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

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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.

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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 h at 50 °C. 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.

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

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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 (Lindman et al., 2010), which improves its subsequent enzymatic hydrolysis in buffered media (Dadi et al., 2006; Shill et al., 2011; Husson et al., 2011; Mizuno et al., 2012; 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 60 °C (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. 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 8 °C, 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 (10 g) was added to a 1-L Erlenmeyer flask containing 100 g melted [Bmim][Cl] at 115 °C, 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 60 °C 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 60 °C. 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