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Directing filtration to narrow molecular weight distribution of oligodextran in an enzymatic membrane reactor

Ziran Su [a,b], Jianquan Luo [a,b*], Manuel Pinelo [c] and Yinhua Wan [a,b*]

[a] *State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, PR China*

[b] *University of Chinese Academy of Sciences, Beijing 100049, PR China*

[c] *Department of Chemical and Biochemical Engineering, Technical University of Denmark, 2800, Kgs. Lyngby, Denmark*

jqluo@ipe.ac.cn (J. Luo)

yhwan@ipe.ac.cn (Y. Wan)

**Corresponding authors. Tel/Fax: +86-10-62650673*

Abstract

Oligodextrans with molecular weight (Mw) within the range of 5.0-8.0 kDa have great commercial potential as precursors of iron-dextran for anemia treatment. Traditional oligodextran production consists of sucrose fermentation, acid hydrolysis and ethanol precipitation, which results in an uneven Mw product, hypersaline wastewater discharge and potential safety hazards. In this work, a novel enzymatic membrane reactor (EMR) system to produce oligodextran is proposed, whereby *in-situ* product recovery can be manipulated to control the Mw distribution of the resulting products. Results showed that the membrane material played an important role in the permeate flux and transmission of oligodextran. Among the tested membranes, a 20 kDa polyethersulfone (PES) membrane was found to be optimal for building up the EMR, as it successfully controlled the oligodextran Mw within the desired range with a relatively narrow distribution and high productivity. Moreover, high transmembrane pressures (3 bars) and low stirring rates (160 rpm) promoted yields beyond 50% in 120 min. Higher permeate fluxes prevented further product hydrolysis and enhanced the yield. However, the resulting concentration polarization (CP) should be minimized to reduce accumulation of large oligodextran molecules on the membrane surface, which might diffuse through the membrane and thus broaden the Mw distribution of the products in the permeate. Both dextranase and dextran caused membrane irreversible fouling. The fouling caused by the enzymes not only favored the enzyme immobilization itself, but also contributed to narrow the membrane pore size

distribution. As a result, a higher uniformity of oligodextran products compared with the pristine EMR was obtained, especially at the beginning of operation with EMR (which was improved by 22%). It was concluded that selecting the suitable membrane type and permeate flux, maximizing the shear rate, and narrowing the membrane pore size distribution were effective strategies to obtain high-quality oligodextran products by EMRs.

Key words: Ultrafiltration; oligosaccharide; dextranase; membrane fouling; *in-situ* product separation

1. Introduction

Linked by alpha-1,6 glucosidic bonds, dextran is one of the most known polysaccharides made of repeating glucose units and is widely used in food and medicine [1]. Dextran molecules exhibit particular properties depending on the molecular weight (Mw). Especially oligodextran with certain Mw (5.0-8.0 kDa) has superior properties for maintaining blood pressure, extending blood vessels and for being able to form iron complexes for clinical usage [1-3]. Traditional oligodextran production consists of sucrose fermentation, acid hydrolysis and ethanol precipitation. *Leuconostoc mesenteroides* converts sucrose to macrodextran molecules with Mw over several million Dalton [4-6]. Acids are thereafter used to break the alpha-1, 6 glycosidic bonds randomly, generating smaller pieces of dextran. Several alcohol sedimentation steps are subsequently required for purifying oligodextrans at several Mw ranges after acid hydrolysis [7]. Such method can result in the occurrence of chlorinated species amongst the oligodextran products, which may cause serious damage to human beings. Additionally, considerable amounts of ethanol are consumed during oligodextran purification, which has to be recovered and recycled. More importantly, the obtained oligodextrans have a wide distribution of Mw, which may result in immune problems in clinical usage [8].

Production of oligodextran by enzymatic hydrolysis can be considered safer, as it occurs at milder conditions and involves less wastewater generation. Dextranase from *Penicillium sp.* can effectively break the alpha-1,6 glycosidic bonds of the dextran molecules under mild aqueous environment [9]. No extra chemicals need to be added to the reaction system, which also contributes to make the process greener. While studying a dextransucrase–sucrose system (synthesis of dextran), Gan et al. found that an early addition of dextranase resulted in the production of low Mw of oligodextran products [8]. Likewise, Iqbal and co-workers reported that dextranase hydrolysis generated less dispersed oligodextran compared to acid hydrolysis, which made oligodextran production of clinical grade possible [10]. Additionally, Goulas et al. demonstrated that low enzyme concentrations could generate narrower oligodextran distributions during enzymatic hydrolysis [11].

Besides optimization of the enzymatic reaction, membrane technology, especially ultrafiltration (UF), has been previously used to narrow the Mw distribution of oligodextran due to its low energy requirement and sustainability [12]. Early in 1999, an UF membrane was used to separate certain clinical oligodextran products after enzymatic hydrolysis [13]. Later research

further developed a continuous membrane reactor in which Mw distribution could be controlled by adjusting enzyme concentration and retention time [14]. Then Torras and co-workers optimized the dextranase loading to be immobilized on a polymeric membrane for controlling the products Mw [15]. Pu et al. also found that a UF-diafiltration process could greatly narrow the Mw distribution of clinical dextran [16]. In most of the previous studies, however, enzymatic hydrolysis and UF were performed separately. Actually, enzyme reuse and *in-situ* product recovery could be carried out simultaneously by coupling enzyme and membrane technology in the same system, an enzymatic membrane reactor (EMR). Among other advantages, such solution could probably reduce product inhibition of the enzyme and avoid further hydrolysis of target products. Previous studies on the topic involving the use of EMR mainly focused on the optimization of enzymatic hydrolysis rather than paying attention to the membrane separation process, which is indeed the product quality-limiting step. During membrane filtration, concentration polarization (CP) is considered an obstacle, resulting in the decrease of permeate flux and selectivity [12, 17]. However, the increase in concentrations of substrate and enzyme on membrane surface could also favor solute transmission through the membrane, hence increasing enzymatic reaction efficiency. Membrane fouling, however, could not only decrease permeate flux and thus increase the retention time of substrates, but also might reduce the transmission of target products through the membrane [18].

In present work, aiming at producing oligodextran with Mw between 5.0-8.0 kDa at a narrow Mw distribution, an EMR was designed to enable enzyme reuse and *in-situ* product recovery. Large dextran molecules with initial Mw of 40 kDa were first hydrolyzed by dextranase. The effect of the membrane material, molecular weight cut-off (MWCO), transmembrane pressure (TMP) and shear rate (i.e. agitation speed) were evaluated in terms of oligodextran Mw and its distribution in permeate, permeate flux, irreversible fouling and yield. Since membrane fouling is inevitable during membrane separation, based on the understanding of fouling mechanisms of the established EMR, dextranase was partly immobilized on the UF membrane in advance by a simple filtration ("fouling-induced" enzyme immobilization [19, 20]). We hypothesized that the "enzyme fouling" layer could not only increase enzyme stability and accelerate online product separation, but also narrow the distribution of membrane pore size (larger pores are more prone to be blocked since the higher local flux around the larger pores would induce more serious fouling formation) [21, 22] and improve the separation selectivity.

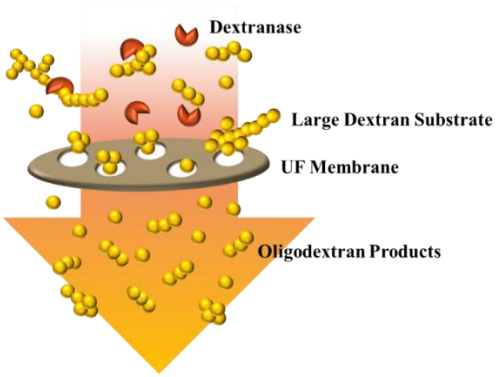

Fig. 1. Illustration of processes taking place in the enzymatic membrane reactor. Dextranase breaks the α-1,6 glucosidic bonds on dextrans randomly and generates smaller dextran molecules with different molecular weights. A membrane with a particular pore size distribution is used under certain pressure to separate the target molecules while retaining large dextran fractions and enzymes. Target oligodextrans (5.0-8.0 kDa) are removed from the reactor to avoid further hydrolysis.

2. Methods and materials

2.1. Materials

Dextran substrate (DXT40K) with Mw 40 kDa was purchased from Sinopharm Co Standard. Dextran Benchmark with Mw 0.36, 5, 12, 25, 50, 80 and 150 kDa, dextranase (EC 3.2.1.11, dry powder from *Penilisilium. Sp.*) and Bradford reagent used for the protein assay were purchased from Sigma-Aldrich Co.. The ultrapure water used for dissolving substrate and enzymes was generated by a Millipore Milli-Q Advantage A10 water purification system (pH 6.0±0.5). Other chemicals were of analytic grade. Information of UF membranes with different materials and MWCO used in the experiments is listed in **Table 1.**

Table 1 Properties of membranes used in the experiments

Membrane type	Skin layer material	MWCO (Da)	Manufacture
RC5	Regenerated cellulose	5,000	Millipore
RC10	Regenerated cellulose	10,000	Alfa Laval
PES10	Polyether sulfone	10,000	AMFOR
PES20	Polyether sulfone	20,000	AMFOR
PES30	Polyether sulfone	30,000	AMFOR
PAN10	Polyacrylonitrile	10,000	AMFOR

2.2. Experimental procedure

Enzymatic hydrolysis reactions were firstly carried out in beaker to obtain preliminary information about the Mw variations of dextran under different dextran/dextranase ratios (25 °C,

160 rpm agitation, pH 6.0 ultrapure water as solvent, chemical dosage was listed in **Table S1**). 0.5 ml samples were collected every 30 min and placed in a boiling water bath for 1 min to deactivate the enzymes.

After a suitable dextran/dextranase ratio was selected, an EMR was constructed and operated at room temperature (25 °C). The fresh membranes were immersed into 5% NaCl (regenerated cellulose membranes) or 0.01 M NaOH (other membranes) solution for 30 min to remove the preservatives, according to the instructions of the manufacturers. The clean membranes were then placed at the bottom of a dead-end stirred cell (Amicon 8050, Millipore, U.S.A) with an effective membrane surface area of 13.4 cm^2 or a home-made cell with an effective membrane surface of 4.52 cm^2, standing above the magnetic stirrer. A constant pressure was generated by filling nitrogen gas into the cell while a constant flux was provided by LC-20AT pump (SHIMADZU). The schematic diagram of these two systems was shown in **Fig. S1**. The membrane was compacted at 4.5 bar for 30 min with pure water. Dextran substrate and dextranase were mixed in the cell which was continuously fed with pure water under different pressure and agitation speed. A cylinder was used for measuring and collecting the permeate samples every 30 min. All the collected samples were heated in boiling water for 1 min and then stored at 4 °C for analysis.

For the experiments dealing with the study of fouling mechanisms, 50 mL 0.05 g/L dextranase and 50 g/L 20 kDa dextran (Sinopharm Co.) were respectively added into the filtration cell equipped with PES20 membrane. Moreover, 50 g/L DXT40K was hydrolyzed by 0.05 g/L dextranase for 30 min, 60 min, 90 min and 180 respectively. After reaction, the samples were heated in boiling water for enzyme deactivation and then added into the filtration cell where the PES20 membrane was placed. For these filtrations (each feed was 50 mL), pure water was continuously fed into the cell under 4.5 bar and 2000 rpm for 2 hours. Water permeability before and after filtration was measured in order to assess the irreversible fouling.

For the "fouling-induced" enzyme immobilization process, a fresh membrane was placed at the bottom of the filtration cell, and 2.5, 5.0 and 10 mg dextranase in 50 ml pure water were added respectively. The filtration was conducted in concentration mode under 4.5 bar and 100 rpm. After 48 ml of solution permeated through the membrane, the filtration was stopped. 0.5 ml of retentate and permeate were respectively collected for protein concentration determination.

The rest of retentate and permeate were mixed with 50 ml 50 g/L dextran substrates for the hydrolysis and separation in this special EMR under 4.5 bar and 2000 rpm.

2.3. Analysis methods

Gel permeation chromatography (GPC) was used to test the average Mw of products and their distribution. Samples were eluted under 1 mL/min in ultrapure water, 40 °C. A refractive index detector coupled with the G4000PWXL column from Shimadzu was used for testing the samples. Peaking information including Mw and dispersity (D=Mw/Mn, Mw is weight-average molar mass, Mn is number-average molecular weight) of each sample were determined with help of the Shimadzu LC solution software. A smaller D value represents a higher uniformity of oligodextran products. The benchmark of the dextran molecules in the range between 0.36 and 150 kDa is given in **Fig. S2.** The average Mw was the arithmetic mean Mw of the four collected samples during the 120 min of hydrolysis.

The phenol-sulfuric acid method was used for testing the total saccharides concentration in the permeate for the different enzymatic systems. Samples were diluted by 500 times with pure water before testing. Each 2 mL diluted sample was added to a 10 mL glass tube. 1 mL of 6% (v/v) phenol and 5 mL of sulfuric acid were mixed with the samples. Then the mixtures were shaken for 30 s and let stand for other 30 min. The sample absorbance was tested in proper cuvettes under 490 nm in a UV-2100 Spectrophotometer. Glucose solutions with concentrations between 0.01 and 0.07 g/L were used for calibration (**Fig. S3a**). The obtained saccharides concentrations were the average saccharides concentrations corresponding to each time interval (0-30 min, 30-60 min, 60-90 min and 90-120 min).

The enzyme activity and oligodextran production rate were tested by using 3, 5-dinitrosalicylic (DNS) acid reagent. 5 mg free dextranase in 30 ml H_2O was stirred under 160, 1000 and 2000 rpm (25 °C) respectively. 1 ml of enzyme solution was taken every 30 min, and then mixed with 10 ml 4 g/L DXT40K for 25 min followed by deactivation in boiling water. Reducing sugar concentration in the solution was measured by using DNS reagent at 540 nm. The enzyme activity was defined as the amount of isomaltose (measured as maltose with unit of μmole) per unit of enzyme mass (mg) after 1 minute at 25 °C. In the oligodextran production rate testing, 4 g/L DXT40K solution (50 ml) was mixed with 0.5 mL 0.2 g/L dextranase. The reactions were performed for 2 hours under 160, 1000 and 2000 rpm, respectively (25 °C). The oligodextran production rate was defined as the cumulative maltose amount (umol) per unit of

enzyme mass (mg) at corresponding reaction time interval. The reducing sugar was tested by the same method mentioned above. Maltose solutions with concentrations between 0.2 and 0.8 g/L were used for calibration (**Fig. S3b**). The protein concentration in enzyme solution was measured by Bradford assay with a spectrophotometer at 595 nm (**Fig. S3c**).

2.4. Characterization of membrane

The morphology of pristine and "fouled" PES20 membranes was characterized by field emission scanning electron microscopy (FESEM, Hitachi S480, Japan) with an acceleration voltage of 5.00 kV after the samples being sprayed with a thin gold layer (around 20 nm). Membrane surface porosity and pore size distribution were calculated from SEM images by Image J software.

2.5. Calculations

2.5.1. Membrane water permeability and permeate flux

Permeate flux (J_p) was calculated as:

$$J_p = \frac{V_p}{t \bullet A_m} \quad \textbf{(1)}$$

Membrane water permeability (L_p) was calculated by the following equation:

$$L_p = \frac{J_p}{TMP} \quad \textbf{(2)}$$

where V_p is the permeate volume corresponding to certain time (t) during the testing, A_m is the effective filtration area, TMP is the transmembrane pressure.

The average permeate flux in an EMR was measured at four reaction time intervals (0-30 min, 30-60 min, 60-90 min and 90-120 min) respectively and calculated by **Eq. (1)**.

Irreversible fouling was defined as the percentage of water permeability loss:

$$F_{irr} = \frac{L_{pi} - L_{pa}}{L_{pi}} \times 100\% \quad \textbf{(3)}$$

where L_{pi} and L_{pa} are the L_p before and after filtration, respectively.

2.5.2. EMR yield

The volume of permeate samples was measured every 30 min. The amount of saccharides in each permeate sample was calculated from the saccharides concentration (C_p) and volume in the permeates:

$$M_p = C_p \times V_p \quad \textbf{(4)}$$

In the water feeding system, the initial mass of dextran was settled, and the yield was calculated as :

$$Y = \frac{M_p}{Mt} \quad (5)$$

where M_p is the sum of saccharides mass in permeate, and M_t is the total mass of saccharides added in the reaction system.

2.5.3. Enzyme loading efficiency

The immobilized amount of enzyme was calculated by

$$M_{imm} = C_{ini.} \times V_{ini.} - C_{per.} \times V_{per.} - C_{ret.} \times V_{ret.} - C_{rin.} \times V_{rin.} \quad (6)$$

where M_{imm} is the immobilized mass of enzyme, $C_{ini.}$, $C_{per.}$, $C_{ret.}$ and $C_{rin.}$ represent enzyme concentrations in the feed, permeate, retentate and rinsing solution respectively. V is the volume of the solutions corresponding to the concentration.

The loading efficiency is defined as:

$$\text{Loading} = \frac{M_{imm}}{C_{ini.} \times V_{ini.}} \times 100\% \quad (7)$$

3. Results and discussion

3.1. Optimizing operational variables in the EMR

3.1.1 Effect of substrate and enzyme ratio

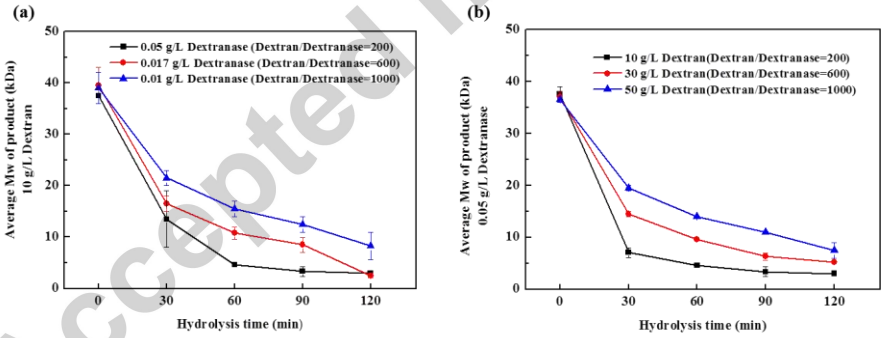

Fig. 2 Effect of concentrations of dextran and dextranase on the average Mw variations in a batch hydrolysis system. (a) 10 g/L dextran substrate with different dextranase concentrations, (b) 0.05 g /L dextranase with different dextran concentrations.

Within certain reaction time, substrate and enzyme concentrations are the main variables affecting the average Mw of products [23]. This was confirmed by the results in **Fig. 2**, where it can be seen that larger dextran/dextranase ratios resulted in slower decreases of Mw in the batch reaction system. For a low dextran/dextranase ratio of 200, the average Mw declined fast within the first 30 min from 37.5±1.5 to 7.1±1.0 kDa and slowed down from 30 min to 120 min. The

average Mw of the final oligodextran product in this case was 3.0±0.2 kDa after 120 min hydrolysis, which was lower than the desired one (5.0-8.0 kDa).

The main goal of this work was to establish an EMR that can carry out simultaneous product separation during hydrolysis. For that purpose, the production rate of target oligodextrans within Mw 5.0-8.0 kDa should match the rate of product removal of the membrane (i.e. reaction and separation should be synchronous). If the product filtration is not quick enough, the products would be further hydrolyzed to smaller pieces, leading to product quality decline. In order to better match the kinetis of the enzymatic reaction and the filtration, a high dextran concentration (50 g/L) and a high ratio of dextran/dextranase (1000) were chosen for the following experiment.

Based on the above results, 50 g/L dextran (DXT40K) together with 0.05 g/L dextranase were mixed in the stirred cell equipped with a PES20 membrane and continuously fed with pure water (pH 6.0±0.5) under 3 bar. The average Mw of the products collected in the permeate side was 7.6±0.2 kDa, which fell within the desired range (5.0-8.0 kDa) but was much lower than the nominal MWCO of the PES20 membrane. This was probably due to the pore narrowing and blocking caused by fouling formation and membrane compaction [24].

Because of the randomness of enzyme hydrolysis, it is possible that the rejected target oligodextrans might be further degraded by the enzymes leading to an undesired wide Mw distribution in the permeate at the beginning (**Fig. S4**). An attempt to incorporate a continuous dextran substrate feeding was then made to prevent further hydrolysis of target oligodextran products, also taking into account that dextranase is prone to hydrolyze larger dextran molecules [7]. However, as shown in **Fig. S4**, continuous substrate feeding under a constant TMP resulted in more accumulation of large dextran molecules, which in turn caused a severe CP layer on the membrane surface. As a consequence, more diffusion of large dextran molecules through the membrane occurred, which led to an even wider Mw distribution of products. From these results it was deduced that the strategy of controlling products average Mw and narrowing down their distribution should be rather focused on increasing the membrane transmission and selectivity, in order to ensure the removal of the desired products as soon as possible and to obtain maximum rejection of large dextrans. Therefore, membrane pore size and materials which are mainly responsible for the filtration behavior were further investigated to optimize this online separation process.

3.1.1. Effect of MWCO and membrane materials

UF membranes with MWCO of 5-20 kDa and different skin layer material were screened to control the average Mw and distribution of products. In general, all the EMRs decreased the average Mw and distribution of products as compared to batch reaction system (**Fig. 3a, b**), however the results could not only be explained by the MWCO differences among the membranes. The RC5 membrane with the smallest pore size had the lowest water permeability and permeate flux (**Fig. 3c, d**). As a result, it produced the most uniform and smallest oligodextran molecules (1.7±0.1 kDa). However, with the same nominal MWCO, the average Mw of products and their Mw distribution in the PES10-EMR were much lower than those in the RC10-EMR and PAN10-EMR, which was even close to those in the PES20-EMR. Such results suggested that the membrane material rather than the MWCO was the most determining factor during product separation for the membranes with larger pore size. It was speculated that the oligodextran molecules with abundant hydroxyl groups had stronger interaction with the hydrophilic RC and PAN membranes, inducing more transmission of large dextrans through the membrane [25]. On the other hand, the permeate flux in the PAN10-EMR was much lower than that in RC10-EMR and PES10-EMR (**Fig. 3c**). It is well known that the permeate flux is governed by both membrane and fouling resistances [18]. The PAN10 membrane had the highest permeability (lowest membrane resistance), and the irreversible fouling detected (permeability loss after filtration) was only slightly higher than the others (**Fig. 3d**). This implied that the CP or reversible fouling in the PAN10-EMR was most serious, which was caused both by the accumulation of dextrans on the membrane surface and by the strongest interaction between dextran and PAN membrane material which is rich in carboxyl groups (nitrile groups would be hydrolyzed to carboxyl groups). The larger average Mw of the resulting products in permeate and the wider Mw distribution for the PAN10-EMR than those for other EMRs (**Fig. 3a, b**) supported the above hypotheses.

The average Mw of the products from PES20-EMR was right within the desired range (7.6±0.2 kDa), while the Mw distribution decreased from 3.59 at 30 min to 2.50 at 120 min. Moreover, the permeate flux for the PES20-EMR during filtration was the highest. Such high flux indicated that the PES membrane (less hydrophilic than the others) could not only reject more large oligodextran molecules resulting in a relative low average Mw and narrow distribution of the products, but also had a thinner CP layer on the membrane surface due to the

weaker interaction with dextrans (leading to a higher permeate flux). Therefore, the PES20-EMR outperformed the others in terms of product quality and permeate flux, which was selected for the further studies.

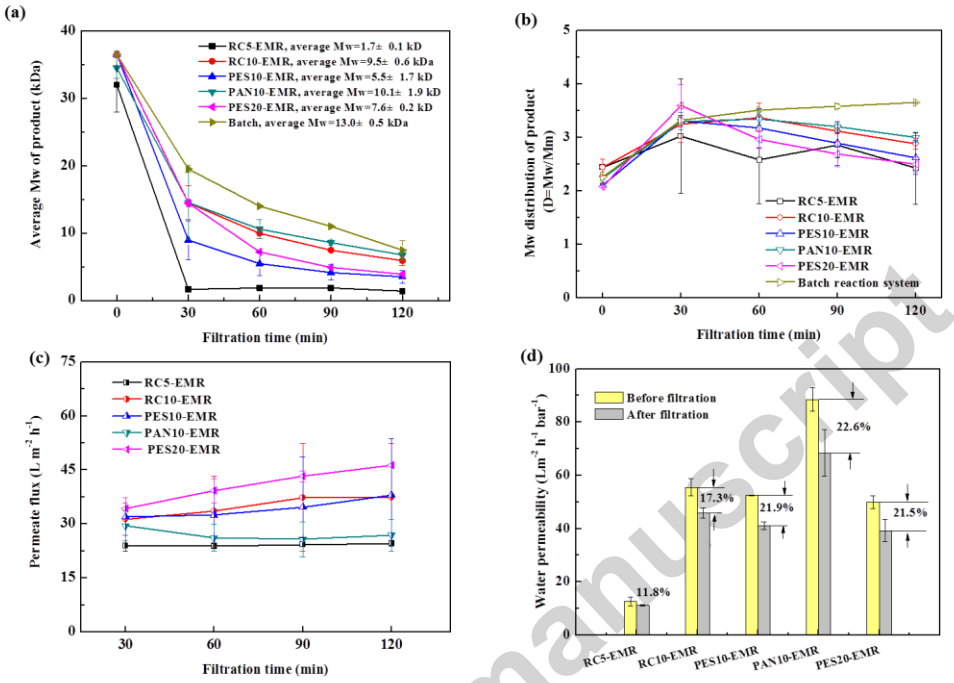

Fig. 3 Effect of membrane materials and MWCO on the performance of EMRs.
(a) Average Mw, (b) Mw distribution of oligodextran molecules in permeate, (c) permeate flux with time and (d) irreversible fouling after filtration for different EMRs.

3.2 Process optimization

Resistance-in-series model is widely used in describing UF process [18, 26] which can be expressed as follows:

$$J = \frac{TMP - \Delta\Pi_b}{\mu(R_m + R_{rf} + R_{if})} \quad \textbf{(8)}$$

Where $\Delta\Pi_b$ is the osmotic pressure difference between the bulk and the permeate (Pa), μ is the solvent viscosity (Pa•s), R_m is the membrane resistance (m^{-1}), R_{rf} and R_{if} are the resistances caused by reversible fouling and irreversible fouling respectively (m^{-1}).

The permeate flux is proportional to the driving force and negatively correlated to the viscosity as well as filtration resistances. Theoretically, higher TMP can increase the permeate flux until the limiting flux is obtained. Higher permeate flux would first produce more solvent convection, resulting in higher solute rejection (dilution effect), but further increasing permeate flux may cause severe CP and membrane fouling, leading to unexpected results. On the other

hand, the higher shear rate induced by agitation on the membrane surface is expected to attenuate the CP and membrane fouling, resulting in higher permeate fluxes and solute rejections under constant TMP mode [27-29]. These theories have been intensively studied in the single membrane filtration. Since the filtration behavior also affects the enzymatic reaction efficiency, it is far more complicated to study such effects in an EMR.

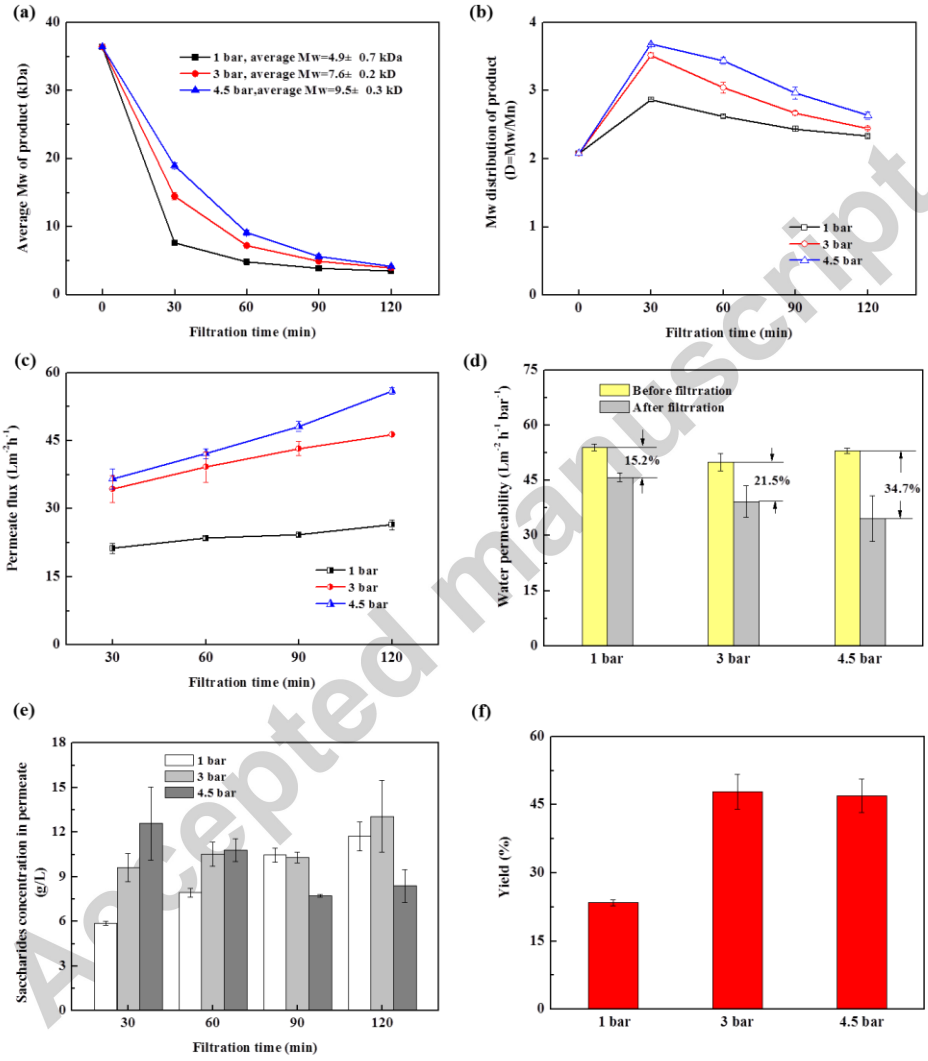

Fig. 4 Effect of TMP on oligodextran production in a PES20-EMR at 160 rpm.

(a) Average Mw, (b) Mw distribution of oligodextran molecules in permeate, (c) permeate flux with time, (d) irreversible fouling, (e) saccharides concentration in permeate and (f) yield of oligodextran in permeate.

3.2.1 Effect of TMP

Fig. 4 illustrates effect of TMP on oligodextran production in a PES20-EMR at 160 rpm. A low pressure (1 bar) resulted in the smallest average Mw (4.9±0.7 kDa) and also the most

uniform products (**Fig. 4a, b**). Long retention time due to low permeate flux (**Fig. 4c**) enabled sufficient hydrolysis of substrate, thus lowering the product Mw. On the other hand, a low permeate flux produced the least irreversible fouling (**Fig. 4d**), and thus the effect of fouling layer on oligodextran transmission through the membrane was negligible, resulting in an increasing oligodextran concentration in permeate with time (**Fig. 4e**). However, the low permeate flux also limited the oligodextran yield, and the lowest yield was obtained at 1 bar after operation for 120 min (**Fig. 4f**). With increase of TMP, the permeate flux in the EMR was greatly improved, leading to severer CP [30] and shorter retention time. Accordingly, less hydrolysis time and stronger concentration-gradient diffusion across the membrane at higher TMP produced a product with higher average Mw (7.6±0.2 kDa at 3.0 bar, 9.5±0.3 kDa at 4.5 bar) and wider Mw distribution (**Fig. 4a, b**). The formation of a more prominent CP layer at higher TMP might induce two opposite effects on oligodextran transmission through the membrane: first, more accumulation of oligodextran in the CP layer could accelerate its diffusion across the membrane; second, a more serious CP could result in a higher irreversible fouling (i.e. pore narrowing and blockage), thus increasing oligodextran rejection. The former was more obvious at the beginning of the operation and at low TMP ranges, and could explain why the saccharides concentration in the permeate was increasing with TMP at 30 and 60 min. The effect of irreversible fouling on oligodextran transmission was dominant at 90 and 120 min, which led to lower saccharides concentration in the permeate at 4.5 bar (**Fig. 4e**). Finally, since the yield was calculated by multiplying permeate volume and saccharides concentration then dividing total added saccharides amount (**Eq. 5**), it became almost the same at 3.0 and 4.5 bar (**Fig. 4f**), which was much higher than that at 1.0 bar because of its lowest permeate flux (**Fig. 4c**).

It is worth mentioning that under constant TMP mode, the permeate flux was changing which might affect the validity of the above discussion. Thus, the effect of permeate flux on the oligodextran production in a PES20-EMR was investigated under constant flux mode. Based on the results in **Fig. 4c**, the permeate flux of 16, 27 and 40 $Lm^{-2}h^{-1}$ was applied. As shown in **Fig. S5**, the higher permeate flux resulted in higher product Mw and wider Mw distribution which was similar to the results in **Fig. 4**. However, the initial pressure was quite high and then decreasing under constant flux mode. The permeability loss was 24.1%, 31.3% and 56.5% at 16, 27 and 40 $Lm^{-2}h^{-1}$ respectively, indicating that severer irreversible fouling was formed at higher permeate flux. Although the CP-induced diffusion enhancement still existed, this denser fouling

layer and higher permeate flux (dilution effect [18]) would increase the oligodextran rejection, thus decreasing the saccharides concentrations in permeate at higher permeate flux. Thus, the product yield was not improved when the permeate flux increased from 16 to 27 $Lm^{-2}h^{-1}$. Actually, the permeate flux should be a little lower at the beginning of the reaction because the target product was few while the CP should be controlled, and then the permeate flux ought to be increased when more oligodextran molecules were generated. Therefore, constant TMP mode resulting in an increase of flux with time was considered as "self-regulation" process, which might be more suitable for this application.

According to the above discussion, a higher TMP was desirable in order to avoid further product hydrolysis and promote high yield, but CP should be minimized to reduce accumulation of large oligodextran molecules on the membrane surface, which might diffuse through the membrane and enlarge the Mw distribution of the products in permeate. Moreover, more serious CP would cause higher irreversible fouling, which also should be controlled to maximize the transmission of target products. Therefore, effect of agitation speed (i.e. shear rate) on the EMR performance at a high TMP of 4.5 bar was evaluated in the following study.

3.2.2 Effect of agitation speed

As expected, higher shear rate induced by agitation reduced the average Mw of products and their Mw distribution, and also increased the permeate flux (**Fig. 5a, b, c**). These positive effects were attributed to a thinner CP layer at higher agitation (stronger shear-induced back diffusion). Indeed, the accumulation of large oligodextran molecules on the membrane would be remarkably alleviated, and their diffusion through the membrane would be also reduced at higher agitation speed [31]. The large oligodextrans possibly had more time to react with the enzymes in the reactor and were hydrolyzed to the target product [32], resulting in lower product Mw and distribution. Irreversible fouling only decreased slightly from 34.7% to 31.8% when the agitation enhanced from 160 to 1000 rpm (**Fig. 5d**), and it even aggravated to 40.9% at 2000 rpm. There is no doubt that the thinner CP layer promoted by higher shear rate would alleviate membrane fouling, however, at the same operating time, the larger permeate flux caused by higher agitation under constant TMP allowed much more solution passing through the membrane, implying that the substrate, product and enzyme could foul the membrane to a higher extent.

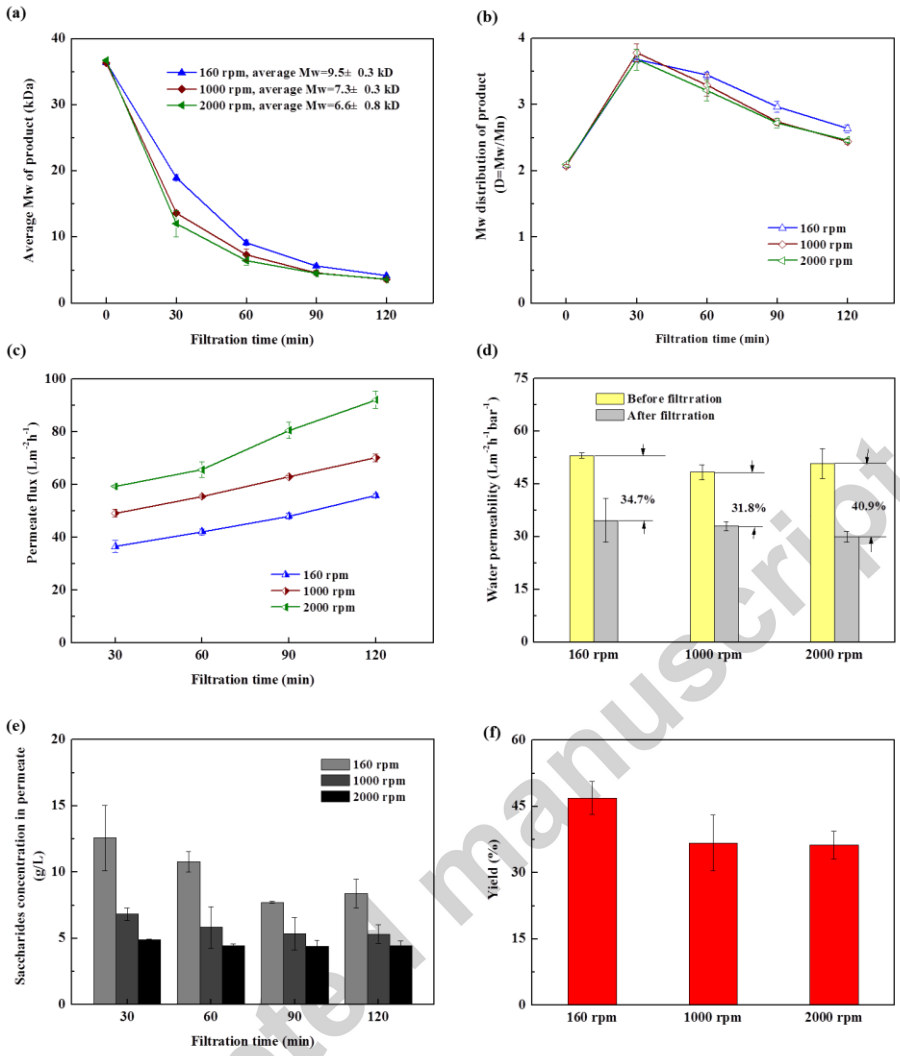

Fig. 5 Effect of agitation speed on oligodextran production in a PES20-EMR at 4.5 bar. (a) Average Mw, (b) Mw distribution of oligodextran molecules in permeate, (c) permeate flux with time, (d) irreversible fouling, (e) saccharides concentration in permeate and (f) yield of oligodextran in permeate.

As above mentioned, less accumulation of oligodextran on the membrane at higher agitation decreased the diffusion of oligodextran through the membrane. On the other hand, higher permeate flux (**Fig. 5c**) also increased the oligodextran rejection due to the "dilution effect" [18]. Consequently, the saccharides concentration in permeate decreased with increase of agitation speed (**Fig. 5e**). Moreover, the saccharides concentration in permeate diminished with increasing operating time probably because the substrate concentration in the EMR was decreasing under the water feeding mode while irreversible fouling was forming. The oligodextran yield was determined by the permeate flux and saccharides concentration, and the yield at 160 rpm was the highest attributed to its highest saccharides concentration in permeate (**Fig. 5f**). The higher

permeate flux at 2000 rpm was offset by its lowest saccharides concentration, and that was why the yield did not further decline compared with that at 1000 rpm.

Besides the filtration behavior, the lower saccharides concentration in the permeate at higher agitation might be caused by the slower enzymatic hydrolysis of the substrate, as higher shear force possibly changed enzyme conformation and decreased enzyme activity [33]. To evaluate this hypothesis, the effect of the agitation speed on the enzyme activity was then investigated. Results in **Fig. 6a** showed that the enzyme activity at increasing incubation time (30-120 min) and different agitation speeds (160-2000 rpm) was similar, indicating that the dextranase was quite stable at high shear stress [34-36]. Moreover, the oligodextran production rate did not change significantly with time at different agitations without product removal (**Fig. 6b**), implying that product inhibition was negligible during the 120 min dextran hydrolysis.

Based on the above results, it could be concluded that higher TMP and shear rate resulted in higher permeate flux and narrower Mw distribution of products, while the oligodextran yield is decreased. Higher permeate flux was preferred to timely remove the product in an EMR, while the effect of permeate flux on the oligodextran rejection should be considered [37] because the higher rejection greatly decreased the saccharides concentration in permeate, probably resulting in a yield decline. When the membrane pore size distribution was wide, controlling the CP layer was important because the unwanted dextran molecules accumulating in the CP layer would diffuse through the membrane from the large pores. Thus, selecting a suitable membrane material and controlling permeate flux, shearing velocity, and narrowing membrane pore size distribution are effective strategies to obtain high-quality oligodextran products by EMRs.

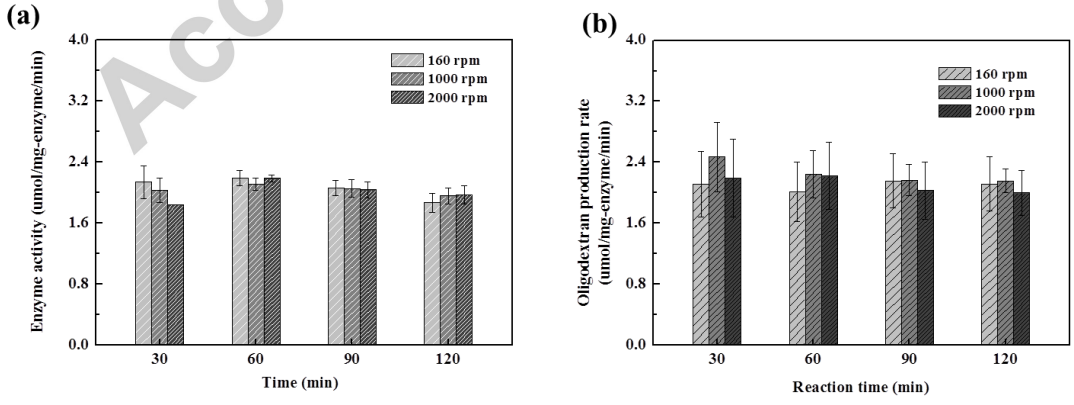

Fig. 6 Effects of agitation speed on (a) enzyme activity and (b) oligodextran production rate.

3.3 Fouling mechanism discussion

Membrane fouling is inevitable when operating in an EMR and has a high impact on the filtration behavior and product quality. To control and even utilize membrane fouling to our advantage, the fouling mechanisms in an EMR and their effects should be analyzed for each of the compounds occurring in the mixture that will be in contact with the membrane. It has been reported that PES membranes could be fouled by adsorption of dextran, which attaches to the membrane by Van der Waals interactions and hydrogen bonds [29, 38]. Moreover, it was well known that proteins are easy to deposit in/on PES membranes by hydrophobic adsorption. Thus, the effect of substrate and enzyme on membrane irreversible fouling was systematically investigated.

3.3.1 Effect of foulant type

As shown in **Fig. 7**, 40.9% of water permeability loss was observed after the filtration of a dextran/dextranase mixture. Two more filtrations with only dextranase or dextran (same concentration as the mixture) were carried out under the same operating conditions (continuously fed with water under 4.5 bar, 2000 rpm), and 22.5% and 21.8% of water permeability decline was found in the enzyme-only and dextran-only filtration systems, respectively. By these experiments it was proved that both of the enzyme and dextran molecules produced membrane irreversible fouling in an EMR.

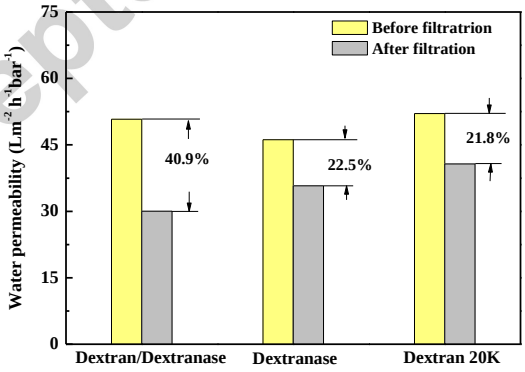

Fig. 7 Effect of dextran and dextranase on water permeability decline of PES20 membrane at 4.5 bar and 2000 rpm.

It is worth mentioning that the concentration of dextran tested in the experiments (50 g/L) was 1000 times higher than dextranase concentration (0.05 g/L) but they caused similar membrane permeability reduction after filtration. Such result suggested that the enzymes

(proteins) had larger affinity for PES membranes than dextran, which is a fact previously observed [29]. Hydrophobic and electrostatic interactions between protein and membrane have been widely investigated [39, 40]. Dextranase with an isoelectric point of 3.88-4.85 was negatively charged in a neutral aqueous solution [9, 35], and thus there was a negligible electrostatic attraction of dextranase with a negatively charged PES membrane. Therefore, hydrophobic rather than electrostatic interactions were probably responsible for the enzyme fouling in this case.

3.3.2 Effect of oligodextran Mw

It was reported that dextran Mw affected the fouling behavior on PES membranes [41]. Large dextran molecules with more contact points are expected to induce more severe fouling in/on the membrane. In this work, oligodextran molecules with different Mws were generated in the EMR during reaction, and thus it was relevant to study the effect of oligodextran Mw on fouling formation. First, DXT40K substrate was hydrolyzed by dextranase at different times, and the obtained oligodextran solutions with different average Mws (enzymes were previously deactivated) were then filtrated through the PES membrane under the same operating conditions. As shown in **Fig. 8**, when the average oligodextran Mw increased, the permeate flux decreased and the irreversible fouling became more serious. It was reasonable that higher rejection of larger oligodextran molecules led to thicker CP layer on the membrane, which in turn facilitated irreversible fouling formation. It was hypothesized that large dextran molecules at the beginning of the hydrolysis dominated the fouling formation during the reaction-separation process. Interestingly, when comparing the permeability loss in **Fig. 7 and Fig. 8**, it was found that the irreversible fouling in the EMR (40.9%) was much lower than that observed during filtration of oligodextran with average Mw of 11 to 20 kDa (around 60%), implying that the large dextran molecules deposited on membrane could be hydrolyzed by the active enzyme, thus reducing the fouling evolution in an EMR.

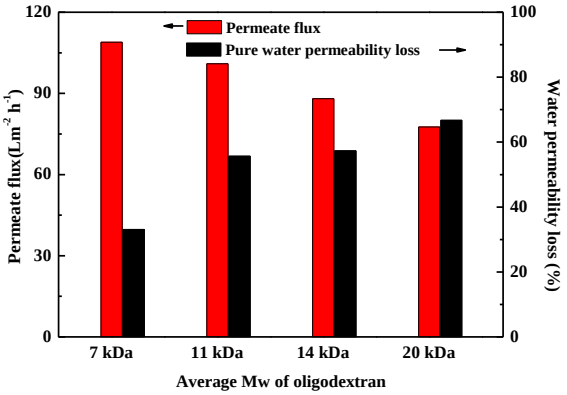

Fig. 8 Effect of dextran Mw on permeate flux and irreversible fouling of PES20 membrane at 4.5 bar and 2000 rpm.

3.4 "Fouling-induced" enzyme immobilization

Although oligodextran Mw distribution could be narrowed down by optimizing permeate flux and controlling the CP layer, it was still very wide at the beginning of operation (**Fig. 4b and 5b**), which was mainly ascribed to the uneven pore size distribution of the commercial membrane [42]. A self-regulating process consisting of adding certain agents capable of accessing the large pores of the membrane and might "partly" block them, leading to a reduction in membrane pore size was evaluated [43]. Theoretically, larger membrane pores would have higher local permeate flux, resulting in more serious fouling formation, while smaller pores would be less fouled [22]. Consequently, the membrane pore size distribution is expected to be narrowed during filtration, and that was possibly the reason why the oligodextran Mw distribution was decreasing from 30 min to 120 min in the cases showed in **Fig. 4b and 5b**. We described above how dextranase contributed more than 50% of the irreversible fouling found in the PES20-EMR membrane (**Fig. 7**), and it was reported that enzymes could be immobilized by the fouling formation on/in the membrane [44]. Inspired by these results, we attempted to achieve enzyme immobilization and pore size distribution narrowing by "enzyme fouling" formation, and establish a novel EMR for producing oligodextran with narrower Mw distribution (**Fig. 9**).

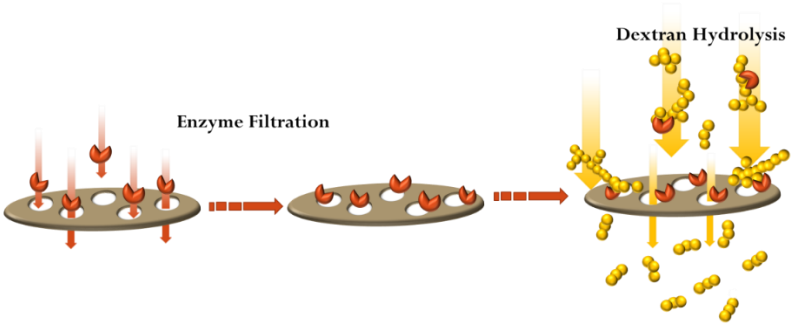

Fig. 9 Schematic diagram of an EMR prepared by "fouling-induced" enzyme immobilization

Dextranase was firstly filtrated under concentration mode to partly blocking the large pores of the membrane. PES membranes with MWCO of 20 kDa and 30 kDa were used in the experiments. As shown in **Fig. 10**, the surface morphology of the PES20 membrane was significantly changed after enzyme filtration, and an "enzyme fouling" layer was formed on the membrane. The membrane pore size distribution was also narrowed because some large pores disappeared and the percentage of the 20 nm pores increased from 57% to 70% (**Fig. 10c and 10d**), confirming that the enzymes were capable of blocking the larger pores during filtration.

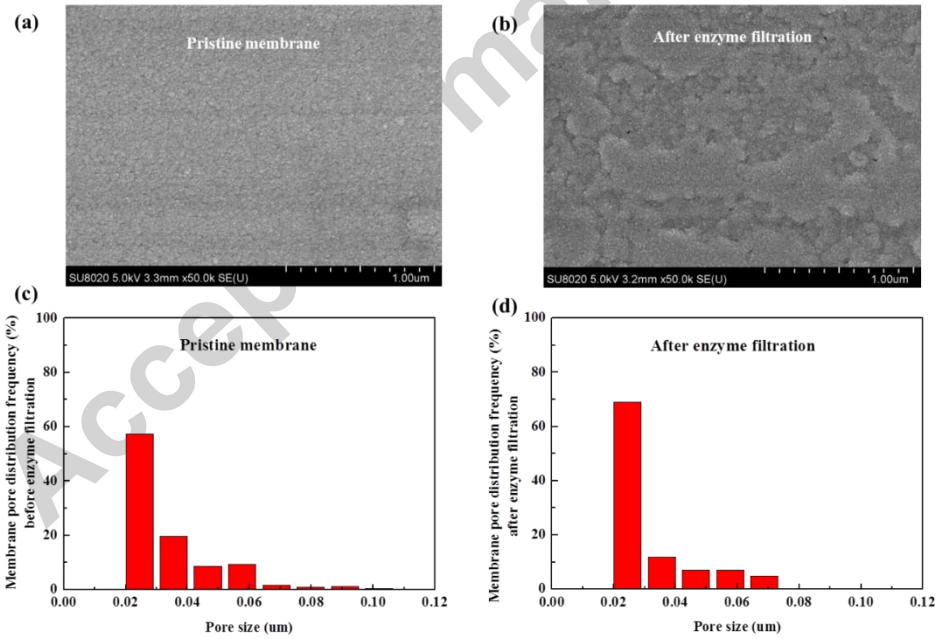

Fig. 10 SEM images of PES20 membrane (a) before and (b) after enzyme filtration; pore distribution frequency (c) before and (d) after enzyme filtration.

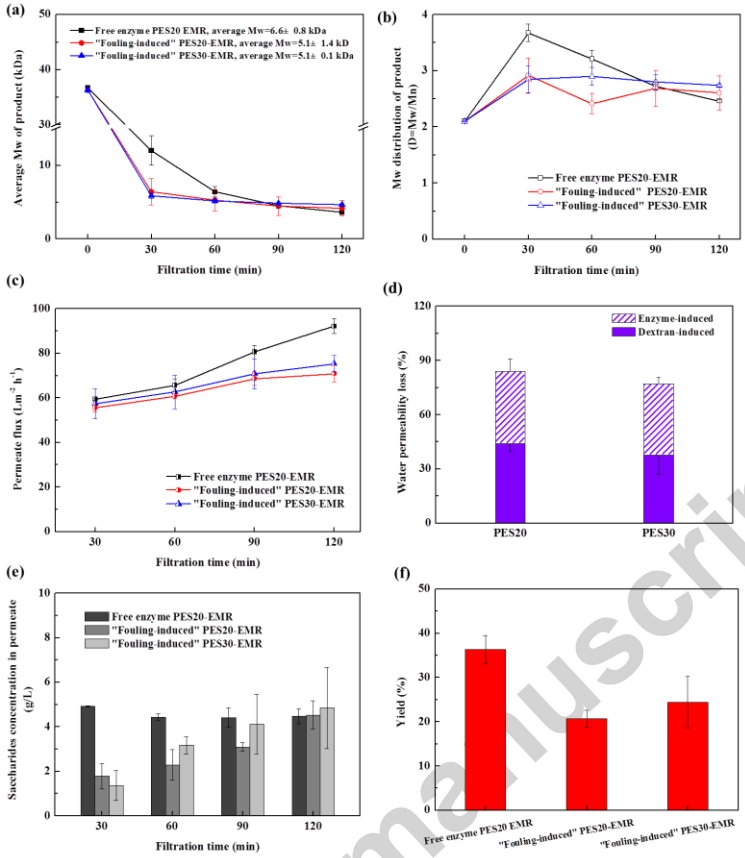

Fig.11 Effect of "fouling-induced" enzyme immobilization on oligodextran production in PES-EMRs at 4.5 bar and 2000 rpm. (a) Average Mw, (b) Mw distribution of oligodextran molecules in permeate, (c) permeate flux with time, (d) irreversible fouling, (e) saccharides concentration in permeate and (f) yield of oligodextran in permeate.

After enzyme filtration, both the retentate and permeate of the enzyme solution were collected and mixed with the substrate, and the EMR was then operated at 4.5 bar and 2000 rpm. There was no dextranase activity detected in the permeate after enzyme filtration, however, less than 30% of protein was loaded on the membrane (detected by Bradford assay, Fig. S6), indicating that most of enzymes were still retained by the membrane and dispersed in the reactor. As seen in **Fig. 11**, the product Mw kept quite stable and was around 5.1 kDa for the PES20-EMR and PES30-EMR membranes with the "enzyme fouling" layer, and the Mw distribution was also obviously improved at 30-90 min, indicating that tailoring membrane pore size by "fouling-induced" enzyme immobilization was really beneficial to the product quality. Additionally the enzyme fouling treatment only reduced the permeate flux slightly as compared with the pristine EMR (**Fig. 11c**). The enzyme treatment, however, could not avoid the further

fouling formation by oligodextrans, which resulted in a super high permeability loss (around 80%, **Fig. 11d**). Moreover, as shown in **Fig. 11e**, the reduction of pore size by "enzyme fouling" decreased the oligodextran transmission through the membrane at the beginning of the EMR operation (30-90 min), causing lower oligodextran yield for the EMR in which the "enzyme fouling" layer was induced (**Fig. 11f**). It is worth mentioning that with the "fouling-induced" enzyme immobilization, the oligodextran yield of the PES30-EMR was only a little higher than that of the PES20-EMR, implying that the "enzyme fouling" layer was determinant in the separation of product in this case.

Therefore, membrane fouling was not only used as a strategy for enzyme immobilization [19, 20, 44], but could also be useful to narrow membrane pore size and regulate its distribution, which was a promising strategy for the design of an EMR able to produce oligosaccharides and peptides with a narrower Mw distribution.

4. Conclusion

An EMR allowing dextran hydrolysis and *in-situ* product recovery with improved Mw distribution was developed in this study. When the membrane MWCO was larger than 5 kDa, the skin layer material played a more important role in the filtration as compared to the actual MWCO. A PES membrane with a nominal MWCO of 20 kDa outperformed the others since the more hydrophilic membranes (RC and PAN) had stronger interaction with dextran molecules, which led to formation of a more severe CP layer. Such a result makes us infer that the membrane material plays a particularly relevant role in the filtration of dextran (whose functional groups can interact with the main commercial membrane materials available nowadays). A more developed CP layer at higher TMPs and lower agitation rates induced more permeation of large dextran molecules through the membrane, leading to higher average Mw of products and wider Mw distribution. Moreover, serious CP seemed to increase membrane irreversible fouling, which enhanced oligodextran rejection. On the other hand, higher TMP and agitation resulted in higher permeate flux and narrower Mw distribution of products, while the oligodextran yield might be decreased. Higher permeate flux was preferred to timely remove the product in an EMR, while the effect of permeate flux on the oligodextran rejection should be considered.

Both enzyme and dextran caused membrane irreversible fouling in an EMR, and the larger oligodextran molecules produced more fouling. Since the higher local flux around the larger

membrane pores would induce more serious fouling formation, a "fouling-induced" enzyme immobilization strategy (i.e. a simple filtration of enzyme solution in advance) enabled to narrow the membrane pore size distribution of the EMR, leading to a relative constant Mw of products in permeate with narrower Mw distribution compared with the pristine EMR.

The strategy consisting of using a protein (enzyme) to foul the membrane and in turn to make more uniform the pore size distribution of the membrane has provided very promising results in this study. We think that such simple strategy could be easily implemented in other processes in which a narrower Mw distribution of products is required. A more detailed study about the influence of enzyme features e.g. size, functional groups present, etc., and operational variables like enzyme concentration and pressure during the "fouling-induced" immobilization could help generalize this strategy and extrapolate it to other processes of industrial interest.

Acknowledgements

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Highlights

- Membrane material plays a key role in oligodextran transmission in an EMR.
- Increasing CP induces higher average Mw and wider Mw distribution of product.
- Fouling, oligodextran rejection, quality and yield are affected by permeate flux.
- Both dextranase and dextran produce membrane irreversible fouling.
- Fouling-induced enzyme immobilization narrows membrane pore size distribution.

Graphic abstract