each membrane was determined from the corresponding mass-balance in enzyme activity between the retentate and the permeate fractions, respectively.

2.6. Determination of enzyme activity

Into a screw-capped vial, 4 mL of 2% (w/v) RC or cellobiose solutions in 50 mM citrate buffer pH 4.8 were placed, and maintained under magnetic stirring in a glycerol bath for 15 min at 50ºC. For each case, the reaction was then started by adding the corresponding cellulase or cellobiase solution (100 μL) from the retentate or permeate fractions, which was magnetically stirred for 4 h. At regular time intervals, 65 μL-aliquots were taken from the reaction medium and suspended in 10 mM bicarbonate buffer, pH 9.8 (1.035 mL) to stop the reaction. The samples were then centrifuged at 13 000 rpm for 10 min. The resulting clear phase was used to quantify the total reducing sugars by the dinitrosalicylic acid (DNS) method. One unit of cellulase activity was defined as the amount of enzyme that produces 1 μmol of reducing sugars per minute. One unit of cellobiase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of cellobiose per minute. All experiments were carried out in duplicate.

2.7. HPLC analysis of sugars

The quantification of glucose and cellobiose concentrations in the enzymatic reaction samples were determined in a Shimadzu HPLC equipped with a multi-channel (LC-20AD) pump, oven and light scattering (ELSD-LT II) detector. A Rezex RCM-monossacharide-Ca^{2+} column (300 × 7.8 mm, Phenomenex) was used as the stationary phase at 60°C. Analyses were performed in isocratic conditions (0.6 mL min^{-1} flow rate) using water as a mobile phase. The glucose (11 min retention time) and cellobiose (9 min retention time) peaks were identified and quantified from the corresponding calibration straight lines, using xylitol (23 min retention time) as the internal standard.

3. Results and discussion

3.1 Retention capacity of ultrafiltration membranes.

The polymeric and ceramic membranes usually applied for enzymatic reactors show different properties with regards to thermal and/or chemical resistance, mechanical stability in hot/wet environments, long-term performance, etc. (Lozano *et al*., 1987, Mori *et al*., 2005; Rios *et al.*, 2007, Andric *et al*., 2010). For the enzymatic saccharification of cellulose, the ability of the membrane to retain the enzyme for recovery and reuse is considered as a key parameter for the economic sustainability of the process. In this context, the suitability of both PES polymeric and ceramic membranes for enzyme retention was tested by using the reaction systems depicted in Fig. 1 and 2, respectively. At this point, it should be mentioned that Celluclast 1.5L® is a commercially available *T. reesei* cellulase preparation containing several glucohydrolases, *i.e*. CBH I, CBH II, and EGs with molecular weights that range from 23 to 94 kDa (Vinzant *et al*., 2001, Rosgaard *et al*., 2007; Lehmann *et al*., 2012), while Novozym 188 is a beta-glucosidase preparation from *A. niger* with a molecular weight of 118 kDa (McCleary and Harrington, 1988). As a function of these enzyme molecular weights, two different PES polymeric membranes (5 and 10 kDa cut-off) and a ceramic membrane (5 kDa cut-off) were used, determining their respective enzyme retention capacities in several ultrafiltration assays for each enzyme preparation, and quantifying the enzyme activity for both retentate and filtrate fractions. As can be seen in Table 1, the amount of retained enzyme was higher than 98% for all cases, which shows the excellent suitability of these membranes for enzyme recovery and reuse in further enzymatic saccharification cycles.

3.2. Enzymatic membrane reactor for saccharification of RC under semi-continuous operation mode.

The suitability of regenerated cellulose (RC) from a [Bmim][Cl] solution for full enzymatic depolymerization into its glucose units is a key step for the overall performance of enzymatic membrane reactors. Figure 3 shows the time course profile of total reducing sugars, glucose and cellobiose in a 60 mL tank-reactor medium for enzymatic saccharification of RC. As can be seen, the synergic action of cellulase and cellobiase lead to a continuous increase in glucose concentration, while the cellobiose was only observed at the beginning of the reaction course, when the RC concentration was high, after which it was hydrolyzed as fast as it was formed. Thus, the enzymatic saccharification proceeded at an overall glucose production rate of 0.5 mmol min^{-1} mg^{-1} prot., which can be considered fast because 90% cellulose hydrolysis was observed after 1 h reaction time, and practically full hydrolysis was attained after 4 h, which demonstrates the excellent suitability of this RC substrate. Furthermore, these profiles are practically identical to those previously obtained for a 4 mL reaction medium volume (Lozano *et al*., 2012), demonstrating the reproducibility of the kinetic profiles during the enzymatic saccharification of cellulose towards scaling-up processes. These results are clearly related to weakening of the cellulose crystallinity due to disruption of the fiber organization produced during by the dissolution/precipitation pretreatment, which resulted in a more accessible substrate for efficient enzymatic hydrolysis (Dadi *et al*., 2006; Lozano *et al*., 2012; Shill *et al.*, 2011; Husson *et al*., 2011, Mizuno *et al.,* 2012; Uju *et al*., 2013).

In an attempt to improve the catalytic efficiency for the enzymatic saccharification of RC, the effect of cellulase/cellobiase loading on the overall glucose production rate was also studied (see Table 2). As can be seen, the best results were obtained when a cellulase/cellobiase loading of 38 and128 (U g^{-1} cellulose), respectively, was used, a continuous decrease in the overall glucose production rate by increasing the

cellulase/cellobiase loading being observed. As observed in Figure 3, the assayed 38/128 (U g^{-1} cellulose) cellulase/cellobiase loading seems to be appropriate for providing rapid cellobiose hydrolysis, which should avoid the cellulase inhibition (Singhania *et al.,* 2013). In the same context, the observed decrease in the overall glucose production rate by increasing cellulase/cellobiase loading could be explained by the jamming effect, whereby an excess of enzyme molecules binding adjacent onto the same small surface area of cellulose would lead to not all the bound enzymes proceed at the same rate, resulting in a reduced overall reaction rate (Bomarius *et al.,* 2008). Based on these results, a 38/128 (U g^{-1} cellulose) cellulase/cellobiase loading was selected for further experiments for the saccharification of cellulose in membrane reactors.

The economic sustainability of any process for the full saccharification of cellulose depends on the full recovery and reuse of the cellulose depolymerizing enzymes. In this context, an enzymatic membrane reactor based on a 60-mL stirred tank connected to a PES ultrafiltration unit by a recirculation pump (see Fig. 1) was tested for the hydrolysis of RC under semi-continuous operation mode. The approach consisted of repeated cycles of a biocatalytic step for hydrolysis of the RC substrate followed by a down-stream step for removing glucose solution through the UF unit (up to 75% reactor volume). Each new cycle was started by refilling the reactor to a 60 mL final volume with fresh 2% (w/v) RC suspension in 50 mM citrate buffer pH 4.8 (see Materials and Method section).

Fig. 4A shows the evolution of permeate flux, glucose concentration and degree of cellulose hydrolysis at the reactor outlet during six consecutive operation cycles of enzymatic saccharification of regenerated cellulose and glucose recovery by ultrafiltration through 5 kDa cut-off PES membranes. As can be seen, both the glucose concentration and cellulose hydrolysis degree were maintained unchanged at approx.110 mM and 94%, respectively, which points to the excellent suitability of this PES membrane for the recovery and reuse the cellulase/cellobiase mixture. However, the permeate flow rate

across this 5 kDa-cut-off membrane gradually fall from 14.2 to 11.3 (L h^{-1} m^{-2}), which could be related to the fouling of the membrane by short glucose oligomers, which cannot be hydrolyzed to their glucose units. The increase in the molecular weight cut-off of the membrane to 10 kDa resulted in a clear improvement in the membrane reactor performance. As can be seen in Figure 4B, both the glucose concentration and the permeate flow rate remained unchanged for 9 operation cycles. Furthermore, both the resulting glucose concentration (approx. 113 mM) and permeate flow rate (approx. 24.7 L h^{-1} m^{-2}) determined at the outlet of this 10 kDa cut-off membrane were slightly higher than those obtained for the 5 kDa cut-off membrane. As membrane fouling did not occur, the cellulosic substrate can be hydrolyzed at the same level as in the stirred-tank reactor without ultrafiltration unit (see Fig. 3). Moreover, the constant hydrolysis yield obtained during semi-continuous operation with the 10 kDa cut-off membrane reactor reflected the excellent operational stability of these enzymes for reuse, allowing 95 % cellulose hydrolysis degree during many repeated catalytic cycles of 4 h reaction. These results underline the excellent suitability of these PES membranes even if this was not fully demonstrated in the case of 5 kDa cut-off PES.

The combination of cellulase and cellobiase for cellulose saccharification in PES membrane reactors has also been reported (Gan *et al*., 2002, Liu *et al*., 2011). However, the recalcitrance of the assayed cellulosic substrate resulted in a low enzymatic hydrolysis yield, up to 14 g L^{-1} after 50 h reaction (Gan *et al*., 2002), although this improved to 92% hydrolysis yield for a 1 h reaction period when a water soluble cellulose derivative, like carboxymethyl cellulose, was used as substrate (Liu *et al*., 2011). The operational stability of enzymes using these approaches has not been reported.

3.3. Enzymatic membrane reactor for saccharification of RC under continuous operation mode.

By using a ceramic membrane reactor design as depicted in Fig 2, the enzymatic saccharification of regenerated cellulose was carried out under continuous operation mode at different substrate concentrations (0.8, 1.2, 1.6 and 2.0 % w/v, respectively) and for 1.5 or 4 h residence time, (see Material and Methods sections for further details). In all cases, the reactor was first operated without recirculation/filtration flow, until the content in the insoluble RC suspension was reduced by the enzyme action. When a cellulose suspension was directly recirculated and ultrafiltered, the UF unit was immediately blocked because of the deposition of solid material on the membrane surface. Figure 5 depicts the profiles of both the reducing sugar concentration and the permeate flux determined at the outlet of the ultrafiltration unit. As can be seen, all the profiles remained practically unchanged for a period similar to the residence time, which, once again, demonstrates the suitability of the pre-treatment of cellulose for obtaining a suitable substrate for straightforward enzyme hydrolysis. In all cases, the resulting reducing sugar concentrations and cellulose hydrolysis degree higher than 95 % agreed with the hydrolysis profile obtained in Figure 3. Furthermore, it should be noted how these 5 kDa-cut-off ceramic membranes provide a permeation flux similar to that obtained for the case of 10 kDa cut-off PES membrane, which could be considered as a positive criterion for its selection in any scaling up, because of the better mechanical properties of ceramic membranes compared with PES ones. Belafi-Bako *et al.* (2006) pointed out to the importance of the mechanical resistance of membranes used in the enzymatic saccharification of cellulose. These authors used a porous stainless steel membrane covered by a non-woven textile layer into a stirred-tank reactor to carry out the hydrolysis of Solka Floc cellulose using Celluclast 1.5L® as biocatalyst, obtaining up to 53 % cellulose hydrolysis in continuous operation for 20 h, which then fall gradually.

4. Conclusions

This work emphasizes the excellent suitability of the resulting amorphous cellulosic substrate from IL pre-treatment for full enzymatic saccharification under continuous or semi-continuous operation modes. Membrane reactor technology is shown to be a perfect tool for the enzymatic hydrolysis of these regenerated cellulosic substrates, providing an efficient approach for enzyme recovery and reuse. Both 10 kDa cut-off PES membranes and 5 kDa cut-off ceramic membranes provided full retention of cellulase and cellobiase enzymes, showing an excellent operational stability for enzyme reuse. This paper demonstrates an easy and sustainable approach to convert cellulose into glucose suitable for scaling-up.

5. Acknowledgements

This work was partially supported by MINECO, Spain (Ref: CTQ2011-28903-C02-02), and SENECA Foundation, Spain (Ref: 08616/PI/08) grants. We thank Ramiro Martinez (Novozymes España. S.A.) for a gift of Celluclast 1.5L® and Novozyme 188, as well as David A. Martinez for the technical assistance during the preparation of regenerated cellulose.

6. References

1. Abels, C.; Carstensen, F.; Wessling, M. 2013. Membrane processes in biorefinery applications. J. Membr. Sci., 444, 285-317.

2. Andric, P., Meyer, A.S., Jensen, P.A. Dam-Johansen, K. 2010. Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis II. Quantification of inhibition and suitability of membrane reactors. Biotechnol. Adv., 28, 407-425

3. Belafi-Bako, K., Koutinas, A., Nemestothy, N., Gubicza, L., Webb, C., 2006. Continuous enzymatic cellulose hydrolysis in a tubular membrane bioreactor. Enzyme Microb. Technol., 38, 155-161.

4. Bommarius, A.S., Katona, A., Cheben, S.E., Patel, A.S., Ragauskas, A.J., Knudson, K., Pu, Y., 2008. Cellulase kinetics as a function of cellulose pretreatment. Metabol. Eng. 10, 370–381.

5. Brandt, A., Grasvik, J., Hallett, J.P., Welton, T. 2013. Deconstruction of lignocellulosic biomass with ionic liquids. Green Chem., 15, 550-583.

6. Dadi, A.P., Varanasi, S., Schall, C.A., 2006. Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step. Biotechnol. Bioeng., 95, 904-910.

7. Franssen, M.C.R., Steunenberg, P., Scott, E.L., Zuilhof H., Sanders, J.P.M. 2013. Immobilised enzymes in biorenewables production. Chem. Soc. Rev., 42, 6491-6533

8. Galbe, M. Zacchi, G. 2012. Pretreatment: The key to efficient utilization of lignocellulosic materials. Biomass Bioenerg., 46, 70-78.

9. Gan, Q., Allen, S.J., Taylor, G., 2002. Design and operation of an integrated membrane reactor for enzymatic cellulose hydrolysis. Biochem. Eng. J., 12, 223–229.

10. Hamada, Y., Yoshida, K., Asai, R., Hayase, S., Nokami, T., Izumi, S., Itoh, T. 2013. A possible means of realizing a sacrifice-free three component separation of lignocellulose from wood biomass using an amino acid ionic liquid. Green Chem., 15, 1863-1868.

11. Hong, F., Guo, X., Zhang, S., Han, S. F., Yang, G., Jonsson, L.J. 2012. Bacterial cellulase production from cotton-bassed wate textiles: Enzymatic saccharification enhanced by ionic liquid pretreatment. Bioresour. Technol., 104, 503-508.

12. Husson, E. Buchoux, S., Avondo, C., Cailleu, D., Djellab, K., Gosselin, I., Wattraint, O., Sarazin, C. 2011. Enzymatic hydrolysis of ionic liquid-pretreated celluloses: Contribution of CP-MAS ^{13}C NMR and SEM. Bioresour. Technol., 102, 7335–7342.

13. Lehmann, C., Sibilla, F., Maugeri, Z., Streit, W.R., de Maria, P.D., Martinez, R., Schwaneberg, U. 2012. Reengineering CelA2 cellulase for hydrolysis in aqueous solutions of deep eutectic solvents and concentrated seawater. Green Chem., 14, 2719-2726.

14. Lindman, B., Karlstom, G. Stigsson, L. 2010. On the mechanism of dissolution of cellulose. J. Mol. Liq., 156, 76-81.

15. Liu, J., Lu, J., Cui, Z., 2011. Enzymatic hydrolysis of cellulose in a membrane bioreactor: assessment of operating conditions. Bioproc. Biosyst. Eng., 34, 525–532.

16. Lozano, P., Manjon, A., Romojaro, F., Canovas, M., Iborra, J.L. 1987. A cross-flow reactor with immobilized pectolytic enzymes for juice clarification. Biotechnol. Lett., 9, 875-880.

17. Lozano, P., Bernal, B., Bernal, J.M., Pucheault, M., Vaultier, M. 2011. Stabilizing immobilized cellulase by ionic liquids for saccharification of cellulose solutions in 1-butyl-3-methylimidazolium chloride. Green Chem., 13, 1406–1410.

18. Lozano, P., Bernal, B., Recio, I., Belleville, M.P. 2012. A cyclic process for full enzymatic saccharification of pretreated cellulose with full recovery and reuse of the ionic liquid 1-butyl-3-methylimidazolium chloride. Green Chem., 14, 2631-2637.

19. McCleary, B.V., Harrington, J. 1988. Purification of beta-D-glucosidase from *Aspergillus niger*. Methods Enzymol. 160, 575-583.

20. Mizuno, M., Kachi, S., Togawa, E., Hayashi, N., Nozaki, K., Itoh, T., Amano, Y. 2012. Structure of Regenerated Celluloses Treated with Ionic Liquids and Comparison of their Enzymatic Digestibility by Purified Cellulase Components. Aust. J. Chem. 65, 1491-1496.

21. Mori, M, Gomez Garcia, R., Belleville, M.P., Paolucci-Jeanjean, D., Sanchez J., Lozano, P., Vaultier, M., Rios. G.M. 2005. A new way to conduct enzymatic synthesis in an active membrane using ionic liquids as catalyst support. Catal. Today, 104, 313–317.

22. Ohira, K., Abe, Y., Kawatsura, M., Suzuki, K., Mizuno, M., Amano, Y., Itoh, T. 2012. Design of cellulose dissolving ionic liquids inspired by Nature. ChemSusChem., 5, 388-391.

23. Ouellet, M., Datta, S., Dibble, D.C., Tamrakar, P.R., Benke, P.I., Li, C.L., Singh, S., Sale, K.L., Adams, P.D. Keasling, J. D., Simmons, B.A., Holmes, B.M., Mukhopadhyay, A. 2011. Impact of ionic liquid pretreated plant biomass on *Saccharomyces cerevisiae* growth and biofuel production. Green Chem., 13, 2743–2749.

24. Paolucci-Jeanjean, D., Belleville, M.P., Rios, G.M. 2001. A comprehensive study of the loss of enzyme activity in a continuous membrane reactor – application to starch hydrolysis. J. Chem. Technol. Biotechnol., 76, 273-278.

25. Rios, G.M., Belleville, M.P., Paolucci-Jeanjean, D. 2007. Membrane engineering in biotechnology: quo vamus?. Trends Biotechnol., 25, 242-246.

26. Rosgaard, L., Pedersen, S., Langston, J., Akerhielm, D., Cherry, J.R., Meyer, A.S. 2007. Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates. Biotechnol. Prog. 23, 1270-1276.

27. Salvador, A.C., Santos, M.C., Saraiva, J.A. 2010. Effect of the ionic liquid [Bmim][Cl] and high pressure on the activity of cellulase. Green Chem., 12, 632-635

28. Shill, K., Padmanabhan, S., Xin, Q., Prausnitz, J.M., Clark, D.S., Blanch, H.W., 2011. Ionic liquid pretreatment of cellulosic biomass: Enzymatic hydrolysis and ionic liquid recycle. Biotechnol. Bioeng. 108, 511–520.

29. Singhania, R.R., Patel, A.K., Sukumaran, R.K., Larroche, C., Pandey, A. 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour. Technol. 127, 500-507.

30. Swatloski, R.P., Spear, S.K., Holbrey, J.D., Rogers, R.D. 2002. Dissolution of cellulose with ionic liquids. J. Am. Chem. Soc., 124, 4974-4975

31. Turner, M.B., Spear, S.K., Huddleston, J.G., Holbrey, J.D., Rogers, R.D. 2003. Ionic liquid salt-induced inactivation and unfolding of cellulase from *Trichoderma reesei*. Green Chem., 5, 443-447.

32. Uju, Nakamoto, A., Shoda, Y., Goto, M., Tokuhara, W., Noritake, Y., Katahira, S., Ishida, N., Ogino, C., Kamiya, N. 2013. Low melting point pyridinium ionic liquid pretreatment for enhancing enzymatic saccharification of cellulosic biomass. Bioresour. Technol., 135, 103-108.

33. Vinzant, T.B., Adney, W.S., Decker, S.R., Baker, J.O., Kinter, M.T., Sherman, N.E. Fox, J.W., Himmel, M.E. 2001. Fingerprinting *Trichoderma reesei* hydrolases in a commercial cellulase preparation. Appl. Biochem. Biotechnol. 91-93, 99-107

34. Zhao, B., Greiner, L, Leitner, W. 2012. Cellulose solubilities in carboxylate-based ionic liquids. RSC Adv. 2, 2476-2479.

35. Zhao, H., Jones, C.L., Baker, G.A., Xia, S.Q., Olubajo, O., Person, V.N. 2009, Regenerating cellulose from ionic liquid for an accelerated enzymatic hydrolysis. J. Biotechnol., 139, 47-54.

Table 1. Cellulase and beta-glucosidase retention for different UF membranes

Enzyme	Membrane / Cut-Off	Enzyme Retention [2] (%)
Cellulase complex (Celluclast 1.5L®)	PES[1] / 10 kDa	99.8 ± 0.2
	PES / 5 kDa	100 ± 0.1
	Ceramic / 5 kDa	99.4 ± 0.3
Cellobiase (Novozym 188)	PES / 10 kDa	98.4 ± 0.5
	PES / 5 kDa	99.4 ± 0.4
	Ceramic / 5 kDa	98.1 ± 0.3

[1] Polyethersulfone; [2] Difference between enzyme activities in permeate and retentate fractions.

Table 2. Effect of cellulose and cellobiase loadings on the overall glucose production rate for saccharification of regenerated cellulose into a 60 mL stirred tank reactor at 50ºC.

Cellulase (U g^{-1} cellulose)	Cellobiase (U g^{-1} cellulose)	Overall Glucose Production Rate (μmol min^{-1} mg^{-1} prot.)
38	128	0.50
57	192	0.34
76	256	0.27
152	512	0.18

LEGENDS TO FIGURES

Figure 1. Schematic representation of the enzymatic membrane reactor based on polymeric membranes, operating under semi-continuous mode.

Figure 2. Schematic representation of the enzymatic membrane reactor based on ceramic tubular membranes, operating under continuous mode. See Fig 1 for further details.

Figure 3 Time-course profiles of total reducing sugars (●), glucose (▲) and cellobiose (◆) released from regenerated cellulose (2% w/v) by the combined action of cellulase (38 U g^{-1} cellulose) and cellobiase (128 U g^{-1} cellulose) in a 60-mL stirred tank reactor at 50ºC.

Figure 4 Evolution of permeate flux, glucose concentration and cellulose hydrolysis degree at the reactor outlet during semi-continuous operation cycles for the enzymatic saccharification of regenerated cellulose (RC), followed by glucose separation through PES ultrafiltration membranes (**A**: 5 kDa cut-off; **B**: 10 kDa cut-off). The enzymatic hydrolysis of RC (2% w/v, 60 mL) was carried out by the catalytic action of cellulase (38 U g^{-1} cellulose) and cellobiase (128 U g^{-1} cellulose) for 4 h at 50ºC. The separation step was carried out by tangential ultrafiltration at 120 mL min^{-1} recirculation flow rate, 1.2 bar transmembrane pressure and 50ºC.

Figure 5 Time course profiles of permeate flux, total reducing sugars and cellulose hydrolysis degree at the reactor outlet for the continuous hydrolysis of RC carried out by the simultaneous action of cellulase (38 U g^{-1} cellulose) and cellobiase (128 U g^{-1} cellulose) in a tangential flow ceramic membrane operating at 215 mL min^{-1} recirculation flow and at 50ºC. **A**: (■), 0.8 % (w/v) cellulose, 1.5 h residence time; (●), 1.2 % (w/v) cellulose, 1.5 h residence time. **B**: (▲), 1.6 % (w/v) cellulose, 4 h residence time; (◆), 2 % (w/v) cellulose, 4 h residence time.

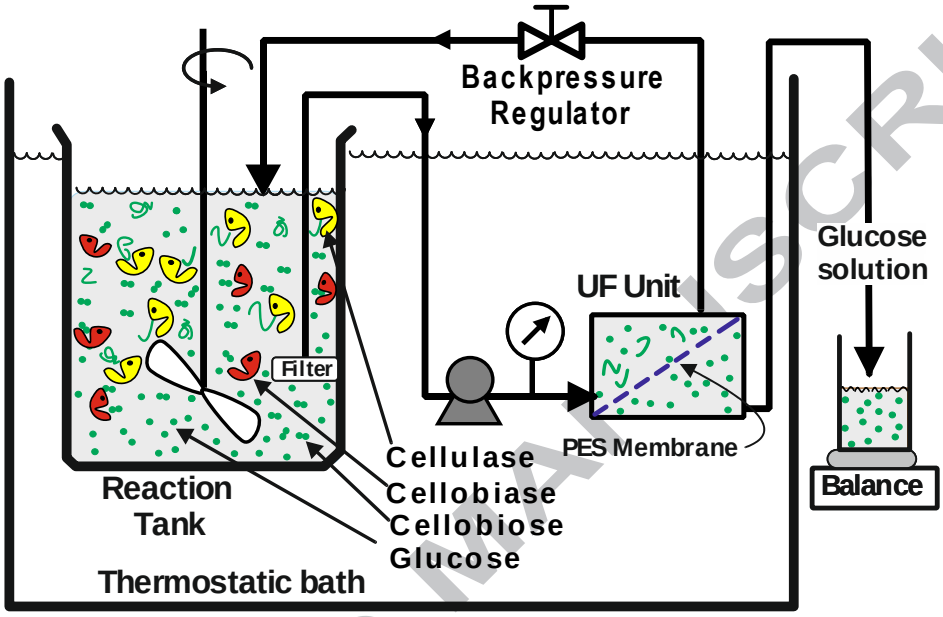

Figure 1 Lozano et al

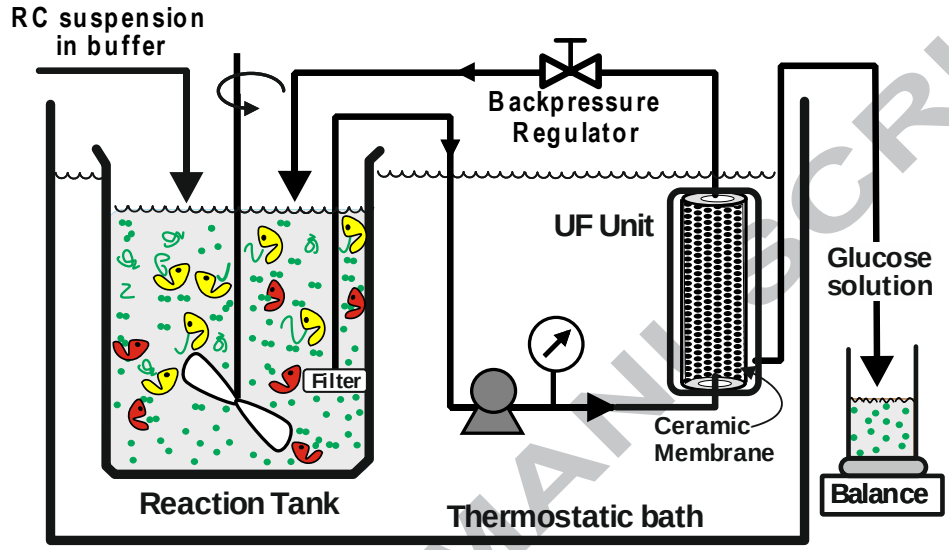

Figure 2 Lozano et al

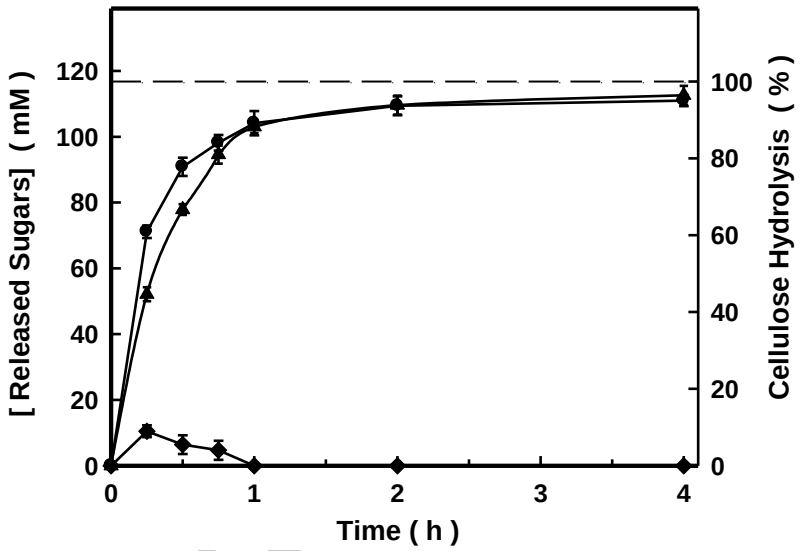

Figure 3

Lozano et al

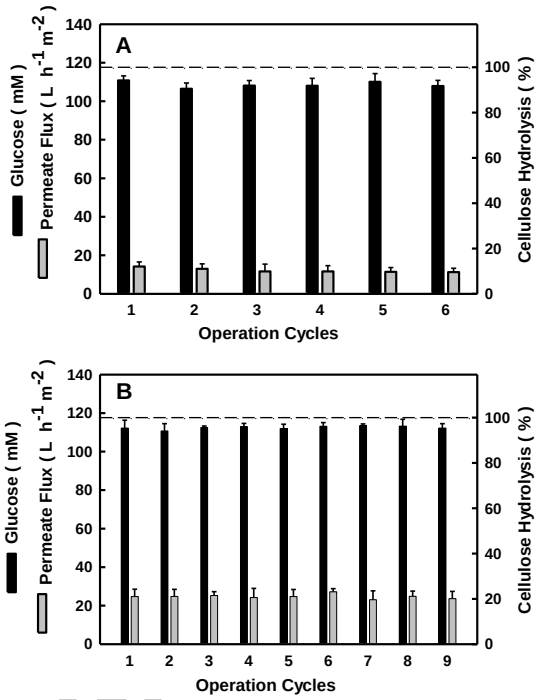

Figure 4

Lozano et al

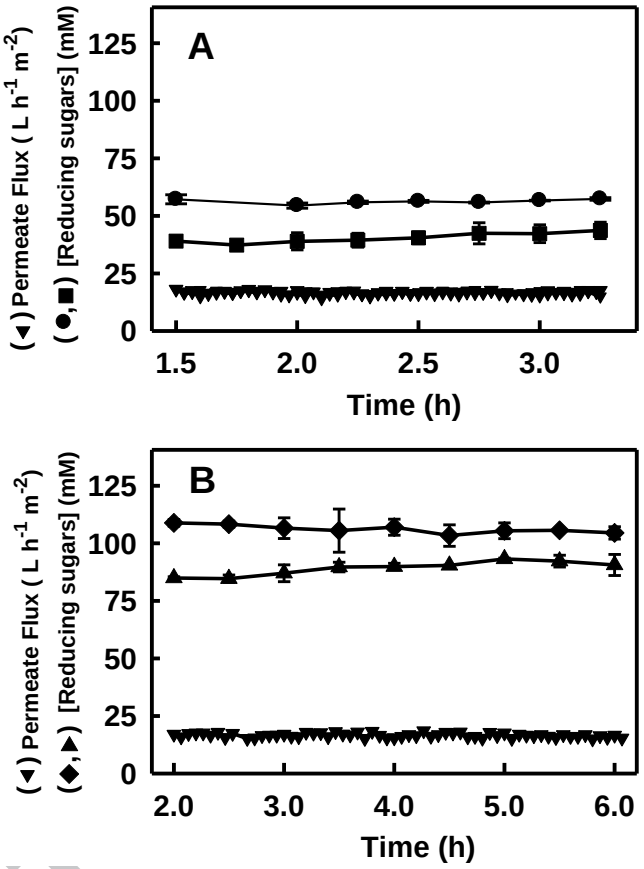

Figure 5 Lozano et al