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Rapid Transgalactosylation Towards Lactulose Synthesis in a Small-scale Enzymatic Membrane Reactor (EMR)

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A small-scale EMR system was developed with a reliable control design giving the possibility for continuous operation at constant flux (constant hydraulic residence time, t_{HRT}). A PID controller was implemented to control the flux during continuous operation. In this study, lactulose yield is shown to depend on the molar ratio of lactose to fructose, m_L/m_F under batch operation. Specific yield in continuous operation of lactulose synthesis was higher than in batch mode. In batch process, the produced lactulose underwent secondary hydrolysis which led to a rapid reduction of the lactulose concentration. In continuous processes, a shorter t_{HRT} obtained higher specific yield. Nevertheless, it acquired a particular drawback as fouling occurred on top of the membrane and could inevitably influence the operational time span of the membrane. Conclusively, fouling and yield have to concomitantly be taken into consideration for continuous production of lactulose in an EMR system.

1. Introduction

Transgalactosylation is a typical reaction which involves the transfer of galactosyl groups into specific galactosyl acceptors (Withers, 2001). As a product of transgalactosylation, lactulose (4-O-β-D;galactopyranosil-D-fructofuranose) that is built from one molecule of galactose and one molecule of fructose has received an increasing attention due to its roles in dairy industry, acting as a prebiotic (Schuster-Wolff-Bühring et al., 2010). Generally, there are two enzyme classes that can assist lactulose synthesis, such as glycosyltransferases and glycosidases. Glycosidases are more relevant for industrial applications (e.g., for the hydrolysis of lactose) as they are commercially available and relatively inexpensive (Perini et al., 2013). In addition to that, glycosyltransferases normally need cofactors to maintain the catalytic activities of the enzymes. Hereby, the catalyzed reactions are regarded as complex reactions (van Rantwijk et al., 1999). As the lactose is hydrolyzed, the concentration of the product (i.e., lactulose) will peak when the probabilities of fructose as galactosyl acceptor are higher than water. However, at its highest concentration, lactulose is prone to undergo secondary hydrolysis. Hence, the yield of lactulose is determined by the availability of fructose and the possibility of continuous removal of lactulose during the reaction.
The transfer of a galactosyl group from a disaccharide to a low-molecular weight monosaccharide (to fructose in case of lactulose synthesis) and to other di-/trisaccharides in case of galactooligosaccharides (GOS) syntheses has been extensively investigated. However, most of the reported studies were carried out under batch operations (Schuster-Wolff-Bühring et al., 2010). Therefore, the optimum time to stop the transgalactosylation was not longer than 8 h to avoid the secondary hydrolysis taking place. This was consequently inefficient as the enzyme consumption was considerably high (Lee et al., 2004; Meyer et al., 2004; Hua et al., 2012). The application of the enzymatic membrane reactor (EMR) can be the alternative to tackle the delicate hindrance of lactulose synthesis due to secondary hydrolysis as it allows continuous removal of products and retains the enzyme molecules inside the reactor. Hence, in the present study

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parallel small-scale EMRs supported with a reliable and robust control system have been developed and used to synthesize lactulose in continuous processes with different t_{HRT} values.

2. Materials and Methods

2.1. Chemicals

The enzyme β-galactosidase from *Kluyveromyces lactis* (EC Number 232-864-1, G3665), acetonitrile (271004), 2-Nitrophenyl β-D-galactopyranoside (ONPG, 73660), 2-Nitrophenol (ONP, 19702), lactulose (61360), D-fructose (F0127), lactose (17814) were purchased from Sigma-Aldrich, Germany. All other chemicals were analytical grade.

2.2 Construction of the small-scale EMR and controller design

The scheme of the EMR system is shown in Figure 1. For a single reactor, it consisted of a pressure-stable glass container that was purchased from Merck Millipore Darmstadt, Germany, whereas the body (holder) was a geometrically modified XFUF-047 dead-end test cell. The UF membrane of polyethersulfone (PES) with a molecular weight cut-off (MWCO) of 10 kDa was obtained from Microdyn-Nadir GmbH, Germany. It was sufficient to retain the enzyme molecules inside the reactor, as the reported molecular weight of the enzyme (i.e., assumed as a tetramer) was about 465 kDa (Juers et al., 2001). The proportional pressure regulator–MPPE was purchased from Festo AG & Co.KG, Germany and to quantify the permeate, a Kern EW 620-3 NM precision balance was purchased from KERN & SOHN GmbH, Germany.

Laboratory Virtual Instrument Engineering Workbench (LabVIEW) software was employed to control such reactors and save all the data needed for analysis. Data collection was supported by several NI modules (i.e., NI 9201, 9264 and 9870). These modules were mounted on cRIO-9076 Integrated 400 MHz Real-Time Controller and LX45 FPGA chassis system produced by National Instruments, Germany.

The filtration process in relation to controller output (CO, bar), and process variable (PV, L/(m².h)) shows a non-periodic response that follows a PT_1T_0 model as process dynamics (Schwarze, 1962) and has been reported elsewhere (Lyagin et al., 2010). This model is generally recognized as general first order plus dead time (FOPDT) model. In addition to that, to control such process dynamics, a common Proportional-Integral-Derivative (PID) controller was implemented in the system. PID parameters were tuned according to the method described by Kuhn (1995). This is an open loop tuning, which considered is not time consuming, as one does not need to wait for several stable oscillations like in a closed loop tuning approach.

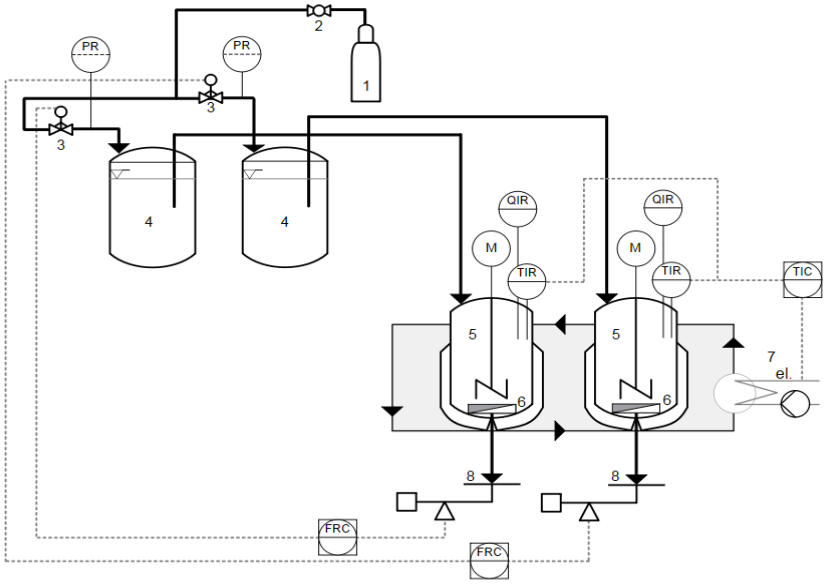

Figure 1: Schematic of two parallel small-scale EMRs; (1) N_2 bottle, (2) pressure reducer, (3) proportional pressure regulator, (4) substrate tank, (5) reactor, (6) flat-sheet PES membrane, (7) heating system, (8) precision balance. Q = quality parameter, pH. Dashed lines indicate the controlling lines

2.3 Constant flux operation

The flux (and thus also t_{HRT}) was controlled in the continuous process. The procedure is described as follows and it always refers to Figure 1. When the setting value (SV) of flux is inserted into the LabVIEW

program, the program will automatically send an analogue input commanding the proportional pressure regulator (no. 3) to open its valve allowing a particular pressure released from N_2 bottle (no.1). As the substrate tank is pressurized (no. 4), substrate is fed into the reactor (no. 5). Since the reactor is fully filled, additional volume from the substrate tank consequently drives the same amount of liquid out of the reactor via the membrane which is then collected by the precision balance (no. 8). In the LabVIEW program this permeate mass is converted to its volume (using the density of the solution) and eventually into the real flux (process variable, PV) by inserting a mathematical expression corresponding to the volume of permeate, membrane effective area and time of filtration. This loop is precisely carried out in one second. The difference between PV and SV is transferred to build an error which is substantially used to give the subsequent command to the proportional pressure regulator (no. 3) again in order to release some amount of pressure. This cycle goes along the transgalactosylation, giving the possibility to control the flux.

2.4 Bioconversion of lactose

The transgalactosylation towards lactulose synthesis was carried out in a reactor working volume of 90 mL with modified batch and continuous process. In modified batch operation, a series of reactions was carried out without disposing the enzymes. A 1.0 mL sample on the permeate side was withdrawn and compensated by the same amount (i.e., 1.0 mL) of fresh substrate transferred into the reactor. This was done by applying particular pressure in the substrate tank manually. The bi-substrate was a combination of lactose and fructose, where the total sugar concentration was 500 g/L. The reaction was carried out in 50 mM buffer phosphate pH 6.8, stirred at 200 rpm and at a constant temperature of 40 °C (±1 °C). In the modified batch processes, the effects of initial molar ratio of lactose to fructose (m_L/m_F) were studied for 12 h of reaction. In continuous processes, the PID controller was applied to control the fluxes. Using the optimum m_L/m_F ratio, lactulose syntheses were conducted at different t_{HRT} values for 35 h. In addition to that, transmembrane pressures (TMPs) were also evaluated under continuous lactulose syntheses. For the discussion, measured parameters were expressed as lactulose yield (%) and specific yield ($Y_{spec.}$, $mg_{lactulose}/U_{enzyme}$) as following:

Lactulose yield (%): $$\left(\frac{C_L}{C_{I,L}}\right)\times 100\% \quad (1)$$

Specific yield (Y_{spec}, $mg_{lactulose}/U_{enzyme}$): $$\left(\frac{C_L}{U_{enzyme}}\right)\times V\times 1000 \quad (2)$$

where $C_{I,L}$: initial concentration of lactose, C_L: concentration of lactulose at certain sampling time (g/L), V: permeate volume (L), U: enzyme concentration (U).

2.5 Measurement of enzyme activity and determinations of mono- and disaccharides

ONPG was used as substrate for determining β-galactosidase activity. The procedure was similar to the previous method reported by Hua et al. (2012). One unit (1 U) of enzyme activity is defined as the enzyme required for liberating the equivalent 1.0 µmol ONP per minute at 30 °C, and pH 6.8.

Determination of sugars (lactose, lactulose and fructose) was done by means of an HPLC (Knauer GmbH, Germany), equipped with a Vertex plus 250 x 4.6 mm Eurospher II 100-3 NH_2 column. The mobile phase was a mixture of acetonitrile and water (75:25 % v/v), pumped at a constant flow rate of 1.0 mL/min, giving a pressure range of 57-59 bar. Samples were diluted with pure water for four times and subsequently diluted again with the mobile phase at ratio of 1:1 prior to injection. Finally, a 20 µL diluted sample was thoroughly injected into HPLC with a retention time of 30 minutes. The calibration lines for sugars (lactose, lactulose and fructose) were made in the concentration ranges of 0-50 g/L with $R^2 > 0.996$.

3. Results and Discussion

3.1 Stability of the controller

Within this study, for tuning the PID controller a method introduced by Kuhn (1995) was adopted, which basically generates the data according to an open loop approach. The P, I and D parameter values were 0.0075; 0.36 min; 0.0873 min for the P, I and D parameter, respectively. The stability of the controller was tested by varying the setting value (SV) in a series (i.e., 30; 0; 50; 0; 75 L/(m².h)). The solution used was bi-substrate, a combination between lactose and fructose with total concentration of 500 g/L mimicking the transgalactosylation reaction composition. Instead of directly increasing the SV from 30 to 50 and to finally 75 L/(m².h), the value of 0 L/(m².h) was inserted in between. The aim of this setting was to confirm the adaptability of the PID controller to such extreme conditions (i.e., 0 L/(m².h)) without producing overshoot responses or lagged settling times. As seen in Figure 2, the CO was not jerky and consequently the PV responses were stable without producing any overshoot response. The calculated error between SV and

PV was less than 5 %. This result was comparable to the study of tuning PI/D parameters in a closed loop method reported by Skogestad (2003) that was subsequently cited by Shamsuzzoha and Skogestad (2010). Within their studies, the PI/D controller settings were gradually calculated from the overshoot data, time to reach overshoot and relative steady state output (Shamsuzzoha and Skogestad, 2010) which were assumed to be tedious.

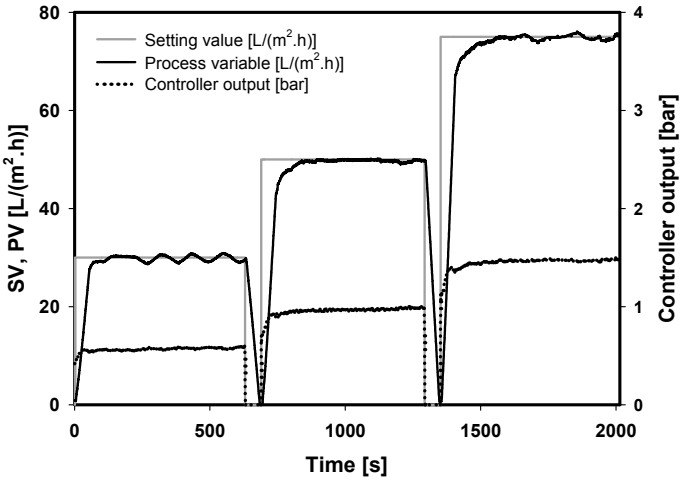

Figure 2: Stability of PID controller under varied SVs in a series

3.2. Bioconversion of lactose and fructose

In Figure 3, an increased m_L/m_F ratio promotes hydrolysis over transgalactosylation. Under these conditions (m_L/m_F ratio = 1.0 and 2.0), lactulose productions were found to decrease, while GOS productions were not affected (data not shown). At a lower level of m_L/m_F ratio (i.e., 0.5), GOS production was remarkably inhibited, and it subsequently increased the lactulose yield (GOS production specified by the number of peaks appeared in HPLC chromatogram). When the m_L/m_F ratio was set to 0.5, a maximum lactulose yield of 6.85 % (16.70 ± 0.34 g/L) was obtained, whereas for ratios of 2 and 1 the lactulose yields afforded were about 2.18 % (8.64 ± 0.37 g/L) and 4.30 % (14.10 ± 0.23 g/L), respectively. The propensities of the effects of m_L/m_F ratio in this study were in-line with a study from Guerrero et al. (2011). Moreover, the level of lactulose reported by Mayer et al. (2004) using CelB from *Pyrococcus furiosus* was in a similar range (i.e., 16 g/L) with this study. At an m_L/m_F ratio of 0.25, synthesis of lactulose was also conducted. However, the level of lactulose yield was higher but not significantly. The large amount of galactosyl acceptor (i.e., fructose) seems to be advantageous for lactulose synthesis, as it has better probability to compete with water to react with the galactosyl-enzyme complex. The effects of m_L/m_F ratio were also reported by Lee et al. (2004) that synthesized lactulose using permeabilized cells of *Kluyveromyces lactis*.

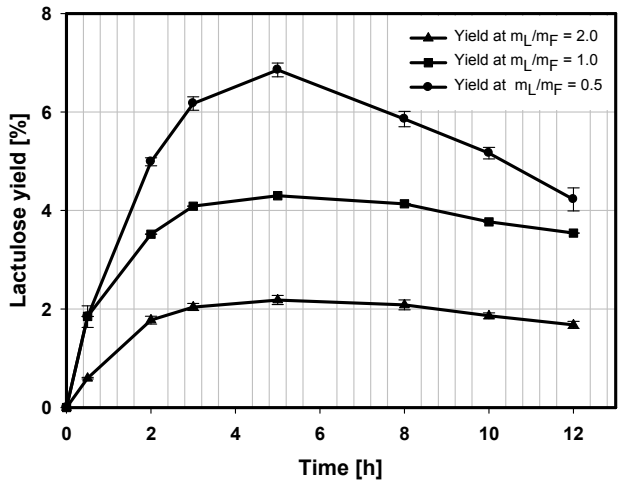

Figure 3: Effects of m_L/m_F ratios on the lactulose productions, $V_{reactor}$ = 90 mL, [E] = 300 U, [sugars] = 500 g/L, phosphate buffer pH 6.8, 40 °C, stirred at 200 rpm

In case of specific yield, lactulose production under continuous operation was higher than in a batch mode. As seen in Figure 4, the maximum $Y_{spec.}$ of the batch process was about 5 $mg_{lactulose}/U_{enzyme}$ at 5 h. It was obvious that in batch mode secondary hydrolysis predominated after the lactulose concentration reached its peak, leading to the reduced specific yield in a long reaction course. Therefore, in batch operation, every 5 h the reaction has to be stopped in order to obtain higher specific yield and thus productivity. On the other hand, for continuous processes, different t_{HRT} values gave a significant difference of $Y_{spec.}$. A shorter t_{HRT} (i.e., 5 h) obtained higher $Y_{spec.}$ of 24.40 $mg_{lactulose}/U_{enzyme}$ at 35 h, whereas at 7 h t_{HRT}, the $Y_{spec.}$ was 17.83 $mg_{lactulose}/U_{enzyme}$ at the same reaction course. Steady state was reached approximately after 12 h under continuous conditions (data not shown). As mentioned previously, every 5 h the batch reaction has to be stopped to obtain a higher specific yield. Therefore, by summarizing the overall procedures (start-up and end activities) of lactulose and GOS syntheses, batch production is presumably less preferred from the industrial point of view as the resulting space-time yield is low.

In continuous process, as a consequence of a constant flux operation using membrane separation, TMP increases over the reaction course due to fouling. The increase of TMP was higher at t_{HRT} of 5 h as compared to t_{HRT} of 7 h (Figure 5). This was due to a higher permeate flux resulting from a shorter t_{HRT} leading to the possibility of higher rate of material (foulant) to be deposited on top of the PES membrane. Similar results have been reported elsewhere, and enzyme molecules were considered to be contributive for such fouling (Lyagin et al., 2010).

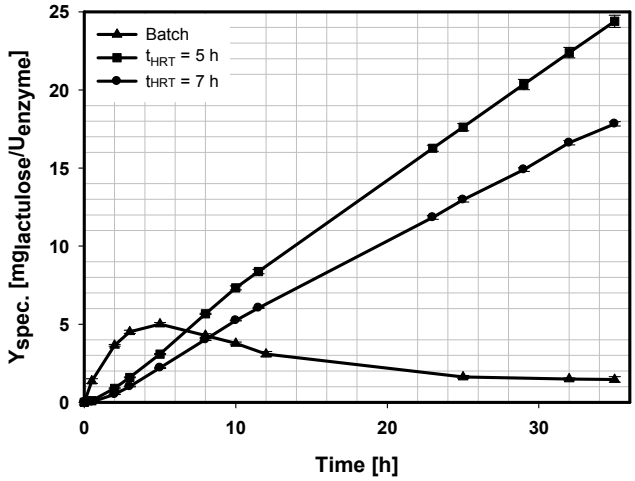

Figure 4: Specific yields of lactulose syntheses during batch and continuous processes. $V_{reactor}$ = 90 mL, [E] = 300 U, [sugars] = 500 g/L, phosphate buffer pH 6.8, 40 °C, stirred at 200 rpm

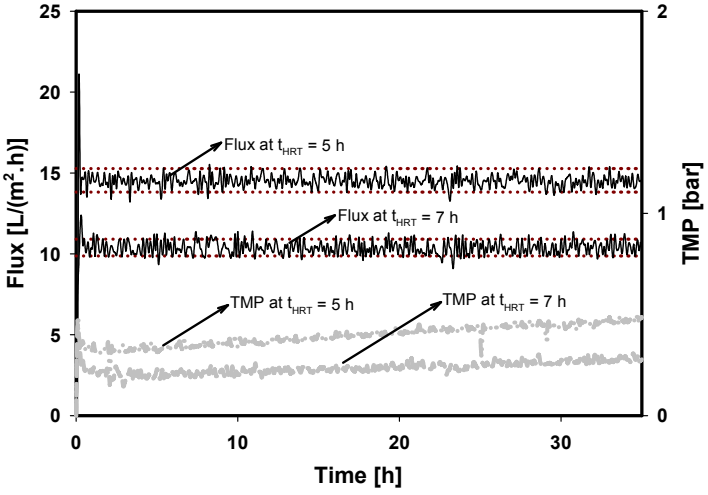

Figure 5: Profiles of flux and TMP evolutions during continuous lactulose syntheses at different t_{HRT} values. $V_{reactor}$ = 90 mL, [E] = 300 U, [sugars] = 500 g/L, phosphate buffer pH 6.8, 40°C, stirred at 200 rpm. Dashed lines indicate ± 5 % error

4. Conclusions

In this study, reliable PID tuning parameters were obtained to support the operation of enzymatic membrane reactors (EMRs) at constant flux and thereby constant hydraulic residence time. The developed EMRs have been successfully used to negate the hindrance of lactulose synthesis due to secondary hydrolysis under batch operation. The specific yields of lactulose syntheses under continuous operations were significantly higher compared to the batch process. Under continuous operation, it was shown that the increase of the TMP for t_{HRT} of 5 h was higher than t_{HRT} of 7h. Nevertheless, higher specific yield was obtained from a shorter t_{HRT}. From this situation, one has to ponder the operational time span of the membrane by balancing (i) the probabilities of fouling to take place on the UF membrane and (ii) the expected yield of lactulose synthesis in a continuous process. Through this study, optimization of continuous synthesis of lactulose is still possible, e.g., by investigating optimum condition for stirring, proportional m_L/m_F ratios and t_{HRT} values or the applications of different ionic strengths in the continuous process.

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