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Application of ultrafiltration and nanofiltration for recycling cellulase and concentrating glucose from enzymatic hydrolyzate of steam exploded wheat straw

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1. Introduction

Biological conversion of renewable, cheap and widely available lignocellulosic biomass for producing useful bioproducts and/or biofuels has received considerable attention around the world due to the depletion of unrenewable fossil fuels and resultant severe environmental pollution and emission of greenhouse gas (Gnansounou and Dauriat, 2010; Spatari et al., 2010). Bioconversion process of cellulotic biomass mainly consists of four steps, i.e. pretreatment, enzymatic hydrolysis, microbial fermentation and product separation (Hahn-Hägerdal et al., 2006). Pretreatment is a prerequisite for enzymatic hydrolysis on the basis of the fact that pretreatment could alter the structure of cellulosic material and make it more susceptible to enzymatic attack (Sousa et al., 2009; Yang and Wyman, 2008). Although a variety of pretreatment methods have been developed, taking into consideration of higher pretreatment efficiency and lower operation cost, steam explosion and dilute acid pretreatments remain the two most commonly used methods (Chandra et al., 2007; Kumar et al., 2009b). Both methods mainly dissolve hemicellulose while leaving most of cellulose and lignin in the pretreated solids, of which cellulose can be enzymatically digested by cellulase to produce glucose for subsequent microbial fermentation (Alvira et al., 2010). Two challenges remains to be resolved after enzymatic hydrolysis, one is that in the solid phase of enzymatic hydrolyzate, cellulase adsorbing on the solid residue should be desorbed for recycling. The other is that in the liquid phase of enzymatic hydrolyzate, cellulase should be separated from glucose for subsequent rounds of hydrolysis so as to reduce the hydrolysis cost and glucose should be concentrated in order to increase the product concentration and improve the fermentation efficiency of microbial fermentation step (Qi et al., 2011a), because the glucose concentration in the hydrolyzate was low due to the presence of end-product inhibition (Holtzapple et al., 1990). Although enzymatic hydrolysis can be integrated with microbial fermentation in one reactor, referred to be as simultaneous saccharification and fermentation (SSF), to reduce the inhibition of glucose, the incompatibility of optimal temperature of enzymatic hydrolysis and microbial fermentation and the inability to recycle microbial cells make SSF at a disadvantage compared to separate hydrolysis and fermentation (SHF) (Ölofsson et al., 2008).

Pressure-driven membrane separation process is a mature technology and has been widely used in various industries, such as chemical, food and pharmaceutical industries, etc. for separation, purification and concentration of desired products because of its high recovery or removal efficiency, the reusability of produced water, the low energy input, the moderate operating conditions and the easy integrating with other operating units (Strathmann, 2001; Wiesner and Aptel, 1996). In recent years, a large number of potential applications have been identified, which will further expand the market demand for pressure-driven membrane process.
if these applications are exploited on an industrial scale. Ultrafiltration (UF) and nanofiltration (NF) are two types of pressure-driven membrane process. UF membranes can retain macromolecules with molecular weight ranging from 1000 to 100,000 g/mol and find its wide application in separation of biological products, in particular protein. NF has a molecular weight cutoff (MWCO) varied between 150 and 1000 g/mol, allowing to retain soluble compounds with molecular weight up to 150–250 g/mol and divalent ions at high levels. NF has been used to fractionate and concentrate saccharides and fermentation broth (Goulas et al., 2002; Morao et al., 2006; Qi et al., 2011b).

The first challenge faced in the enzymatic hydrolysis step can be resolved by a variety of desorption methods including the use of surfactant, alkali, urea and buffer with varying pH (Jackson et al., 1996), while the second challenge is expected to be tackled by integrated UF and NF technology. A combined technology, therefore, is proposed for treatment of enzymatic hydrolyzate for improving the economic viability of enzymatic hydrolysis of lignocellulosic biomass through co-producing other value-added chemicals. Fig. 1 shows the schematic representation of proposed process. A liquid phase containing cellulase and glucose and a solid phase containing cellulase will be obtained by filtering the enzymatic hydrolyzate. The cellulase in liquid phase can be retained by UF and small molecule glucose passes through UF membrane and enters the permeate. The UF permeate is further treated by NF to concentrate glucose while producing reusable water. After desorbing cellulase, the solid residue mainly composed of lignin could undergo chemical processes to produce value-added products, such as adhesives, conducting polymers, fiber and epoxy resin, etc. (Doherty et al., 2011; Kumar et al., 2009a). The cellulase desorbed from solid residue as well as the cellulase retained by UF membrane can be used in subsequent round of enzymatic hydrolysis, as reported in our previous work (Qi et al., 2011a). To the best of our knowledge, this is the first time to propose an overall route map for application of membrane separation technology to treat lignocellulosic hydrolyzate.

In the present work, the use of UF and NF for treatment of the liquid phase of enzymatic hydrolyzate of steam exploded wheat straw (SEWS) was proposed and investigated for the purpose of recycling cellulase in the UF stage and concentrating glucose and producing reusable water in the NF stage. Three UF membranes and two NF membranes with varied MWCO were evaluated in terms of solute rejection, membrane permeability and antifouling performance.

![Fig. 1. Schematic representation of treatment of enzymatic hydrolyzate of lignocellulosic feedstock by combined use of UF and NF while co-producing other value-added products.](image-url)

### 2. Methods

#### 2.1. Wheat straw, cellulase, membranes

Steam exploded wheat straw (SEWS) was provided by Henan Tianguan Group, China. The composition of the pretreated wheat straw (on dry weight basis) was as follows: cellulose, 32.4%; xylan, not detected; acid-soluble lignin (ASL), 4.5%; acid-insoluble lignin (AIL), 41.2%; ash, 13.6%. Pretreatment of wheat straw was performed under following conditions: wheat straw was first chipped into pieces and then grinded to 20 mesh, followed by steam explosion at 220 °C for 7 min.

Cellulase was obtained from Genencor Bio-Products Co. Ltd., Wuxi, China. Its filter paper activity (FPA), β-glucosidase activity and protein concentration were 110 FPU/ml, 20.8 CBU/ml and 73.3 mg/ml, respectively.

Three UF membranes, named as PES5, PES10 and PES30 in MWCO order, and two NF membranes were evaluated in the present study. The typical characteristics of the UF and NF membranes obtained from the manufacturers’ data sheet and published literature (Luo et al., 2011; Zhu et al., 2007) are summarized in Table 1.

#### 2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of SEWS was carried out in a 2 l fermentor (BioFlo 110, New Brunswick Scientific, USA) with a working volume of 1.5 l at 50 °C and 200 rpm. After mixing pretreated wheat straw at 10% concentration (w/v, on dry weight basis) with distilled water, the fermentor was autoclaved at 121 °C for 60 min. When the fermentor was cooled to 50 °C, cellulose of 20 FPU/g cellulose was added and enzymatic hydrolysis started. The pH during the hydrolysis process was adjusted to 4.8. Samples were taken at intervals and centrifuged at 7000 rpm for 15 min. The supernatants obtained were used for the analyses of glucose and protein content and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as well as for the following membrane filtration experiments.

#### 2.3. Membrane filtration set-up and procedure

The membrane filtration experiments were performed in a stirred cell using a continuous concentration mode, i.e. feed was continuously pumped into the cell. The schematic diagram of the filtration set-up was described in detail elsewhere (Luo et al., 2009). The filtration module was a self-made magnetic stirred cell
with a working volume of 13.5 ml. The suspended bar impeller was driven by a magnetic agitator with a hot plate (Xuanwo 85-2, Shanghai Sile Equipment Co. Ltd., China). The bottom of the cell could be fitted with a membrane disc of 25 mm in diameter with an effective membrane surface area of 4.52 m$^2$. The cell was immersed in water bath to control the operation temperature at 25 °C. Through a switch valve (V-7, Pharmacia, Sweden), feed or deionized water was pumped at constant flow rate into the filtration cell using a high performance liquid chromatography (HPLC) pump (LC-20AT, Shimadzu Corp., Kyoto, Japan). The transmembrane pressure (TMP) of the filtration process was continuously monitored by a pressure sensor (MLH040BSB09A, Honeywell, USA) and logged into a computer.

A standard protocol for UF and NF experiments were composed of three steps. At first, deionized water was pumped into the cell to measure the water permeability of the membrane. Secondly, the cell was emptied and filled with feed to perform the continuous concentration experiments until the TMP reached 4.5 bar for UF or 35 bar for NF (near the upper pressure limit of UF and NF membranes, respectively). The cumulative permeate was collected and analyzed for contents of protein and glucose and conductivity. The pH of permeate was measured for UF operation, whereas for NF, the pH of the concentrate was measured. In the third step, when the filtration experiment was finished, the membrane was rinsed with deionized water to eliminate any visible deposits, and then water permeability was measured again in order to determine the irreversible fouling (IF) of the membrane.

A new membrane was used in each experiment. The new membrane was first dipped in 50% (v/v) ethanol solution for 5 s, followed by washing with deionized water to loosen the shrunken membrane pores due to long-term storage. The membrane was subsequently soaked in deionized water for at least 24 h until used in the filtration experiment. Prior to the conduct the standard protocol, the soaked membrane was compacted inside the cell by filtering deionized water at relatively high permeate flux until TMP reached constant (4.5 bar for UF membrane, 35 bar for NF membrane) so as to minimize pressure effect on the membrane performance in the filtration tests.

### 2.4. Analytical methods

The chemical composition of pretreated wheat straw was determined according to the method developed by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2006). Both filter paper activity and $\beta$-glucosidase activity were determined using standard method described by Ghose (1987). One unit of filter paper activity (FPU) is defined as the amount of enzyme that forms 1 μmol of glucose (reducing sugars as glucose) per minute from filter paper. One unit of $\beta$-glucosidase activity (CBU) is defined as the amount of enzyme that produces 2 μmol of glucose per minute from cellobiose. Reducing sugar was quantified using the 3,5-dinitrosalicylic acid reagent (DNS) method with glucose as standard (Miller, 1959). Protein concentration was measured by the Bradford protein assay using bovine serum albumin (BSA) as standard (Bradford, 1976). The conductivity was measured by a conductivity meter (DDS-70A, Shanghai Precision and Science Instrument Co. Ltd., China). The pH was measured by a pH meter (PHS-2F, Shanghai Precision and Science Instrument Co. Ltd., China).

Glucose content was analyzed by HPLC (LC-20AT, Shimadazu Corp., Kyoto, Japan) equipped with a refractive index (RI) detector (RID-10A, Shimadzu Corp., Kyoto, Japan) and Shimadzu Shimpack-SPR-H column (300 × 7.8 mm). Four millimolar perchloric acid was used as the mobile phase at a flow rate of 0.6 ml/min and the column temperature was maintained at 40 °C.

SDS–PAGE was performed according to the methods described by Wang and Fan (2000). Resolving gel consisted of 12.5% polyacrylamide in Tris–HCl (1.5 M, pH 8.8), while stacking gel consisted of 4.5% polyacrylamide in Tris–HCl (1.0 M, pH 6.8). The sample solution containing 2% (w/v) SDS, 5% (v/v), 25% (v/v) glycerol, 60 mM/L Tris–HCl (pH 6.8), 14.4 mM/L 2-mercaptoethanol and 0.1% (w/v) bromophenol blue, was diluted 5 times with protein and then boiled for 5 min. The injection volume was 10 μl. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 10% (v/v) acetic acid/45% (v/v) methanol for 30 min at room temperature, thereafter, destained with 10% (v/v) methanol/10% (v/v) acetic acid solution.

### 2.5. Data processing

The hydrolysis yield is defined as follows:

\[
\text{Hydrolysis yield} \,(\%) = \frac{C_{\text{glu}} \times 0.9}{C_{\text{sub}} \times 32.4\%} \times 100
\]

where $C_{\text{glu}}$ is the glucose concentration (g/l) produced during hydrolysis process, $C_{\text{sub}}$ is the substrate concentration (100 g/l), 0.9 is the conversion factor considering water addition during cellulose hydrolysis. 32.4% is the cellulose content of pretreated straw.

The pure water permeability ($L_p$) of membrane is expressed as follows:

\[
L_p = \frac{J}{\text{TMP}}
\]

where $J$ is the permeate flux of deionized water (l/m$^2$ h), TMP is transmembrane pressure (bar).

The volume concentration factor (VCF) is defined as Eq. (3):

\[
\text{VCF} = \frac{V_f}{V_c}
\]

where $V_f$ is the feed volume, $V_c$ is the concentrate volume (cell volume), 13.5 ml.

The percentage recovery of the solute in concentrate is represented as follows:

\[
\text{Percentage recovery} \,(\%) = \left(1 - \frac{X_pV_f}{X_cV_c}\right) \times 100
\]

where $X_p$ and $X_c$ are the protein concentration, or glucose concentration, or conductivity in permeate and feed, respectively. $V_f$ is the permeate volume.

Irreversible fouling (IF) is defined as the pure water permeability decrease after filtration experiment divided by initial water permeability, as illustrated in the following:

\[
\text{IF} \,(\%) = \frac{L_{pb} - L_{pa}}{L_{pb}} \times 100
\]

where $L_{pa}$ and $L_{pb}$ are the pure water permeabilities before and after filtration experiments, respectively.

All experiments were performed in duplicate and the average values were reported. Experimental data were statistically evaluated using SPSS 15.0 software.
3. Results and discussion

3.1. Cellulose hydrolysis and cellulase adsorption during hydrolysis of SEWS

Available literature demonstrated the detrimental effect of lignin on cellulose conversion and cellulase adsorption during enzymatic hydrolysis of pretreated lignocellulosic biomass due to its nonproductive binding properties for cellulase (Palonen et al., 2004). If the substrate contained more lignin, lower hydrolysis yield and more cellulase adsorption would be expected. The high lignin content in SEWS (ASL + AIL, 45.7%), therefore, would necessarily affect the hydrolysis yield of cellulose and cellulase adsorption and desorption profile during hydrolysis process.

The concentrations of glucose and protein in the reaction suspension were monitored during the entire hydrolysis process and the results are shown in Fig. 2(a). Rapid protein adsorption occurred during the first 3 h hydrolysis with 57.9% of initially added cellulase protein adsorbed on SEWS. Over the same period, only 31.4% of cellulose was digested. With progress of hydrolysis from 3 to 12 h, the percentage protein adsorbed on substrate remained nearly constant while the cellulose hydrolysis yield increased more than two-fold from 31.4% to 64.9%. After 12 h of hydrolysis, the cellulase absorbed on SEWS was gradually released into the reaction suspension. The protein in the suspension increased from 40.6% at 12 h to 50.4% at 24 h with cellulose hydrolysis yield increasing from 64.9% at 12 h to 81.3% at 24 h. When hydrolysis further proceeded from 24 to 48 h, the percentage protein adsorbed on solid residue was kept at 49.6%. While at the same time, the percentage cellulose being hydrolyzed increased from 81.3% to 84.5%. The phenomenon that substantial amount of protein was associated with hydrolyzed residue during this period likely resulted from the non-specific binding of abundance of lignin in the substrate residue for cellulase with most of cellulose saccharified.

SDS–PAGE analysis of enzymatic hydrolyzate during 48 h of hydrolysis of SEWS also confirmed the adsorption–desorption profile occurred during hydrolysis process. As can be seen from Fig. 2(b), the cellulase had a wide molecular weight distribution with the molecular weight of major components (CBH I and CBH II, abbreviated from cellobiohydrolase I and cellobiohydrolase II, respectively) lied in the range from 55,000 to 68,000 g/mol, which was in accordance with previous reports (Tu et al., 2007; Yang et al., 2010). The relative intensity of near 66,000 g/mol band decreased in the first 12 h of hydrolysis, followed by a gradual increase until the end of hydrolysis, clearly indicating the adsorption of cellulase on substrate was a prerequisite for hydrolyzing cellulose and desorption of adsorbed cellulase occurred with progress of hydrolysis.

As described above, after 48 h of hydrolysis, about 50% of initially added cellulase protein was present in the supernatant, where the glucose concentration was as low as 30.4 g/l. Therefore, it is of practical significance if the cellulase could be separated from glucose for recycling cellulase in hydrolysis process, while the glucose could be concentrated for subsequent microbial fermentation. As a result, the cost of cellulase hydrolysis could be decreased and product concentration and fermentation efficiency of microbial fermentation could be increased. In the following sections, the feasibility of recovering cellulase from enzymatic hydrolyzate of SEWS by UF and concentrating glucose by NF was examined in detail.

3.2. Recovering cellulase by UF

Treatment of enzymatic hydrolyzate of SEWS by three PES UF membranes with different MWCOs in continuous concentration mode was carried out at two different permeate fluxes in order to select the suitable UF membrane and operating conditions for recovering cellulase. The suitable UF membrane should have the performances of high rejection of protein but low rejection of glucose, high flux and high antifouling. Table 2 shows the properties of raw hydrolyzate and UF permeate. PES5 retained most of protein but allowed glucose to largely passed through the membrane when the membrane was tested at permeate flux of 26.5 l/m² h. As for PES10 and PES30 membranes, glucose almost completely transmitted through both membranes, in contrast, the majority of protein was rejected regardless of permeate flux used. Conductivity analysis of raw hydrolyzate and UF permeate showed that the salts could almost completely pass through the PES10 and PES30 membranes. It was worthy mentioning that the protein transmission of the PES30 membrane was lower than of the PES10 membrane at the same permeate flux despite its MWCO was larger than the PES10 membrane (Table 1). The lower transmission of protein through PES30 membrane could be attributed to protein adsorption at the wall of membrane pores, thus narrowing or blocking the membrane pores and consequently leading to its pore size smaller than that of the PES10 membrane. The pH values of the permeates obtained from the three UF membranes under different conditions were nearly the same as that of enzymatic hydrolyzate.

The TMP profile during continuous concentration process of enzymatic hydrolyzate by three different UF membranes is shown in Fig. 3. The PES5 membrane had the highest TMP among the three tested membranes even though the filtration experiments

![Fig. 2. Cellulase partition during enzymatic hydrolysis of SEWS (a) and SDS–PAGE analysis of enzymatic hydrolyzate (b).](image-url)
were performed at 26.5 l/m² h. The PES5 membrane could retain higher amount of glucose and salts compared to other two membranes (Table 2), thus leading to gradual accumulation of glucose and salts in the retentate with an increase of VCF, which in turn resulted in higher osmotic pressure. In addition, the highest intrinsic membrane resistance due to its smallest MWCO among the three tested UF membranes also contributed much to its distinct TMP profile. All these could explain why the PES5 membrane reached the pressure limit of 4.5 bar fastest among the three UF membranes under the same operation conditions. When PES10 and PES30 membranes were used for concentrating enzymatic hydrolyzate, glucose could transmit through both membranes completely (Table 2), reducing the osmotic pressure. The TMPs of both membranes were relatively lower than that of the PES5 membrane at the permeate flux of 26.5 l/m² h. Fig. 3 also shows an unexpected phenomenon that the PES10 membrane with lower MWCO showed lower TMP with increasing VCF than the PES30 membrane with higher MWCO at the same permeate flux. The reason was likely due to the PES30 membrane more easily fouled by the proteins present in the solution, as discussed below. Another possible reason could be attributed to the higher level of retention of salts and protein compared to PES10 membrane (Table 2), leading to higher osmosis pressure and more severe concentration polarization, respectively. During continuous concentration process until VCF reached 5, the PES10 membrane gave the lowest TMP when performed at 26.5 l/m² h, followed by the PES30 at 26.5 l/m² h and PES10 at 53.1 l/m² h.

The anti-fouling performance of membrane is one of the most important factors to be considered in selecting desirable membrane because membrane fouling is responsible for TMP increase in constant flux mode. Fouling can be classified into reversible fouling and irreversible fouling (IF). The former stems from concentration polarization and cake layer and can be removed by backwashing, whereas the latter is caused by deposit of solute or particle into membrane pores and can be removed by chemical cleaning. Fig. 4 shows the pure water permeability decline after filtration. IF for PES10 membrane was 43.7% at 26.5 l/m² h, the lowest among the membranes and operating conditions examined, exhibiting the best anti-fouling performance. The highest IF of 66.6% for PES30 membrane when operated at 26.5 l/m² h indicated the severe fouling resulting from occlusion of membrane pores mainly due to its larger MWCO than molecular weight of some components of cellulase. The middle level of IF for PES5 at 26.5 l/m² h and PES30 at 53.1 l/m² h, 50.8% and 48.7%, respectively, could be attributed to the short filtration time indicated by the low VCF (Fig. 3).

Table 3 summarizes the percentage recoveries of protein and glucose and average TMP during UF process. Although PES5 membrane at 26.5 l/m² h and PES30 membrane at 53.1 l/m² h gave high protein recovery (89.41% and 87.5%, respectively), the high glucose recovery (63.1% and 41.4%, respectively) and high average TMP (3.99 and 3.80 bar, respectively) made both unsuitable for continuously recovering cellulase protein. When filtering enzymatic hydrolyzate using the PES10 at 25.6 l/m² h, the glucose recovery (16.7%) was the same as the PES10 at 53.1 l/m² h; the protein recovery (73.9%) was a little lower than the PES10 at 53.1 l/m² h (79.7%) and PES30 at 25.6 l/m² h, 79.7%. While the average TMP of the PES10 at 25.6 l/m² h was the lowest. The PES10 filtered at 25.6 l/m² h, therefore, was selected for recovering cellulase from enzymatic hydrolyzate.

In the UF stage, the cellulase in the hydrolyzate suspension was recovered in the retentate and could be used for subsequent round of hydrolysis of cellulosic substrate. Glucose completely transmitted through the UF membrane and could be collected in the permeate, which could be further concentrated by NF to increase glucose concentration.
3.3. Concentrating glucose by NF

The UF permeate from PES10 membrane operated at 26.5 l/m² h was treated by two NF membranes at two permeate fluxes and the results are shown in Table 4. Glucose, protein and salts were accumulated in the concentrate of both NF membranes. The highest glucose concentration of 110.2 g/l, 3.5 times more than original UF permeate, was obtained when using NF270 membrane at 13.3 l/m² h. The pH values of concentrates from NF90 and NF270 membranes showed insignificant difference compared to that of the original UF permeate.

The TMP profile during NF treatment of UF permeate is shown in Fig. 5. The larger the MWCO of NF membrane and the lower the permeate flux, the higher the VCF. A VCF of 2.89 was obtained when NF270 membrane was operated at 13.3 l/m² h. The fast rise in the TMP could be attributed to three factors. One was that the buildup of glucose and salts in the retentate of both membranes (Table 5) led to high osmotic pressure. Another was that the complete rejection of protein by NF90 and NF270 (Table 5) formed a concentration polarization layer. The third could be much severe membrane fouling. As illustrated in Fig. 6, IF for NF90 and NF270 at two permeate fluxes ranged from 60.0% to 69.4%, and from 83.3% to 86.5%, respectively.

In the NF stage, when the ultrafiltered hydrolyzate was treated by NF270 at 13.1 l/m² h, the glucose concentration increased to 110.2 g/l from 30.2 g/l, while the produced permeate could be collected for water reuse and recycling.

4. Conclusions

This work demonstrated the feasibility of recycling cellulase and concentrating glucose from lignocellulosic hydrolyzate by a two-stage membrane process with combination of UF and NF. In the first stage, the PES10 ultrafiltration membrane when operated at 25.6 l/m² h was suitable to recover cellulase protein because of its good anti-fouling performance and allowance of complete transmission of glucose. In the second stage, the NF270 nanofiltration membrane when operated at 13.3 l/m² h could effectively concentrate glucose and gave high glucose concentration in the retentate.

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