DEVELOPMENT OF CONTINUOUS LACTULOSE PRODUCTION IN PARALLEL MEMBRANE REACTORS

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This work is dedicated to my beloved ones: my parents and siblings (incl. their spouses and children).

Azis Boing Sitanggang, M.Sc.

“Not having heard something is not as good as having heard it; having heard it is not as good as having seen it; having seen it is not as good as knowing it; knowing it is not as good as putting it into practice”
Taken from XUNZI (Xun Kuang, 310-235 BC)

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements.

Azis Boing Sitanggang, M.Sc.
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Abstract

The automated and parallel small-scale reactors are considered as an efficient and a time-saving tool since these reactors can facilitate a straightforward bioprocess development to reach its commercial success. Within this study, a screening and characterization system for continuous biocatalytic processes that had been coined by Lyagin et al. (2010, 2015) was upgraded. The previously established program in Visual Designer^{TM} 4.0 was successfully transferred into the Laboratory Virtual Instrument Engineering Workbench (LabVIEW^{TM}) program with a completely new structure of the data acquisition system (i.e., hardwares). By these changes, the maximum number of parallel reactors that could be controlled (i.e., constant flux and enzyme dosing) could be increased from only two (Lyagin et al., 2010, 2015) to twelve parallel reactors. By the application of the PID controller with fast setting, the control accuracy of a constant flux operation was more than 95 %. In addition, an automated protocol of the enzyme addition has also been developed with a control error of less than 2 %.

For its applications, the automated EMR system is suited for process characterizations of newly isolated/improved/genetically modified enzyme-based biotransformations. These are the reactions, where (i) the enzymes are freely dissolved in the reaction media, (ii) products are susceptible to undergo further degradation by the same enzyme and (iii) either product or substrate inhibition is pronounced during the reaction. As a model reaction, continuous synthesis of lactulose using β-galactosidase in the presence of lactose and fructose was investigated. Under batch operation, the specific productivity P_{spec} of lactulose production using *K. lactis* β-galactosidase decreased abruptly from 2.72 to 0.04 mg/($U_{[E]}$h) over 35 h. This was presumably caused by the action of β-galactosidase that performed secondary hydrolysis upon the produced lactulose. The continuous operation of an EMR system led to continuous removal of lactulose in the reactor. Hereby, the degradation of lactulose due to secondary hydrolysis could be tackled. Continuous lactulose synthesis in the EMR system yielded relatively constant P_{spec} values, approximately 0.70 and 0.50 mg/($U_{[E]}$h) at τ = 5 and 7 h, respectively.

Besides comparing batch and continuous production of lactulose, this study also addressed three main issues which are commonly encountered in membrane-assisted enzymatic reactions, such as membrane-enzyme electrostatic interaction, optimum operating conditions (with industrially relevant parameters) and maintenance of the catalytic activity during a long-term synthesis. The optimum pH value for *K. lactis* and *A. oryzae* β-galactosidase are different for lactulose synthesis. There will be a shifting on electrostatic interaction between the membrane surface and the enzyme molecules whenever *K. lactis* β-galactosidase is substituted by *A. oryzae* β-galactosidase. Consider-

ing *A. oryzae* β-galactosidase charge distribution at pH 4.6 is largely positive, a negatively charged PES membrane was not suited for continuous lactulose synthesis. With this membrane, a rapid increase in transmembrane pressure ΔP was pronounced. The UFX10 membrane MWCO = 10 kDa is permanently hydrophilic and exhibited the repulsion of the enzyme molecules. During the synthesis, the transmembrane pressure ΔP increase was not significant. The UFX10 membrane was therefore, selected for further investigations. The operating conditions of lactulose synthesis were validated and the values turned out to be: enzyme concentration $[E]$ = 10 U/mL, molar ratio of lactose to fructose $m_L/m_F = \frac{1}{4}$, hydraulic residence time τ = 9 h (or J_{SP} = 8.07 L/(m^{2}h)) and the incubation temperature T = 40 °C. Moreover, a long-term synthesis of lactulose was performed for 28 days. The enzyme activity was controlled by the addition of fresh enzyme as much as 10 % from the initial concentration every 48 h. The average lactulose outlet concentration C_{Lu} could be kept constant at 8.67 g/L. It is worth mentioning that this study was the longest continuous lactulose synthesis ever reported in fully automated membrane reactor. The established EMR system was proven to be a useful instrument for the characterizations of continuous lactulose synthesis. On the other hand, the EMR system can bridge the gap between typical laboratory scale where the reactions are mostly carried out in batch mode and industrial scale where reactions are often continuously operated especially for serial reactions.

Keywords: enzymatic membrane reactor (EMR), ultrafiltration (UF), continuous stirred tank reactor (CSTR), membrane, transgalactosylation, lactulose, lactose, β-galactosidase, enzyme.

Zusammenfassung

Automatisierte, parallel betriebene kleine Reaktoren sind eine effiziente und zeitsparende Möglichkeit um eine einfache Bioprozessentwicklung und somit wirtschaftlichen Erfolg zu realisieren. Im Rahmen dieser Studie wurde ein System für das Screening und die Charakterisierung kontinuierlicher biokatalytischer Prozesse basierend auf vorigen Arbeiten Lyagin et al. (2010, 2015) erweitert und aktualisiert. Das zuvor in Visual Designer^{TM} 4.0 entwickelte Programm wurde mit einer völlig neu strukturierten Datenerfassung (Hardware) in das Programm Laboratory Virtual Instrument Engineering Workbench (LabVIEW^{TM}) übertragen. Durch diese Veränderungen wurde die maximale Anzahl an steuerbaren Reaktoren (d.h. konstanter Fluss und Enzymdosierung) von nur zwei (Lyagin et al., 2010, 2015) auf zwölf parallele Reaktoren erhöht. Durch die Nutzung einer PID-Regelung konnte eine Regelgenauigkeit des Flusses im kontinuierlichen Betrieb von mehr als 95 % erreicht werden. Zusätzlich dazu wurde ein automatisiertes Protokoll für die Enzymzugabe mit einem Regelungsfehler von weniger als 2 % entwickelt.

Das automatisierte EMR System ist für die Prozesscharakterisierung von neu isolierten bzw. entwickelten oder genetisch modifizierten enzymbasierten Biotransformationen geeignet, besonders für Reaktionen, bei denen: (i) das Enzym frei in dem Reaktionsmedium solubilisiert wird, (ii) die Produkte für weitere Abbauvorgänge durch dasselbe Enzym anfällig sind oder (iii) entweder das Produkt oder das Substrat die katalytische Aktivität des Enzyms inhibiert. Als Reaktionsmodell wurde in Gegenwart von Lactose und Fructose die kontinuierliche Synthese von Lactulose mittels ß-Galactosidase untersucht. Die spezifische Produktivität P_{spec} der Lactuloseproduktion im Batch-Verfahren mittels *K. lactis* β-Galactosidase nahm drastisch über 35 Stunden Reaktionszeit von 2,72 auf 0,04 mg/($U_{[E]}$h) ab. Dies wurde möglicherweise durch die Sekundärhydrolyseaktivitäten der β-Galactosidase während der Lactuloseproduktion verursacht. Der kontinuierliche Betrieb des EMR Systems führt dagegen zur kontinuierlichen Abfuhr der Lactulose aus dem Reaktor und verhindert somit den Lactuloseabbau durch Sekundärhydrolyse. Über 35 Stunden ergab die kontinuierliche Lactulose-Synthese in dem EMR System eine relativ konstante Ausbeute von etwa 0,70 und 0,5 mg/($U_{[E]}$h) mit hydraulischen Verweilzeiten von τ = 5 und 7 Stunden.

Neben dem Vergleich zwischen Batch- und kontinuierlichen Verfahren zur Lactuloseproduktion befasste sich diese Arbeit mit drei Hauptthemen, die üblicherweise in membranbasierten enzymatischen Reaktionen auftauchen: (i) elektrostatische Membran-Enzym Interaktionen, (ii) optimale Reaktionsbedingungen (mit industriell relevanten Parametern) und (iii) die Regulierung der katalytischen Aktivitäten bei langanhaltender Synthese. Die optimalen pH-Werte für *K. lactis* und *A. oryzae* β-Galactosidase sind für die Synthese von Lactulose unterschiedlich. Wenn *K. lactis*

β-Galactosidase durch *A. oryzae* β-Galactosidase ersetzt wird, verändern sich die elektrostatischen Wechselwirkungen zwischen der Membranoberfäche und den Molekülen des Enzyms. Da die Ladungsverteilung von *A. oryzae* β-Galactosidase stark positiv ist (pH-Wert 4,6), wurde eine negativ geladene PES Membran als ungeeignet für die kontinuierliche Lactulose-Synthese im EMR System eingestuft. Dadurch kann auch die rasche Steigerung des Transmembrandruckes ΔP während der Synthese mit dieser Membran erklärt werden. Die hydrophile UFX10 Membran mit MWCO 10 kDa dagegen stieß die Enzymmoleküle ab und verursachte nur eine insignifikante TMP Steigerung. Diese Membran wurde daher für weitere Untersuchungen zur Bestätigung der Betriebsbedingungen ausgewählt, welche folgende optimalen Werte ergab: Enzymkonzentration $[E]$ = 10 U/mL, Molverhältnis von Lactose zu Fructose $m_L/m_F = \frac{1}{4}$, hydraulische Verweilzeit τ = 9 h (or J_{SP} = 8.07 L/m^{2}h) und Inkubationstemperatur T = 40 °C. Eine Langzeitsynthese von Lactulose wurde für einen Zeitraum von 28 Tagen mit einer durchschnittlichen Konzentration von 8,67 g/L Lactulose im Ablauf des Reaktors durchgeführt. Die Enzymaktivität wurde alle 48 Stunden durch Zugabe von frischem Enzym mit 10 % der Ausgangskonzentration reguliert. Zu erwähnen ist insbesondere, dass diese Arbeit die längste jemals publizierte kontinuierliche Lactulose-Synthese im voll automatisierten Membranreaktor ist. Somit wurde erwiesen, dass das EMR-System ein praktisches Instrument für die Charakterisierung der kontinuierlichen Lactulose-Synthese ist und zudem die Lücke zwischen typischem Batch-Verfahren (meist Labormaßstab) und kontinuierlichen Verfahren (Industriemaßstab) überbrücken kann.

Stichworte: enzymatischer Membranreaktor, Ultrafiltration, Rührkessel, Membran, Transgalactosylation, Lactulose, Lactose, β-Galactosidase, Enzyme.

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Nomenclature

Roman Symbols

A_{eff}	Effective membrane area [m^2]
C	Concentration [g/L] [M]
d_h	Hydraulic diameter [nm]
$[E]$	Enzyme concentration [U/mL]
$[E_s]$	Enzyme stock concentration [U/mL]
E	Enzyme amount [U]
e	Error of controller and measurement [%]
F_{ad}	Adhesion force [N]
$\frac{J}{J_0}$	Normalized flux [-]
J	Flux [L/(m^2h)]
J_0	Initial flux [L/(m^2h)]
K	Proportional constant for controller [L/(m^2h bar)]
ℓ_p	Membrane permeability [L/(m^2h bar)]
M	Molecular masss [gr/mol]
m_L/m_F	Molar ratio lactose to fructose [-]
n	Dosing event, number of reactors, stirrer speed [rpm]
R	Radius of the microsphere [m]
R_f	Fouling/cake resistance [m/kg]
R_m	Membrane resistance [1/m]

T	Temperature [°C]
t	Reaction (Filtration) time [s], [d]
T_0	Dead time [s]
T_1	Time constant [s]
T_Σ	Summation of time constant [s]
$\dot{V}$	Volumetric flow rate [L/h]
V	Volume [L]
W_{ad}	Adhesion work [J/m^2]
W	Weight [kg]

Greek Symbols

ε	Proportional constant for controller [-]
ρ	Density [kg/m^3]
τ	Residence time [h]

Subscripts

aD	actual dosing
ads	adsorbtion
Ao	*Aspergillus oryzae*
Do	dosing
e	enzyme
e	fouling
i	initial in $C_{i,Lo}$, inflow in $\dot{V}_i$
irr	irreversible
Kl	*Kluyveromyces lactis*
Lo	lactose
Lu	lactulose
o	outflow

p permeate

R elapsed reaction in t_R and reactor in V_R

re reversible

S substrate in C_S and stock in $[E_s]$

sD set dosing

w water

Acronyms / Abbreviations

CO Controller Output

EMR Continuous Stirred Tank reactor

EMR Enzymatic Membrane Reactor

HRT Hydraulic Residence Time

$MWCO$ Molecular Weight Cut-Off

NC Normally Closed

NO Normally Open

ONP 2-nitrophenol

$ONPG$ 2-nitrophenyl β-D-galactopyranose

PBR Packed Bed Reactor

PC Personal Computer

PES Polyethersulfone

PID Proportional Integral Derivative

PPR Proportional Pressure Regulator

PV Process Variable

SP Set Point

SR Step Response

TMP Transmembrane Pressure

Chapter 1

INTRODUCTION AND MOTIVATION

1.1 Background

The effective catalytic properties[1] (i.e., stereo- and regio-selectivity, specifity and ability to operate under mild conditions) of enzymes provide tremendous opportunities for various industries to carry out efficient and economical biocatalytic conversions (Kirk et al., 2002; Lye et al., 2003). Especially in the areas of food and feed, drugs, cleaners and minor in agricultural chemicals (herbicides and intermediates), various hydrolytic and isomerization enzymes like lipases, proteases, cellulases, ismoerases and transferases are currently of great interests (Krishna, 2002; Kirk et al., 2002; Schmid et al., 2002; Zaks, 2001).

A process development of a bioconversion using an enzyme always starts with biocatalyst identification and production (Lye et al., 2003) (Figure 1.1). By means of genetic techniques, such as isolation, improvement and/or immobilization steps are done to prepare economically prospective biocatalysts. The improvements are needed for naturally occuring enzymes especially when an industrial process requires the biocatalyst to work under unusual operating conditions (i.e., non-natural substrates, extreme pH and temperature, pressure, etc.) (Dalby, 2007). Before the application of an enzyme in a full-scale operation, biocatalytic reactions at small-scales (1 to 10^5 μL) are generally needed as an initial phase of the process development. The experiments with these smaller scales substantially demand increased necessities of suited micro-to-small scale-up techniques (Lye et al., 2003).

Small scale reactors are generally used to reduce the amount of reactants, samples as well as biocatalysts. It is worth mentioning that small reactors possess a number of benefits in terms of reaction performances, as high rates of mass and heat transfer and extremely large surface-to-volume ratios allow the chemical reactions to be faster (Jensen, 2001; Wörz et al., 2001). However, according to Betts and Baganz (2006), there is a trade-off in information outputs at different experiment scales. As the scale is getting smaller (micro plates to shake flasks), a higher experimental throughput is available, but with a less process information due to the reduced

[1] Naturally occuring enzymes are capable of accelerating reaction rates up to 10^7-fold (Radzicka and Wolfenden, 1995)

monitoring and control (see Figure 1.1). To overcome this constraint, the automated micro to small scale reactors are developed. As these reactors are considered to be a time-saving tool, it is expected that these reactors can facilitate a straightforward bioprocess development to reach its commercial success (Puskeiler et al., 2005).

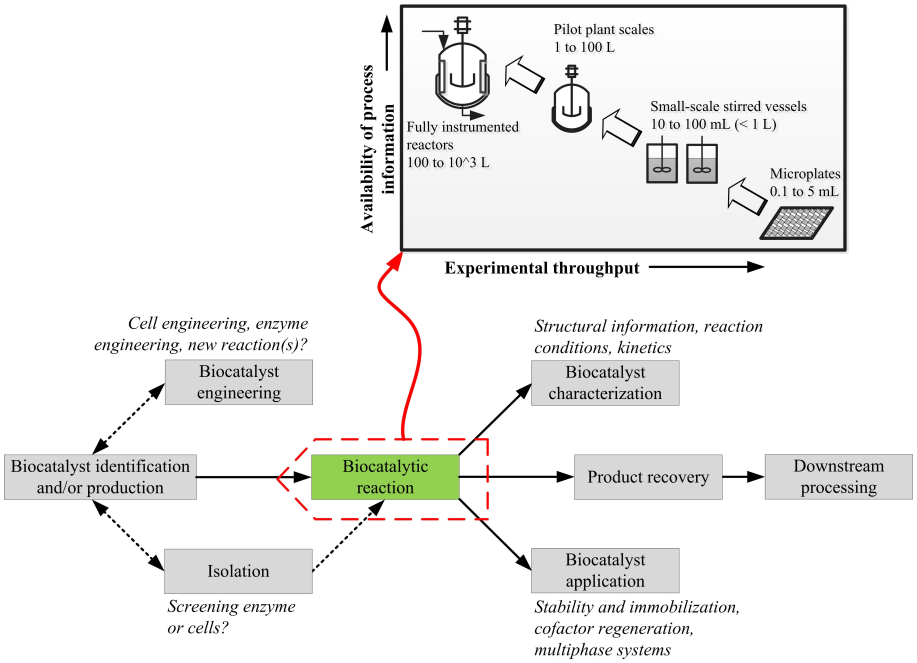

Figure 1.1 A general process development of bioconversion using cells and enzymes: Scale-dependent trade-off in information outputs *vs*. experimental throughput (Betts and Baganz, 2006; Lye et al., 2003).

To catalyze a biochemical reaction, an enzyme can be used either in native (free enzyme) or immobilized form. The immobilization of an enzyme, to some extent can increase the protein solubility, convenience in handling (separation from reaction mixture), thermal and pH stability and can also permit the use of varied reactor configurations (Ansari and Husain, 2012; Garcia-Galan et al., 2011). However, one possible drawback of the immobilization is that recovered activity sometimes can be as little as 10 % (Kamrat and Nidetzky, 2007; Mateo et al., 2007). Besides saving the extra cost and time for the immobilization, the utilization of free enzyme can also mitigate mass-transfer limitations of substrate(s) to access the active sites of the enzyme. With this native form, however, reduced activity due to heat and mechanical stress is also encountered during a prolonged reaction (Thomas and Geer, 2011; Tao et al., 2011).

Typical batch-wise operations are often found in enzymatic processes. Whenever substrate or product inhibition is pronounced, the enzyme will suffer and it may eventually lead to a lower reaction yield. Several products of the reactions are susceptible to undergo further degradations by the pertinent enzymes especially for serial reactions (van Rantwijk et al., 1999). In addition to this, the time spent to start and end the production cycle also adds to the operational cost. Hence, performing continuous operations are sometimes more preferred (Gan et al., 2002).

There are many reactor configurations that can be designed to perform a continuous enzymatic reaction. Packed bed reactors (PBRs) (Lin, 1972) and enzymatic membrane reactors (EMRs) (Prazeres and Cabral, 1994; Rios et al., 2004) are the most used configurations besides recent developments in micro-channels (MCs) (Miyazaki and Maeda, 2006). Especially in PBRs and MCs, the enzymes are immobilized in the carriers (i.e., silica nanospheres (Luckarift et al., 2004)) and packed inside the column or attached to the inner wall surfaces of the channels. When the enzymes are covalently bonded with activated support[2], MC enzymatic reactors are mainly used in biosensors, medical diagnostic and therapy (Křenková and Foret, 2004). There are also possibilities to immobilize the enzymes on/in membranes (i.e., ultrafiltration, nanofiltration) (Rios et al., 2004). Reactive membranes can be tailored by immobilizing the proteins through chemical bonding, physical adsorption (exposed to skin layer) and entrapment within the membrane support (Belleville et al., 2001; Butterfield et al., 2001; de Cazes et al., 2014; Luo et al., 2014a; Sen et al., 2011). In a size exclusion EMR system, as the molecular weight cut-off (MWCO) of the membrane is smaller than the molecular weight of the enzyme, the enzyme molecules can be retained inside the reactor during continuous operation. The smaller products (and also possible for the substrate(s)) can be continuously withdrawn. Hence, besides facilitating a continuous bioconversion by simply dissolving the enzyme into reaction medium, the EMR also acts as a separation unit. Highlighting on this beneficial operation mode, this study mainly concerned on the possibility of developing a biocatalysis process using an automated small scale EMR system.

1.2 State of the art: Reactor and reaction

1.2.1 Small scale commercial and modified EMRs

To date, there are many developed EMR designs that have been published (see also Table 2.2 in Section 2.2.3) (Carstensen et al., 2012; Giorno and Drioli, 2000; Greiner et al., 2003; Lozano et al., 2014; Prazeres and Cabral, 1994). Especially for free-enzyme systems, the designs are basically developed from (i) dead-end, (ii) recycle (cross-flow) filtration set-up or (iii) membrane aeration type. These reactors are operated in continuous mode with controlled temperature and pressure (gas inlet) with or without the regulation of the flow rate (Prazeres and Cabral, 1994).

For commercially available EMRs (filtration reactors) with smaller working volumes (less than 1 L), the most widely used (not limited to, but most popular in recent publications) are Amicon® stirred cell and Solvent resistant stirred cell (XFUF-047, XFUF-076) by Merck Millipore and Crossflow concentrator by Sartorius Stedim Biotech GmbH (see Figure 1.2). The Amicon® stirred cell volume ranges from 3 to 400 mL with effective membrane areas of 0.9-41.8 cm^2. A volume range of 75 to 300 mL is facilitated by Millipore solvent resistant stirred cell with membrane areas of 15-40 cm^2 and maximum pressure of 6.2 bar (90 psi). These two filtration test cells work in dead-end filtration mode and are popularly used especially in laboratory scale

[2]Activated support is a term for the support that has been reacted with functional groups (i.e. epoxy) in order to have electrophilic groups which later in the coupling step, can react with nucleophiles of the proteins (Guisán, 1988)

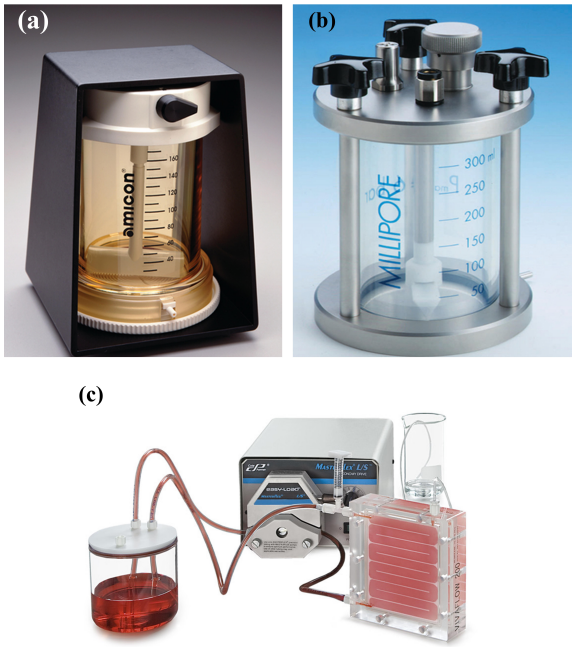

Figure 1.2 Typical commercial filtration units: (a) Amicon®, (b) Solvent resistant stirred cell and (c) Crossflow concentrator. Figures are courtesy of Merck Millipore (a and b) and Sartorius Stedim Biotech GmbH (c).

(Chen and Columbia, 2011; Olano-Martin et al., 2001; Prazeres and Cabral, 1994). For cross-flow filtration mode, the disposable and reusable crossflow concentrator by Sartorius have relatively high flow rates of 200-400 mL/min.

Henley et al. (1980) reported the application of a membrane reactor for the enzymatic saccharification of cellulose. A stirred UF cell (Amicon model 202) with an Amicon XM50 membrane was connected with a CSTR. Basically, two reactors were used, one for the reacting compartment and the rest for the separation purposes. This design was not efficient, besides a higher number of reactors, more pumps (n = 4) were needed to control the level of liquid in the second reactor and to have a constant permeate flow rate (see Figure 1.3a). Lee and Kim (1993) used a kit of Amicon Model 8200, equipped with a water jacket for enzymatic hydrolysis of cellulose. An Amicon membrane MWCO = 10 kDa was used. Both reaction and separation process were done in one compartment. However, two peristaltic pumps were still employed to continuously feed the buffer (cellulose was fed using a solid feeder to avoid clogging inside the tube) and withdraw the products (Figure 1.3b). Gan et al. (2002) modified an Amicon dead-end filtration cell (Amicon PM10) with a maximum volume of 2.5 L. A flat sheet polysulfone UF membrane MWCO = 10 kDa with a diameter of 150 mm (effective membrane area = 177 cm^2) was installed at the bottom of the filtration cell. The EMR system was used for the same reaction mentioned above (Henley et al., 1980; Lee and Kim, 1993). The enzymatic reaction and the separation unit were done in the same compartment. Through modifications, an efficient transport of buffer and the product permeation

were done by a single control of an inlet gas (Figure 1.3c). This reactor, therefore, could be operated either at constant transmembrane pressure or flux.

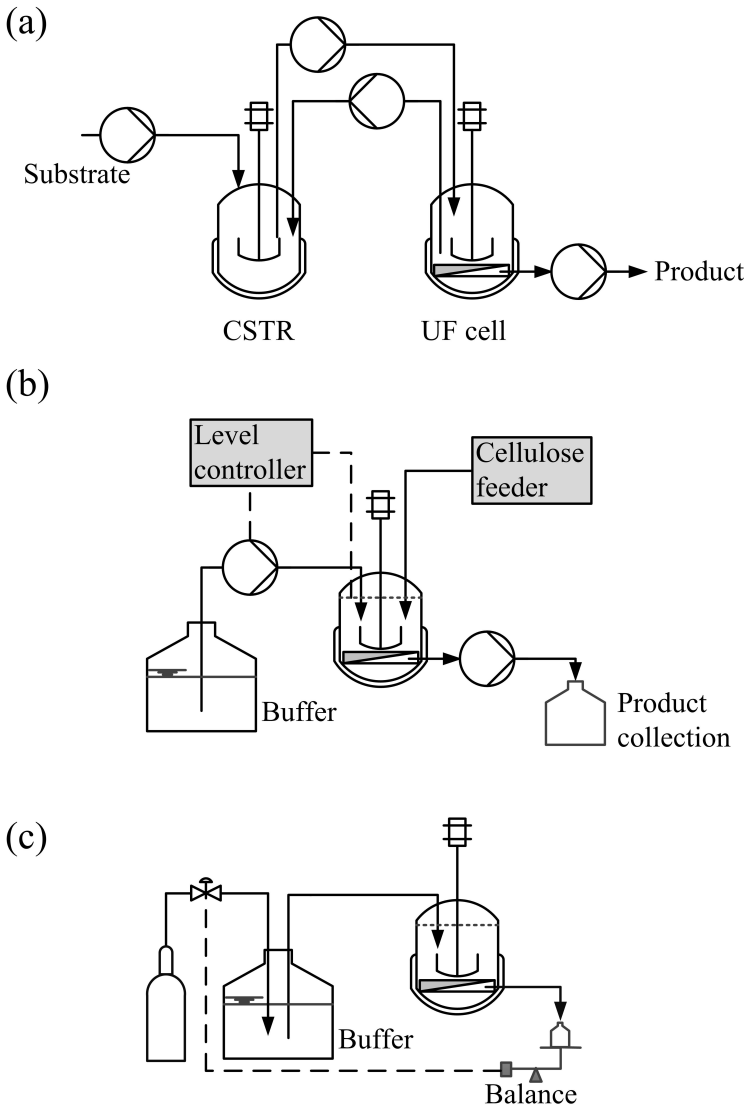

Figure 1.3 Development of EMR based on dead-end filtration mode according to (a) Henley et al. (1980), (b) Lee and Kim (1993) and (c) Gan et al. (2002).

Recently, Lyagin (2014) developed a screening and characterization system for continuous biocatalytic processes. An XFUF-047 Millipore solvent resistant stirred cell was modified to facilitate the insertions of pH and temperature sensor, substrate and enzyme inlet into the reactor (see Figure 1.4). The reaction and the separation were done in one compartment (similar to Gan et al. (2002)). This developed configuration had a relatively small volume ($\leq$ 100 mL) with a low hold-up volume (< 0.5 mL). The possibilities of both operations (constant flux or presssure) were amenable by the precise control of the proportional pressure regulator (Lyagin et al., 2010). Furthermore, Lyagin et al. (2015) also reported the applications of micro-solenoid valves to dose the

enzyme periodically into the reactor to have a maintained enzyme activity for two-weeks continuous reaction. The developed screening and characterization system was therefore, claimed to be a useful tool for comprehensive process description as well as for optimization and reliable process design. Looking into the details of the whole EMR system reported by Lyagin et al. (2015) and from the described data acquisition and software used (i.e., Visual Designer^{TM} 4.0) (Lyagin et al., 2010), the maximum number of parallel reactors that can be controlled (i.e., constant flux and enzyme dosing) was only two reactors. Considering the concept of the EMR system that had been coined by Lyagin et al. (2015) was 'attractive', this study was aimed to upgrade the control or process execution of that EMR system. One of the goals of this study was to have a developed robust program that can mostly mimic the process execution as reported by Lyagin et al. (2015) with a possibility of increasing the number of parallel reactors (more than ten reactors). An automated reactor system that has a higher number of parallel reactors is of importance as it can be used for an efficient and fast biotransformation process development (Betts and Baganz, 2006; Lye et al., 2003; Weuster-Botz, 2005). With the new control system, the addition of more reactors into the system was expected to not influence the parallelity (i.e., performance similarity) amongst the reactors themselves.

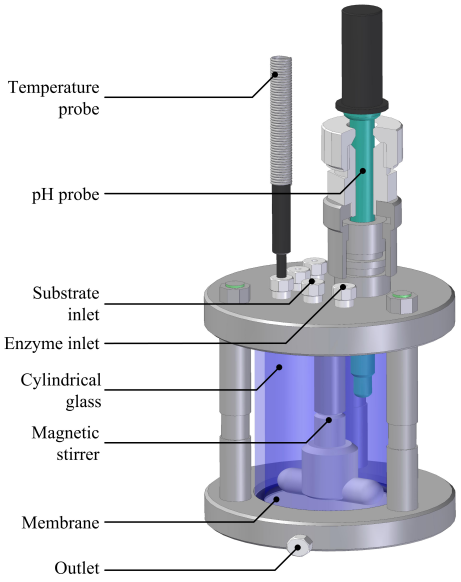

Figure 1.4 Developed configuration of filtration reactor which is analogous to XFUF-047 Millipore solvent resistant stirred cell (Lyagin, 2014).

1.2.2 Lactose and synthesis of lactulose

According to the report of Food and Agriculture Organization (FAO) (http://faostat.fao.org/), in 2012, the world-wide cheese production (based on goat, buffalo, sheep and cow milk) was about 20.5 million tonnes. Kosikowski (1979) reported that for making 1 kg of cheese, 9 kg of whey was disposed. Cheese-whey generally contains nutrients, such as lactose (4.5-5 % w/v), soluble proteins

(0.6-0.8 % w/v), lipids (0.4-0.5 % w/v) and mineral salts (8-10 % of dried extract) (Siso, 1996). Approximately 180 million tonnes of whey were produced in 2012 which contained approximately 8-9 tonnes of lactose. Although several possibilities for cheese-whey exploitations have been assayed over the last 50 years, around 30-50 % of the world cheese-whey productions were not treated, but discarded as effluents (Alonso et al., 2011; Siso, 1996). Therefore, there is a commercial interest to produce higher economic value substances based on whey of which lactose utilization is one possibility.

Lactose (4-*O*-β-D-galactopyranosyl-D-glucose) that consists of one molecule D-galactose and D-glucose can be purified from cheese-whey by crystallization (Siso, 1996). Lactose is mainly used as an ingredient in foods, infant formula, bakery and confectionery products and it has been extensively employed as diluent in tablets and carrier of medicines in the pharmaceutical industries (Gutiérrez et al., 2012). There is only a small amount of lactose used for food productions and biomedical applications. This is due to its low sweetness and solubility, including the intolerance of some population segments (Gutiérrez et al., 2011). To increase the economic value of lactose by producing valuable lactose derivatives which can be broadly applied in the food, pharmaceutical and chemical industries, has been a motivation of various studies (Gänzle et al., 2008). Several value added derivatives of lactose can be seen in Figure 1.5.

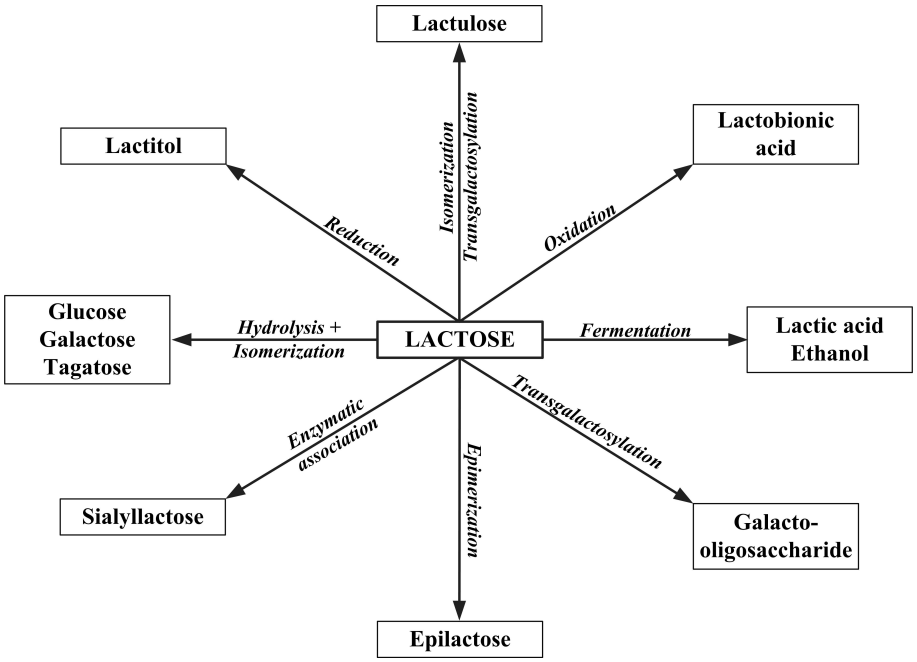

Figure 1.5 Commercially produced lactose derivatives and the related processes (Gänzle et al., 2008; Siso, 1996; Zadow, 1984).

As a derivative of lactose, lactulose[3] (4-*O*-β-D-galactopyranosyl-D-fructose), a synthetic disaccharide (does not occur naturally), is formed from one molecule of fructose and one molecule

[3]Lactulose was first synthesized in 1930 through alkaline isomerization of lactose at elevated temperature (Montgomery and Hudson, 1930)

of galactose via β-1,4-glycosidic bond (Wang et al., 2013a). The discovery of lactulose production is important in the dairy industry, which provides a strong support for the utilization of whey or lactose. Lactulose is fortuitously synthesized in small amount during milk processing. The dissolved salt system of milk (consisting mainly of phosphates, citrates, (bi) carbonates) is a buffered solvent favourable for the formation of lactulose from lactose in the course of milk heat treatment (Montgomery and Hudson, 1930).

Lactulose has received an increasing attention due to its roles in dairy industry, acting as a prebiotic[4] (Panesar and Kumari, 2011). As an ingredient of functional foods, lactulose is reported to have a number of physiological effects on humans, such as enhancing colonic motility, enriching the growth of probiotic bacteria, improving mineral absorptions, reducing the growth of putrefactive bacteria and acting as a laxative agent in the treatment of constipation (Olano and Corzo, 2009; Schumann, 2002; Seki et al., 2007).

Lactulose can be produced either by the chemical isomerization (using acid or base) or enzymatic synthesis (see Section 2.3). The chemical isomerization generally possesses several drawbacks, such as colored by-products, waste management issues and poorly specific reaction (Aider and de Halleux, 2007; Guerrero et al., 2011; Panesar and Kumari, 2011; Schuster-Wolff-Bühring et al., 2010). In addition to that, several time consuming steps, such as: acidification step, concentration, chromatography separation and further purification with ethanol or methanol, are generally acquired for downstream processes to recover lactulose from the mixture (Schuster-Wolff-Bühring et al., 2010). In contrast, the enzymatic-based lactulose synthesis has been reported to be more environmentally friendly and to require less laborious steps in product purification (Panesar and Kumari, 2011). Considering these facts, the enzymatic-based lactulose synthesis is an attractive alternative.

When lactulose is produced for functional food purposes, the used enzymes are restricted only to those which have GRAS (generally recognized as safe) FDA status (Rodríguez et al., 2006). Although the enzyme recombinant cellobiose 2-epimerase (see Table 2.7) has been proven to increase the yield of lactulose synthesis using a single substrate lactose (also in case of recombinant CelB (Mayer et al., 2010) and recombinant β-galactosidase from *Sulfolobus solfataricus* (Kim et al., 2006)), the enzymes preparations have not been listed as GRAS and are not commercially available. This makes the enzymes not ready yet for food industrial applications. There are only several β-galactosidase preparations from microorganisms that are commercially available and have been considered safe. These are from yeast and fungi, such as: *Kluyveromyces lactis*, *K. fragilis*, *Aspergillus niger* and *A. oryzae* (Ladero et al., 2000; Rodríguez et al., 2006; van Ooyen et al., 2006).

Additionally, most of the reported lactulose productions (from bi-substrate lactose and fructose) were run in batch procedures with typical working volumes of 1-500 mL (see Table 2.6). In batch operation, one of the bottlenecks for a higher productivity of lactulose synthesis using GRAS enzymes is secondary hydrolysis. The concentration of lactulose will peak when the probability of fructose as a galactosyl acceptor is higher than that of water (see Figure 2.7). As secondary hydrol-

[4]Friedrich Petuely (born in June 15, 1922 in Wien, Austria) successively discovered that lactulose can act as a growth factor for bifidobacteria and used it for chronic constipation.

ysis is kinetically controlled (Mayer et al., 2004), lactulose at its highest concentration is prone to undergo the secondary hydrolysis by the action of the pertinent enzyme (i.e., β-galactosidase) (van Rantwijk et al., 1999). A strategy where a continuous removal of the produced lactulose during the reaction can be an alternative to tackle this drawback. Hence, the developed EMR system could facilitate the investigations of the process engineering aspects of continuous lactulose production using GRAS enzymes. The continuous synthesis of lactulose with this reactor might provide at least three benefits, such as (i) circumvention of secondary hydrolysis by performing a continuous operation without tedious enzyme immobilization procedures, (ii) facilitation of continuous separation of the product from the enzyme, which also means a further enzyme utilization by cutting unproductive time and (iii) possibility of a long-term continuous operation that is also related to cutting unproductive time for the end and start-up procedures in each batch cycle.

1.3 Scope of the research and the outline of the thesis

1.3.1 Scope of the research

To sum-up all the elucidations mentioned above, this study was firstly aimed to upgrade the control or process execution of the EMR system that has been reported by Lyagin et al. (2015). The main task was to transfer the previously established program in Visual Designer^{TM} 4.0 into the Laboratory Virtual Instrument Engineering Workbench (LabVIEW^{TM}) program. Moreover, the data acquisition system (i.e., hardwares) was also upgraded to have a possibility of a higher number of parallel reactors installed. As a rule of thumb, by this upgrading, a constant flux operation and the ability to dose the enzyme periodically must be amenable, besides on-line pH and temperature monitoring. Secondly, to show the applicability of the EMR system, the process engineering aspects of lactulose production were investigated. The comparison between batch and continuous synthesis was studied. Moreover, the relevant operating conditions were optimized and used to perform a long-term lactulose synthesis with a maintained enzyme activity.

1.3.2 Outline of the thesis

The structure of the thesis is shown in Figure 1.6. The thesis starts with Chapter 1 where the Background, State of the art and the Scope of the research are presented. For the Literature Review of this thesis (Chapter 2), two topics: enzymatic membrane reactor and lactulose production are presented (the contents of these reviews are going to be submitted). In Chapter 3 (Materials and Methods), the contents are about the chemicals, the equipments and the methods that are used during the study. In Chapter 4 as the first part of the Results and Discussion, the discussion is mainly focused on the implementation of the robust feed-back controller (i.e., PID controller) in the EMR system for controlling flux and the facilitation of the automated protocol of the enzyme dosing. The stability of PID controller is evaluated in serial stepping fluxes (Sitanggang et al., 2014b). The results of the automation studies have been published in Sitanggang et al. (2014a,b,

2016). As the second part of the Results and Discussion, in Chapter 5, the new process execution (i.e., developed PID controller) is used to perform continuous lactulose production. The comparison of lactulose production in batch and continuous mode using commercial *K. lactis* β-galactosidase is also discussed. The output of this invesigation has been published in Sitanggang et al. (2014a,b). Moreover, the optimum operating conditions for continuous lactulose synthesis using *K. lactis* and *Aspergillus oryzae* β-galactosidase are also presented within Chapter 5. By this latter studies, the results have been published in Sitanggang et al. (2015, 2016). At the end of Chapter 5, the application of the automated enzyme dosing feature in EMR system is proven to maintain the enzyme activity during a long-term continuous lactulose production using commercial *A. oryzae* β-galactosidase (Sitanggang et al., 2016). Finally, in Chapter 6, the Summary and Future Outlook of the present study are briefly presented.

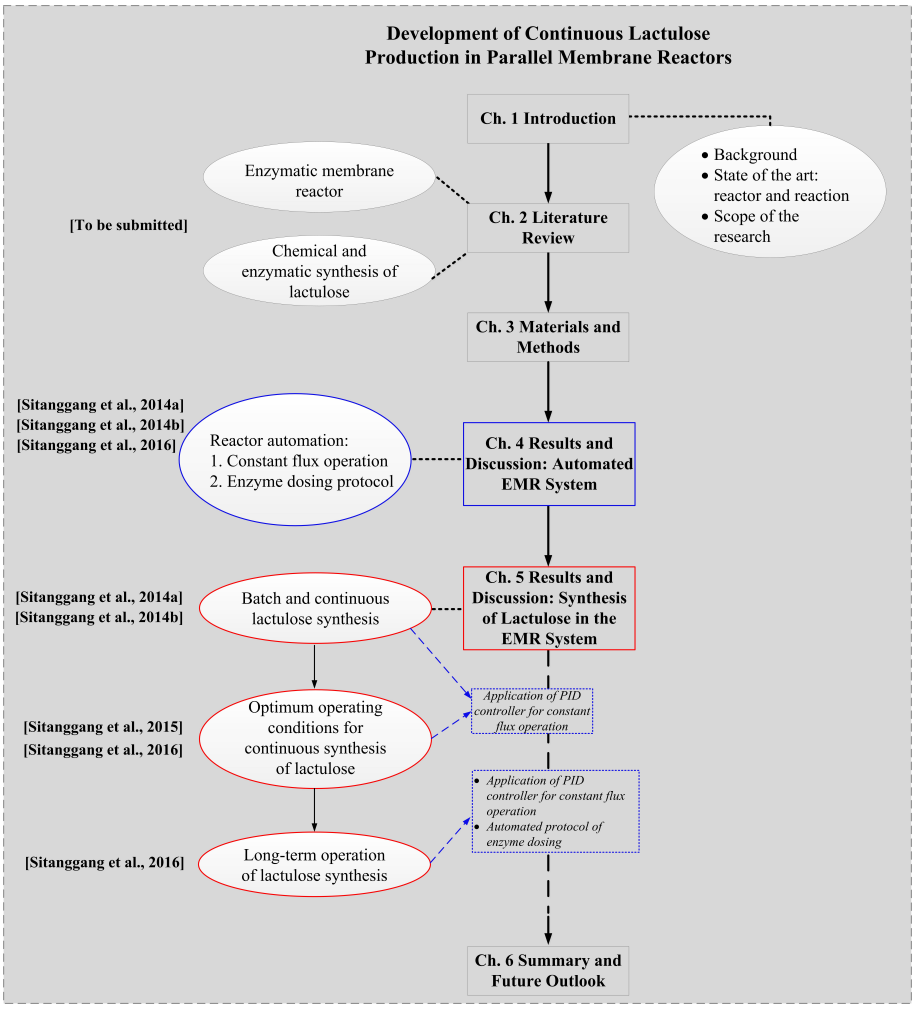

Figure 1.6 Structure of the thesis.

Chapter 2

LITERATURE REVIEW

2.1 Enzyme-based bioconversion: Market shared and the importance of continuous operation

Biocatalysis is defined as any process that involves enzymes or cells to catalyze the bioconversion of a substrate into useful products either in homo- or heterogenous systems (Prazeres and Cabral, 1994). Today, the applications of the enzymes for biotransformations can be found in agro-food sectors, pharmaceutical, energy and chemical industry (Giorno and Drioli, 2000). The enzyme-promoted bioconversion is more preferred than the other conventional means due to the mild conditions (low temperature, efficient energy consumption, environmentally friendly) as well as high quality of products produced (Kirk et al., 2002). To help the penetration of enzymes in various areas, an increasing number of studies has been done to perform controlled operations of the enzyme-catalyzed processes in terms of stable enzyme properties (natural or modified), approved and safe preparations of enzymes and stable product qualities (Estell, 1993; Koeller and Wong, 2001; Schmid et al., 2001).

The distributions of the enzyme markets by applications and classes are presented in Figure 2.1. The global market revenue for industrial enzymes was $4.1 billion in 2012, $4.41 in 2013 and by 2020 is expected to increase to be more than $7.5 billion according to the market research conducted by Grand View Research[1] (GrandViewResearch, 2014). Based on the region, North America plays a major role by having 37.37 % of the market followed by Europe, Asia pacific and others. By the increase of global population and lifestyle trends have had a positive impact on global processed foods demand. With the increasing demands for feeding the population, the demand for the enzymes in food industry is expected to be higher compared to others. Therefore, in terms of application areas, food and beverages possess the highest enzyme market share, followed by detergent, animal feeds and biofuels, respectively (Figure 2.1a). Corresponding to this data,

[1]This data was taken from Grand View Research market research entitled Enzymes Market Analysis by Product (Carbohydrase, Proteases, Lipases, Polymerases and Nucleases) and Segment Forecasts to 2020. It was received through personal communication to John Scott, a corporate sales specialist of Grand View Research, USA.

carbohydrases and peptidases represent the largest segments in the global market (Schmid et al., 2002; Zaks, 2001).

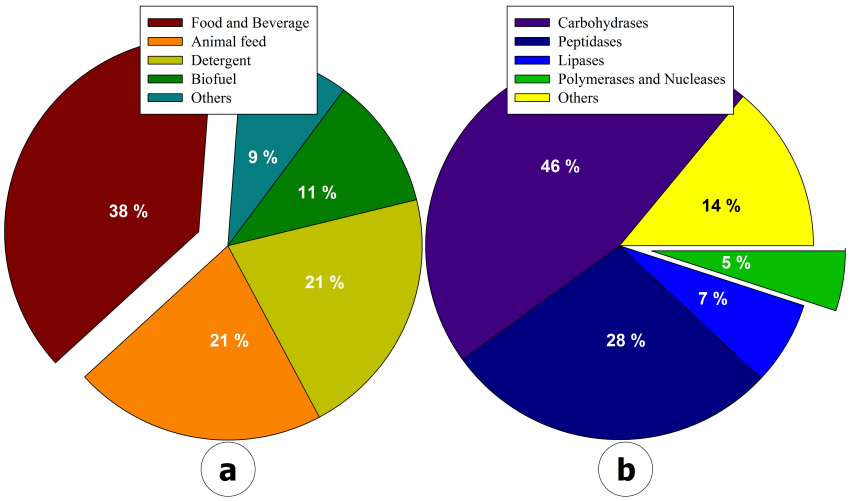

Figure 2.1 Global enzyme market share by (a) applications and (b) by enzyme classes. Accessed from: http://www.grandviewresearch.com (GrandViewResearch, 2014).

During bioconversion, the enzymes can be utilized either in native (free form) or immobilized form. As mentioned in Section 1.1, the immobilization of an enzyme, to some extent can increase the protein solubility, convenience in handling (separation from the reaction mixture), thermal and pH stability and can also permit the use of varied reactor configurations (Ansari and Husain, 2012; Garcia-Galan et al., 2011). However, one possible drawback of the immobilization is that the recovered activity sometimes can be as little as 10 % (Kamrat and Nidetzky, 2007; Mateo et al., 2007). Besides saving the extra cost and time due to the immobilization, the utilization of free enzyme can also negate mass-transfer limitations of substrate(s) to access the active sides of the enzyme. With this native form, however, reduced activity due to the heat and mechanical stress is also encountered during a prolonged reaction (Thomas and Geer, 2011; Tao et al., 2011).

Most of the enzyme-catalyzed processes are still run batch-wise. Batch operation is sometimes hindered by the occurrence of substrate and/or product inhibition (i.e., hydrolysis of cellulose). This can lead to a lower productivity as well as a higher number of processing cycles. Additionally, the other disadvantages of batch reactions can be the batch-to-batch oscillations, high labor costs, intricate start-up and end procedures including the need to recover the enzymes after each batch (Prazeres and Cabral, 1994). Reciting the strict regulations of producing fine chemicals in food (esp. functional foods, food additives) and in synthetic biology (for pharmaceutical industries like productions of drugs, antibiotics, amino acids, anti-inflammatories, etc.), the need of stable products qualities and the use of considerably safe enzymes preparations are obligatory. To meet the demands of stable products qualities, performing continuous operations are sometimes more preferred rather than the batch ones (see also Table 2.2).

2.2 Enzymatic Membrane Reactor (EMR)

2.2.1 Aspects in performing continuous bioconversion in EMR

A continuous catalytic process (PBR, MC, CSTR and EMR) can be an option to reduce the oscillations in product qualities in batch operation. However, the additional cost to recover the enzyme at the end of operation (CSTR, MC) or for the enzyme immobilization (PBR, MC) may significantly increase the total operational cost. In EMRs, the enzymes can be used either in native or immobilized form. The immobilization of an enzyme directly on the membrane can be achieved by chemical bonding (covalent bond), physical adsorption or electrostatic attraction (Butterfield et al., 2001; Wu et al., 2005). The immobilization locations can be on the active layer of the membrane, inside/along the pore channels or inside the porous support of the membrane. In native form, the enzyme is normally suspended in the reaction medium, retained inside the reactor system mostly by size exclusion and/or electrostatic repulsion (Freixo and de Pinho, 2002; Giorno and Drioli, 2000; Olano-Martin et al., 2001; Nembri et al., 1997; Prazeres and Cabral, 1994). Whenever the purpose of reaction is to hydrolyze a macromolecule, the educt can be retained inside the reactor as well, while the product(s) may flow through the membrane (Giorno et al., 2007). Briefly, with this EMR specific mode, a continuous process with a simpler preparation can be established (Rios et al., 2004).

The proper selection of a suited membrane for designing an EMR is a major factor for a successful continuous operation. This selection can be made based on the molecular size of the enzyme, substrate(s) and product(s) as well as physico-chemical properties of those compounds in solution. The polymers for both membrane skin and support have to be stable at pH and temperature where the optimum catalytic activity of the enzyme appears. The optimum pH and the corresponding enzyme, substrate and product charge have to be considered when chosing the proper membrane materials as they are closely related to the fouling behavior (discussed in Subsection 2.2.5). For the membrane type, ultrafiltration (UF) membranes are widely used for designing EMR systems. The enzymes generally have molecular weights between 10 and 150 kDa. With a known enzyme molecular weight, a proper membrane MWCO can be selected to ensure a near-complete rejection of the enzyme molecules. Several examples of enzymes rejections ($\mathfrak{R}$) by UF membranes are shown in Table 2.1. Nanofiltration (NF) membranes can also be used in EMRs for relatively small molecular weight enzymes (Nidetzky et al., 1996). In addition, as a secondary separation unit, NF membranes are used to concentrate compounds of interest. Cited as an example, Luo et al. (2014d) has reported an integrated membrane system for the biocatalytic production of 3-sialyllactose from dairy by-products. In their study, a UF membrane was used to retain the enzyme while sialic acid, sialyllactose and lactose permeated. Due to a small difference in molecular weight and the opposite electric charge between sialyllactose (633.55 Da, charge: -) and lactose (342.30 Da, charge: neutral), NF membrane NTR7450 (MWCO 600-800 Da, polyethersulfone, negatively charged) was found to be the suited membrane type (Luo and Wan, 2013).

Table 2.1 Several examples of enzymes rejections ($\mathfrak{R}$) by UF membranes.

Enzyme	Membrane	MWCO	Material	$\mathfrak{R}$ (%)	Reference
Novozyme Biomass-kit	ETNA01PP	1 kDa	Composite fluoropolymer	81	Li et al., 2014
	ETNA10PP	10 kDa	Composite fluoropolymer	78	
	UFX10	10 kDa	Polysulfone, hydrophilic	83	
	UP010P	10 kDa	Polyethersulfone	83	
Cellulase complex (Celluclast 1.5L®, Novozymes S.A, Spain)	UP010P	10 kDa	Polyethersulfone	99.8	Lozano et al., 2014
	UP005P	5 kDa	Polyethersulfone	100	
	Ceramic	5 kDa	TiO_2	99.4	
Cellobiase (Novozym 188®, Novozymes S.A, Spain)	UP010P	10 kDa	Polyethersulfone	98.4	Lozano et al., 2014
	UP005P	5 kDa	Polyethersulfone	99.4	
	Ceramic	5 kDa	TiO_2	98.1	
A. niger F3 strain phytase	UP010P	10 kDa	Polysulfone, hydrophilic	99.95	Rodríguez-Fernández et al., 2013

2.2.2 EMR Designs

An EMR design is generally developed by considering whether an enzyme is used in native or immobilized form. The molecular size of the enzyme, substrate and product have to be considered for the selection of membrane MWCO. The placement of the membrane can be varied, such as directly at the bottom of the reactor, submerged inside the reactor and outside of the reactor (external loop). The biocatalytic medium, either aqueous or solvent may also contribute to the configuration of an EMR. Furthermore, CSTR and plug flow/tubular reactor are the common reactor types used. The reported EMRs can be generally grouped based on the functions of the membrane, hydrodynamics and the filtration characteristics, such as: (i) dead-end EMR (incl. submerged EMR), (ii) (recycle) cross-flow EMR, (iii) dialysis EMR and (iv) interfacial EMR (refer to Figure 2.2 and Figure 2.3).

Membrane as a separation unit or as catalytic area and separation unit

Within this group, the enzymes can be utilized either in native (size exclusion) or immobilized form. The dead-end and cross-flow membrane reactor are the common configurations. Dead-end EMR is the most widely used configuration especially at laboratory scale. This configuration is normally used for testing the filtration characteristics of solutions, operational concepts of enzymatic reactions, etc (Chen and Columbia, 2011; Olano-Martin et al., 2001; Prazeres and Cabral, 1994). The catalytic reaction and separation unit are done in the same compartment with mostly flat-sheet membrane configuration. The pressure is set in the reactor to achieve permeation of solutes and the reactor can be operated either at a constant transmembrane pressure or a constant flux. In submerged membrane reactors, the membrane is immersed into the reacting solution to facilitate products permeation (Carstensen et al., 2012). Hollow fiber membranes are normally used to increase the membrane surface (Chakraborty et al., 2012). Although the application of this

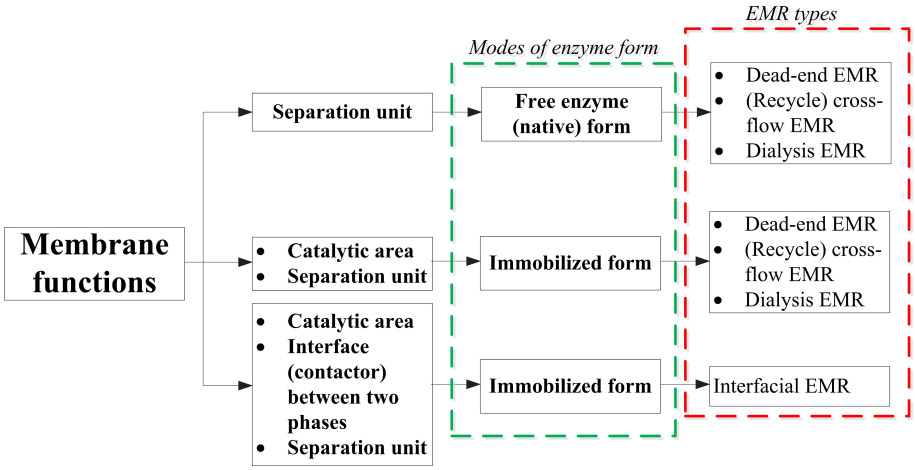

Figure 2.2 EMR configurations based on the membrane functions, hydrodynamics and the filtration characteristics.

design is inherently a good alternative in whole-cell based bioprocesses (Carstensen et al., 2012), limited reports are found for enzymatic reactions (Stark and von Stockar, 2003).

In (recycle) cross-flow membrane reactors, the feed stream flows parallel to the membrane surface. Especially for hydrolysis of macromolecules, the tangential velocity is necessary to reduce the deposition of foulants from the membrane. The common membrane configurations are tubular and hollow fiber membranes with several minor applications using flat-sheets. The retentate can be recycled again into the reactor to have a longer reaction time with the enzyme prior to filtration. This is helpful, especially for immobilized systems. An increased number of recycles can provide a longer contact between substrate and enzyme molecules (Akay et al., 2002; Yu et al., 2010).

In dialysis membrane reactors, after having a contact with the enzyme, the product is transported to the other side of the membrane by diffusion due to the resulted concentration gradients. This diffusion-based transport is also a disadvantage of this system as the mass transport is less efficient (Prazeres and Cabral, 1994). To prevent the convective flow of the solutions, the transmembrane pressure has to be carefully balanced, i.e., the streams are circulated at approximately the same flow rate and pressure (Lin and Yang, 2003b). The common configuration for dialyzer reactors is hollow fiber where the enzyme is normally immobilized (Lin and Yang, 2003a,b). Whenever the enzyme is entrapped in the aqueous core of reversed micelles or Pickering emulsions, this reactor design can be used for the conversion of nonpolar compounds (Chiang and Tsai, 1992).

Membrane as catalytic area, interface and separation unit

The utilization of the enzymes in interfacial EMRs is mostly through the immobilization on the surface of the membrane skin layer, support or inside the support layer. The common membrane configurations are tubular and hollow fiber. A contact between enzyme and substrate is facilitated at the membrane matrix. Herein, due to the difference in substrate and product polarity, two streams are available in the reactor system (biphasic) (Giorno et al., 2007; Long et al., 2005). Many factors

influence its successful operation and these include characteristics of the enzyme, membrane, circulating fluids and other operating conditions (Agustian et al., 2011). The applications of interfacial EMRs are in the field of enzymatic resolution of racemic compounds or conversion of nonpolar compounds where the (by-)products are normally water soluble (Agustian et al., 2011; Drioli et al., 2005; Jochems et al., 2011).

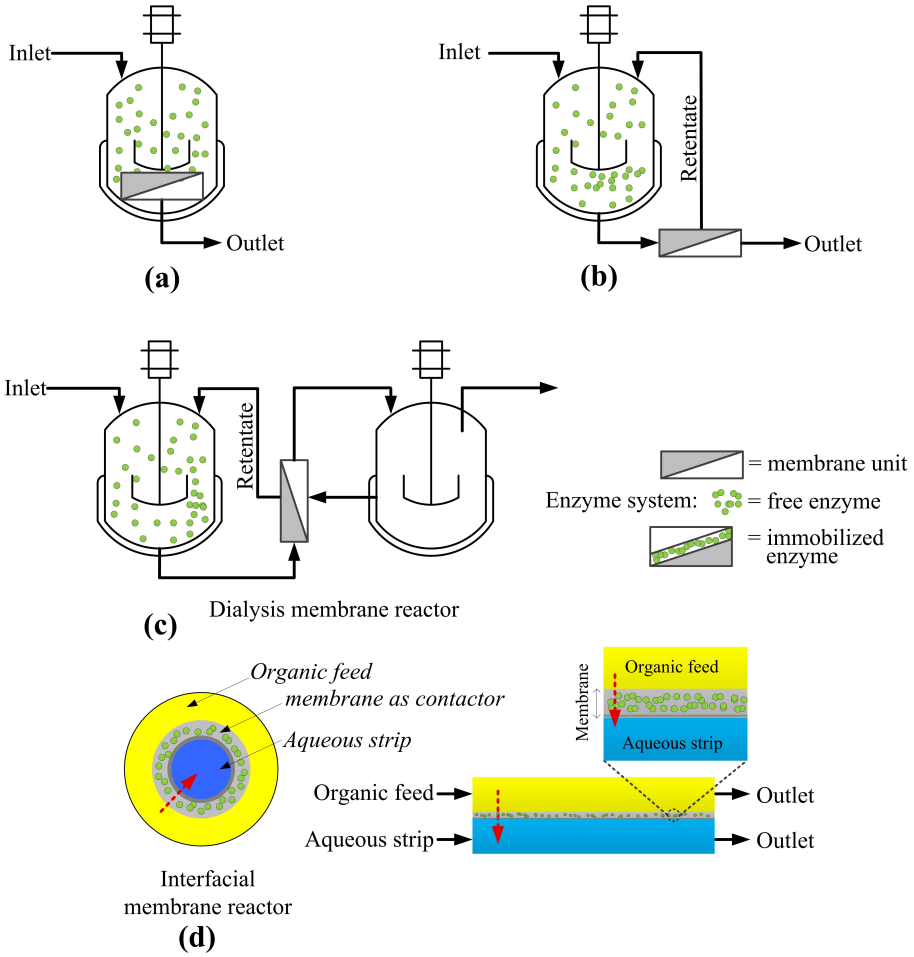

Figure 2.3 Illustrations of common EMR configurations: (a) dead-end, (b) cross-flow, (c) submerged and (d) interfacial membrane reactor.

2.2.3 Applications of EMRs

In 1972, the first successful combination of a biocatalytic reaction and a separation process in a single reactor system was demonstrated by Rony (1972). Enzymes, i.e., alkaline phosphatases were contained in semipermeable hollow fibers. Starting from this year, an increasing number of publications regarding the utilizations of EMRs in assorted fields is apparent. As can be seen in Figure 2.4, in the first-ten years (1972-1982), the number of publications fell in the range of 2-9 articles per year. In 2010s, the number of publications was approximately ten-fold. This shows that EMR has gained an elevating research interest.

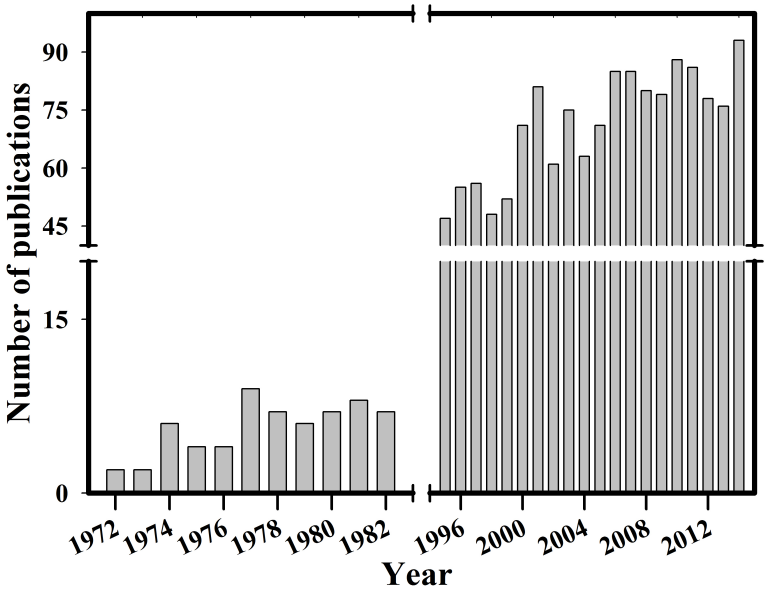

Figure 2.4 Number of publications related to EMRs in 1972-2014. The data was collected by inserting keywords: (i) enzyme reactor and (ii) membrane in the search engine of http://apps.webofknowledge.com. The data bases included Web of Science™ Core Collection, Current Contents Connect®, MEDLINE®, Inspec® and SciELO Citation Index.

As mentioned earlier, applications of biocatalysts are common in the agro-food sectors, pharmaceutical, energy and chemical industry and environmental sectors (Cao et al., 2007; Fjerbaek et al., 2009; Iorio et al., 1994; Giorno and Drioli, 2000; Machsun et al., 2010; Rios et al., 2004). The uses of EMRs in food, pharmaceutical and biomedical are mainly in hydrolysis of macromolecules–proteins, polysaccharides, lipids (Giorno and Drioli, 2000). Especially in the area of food and beverages, EMRs are used for the extraction and clarification processes during the manufacturing of juices and wines (Rodriguez-Nogales et al., 2008), reducing lactose content in milk and whey and its conversion into digestible or non-digestible sugars, i.e., prebiotic (Botelho-Cunha et al., 2010; Ebrahimi et al., 2010) and hydrolysis of proteins into peptides that have antioxidant properties (Mazzei et al., 2010; Zhao et al., 2013). Several reported applications of EMRs are listed in Table 2.2.

For pharmaceutical applications, EMRs are generally used to degrade various proteins into bioactive peptides (i.e., peptides with angiotensin converting enzyme (ACE) inhibitory activity), production of vitamin percursors and other chiral drugs that have analgesic, antipyretic and anti-inflammatory effects (Cantarella et al., 2010; Welderufael and Jauregi, 2010; Sie Yon et al., 2013).

The applications of EMRs in energy sectors are mainly in biodiesel productions from fat, lard, tallow, and vegetable oils, such as sunflower, palm, rapeseed, soybean, castor and *Jatropha curcas* oil (Fjerbaek et al., 2009; Machsun et al., 2010). Transesterification of triglycerides produces fatty acid alkyl esters (FAAE) and glycerol that can be used as biodiesel fuel for diesel engines (Chisti,

2007; Fjerbaek et al., 2009; Meher et al., 2006). The reaction still needs small amounts of water (0.1-20 % wt water) as a lubricant for enzyme conformation in order to function optimally (Fjerbaek et al., 2009; Mata et al., 2012). The ultimate advantage of enzyme-based transesterification in combination with membranes is that continuous products (i.e., glycerol and FAAE) removal can lead to a higher product yield and quality. However, the cost of enzymes and low mass transfer (when enzymes are immobilized) can significantly add to the capital cost. In addition, the reduction of enzyme activity over time (i.e., by thermal inactivation) is also included in one of the drawbacks of EMR-based transesterifications (Fjerbaek et al., 2009). For the other applications, the EMRs are also used to degrade toxic compounds in waste water (esterogens, bisphenol A, etc.) whenever the whole cell catalysis is not efficient in waste water treatment plant (WWTP) (Escalona et al., 2014; Lloret et al., 2012).

Table 2.2 Applications of EMRs in agro-food, pharmaceutical and biomedical, environmental and energy sector.

EMR design	Enzyme	Purpose	Reference
1. Agro-food sector			
Interfacial / Ceramic tubular membrane (20 kDa)	β-galactosidase, Maxilact ®	Production of galactosyl-oligosaccharides (GOS)	Ebrahimi et al., 2010
Cross-flow / Ceramic tubular membrane (8, 15 and 50 kDa)	β-galactosidase	Production of chemically modified starches and their hydrolysates	Sulej-Chojnacka et al., 2010
Dead-end / Flat-sheet membrane (20 kDa)	Dextrozyme (a mixture of glucoamylase and pullulanase)	Hydrolysis of maltose	Findrik et al., 2010
Interfacial / Hollow fiber capillary membrane (30 kDa)	β-glucosidase	Hydrolysis of oleuropein for productions of 1 (3,4-DHPEA-EA) and 2 (3,4-DHPEA-EDA) as antioxidants	Mazzei et al., 2010
Cross-flow / Micro-channel with flat-sheet membrane (10 kDa)	Pectin lyase, Novozymes A/S	Production of smaller pectin fragments with a double bond on their non-reducing ends	Alam et al., 2011
Dead-end / Flat-sheet membrane (10 kDa)	Cellulase	Hydrolysis of cellulose to produce reducing sugars	Liu et al., 2011b
Cross-flow / Flat-sheet membrane (10 kDa)	Alcalase 2.4 LF	Production of modified gluten for food industry	Cui et al., 2011
Cross-flow / Ceramic tubular ultrafine porous ZrO_2 (0.05 μm)	Pepsin	Production of fish protein hydrolysates	Benhabiles et al., 2012
Cross-flow / Flat-sheet membrane (3, 5 and 10 kDa)	Alcalase 2.4 FG	Production of casein bioactive phosphopeptides	Zhao et al., 2013
Cross-flow / Flat-sheet UF (5, 10, 20, 30 kDa) and NF membrane (200, 600 and 1000 Da)	Mutated sialidase, Tr6	Production of 3-sialyllactose as prebiotic	Luo et al., 2014d

EMR design	Enzyme	Reaction	Reference
Cross-flow / Hollow fiber membrane (32 kDa)	Cyclodextrin glycosyltransferase (CGTase)	Synthesis of cyclodextrin as chemical enhancer in food industry (emulsifiers, antioxidants and stabilizing agent)	Sakinah et al., 2014
Dead-end / Flat-sheet membrane (1, 5 and 10 kDa)	Flavourzyme^{TM}, Novozymes	Hydrolysis wheat gluten for production of amino acids and peptides	Berends et al., 2014
2. Pharmaceutical and biomedical treatments			
Cross-flow / Flat-sheet membrane (1 kDa)	Protease N-Amano	Production of peptides with angiotensin converting enzyme (ACE) inhibitory activity	Welderufael and Jauregi, 2010
Cross-flow / Flat-sheet NF membrane	β-galactosidase	Production of galacto-oligosaccharides (GOS) for pharmaceutical purposes	Botelho-Cunha et al., 2010
Dead-end / Flat-sheet membrane (10 kDa)	Nitrile hydratase and amidase	Production of nicotinamide as vitamin percursor	Cantarella et al., 2010
Dead-end / Flat-sheet membrane (10 kDa)	Alcohol dehydrogenase and glucose dehydrogenase	Reduction of 2-octanone to produce enantiopure alcohols	Kohlmann et al., 2011
Interface / Tubular membrane	Lipase	Production of high purity (S)-ibuprofen acid	Lau et al., 2011
Cross-flow / Spiral-wound membrane (3 and 5 kDa)	Alcalase and neutrase, NOVO Industry A/S	Production of antihypertensive peptides from corn proteins	Huang et al., 2011
Interface / Hollow fiber membrane (50 kDa)	Lipase (Type VII)	Dynamic kinetic resolution of ibuprofen ester for production of chiral drugs	Sie Yon et al., 2013
Dead-end / Track-etched microporous membrane electrodes (Nucleporetrack^{TM}-etched membrane filter, 0.4μm)	Glucose oxidase	Biosensor for glucose analysis	Mizuguchi et al., 2013
Dead-end / Flat-sheet membrane (50 kDa)	Alcohol dehydrogenase and L-glutamate dehydrogenase	Conversion of NAD^+ into NADH	Luo et al., 2013
Cross-flow / Flat-sheet membrane (1 and 5 kDa)	Porcine pancreatin	Preparation of bioactive peptides, angiotensin-converting enzyme-inhibitory peptides, casein phosphopeptides and antimicrobial peptides	Wu et al., 2013
Dead-end / Flat-sheet membrane (10 kDa)	benzoylformate decarboxylase	Synthesis of (S)-2-hydroxypropiophenone ((S)-2-HPP) for the synthesis of pharmaceutical agents	Valinger et al., 2014
Dead-end / Flat-sheet membrane (10 kDa)	Heparin lyase	Production of low-molecular-weight heparin (LMWH) tinzaparin	Fu et al., 2014
Cross-flow / Flat-sheet membrane (3 kDa)	Alcalase 2.4 LFG	Production of antihypertensive peptides from *Porphyra yezoensis* proteins	Qu et al., 2015

EMR design	Enzyme	Reaction	Reference
Interface / Hollow fiber dense membrane	Candida antarctica lipase B (CALB)	Production of amides that are precursors for peptide and lactam synthesis	Luque et al., 2014
3. Environmental, biodiesel, energy productions and others			
Dead-end / Flat-sheet membrane (300 kDa)	Lipase	Biodiesel production	Machsun et al., 2010
Cross-flow / Hollow fiber membrane (10 kDa)	Celluclast® 1.5 L	Hydrolysis of cellulose for bioethanol production	Hwang et al., 2014
Cross-flow / Flat-sheet membrane (10 kDa)	Cellulase	Saccharification of ionic liquid-pretreated microcrystalline cellulose for bioethanol production	Lozano et al., 2014
Cross-flow / Tubular ceramic membrane (15 kDa)	Cutinase	Transesterification of triolein for production of biodiesel	Badenes et al., 2011
Cross-flow / Flat-sheet membrane (25 and 50 kDa)	Lipase, Novozyme ® 435	Biodiesel production	Ko et al., 2012
Cross-flow / Flat-sheet NF membrane (200 Da)	Acylase (quorum quenching enzyme)	Reducing secretion of EPS for controlling membrane biofouling	Kim et al., 2011a
Cross-flow / Flat-sheet membrane (10 kDa)	Peroxidase	Generation of the Mn^{3+}-malonate complex for oxidizing industrial wastes	Taboada-Puig et al., 2011
Cross-flow / Flat-sheet membrane (10 kDa)	Laccase	Degradation of estrogens in waste water treatment plants	Lloret et al., 2012
Cross-flow / Flat-sheet NF membrane (200 and 300 Da)	Horseradish peroxidase	Removal of bisphenol A (BPA)	Escalona et al., 2014
Cross-flow / Tubular ceramic membrane (0.2 and 1.4 μm)	Laccase	Tetracycline degradation	de Cazes et al., 2015

2.2.4 Advantages and disadvantages of EMRs

One of the essential advantages of EMR is the facilitation of a continuous process. The operation of EMRs merely requires a shorter time of start-up and end procedures (Rios et al., 2004). Thus, the reduction of non-productive operational time can lead to a reduced processing cost. As common to continuous processes, the intensive use of the enzymes will yield an increased productivity and economic viability (Prazeres and Cabral, 1994). The separation of product (as permeate) from the reaction mixture is advantageous, especially for those reactions where the product and/or substrate inhibition and degradation of the intermediate product by pertinent enzymes are pronounced (Gan et al., 2002). Additionally, this continuous product removal can also be beneficial, as a favorable shift of the equilibrium towards product side can take place (van der Padt and van 't Riet, 1991). The use of several membranes in series with different MWCOs also contributes to the product selectivity. The enrichment/concentration of the non-permeable products from the final

membrane separation step can be done (Berends et al., 2014; Wu et al., 2013; Zhao et al., 2013). The immobilized enzyme inside the membrane support (see Figure 2.3d) can facilitate bi-phasic reactions especially in pharmaceutical and biomedical treatments (Lau et al., 2011; Valinger et al., 2014; Sie Yon et al., 2013). The emulsion problems (i.e., phase separation, etc.) will be mitigated and thus, a higher agitation intensity and energy input can be avoided. The overall comparison between advantages and disadvantages of EMR applications can be seen in Table 2.3.

Table 2.3 The advantages and disadvantages of EMRs (Prazeres and Cabral, 1994; Rios et al., 2004).

Advantages	Disadvantages
Facilitation of continuous process (reducing operational time and cost)	Unfavorable adsorption of enzyme
Higher productivity (intensive use of enzyme)	Shear (and thermal)-induced enzyme inactivation
Better control possibilities	Loss of enzyme activators or cofactors
Shift of chemical equilibrium to product(s)	Fouling and concentration polarization
Improved rates in product- and/or substrate-inhibited reactions	
Enrichment/ concentration of products/ control of product properties (selectivity by membrane)	Enzyme leakage
Possibility of bi-phasic reaction	

The applications of EMRs at industrial scale are sometimes hindered due to the decay of enzyme inherent catalytic activity over the time (Rios et al., 2004). This decay might be caused by thermal-, shear-induced inactivation or leakage. The influence of shear rate on the enzyme activity has been reported by Thomas and Geer (2011). Generally, the activity is a property that is strongly dependent on the conformation of the protein. The activity can reduce when the enzyme molecules are appropriately oriented in the shear (hydrodynamic)-field causing the break of tertiary structures of the protein (Elias and Joshi, 1998; Lencki et al., 1993). Through immobilization, thermal stability of the enzymes can be increased (Ong et al., 2008). However, a decay of activity may follow after a period of utilization and, hence, the life span of the system will be strongly affected. In case of free-enzyme systems, the enzyme leakage may occur depending on its form and the distribution of membrane pore sizes (refer to Table 2.1 for the enzymes rejections of different membrane MWCOs). Especially in bi-phasic reactions, a slower diffusion rate of substrate to access the active sides of enzymes can lead to a lower amount of product produced. In addition, fouling is generally pronounced, especially when the EMRs are used for the degradation of macromolecules. Several treatments can be carried out to reduce fouling, such as filtration breaks, backflush and frequent cleanings (chemical cleanings) (Drews, 2010). These treatments eventually can influence the productivity of the processes or increase the related operational costs.

2.2.5 EMRs as a pressure-driven membrane technology: Operation, fouling and its circumventions

To have a convective transport in membrane operation, for instance in porous membranes, a sufficient pressure difference ΔP is needed. As mentioned previously, the applications of UF and/or NF membranes in EMRs are normally used to retain the enzymes (and also possible for substrate) on the feed side (i.e., free enzyme systems) or to facilitate the contact between substrate and immobilized enzyme while allowing the permeability of the solvent and solute (products, reactant) to the other side of the membrane. The calculation of flux J can be written as:

$$J = \ell_p \cdot \Delta P \tag{2.1}$$

where ℓ_p is membrane permeability. The total hydraulic resistance R_t can be determined as:

$$R_t = \frac{1}{\ell_p \cdot \eta} \tag{2.2}$$

where η is the dynamic viscosity of permeate. The total hydraulic resistance is the summation of several subresistances, such as membrane resistance R_m and resistance due to fouling R_f which can be divided into reversible R_{re} and irreversible resistance R_{irr}. Eq. 2.1 can be rewritten into the following resistance-in-series model (Bouhabila et al., 2001):

$$J = \frac{\Delta P}{\eta (R_m + R_{re} + R_{irr})} \tag{2.3}$$

In continuous operation of ideal reactors (PFR, CSTR), we may refer to space-time (hydraulic residence time τ) and space-velocity ($s = 1/\tau$) as proper performance measures. Space-time is defined as time required to process one reactor volume of feed measured, whereas space-velocity is the number of reactor volumes of feed at specified conditions which can be treated in a time unit (Levenspiel, 1999). This can be expressed as:

$$\tau = \frac{1}{s} = \frac{C_{S0} \cdot V_R}{F_{S0}} = \frac{V_R}{\dot{V}} \tag{2.4}$$

where C_{S0} = molar inlet concentration of substrate, V_R = reactor volume, F_{S0} = inlet molar feed rate and $\dot{V}$ = volumetric flow rate. Taking the relationship between flux J and membrane area A_{eff} ($J = \dot{V}/A_{eff}$) and assuming V_R is constant, at a constant flux operation the residence time τ is also constant. With a constant residence time one can easily evaluate how quickly/slowly the compositions inside the reactor shift to a change in removal rate.

$$\tau = \frac{V_R}{J \cdot A_{eff}} \tag{2.5}$$

During the operations of EMRs, the membranes can suffer from two distinctive phenomena: concentration polarization and fouling. Accumulation of chemical species at the boundary adjacent

to the membrane surface during an EMR operation can cause concentration gradients between membrane surface and bulk solution and induces concentration polarization. This can cause back diffusion of chemical species to the bulk solution (Drews, 2010; van den Berg and Smolders, 1989). Fouling is associated with the deposition (caking), adsorption, gel formation and pore blocking of particles on the membrane surface or inside the pores. For a relatively strong adsorption of particles inside pore channels, this might lead to a progressive reduction in the flux (Prazeres and Cabral, 1994). Considerably, caused by fouling, an increasing resistance leads to either less flux at a given constant pressure or a higher pressure if flux is kept invariant. In return, this may significantly affect the production efficiency and increase the energy consumption for membrane cleaning.

The nature of membrane fouling, especially in membrane reactors is determined by feed composition, membrane properties and hydrodynamic conditions (see Figure 2.5) (Drews, 2010; Tang et al., 2011). During the operation of EMRs, especially in dead-end filtration mode, macromolecules such as polysaccharides, lipids and proteins (incl. enzyme molecules) tend to form a cake layer on membrane surface. Whenever an extensive three-dimensional cross-linked structure by some of these macromolecules is pronounced, a gel layer is formed (Wang and Waite, 2009). For example, at very high concentrations of proteins close to the membrane (due to concentration polarization), a gel layer due to strong inter-colloidal attraction or specific interaction can be formed. A gel formation is also possible for some polysaccharides in the presence of calcium (Wang and Waite, 2008). In some references, the term “cake layer” is reserved for non-gelling fouling layers (Tang et al., 2011).

In size exclusion EMR, very often the optimum pH value of the enzyme (when it is buffered) is not suited for an electrostatic (charge-charge) interaction towards repulsion (Boye et al., 2010; Wang and Tang, 2011). Adsorption and eventually the deposition of the enzyme molecules onto the membrane occurs during the process. It is common that proteins with opposite surface charge compared to that of the membrane have the strongest adsorption (Beier et al., 2007).

In order to maintain the usage of the membrane (i.e., reducing membrane cleaning activities), the selection of a membrane with appropriate physical properties is needed to design an EMR. Membrane properties, such as surface charge properties, hydrophobicity and roughness can significantly affect the fouling propensity (Elimelech et al., 1997; Vrijenhoek et al., 2001). In general, smooth, low surface charge, and more hydrophilic membranes tend to show better anti-fouling properties. These anti-fouling properties are only effective in the initial operation stage since the dominating phenomenon is foulant-membrane interaction. For a long-term operation, these membrane anti-fouling properties are less considered as the dominating phenomenon shifts from foulant-membrane to foulant-foulant interaction (Tang et al., 2009; Wang et al., 2013b).

Operating parameters, such as shear stress, pressure/permeate flux and temperature can influence the mass transfer rate and hydrodynamics over the membrane surface (Zhang et al., 2015). A higher propensity of fouling is generally amenable at a higher membrane flux and/or a lower cross flow. At a high flux, an increased convective transport can lead to a higher rate of foulants deposition onto the membrane surface of a dead-end EMR. In a cross-flow EMR, the cross-flow velocity

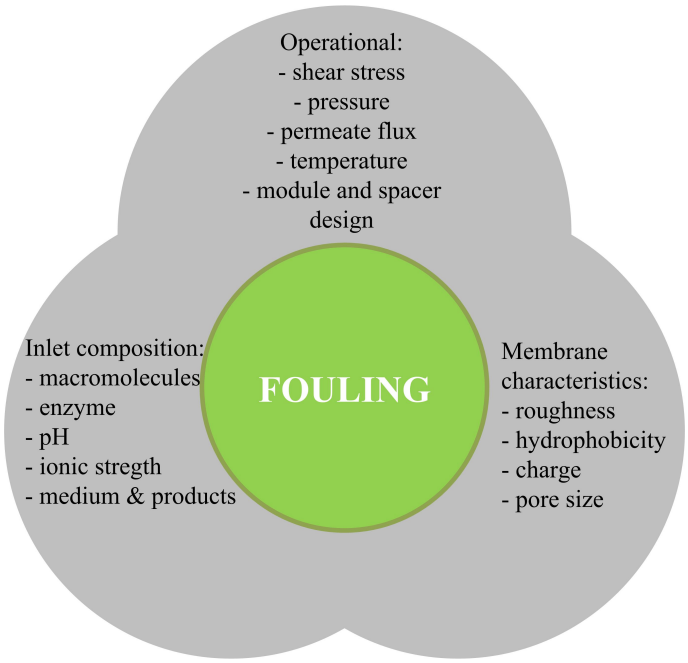

Figure 2.5 Factors that contribute to fouling in EMRs (Tang et al., 2011; Zhang et al., 2015).

is important as it affects the lifting rate of foulants from the membrane surface by introducing back-transport (Tang et al., 2011). However, a very high cross-flow velocity can cause large pressure drops, local membrane fouling and high pump energy consumption which can consequently shorten the membrane life span and increase the operation cost (Ding and Jaffrin, 2014). In some cases, a constant-flux operation in EMR was realized at a constant transmembrane pressure which was below a critical value[2]

The strategies to minimize fouling in MBR can be practically applied in EMR, such as: (i) avoidance and (2) remediation strategy (van Der Bruggen et al., 2003). Considerations upon inlet composition, membrane characteristics and operational procedures can be grouped as avoidance strategy. The critical flux concept is primarily included to avoid fouling. In case of WWTP, which is sometimes combined with EMR operation (de Cazes et al., 2015; Escalona et al., 2014), several avoidance strategies are done in pretreatment, such as coagulation, adsorption and preoxidation using ozone, permanganate and chlorine (Gao et al., 2011; Kim et al., 2011b). Other avoidance strategies that can be introduced in EMRs are an increased shear rate on the membrane surface, modifying pH and/or ionic strength, applying ultrasonic fields, etc. (Luo et al., 2014c). However, the conditions for these strategies should be carefully optimized as they may induce the enzyme conformational changes.

Cleaning through physical means, such as backflushing or relaxation under crossflow conditions is classified into the second group. Traditionally, fouling types which can be avoided by backflushing or relaxation are reversible fouling including physical adsorption fouling. It is of importance

[2]Critical flux concept in pressure-driven membrane processes has been stated as a threshold flux which fouling is negligible and above which fouling tends to be severe (Field et al., 1995).

that backflushing should not be performed at a high pressure in view of the limited mechanical stability of the UF and NF flat-sheet membranes where thin-film composite membranes are used (van Der Bruggen et al., 2003). The remediation strategy through chemical cleaning to remove irreversible fouling (Drews, 2010; Lyko et al., 2008) can also be done at regular time. Large differences in the cleaning frequencies can be found, ranging from daily to yearly, depending on the concentration of foulants (period of operation) and the pretreatments, i.e., in case of WWTP. Through chemical cleaning, most of the foulants that have accumulated during the operation can be removed. The initial permeability is almost recovered and only a small portion of permeability cannot be recovered which adds to irrecoverable fouling (Kraume et al., 2009).

2.3 Lactulose production: Overview of chemical and enzymatic synthesis

Production of lactulose has recently received a growing interest. Lactulose has been reported as a prebiotic and to have a number of physiological effects on humans (Schumann, 2002; Seki et al., 2007). Herein, the synthesis of lactulose is considered to be an additional solution to elevate the utilization of lactose (see Subsection 1.2.2). Within this Section 2.3, a short review of lactulose production based on chemical and enzymatic synthesis is presented.

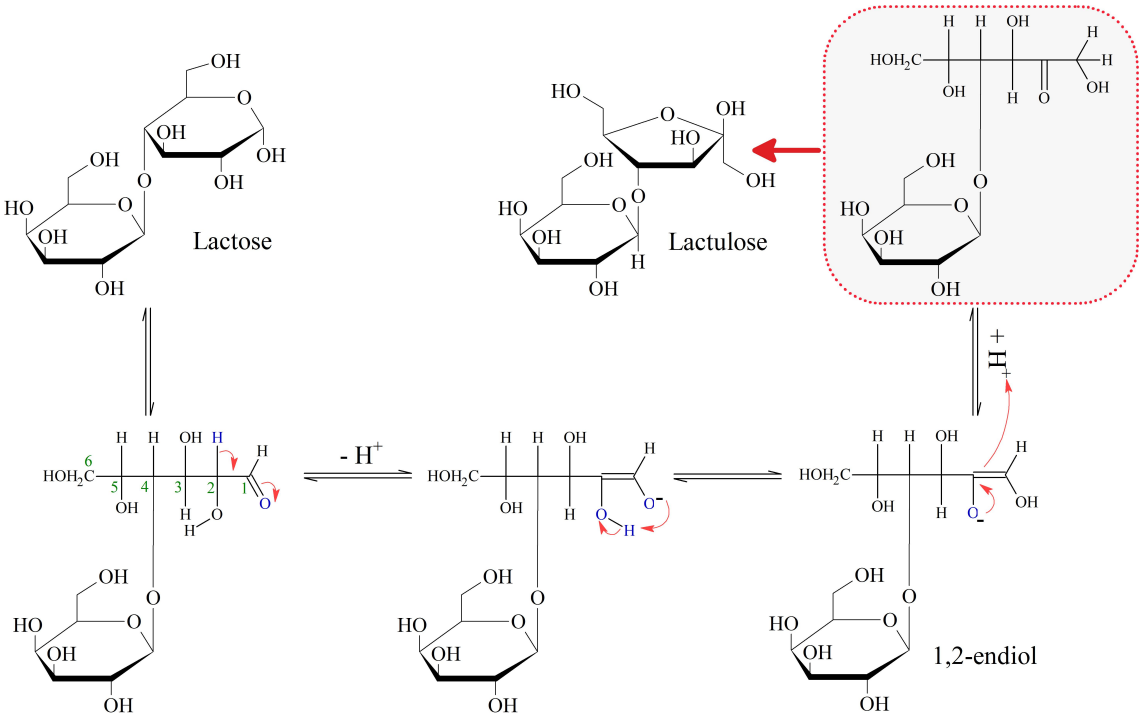

Figure 2.6 Lobry de Bruyn–Alberda van Ekenstein (LA) arrangement (Schuster-Wolff-Bühring et al., 2010).

2.3.1 Chemical isomerization based lactulose production

Lactulose can be produced either by chemical isomerization or enzymatic synthesis. The chemical isomerization can be done using acids (lime, sulphuric acid), bases (triethylamine, sodium hydroxide, magnesium oxide and sulfites) and amphoteric catalysts (hydroxyls, sulphites, and borates) (Aider and de Halleux, 2007). This isomerization proceeds through Lobry de Bruyn–Alberda van Ekenstein (LA)[3] arrangement where glucose moiety is isomerized into fructose (Schuster-Wolff-Bühring et al., 2010) (Figure 2.6). To simplify the arrangement of glucose into fructose in Figure 2.6, the glucose molecule is shown in Fisher projection. First of all, carbon number 2 (C2) from the glucose moiety is deprotonated giving C1=C2. As a consequence, the C1=O bond is degraded, leaving the O atom in a reactive state. Due to its negativity, the O atom attracts the adjacent H atom (-OH from C2). Subsequently, the C1 is stabilized, leaving the O atom at C2 in a reactive state. This intermediate is called 1,2-enediol. Through re-arrangement of C1=C2 into C1-C2, eventually fructose moiety is produced yielding a double bond between C2=O (Angyal, 2001).

Table 2.4 Recent (electro-)chemical isomerizations of lactulose production.

Catalysts	Temp. (^{o}C)	pH	Time (min)	Substrate (%)	Yield (%)	Reference
1. Catalyst isomerization						
Sodium hydroxide–boric acid	70	11	120	10	77-80	Zokaee et al., 2002
Boric acid	70	11	-	20	75	Kozempel and Kurantz, 1994
Sodium hydroxide	70	11	60	10	25.4	Hashemi and Ashtiani, 2010
Calcium carbonate	96	6.7	120	4	18-21	Paseephol et al., 2008
Sodium carbonate	90	-	20.41	10	29.6	Seo et al., 2015
Sodium carbonate–bicarbonate	60	10	3	10	18.75	Moreno et al., 2003
Alkaline–sepiolite	90	8	60	5	20	de la Fuente et al., 1999
Natural sepiolite	100	6.2-6.5	1	5	20.8	Troyano et al., 1996
2. Electrochemical isomerization						
Electro-catalytic synthesis: Froude number (Fr) = 2.05×10^{-2},λ = 4.64 mS/cm, i = 4.86 mA/cm^2 and U = 30 V	0-10	10.30	2	10	25	Aissa and Aider, 2013
Electro-activation reactor: current = 200 mA, 0.3 M Na_2SO_4, 0.05 M $CaCl_2$	10	11	30	30	30	Aissa and Aider, 2014

[3]The Lobry de Bruyn–Alberda van Ekenstein arrangement was discovered in 1885 by Cornelis Adriaan Lobry van Troostenburg de Bruyn and Willem Alberda van Ekenstein, chemists from The Netherlands.

The alkaline isomerization of lactose into lactulose using sodium hydroxide or tertiary amine coupled with a complexing agent (i.e., aluminate or borate) permits the yields of lactulose formations as high as 70-80 % (Hicks and Parrish, 1980). However, this can only be achieved when a large excess of sodium alumina or boric acid is supplemented to the reacting medium (Hicks and Parrish, 1980; Zokaee et al., 2002). Small amounts of galactose, epilactose, tagatose and formic acid are also formed in the reaction mixture (Schuster-Wolff-Bühring et al., 2010). As a matter of fact, aluminate and borate, however, are difficult to be removed from the reaction mixture, thus, giving a complexity in the separation and purification steps. In the Russian Patent No. 2101358 1994 (Method for lactulose syrup production), anion exchange resins were used to facilitate isomerization of lactose into lactulose by utilizing OH ion exchange between solution in reaction mixture and resins. One of the advantages of this process is no addition of catalysts which consequently simplifies the separation and purification processes (Aider and de Halleux, 2007; Panesar and Kumari, 2011; Schuster-Wolff-Bühring et al., 2010).

The electro-activation synthesis of lactulose based on the electrocatalytic method has been recently reported. This approach is considered to be an energy saving and reagentless technology by self-generating high alkalinity of the reaction medium (Aider and Gimenez-Vidal, 2012; Aissa and Aider, 2013). However, the yield of this synthesis is still lower compared to that of chemical isomerization with a complexing agent. Several (electro-)chemical isomerization-based lactulose productions can be seen in Table 2.4.

Table 2.5 Downstream process steps and their physical impacts on purification of lactulose resulted from lactose isomerization (Schuster-Wolff-Bühring et al., 2010).

Process step	Impact
Acidification (pH 4-8)	- Disintegration of lactulose-chelate complex - Precipitation of chelating agents - Precipitation of alkalizers as salts
Concentration, cooling $\mapsto$ separation	- Precipitation of chelating agents - crystallization of lactose
Chromatography	- Removal of salts, deionization - Removal of boric acid - Removal of lactulose
Extraction with ethanol and methanol	- Separation of lactose

To design a lactulose production process based on chemical isomerization must be done in a comprehensive way considering an isomerization and a post isomerization stage. In an isomerization stage, the selection of catalyst should be based on its turnover number, toxicity, cost and supply and easiness to be removed from the mixture (Aider and de Halleux, 2007). Improper selection of the catalyst may greatly influence the complexity in the post isomerization stage (separation and purification of lactulose). Due to a poorly specific reaction, generations of by-products are evident. Herein, besides the tedious procedure of removing catalyst (incl. complexing agent), additionally, further purification is still needed to remove the other monosaccharides (Aider and

de Halleux, 2007; Guerrero et al., 2011; Panesar and Kumari, 2011; Schuster-Wolff-Bühring et al., 2010). An overview of post isomerization steps in the processing of lactulose mixture is given on the Table 2.5.

2.3.2 Enzyme based lactulose synthesis

Considering the catalysts used for the chemical isomerization processes must have properties, such as: low cost, easy to remove from the medium, eco-friendly, safe and non-toxic, the enzymatic-based lactulose synthesis is an attractive alternative (Panesar and Kumari, 2011; Schuster-Wolff-Bühring et al., 2010). Lactulose is synthesized through transgalactosylation reaction in the presence of lactose (as galactosyl donor) and fructose (acceptor). In general, there are two suitable enzyme classes that are capable for lactulose synthesis, namely glycosyltransferases and glycosidases (Mayer et al., 2004). From the two classes, glycosidases are more relevant for the industrial applications (e.g., β-galactosidase) as they are commercially available, relatively inexpensive and already widely used in the food industry especially for lactose free milk products (Hua et al., 2010).

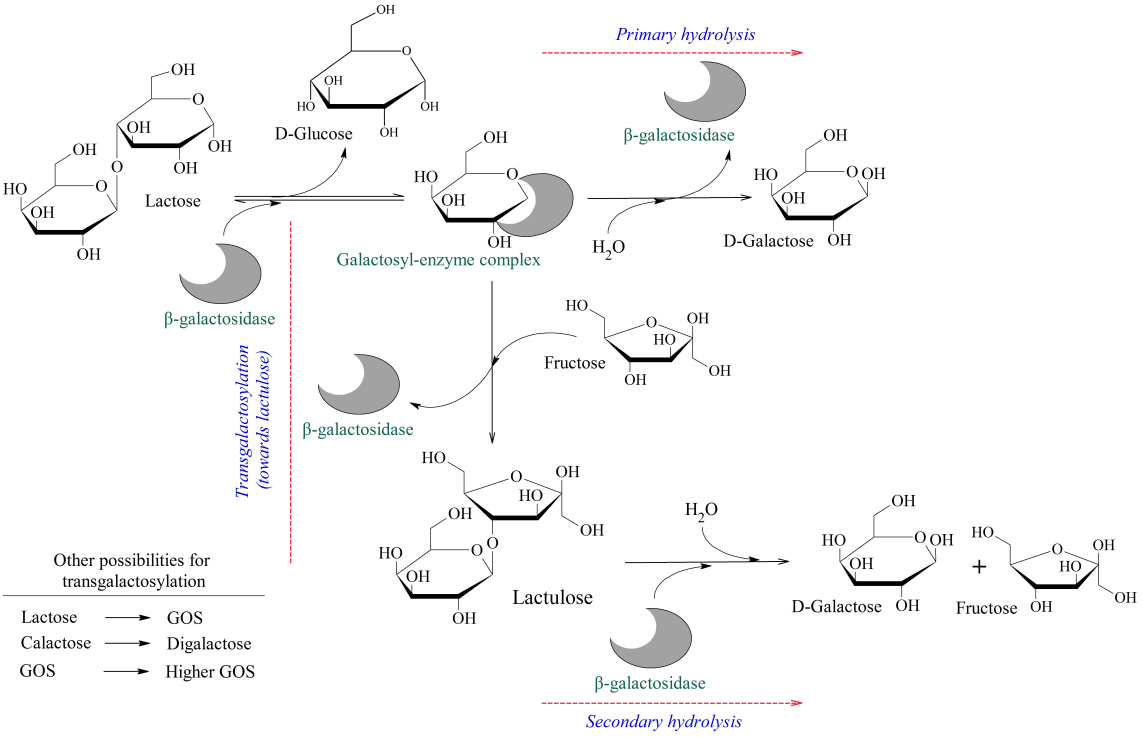

Figure 2.7 Possible reactions and products of lactose transgalactosylation in the presence of fructose catalyzed by β-galactosidase. (Mayer et al., 2004; Mayer et al., 2010; Shen et al., 2012; Sitanggang et al., 2015).

The complete lactose (primary) hydrolysis using β-galactosidase is achieved when water acts as nucleophile. The hydrolysis yields one molecule of galactose and glucose for each molecule of lactose. Transgalactosylation especially for galactooligosaccharide (GOS) production is a side reaction in lactose hydrolysis. Lactose can also compete with water to react with the available galac-

tosyl moieties in the mixture (Mahoney, 1998). The ability of the enzymes (e.g., β-galactosidase) to transfer galactosyl moieties to other hydroxil containing nucleophiles is then realized to produced lactulose (see Figure 2.7) (Lee et al., 2004; Sitanggang et al., 2014a, 2015). The synthesis of lactulose by the enzymes (e.g., β-galactosidase) from lactose and fructose is a serial reaction since the partial hydrolysis to provide the galactosyl-enzyme complex is prerequisite. In batch synthesis, it is worth-mentioning that lactulose at its highest concentration is prone to undergo a secondary hydrolysis by the action of the pertinent enzyme (e.g., β-galactosidase) (Mayer et al., 2004; van Rantwijk et al., 1999).

Table 2.6 Lactulose synthesis using glycosidases in the presence of lactose and fructose. a* = whey and fructose, a** = dual enzymatic system with a complementary enzyme glucose isomerase of 0.05 g/mL, C_s = substrate conc., C_{Lu} = lactulose conc., $[E]$ = enzyme conc., V_R = reactor volume, EMR = enzymatic membrane reactor, MC = microchannel, PBR = packed bed reactor.

Enzyme source	Operation	$[E]$	C_s (g/L)	C_{Lu} (g/L)	Yield (%)	Reference
S. fragilis	Batch	-	-	9	-	Marja and Kauppinen, 1978
L. acidophilus NRRL 4495	Batch, V_R = 2 mL	4 U/mL	600	25	6.25	Hasem et al., 2013
S. solfataricus	Batch	3 U/mL	600	50	12.5	Kim et al., 2006
P. furiosus	Batch	50 nkat/mL	304	16	47.06	Mayer et al., 2004
P. furiosus	Continuous (EMR), V_R = 10 mL, flow rate = 1 V_R/h	495 nkat/mL	304	14	41.2	Mayer et al., 2010
P. furiosus	Continuous (PBR), V_R = 20 mL, flow rate = 3.4 V_R/h	690 nkat/mL	304	15	44.12	Mayer et al., 2010
B. circulans	Batch, V_R = 100 mL	200 IU/g	500	1-2	0.6	Guerrero et al., 2011
K. lactis	Batch, V_R = 10 mL	-	600	20	5	Lee et al., 2004
K. lactis	Batch	0.1 g/mL	900	151	18.88	Hua et al., 2010[a**]
K. lactis	Batch, V_R = 100 mL	200 IU/g	500	5	1.53	Guerrero et al., 2011
K. lactis	Batch, V_R = 10 mL	3 U/mL	400	15.4	7.7	Shen et al., 2012
K. lactis	Continuous (MC), flow rate = 2.5 μ/min	-	400[a*]	1.42	0.71	Song et al., 2012

Enzyme source	Operation	$[E]$ conc.	C_s (g/L)	C_{Lu} (g/L)	Yield (%)	Reference
K. lactis	Batch	12 U/mL	600	15.8	3.95	Song et al., 2013a
K. lactis	Continuous (PBR), flow rate = 0.5 mL/min	12 U/mL	400^{a*}	19.1	9.55	Song et al., 2013b
K. lactis	Batch	0.06 mL	350	8	3.2	Hua et al., 2013
K. lactis	Batch	3 U/mL	480	10.4	3.47	Khatami et al., 2014
K. lactis	Batch, V_R = 90 mL	3.3 U/mL	500	16.8	6.85	Sitanggang et al., 2014a,b
K. lactis	Continuous (EMR), V_R = 90 mL	3.3 U/mL	500	14.5	5.95	Sitanggang et al., 2015
A. oryzae	Batch, V_R = 30 mL	50 nkat/mL	304	10	29.41	Mayer et al., 2004
A. oryzae	Batch, V_R = 100 mL	200 IU/g	500	20	6.10	Guerrero et al., 2011
A. oryzae	Continuous (EMR), V_R = 90 mL	3.0 U/mL	500	8.8	5.47	Sitanggang et al., 2016

The enzymatic synthesis of lactulose from lactose and fructose has been firstly reported in 1978 using β-galactosidase from *S. fragilis* (Marja and Kauppinen, 1978). Ever since, an increased number of following studies has been reported (Olano and Corzo, 2009). Table 2.6 shows an overview of studies reported for enzymatic synthesis of lactulose. Besides fructose, other nucleophiles such as: water, lactose and GOS are also present during lactulose preparation in aqueous system. Thus, the reaction selectivity can be influenced by the competition amongst these acceptors which is considerably influenced by the operating conditions (e.g., substrate composition, substrate and enzyme concentration, temperature and pH of the medium) (Ait-Aissa and Aider, 2014; Panesar and Kumari, 2011; Schuster-Wolff-Bühring et al., 2010).

Lee et al. (2004) reported the production of lactulose using β-galactosidase in permeabilized cells of *K. lactis*. Starting from a substrate concentration of 375 g/L, the amount of lactulose was enhanced 0.03 g per g substrate (lactose + fructose) added. Hua et al. (2010) increased the substrate concentration up to 1.3 g/mL to reduce the water activity a_w during the synthesis. The obtained yield was about 40 g/L using *K. lactis* β-galactosidase. Moreover, with 5 % of water was replaced by cyclohexane, the yield was enhanced by 2.5-fold. It must be noted that limited a_w can reduce the rate of primary hydrolysis. The reaction selectivity increases as water is hindered to act as nucleophile. Corresponding to this, specific molar ratio of lactose to fructose m_L/m_F in the bi-substrate is also necessary for optimum lactulose synthesis and the value is varied depending on the sources of

the enzymes (Panesar and Kumari, 2011). A lower m_L/m_F is generally favorable to obtain an increased lactulose concentration, as the probability of fructose to act as galactosyl acceptor is higher compared to water (Sitanggang et al., 2015). In a batch operation, Guerrero et al. (2011) indicated an optimum m_L/m_F = 1/8 for lactulose synthesis using *A. oryzae* β-galactosidase. At an m_L/m_F = 1/15, immobilized hyperthermostable β-glycosidase from *P. furiosus* (CelB) produced the maximum concentration of lactulose, up to 16 g/L (Mayer et al., 2010). Using β-galactosidase from *S. solfataricus*, Kim et al. (2006) reported that high concentrations of lactulose were obtained at m_L/m_F ratios in a range of 2/1 to 1/2. From a study conducted by Song et al. (2013b), at an m_L/m_F = 1/2, a higher lactulose concentration (i.e., 10.95 g/L) was afforded using immobilized *K. lactis* β-galactosidase. At a very low m_L/m_F can lead to fructose inhibition for *A. oryzae* β-galactosidase and CelB (Mayer et al., 2004). Moreover, with a higher m_L/m_F, lactose is pronounced to act as galactosyl acceptor. Thus, production of GOS is favorable in detriment of lactulose (Guerrero et al., 2011).

The enzyme concentration also markedly influences the reaction selectivity. Sitanggang et al. (2016) reported that at a higher enzyme concentration ($\geq$ 15 U/mL), both primary (hydrolysis of lactose) and secondary hydrolysis (hydrolysis of the resulted lactulose) were performed faster. Additionally, Sitanggang et al. (2016) also indicated that the ratio between enzyme concentration and initial amount of lactose has to be considered to improve the reaction selectivity. The use of a complementary enzyme to isomerize the produced glucose to fructose is also a prominent idea. Dual-enzymatic reaction (galactosidase + glucose isomerase) in organic-aqueous system (cyclohexane 5 %) has been reported by Hua et al. (2010) to increase lactulose concentration up to 151 g/L.

Table 2.7 Isomerization of lactose into lactulose using *C. saccharolyticus* recombinant cellobiose 2-epimerase.

Operation	Enzyme conc.	Substrate (g/L)	Lactulose (g/L)	Yield (%)	Comments	Reference
Batch, V_R = 2 mL	150 U/mL	700	408	58.30	*C. saccharolyticus* DSM 8903, *E. coli* ER2566 and plasmid pET-28a(+) were used as the sources of genomic DNA, host cells and expression vector.	Kim and Oh, 2012
Batch, V_R = 2 mL	150 U/mL	700	614	87.71	Addition of borate with borate to lactose molar ratio of 1:1.	Kim et al., 2013
Batch, V_R = 25 mL	39.5 $\mu kat_{epilactose}$	48.5	28.0	57.73	The strains *E.coli* XL-1 Blue and *E. coli* BL21 (DE3) were used for cloning and expression.	Rentschler et al., 2015
Batch	12.5 U/mL	600	390.59	65.1	Production was done using recombinant *E. coli* cells with addition of borate (120 g/L).	Wang et al., 2015

Since it is kinetically controlled, secondary hydrolysis proceeds after a highest lactulose concentration has been reached. In batch operations, this phenomenon has been well investigated. Lee et al. (2004) have previously reported that a longer reaction time was needed to reach maximum lactulose concentration for a higher initial lactose concentration. Moreover, at a prolonged reaction, lactulose concentration decreased gradually after peaking at optimum reaction time. The similar observation was also documented by Hua et al. (2010). After reaching a concentration of 151 g/L at 2 h, lactulose concentration reduced abruptly down to 50 g/L at 4 h. As a bottleneck of batch lactulose preparation, to avoid secondary hydrolysis, the reaction has to be stopped at the optimum reaction time. This may add to unproductive operation time and eventually an increased total production cost (Sitanggang et al., 2014a,b). Besides a number of repetitions for start and end-up activities, extra works have also to be done to recover the enzyme from the reaction mixture or for the immobilization. To concomitantly circumvent secondary hydrolysis and additional unproductive time, synthesis lactulose can be done continuously in enzymatic membrane reactor (EMR), microchannel (MC) and packed bed reactor (PBR) (Mayer et al., 2010; Sitanggang et al., 2014a,b, 2015, 2016; Song et al., 2012). Especially in EMR, through size exclusion, the enzyme molecules can be retained inside the reacting compartment by the membrane while having product, by-products and substrate permeation. Through continuous removal, the produced lactulose is less exposed to the pertinent enzyme and thus, escapes from further degradation (Sitanggang et al., 2014a). Nevertheless, the enzyme activity may reduce due to electrostatic interaction between enzyme and membrane surface, mechanical and thermal inactivation (Sitanggang et al., 2015). In EMR, Mayer et al. (2010) reported that lactulose concentration was nearly constant within 1 day operation using CelB. After 4 days, the concentration rapidly decreased to almost zero. With the same reactor design, lactulose concentration also reduced by 31 % within 7 days operation using *K. lactis* β-galactosidase (calculated based on the maximum lactulose concentration obtained) (Sitanggang et al., 2015). The loss of activity due to enzyme wash out was evitable since the membrane MWCO used was exceedingly smaller compared to the enzyme molecular weight (Sitanggang et al., 2015). As the inactivation closely relates to the end-productivity of the process, the "fresh" enzyme must be added to the reactor. To facilitate this, Sitanggang et al. (2016) developed an automated enzyme addition control within a dead-end EMR system. By adding enzyme in every 48 h, lactulose outlet concentration was kept constant at 8.8 g/L for 28 days using *A. oryzae* β-galactosidase.

Most of the studies showed that the maximum yields of enzymatic preparations of lactulose using non-recombinant enzymes were majority below 10 %[4] based on initial concentration of lactose (see Table 2.6). With recombinant enzymes (Kim et al., 2006; Mayer et al., 2004; Mayer et al., 2010) the yield was enhanced up to 47 %. Driven by this relatively low reaction yield and increased necessity of lactulose in the functional food markets, further investigations for more economical

[4]Study conducted by Mayer et al. (2004) to synthesize lactulose using *A. oryzae* β-galactosidase had an m_L/m_F of 1/15, giving initial lactose concentration was only 34 g/L with total substrate concentration (lactose + fructose) of 304 g/L. The yield of lactulose production was about 29.41 %. In addition, Hua et al. (2010) used dual-enzymatic system in organic-aqueous two-phase media that reported a yield of 19 %.

enzyme-based lactulose preparations were attempted. The enzyme cellobiose 2-epimerase has been reported having the ability to isomerize the glucose moiety in disaccharide cellobiose into mannose or fructose (Park et al., 2011). Taking this property into consideration, the glucose moiety in lactose might possibly be isomerized into fructose moiety in order to produce lactulose and a by-product epilactose (4-O-β-D-galactosyl-D-mannose). The first isomerization of lactose into lactulose using cellobiose 2-epimerase has been proven and reported by Kim and Oh (2012). The obtained lactulose concentration was 408 g/L after 2 h reaction with initial lactose concentration of 700 g/L. By following this strategy, other studies were conducted as well to produce lactulose using recombinant cellobiose 2-epimerase and these are shown in Table 2.7. To further increase the yield up to 88 %, borate was added as complexing agent into the reacting solution (molar ratio of borate to lactose was 1:1) (Kim et al., 2013). With this relatively new strategy, the conversion yields between enzyme- and chemical isomerization based lactulose synthesis are more or less in the same magnitude.

Chapter 3

MATERIALS AND METHODS

3.1 EMR system configuration

3.1.1 Designs and materials

In the EMR system, two parallel reactors were constructed to make it possible to obtain reproducible results in a short time. The schematic process flowsheets of the EMR system are shown as: (a) simplified EMR system, where the design was aimed to just facilitate continuous feeding of substrate and separation purposes (Figure 3.1) and (b) EMR system where the feature of the enzyme dosing was also embedded (Figure 3.2).

A single reactor system consisted of the following materials (refer to Figure 1.4 and 3.2):

1. Borosilicate glass container with P_{max} = 6 bar (Merck Millipore Darmstadt, Germany)
2. pH meter pH 3310 (WTW GmbH, Germany)
3. Pressure-stable pH electrode made of glass, P_{max} = 6 bar, T_{max} = 130 °C (ProSense BV, The Netherlands)
4. Kern EW 620-3NM precision balance (Kern & Sohn GmbH, Germany)
5. Proportional pressure regulator (PPR) MPPE-3-1/4-6-010-B with accuracy of ± 20 mbar (Festo AG & Co. KG, Germany)
6. Micro-solenoid valves: (i) normally closed (2/2 NC) and (ii) normally open (2/2 NO) were used for controlling the flow of enzyme and substrate, respectively and with response time < 10 ms, dead volume < 13 μL (ASCO Numatics GmbH, Germany)
7. Heating system (Haake D1, Germany)
8. 2 MAG MIX 1 stirrer (2mag AG, Germany)
9. UF membranes with A_{eff} = 12.38 x 10^{-4} m^2 . Four different UF membranes were used: (1) polyethersulfone PES, (2) polysulphone UFX10, (3) cellulose acetate RC70PP and

(4) fluoro polymer ETNA10PP (see Table 3.1). Prior to use, each membrane was washed with ultrapure water at a constant pressure of 0.5 bar for 1 h.

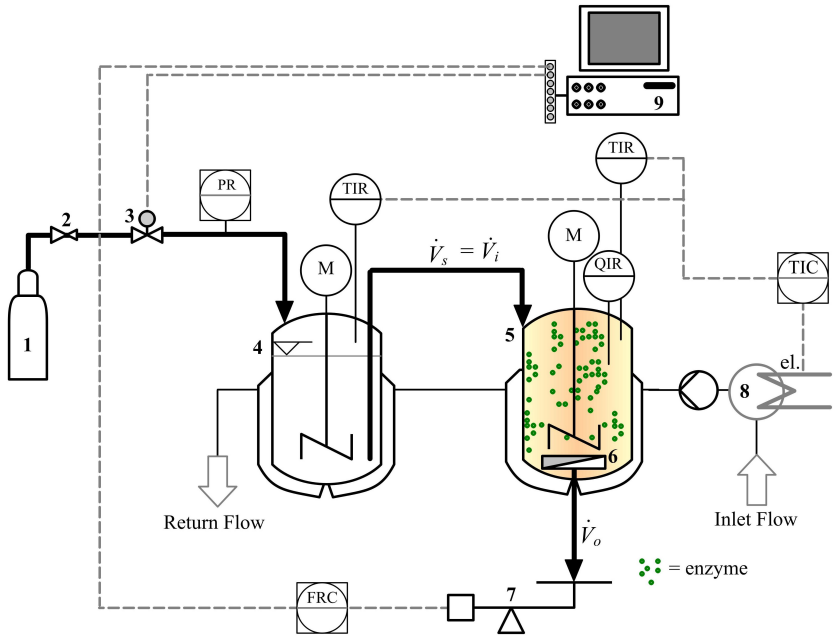

Figure 3.1 Simplified EMR system: (1) N_2 bottle, (2) pressure reducer, (3) PPR, (4) substrate tank, (5) reactor, (6) flat-sheet UF membrane, (7) precision balance, (8) heating system, (9) PC. Q = quality parameter, pH.

Table 3.1 Membrane types and properties used in this study.

No	Material	Notation	MWCO (kDa)	Stability	Company
1	Polyethersulfone	PES	5, 10, 20	pH = 0-14, T = 5-95 °C	Microdyn-Nadir GmbH, Germany
2	Polysulphone	UFX10	10	pH = 1-13, T = 0-75 °C	
3	Cellulose acetate	RC70PP	10	pH = 1-10, T = 0-60 °C	Alfa Laval Mid Europe GmbH, Germany
4	Fluoro polymer	ETNA10PP	10	pH = 1-11, T = 0-60 °C	

3.1.2 Control strategy

Besides registering and saving the information outputs for pH, temperature and transmembrane pressure, the EMR system could facilitate continuous operations where (i) the substrate and permeate flow were kept at the same level at various hydraulic residence times and (ii) the enzyme addition could be performed periodically. A constant permeate flux was maintained through a feed-back controller. Both data acquisition (DAq) and control design were supported by several

National Instruments (NI) modules, summarized in Table 3.2. A proportional-integral-derivative (PID) controller was developed by using Laboratory Virtual Instrument Engineering Workbench (LabVIEW^{TM}) software, NI, Germany. The tuning of PID parameters was done according to the close-loop method reported elsewhere (King, 2006; Kuhn, 1995).

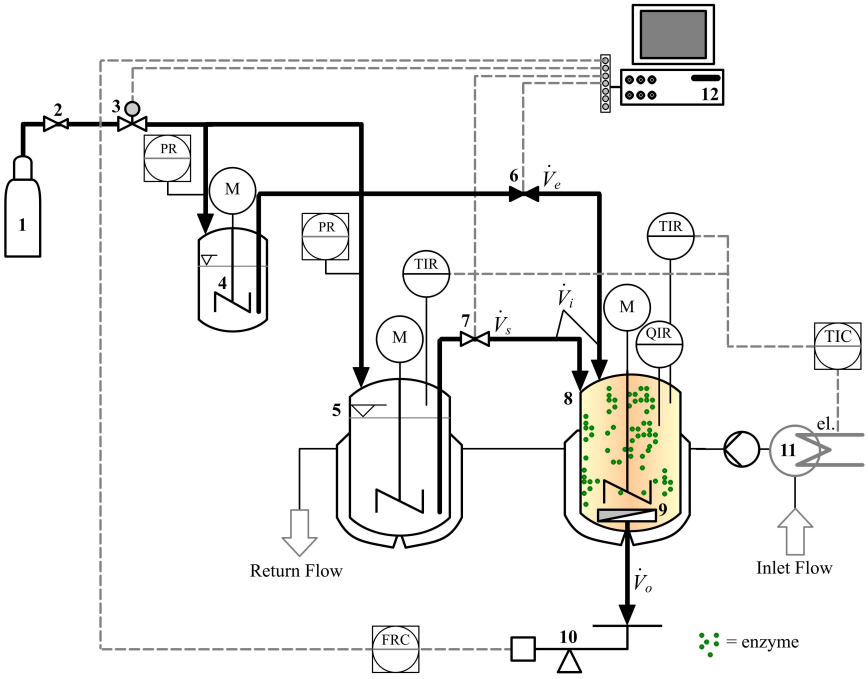

Figure 3.2 EMR system with embedded enzyme dosing: (1) N_2 bottle, (2) pressure reducer, (3) PPR, (4) enzyme tank, (5) substrate tank, (6) 2/2 NC valve, (7) 2/2 NO valve, (8) reactor, (9) flat-sheet UF membrane, (10) precision balance, (11) heating system, (12) PC. Q = quality parameter, pH.

Table 3.2 Modules and chassis used for data acquisition in EMR system.

No	Type	Comments
1	NI 9870, 4-port, RS232 serial interface	Data transmission from precision balances
2	NI 9264 16-channel +/- 10 V analogue output	Controlling PPRs
3	NI 9207 16-channel current/voltage analogue input	Controlling PPRs
4	NI 9476 32-channel 24 V sourcing digital output	Controlling 2/2 NO and 2/2 NC valves
5	cRIO-9076 Integrated 400 MHz Real-Time Controller and 2M Gate FPGA chassis	-

3.2 Synthesis of lactulose

3.2.1 Chemicals

The enzyme β-galactosidase (EC Number 232-864-1) from *Kluyveromyces lactis* and *Aspergillus oryzae* (M_{Kl} = 117 kDa and M_{Ao} = 107 kDa)[1], acetonitrile, 2-nitrophenyl β-D-galactopyranoside (ONPG), 2-nitrophenol (ONP), lactulose (M_{Lu} = 342.30 Da), D-fructose (M_F = 180.16 Da) and lactose (M_{Lo} = 342.30 Da) were purchased from Sigma-Aldrich, Germany.

3.2.2 Determination of enzyme activity

The activity of β-galactosidase from *K. lactis* was measured according to Hua et al. (2010) using ONPG as the substrate. The enzyme β-galactosidase generally hydrolyzes ONPG to yield ONP. The color of the ONP solution is yellow and can be monitored using a spectrophotometer at 420 nm (Specord® 210 Plus – Analytik Jena AG, Germany). One unit (1 U) of the enzyme activity is defined as the amount of enzyme required to liberate the equivalent 1 mol ONP/min at 30 °C and pH 6.8.

The activity of β-galactosidase from *A oryzae* was determined based on Sigma-Aldrich Enzymatic Assay. A 10 mM ONPG solution was prepared in 20 mM phosphate-citrate buffer (pH 4.5). After incubation at 30 (± 1) °C for 10 min, the reaction was stopped by adding 200 mM borate buffer (pH 9.8). The color of the ONP solution is yellow and can be monitored at 410 nm using the same spectrophotometer mentioned above. One unit (1 U) of the enzyme activity is defined as the amount of enzyme required for hydrolyzing 1 µmol ONPG to ONP and D-galactose per minute at pH 4.5 and 30 °C.

3.2.3 Study of lactulose synthesis

Lactulose (refer to Figure 2.7) was synthesized from lactose and fructose in the developed EMR system under batch (using *K. lactis* β-galactosidase) and continuous mode (using *K. lactis* and *A oryzae*, respectively). Under batch operation, a number of reactions was carried out without removing the enzyme molecules from the reactor. A 1.0 mL sample was taken from the permeate side and compensated by the same amount (i.e., 1.0 mL) of fresh substrate conveyed into the reactor at 0.5; 2: 3; 5; 8; 10; 12; 25 and 32 h. In order to enable each sampling procedure, the pressure (i.e., 0.5 bar) was applied in the substrate tank manually. Although an exchange between inlet and permeate took place in the system, the operation was still considered as a batch reaction as it had a very long residence time (i.e., at least 90 h). The effects of several parameters, such as total sugars concentration C_S (the sugars were dissolved in 30 mM phosphate buffer pH 6.8, buffer was prepared with ultrapure water with a resistivity of 18.3 M cm, produced by the purification system Merck Millipore Milli-Q Gradient), enzyme concentration [E] and molar ratio of lactose to fructose m_L/m_F on the lactulose production were investigated in batch operation using *K. lactis*

[1] calculated based on Subsection 3.2.4

β-galactosidase. In addition to this, a comparison between continuous and batch lactulose synthesis was also conducted (up to 35 h reaction). Thermal inactivation study of *K. lactis* and *A. oryzae* β-galactosidase were also studied under non-reactive conditions. The enzymes were diluted into the corresponding buffered solutions (*K. lactis* β-galactosidase in 30 mM phosphate buffer pH 6.8; *A. oryzae* β-galactosidase in 150 mM citrate-phosphate buffer pH 4.6) and incubated at various temperatures (30 to 60 °C) at designated times. Prior to a long-term continuous synthesis of lactulose using *A. oryzae* β-galactosidase, the influences of operating parameters on lactulose synthesis were optimized, such as: enzyme concentration [E], molar ratio of lactose to fructose m_L/m_F, hydraulic residence time τ and incubation temperature T. Reaction performances were assessed in terms of lactulose concentration C_{Lu} (directly from HPLC measurements (refer to Section 3.2.5), reaction yield Y (%), selectivity S ($\text{mol}_{Lu}/\text{mol}_{Cons.Lo}$) and specific productivity P_{spec} (g/U_E h).

$$Y = \frac{C_{Lu}}{C_{i,Lo}} \tag{3.1}$$

$$S = \frac{\left(\frac{C_{Lu} \times V_i}{M_{Lu}}\right)}{\left(\frac{\Delta C_{Lo} \times V_i}{M_{Lo}}\right)} \tag{3.2}$$

$$P_{spec} = \frac{C_{Lu} \times V_i}{E \times t_i} \tag{3.3}$$

where C_{Lu} = lactulose outlet concentration (g/L), $C_{i,Lo}$ = initial lactose concentration (g/L), ΔC_{Lo} = consumed lactose concentration (g/L), V_i= permeate volume collected until t_i (for batch mode V_i = 90 mL), t_i = time (h), E = enzyme amount (U) ($E = [E] \times V_R$),M_{Lu}, M_{Lo} = molecular mass of lactulose and lactose (Da), respectively .

3.2.4 Calculation of protein charge distribution and enzyme molecular weight

The codes for accessing amino (AA) acids sequences of eight β-galactosidase (EC 3.2.1.23) producing *A. oryzae* strains and one β-galactosidase producing *K. lactis* were obtained from http://www.brenda-enzymes.org (This data is curated by Dept. of Bioinformatics and Biochemistry, Technische Universität Braunschweig, Germany). Using the Basic Local Alignment Search Tool (BLAST) provided on http://www.uniprot.org (The Universal Protein Resource, UniProt), the complete AA query sequences of β-galactosidase from those strains were collected. The protein charge distribution (incl. enzyme molecular weight) of *A. oryzae* (mean value) and *K. lactis* β-galactosidase could be calculated based on those AAs at different pH values using software provided on http://protcalc.sourceforge.net (The Scripps Research Institute, USA). The calculation is based on the ionizations of amino acids (i.e., aspartic, glutamic acid, lysine, arginine and histidine), N and C termini at varied physiological pHs (pH 2.0 – 12.0). The same software has also been used elsewhere for calculating molecular weights of several proteins (Paul et al., 2015).

3.2.5 Determination of disaccharides and density

For disaccharide analysis, an HPLC was used, equipped with a Vertex Plus 250 x 4.6 mm Eurospher II 100-3 NH_2 column (Knauer GmbH, Germany), a WellChrom K-2300 RI detector and a K.1001 pump combined with an electric valve drive. The evaluations of the resulting chromatograms were done by the software Eurochrom 2000. The mobile phase was a mixture of acetonitrile and water (75 %: 25 %) with an isocratic gradient, pumped at a constant flow rate of 1.0 mL/min. Column temperature was set to 30 °C. To achieve a proper resolution, the samples were diluted with ultrapure water at a ratio of 1:4 prior to injection. Finally, 20 μL of the diluted sample was injected into the HPLC. The retention time was 30 min. From two reactors which were operated under the same reaction conditions, samples were taken at designated times and analyzed twice or thrice. Therefore, every single point plotted in the figures was a mean of at least 4 values (2 reactors and 2 duplicate injections) shown with its corresponding standard deviation.

For the density measurement, a Density and Sound Velocity Meter DSA 5000M (Anton Paar, Germany) was used.

3.2.6 Determination of adhesion force

The adhesion force F_{ad} (N) of (1) *K. lactis* β-galactosidase *vs.* PES membrane MWCO = 10 kDa (30 mM phosphate buffer pH 6.8, T = 40 °C) and (2) *A. oryzae* β-galactosidase *vs.* PES membrane MWCO = 10 kDa (150 mM phosphate-citrate buffer pH 4.6, T = 40 °C) were measured using atomic force microscopy (AFM) (Asylum Research MFP3D Classic, Santa Barbara, Canada).

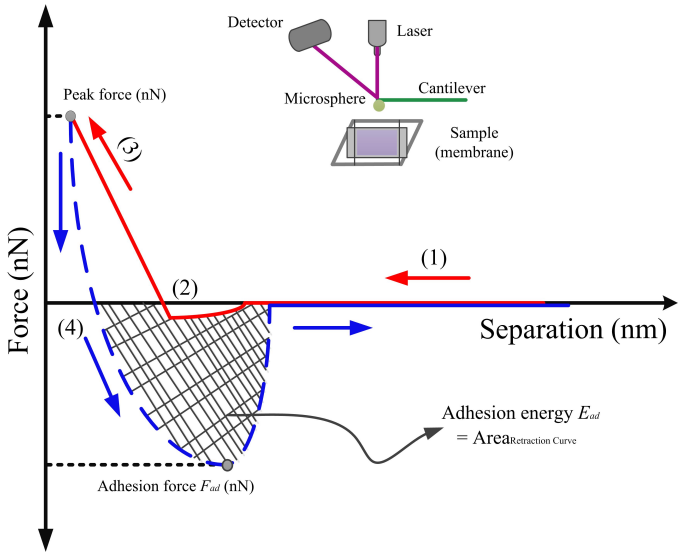

Figure 3.3 Typical experimental force–separation curve between a microsphere and a membrane surface. Numbers (1 to 4) mark the single steps as described in the text (Füllbrandt et al., 2015; Variola, 2015).

The collodidal probes for the AFM measurements were prepared by sintering silica gel spherical (radius = 3.35 μm, Bangs Laboratories, Inc., USA) onto HQ:CSC18/TIPLESS/CR-AU

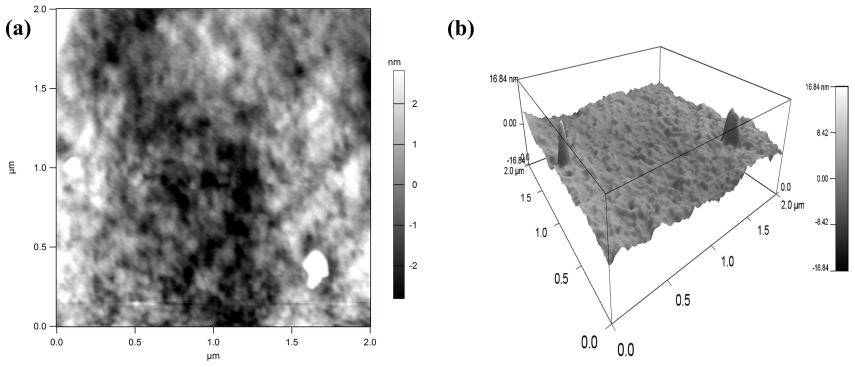

Figure 3.4 2D (a) and 3D (b) AFM images of virgin PES membrane MWCO = 10 kDa.

cantilevers (Cantilever B, Micromash). Hereby, a two-component epoxy adhesive (UHU plus endfest 300, UHU GmbH, Bühl, Germany) was used. The cantilevers were moved with a micromanipulator so that their far end was brought in contact with the glue. Afterwards, an individual silica microsphere could be picked up. The cantilevers were then left for at least 12 h at room temperature (so that the glue could harden) and then cleaned in an air plasma chamber for 20 minutes before use. The attached silica microspheres on the tip of cantilevers were divided into two groups, soaked in enzyme solution of *K. lactis* β-galactosidase ($[E]$ = 12 U/mL, 30 mM phosphate buffer pH 6.8) and *A. oryzae* β-galactosidase solution ($[E]$ = 36 U/mL, 150 mM phosphate-citrate buffer pH 4.6) at room temperature for 24 h. The excessive amounts of the enzyme concentrations were to ensure sufficient amounts of enzyme molecules adsorbed onto the silica microspheres. Prior to the measurement, the cantilever deflection (inverse optical lever sensitivity, InvOLS) was calibrated on a hard surface and the exact spring constant was determined by the thermal noise method (Wang et al., 2013b), which is a built-in procedure in the MFP-3D instrument. The reference spring constant of the cantilever is given as 0.03 N/m. A force map consisting of 100 force curves were recorded for each sample.

A typical force–distance curve for an AFM measurement is shown in Figure 3.3. The steps are basically (1) the microsphere approaches to the membrane surface, (2) the jump-to-contact point, where the microsphere is attracted toward the surface, followed by (3) sample indentation and cantilever deflection and (4) the retraction of the microsphere, which might be hindered by the adhesive force. The analysis of a single adhesion force was done based on Derjaguin–Muller–Toporov (DMT) model. The correlation between F_{ad} and adhesion work W_{ad} (J/m^2) is described through a simple analytical equation as follows (Butt et al., 2005):

$$F_{ad} = c\pi R W_{ad} \tag{3.4}$$

where R = radius of microsphere (μm), c is a constant (≈ 2 for DMT model) (Butt et al., 2005; Füllbrandt et al., 2015; Wang et al., 2013b). Additionally, from the surface topography analysis, the measured roughness (RMS) of PES membrane MWCO = 10 kDa was 1.580 nm with the scanned size of 2.0 x 2.0 μm (see Figure 3.4).

Chapter 4

Results and Discussion–Automated EMR System: Constant Flux Operation and Protocol of Enzyme Dosing

Within this Chapter 4, the main discussion is focused on the establishment of the automated EMR system. A robust feed-back controller (e.g., PID controller) was developed by combining a DAq system (see Table 3.2) and a developed LabVIEW^{TM} program that serves as hardware communications, data logging as well as interfacing. By the application of the PID controller, the EMR system could be operated at various constant fluxes and the enzyme addition could also be performed periodically (Sitanggang et al., 2014a, 2016).

4.1 Strategy for constant flux operation

The operation of a membrane reactor at a constant transmembrane pressure (TMP) ΔP is simpler. Therefore, it is often carried out at lab-scale (Prazeres and Cabral, 1994). Generally, the flux reduces when fouling or cake build up occurs. As a result, the hydraulic residence time τ (time-space) which is one of the key operating parameters in continuous operation gets longer. Especially for EMRs, for reactions that are prone to product inhibition (Murphy et al., 2013), a prolonged residence time consequently leads to a reduced performance of the enzyme (Koeller and Wong, 2001).

Instead, operation at constant flux J (and thus constant τ) is more preferred. To have a constant permeation although fouling occurrs, an increase of transmembrane pressure is required to compensate the elevating filtration resistance. Within Section 4.1, the description of the feed back controller establishment using LabVIEW^{TM} is discussed. The basic operation of PPR was firstly

realized and followed by the on-line permeate flux monitoring. Eventually, by gathering the basic operation of PPR and the on-line flux measurement, a feed-back controller was developed to have a constant flux operation. It is worth mentioning, besides developing a feed back controller (PID controller), the on-line pH and temperature monitoring were also done (see Appendix A.2 for the data collection from pH meter to have pH and temperature value).

The type of EMR used in this study was dead-end EMR with a UF membrane placed at the bottom. Thus, to have a permeation the pressurization must be done in the reactor. As seen in Figure 3.1, one part of the reactor system that could be controlled electrically is the opening of PPR valve (no. 3). Through variations in the opening, different pressures might be introduced respectively into substrate reservoir and reactor (no. 4 and 5). Due to the pressurization in the reactor, a pressure gradient was created between two sides of the membrane (i.e., (i) reactor side and (ii) container that was placed on the balance). The permeation of solvent and solutes could be achieved depending on the value of transmembrane pressure and the membrane properties (i.e., MWCO, hydrophobicity, roughness, etc.). According to the technical information from the company, the PPR valve has an accuracy of ± 20 mbar (Festo AG Co. KG, Germany). In addition, as shown in Figure 4.1, the linearity (R^2) of the PPR was 0.9992. At a low set pressure given, the actual pressure tended to deviate. Cited as an example, at a set pressure of 100 mbar, the actual pressure obtained was 92.7 mbar, thus, giving an error of 7.3 %.

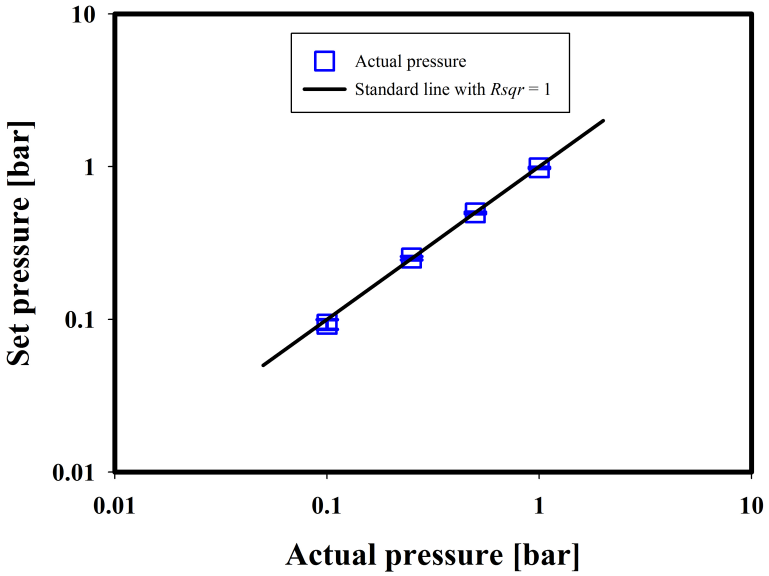

Figure 4.1 Linearity of proportional pressure regulator MPPE-3-1/4-6-010-B.

The calculation of permeate flux[1] was based on permeate weight W_p measured by means of a precision balance. This weight digital information collected by the balance was transferred automat-

[1]Permeate flux refers to the individual flux that is calculated per loop of the data transmission. Filtered flux means a flux value that is an average of certain number n of individual permeate fluxes. Later on, this filtered flux refers to flux of process variable J_{PV}.

ically to the computer through RS232 connection by using a developed program in LabVIEW^{TM}. The loop of the data transmission was set to 1 s (refer to the Appendix A, Figure A.1 to A.6 for the complete developed programs). To have a stable controller output, permeate fluxes were smoothed by filtering the noise due to random fluctuations during the filtration. A moving average was proposed to filter the data, as following:

$$J_{PV} = \frac{1}{n} \sum_{a=i}^{i+n-1} x_a \tag{4.1}$$

where J = output (filtered) data or flux, a = initial data point, i = sequence of data point, x = input data, n = number of individual fluxes being averaged.

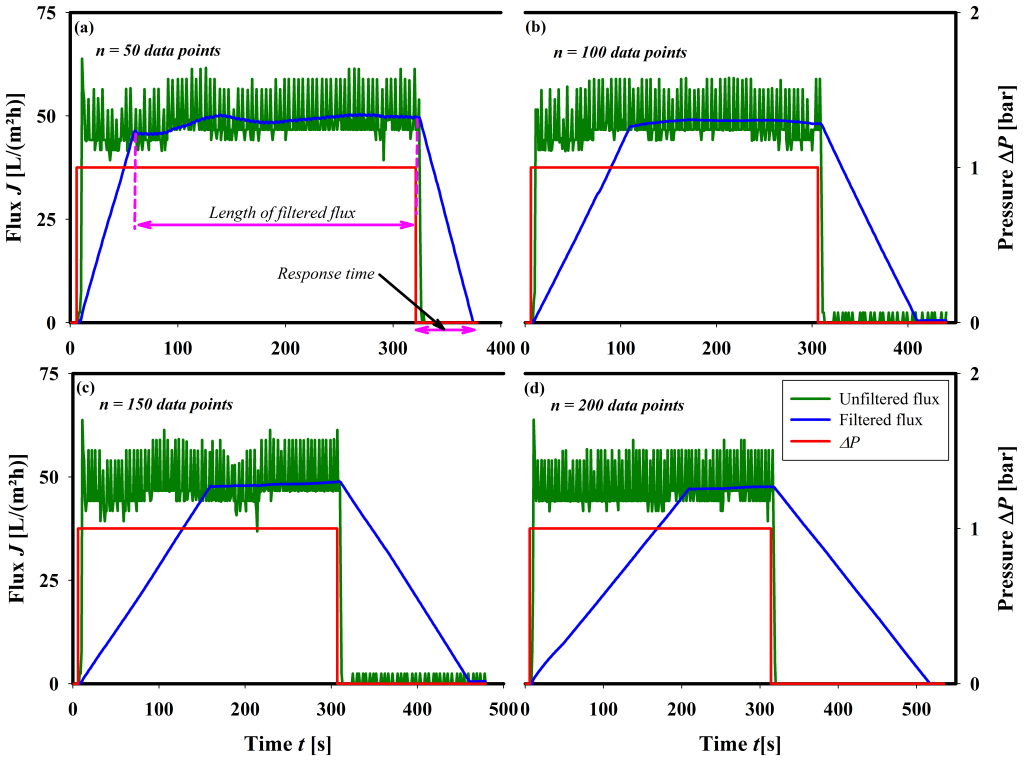

Figure 4.2 The influence of number of data points being averaged n on the filtered flux.

The word moving simply means the replacement of the oldest data point (i.e., flux value) by the newest one. Therefore, the filtered flux data set is constantly moving through out the filtration time. As seen in Figure 4.2, as the number of data points being averaged increased, at the same time interval, the length of the filtered flux reduced. It is the nature of moving average method, that the filtered data is less responsive with a higher value of n. When the inlet pressure was stopped (ΔP = 0 bar), relatively steep declines of the filtered fluxes were observed at n = 50 and 100 data points. In contrast, at n = 150 and 200 data points, the values of the filtered fluxes reduced slower compared to those at n = 50 and 100 data points. Validating the value of n is crucial because it indicates how stable the system is when a feed back controller is utilized (see also Figure 4.4).

As the basic operation of the PPR and the calculation of permeate flux had been established, a PID controller for controlling the opening of PPR valve was possible to be realized. There are two common methods used to interpret the behavior of a controller, physical modeling and experimental identification (Reuter and Zacher, 2004). Physical modeling usually gives better results but it is also time consuming and requires a precise mathematical description of the system (Reuter and Zacher, 2004). In comparison, although the experimental identification is less-accurate, it is relatively simple and less tedious. In this study, the latter method was selected.

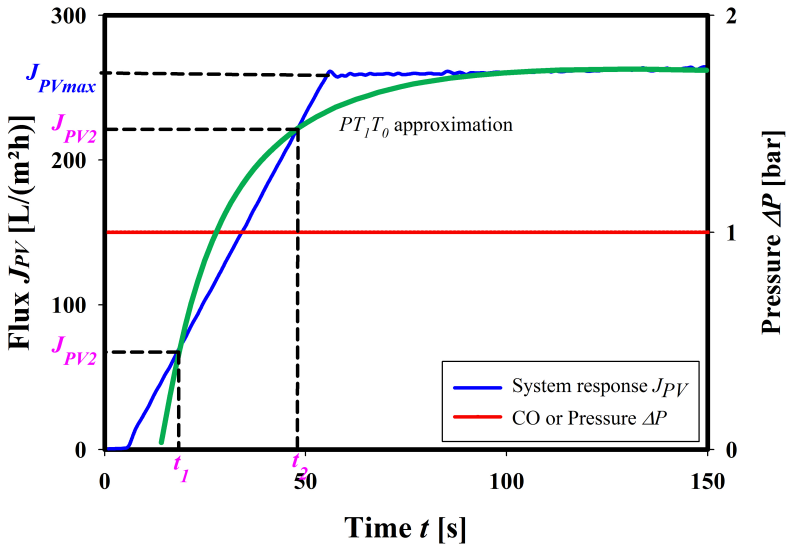

Figure 4.3 Experimental response signal due to a step change in CO and its PT_1T_0 approximation: Filtration of ultra pure water, PES membrane MWCO = MWCO 10 kDa, n = 100 data points, agitation n = 350 rpm, T = 40°C.

Figure 4.3 shows the experimental identification of the relationship between system response or flux of process variable J_{PV} and a change in controller output CO or pressure ΔP during ultrapure water filtration. This filtration showed a non-periodic response that follows a PT_n behavior (Schwarze, 1962). Similar investigations have been reported by Lyagin et al. (2010, 2015). Whenever this system response is approximated using PT_1T_0 model, the approximation line generally intersects the system response at 10 and 90 % of J_{PVmax}. Herein, according to Figure 4.3, these intersections were at t_1 = 10.257 s , J_{PV1} = 26.572 L/(m^{2}h) and t_2 = 51.379 s , J_{PV2} = 239.148 L/(m^{2}h). To tune a feed-back controller, several parameters have to be known, such as: controller gain K and the total time constant T_Σ which is the summation of time constant T_1 and process dead time T_0 (eqs. (4.2), (4.3) and (4.4) ((King, 2006; Kuhn, 1995))).

$$K = \frac{J_{PVmax}}{\Delta P} \tag{4.2}$$

$$T_1 = \frac{t_2 - t_1}{ln(\frac{J_{PVmax} - J_1}{J_{PVmax} - J_2})} \tag{4.3}$$

$$T_0 = T_1 ln(1 - \frac{J_i}{J_{PVmax}}) + t_i \rightarrow i = 1,2 \tag{4.4}$$

At CO or ΔP of 1.0 bar, the calculated K was 265.72 L/(m^{2}h bar)[2] and T_1 and T_0 were 18.72 and 8.28 s, respectively. Murrill et al. (2005) defined T_0 as the time period following an upset during which the response variable is not yet responding. Moreover, T_0 is empirically determined at a point where the response signal reaches 2 % of its maximum value. Following this argument, with maximum response signal J_{PVmax} of 265.72 L/(m^{2}h) and its corresponding 2 % upset J_{PV} of 5.31 L/(m^{2}h), the resulting T_0 was 6.43 s. This value was not too far from the previous calculation according to eq. (4.4).

In order to tune a PID controller, the PID parameters, such as controller gain K_p, integral time-based property T_I and derivative time-based property T_D were calculated according to Kuhn (1995) and shown in Table 4.1 (in German: `T-Summen-Regel`). Briefly, through the application of a tuned PID controller, a constant flux operation was amenable. Hereby, the difference between set point flux J_{SP} and process variable flux J_{PV} during the filtration was minimized (see also Figure 4.6).

Table 4.1 Tuning PID parameters according to Kuhn (1995).

	Control type	Control parameter		
		K_p	T_I	T_D
Normal setting	P	1/K	-	-
	PI	0.5K	$0.5T_\Sigma$	-
	PID	1/K	$0.66T_\Sigma$	$0.167T_\Sigma$
Fast setting	PI	1/K	$0.7T_\Sigma$	-
	PID	2/K	$0.8T_\Sigma$	$0.194T_\Sigma$

In Figure 4.4a, two types of PID controller (for normal and fast setting) were used to have a constant flux during the filtration of ultrapure water at J_{SP} = 25 L/(m^{2}h). The PID controller with fast setting was found to have a smaller over–shoot response (J_{PV}) (Sitanggang et al., 2014a,b). To have a more stable and accurate response, taking the PID controller for fast setting, the influence of n was revisited. As can be seen in Figure 4.4b and also corresponding to Figure 4.2, it was confirmed that a higher value of n led to a poorly reactive response. Within this study, n = 50 data points was selected to filter the flux data points. Furthermore, this setting was tested in the filtration of lactose and fructose with a total concentration of 500 g/L and $m_L/m_F = \frac{1}{2}$ in a series of flux stepping operations (see Figure 4.5). When J_{SP} shifted, the CO changed accordingly to bring J_{PV} as close as possible to J_{SP}. The CO or ΔP was not jerky and expectedly, the response J_{PV} was almost stable with a control error e being less than 3.5 %[3] (Sitanggang et al., 2014a). This

[2]This value was basically the permeability ℓ_p of the PES membrane with MWCO 10 kDa during pure water filtration.

[3]Calculation of the error was done as: $e = \frac{|J_{PV} - J_{SP}|}{J_{SP}} \times 100\%$

result was comparable to the study of tuning PI/D parameters in a closed-loop method reported by Skogestad (2003) and Shamsuzzoha and Skogestad (2010). Considering the robustness of the control design that was developed within present study and by underlying the capacity of hardwares used, a constant flux operation of parallel reactors with a maximum number of 12 reactors was possible.

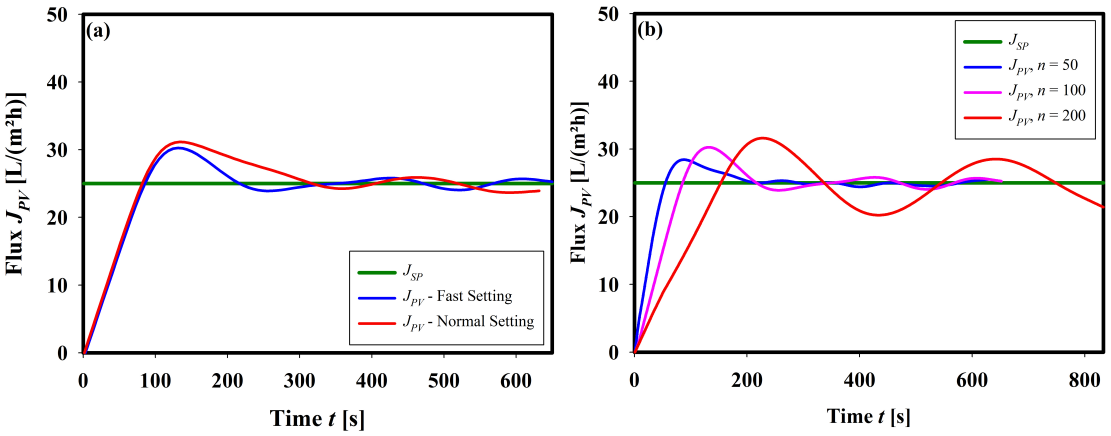

Figure 4.4 (a) Testing PID controllers–normal setting *vs.* fast setting during ultrapure water filtration with n = 100 data points and (b) The influence of data points being averaged n on the stability of J_{PV} with PID controllers for fast setting and J_{SP} = 25 L/(m^{2}h), PES membrane MWCO = 5 kDa, n = 350 rpm and T = 40°C.

4.2 Enzyme dosing procedure

During the filtration, flux J is calculated from the outflow ($\dot{V}$) and the effective membrane area A_{eff}.

$$J = \frac{\dot{V}}{A_{eff}} \tag{4.5}$$

As shown in Figure 3.2, the inflow $\dot{V}_i$ consists of substrate $\dot{V}_s$ and enzyme volumetric flow rate $\dot{V}_e$. At a constant flux operation, in order to avoid additional volume (maintaining V_R constant), $\dot{V}_s$ is stopped when enzyme is added. To do this, the operations of the valves (2/2 NC and 2/2 NO) which are basically an on-off control type, must be embedded within the operation loop of the PID controller (see Figure 4.6). Additionally, one has to be aware that the response time–time needed for both valves to become completely open or closed (when they are energized) has to be lower than the loop time of the PID controller. This is important to ensure the CO or pressure is not jerky. The response time of the used valves is less than 10 ms (based on supplier's information). In this study, the loop time of the PID controller was set to 1 s (see Section 4.1). The overall equation to trigger the opening of enzyme and the closing of the substrate valve during the enzyme addition at a constant flux operation could be summarized as follows:

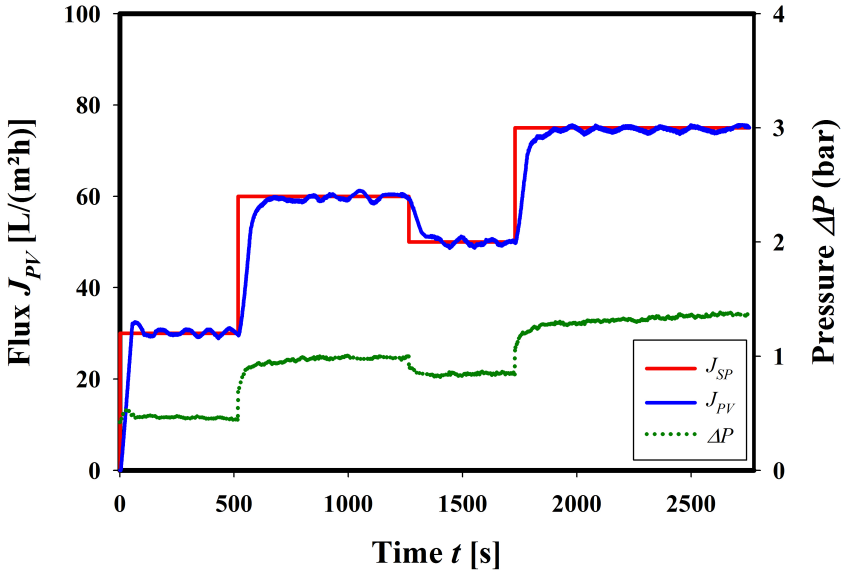

Figure 4.5 J_{PV} with corresponding CO or ΔP as controlled by the PID controller for fast setting with n = 50 data points in a series of J_{SP} operations (30, 60, 50 and 75 L/(m^{2}h)). Solution used was a mixture of lactose and fructose at a level of 500 g/L with $m_L/m_F = \frac{1}{2}$, pH = 6.8, PES membrane MWCO 10 kDa, n = 350 rpm, T = 40°C.

$$\dot{V}_i = \sum \left[(1-\varepsilon)\dot{V}_s + \varepsilon \dot{V}_e\right] = \dot{V}_o \tag{4.6}$$

As the enzyme addition was time-based (periodically), the constant ε was manipulated based on the elapsed reaction time t_R, dosing time t_{Do} and dosing duration t_{Du} (refer to Figure 4.6). The constant ε might take only two values: 0 or 1 (see eq. (4.7)). In every addition of fresh enzyme into the reactor, ε experienced a cycle of $0 \rightarrow 1 \rightarrow 0$. This was then called one dosing event n.

$$\begin{aligned} &\varepsilon = 0 \rightarrow t_R < t_{Do} \\ &\quad\downarrow \\ &= 1 \rightarrow t_{Do} \leq t_R \leq (t_{Do} + t_{Du}) \\ &\quad\downarrow \\ &= 0 \rightarrow t_R > (t_{Do} + t_{Du}) \end{aligned} \tag{4.7}$$

The first dosing accuracy was evaluated using water to replace the enzyme solution and substrate. The investigation was done at 40 °C. Set dosing volume V_{sD} was varied in a range of 0.5 to 10 mL. The water density ρ_w was measured to be 991.507 kg/m^3 at 40 °C. The enzyme tank was placed on the balance, so the reduced weight of the tank should be due to some amount of water (actual dosing volume V_{aD}) pumped into the reactor. Since the protocol of the enzyme dosing was embedded within

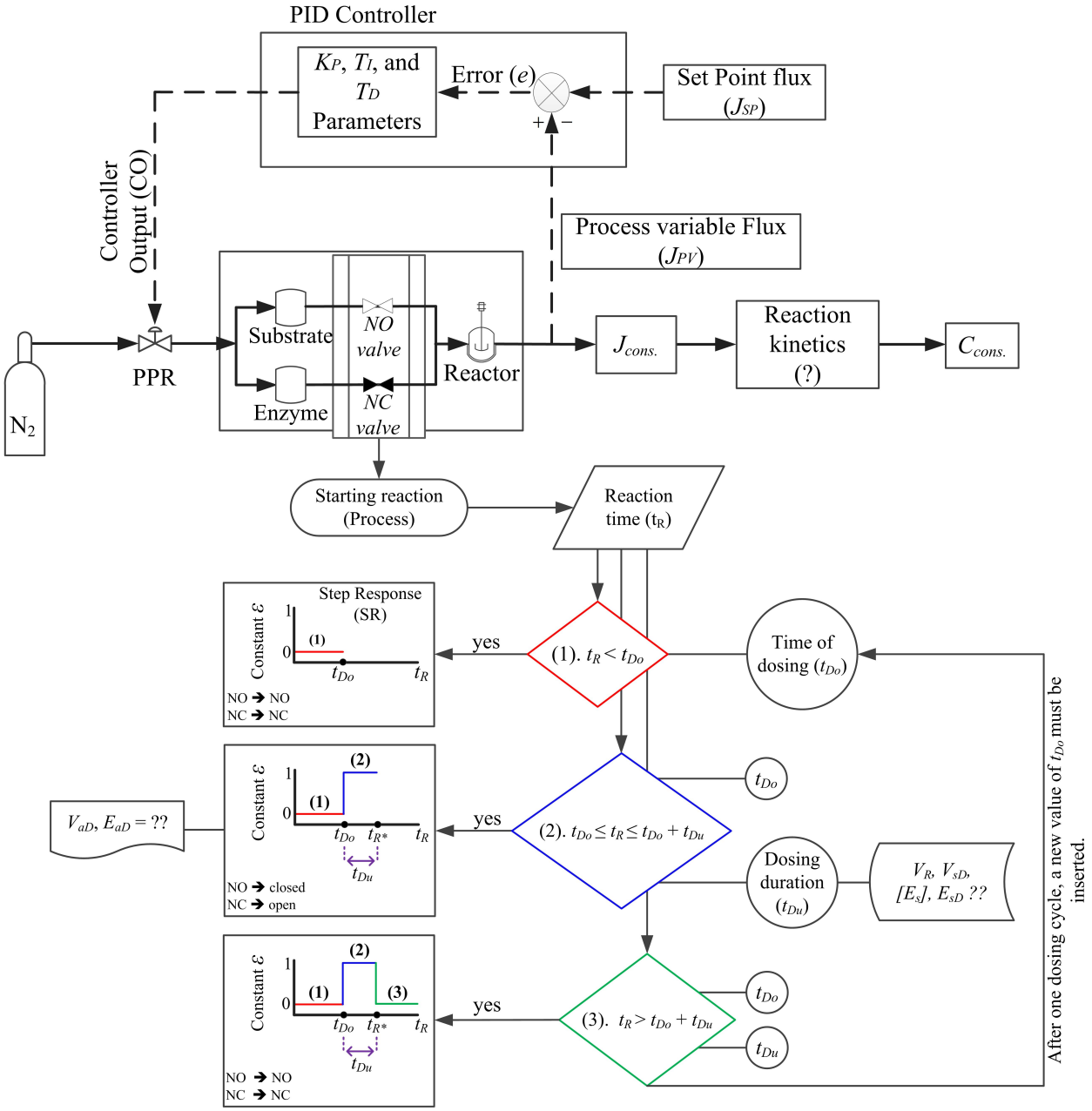

Figure 4.6 Realization of parallel control designs of constant flux and the iteration of enzyme addition using the PID controller.

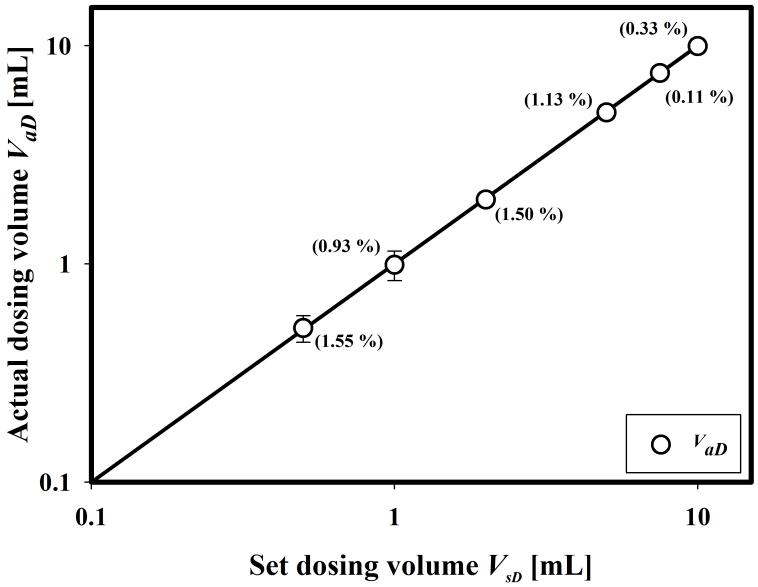

Figure 4.7 Evaluation of dosing accuracy in the developed EMR with average errors in parentheses. Each point was an average of 10 measurements.

the operation of PID controller which had an error of less than 3.5 %, as expected, the inaccuracy of the developed dosing system was also corresponding to this value (Figure 4.7) (Sitanggang et al., 2016). Through a control design developed in this present study, the EMR system can be operated similarly as reported by Lyagin et al. (2010, 2015) in terms of constant flux operation and the possibility of the enzyme dosing periodically (Table 4.2).

Table 4.2 Comparison of developed control or process execution between the present study and Lyagin et al. (2010, 2015).

EMR type	Controller	Error of flux control (%)	Error of enzyme dosing (%)	Max. number of parallel reactors	Complexity in parallelization	Reference
Dead-end EMR	PI/D	< 3	< 3	2	-	Lyagin et al., 2010, 2015
	PID	< 3.5	< 2	12	no complexity	This study

Chapter 5

Results and Discussion–Synthesis of Lactulose in the EMR System

Chapter 5 deals with the investigation of the engineering aspects of continuous lactulose synthesis using the developed EMR system. The benefits of continuous operation (with the developed reactor system) are discussed in comparison to batch one (Sitanggang et al., 2014a,b). The optimized operating conditions for continuous lactulose synthesis using *K. lactis* (Sitanggang et al., 2015) and *A. oryzae* β-galactosidase (Sitanggang et al., 2016) are also presented. Thereby, the developed robust feed-back controller in the EMR system was applied to facilitate a constant flux (thus hydraulic residence time) during the operations. At the end of Chapter 5, a discussion on a long-term continuous synthesis of lactulose is also included. Besides the facilitation of constant flux operation, the previously developed control was also used to dose the enzyme into the reactor periodically to have a maintained enzyme activity.

5.1 Synthesis of lactulose: Batch *vs*. continuous operation

In Sitanggang et al. (2014a,b), the optimum operating conditions for batch lactulose production using *K. lactis* β-galactosidase were reported. In batch operation, although a synthesis at a higher substrate (lactose + fructose) concentration constantly obtained a higher lactulose concentration, the reaction yield was not significantly increased. By increasing a substrate concentration C_S from 300 to 500 g/L ($m_L/m_F = \frac{1}{1}$), the reaction yield only increased from 0.03 to 0.034 $g_{Lactulose}/g_{Initiallactose}$ (Sitanggang et al., 2014a). For the enzyme concentration $[E]$, the optimum value was found to be 3.33 U/mL. At a higher $[E]$ (i.e. 5.56 U/mL), hydrolysis was preferred over synthesis. After a maximum concentration of 13.77 g/L was reached at 3 h with $[E]$ = 5.56 U/mL, lactulose concentration reduced by more than 50 % within 12 h (Sitanggang et al., 2014a). Furthermore, the optimum value of molar ratio of lactose to fructose m_L/m_F was $\frac{1}{2}$. It must be noted that higher

amounts of lactose can increase the probabilities of lactose to act as galactosyl acceptor (Guerrero et al., 2011). Galactooligosaccharide (GOS) production is thus, more preferred over lactulose synthesis. Higher amounts of fructose is expected to increase the probabilities of fructose to act as nucleophile. However, fructose has been reported to inhibit the catalytic activity of β-galactosidase during lactulose synthesis (Mayer et al., 2004). Briefly, with the optimum operating conditions, such as: C_S = 500 of g/L, $[E]$ = 3.33 U/mL and $m_L/m_F = \frac{1}{2}$, a maximum lactulose concentration of 16.70 g/L was obtained at 5 h reaction during batch synthesis (see Figure 5.1a).

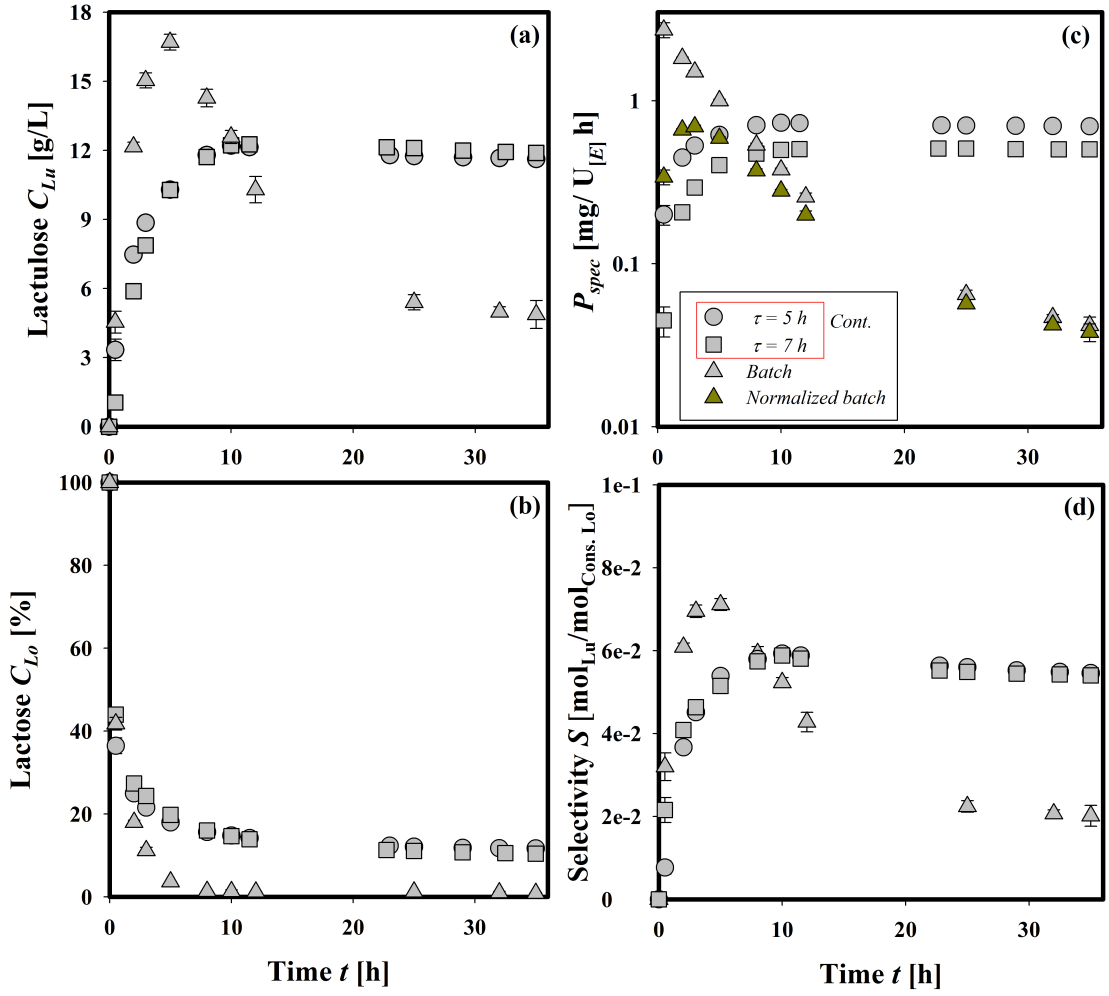

Figure 5.1 The profiles of lactulose concentration C_{Lu} (a), lactose concentration C_{Lo} (b), specific productivity P_{spec} (c) and reaction selectivity S (d) during batch and continuous lactulose production using *K. lactis* β-galactosidase, C_S = 500 g/L, $[E]$ = 3.33 U/mL, $m_L/m_F = \frac{1}{2}$, phosphate buffer pH 6.8, n = 200 rpm, T = 40 °C.

Although the maximum lactulose concentration in batch was higher compared to continuous operation (i.e., τ = 7 h), this value reduced down to 4.87 g/L within 35 h. This was due to secondary hydrolysis that was performed by β-galactosidase (van Rantwijk et al., 1999). For continuous

operations, the highest lactulose concentrations were obtained at 10 and 12 h, respectively for $\tau = 5$ and 7 h. The values were almost similar, approximately 12 g/L with the lactulose concentration at $\tau = 7$ h was slightly higher (2 %). The concentrations of lactulose for these continuous operations were almost constant up to 35 h. It is clear that the secondary hydrolysis which is one of the drawbacks of batch lactulose synthesis could be circumvented by performing a continuous operation with the developed EMR system. In addition, the primary hydrolysis also took place rapidly in the batch operation, leading to a 2 % of the remaining lactose concentration C_{Lo}. This value was lower compared to the continuous operations (11.70 and 10.60 %, respectively for $\tau = 5$ and 7 h) (Figure 5.1b).

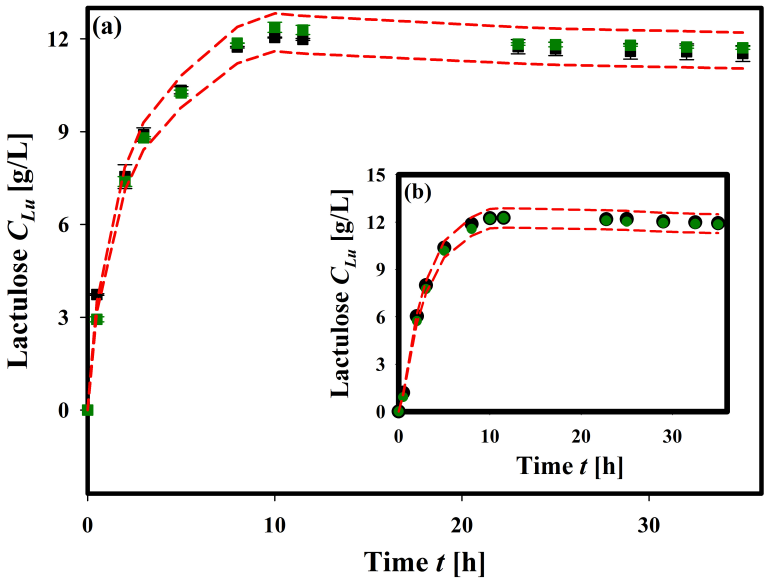

Figure 5.2 Performance of the EMR system–similarity of parallel reactors (a) $\tau = 5$ and (b) $\tau = 7$ h. The dashed red lines represent $\pm$ 5 % of average C_{Lu}, green and black symbols represent data points from reactor 1 and 2, respectively.

In general, with a shorter residence time, a higher volume of permeate is collected over the time. Although the lactulose concentrations were almost similar, the specific productivity P_{spec} at $\tau = 5$ h was therefore higher than that at 7 h (0.70 and 0.50 mg/($U_{[E]}$h), respectively) (Figure 5.1b). P_{spec} in batch process at 0.5 h was four-fold higher than the maximum P_{spec} in continuous operation (i.e., at $\tau = 5$ h). This might lead to a conclusion that batch was preferable over continuous operation. The unproductive time for start-up and end activities (preparation, filling, heating, cleaning, etc.) in batch operation was approx. 3.5 h. Whenever this value was considered in every batch cycle, the maximum P_{spec} was nearly similar to continuous operation, $\sim 0.70 mg/(U_{[E]}\text{h})$ (see the curve of normalized batch in Figure 5.1c). Moreover, if the overall specific productivity between batch (i.e., each batch was stopped every 5 h with C_{Lu} = 16.70 g/L) and continuous synthesis (i.e., $\tau = 7$ h, C_{Lu} = 12 g/L) were evaluated for 35 h reaction, the overall specific productivity of continuous

operation was more than five-fold higher compared to the batch one. Within seven cycles of batch operation, the total unproductive time was about 24.5 h. Additionally, since the enzyme could not be recovered in each cycle (i.e., freely suspended system), the total amount of enzyme needed was about 2100 U. In contrast, the unproductive time for continuous operation was only 3.5 h with the enzyme total amount of 300 U. Besides the circumvention of secondary hydrolysis, the continuous operation in the developed EMR system was also proven to mitigate the unproductive time as well as the excessive enzyme amount needed like in batch lactulose synthesis (refer to the last paragraph of Section 1.2.2). In addition to this, the reaction selectivity values of continuous operations were also higher compared to batch operation (Figure 5.1d).

The continuous synthesis of lactulose was done in two parallel reactors. The dissimilarity of both reactors was also evaluated based on the difference in lactulose concentrations (see Figure 5.2). For both residence times (τ = 5 and 7 h), the dissimilarity of the reactors was less than 5 %. As this dissimilarity indicates the robustness of the developed feed-back controller for ensuring a constant flux operation, the number of parallel reactors in the EMR system can be prospectively increased to the maximum control capacity (n = 12 reactors, see Section 4.1).

5.2 Engineering aspects of continuous synthesis of lactulose in EMR system

The optimum operating conditions for continuous synthesis of lactulose in EMR system using *K. lactis* β-galactosidase have been reported in Sitanggang et al. (2015). These conditions were C_S = 500 g/L, $[E]$ = 3.33 U/mL, $m_L/m_F = \frac{1}{2}$, τ = 9 h, phosphate buffer pH 6.8, n = 350 rpm, T = 40 °C and PES membrane MWCO = 10 kDa. Within Section 5.2, the discussion of the engineering aspects of lactulose continuous production using *A.oryzae* β-galactosidase in EMR system is selected. This is due to the fact that the results of the investigations (i.e., the engineering aspects of lactulose continuous synthesis using *A.oryzae* β-galactosidase) were more comprehensive. The investigations covered three main issues which are usually encountered in membrane-assisted enzymatic reactions, such as: membrane–enzyme electrostatic interaction, optimum operating conditions (with industrially relevant parameters) and maintenance of the enzyme activity in a long-term operation (Sitanggang et al., 2016).

5.2.1 Membrane–enzyme interaction and membrane selection

Interactions between membrane and enzyme (protein) at different pHs

The interactions between proteins and (synthetic) membranes in certain acidity and ionic conditions have been studied for decades and used for separations and purifications of proteins (Zydney, 2009). By the differences in molecular size, solubility, pH and ionic strength of medium and membrane charge, the degree of membrane based protein separations are varied (Ghosh and Cui, 2000; Levitsky et al., 2014). Especially by the alterations of pH and ionic strength, due to the

protein folding and the electrostatic interactions between proteins and the membrane surfaces, the repulsion or adsorption of the protein molecules onto the membrane surfaces can be favored. As mentioned previously, besides for the separation of the product(s), the function of the membrane is also to retain the enzyme molecules inside the size exclusion EMR (Prazeres and Cabral, 1994). However, the optimum pH of the enzyme (when it is buffered) is often not suited for an electrostatic (charge-charge) interaction towards the repulsion (Boye et al., 2010; Das et al., 2009; Wang and Tang, 2011). The adsorption and eventually the deposition of the enzyme molecules onto the membrane may occur during the operation. In order to maintain the usage of the membrane (i.e., reducing membrane cleaning activities), the selection of a suited membrane (with appropriate physical characteristics, such as surface charge, hydrophobicity, roughness, etc.) that can minimize a charge-charge interaction towards the adsorption at the enzyme optimum pH is needed.

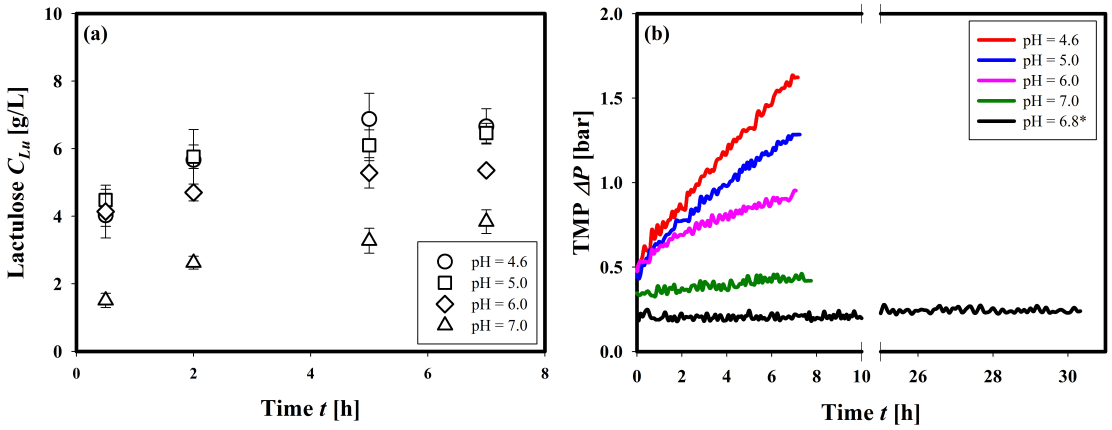

Figure 5.3 (a) The effect of pH on continuous synthesis of lactulose and (b) transmembrane pressure during continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, $m_L/m_F = \frac{1}{8}$, $[E]$ = 3 U/mL, τ = 7 h, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C, PES membrane MWCO = 10 kDa. In Sitanggang et al. (2015) at pH = 6.8*, the other operating conditions for continuous synthesis of lactulose using *K. lactis* β-galactosidase were C_S = 500 g/L, $m_L/m_F = \frac{1}{2}$, $[E]$ = 3.33 U/mL, τ = 7 h, n = 350 rpm, T = 40 °C, PES membrane MWCO = 10 kDa.

To confirm that *A. oryzae* β-galactosidase works optimally in acidic conditions (Guerrero et al., 2011; Mayer et al., 2004), the synthesis of lactulose was performed at different pHs. As can be seen in Figure 5.3a, the maximum C_{Lu} was obtained when the pH of the medium was 4.6 or 5.0. This pH range (4.5-5.0) is in agreement with the published literatures where *A. oryzae* β-galactosidase was used for lactulose and galactooligosaccharides (GOS) production (Guerrero et al., 2011; Huerta et al., 2011; Mayer et al., 2004). Within 7 h operation, the transmembrane pressures (TMPs) ΔPs at pH 4.6 and 5.0 increased rapidly which is generally viewed adverse for the membrane life-span or frequent cleanings are necessary (see Figure 5.3b). Interestingly, as the pH brought to neutral, the increase of the TMP was not significant.

The *A. oryzae* β-galactosidase has a molecular mass between 100 and 110 kDa (calculation based on AA sequences as described in Section 3.2.4) which is distinctively larger than the MWCO of the used PES membrane (i.e., 10 kDa). An increased TMP during the membrane operation can be due to convective forces that facilitate the deposition of the enzyme molecules onto the membrane surface (Luo et al., 2013, 2014a). In case of the rapid increases of the TMPs at pH 4.6 and 5.0, besides convective forces and possible protein-protein interactions (Filipe and Ghosh, 2005), another plausible cause was the electrostatic interaction between the enzyme and the membrane surface. The influences of the charge-charge interaction for the increase of the TMP at pH 4.6 and 5.0 were presumably predominant compared to the applied convective forces (i.e., inlet pressure) as at other pHs also experienced the same flux condition.

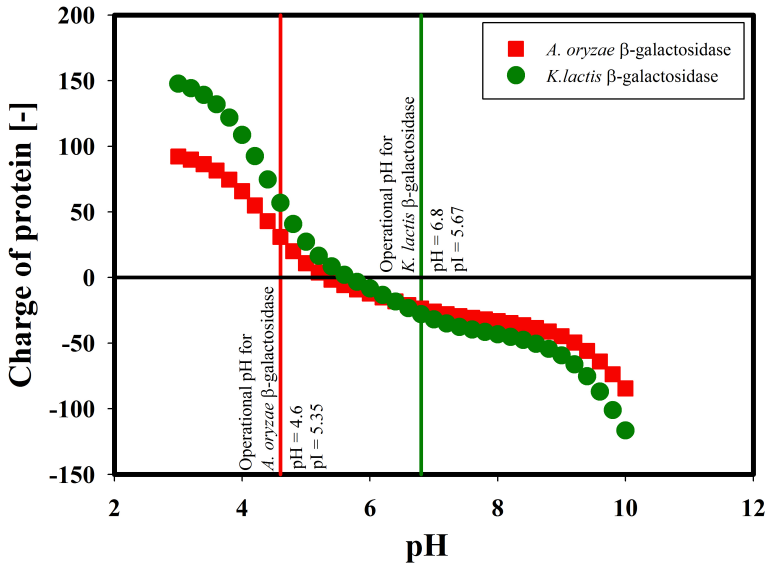

Figure 5.4 The protein charge distributions between *A. oryzae* and *K. lactis* β-galactosidase (The accession codes for calculating protein charge distribution of *A. oryzae* β-galactosidase were Q2UCU3, Q2U6P1, Q2UMD5, Q2U7N3, I8U3W6, I8U2V1, W5ZSH9 and B7VU80, whereas for *K. lactis* β-galactosidase was P00723. The accession codes were taken from http://www.brenda-enzymes.org).

Comparing the TMPs between pH 4.6 and a continuous lactulose synthesis using *K. lactis* β-galactosidase at pH 6.8 (Figure 5.3b), the TMP increase was insignificant at pH 6.8. The PES membrane (Microdyn-Nadir GmbH, Germany) was reported to be a negatively charged membrane (de la Torre et al., 2009; Kaya et al., 2006; Suárez et al., 2012). This property consequently influences the types of the electrostatic interactions between the membrane and the enzyme at different pH values. In Figure 5.4, the protein charge distributions between *A. oryzae* and *K. lactis* β-galactosidase are presented. The *A. oryzae* and *K. lactis* β-galactosidase isoelectric point (pI) were 5.35 and 5.67, respectively. The charge of the protein is generally negative when the pH of the solution is higher than the pI and vice versa. For the synthesis of lactulose using *K. lactis*

β-galactosidase at pH 6.8, the charge of the protein was about -28. Thus, a negatively charged PES membrane led to the repulsion of the enzyme molecules. As a consequence, the TMP increase was nearly insignificant for 32 h (Sitanggang et al., 2015). In contrast, for the synthesis at pH 4.6, the protein charge of *A. oryzae* β-galactosidase was 30.85. This could lead to an electrostatic interaction towards the adsorption of the enzyme molecules onto the membrane surface (Sitanggang et al., 2016). On the other hand, at pH 7.0, the charge of *A. oryzae* β-galactosidase was almost similar to *K. lactis* β-galactosidase. Therefore, the similar electrostatic interaction towards the repulsion was also expected. It is common that a protein which has an opposite charge to the membrane surface has the strongest adsorption (Beier et al., 2007).

To support the explanation above, the adhesion force F_{ad} of the PES membrane *vs. K. lactis* β-galactosidase and the PES membrane *vs. A. oryzae* β-galactosidase were measured by means of AFM (see Section 3.2.6). Generally speaking, the adhesion force is a comprehensive combination of electrostatic interactions, hydrogen bonding, van der Waals interactions and other forces caused by acid-base interactions (Butt et al., 2005; Wang et al., 2013b). A higher F_{ad} between two materials associates with a higher magnitude of an attraction (Butt et al., 2005). In Figure 5.5, the average F_{ad} of the PES membrane *vs. A. oryzae* β-galactosidase was five-fold higher than the PES membrane *vs. K. lactis* β-galactosidase (i.e., 2.53 and 0.47 nN, respectively). Therefore, the electrostatic interaction towards the adsorption of the *A. oryzae* β-galactosidase enzyme molecules onto the PES membrane surface was more pronounced.

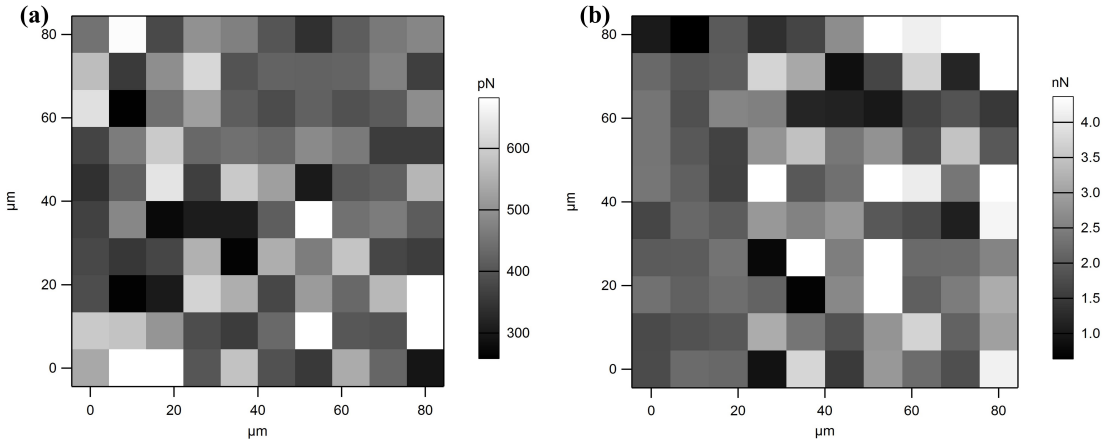

Figure 5.5 The adhesion force F_{ad} map of (a) the PES membrane (MWCO = 10 kDa) *vs. K. lactis* β-galactosidase (30 mM phosphate buffer pH 6.8, T = 40 °C) and (b) the PES membrane (MWCO = 10 kDa) *vs. A. oryzae* β-galactosidase (150 mM phosphate-citrate buffer pH 4.6, T = 40 °C) with a map size of 80 x 80 μm.

Selection of suited membrane type for lactulose synthesis

Since the 10 kDa PES membrane was not suited for continuous synthesis of lactulose using *A. oryzae* β-galactosidase at pH 4.6, other membrane types were screened. The related physical properties of the other membranes are shown in Figure 5.6 (Sitanggang et al., 2016). The highest

water flux was obtained with the UFX10 membrane. This membrane is made from polysulfone which is permanently hydrophilic (supplier's information). As seen also in Figure 5.6, the contact angle cannot be considered as the only factor for explaining the pure water flux. According to Nabe et al. (1997), surface roughness is also an important membrane property to determine pure water flux whereby a smoother membrane normally possesses a higher flux. From literature, the measured roughness of those membranes can be written as: poly-(ether)sulphone < regenerated cellulose < fluoro polymer (Evans et al., 2008; Macedo et al., 2015; Weis et al., 2005). Herein, the surface roughness of each membrane material was in accordance with its pure water permeability.

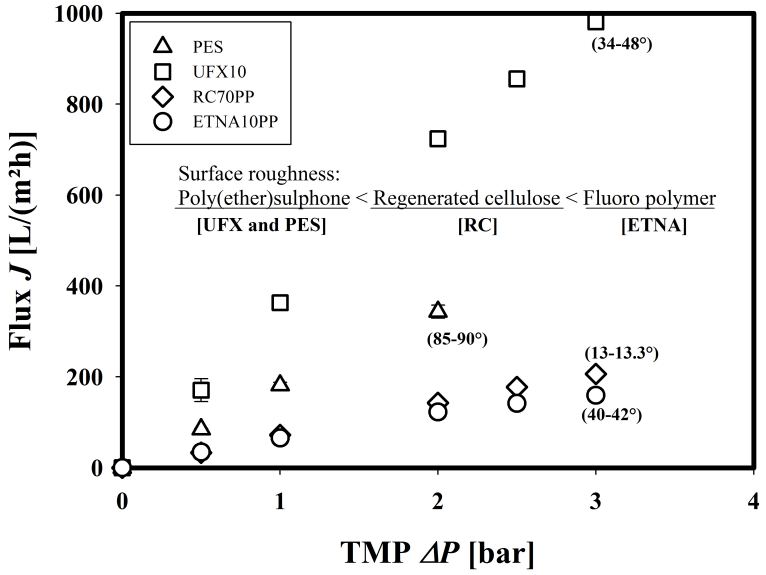

Figure 5.6 The water fluxes of different membrane types. Numbers inside parentheses refer to contact angles from literatures (Beier et al., 2007; Cho et al., 2000; Koivula et al., 2011; Liu et al., 2011a; Nabe et al., 1997; Ulbricht and Belfort, 1996).

These membranes (i.e., PES, UFX10, RC70PP and ETNA10PP with the same MWCO = 10 kDa and also PES with MWCO = 20 kDa) were used for continuous synthesis of lactulose (Figure 5.7a). With the 10 and 20 kDa PES membrane, the lactulose concentrations were similar. This indicates the enzyme wash-out did not occur with a higher cut-off value. For the UFX10 and RC70PP membrane, the lactulose concentrations were not significantly different but the values were lower compared to either 10 or 20 kDa PES membrane.

According to Portugal et al. (2006, 2008), during ultrafiltration, the structural changes of an enzyme which are linked to its catalytic activity, are controlled by the electrostatic protein-membrane interactions. Both UFX10 and RC70PP membrane repelled the enzyme molecules as indicated by the insignificant increases of the TMPs (Figure 5.7b). This repulsion might lead to the folding or unfolding of the protein structures that eventually reduced the enzyme activity, whereas for the PES membrane, an enzyme layer (due to adsorption) could avoid the exposure of the remaining enzyme molecules to the membrane surface. This might mitigate the further enzyme

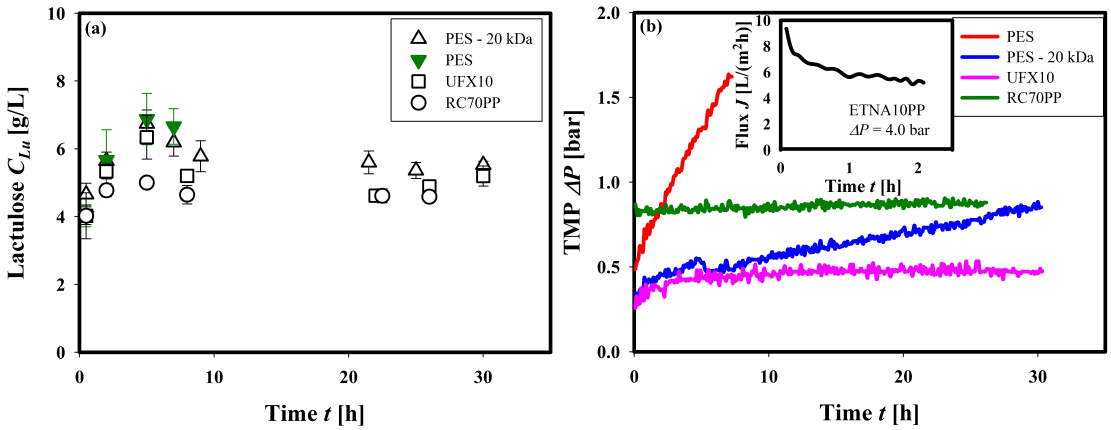

Figure 5.7 The influence of membrane types on (a) lactulose concentration and (b) transmembrane pressure during continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, $m_L/m_F = \frac{1}{8}$, $[E]$ = 3 U/mL, τ = 7 h, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C. Unless mentioned otherwise, the membrane MWCO = 10kDa.

inactivation. This built enzyme layer could concomitantly describe the higher lactulose productions and the rapid increases of the TMPs with the PES membrane for both cut-offs (see Figure 5.7b). In case of the ETNA10PP membrane, a constant flux operation at τ = 7 h could not be performed as the TMP drastically increased close to 5 bar (P_{max} of the reactor = 6 bar). The operation under constant pressure of 4 bar afforded an initial flux of 10 L/(m^{2}h) or τ = 9 h. This flux steeply reduced down to 50 % within 2 h. Due to the lowest initial TMP and the relatively insignificant increase of the TMP with the UFX10 membrane, this membrane type was used for further investigations.

5.2.2 Influences of operating conditions on continuous synthesis of lactulose

Effect of enzyme concentration

During continuous synthesis of lactulose from lactose and fructose, the enzyme hydrolyzes lactose and forms a complex with galactosyl moiety. Thus, the nucleophile (i.e., fructose) will receive this galactosyl moiety (Shen et al., 2012). A lower enzyme concentration $[E]$ is generally insufficient to obtain a higher lactulose concentration C_{Lu}. This is due to a lower amount of galactosyl moieties available within the reacting solution (Sitanggang et al., 2014a). As shown in Figure 5.8a and c, the lowest C_{Lu} and reaction yield Y were obtained at the lowest $[E]$. Nevertheless, the reaction selectivity S was the highest under this condition (Figure 5.8d).

The maximum C_{Lu} was obtained at $[E]$ = 10.0 U/mL. At the highest $[E]$, i.e., 15.0 U/mL, C_{Lu} was not further enhanced. It is worth mentioning that primary hydrolysis (hydrolysis of lactose) was proceeded faster at this condition, as indicated by the highest lactose consumption ΔC_{Lo} (Figure 5.8b). In batch production, lactulose concentration has been enhanced by the increase of

the enzyme concentration. However, the synthesis reached a highest performance (i.e., lactulose concentration) at an optimum enzyme concentration (Kim and Oh, 2012; Lee et al., 2004).

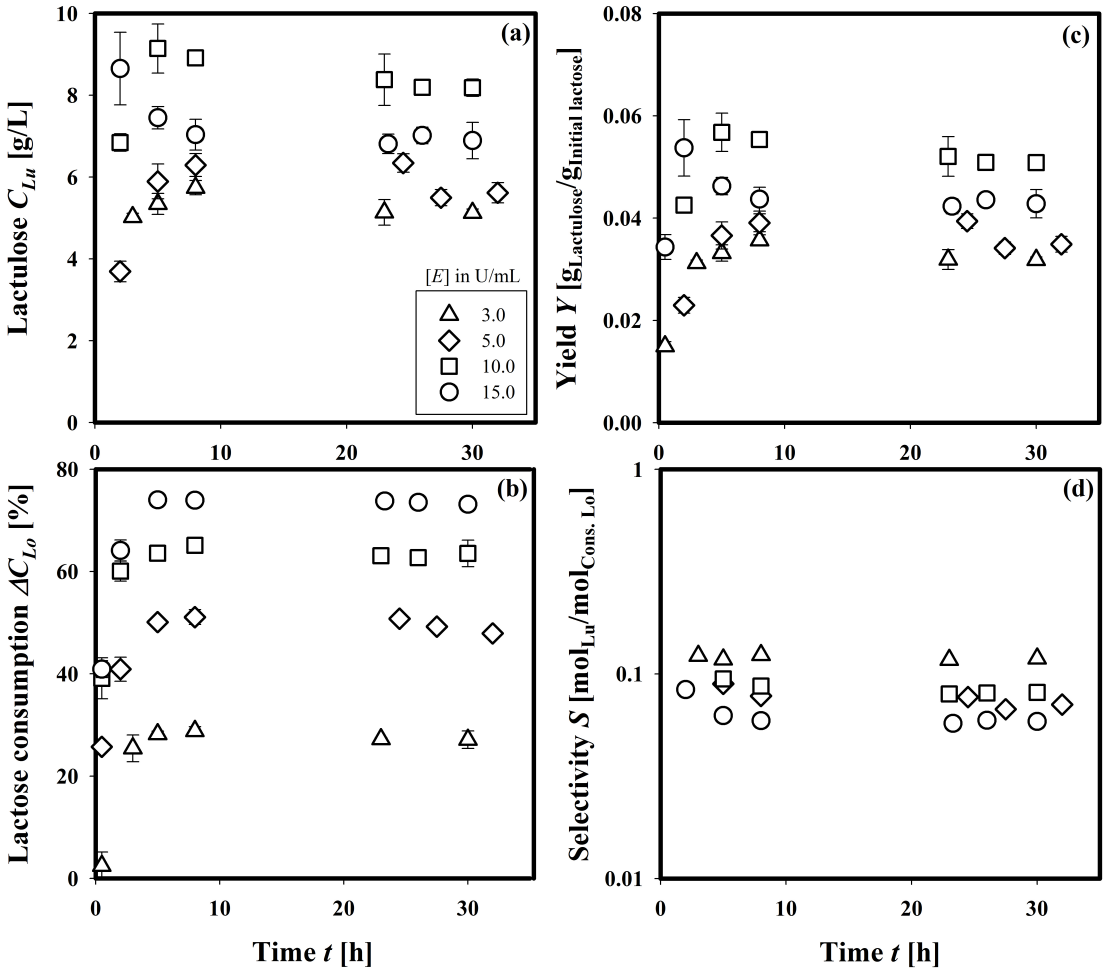

Figure 5.8 The effect of enzyme concentration on (a) lactulose concentration, (b) lactose consumption, (c) reaction yield and (d) selectivity during continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, $m_L/m_F = \frac{1}{4}$, τ = 7 h, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C, UFX membrane MWCO = 10 kDa.

Effect of molar ratio of lactose to fructose

A specific value of m_L/m_F is of importance for an optimum lactulose synthesis and this value generally depends on the type and source of the used enzyme (Panesar and Kumari, 2011; Schuster-Wolff-Bühring et al., 2010). A lower m_L/m_F is to some extent advantageous: (i) fructose can have a higher probability compared to water to act as galactosyl acceptor (Guerrero et al., 2011; Sitanggang et al., 2014a,b) and (ii) the secondary hydrolysis can be suppressed by shifting the direction of the reaction to the left side (to lactulose) (refer to Figure 2.7) (Sitanggang et al., 2015).

As shown in Figure 5.9a, the lactulose concentration with an m_L/m_F of $\frac{1}{4}$ was higher compared to the other m_L/m_F values. At the highest m_L/m_F (or at the highest $C_{i,Lo}$), the synthesis of GOS was favored in detriment of lactulose (indicated by additional peaks found in HPLC chromatogram). Therefore, the lowest reaction yield was obtained (Figure 5.9c). The highest reaction yield and selectivity value were obtained with an m_L/m_F of $\frac{1}{8}$ (the selectivity value was similar with an m_L/m_F of $\frac{1}{4}$, see Figure 5.9c and d).

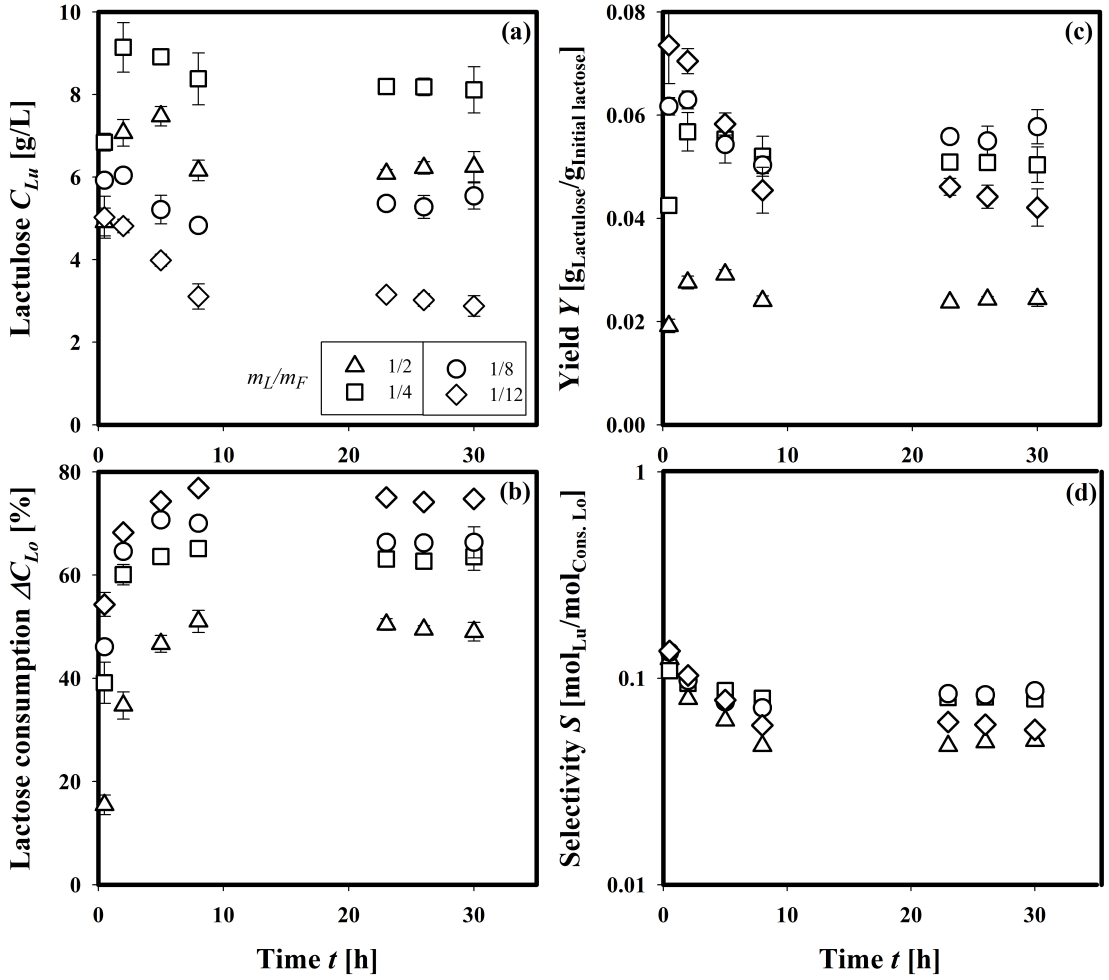

Figure 5.9 The effect of molar ratio of lactose to fructose on (a) lactulose concentration, (b) lactose consumption, (c) reaction yield and (d) selectivity during continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, [E] = 10.0 U/mL, τ = 7 h, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C, UFX membrane MWCO = 10 kDa.

A further increase in the fructose concentration, i.e., at m_L/m_F of $\frac{1}{12}$ could be detrimental because the activity of β-galactosidase might be inhibited (see Figure 5.9a, b and c) (Mayer et al., 2004; Panesar and Kumari, 2011). With this m_L/m_F, the highest lactose consumption was also obtained (Figure 5.9b). By using *A. oryzae* β-galactosidase in batch operation, Guerrero et al. (2011) also mentioned that at the higher values of m_L/m_F (more than $\frac{1}{1}$), the syntheses of GOS

were favored instead of lactulose. The optimum m_L/m_F reported was $\frac{1}{4}$ which is the same as in the present study. The reaction yield in this study was also in a good accordance with that reported by Guerrero et al. (2011), approximately 6 %.

From the evaluation of two operating conditions mentioned above (i.e., [E] and m_L/m_F), another parameter, i.e., ratio of enzyme amount to initial lactose ($E/C_{i,Lo}$, U/g$_{lactose}$) can be further discussed. As shown in Figure 5.10, the optimum $E/C_{i,Lo}$ was about 62 U/g$_{lactose}$. At the ratios higher than 62 U/g$_{lactose}$ were baneful for lactulose productions, especially for the highest one (146 U/g$_{lactose}$). In addition, the hydrolysis was preferred over the synthesis at 146 U/g$_{lactose}$. Chockchaisawasdee et al. (2005) also reported that at high $E/C_{i,Lo}$ synthesis of GOS in a membrane reactor was also inefficient.

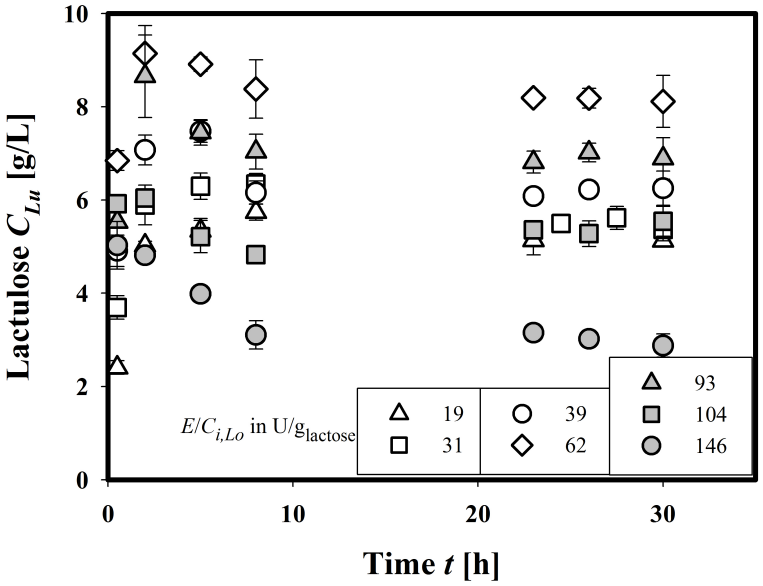

Figure 5.10 The effect of ratio of enzyme amount to initial lactose on lactulose concentration during continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, [E] = 10.0 U/mL, τ = 7 h, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C, UFX membrane MWCO = 10 kDa.

Effect of hydraulic residence time

In Figure 5.11, it is clearly seen that residence time also influenced the performances of lactulose synthesis. A higher dilution rate consequently led to a shorter time for the enzyme to perform lactose hydrolysis. Thus, a lower amount of galactosyl moieties was available during the transgalactosylation. This consequently resulted in a lower lactulose concentration (Mayer et al., 2010; Song et al., 2013b). As shown in Figure 5.11a and c, the lactulose concentration and reaction yield at τ = 3 h were lower than that at 5, 7 and 9 h. A longer residence time was found to be favorable, i.e. τ = 9 h to obtain a higher lactulose concentration. However, when τ was prolonged above this

value (i.e., 12 h) the performance of the reaction was reduced, as reflected by the lowest lactulose concentration and reaction yield obtained.

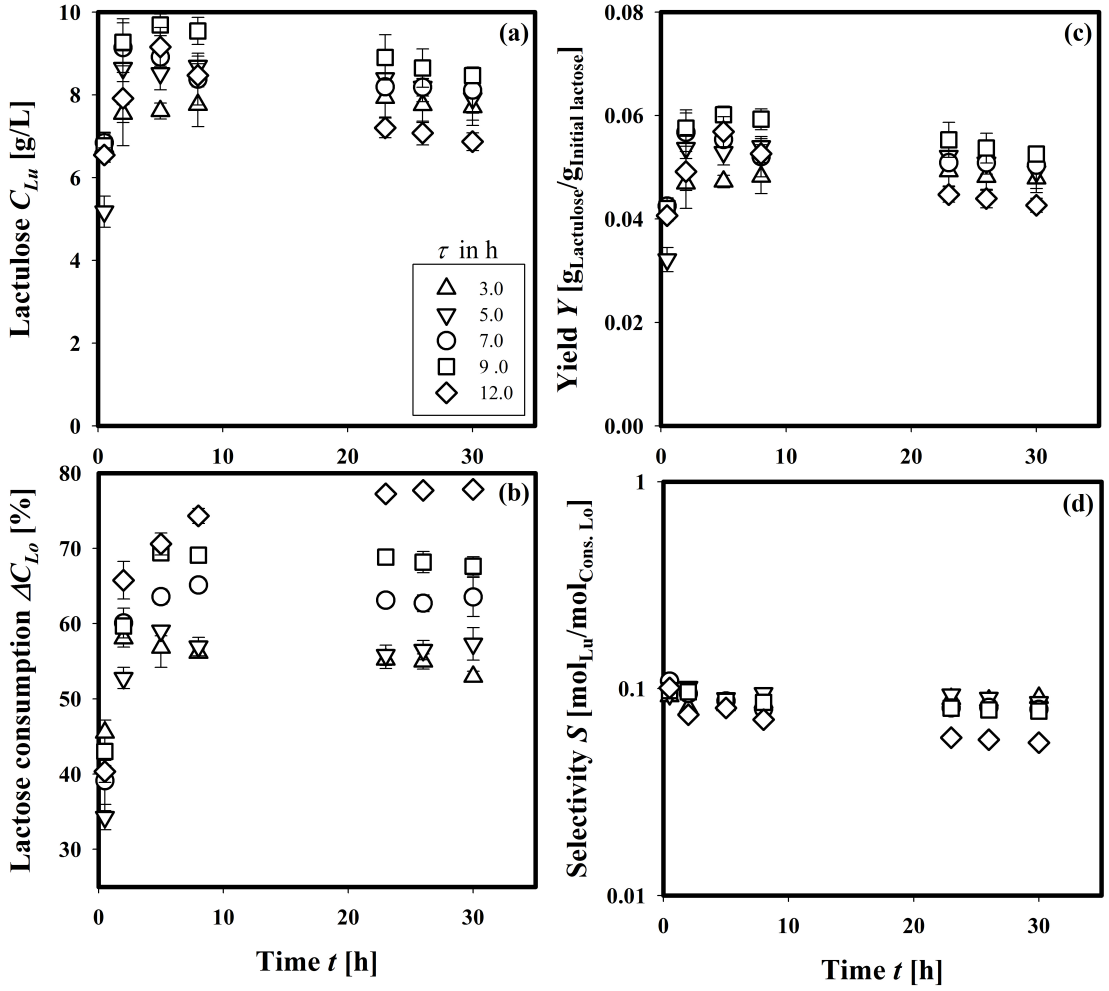

Figure 5.11 The effect of hydraulic residence time on (a) lactulose concentration, (b) lactose consumption, (c) reaction yield and (d) selectivity during continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, [E] = 10.0 U/mL, $m_L/m_F = \frac{1}{4}$, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C, UFX membrane MWCO = 10 kDa.

In batch production, after a maximum lactulose concentration is achieved, the produced lactulose undergoes a secondary hydrolysis. By the pertinent enzyme, this reaction yields fructose and galactose back (Hua et al., 2010; Lee et al., 2004). It is one of the drawbacks encountered in batch operation which can be tackled by performing a continuous lactulose synthesis (refer to Figure 5.1). In case of the operation at τ = 12 h (and presumably also for values longer than this), a longer time for the produced lactulose (and also for other substance such as GOS) residing in the reactor might facilitate the secondary hydrolysis. Additionally, this could also lead to a higher

lactose hydrolysis (thus a higher lactose consumption, see Figure 5.11b) and the lowest reaction selectivity (Figure 5.11d).

Effect of reaction temperature and thermal inactivation of the enzyme

In batch operation, Lee et al. (2004) reported the lactulose concentrations using *K. lactis* β-galactosidase were insignificantly different at 40 to 60 °C with an incubation time less than 8 h. Guerrero et al. (2011) also mentioned that in the same temperature range, there was not any difference between lactulose productions using *A. oryzae* β-galactosidase within 6 h. In this present study, the lactulose concentration at 40 °C was significantly different compared to 50 and 60 °C (see Figure 5.12a). The reactions were carried out for more than 24 h where the thermal inactivation might be pronounced. Especially at 60 °C, the concentration declined rapidly down to approximately 70 % within 30 h (calculated based on the maximum lactulose concentration).

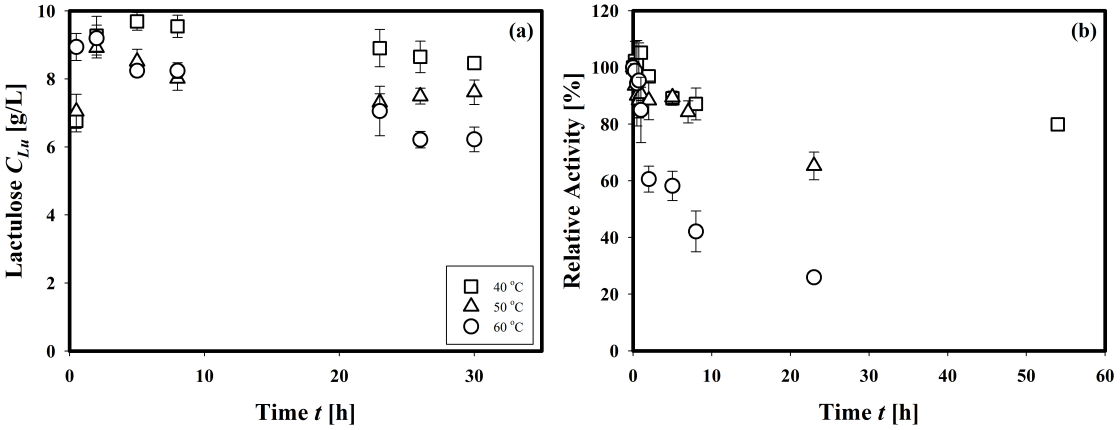

Figure 5.12 (a) The effect of incubation temperature on lactulose concentration during continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, [E] = 10.0 U/mL, $m_L/m_F = \frac{1}{4}$, τ = 9 h, 150 mM citrate-phosphate buffer, n = 350 rpm, UFX membrane MWCO = 10 kDa; (b) The inactivation study of *A. oryzae* β-galactosidase under non-reactive conditions.

Thermal inactivations of *A. oryzae* β-galactosidase were investigated at 40, 50 and 60 °C under non-reactive conditions (see Section 3.2.3). It is clearly seen the inactivation was higher as the incubation temperature was elevated (Figure 5.12b). After 24 h, the remaining activity for 40, 50 and 60 °C were 80, 68 and 20 %. The highest inactivation rate at 60 °C could correspond to a rapid reduction of lactulose concentration shown in Figure 5.12a.

Under non-reactive conditions, *A. oryzae* β-galactosidase was more thermally stable than *K. lactis* β-galactosidase (for the thermal inactivation data of *K. lactis* β-galactosidase, refer to the supplementary data in Sitanggang et al. (2015)). With a constant agitation, i.e., 350 rpm, the remaining activity of *K. lactis* β-galactosidase was reduced by up to 90 % within 1 h at 40 °C (Sitanggang et al., 2015). For *A. oryzae* β-galactosidase at almost similar conditions (T = 40 °C,

350 rpm), the enzyme activity reduced by 20 % within 24 h. Huerta et al. (2011) also reported the thermal inactivation of *A. oryzae* β-galactosidase under non-reactive conditions, where the remaining activity of the enzyme was around 60 % within 40 h incubation at 50 °C.

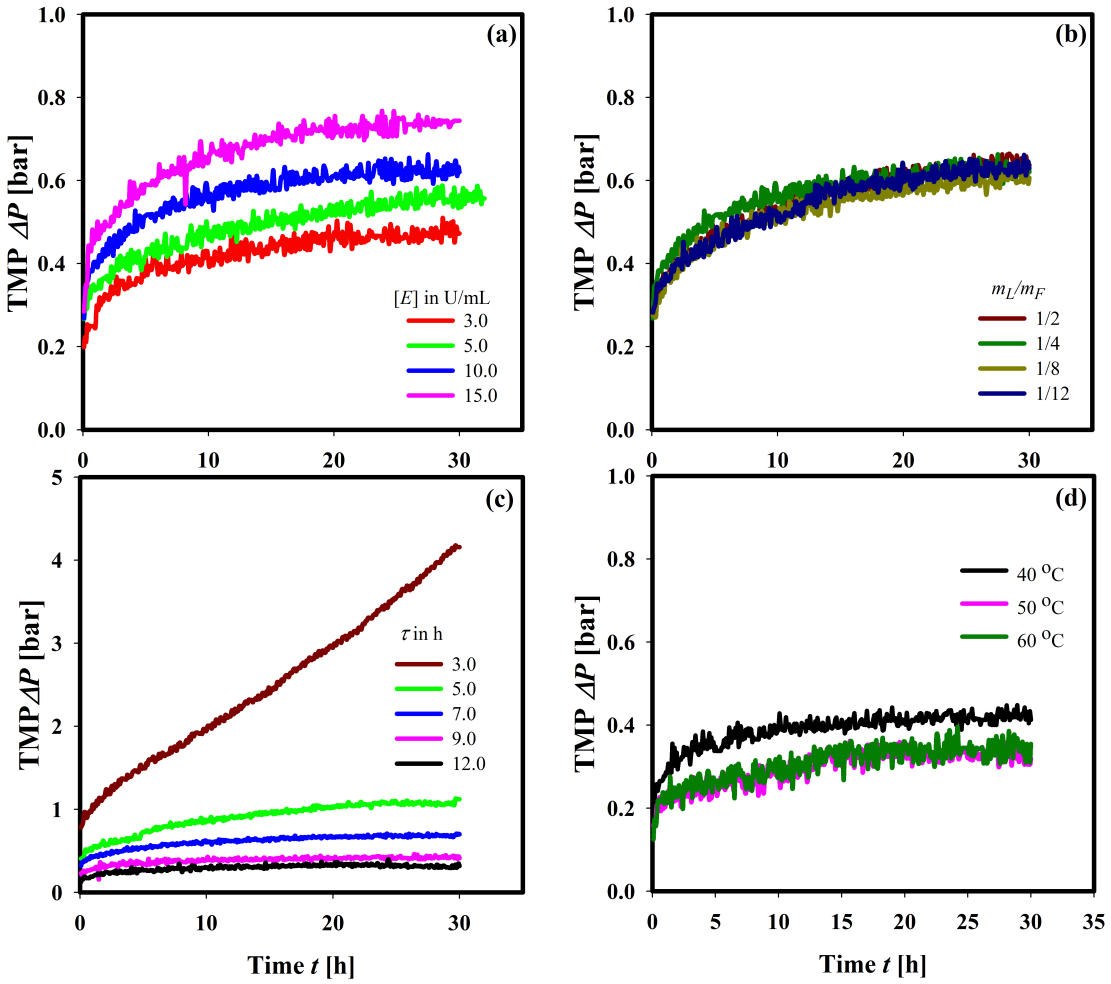

Figure 5.13 The influence of enzyme concentration (a), molar ratio of lactose to fructose (b), hydraulic residence time (c) and incubation temperature (d) on the transmembrane pressure during continuous lactulose synthesis. The conditions are the same as mentioned in Figure 5.8 (a), Figure 5.9 (b), Figure 5.11 (c) and Figure 5.12a (d).

The evaluation of transmembrane pressures during continuous lactulose synthesis at different operating conditions

As presented in Figure 5.13a, b, c and d, the TMP was influenced by the enzyme concentration $[E]$, residence time τ and incubation temperature T. In case of the operations at different molar ratios of lactose to fructose m_L/m_F ($\frac{1}{2}$ to $\frac{1}{12}$), the difference between the TMPs were insignificant.

This was due to the smaller molecular mass of the substrates and the products of hydrolysis and transgalactosylation compared to the cut-off of the membrane (i.e., 10 kDa) (see Section 3.2.1).

During a cake filtration at a constant flux, the TMP increases due to the cake deposition. For a free-enzyme system, the enzyme is presumed to be deposited on the membrane (Beier et al., 2007; Luo et al., 2013). By increasing the enzyme concentration consequently led to a higher TMP (Figure 5.13a). At a shorter hydraulic residence time, a higher convective transport which could lead to a higher enzyme deposition onto the membrane surface was inevitable. Therefore, the TMP was higher compared to the longer ones. Within 30 h, the TMP has reached to more than 4 bar at τ = 3 h. Furthermore, the temperature was also observed to influence the TMP (Figure 5.13d). The TMPs at T = 50 and 60 °C were not significantly different but were higher compared to T = 40 °C. In general, at a constant flux operation, the reduction in viscosity and the increase in diffusivity of materials that are transported through the membrane at an elevated temperature could yield a lower TMP during the filtration (Vladisavljević et al., 1992; Kallioinen et al., 2007).

5.2.3 Long-term continuous lactulose synthesis

A prolonged continuous synthesis of lactulose was carried out at optimum operating conditions, as follows: [E] = 10 U/mL, $m_L/m_F = \frac{1}{4}$, τ = 9 h (or J_{SP} = 8.07 L/(m^{2}h)) and T = 40 °C (Sitanggang et al., 2016). With reference to the costs of raw materials[1] and the downstream processing, the lactulose concentration was chosen as a parameter for selecting the optimum operating conditions.

The enzyme inactivation was pronounced during the prolonged synthesis. The lactulose concentration reduced markedly after 30 h. Moreover, at 100 and 168 h, the reductions were about 28.7 and 34.4 %[2], respectively (Figure 5.14). If one assumes that the maximum lactulose concentration indicates the initial enzyme activity, then the enzyme activity reduced slower during the synthesis than in the non-reactive conditions (Figure 5.14). It is generally reported that sugars are frequently enzyme stabilizers (Prasad and Roy, 2010). Mayer et al. (2010) reported a production of lactulose in an EMR using free β-glycosidase from *P. furiosus* (CelB). In their study, with an optimum $m_L/m_F = \frac{1}{15}$ and τ = 1 h, the maximum lactulose concentration at "steady state" was about 14 g/L. However, this value rapidly decreased after 96 h down to almost zero. The calculated half-life of the enzyme was about 36 h. In addition, they have also investigated a lactulose synthesis in a PBR using immobilized β-glycosidase from *P. furiosus* (CelB). The stability of the immobilized enzyme was promising, as a nearly constant lactulose concentration of 14 g/L was obtained for 14 days operation. However, from an economic point of view, the enzyme is not ready for industrial applications. Besides being not commercially available, the enzyme preparation has not been approved to have GRAS status. These considerations outweigh the fact that this study has a lower productivity in comparison to Mayer et al. (2010) and other studies where recombinant enzymes were also used to synthesize lactulose.

[1] According to the Sigma-Aldrich–Merck KGaA, Darmstadt, Germany, the price ratio between lactulose to lactose and fructose $\frac{\$_{Lu}}{(\$_{Lo}+\$_F)}$ is 44.66.

[2] calculated based on the maximum lactulose concentration obtained.

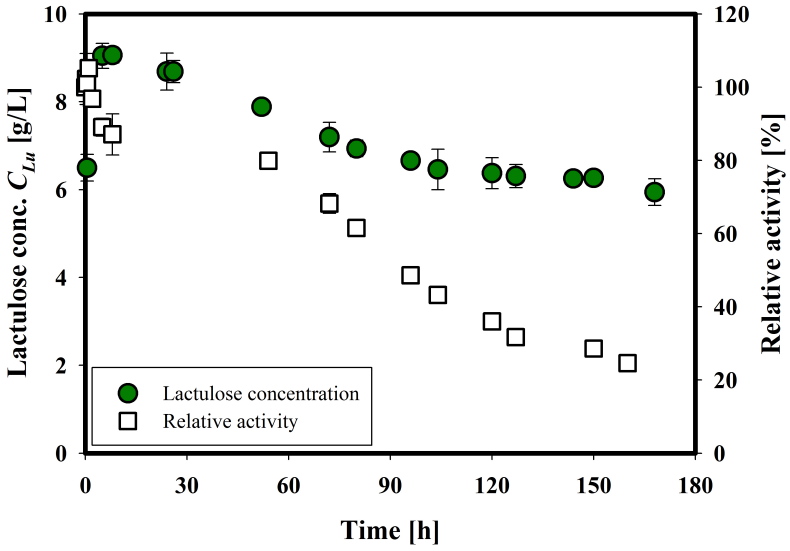

Figure 5.14 The profile of the lactulose concentration during a prolonged continuous synthesis of lactulose using *A. oryzae* β-galactosidase in EMR system with conditions: C_S = 500 g/L, $[E]$ = 10.0 U/mL, $m_L/m_F = \frac{1}{4}$, τ = 9 h, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C, UFX membrane MWCO = 10 kDa and relative activity of *A. oryzae* β-galactosidase under non-reactive conditions at T = 40 °C.

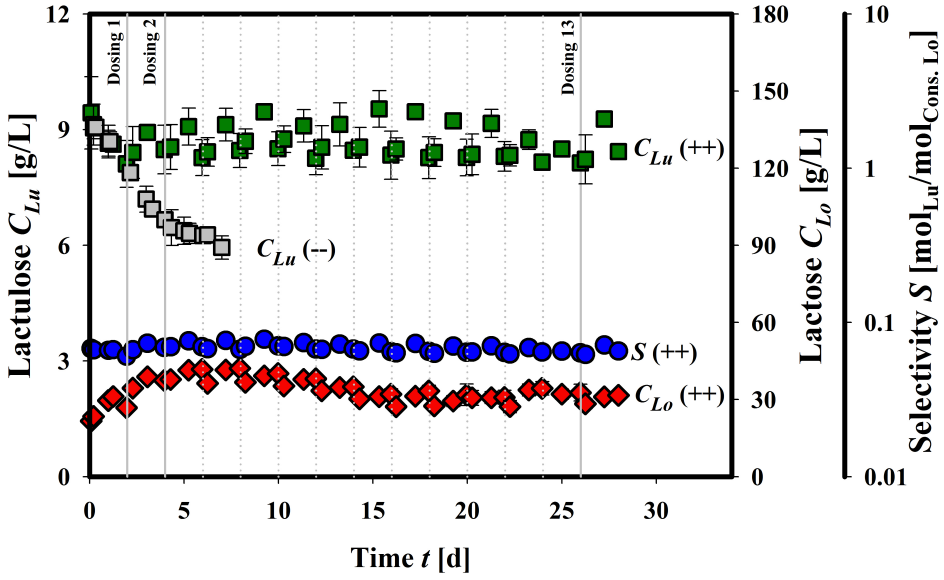

Figure 5.15 Lactulose concentrations between non-maintained and maintained *A. oryzae* β-galactosidase activity in EMR system during a a prolonged synthesis; C_{Lu} (- -) = profile of lactulose concentration during a prolonged synthesis without enzyme addition and C_{Lu} (+ +) = lactulose concentration with enzyme addition (enzyme dosing: 10 % of initial enzyme amount every 48 h). Conditions: C_S = 500 g/L, $[E]$ = 10.0 U/mL, $m_L/m_F = \frac{1}{4}$, τ = 9 h, 150 mM citrate-phosphate buffer, n = 350 rpm, T = 40 °C, UFX membrane [MWCO = 10 kDa.

A long-term continuous synthesis of lactulose was performed with the addition of fresh enzyme into the reactor periodically. Based on the reduction of lactulose concentration by 10 % within approx. 48 h (see Figure 5.14), 10 % of the initial enzyme amount was added every 48 h. This consideration was based on the fact that hydrolysis was preferred over the synthesis with an excess amount of enzyme (see Section 5.2.2 for the effect of enzyme concentration).

As shown in Figure 5.15, the inter-day reproducibility of the lactulose concentrations within 48 h between previous experiment C_{Lu} (- -) (depicted from the data in Figure 5.14) and long-term one C_{Lu} (+ +) was in a good accordance. As expected, through the enzyme addition right after 48 h, C_{Lu} (+ +) increased whereas for the previous experiment C_{Lu} (- -) continuously decreased. The subsequent additions (n = 13) were done at an interval of 48 h. For 28 days, C_{Lu} (+ +) could be maintained at a mean value of 8.67 g/L with a selectivity range of 0.06 – 0.07 $mol_{Lu}/mol_{Cons.Lo}$. The outlet lactose concentration C_{Lo} (+ +) was about 34 g/L, giving ΔC_{Lo} (+ +) of 78 %. This study is the longest one ever reported for continuous lactulose synthesis using a membrane reactor with a maintained enzyme activity and in the context of constant flux operation and thus constant volumetric productivity (Sitanggang et al., 2016).

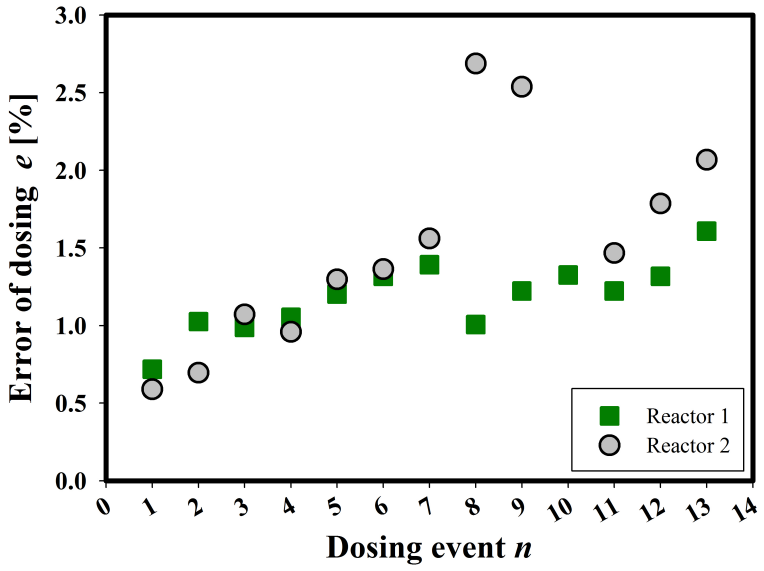

Figure 5.16 Evaluation of the dosing accuracy during long-term continuous synthesis of lactulose.

A second evaluation of dosing accuracy was also done during this long-term operation. The measured density of the enzyme solution ρ_e was 1011.567 kg/m^3 (at 30 °C), whereas the substrate solution density ρ_s was 1182.66 kg/m^3 (at 40 °C). By recording the permeate weight W_p on the precision balance after one dosing event n was finished (permeate density ρ_p was assumed to be similar to the substrate solution density ρ_s), V_{aD} (and the enzyme amount E_{aD}) could be calculated ($E_{aD} = [E]_s \times \frac{W_p}{\rho_p}$). Thus, the accuracy (i.e., error of dosing e) of the dosing system could be

determined. As shown in Figure 5.16, the average e for $n = 13$ was less than 2 %[3] (Sitanggang et al., 2016). This value was in accordance with the first dosing evaluation presented in Section 4.2. Moreover, the average dissimilarity of the dosing accuracy between parallel reactors used was less than 1.5 %.

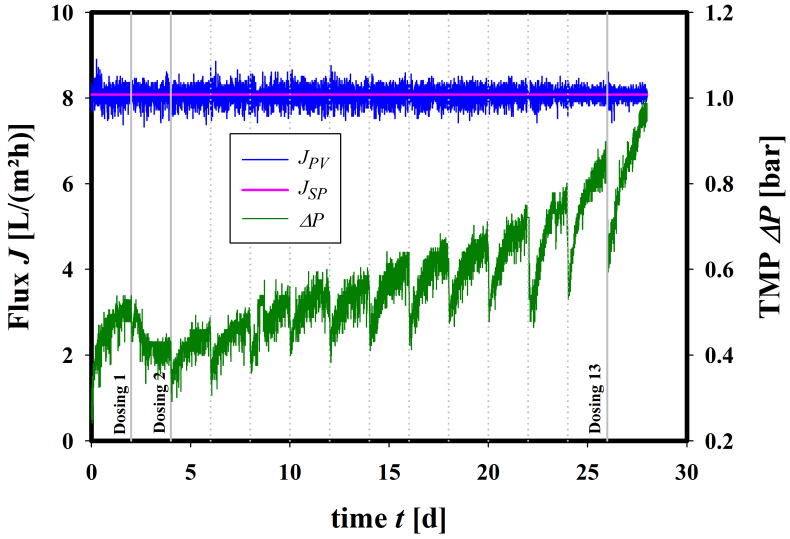

Figure 5.17 Transmembrane pressure ΔP and flux J_{PV} profile during long-term continuous synthesis of lactulose at $J_{SP} = 8.07$ L/(m^{2}h).

During a long-term operation, the TMP increased from approx. 0.3 bar to 1.0 bar. A progressive increase of the enzyme amount in the reactor throughout the synthesis could lead to an increasing microscopic enzyme layer built onto the membrane surfaces. This led to a higher R_t or reduced ℓ_p. Prior to the addition of the fresh enzyme (i.e., 1 h before), the operation of the EMR system was always stopped (i.e., the PID controller was inactivated and the inlet pressure was reduced to 0 bar). The freshly prepared enzyme solution was poured into the enzyme tanks. Then, the system was operated again with the same conditions as previously mentioned (i.e., $J_{SP} = 8.07$ L/(m^{2}h)). The whole procedures (i.e., to shutdown the PID controller $\rightarrow$ refilling the enzyme tanks $\rightarrow$ the activation of the PID controller) were repeated in every cycle of dosing and took approximately 3 to 4 minutes. These procedures were done due to the limitation of the temperature controller in providing a temperature range of 2-8 °C and very unlikely occured at industrial scale. According to Drews (2010) and Kraume et al. (2009), the filtration breaks or membrane relaxation could counteract reversible, residual and some influence of irreversible fouling at constant flux operation of a membrane reactor. Due to this individual filtration break (i.e., 3 to 4 minutes), the membrane experienced a relaxation and consequently, the TMP was reduced in every cycle of dosing (see Figure 5.17).

[3]Error $e = \left|1 - \frac{E_a D}{E_s D}\right| \times 100\%$

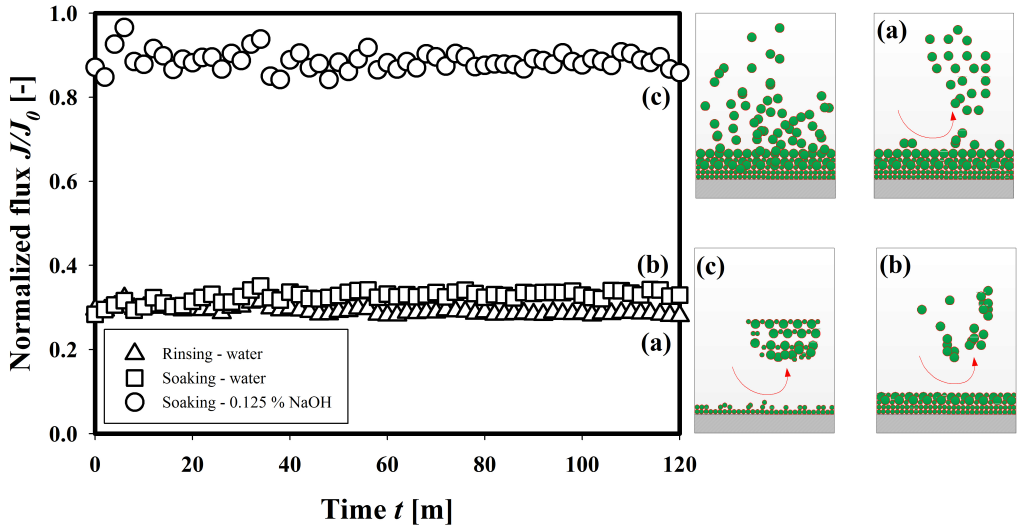

Figure 5.18 The efficiency of serial cleanings of the membranes used in long-term operation: (a) rinsing with ultrapure water, (b) soaking with ultrapure water and (c) soaking with 0.125 % NaOH (chemical cleaning).

The effectiveness of physical cleanings and chemical cleaning were also investigated on the used UFX10 membranes (Figure 5.18). The cleanings were done successively from: rinsing with ultrapure water → soaking with ultrapure water → chemical soaking. In the first cleaning (rinsing), the membranes were not removed from the reactor. However, all other parts, i.e., stirrer, borosilicate glass, were cleaned with ultrapure water. After the cleaning, the reactors were filled with ultrapure water and stirred at 350 rpm, T = 40 °C for 15 minutes. This procedure was repeated thrice prior to ultrapure water flux determination at ΔP = 0.5 bar. Through this step, the recovered flux was 29.14 %.

In the following physical cleaning, the rinsed membranes were delicately stored in 90 mL of ultrapure water at T = 40 °C without being stirred for 24 h. This was done to evaluate the influence of soaking time on the removal of the enzyme compact microscopic layer. Prior to ultrapure water flux determination, the soaked membranes were gently washed. This cleaning was found to be inefficient since the resulted flux was only increased by 3 % from the previous cleaning. Eventually, the membranes were soaked again as explained in the previous step, by replacing ultrapure water with 0.125 % NaOH. This chemical cleaning could recover 88.57 % of the original flux (J_0 = 150.18 L/(m^{2}h)). Through a combination of the physical cleanings and a simple chemical cleaning, the irrecoverable flux for the used UFX10 membranes was about 12 %.

Chapter 6

SUMMARY AND FUTURE OUTLOOK

6.1 Summary

The automated and parallel small-scale reactors are considered as an efficient and a time-saving tool since these reactors can facilitate a straightforward bioprocess development to reach its commercial success (Puskeiler et al., 2005). Within this study, a screening and characterization system for continuous biocatalytic processes that had been coined by Lyagin et al. (2010, 2015) was upgraded. The previously established program in Visual Designer^{TM} 4.0 was successfully transferred into the Laboratory Virtual Instrument Engineering Workbench (LabVIEW^{TM}) program with a completely new structure of the data acquisition system (i.e., hardwares). By these changes, the maximum number of parallel reactors that could be controlled (i.e., constant flux and enzyme dosing) was increased from only two (Lyagin et al., 2010, 2015) to twelve parallel reactors. By the application of the PID controller with fast setting (Kuhn, 1995), the control accuracy of a constant flux operation was more than 95 %. In addition to this, an automated protocol of the enzyme addition has also been developed with a control error of less than 2 % (see Table 4.2).

The developed reactor system was used to perform batch and continuous synthesis of lactulose. By performing a continuous operation, the secondary hydrolysis which is one of the drawbacks of batch lactulose synthesis could be circumvented (see Section 5.1). Besides the circumvention of secondary hydrolysis, the continuous operation in the developed EMR system was also proven to mitigate the unproductive time as well as the excessive enzyme amount needed like in batch lactulose synthesis and eventually led to a higher specific productivity (Sitanggang et al., 2014a,b).

The engineering aspects for continuous lactulose synthesis using *K. lactis* and *A. oryzae* β-galactosidase were thoroughly investigated using the developed reactor system (Sitanggang et al., 2015, 2016). In case of continuous synthesis of lactulose using *A. oryzae* β-galactosidase, the investigations covered three main issues which are usually encountered in membrane-assisted enzymatic reactions, such as: membrane–enzyme electrostatic interaction, optimum operating conditions (with industrially relevant parameters) and maintenance of the enzyme activity in a long-term operation. The membrane selection was based on the electrostatic interaction between the membrane surface and the enzyme molecules. By this, UFX10 MWCO = 10 kDa was selected

due to the electrostatic interaction towards the repulsion and the small initial transmembrane pressure resulted. A prolonged continuous synthesis of lactulose was carried out at optimum operating conditions, as follows: enzyme concentration [E] = 10 U/mL, molar ratio of lactose to fructose $m_L/m_F = \frac{1}{4}$, hydraulic residence time τ = 9 h (or flux J_{SP} = 8.07 L/(m^{2}h)) and temperature T = 40 °C. For one week reaction, the reductions of lactulose concentration were about 28.7 and 34.4 %[1] at 100 and 168 h, respectively (Figure 5.14). A long-term continuous synthesis of lactulose was performed with an addition of the fresh enzyme into the reactor periodically (i.e., 10 % of the initial enzyme amount was added every 48 h). For 28 days, lactulose concentration could be maintained at a mean value of 8.67 g/L with a selectivity range of 0.06 – 0.07 $mol_{Lu}/mol_{Cons.Lo}$. This study is the longest one ever reported for continuous lactulose synthesis using a membrane reactor with a maintained enzyme activity and in the context of constant flux operation and thus constant volumetric productivity (Sitanggang et al., 2016).

To briefly summarize, the EMR system can bridge the gap between typical laboratory scale where the reactions are mostly carried out in batch mode and industrial scale which reactions are often continuously operated especially for serial reactions. Moreover, the developed reactor system has been highlighted to be useful especially for process characterization of continuous enzyme based biotransformations (also possible for whole cells) where: (i) the enzymes are freely dissolved in the reaction media, (ii) the products are susceptible to undergo further degradation by the same enzyme and (iii) either product or substrate inhibition is pronounced during the reaction.

6.2 Future outlook

The developed EMR system in this study can be further used, modified and developed to present its various applications especially in the field of biotechnology, food science and technology or even in chemistry. Brief descriptions for these possibilities are introduced below.

6.2.1 Further strategy for lactulose production

Through the inherent catalytic ability of cellobiose 2-epimerase, lactulose can be synthesized directly from lactose with an identical reaction yield compared to chemical isomerization method (see Section 2.3.2, Table 2.4 and 2.7). However, when lactulose is produced for functional food purposes, the used enzymes are restricted only to those which have GRAS (generally recognized as safe) FDA status (Rodríguez et al., 2006). There are only several β-galactosidase preparations from microorganisms that are commercially available and have been considered safe. These are from yeast and fungi, such as: *Kluyveromyces lactis*, *K. fragilis*, *Aspergillus niger*, and *A. oryzae* (Ladero et al., 2000; Rodríguez et al., 2006; van Ooyen et al., 2006). Hence, to sum up all the elucidations in enzymatic preparation of lactulose, there are two efficient options available. Firstly, by keep utilizing the inherent catalytic properties of cellobiose 2-epimerase. With this, further studies should be conducted concerning the safety issue of the enzyme preparation as well as its

[1] calculated based on the maximum lactulose concentration obtained.

commercial availability. Moreover, the biochemical engineering characterizations of this enzyme in terms of continuous lactulose synthesis using the developed EMR system are also necessary as the reported studies were only conducted at batch operations (Kim and Oh, 2012; Kim et al., 2013; Rentschler et al., 2015; Wang et al., 2015). Secondly, following the study conducted by Hua et al. (2010) where dual-enzymatic synthesis of lactulose in organic-aqueous two-phase media was done. The enzymes preparations (*K. lactis* β-galactosidase and glucose isomerase) have been considered as safe. However, to circumvent secondary hydrolysis and mitigate the unproductive time, the application of the EMR system to perform continuous operation can also be taken into consideration.

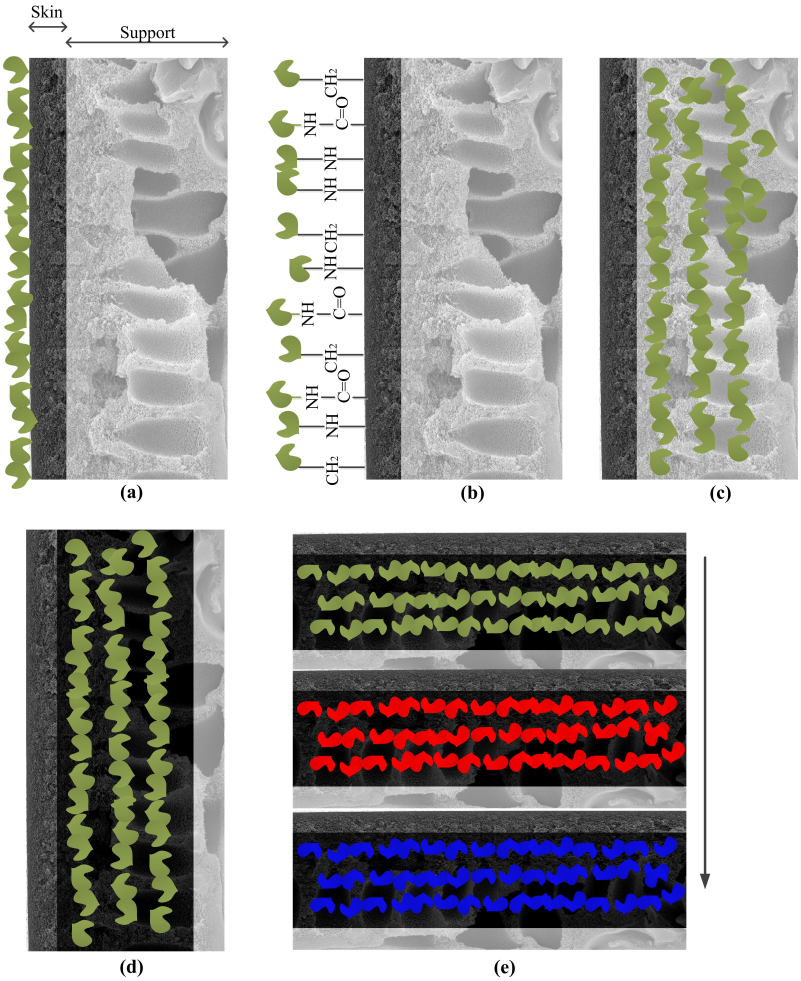

Figure 6.1 Concepts of enzyme immobilization on or inside the membrane: (a) adsorption on skin layer, (b) covalently linked on skin layer, (c) entrapment inside spongy support layer, (d) entrapment inside spongy support layer and coated with other material (i.e., dopamine), (e) different enzymes entrapped inside different spongy support layers of membranes and stacked together (Jochems et al., 2011; Luo et al., 2013, 2014b, 2015).

6.2.2 Biocatalytic membrane reactor

The polymeric membrane can be used as a support for enzyme immobilization. The immobilization can be done either on the skin layer or inside the membrane support (Luo et al., 2013) (see Figure 6.1). The immobilization of an enzyme on the membrane skin layer can be simply done by a pressure driven process where the enzyme is non-covalently linked to the membrane (i.e., adsorption) or by establishing covalent bonds. In the latter system, the presence of binding sites on the membranes is a prerequisite. These binding sites are functional groups (i.e., epoxy, amid, imidazol, carboxyl, etc.) which are already present or can be introduced on the membrane skin layer (Jochems et al., 2011). Sometimes, the introduction of a spacer/linker (i.e., glutaraldehyde, carbodiimide, diamine, etc.) is necessary to increase the distance between the enzyme and the membrane. This distance can to some extent reduce the conformational change due to the direct immobilization process and also increase the accessibility of substrate to the active sites (Vishwanath et al., 1995). The immobilization of the enzymes on the support layer of the membrane is preferable because of a higher enzyme loading and a better reuse stability (Luo et al., 2013). Moreover, this support layer can be coated by a polymer, i.e., dopamine to prevent the enzyme loss during the operation. This immobilization procedure can still maintain the separation capacity of the skin layer as it is unmodified (Luo et al., 2014b). Through this established immobilization procedure, sequential immobilizations can be done in the support layers of different membranes stacked together. Hereby, multi-enzymatic cascade reactions can be done in a single process (i.e., production of methanol from CO_2) (Luo et al., 2015).

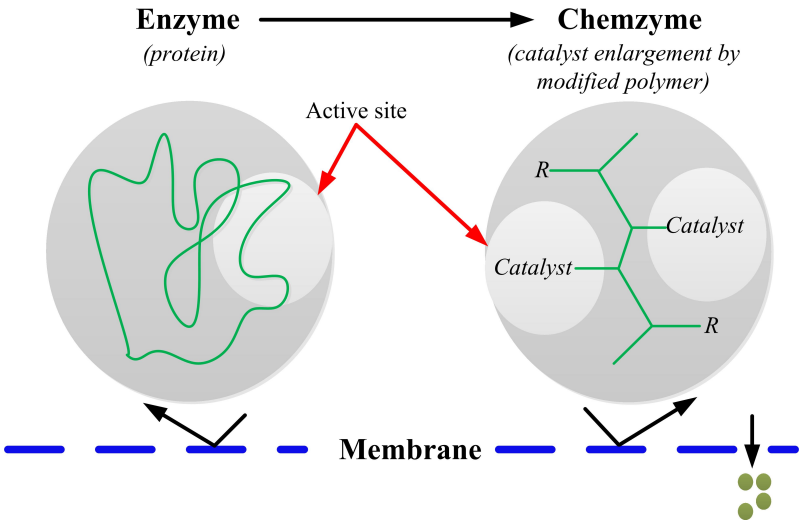

Figure 6.2 Concept of molecular weight enlargement of transition metal catalysts and organocatalysts according to Janssen et al. (2011) and Wöltinger et al. (2005).

6.2.3 Chemzymatic membrane reactor

The stability and the activity of an enzyme always depend on the solution chemistry (pH, ionic strength), operating temperature, mechanical stress (agitation) and the presence of co-factors and inhibitors (product and/or substrate) (Dalby, 2007; Estell, 1993; Aehle and Misset, 2008). At some point, the use of a particular homogenous catalyst in conversion of economically viable substance is preferred due to a higher turn over number and broader applications (aqueous and solvent system). There are many solvent-stable polymer membranes that have been commercially produced at present (Ulbricht, 2006; der Bruggen et al., 2002), owing to the possibility for homogenous catalyst utilization in membrane reactor. Due to low molecular weights of the homogenous catalysts (< 2000 Da), the enlargement strategies are needed to retain the catalysts during the operation (Wöltinger et al., 2005). Typical established possibilities used for an enlargement of a catalyst can be core-functionalized dendrimer, hyperbranched polymer, dendron, polyhedral oligomeric silsesquioxanes (POSS) (for a comprehensive review see Janssen et al. (2011)). Herein, the enzyme utilization in the EMR system can be shifted to chiral modified polymers which are called chemzymes (Wöltinger et al., 2005) (see Figure 6.2). This strategy has been commercially applied by German-based company Degussa AG Exclusive Synthesis & Catalysts for the reduction of tetralone by polysiloxane or polystyrene-bound oxazaborolidine, synthesis of homogeneous oligo(l-leucine) and other fine chemicals (Wöltinger et al., 2005).

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Appendix A

Developed Control Designs of EMR System Using LabVIEW Software

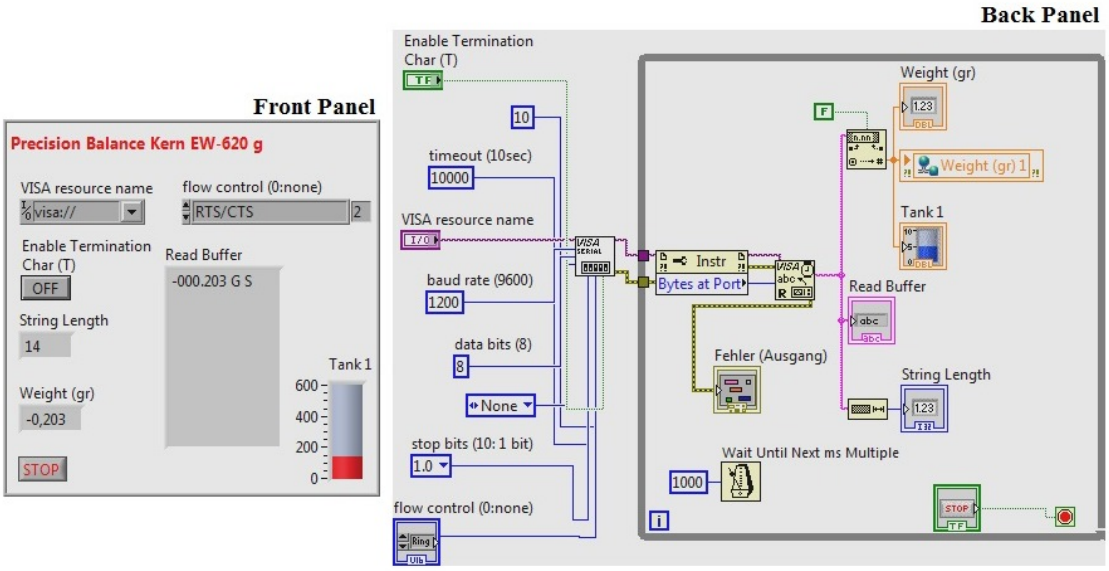

Figure A.1 Front and back panel of the LabVIEW program to extract the information from precision balance Kern EW 620-3NM.

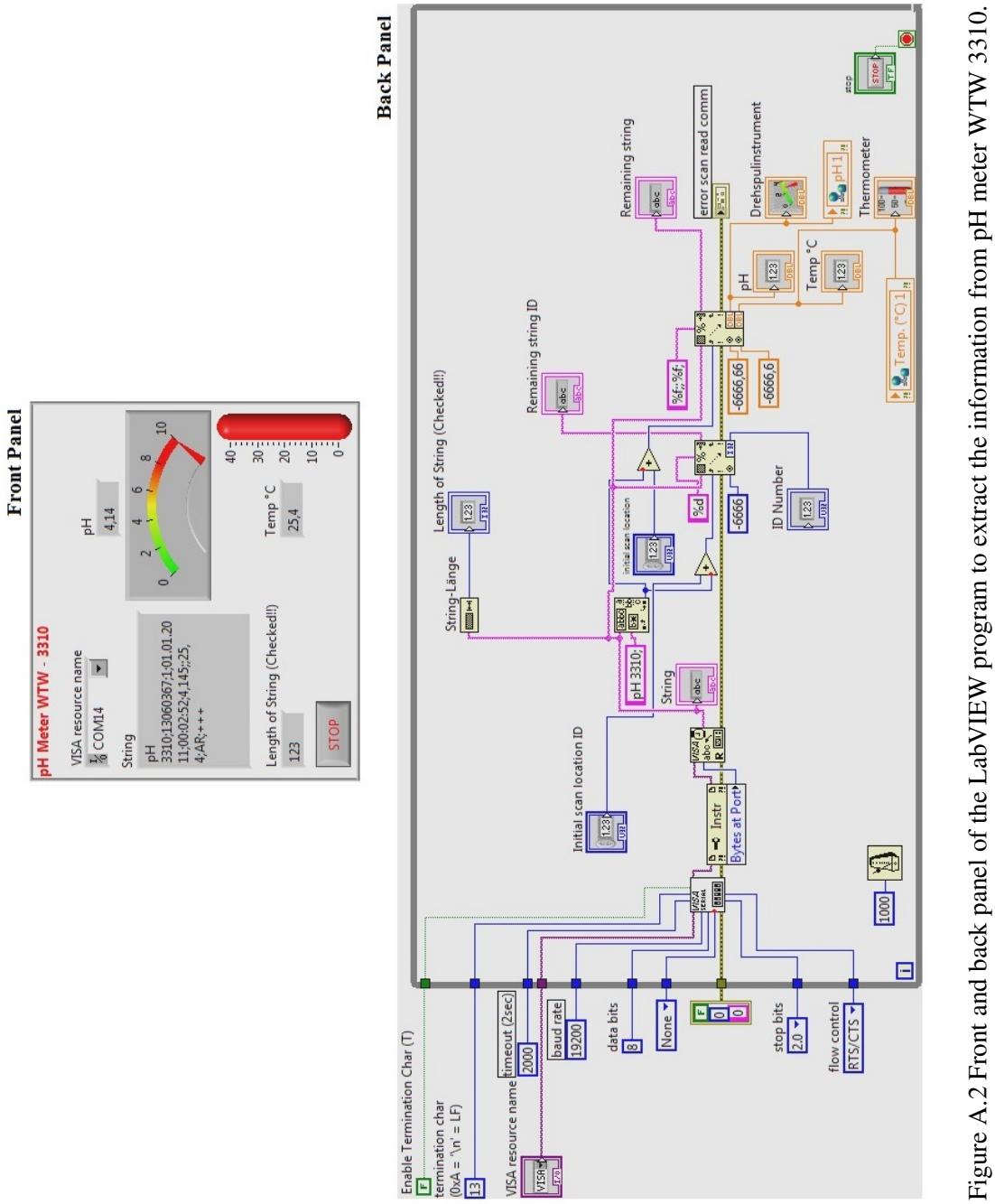

Figure A.2 Front and back panel of the LabVIEW program to extract the information from pH meter WTW 3310.

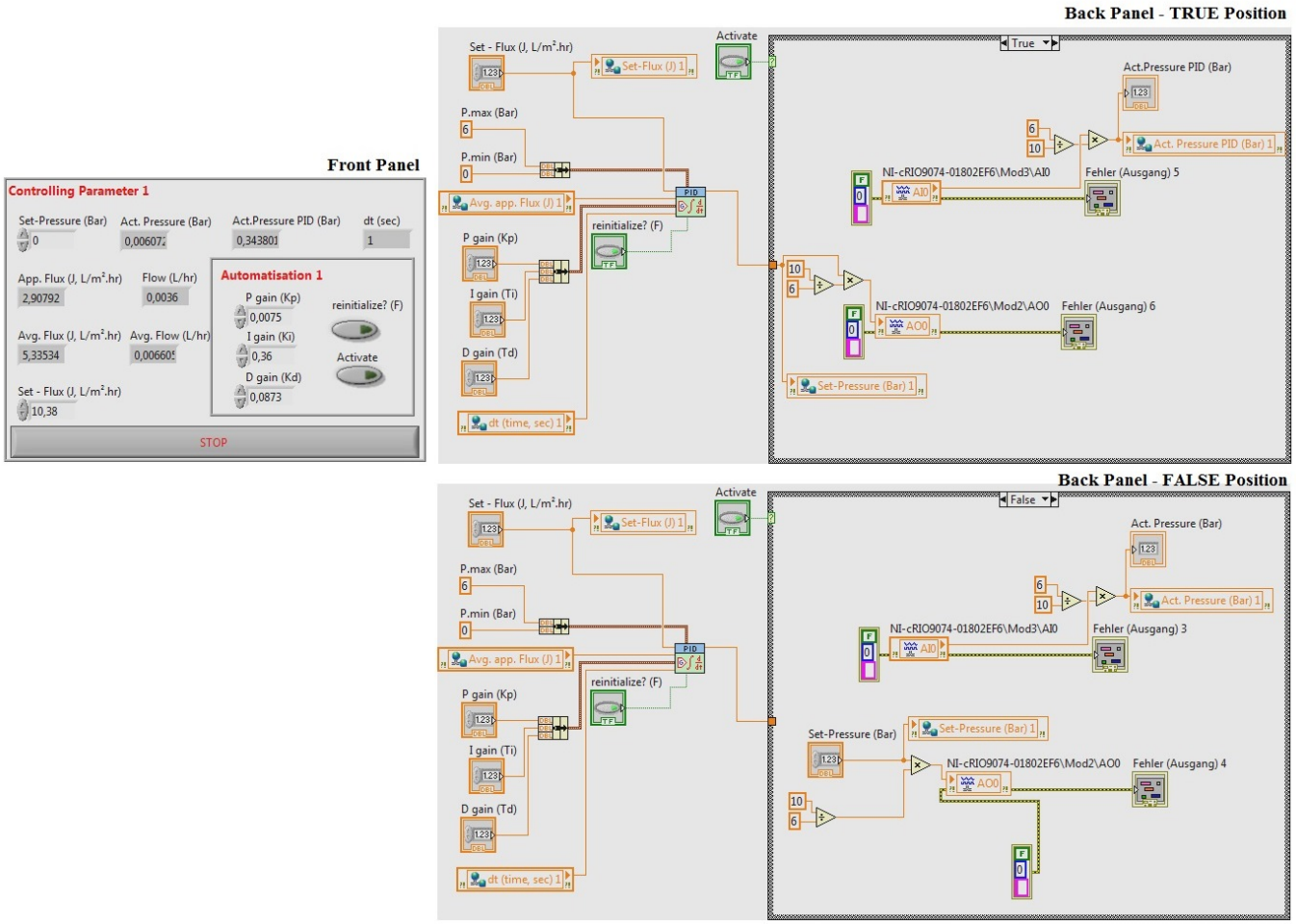

Figure A.3 Front and back panel (True and False position) of the LabVIEW program to run the PID controller.

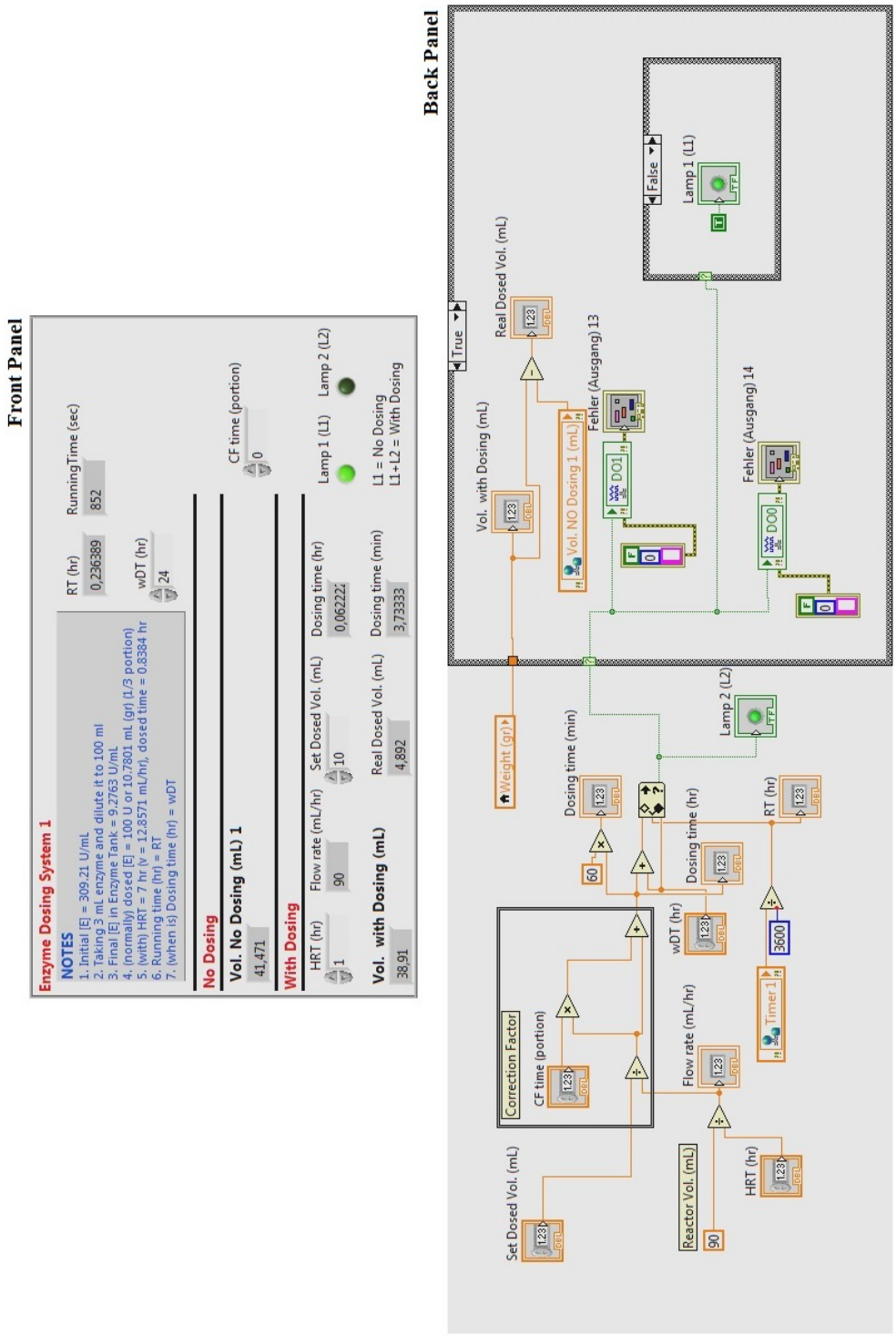

Figure A.4 Front and back panel of the LabVIEW program for automated protocol of enzyme dosing.

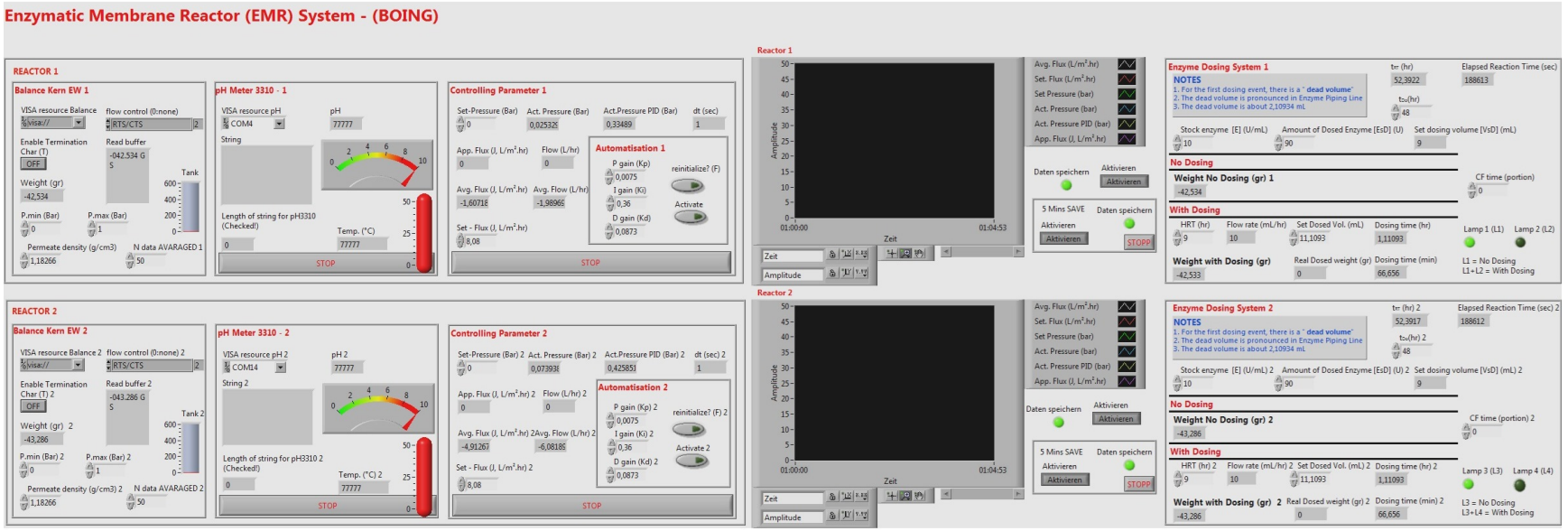

Figure A.5 Front panel of the LabVIEW program for the whole control designs of EMR system.

Figure A.6 Back panel of the LabVIEW program for the whole control designs of EMR system.

Appendix B

Published Articles

List of the published articles used for this cumulative thesis:

1. Sitanggang A.B.; Drews, A.; Kraume, M., 2014. Rapid transgalactosylation towards lactulose synthesis in a small-scale enzymatic membrane reactor (EMR). Chemical Engineering Transactions, 38: 19-24.

2. Sitanggang A.B.; Drews, A.; Kraume, M., 2014. Continuous synthesis of lactulose in an enzymatic membrane reactor reduces lactulose secondary hydrolysis. Bioresource Technology, 167: 108-115.

3. Sitanggang A.B.; Drews, A.; Kraume, M., 2015. Influences of operating conditions on continuous lactulose synthesis in an enzymatic membrane reactor system: A basis prior to long-term operation. Journal of Biotechnology, 203: 89-96.

4. Sitanggang A.B.; Drews, A.; Kraume, M., 2016. Development of a continuous process for enzyme-catalyzed lactulose synthesis. Biochemical Engineering Journal, 109: 65-80.