In order to meet the soaring global energy demand, fossil energy and fuel sources have been over-exploited at an extraordinary rate, imposing dramatic climate changes and threatening energy security and economic stability. These general environmental, socio-political, and economic concerns have veered the global attention toward the production and application of alternative renewable energy and fuel sources such as biofuels. Bioethanol has long been one of the main biofuels of interest that can be produced sustainably from a variety of feedstocks. However, the choice of raw material for bioethanol production has been a matter of controversy in the past decades. Lignocellulosic materials (e.g., agricultural residues) are potential candidates for sustainable bioethanol production (second generation bioethanol). However, in order to have a commercially feasible process, the complexities associated with lignocellulosic bioethanol production in pretreatment, hydrolysis, fermentation and downstream processing stages should be alleviated.

Membrane bioreactors with their great capabilities in semi-selective separation of different medium constituents are promising options for making a breakthrough in lignocellulosic biorefinery processes. Therefore, in this thesis, immersed membrane bioreactors that are well-developed technologies used for long in water and wastewater treatment processes, were used to tackle challenges and enhance lignocellulosic bioethanol production. In this regard, pressure-driven immersed membrane bioreactors were used to intensify lignocellulosic bioethanol production process by facilitating continuous hydrolysis and fermentation, cells and suspended solids separation, sugars and ethanol recovery, and physical bacteria decontamination. Furthermore, the new concentration-driven membrane technique of reverse membrane bioreactor was introduced in this thesis and practiced for simultaneous substrate utilization, inhibitor detoxification and lignocellulosic bioethanol fermentation.
Thesis for the Degree of Doctor of Philosophy

**Immersed flat-sheet membrane bioreactors for lignocellulosic bioethanol production**

Amir Mahboubi Soufiani
Immersed flat-sheet membrane bioreactors for lignocellulosic bioethanol production

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Cover photo: A simple depiction presenting cells, suspended particles and an Integrated Permeate Channel (IPC®) membrane envelope (Image © Blue Foot Membranes)

Borås, 2019
Abstract

The rising awareness of the environmental, economic and socio-political impacts of over-exploitation of fossil-based fuel and energy sources, have motivated the transition toward more sustainable and renewable energy sources. Lignocellulosic materials (e.g. agricultural residues) are potential candidates for sustainable bioethanol production that contributes to the replacement of fossil fuels. However, to have an economically feasible and commercialized process, issues associated with lignocellulosic bioethanol production in upstream, fermentation and downstream processing stages should be alleviated. Membrane bioreactors with their great capabilities in semi-selective separation are promising options for making a breakthrough in lignocellulosic biorefinery processes. Therefore, in this thesis, different membrane modules and immersed membrane bioreactors (iMBRs) set-ups were developed and applied to take advantage of this long-matured water and wastewater treatment technique in remediation of challenges in the lignocellulosic bioethanol production.

Thus, In order to intensify and optimize the lignocellulosic bioethanol production process, pressure-driven flat sheet microfiltration iMBRs were integrated into different processing stages. The application of a continuous iMBR led to a high ethanol productivity and yield (83% of theoretical yield) at high suspended solid content (up to 20% w/v) of wheat straw hydrolysate, and successful bacterial contamination separation from yeast (up to 93% removal). Moreover, using double-staged iMBRs for continuous hydrolysis-filtration and co-fermentation-filtration led to an effective separation of lignin-rich solids (up to 70% lignin) and sugar streams from the hydrolysate, and yeast cells from the fermentation product stream, stable long-term filtration performance (up to 264 h) at filtration flux of 21.9 l.m⁻².h⁻¹. In this thesis, filtration performance was thoroughly investigated, and effective physical fouling preventive approaches were applied to guarantee continuous bioprocessing. In addition, in order to remediate issues related to high content of inhibitors and presence of sequentially-fermented hexose and pentose saccharides in lignocellulosic fermentation, the cell-confinement approach of reverse membrane bioreactor (rMBR), which merges the benefits of iMBRs and cell encapsulation techniques, was introduced and applied in this thesis. It was observed that the high local cell density and diffusion-based mass transfer in the rMBR promoted co-utilization of sugars, and boosted cell furfural detoxification at concentrations of up to 16 g.l⁻¹. Moreover, considering the needs of rMBR processes for cell recirculation, membrane envelope degassing, and media conditioning, a novel membrane module was designed, developed, and patented in this thesis work.

Keywords: Lignocellulosic bioethanol, immersed membrane bioreactor, membrane fouling, reverse membrane bioreactor
List of publications

The results presented in the following articles form the basis of this thesis:


Statement of contribution

Amir Mahboubi’s contribution to the above-mentioned publications is as follows:

**Paper I.** Responsible for all the experimental work, data analysis, and manuscript preparation and revision

**Paper II.** Responsible for the idea development, part of the experimental work, data processing, and analysis and writing of the manuscript

**Paper III.** Responsible for developing the idea and designing the experiments, involvement in the experimental work, data analysis, writing of the manuscript and revision

**Paper IV.** Responsible for part of the idea development, all literature survey, data collection, writing of the manuscript and revision

**Paper V.** Responsible for the design and execution of the experimental work, data analysis, and manuscript preparation and revision

**Paper VI.** Responsible for the design of the experiment and part of the experimental work, data analysis, and manuscript preparation and revision

**Additional publications that are not included in this thesis**

**Patent:**


**Articles:**


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<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BW</td>
<td>Backwash</td>
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<tr>
<td>CBP</td>
<td>consolidated bioprocessing</td>
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<td>CP</td>
<td>concentration polarization</td>
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<tr>
<td>DDGS</td>
<td>dried distiller's grain with solubles</td>
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<tr>
<td>EEA</td>
<td>European Energy Agency</td>
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<tr>
<td>EPS</td>
<td>extracellular polymeric substances</td>
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<td>FPU</td>
<td>filter paper units</td>
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<td>FS</td>
<td>flat sheet</td>
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<td>GHG</td>
<td>greenhouse gas</td>
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<td>HF</td>
<td>hollow fiber</td>
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<td>HMF</td>
<td>5-hydroxymethylfurfural</td>
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<td>HRT</td>
<td>hydraulic retention time</td>
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<td>IEA</td>
<td>International Energy Agency</td>
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<tr>
<td>iLUC</td>
<td>indirect land-use change</td>
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<tr>
<td>iMBR</td>
<td>immersed membrane bioreactor</td>
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<tr>
<td>IRENA</td>
<td>International Renewable Energy Agency</td>
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<tr>
<td>IPC</td>
<td>Integrated Permeate Channel</td>
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<tr>
<td>LMH</td>
<td>liter per square meter per hour (flux)</td>
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<tr>
<td>MBR</td>
<td>membrane bioreactor</td>
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<tr>
<td>MF</td>
<td>microfiltration</td>
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<tr>
<td>MLSS</td>
<td>mixed liquor suspended solids</td>
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<tr>
<td>Mtoe</td>
<td>million tons of oil equivalent</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>NF</td>
<td>nanofiltration</td>
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<td>NSSF</td>
<td>nonisothermal SSF</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PES</td>
<td>polyethersulfone</td>
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<td>plate and frame</td>
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<tr>
<td>PVC</td>
<td>polyvinyl chloride</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<tr>
<td>RED</td>
<td>Renewable Energy Directive</td>
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<tr>
<td>rMBR</td>
<td>reverse membrane bioreactor</td>
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<tr>
<td>RO</td>
<td>reverse osmosis</td>
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<tr>
<td>SMP</td>
<td>soluble microbial products</td>
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<tr>
<td>SHF</td>
<td>separate hydrolysis and fermentation</td>
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<tr>
<td>SS</td>
<td>suspended solids</td>
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<tr>
<td>SSCF</td>
<td>simultaneous saccharification and co-fermentation</td>
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<tr>
<td>SSF</td>
<td>simultaneous saccharification and fermentation</td>
</tr>
<tr>
<td>SW</td>
<td>spiral wound</td>
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<tr>
<td>TMP</td>
<td>transmembrane pressure</td>
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<tr>
<td>TRL</td>
<td>technology readiness level</td>
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<tr>
<td>TB</td>
<td>tubular</td>
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<tr>
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<td>ultrafiltration</td>
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<tr>
<td>UN</td>
<td>United Nations</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast extract-peptone-dextrose</td>
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The journey

My research journey has had lots of twists and turns, and that made it very special. Here, I have tried to put together a fast forward review of the past years. I started my bachelor studies in my hometown, Isfahan, Iran, at Isfahan University of Technology (IUT) in the field of material engineering (metallurgy) in 2002. By the end of my bachelor studies, I was determined that I wanted to pursue my studies in material science. So I continued my MSc in material engineering at IUT, working on advanced material production techniques until 2010. As I experienced a very fruitful M.Sc. studies, doing a PhD was not a matter of doubt. However, I had bigger dreams; I wanted to do my Ph.D. studies abroad to have a broader insight of the field of research that I was involved in. This intention turned the period of 2010 to 2013 into such a bumpy ride with many ups and downs. In those days, getting a Ph.D. position in a sought-after university abroad was the simplest part of the process for an Iranian student. However, many of these students never got to see the destination universities. Here is the sad part, sometimes geographical and socio-political obligations, that you have not been a part of forming them, decide your future for you. Long story short, the period of 2010–2013 was full of granted Ph.D. positions and entry visa rejections, keeping me stranded between temporary jobs, from being a lab technician to a language teacher. As I had no plans to quit studying, based on my brother’s advice as he had previously studied at the University of Borås, I made the tough decision to study for another M.Sc. degree in resource recovery. This opened a new chapter in my life. Although I believe that there is nowhere better to study resource recovery than Sweden, in the beginning the field was totally new for me so there was a lot to catch up with. Soon I found myself on track again and couldn’t be happier with the choice I had made. I, who had a background in metallurgy, now had found a totally different new interest among the subjects covered during the master’s program: biotechnology. After the first year of MSc studies, I was sure that biotechnology is my thing, so I did my master’s thesis on fungal bioconversion of dairy waste. Midway through the thesis work, as advised by Professor Mohammad Taherzadeh, I put together a proposal on the application of membrane bioreactors (MBRs) for bioethanol production from lignocellulosic materials and applied for a Ph.D. position offered by the Flemish Institute for Technological Research (VITO NV), Belgium. This signaled another turning point in my research life, and it turned out to be the best one. In November 2014, I travelled to VITO NV, with the help and support of Wim Doyen and Heleen De Wever, my promoters at VITO, I defended the proposal, was granted the position and that’s how the Ph.D. life began. In 2015, I started my Ph.D. in industrial biotechnology at the University of Borås and entered the wondrous world of membrane science. As a toddler researcher, new to membranes and membrane bioreactors, I benefitted from the support, guidance and trust of great supervisors and colleagues that held my hands, walked along with me, and helped me to know my way around. Now, I am an
enthusiastic 4-year-old MBR researcher, eager to explore and learn more to dedicate more to the field of membrane science. The journey is to be continued …
Chapter 1

INTRODUCTION

In order to meet the soaring global energy demand, fossil energy and fuel sources have been exploited at an extraordinary rate. This has brought about general environmental, socio-political, and economic concerns regarding the impacts of using fossil fuels on climate change, energy dependency (security), and economic stability. In order to alleviate the issues confronted using non-renewable fossil sources, the global attention has veered toward the production of alternative environmentally-friendly renewable energy and fuel sources [1]. In this regard, in order to phase out fossil fuels used in road transport and reduce carbon emissions, vehicles using renewable fuels such as biofuels, hydrogen, electricity, etc. should be benefited from. Bioethanol has long been one of the main biofuels of interest that can be produced sustainably from a variety of feedstocks. However, the choice of raw material for bioethanol production has been a matter of controversy in the past decades. Although the commercial production of first generation bioethanol benefits from a rather simple and well-matured process, its future application has been doubted as the utilized sugar- and starch-based feedstocks (e.g., sugarcane and corn) compete with human food and animal feed, and its expansion increases the risk of indirect land-use change (iLUC). In this regard, in 2009 and later in 2018, the EU issued Renewable Energy Directive (RED I and II) mandates, not only to increase the share of renewable energy in the total energy used in the member states but also to put a cap on the consumption of first generation biofuels and motivate the application of advanced biofuels, such as second generation bioethanol, in the transport sector [2, 3].

Second generation bioethanol is produced from lignocellulosic materials such as agricultural and forest residues, and municipal and industrial wastes. The complex lignocellulosic bioethanol production process is comprised of pretreatment, hydrolysis, fermentation, and product recovery stages. Although, lignocellulosic materials are considered relatively abundant, cheap, and not in competition with food and feed, their recalcitrant nature and process complications hinder the technological advancement to worldwide commercialization. The challenges with lignocellulosic bioethanol production processes can be traced
through the chemical and energy intensive pretreatment process, sugar degradation and generation of inhibitory compounds, solid-liquid separation, lignin recovery, enzyme demand and cost, low sugar release rate, prioritized utilization pentose and hexose sugars, low ethanol yield and productivity, and ethanol recovery and purification [4]. Therefore, new technological breakthroughs are needed in order to shift from first to second generation bioethanol. In this thesis, membrane bioreactors have been used as the winning card in lignocellulosic bioethanol production to tackle technical issues hindering process feasibility.

Membrane technology has long been associated with water and wastewater treatment [5], due to the superior performance of semi-selective synthetic membranes in the separation and concentration of different medium constituents (components). The integration of membrane modules and bioreactors at different stages of lignocellulosic bioethanol production process can enhance the process feasibility by assisting continuous fermentation and hydrolysis, in situ product recovery, cell concentration and reuse, enzyme retention, sugar concentration, inhibitor removal, etc. In this thesis, continuous pressure-driven immersed membrane bioreactors (iMBRs) were used to increase the feasibility of lignocellulosic bioethanol production by merging and intensifying different processing stages associated with hydrolysis and fermentation, to operate hydrolysis and fermentation in a continuous mode to increase product recovery rate, to retain a high concentration of cells, prevent cell washout, and benefit from high yield and bioconversion rate, and to concentrate and separate lignin-rich solids. In addition, iMBRs were applied to address the challenges associated with bacterial contamination in fermentation systems through physical decontamination. Furthermore, in order to remediate the issues with sequential substrate utilization and fermentation inhibition by the common inhibitory compounds in lignocellulosic hydrolysate, the new membrane technique of reverse MBR (rMBR) was introduced in this thesis; thereafter, its effects on simultaneous sugar consumption and cell inhibitor tolerance and detoxification capacity was investigated.

1.1 Aims of the studies

The aims of the research studies conducted in this thesis were to tackle some of the commonly confronted issues in lignocellulosic bioethanol production process by modifying the conventional process stages with the introduction of membrane bioreactors. The research phases can be divided into three main parts:

- Development of a robust continuous hydrolysis-filtration and fermentation-filtration lignocellulosic bioethanol production using immersed membrane bioreactors (iMBRs) to intensify the processing stages and to have clear feed, product and by-product streams. Modification of iMBRs used for lignocellulosic bioethanol production to sustain filtration performance under high cell and suspended solid concentrations.
• Investigation of the effectiveness of physical bacterial contamination removal from fermentation systems through the application of iMBRs.

• Introduction and application of diffusion-based rMBRs for biological treatment of complex feed streams such as lignocellulosic hydrolysate that contain sequentially-consumed substrates and inhibitory compounds.

1.2 Thesis outline
This thesis includes five main chapters:

• Chapter 1 introduces the research topic and briefly describes the thesis motivation and research goals.

• Chapter 2 presents first and second generation bioethanol production and elaborates on different processing stages of pretreatment, hydrolysis, and fermentation for lignocellulosic bioethanol production.

• Chapter 3 presents the basics of membrane technology and its application in membrane bioreactors, with the focus on pros and cons of the application of immersed membrane systems.

• Chapter 4 includes different application approaches and benefits of intensification and optimization of lignocellulosic bioethanol production using pressure-driven iMBRs.

• Chapter 5 describes the principles and application potentials of the new technique of reverse MBR and presents the results on simultaneous substrate consumption and inhibitor detoxification during fermentation in an rMBR.

• Chapter 6 presents the conclusions drawn from this thesis work and suggestions for future research.
Chapter 2

LIGNOCELLULOSIC BIOETHANOL PRODUCTION

The growing evidence on the contribution of fossil-based fuel and energy to climate change and energy dependency has increased global awareness of the need for transition to low carbon economy. These concerns have been vividly reflected upon in the Sustainable Development Goals 7 and 13 in the 2030 Agenda for Sustainable development by the United Nations [6] and the interacting policies legislated by the European Union (EU) on the share of renewable energy sources in total energy consumption. In 2009, the EU set the directive RED I 2009/28/EC to increase and reach a 20% share of renewable energy in the total energy consumption by 2020 [2]. By the end of 2017, the share of renewable energy in EU had already reached 17.5% [7]. The EU proved its determination in shifting toward more sustainable renewable energy sources by topping the goal set in 2009 by about 12% in the revised directive RED II 2018/2001/EU [3]. In this regard, Sweden reached 54.5% renewable energy inclusion in its total gross energy consumption in 2017, surpassing the 49% goal set for 2020, and has targeted becoming carbon dioxide neutral by 2050 [8]. In addition, Sweden has an ambitious goal of having a complete fossil-free vehicle fleet by 2030 [9]. In order to phase out fossil fuels used in road transport, the use of vehicles running on renewable fuels such as hydrogen, electricity, and biofuels is to be encouraged. These defined environmental, economic, and socio-political incentives have also motivated the application of bioenergy sources. The share of bioenergy in EU has increased from about 71.8 Mtoe in 2005 to 119.7 Mtoe in 2017 and is estimated to reach 139.5 Mtoe by 2020 [10]. As one of the main sectors contributing to greenhouse gas (GHG) emissions, RED I defined and encouraged that 10% of the road transport energy is to be provided using biofuels by 2020 [2]. In a bigger picture, the International Renewable Energy Agency (IRENA) has estimated that 22% of the transport energy will come from biofuels by 2050 [11]. This means a rise from about 130 billion liters per year of transport liquid biofuels used in 2017/2018 to about 650 billion liters per year in 2050 [11]. This vision of the future demand proves the need for further developments in the biofuel production sector in the upcoming decades.
Industrial-scale commercial production of first generation biofuels from sugar- and starch-based feedstocks such as corn, wheat, sugarcane, and sugar beet has long been successfully practiced. However, the debate on food/feed versus fuel, and the effect on indirect land-use change, has gradually faded the role of first generation biofuels in the future biofuel market. In this regard, the EU capped the first generation biofuels transportation energy contribution to 7%, while the total energy share of biofuels is to be increased to 14% by 2030 [3]. Moreover, RED II motivates the replacement of first generation biofuels with advanced biofuels [3]. Bioethanol is a promising liquid biofuel for transportation that is projected to contribute about 7.3 Mtoe to EU bioenergy usage by 2020 [10]. Second generation bioethanol is one of the advanced biofuels produced from lignocellulosic materials such as agricultural residues. Lignocellulosic bioethanol alleviates the problems associated with the iLUC, and feedstock competing with food and feed confronted by first generation bioethanol production. As reported by the United Nations [12], by 2015 the world installed capacity for second generation bioethanol production has been around 1.39 billion liters. However, complexities in the lignocellulosic biofuel production process has limited its production, application, and market share compared to first generation bioethanol. As the lignocellulosic bioethanol production process still requires extensive process optimization, design, and process reliability improvements, the Technology Readiness Level (TRL) is evaluated at level 8 (First-of-a-kind commercial) [13]. According to the European Energy Agency (EEA), along with the risk of GHG emissions due to iLUC and high abatement costs associated with biofuels, the slow pace of development in second generation biofuels production is one of the main reasons contributing to slow growth in the portion of renewable energy used by the transport sector [14]. As presented in Figure 2.1, by 2017, a small fraction of European bioethanol production was derived from lignocellulosic bioethanol and other advanced biofuel feedstock (defined in RED I and II-part A annex IX) [15]. Therefore, efforts have been made in this thesis to open new grounds for technological improvements in lignocellulosic bioethanol production using membrane bioreactors.

Figure 2.1. The share of feedstock used and bioethanol produced in Europe by ePURE members in 2017 [15].
2.1 First generation bioethanol production

First generation bioethanol uses starch- or sugar-based crops as the feedstock for ethanol production. The feedstock used depends on crop abundancy based on location, climate, and production potentials. The two major players in the first generation bioethanol market are Brazil and the United States that use sugarcane and corn for ethanol fermentation, respectively. On the other hand, in Europe, the main feedstocks are cereals and sugar beet [16]. When the production of bioethanol doubled from 2005 to around 85.6 billion liters in 2010, Brazil and the United States were the main contributors and by 2016, they accounted for 73% of the production market [12].

The type of feedstock used defines the complexity and the number of treatment stages required for ethanol fermentation. While sugar-rich extract derived from sugarcane can be directly used for fermentation, starch-based feedstocks demand hydrolysis stages to release fermentable sugars prior to fermentation. Starchy materials are mainly comprised of two structural polymers, amylose and amylopectin, made up of glucose units. In order to ferment starch, the common approach is to liquefy starch using $\alpha$-amylase at elevated temperature, followed by saccharification by the application of glucoamylase [17]. In 2017, around 76% of the bioethanol produced in Europe (ePure members) was from corn (39%), wheat (30%), and other cereals (7%), along with 20% sugar-ethanol contribution for a total of 5.6 billion liters (Figure 2.1) [15]. Approximately, 13.22 million tons of cereals and sugar-based feedstocks were utilized for the aforementioned amount of renewable ethanol production [17].

Although the production of 1st generation bioethanol has long been successfully commercialized, there are controversial issues hindering its long-term application in the transportation market. One of these sustainability issues is the pressure on land-use in order to intensify crop production through direct and indirect land-use change [12, 18]. As agricultural lands are provided by removing forests to meet feedstock demands, CO$_2$ is emitted in substantial amounts in to the atmosphere [18]. Change of arable agricultural land used for cultivation of food and feed crops in favor of producing energy crops comes at the cost of food and feed scarcity and rise in price [19]. Feedstock cost can comprise up to 70% of the production cost in 1st generation bioethanol production [20]. Moreover, as throughout the feedstock production process, fossil fuels, and considerable amounts of fertilizers and irrigation water are applied, limited GHG emission reduction benefits are experienced [19, 20]. Considering the aforementioned environmental, economic, and ethical aspects revolving around first generation bioethanol, and in order to have no or low emissions due to indirect land-use change, production of second and third generation bioethanol have been the focus of great research in current decades.
2.2 Second generation bioethanol production

Lignocellulosic biomass, including agricultural and forest residues, is a promising feedstock for the sustainable production of second generation bioethanol [21]. In addition to being relatively cheap and abundant in different types, based on geographical location, their provision does not impose additional demands for land. Unlike, crop-based biomass used for 1st generation bioethanol production, lignocellulosic biomass does not compete with food and feed [22, 23]. Apart from all the advantages, lignocellulosic materials require extensive upstream treatment prior to fermentation compared to sugar- and starch-based materials. Lignocelluloses owe their recalcitrance to the tight and complex association of the three main structural polymers, cellulose, hemicellulose, and lignin in the plant cell wall [24]. Therefore, to access and release the fermentable sugars in the lignocellulosic biomass, their rigid structure needs to be destructed and dissociated prior to fermentation.

2.3 Lignocellulosic materials

Based on the plant species (e.g., softwood and hardwood) and growth conditions, lignocelluloses contain 10-35% lignin [25] and up to 70% cellulose and hemicellulose [26]. Plant cell walls are mainly composed of cellulose, a polysaccharide composed of cellobiose units formed by two glucose monomers having a β-1,4 glucosidic bond, hemicellulose, a mixed polysaccharide of pentoses (5 carbon monosaccharides such as arabinose and xylose) and hexoses (6 carbon monosaccharides such as glucose and mannose) monosaccharides and lignin, a complex of linked phenolic polymers [27, 28]. Cellulose microfibrils are mostly crystalline and composed of linear cellulose polymers that are tightly kept together with hydrogen bonding [29]. Compared to cellulose, hemicelluloses have a lower molecular weight and, depending on the plant species, are comprised of a variety of different saccharides made of galactose, glucose, mannose, arabinose, and xylose [27, 30]. While mannose and glucose (hexoses) comprise most of the hemicellulose in softwood (e.g. pine and spruce), xylose is the main monosaccharide other than glucose in hardwood (e.g. birch) [31]. In wheat straw used as the feedstock for fermentation in this research, xylose is the main pentose monosaccharide present in the hemicellulose (Papers I, II and VI). Another common difference between softwoods and hardwoods is that the former contain higher lignin content (25-30%wt) than the latter (20-25%wt) [32]. Lignin is relatively hydrophobic and tightly associated with cellulose and hemicellulose through hydrogen and covalent bonds. After cellulose, lignin is the most abundant polymer in vascular plants and contributes greatly to plant cell wall rigidity, resistance against biodegradation, and water transport. Lignin is a three-dimensional polyphenolic polymer with a highly amorphous nature, built up of monomers derived from p-coumaryl, coniferyl, and sinapyl alcohols [33-35]. In addition to the mentioned
structural components lignocellulosic materials contain different amounts (2-10%wt) of non-structural extractive compounds such as wax, resins, fat, phenolics, and ash (inorganics) [36].

Considering that every kg of wheat grain can yield an average of 1.3-1.4 kg of wheat straw, and that it is estimated that 2,175 million tons of wheat grain will be produced in 2019/2020, wheat straw could be a promising lignocellulosic substrate for bioethanol production [37, 38]. However, in order to obtain fermentable hexose and pentose sugars and biologically convert lignocellulose to bioethanol, lignocellulosic biomass should first be pretreated and hydrolyzed. In the following sections, the principles of common pretreatment and hydrolysis approaches are discussed with the focus on pretreated wheat straw slurry and hydrolysate used as substrate in this thesis (Papers I, II and VI).

2.4 Pretreatment and hydrolysis of lignocellulosic material

2.4.1 Pretreatment

The main upstream processing stage of lignocellulosic bioethanol production is pretreatment. The main goals sought in biomass pretreatment are the removal, redistribution or detachment of lignin, removal or solubilization of hemicellulosic saccharides, reducing cellulose crystallinity, and increasing structural porosity [39]. A successful pretreatment approach should open the lignocellulosic microstructure for better enzyme accessibility and hydrolysis, while avoiding sugar loss through the degradation and formation of fermentation hindering inhibitory by-products [40]. As pretreatment accounts for a great portion of the cost of lignocellulosic bioethanol production, its cost-effectiveness is of primary importance [39]. Depending on the type of feedstock, hydrolysis, and fermentation conditions, and other process limiting factors, pretreatment can include a mixture of physical, chemical, and biological processes.

Processes including chipping, grinding, milling, pyrolysis, and irradiation have been employed for the physical pretreatment of lignocellulosic materials [41]. The aforementioned physical treatments can enhance the enzymatic hydrolysis by making structural alternations to the lignocellulosic material by reducing the particle size, increasing the surface area and porosity, and above all, reducing the crystallinity [40, 41]. In addition, using a combination of physical and chemical pretreatments is also commonly practiced [41]. Steam explosion, ammonia fiber explosion, and CO₂ explosion are some commonly practiced physico-chemical pretreatment methods that benefit from the effect of a combination of high pressure and decompression together with high temperature and chemicals [40, 42]. Moreover, in order to enhance the enzymatic hydrolysis of cellulose, hemicellulose and lignin can be degraded or removed using microorganisms that leave cellulose rather intact. However, biotreatment of lignocellulosic material occurs at a relatively low rate [41]. The highest diversity is found among chemical pretreatment approaches
including ozonolysis, alkaline hydrolysis, oxidative delignification, organosolv process, acid hydrolysis, etc. Although powerful in hydrolysis of carbohydrates, concentrated acids such as H₂SO₄ are corrosive to the reactors and other equipment, and should be recovered to have a cost effective process [43]. However, as a successful alternative, dilute acid pretreatment can be applied at a milder condition to have an optimal conversion rate of cellulose and hemicellulose to their building blocks [42]. The wheat straw slurry used in this thesis (Papers I, II and VI) as the base lignocellulosic substrate was obtained in Sweden and was dilute-acid pretreated using 0.3-0.5% H₂SO₄ for 8 min at 185°C at SEKAB (Örnsköldsvik, Sweden). The dilute-acid treated wheat straw slurry used in this thesis was greatly particulated and viscous (Figure 2.2); therefore, to mitigate the technical problems confronted during the continuous fermentation, the slurry was further diluted 1:2 (Papers I and VI), 1:4 (Paper I) and 1:8 (Paper II) with distilled water. The composition of the 1:2 diluted pretreated wheat slurry is presented in Table 2.1.

![Figure 2.2. Images of (a) as-received pretreated wheat straw slurry and (b) large solid particles separated from the slurry using a kitchen sieve.](image)

**Table 2.1. The composition (g.l⁻¹) of pretreated and hydrolyzed wheat straw slurry (Paper I).**

<table>
<thead>
<tr>
<th>Composition of treated slurry (g.l⁻¹)</th>
<th>Acetic acid</th>
<th>Furfural</th>
<th>Glucose</th>
<th>HMF</th>
<th>Xylose</th>
<th>Xylitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-pretreated (as-received)</td>
<td>3.40±0.10</td>
<td>3.09±0.01</td>
<td>3.85±0.08</td>
<td>0.41±0.04</td>
<td>18.36±0.12</td>
<td>4.61±0.08</td>
</tr>
<tr>
<td>Pretreated and enzymatically hydrolyzed</td>
<td>4.55±0.17</td>
<td>2.93±0.01</td>
<td>50.03±0.13</td>
<td>0.35±0.03</td>
<td>26.16±0.07</td>
<td>4.94±0.05</td>
</tr>
</tbody>
</table>

### 2.4.2 Hydrolysis

In order to proceed with lignocellulosic ethanol fermentation, monomeric sugars building cellulose and hemicellulose should be released and solubilized. In this section, the two commonly used hydrolysis approaches of enzymatic and acid hydrolysis are briefly discussed.

Application of acid to the lignocellulosic material can play the roles of both pretreatment and hydrolysis. Acid treatment can lead to degradation and hydrolysis of both hemicellulose and cellulose, and
solubilization of the acid soluble fraction of lignin (ASL). Both concentrated and dilute acids (H₂SO₄, HCl, H₃PO₄ etc.) have been used for the hydrolysis of lignocelluloses. Although concentrated acid treatment can result in high hydrolysis yield (more than 90% sugar release), cost intensive acid recovery and harsh corrosive treatment conditions have hindered its widespread application [44]. On the other hand, dilute acid treatment, which can be used both as a stand-alone and in a stepwise process followed by enzymatic hydrolysis, has gained great attention. Apart from the benefits of reduction in acid consumption, dilute acid hydrolysis results in low total sugar release and degradation of sugars to fermentation inhibitory compounds [44, 45].

The other commonly applied hydrolysis approach is enzymatic hydrolysis, which involves the utilization of cellulolytic or a mixture of cellulolytic and hemicellulolytic enzymes. Enzymatic hydrolysis is usually conducted at a rather mild pH (4.8-5) and low temperatures (45-50°C). However, in order to have a successful hydrolysis, yielding the highest possible soluble sugars, a pretreatment stage is required to open up the lignocellulosic microstructure making the carbohydrates more accessible to the enzyme [46]. The main benefits of enzymatic hydrolysis over acid hydrolysis are that mild conditions (pH, temperature, and pressure) are required, high hydrolysis yield and sugar release are achievable, and low or no fermentation inhibiting products are formed [24]. However, increase in the hydrolysis products (e.g., glucose and cellobiose) can hinder the enzymatic activity, hydrolysis occurs at a lower rate compared to acid application, and unproductive and unwanted attachment of enzyme to lignin is experienced [47]. The cost of enzymes is another concern when it comes to the economy of the hydrolysis process. In this thesis, the Cellulase Cellic Ctec2 enzyme (Novozymes, Denmark) was applied according to the procedure implied by Ishola et al. [48] for enzymatic hydrolysis of wheat straw slurry. In this regard, considering the activity of the enzyme measured according to the NREL protocol [49], enzymatic hydrolysis was performed using enzyme loadings up to 15.7 filter paper units (FPU).g SS⁻¹. The composition of hydrolyzed 1:2 diluted wheat straw hydrolysate is featured in Table 2.1.

### 2.4.3 Inhibitory by-products of pretreatment and hydrolysis

The main goal sought by pretreatment and hydrolysis of lignocellulosic biomass is maximum release of structural sugars. However, depending on the type of pretreatment and hydrolysis applied, along with the production of fermentable sugars, different by-products are released or produced. The by-products mainly include weak carboxylic acids, furan aldehydes, and phenolic compounds. These compounds can inhibit both yeast growth and fermentation. It has been reported that when yeast cells are in direct contact with these inhibitory compounds, the lag phase increases, some catabolic enzymes such as pyruvate dehydrogenase, acetaldehyde dehydrogenase, etc. are negatively affected, cell viability decreases,
intracellular pH drops and cell membrane integrity is disturbed [50, 51]. Some of the inhibitors found during the lignocellulosic biomass pretreatment and hydrolysis and their origin are presented in Figure 2.3.

![Diagram of lignocellulose metabolic pathways](image)

**Figure 2.3.** Some of the main inhibitory compounds (dashed lines) originating from pretreatment and hydrolysis of lignocellulosic biomass (*Paper VI*).

The inhibitory compounds are mainly degradation products of hemicellulosic and cellulosic monomers, wood extractives, and phenolic derivatives [52, 53]. These inhibitory compounds are either converted through *in situ* detoxification (e.g., furan aldehydes), or not bio-transformed under anaerobic condition (e.g., acetic acid) by *Saccharomyces cerevisiae* [54-56]. *S. cerevisiae* is the most dominant microorganism for industrial ethanol fermentation due to its high capacity for ethanol fermentation along with high ethanol tolerance [57, 58]. Furfural and 5-hydroxymethylfurfural (HMF) are the main furan aldehydes in lignocellulosic hydrolysate, which are degradation products of pentose and hexose sugars, respectively. Further degradation of furan aldehydes results in production of formic and levulinic acid [59]. The dilute acid pretreated wheat straw slurry used in this thesis contained around 6 g.l⁻¹ furfural and 0.8 g.l⁻¹ HMF. In specific fermentation conditions, even as low as 1 g.l⁻¹ of furfural can hinder the cell activity [50]. The dilute acid pretreated wheat straw slurry was diluted to different degrees, and used as the substrate for hydrolysis and fermentation in *Papers I, II* and *VI*.

Yeast is capable of detoxifying the fermentation medium by converting furfural and HMF into the less inhibitory compounds furfuryl alcohol and 2,5-bis-hydroxymethylfuran, respectively [60]. Furfural and HMF are considered as electron sinks (acceptors); therefore, in their presence, the cell glycerol production drops [61]. It is reported that in the biotransformation of furan aldehydes to alcohols, NAD(P)H dependent alcohol dehydrogenases are involved [51]. At lower furfural concentrations, NADH is used as a reducing
agent [62, 63]. As HMF is converted at a lower rate and is less toxic to yeast than furfural [64], in this thesis the main focus has been on the effect of furfural on cells and fermentation (Papers V and VI). Overall, the system’s robustness in in situ detoxification of furan aldehydes depends directly on the cell to inhibitor ratio [24]. Therefore, yeast retention and high local cell density in the fermentation reactor is of great importance when hydrolysates containing different levels of inhibitors are handled. In this regard, in Papers I and II, continuous fermentation of wheat straw hydrolysate, containing about 0.75-3 g.l\(^{-1}\) furfural and 0.1-0.4 g.l\(^{-1}\) HMF, was practiced. Moreover, in Papers V and VI, in order to investigate the inhibitor tolerance and detoxification capacity of yeast at high local cell density in a rMBR (Paper IV), fermentation was performed using xylose-glucose semi-synthetic media and wheat straw hydrolysate containing 2.5-10 g.l\(^{-1}\) (Paper V) and 1.7-20 g.l\(^{-1}\) (Paper VI) of furfural, respectively.

Carboxylic acids can also be present at different levels in lignocellulosic hydrolysate. Acetic acid, a product of deacetylation of hemicellulose and to a smaller extent lignin, is the main carboxylic acid that is found in the hydrolysate. As mentioned, levulinic and formic acid can also be present in lignocellulosic hydrolysate if furan aldehydes are dehydrated at a high temperature and an acidic condition during pretreatment. While formic acid can be generated as both furfural and HMF are degraded, HMF is the precursor for levulinic acid formation [26, 41, 50, 65]. Other weak acids originating from wood extractives can also be present in the hydrolysate in small amounts [66]. The presence of a high concentration of organic acids can have a negative impact on cell growth and ethanol fermentation. The problem with carboxylic acid inhibition occurs when high concentrations of undissociated acids are present. Undissociated acids that are soluble in lipids diffuse through the cell membrane, experience the near neutral intracellular pH, and dissociate while releasing hydrogen ions, thus plunging the pH. Therefore, to restore the cytosolic pH, an ATP dependent transporters consume the cell energy [67]. As mentioned, the inhibitory effect of the acid is directly dependent on medium pH and acid pK\(_a\). Although lignocellulosic hydrolysates usually have a higher content of acetic acid, formic acid due to its smaller molecular size and lower pK\(_a\), and levulinic acid due to its higher lipophilicity, impose higher inhibition [50, 68].

Phenolic compounds in lignocellulosic hydrolysate are generated from the breakdown of lignin and alkaline extractives [69]. The amount and kind of phenolic compounds found in the hydrolysate depend on the type of raw biomass, and the pretreatment and hydrolysis approach [61]. It has been reported in the literature that S. cerevisiae is capable of converting and detoxifying some of the phenolic compounds such as ferulic and cinnamic acid, which are inhibitory to fermentation [70].
2.5 Fermentation of lignocellulosic material

In comparison to the first generation bioethanol, lignocellulosic bioethanol fermentation processes have greater complexities. The efficiency of lignocellulosic ethanol fermentation is mainly dependent on the type of lignocellulosic biomass, type and intensity of the pretreatment and hydrolysis, fermenting microorganism, and fermentation mode. In the following sub-sections, the characteristics of the microorganisms, and pros and cons of different fermentation modes used for the production of lignocellulosic bioethanol are reviewed.

2.5.1 Fermenting microorganism

During pretreatment and hydrolysis of lignocellulosic materials, different hexoses (glucose, galactose, mannose, rhamnose, and fucose), pentoses (xylose and arabinose), undesirable and inhibitory lignin residues, furan aldehydes, weak organic acids, and phenolic compounds can be released. Therefore, unlike in crop-based ethanol fermentation, where glucose is the only monomeric sugar released, in fermentation of lignocellulosic substrates, the microorganism of choice should be able to utilize both pentose and hexose sugars in inhibitory media [71]. In order to have an efficient lignocellulosic ethanol fermentation, the ethanologenic microorganism should have the ability to consume a wide range of substrates, have relatively high tolerance to ethanol, have high inhibitor tolerance and in situ detoxification ability, show robustness when experiencing sudden changes in pH, temperature and salt/sugar concentration and above all, provide high ethanol yield. This imposes great demands on the characteristics of the potential microorganism used for lignocellulosic ethanol production.

*S. cerevisiae* has long been considered as the dominant microorganism when it comes to industrial crop-based ethanol production. The wild *S. cerevisiae* is the prime microorganism in bakeries and breweries, has high ethanol production and tolerance capacity, and is capable of withstanding rather unpleasant fermentation conditions such as the presence of inhibitors and sudden changes in the pH (acidity), temperature, salt and sugar concentrations [71, 72]. The wild-type *S. cerevisiae* can consume a variety of hexose monomers such as glucose, fructose, galactose, mannose, and some of their disaccharides such as sucrose and maltose; however, it either does not utilize pentoses such as xylose and arabinose or the consumption occurs at a very low rate [71, 73, 74]. The famous ethanologenic bacteria, *Zymomonas mobilis*, also suffers from the same restrictions in pentose uptake [71]. Although some yeast strains such as *Pachysolen tannophilus*, *Candida shehatae*, and *Pichia stipites* are able to consume pentoses, their industrial scale application is limited by their low ethanol production and ethanol tolerance, sensitive micro-aeration requirement, and poor inhibitor and low pH tolerance [75, 76]. As natural microorganisms cannot efficiently ferment lignocellulosic hydrolysate, by the help of metabolic engineering, essential traits that are to be possessed have been transferred to the fermenting microorganism. Therefore, in the past four decades, there
has been extensive research on engineering a variety of microorganisms with features required for lignocellulosic ethanol fermentation. The targeted traits mainly revolve around the ability for hydrolysis of cellulose and hemicellulose, having high tolerance to low pH, high temperature and inhibitory media, and simultaneous sugar utilization [71]. The strain of *S. cerevisiae* employed for the fermentation of wheat straw hydrolysate in Papers I, II, V and VI was a xylose-consuming recombinant. However, for this strain, as for some other reported xylose-consuming *S. cerevisiae*, the same cell membrane protein transporters are used for glucose and xylose transportation to the intracellular space [77-81]. As a result, xylose transportation and consumption only occur if the hexose (glucose, mannose, etc.) concentration is low enough [77-80]. This preference in sugar utilization hinders simultaneous sugar consumption, thus, challenging the lignocellulosic ethanol fermentation productivity [81]. The new concept of rMBR, introduced in Paper IV, presents the principles of a membrane cell retention technique that can assist co-utilization of monomeric sugars in complex substrates such as lignocellulosic hydrolysate. This approach was applied in Papers V and VI for the fermentation of xylose-glucose-furfural semi-synthetic media and the liquid fraction of wheat straw hydrolysate, where simultaneous sugar consumption and inhibitor detoxification were studied.

### 2.5.2 Batch, fed-batch, and continuous fermentation

Due to the presence of inhibitory compounds, different monomeric sugars and high concentration of suspended particles in lignocellulosic hydrolysate, the choice of cultivation method is of great importance. The potential of batch, fed-batch, and continuous fermentation modes for lignocellulosic bioethanol production is considered in this section.

Considering the simplicity and cost of operation, batch fermentation has for long been the preferred choice of technology in large-scale alcoholic plants. However, in every cycle of batch fermentation, broth should be harvested, equipment should be cleaned, sterilized and refilled, new cell seed culture provided etc. This makes batch fermentation a labor intensive, time consuming, and low productivity process. To enhance the productivity and bioconversion rate in a batch process, cells should be separated and reused for consecutive cycles. Conventionally, cell recycling is done through centrifugation and sedimentation approaches [82].

Since suspended cells are all exposed to the same concentrations of medium constituents in batch fermentation, the fermentation of inhibitory media such as dilute acid treated lignocellulosic hydrolysate is problematic. Depending on the type and concentration of the inhibitory compounds in the lignocellulosic hydrolysate, yeast cells can either detoxify the media, which will cause a long lag phase, or be completely toxified, which leads to process failure [65, 68, 83]. Using yeast with a higher tolerance to inhibitors, a higher yeast cell concentration and detoxifying the medium prior to fermentation could contribute to the
robustness of batch fermentation of lignocellulosic hydrolysate. In Papers V and VI, batch fermentation of semi-synthetic inhibitory media and wheat straw hydrolysate was performed using high locally concentrated yeast cells, respectively. In these batch cultivations, cells were completely separated from the feed media and product stream, allowing them to be reused for several consecutive batches. Moreover, high local cell concentrations of around 65 g.l\(^{-1}\) (total cell concentration in the reactor 0.8±0.1 g.l\(^{-1}\) (Paper V) and 1.9 ± 0.2 g.l\(^{-1}\) (Paper VI)), were provided to enhance the inhibitor tolerance and detoxification rate, and eliminate the need for hydrolysate detoxification prior to fermentation. In these robust batch fermentations, different sugars were simultaneously utilized and ethanol was produced, while the medium containing 2.5-10 g.l\(^{-1}\) (Paper V) and 1.7-20 g.l\(^{-1}\) (Paper VI) of furfural was actively detoxified.

Another approach to increase the process volumetric productivity is to conduct fermentation in a continuous mode. In continuous fermentation, the bioreactor is continuously fed with the substrate at the same rate as the product is removed, therefore, higher productivity and less ethanol inhibition are experienced compared to batch fermentation. Moreover, the process can be better controlled, and there are less maintenance and operational requirements. However, as the yeast cells are removed along with the product, the highest operational dilution rate and subsequently, volumetric productivity, are limited by the yeast’s maximum growth rate. If a continuous fermentation that is at steady-state \(\frac{dx}{dt} = 0\) (where \(x\) is the cell concentration and \(t\) is time) has a high concentration of substrate \((S>>K_s)\), uses sterile feed, and has inconsiderable cell death, according to the Monod growth model (2.1) [84], the maximum specific cell growth rate \(\mu_{\text{max}}\) and dilution rate \(D\) are equal (2.2):

\[
\frac{dx}{dt} = \text{cell growth} - \text{cell washout} = x\mu - xD
\]

\[
\mu = \frac{\mu_{\text{max}}S}{S + K_s}
\]

\[
\frac{dx}{dt} = 0 \text{ and therefore } D = \mu_{\text{max}}
\]  

Where \(\mu_{\text{max}}\) is the yeast maximum specific growth rate (h\(^{-1}\)), \(S\) is the concentration of the limiting substrate (g.l\(^{-1}\)), \(\mu\) is the yeast specific growth rate, and \(K_s\) is the half saturation constant (g.l\(^{-1}\)).

Although volumetric productivity and dilution rate are directly related, in order to prevent cell washout and process failure, the dilution rate cannot go beyond \(\mu_{\text{max}}\). Considering that lignocellulosic hydrolysate is an inhibiting medium that reduces yeast’s growth rate [85], to prevent washout during continuous cultivation, lower dilution rates should be used, which negatively impacts the ethanol volumetric productivity [62]. Considering the abovementioned challenges, uncoupling the dilution rate and cell growth can boost the volumetric productivity of the process. Compared to batch fermentation of lignocellulosic hydrolysate, in
continuous mode, based on the detoxification capacity of yeast and application of appropriate dilution rates, the concentration of inhibitors in the bioreactor can be controlled. An approach to increase the detoxification rate is to have a higher cell density by cell retention, immobilization, or recirculation [86-89]. However, it should be considered that long term continuous cultivations are more prone to bacterial contamination than batch cultures [90]. Contamination is likely to be introduced into continuous cultivation systems by non-sterile instruments, reactors, pipelines of the feed stream, added chemicals and nutrients, or through recycling yeast [91, 92].

Fed-batch cultivation possesses the advantages of both batch and continuous cultivation modes. In this fermentation mode, first, yeast is batch cultivated in a rather small volume and then the bioreactor is fed at different regimes with fresh feed until the maximum reactor working volume is reached [93]. Through fed-batch fermentation of dilute acid treated hydrolysate, yeast is not exposed to intolerable inhibitory content [94], no cell washout occurs, and apparently the constant low substrate concentration will mitigate glucose repression and prioritize consumption of sugars.

2.5.3 **SSF, SHF, and CBP**

Methods used for the application and integration of enzymatic hydrolysis and fermentation of lignocellulosic material can be divided into three main groups: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and consolidated bioprocessing (CBP) (also known as direct microbial conversion) [71]. Based on process customization and addition/removal of process stages, these three main groups have been sub-categorized to simultaneous saccharification and co-fermentation (SSCF), nonisothermal simultaneous saccharification and fermentation (NSSF) [95] and simultaneous saccharification, filtration and fermentation (SSFF) [96], etc. Among these processing configurations, SHF and SSF are the most studied and explored.

In SHF, enzymatic hydrolysis of lignocellulosic substrate is performed using cellulases and/or hemicellulases, separate from the bioreactor where fermentation is conducted. Using this approach, hydrolysis and fermentation can be performed at different optimum pH and temperature [20]. However, as the hydrolysis is performed in batch, the increase in the content of cellulose breakdown products, cellobiose and glucose, may inhibit the enzymatic activity [97]. In addition, as the complete enzymatic hydrolysis cycle may take up to four days, contamination can be an undesirable issue that can arise [24, 98]. As hydrolysis and fermentation require separate units, the cost of the large-scale application is an additional concern [24].

In Paper I, a modified version of SHF was applied as first, the wheat straw slurry was enzymatically hydrolyzed at 50°C and pH 5 for 24 h and then, autoclaved before continuous feeding to the fermentation...
MBR. In this work (Paper I), only co-fermentation and filtration were conducted in continuous mode. As in the fermentation stage, xylose-consuming *S. cerevisiae* was used to ferment both hemicellulosic and cellulotic pentoses and hexoses the process was referred to as co-fermentation [24, 95].

On the other hand, in SSF, hydrolysis and fermentation take place in the same unit. Therefore, the end-product enzyme inhibition is mitigated as the sugars released are continuously fermented to ethanol by the ethanologenic microorganism [99]. Compared to SHF, SSF is reported to result in higher ethanol yield [100-103]. Although the presence of ethanol may remediate the contamination issue, high concentrations may inhibit the enzymatic activity [104]. However, compared to SHF, SSF is performed at suboptimal conditions as hydrolysis and fermentation have different temperature and pH requirements. Although the optimum temperature for fermentation by *S. cerevisiae* and hydrolysis by cellulase are 30-35°C and 45-50°C, respectively [71], SSF is commonly conducted at 35°C [105, 106]. Furthermore, since the lignocellulosic substrate being enzymatically hydrolyzed usually contains a high content of particulate solids, it is hard to separate and reuse the yeast. This means every new batch of experiments requires fresh yeast preculture propagation and preparation [99]. In order to improve the shortcomings of the SSF method, different process modifications have been practiced.

A modification to the conventional SSF is nonisothermal simultaneous saccharification and fermentation (NSSF), which provides simultaneous saccharification and fermentation in separate reactors. With this approach, both hydrolysis and fermentation can be conducted at their optimum pH and temperature; therefore, enzyme activity can be used at full potential, and maximum yield can be reached in shorter time intervals than SSF [24]. Another improvement to SSF is simultaneous saccharification and co-fermentation (SSCF), where a single microorganism such as xylose-consuming *S. cerevisiae* is used in a single stage hydrolysis and fermentation bioreactor for co-fermentation of hexose and pentose sugars [107, 108]. Conventionally, pentoses resulting from the hydrolysis of hemicellulose during the pretreatment of hardwood and agricultural residues, such as wheat straw, are separated and could be further fermented to ethanol in a separate bioreactor [24]. However, in order to combine the advantages of SHF and SSF, the addition of a post-hydrolysis filtration stage, prior to fermentation, has been studied. This process, regarded as SSFF, includes a hydrolysis stage at an optimum condition followed by a filtration stage to remove the particle-free sugar-rich medium that is continuously fed to the fermentation reactor [96, 109]. In this batch SSFF process, end-product inhibition does not occur as sugars released due to saccharolytic activity of the enzyme are constantly removed. As in SSFF the fermentation broth is constantly recirculated back to the hydrolysis reactor, in order to prevent cell washout and ease product-cell separation, cells should be retained in the fermentation reactor. Therefore, the microorganism used should be either flocculative, encapsulated, immobilized on a surface or porous matrix, or retained using a membrane, etc. SSFF proved to be an upgrade
to SSF when used for hydrolysis and fermentation of wheat straw hydrolysate, as higher ethanol yields were achieved and cells were efficiently retained in the fermenter to be used for several consecutive batches [110].

Like SSF, in CBP, hydrolysis and fermentation occur in the same reactor using single or double microbial cultures. However, in this method, the microorganism(s) secrete(s) saccharolytic enzymes to hydrolyze the substrate and subsequently ferment ethanol from released sugars (no need for external enzyme addition) [111]. In addition to having high ethanol tolerance, the microorganisms used for this purpose should possess high cellulolytic enzyme secretion and ethanol production ability. However, the application of a single microorganism such as *Clostridium thermocellum* or *Neurospora crassa* for CBP of lignocellulosic feedstocks has proven to be a slow process, yielding low amount of ethanol, along with undesirable by-products [24]. Therefore, in order to have an efficient CBP of lignocellulosic substrate, microorganisms capable of high ethanol production or those with high cellulolytic enzyme production abilities should be genetically modified to possess desired traits [112, 113].

Regarding the approaches taken for the integration of enzymatic hydrolysis and fermentation in this thesis, in **Paper I**, dilute acid pretreated wheat straw was first hydrolyzed then co-fermented, and the ethanol-rich stream filtered in a continuous mode. In this fermentation unit, a high suspended solid (SS) mixture of *S. cerevisiae* and whole hydrolysate were present. In addition, by applying the same set-up and using a semi-synthetic xylose-glucose media, a successful co-fermentation was performed resulting in the utilization of more than 97% of xylose and all the glucose content. Moreover, the total ethanol yield on xylose and glucose reached 89% of the theoretical, representing a productivity of 4.6 g.l⁻¹.h⁻¹. In order to enhance the processing conditions and intensify the lignocellulosic ethanol hydrolysis and fermentation stages, in **Paper II**, a double-staged continuous hydrolysis-filtration and co-fermentation-filtration was proposed and practiced. This system merges the advantages of SHF and SSCF. In this regard, hydrolysis was performed at the desirable temperature and pH, and the end-product inhibition was eliminated as the lignin-rich solid residuals (up to 70% lignin) were separated from the released sugars through continuous filtration. Continuous hydrolysis of wheat straw slurry, solid particle retention, and sugar removal led to a volumetric sugar release rate of about 1.4-3.3 g.l⁻¹.h⁻¹. Simultaneously, the fermentation reactor was continuously fed with fresh medium, and particle-free ethanol-containing filtrate was recovered, increasing the downstream processing efficiency. During fermentation, there was no cell washout at any dilution rate, and cells were retained and concentrated in the bioreactor. This enhances the co-utilization of pentoses and hexoses, and inhibitor tolerance and *in situ* detoxification. Complete cell retention gives the possibility to conduct continuous fermentation at a desired dilution rate to reach higher ethanol productivity.
Chapter 3

MEMBRANE BIOREACTORS

Membrane bioreactor can be defined as a membrane module integrated with a reactor containing biological catalysts [114]. Membrane separation technologies and MBRs have long attracted attention in water and wastewater treatment [115-118]. In the late 1960s, the first commercially developed MBRs were used for ship-board sewage treatment [5]. As reported by Hardt et al. [119], around the same time bench-scale membrane systems were combined with conventional activated sludge process for wastewater treatment. Since then membranes and MBRs have found diverse applications in a wide range of engineering processes [5, 120-124] from in situ product recovery [125, 126] to desalination, and agricultural and industrial wastewater treatment [123, 127-130]. The main advantages of membrane bioreactors which have led to their widespread application are that: they have a small footprint as they intensify the process by reducing the processing stages, assist the retention and reuse of the biocatalyst, help removing the product of bioreactions leading to less product inhibition, and provide the ability to have a continuous operation with higher productivity [118]. Membrane separation is favored in biotechnological processes, as the separation is performed at mild conditions (e.g., temperature), without the addition of external chemicals; therefore, products and biocatalysts are not degraded, deactivated, or denatured [131]. In MBRs, membranes are mainly applied to support, immobilize, recycle, or separate biocatalysts including enzymes, bacteria, fungi, yeast, mammalian cells, etc., or to assist in situ recovery of reaction products (metabolites) as a simple separation unit [4, 126, 132, 133].

In this chapter, membranes, membrane modules and processes, MBR configurations, modes of filtration, and issues regarding membrane fouling are briefly discussed. In this regard, membrane modules and MBR set-ups developed in this thesis for lignocellulosic bioethanol production are presented in detail. In addition, the effectiveness of fouling preventive approaches of backwashing and air/gas scouring in membrane surface cleaning and sustaining filtration performance is evaluated.
3.1 Membranes, modules, and processes

In the following sections membranes, membrane processes, modules and configurations, including the ones applied in this thesis, are briefly described.

3.1.1 Membranes and membrane processes

As defined by Giorno et al. [134], membranes are discontinuous barriers (phases) separating two phases, permitting the transfer of matter, energy, and information in between phases based on their selective or nonselective properties. Based on their exceptional separation capabilities, membranes and membrane processes have found their way in a wide range of industrial applications such as water and wastewater treatment, and production of dairy, food, beverage, and pharmaceuticals [5, 135, 136]. The application of synthetic membranes in biological processes is defined based on their ability to selectively retain specific components of the media, while being permeable to the rest. In membrane processes, components of the feed mixture are divided into two groups: part of the feed that passes through the membrane, called permeate or filtrate, and the fraction that is retained by the membrane, called retentate. The membrane can owe its selectivity to its pore size and structural morphology, charge, affinity or hydrophobicity [5]. The driving force for the permeation and transport of compounds through a membrane can be the presence of pressure, concentration, temperature or electrical potential gradient(s) across the membrane surface. The magnitude of this gradient is one of the main parameters defining the rate of component transport through the membrane [134].

Membranes can be solid or liquid, and made of biological, organic, and/or inorganic materials. Based on the structural formation, membranes can be divided into symmetric or asymmetric and homogeneous or heterogeneous [120]. As the term “membrane” covers a wide range of materials and structures from coatings and packaging materials to highly selective synthetic membranes, it is preferable to categorize membranes based on their functions. Membranes can be classified as porous and dense. Depending on the application, porous membranes can act as a conventional filter or sieve, or a medium bringing two phases in contact. These synthetic membranes are solid, made of polymers, glasses, metals, or ceramics and have a rather defined pore (or hole) diameter [120, 137]. The membranes are further grouped into macro-, meso-, micro- or non-porous membranes with pore sizes of 10 µm-50 nm, 2-50 nm, 0.1-2 nm and no permanent pores, respectively. In dense membranes, solution-diffusion through free volumes in the structure determines the separation mechanism [138]. In general, pressure-driven membrane processes can be further classified based on the membrane’s pore size, transmembrane pressure (TMP) (i.e., pressure difference built over the membrane for filtrate removal), and separation principle, to microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO) [120]. As the main focus of this thesis, the discussions made are based on the functions and applications of microfiltration membranes.
Microfiltration involves the application of membranes with an average pore size of 0.1-10 µm to physically separate components of a mixture. Microfiltration can either act as a sieve for the separation of suspended particles, colloids, and microorganisms, etc. or to compartmentalize two miscible and/or immiscible phases/mixtures [139]. One of the main benefits of microfiltration over other pressure-driven membrane processes is that the filtration can be performed by the application of a small pressure difference (<0.3 bar) over the membrane surface [140]. Mainly, two factors define the effectiveness and cost of microfiltration: filtrate flux through the membrane relative to the applied TMP (permeability) and selectivity of the microfiltration based on rejection or retention of compounds by the membrane. Many parameters such as feed/medium quality (pH, temperature, concentration, particle content, etc.), membrane quality and characteristics (pore size, surface charge, hydrophobicity, etc.) and filtration parameters (TMP, filtration regime, etc.) should also be taken into consideration in a microfiltration process. Microfiltration has been widely employed in water and wastewater treatment for producing particle-free water with less turbidity, removing bacteria and protozoa, and assisting nutrient removal in aerobic MBRs, in the milk and dairy industry for separation of micellar casein, whey fat, and reducing bacterial concentration, as well as in fruit juice and wine clarification, etc. [135, 140-143].

For purposes of this thesis, different microfiltration membranes were applied. In Papers I and II, a hydrophilized polyethersulfone (PES) membrane with a nominal pore size of 0.3 µm was applied. In these research phases, the membrane was used to remove the residual lignin-rich solids from the wheat straw hydrolysate in continuous hydrolysis and retain the yeast cells for high cell density continuous fermentation. In Paper III, the goal was to effectively separate bacterial contamination from yeast cells in a continuous fermentation and filtration. The yeast *S. cerevisiae* had an average diameter of 2.5±0.2 µm, while the bacillus *Enterobacter cloacae* (bacterial contamination) had an average length of 0.7–1.2 µm and diameter of 0.3–0.5 µm. In this regard, in order to investigate the effect of the membrane pore size on yeast-bacteria filtration and separation, open pore PES membranes with average pore sizes of 1.0 and 2.4 µm were used. As discussed in section 4.3.2, by the application of a 2.4 µm membrane and adjusting the filtration parameters, yeast cells were successfully retained in the reactor, while bacteria were washed out through the membrane. In Papers V and VI, membrane panels were used to encase and retain the yeast cells and separate them from the bulk feed media. In these research phases, the microfiltration membrane acted as a biomass contactor that brings cell biomass and feed media in contact through membrane pores, while preventing mixing of the phases. PES membranes with a pore size of 0.3 µm were used to confine the yeast cells. Unlike the microfiltration membranes used in Papers I, II, and III that were used in pressure-driven filtration, in Papers V and VI, microfiltration membranes were used in a concentration-driven separation process, where pores act as open channels for diffusion of different compounds in between the two compartments.
3.1.2 Membrane modules

In order to use a membrane for filtration purposes, the membrane should be formed, framed, packed, and/or housed into a unit. These fabricated membrane units are called membrane modules. Depending on the configuration of the membrane in a module, the membrane modules can be divided into three main categories of: flat sheet (FS), tubular (TB), and hollow fiber (HF). The module categories can be further sub-classed based on the application of pressure or vacuum for filtration. For example, in FS plate and frame (PF) and spiral wound (SW) modules, positive pressure is used for filtration, while in FS immersed panels, under-pressure (vacuum) is the main filtration driving force. Table 3.1 lists some basic membrane module configurations and their examples. The idea of the application of immersed FS modules in MBRs was developed by KUBOTA® and Yamamoto et al. [144] by the late 1980s. The majority of the wastewater treatment MBRs use immersed HF or FS membrane modules as they are not capital and energy intensive [5, 140].

Table 3.1. Common membrane configurations, modules, and commercial examples (membrane modules commercially used in MBR are also indicated) [5, 140].

<table>
<thead>
<tr>
<th>Membrane configuration</th>
<th>Module</th>
<th>Commercial examples</th>
<th>MBR application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hollow fiber (HF)</td>
<td>Immersed</td>
<td>GE ZeeWeed®, Mitsubishi Sterapore™,</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>In pressure vessel</td>
<td>Asahi Microza®, GE ZeeWeed®</td>
<td>-</td>
</tr>
<tr>
<td>Flat sheet (FS)</td>
<td>Immersed</td>
<td>Kubota, Toray Membray, Microdyn Bio-cel®</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Spiral wound</td>
<td>Hydranautics ESPA®, Toray R0membra</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Plate and frame</td>
<td>Electrocell Syn cell®</td>
<td>-</td>
</tr>
<tr>
<td>Tubular (TB)</td>
<td>Pressure filtration</td>
<td>Koch Abcor™</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Vacuum filtration</td>
<td>Pentair Airlift™</td>
<td>+</td>
</tr>
</tbody>
</table>

3.1.3 Flat sheet membrane modules used in this thesis

In order to benefit from the advantageous properties of FS immersed membrane modules in the production of lignocellulosic ethanol, customized FS membrane modules were developed and applied for hydrolysis and fermentation in Papers I, II, III, V, and VI. The modules were 2nd generation Integrated Permeate Channel (IPC) membrane panels, which were specifically tailored for this thesis work at the Flemish Institute for Technological Research (VITO NV, Belgium) to fit 2-4 liter lab-scale reactors. As presented in Figure 4, the panels include two layers of hydrophilized PES microfiltration membrane casted on each side of a spacer-fabric support (weft-type of polyester). One of the great advantages of these IPC panels is their great strength in preserving integrity when extensive backwashing/backflushing is required for membrane
surface cleaning. These membrane panels owe their superior robustness to the strong mechanical anchorage of the membrane layer to the inter-tangled spacer-fabric (Figure 3.1) [145]. The membrane panel also benefits from a hollow space between the membrane layers with high porosity (85%). The total area of the PES membrane coating provided on each panel is 68.6 cm². For the 0.3 µm pore size membrane, the clean water permeability was 3000-4000 l.h⁻¹.m⁻².bar⁻¹. As can be seen in Figure 3.1, the prepared membrane envelope (membrane coated spacer-fabric) is fitted into a 3D printed frame, including two inbuilt air/gas passage systems (green lines presented on the panel) to twelve diffusers (6 on either side) at the bottom of the frame. The inbuilt diffusers provide better air/gas scouring by releasing and directing bubbles adjacent to the membrane surfaces in between the envelopes. The IPC membrane panels were then placed in a PVC spacer box that was developed to act as a riser compartment providing air/gaslift conditions and to reduce the membrane channel width (the width of the gap between the membrane surface and the walls of the spacer box) and enhance the effectiveness of the air/gas scouring and medium agitation. The IPC™ technology has recently been used by Blue Foot Membranes® in the production of FS microfiltration membrane units on a commercial scale (Figure 3.2).

Figure 3.1. Illustrations of the membrane-coated spacer-fabric, IPC membrane panel, spacer box, and the integration of the module into the bioreactor.

Figure 3.2. The lab-scale (a) and commercial-scale IPC membrane modules (image © Blue Foot Membranes®).

Prior to every hydrolysis and fermentation experiment, the submerged membrane panels were chemically cleaned and sterilized, according to the protocol recommended by VITO NV and Ishola et al. [48]. The IPC
panels were used both in pressure-driven (Papers I, II, and III) and concentration-driven (Papers V and VI) experiments. In this regard, in Papers I, II, and III, by the application of negative pressure in between the membrane layers using a peristaltic pump, the permeate was recovered in the hollow area of the panels and removed through the permeate line, while cells and other suspended solids were retained on the shell side of the reactor. On the other hand, in Papers V and VI, the volume in between the membrane layers in the panel was used for housing and confining the yeast cells.

3.2 MBR configurations
In order to benefit from the synergistic effect of selective separation and bio-chemical reaction, different MBR configurations were created from the integration of membrane modules into bioreactors [146]. Generally, based on the positioning of the membrane module, MBR configurations can be categorized as either immersed (also known as submerged) (iMBR) or external loop (also known as sidestream) (Figure 3.3).

![Figure 3.3. Schematic of the three main MBR configurations of (a) immersed MBR, (b) external immersed MBR and (c) side-stream MBR (Paper IV).](image)

In iMBRs, usually the flat sheet, hollow fiber, or capillary membrane modules are immersed vertically (capillary has also been used horizontally) into the bioreactor or a separate compartment connected to the main bioreactor [5, 126, 147]. In contrast, in sidestream MBRs, the membrane module is placed external to the bioreactor, and high volumes of reactor medium are pumped in an external loop, through the membrane.
module, tangential to the membrane surface (Figure 3.3) [117, 148]. Membrane modules such as spiral wound and tubular have attracted great attention for application in sidestream MBRs. Carstensen et al. [126], Ylitervo et al. [124], Judd [5], and Judd [149] have thoroughly reviewed the differences in principles and applications of immersed and external loop MBRs.

The presence of pressure and/or concentration gradient over the membrane are the main separation driving forces in MBRs (Figure 3.4) [5]. In pressure-driven immersed membrane systems, the application of a negative pressure (vacuum or under-pressure) on the filtrate side initiates filtration or product recovery, while the positive pressure or over-pressure caused by a high velocity flow of the medium over the membrane surface is the main cause of filtrate withdrawal in cross-flow membrane modules [126]. In some cases, filtrate removal in an iMBR is performed through reactor pressurization or gravitational-filtration [150, 151]. An extended list regarding the application of different pressure-driven MBRs in bioprocessing, with the focus on bioethanol production, has been provided in Paper IV.

![Figure 3.4. Membrane separation driving forces used in this thesis based on (a) pressure and (b) concentration gradients built across the membrane surface (Paper IV).](image)

### 3.2.1 Immersed MBR versus sidestream MBR

The main purpose of using immersed or external loop MBRs is cell retention and *in situ* recovery of products. This gives the bioprocess the ability to run in continuous mode at a high dilution rate and a high cell concentration to reach high productivity [152, 153]. A continuously operated MBR requires a smaller bioreactor volume and produces a clean product stream that reduces the number of downstream stages, thus, requiring smaller capital and labor costs [126]. In this regard, sidestream MBRs have been evaluated for
continuous product recovery, and yeast and bacteria retention in anaerobic bioprocesses such as fermentation of lactic acid, ethanol, etc. [126, 153]. However, in high cell density aerobic cultures, due to a lack of dissolved oxygen, pH fluctuations, and nutrient limitations, sidestream MBRs have not been considered successful [153]. Moreover, as reported by Chang et al. [154], maintaining the sterility of sidestream units in long-run continuous fermentation is a matter of concern. Although sidestream MBRs were considered a better option for wastewater streams of extreme pH, toxicity, organic content, etc., the most utilized membrane configuration for municipal wastewater treatment is submerged [153].

Considering the energy consumption and costs, as in sidestream MBRs, the reactor medium should be pumped through an external loop, immersed MBRs are considered more efficient, as the module is placed inside the bioreactor [126]. This becomes more complicated considering that many factors, such as cell concentration, culture condition, medium viscosity, and transmembrane pressure required for filtration and membrane surface cleaning are involved in defining the pumping rate and energy required for the recirculation of the media in an external loop. In sidestream MBRs, the tangential flow of the medium caused by the medium recirculation is responsible for the membrane surface cleaning, while for immersed MBRs the membrane surface cleanliness from deposited particles and cake layer is mainly attributed to air/gas scouring and/or backwashing. It has been reported that cleaning the membrane by air scouring rather than by cross flow reduces the MBR process cost significantly [155, 156]. Moreover, cells or enzymes can be damaged if exposed to high mechanical stress induced by substantial cross-flow velocities [157]. Although higher filtrate fluxes can be achieved using cross-flow filtration with less concerns of membrane fouling and cake layer formation, operational energy and space demands are in favor of iMBRs [5, 117, 158].

One of the main advantages of immersed membrane systems is their ease of application. Compared to sidestream MBRs, the immersed membrane configurations have a simpler design, are easy to install, and are compact in size, as the membrane module is immersed in the reactor [126, 147]. Therefore, immersed membrane systems have a lower footprint, power consumption, and investment, operation and maintenance costs as they can be easily controlled, repaired, and replaced [126, 147, 153]. These technical and economical features make iMBRs a better option when retrofitting an existing facility [147]. However, as in iMBRs, the membrane surface is in constant direct contact with the bulk medium, and there is less intense shear stress on the membrane surface compared to the sidestream systems, membrane fouling and cake layer formation are more difficult to control [126]. Moreover, iMBRs can suffer from low surface to volume ratio [153]. In addition, iMBRs are reported to have less flexibility in the type of application (mostly involved in municipal wastewater treatment) compared to sidestream membrane systems [159].
3.2.2 Immersed MBR set-ups used in this thesis

In order to fulfill different research goals sought in this thesis, different iMBR set-ups were used. These set-ups include two pressure-driven iMBRs (Figure 3.5a and b) and a concentration-driven iMBR (Figure 3.6).

Figure 3.5a presents the set-up used in Papers I and III for continuous fermentation of wheat straw slurry and bacterial decontamination, respectively. For experiments in Paper I, the iMBR consisted of two IPC membrane panels placed in a spacer box. In this experiment, as mentioned in section 2.5.3, the whole pre-hydrolyzed wheat straw slurry was continuously fed to the fermentation iMBR, and the ethanol-rich permeate removed and collected in a permeate tank. As high gas sparging rates were applied in this experiment for membrane cleaning and high SS medium mixing, two sequential ice-jacketed ethanol traps...
were attached to the gas outlet. In **Paper III**, the same set-up was applied for continuous physical bacteria decontamination from a buffer solution and semi-synthetic fermentation media.

The scheme of the double-staged iMBR applied in **Paper II** for continuous hydrolysis-filtration and co-fermentation-filtration is illustrated in Figure 3.5b. In this set of experiments, the hydrolysis iMBR was continuously fed with the slurry from a feed tank, and the sugar-containing permeate from the hydrolysis was recovered and fed to the buffer tank. Simultaneously, the fermentation iMBR was continuously fed with the clear hydrolysate, and the particle-free permeate, containing fermentation products, was withdrawn and collected in a permeate tank. This set-up benefited from two submerged IPC panels in each reactor. The data acquisition and control of the bioreactors, pumps, pressure sensors, and flow meters were completely synchronized and automated, using the Mefias® steering and data acquisition software [160] specifically programmed and developed for this research work.

![Figure 3.6](image)

**Figure 3.6.** Schematic presentation of the concentration-driven immersed MBR used in **Paper VI**.

In **Papers V and VI**, the main goal was to have an immersed fermentation set-up that provides diffusion-based mass transfer of compounds through the membrane. In this regard, as presented in Figure 3.6, IPC panels were used for housing the cells in between the membrane layers and separating them from the bioreactor bulk media. The membrane panels immersed in the feed medium were syringe-inoculated with a concentrated yeast cell culture. In **Papers V** two and in **Paper VI** four submerged membrane panels were used. In order to have a homogeneous concentration throughout the fermentation process, the reactor shell-side medium was continuously recirculated.
3.3 Membrane fouling

A common phenomenon that is associated with nearly all membrane separation processes is the decline of permeate flux (permeate) through the membrane over time. The drop in flux during the membrane filtration of a feed solution (mixture of different compounds) compared to pure water flux is due to concentration polarization (CP) and membrane fouling [161]. Concentration polarization happens in all membrane processes as a result of membrane selectivity that leads to the concentration or accumulation of solutes and/or particles adjacent to the membrane surface. The mass transfer boundary layer formed due to CP, contains high concentrations of the solutes and particles that impose an osmotic pressure adjacent to the membrane surface, reducing the effectiveness of the net driving pressure force applied across the membrane for filtration [161]. The CP severity is considerable in pressure-driven processes, however, it can simply be reversed and eliminated by hydrodynamic means such as membrane relaxation i.e., the removal of TMP and therefore flux [161, 162]. The second phenomenon contributing to the reduction in flux is membrane fouling. Fouling can be the result of CP and/or accumulation and buildup of deposited particles, adsorption of macromolecules, and gel formation on the membrane surface or within the membrane structure (pores) (Figure 3.7). The latter cause of fouling can especially be present during microfiltration [163]. The interaction and affinity between the solution/mixture components and the membrane surface may cause the formation of a layer of particles and solutes. This layer can form even in the absence of flux, however, CP can exacerbate the extent of adsorption (and vice versa) and further contribute to filtration hydraulic resistance [161, 162]. The membrane surface-substance attachments can be due to van der Waals attractions, electrostatic forces, or hydrophobic interactions [162]. Blockage or partial closure of the membrane pores during filtration (mainly in micro- and ultrafiltration) can also contribute to fouling. Another common fouling mechanism is the formation of a cake layer on the membrane surface due to the buildup of deposited particulate matter. Depending on the condition, composition, and compactness, the cake layer formed can add a considerable hydraulic resistance during the membrane filtration. Gel formation on the membrane surface due to CP of specific macromolecules (e.g., proteins) can also contribute to fouling [161, 162]. In general, fouling due to the cake and gel layer formation can be induced by the presence of different inorganic compounds such as metal hydroxides (scale precipitates), and organic and biological materials such as proteins, polysaccharides, microorganisms, enzymes, metabolic products, and other macromolecules (extracellular polymeric substances (EPS) and soluble microbial products (SMP)) [162, 164, 165]. Another problematic yet less discussed issue that occasionally deteriorates filtration performance in HF and FS MBRs is clogging. Clogging contributes to the deterioration in filtration performance as solid particles agglomerate at the entrance or within the membrane channels [166].
Figure 3.7. Schematic of general membrane fouling patterns: (a) new membrane, (b) adsorption, (c) pore narrowing, (d) pore blocking, and (e) cake layer formation.

Fouling control is more difficult than CP minimization, as the complexity of fouling preventive and curative approaches differs, depending on the solution that is treated and the hydrodynamics of the membrane filtration process. The drop in the filtration flux will exacerbate by exceeding the critical flux. The flux that causes the transition from CP to cake layer formation and fouling is considered as critical flux. The critical flux, first defined by Field et al. [167], is considered as the flux below which fouling does not occur; hence, flux does not deteriorate over time. Many parameters such as, particle size and concentration, quality of the medium, pH, temperature, viscosity, membrane pore size, membrane surface quality, module type and design, etc. are involved in setting the critical flux for different filtration systems. After operating a membrane system above its critical flux, reducing the TMP does not help in recovering previous membrane filterability [162]. However, there is no guarantee that gradual long-term sub-critical filtrations will keep the membrane clean from irreversible fouling [168].

Unlike CP, fouling can have a reversible and irreversible part. If the foulant is loosely attached to the membrane surface backwashing, air/gas scouring, an increase in the liquid cross-flow velocity or any other means of medium turbulence and shear elevation can help to efficiently clean the membrane surface. However, these physical cleaning strategies are not as functional when the foulant is firmly held by the membrane. Irreversible fouling can be caused by biofilms containing EPS and bacteria, macromolecules tightly bound to the surface, pore blockage by particles and colloids, and dense and compact cake layers [140, 169]. In order to mitigate these membrane fouling effects, the feed mixture can be pretreated, the membrane surface can be modified, membrane cleaning can be employed more frequently, and above all, the hydrodynamics of the filtration system can be optimized [162]. Irreversible fouling, to some extent, can be removed by physical and chemical means; however, accumulation of irreversible foulants after every filtration cycle may finally impose the need for membrane replacement [169].
3.3.1 Constant flux versus constant TMP

The most common modes of membrane filtration operation are constant flux and constant pressure (or TMP). In most immersed membrane filtration systems, the rate of permeate removal through the membrane is fixed, in other words, the filtration occurs under a constant flux mode. On the other hand, for external loop or housed membranes, a constant pressure mode is favored [140]. However, this may differ based on the membrane module, application, and operational conditions. By operating an iMBR in a constant flux mode, flux can be maintained below that of the estimated critical flux and excessive fouling can be avoided. At a low flux, a moderate change in the TMP to sustain the flux is not a matter of concern. However, as fouling cannot be completely avoided in long filtration cycles, increase in the TMP accelerates as filtration proceeds. However, in constant pressure mode, the membrane experiences high initial fluxes, so as the flux declines, satisfactory permeate flow rate is achieved. In this condition, in the initial filtration phases, the back-transport [5, 170] of the particles cannot counter the drag force of the bulk permeate flow toward the membrane surface, and cake layer formation becomes inevitable. In addition, the cake layer can be compacted at high initial fluxes which leads to a quick drop in flux. In order to mitigate fouling in systems operating at a constant pressure, a moderate stepwise increase in the TMP has been practiced. [140]. As in this thesis work, iMBRs were operated at a constant flux (Paper II) or no constant flux or pressure (Papers I and III) modes, only fouling and fouling prevention methods in this regard have been considered.

3.3.2 Fouling prevention in immersed MBRs

In pressure-driven immersed membrane filtration systems working under a constant flux, fouling can start from an initial adsorption of medium compounds on the membrane surface and then proceed, causing a gradual increase in the TMP and finally, a sharp jump in the TMP levels [171, 172]. Microbial compounds, different macromolecules, and chemicals in the feed media can be adsorbed to the membrane surface upon exposure even in the absence of an acting flux. This adsorbed layer is the initial cause of fouling and increase in the hydraulic resistance. The more hydrophilic a membrane is, the less severe the extent of adsorption [171, 172]. Further deposition of particles and other macromolecules on the membrane surface gives marginally progressive increase in the TMP as filtration proceeds. As also observed in Papers I, II, and III, this stage is followed by a sudden and rather exponential rise in the TMP. This sudden jump in the TMP in large-scale as well as lab-scale immersed membrane filtration operating under constant flux is reported to be due to the cake layer compaction [171-173]. By the loss of pressure in the cake layer, the TMP increases to compensate for the loss in flux. The increase in the TMP induces higher pressure on the cake, compressing it downwards toward the membrane surface. The synergistic effect of the TMP on the cake layer compaction and vice versa results in a dramatic jump in the TMP as the pressure threshold is reached [140]. Another
explanation for this sudden jump can be the blockage of membrane pores by particles. As pores become more and more blocked by particles and deposits, and as the flux should be maintained constant, the rest of the open pores may experience local fluxes beyond that of critical flux. This may cause excessive particle deposition and fouling, and consequently an increase in the TMP [168, 174]. Moreover, deposition and adsorption of macromolecules and colloids within the cake layer reduces the cake layer porosity, which can exacerbate the cake layer compaction and the TMP increase [175]. Similar fouling patterns were observed in Papers I, II, and III, when fouling prevention methods applied during filtration were not effective (Figure 3.8). However, preventive and curative measures could be applied in iMBRs to reduce the process losses due to fouling.

![Figure 3.8](image.png)

Figure 3.8. The TMP increasing patterns based on dilution rates (0.25, 0.5 and 0.1 h⁻¹) applied during filtration of the fermentation media containing a mixed culture of yeast and bacteria (Paper III). The upper curve for each marker represents the changes in the TMP during filtration, while other points show the latent effects of backwash.

### 3.3.2.1 Air/gas scouring

One of the most practiced methods to keep the membrane surface clean in an iMBR is air/gas sparging or scouring [5, 140]. The physical cleaning effect is due to the shear stress and liquid agitation induced on the membrane surface as the bubbles rise adjacent to the membrane surface. The turbulence caused in the medium close to the membrane surface assists the backtransport of particles and colloids back to the bulk media, disturbs the CP layer, and reduces the cake layer formation. The physical cleaning is either the effect of the direct contact between the bubble and the membrane or changes in the liquid flow regime over the membrane surface. As the bubble rises, the liquid on top of the bubble is pushed to the sides of the bubble and down to fill the gap produced by the rising bubble (also called the falling liquid film). This changes the
mass transfer patterns if the bubble is close to the membrane surface [176]. Moreover, as this liquid reaches the bottom of the bubble, it swirls and makes eddies in the wake of the bubble, inducing turbulence on the membrane surface [177]. Flat sheet membranes used for iMBRs are stacked in modules parallel to each other, forming channels that are pathways for the rising bubble. Depending on the channel width, the air/gas flow rate and bubble size, the fouling prevention efficiency changes in the iMBRs. In general, larger bubbles and smaller channels between the membrane surfaces give rise to higher shear stress [140]. The IPC membrane modules used in Papers I, II, and III have in-built diffusers on either side of the panel to help with the membrane cleaning by air/gas scouring and medium turbulence. The 12 (6 on each side) available diffusers have a diameter of 0.5 mm. This increases the efficiency of scouring as the bubbles are released in close vicinity of the membrane compared to the more common universal aeration.

As many iMBRs do not use mechanical agitation with impellers, air/gas sparging has a great role in efficient mixing of the media and defining mass transfer patterns. In aerobic iMBRs used for wastewater treatment, the provision of adequate amounts of dissolved oxygen is necessary for efficient nutrient removal [5, 140]. Therefore, if oxygen transfer is hindered by poor mixing and mass transfer, the air flow rate should be increased, which imposes greater costs on the process. In general, in aerobic biological processes using iMBRs, the air demand for scouring of the membrane is constrained by the biological oxygen demand for microbial growth and reactions, and medium mixing [140, 178]. In FS or HF iMBRs, an airlift condition can be provided to enhance the medium mixing and dissolved oxygen level by high velocity medium movement [179]. An airlift MBR consists of an inner compartment (cubic or cylindrical) called the riser, where the membranes are placed. Air/gas sparging from the bottom of the riser gives rise to the medium, while the medium out of the riser flows downward due to the pressure difference. Although high bulk velocities can improve membrane cleaning, microbial flocs can be broken and reduced in size [179, 180]. In Papers I and II, a spacer box was tailored to act as the riser, housing the membrane panels and preparing the air/gaslift condition in the lab-scale reactor.

In order to investigate the effect of air scouring on the filtration performance, aeration rates of 1, 3, and 4 l.min⁻¹ were applied in an airlift iMBR (Paper I) (Figure 3.9). The iMBR contained a xylose-glucose semi-synthetic medium and an initial cell inoculum of 2.3±0.7 g.l⁻¹. The medium was continuously recirculated through the permeate line back to the reactor for 24 h to have complete conversion of substrates to cell biomass and metabolites. In this experiment, two initial fluxes of about 19 and 43 LMH (l.m⁻².h⁻¹) were set. In order to see the actual effect of the air sparging on fouling prevention, filtration cycles were performed without a fixed flux or TMP. As can be seen in Figure 3.9, regardless of the aeration rate, at a dilution rate of 0.22 h⁻¹ (43 LMH), a great jump of 300-400 mbars was experienced in less than 9 h of filtration causing a 13% drop in the permeate flow rate. As discussed, this dramatic inclination in the TMP can be due to
exceeding critical flux and hence cake layer formation and compaction. However, aeration could have an active role in controlling fouling as the flux of 19 LMH (D=0.1 h\(^{-1}\)) was applied. As presented in Figure 3.9, although in the early hours of batch fermentation and filtration (10 h) similarly small TMP changes were observed for air flow rates of 1, 3, and 4 l.m\(^{-1}\), after 24 h, the rise in the TMP was 67% less for 4 l.m\(^{-1}\) compared to 1 l.m\(^{-1}\). This proves the effectiveness of higher aeration rates at sub-critical fluxes.

![Figure 3.9](image.jpg)

Figure 3.9. Changes in the TMP by time for different aeration rates at dilution rates of 0.1 and 0.22 h\(^{-1}\) (Paper I).

In anaerobic bioprocesses, such as ethanol fermentation, gases such as N\(_2\) and CO\(_2\) that provide an anoxic condition can be supplied (or circulated by a pump) in order to have efficient mixing and membrane scouring regardless of the biological oxygen demand. Therefore, in the fermentation experiments performed in this thesis, after a short phase of aeration and yeast culture propagation, membranes were continuously scoured with N\(_2\). Although high gas sparging rates (3-4 l.m\(^{-1}\)) could successfully remediate fouling propensity, a challenge faced during fermentation of wheat straw hydrolysate was excessive ethanol stripping and evaporation (Papers I and II). Therefore, applicable solutions are required to collect and recover the ethanol produced while achieving optimum membrane cleaning and filtration. This problem was remediated in Paper I by using two sequential ice-jacketed ethanol traps (Figure 3.5). The ethanol loss due to changes in the gas flow rate from 1 to 4 l.min\(^{-1}\) and ethanol recovery efficiency using different trap set-ups in Paper I are presented in Figure 3.10.
Intentional ethanol stripping by air or gas has been applied by Kumar et al. [181] to have a condensate with around 56 g.l⁻¹ ethanol, while the broth ethanol concentration was 3.4 times less. However, in Paper I, during the continuous co-fermentation and filtration of xylose-glucose semi-synthetic media, about 150 and 135 g.l⁻¹ ethanol was gathered in two consecutive traps (ethanol recovery rate of up to 1.63 g.h⁻¹), while the ethanol content of the fermentation broth had stabilized at around 32 g.l⁻¹ (Figure 3.11).
3.3.2.2 **Backwashing**

Backwashing (BW) (backflushing) is another physical remedy for dislodging and removing the cake layer from the membrane surface. During backwashing, the direction of the permeate flow is reversed and a portion of the permeate is sent back to the reactor through the membrane pores for a relatively short period. In iMBRs, BW is commonly performed intermittently every 5 to 90 minutes for a period of 0.5-30 seconds [140, 182]. With the application of backwashing, reversible fouling can be mitigated by the removal of the porous cake layer and resuspension of solid deposits. Irreversible fouling has also been reported to be alleviated using BW [182, 183]. However, the effectiveness of backwashing in fouling prevention is hindered as the filtration pressure exceeds that of critical TMP [184]. Although backwashing and air/gas scouring can be effective in reducing the particle deposition and cake layer formation, they have minimal effectiveness when it comes to dense and sticky cakes formed from macromolecules such as EPS and SMP [140]. An extensive review on the effects of backwashing has been provided by Akhondi *et al.* [185].

Backwashing has mostly been associated with HF immersed membranes used for surface water and secondary effluent treatment [123, 185]. However, the IPC membrane panels used in this research possess great strength hence extensive backwashing/backflushing (up to 2.0 bar BW pressure) can be applied. Therefore, they increase the flexibility and range of application of the FS membranes in iMBRs [145]. The common backwashing operational modes used are: (a) fixed time cycle, where BW occurs regardless of changes in the TMP, and (b) fixed maximum TMP, where BW is applied as the TMP max is reached [185].

In the iMBRs used in **Papers I, II, and III**, for the hydrolysis and fermentation of wheat straw slurry using *S. cerevisiae*, and separation of yeast and bacterial contamination, intermittent backwashing and continuous air/gas scouring were used. In all the experiments using BW, a fixed time cycle mode was applied, while each BW regime was modified based on the process requirements (3-5 min cycles with 30 sec BW) (**Papers I, II, and III**). In order to observe the self-sustaining potential of the filtration process in **Papers I and III**, neither flux nor TMP were kept constant. In these iMBRs, the flux used for BW was similar to that of filtration. However, in **Paper II**, a constant flux filtration mode was applied in the double-staged iMBR, using backwashing fluxes twice that of filtration.

In order to observe the effect of BW on bacterial washout ability, a phosphate-buffered saline (PBS) containing 0.05 g.l⁻¹ *E. cloacae* was continuously filtered at a flux of 112 LMH with and without BW (**Paper III**). As illustrated in Figure 3.12, without the application of BW, there was an extreme fouling tendency, resulting in a sharp and rather linear 280 mbar incline in the TMP in less than 2 h. Although the concentration of suspended bacteria dropped by 49% in this time period, there were no signs of bacterial population increase in the permeate tank. Therefore, it could be concluded that cell concentration reduction in the main reactor has most probably been due to the attachment/deposition of bacteria on the membrane surface as the
bulk flow toward the membrane surface suppresses the of backtransport [186, 187]. However, when a 30 sec BW was applied after every 3.5 min of filtration, the cake layer formation was minimized by effective cell resuspension and more than 90% of the initial bacterial inoculum was washed out through the permeate line (Figure 3.12).

![Figure 3.12. Changes in the (a) TMP and (b) the optical density of reactor media (R) and permeate tank (P) during the filtration of bacteria containing PBS medium with and without BW at a dilution rate of 0.5 h⁻¹ (Paper III).](image)

A more specific analysis of the effect of BW on fouling during hydrolysis of wheat straw slurry was performed in Paper II. In Paper II, simultaneous saccharification, filtration, fermentation and filtration was performed at three different fluxes of 21.9, 36.4, and 51.0 LMH. The wheat straw slurry was fed to the hydrolysis iMBR, and the hydrolysate was continuously filtered out to feed the fermentation iMBR. Considering that the immersed membrane is exposed to an ongoing hydrolysis of slurry to hydrolysate, it was important to understand the filtration performance and membrane-fouling propensity in the presence of these two media. Therefore, a step-flux experiment was set up starting at 21.9 LMH and increasing to 36.4 and 51.0 LMH with a stage of flux drop, back to 21.9 LMH, in between every period of flux increase to investigate the residual fouling. This set of experiments was performed to investigate the effect of flux, medium quality (wheat straw slurry or hydrolysate), and BW on the cake layer formation and membrane fouling (Figure 3.13).

In this regard, the total resistance and cake layer resistance were calculated based on equations (3.1) and (3.2), respectively.

\[
J = \frac{TMP}{\mu R_T}
\]  
(3.1)
\[ R_T = R_m + R_C + R_f \]  

Where \( J \) is permeate flux, \( \mu \) is permeate viscosity, \( R_T \) is total resistance, \( R_m \) is membrane resistance (clean water filtration), \( R_C \) is the cake layer resistance, and \( R_f \) is irreversible fouling resistance. As in microfiltration irreversible fouling is considerably smaller than \( R_C \), \( R_C \) was used to represent the resistance due to the cake layer and irreversible fouling [188].

The composition of the media in contact with the immersed membrane directly affects the filtration performance [161]. The effect of the medium quality on the filtration performance is presented in Figure 3.13. As illustrated, the initial TMP is higher for the hydrolysate compared to the slurry at all tested fluxes. Moreover, the latent residual cake layer resistance after stepwise increase and drop in flux is considerably higher during the filtration of the hydrolysate compared to the slurry. For instance, when no BW was applied, the residual cake layer resistance, at the end of the step-flux experiment at 21.9 LMH, was \( 2.11 \times 10^{11} \) m\(^{-1} \) for the slurry, while it was \( 5.23 \times 10^{11} \) m\(^{-1} \) for the hydrolysate. In immersed microfiltration MBRs, changes in the medium SS concentration can be one of the prime reasons for the cake layer formation and increase in the filtration resistance [5, 140]. However, in this case as the SS content of the hydrolysate (6.4 g.l\(^{-1} \)) is nearly half that of the slurry, the higher TMP cannot be directly related to the changes in the medium SS concentration. Therefore, other factors such as particle characteristics, and changes in the medium composition due to the presence of enzyme and released sugars may have contributed to the change in the filtration behavior [189, 190]. Solid particles undergo structural changes through hydrolysis [191], which may affect particle rigidity and size, and consequently, cake layer compressibility [189, 192]. As the particle softness and compressibility increases and the particle surface quality changes (e.g. enzyme attachment), the cake layer becomes more susceptible to compaction. The attachment of cellulases to cellulose [193] and lignin [194] during the wheat straw hydrolysis may change the particle surface quality, and consequently, particle-particle and particle-membrane interactions, leading to changes in the cake layer formation, densification, and overall filtration resistance.
Regarding the effect of filtration flux on fouling propensity, it was observed that at 21.9 LMH, the rate of changes in the TMP was less than 0.03 mbar.min\(^{-1}\) for both media with and without BW, resulting in a maximum filtration resistance of around \(0.3 \times 10^{11}\) m\(^{-1}\) (Figure 3.13). As the permeate flow rate was increased...
from 0.3 to 0.5 l.h\(^{-1}\) (36.4 LMH), the TMP escalation accelerated. The upward trend in the TMP change became more apparent as the flux was increased to 51.0 LMH. At this filtration condition, the rate of change in the TMP tripled compared to 36.4 LMH with 0.24 and 0.41 mbar.min\(^{-1}\) for the slurry and hydrolysate with no BW, respectively. In the 21.9 LMH filtration condition, regardless of the BW application, the membrane surface air/gas scouring and shear stress induced by the medium agitation successfully assisted the backtransport of particles against the convective flow toward the membrane. As the flux is apparently lower than the critical flux for such medium, cake layer compaction and sharp increase in the hydrodynamic resistance was not detected. Despite the low sugar content and volumetric productivity achieved at this flux, long-term continuous hydrolysis and filtration can be guaranteed, benefiting from less process downtime and frequent membrane cleaning.

As the wheat straw slurry and hydrolysate contain substantial amounts of suspended particles, BW was performed for 30 sec, every 4.5 min at double the filtration flux to see the effect on fouling prevention and cake layer removal. As presented in Figure 3.13, filtration with no BW results in progressive TMP increase rates of 0.03, 0.07, and 0.24 mbar.min\(^{-1}\) for the slurry, and 0.02, 0.16, and 0.41 mbar.min\(^{-1}\) for the hydrolysate at fluxes 21.9, 36.4, and 51.0 LMH, respectively. However, with dramatic difference from no BW condition, this rate for both medium did not exceed 0.06 mbar.min\(^{-1}\) at any applied flux. When the hydrolysate was filtered in no BW condition, after the drop of flux from 51.0 to 21.9 LMH, the residual cake resistance was at the highest by \(5.23 \times 10^{11}\) m\(^{-1}\), whereas the one with BW had less than half the resistance \((2.41 \times 10^{11}\) m\(^{-1}\)). It should be considered that the final cake layer resistance after the initial phase of filtration at 21.9 LMH was only \(1.80 \times 10^{11}\) m\(^{-1}\) and \(0.84 \times 10^{11}\) m\(^{-1}\) for the hydrolysate filtered without and with BW, respectively. The results showed that the resistance of the residual cake layer can be reduced up to 54% even at the highest flux by the application of BW. However, regardless of fouling remediation by BW, the higher fluxes of 36.4 and 51.0 LMH caused a progressive development of sticky and compact cake layer. This dense cake layer cannot be effectively removed by air/gas scouring or BW [140, 184]; therefore, a combination of a flux below critical and BW can be a better option in a hydrolysis iMBR running at constant flux.
In bioprocesses, having a high volumetric productivity enhances the economic feasibility of the process. Achieving this goal requires a continuous feeding and product removal process benefiting from high cell densities. Moreover, as downstream processing is responsible for a considerable part of the total process time, energy, and cost, it is of great importance to consider cell separation and product recovery as well as purification methods that can be feasible when applied in large scale [153]. One of the approaches that can enhance the process productivity and therefore, feasibility, is the effective integration of different parts of the production process [195]. A general overview of the applications of pressure-driven immersed and sidestream MBRs in ethanol fermentation using various substrates and microorganisms has been presented in Paper IV.

In this chapter, the potential of pressure-driven iMBR set-ups in intensifying and technically optimizing lignocellulosic bioethanol production process is evaluated. To fulfill this purpose, the effectiveness of different iMBR set-ups and filtration conditions in reducing processing stages, retaining/separating high concentrations of suspended solids and cells inside the reactor, performing in situ recovery of products during continuous hydrolysis and fermentation of pretreated wheat straw, and physically removing the bacterial contamination from fermentation systems were investigated (Papers I, II, and III).

4.1 High cell density in MBRs
In fermentation processes, one of the criteria defining the economic success of the process is the volumetric productivity. Productivity of a bioprocess is directly related to cell concentration, cell activity, product concentration, and dilution rate [196]. Considering the cells specific activity \( v_p \), the higher the cell concentration \( X \), the higher the productivity \( P = v_p \cdot X \) [197]. In order to benefit from a high bioconversion rate and productivity \( P \), high cell concentrations should be obtained, and cell activity and metabolic
performance should be maintained through continuous the provision of nutrients and environmental condition needs (pH, temperature, etc.) [196, 197]. Continuous fermentation, coupled with cell retention or recycling in MBRs, can provide such conditions. In MBRs, permeate flow rate is equal to the feeding rate, which defines the dilution rate (D). Moreover, as discussed in section 2.5, by using MBRs in continuous fermentation, cell washout can be eliminated and the dilution rate can be selected regardless of the cell growth rate. As considered in equation (4.1), increasing the dilution rate can lead to an increase in the cell activity, product concentration and consequently volumetric productivity [196, 197].

\[ P = \nu_p X = pD \]  

(4.1)

Where \( p \) is the product concentration and \( D \) is the dilution rate.

In this regard, MBRs have been considered in laboratory and industrial scale to enhance the productivity by maintaining high cell densities in the bioreactor while increasing the dilution rate [126, 197]. MBRs using tangential filtration have been successfully applied in high cell density fermentation processes with \( S. \) \textit{cerevisiae} (300-330 g.l\(^{-1}\)) and \( \text{Propionibacterium acidipropionici} \) (100 g.l\(^{-1}\)) for the production of ethanol and propionate at high volumetric productivities of 33 and 14.3 g.l\(^{-1}\).h\(^{-1}\), respectively [198-200]. Moreover, as explained elaborately in Paper IV, during the fermentation of inhibitory media such as lignocellulosic hydrolysate, high local cell concentrations in the bioreactor boost the yeast inhibitor tolerance and detoxification rate. Another benefit of using continuous fermentation by means of MBRs is preventing product (sugars in hydrolysis or metabolites like ethanol in fermentation) inhibition, as the product levels are kept low by continuous \textit{in situ} removal [126].

In \textbf{Paper I}, an iMBR with the set-up presented in Figure 3.5 was used for the continuous fermentation of xylose-glucose semi-synthetic media in order to study the effect of high cell concentration on membrane filtration performance and simultaneous assimilation of xylose and glucose. The initial cell cultures were prepared using yeast extract peptone dextrose (YPD) medium in shake flasks, then added to the iMBR containing YPD broth of 100 g.l\(^{-1}\) sugars (glucose to xylose ratio of 1:1). Following a batch cell propagation stage, continuous fermentation started, feeding the reactor with 50 g.l\(^{-1}\) glucose, 50 g.l\(^{-1}\) xylose, 10 g.l\(^{-1}\) peptone, 5 g.l\(^{-1}\) yeast extract, and 4.75 g.l\(^{-1}\) KH\(_2\)PO\(_4\) at a dilution rate of 0.1 h\(^{-1}\). Regarding ethanol production, and xylose and glucose consumption the system fully stabilized after 8 h of continuous cultivation (Figure 3.11). In this period, the \( S. \) \textit{cerevisiae} concentration increased linearly up to 43 g.l\(^{-1}\) till 72 h before reaching a plateau. In order to call a continuous process stable, the system should maintain its condition for at least 4 to 5 hydraulic retention times (HRT) [201]. This system proved to be perfectly stable till 120 h. The exceptional process stability also applied to the filtration parameters of the TMP and flux. Considering that no constant flux or TMP conditions were applied, stability in the flux and TMP was reached after an initial
rise in TMP levels to 70 mbar. To see the effect of high cell densities and to expose the immersed membrane to a more viscous medium, the dilution rate was more than doubled. Although a high cell concentration of 95 g.l\(^{-1}\) was achieved in this condition, the TMP levelled out at about 175 mbars (Figure 4.1). The acquired results using this iMBR set-up proved that with the help of gas sparging and intermittent BW cycles extensive membrane fouling could be remediated even at yeast concentrations of 43-95 g.l\(^{-1}\), while reaching 97% of xylose and 100% glucose utilization and an ethanol volumetric productivity 4.6 g.l\(^{-1}\).h\(^{-1}\) (Paper I).

![Figure 4.1. Changes in the TMP (a), permeate flow rate (b) and cell concentration (biomass) during batch and continuous fermentation of xylose-glucose semi-synthetic medium (Paper I).](image)

4.2 Lignocellulosic ethanol process intensification using MBRs

Membrane systems can be integrated into bioethanol fermentation processes to assist product recovery and purification, sugar concentration, cell and enzyme retention, and to enhance the overall feasibility of the process [124, 133]. Considering the technical complexities, and energy and capital demanding pretreatment, hydrolysis and fermentation requirements of lignocellulosic bioethanol production, intensification of the process using membrane separation technologies can facilitate, optimize, and reduce the process stages [4]. In order to make the lignocellulosic bioethanol production process economically feasible, membranes could
be integrated throughout the process rather than application to a single stage [202]. These stages have been presented in Figure 4.2.

Figure 4.2. Potential applications of membrane technology in lignocellulosic bioethanol production process (Paper II).

The initial pretreatment stage for lignocelluloses is physical treatment to reduce the feedstock’s particle size that is to be fed to the following pretreatment stages. The reduction in particle size increases the accessible surface area, increasing the efficiency of the physico-chemical pretreatment. The next step is physico-chemical pretreatment to disrupt and open the inner lignocellulosic structure, mainly by removing lignin or solubilizing hemicelluloses. To cut on pretreatment costs, acid or bases used in the pretreatment step can be recovered and reused through membrane filtration [203]. Additionally, depending on the type of pretreatment and the pretreatment by-products, a membrane separation, fractionation and purification process can be applied. For example if the feedstock has been organosolv pretreated, lignin can be purified and removed using UF [204]. On the other hand, if the pretreatment leads to dissolution of hemicelluloses, the remaining solid residuals can be separated and hemicelluloses purified using MF/UF [205]. Moreover, as explained thoroughly in section 2.4, acid pretreatment and hydrolysis may generate inhibitors such as furfural and acetic acid that hinder fermentation but are valuable chemicals. These inhibitory compounds can also be recovered through NF/RO post-pretreatment [204]. As described, by means of different membrane techniques, pretreated slurry can be detoxified, fractionated, and marketed in a context of a biomass-based biorefinery. In order to degrade cellulose, the pretreated slurry undergoes enzymatic hydrolysis. Considering the high cost of enzyme provision, the process can benefit from enzyme retention, recirculation, and reuse [203, 206]. Ultrafiltration has proven to be an effective approach for retention and reuse of cellulolytic enzymes [207]. Therefore, by using a cascade of MF, UF, and NF during and after the hydrolysis, enzyme product inhibition can be omitted, solid residuals can be separated, enzymes recirculated and retained in the hydrolysis reactor, and the resulting sugars can be concentrated [202, 203, 206]. Membrane separation (MF and NF) can also assist the fermentation step to recirculate cells, retain sugars,
and clarify the ethanol-rich stream entering the downstream processing [203]. Cell retention using MF can provide high cell concentration and substrate conversion along with the ability to conduct fermentation in a continuous mode and benefit from high volumetric productivity [202, 203, 206]. Moreover, membrane distillation, pervaporation, and vapor permeation can be integrated with conventional downstream processes to reduce the energy demand, increase the ethanol concentration prior to distillation, and/or help with ethanol dehydration. Other membrane processes, such as NF and RO, can be employed to treat the intake water and the process wastewater [206]. However, there are different advantages and disadvantages in the application of immersed or sidestream membrane units in these hybrid membrane set-ups, of which some are discussed here and in section 3.2. Although side-stream membrane units can reach higher fluxes and membrane modules can be replaced as the operation goes on, there are issues with oxygen limitation in the loop, maintaining sterility, fouling prevention for very viscous broth and broth recirculation energy demand. On the other hand, in immersed membrane units, the process is simple and there is no broth recirculation through an external loop, but the membrane system is considered as inflexible, susceptible to fouling, limited by membrane area per reactor volume, and additional problems induced by highly viscous medium [5, 126, 140, 206]. Therefore, to integrate the iMBRs into the lignocellulosic bioethanol production and to benefit from the advantages of intensifying processing stages, the issues associated with the iMBRs should first be resolved. In Papers I, II, and III, some of the challenges associated with the integration of the iMBRs in lignocellulosic bioethanol production process have been addressed.

As mentioned previously, in Paper I, a modified version of SHF was applied. In the set-up used in Paper I, hydrolysis was performed separately in an optimum condition prior to the fermentation and then yeast and suspended solids, containing mainly lignin, were concentrated in the same iMBR. However, in order to intensify and cover more stages of the lignocellulosic bioethanol production, in Paper II continuous and simultaneous hydrolysis and fermentation were practiced, using two interconnected microfiltration iMBRs. The aim pursued in Paper II was to find the optimum hydrolysis-filtration and fermentation-filtration condition to have an effective separation of lignin-rich solids from the hydrolyzed sugars, and yeast cells from the bioethanol stream, along with stable long-term filtration and enhanced ethanol productivity. In this set of experiments, dilute wheat straw slurry (14±1 g.1⁻¹ SS) was continuously fed to a hydrolysis iMBR and the hydrolysate removed at filtrate flow rates of about 0.3, 0.5, and 0.7 l.h⁻¹ corresponding respectively to fluxes 21.9, 36.4, and 51 LMH. After being fed to a buffer tank, the hydrolysate containing an average of 14.7±0.6 g.1⁻¹ of total sugars (based on xylose and glucose) was continuously fed to the fermentation iMBR, and the product containing stream was removed through the permeate line at the above-mentioned fluxes. The filtration was performed in a constant flux mode with continuous gas sparging and intermittent BW to keep the membrane surface clean and prevent fouling.
Figure 4.3. Changes in the concentration of substrates and metabolites during batch and continuous hydrolysis and fermentation of wheat straw slurry at different permeate fluxes (Paper II).

As discussed, considering the low sugar content of the dilute wheat straw slurry, higher dilution rates (here considered as permeate flux) are favored to enhance the volumetric productivity. This can apply to both the ethanol productivity and fermentable sugar recovery rate during hydrolysis (in case of a constant HRT). Therefore, since the retention of suspended solids and cells in the reactor is ensured with the applied double-staged iMBR system, higher permeate fluxes were favorable. However, the flux applied is limited by the filtration performance and fouling tendency [140]. As illustrated in Figure 4.3, during hydrolysis at different feeding rates, sugar concentrations of 14-15 g.L⁻¹ were yielded. However, while benefitting from similar starting yeast inoculum size, feeding the fermentation iMBR at a high flow rate of 0.7 l.h⁻¹ (51.0 LMH) led to ethanol volumetric productivity of 1.8 g.L⁻¹.h⁻¹ (specific productivity 0.23 g.g⁻¹.h⁻¹), which is 1.4 and 2.3-times higher than that achieved at 0.5 and 0.3 l.h⁻¹, respectively.
Although, in all treatments the continuous hydrolysis and fermentation reached stability and maintained it for more than 7 HRTs, dramatic changes in the filtration performance hinder the benefits achieved through the application of high filtration fluxes (36.4 and 51.0 LMH) (Figure 4.4). In constant flux mode, changes in the quality of the membrane surface (e.g. cake formation) and extent of fouling are projected in the TMP, as it changes to satisfy the set flux. As shown in Figure 4.4, the flux of 51.0 LMH exceeded the critical flux as the TMP increased rather exponentially during hydrolysis and linearly from the very beginning of the continuous process during fermentation. The sharp incline in the TMP to around 130 mbar, corresponding
to a total membrane and cake layer resistance of $1.1 \times 10^{12} \text{ m}^{-1}$, after only 51 h of continuous hydrolysis calls for process termination and membrane cleaning. Although starting at a flux of 36.4 LMH postpones reaching this level of membrane fouling to around 135 h, from 102 h onward severe cake layer formation and compaction brings hydrolysis and filtration to a halt. However, when the sub-critical flux of 21.9 LMH was applied, filtration performance could be sustained up to 11 days with negligible increase in the TMP. Although the TMP soared to 400 mbars (total resistance=$3.4 \times 10^{12} \text{ m}^{-1}$) in 51 h for the flux of 51.0 LMH, lower fluxes showed a rather stable filtration TMP throughout the fermentation process. According to the obtained results for the practiced conditions, for this specific double-staged iMBR system, operating at the flux of 21.9 LMH guarantees a stable continuous hydrolysis-filtration and fermentation-filtration.

In order to benefit from a rich-lignin stream, the SS accumulation in the hydrolysis iMBR was also tracked. As can be seen in Figure 4.5, in all of the treatments, the SS content of the hydrolysis iMBR had more than 63% lignin, nearly double that of the feed wheat straw slurry. This lignin content in the reactor could reach as high as 69 g.L$^{-1}$ and has the potential to be extracted as a valuable by-product of lignocellulosic ethanol fermentation [208].

![Figure 4.5](image-url)

Figure 4.5. Changes in the composition and concentration of suspended solids and lignin in the feed slurry and at different stages of continuous hydrolysis and filtration (without draining) (Paper II). (ASL-acid soluble lignin, AIL-acid insoluble lignin and CH-structural carbohydrates)
4.2.1 The effect of suspended solids on filtration performance

One of the main goals in any pressure-driven membrane-assisted product recovery process is to reach and sustain a high permeate flux to benefit from high productivity [126]. However, CP and fouling mechanisms, such as cake layer formation, challenge achieving such stable filtration state [209]. If all filtration parameters such as the TMP and membrane resistance are kept constant, an increase in the medium viscosity can increase the propensity of fouling in both the immersed and side-stream MBRs. By increasing the medium viscosity, the effectiveness of medium turbulence and shear stress due to air/gas sparging on membrane surface drops, the bubble size reduces (immersed MBRs), efficient mass transfer is hindered (immersed and side-stream MBRs), and higher broth recirculation energy is required (side-stream MBR) [5, 140, 210-212].

One of the medium characteristics that can directly contribute to the changes in viscosity is the suspended solid content, including retained cell (biomass) and residual particulate matter [148]. As reported for mixed liquor suspended solid (MLSS) in MBRs used for wastewater treatment, there seems to be no direct answer for the effect of SS on fouling and reduction in the flux [5, 140]. According to the literature, increasing the MLSS concentration can have a positive, negative, or no perceptible effect on the permeate flux [5, 140, 210-212]. Although different models and practical methods have been presented for estimating the SS-flux relation (some of the models are presented in Paper IV), they are not generally applicable to all MBR systems due to specificity of the assumptions and parameters taken into account [213-216]. Accordingly, the critical SS concentration in every MBR system should be defined independently [140, 217]. It has been reported by Bin et al. [218] that at low SS concentrations, colloidal compounds are the main contributors to the increase in filtration resistance, whereas at high SS, rapid cake layer formation and progression is the noticeable fouling mechanism. Immersed MBRs working at low TMPs are also prone to flux deterioration as the MLSS increases [219, 220].

Considered as SS, cell biomass affects the viscosity of the medium but not in a linear fashion [221]. Considering that one of the main application purposes of microfiltration MBRs is to retain and/or recirculate cells to reduce HRT, increase the dilution rate, and enhance the productivity, high cell concentrations can induce membrane fouling [126, 199]. Therefore, there is a need for a balanced MBR procedure that can sustain both high flux and high cell concentrations in a satisfactory manner.

Changes in temperature can also affect the viscosity of a media [222]. By changing the temperature, the behavior of the fluid medium near the membrane surface is altered [140]. As the temperature elevates, the kinetic energy of the molecules in the medium increases and Brownian movement becomes more significant. This change assists the particle backtransport [140]. If the backtransport velocity of the particles/solutes away from the membrane surface increases in comparison to the permeation velocity/drag toward the membrane, the extent of CP and cake layer formation can be reduced [5, 140].
In this thesis, using a combination of gas sparging, BW and airlift medium mixing, the iMBR set-up performed exceptionally stable at *S. cerevisiae* concentrations of up to 95 g.l⁻¹, without a noticeable drop in the flux or an increase in the TMP (Figure 4.1). The other source of SS in this research work was residual lignin-rich particles. As mentioned previously, based on the plant species and growth condition, lignocellulosic materials can contain between 10-35% lignin [25]. Depending on the type of pretreatment used for the lignocellulosic material, the main fraction of structural lignin can be removed (e.g. organosolve) or partially solubilized as non-fermentable polymeric compounds (e.g., acid treatment) [24]. In dilute acid pretreatment after the degradation of hemicelluloses, the enzymes are added to the more porous lignin-cellulose particles. As reported in *Paper II* (Figure 4.5), since glucose is released due to the cellulases activity, undegraded lignin residuals are left as suspended solids. It should be considered that high SS loading in MBR hydrolysis and fermentation increases the viscosity of the lignocellulosic slurry, imposes mass transfer limitations (reduces homogeneity), increases the unproductive enzyme-lignin attachment, reduces the hydrolysis effectivity, and dampens the membrane surface shear stress [223-225]. Moreover, if cell separation and reuse is targeted, the SS lignocellulosic residuals add to the number of downstream
processing stages and the overall cost. In this regard, the filtration and co-fermentation performance of the iMBR used in Paper I was challenged by being continuously fed with wheat straw hydrolysate containing about 40 g.l\(^{-1}\) SS. The biomass content of the iMBR, composed of yeast cells and lignocellulosic residuals, inclined linearly to about 200 g.l\(^{-1}\) after 48 h of continuous feeding (Figure 4.6). The images of the membrane surface condition after the experiments are presented in Figure 4.7c. Although there is a high fouling and clogging propensity at this SS concentration, the membrane filtration performed surprisingly well due to the synergistic effect of gas scouring, BW, and medium agitation induced by the gaslift condition. Considering that the average SS for wastewater treatment iMBRs is about 0.8-2% w/v, at this high SS (20% w/v) the iMBR (Paper I) showed promising filtration performance by keeping the flux stable (around 18 LMH) and TMP between 25-57 mbar. Although no impeller was used for the mechanical mixing of such thick medium, effective mass transfer was observed. The average ethanol yield obtained during the stabilized continuous fermentation was 0.4 g.g\(_{\text{sugars}}^{-1}\) (79 % of the theoretical yield), reaching a volumetric productivity of 3.29 g.l\(^{-1}\).h\(^{-1}\). In this experiment, up to 73% of the fed xylose and all the glucose were utilized by the xylose-consuming \textit{S. cerevisiae} during continuous fermentation (Figure 4.8). This lower xylose utilization rate can be due to the exposure of suspended cells to the lignocellulosic inhibitors [226]. This enhanced fermentation performance was observed even in the presence of more than 6 g.l\(^{-1}\) acetic acid, 4.7 g.l\(^{-1}\) HMF and less than 1 g.l\(^{-1}\) furfural. Considering that an individual inhibitor such as acetic acid hinders fermentation at such concentrations [227] and that simultaneous presence of these inhibitors brings along higher synergistic toxicity [228], this iMBR fermentation set-up proved to function robustly.

Figure 4.7. (a) Clean IPC membrane panel and membrane panels taken out of the bioreactor, working at (b) 95 g.l\(^{-1}\) yeast cells and (c) 200 g.l\(^{-1}\) SS (cells and lignocellulosic residues).
Contamination removal using immersed MBR

Bacterial contamination in industrial fermentation has long been a major issue hindering the process efficiency [92, 229]. An estimate of 500 different strains of bacteria, contaminating the fermentation process at different stages, have been identified [230, 231]. The source of this contamination can be found in various instruments, reactors, pipelines, added chemicals, and nutrients or even recycled yeast [91, 92]. The bacterial contamination not only causes reduction in the final ethanol yield (up to 30%) and process profitability, but also produces metabolites such as lactic and acetic acid that disturb the yeast metabolic activity and reduce the purity of the product stream [92, 232]. There is a high risk of bacterial contamination when yeast cells are sent out of the main fermenter to be recycled, concentrated, and reused for consecutive batches. Therefore, in order to maintain sterility during fermentation and benefit from *in situ* product recovery, iMBRs are the preferred choice over sidestream ones [126, 206].

To avoid extra contamination-imposed processing costs due to the yeast flocculation, low cell viability, excessive foaming, reduction in ethanol yield, etc., fermentation sterility should be maintained [233]. However, having a long-term contamination-free industrial scale process is costly, laborious, and hard to maintain. One commonly practiced remedy is the application of conventional anti-bacterial agents such as the antibiotic virginiamycin, sodium fluoride, etc. [234-237]. However, these anti-bacterial compounds do not provide long-term contamination control, and their residues may end up in certain fermentation by-
products such as distiller's dried grains with solubles (DDGS), raising health and environmental concerns [238]. As the contaminating bacteria is less resistant to a low pH than \textit{S. cerevisiae}, another decontamination approach has been short-term reduction in the pH (sulfuric acid, pH 1.5-3 for 1-3 h) [233]. However, the downside of the low pH treatment is loss of viability and metabolic activity of the yeast [239, 240].

On the other hand, MBRs have the potential to provide a physical approach for contamination removal. The basis of microfiltration membrane systems is the semi-selective size-exclusion of compounds, cells, and particles. Based on this capability, iMBRs have long been used for the retention of microorganisms [140]. However, in \textbf{Paper III}, the size selectivity of the microfiltration iMBR was used to washout the bacterial contamination, while retaining the larger yeast cells (Figure 4.9a). In addition to physically removing bacterial contamination, high concentrations of yeast can be obtained in a continuous fermentation; consequently, enhanced ethanol yield and productivity can be expected. However, due to the risk of membrane fouling and biofouling [241, 242], the viability of this physical decontamination method depends on proper synchrony between the choice of membrane, fermentation medium, microorganisms, and filtration parameters.

![Figure 4.9](image1.png)

Figure 4.9. (a) The application of iMBR for the physical separation of yeast from bacterial contamination. (b) The optical microscopy image of \textit{S. cerevisiae} and \textit{E. cloacae} in the contaminated fermentation medium (\textbf{Paper III}).

As the customized IPC membrane panels used in \textbf{Paper III} could not withstand adequately high temperatures, they were sterilized chemically. The common bacterial contamination confronted during our MBR fermentation experiments using xylose-glucose media and lignocellulosic hydrolysate was \textit{Enterobacter cloacae}, seen in Figure 4.9b. Therefore, this acetic acid-producing bacteria was isolated at the University of Borås, Sweden, and identified at the Culture Collection University of Gothenburg (Sweden) as CCUG 68890 (\textbf{Paper III}). \textit{E. cloacae} is a bacillus facultative anaerobic gram-negative bacteria that can thrive on glycerol, cellulose, glucose and xylose optimally at 37°C and pH 7.0 [243, 244]. Therefore, in \textbf{Paper III}, an iMBR set-up (same as \textbf{Paper I}) was used to examine the ability of physical decontamination...
of the fermentation system from unwanted *E. cloacae* while retaining *S. cerevisiae*. In order to find the optimum processing conditions for maximal bacterial washout, the effect of different parameters such as membrane pore size and area, medium quality, inoculum size and mixture, and filtration parameters were evaluated.

In this regard, in order to have an understanding of the filtration performance and cell washout during filtration, and to maintain cell viability and concentration [245], filtration experiments were conducted using PBS as the reactor medium. The effect of filtration parameters such as dilution rate (0.25-0.75 h⁻¹), backwash, membrane pore size (1.0 and 2.4 µm), and effective membrane filtration area (0.0069 and 0.0137 m²), and yeast and bacteria concentration (0.02-0.30 g.l⁻¹ bacteria and 0.14-0.60 yeast g.l⁻¹) on bacteria washout, and yeast retention were investigated (Paper III).

One of the main goals of applying MBRs during fermentation is yeast retention. Therefore, considering that the yeast strain used in this work had an average diameter of 2.5±0.2 µm, PES membranes with nominal pore sizes of 1.0 and 2.4 µm were used. It was observed that when the buffer solution inoculated with the yeast was subjected to filtration at both membrane pore sizes at a dilution rate of 0.5 h⁻¹, there were no noticeable changes in the turbidity of the bulk media in the MBR and permeate tank. In addition, the nutrient plate inoculation from the permeate line aliquots showed no signs of cell growth. Therefore, both pore sizes could successfully retain yeast in the iMBR.

Figure 4.10. The effect of membrane pore size (1.0 µm and 2.4 µm) on the changes in (a) the TMP and (b) turbidity of the reactor medium (R) and permeate (P) during filtration of bacteria suspension (Paper III).

Bacterial size is of great importance as it can influence pore blocking and therefore membrane fouling [246]. *E. cloacae* has a length of 0.7–1.2 µm and diameter of 0.3–0.5 µm. When the PBS inoculated with bacteria was subjected to filtration (flux 55 LMH or D=0.25 h⁻¹) using a membrane with 1.0 µm pore sizes, the TMP
rose constantly, while the turbidity of the permeate did not experience any changes, indicating that the bacteria has not been washed out of the MBR (Figure 4.10). In contrast, when using a membrane with 2.4 μm pore size, a stable TMP and flux along with considerable washout of bacteria to the permeate tank was experienced (Figure 4.10). Although, 89% bacterial washout was observed with 0.05 g.l⁻¹ starting bacterial concentration in this condition, an increase to an initial concentration of 0.1 g.l⁻¹ resulted in a significant and progressive increase in the TMP (Figure 4.11). As higher dilution rates during continuous fermentation result in higher productivity, the bacterial washout ability at dilution rates of 0.5 and 0.66 h⁻¹ was also examined. Filtration of a 0.05 g.l⁻¹ bacterial solution at about 112 LMH (D=0.5 h⁻¹) induced excessive fouling as the TMP jumped to 280 mbar in less than 2 h (Figure 3.12). During this filtration period the turbidity of the main reactor medium dropped by 49%, whereas no apparent increase was detected for the permeate. Apparently, in this case, rapid deposition of bacteria on the membrane surface forming a compact cake layer hindered effective bacterial washout [186, 187]. Surprisingly, when a 30 sec backwash cycle was applied every 3.5 min at the dilution rate of 0.5 h⁻¹, bacteria washout was ameliorated, resulting in a TMP fluctuating around 50 mbar in the 6 h filtration period. This change in filtration behavior resulted in the removal of 93% of the initial bacterial inoculum to the permeate tank (Figure 3.12).

![Figure 4.11](image1)

Figure 4.11. The effect of initial bacteria inoculum size (0.05 and 0.1 g.l⁻¹) on (a) TMP and (b) optical density of the medium during filtration (Paper III).

In order to evaluate the effect of membrane area on bacteria washout, experiments were performed at D=0.5 h⁻¹ using single or double membrane panels. Although the total permeate flow rate was the same for both configurations, as the double panel experienced half the flux of the single panel, bacteria washout occurred 1.6-times faster for the single panel. Bacteria removal was also tested in concomitant presence of yeast to have a condition resembling a contaminated fermentation system. It was observed that at D=0.5 h⁻¹ and BW
of 30 sec every 3.5 min, near complete washout of bacterial was reached for both single and double panel iMBR systems.

Figure 4.12. Changes in the TMP based on different starting yeast (Y) and bacteria (B) inoculum size (g.l⁻¹).

Following filtration experiments in PBS, fermentation and filtration of a contaminated growth-supporting semi-synthetic medium was performed. As shown in Figure 3.8, regardless of the dilution rate, in the presence of only bacteria, fouling occurs and the TMP increases even at very low inoculum concentrations (0.001 g.l⁻¹). However, confirming the results achieved in the PBS filtration, in the presence of yeast (mixed culture), even in media with 70 and 200-times larger overall inoculum, less fouling is experienced (Paper III). In order to find the optimum starting bacteria and yeast inoculum sizes that impose the least fouling, additional experiments were conducted with changes in the yeast to bacteria ratio at permeate flux of 16 LMH. As expected, with lowering of the initial bacterial inoculum size, while increasing the yeast content, the filtration performance was improved (Figure 4.12).
REVERSE MEMBRANE BIOREACTOR

By merging the technical advantages of conventional iMBRs and cell encapsulation techniques the new concept of a reverse membrane bioreactor (rMBR) was introduced in Paper IV. Built principally on the combination of the above-mentioned cell retention techniques, in rMBRs, the synthetic membrane layers confine the microorganisms and separate them from the fermentation media. This high local cell density packed in between the immersed membrane layers (submerged module) provides the opportunity for gradual exposure of cells to different substrates and inhibitors in the feed medium based on their concentration difference over the membrane and cell aggregate (Paper IV). Therefore, in this thesis, the concept of rMBR was put into practice to evaluate the potential of a lignocellulosic bioethanol fermentation system functioning based on diffusive mass transfer in enhancement of simultaneous co-utilization of prioritized (sequentially consumed) sugars, and lignocellulosic inhibitor tolerance and detoxification.

Figure 5.1. Schematic of cell position, and feed and product flow in a (a) conventional iMBR and (b) rMBR (Paper IV).
Figure 5.1 and Table 5.1 present the general differences between a conventional pressure-driven iMBR and concentration-driven rMBR with suspended and encased cells, respectively. Paper IV presents a thorough review on rMBR principles and potential biotechnological applications. In this section, the basics of diffusive mass transfer in rMBRs and their application in biofuels, especially lignocellulosic bioethanol production, are briefly discussed.

Table 5.1. Conventional iMBR versus rMBR (Paper IV).

<table>
<thead>
<tr>
<th></th>
<th>Conventional iMBRs</th>
<th>rMBRs</th>
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<tbody>
<tr>
<td>Configuration</td>
<td>Immersed</td>
<td>Immersed</td>
</tr>
<tr>
<td>Main separation driving force</td>
<td>Pressure gradient</td>
<td>Concentration gradient</td>
</tr>
<tr>
<td>Mass transfer mechanism(s)</td>
<td>Convection and diffusion</td>
<td>Diffusion</td>
</tr>
<tr>
<td>Cell/feed medium</td>
<td>Cells mixed with the feed medium</td>
<td>Cells separate from the feed medium</td>
</tr>
<tr>
<td>Cell positioning</td>
<td>Freely suspended in the medium on reactor shell side</td>
<td>Encased between membrane layers</td>
</tr>
<tr>
<td>Product recovery</td>
<td>Through the permeate (filtrate)</td>
<td>Mixed with feed on reactor shell side</td>
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</table>

5.1 Cell encapsulation and its challenges

As rMBRs and cell encapsulation techniques share the same functional principles, a knowledge of cell encapsulation helps to better understand rMBRs. In bioprocesses, in addition to process conditions such as temperature, pH, etc., high cell concentration in the bioreactor is an influential factor defining the productivity and bioconversion rate [196]. In this regard, various natural or artificial cell retention, confinement, or immobilization methods such as flocculation, encapsulation, or application of MBRs have been practiced in order to reach high cell densities in the bioreactors. A natural approach to retain cells in the reactor is flocculation, by which cells form flocs that either settle or float in the reactor, assisting product recovery with minimal cell washout. Cells can also be immobilized artificially by entrapment in a bead or capsule with a gel matrix having agar, polyacrylamide, alginate, collagen, chitosan, etc. as the main constituent [247]. The main purpose of cell encapsulation is to benefit from the exceptional features of high local cell density within a permeable capsule, especially when handling complex feeds such as lignocellulosic hydrolysate. The concentration gradient built over the capsules’ membrane and also through the cell aggregate, from the inner surface of the capsule membrane to the core, provides cells with the ability to simultaneously co-utilize different substrates and to have enhanced performance regarding inhibitor tolerance and detoxification. In case of suspended cells, all cells are exposed to the same concentrations of substrates and inhibitors in the bulk medium, whereas in capsules, diffusive mass transfer defines the concentration of compounds in the cell-surrounding microenvironment [248, 249]. Although the gradual exposure of different sugar sources and inhibitory compounds through diffusion can assist their co-
consumption and detoxification, preparation of these encapsulated microenvironments has its own challenges, hindering their industrial application. Cell encapsulating is a laborious and time-consuming process. Moreover, capsules can break due to heavy agitation in the bioreactor or undergo rupture due to gas production [48, 250]. In case the fermentation medium contains prioritized sugars such as xylose and glucose, cell release from the capsule may cause an imbalance in the simultaneous sugar consumption [251]. Incomplete utilization of xylose has been reported by Ishola et al. [109] using encapsulated yeast cells. Considering the advantages and shortcomings of cell encapsulation, the new technique of rMBR, which includes cell confinement in between two adjacent synthetic membrane layers, was introduced and discussed in Paper IV.

5.2 rMBRs

5.2.1 Principles of diffusion-based separation in rMBRs

Cell confinement by membrane layers divides rMBR systems into three diffusion phases of: cell aggregate (in between membrane layers), bulk feed (reactor shell side), and the membrane. Each of these phases affect the diffusion behavior of the medium constituents and the bioconversion kinetics differently. Therefore, it is essential to study the factors affecting the diffusion of various substances to and from the membrane-confined region. The diffusion pattern of different chemicals in an rMBR system can be influenced by the viscosity, concentration, temperature, membrane porosity, surface charge, hydrophobicity, cell-membrane and cell-cell interactions, etc. (Paper IV). As presented in Figure 5.2, in an rMBR, diffusion can be simplified as, the diffusion of feed compounds (e.g., sugars and inhibitors in lignocellulosic hydrolysate) toward and metabolites (e.g., ethanol and glycerol) away from the membrane surface, diffusion of compounds (substrates and products) through the membrane and diffusion of these compounds in the cell aggregate (cell side) (Paper IV).

Figure 5.2. Diffusion stages and transfer direction of substrates and products in an rMBR (Paper IV).
The concentration gradient of different solutes over the membrane surface drives the diffusion towards or away from the membrane in the bulk feed. This diffusion by random movement of solutes occurs from regions of high concentration to those of lower concentration in order to reach concentration homogeneity and equilibrium. A number of different models that can provide an estimate of the diffusion rate or molar flux in porous and non-porous membranes are provided in Paper IV. Fick’s first law of diffusion, which relate diffusion rate (diffusive flux) of compounds in a steady state to the concentration gradient through the ease at which molecules move in a medium (diffusivity or diffusion coefficient), best represents the diffusion pattern in the rMBRs used in Papers V and VI. In microfiltration membranes, pores provide open channels for diffusion and act as a cell-feed contactor [252]. Although, porous synthetic membranes provide a continuous liquid linkage between the two regions separated by the membrane, quality of the membrane, hydrophobicity, charge, porosity, etc. can affect the diffusion rate of different compounds [252]. According to Fick’s first law, when a steady state is reached and the flux has stabilized, the flux of compounds through the membrane is inversely proportional to the thickness of the membrane (Paper IV).

In an rMBR, the cell-side is the most critical region as the substrate provision through diffusion meets bioconversion. Similar to cell encapsulation [248, 251, 253], this cell-packed region determines the inhibitor tolerance and in situ detoxification, and co-utilization of sugars when conducting rMBR fermentation using lignocellulosic hydrolysate. The diffusion of solutes in the cell-side can be affected by the compactness and thickness of the cell aggregate, the cell-interactions, and presence of microbial compounds such as extracellular polymeric substances (EPS). Paper IV has covered different scenarios of diffusion in yeast/bacterial aggregate or biofilm.

5.2.2 rMBR-assisted biofuel production

As rMBRs can provide bioprocessing with new technological features, they have recently been applied for the production of biogas and bioethanol [48, 254]. In these applications, a variety of different rMBR membrane modules have been used to house cells. Some of these membrane module configurations such as membrane sachets, flat sheet membrane panels, and multi-layer membrane columns are sketched in Figure 5.3. These rMBR immersed modules can be suspended (sachets) [255] or fixed in place (multi-layer packed column and flat sheet panel) [48, 256] (as the set-up in Papers V and VI) in the bioreactor. The rMBR sachet configuration containing a bacterial consortium confined by the PVDF membrane layers has successfully been used for biogas production from syngas or a mixture of syngas and organic substances [254]. Furthermore, this rMBR configuration has been used in bio-methanation of substrates containing inhibitory substances such as D-limonene (compound with hydrophobic nature), protecting cells and leading to a more robust process when compared with freely suspended cells [255, 257]. The multi-layer packed
column rMBR formed of fixed double-layer membrane sandwiches placed vertically as a column has also been used for biogas production [256]. In another research work by Ishola et al. [48], flat sheet panels were used to encase yeast cells for production of bioethanol from semi-synthetic medium and lignocellulosic hydrolysate. Considering that rMBRs have mainly been the focus of biogas production research, investigation on their relatively less studied principles and applications in lignocellulosic bioethanol production has been focused on in Papers IV, V and VI.

![Figure 5.3](image)

**Figure 5.3.** The rMBR set-ups used for the production of biogas and bioethanol: (a) membrane sachets, (b) packed multi-layer membrane column, and (c) flat sheet membrane panels (Paper IV).

### 5.2.3 Diffusion measurement

In order to measure the diffusion rate of compounds through a membrane, different methods have been suggested. Some of these approaches and model equations used to measure the diffusional flow of compounds through a membrane have been elaborated in Paper IV. One of the main techniques used to measure the diffusion rate of solutes through a porous membrane is the application of diffusion cells [252, 258-260]. Diffusion cells are commonly used to measure the diffusion rate of pharmaceuticals and cosmetic compounds through membranes. These diffusion cells can be configured as vertical or side-by-side and ran in static or flow-through modes to measure the finite and infinite dose permeation and flux of compounds through the membrane [252, 258-262].

In order to have an estimate of the diffusion behavior of different compounds involved in the fermentation of wheat straw hydrolysate in a diffusion-driven rMBR, a side-by-side diffusion cell was utilized in Papers V and VI. As illustrated in Figure 5.4, in order to resemble the rMBR, the diffusion cell consisted of a donor chamber, containing compounds in the feed such as xylose, glucose, and furfural, and a receptor chamber, containing fermentation products such as ethanol and glycerol, separated with a single PES membrane layer...
(0.3 µm pore size) isolated from a 2nd generation IPC. In Paper VI wheat straw hydrolysate was used as the donor chamber media with changes in the furfural content (up to 20 g.l⁻¹), while in Paper V, a semi-synthetic media (furfural content of 0-10 g.l⁻¹) was used as the donor media with the ratio of compounds set in accordance to those of the hydrolysate used in Paper I.

Figure 5.4. The scheme of the diffusion cell used in Papers V and VI.

In order to measure the counter-diffusion behavior of compounds through the membrane, sample aliquots were taken at different time intervals from the receptor compartment, and fresh receptor medium was replaced instead (Papers V and VI). The cumulative amount (Q) of each compound passed through a unit area of the membrane (g.cm⁻²) was then calculated using equation (5.1) [263]:

\[ Q = \left( C_n V + \sum_{i=1}^{n-1} C_i S \right) / A \]  

(5.1)

Where S is the sample volume (ml), V is the chamber volume (ml), A is the membrane surface area (cm²), and \( C_n \) is the receptor chamber concentration (g.ml⁻¹) at the nth sampling, and \( \sum_{i=1}^{n-1} C_i S \) is the total amount (g) of a compound diffused through the membrane until the n-1th sampling. As shown in Figure 5.5, the cumulative amount (Q) was plotted against time and considering Fick’s first law of diffusion (Paper IV), the slope of the adapted regression line was determined as the diffusive flux \( (J) \) of a component through the membrane [264]. The diffusive flux of compounds were measured for both the semi-synthetic media (Paper V) and wheat straw hydrolysate (Paper VI).

As can be seen in Figure 5.5a, the 2:1 concentration ratio of glucose to xylose has led to 1.8 times faster diffusion of glucose (0.0033±0.0003 g.cm⁻².h⁻¹) through the membrane. Although the sugar content of the hydrolysate used in Paper VI was nearly double that of the semi-synthetic (Paper V), having the same glucose to xylose ratio, lead to a relatively comparable diffusion rate ratio (Figure 5.5b). However, as co-utilization of sugars is pursued in an rMBR, this faster diffusion may cause prioritized sugar uptake as xylose utilization may be suppressed until glucose is highly depleted [74, 265, 266].
Figure 5.5. Changes in the accumulated amount of different substrates and metabolites involved in the fermentation of (a) the semi-synthetic media (Paper V) and (b) wheat straw hydrolysate (g.cm$^{-2}$) (Paper VI) by time in the diffusion cell.

As the exact local concentration of fermentation products such as glycerol and ethanol in and around the cell aggregate confined by the membrane layers is not known, an initial content of 15 g.l$^{-1}$ ethanol and 5 g.l$^{-1}$ glycerol were provided in the receptor, resulting in fluxes of 0.0024-0.0029 and 0.0008-0.0009 g.cm$^{-2}$.h$^{-1}$,
respectively (Figure 5.5). Moreover, in the semi-synthetic media the diffusion rate of furfural increased four times from 0.0006 g.cm⁻².h⁻¹ at an initial furfural concentration of 2.5 g.l⁻¹ to reach 0.0024 g.cm⁻².h⁻¹ at 10 g.l⁻¹. As the concentration gradient defines the diffusive flux (Fick’s first law of diffusion), this proportional change in flux was also observed when the furfural content of hydrolysate was increased from around 1.7 g.l⁻¹ (0.0004 g.cm⁻².h⁻¹) to 20 g.l⁻¹ (0.0041 g.cm⁻².h⁻¹) (Figure 5.5). These diffusion results were further compared in Papers V and VI with the estimated consumption, conversion or production rates of substances in the rMBR fermentation using feed media with the same compositions.

5.2.4 Lignocellulosic ethanol production using rMBRs

Among the main concerns during the fermentation of lignocellulosic hydrolysates are the presence of different prioritized sugars and inhibitory compounds. As presented in details in Paper IV, rMBRs have the potential to provide the microenvironment required for improving the cells’ inhibitor tolerance and substrate co-utilization. In cell aggregates having an average thickness of several millimeters or more, such as the ones provided in capsules, flocs, or rMBRs, the diffusion of compounds through the aggregate dictates the concentration of substrates and inhibitors that cells are exposed to during the fermentation of lignocellulosic hydrolysate. Therefore, the concentration gradient in the cell aggregate can hypothetically divide the cell aggregate into different regions with cells of different metabolic activity. As illustrated in Figure 5.6 (Paper IV), in a non-inhibitory medium containing a single substrate such as glucose, the cells placed closer to the membrane surface (cell aggregate frontier) are exposed to a high concentration of glucose. As these cells get involved in rapid consumption of glucose, if the diffusion rate of glucose is insufficient, the yeast cells placed deeper in the cluster experience low nutrient levels or starvation. However, in complex feeds such as lignocellulosic hydrolysate, different hexose (e.g., glucose) and pentose (e.g., xylose) saccharides are present for which consumption is prioritized by yeast cells. In this regard, in a freely suspended cell system, slow xylose uptake is experienced, both in the presence of high glucose content or after its total depletion [251, 267, 268]. However, when an aggregate of xylose-consuming yeast is encapsulated or encased in an rMBR system, cells placed in the regions adjacent to the membrane surface consume glucose and cells closer to the core of the aggregate, which experience low concentrations of glucose (glucose deprived or starved regions), get involved with xylose utilization (Figure 5.6c). As reported by Westman et al. [251], while glucose conversion rate by cells closer to the capsule surface is defined by the diffusion rate, the consumption rate defines the xylose uptake for cells placed deeper in the encapsulated cell aggregate.
Figure 5.6. Schematic figure on the medium sugar and inhibitor profile and their effects on cell metabolism in an rMBR system: (a) non-inhibitory medium with the presence of a single sugar source, (b) inhibitory medium with the presence of a single sugar source, and (c) non-inhibitory medium with the presence of two prioritized sugar sources (Paper IV).

However, the scenario changes when the media contain inhibitors such as furan aldehydes that can be detoxified by cells. In this condition, the cells at the outer surface of the cell aggregate are exposed to a highly inhibitory medium (the same as what suspended cells experience). The first priority for cells in these regions is to detoxify the medium to a less inhibitory level to maintain their metabolic and physiological activities. Therefore, most of the sugars diffuse further through the cell aggregate, where the above-mentioned sugar consumption steps occur [248, 253]. Moreover, following the principles applied in cell encapsulation, cell retention in an rMBR can increase the inhibitor tolerance by manipulating the cells’ counter stress responses and mass transfer patterns (Paper IV) [26, 41, 248, 253, 269]. In this regard, if proper conditions regarding the balance between the sugars and inhibitors concentration, and cell concentration and cell aggregate thickness, are met in an rMBR system treating the lignocellulosic hydrolysate, simultaneous sugar consumption and inhibitor in situ detoxification and ethanol production can be achieved.

In Paper V, the practicality of the hypothesis put forward was examined by rMBR fermentation of xylose-glucose semi-synthetic media containing different levels of furfural, resembling those of lignocellulosic hydrolysate. The composition and concentration of the semi-synthetic medium were set in accordance with the wheat straw hydrolysate used in Paper I, with changes in the furfural content (0-10 g.l⁻¹).

Considering the glucose diffusion and consumption rate during the rMBR fermentation, the results showed surprisingly that in the rMBR set-up the utilization rate of glucose for a medium containing 2.5 g.l⁻¹ furfural was similar to that of the non-inhibitory medium (Figure 5.7). As the diffusion rate recorded in the diffusion cell was two times more than that of the glucose consumption rate in the rMBR (Figure 5.8), the limiting factor should be hindrance of diffusion in the cell aggregate or the bioconversion kinetics. A similar trend
was observed for xylose consumption, with diffusion in the diffusion cell being five-times higher than the utilization rate in the rMBR (Figure 5.8). However, the increase in the furfural content up to 10 g.l⁻¹, dramatically dropped (more than 90%) the glucose and xylose utilization (Figure 5.7). The reasons for the poor metabolic performance of the inhibitor-stricken cells are thoroughly described in Paper V.

Figure 5.7. Changes in the amount of substrates and metabolites consumed, converted, or produced during the rMBR fermentation (Paper V).
Figure 5.8. The comparison of the diffusive flux of different compounds in the diffusion cell with their consumption, conversion or release rate in rMBR (Paper V).

The diffusion behavior of furfural and cell detoxification capabilities in the rMBR system determine the robustness of the lignocellulosic ethanol fermentation process. Benefitting from the conditions provided in the rMBR, at a cell concentration of 0.82 g.l⁻¹, the furfural bioconversion occurred at the same pace as its diffusion through the membrane at furfural concentrations of 2.5 and 5 g.l⁻¹ (Figure 5.8). This exceptional behavior proves that as the furfural detoxification rate increases with the increase in the diffusion rate through the membrane, the limiting factor in furfural conversion in the rMBR containing concentrations between 2.5 to 5 g.l⁻¹ has been the diffusion rate. In the condition with the presence of 2.5 g.l⁻¹ of furfural, the discussed diffusion regions are formed in the rMBR as all the furfural diffused is converted and has no interference with the consumption of glucose and xylose diffusing deeper in the cell aggregate. Therefore, in no or low inhibitory conditions, the cells closer to the surface of the cell aggregate utilize glucose, while deeper in the aggregate, cells experiencing less catabolic repression consume xylose simultaneously. This hypothetically divides the cell aggregate into a high-inhibitor/detoxification region, a low-inhibitor/glucose consumption region, and a low-inhibitor and glucose/xylose consumption region. However, as the inhibitor content is increased to 10 g.l⁻¹, the diffusion rate becomes four-times faster than that of 2.5 g.l⁻¹, and the fermentation bottleneck becomes the slower bioconversion rate compared to the diffusion (Figure 5.8). In this high inhibitor diffusion to conversion ratio, the furfural infiltrates deeper toward the core of the cell aggregate, disturbs the rMBR fermentation balance, and dramatically plunges the substrate utilization and ethanol fermentation. However, the discussed scenarios can simply vary depending on the microorganism.
inoculum size, the concentration of substrates and inhibitors, membrane quality and porosity, membrane module dimensions, etc.

In this regard, in Paper VI, the diffusion based bioconversion of wheat straw hydrolysate containing up to 20 g.l⁻¹ of furfural in an rMBR set-up benefitting from twice the initial sugar content (same glucose to xylose ratio), membrane area (four IPC panels), and inoculum size compared to the aforementioned semi-synthetic rMBR (Paper V) was investigated.

Considering the 1.8-times faster diffusion rate of glucose than xylose (Figure 5.5b), xylose was consumed 2.7-times slower than glucose during rMBR fermentation of the as-received hydrolysate. Although, glucose and xylose consumption rates were lower than the estimated diffusion rates (Figure 5.5b), about 87% of the initial sugars were consumed at a specific consumption rate of 0.43 g.g⁻¹.h⁻¹ (Figure 5.9). In this rMBR test, at an initial furfural concentration of 1.7 g.l⁻¹ (furfural to cell ratio of 0.93:1), a complete furfural detoxification was obtained within 40 h of fermentation (Figure 5.9). According to the hypothetical scenarios presented in Paper V, in this sub-inhibitory condition the low diffusion rate of furfural only affects a fraction of cells located in the surface of the cell aggregate, while cells placed in the deeper layers continue to thrive on the nutrients. This resulted in a high ethanol yield of 0.48 g.g⁻¹ sugars⁻¹ corresponding to an ethanol release rate of 0.0025 g.h⁻¹.cm⁻² into the reactor bulk media. The achieved high ethanol yield may be due to the minor disturbance in cell reproduction and biosynthesis activities and drop glycerol production [26, 52, 270, 271]. Increasing the initial furfural content of the hydrolysate to about 9 g.l⁻¹ (furfural to cell ratio of 5:1) was followed by the reduction in the overall sugar consumption to about 42% (Figure 5.9). In this condition as furfural diffuses 4.2-times faster than in the as-received hydrolysate (Figure 5.5b), more layers of cells in the cell aggregate are involved in furfural detoxification and less total sugar is utilized. However, this sugar utilization rate is still 10-times faster than that experienced in Paper V when using a semi-synthetic medium containing 10 g.l⁻¹ of furfural. Despite the relatively high furfural concentration and associated issues like reduction in cellular energy, and accumulation of alcohol dehydrogenase [270, 271], a considerably high ethanol yield of 0.36 g.g⁻¹ sugars⁻¹ (specific productivity of 0.07 g.g⁻¹.h⁻¹) was obtained. In this rMBR set-up encased cells performed exceptionally by detoxifying about 89% of the added furfural at specific conversion rate of 0.05 g.g⁻¹.h⁻¹ (Figure 5.9). For the rMBR fermentation set-up used in Paper VI, the inhibition threshold was reached at furfural to cell ration of 9.5:1 (16 g.l⁻¹ of furfural). Surprisingly, even in this highly inhibitory condition, cells sustained their viability up to 40 h, converting 38% of the initial furfural, before the metabolic activities such as detoxification and sugar utilization come to a halt. The effect of the inoculum size, furfural to inoculum ratio, membrane area and membrane panel size on the performance of an rMBR are further discussed in Paper VI.
Figure 5.9. Changes in the amount of glucose and xylose consumed, ethanol produced and furfural converted in the rMBR fermentation of wheat straw hydrolysate containing 1.7, 9, 16, and 20 g/l of furfural.

5.2.5 Development of a new flat sheet membrane module for rMBR applications

In order to have an optimized rMBR fermentation process with improved co-consumption of substrates and inhibitor detoxification, the rMBR membrane module should be technically customized to fulfill the needs of the scientific theories put forward in Paper IV. Based on the principal requirements of an rMBR fermentation system (Paper IV) and the experiments conducted (Papers V and VI), the obstacles in the application of the 2nd generation IPC for rMBR processes were diagnosed. Accordingly, some of the parameters that should be considered in the selection or development of the rMBR modules for fermentation of complex feed such as lignocellulosic hydrolysate are considered in this section.

The rMBR membrane module should have the flexibility of operation in closed or open channel modes. This means cells can either be confined/retained in the module (closed channel) as performed using 2nd generation IPCs, or recirculated in an external loop to and from an interconnected cell culture reactor. If this option is provided, cells can be inoculated to the panel, harvested from the panel, or even replaced with a fresh culture without any interference from the bulk bioreactor feed. Moreover, as production of gaseous compounds such as CO₂ during fermentation in between the hydrophilic membrane layers may disturb the
diffusion pattern, periodical recirculation of cell aggregate to an external tank can help degassing the system. This option can also be of great advantage when rMBRs are applied for anaerobic digestion purposes. In addition, by this recirculation, the cell environment with regards to pH, nutrients, etc. can be adjusted favorably to sustain cell viability, external to the reactor as the fermentation is ongoing. This is of great importance when fermentation of inhibitory media is concerned.

Furthermore, to build rather separate concentration-divided regions in the cell aggregate to have the optimal response regarding simultaneous utilization of substrates and inhibitor in situ detoxification, the dimensions of the hollow channel confined by the membrane is critical. The compactness and thickness of the encased cell aggregate directly affects the diffusion and bioconversion rate of the medium components, and defines the success rate of an rMBR fermentation process (Papers IV, V and VI). Additionally, it is important that cells are evenly distributed throughout the module to cover the maximum contact area provided by the membrane. Therefore, the dimension and orientation of the cell housing channels in an rMBR module should be tailored to that of the application needs.

![Image of 3rd generation IPC membrane module](image)

Figure 5.10. The 3rd generation IPC membrane module (biomass membrane contactor) designed and developed during this thesis for rMBR applications. The images present the panel design, and development stages, as well as the final membrane coated perforated multi-wall sheet panel [272].

In case the above-mentioned characteristics are provided in an rMBR membrane module, the fermentation media can be replaced with fresh feed for several batches while cells are reused. Considering the general requirements of an rMBR membrane module put forward, a novel membrane panel was designed,
developed, and patented during this thesis at VITO NV, Belgium [272]. As shown in Figure 5.10, the developed 3rd generation IPC membrane module or “Biomass Membrane Contactor” consists of a perforated polycarbonate multi-wall sheet as the main skeleton, with 0.1 µm pores size PVDF membrane coated on either side of the panel (with a pure water permeability of 1300±100 l.h⁻¹.m⁻².bar⁻¹). The panel includes side frames connecting the consecutive channels horizontally or vertically to facilitate the passage of fluids or biomass in a zigzag manner. The design supports the internal recirculation of cells or fluids, and the channel size (cross-section) can easily be increased or decreased depending on the application.
CONCLUDING REMARKS AND FUTURE DIRECTIONS

The findings in this thesis proved the integration and application potential of immersed pressure-driven and concentration-driven MBRs in lignocellulosic bioethanol production. The general conclusions drawn can be listed as follows:

- The continuous fermentation iMBR set-up used for bioethanol production from wheat straw hydrolysate could operate at unconventionally high concentrations of suspended solids up to 20% w/v without significant deterioration of the filtration properties. This exceptional filtration performance was also observed in filtration of fermentation broth containing up to 9.5% w/v yeast cells. The retention of high cell density in the iMBR enhanced the sugar consumption (total glucose and up 83% xylose), leading to high ethanol volumetric productivities of up to 4.6 g.l\(^{-1}\).h\(^{-1}\). This successful high-productivity continuous performance signals enhanced process feasibility by the integration of flat sheet microfiltration iMBRs into the commercial production of lignocellulosic bioethanol.

- Application of a double-staged iMBR for continuous hydrolysis-filtration and co-fermentation-filtration facilitates the separation of released sugars from lignin-rich solids during hydrolysis, cell concentration, and in situ recovery of products during fermentation, reducing the intensity of the downstream processing. Considering the practiced conditions in this thesis, this set-up can effectively intensify the lignocellulosic ethanol production process at a flux of 21.9 LMH for long-term continuous hydrolysis and fermentation (up to 264 h) with negligible changes in the filtration performance. Moreover, lignin residuals can be successfully concentrated (up to 70% of medium SS content) for future valorization.

- As it was observed that flux plays a more important role in fouling and cake layer formation than other factors such as SS, effective BW regimes, air/gas sparging, and flux control can be applied to
keep the membrane surface clean for long-term filtrations to reduce the process downtime, membrane intensive cleaning requirements, and operational costs.

- The iMBR set-up used in this thesis proved to be a promising approach for bacteria decontamination from fermentation systems if filtration parameters and culture condition are well synchronized. In non-growth media, enhanced physical bacteria decontamination can be reached (93% or more removal); however, profound investigation is required to optimize the iMBR-assisted bacteria removal from the actual fermentation broth.

- Based on the results acquired from the rMBR fermentation experiments, it can be concluded that this approach in cell confinement remediates the limitations in industrial application of cell encapsulation, and has the potential to tackle some of the issues confronted with lignocellulosic bioethanol production such as the presence of prioritized sugars and inhibitory compounds. The diffusive mass transfer over the membrane and the cell aggregate led to enhanced simultaneous utilization of xylose and glucose. In addition, cells’ furfural tolerance and in situ detoxification capabilities were boosted in highly inhibitory media containing up to 16 g.l⁻¹ of furfural.

**FUTURE DIRECTIONS**

The research conducted in this thesis, broadens the understanding of the application potential of immersed MBRs in lignocellulosic bioethanol production. Based on the findings reported in this thesis, complementary follow-up research work can be suggested to further expand the knowledge on the possibilities of the integration of membrane technology into 2nd generation bioethanol production.

- In order to further intensify and enhance the technical and economic feasibility of the lignocellulosic bioethanol production process, coupling the double-staged iMBR with a cascade of membrane units providing enzyme retention, sugar concentration, inhibitor separation, and ethanol concentration should be investigated.

- Fouling behavior, cake layer quality and formation stages, and the interactions of yeast and lignin-rich suspended solids with the immersed membrane during the hydrolysis and fermentation of lignocellulosic materials require further in depth research (microscopic analysis of membrane surface).

- Techno-economic analysis of the application of continuous iMBRs for 2nd generation bioethanol production and its comparison to the benchmark technology should be performed.

- A profound study of the effects of membrane filtration parameters (pore size, membrane quality, flux, BW, etc.) and membrane-bacteria-yeast interactions at the microscopic level should be
performed in order to optimize the physical decontamination of fermentation systems using iMBRs.

- The application of rMBRs should be further expanded from lignocellulosic bioethanol production to bioconversion of other complex feed streams (e.g., food waste) containing different substrates and inhibitory compounds.
- The potential of the newly developed biomass membrane contactor (3rd generation IPC) in rMBRs used for biological treatment of different feedstock requires further evaluations.
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References


185. Akhondi, E., et al., The performance and fouling control of submerged hollow fiber (HF) systems: A review. Applied Sciences (Switzerland), 2017. 7(8).


Continuous bioethanol fermentation from wheat straw hydrolysate with high suspended solid content using an immersed flat sheet membrane bioreactor

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HIGHLIGHTS

• Immersed membrane bioreactor was used for continuous lignocellulosic bioethanol fermentation.
• The iMBR was optimized to work flawlessly at high suspended solid content (20%).
• Enhanced co-consumption of total glucose and up 83% xylose content was achieved.
• A bioethanol content of up to 83% of the theoretical yield was obtained.

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ABSTRACT

Finding a technological approach that eases the production of lignocellulosic bioethanol has long been considered as a great industrial challenge. In the current study a membrane bioreactor (MBR) set-up using integrated permeate channel (IPC) membrane panels was used to simultaneously ferment pentose and hexose sugars to ethanol in continuous fermentation of high suspended solid wheat straw hydrolysate. The MBR was optimized to flawlessly operated at high SS concentrations of up to 20% without any significant changes in the permeate flux and transmembrane pressure. By the help of the retained high cell concentration, the yeast cells were capable of tolerating and detoxifying the inhibitory medium and succeeded to co-consume all glucose and up to 83% of xylose in a continuous fermentation mode leading to up to 83% of the theoretical ethanol yield.

1. Introduction

In order to meet the global energy and fuel demands, fossil sources are extremely stressed heading towards early depletion by over excavation. Considering these energy concerns plus the proven environmental impacts of using fossil fuels (Nigam and Singh, 2011), production of alternative renewable more environmentally friendly fuels such as bioethanol has been given substantial research attention. However, the production of biofuels has been limited by choice of raw material, process costs and production scale (Börjeson et al., 2012; Gnansounou, 2010; Naik et al., 2010). First generation bioethanol is mainly being produced from starch and sugar based raw materials such as corn and sugarcane that are of high human food and animal feed value that make their use for bioethanol production ethically controversial. However, second generation bioethanol is produced from lignocellulosic biomass such as agricultural and forest residues that are potential candidate raw materials for sustainable bioethanol production (Brown and Brown, 2013). The fact that lignocellulosic materials are renewable, abundant, available in different forms and types depending on the geographical location, cheap and not considered as food or feed have put 2nd generation bioethanol production in the centre of research attention for several decades (Limayem and Riche, 2012; Maurya et al., 2015).

Lignocellulosic materials are structurally composed of cellulose, hemicellulose and lignin. Depending on the type of lignocellulosic raw material, different hexoses (glucose, mannose etc.) and pentoses (xylose, arabinose etc.) can be released and fermented to ethanol. The advantage of using lignocellulosic raw materials as a source of bioethanol is the higher sugar content compared to conventional raw materials.
hydrolysate may contain different concentrations of cell inhibitory compounds such as furans (furfural, 5-hydroxymethyl furfural (HMF)), phenolic compounds and carboxylic acids (acetic acid etc.). Among ethanol fermenting microorganisms yeast *Saccharomyces cerevisiae* has long been the most favorable choice for industrial bioethanol fermentation due to its high ethanol productivity and tolerance (Casey and Inglewed, 1986; Ghareib et al., 1988; Stanley and Hahn-Hägerdal, 2010; You et al., 2003). In addition, some yeast strains have the ability to convert furfural and HMF to less inhibitory chemicals such as furfuryl alcohol (Li et al., 2009; Liu, 2011; Tian et al., 2011; Ylitervo et al., 2013). An industrial concern in production of lignocellulosic ethanol is co-utilization or in other words, simultaneous consumption of the main sugars, glucose and xylose. The wild-type *S. cerevisiae* strain is a prime glucose consumer, therefore it shows no utilization of pentose sugars such as xylose (Sánchez Nogué and Karhumaa, 2015; Van Zyl et al., 1989; Zaldivar et al., 2001). The genetically engineered recombinant *S. cerevisiae* strains can uptake pentoses (Sánchez Nogué and Karhumaa, 2015; Stanley and Hahn-Hägerdal, 2010), however, xylose consumption only occurs when the media is depleted from glucose (Bertilsson et al., 2008; Hamacher et al., 2002; Meinander et al., 1999; Sedlak and Ho, 2004). This limitation causes stepwise preferential consumption of glucose and then xylose in a fermentation medium containing a mixture of sugars such as lignocellulosic hydrolysate. Therefore, in order to have closer to complete sugar utilization, higher inhibitor tolerance and higher conversion rate during fermentation of lignocellulosic hydrolysate, high cell concentration in the bioreactor has to be guaranteed. In this regard, one of the most effective remedies for cell retention is using a membrane bioreactor (MBR) (Mahboubi et al., 2016; Ylitervo et al., 2013).

Membranes are generally used in a wide range of separation and filtration applications due to their superior ability in separating different constituents (chemical compounds, cells etc.) of a mixed media. Membrane material, pore size, and morphology define its charge, hydrophobicity and affinity to different compounds due to an applied pressure or concentration gradient acting as the driving force over the membrane (Judd and Judd, 2011; Mahboubi et al., 2016). In biological processes membranes are integrated with bioreactors mainly with the aim to ease product separation and recovery or to reach high cell concentrations (Carstensen et al., 2012; Mahboubi et al., 2016).

There are two general configurations for the pressure driven MBRs: immersed/submerged (iMBR) (membrane module immersed either in the main bioreactor or a separate compartment) and side-stream/external loop (sMBR) (module placed in an external chamber). In the iMBR application of a negative pressure (under-pressure) on the filtrate side defines the transmembrane pressure (TMP) and the separation driving force (Carstensen et al., 2012). Although higher permeate (filtrate) fluxes can be obtained while having less concerns for membrane fouling due to cake layer formation in a sMBR, iMBRs are favored regarding energy and space needed and also ease of operation (Judd and Judd, 2011; Radjenović et al., 2008; Singhania et al., 2012). Considering the high cell density achieved in MBRs, continuous ethanol fermentation can be performed at high dilution rate (the ratio of feed flow rate to reactor working volume) and productivity without being concerned with undesirable cell washout. A thorough list of final cell concentrations achieved using different membrane modules for bioethanol production is presented in a review by Mahboubi et al. (2016).

In contradiction to sMBRs where fermentation broth is recirculated at high shear stresses across the membrane surface, in iMBRs the membrane module is submerged into a mixture of cells and fermentation medium where broth conditions become of extensive importance in defining membrane filtration performance and the extent of fouling (Mahboubi et al., 2016). Utilization of feed streams such as lignocellulosic hydrolysate containing high concentrations of suspended solids (SS) e.g. undigested undissolved lignin particles is considered a hurdle to MBR assisted ethanol fermentation. Through the lignocellulose pretreatment process lignin residues (non-fermentable polymeric compounds) are released, increasing the viscosity of the slurry (Verardi et al., 2012; Zhao et al., 2012). In an IMBR assisted fermentation process a raise in the viscosity level not only hinders proper medium mixing and mass transfer but also contributes to membrane fouling by inducing cake layer formation, reduction in shear stress on the membrane surface by air/gas sparging or medium agitation (Galbe and Zacchi, 2002; Judd and Judd, 2011; Liu et al., 2015; Sassner et al., 2006; Verardi et al., 2012).

In order to have a reliable lignocellulosic bioethanol production approach, a robust fermentation process is needed to tackle the above mentioned issues. In this regard, in the current study an IMBR set-up was applied in a continuous bioethanol fermentation process for both semi-synthetic media and lignocellulosic hydrolysate using a strain of xylose-consuming recombinant yeast aiming at enhanced co-utilization of xylose and glucose in a high suspended solid medium. The functioning IMBR system using integrated permeate channel (IPC) membrane panels was modified to work at very high suspended solid concentrations in the bioreactor and feed stream, while preserving membrane functionality through stabilization of filtration parameters and membrane fouling prevention. Moreover, modifications were made to the fermentation conditions integrated with filtration adjustments to boost the co-bioconversion of the pentose and hexasaccharides in the semi-synthetic substrate and lignocellulosic hydrolysate to bioethanol and to have optimal ethanol recovery from the system through in situ membrane assisted product recovery and additional ethanol traps.

2. Materials and methods

2.1. Lignocellulosic slurry and enzymatic hydrolysis

The lignocellulosic substrate used in this experiment was a xylose-rich agricultural residue of wheat straw obtained in Sweden. For the pretreatment, the wheat straw was treated with dilute acid (0.3–0.5% H₂SO₄ for 8 min at 185 °C at SEKAB E-Technology (Örnsköldsvik, Sweden)). The pretreated wheat straw slurry was kept in the cold room (4–5 °C) prior to use. Therefore, in order to be able to reduce the SS concentration and ease the application of the substrate during fermentation the viscous as-received substrate was diluted to half its original concentration with Milli-Q water. The composition of the diluted as-pretreated lignocellulosic material is presented in Table 1. The diluted slurry had a pH of about 2.6 and contained 9.55% suspended solids (SS) and 12.10% total solids.

The wheat straw slurry was further enzymatically hydrolyzed using *Cellulase Ccret2* enzyme (Novozymes, Denmark) to have the monomeric sugars from cellulose and remaining hemicellulose released and available to cells for fermentation according to the procedure implied by Ishola et al. (2015). The activity of the enzyme was measured according to NREL protocol (Adney and Baker, 2008) to be around 180 FPU ml⁻¹ and subsequently 14 FPU g SS⁻¹ was used for hydrolysis. The concentration of different compounds in the dilute, pretreated and hydrolyzed wheat straw slurry is featured in Table 1.

2.2. Yeast strain and inoculum preparation

The microorganism used in this study was a recombinant xylose-utilizing strain of *Saccharomyces cerevisiae* (Ishola et al., 2015).
The maximum growth rate of this strain in a xylose-glucose semi-synthetic medium was measured to be 0.32 h⁻¹ while it was 0.25 h⁻¹ for glucose semi-synthetic medium at pH 5 and a temperature of 30 °C. The incubation and storage of yeast before use was performed according to Ishola et al. (2015).

In order to prepare the required yeast inoculum for continuous fermentation, precultures were prepared in loop inoculated 250 ml Erlmeneyer flasks containing 100 ml of yeast extract peptone dextrose (YPD) broth consisting of 25 g l⁻¹ glucose, 25 g l⁻¹ xylose, 20 g l⁻¹ peptone and 10 g l⁻¹ yeast extract kept in a shaking water bath (Grant OLS 200, Grant instrument Ltd, UK) at 30 °C and 125 rpm for 48 h. The cultures held a biomass content of 7.5 ± 1.6 g l⁻¹ after 48 h of cultivation and were then used to inoculate the MBR.

### 2.3. Integrated permeate channel (IPC) flat sheet membrane panels and spacer box

#### 2.3.1. 2nd generation IPC membrane panels

The 2nd generation IPC membrane panels used in this research work had double filtration layers casted onto a weft-type of polyester spacer-fabric support, customized and developed at the Flemish Institute of Technological Research (VITO NV, Belgium). These IPC membrane panels developed possess a significant strength in preserving structural integrity during extensive backwash cycles because of their superior adhesion properties of the robust membrane layers to the 3D complex inter-tangled spacer-fabric (Doyen et al., 2010). The hollow area with very high porosity (85%) provided within the intertwined fabric is used for the withdrawal of the filtrate/permeate. Moreover, unlike many other commercial membrane panels available, the novel IPCs come with an inbuilt air/gas channeling with 12 diffusers (6 on each side) of each 0.5 mm in diameter at the bottom of the panel. This enables more inbuilt air/gas channeling with 12 diffusers (6 on each side) of each 0.5 mm in diameter at the bottom of the panel. This enables more efficient single panel aeration and membrane surface cleaning from fountains in comparison to universal aeration in ordinary iMBRs.

The total area covered per panel was 68.6 cm² specifically tailored to fit in the spacer box placed in the 2 L bioreactor (Bistat Bplus, Sartorius BBI Systems GmbH, Germany). The membrane coating used was based on hydrophobilized polyethersulfone (PES) with a mean pore size of ~0.3 μm and a clean water permeability of 3000–4000 l h⁻¹ m⁻² bar⁻¹.

In order to sterilize the MBR prior to each fermentation cycle, the bioreactor, tubings etc. were first autoclaved at 121 °C for 20 min and then the membrane panels and spacer box were placed into the bioreactor and chemically cleaned and disinfected using the stepwise process recommended by VITO NV and applied by Ishola et al. (2015).

#### 2.3.2. Air/gaslift spacer box

A special cubic PVC spacer box of 117.5 × 90 × 87 mm³ was designed and manufactured at VITO NV, Belgium, to house up to four IPC membrane panels acting as the middle riser compartment providing the membrane bioreactor with a favorable air/gaslift condition (Fig. 1). The spacer box has 4 membrane housing compartments which are separated with interspace planes (distance between the membrane surface and the plate was 5.5 mm) that guarantee the same flow conditions at both sides of each membrane panel. Homogeneous flow conditions are induced by circulation of gas across the aeration holes at the bottom of the membrane panels. The flow of gas/liquid mixture in the newly developed air/gaslift membrane bioreactor is presented in Fig. 1. The air/gaslift condition is induced inside the spacer box to enhance mass transfer and mixing in the cultivation media while working at high viscosity and SS content. The membrane cleaning efficiency is improved by focusing the sparged air/gas along the membrane surface inside the narrow membrane surface/plate interspace and the extent of the shear stress required for breaking cell flocs while using the highly flocculative recombinant yeast strain.

### 2.4. Membrane bioreactor design, set-up and operation

The schematic of the air/gaslift iMBR set-up used in the current study is presented in Fig. 1. The MBR consists of two 2nd generation IPC membrane panels placed in the spacer box middle riser compartments, immersed in a 2 l water-jacketed bioreactor (Bistat B plus, Sartorius BBI Systems GmbH, Germany) (Fig. 1). During the experiment the fermentation related parameters such as temperature, pH, liquid level in the reactor, foaming control and air/nitrogen flow rate were constantly controlled and adjusted by the Bistat B plus fermentation controlling unit (Fig. 1). During continuous fermentation, the substrate was continuously pumped from the feed reservoir using a Watson-Marlow 403U/R1 peristaltic pump (Watson Marlow, United Kingdom) through a 710 Atrato ultrasonic flowmeter (Titan Enterprises Ltd., United Kingdom) to the MBR (Fig. 1). By the application of an under pressure to the permeate side of the membrane by means of a Watson-Marlow 403U/R1 peristaltic pump the required TMP was built up and the filtrate was withdrawn. The pressure on the permeate line and the flow rate of permeate was further measured by means of a Microfluidic pressure sensor MPS3 (Elvenflow, France) and Atrato ultrasonic flowmeter respectively, before being collected in the permeate tank (Fig. 1). As the MBR set-up is not running in constant flux mode, a tubing line was drawn from the bottom of the bioreactor to an overflow/bleeding tank (Fig. 1). In case the liquid level surpassed a fixed desired initial level, either unintentionally due to filtration failure, or in case of deliberate cell/SS bleeding, the level probe signaled the fermentation control unit and an adequate amount of medium was automatically drained. The backwashing cycle details were signaled to the permeate pump using a Schneider Zelio logic relay (Schneider Electric Automation GmbH, Germany). The pressure sensor reader and flowmeters were connected to a computer where received data were logged for further analysis (Fig. 1).

As great amounts of air/gas sparging is required to keep the membrane clean and to have a decent mixing of the high SS medium for adequate mass transfer, considerable amounts of the final product ethanol are vaporized and stripped from the MBR through the condenser line. In order to recover the ethanol exiting the bioreactor two sequential ice-jacketed ethanol stripping traps of –0.3 °C each containing 1 l of deionized water were prepared. The line exiting the condenser entered the first trap and ethanol-poor gas left the second trap. Samples were taken from both

### Table 1

<table>
<thead>
<tr>
<th>Composition of treated slurry (g l⁻¹)</th>
<th>Acetic acid</th>
<th>Furfural</th>
<th>Glucose</th>
<th>HMF</th>
<th>Xylose</th>
<th>Xylitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-pretreated (as-received)</td>
<td>3.40 ± 0.10</td>
<td>3.09 ± 0.01</td>
<td>3.85 ± 0.08</td>
<td>0.41 ± 0.04</td>
<td>18.36 ± 0.12</td>
<td>4.61 ± 0.08</td>
</tr>
<tr>
<td>Pretreated and enzymatically hydrolyzed</td>
<td>4.55 ± 0.17</td>
<td>2.93 ± 0.01</td>
<td>50.03 ± 0.13</td>
<td>0.35 ± 0.03</td>
<td>26.16 ± 0.07</td>
<td>4.94 ± 0.05</td>
</tr>
<tr>
<td>Pretreated, hydrolyzed and autoclaved</td>
<td>4.19 ± 0.05</td>
<td>2.98 ± 0.03</td>
<td>51.39 ± 0.03</td>
<td>0.38 ± 0.01</td>
<td>25.45 ± 0.43</td>
<td>4.93 ± 0.33</td>
</tr>
</tbody>
</table>
ethanol traps at different time intervals in order to measure the recovered ethanol content.

2.5. Batch tests to determine optimal operating conditions

The fermenter was filled with 1.9 L of autoclaved broth containing 50 g l⁻¹ glucose, 50 g l⁻¹ xylose, 10 g l⁻¹ peptone, 5 g l⁻¹ yeast extract and 4.75 g l⁻¹ KH₂PO₄. After inoculation with 300 ml of the prepared preculture to a starting cell concentration of 2.3 ± 0.7 g l⁻¹, pH was corrected to 5, and 0.15 ml of fatty acid ester antifoam was added. During the batch phase, permeate recirculation was applied to expose the membrane to a gradually increasing SS content in the medium. Different combinations of initial permeate flux (dilution rates of 0.1–0.22 h⁻¹), and air sparging rate (1–4 l min⁻¹) were tested during 24-h batch runs to evaluate the performance for membrane filtration and the efficiency of the membrane cleaning approaches. Process performance was monitored in terms of permeate flux, TMP and cell dry weight.

2.6. Continuous fermentation of xylose-glucose semi-synthetic medium

The MBR was started up in batch mode as described in Section 2.5, and was then shifted to continuous operation. Typically, a 96 h continuous fermentation cycle was conducted using a YPD broth with the composition mentioned in Section 2.5 as feed. Continuous fermentation commenced after the batch phase. Nitrogen gas was sparged in the bioreactor instead of air in order to have an anaerobic condition for maximum ethanol production during continuous fermentation. The filtration regime consisted of a 4 min filtration cycle followed by a 30 s backwash cycle reversing the flow at the same rate as the forward filtration flow in order to benefit from the double cleaning effect of the gas sparging and backwashing. Samples were withdrawn at different time intervals from the main bioreactor, permeate line and applied ethanol traps to analyze the changes in the concentrations of different substrates and metabolites. In addition, in order to screen the changes in the cell concentration, cell dry weight measurement was performed.

2.7. Continuous fermentation of wheat straw hydrolysate

The acid pretreated and enzymatically hydrolyzed wheat straw (Table 1) was sieved after hydrolysis with an ordinary kitchen sieve to remove extra-large undigested residues in order to prevent tube clogging. The issue confronted when the slurry is to be pumped continuously to the reactor is that, as there is a weak flow through the tubes, sedimentation of bigger particles occurs that leads to blockage of tubes and inlet ports. Therefore, for the continuous fermentation of the hydrolysate slurry the feed reservoir was diluted to half its concentration (1/4 of the as-received concentration) and was constantly agitated with a magnetic stirrer at a stirring rate of 650 rpm at 30 °C to have a homogeneous feed and keep the solids suspended.

The continuous fermentation of wheat straw hydrolysate to bioethanol started after an initial batch phase (Section 2.5) with permeate recirculation at an aeration rate of 4 l min⁻¹ and initial flow rate representing a dilution rate of about 0.1 h⁻¹ as higher dilution rate (0.2 h⁻¹) results in extensive membrane fouling. Dur-
ing the fermentation cycles when four times diluted slurry was used as feed, the dilution rate was increased and set on ~0.12 h⁻¹ to be able to provide the growing cells with the required nutrients and to control the SS content of the medium, while the permeate flow was kept unchanged. The excess medium accumulated in the MBR was detected with a real-time level probe and removed by bleeding cells and suspended solid particles. This suspended solid bleeding from the system kept the concentration of suspended solid in the MBR comparatively lower compared to that of the half diluted feed.

2.8. Analytical methods

The concentration of different compounds and metabolites in the hydrolysate and cultivation medium was analyzed by means of high performance liquid chromatography (HPLC) (Walters 2695, Walters Corporation, Milford, USA). A hydrogen-based column (Aminex HPX-87H, Bio-Rad, Hercules, USA) working at 60 °C with 5 mM H₂SO₄ eluent flowing at 0.6 ml min⁻¹ was used for detection and quantification of the sugars and the inhibitory compounds as well as ethanol and glycerol.

Cell dry weights were determined by removing 5 ml of the medium at different time intervals, centrifuging at 3000g for 5 min, removing the supernatant and drying the cell pellet in 70 °C oven for 24 h.

The comparison of the results was performed by statistical analysis of the data acquired by the help of the software package MINITAB 17. Analysis of variance (ANOVA) was performed on the results using general linear models with 95% confidence interval followed by pairwise comparisons using Tukey’s test. It is to be noted that the experiments have been conducted in duplicates and the error bars presented on the graphs represent two standard deviations.

3. Result and discussion

In the current study, initially the desirable membrane filtration conditions for continuous ethanol fermentation were investigated in a batch fermentation mode with permeate recirculation and then the set-up was applied in continuous fermentation of xylose-glucose semi-synthetic medium and lignocellulosic hydrolysate with high suspended solid content. The step up procedure leading to robust lignocellulosic bioethanol production by means of iMBR is fully described in the following sections.

3.1. Optimization of the operation conditions in batch mode

In order to optimize the membrane assisted product recovery process, different factors affecting membrane filtration performance of iMBRs such as the intensity of gas sparging and filtration rate (permeation flux) were investigated.

One of the most effective methods for continuous physical cleaning of membrane and fouling prevention for iMBRs is air/gas sparging (Judd and Judd, 2011; Yoon, 2015). Due to the shear stress and medium turbulence created on and around the membrane surface as a result of the presence of ascending air/gas bubbles, deposition of colloidal compounds and solid particles forming a cake layer on the membrane surface is prevented (Yoon, 2015). Moreover, as most iMBRs do not benefit from an impeller stirring system, the air/gas sparging regime in an iMBR defines the mixing efficiency and mass transfer patterns in the reactor. In this regard the effects of different aeration rates of 1, 3 and 4 l min⁻¹ on the functionality of the membrane was investigated through controlling and comparing the changes in TMP, and permeate flux in a 24 h period following reactor inoculation (Fig. 2). In order to omit the effects of different suspended solid content on the membrane filtration performance the initial media contained the same amount of nutrients and initial cell density (2.3 ± 0.7 g l⁻¹) in all experiments. The medium was continuously recirculated to the main reactor through the permeate line while air was sparged by means of the infusers in the membrane panel for 24 h in order to have full conversion of nutrients to metabolites and biomass and also to observe the effects of rise in cell biomass concentration on membrane separation performance.

The filtration capability of an immersed membrane is directly affected by the condition of its surface. In the present study neither flux- nor TMP-control were applied, therefore the main effort was to provide conditions that the filtration parameters experience little variations as possible during the test period. In order to have an effective physical cleaning of the membrane and prevent fouling continuous air/gas sparging and backwashing was applied. As illustrated in Fig. 2a, for the dilution rate of 0.1 h⁻¹ and with the low aeration rate of 1 l min⁻¹ the TMP starts escalating linearly to about 90 mbar after 12 h of relatively constant value. This could be both due to gradual cake layer build up on the membrane surface due to ineffective air sparging and/or increase in the cell concentration in the medium and change of medium viscosity. Hence it is not surprising that the rise in the TMP is smaller for the higher air sparging rates of 3 and 4 l min⁻¹. The statistical analysis of the final TMP values confirms that there is a significant difference between final TMP readings of 11 min⁻¹ versus 31 min⁻¹ (P-value = 0.009) and 41 min⁻¹ (P-value = 0.007). However, the changes in the permeate flux rate were similar with no significant difference (P-value = 0.427) (Fig. 2a). As in continuous processes higher dilution rates are more favorable in obtaining high productivity rates, the initial permeate flux was more than doubled in order to examine membrane performance. As can be seen in Fig. 2a, both 3 and 4 l h⁻¹ aeration rates proved to be non-effective for sufficient membrane cleaning. In less than 12 h from the start of cultivation the TMP rose to more than 400 and 300 mbar respectively. This extensive membrane fouling caused about 13% drop in the flux for both aeration conditions in 10 h (Fig. 2b).

However, air or gas sparging does not only target membrane cleaning and medium mixing. The changes in the aeration rate in aerobic cultivation systems also changes the amount of oxygen provided to the cells, and may induce enhanced cell growth. Although this cell growth and increase in the suspended solid content of the medium is beneficial to the bioconversion rate of substrate to desirable product, it may be detrimental to membrane filtration performance by increasing the viscosity of the medium (Verardi et al., 2012; Zhao et al., 2012) and particle deposition on the membrane surface in addition to mass transfer hindrance (Judd and Judd, 2011). In anaerobic MBRs such as used here for ethanol fermentation, changes in gas sparging rates may cause ethanol losses through stripping and excessive evaporation. Therefore, applicable remedies are required to collect and recover the ethanol produced while achieving optimum membrane cleaning and filtration.

In order to recover the ethanol evaporated from the MBR, the gas escaping through the condenser outflow line was sparged in one or two subsequent water traps kept at ambient or ice cold temperature. Fig. 3a presents the changes in the ethanol content of the bulk medium in the bioreactor and the ethanol traps in a 24 h permeate recirculation cycle in batch mode. As can be seen the lower the aeration rate the higher the content of the ethanol remaining in the main bioreactor and the lower in the ethanol traps. The total cumulative amount of ethanol (in bulk medium plus recovered) showed little difference (Fig. 3b). To benefit from the high air sparging and membrane cleaning capacity and at the same time prevent ethanol loss double ice-jacketed water traps were utilized
to maximize ethanol recovery and minimize the escape of dissolved ethanol in the traps. It was indeed observed that using double traps at an aeration rate of 3 l min\(^{-1}\) lead to a noticeable (P-value = 0.005) 26% rise in the total ethanol collected in 24 h compared to a single trap (Fig. 3b). Moreover, when long continuous fermentation cycles are targeted, efficient ethanol stripping from the bioreactor is essential in order to alleviate product inhibition effects on yeast cell metabolism (Taylor et al., 2010). Higher aeration rates lead to more effective airlift conditions and also aid the oxygen mass transfer through the medium leading to higher cell growth rate. This is evidenced by the final cell concentrations obtained in the MBR. After 24 h of permeate recirculation these were 9.9 ± 0.1, 12.0 ± 1.0 and 14.0 ± 0.0 g l\(^{-1}\) for 1, 3, and 4 l min\(^{-1}\) aeration rates respectively.

Overall, the higher aeration rate of 4 l min\(^{-1}\) not only helped better membrane cleaning, sustained membrane filtration performance and allowed higher product recovery but also enhanced oxygen transfer in the inoculum growth phase and medium mixing as the SS content of the media increases. Hence, this was considered the optimal condition for subsequent testing.

Fig. 2. (a) Changes in the TMP by time for different aeration rates (b) Variations in permeate flux by time at different aeration rates.
3.2. Continuous ethanol fermentation from xylose-glucose semi-synthetic media

For continuous ethanol fermentation using a semi-synthetic feed, the MBR containing xylose-glucose semi-synthetic medium was prepared as described in Section 3.1, followed by permeate recirculation at an average permeate flux of 18.22 l h$^{-1}$ m$^{-2}$ ($D = 0.1$ h$^{-1}$).

As can be seen in Fig. 4a, the recombinant yeast was capable of co-utilizing of xylose and glucose. Glucose was completely consumed in 12 h and 58% of the xylose in 24 h to produce biomass and metabolites. After 24 h of batch cultivation, continuous

![Graph a](image1.png)

**Fig. 3.** (a) Changes in the ethanol concentration in the reactor (dotted lines) and in the ethanol trap(s) (solid lines) due to the application of different air sparging regimes (b) Changes in the cumulative amount of ethanol produced both remaining in the reactor and recovered in the ethanol trap(s).
cultivation was started and permeate extracted (Fig. 1). As illustrated in Fig. 4a, the concentration of metabolites and substrates in the main bioreactor nearly stabilized around 32 h while the ethanol content of the traps showed a linear increase. Between 48 and 120 h, the yeast strain perfectly co-utilized 97% of xylose and 100% glucose. During this period a collective amount of 23.75 g of xylose remained in the reactor and the permeate tank. This enhanced simultaneous consumption of xylose and glucose contributed to a great boost in ethanol yield of 0.45 g ethanol per g substrate. This yield corresponds to 89% of the theoretical yield and an ethanol volumetric productivity rate of 4.62 g ethanol per liter of the bioreactor volume per hour. As featured in Fig. 4b, the cell concentration in the MBR had a considerable rise till 72 h reaching $43.30 \pm 1.30 \text{ g l}^{-1}$, and then remained stable till 120 h. This represents a biomass yield on substrate of 0.02 g g$^{-1}$ during this 72 h of continuous feeding. The filtration system showed a

![Fig. 4. Changes in the (a) concentration of substrates and metabolites, (b) TMP and biomass concentration, and (c) permeate flux and membrane resistance during batch and continuous fermentation of xylose-glucose semi-synthetic medium.](image-url)
stable performance in this first continuous phase (Fig. 4b and c). The initial rise of the TMP and membrane resistance in the batch phase and the beginning of continuous phase can be a result of constant increase in the cell concentration and associated medium viscosity increase. However, as the biomass content of the medium reaches a plateau so does the TMP and membrane resistance to permeate flow. This stabilization of TMP can be due to the presence of a dynamic cake layer where there is a balance between SS joining and leaving the cake layer (Yoon, 2015). The acquired result on membrane filtration status proves that the gas sparging and frequent backwash cycles applied as membrane fouling preventative remedies have been able to control the cake layer formation on the membrane surface at a yeast concentration of 43 ± 1 g l⁻¹.

From 120 h onwards, the dilution rate was doubled. This boosted the biomass content in the bioreactor up to 95 ± 0.20 g l⁻¹ at 175 h. Even at this high concentration of suspended solid (yeast cells) (Fig. 4b) it was observed that the membrane filtration system could be stabilized at a TMP leveling out at 173 ± 7 mbar (membrane resistance of 0.005 h m⁻² bar l⁻¹) and nearly constant permeate flux in an iMBR set up that is working on neither fixed flow nor fixed TMP modes.

The metabolic activity of the recombinant yeast strain at this elevated feeding rate was also evaluated (Fig. 4a). It was observed that even at this higher continuous feeding rate the yeast was capable of maintaining its metabolic activity. The ethanol content of the culture media in the bioreactor remained at around 33 g l⁻¹ while the ethanol content of the traps significantly increased to about 149 and 135 g l⁻¹ in trap 1 and 2 respectively. It has been reported in different literature that specifically in anaerobic fermentation conditions, consumption of xylose deteriorates due to an excessive redox imbalance (Matsushika et al., 2009). Moreover, it has been proven that although high glucose content suppresses xylose utilization by yeast, presence of a certain amount of glucose in the medium is necessary to guarantee xylose up take by the cells (Ha et al., 2011; Westman et al., 2014). However, the trend of co-consumption of xylose and glucose was perfectly sustained in our experiments. The remaining xylose in the cultivation medium was 0.76 ± 0.12 g l⁻¹. The total xylose remaining in the reactor and

![Fig. 5. Changes in the concentration of different (a) substrates and metabolites, and (b) inhibitory compounds during continuous fermentation of lignocellulosic hydrolysate. Changes in the (c) TMP and biomass concentration and (d) permeate flux and membrane resistance by time during fermentation.](image-url)
permeate tank proved that in the 55 h continuous cultivation at the higher feeding rate around 98.62% xylose has been converted. The yield of biomass on substrate in this phase was similar as in the previous phase and about 0.02 g per g substrate fed.

3.3. Continuous ethanol fermentation from wheat straw hydrolysate

In the experiment with lignocellulosic hydrolysate, the MBR was continuously fed with wheat straw hydrolysate after the 24 h batch cultivation step. During the 24 h batch phase the yeast was capable of consuming all glucose in the medium in 8 h while around 91% of the initial xylose was utilized in 24 h. The total amount of ethanol produced, including the content of the reactor and the traps, was around 45 g l⁻¹, representing a yield of 0.44 g per g sugars, while this was about 0.12 g per g sugars for cell biomass. Following the start of continuous cultivation the xylose content of the media experienced a marginal rise to 7.59 ± 0.10 g l⁻¹, while glucose was completely consumed (Fig. 5a). Considering the xylose and glucose content of the hydrolysate (81.62 ± 0.02 g l⁻¹) and the accumulated xylose in the bioreactor and permeate tank after 72 h of cultivation, it can be observed that in the continuous phase about 73% and in total 80% xylose has been utilized. Continuous fermentation of lignocellulosic hydrolysate in the current MBR system has proven to be successful in production of bioethanol at a high conversion rate. During the 48 h continuous feeding phase the ethanol concentration of the medium inclined from 17.16 ± 0.31 g l⁻¹ to about 25.73 ± 0.10 g l⁻¹. However, the changes in the concentration of ethanol recovered in the ethanol traps followed noticeable upward trends throughout the cultivation (Fig. 5a). Considering the 0.51 g g⁻¹ theoretical yield of ethanol on glucose and xylose, the achieved 0.40 ethanol yield accounts for nearly 79% of the theoretical yield. The volumetric productivity rate of ethanol in the current MBR fermentation system reached as high as 3.29 g per liter of bioreactor volume per hour.

Although the xylose content of feed hydrolysate (Table 1) is less than the semi-synthetic feed medium (Section 3.2), lower xylose conversion rate experienced may be a result of cells being exposed to an inhibitory condition (Ylitervo et al., 2014). The changes in the concentration of the inhibitory compounds fed along with the hydrolysate are presented in Fig. 5b. The concentration of acetic acid constantly increased, rising to 6.14 ± 0.10 g l⁻¹ after 72 h. As such carboxylic acids are considered as non-convertible inhibitory compounds by yeast during anaerobic fermentation of lignocellulosic substrates, their concentration in the medium is of critical importance to cell carbon source utilization and metabolite production (Westman et al., 2012). This relatively high concentration of acetic acid could not hinder the metabolic activity of yeast cells to a large extent, although this concentration has been reported in literature to be detrimental to cell performance during fermentation (Narendranath et al., 2001). Some strains of yeast have the
Fig. 6. Changes in the concentration of different (a) substrates and metabolites, and (b) inhibitory compounds during continuous fermentation of half diluted lignocellulosic hydrolysate. Changes in the (c) TMP and biomass concentration and (d) permeate flux and membrane resistance by time during fermentation.
ability to perform in situ detoxification of some furan aldehydes such as furfural and HMF, and convert them into less toxic substances such as furfuryl alcohol (Liu, 2011; Ylitervo et al., 2013). However, the effectiveness of yeast detoxification depends on different factors such as cell concentration in the cultivation medium, concentration of inhibitory compounds etc. (Mahboubi et al., 2016; Ylitervo et al., 2013). In the current study, using an MBR, the conditions have been provided to keep the furfural level relatively constant (at about 0.05 g l\(^{-1}\)) probably both by cell detoxification and removal through the permeate line. On the other hand, after an initial accumulation of HMF (0.33 ± 0.01 g l\(^{-1}\) at 48 h) in the reactor, the cells have been successful in progressively reducing the content of this inhibitor (Fig. 5b).

The performance of the air/gaslift iMBR set-up used in this research was challenged by feeding the reactor continuously with lignocellulosic hydrolysate containing 40.41 ± 0.46 g l\(^{-1}\) of suspended solids. By the start of continuous feeding, the suspended solid concentration of the reactor medium followed a sharp increase till the end leading to a high final SS content of 20%. Filtration performance of the immersed membrane bioreactor in this high SS viscous medium is surprisingly stable. As illustrated in Fig. 5c, very little change is observed in the TMP moving from batch to continuous mode. Values remain as low as 36 mbar and only start to slightly increase at higher concentrations of SS (149.40 ± 0.20 g l\(^{-1}\)) around 56 h. This change has also been presented in form of membrane resistance (Fig. 5d). Also the permeate flux remained stable. This remarkable filtration performance of the membrane in this high SS medium can be attributed to synergy provided between the air/gas sparging regime, backwash cycle and agitation induced by medium mixing assisted by the air/gaslift condition. Considering the changes in the concentrations of different metabolites and substrates (Fig. 5a) and the fact that there is no impeller stirring involved, it can be concluded that the air/gaslift condition provided by the integration of the spacer box to the MBR has ensured an enhanced mixing of the medium and mass transfer in high SS media.

The issue faced while working with a concentrated feed with high suspended solid content was the sedimentation of solid lignocellulosic residuals throughout the feeding line leading to inevitable frequent tube clogging. Therefore, in order to alleviate the problem, and also to investigate the effects of feed suspended solid content on membrane performance and to reduce the speed of accumulation of solids in the bioreactor, the feed was diluted to half its previous concentration (20 ± 1.13 g l\(^{-1}\) SS). Moreover, in order to provide the growing yeast cells with required nutrients the dilution rate was increased to 0.12 h\(^{-1}\). Considering the increase in the feeding rate and in order to ease the control of the suspended solid level in the MBR, using a level probe, 0.048 ± 0.002 l h\(^{-1}\) of the reactor medium was withdrawn and collected in an overflow tank. During the continuous cultivation phase the glucose content was fully depleted while the concentration of residual xylose increased marginally from 1.7 ± 1.03 g l\(^{-1}\) at 24 h to 3.56 ± 0.68 g l\(^{-1}\) at 72 h (Fig. 6a). This slight increase in the concentration of xylose during continuous cultivation may be due to deliberate cell bleeding from the reactor. Taking into account the total residual xylose gathered in the main reactor, permeate tank and overflow tank the percentage of xylose converted during continuous cultivation phase and the overall 72 h of the run was about 75 and 83% respectively. Unlike the abovementioned experiment with a concentrated feed, the ethanol concentration in the main cultivation medium gradually dropped from 20.89 ± 2.48 g l\(^{-1}\) after 24 h of batch stage to 11.34 ± 1.59 g l\(^{-1}\) by the end of the continuous cultivation (Fig. 6a). This drop in ethanol content may be due to higher dilution rate plus cell bleeding resulting in inadequate residence time for full conversion nutrients or due to low carbon source content of the feed. However, as ethanol was constantly being stripped from the reactor medium, the concentration of ethanol in the traps increased progressively. The total ethanol produced and recovered in the continuous phase of this experiment with a diluted feed was only around 59% of that of the concentrated feed. However, the ethanol yield from this MBR set-up was still as high as 0.42 g ethanol per g substrate added (83% of the theoretical yield of ethanol on xylose and glucose). The ethanol volumetric productivity rate in this case was around 2.11 g per liter volume of the bioreactor per hour. As presented in Fig. 6b, the concentration of inhibitory furfural and HMF were kept at stable low levels (less than 0.9 g l\(^{-1}\)) in the membrane bioreactor after an initial rise during the first 12 h of continuous feeding. However, the concentration of acetic acid in the media peaked around 2.52 ± 0.48 g l\(^{-1}\) at 54 h.

The immersed membrane filtration set-up again showed an excellent performance. It was observed that after an initial increase in the TMP during permeate recirculation, the TMP level dropped to around 59.14 ± 7.85 mbar after the start of hydrolysate feeding (Fig. 6c). This initial rise in the membrane resistance (0.0055 m² bar l\(^{-1}\)) in batch phase may be due to the higher initial starting flux (21.14 ± 0.73 l h\(^{-1}\) m\(^{-2}\)) (Fig. 6d) comparing to the previous experiments (Fig. 5d). Moreover, the changes in TMP and membrane resistance before and after the batch phase could be attributed to the alternation in medium characteristics such as viscosity and addition of large suspended particles in the feed. As can be seen in Fig. 6d, the initially set permeate flux of 21.14 ± 0.73 l h\(^{-1}\) m\(^{-2}\) dropped about 7.2% after 24 h and stabilized during continuous cultivation at 18.95 ± 2.19 l h\(^{-1}\) m\(^{-2}\). As predicted, both flux and TMP stabilized when the concentration of suspended solids reached a plateau as a result of medium withdrawal. With the application of intermittent medium removal, the SS loading was indeed limited to 0.85 g SS h\(^{-1}\).

4. Conclusion

Considering the complexities in production of 2nd generation bioethanol, the iMBR set-up used in this study proved to be a perfect choice for continuous fermentation of lignocellulosic bioethanol. The iMBR operated at high SS concentrations up to 20% without significant deterioration of the filtration properties, while benefitting from high cell concentration, inhibitor tolerance, enhanced co-consumption of total glucose and up 83% xylose content and a bioethanol content of up to 83% of the theoretical yield. The iMBR set-up used for bioethanol production in this research paves the way for reaching higher feasibility in industrial production of 2nd generation bioethanol.

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References


Intensification of lignocellulosic bioethanol production process using continuous double-staged immersed membrane bioreactors

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Abstract

Processing complexities associated with different lignocellulosic bioethanol production stages have hindered reaching full commercial capacity. Therefore, in this study efforts were made to remediate some issues associated with hydrolysis and fermentation, by the integration of immersed membrane bioreactors (iMBRs) into lignocellulosic bioethanol production process. In this regards, double-staged continuous saccharification-filtration and co-fermentation-filtration of wheat straw slurry was conducted using iMBRs at filtration fluxes up to 51.0 l.m\(^{-2}\).h\(^{-1}\) (LMH). The results showed a stable long-term (264 h) continuous hydrolysis-filtration and fermentation-filtration with effective separation of lignin-rich solids (up to 70% lignin) from hydrolyzed sugars, and separation of yeast cells from bioethanol stream at an exceptional filtration performance at 21.9 LMH. Moreover, the effect of factors such as filtration flux, medium quality and backwashing on fouling and cake-layer formation was studied. The results confirmed the process intensification potentials of iMBRs in tackling commonly faced technical obstacles in lignocellulosic bioethanol production.

Keywords: lignocellulosic bioethanol, process intensification, immersed membrane bioreactors, membrane fouling.
1 Introduction

The growth in the global energy and fuel demands, along with the rising awareness of the environmental, economic and socio-political impacts of excessive usage of fossil-based fuel and energy sources, have motivated transition to more sustainable and renewable energy sources. This willingness to fight dramatic climate changes by moving towards a low carbon economy is clearly reflected in the Renewable Energy Directives (RED I and II) set by the European Union to increase the share of renewable energy in total energy consumption to 32% by 2030 (RED I 2009/28/EC, 2009; RED II 2018/2001/EU, 2018).

Transport biofuels such as bioethanol are an undeniable part of this shift towards renewable fuel sources. As reported by ePURE (2019), about 96% of the bioethanol produced in EU in 2017 by the main bioethanol producers has been from starch- (e.g. wheat) and sugar-based (e.g. sugar beet) feedstock (first generation bioethanol). Although, first generation bioethanol has long reached industrial-scale commercial production due to technological maturity and the rather simple processing pathway, the feedstock used for this purpose competes with human food and animal feed, and the practice imposes stress on indirect land-use change (iLUC) (European Commission, 2012; Fivga et al., 2019). Therefore, efforts have focused on replacing first generation biofuels with advanced biofuels such as lignocellulosic bioethanol (RED II 2018/2001/EU, 2018). Second generation bioethanol is produced from lignocellulosic biomass such as agricultural and forest residues that are abundant, cheap, not in competition with food/feed and have a low risk of iLUC (Fivga et al., 2019; Limayem & Ricke, 2012; Maurya et al., 2015). Wheat straw used in this study is one of the most researched lignocellulosic feedstock for bioethanol production (Ali et al., 2012; Ishola et al., 2015; Karagoz & Özkan, 2014; Nielsen et al., 2017; Saha et al., 2015; Toquero & Bolado, 2014). Considering the estimated 2.17 billion tons of wheat grain production for 2019/2020 and the 1.3-1.4 ratio of wheat straw generated to wheat grain harvested, wheat straw possesses a great potential for commercial lignocellulosic bioethanol production (International Grains Council, 2019; Saha et al., 2015).

Regardless of the 1.39 billion liters of world installed capacity by 2015 (United Nations, 2016), the unstable performance due to processing complexities has kept the commercialization of lignocellulosic bioethanol at a Technology Readiness Level (TRL) of 8 (IEA, 2019). Due to the recalcitrant structure of lignocellulosic material made of a matrix of cellulose, hemicellulose and lignin, the conventional lignocellulosic bioethanol production process includes four stages of pretreatment, hydrolysis, fermentation and product recovery. Therefore, in order to have a technically and economically feasible lignocellulosic bioethanol production process, efforts should be made to improve the process design towards enhanced yield and productivity, and effective separation (recovery) of solid residuals, cells, product and by-products. In this regard, integration of membrane separation technology in lignocellulosic bioethanol production can open new grounds for
technological improvements and process intensification (Dey et al., 2018; Hajilary et al., 2019; Lipnizki, 2010; Saha et al., 2017).

As presented in Figure 1, membrane units can be coupled with pretreatment units in order to recover applied chemicals (e.g. acids and bases), remove generated inhibitors (e.g. furfural), and based on the type of pretreatment, separate liquid and solid fractions containing cellulose, hemicellulose and/or lignin (Egüés et al., 2012; Hai et al., 2015; Hajilary et al., 2019; Lipnizki, 2010). If used in combination with enzymatic hydrolysis, different types of membrane filtration units can facilitate continuous hydrolysis (Malmali et al., 2015; Stickel et al., 2018), enzyme retention and reuse (Mores et al., 2001), sugar concentration (Malmali et al., 2014), and suspended solid separation (Jørgensen & Pinelo, 2017; Saha et al., 2017). In addition, membrane processes such as micro- and nanofiltration can be merged into the fermentation stage to provide the ability for continuous fermentation (high volumetric productivity), cell retention and recirculation (Escobar et al., 2001), sugar retention and particle-free in situ product recovery (less downstream processing required) (Carstensen et al., 2012; Lipnizki, 2010; Saha et al., 2017).

Depending on how these membrane units are integrated with the above mentioned processing units (e.g. bioreactors), they can have immersed or sidestream configurations (Carstensen et al., 2012). The application of sidestream MBRs has frequently been reported for lab- and pilot-scale cell recirculation and in situ product recovery during fermentation (Ben Chaabane et al., 2006; Escobar et al., 2001; Melzoch et al., 1991; Taniguchi et al., 1997). Although sidestream (external loop) membrane units can provide higher fluxes, they are affiliated with issues such as maintaining sterility, high-energy requirement for medium recirculation in an external loop etc. On the other hand, in immersed membrane units, the process is simple and there is no broth recirculation through an external loop. However, immersed membrane systems are considered as inflexible, limited by specific membrane area per reactor volume, and susceptible to fouling and additional problems induced by highly viscous medium (Carstensen et al., 2012; Judd, 2011; Lipnizki, 2010; Yoon, 2015). Although higher filtration fluxes can be achieved using cross-flow filtration with less concerns of membrane fouling and cake layer formation, operational energy and space demands are in favor of iMBRs (Judd, 2011; Radjenović et al., 2008; Singhania et al., 2012). As reported, immersed membrane systems are simpler in design, easy to install and compact in size (Carstensen et al., 2012; Koltuniewicz, 2016), hence, they have lower footprint, energy consumption, and operation and maintenance costs (Carstensen et al., 2012; Dhariwal, 2007; Koltuniewicz, 2016). The mentioned technical and economical features make iMBRs a better choice when it comes to retrofitting an existing facility (Koltuniewicz, 2016). Although, iMBRs have for long been used for water and wastewater treatment purposes (Giorno & Drioli, 2010; Judd, 2011), they are reported to be less flexible in the type of application (Yang et al., 2006). Additionally, as in iMBRs less intensive shear stress is provided for membrane surface cleaning compared to tangential flow sidestream
MBRs, the main issue with iMBRs is membrane fouling (Carstensen et al., 2012). Different types of membrane fouling that can contribute to loss of permeability and drop in flux are adsorption of medium constituents on the membrane surface, pore narrowing, pore blocking and cake layer formation. Therefore, in order to have an economically feasible and technically robust second generation bioethanol production, effective fouling prevention methods should be implied. Different approaches have been considered for preventing and curing fouling in iMBRs. One the most practiced methods to keep the membrane surface clean in an iMBR is air/gas sparging/scouring (Judd & Judd, 2011; Yoon, 2015). The physical cleaning effect is due to the shear stress and liquid agitation induced on the membrane surface as the bubbles rise adjacent to the membrane surface. Backwashing (BW) (backflushing) is another physical remedy for dislodging and removing cake layer from the membrane surface. During backwashing, the direction of the permeate flow is reversed and a portion of permeate is sent back to the reactor through the membrane pores for a relatively short period (Basu, 2016; Yoon, 2015).

This study focuses on establishing an innovative interconnected double-stage immersed MBR system for intensification of lignocellulosic bioethanol production process. This set-up aims to enhance and intensify the process, and tackle the common associated processing obstacles by merging several processing stages into a continuous hydrolysis, fermentation and filtration system, effectively separating suspended solids, yeast and product streams in a fully automated system. In this regard, using pretreated wheat straw slurry as the substrate, the performance of the double-staged simultaneous saccharification-filtration and co-fermentation-filtration at different filtration fluxes up to 51.0 LMH was evaluated. In addition, the effect of different factors such as filtration flux, medium quality and backwashing on fouling propensity and cake-layer formation behavior of the iMBR during continuous filtration was thoroughly studied.
2 Materials and methods

2.1 Cell pre-culture preparation and enzymatic hydrolysis

The lignocellulosic substrate used was dilute-acid pretreated wheat straw (0.3-0.5% H₂SO₄, 8 min at 185°C) provided by SEKAB E-Technology (Örnsköldsvik, Sweden). Wheat straw was chosen as the model lignocellulosic biomass in this research due to its abundance and low cost (International Grains Council, 2019; Saha et al., 2015). As the as-received wheat straw slurry contained large residual particles of over several centimeters, in order to ease continuous feeding of the slurry, prior to hydrolysis, the slurry was diluted to 1/8 of its original concentration with Milli-Q water, was sieved with a kitchen sieve, and autoclaved after pH adjustment to 5.0 with 10 M NaOH. As the activity of the enzyme Cellulase Cellic Ctec2 (Novozymes, Denmark), measured according to NREL protocol (Adney & Baker, 2008), was 130 filter paper units (FPU).ml⁻¹, enzyme loading of 15.6 FPU.g⁻¹SS was used for hydrolysis of the pretreated wheat straw slurry. The optimum temperature recommended for enzymatic hydrolysis using cellulases is 45-50°C for a period of 24 h. However, as hydrolysis was performed in a continuous mode in an iMBR, the enzymatic hydrolysis was performed at 35°C to maintain the temperature close to that favored by yeast (continuous feeding) and to sustain the membrane panels’ condition in long run. In order to have an estimate of the expected sugar yield, enzymatic hydrolysis of the slurry was conducted in 250 ml Erlenmeyer flasks containing 100 ml of slurry in a shaking water bath (Grant OLS 200, Grant instrument ltd, UK) at 50°C for 24 h. The composition of the wheat straw slurry and the hydrolysate are presented in Table 1.

The yeast strain used in this study was a recombinant xylose-utilizing Saccharomyces cerevisiae. The yeast was loop-inoculated on yeast extract-peptone-dextrose (YPD) plates containing 20 g.l⁻¹ agar, 10 g.l⁻¹ glucose, 10 g.l⁻¹ xylose, 10 g.l⁻¹ yeast extract and 10 g.l⁻¹ peptone, incubated for 2-3 days at 30°C and then stored at 4°C until use. In order to prepare required yeast precultures for iMBR continuous fermentation, 250 ml cotton-plugged Erlenmeyer flasks containing 100 ml of medium composed of 12.5 g.l⁻¹ glucose, 12.5 g.l⁻¹ xylose, 5 g.l⁻¹ yeast extract and 10 g.l⁻¹ peptone, were loop-inoculated, and placed in a shaking water bath (30°C and 120 rpm) for 48 h. Three flasks (300 ml) containing yeast concentration of 6.56±0.3 g.l⁻¹ were further added to the fermentation iMBR containing 2.2 l of medium with 25 g.l⁻¹ glucose, 25 g.l⁻¹ xylose, 5 g.l⁻¹ peptone, 2.5 g.l⁻¹ yeast extract and 2.38 g.l⁻¹ KH₂PO₄. Following inoculation, cell propagation was conducted prior to the start of continuous fermentation and filtration for 24 h in the fermentation reactor by air sparging (4 l.min⁻¹) at a controlled pH of 5.0.
2.2 Membrane module and membrane bioreactor set-up

The second generation integrated permeate channel (IPC) membrane modules customized and developed by the Flemish Institute of Technological Research (VITO NV, Belgium) and used in this study have two membrane layers coated on each sides of a 3D spacer-fabric support. The IPC membrane modules have hydrophilized polyethersulfone (PES) membranes with a nominal pore size of 0.3 µm and a filtration surface area of 68.6 cm² per panel. The iMBRs used for hydrolysis and fermentation in this study each contained two immersed IPC panels. The application of IPC membrane panels is advantageous in immersed membrane systems as the strong anchoring of the membrane layers onto the spacer fabric makes them ideal for intensive backwashing (Doyen et al., 2010).

The schematic of the double-staged iMBR used in this study for continuous hydrolysis and fermentation is presented in Figure 2. The two membrane panels used in each iMBR were placed parallelly in a spacer-box providing an air/gaslift condition for better medium mixing and mass transfer in addition to directing the air/gas bubble towards the membrane surface for better scouring. The bioreactors used were 4.0 l Belach WebAnt® reactors (Belach Bioteknik AB, Skogås, Sweden) with 2.5 l working volume. Prior to every hydrolysis and fermentation cycle, all equipment (reactors, tubing etc.) were autoclaved. Following that, IPC membrane panels were installed in the bioreactors and chemically cleaned and disinfected according to Mahboubi et al. (2017).

2.3 Interconnected double-staged immersed membrane bioreactor

As the startup phase, both enzymatic hydrolysis and fermentation were conducted in batch in the MBRs prior to the initiation of the continuous process. The batch hydrolysis and fermentation were carried out at temperatures of 35°C and 30°C for 48 and 24 h, respectively. Based on the set temperature for hydrolysis, to provide a starting retention time of about 48 h for maximum sugar release, the batch hydrolysis was started 24 h before the initiation of batch fermentation. Moreover, during continuous hydrolysis, the enzyme was added to the feed tank and the size of feed tank was adjusted relatively to have a hydraulic retention time of 42 to 46 h for different feeding rates (HRT is calculated based on retention time in feed tank and hydrolysis reactor). As presented in Figure 2, during the continuous hydrolysis-filtration and fermentation-filtration, the hydrolysis iMBR was continuously fed with the slurry from the feed tank, permeate from the hydrolysis was then removed and fed to the buffer tank and subsequently fed to fermentation iMBR. The particle-free permeate, containing fermentation products, was withdrawn from the fermentation iMBR and collected in a permeate tank. In order to analyze the changes in the concentration of cells, SS, lignin, ash and metabolites samples were withdrawn every 8 and 16 h from both iMBRs. Moreover, to partially compensate the lack of nutrients in the hydrolysate, 10 ml of a nutrient solution was added to the
fermentation bioreactor every 24 h, increasing the momentary concentration of yeast extract, (NH₄)₂SO₄ and KH₂PO₄ to 2.5, 2.5 and 3.5 g.l⁻¹, respectively. As experienced in our previous work (Mahboubi et al., 2017), excessive air/gas sparging for membrane surface cleaning and medium mixing results in ethanol stripping from the iMBRs. Therefore, the amount of ethanol escaped through the condenser outlet was measured and taken into account in ethanol volumetric and specific productivity and yield calculations (concentration graphs in Figure 4 only present the amount of ethanol in the reactor medium).

The data acquisition and control of the liquid level in the bioreactors, pumps involved in feeding and permeate removal, pressure sensors and flow meters were completely synchronized and automated, using the LabView-based Mefias® software (Doyen et al., 2003) specifically customized and developed by VITO for this research work. In addition, parameters such as temperature, pH, foaming (fatty acid ester antifoam was applied) and air/nitrogen flow rate were controlled and adjusted by the WebAnt® controlling unit (Belach Bioteknik AB, Skogås, Sweden). Throughout the continuous processes, Watson-Marlow 403/R1 peristaltic pumps (Watson Marlow, United Kingdom) were used for feeding or permeate removal. Moreover, liquid flowrates, pressure on the main reactors and permeate lines were provided to Mefias® by 710 Atrato ultrasonic flowmeters (Titan Enterprises Ltd., United Kingdom) and PMC131 pressure sensors (Endress+Hauser AB, Solna, Sweden), respectively. By the help of the Mefias® control software, the continuous double stage hydrolysis and fermentation was performed in a constant-flux mode at three different permeate flux rates of 21.9, 36.4 and 51.0 l.m⁻².h⁻¹ (LMH), corresponding to flow rates of 0.3, 0.5 and 0.7 l.h⁻¹ and dilution rates of 0.12, 0.20 and 0.28 h⁻¹, respectively. In order to prevent cake layer formation and fouling during continuous filtration, in addition to air/gas sparging, intermittent backwash was applied for 0.5 min in every 5 min filtration cycle with a flux double that of filtration.

2.4 Step-flux experiment for membrane fouling measurement
In order to investigate the extent of cake layer formation and reversibility of membrane fouling during continuous hydrolysis and filtration, wheat straw slurry and hydrolysate were separately subjected to stepwise filtration cycles (step-flux) at three different fluxes of 21.9, 36.4 and 51.0 LMH at 35°C. The filtration cycle included 1 h of filtration at each of the aforementioned fluxes with permeates recirculation, followed by 30 min filtration at the initial flux (21.9 LMH). In order to study the effectivity of backwashing on fouling mitigation, this stepwise trend was repeated for both medium at all fluxes with and without backwashing. Clean water filtration performance before the filtration cycles was considered with the same step-flux regime to measure the filtration TMP and clean membrane resistance. The changes in TMP, total
filtration resistance and cake layer resistance were determined for all fluxes with and without backwashing. The amount of total resistance and cake layer resistance were calculated according to equations (1) and (2).

\[
J = \frac{\text{TMP}}{\mu R_T}
\]

\[
R_T = R_m + R_C + R_f
\]

Where \( J \) is the permeate flux, \( \mu \) is the permeate dynamic viscosity, \( R_T \) is the total resistance, \( R_m \) in the membrane resistance (clean water filtration), \( R_C \) is the cake layer resistance and \( R_f \) is the resistance due to irreversible fouling.

As the sugar content (Brix degree) of the hydrolysate is very low to make perceptible changes in medium viscosity and considering the hydrolysis temperature of 35°C, \( \mu \) of water at 35°C (0.789×10^{-3} \text{ Pa.s}) was considered for the micro-filtered permeate. As in microfiltration irreversible fouling (\( R_f \)) is considerably smaller than \( R_C \), \( R_C \) is used to represent the resistance due to cake layer formation and irreversible fouling (Choo & Lee, 1996).

2.5 Analytical methods

High performance liquid chromatography (HPLC) (Waters 2695, Waters Corporation, Milford, USA) was used to determine the concentration of different substrates and metabolites in the hydrolysate and fermentation medium. The HPLC unit used a hydrogen based ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules, USA) working at 60°C with 5 mM H2SO4 eluent flowing at 0.6 ml.min^{-1}.

Changes in the cell dry weight (CDW) during fermentation and suspended solid content during hydrolysis were measured according to the procedure used by Mahboubi et al. (2017). The measurement of acid insoluble lignin (AIL), acid soluble lignin (ASL) as well as structural carbohydrates and ash content of the suspended solids of the initial wheat straw slurry and hydrolysate during continuous hydrolysis and filtration were estimated using the NREL protocol by Sluiter et al. (2011). The experiments and measurements were performed in duplicates and the error bars shown on graphs present two standard deviations.

3 Results and discussion

The aim of the application of this newly developed double-staged simultaneous hydrolysis-filtration and co-fermentation-filtration system was to reduce the processing stages of lignocellulosic bioethanol production process from wheat straw by merging hydrolysis, sugar recovery, lignin concentration and recovery,
fermentation, yeast retention and ethanol recovery in an automated continuous process using iMBRs. In this regard, in order to prove the proposed concept of continuous double-staged iMBR for long-term stable hydrolysis-filtration and fermentation-filtration, different filtration conditions were practiced, and the outcomes were thoroughly analyzed.

### 3.1 Double-stage continuous hydrolysis-filtration and fermentation-filtration

A simple schematic of the double-staged iMBR is illustrated in Figure 2. After 48 h of batch hydrolysis, dilute acid treated wheat straw slurry of 14.0±1.5 g.l⁻¹ SS was continuously fed to a hydrolysis iMBR and the particle-free hydrolysate removed at filtration flow rates of 0.3, 0.5 and 0.7 l.h⁻¹, corresponding respectively to fluxes 21.9, 36.4 and 51 LMH. In order to have flexibility in operation, the hydrolysis permeate was then fed to a buffer tank. According to the filtration flux applied, the HRT of the hydrolysis iMBR was corrected to have relatively similar hydrolysis time for all treatments. The hydrolysate containing an average of 14.65±0.59 g.l⁻¹ of total sugars (based on xylose and glucose) was then continuously fed to the fermentation iMBR and the ethanol-containing stream was removed from the reactor through the permeate line at similar fluxes as for hydrolysis.

Considering the difficulties in pumping highly particulated lignocellulosic slurry to the hydrolysis reactor, the slurry should be diluted prior to a continuous hydrolysis (Andrić et al., 2009; Malmali et al., 2015). Although it has been reported by Andrić et al. (2009) that continuous hydrolysis of pretreated wheat straw slurry can double the sugar volumetric productivity compared to batch hydrolysis, over dilution of the feed slurry can hinder the effectivity of continuous hydrolysis. Therefore, considering the low sugar content of the diluted wheat straw slurry used in this study, higher dilution rates (feeding and permeate flowrates) are favored to enhance volumetric productivity. This applies to both ethanol productivity during fermentation and fermentable sugar recovery rate during hydrolysis (in case of a constant HRT). Therefore, considering that in the iMBR the suspended particles and cells are fully retained in the bioreactor, higher permeate fluxes were desirable. However, as in all membrane-based systems, flux increase is constrained by limitations in filtration performance and fouling tendency (Yoon, 2015).

As illustrated in Figure 3, an average total sugar concentration of 14.65±0.59 g.l⁻¹ (based on glucose and xylose) was yielded. During hydrolysis, less glucose release was observed for slurry feeding rate of 0.3 l.h⁻¹. However, in all continuous treatments the sugar release rate reached a stable value at about 70-80% of the previously performed batch hydrolysis (Table 1) (Ishola et al., 2015; Mahboubi et al., 2017). Cell propagation was first performed in batch for 24 h, to increase the cell culture to about 6.01±0.28 g.l⁻¹ prior to continuous fermentation and filtration (Figure 3). The ethanol content in the batch reached as high
as 11.82±0.16 g.l⁻¹ after 8 h. As continuous hydrolysate feeding started, the ethanol content of the media dropped due to high intensity nitrogen sparging and low rate of ethanol production (low sugar and nutrient content of the hydrolysate), and then stabilized for feeding flow rates of 0.3, 0.5 and 0.7 l.h⁻¹ at 4.64±0.37 g.l⁻¹, 5.55±0.59 g.l⁻¹ and 5.40±0.22 g.l⁻¹ after 46 h, respectively. While benefitting from similar initial yeast inoculum concentration, feeding the fermentation iMBR at a high flow rate of 0.7 l.h⁻¹ (51.0 LMH) led to a volumetric productivity of 1.8 g.l⁻¹.h⁻¹ (specific productivity 0.23 g.g⁻¹.h⁻¹) that is 1.4 and 2.3-times more than that achieved at lower feeding rates of 0.5 and 0.3 l.h⁻¹, respectively. The lowest volumetric productivity achieved (0.8 g.l⁻¹.h⁻¹) in this experiment with feeding flow rate of 0.3 l.h⁻¹, is relatively comparable to results achieved by Mahboubi et al. (2017), using four-times more concentrated wheat straw hydrolysate as the substrate in a continuous iMBR. It should be noted that, in the determination of productivities, the amount of ethanol stripped from the reactor due to gas sparging and the net fluxes considering BW have been taken into account.

Although, the continuous hydrolysis and fermentation reaches stability and maintains it for at least 7 HRTs for all three applied fluxes fulfilling the requirements for a stable continuous fermentation (Macauley-Patrick & Finn, 2008), dramatic changes in the filtration performance hinders the benefits achieved through the application of high filtration fluxes (36.4 and 51.0 LMH) (Figure 4). In constant flux mode, changes in the membrane surface quality and extent of fouling is projected in the TMP as it changes to satisfy the need for the set flux (Yoon, 2015). Moreover, every membrane filtration system has its own critical flux, above which, progressive membrane fouling is inevitable (Field et al., 1995). Therefore, for a stable continuous long-term hydrolysis, fermentation and filtration, it is of critical importance to know the filtration operational limits that are coupled with bioconversion process. As shown in Figure 4a and b, in both hydrolysis and fermentation iMBRs, flux of 51.0 LMH has clearly exceeded the system’s critical flux. As can be seen, the TMP throughout hydrolysis and filtration at 51.0 LMH increased exponentially for about 130 mbars in only 51 h. This change in TMP was accompanied with the jump in total permeation resistance from 1.5×10¹¹ m⁻¹ to 1.1×10¹² m⁻¹. As the sharp inclination started from the very beginning of the hydrolysis when the medium contained less than 8 g.l⁻¹ of SS, the main fouling promoter has been the high flux that induces cake layer propagation and compaction rather than the linear rise (1.11 g.l⁻¹.h⁻¹) in the SS content. The dramatic change in filtration performance can also be traced during the fermentation and filtration at 51.0 LMH (Figure 4b). Although, as mentioned, higher feeding rate of 0.7 l.h⁻¹ enhances the process profitability due to higher volumetric productivity, it cannot be credited as a self-sustained iMBR process. Due to the difference in the quality of the media being filtered, the behavior and severity of membrane fouling was different for hydrolysis and fermentation at the same flux of 51.0 LMH. From the start of fermentation and filtration, the TMP dramatically increased in a linear manner (0.02 bar.h⁻¹) to 340 mbar after only 19 h. Thereafter, as the cake layer had fully developed on the membrane and compacted, the
extent of fouling progression declined 10-times (0.002 bar·h⁻¹). The experiment was stopped by reaching a TMP of 400 mbars corresponding to a resistance of 3.4×10¹² m⁻¹. This undesirable deterioration of membrane filtration performance under the applied condition imposes new operational costs to a continuous hydrolysis process, as it demands frequent process termination and substantial membrane cleaning. The application of a flux of 36.4 LMH during the hydrolysis and filtration postpones reaching critical fouling levels up to around 135 h. The TMP pattern of the hydrolysis iMBR presents the classic three-stages of the fouling pattern observed in immersed membrane filtration systems operating under constant flux mode (Yoon, 2015). At this filtration flux, the TMP increases linearly from 0.011 to 0.045 bar in 102 h followed by a sharp jump of 100 mbar in only 33 h. As the operational conditions did not change during the filtration period while the membrane was exposed to 0.9 g·l⁻¹·h⁻¹ increase in suspended particles, reaching 99.21±0.54 g·l⁻¹ SS after 102 h, the sudden jump in TMP can be best justified by cake layer compaction. It should be noted that this iMBR set-up has been exposed to extremely high SS concentration that is uncommon in conventional iMBR systems (Judd, 2010). In this constant flux mode operation, due to progressive particle deposition on the membrane surface, cake layer develops, imposing pressure loss through the cake. As this pressure loss reaches critical levels, the cake layer is compressed downwards towards the membrane (highest pressing force experienced at the bottom of the cake layer). As the cake layer becomes more compact, the TMP is increased to compensate the loss of permeate flux through the membrane and this increase in TMP exacerbates cake layer compaction. This vicious cycle between the TMP rise and cake layer compression motivates the sudden jump observed in TMP (Chang et al., 2006; Fane, 2007; Park et al., 2006). As extreme initial particle deposition occurs at greater fluxes beyond the systems critical flux, these stages are accelerated at 51.0 LMH compared to 36.4 LMH (Figure 4a). Other put forward theories such as pore loss model or area loss model can also describe the fouling behavior observed (Cho & Fane, 2002; Ognier et al., 2004; Ye et al., 2006). These models mostly explain the observed trend in TMP change based on the available pores or available membrane area for filtration at different stages of the operation. In the initial stages of filtration in constant flux mode, when pores are blocked or available filtration surface is reduced due to particle deposition, permeation occurs readily through the non-affected pores to maintain the flux. However, as pore blockage proceeds, higher flows should be sent through reduced number of pores. At a threshold, this causes the local flux through the open pores to exceed the critical flux, accelerated particle deposition to occur and the membrane resistance and consequently TMP to increase abruptly (Cho & Fane, 2002; Yoon, 2015). In the experiment using a flux of 36.4 LMH during hydrolysis, the filtration resistance increased more than four-times to 6.8×10¹¹ m⁻¹ in a duration of 102 h, whereas, from 102 to 135 h the resistance was nearly doubled due to cake layer compaction. This progressive cake layer formation and compaction proceeded irreversibly regardless of SS draining that was applied at 102 h to keep the SS content extremely high (99.51± 0.20 g·l⁻¹) (Figure 4a). However, filtration of the fermentation broth at 36.4
LMH had a different trend compared to that of hydrolysis. In this regard, after an initial steep increase in TMP (0.002 bar.h\(^{-1}\)) up to around 19 h, the TMP reached a plateau at around 0.04 bar, experiencing marginal change (0.042 mbar.h\(^{-1}\)) till the end of the experiment. This initial increase in filtration resistance from around \(0.31 \times 10^{11} \text{ m}^{-1}\) to \(4.0 \times 10^{11} \text{ m}^{-1}\), that was also observed in extremes at 51.0 LMH, can be due to the synergistic effect of high flux and medium components. The nature of the fermentation medium is different from that of the hydrolysate, as the presence of microbial products and unmetabolized nutrients (e.g. peptone) can alter membrane and cell surface characteristics and consequently, cell-cell and cell-membrane interactions (Russotti et al., 1995). This may induce higher adsorption and deposition of particles/cells on a more sticky cake layer (Wu et al., 1995; Yoon, 2015).

On the contrary, when sub-critical flux of 21.9 LMH was applied, the performance of hydrolysis and filtration could be sustained up to 11 days with negligible increase in TMP (Figure 4a). In this long-term stable hydrolysis, due to the moderate flux applied, a dynamic balance had been established between drag forces towards the membrane surface, motivating particle deposition (bulk permeate flow), and forces promoting particle and cake layer detachment and resuspension (BW, gas sparging and backtransport). Surprisingly, the total change in the TMP throughout the 264 h continuous hydrolysis and filtration was less than 10 mbar leading to a final resistance of \(3.8 \times 10^{11} \text{ m}^{-1}\). As the continuous hydrolysis and filtration stayed at a steady state for about 14 HRTs regarding released sugar rate and filtration operation, after 142 h (57.5±0.06 g.l\(^{-1}\) SS) intermittent medium draining (14 ml.h\(^{-1}\)) was applied to keep the SS content of the medium at around 5.3% (Figure 4a). Stickel et al. (2018) reported that when a cross-flow tubular filtration unit was used for continuous hydrolysis of corn stover slurry of 2.5-5% SS the filtration system worked for 80 h but with frequent permeation deterioration. The exceptional filtration performance at 21.9 LMH was also observed for fermentation and filtration (Figure 4b). This was confirmed by the minimal change in TMP and a final filtration resistance of 20% of that of 36.4 LMH. As compared to feed flow rates of 0.5 and 0.7 l.h\(^{-1}\), at 0.3 l.h\(^{-1}\) yeast cells are provided with nutrients at a lower rate resulting in lower maximum cell concentration (7.38±0.01 g.l\(^{-1}\)). As seen by the trend of changes in biomass and ethanol concentration, low nutrient supplementation, both through hydrolysate feeding, and external nitrogen source and salt provision, along with the risk of system contamination in long continuous fermentation practices may lead to loss of cell viability and fermentation performance (Figure 3 and 5b). Therefore, at low feeding rates, yeast sugar and nutrient deprivation should be avoided by either sugar concentration prior to fermentation or ex situ supplementation (Malmali et al., 2014). Considering the robustness in continuous hydrolysis, fermentation and filtration, for this specific double-iMBR system, the flux 21.9 LMH proved to be the optimal option compared to the two other filtration conditions. This feeding and permeation flow rate can assure long-term bioconversion and filtration with less process downtime, membrane physical and chemical cleaning requirements, filtration and BW energy demands and, consequently, operational cost.
One of the main benefits of the applied double-stage iMBR in this research work is the ability to separate lignin-rich suspended solids, sugars, yeast cells and fermentation product streams from one another in a single set-up. The micro-filtered clear sugar-rich stream is fed to the fermentation, avoiding cell mixture with lignocellulosic residual solids. This provides the opportunity to reuse excess cell content for different applications. Moreover, clean particle-free product stream obtained from the fermentation iMBR sent for concentration and purification, will reduce the cost of downstream processing (Hajilary et al., 2019; Lipnizki, 2010). One important by-product of the double-stage, continuous iMBR is lignin-rich SS acquired during hydrolysis. As lignin has the potential to be used in a wide variety of applications (Wang et al., 2019), it is of great importance to try to concentrate and separate it in the lignocellulosic ethanol production process. Therefore, in order to benefit from a rich-lignin stream, the SS accumulation in the hydrolysis iMBR was tracked (Figure 5). As it can be seen in Figure 5, regardless of the treatment flux, the SS content of the hydrolysis iMBR contained at least 63% lignin, nearly double that of the feed wheat straw slurry. As expected acid insoluble lignin (AIL) comprised the main fraction of the SS with ash content in the second place. The lignin content in the reactor could reach as high as 69 g.l\(^{-1}\) and has the potential to be extracted as a valuable by-product.

3.2 The effect of medium, backwashing and permeate flux on filterability during hydrolysis

In order to study the effect of medium quality, permeate flux and backwashing on membrane fouling, wheat straw slurry and hydrolysate were subjected to a step-flux experiment at different fluxes of 21.9, 36.4 and 51.0 LMH (at 35°C). As noted in section 2.4 and illustrated in Figure 6, the filtration cycle included 1 h filtration at each of the aforementioned fluxes followed by 30 min filtration at the initial flux (21.9 LMH). In this regard, the rate of changes in TMP and resistance at different filtration fluxes and the latent resistance due to residual fouling at initial flux after every filtration cycle were set as the basis for comparison. Moreover, in order to have a clear view of the contribution of cake layer formation to overall resistance, clean water filtration was performed in the same sequence to achieve \(R_m\) (Figure 6a).

3.2.1 The effect of medium

The composition of the media in contact with the immersed (submerged) membrane directly affects the filtration performance (Field, 2010). As in this work pre-treated wheat straw slurry was continuously fed and particle-free fraction of the hydrolysate withdrawn from the iMBR, the interaction of hydrolyzed and non-hydrolyzed medium with the membrane was of great importance. The effect of media composition can be clearly observed in Figure 6a and b where the changes in TMP during filtration of wheat straw slurry and hydrolysate are compared. As can be seen, at all three tested permeate fluxes, regardless of the application of BW, the starting TMP is higher for the hydrolysate (Table 2). This higher starting TMP can be due to a
more extensive adsorption of medium components on the membrane surface at initial phases of membrane-medium contact (Yoon, 2015). Moreover, the latent residual cake layer resistance after stepwise increase and drop in flux is considerably greater during filtration of the hydrolysate compared to the slurry (Table 2). For instance, when no BW was applied, the residual cake layer resistance at first, second and third filtration cycles at 21.9 LMH were $0.86 \times 10^{11}$, $1.36 \times 10^{11}$ and $2.11 \times 10^{11}$ m$^{-1}$ for the slurry, respectively, while they were $1.80 \times 10^{11}$, $3.11 \times 10^{11}$ and $5.23 \times 10^{11}$ m$^{-1}$ for the hydrolysate. Commonly, in immersed microfiltration MBRs changes in the medium SS concentration is one of the reasons for cake layer formation and increase in filtration resistance (Judd, 2011; Yoon, 2015). However, in this case as the SS content of the hydrolysate (6.4 g.l$^{-1}$) was nearly half that of the slurry, the higher starting TMP cannot be directly related to changes in medium SS concentration and viscosity. Therefore, several other factors such as particle characteristics, enzyme addition and sugar release may have contributed to this change in filtration behavior. The sugar content (based on xylose and glucose) of the hydrolysate (around 15 g.l$^{-1}$) is more than double that of the slurry, however, this rise in sugar level cannot impose a significant change to the medium viscosity or cake layer resistance in a microfiltration process. As reported by Toquero and Bolado (2014), the structure of wheat straw solid particles may undergo changes through pretreatment and hydrolysis as the structural carbohydrates, partly responsible for particle resilience, are degraded. There is the possibility that as the rigidity of the particulated solids in the medium drop, cake layer formed on the membrane surface becomes more susceptible to compaction leading to rise in TMP. A higher initial TMP of filtration at any applied flux was observed for hydrolysate than the slurry (Table 2), although, the same increase in TMP was experienced throughout filtration for both media when BW was applied. Regardless of the filtration condition, the exposure of the membrane to the filtration media is the first step towards fouling that involves the adsorption of compounds with highest affinity to the membrane surface and inside the pores. Depending on the membrane surface characteristics and medium properties, the adsorbed layer composed of metabolized cell products and unmetabolized medium components may cause an initial increase in TMP and build a jelly/slimy basis for the attachment and deposition of colloidal compounds and suspended particulate matter further through the filtration process. Cellulases are enzymes that are employed for the degradation of cellulose to glucose units. Considering that cellulases can bound to cellulose (Malmali et al., 2015) or form non-productive binding to lignin through hydrophobic interactions (Saini et al., 2016), they may change the surface quality of particles and consequently, effect the interaction of the particles and the membrane surface. However, a conclusive judgment in this regard requires further membrane surface and pore microscopic imaging and analysis.

3.2.2 The effect of flux

Permeate flux is one of the main factors directly influencing the productivity and therefore, the economic feasibility of an MBR process. As at a certain product concentration, the higher the permeate flux the greater
the volumetric productivity, in all MBR applications, elevation in flux is desirable unless it leads to severe membrane fouling. In the current study, as the total sugar concentration of the wheat straw hydrolysate was relatively low (15 g.l\(^{-1}\)), a low HRT could compensate the cells’ need of a carbon source during fermentation. Therefore, the effect of different fluxes (21.9, 36.4 and 51.0 LMH) that were used for double-staged iMBRs on cake layer formation and membrane fouling were investigated. It was observed that at a flux of 21.9 LMH, the rate of changes in the TMP was imperceptible at less than 0.03 mbar.min\(^{-1}\) for both media with or without backwashing (Table 2). Application of this flux led to marginal increase in the total permeation resistance with the maximum at around 0.3×10\(^{11}\) m\(^{-1}\) for both medium conditions with no BW. As the permeate flow rate was increased from 0.3 to 0.5 l.h\(^{-1}\) (34.6 LMH), the TMP escalation accelerated. While the increase in the cake layer resistance for the slurry no BW and hydrolysate with BW were the same (0.5×10\(^{11}\) m\(^{-1}\)), the maximum and minimum elevation was observed for hydrolysate no BW and slurry with BW at 1.20×10\(^{11}\) and 0.27×10\(^{11}\) m\(^{-1}\), respectively. The upward trend in TMP change due to excessive cake layer formation became more apparent as the flux was increased to 51.0 LMH. At this filtration condition, the rate of change in TMP more than tripled that of 34.6 LMH with 0.24 and 0.41 mbar.min\(^{-1}\) for slurry and hydrolysate with no BW, respectively. By comparing the changes in fouling tendency and cake layer formation induced by changes in the flux, it can be observed that in no BW conditions the filtration parameters are better sustained at 21.9 LMH. In this filtration condition, regardless of BW application, the membrane surface air/gas scouring and shear stress induced by medium agitation has successfully assisted the back transport of particles against the convective flow towards the membrane. Although in this condition formation of a dynamic cake layer on the membrane surface is observed, as the flux is lower than the apparent critical flux for filtration of such medium, cake layer compaction and sharp increase in cake layer resistance was not detected. Despite the low sugar content and volumetric productivity achieved at this flux, long-term continuous hydrolysis and filtration can be guaranteed, benefiting from less process downtime and frequent membrane cleaning. However, increase in the flux up to 51.0 LMH comes at the cost of passing critical flux threshold, excessive cake layer formation and compaction, and fouling exacerbation. The effect of flux has been more profound when it comes to wheat straw hydrolysate filtration.

### 3.2.3 The effect of backwashing

Backwash or backflush is applied in order to remove surface cake layer and resuspend deposited particles, by simply reversing the flow and redirecting a fraction of the permeate towards and through the membrane. Through this approach, the loose and uncompact portion of the cake layer can be dislodged. (Yoon, 2015). As the lignocellulosic pretreated slurry and hydrolysate contains large amounts of suspended particles, in this work the effect of BW on cake layer removal and fouling prevention was examined. In this regard, slurry and hydrolysate were filtered with and without BW. The BW was performed for 0.5 min in every 5 min filtration cycle at double the flux of permeate withdrawal. As presented in Figure 6a and b, and Table
filtration with no BW resulted in progressive TMP increase rate of 0.03, 0.07 and 0.24 mbar.min$^{-1}$ for slurry, and 0.02, 0.16 and 0.41 mbar.min$^{-1}$ for the hydrolysate at 21.9, 34.6 and 51.0 LMH, respectively. However, with dramatic difference from no BW condition, this rate for both medium did not exceed 0.06 mbar.min$^{-1}$ at any applied flux. Comparing the cake layer resistance at 21.9 LMH in the beginning and after every stepwise change in filtration flux, proves that although BW had greatly enhanced fouling prevention through cake layer exfoliation compared to no BW condition, still at high fluxes latent cake exists on the membrane. As an example, when hydrolysate was filtered in no BW condition, after the drop of flux from 51.0 to 21.9 LMH, residual cake resistance was at highest by $5.23 \times 10^{11}$ m$^{-1}$, whereas the one with BW had less than half the resistance ($2.41 \times 10^{11}$ m$^{-1}$). It should be considered that the final cake layer resistance after the initial phase of filtration at 21.9 LMH had only been $1.80 \times 10^{11}$ m$^{-1}$ and $0.84 \times 10^{11}$ m$^{-1}$ for hydrolysate without and with BW, respectively. Although application of BW has been promising in mitigating cake layer formation in either of the filtered media, its effectivity at fluxes over the critical flux is a matter of question. The results showed that the resistance of the residual cake layer could be reduced up to 54% by BW at the highest flux. However, regardless of fouling remediation by BW, the higher fluxes of 36.4 and 51.0 LMH caused progressive development of compact cake layer. This dense cake layer could not be effectively removed by air/gas scouring or BW (Yoon, 2015), therefore, a combination of a flux below critical and BW is the better option in a continuous hydrolysis iMBR running at constant flux.

4 Conclusions

The double-staged iMBRs applied in this research work was successfully used for continuous hydrolysis with separation of released sugars from lignin-rich solid residual, and fermentation with cell concentration and in situ recovery of fermentation products. Immersed membrane bioreactors performed effectively at a flux of 21.9 LMH up to 264 h of continuous hydrolysis and fermentation with imperceptible changes in filtration performance at high medium suspended solid content. During the continuous hydrolysis, lignin residuals were successfully concentrated, forming up to 70% of medium SS content. Although, increase in the filtration flux to 36.4 and 51.0 LMH resulted in higher ethanol productivity, membrane filtration experienced progressive fouling and TMP elevation, proving that the applied fluxes have exceeded the critical flux of the system. The step-flux experiment that was performed using wheat straw slurry and hydrolysate at three different fluxes, with and without backwash, showed the effect of medium quality, backwash and flux on changes in filtration resistance, cake layer formation and fouling. It can be concluded that using feeding and permeation flow rate of 0.3 l.h$^{-1}$ (21.9 LMH), can assure long-term lignocellulosic
bioethanol production process with less process downtime, membrane physical and chemical cleaning requirements, filtration and BW energy demands and, consequently, operational cost.

Acknowledgements

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References

Dhariwal, A. 2007. The significance of Submerged Ceramic Membrane systems for production oriented Bioprocesses, Universität Saarbrücken.
Doyen, W., Van Hoof, E., Molenberghs, B., Dotremont, C. 2003. A new approach to pilot installation automation, the ingeneous solution you have been looking for, European Desalination Society Newsletter, pp. 4-7.
ePURE. 2019. European renewable ethanol - key figures 2017. ePURE european renewable ethanol.


Table 1. The composition of wheat straw pretreated slurry and hydrolysate. Hydrolysis was conducted in shake flasks in optimum condition (50°C, pH 5.0, 24 h)

<table>
<thead>
<tr>
<th>Composition (g.l⁻¹)</th>
<th>Acetic acid</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Furfural</th>
<th>HMF</th>
<th>SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreated slurry</td>
<td>1.06±0.06</td>
<td>0.93±0.01</td>
<td>4.45±0.01</td>
<td>1.05±0.05</td>
<td>0.11±0.01</td>
<td>14.00±1.53</td>
</tr>
<tr>
<td>Hydrolysate</td>
<td>0.95±0.02</td>
<td>13.40±0.03</td>
<td>6.79±0.11</td>
<td>0.68±0.01</td>
<td>0.09±0.00</td>
<td>5.57±0.92</td>
</tr>
</tbody>
</table>
Table 2. Changes in TMP, total and cake layer resistance during step-flux experiment of wheat straw slurry and hydrolysate at different fluxes with and without backwashing.

<table>
<thead>
<tr>
<th>Flux (l.m(^{-2}).h(^{-1}))</th>
<th>21.9</th>
<th>36.4</th>
<th>21.9</th>
<th>51.0</th>
<th>21.9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
<td>Start</td>
<td>End</td>
<td>average</td>
</tr>
<tr>
<td>TMP (mbar)</td>
<td>Start</td>
<td>End</td>
<td>average</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clean water</td>
<td>3.0</td>
<td>3.1</td>
<td>5.0</td>
<td>5.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Slurry no BW</td>
<td>5.8</td>
<td>7.3</td>
<td>12.8</td>
<td>16.8</td>
<td>9.0</td>
</tr>
<tr>
<td>Slurry with BW</td>
<td>6.0</td>
<td>6.6</td>
<td>11.4</td>
<td>13.6</td>
<td>7.7</td>
</tr>
<tr>
<td>Hydrolysate no BW</td>
<td>10.5</td>
<td>11.9</td>
<td>17.4</td>
<td>27.1</td>
<td>17.5</td>
</tr>
<tr>
<td>Hydrolysate with BW</td>
<td>7.4</td>
<td>7.2</td>
<td>14.2</td>
<td>18.4</td>
<td>10.9</td>
</tr>
</tbody>
</table>

| total resistance (R\(_t\)) (m\(^{-1}\)×10\(^{11}\)) |
|--------------------------------|------|------|------|------|------|------|------|------|
| Clean water (R\(_m\))          | 0.62 | 0.64 | 0.61 | 0.64 | 0.48 | 0.61 | 0.65 | 0.49 |
| Slurry no BW                   | 1.19 | 1.50 | 1.58 | 2.07 | 1.84 | 1.94 | 3.23 | 2.60 |
| Slurry with BW                 | 1.24 | 1.36 | 1.41 | 1.68 | 1.58 | 1.71 | 2.02 | 1.91 |
| Hydrolysate no BW              | 2.15 | 2.45 | 2.15 | 3.35 | 3.59 | 3.56 | 5.73 | 5.72 |
| Hydrolysate with BW            | 1.51 | 1.48 | 1.76 | 2.27 | 2.23 | 2.44 | 2.79 | 2.91 |

| cake layer resistance (R\(_c\)) (m\(^{-1}\)×10\(^{11}\)) |
|--------------------------------|------|------|------|------|------|------|------|------|
| Slurry no BW                   | 0.57 | 0.86 | 0.97 | 1.43 | 1.36 | 1.33 | 2.58 | 2.11 |
| Slurry with BW                 | 0.62 | 0.72 | 0.80 | 1.04 | 1.10 | 1.09 | 1.37 | 1.41 |
| Hydrolysate no BW              | 1.54 | 1.80 | 1.54 | 2.71 | 3.11 | 2.95 | 5.07 | 5.23 |
| Hydrolysate with BW            | 0.90 | 0.84 | 1.15 | 1.63 | 1.75 | 1.83 | 2.14 | 2.41 |
Figure 1. Integration and application possibilities of membrane technology in lignocellulosic bioethanol production process.
Figure 2. The scheme of the experimental set-up of the double-staged immersed membrane bioreactor applied for continuous hydrolysis and fermentation: (PT) pressure sensor, (LT) level transmitter and (FT) flow meter.
Figure 3. Changes in the concentration of substrates and metabolites during batch and continuous hydrolysis and fermentation of wheat straw slurry at different permeate fluxes of 21.9, 36.4 and 51.0 LMH.
Figure 4. Changes in the TMP, concentration of suspended solids and cell biomass at different constant filtration fluxes of 21.9, 36.4 and 51.0 LMH (a) during continuous hydrolysis (b) and fermentation of wheat straw slurry.
Figure 5. Changes in the composition and concentration of suspended solids and lignin in the feed slurry and at different stages of continuous hydrolysis and filtration (without draining). (ASL-acid soluble lignin, AIL-acid insoluble lignin and CH-structural carbohydrates)
Figure 6. Changes in TMP, during step-flux experiment using pure water (a), wheat straw slurry (b) and hydrolysate (c) at different fluxes of 21.9, 36.4 and 51.0 LMH, with and without backwashing.
Removal of Bacterial Contamination from Bioethanol Fermentation System Using Membrane Bioreactor

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Abstract: A major issue hindering efficient industrial ethanol fermentation from sugar-based feedstock is excessive unwanted bacterial contamination. In industrial scale fermentation, reaching complete sterility is costly, laborious, and difficult to sustain in long-term operation. A physical selective separation of a co-culture of \textit{Saccharomyces cerevisiae} and an \textit{Enterobacter cloacae} complex from a buffer solution and fermentation media at dilution rates of 0.1–1 1/h were examined using an immersed membrane bioreactor (iMBR). The effect of the presence of yeast, inoculum size, membrane pore size, and surface area, backwashing and dilution rate on bacteria removal were assessed by evaluating changes in the filtration conditions, medium turbidity, and concentration of compounds and cell biomass. The results showed that using the iMBR with dilution rate of 0.5 1/h results in successful removal of 93% of contaminating bacteria in the single culture and nearly complete bacteria decontamination in yeast-bacteria co-culture. During continuous fermentation, application of lower permeate fluxes provided a stable filtration of the mixed culture with enhanced bacteria washout. This physical selective separation of bacteria from yeast can enhance final ethanol quality and yields, process profitability, yeast metabolic activity, and decrease downstream processing costs.

Keywords: membrane bioreactor; filtration; bacterial decontamination; fermentation

1. Introduction

A major issue hindering efficient industrial fermentation of sugar-based feedstock into ethanol is unwanted bacterial contamination [1,2]. The bacterial contamination can be introduced into the fermentation system from different hardware components such as instruments, reactors, pipelines of the feed stream, and added chemicals, and nutrients. These bacterial contaminations can also be introduced to the system by recycling yeast [2,3]. Approximately 500 different strains of bacteria have been isolated from different stages of a fermentation process, which predominantly are lactic acid producing bacteria as they have a relatively better tolerance for low pH environments as well as higher ethanol concentrations [4,5].

The presence of bacterial contamination in a fermentation process can noticeably decrease the final ethanol yields and process profitability. In continuous, semi-batch or batch mode fermentation processes, the substrate and nutrient sources are provided for the yeast \textit{Saccharomyces cerevisiae} to thrive in and to produce ethanol. However, the competition between yeast and bacteria to utilize nutrients to survive, grow, and reproduce leaves inadequate levels of food components for the yeast,
resulting in a considerably lower ethanol yield. Moreover, the contaminating microorganisms produce metabolites such as lactic and acetic acids that disturb and inhibit the metabolic activity of yeast, as well as reduce the quality of the final product [2]. In certain cases, the bioethanol yield may decrease as much as 30% due to bacterial metabolites, causing a major loss for the ethanol producers [6]. In addition, in processes where yeast cells are recycled in consecutive fermentation batches, a high level of bacterial contamination may lead to yeast flocculation, resulting in poor mass transfer, low cell viability, and reduction in ethanol yield. This could also lead to excessive foam formation, and the need of antifoam agents that increase the overall costs [7].

In industrial large-scale fermentation, a fully contamination-free process is difficult to achieve. Complete sterilization and maintaining sterility of the feedstock and instruments are costly and laborious processes. In order to alleviate the contamination issue and control bacterial growth in fermentation systems, conventional anti-bacterial agents such as the antibiotic virginiamycin, sodium fluoride and hydrogen peroxide have been added at different stages of the process [8–11]. However, these anti-bacterial agents have not proven to be fully effective for long-term bacterial control and system disinfection. Moreover, the presence of antibiotic residues in certain by-products of the ethanol fermentation process such as Distiller’s Dried Grains with Solubles (DDGS) result in environmental and health concerns that render their commercialization problematic. Antibiotic residues in DDGS used as animal feed risk ending up in animal products such as milk, or if used as fertilizers, may cause resistance in the bacterial cultures grown on the crops [12].

Another approach to partially decontaminate the fermentation system involves the use of acids, e.g., sulfuric acid, in order to temporarily (1–3 h) lower the pH to about 1.5–3 for the treatment of a diluted yeast culture [7]. Contaminating bacteria (with favorable pH 6) are less resistant to lower pH than yeast (favor pH above 4), therefore, a pH drop in the beginning of the fermentation cycle ameliorates issues related to bacterial activity. However, this approach may also deteriorate the yeast’s viability and metabolic activity [13,14].

Membrane bioreactor (MBR) systems could potentially solve the contamination issue by means of a physical approach. Membrane-related applications are generally based on the ability of the semi-selective membrane to efficiently separate different compounds and/or cells/particles on size-exclusion and affinity mechanisms since they are permeable to some components while retaining others [15]. Based on this capability of synthetic membranes, it is suggested that a selective washout of bacterial contamination, while retaining the larger yeast cells, could be achieved through proper choice of membrane pore size, membrane material hydrophobicity and affinity for different cells and chemical compounds. By this approach, not only the bacterial contamination in the fermentation process can be physically removed, but also higher ethanol yield and productivity can be expected, as high yeast cell concentrations are present in the medium during a continuous fermentation. However, as the suspended bacteria can cause bio-fouling due to internal and external membrane pore blockage and/or biofilm formation, the viability of this physical decontamination method needs to be studied in more detail [16,17].

In this paper, the capability of an immersed MBR (iMBR) set-up to actively decontaminate a fermentation system from unwanted bacteria while retaining yeast is examined for a Saccharomyces cerevisiae-Enterobacter cloacae model system. In this regard, the effects of different filtration parameters (like transmembrane pressure, permeate flow rate, backwash flowrate, and backwash pressure difference), membrane characteristics (like pore size and effective membrane filtration area), media (phosphate buffered saline (PBS) and glucose semi-synthetic medium), pH, and inoculum size on bacterial decontamination behavior are evaluated.
2. Materials and Methods

2.1. Microorganisms

The yeast *Saccharomyces cerevisiae* and a bacterial complex of *Enterobacter cloacae* were used as fermentation agent and bacterial contamination, respectively. Throughout the text the bacterial complex is referred to as bacteria.

2.1.1. Saccharomyces Cerevisiae

The yeast strain *S. cerevisiae* CBS 8066, provided by Centraalbureau voor Schimmelcultures (Delft, The Netherlands), was loop-inoculated on sterile Yeast extract Peptone Dextrose (YPD) agar plates consisting of 20 g/L peptone, 10 g/L glucose, 20 g/L agar, and 10 g/L yeast extract, incubated for 24 h at 30 °C, and then kept at 4 °C until use.

2.1.2. Enterobacter Cloacae

The bacteria used were an *Enterobacter cloacae* complex. This bacillus acetic acid-producing bacterial contamination was first isolated from a xylose-glucose fermentation system at the University of Borås, Sweden, and was further identified at the Culture Collection University of Göteborg (Sweden) as CCUG 68890. The identified *E. cloacae* complex consists of nearly 30 different bacterial species with *Enterobacter cloacae* being dominant. *E. cloacae* is a facultative anaerobic gram-negative, rod-shaped bacteria that grows well in mesophilic conditions (37 °C) and at a pH around 7.0. *E. cloacae* is capable of consuming a variety of carbon sources such as glycerol, cellulose, glucose, and xylose [18,19]. The *E. cloacae* complex was kept on nutrient agar plates consisting of 5 g/L peptone, 5 g/L NaCl, 15 g/L agar, and 3 g/L yeast extract and stored at 4 °C until use.

2.2. Culture Preparations

A synthetic nutrient medium consisting of 30 g/L glucose, 10 g/L peptone, 5 g/L yeast extract and 3.5 g/L KH₂PO₄, was used for the cultivation of yeast and bacteria precultures in shake flasks. The nutrient broth and KH₂PO₄ were autoclaved separately, and mixed under sterile conditions before the addition of yeast/bacteria inoculum. Originally, the pH of the medium was 5, but for some preparations, the pH was adjusted to 7 by adding 10 M NaOH for bacteria cultivation.

2.3. Maximum Growth Rate Measurement

An understanding of the maximum growth rate of yeast and bacteria is of great importance when defining the maximum dilution rate of a continuous fermentation process. Therefore, the growth rates of yeast at pH 5 and bacteria at pH 5 and 7 were investigated. For shake flask cultivations, 10 mL of PBS (8 g/L NaCl; 0.2 g/L KCl; 1.42 g/L Na₂HPO₄; 0.24 g/L KH₂PO₄) was loop-inoculated with a colony of yeast and/or bacteria and placed in a shaking water bath (Grant OLS 200, Grant Instrument Ltd., Cambridge, UK) at 30 °C/125 rpm for 30 min. Subsequently 100 mL of nutrient broth was inoculated with 1 mL of the preculture prepared in 250 mL Erlenmeyer flasks and placed in a shaking water bath. Frequent samplings were done at different time intervals, and the absorbance of the cell samples was measured with a spectrophotometer against a PBS blank. Readings were plotted against time, and the slope of the graphs in the exponential growth phase was measured to define the maximum growth rate (Section 3.1).

2.4. Yeast and Bacteria Size Determination

Estimation of the dimensions of both yeast and bacteria was essential to decide on the pore size of the membranes to be used for filtration purposes. In order to evaluate the average size of both yeast and bacteria, samples were taken with an inoculation loop from the culture broth, Gram stained [20], and observed and scaled under a light microscope at ×1000 magnification. A Burker’s counting
chamber was used to count the yeast cells. Size distribution charts were prepared from the gathered data to calculate the average dimensions of yeast and bacteria.

2.5. Membrane Bioreactor Set-Up and Operation Conditions

2.5.1. Immersed Membrane Bioreactor

The general schematic of the membrane bioreactor set-up is illustrated in Figure 1. The membrane panels used for MBR filtration were 2nd generation flat-sheet Integrated Permeate Channel (IPC) panels [21] with polyester spacer-fabric support, developed by the Flemish Institute for Technological Research (VITO NV, Mol, Belgium). For better oriented aeration and membrane fouling prevention the panels had inbuilt air/gas diffusers (6 on each side) of 0.5 mm in diameter, at the bottom of the panel. The total membrane-coated area per panel was 68.6 cm². Membranes were made from hydrophilized polyethersulfone (PES) possessing a clean water permeability of 3000–4000 L/h·m²·bar. IPC membranes with membrane layers with mean pore sizes in the microfiltration range of, respectively, 1 µm and 2.4 µm were used. The 2nd generation IPC panels were integrated inside a 1.5 L bench-top reactor for single panel experiments and a 2 L water-jacketed bioreactor (Biostat B plus, Sartorius BBI Systems GmbH, Melsungen, Germany). A special spacer box for double panel experiments was used in order to improve membrane cleaning and medium mixing efficiencies. The experiments were conducted with both single and double membrane panels to enable observation of the effect of membrane surface area on filtration and bacterial washout performances.

Figure 1. Immersed MBR setup: (a) iMBR (b) feed tank (c) permeate tank (d) pumps (e) flowmeters (f) pressure sensor (g) pressure sensor reader (h) relay (i) control unit (j) computer (k) pH electrode (l) temperature sensor (m) spacer box. * Units marked in italic are only present in the double panel setup.

The applied MBR was operated at a constant flux filtration mode. However, since the initial flow was set by implementing a specific pump rotation speed, small drops in flow could occur during the tests. The filtrate flow rate and the TMP were monitored by means of a 710 Atrato ultrasonic flowmeter (Titan Enterprises Ltd., Sherborne, UK) and a Microfluidic pressure sensor MPS3 (Elveflow, Paris, France), respectively. The recorder flow rates have not been presented in the figures as constant flux filtration mode was applied. The raw data provided from the measurement devices were logged and processed in a computer connected to the measurement instruments. The reactor was fed from the feed tank and the filtrate was removed from the hollow space between 2 membrane layers of the IPC membranes using Watson-Marlow 403U/R1 peristaltic pumps (Watson Marlow, Wilmington, MA, USA). In the cases where backwashing was applied, the cycle was controlled through a Schneider Zelio logic relay (Schneider Electric Automation GmbH, Lahr, Germany) attached to the permeate.
pump. Two different cycles of backwash were investigated for their effect on reversible fouling: A 4-min cycle (30 s of backwash + 3.5 min of permeation) and a 3-min cycle (30 s of backwash + 2.5 min of permeation). Backwash flow rates were the same as the forward flow rates.

In experiments where the 1.5 L bench reactor was used, the temperature was controlled at 30 °C through a heating jacket, pH was monitored by the control unit and was manually adjusted by the addition of 10 M NaOH. In examinations with the 2 L water-jacketed bioreactor, temperature, pH and aeration were monitored through a Biostat B plus fermentation controlling unit.

In fermentation cycles, foaming was controlled manually by adding fatty acid ester anti-foam, and the reactor was aerated with 250 L/h N\textsubscript{2} to provide anaerobic conditions. When PBS medium was used, the reactor was aerated with 250 L/h air through a rotameter.

Before each run, reactors and tubings were autoclaved at 120 °C for 20 min, the membrane was subsequently chemically cleaned with sterilization solutions of 2% NaOH, 1% H\textsubscript{3}PO\textsubscript{4}, and 200 ppm NaOCl at 45 °C each for 30 min followed by rinsing with sterile distilled water.

2.5.2. Evaluation of Retention and Washout of Microorganisms

In order to evaluate the ability of the membrane filtration to selectively retain yeast and remove bacteria, membrane filtration at different filtration conditions was performed in sterile PBS buffer. PBS was used as the bioreactor medium to maintain the viability of the cells during the filtration cycle in both 1.5 L and 2 L MBRs.

The reactor was inoculated with yeast, bacteria, or yeast and bacteria to observe the influence of the presence of the cells on the membrane filtration capabilities and to measure the extent of filterability and separation of microorganisms. The inoculum preparation was as described in Section 2.2. After the precultures were prepared in shake flasks, the content was centrifuged at 3000 \times g for 5 min. The supernatant was then removed and the cells were re-suspended in the same amount of PBS to avoid a color change that would have disturbed spectrophotometry readings. To ensure homogeneity of the zero-hour samples taken for turbidity measurements, feeding and permeate withdrawal was started after the medium and the inoculum had been mixed for 5 min. A range of dilution rates were tested: 0.25–0.66 1/h, when the reactor working volume was 1.5 L, and 0.5–0.75 1/h when the working volume was 2 L. Tested biomass concentrations were 0.3–0.02 g/L for bacteria and 0.6–0.135 g/L for yeast.

2.5.3. MBR Fermentation and Filtration

Regarding continuous fermentation and bacteria filtration (washout) conditions, the sterile synthetic medium consisting of glucose, peptone, yeast extract and K\textsubscript{2}HPO\textsubscript{4} was used in two different concentrations: 30 g/L Glucose + 10 g/L Peptone + 6 g/L Yeast Extract + 3.5 g/L K\textsubscript{2}HPO\textsubscript{4} and 20 g/L Glucose + 1 g/L Peptone + 1 g/L Yeast Extract + 3.5 g/L K\textsubscript{2}HPO\textsubscript{4}. Before starting the experiment, the K\textsubscript{2}HPO\textsubscript{4} and nutrient solutions were autoclaved separately and were mixed inside the reactor. The pumps for permeation and feeding were started after the medium and the inoculum were mixed for 5 min to ensure homogeneity of the zero-hour samples. Inoculum preparation was as described in Section 2.2, and the contents of the shake flasks were used to inoculate the reactor. The filtration behavior during ethanol fermentation of the bacteria-contaminated medium was performed at a permeate flux range representing dilution rates of 0.25 and 0.5 1/h using the 1.5 L reactor and 0.11–1 1/h for the 2 L reactor. Cell concentrations of ~8 \times 10^{-6}–0.039 g/L (~1.1 \times 10^5–0.6 \times 10^6 CFU) of bacteria and 0.013–0.183 g/L (~0.03 \times 10^6–0.51 \times 10^6 CFU) of yeast were used to determine the optimum bacteria washout capabilities at different fermentation and filtration conditions.

2.6. Analytical Methods

The changes in the concentration of bacteria and yeast in shake flasks, MBR and permeate tank were monitored through optical density measurements at 600 nm in the linear range in a Libra S60 (Biochrom Ltd., Cambridge, UK) spectrophotometer. For growth-rate measurement and MBR
Fermentation samples, changes in the turbidity were checked by centrifuging the sample \((3000 \times g, \ 5\ \text{min})\), removing the supernatant, re-suspending cells in the initial feed medium, and checking the absorbance of the samples against the relevant pure feed medium. In the cases where bacteria and yeast cultures were to be added together, the turbidity of the bacteria was measured first, and then the turbidity of the mixed culture. Samples were taken on average every 20 min from the reactor and permeate tank, and readings were plotted against time.

The concentration of different nutrients and metabolites during preculture preparation and MBR fermentation cycles were analyzed using high performance liquid chromatography (HPLC). Samples taken at different time intervals were centrifuged at \(15,000 \times g\) for \(5\ \text{min}\), and the supernatant was used for HPLC analysis. The HPLC (Waters 2695, Waters Corporation, Milford, CT, USA) had a hydrogen-based column (Aminex HPX-87H, Bio-Rad, Hercules, CA, USA) working at \(60^\circ C\) with \(5\ \text{mM H}_2\text{SO}_4\) eluent flowing at \(0.6\ \text{mL/min}\), sufficient for detection and quantification of sugars and fermentation metabolites such as glucose, ethanol, glycerol, and acetic acid.

Cell dry weight (CDW) measurement was performed by collecting \(5\ \text{mL}\) of the culture in duplicate at different time intervals, centrifuging at \(3000 \times g\) for \(5\ \text{min}\), replacing the supernatant with Milli-Q water and repeating the process, and then drying the cell pellet in previously dried and weighed test tubes at \(70^\circ C\) for \(24\ \text{h}\).

Cell counts were performed as follows. For yeast cells, \(10^{-1}\) diluted and non-diluted samples of \(0.2\ \text{mL}\) were inoculated on YPD plates. Duplicates were incubated for \(24\ \text{h}\) at \(30^\circ C\). For bacteria, nutrient agar plates were inoculated with \(50\ \mu\text{L}\) samples taken at \(0, 5\) and \(10\ \text{h}\), or serial dilutions thereof. Duplicates were incubated for \(24\ \text{h}\) in \(30^\circ C\).

3. Results and Discussion

In this study, the potential of an iMBR set-up for yeast retention and washout of bacteria was evaluated. First, filtration performance of yeast, bacteria or a mixture of both microorganisms was investigated in a synthetic buffer medium. Then, the optimal conditions were applied in continuous fermentation experiment with the mixed culture on semi-synthetic medium.

3.1. Growth Rate Measurement of Yeast and Bacteria

The specific growth rate of microorganisms has a significant role in defining the optimum dilution rate for effective bacteria removal. For yeast and \(E.\ cloacae\) the maximum growth rates were determined to be 0.63, 0.47, and 0.78 \(1/\text{h}\), representing 1.0, 1.47, and 0.89 \(\text{h}\) doubling times for yeast at its optimum pH 5, and for bacteria at pH 5 and the optimum pH 7, respectively (see Figure 2). Considering continuous fermentation in a steady-state condition (1) using sterile feed and having negligible cell death, cells are removed from the reactor equal to their growth rate. That means, according to the Monod growth model (2) [22], cell growth rate \((\mu)\) is equal to the dilution rate \((D)\):

\[
\frac{dx}{dt} = \text{cell growth + cell washout} = x\mu + xD
\]

In steady-state continuous fermentation:

\[
\frac{dx}{dt} = 0 \quad \text{and therefore} \quad D = \mu \quad (1)
\]

\[
\mu = \frac{\mu_{\text{max}}S}{S + K_s} \quad (2)
\]

where \(\mu\) is the cell growth rate, \(\mu_{\text{max}}\) the cell maximum growth rate \((1/\text{h})\), \(S\) the concentration of the limiting substrate \((\text{g/L})\), \(K_s\) the half saturation constant \((\text{g/L})\).

If high concentration of substrate is available \((S \gg K_s)\) the dilution rate nearly equals \(\mu_{\text{max}}\). When the dilution rate is higher than \(\mu_{\text{max}}\) washout of bacterial cells will occur. In addition, because
at pH 5 (the favorable pH for fermentation) the bacteria have a slower maximum growth rate than S. cerevisiae, a selective microorganism separation can in principle be achieved through the proper choice of dilution rate.

![Graph](image)

**Figure 2.** Changes in the optical density (λ = 600 nm) of the media for bacterial cultivation at pH 5 (B5), pH 7 (B7) and yeast cultivation at pH 5 (Y5).

### 3.2. MBR Filtration Using Buffer as Feed Solution

MBR fermentation systems have significant advantages over conventional fermentation modes as they can provide high cell concentrations in the reactor that allow efficient conversion of substrates into products and also higher tolerance to cell inhibitory compounds [23]. Moreover, higher dilution rates can be achieved since undesired cell washout is prevented [24]. However, higher dilution rates require higher permeate fluxes through a given membrane surface area which increases membrane fouling tendency [25]. Therefore, prior to fermentation runs, filtration tests were implemented using PBS as the reactor medium to maintain viable cells at constant cell numbers [26]. The effect of filtration parameters such as initial transmembrane pressure (TMP) and permeate flux, backwash cycles, and membrane characteristics such as pore size and effective membrane filtration area on cell washout and retention behavior were investigated.

#### 3.2.1. Yeast Retention in the iMBR

As yeast retention in the MBR was desired, and the yeast’s average diameter is 2.5 ± 0.2 µm, membranes with pore sizes of 1 µm and 2.4 µm were applied. While in conventional continuous bioreactors $D > \mu_{max}$ is an unfavorable condition leading to yeast washout, this is not the case in a MBR, where the membrane guarantees total yeast cell retention. Therefore, dilution rates well above the $\mu_{max}$ of yeast were also evaluated. Figure 3a,b present the changes in the turbidity of reactor and permeate medium and TMP during the filtration of yeast suspension. For both membrane pore sizes, nearly stable TMP and permeate flow rates were recorded. This indicates that there was no considerable fouling or cake layer formation on the membrane surface in this filtration condition with 0.5 1/h dilution rate and 0.7 ± 0.14 g/L initial yeast concentration. Furthermore, there was no noticeable change in the permeate and MBR medium turbidity, or in permeate plate counts, confirming that yeast was completely retained inside the reactor. Hence, membranes with both pore sizes were tested for the bacteria removal evaluations.
3.2.2. Bacteria Removal in the iMBR

Bacterial size (length of 0.7–1.2 μm and diameter of 0.3–0.5 μm) has a significant importance for membrane fouling and pore clogging [27]. In addition to a variation in membrane pore sizes, different permeate fluxes, and TMP were examined to achieve the maximum percentage of bacteria removal with the shortest cycle time possible.

3.2.2.1. The Effect of Membrane Pore Size on Bacteria Removal

The effect of membrane pore sizes (1 μm and 2.4 μm) was examined at an average initial permeate flux of 55.01 ± 0.85 L/h·m² (0.25 1/h dilution rate). As shown in Figure 3c,d, there was only a slight decrease in the concentration of bacteria in the reactor medium with the 1 μm pore size membrane, while the turbidity of the permeate stayed at zero. This indicates that bacteria could not be readily washed out across the membrane pores and their deposition on membrane surface and pores caused pore clogging and/or membrane fouling, as evidenced by the increased TMP. While it has been reported...
that the flexibility of the peptidoglycan cell wall of Gram-negative bacteria (e.g., *E. cloacae*) may allow deformation and penetration across the membrane to some extent [28,29], the results of our tests proved otherwise. In contrast, with the membrane of 2.4 µm pore size, a noticeable decreasing and increasing trend in the concentration of bacteria in the reactor medium and permeate was observed, respectively. Moreover, a stable TMP during filtration indicates that bacteria were effectively washed out without severe membrane-bacteria interaction. This membrane was, therefore, chosen for all other experiments.

3.2.2.2. The Effect of Initial Inoculum Size on Bacterial Removal

In industrial fermentation systems, the initial concentration of bacterial contaminants is about $10^5$–$10^8$ colony forming units (CFU) per ml [4,30]. However, if the process is ran in batch mode the propagation of contamination occurs at an accelerated pace and this leads to the loss of nutrients, low product yield and product quality deterioration. In order to have an understanding of the maximum starting inoculum size which does not cause extensive membrane fouling and can be washed out effectively before complete bacterial breakout, filtration performance was evaluated at a starting dilution rate of 0.25 1/h by inoculating the MBR containing PBS with either 0.1 g/L (~1.4 $\times$ $10^9$ CFU) or 0.05 g/L of *E. cloacae*.

The results showed a remarkable 89% reduction in bacteria concentration when the inoculum concentration was 0.05 g/L (Figure 3e). However, a two-fold higher inoculum concentration of 0.1 g/L caused progressive elevation in TMP (Figure 3f).

3.2.2.3. The Effect of Dilution Rate, Backwash and Membrane Surface Area on Bacteria Removal

A higher dilution rate and filtration flux is desirable because it raise the productivity in continuous fermentation processes [24,31]. However, for every membrane filtration system there is a critical flux below which extensive cake layer formation and fouling can be prevented [32]. Hence, the optimum operable flux for a given iMBR set-up has to be determined. To this end, various dilution rates of 0.25, 0.5, 0.66 1/h were examined using the same inoculum size of 0.05 g/L with and without backwashing. While a dilution rate of 0.25 1/h (permeate flux ~55 L/h·m²) yielded favorable results as mentioned in Section 3.2.2.1, this was no longer the case at the higher permeate fluxes (~112–145 L/h·m²).

These induced greater fouling tendency as the TMP showed a dramatic jump up to 280 mbar in only 2 h (Figure 4b). Apparently, quick formation of a cake layer hindered effective removal of bacteria, because only 49% reduction in the reactors initial bacterial population was observed (Figure 4a). As there are minimal changes in the turbidity of the permeate (Figure 4a), the reported decrease in bacteria concentration in the main reactor must probably be attributed to the attachment/deposition of bacterial cake on the membrane surface [33,34].

The surface condition of an immersed membrane directly affects its filtration capability [25]. In order to keep the membrane surface clean and prevent fouling, backwashing is an effective physical cleaning remedy [34]. Hence, 2 different backwashing regimes were applied: 3.5 min forward flow, and 0.5 min backwash (4 min cycle) at a dilution rate 0.5 1/h (109 L/h·m²), and a more frequent backwash of 2.5 min forward flow plus 0.5 min backwash (3 min cycle) at a higher dilution rate of 0.66 1/h (144 L/h·m²). In the latter case, TMP stayed nearly constant at 90 mbar following an increase and flowrate was stable (Figure 4d), while it did not result in a considerable drop in bacteria concentration (34%) (Figure 4c). Consequently, a more frequent cleaning of the membrane surface could not compensate the effect of a higher dilution rate of 0.66 1/h compared to 0.5 1/h in the tested range and at the tested backwash rates. Therefore, a 0.5 1/h dilution rate combined with a 4 min backwash cycle proved to be the most desirable out of the tested filtration conditions leading to an optimum bacteria removal of 93%.
In the next step, single and double membrane panels were applied under the same conditions (dilution rate of 0.5 L/h, backwash cycle of 4 min), with 0.042 g/L (~0.6 × 10^9 CFU) and 0.033 g/L (~0.5 × 10^9 CFU) initial bacteria concentration (Figure 5). Since permeate flow was the same in both conditions, the flux applied on a single panel (145 L/h·m²) membrane was twice that of the double panel (73 L/h·m²). The same extent of bacteria removal was achieved 1.6-times faster for a single panel (Figure 5). While the initial drop in reactor biomass levels was similar in both cases, the corresponding increase in permeate optical density was higher in the single panel test, which might be related to the higher fluxes and convective flow.

![Figure 4](image-url)
3.2.2.4. The Effect of Concomitant Presence of Bacteria and Yeast on Bacteria Removal

In a condition resembling that of a contaminated fermentation system, bacteria removal was examined in concomitant existence with yeast. The results showed that nearly complete bacteria removal was achieved successfully over 6 h of filtration (Figure 6a) with a single membrane panel, and the previously proven favorable filtration conditions of 0.5 l/h dilution rate and 4 min backwash cycle. Moreover, a slight increase in TMP (initial increase before reaching a plateau) was observed at an inoculum concentration of bacteria and yeast of 0.067 g/L and 0.173 g/L, respectively (Figure 6b). This exceptionally good bacterial washout in the presence of yeast could be due to the interaction of yeast with the membrane surface or between yeast and bacteria. However, a definite answer cannot be given at this point without thorough membrane surface imaging and analysis. Moreover, when the membrane surface area was doubled using two IPC membrane panels while providing the initial overall permeate flux, complete bacteria washout was achieved in 2 h, which is significantly faster than for a single panel (6 h) (Figure 7). However, it is noteworthy that having the same flow rate, while increasing the surface area could become problematic for bacterial washout if a proper relation between the bacteria (any suspended particulated matter) permeation velocity (drag) towards the membrane surface and back-transport velocity away from the surface is not met [25]. Therefore, reduction in flux by increasing the membrane surface area can proceed to the point that back-transport mechanisms do not dominate the direction of the movement of bacteria. This was also observed for the filtration of bacterial culture (Section 3.2.2.3), where increasing membrane surface area at a fixed permeate flow rate deteriorated washout (Figure 5).
Figure 6. Changes in the (a) turbidity of the reactor (R) and permeate line (P) and (b) TMP during filtration of a mixed culture of bacteria and yeast in PBS medium. (The upper curve represents the changes in TMP during forward filtration, and dispersed points under that curve are the latent effects of the BW pressure readings).

Figure 7. The effect of membrane area on filtration performance and bacterial washout in a mixed culture: Changes in the turbidity of the reactor (R) and permeate line (P) using a single (S.P.) or double (D.P.) IPC panel with the same initial permeate flux.
3.3. The Effect of the Presence of Yeast and Bacteria on the Fermentation Process

Fermentation experiments were conducted to observe the growth characteristics and metabolite productions of both bacteria and yeast as well as to determine the effect of metabolic activity of one microorganism on that of the other. As is in batch fermentations, these experiments also provided an insight in yeast behavior under accumulated acetate levels. Separate bacteria or yeast cultures, or combined bacteria and yeast cultures were cultivated in batch mode with semi-synthetic medium (Section 2.5.3) (Figure 8).

Figure 8. Changes in (a) cell dry weight and (b) substrate and metabolites in the reactor during batch fermentation. The figure legends Y, B and Y-B represent samples containing only yeast, only bacteria, and both bacteria and yeast, respectively. The error bars represent ±2 standard deviations.

Figure 8. Changes in (a) cell dry weight and (b) substrate and metabolites in the reactor during batch fermentation. The figure legends Y, B and Y-B represent samples containing only yeast, only bacteria, and both bacteria and yeast, respectively. The error bars represent ±2 standard deviations.
Through dry mass measurements, it was observed that, although yeast started growing without a lag phase, noticeable change in E. cloacae only occurred after the first 6 h from the start of the cultivation (Figure 8a). Considering that in a mixed culture there is competition over nutrient consumption, the final biomass concentration acquired in pure yeast culture (25.5 g/L) was higher than that of the mixed culture (22 g/L). As seen in Figure 8b, this nutrient consumption competition is mostly focused on the present nitrogen sources (yeast extract, peptone etc.) as the changes in the glucose concentration during cultivation of only bacteria was minimal (4.5%) (Figure 8b). Where yeast and bacteria are cultivated together or when yeast is cultivated solely, more than 96% of the glucose content of the reactor is depleted within the first 10 h. The trend of glucose consumption for the mixed culture followed a less steep decline with more residual glucose (4%) after 10 h of cultivation. This could be the effect of acetic acid produced by bacteria on yeast energy level (in form of ATP) and therefore cell growth [35]. The competition over the nutrients is the reason for the 2-fold higher acetate concentration in the bacterial culture (0.48 g/L) compared with mixed culture (Figure 8b). In addition, it could be perceived that the increase in cell dry mass in the mixed cultures after the 6th hour is attributed to yeast growth (Figure 8a).

As illustrated in Figure 8b, regardless of the presence of bacteria in the medium, nearly similar final (10 h) concentrations of ethanol (9 g/L) were achieved. However, in the culture with only yeast the maximum level of ethanol is reached after 8 h. It is concluded from the changes in metabolites during bacteria cultivation that the complex inoculum mostly included homofermentative bacteria producing acetic acid (Figure 8b). No ethanol and lactic acid was detected.

3.4. Bacteria Decontamination during Fermentation in MBR

In the previous sections, filtration of microbial cell suspensions was studied in PBS medium, which preserves cell viability while negligible growth occurs. The ultimate goal is, however, to investigate bacterial washout under actual fermentation conditions, on a growth-supporting semi-synthetic medium.

The Effect of Inoculum Size and Dilution Rate on Bacteria Decontamination

In the first stage, the reactor was only inoculated with only bacteria (0.001 g/L ≈ 0.1 × 10⁹ CFU) and different dilution rates of 1, 0.5, and 0.25 L/h were applied during filtration to investigate the effect of permeate flux on bacteria removal. Figure 9a shows that in all conditions the pressure began to build up between 4–6 h from the start of the tests, while the rate of TMP increase is faster at higher dilution rates (Figure 9a). Therefore, the lowest dilution rate of 0.25 L/h (permeate flux ~55 L/h·m²) was chosen for further tests.

Next, fermentation of a mixed culture (bacteria and yeast) was investigated. Conform the PBS filtration results, the final TMP values were lower for mixed cultures (Figure 9c) than for pure bacterial cultures (Figure 9a), even though the overall initial inoculum size of the mixed cultures (0.07–0.211 g/L) (separate bacteria and yeast inoculum size is presented on the figure) (Figure 9c) was 70 and 200-times higher (Figure 9a).

The changes in the concentration of biomass in the reactor and the changes in the acetate concentration are presented in Figure 9b. As can be seen, biomass content constantly inclined. In addition to yeast, bacteria must also have accounted for this growth in biomass as evidenced by the increase in the acetate content of the media (Figure 9b). It is, however, anticipated that the lower TMP in mixed culture could be due to lower growth of bacteria due to competition over nutrients in addition to the mitigating effect of yeast on membrane surface cake layer formation [36]. Therefore, bacteria removal prior to bacteria growth breakout should be aimed for to reduce membrane fouling and TMP rise (Figure 9).

Additional experiments were executed with relatively higher yeast and smaller bacterial inoculum and at permeate fluxes of 16 L/h·m² (dilution rate 0.11 L/h) and 73 L/h·m² (dilution rate 0.5 L/h) (Figure 10).
Figure 9. Changes in (a) TMP based on Dilution Rate (D.R.): 0.25, 0.5, and 1 1/h in double panel, (b) biomass concentration in the reactor and acetate concentrations inside the reactor (R) and permeate tank (P) and (c) TMP comparison based on initial inoculum concentration in the reactor; with bacteria 0.026 g/L and yeast 1.85 g/L, bacteria 0.023 g/L and yeast 0.047 g/L (D.R. 0.25 1/h and flux 73 L/h·m²) in single panel. (The upper curves for each marker represents the change in TMP during forward filtration, and dispersed points under that curve are the latent effects of the BW pressure readings).
As expected, the condition with lowest bacteria (0.0001 g/L) and highest yeast (0.075 g/L) concentration showed best filtration performance (Figure 10a). This may imply that if the initial bacteria concentration is less than 0.1% of that of yeast, bacteria washout during filtration can be efficiently conducted and chemical decontamination steps can be reduced or even skipped. While the filtration results for a non-growing culture in PBS give the notion that the dilution rate of 0.5 1/h provides better bacterial washout, this is not the case during actual fermentation conditions. After 6 h of fermentation and filtration, excessive fouling occurred. (Figure 10b). By present assessment, the bacteria washout performance can be sustained throughout fermentation and filtration, only if filtration regime is improved. Further, in-depth research is required on filtration performance through optimization of backwash conditions and applying constant flux or TMP operations. While higher fluxes contribute to more initial washout, they increase the fouling tendency. Considering that, more fouling may lead to more retention, generalization of this physical bacteria decontamination approach to different fermentation systems requires a profound understanding of yeast-bacteria-membrane interactions contributing to fouling at microscopic level.

![Figure 10. Changes in (a) TMP based on different starting inoculum size (dilution rate 0.11 1/h) and (b) TMP based on dilution rate with filtration in double panel.](image)

4. Conclusions

The iMBR set-up proved to be a promising replacement for conventional chemical bacteria decontamination from fermentation systems. Pure filtration of non-growing cells led to successful
removal of 93% and nearly total amount of contaminating bacteria when only bacteria or both bacteria and yeast were present, respectively. While bacterial washout during fermentation was not as efficient as in a non-growth buffer medium, stable filtration and bacteria removal can be achieved by using lower dilution rates and at a higher ratio of yeast to bacteria. This work showed that effective filtration can occur under a certain restricted filtration regime and culture condition. While, it was observed that by synchronizing filtration parameters (dilution rate, flux, etc.) and culture condition (inoculum size, mixed culture etc.) enhanced physical bacteria decontamination may be reached, extensive research is required to reach a stable situation where active washout at high fluxes can be obtained before cake layer build-up.

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References


Reverse membrane bioreactor: Introduction to a new technology for biofuel production

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Abstract

The novel concept of reverse membrane bioreactors (rMBR) introduced in this review is a new membrane-assisted cell retention technique benefiting from the advantageous properties of both conventional MBRs and cell encapsulation techniques to tackle issues in bioconversion and fermentation of complex feeds. The rMBR applies high local cell density and membrane separation of cell/feed to the conventional immersed membrane bioreactor (iMBR) set up. Moreover, this new membrane configuration functions on basis of concentration-driven diffusion rather than pressure-driven convection previously used in conventional MBRs. These new features bring along the exceptional ability of rMBRs in aiding complex bioconversion and fermentation feeds containing high concentrations of inhibitory compounds, a variety of sugar sources and high suspended solid content. In the current review, the similarities and differences between the rMBR and conventional MBRs and cell encapsulation regarding advantages, disadvantages, principles and applications for biofuel production are presented and compared. Moreover, the potential of rMBRs in bioconversion of specific complex substrates of interest such as lignocellulosic hydrolysate is thoroughly studied.

Keywords: Membrane bioreactor, Reverse membrane bioreactor, Diffusion, Bioconversion, Inhibitory compounds, Suspended solid, Fouling, Biofilm

1. Introduction

Membranes and membrane related technologies have now been around for long, attracting the most attention in wastewater treatment and water quality improvement technologies (Lin et al., 2012; Peters,
The centre of focus throughout this review is membrane-assisted cell retention. This technique uses a selective synthetic membrane to retain cells and specific chemical compounds in the bioreactor while allowing some low molecular weight solutes (depending on membrane properties) to diffuse freely through the membrane (Tampion and Tampion, 1987). Membrane applications are generally based on the ability of the membrane to efficiently separate different compounds and/or cells/particles, being selectively permeable to some substances while retaining others. In this context compounds are divided in two groups, i.e. the compounds that pass through the membrane end up in permeate (also called filtrate), the ones that are retained in the retentate. The selective behaviour of different membranes originates from membrane pore size and morphology, and other characteristics such as membrane charge, affinity or hydrophobicity (Judd and Judd, 2011). Membrane separation mainly occurs through application of pressure and/or concentration gradient as the separation driving force over the membrane (Judd and Judd, 2011) (Fig. 1). This is a criterion for categorizing membrane systems on basis of the separation driving force into pressure or diffusion (concentration gradient) driven.

In biological processes where membranes are integrated with the main bioreactor either for filtration, product recovery, or cell separation or retention, the MBR configuration plays a determining role. As mentioned by Judd and Judd (2011), MBR configuration covers both the integration of the membrane with the bioreactor and also the set-up of the membrane module in relation with the bioreactor. In general the configuration of various conventional MBRs sits under one of the two categories of immersed (iMBR), also known as submerged MBR, and side-stream (external loop) sMBR (Fig. 2). The submerged membrane module in iMBRs can be submerged either in the bioreactor or in a separate compartment connected to the main reactor through an external loop (Carstensen et al., 2012; Judd and Judd, 2011). Considering system energy balance, in comparison to sMBRs, iMBRs are more energy-saving as the module is placed in the bioreactor. In contrast, the sMBR set-up requires pumping of great medium volumes through an external membrane module housing in a cross-flow filtration system (Hai et al., 2013; Radjenović et al., 2008). Profound reviews of MBR principles and applications and also the differences between iMBR and sMBR in performance, operation and application are well covered in reviews by Carstensen et al. (2012), Ylitervo et al. (2013a), Judd and Judd (2011) and (Judd, 2008).

Regarding cell positioning in conventional MBRs, in iMBRs cells are kept inside the main bioreactor in a mixture with the feed medium, while in sMBRs cells are pumped through the external membrane module and then recirculated back to the main bioreactor. The ability of MBRs in retaining high cell concentrations in the bioreactor facilitates the in situ product recovery in biofuel production (Carstensen et al., 2012; Ylitervo et al., 2013a). Several examples of final cell concentrations (cell biomass) achieved by applying different MBRs for bioethanol production are presented in Table 1.

Conventionally, both sMBR and iMBR processes work based on the application of pressure difference (over-pressure or under-pressure). These pressure driven MBRs have long been in use for a wide range of applications from wastewater treatment to ethanol fermentation (Carstensen et al., 2012; Judd and Judd, 2011; Ylitervo et al., 2013a; Yoon, 2015). In the sMBRs filtration or product recovery happens through pumping the cultivation medium over and parallel to the membrane surface through a membrane compartment/unit, where permeate is withdrawn, set in an external loop to the main bioreactor (Carstensen et al., 2012). On the other hand, iMBRs have the membrane module
immerged in the main bioreactor or an external compartment having the filtration or metabolite/product recovery in place by application of under-pressure. The immersed and side-stream configurations have been long taken into practice in various continuous, fed batch and batch fermentation processes (Carstensen et al., 2012; Judd and Judd, 2011). In order to have an overview of MBR assisted bioprocesses, extended examples of the applications of iMBR and sMBR in ethanol fermentation processes taking into account the type of microorganism and substrate used and final retained biomass content are presented in Table 1. However, biofuel production through bioconversion of complex substrates containing inhibitory compounds, high suspended solid (SS) content and several different sugar sources by means of the new rMBRs technology is yet to be explored.

1.2. Cell encapsulation

In order to benefit from a high bioconversion rate and productivity, in addition to optimisation of the process conditions such as pH and temperature, maintaining a high cell density in the bioreactor is of great importance (Westman and Franzén, 2015). In this regard, various approaches of natural (e.g. flocculation) and artificial cell immobilization (e.g. cell encapsulation and the application of MBRs) have been taken into consideration in order to have enhanced productivity and maintain high cell concentration in the bioreactor while increasing the substrate feeding rate. Cell immobilization can happen by natural cell immobilization through which cells tend to form flocs and start to settle or float in the bioreactor. In addition, cells can also be artificially
### Table 1
An overview of the ethanol fermentation processes involving submerged or external-loop MBRs.

<table>
<thead>
<tr>
<th>Membrane bioreactor design</th>
<th>Membrane configuration/quality</th>
<th>Pore size/MWC</th>
<th>Microorganism</th>
<th>Biomass (g/l)</th>
<th>Productivity/product concentration</th>
<th>Working volume or reactor size (l)</th>
<th>Medium (g/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>External cross-flow</td>
<td>Polyamide</td>
<td>0.02 μm</td>
<td>Zymomonas mobilis ATCC4126</td>
<td>40</td>
<td>120–200 g/l.h</td>
<td>–</td>
<td>Glucose 120–150</td>
<td>Lee et al. (1980)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Short hollow fibre cartridge</td>
<td>50,000 MWC</td>
<td>Kluyveromyces fragilis NRRL 2415</td>
<td>90</td>
<td>240 g/l.h</td>
<td>0.5–3.1</td>
<td>Lactose 150</td>
<td>Cheryan and Mehaia (1983)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Flat cellulose acetate sheet</td>
<td>20,000 MWC</td>
<td>Saccharomyces cerevisiae H1022</td>
<td>90</td>
<td>44 g/l.h</td>
<td>2.51</td>
<td>Glucose 182</td>
<td>Hoffmann et al. (1985)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Carbon coated zirconium oxide</td>
<td>0.14 μm</td>
<td>Saccharomyces cerevisiae 7013</td>
<td>300</td>
<td>33 g/l.h</td>
<td>2.41</td>
<td>Glucose 150</td>
<td>Lafforgue et al. (1987)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Flat (Pellicon-cassetts Millipore)</td>
<td>0.2 μm</td>
<td>Zymomonas mobilis ZM6</td>
<td>18.5</td>
<td>49.5 g/l.h</td>
<td>3.51</td>
<td>Glucose 100</td>
<td>Hoffmann et al. (1988)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Capillary (Enka HB 3355E)</td>
<td>0.2 μm, 0.3 μm</td>
<td>Zymomonas mobilis ZM4</td>
<td>75 g/l</td>
<td>5 l</td>
<td></td>
<td>Glucose 100</td>
<td>Chun and Rogers (1988)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Hollow fibre polysulfone</td>
<td>30,000 MWC</td>
<td>Saccharomyces cerevisiae (ATCC 31821)</td>
<td>160</td>
<td>31 g/l.h</td>
<td>0.25–0.5</td>
<td>Lactose 64</td>
<td>Mehaia and Cheryan (1990)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Tubular polyphenyleneptialamide</td>
<td>25,000 MWC</td>
<td>Saccharomyces cerevisiae SL100</td>
<td>10–60</td>
<td>15 g/l.h</td>
<td>0.15–0.33</td>
<td>Sucrose 50–300</td>
<td>Melzoch et al. (1991)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Ceramic</td>
<td>0.45 μm</td>
<td>Kluyveromyces marxianus Y-113</td>
<td>109</td>
<td>6.2 g/l.h</td>
<td>0.75</td>
<td>Lactose 64</td>
<td>Tin and Mawson (1993)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Multi-tubular ceramic</td>
<td>0.05 μm</td>
<td>Saccharomyces cerevisiae</td>
<td>33.2 g/l</td>
<td>3</td>
<td></td>
<td>Molasses</td>
<td>Kaseno and Kokugan (1997)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Ceramic</td>
<td>–</td>
<td>Saccharomyces cerevisiae</td>
<td>11.5 g/l</td>
<td>7000</td>
<td></td>
<td>Corn starch hydrolysate</td>
<td>Escobar et al. (2001)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Durapore filter (Millipore)</td>
<td>0.45 μm</td>
<td>Saccharomyces cerevisiae (ATCC 9658)</td>
<td>12</td>
<td>–</td>
<td>–</td>
<td>Corn steep liquor</td>
<td>Linden et al. (1999)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Tubular ceramic (Orelis Rhodia TAMi)</td>
<td>150,000 MWC</td>
<td>Saccharomyces cerevisiae CBS 8066</td>
<td>59–156.89</td>
<td>41–65 g/l</td>
<td>1.5–4.5</td>
<td>Glucose 500</td>
<td>Ben Chaabane et al. (2006)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Tubular polyethylene (PE)</td>
<td>0.2 μm</td>
<td>Saccharomyces cerevisiae CCLC 53310</td>
<td>6.4</td>
<td>31.1 g/l</td>
<td>0.3</td>
<td>Softwood hydrolysate</td>
<td>Ishola et al. (2013b)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Sartorius</td>
<td>0.45 μm</td>
<td>Saccharomyces cerevisiae NCIM 3090</td>
<td>–20.1</td>
<td>55 g/l</td>
<td>5</td>
<td>Rice straw hydrolysate</td>
<td>Zahed et al. (2015)</td>
</tr>
<tr>
<td>External cross-flow</td>
<td>Polyethylene (PE) (Microdyn-Nadir)</td>
<td>0.2 μm</td>
<td>Saccharomyces cerevisiae T 0936</td>
<td>6</td>
<td>35 g/l</td>
<td>0.6</td>
<td>Wheat straw hydrolysate slurry</td>
<td>Ishola et al. (2015b)</td>
</tr>
<tr>
<td>External (PF)</td>
<td>Composite plate polydimethylsilicon</td>
<td>–</td>
<td>Saccharomyces cerevisiae (4.49–5.37)</td>
<td>0.692–1.90 g/l.h</td>
<td>4</td>
<td></td>
<td>Glucose 20–70</td>
<td>Ding et al. (2012)</td>
</tr>
</tbody>
</table>

(continued on next page)
## Table 1 (continued)

<table>
<thead>
<tr>
<th>Membrane bioreactor design</th>
<th>Membrane configuration/quality</th>
<th>Pore size/MWC</th>
<th>Microorganism</th>
<th>Biomass (g/l)</th>
<th>Productivity/product concentration</th>
<th>Working volume or reactor size (l)</th>
<th>Medium (g/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>External (PF) Poly dimethylsiloxane</td>
<td>20,000 MWC</td>
<td>Alcohol active dry yeast (ADY)</td>
<td>15.8</td>
<td>1.51 g/l.h</td>
<td>5</td>
<td>Glucose 43</td>
<td>Chen et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>Submerged Ceramic Al₂O₃</td>
<td>0.3 μm</td>
<td>Saccharomyces cerevisiae</td>
<td>58</td>
<td>13 g/l</td>
<td>1.5</td>
<td>Glucose 100</td>
<td>Park and Kim (1985)</td>
<td></td>
</tr>
<tr>
<td>Submerged Stainless steel tubes</td>
<td>2, 10 μm</td>
<td>Saccharomyces cerevisiae ATCC 244858</td>
<td>50–150</td>
<td>20 g/l.h</td>
<td>1</td>
<td>Glucose 100</td>
<td>Chang et al. (1993)</td>
<td></td>
</tr>
<tr>
<td>Submerged Ceramic Al₂O₃</td>
<td>5 μm</td>
<td>Saccharomyces cerevisiae</td>
<td>207</td>
<td>1–4 g/l.h</td>
<td>2</td>
<td>–</td>
<td>Suzuki et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Submerged Stainless steel tubes</td>
<td>2 μm</td>
<td>Saccharomyces cerevisiae</td>
<td>42</td>
<td>17 g/l.h</td>
<td>1.8</td>
<td>Tapioca hydrolysate</td>
<td>Lee et al. (1994)</td>
<td></td>
</tr>
<tr>
<td>Submerged Asymmetric ceramic α-Al₂O₃/β-Al₂O₃</td>
<td>0.005/0.3 μm</td>
<td>Saccharomyces cerevisiae ATCC 244858</td>
<td>0–60</td>
<td>40–61 g/l</td>
<td>2</td>
<td>–</td>
<td>Zhang et al. (1998b)</td>
<td></td>
</tr>
<tr>
<td>Submerged Ceramic Al₂O₃</td>
<td>0.2 μm</td>
<td>Saccharomyces cerevisiae K 901</td>
<td>236</td>
<td>13.1 g/l.h</td>
<td>1.5</td>
<td>Glucose 100</td>
<td>Otashi et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>Submerged Ceramic tube</td>
<td>0.3 μm</td>
<td>Saccharomyces cerevisiae</td>
<td>1.5 × 10⁸ cell/ml</td>
<td>16.9 g/l.h</td>
<td>1.5</td>
<td>Lignocellulosic hydrolysate</td>
<td>Lee et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>Submerged Fluoro polymer</td>
<td>2 μm</td>
<td>Saccharomyces cerevisiae</td>
<td>15</td>
<td>–</td>
<td>0.14</td>
<td>Molasses</td>
<td>Thiovander (2012)</td>
<td></td>
</tr>
<tr>
<td>Submerged Integrated Permeate Channel (IPC) (Vito NV)</td>
<td>0.3 μm</td>
<td>Saccharomyces cerevisiae CBS 8066</td>
<td>&gt;50</td>
<td>7.94 g/l.h</td>
<td>0.6</td>
<td>Lignocellulosic hydrolysate</td>
<td>Yiltevo et al. (2014)</td>
<td></td>
</tr>
<tr>
<td>Submerged Hollow fibre cartridge (ZeeWeed)</td>
<td>0.08–0.1 μm</td>
<td>Saccharomyces cerevisiae (Baker’s yeast)</td>
<td>30</td>
<td>62 g/l</td>
<td>1</td>
<td>Glucose 100–175</td>
<td>Radoaj and Dinosady (2014)</td>
<td></td>
</tr>
<tr>
<td>Submerged (PF) Hollow fibre Rubber Silicon Silicalite</td>
<td>–</td>
<td>Saccharomyces cerevisiae NRRL-Y-2034</td>
<td>8.10</td>
<td>7.3 g/l.h</td>
<td>0.42</td>
<td>Glucose 100</td>
<td>Cho and Hwang (1991)</td>
<td></td>
</tr>
<tr>
<td>Submerged (PF) Hollow fibre</td>
<td>–</td>
<td>Saccharomyces cerevisiae ~14.6</td>
<td>11.4 g/l.h</td>
<td>–</td>
<td>–</td>
<td>Glucose</td>
<td>Kargupta et al. (1998)</td>
<td></td>
</tr>
<tr>
<td>Submerged (PF) Silicalite-Silicon Rubber</td>
<td>–</td>
<td>Saccharomyces cerevisiae (Baker’s yeast)</td>
<td>10</td>
<td>–</td>
<td>0.15</td>
<td>Glucose 200</td>
<td>Iekami et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>Hollow fibre fermenter Poly sulfone</td>
<td>10,000 MWC</td>
<td>Saccharomyces cerevisiae ATCC 4126</td>
<td>3.5 × 10⁹–10¹⁰ cell/ml</td>
<td>17–26 g/l.h</td>
<td>–</td>
<td>Glucose 120–150</td>
<td>Inloes et al. (1983)</td>
<td></td>
</tr>
<tr>
<td>Hollow fibre fermenter Hollow fibre (Romicon)</td>
<td>50,000 MWC</td>
<td>Saccharomyces cerevisiae NRRL-Y-132</td>
<td>260</td>
<td>17 g/l.h</td>
<td>0.625</td>
<td>Glucose 100</td>
<td>Mehaia and Cheryan (1984)</td>
<td></td>
</tr>
<tr>
<td>Hollow fibre fermenter Poly sulfone</td>
<td>10,000 MWC</td>
<td>Saccharomyces cerevisiae ATCC 4126</td>
<td>–</td>
<td>133 g/l.h</td>
<td>–</td>
<td>Glucose 100</td>
<td>Inloes et al. (1985)</td>
<td></td>
</tr>
<tr>
<td>Hollow fibre fermenter Polyamide</td>
<td>10,000 MWC</td>
<td>Saccharomyces cerevisiae ATCC 244858</td>
<td>6</td>
<td>10 g/l.h</td>
<td>0.04</td>
<td>Glucose 100</td>
<td>Park and Kim (1985)</td>
<td></td>
</tr>
<tr>
<td>Hollow fibre fermenter Polypropylene Celgard X-20</td>
<td>–</td>
<td>Saccharomyces cerevisiae NRRL-Y-132</td>
<td>100–300</td>
<td>31.6 g/l</td>
<td>0.075</td>
<td>Glucose 300</td>
<td>Kang et al. (1990)</td>
<td></td>
</tr>
</tbody>
</table>

PF: pervaporation fermentation, MWC: molecular weight cut-off.
immobilized either through entrapment in gel matrices (gel capsules or beads) or retention in MBRs, generally referred as cell encapsulation (Fig. 3) (Tampion and Tampion, 1987). The main compounds involved in gel formation are agar, alginites, collagen, kappa-carrageenan, agarose, chitosan and polyacrylamide (Tampion and Tampion, 1987). One of the highlighted cell immobilization techniques that provides bioconversion and fermentation processes with exceptional functional features is cell encapsulation (Westman et al., 2012a, 2012b, 2014a). The principal aim of cell encapsulation is to provide very high local cell concentration within a capsule. This high local cell concentration and the substrate concentration gradient present in capsules, from the capsule surface to the core, give the entrapped cells the ability to co-consume different substrates. This is also followed by extraordinary performance of encapsulated cells in medium inhibitor tolerance and detoxification (Westman et al., 2012a, 2012b). The dominant mass transfer method in cell capsules is diffusion as direct convection does not apply inside the capsules. The diffusion behaviour of different compounds also contributes to the superior characteristics observed through fermentation using encapsulated cells (Westman et al., 2012a, 2012b). The principle of cell encapsulation, that is providing high local cell density through cell confinement by an external membrane while having diffusion as the dominant mass transfer mode, is used as the backbone of the rMBR system.

1.3. Challenges with MBRs and cell encapsulation

Diverse techniques of cell retention and immobilization such as membrane cell recycling and retention, and cell immobilization through encapsulation and flocculation have been applied in bioreactors aiming mainly at obtaining higher productivity and bioconversion rates, and benefiting from the ease of product separation from cells and cell reuse (Carstensen et al., 2012; Westman et al., 2012b). As high cell concentration is achieved and cell washout prevention is assured through the application of MBRs, the bioreactor can run in continuous mode at high dilution rate and low hydraulic retention time. However, successful membrane-assisted cell retention and/or cell immobilization by cell encapsulation does not always guarantee a successful fermentation.

It is to be considered that the condition of the utilized feed (substrate) also determines the outcome of the MBR bioconversion process. For example fermentation of media containing inhibitory compounds (furan aldehydes, carboxylic acids, etc.) can cause problems since toxic compounds may affect the microorganism’s physiological and metabolic activity in a negative way. Utilization of high suspended solids (SS) viscous substrates and feed streams containing different types of sugars (pentoses, hexoses, etc.) is still a great hurdle (Klinke et al., 2004). Conventional MBRs lack the potential to positively enhance the cell inhibitor detoxification ability and simultaneous sugar utilization potential of cells. Although high cell concentration is provided in the MBR, cells are suspended in the main reactor and exposed to uncontrolled concentrations of toxic compounds and various sugar sources. The above mentioned issues are unfavourably confronted when the purpose is to produce second generation ethanol from lignocellulosic materials (Bertilsson et al., 2008; Klinke et al., 2004; Laluce et al., 2012). Due to their recalcitrant structure comprised of lignin, hemicellulose and cellulose, lignocellulosic materials show great resistance to enzymatic hydrolysis (Taherzadeh and Karimi, 2008). Therefore, to have the sugars released and prepared for bioconversion by the microorganism the lignocellulosic materials should be pre-treated (Taherzadeh and Karimi, 2008). The pre-treatment stage is where inhibitory and toxic compound such as furan aldehydes, phenolic compounds and carboxylic acids are produced (Almeida et al., 2007; Klinke et al., 2004; Taherzadeh et al., 1997; Taherzadeh and Karimi, 2008; Zaldivar et al., 2001) hindering fermentation. These inhibitory compounds suppress ethanol fermentation by increasing the lag phase, decreasing cell viability, stopping bioconversion by inhibition of catabolic enzymes, decreasing intracellular pH, disturbing cell membrane integrity, etc. when directly in contact with freely suspended cells in the medium (Almeida et al., 2007). Moreover, saccharides released by means of lignocellulosic pre-treatment consist of pentoses (xylose, arabinose, etc.) and hexoses (glucose, mannose, galactose, etc.) extracted mainly from hemicellulose and cellulose respectively, with the extent depending on the type of lignocellulosic source (softwood, hardwood, etc.) (Taherzadeh and Karimi, 2008). The actual problem occurs as wild ethanol fermenting microorganisms have poor performance in co-consumption of hexose and pentose sugars. This results in initial hexose utilization followed by pentose consumption once the hexose is depleted (Sánchez Nogué and Karhumaa, 2015; Stanley and Hahn-Hägerdal, 2010b). In general, due to the above mentioned factors, the bioconversion rate of lignocellulosic substrates to ethanol is...
the novel rMBR process cells are encased between synthetic membrane layers that were prepared separately and then inoculated with growed Saccharomyces cerevisiae.

Another obstacle requiring engineering solutions is the high suspended solid content of specific fermentation feeds such as lignocellulosic hydrolysate. Lignocellulosic material, depending on the plant species (ex. softwood and hardwood), contains different percentages of lignin (10–35%) (Zhao et al., 2012). Lignocellulose pre-treatment processes that aid sugar release and also enhance enzymatic accessibility of the lignocellulosic raw material result in production of lignin residues with non-fermentable polymeric compounds (Zhao et al., 2012). These residual lignin particles bring along several problems. Accumulation of residual lignin in batches adds to the medium viscosity of the lignocellulosic hydrolysate slurry due to high suspended solid content (Verardi et al., 2012; Zhao et al., 2012). It has been claimed that up to 30% w/w solids loading in the pre-treated hydrolysate is required to guarantee an acceptable ethanol concentration (4–5 wt%). However, rising the solid loading in a hydrolysis and fermentation process increases the viscosity of the medium causing mass transfer limitations in pressure driven MBRs deteriorating membrane performance by cake layer formation and consequently MBR failure (Section 2.3). Moreover, high SS concentration hinders enzymatic performance, increasing the inhibitory effects of intermediate compounds and decreasing the ease of mixing of the broth (homogeneity) (Sassner et al., 2006; Verardi et al., 2012). In addition, in case cell reuse is pursued for consecutive batch processes, this increase in solid residues increases the number of stages and cost of downstream processes for the separation of cells from solids both in MBR and cell encapsulation systems. When it comes to MBR fermentation the SS level of the feed is of critical importance as it may contribute to the membrane fouling propensity. As presented in the work by Liu et al. (2015) high SS broth with elevated viscosity reduces the effect of the shear stress induced by air bubbles on the surface of the membrane required for fouling prevention. Furthermore, cake layer formation by solid particles is exacerbated as the SS content increases (Judd and Judd, 2011). Adding to that is the solid residual accumulation in the bioreactor if the process is to be run for repeated batches (Galbe and Zacchi, 2002). In the cross-flow sMBRs, in addition to cake layer formation, a high viscosity increases the energy required for pumping the fermentation broth from the bioreactor through the membrane module in a closed loop (Ishola et al., 2013b). However, an increase in viscosity can be advantageous in special cases. Ishola et al. (2013a) proved that increasing the SS loading from 8 to 12% in simultaneous saccharification and fermentation (SSF) of a non-sterile lignocellulosic hydrolysate reduced the bacterial contamination activity and increased the ethanol yield.

2. Reverse membrane bioreactors (rMBR): principles and applications

The reverse membrane bioreactor (rMBR) is a recently introduced novel immersed membrane configuration used for production of biofuels such as methane and ethanol (Ishola et al., 2015a; Youngsukkasem et al., 2015). The principal difference between the conventional iMBR and the rMBR process is that, in the latter the cells are immobilized in between membrane layers (Fig. 4b), separated from the actual feed medium, whereas for the conventional iMBR the cells are suspended in the medium in direct contact with the feed (Fig. 4a). In the novel rMBR process cells are encased between synthetic
membrane layers in the form of membrane sachets, compact multi-layer membrane columns, Integrated permeate channel (IPC) flat sheet membranes (Doyen et al., 2010) and other membrane encapsulation configurations (Fig. 5). General differences between rMBRs and conventional MBRs are presented in Table 2.

In contrast to pressure driven submerged and side-stream MBR processes, in rMBR, active liquid permeation has been replaced by substrate diffusion through the membrane to the cell side and in the opposite direction for the metabolic products. In rMBRs, the synthetic membrane plays a similar role as the plant cells membrane, separating the membrane confined medium (cytoplasm in plant cells) and cell interior components (Golgi, mitochondrion, nucleus, etc. in plant cells) from the surrounding medium, only letting specific nutrients to pass through due to the concentration gradient over the membrane. Moreover, in rMBRs a synthetic membrane plays the same role as the membrane capsule in encapsulation. A thorough analysis of the similarities in principles and functions of cell encapsulation and rMBR is provided in Sections 2.1 and 2.2. Membrane compartments housing microorganisms in rMBRs could be suspended (floating) in the reactor as closed membrane sachets (Youngsukkasem et al., 2013a), or be fixed in place such as compact multi-layer membrane columns (Youngsukkasem et al., 2013b) or flat sheet membrane panel (Ishola et al., 2015a) (Fig. 5). In order to benefit from rapid bio-methanation of syngas and co-digestion of syngas and organic substances, Youngsukkasem et al. (2015) successfully used closed sachets made of flat plain PVDF (polyvinylidene fluoride) membrane sheets to entrap methanogenic bacteria in an rMBR. Syngas is a gas mixture mainly composed of carbon dioxide, carbon monoxide and hydrogen formerly made from controlled combustion of coal, biomass, etc. in presence of steam that can be used as chemical precursor for other chemical processes. In this experiment the sludge encased in the sachets was capable of converting the fed syngas comprised of H₂, CO₂ and CO into biogas in a short retention time of 1 day. It was reported in the study that thermophilic conditions (55 °C) and co-digestion using membrane sachets contributed to higher methane yield (Youngsukkasem et al., 2015). In another attempt, the effect of organic loading rate (OLR) on the performance of methanogenic freely suspended and encased (in sachet) bacteria was examined (Youngsukkasem et al., 2013a). It was observed that the bacteria entrapped in sachets were still viable and active at an OLR of 15 g COD/L.day⁻¹, whereas at a loading rate of 7.5 g COD/L.day⁻¹ the system including freely suspended cells totally failed. Also membrane sachets (Fig. 5a) used in rMBR configuration prevented specific inhibitory

### Table 2

<table>
<thead>
<tr>
<th>Configuration(s)</th>
<th>Main separation driving force</th>
<th>Mass transfer mechanism(s)</th>
<th>Cell/feed medium</th>
<th>Cell positioning</th>
<th>Product recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional MBRs</td>
<td>Immersed or external cross-flow</td>
<td>Pressure gradient</td>
<td>Convection and diffusion</td>
<td>Cells mixed with the feed medium</td>
<td>Freely suspended in the reactor shell side</td>
</tr>
<tr>
<td>rMBRs</td>
<td>Immersed</td>
<td>Concentration gradient</td>
<td>Diffusion</td>
<td>Cells separate from the feed medium</td>
<td>Encased between membrane layers</td>
</tr>
</tbody>
</table>

![Fig. 5. Different rMBR setups used for production of biogas: a) membrane sachet bioreactor, b) fixed compact multi-layer membrane column bioreactor, and ethanol: c) IPC membrane panels.](image-url)
compounds to come in contact with the acting microorganism (Wikandari et al., 2014). This effect also applies to the hydrophilic capsule membrane in cell encapsulation (Pourbafrani et al., 2007a). As studied by Wikandari et al. (2014), in the case of β-limonenone, known as a potential inhibitor for the bio-methanation process, hydrophilic PVDF membrane saclets were impermeable to the naturally hydrophobic β-limonene, while glucose and volatile fatty acids present in the medium penetrated through the membrane layer to the cell side. Robust methane production in an inhibitory medium by cells encased in membrane saclets in an rMBR has also been experienced by Youngsukkasem et al. (2013a) and the results were compared with that of freely suspended cells.

Another newly developed rMBR configuration for biogas production is the compact multi-layer membrane column (Fig. 5b). In this concept, a number of double layer steel fixtures including membranes on both faces, are packed and submerged in a column bioreactor. The liquid medium, either as the substrate or as the carrier of syrags or biogas (methane and carbon dioxide), flows upward through the column, and allows the exchange of the substrates and products along the membrane surface of the membrane packs housing the microorganism. This type of rMBR was used for biogas production by Youngsukkasem et al. (2013b), where they examined the performance of different membranes of hydrophobic polyamide 46 (PA), hydroxyethylated polyamide 46 (HPA) and PVDF regarding cell retention and diffusive transfer of compounds.

It is noteworthy that in the above mentioned biogas production setups (Fig. 5a and b) a semi-rMBR system exists as the feed is being recirculated in the bioreactor. In these conditions there is liquid and/or gas feed flow over the exterior surface of the membrane saclets and packed layer. Therefore, mass transfer on the feed side is assisted by convection and also diffusion in a thin stagnant liquid layer on the surface of the membrane. However, on the interior surface of the membrane (cell side) it is only diffusion that dominates the transfer of compounds from the membrane to cells and in the opposite direction for the products.

Regarding bioethanol production, in a recent research work Ishola et al. (2015a) used integrated permeate channel (IPC) flat sheet membrane panels (Doyen et al., 2010) for housing recombinant S. cerevisiae cells in an rMBR set up (Fig. 5c). In this regard, the functionality of the rMBR technique was evaluated in bioethanol fermentation from the liq-uid fraction of wheat straw hydrolysate containing different sugar sources and inhibitory compounds. It was reported that the IPC rMBR configuration had a significant positive effect of simultaneous utilization of xylose and glucose, and in situ detoxification of furfural and hydroxy- methyl furfural (HMF). The result indicated complete consumption of glucose and 87% utilization of xylose by the yeast leading to an ethanol yield of 83% of the theoretical yield. Although biogas production by means of different rMBRs has recently been a focus of exploration, the application of rMBR in bioethanol production is relatively an undiscovered area of research yet to be explored.

The scientifically and technologically newly developed rMBR concept, representing membrane bioreactors performing on basis of diffusion with cells encased in between membrane layers, has a great potential to be experimentally explored for biosynthesis of biochemical (organic acids, propanol, butanol, etc.) and biofuels (bioethanol, biogas and biodiesel) form feed streams with complex mixture of sugars and inhibitors such as lignocellulosic material, citrus waste and other carbohydrate-rich waste streams. In the following Sections 2.1–2.3, the principles of rMBR and its applications in increasing cell inhibitor tolerance, simultaneous consumption of different sugar sources and dealing with high suspended solid feeds are elaborated in details. Also the advantages and shortcomings of rMBRs in comparison with the conventional MBRs, cell encapsulation and flocculation are thoroughly studied.

2.1. Inhibitor tolerance

One of the potential areas where rMBR technology can be effectively applied is the bioconversion of inhibitory feed streams. In this section the mechanism by which the rMBR set up can improve the cells inhibitor tolerance and in situ detoxification is discussed in details. Among the main issues confronted during the fermentation of complex substrates such as lignocellulosic hydrolysate and citrus waste to biofuels, specifically bioethanol, is the presence of inhibitory compounds in the medium. These inhibitors are either convertible through in situ detoxification (ex. furan aldehydes) or non-convertible under anaerobic condition (ex. phenolic compounds) by the acting microorganism(s). The main industrially used microorganism for ethanol fermentation is the yeast Saccharomyces cerevisiae. S. cerevisiae has long been the microorganism of choice in ethanol production industries as it has high capacity of ethanol fermentation along with high ethanol tolerance (Casey and Ingledew, 1986; Ghareib et al., 1988; Stanley et al., 2010a; You et al., 2003). Moreover, some strains of yeast are capable of in situ detoxification and conversion of some inhibitory compounds such as 5-hydroxymethyl furfural (HMF) and furfural into less toxic chemicals such as furfuryl alcohol (Li et al., 2009; Liu, 2011; Tian et al., 2011). However, high inhibitory level in the feed and low cell density in the bioreactor decreases the success level of in situ detoxification. A well-developed bioreactor housing high yeast cell concentration and low toxicity level of the feed stream has great propensity to conduct fermentation along with in situ detoxification. In order to provide the mentioned condition during fermentation different remedies can be taken into practice e.g.; using a MBR (Yitetro et al., 2013b) or cell encapsulation (Westman et al., 2012a) for higher cell density, running fermentations in fed-batch mode to control the inhibitory level (Taherzadeh et al., 1999), engineering of recombinant cells with higher inhibitor tolerance and ability to convert inhibitors (Koppram et al., 2012).

Encapsulated yeast can effectively ferment inhibitor-containing lignocellulosic hydrolysates that are considered extremely toxic for a freely suspended cell system (Talebnia and Taherzadeh, 2006). Cell encapsulation provides high local cell concentration that increases the inhibitor tolerance of the cells as claimed by Westman et al. (2012a). The rMBR by retaining cells in a confined space between membrane surfaces simulate that of encapsulation condition (Ishola et al., 2015a). There are several different mechanisms contributing to better in situ detoxification and higher inhibitor tolerance by encapsulated yeast or by cells retained in an rMBR. These remedial mechanisms owe their increased inhibitor tolerance to manipulated cell stress and mass transfer patterns in the cell proximity due to high local density.

Cells subjected to encapsulation or retained in an rMBR, are intentionally kept in a limited space for the sake of high local cell density. The yeast cell concentration in capsules can increase up to 309 g/l of capsule volume (Cheong et al., 1993). As a result of high local cell density, these cells experience stress inducers such as low nutrition level or nutrition starvation. Cells located deeper into the cell aggregate are exposed to a lower level of nutrients such as glucose that is required as carbon and energy source due to mass transfer and diffusion limitations. In addition, it has been reported that the stress implied during encapsulation is accompanied by counter stress responses by the cells through expression of stress related genes (Klinke et al., 2004; Taherzadeh and Karimi, 2008). It has been observed by Sun et al. (2007) and Talebnia and Taherzadeh (2007) that the level of trehalose, involved in cell stress tolerance, rises in encapsulated cells. This initial stress response induces a protective effect and gives the cells the ability to withstand the upcoming stress by the inhibitory compounds. In a work by Yitetro et al. (2011) it has been proven that this initial stress response could also help cells to better withstand thermal stresses. This reasoning supports the idea that the inhibitor tolerance does not necessary occur in cell encapsulation by the capsule membrane but with cells tightly kept together (Westman et al., 2014b). The above put forward discussion forms the basics of cell inhibitor tolerance in rMBR cell confinement (Fig. 6b).

Other techniques of cell immobilization that pursue the goal of keeping cells together such as flocculation also result in an increased inhibitor tolerance. However, in the case of flocculation, high sugar content of the medium, for example in batch fermentations, blocks flocculation.
When the lectin-like cell wall proteins (flocculins) that have the mission to attach to carbohydrate residues (e.g. mannose) on the cell wall of neighbouring cells are blocked by the saccharides present in the feed, flocculation does not occur until the end of fermentation. For example for mannase there are both reports on flocculation inhibition and enhancement (Maril and Vranes, 2007; Westman et al., 2014b). This is also considered as a benefit in breweries as it gives better product recovery efficiency by having yeast coagulated in flocs. It is noteworthy that ordinary lab yeast strains are poor or non-flocculants due to defective or missing genes required for flocculation. Although strong flocculation is accompanied by mass transfer resistance and slower glucose consumption in non-inhibitory medium compared to free suspended cells, strong flocculation in the inhibitor containing medium helps withstanding higher ethanol and toxic compound level and also faster glucose and mannose consumption compared to suspended cells. Westman et al. (2014b) studied ethanol fermentation through bioconversion of spruce dilute acid lignocellulosic hydrolysate by three recombinant mutants of S. cerevisiae having different flocculation strength, all being originated from the strain CEN.PK 113-7D, and the performance was compared with free suspended cells. It was reported that strong flocculation has the same effect as cell encapsulation when fermenting lignocellulosic hydrolysate containing different inhibitors (Westman et al., 2014b).

In addition to starvation stress, an inhibitor concentration gradient in the cell aggregate from the surface cells (directly exposed to high content of inhibitor) to cells placed deeper into the cell cluster (exposed to sub-inhibitory concentrations), is the other mechanism contributing to enhanced inhibitor tolerance and process robustness. In capsules, flocs or MBR systems that contain cell clusters of several millimetres or more in thickness, diffusion limitation defines the concentration of different nutrients and toxic compounds that cells are exposed to. It has been reported that in flocks larger than 100 μm, mass transfer limitations lead to low biomass growth and ethanol production (Talebnia and Taherzadeh, 2007).

In these diffusion driven systems, cells closer to the capsule or floc surface or adjacent to the membrane surface in an MBR experience extremely harsh inhibitory conditions. In inhibitor containing mediums, the cells at the frontier get involved with the detoxification of readily convertible inhibitors such as furan aldehydes, and reduce the amount of inhibitors diffusing deep into the cell cluster leaving the cells in the interior unaffected and active for fermentation. In this condition, cells near the cluster centre benefit from the sugar sources that have not been consumed by the surface cells involved in detoxification (Westman et al., 2012a, 2014b). However, as the mass transfer barrier still exists, having diffusion as the dominant mechanism, a low rate of cell growth and fermentation occurs near the base/centre/core of the cell cluster. This slow growth near the centre has its own benefits. It has been proven that slow growing yeast has enhanced stress resistance that sequentially leads to increased inhibitor tolerance (Elliott and Putscher, 1993). This lower growth rate in the inner cell layers can also be justified as the limited amount of sugar that reaches deep into the cluster as utilized as maintenance energy rather than budding and growth (Westman et al., 2012a).

A schematic picture of the cell metabolic activity which depends on the cell location in the cluster and medium condition in an rMBR is presented in Fig. 6. As it can be seen in non-inhibitory fermentation medium, cells close to the membrane surface are involved in sugar consumption and cells placed further from the surface experience low nutrient concentrations. For the inhibitor containing medium, the cells at the surface have the role of inhibitor detoxification while cells deeper in the cluster have the opportunity of fermenting the present sugars.

The above mentioned remedies do not protect cells or increase the inhibitor tolerance to non-convertible inhibitors such as carboxylic acids and some phenolic compound present in lignocellulosic hydrolysate (Westman et al., 2012a). As presented by Vilela-Moura et al. (2011), in anaerobic fermentation the presence of glucose represses acetic acid metabolism. As predicted, the microorganism utilizes less glucose in media containing inhibitors than in non-inhibitory ones. This applies to both high local cell density systems such as encapsulation and freely suspended cells (Westman et al., 2012a).

On the other hand, in MBRs with freely suspended cells, although yeast cells are capable of detoxifying convertible inhibitors, fermentation robustness is not preserved. In this condition, inhibitor tolerance enhancing mechanisms are not active as all the cells in the medium are exposed to the same inhibitory compound with similar concentration at once (Westman et al., 2012a; Ylitervo et al., 2013b). This is due to better convectional mass transfer through stirring, gas purging or other agitation methods. Conventional submerged and external-loop MBRs provide desirable medium mixing (good mass transfer) in addition to retaining high concentrations of cells in the bioreactor. As experienced by Ylitervo et al. (2013b), up to 17 g/l of furfural was detoxified using high cell density of 180 g/l by means of cross-flow tubular sMBR. However, in fermentation conditions where cells are not retained (considerably low densities), there would be a long lag phase, low bioconversion rate or even in case of high inhibitor concentration, fermentation halts, until all convertible inhibitory compounds have been detoxified to less toxic ones. In addition, as suspended cells are constantly subjected to toxic medium e.g. lignocellulosic hydrolysate,
the length of time that cells can be reused is closely dependent on the level of toxicity and cell robustness (Talebnia et al., 2005). As investigated by Westman et al. (2012a) in an inhibitor containing defined medium with furan aldehydes and carboxylic acids the rate of glucose consumption in the initial stage of fermentation for freely suspended cells and encapsulated yeast cells were 40 and 80% of that of a non-inhibitory medium respectively. Taking the above mentioned reasoning into consideration, the rMBR is the preferred choice over the conventional sMBR and iMBR systems when it comes to biological conversion of inhibitory containing complex feeds such as lignocellulosic hydrolysate.

2.2. Simultaneous sugar consumption

The other main issue confronted in fermentation of complex feeds is the concomitant presence of different sugars for example pentoses (monosaccharides having five carbon atoms) and hexoses (monosaccharides with six carbon atoms). The problem arises from the point that the wild-type \textit{S. cerevisiae} strains either consume pentoses at a very low rate or do not utilize pentoses at all (Sánchez Nogue and Karhumaa, 2015; Van Zyl et al., 1989; Zaldívar et al., 2001). The prime remedy in this regard is having genetically manipulated recombinant \textit{S. cerevisiae} strains that are capable of pentose (xylose, arabinose, etc.) uptake (Sánchez Nogue and Karhumaa, 2015; Stanley and Hahn-Hägerdal, 2010b). However, due to the fact that there are no inherent pentose transporter proteins in the cell membrane, xylose and/or arabinose transportation to the intracellular space only happens if the concentration of hexoses (glucose, mannose, etc.) is low enough (Bertilsson et al., 2008; Hamacher et al., 2002; Meinaender et al., 1999; Sediak and Ho, 2004).

A breakthrough has been made by keeping cells in close proximity for example in the case of cell encapsulation and application of rMBR (Ishola et al., 2015a; Westman et al., 2014a). Cell encapsulation has proven to be a successful approach when co-utilization of sugars is sought. Westman et al. (2014a) have reported a 220-fold increase in xylose consumption rate, 50% more xylose uptake and 15% more ethanol production for encapsulated recombinant (genetically modified) \textit{S. cerevisiae} compared to the same condition with freely suspended cells. Encapsulated cells also showed 7% more ethanol production in a lignocellulosic hydrolysate medium (containing inhibitors) than the suspended cells. Moreover, Ishola et al. (2015a) experienced a successful co-utilization and fermentation of glucose and xylose present in lignocellulosic hydrolysate to 83% of the theoretical ethanol yield. This arises from the diffusive nature of mass transport in cell encapsulation and also other fermentation processes benefiting from high localized cell density such as rMBR. The diffusion pattern, somehow, has the same pattern as described for the inhibitor concentration gradient (Fig. 6b). In this condition when cells are packed together either in a capsule or encased in between membrane layers in a reverse membrane bioreactor, depending on the cells location from the surface of the capsule or inside synthetic membranes in an rMBR, are exposed to different nutrient levels and possess diverse cell physiology (Westman et al., 2012b). The cells close to the surface experience high hexose (specific glucose) concentrations, which is in the first priority of consumption for recombinant cells. In this condition pentoses (specific, xylose) diffuse through the cell layers to cells placed in deeper positions. Cells close to the core of the cell aggregate are in the state of glucose starvation, while xylose content is high in those regions. Therefore, these cells are involved with xylose fermentation as there is no or very little glucose present in the bioreactor exposed to both hexoses and pentoses simultaneously. In this condition, there is very low or no xylose consumption before glucose is completely depleted in the medium (Chandrakant and Bisaria, 1998). In case xylose is the only carbon source in the fermentation medium the rate of xylose consumption in both freely suspended cell system and encapsulated yeast cells is the same (Westman et al., 2014a; Westman and Franzén, 2015). The other issue to be dealt with, when considering co-utilization of sugars in freely suspended cell systems, is slow xylose uptake after total glucose depletion (Kupper et al., 2005; Westman et al., 2014a). The slow metabolism of xylose after full consumption of glucose in the medium may be due to lack of intermediary metabolites required for pentose metabolism and the related phosphate pathway, and severe redox imbalance (Ha et al., 2011). After glucose depletion cells have to quickly adapt themselves with a drop in NADPH and NAD+ due to anaerobic consumption of xylose (Ha et al., 2011). Shortly after total glucose consumption, xylose metabolism stops in suspended cell cultures (Westman et al., 2014a). However, this adaptation happens smoothly for cells in the inner layers of cell clusters in encapsulation and rMBR as they are exposed to xylose from the beginning of the fermentation process.

In addition to the put forward advantages of an rMBR system in simultaneous sugar consumption, the consequences accompanying the low pace of mass transfer should also be considered. In case of poor or slow sugar transfer through the microbial aggregate, starving cells near the centre of the aggregate go through the stationary and consequently death phase. In order alleviate such conditions, circulation of cells and the feed medium in- and outside the membrane package can be considered as an option. In order to have a pure diffusion dominated mass transfer the circulation pace should induce the same amount of pressure drop on both sides so that pressure equality conditions are applied.

2.3. Viscosity and suspended solid content

One of the main concerns in membrane filtration, membrane-assisted product recovery and in general membrane bioprocesses driven by pressure is to maintain a reasonable permeate flux through the membrane in order to benefit from high productivity of permeate or filtrate (Carstensen et al., 2012). However, membrane flux deterioration due to different fouling mechanisms such as cake layer formation and concentration polarization is a common phenomenon hindering the process efficiency (Park et al., 1997). Concentration polarization as defined by Judd and Judd (2011) is the concentration of rejected solute and precipitation of poorly soluble inorganic macromolecular compounds on the surface of the membrane, while cake formation is known as accumulation of rejected solids on the membrane. This may be exacerbated by reaching the critical flux or having changes in the medium condition, as in the case of increase in medium viscosity. The concept “critical flux” was introduced by Field et al. (1995) as the flux below which reduction in flux with time does not take place. Fluxes exceeding the critical flux lead to membrane fouling. In conventional MBRs, the flux of permeate through the membrane is negatively affected by the viscosity of the medium, in case all other factors such as transmembrane pressure (TMP) (the average of feed pressure minus permeate pressure) and membrane resistance are kept constant (Yoon, 2015). Additionally, the viscosity of the feed medium is in direct relationship with the biomass (cell) and solid content of the liquid medium and determines the fouling propensity, the flux through the membrane and the gas/air bubble size of pressure driven submerged and side-stream MBRS (Lee and Yeom, 2007; Wicaksana et al., 2006). As noted by Sarbatly and England (2004) for starch hydrolysis in sMBR, starch hydrolysis process the starch milk mass has to be kept at a weight concentration of 10% in order to control viscosity rise and prevent fouling. Furthermore, increase in the viscosity of the medium reduces the effectivity of shear stress induced by gas sparging on the fouling resistance (van den Brink et al., 2011). Itonaga et al. (2004) have concluded that the changes in viscosity level for feed water in MBR municipal wastewater treatment is marginal up to a certain suspended solid (SS)
The diffusive behaviour of mass transfer in rMBRs is directly affected by changes in the rheological properties of the medium. In diffusion driven rMBRs the suspended solid content of the medium is less problematic in comparison with the conventional pressure driven process as there is no need for a convective flux of compounds through the membrane. This property of rMBRs avoids membrane function deterioration due to SS related problems such as cake layer formation on the membrane surface. Therefore, increase in suspended solid content of the medium although detrimental to the diffusion rate of compounds in the feed medium, does not cause membrane fouling by cake layer formation. As rMBR bioconversion systems are strongly dependent on the diffusion of various solutes through the membrane and membrane to the cell side and in reverse for metabolic products, viscosity and the parameters influencing it such as working temperature and medium solid content require profound investigation.

The suspended solid content of the medium plays an important role in defining the viscosity of the medium (Hai et al., 2013). In pressure driven MBR systems there seems to be a complex relation between the fouling susceptibility and the SS concentration of the medium (Judd and Judd, 2011; Yoon, 2015). It has been reported in the literature involved with wastewater treatment that, the increase in the mixed liquor suspended solid (MLSS) can have negative, imperceptible or even positive (in cases with low initial SS content) impact on permeate flux (Chang and Kim, 2005; Çöcek et al., 1999; Hong et al., 2002; Le-Clech et al., 2003; Lesjean et al., 2005; Rosenberger et al., 2005; Yoon, 2015). Through practical experimentation, different methods of predicting the relation between the SS content and permeate flux through the membrane have been proposed. Most of these equations have specific conditions applied for deriving the equations leading to their limited applicability for all MBR systems as some parameters are considered in one and disregarded in other (Table 3) (Fang and Shi, 2005; Krauth and Staab, 1993; Sato and Ishii, 1991; Shimizu et al., 1996). A comprehensive list of these approaches has been provided by Judd and Judd (2011). Specific to each MBR, there is an upper limit to the SS content of the medium. In SS concentrations exceeding that of the limit, contribution of the SS to fouling becomes problematic (Meng et al., 2007; Yoon, 2015).

Another factor contributing to changes in the viscosity is the cell concentration/density in the bioreactor (Reuß et al., 1979; Shimmons et al., 1976). However, the increase in cell density-viscosity is not linear (Shimmons et al., 1976). As presented by Bhave and Todaro (1996), in cross-flow filtration on fermentation broth, the flux across the membrane declines by the increase in yeast cell concentration. In addition, in sMBRs and iMBRs used either for fermentation or wastewater treatment purposes, cell retention is of a great importance as cell concentration defines hydraulic retention time, dilution rate, the bioreactor size and the productivity of the process (Carstensen et al., 2012; Lafforgue-Delorme et al., 1994). High cell concentration due to cell recirculation and retention in MBRs contributes to the increase in medium viscosity and may consequently lead to reduction in medium flow or fouling (Lafforgue-Delorme et al., 1994; Lafforgue et al., 1987; Yoon, 2015). Puzanov (1999) proved that in lactic acid fermentation in a cell recycling MBR, at cell densities above 130 g/l the rheological behaviour of the fermentation broth changed from Newtonian to pseudoplastic. In Newtonian liquids viscosity is independent of the flow rate, where for pseudoplastics the viscosity decreases with increased shear stress (Kriebel and Whitwell, 1949).

One of the parameters affecting both conventional MBR and rMBR processes is the working temperature. Decrease in temperature increases the viscosity of Newtonian fluids (Kumaran, 2010). The rule of thumb in this regard is that with every degree increase in temperature in normal conditions the viscosity drops about 3%. As claimed by Kumaran (2010), the more viscous a fluid is the more temperature dependent the viscosity becomes.

### Table 3

<table>
<thead>
<tr>
<th>Equation</th>
<th>Variables</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>( J = J_0 + \frac{\Delta \rho}{\rho \cdot MLSS} )</td>
<td>( J = ) specific flux rate (m(^3)·m(^{-2})·h(^{-1})), ( J_0 = 0.00001072 \cdot P_T M \cdot \rho \cdot MLSS \cdot \Delta \rho/\rho \cdot MLSS )</td>
<td>Krauth and Staab (1993)</td>
</tr>
<tr>
<td>( R_c = \Delta P/\eta = R_m + R_p + R_c )</td>
<td>( R_c = ) Filtration resistance of activated sludge (m(^{-1})), ( \Delta P = ) trans-membrane pressure gradient (N·m(^{-1})), ( \eta = ) viscosity of permeate (N·m(^{-1})), ( J = ) permeation flux (m(^3)·m(^{-2})·h(^{-1})), ( R_m = ) intrinsic membrane resistance, ( R_p = ) pore fouling resistance, ( R_c = ) cake layer resistance</td>
<td>Fang and Shi, 2005</td>
</tr>
<tr>
<td>( J_0 = V_L - K' \cdot \phi \cdot \rho \cdot MLSS^{0.5} )</td>
<td>( J_0 = ) steady-state filtration flux (m(^3)·m(^{-2})·d(^{-1})), ( K' = ) filtration constant, defined by equations (kg·m(^{-3})·m(^{-1})), ( MLSS = ) dried sludge concentration (mixed liquor suspended solids) (kg·m(^{-3})), ( \phi = ) geometric hindrance coefficient of membrane module, ( \rho = ) viscosity of filtrate (Pa·s(^{-1})), ( V_L = ) lift velocity (m·s(^{-1}))</td>
<td>Shimizu et al. (1996)</td>
</tr>
<tr>
<td>( R = 842.7 \cdot \Delta P/\eta \cdot (\rho \cdot MLSS)^{0.26} \cdot (\rho \cdot COD)^{0.26} )</td>
<td>( R = ) Filtration resistance (m(^{-1})), ( \Delta P = ) trans-membrane pressure gradient (N·m(^{-1})), ( \rho = ) density of the activated sludge (kg·m(^{-3})), ( MLSS = ) inorganic solids (kg·m(^{-3})), ( COD = ) Chemical Oxygen Demand (g·l(^{-1}))</td>
<td>Sato and Ishii, 1991</td>
</tr>
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</table>
In another way, changes in temperature can affect the behaviour of particles in the fluid near the membrane surface. Increase in temperature increases the kinetic energy of the molecules constituting the liquid medium resulting to a more significance in Brownian movement of the particle in the medium. Considering the Brownian diffusion of suspended particles near the membrane surface in MBRs (Yoon, 2015), by reducing the temperature, the particle back transport velocity decreases in a linear manner which results in the extent of cake layer formation (Judd and Judd, 2011). The velocity of particles/solutes deposition on the membrane surface is determined considering the difference between permeation velocity/drag (flux), towards the membrane, and back-transport velocity away from the membrane caused by Brownian diffusion, shear-induced diffusion, charge repulsion, etc. (Fig. 7) (Yoon, 2015).

From the biological aspect, temperature reduction and elevation is limited to a range that does not affect the biological activities of the microorganisms (Folasade, 2006; Singh and Viraraghavan, 2003).

3. Diffusion in MBRs

The role of diffusion in the general mass transfer pattern in conventional pressure driven MBRs greatly differs from that of the rMBRs. In pressure driven MBR processes, diffusion through the membrane occurs as a result of the pressure difference applied over the membrane leading to permeation or retention of certain compounds on basis of different factors such as size and polarity. The most highlighted role that mass transfer by diffusion plays in these conventional MBRs is in a delicate balance between permeation or retention of certain compounds on basis of different factors hindering the diffusion of compounds to and from the cells is of great importance. Diffusion of chemical compounds in feed-side, membrane and cell-side (Fig. 8) depends on medium viscosity, membrane characteristics (pore size, polarity, etc.) and cell biofilm respectively.

The diffusion of feed medium chemical compounds e.g. saccharides and inhibitory compounds and metabolic products in rMBR are considered at three separate phases (Fig. 8):

I. Diffusion of compounds on the feed-side (substrate) towards the membrane surface and in reverse for products.

II. Diffusion of compounds, either substrates or metabolites, through the membrane layer.

III. Diffusion of feed components and products (through the biofilm layer) on the cell side.

3.1. Diffusion in rMBRs

The diffusion related phenomena form the basis of the rMBR system and play a major role in cell inhibitor tolerance and co-utilization of sugars. In an rMBR set up there are three major phases of feed bulk, membrane and cell aggregate which directly affect the diffusion behaviour of different compounds by acting as resistant to easy diffusion. Each of the three regions acts similar to a resistant in an electric circuit that slows down the electron transfer through the circuit at different degrees. Therefore, in order to find out the stage acting as the bottleneck regarding the diffusion rate and compound flow, possible diffusion patterns in each region have to be studied profoundly. The diffusion rate of different compounds (substrate and metabolites) through the feed-side, membrane and cell-side defines the bioconversion rate. In this regard, the study of diffusion mechanisms in an rMBR system and also factors hindering the diffusion of compounds to and from the cells is of great importance. Diffusion of chemical compounds in feed-side, membrane and cell-side (Fig. 8) depends on medium viscosity, membrane characteristics (pore size, polarity, etc.) and cell biofilm respectively.

On the contrary, diffusion is the main mass transfer mechanism in rMBRs. The substrate and products are transported by diffusion in and out of the membrane-confined space. It is noteworthy that, in special rMBR cases shear stress on the membrane/feed-side surface also induces higher diffusion rate (Youngsukkasem et al., 2013b, 2015). The following is a brief description of the diffusion process and its mechanism in rMBRs.

3.1.1. Diffusion of compounds on the feed-side

The first and last stage of diffusion in an rMBR system is the transfer of substrates and metabolites to and from the membrane surface to the bulk feed-side respectively (Fig. 8). The diffusion of chemical compounds (solute) in a liquid medium occurs as a result of the presence of a concentration gradient. The further a system is from concentration homogeneity and equilibrium, the stronger is the concentration driving

Fig. 7. Forces applied to a spherical charged particle in a viscous medium near a flat porous surface in a laminar flow (Yoon et al., 1999).

Fig. 8. Diffusion stages of substrates (sphere) and products (star) in an rMBR set up.
Table 4
Relevant equations for measuring the diffusion behaviour of compounds in different stages of an rMBR system.

<table>
<thead>
<tr>
<th>Diffusion stage</th>
<th>Equation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fick’s first law (steady state)</td>
<td>( J_A = -D_{AB} \frac{dC_A}{dx} )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( D_{AB} = ) diffusivity of compound A in B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( x = ) diffusion distance</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( c_A = ) concentration of A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( J_A = ) molar flux (diffusion rate) of compound A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fick’s second law (non-steady state)</td>
<td>( \frac{dC_A}{dt} = D_{AB} \left( \frac{d^2C_A}{dx^2} \right) )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( t = ) time interval</td>
<td></td>
</tr>
<tr>
<td>Equimolar counter-diffusion</td>
<td>( J_A = -J_B = D_{AB} \frac{dC_A}{dx} )</td>
<td>(American Society of Heating, 2005)</td>
</tr>
<tr>
<td></td>
<td>( J_A = ) molar flux (diffusion rate) of compound A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( J_B = ) molar flux (diffusion rate) of compound B</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxwell-Stefan diffusion</td>
<td>( -\frac{x_A}{RT} \frac{d\mu_A}{dx} = \frac{x_B}{RT} \frac{d\mu_B}{dx} - \frac{N_B}{N_A} ) ( \frac{x_B N_A - x_A N_B}{c_i D_{AB}} )</td>
<td>(Leonardi and Angeli, 2010)</td>
</tr>
<tr>
<td></td>
<td>( N_B = ) molar flux</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( x_B = ) molar fraction of components</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( R = ) universal gas constant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( T = ) temperature</td>
<td></td>
</tr>
<tr>
<td>Feed-side</td>
<td>( D_{AB} = \frac{RT}{N_A} \frac{1}{6 \pi \eta A} )</td>
<td>(Einstein, 1905, Miller, 1924, Sharma and Yashonath, 2006)</td>
</tr>
<tr>
<td>Stokes-Einstein equation</td>
<td>( \frac{D_{AB}}{R T} = \frac{1}{6 \pi \eta A} )</td>
<td></td>
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<td></td>
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<tr>
<td>Wilke-Chang equation</td>
<td>( x = ) association parameter defining the effective molecular weight of the solvent (for non-associated solvents ( x = 1 ) and for water ( x = 2.6 )) ( M_B = ) molecular weight of the liquid medium</td>
<td>(Wilke and Chang, 1955)</td>
</tr>
<tr>
<td></td>
<td>( D_{AB} = \frac{C}{(V A M_B)^{1/3}} \left{ \frac{R T^3}{162 \pi^2 N_A^2 \eta^2} \right}^{1/3} (f) )</td>
<td></td>
</tr>
<tr>
<td>Polson equation</td>
<td>( M_B = ) molecular weight of solute</td>
<td>(Polson, 1950)</td>
</tr>
<tr>
<td></td>
<td>( f = ) frictional constant per mole and for a spherical unhydrated molecule the frictional constant is ( f_0 )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C = ) constant and its value can be calculated from diffusion constant and molecular weight data of proteins with low ( f/f_0 ) values reported by Svedberg et al. (1940).</td>
<td></td>
</tr>
<tr>
<td>Diffusion of solute in porous solid (Fick’s first law)</td>
<td>( \int_0^h \frac{J dx}{h} = - \int_i^C \frac{dC dx}{h} ) ( J = - \frac{1}{h} \int_i^C D dx ) ( J \neq \frac{1}{h} )</td>
<td></td>
</tr>
<tr>
<td>Steady state diffusion through the membrane (no membrane hindrance)</td>
<td>( J_A = \frac{eD}{\tau} \frac{(C_{A0} - C_{A})}{(x_i - x)} ) ( x_i, x = ) distance over which diffusion is being measured</td>
<td>(Beck and Schultz, 1972, Cussler, 2009)</td>
</tr>
<tr>
<td>Steady state diffusion through the membrane (with membrane hindrance)</td>
<td>( J_A = \frac{e D_{eff}}{\tau} \frac{(C_{A0} - C_{A})}{(x_i - x)} ) ( d_s = ) solute diameter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( d_p = ) pore diameter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( c = ) concentration of the compound</td>
<td></td>
</tr>
<tr>
<td>Membrane</td>
<td>( D_{eff} = D \left( \frac{x_i - d_i}{d_s} \right)^m ) ( m = ) constant</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( D = ) diffusivity of the solute in the liquid in the pores</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( D_{eff} = ) effective diffusivity hindered by membrane/solute interactions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( d_s = ) solute diameter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( d_p = ) pore diameter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( c = ) concentration of the compound</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( x = ) distance over which diffusion is being measured</td>
<td></td>
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<tr>
<td></td>
<td>( \tau = ) when the pore diameter is significantly bigger than the solute diameter</td>
<td></td>
</tr>
</tbody>
</table>
### Table 4 (continued)

<table>
<thead>
<tr>
<th>Diffusion stage</th>
<th>Equation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion in porous membrane</td>
<td>( J_s = \frac{D A K}{h} c )</td>
<td>( \text{Pellett et al., 1997} )</td>
</tr>
<tr>
<td>(Derivation of Fick’s first law of diffusion)</td>
<td>( K = \text{membrane-vehicle partition coefficient} ) ( c ) = solute concentration in solution ( h ) = diffusion distance</td>
<td></td>
</tr>
<tr>
<td>Cumulative compound release (diffusion cell)</td>
<td>( Q = \left( \epsilon_s V + \sum_{i=1}^{n-1} c_i S \right)/A )</td>
<td>( \text{Jung et al., 2012, Ng et al., 2010} )</td>
</tr>
<tr>
<td>A = membrane surface area ( c_i = \text{concentration of the compound at the nth sampling} ) ( V = \text{volume of the diffusion cell receptor} ) ( \sum c_i = \text{sum of the concentrations till n-1 sampling} ) ( S = \text{sampling volume} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffusivity in membrane (double diffusion cell)</td>
<td>( D_{ab} = \frac{V}{\pi A t} \ln \left( \frac{C_f^2 - C_0^2}{C_f^2 - C_1^2} \right) )</td>
<td>( \text{Singh et al., 1996} )</td>
</tr>
<tr>
<td>( V = \text{cell volume} ) ( \delta = \text{membrane thickness} ) ( t = \text{time} ) ( C = \text{concentration of solute at different run intervals (at } t = 0, C_0 \text{)} ) in cells 1 and 2. Here cell 1 is considered as the donor and cell 2 as the receptor cell.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative diffusion (double diffusion cell)</td>
<td>( Q = \frac{AD C_0^2 \delta}{2} \left( 1 - \delta^2 \right) )</td>
<td>( \text{Bassi et al., 1987, Hannoun and Stephanopoulos, 1986} )</td>
</tr>
<tr>
<td>( t_0 = \frac{\delta}{\delta_0} ) ( Q = \text{total amount of solute diffused by time } t ) ( C_0^2 = \text{initial concentration of the solute in the donor cell} ) ( \delta = \text{membrane thickness} ) ( t_0 = \text{lag phase (time required for reaching diffusive equilibrium)} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>The relationship between temperature, viscosity and diffusion in water</td>
<td>( \text{constant} = \frac{D_w \mu}{T} )</td>
<td>( \text{Stewart, 2003} )</td>
</tr>
<tr>
<td>( D_w = \text{diffusivity in water} ) ( \mu = \text{viscosity} ) ( T = \text{temperature} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porosity of a biofilm</td>
<td>( \varepsilon = 1 - \frac{D_w \mu}{T} )</td>
<td>( \text{Melo, 2005} )</td>
</tr>
<tr>
<td>( \varepsilon = \text{porosity} ) ( \rho_d = \text{dry biomass to the volume of dry material} ) ( \rho_{dw} = \text{dry mass per unit wet volume} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time for the solute to attain about 90% of its concentration over the biofilm (( t_{90} ))</td>
<td>( t_{90} = 1.03 \left( \frac{R^2}{D_{eff}} \right) )</td>
<td>( \text{Stewart, 2003} )</td>
</tr>
<tr>
<td>Time for the solute to attain about 90% of its concentration in the centre of the biofilm (Cell cluster) (( t_{90c} )) (reaction and adsorption of compound in the biofilm are not considered)</td>
<td>( t_{90c} = 0.37 \left( \frac{R^2}{D_{eff}} \right) )</td>
<td>( \text{Stewart, 2003} )</td>
</tr>
<tr>
<td>( D_{eff} = \text{effective diffusivity in the biofilm} ) ( R = \text{cell cluster radius} ) ( l = \text{biofilm thickness} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell-side</td>
<td>( a = \left( \frac{D_{eff} S_{0}}{K_0} \right)^{1/2} )</td>
<td>( \text{Stewart, 2003} )</td>
</tr>
<tr>
<td>( a = \text{penetration depth} ) ( K_0 = \text{half-saturation coefficient (value of substrate when } \mu \text{ is half } \mu_{\text{max}} ) ( S_0 = \text{solute concentration at the biofilm interface} ) ( k_0 = \text{reaction rate of the solute} ) ( X = \text{density of cells in the biofilm} ) ( \mu_{\text{max}} = \text{maximum growth rate} ) ( Y_{Xs} = \text{yield of biomass on the solute} )</td>
<td></td>
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</tbody>
</table>
force. In order to reduce the concentration difference, molecules of the solute move towards the low concentration area by random movement. In this regard, the diffusion rate (molar flux) can be defined by Fick’s first and second law (Table 4, Eqs. (1) and (2)). Fick’s law relates the diffusion rate ($J$) to the diffusivity (diffusion coefficient) ($D$) of a compound in a medium. Diffusivity is defined as the ease with which molecules of a solute move in a medium. In the conditions where molecule/molecule and/or molecule/membrane forces are high $D$ acts as a variable (Poling et al., 2001). For the condition that there is one dimensional diffusion and $D$ is independent of distance ($x$), time ($t$) and concentration ($c$), Fick’s laws are presented in Table 4.

In case a medium is comprised of two components of $A$ and $B$, equimolar counter-diffusion and Maxwell-Stefan diffusion models can also be applied to track diffusion patterns of compounds. Equimolar counter-diffusion describes the condition in which components $A$ and $B$ are diffusing in opposite directions with the same molar flux, while Maxwell-Stefan diffusion model can be applied when a component diffuses and the other remains or in the case that there is multi-component diffusion (Leonardi and Angeli, 2010) (Table 4, Eqs. (3) and (4)).

A number of parameters such as solute molecular volume and weight, liquid viscosity and process temperature affect the diffusivity of a compound in a liquid medium. Regarding the estimation of diffusivity ($D$) of a compound in a liquid, different models have been developed to correlate solute diffusivity to liquid properties such as Stokes-Einstein, Wilke-Chang and Polson equations (Table 4, Eqs. (5), (6) and (7)).

Therefore, in rMBR systems by the help of the above mentioned models an estimate of the diffusion rate of different compounds through the feed to the membrane surface and also products from the membrane surface to the bulk feed-side can be obtained. For further information in this regard, a profound discussion on the diffusion of compounds in liquid medium can be found in the work by Cussler (2009).

### 3.1.2. Diffusion of compounds through the membrane

In rMBR processes, the porous membrane is considered as a bio-mass/liquid (cell/feed) contactor (Fig. 8). As noted by Jung et al. (2012), the synthetic porous membrane has the role of a continuous linking channel. Depending on the membrane quality and production method, membranes possess different porosity, tortuosity, hydrophilicity, etc. On basis of these parameters, different diffusion patterns for chemical compounds through the membrane are expected. In rMBRs, penetration of chemical compounds through the membrane is concentration gradient dependent. Greater concentration gradients lead to higher flux of the compounds through the membrane. In the case of pressure driven MBRs, this permeation rate is dependent on transmembrane pressure.

The defining factors during membrane diffusion measurements are membrane hydrophilicity, physical and chemical properties of the compound, interaction of the membrane and compounds, etc. (Jung et al., 2012). In membrane separation techniques such as pervaporation where non–porous or very dense selective membranes are used, polymer quality (glass transition temperature, main chain flexibility and side-groups, polymer crystallinity, free volume, etc.), nature of penetrant compounds (molecular weight, size, shape, etc.) and process condition (temperature, etc.) are of critical importance (Berens and Hopfenberg, 1982; Chen et al., 2001; Steingiser et al., 1987; Stern et al., 1987). However, in rMBR systems porous membranes are utilized, where pores are considered as facile compound diffusion pathways/channels connecting the two media. In such set ups, after reaching a steady state, as flux becomes stable and does not change with time, according to Fick’s first law, flux ($J$) through the membrane will be inversely proportional to membrane thickness ($h$) as is presented by Eq. (8) (Table 4). More detailed diffusion models for the diffusion of a solute in a porous solid such as a membrane have been presented that illustrate the dependence of the flux of the solute to membrane porosity ($ε$) and tortuosity ($τ$) (Table 4, Eqs. (9) and (10)) (Beck and Schultz, 1972; Cussler, 2009). Another approach to measure the diffusion rate through a membrane has been simply put by Pellett et al. (1997), as a derivative of Fick’s first law of diffusion (Table 4, Eq. (12)).

One of the main approaches applied for measuring the diffusion rate of different compounds through a porous membrane is using diffusion cells. Among diffusion cells, Franz cell set up has had extended experimental application (Clément et al., 2000; Jung et al., 2012; Ng et al., 2010; Shah et al., 1999). The studies manoeuvring over membrane diffusion measurements by diffusion cells have mainly aimed at measuring the diffusion rate of pharmaceutical and cosmetic chemical compounds (Bonferton et al., 1999; Clément et al., 2000; Hadgraft and Ridout, 1987; Jung et al., 2012; Ng et al., 2010; Shah et al., 1999). Depending on the operation mode and cell configuration, diffusion cells are divided into static and continuous flow cells (flow-through cell). Static cells are available in vertical and side-by-side configurations and are used for finite dose permeation, steady flux of compound and compound uptake into the membrane measurements, whereas flow-through cells come in are to mimic blood vessels, finite and infinite dose permeation (Anon., 2014; Ng et al., 2010). These diffusion cells can be utilized to estimate the diffusion rate of different compound through the membrane in diffusion driven rMBRs. Principally, cumulative amount ($Q$) of compound released by passing through the membrane in a diffusion cell during time is calculated (Table 4, Eq. (13)). The slope of the graph $Q$ versus $1/\sqrt{t}$ represents the release (diffusion) rate of the solute (Jung et al., 2012; Ng et al., 2010). However, Eq. (13) is valid if only diffusion of one single compound is being measured, the diffusion coefficient ($D$) of the compound stays constant and the diffusing compound has no interaction with the membrane.

In case a side-by-side (double cell) diffusion cell is being used for the measurement of the diffusivity in membranes, an alternative approach is taken as in Eq. (14) (Table 4). Singh et al. (1996) used a side-by-side diffusion cell to measure the diffusivity of silver bromide (AgBr) through a glass membrane to purify lead bromide (PbBr$_2$). They measured the diffusivity ($D$) from the slope of $\ln (\frac{C_2}{C_1} – \frac{C_2}{C_1})$ versus time plot (Table 4, Eq. (15)). However, unlike Eq. (14), the effects of porosity and tortuosity have been neglected for the glass membrane used in this study. In another work, Bassi et al. (1987) measured the effective diffusion coefficient ($D$) of lactose and lactic acid through a 3% agarose

<table>
<thead>
<tr>
<th>Diffusion stage</th>
<th>Equation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product (metabolite) concentration in biofilm regions that there is substrate (solute) depletion</td>
<td>$P = \frac{D_{lm} Y_{sn}}{D_{op}} + P_0 \quad (22)$</td>
<td>(Stewart, 2003)</td>
</tr>
</tbody>
</table>
membrane (pore size 0.1–1.0 µm) in a side-by-side diffusion cell using the formula developed by Hannoun and Stephanopoulos (1986) (Table 4, Eq. (16)).

### 3.1.3. Diffusion of compounds on the cell-side

The most important stage of diffusion that is the backbone of many of the applications of rMBRs, is diffusion in the cell-side (Fig. 8). In rMBRs the cells packed together in between membrane layers provide high local cell density. The diffusion rate and behaviour of compounds in the cell-side and through the imposed cell aggregate determine cell inhibitor tolerance and in situ detoxification, and co-utilization of sugars (Westman et al., 2012a, 2014a, 2014b). As bioconversion happens in the membrane-confined cell-side, diffusion rate of substrates and products in this region plays a crucial role in defining the bioconversion rate and process yield. The difference in diffusion of compounds in cell-side in comparison with diffusion in feed-side and membrane is that in the former condition two phenomena of mass transfer and reactions occur simultaneously. Therefore, diffusion patterns and models presented for the cell-side (biofilm side) take into account the effect of reaction as well as effective diffusion. In this regard, as reported by Melo (2005) there seems to be a two way effect as the physical structure of the cell aggregate or biofilm depends on the internal mass transfer and the structure affects the diffusion characteristics of compounds. In order to better understand the role of biofilm and the effect of biofilm formation on the diffusion of compounds on the cell-side in an rMBR system, a basic knowledge on biofilms nature, formation and prevention is required.

Biofilms in general are micro-colonies of microorganisms attached to a surface and embedded in a gel like extracellular polymeric substance (EPS) matrix (Fig. 9). Biofilm is the dominant life form of bacteria guaranteeing their survival in comparison to free floating cells (Marić and Vrančić, 2007). These biofilms are comprised of cells and a network of EPS with water channels in between the cell colonies responsible for water, nutrient and metabolite delivery in and out of the biofilm. The extracellular matrix is a combination of 90% water and 10% extracellular polymeric substances (EPS) (Rendueles et al., 2013).

In addition to bacteria, different strains of yeast such as Candida albicans, Candida glabrata, S. cerevisiae, etc. also have the capability to adhere to abiotic surfaces e.g. membranes, cells and tissues (Short, 2011; Verstrepen and Klis, 2006). Special proteins on the yeast cell wall surface are responsible for the attachment to other cells and also abiotic surfaces are called “adhesins”.

EPS play an essential role in biofilm formation accounting for, depending on the type of the microorganism, from 75 to 90% of the biofilm with the rest being composed of cells (Marić and Vrančić, 2007). The term EPS (high-molecular weight mixture of polymers) is noted to cover a wide range of macromolecules such as proteins, carbohydrates, nucleic acids, lipids, etc. on and around the cell exterior wall (not all necessarily directly anchored to the cell wall) covering colonies of cells and maintaining the stability of cell aggregate in the biofilm (Flemming and Wingender, 2001; Sheng et al., 2010). EPS mainly comprises of proteins and carbohydrates. Carbohydrates, having more hydrophilic tendency, are mainly responsible for cell adhesion to the membrane.

#### 3.1.3.1. Fouling due to biofilm formation in MBRs

Different parameters influence the EPS content in conventional pressure driven MBRs process, to name some: shear stress near the membrane surface by gas sparging and other means which induce turbulence, the substrate condition regarding nutrient content and organic loading rate. For example, the yeast strain C. albicans is said to form more EPS in an agitated cultivation medium than in a stationary condition (Henriques, 2005). In wastewater treatment MBRs, solid retention time (SRT) is of great importance for the EPS concentration in the medium (Brookes et al., 2003).

As mentioned, membrane fouling is a major issue in MBR processes. The soluble part of the EPS, defined as soluble microbial products (SMP), is adsorbed and deposited on the membrane surface forming a gel layer blocking pores and the flow through the membrane and providing the basis for the formation of a biofilm on the membrane (cell/cell attachment of the microorganisms and EPS matrix formation) (Rosenberger et al., 2005; Vanysacker et al., 2013). In a study by Vanysacker et al. (2013) fouling of PVDF, polysulphone and polyethylene membranes by Escherichia coli and Pseudomonas aeruginosa biofilms were investigated. It was observed that the more hydrophilic a membrane is the less biofilm associated fouling occurs in an aqueous solution.

The problem with biofilm formation in conventional MBRs and specially in rMBRs is that, the EPS gel housing the cells can be a major obstacle to nutrient delivery (the dominant mechanism of nutrient delivery is diffusion through water channels in the EPS matrix) to cells and cell assisted bioconversion and also a barrier for permeation.

![Fig. 9. Biofilm formation in an rMBR system.](image-url)
through membrane in pressure driven processes. Considering the role of EPS in cell/cell attachment, in some MBR systems, disruption and decrease in EPS content is assumed to negatively affect MBR performance, however, this has not been proved (Jang et al., 2005).

As supported by Li et al. (2014) biofilm related fouling and internal pore blocking in MBRs only requires chemical cleaning. Removal of the biofilm in down time of the MBR is also a hurdle as chlorine, bactericidies and other cleaning-in-place methods (CIP) do not necessarily work efficiently in EPS removal as they mainly affect the bacteria (Armstrong et al., 2011).

3.1.3.2. Biofilm prevention. As the presence of biofilm can hinder the performance of both the conventional MBRs and rMBRs there is need for in depth studies on preventive methods and approaches. In order to facilitate diffusion of different compounds through the membrane layer and to the cell membrane surface, it is essential to keep the extent of biofilm formation at low levels. In an rMBR system, cells are intentionally kept in close proximity in-between membrane layers. This induces the propensity of biofilm formation in rMBR systems. However, this biofilm formation is undesirable as it limits the mass transfer by diffusion only to the water channels present in the EPS matrix. Different environmental factors such as nutrient and oxygen content, pH and temperature of the medium can affect biofilm structure and the extent of biofilm formation. In this regard, the effect of phosphate concentration on biofilm formation has been investigated by Kim Kwang and Frank (1995). As presented by Kim Kwang and Frank (1995) the presence of trehalose and mannose enhances biofilm formation. Furthermore, it has been claimed that, glucose availability has a direct impact on biofilm thickness (Marić and Vraneš, 2007). In addition, oxygen content, temperature and pH of the medium affect cell adhesion. Although some bacteria can form biofilms at acidic conditions, considerably low medium pH reduces cell mobility required for initiating adhesion (Kim Kwang and Frank, 1995). Low medium oxygen content and high temperature are said to be detrimental to cell/surface adhesion and cell/cell connection, respectively (Kim Kwang and Frank, 1995). Metal ion concentration of the medium is also a defining factor to be taken into account as EPS binds with the cell wall through building ion bridging with multivalent metals such as Ca$^{+2}$ and Mg$^{+2}$. Therefore, less metal content leads to less EPS resulting in less onerous biofilm (Sheng et al., 2010).

All in all, in order to have healthy functioning rMBR the biofilm formation should be controlled at an acceptable low level. The pursued reduction in biofilm formation tendency may be achieved through different approaches:

1. Cell/cell communication blockage: To initiate a cell/cell attachment and cell/surface adhesion, cells should communicate through signaling molecules, blocking this communication pathway may be a solution for preventing biofilm formation (Marić and Vraneš, 2007).
2. EPS enzymatic hydrolysis: In order to break down and disrupt a biofilm layer, enzymatic hydrolysis and degradation of extracellular polymeric matrix is another applicable option (Marić and Vraneš, 2007).
3. Cell secreted anti-biofilm polysaccharides: Another option is presented by the microorganism as there are different sorts of bacteria that secrete anti-biofilm polysaccharides blocking the proteins on the cell wall that attach to sugars on other cell surfaces and inhibit biofilm formation (Rendueles et al., 2013).
4. Genetic inactivation: repression of genes involved in EPS formation, cell/cell and cell/abiotic surface attachment is another approach to alleviate biofilm related issues (Marić and Vraneš, 2007; Verstrepen and Klis, 2005).
5. Environmental stress factors: Environmental stress factors such as the change in the medium carbon and nitrogen content e.g. nutrient starvation, pH and ethanol content can trigger the yeast cell adaptation technique from adhesive to non-adhesive and vice versa (Verstrepen and Klis, 2006). However, the behaviour is still to be studied as for example different glucose concentrations in the medium can play roles in both enhancing and hindering adhesion (Verstrepen and Klis, 2006).

6. Cell cultivation condition: In order to reduce the amount of EPS by controlling the process conditions, it has been claimed by Sheng et al. (2010) that aerobic cultivation conditions lead to a large content of EPS, while anaerobic conditions reduces EPS production and sometimes lead to disintegration of the biofilm (Judd and Judd, 2011).

3.1.3.3. The effect of biofilm formation on diffusion in rMBR. It has been reported that the dry density of the biofilm is in close relationship with effective diffusivity as increase in one negatively affects the other (Beyenal and Lewandowski, 2002; Fan et al., 1990; Stewart, 1998, 2003). However, there are still uncertainties in generalization of such matter (Casey et al., 2000; Zhang et al., 1998a). Biofilms are composed of 90–95% water. The water channels (macro-pores) of different length, diameter and tortuosity have the responsibility of carrying compounds in and out of the biofilm. There are macro- and micro-pores in between cell/EPS and inside cell/EPS aggregates respectively (Melo, 2005). As biofilms are mostly water, the first step in estimation of the effective diffusivity in biofilms is to have the diffusivity of different compounds in water (Stewart, 2003). The data on diffusion of many compounds in water is readily available. However, missing calculations for unknown diffusivities in water can be made through different models presented in the previous sections such as Wilke-Chang (Perry and Chilton, 1973; Wilke and Chang, 1955). It is noteworthy that, as mentioned before, the diffusivity in water, as well as other media, depends on temperature and viscosity (Stewart, 2003). The relationship between temperature, viscosity and diffusion in water is presented in Eq. (17) in Table 4.

Cells are concentrated and packed in very close proximity in between membrane layers resembling an imposed biofilm. The highly compact biofilm matrix reduces the diffusivity of compounds due to increased tortuosity. This means that due to the presence of different obstacles, such as cells, EPS, abiotic particles and gas bubbles in the biofilm, compounds are forced to take longer diffusion paths than when having free unrestricted diffusion (Stewart, 2003; Zalc et al., 2004). Therefore, the effect of tortuosity ($\tau$) and porosity ($\varepsilon$) of the biofilm should be considered while measuring the effective diffusivity ($D_{eff}$) of compounds in a biofilm ($D_{w}$ is the diffusivity of the compound in water). According to Melo (2005) the porosity of a biofilm can be calculated from density values (Table 4, Eq. (18)).

Diffusion is limited in biofilms by the diffusion distance and decrease in fluid flow (in case of bulk flow) (Stewart, 2003). According to Stewart (2003), the time required for a diffusive equilibrium in biofilms is proportional to the square root of the diffusion distance. Moreover, the time for the solute added to a liquid medium, containing a biofilm, to attain about 90% of its concentration over $t_{90}$ and in the centre of the biofilm (Cell cluster) ($t_{90c}$) can be estimated (Table 4, Eqs. (19) and (20)).

These equations have been built assuming that the solute does not react or is not absorbed in the biofilm, the transfer of compounds to the biofilm surface occurs with no resistance and the biofilm thickness is uniform along the substrate. These equations are not always valid in real biofilm diffusion conditions as usually sugars are utilized by cells and consequently there would be a concentration gradient through the biofilms and this is in contradiction with the assumptions for $t_{90}$ and $t_{90c}$ (Stewart, 2003). It should be noted that when a solute reacts in a biofilm, it may not pass through the biofilm as all may be utilized by cells. In conditions that the solute undergoes reaction in a biofilm and the rate of reaction is independent of solute concentration, the depth that a solute can penetrate in a biofilm can be estimated through Eq. (21) (Table 4). These formulae have been driven on basis of zero-order kinetics, which means that the reaction rate is independent of

\[ D_{eff} = \frac{D_{w}}{\varepsilon (1-\varepsilon) \tau^2} \]
solute concentration (for Monod and Michaelis-Menten models that is $S \gg K_m$) (Stewart, 2003).

The other issue faced in mMBRs is that while the substrate diffusing in the biofilm is being taken up by cells while participating in reactions, the metabolites produced also diffuse in reverse from the biofilm towards the feed-side due to concentration gradient. A simplified equation is used by Stewart (2003) to obtain the concentration of the product in locations in the biofilm where the reacting substrate has depleted (Table 4, Eq. (22)). In this equation it has been assumed that product formation is in a stoichiometric relation with substrate consumption.

4. Concluding remarks

Membrane bioreactors have long been used in different biological processes. Depending on the feed medium and condition, process parameters and the final product a number of different membranes and membrane module configurations can be taken into practice. In this regard, the newly introduced concept of mMBR opens new horizons for further research and application development of MBRs. The mMBRs feature exceptional properties such as high local cell density, diffusive nature of compound separation and the ability of cell separation and reuse. These unique specifications bring along the potential for the bioconversion of complex substrates that contain high concentration of inhibitory compounds, different sugar sources and high suspended solid levels difficult to be handled by conventional pressure driven MBRs.

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References


Ishola, M., Ylitervo, P., Taherzadeh, M., 2015a. Co-utilization of glucose and xylose for en-
Ishola, M., Babapour, A.B., Gavitar, M.N., Brandberg, T., Taherzadeh, M.J., 2013a. Effect of
Ikegami, T., Yanagishita, H., Kitamoto, D., Negishi, H., Haraya, K., Sano, T., 2002. Concentra-
Ishola, M., Brandberg, T., Taherzadeh, M.J., 2015b. Simultaneous glucose and xylose uti-
Ishola, M., Jahandideh, A., Haidarian, B., Brandberg, T., Taherzadeh, M.J., 2013b. Si-
Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and
Koppram, R., Albers, E., Olsson, L., 2012. Evolutionary engineering strategies to enhance
Lafforgue, C., Malinowski, J., Goma, G., 1987. High yeast concentration in continuous fer-
Koppman, R., Albers, E., Olsson, L., 2012. Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce bio-
Krauth, K., Staab, K.F., 1993. Pressurized bioreactor with membrane filtration for waste-
Krauth, K., Staab, K.F., 1993. Pressurized bioreactor with membrane filtration for waste-
Krauth, K., Staab, K.F., 1993. Pressurized bioreactor with membrane filtration for waste-
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Diffusion-based reverse membrane bioreactor for simultaneous bioconversion of high-inhibitor xylose-glucose media

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A R T I C L E   I N F O
Keywords: Reverse membrane bioreactor Concentration gradient Diffusion rate Fermentation Inhibitor detoxification

A B S T R A C T
Two of the main hurdles in industrial production of second generation bioethanol are the high content of inhibitory compounds and presence of sequentially fermented hexose and pentose saccharides in the feedstock. In order to tackle these issues, the novel cell confinement approach in a reverse membrane bioreactor (rMBR), used in this study, proved to be promising for robust fermentation of high-inhibitory xylose-glucose media simulating a lignocellulosic hydrolysate. The high local cell concentration and concentration-driven diffusion-based mass transfer conditions in rMBR enhanced simultaneous utilization of sugars and boosted cell furfural tolerance/detoxification capacity. The diffusion rates of all compounds through the membrane were measured in a diffusion cell and in an rMBR. In the rMBR, yeast cells could readily convert high content of furfural (10 g/l) that is toxic to freely-suspended cells. Moreover, in the presence of 2.5 g/l of furfural, cells had the same performance as in medium with no inhibitor and could simultaneously convert glucose, xylose, and furfural with the latter two at the same rate with no lag phase. The performance of rMBR in remediating issues revolving around lignocellulosic bioethanol production covers the shortcomings of the conventional encapsulation technique and opens new areas of application for diffusion-based bioconversion systems.

1. Introduction
Although the application of MBRs in wastewater treatment dates back to late 1960s [1], their range of application has recently expanded to a great number of engineering processes from filtration to complex membrane bioreactors (MBR) [2,3]. Other than wastewater treatment, in recent decades, with the increasing demand for production of fuel from renewable sources to replace the depleting and environmentally polluting fossil-based fuels [4], there has been a great interest to use MBRs for biofuel production [5].

Bioethanol has been a biofuel of great interest to be produced and recovered feasibly using MBR technology [6]. In recent years, production of 2nd generation bioethanol from processing lignocellulosic materials (agricultural residues etc.) that are relatively cheap, abundant and from non-food or feed sources has gained great attention [7–9]. However, 2nd generation bioethanol fermentation has been limited by the process costs and production scale [10–12]. Lignocellulosic materials have a recalcitrant structure, mainly made up of cellulose, hemicellulose and lignin, that first needs to be opened up by intensive physical, thermal or thermochemical pretreatment, followed by enzymatic hydrolysis prior to fermentation [13]. During pretreatment, different hexose (glucose, mannose etc.) and pentose monosaccharides (xylose, arabinose etc.) and cell-inhibitory degradation by-products such as furan aldehydes (furfural and 5-hydroxymethyl furfural (HMF)) are produced [13–15]. On the other hand, wild-type yeasts cannot utilize pentose sugars and xylose-consuming recombinant yeasts consume sugars sequentially, i.e. utilize glucose first and then xylose only in glucose-deprivation conditions [16]. In addition, some strains of yeast are capable of converting some inhibitors such as furfural and HMF to the less inhibitory furfuryl alcohol and HMF alcohol, respectively [17,18]. However, presence of high content of furans during fermentation disturbs the cell’s normal metabolic and physiologic condition by inhibiting cell growth and inactivating enzymes, changing cell membrane permeability and disturbing the cell redox balance [14,19]. In fermentation systems containing inhibitors, different sugars and freely suspended cells, all cells are exposed to the same level of medium constituents. This leads to a long lag phase (sometimes linked to medium detoxification) followed by priority-based substrate consumption. However, in recent years it has been reported that enhanced

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inhibitor tolerance and simultaneous utilization of different sugars can be achieved by providing high cell concentration microenvironments using cell encapsulation and/or flocculation techniques [20,21]. This cell-dense microenvironment controls the rate at which cells are exposed to different medium components attributing its success to the concentration gradient built over the membrane and cell aggregate [6]. However, several issues in the preparation and application of capsules have limited their scale and area of application. The process of cell encapsulation is time consuming and laborious [22]. Moreover, poor preparation can cause cell attachment to the outer surface of the capsules and also capsule breakage due to extensive gas formation in the capsule or high shear stress due to agitation can lead to cell escape [23,24]. These issues can be remediated using the new membrane cell confinement technique of rMBR [6].

The rMBR is a newly introduced type of immersed membrane bioreactor (iMBR) that has recently been applied in closed sachet [25] and multi-layer membrane column [25] configurations for biogas and in flat-sheet membrane panel configuration [24] for bioethanol production. While in the conventional iMBR, cells are suspended in the bulk medium and convective product separation happens through building a pressure gradient over the membrane surface, in rMBRs cells are confined between membrane layers and diffusional mass transfer happens in and out of the membrane bound area due to the presence of a concentration gradient [6]. As discussed in our previous review work [6], merging the benefits of conventional MBRs and cell encapsulation into the rMBR technique provides us with a promising approach for treatment of complex feed streams containing inhibitory compounds and mixtures of different sugars.

In this work, by benefiting from the membrane-assisted cell confinement technique of rMBR, we have tried to tackle some issues affiliated with 2nd generation bioethanol production by studying the possibility of simultaneous consumption of pentose and hexose saccharides along with detoxification of inhibitory compounds during fermentation. First the diffusion behavior of different chemical compounds was measured in semi-synthetic media representing lignocellulosic hydrolysate using a side-by-side diffusion cell. Then, rMBR fermentations were conducted at different concentrations of inhibitory compounds to observe the effect of environmental stress on cell metabolic activity by monitoring rates of consumption, production and detoxification of different compounds. The results of this study evaluate rMBRs capability of assisting the bioconversion of lignocellulosic material to bioethanol from an unconventional and interesting view point.

2. Materials and methods

2.1. Diffusion rate measurement

A diffusion cell (Side-Bi-Side, PermeGear Inc., Hellertown, PA, USA) was applied in order to have an understanding of the diffusion rate and flux of different compounds involved in fermentation through the membrane used for cell encapsulation. A simple scheme of the diffusion cell is presented in Fig. 1. The diffusion cell consists of a donor and a receptor chamber each of 60 ml volume connected through an opening (orifice) of 30 mm diameter (area 7.07 cm²). The diffusion cell is water-jacketed and the temperature is maintained at 30 °C (chosen fermentation temperature) by a water-circulating water bath. In order to simulate the conditions used for actual rMBR fermentation cycles, single membrane layers were isolated from 2nd generation IPC (Integrated Permeate Channel) dual layer membranes. An IPC membrane typically contains two Polyethersulfone (PES) membrane layers, with an average pore size of 0.3 μm. Single membrane layers were obtained by slicing such IPC membrane into half. These were used to separate the donor (contains the diffusant(s)) and receptor (contains no or very low concentration of the diffusant(s)) compartments in the diffusion cell. In order to have homogeneous concentrations at all time, both receptor and donor cells were stirred at 500 rpm using a double-core H-series magnetic stirrer (PermeGear Inc., Hellertown, Pennsylvania).

In order to simulate the rMBR fermentation conditions and to measure the counter-diffusion behavior of different compounds, the donor compartment mainly contained the substrates glucose, xylose and furfural while the receptor cell had only ethanol and glycerol. The used membrane layers were first soaked in NaOH 2% for 15 min and then rinsed with distilled water, before and after each diffusion cycle. The diffusion cycle was 12 h with 2 h sampling intervals. At every sampling 1 ml aliquot was withdrawn from the receptor cell and replaced with the same amount of fresh receptor medium. The changes in the concentrations of compounds in the receptor cell were measured using high performance liquid chromatography (HPLC) (Walters 2695, Walters Corporation, Milford, USA) (Section 2.4). To have steady state diffusion across the membrane, sink conditions should be provided in the diffusion cell i.e. the receptor cell is kept at zero concentration of diffusants [26]. However, this cannot be completely achieved in a static diffusion cell, therefore, the sink condition has been redefined as the condition at which the diffusant concentration in the receptor cell is less than 10% of its saturation solubility concentration [26]. The concentration of compounds in the donor compartment was chosen to be comparable to that of acid pretreated and enzymatically hydrolyzed wheat straw hydrolysate [27].

After each concentration measurement, the cumulative amount released per unit area of membrane (Q) was calculated for different compounds using Eq. (1) according to K.D. Thakker and W.H. Chern [28]:

\[
Q = \frac{C_0 V + \sum_{i=1}^{n-1} C_i S_i}{A}
\]

(1)

Where Q is the cumulative amount of a compound passed through the surface area of the membrane (g/cm²), S is the sample aliquot volume (1 ml), V is the volume of each chamber (60 ml), A is the membrane surface area (7.07 cm²), Cn is the receptor cell concentration (g/ml) at the nth sampling and \(\sum_{i=1}^{n-1} C_i S_i\) is the total amount of a compound released from the 1st to the n-1th sampling intervals.

Graphs of the cumulative amount (Q) versus time were plotted and a regression line was estimated for the linear region of the graph. As the sink conditions exist, the slope of the adapted regression line represents flux (J) of a component per unit area of membrane surface [29].

The apparent permeability coefficient (Kp) of compounds through the membrane layer was estimated using Eq. (2) [30]:

\[
K_p = \left(\frac{1}{A C_0}\right) \frac{dM}{dt}
\]

(2)

Where A is the membrane surface area, C0 is the initial concentration of the compound in the donor cell and (dM/dt) is the flux of the compound through the membrane.
2.2. Pre-culture and reactor medium preparation

In this study, a recombinant xylose-utilizing strain of Saccharomyces cerevisiae [24] was used as the acting microorganism. The maximum growth rate of this strain was observed at pH 5 and a temperature of 30 °C. The yeast was inoculated on yeast extract peptone dextrose (YPD) plates [24] and stored at 4 °C till use.

In order to obtain a high concentration of yeast cells to be injected in the hollow inter-membrane layer space of the IPC membrane, cell pre-cultures were prepared prior to the rMBR experiment. In this regard, two 1-l Erlenmeyer flasks containing 400 ml of broth consisting of 20 g/l glucose, 10 g/l xylose, 5 g/l peptone and 5 g/l yeast extract were loop inoculated with yeast and kept in a shaking water bath (Grant OLS 200, Grant instrument ltd, UK) at 30 °C and 115 rpm for 48 h. The sugar content of inoculum was totally depleted before the transferring inoculum into the membrane panel. The total 800 ml of culture medium was concentrated 16 times to 50 ml by centrifugation at 5000 × g for 2 min and removal of supernatant. The concentrated inoculum contained 65.99 ± 7.72 g/l yeast. A total of 25 ml of the inoculum was injected in the membrane-confined space of two membrane panels representing a cell concentration of 0.82 ± 0.09 g/l (considering overall MBR volume).

For the batch mode rMBR fermentation, a semi-synthetic xylose-glucose medium containing 26 g/l glucose, 13 g/l xylose, 1.5 g/l yeast extract, 0.5 g/l KH₂PO₄, 3 g/l (NH₄)₂SO₄ with addition of 0, 2.5 g/l, 5 g/l, 10 g/l of furfural was used. In order to prevent foaming and bacterial contamination 0.15 ml of fatty acid ester antifoam and 5 g/l, 10 g/l of furfural was used. In order to prevent foaming and bacterial contamination 0.15 ml of fatty acid ester antifoam and 5 g/l, 10 g/l of furfural was added. Fermentation cycles of 48 h were all conducted in duplicates. Samples were withdrawn at different time intervals (4, 8, 12, 24, 36 and 48 h) from the bioreactor to track the changes in the concentrations of different substrates and metabolites.

2.3. Flat-sheet membrane panels and membrane bioreactor set-up

In the current research work, membrane panels containing 2nd generation Integrated Permeate Channel (IPC) were used which were customized and developed especially for this research at the Flemish Institute of Technological Research (VITO NV, Belgium). These customized membrane panels contain double membrane layers each about 0.3 μm thick and casted on an inter-tangled polyester spacer-fabric support [31]. The inter-membrane hollow space in these IPC panels is provided by the 2 mm distance between the two adjacent membrane layers with a very high open volume (85%) resulting in a very low pressure drop. This space is a consequence of the used spacer-fabric support. The IPC membranes are commonly used in MBRs for the withdrawal of the filtrate/permeate. However, in this work the hollow space was used for addition, removal and confinement of high concentration of yeast between the adjacent membrane layers. Each IPC panel had 12 inbuilt 0.5 mm in diameter gas/air diffusers (6 on each side) positioned at the bottom of the panel that can be used for medium mixing and membrane surface cleaning from fouling at the outside of the panel. The total area of the two membrane layers of each IPC membrane panel used in this study was 68.6 cm². The membrane layers were made from a polyethersulfone (PES)/Polyvinylpyrrolidone (PVP) polymer mixture and have an average pore size of 0.3 μm and 3000–4000 l/h. m².bar of clean water permeability.

The rMBR used in this study consisted of two such customized 2nd generation IPC membrane panels immersed in a 31 water-jacketed bioreactor (Biostat Bplus, Sartorius BBI Systems GmbH, Germany).

Prior to each rMBR fermentation cycle, the bioreactor and tubings were separately autoclaved (at 121 °C for 20 min). Following that, membrane panels were attached in the reactor and cleaned and sterilized using NaOH 2%, H₃PO₄ 1% and NaOCl 200 ppm solutions in a stepwise process [24] as suggested by VITO. The cleaning and sterilization solutions were recirculated in the hollow space in between membrane layers to guarantee complete sterility.

After the sterilization of the MBR, initially, two membrane panels where syringe-inoculated with 25 ml of concentrated cell culture and the cultivation medium was added to the reactor. In order to make sure that the reactor medium is well mixed, a zero-hour sample was taken from the reactor after 10 min. During the run the reactor was sparged with 0.5 vvm of nitrogen gas through the external gas diffusers of the panels to have anaerobic fermentation conditions plus minimal mixing. Throughout the experiment the temperature and pH of the media in the reactor were maintained at around 30 °C and 5.0 (using NaOH 2 M), respectively. The Biostat B plus fermentation controlling unit was used to control fermentation related parameters such as temperature, pH and nitrogen flow rate during the experiment. The fraction of the ethanol produced and stripped from the MBR through the condenser by gas sparging was rec006fed through a 0.5 l water-ethanol trap.

2.4. Analytical methods

The detection and quantification of the changes in the concentration of different substrates (glucose, xylose and furfural) and metabolites (ethanol and glycerol) in the rMBR and the diffusion cell was analyzed using high-performance liquid chromatography (HPLC) (Waters 2695, Waters Corporation, Milford, USA), and a hydrogen-based column (Aminex HPX-87H, Bio-Rad, Hercules, USA) having a working temperature of 60 °C with 5 mM H₂SO₄ eluent flowing at 0.6 ml/min.

Determination of inoculum cell dry weight was done by withdrawing 3 ml of the concentrated inoculum medium, centrifuging at 3000 × g for 2 min, removing the supernatant and replacing it with milli-Q water and repeating centrifugation. This cycle was repeated twice and then cell pellets were dried in a 70 °C oven for 24 h.

The software package MINITAB 17 was used for statistical analysis of the obtained data. In order to investigate the significance deference level between the compared data, the Analysis of variance (ANOVA) was performed using general linear models with 95% confidence interval followed by pairwise comparisons by Tukey’s test. The error bars represent twice the sample standard deviation (95% confidence interval) of the duplicated experiments.

3. Results and discussion

In this study, initially the diffusion rates of different compounds involved in fermentation through the single membrane layers from an IPC membrane were measured using a diffusion cell. These were further compared with the flux of compounds during rMBR fermentation. In addition, media with different levels of inhibitory compound and sugars were used for fermentation and the effect of using an rMBR set-up on enhancing diffusion-based detoxification of high inhibitory media along with co-utilization and fermentation of prioritized sugars were analyzed and presented.

3.1. Diffusion rate of compounds through single membrane layers

A side-by-side diffusion cell was used in order to have a better understanding of the diffusion rates of different chemical compounds involved in the fermentation process of lignocellulosic materials in an rMBR. The results of the counter-diffusion of substrates and metabolites involved in the targeted fermentation process are presented in Fig. 2. According to Fick’s first law of diffusion [6], the slope of the regression lines plotted for the changes in the amount of compound passed through the unit area of the membrane per time represents the flux or in other words the diffusion rate of a compound through the membrane. This has been presented for the compounds of interest and different initial concentration in Fig. 2.

The initial ratio of glucose to xylose was kept at 2:1 for all samples based on hydrolyzed wheat straw glucose/xylose ratio recorded in our previous work [27]. This is reflected in the 1.8 times faster diffusion of
glucose \((0.0033 \pm 0.0003 \text{ g/cm}^2\text{ h})\) than xylose \((0.0018 \pm 0.0001 \text{ g/cm}^2\text{ h})\) through the membrane. The significantly higher \((p-value = 0.000)\) diffusion rate of glucose plus the fact that glucose is favored as a substrate over xylose for yeast reduces the probability of having same co-utilization for these sugars in an rMBR. Changes in the accumulated amount of glycerol and ethanol as the products of fermentation are presented in Fig. 2. The exact concentration of these products in the intra-membrane layer hollow space cannot be exactly estimated due to membrane module limitations. However, there is the possibility of having high local concentration of metabolites in the cell aggregate. With choosing an initial concentration 15 g/l ethanol and 5 g/l glycerol in the receptor compartment of the diffusion cell, fluxes of 0.0024 \pm 0.0001 and 0.0009 \pm 0.0004 g/cm^2.h were acquired, respectively. The trend of increase in the concentration of furfural in the receptor cell was also linear and the diffusion rate mostly proportional to the starting concentration as it four-folded from 0.0006 g/cm^2.h for 2.5 g/l of initial furfural to 0.0024 \pm 0.0001 g/cm^2.h for that of 10 g/l.

3.2. Diffusion and conversion rates of compounds in rMBR

In order to build a balance between the diffusion rate and bioconversion rate of substrates and metabolites, and to have enhanced simultaneous sugar consumption and inhibitor detoxification, rMBR fermentation experiments were conducted. The changes in the amounts of compounds and their fluxes through the membrane layer during rMBR fermentation are presented in Fig. 3 and Table 1, respectively.

Considering the changes in the diffusion and utilization rates of glucose during fermentation, a surprisingly comparable performance was observed for media with zero and 2.5 g/l initial furfural (Fig. 3a). In addition, the most noticeable change in the concentration of glucose (about 5 g/l) was observed for the aforementioned cultivations with no significant difference \((p-value = 0.465)\) in the flux \((0.0017 \pm 0.0004 \text{ g/cm}^2.h)\). As reported by M. Ishola et al. [24], the flux of glucose in an rMBR containing xylose-glucose semi-synthetic media and wheat straw hydrolysate with about 6 and 50 g/l of initial glucose was 0.0006 and 0.0025 g/cm^2.h, respectively. As the initial furfural content increased to 5 and 10 g/l, the flux of glucose showed 70% and 92% drop compared to that of the diffusion cell, respectively (Fig. 4). As the diffusion of glucose happens at the same rate in all preparations, changes in the flux of glucose are solely attributed to the cell metabolic and physiologic condition in the absence or presence of furfural [32]. As the diffusion rate of glucose in the diffusion cell (Section 3.1) was at least twice higher than that measured in the rMBR, the rate limiting factor in the consumption of glucose, even in the non-inhibitory condition, can be assumed to be either bioconversion kinetics or diffusion hindrance of the packed cells. However, as there is no real-time access to the intra-membrane space and no exact estimation of compounds concentrations to define the extent of diffusion, a firm
conclusion cannot be drawn. To investigate the above-mentioned hypothesis, a thorough study of the diffusion in the cell-aggregate (induced biofilm) confined in between membrane layers is necessary.

Similar to the trend observed for glucose consumption in the case of non-inhibitory and 2.5 furfural media (Fig. 3a), xylose diffusion rates (Table 1) in these two preparations (p-value = 0.095), are similar although five-times less than measured in the diffusion cell (Fig. 4). This difference can be due to the poor mixing in the rMBR compared to the diffusion cell compartments and also the resistance to diffusion of compounds imposed by the packed cells. By benefitting from an inoculum size and membrane area of 10-times and 5.5-times that used in our research work, respectively, a higher xylose conversion flux of about 0.001 g/cm².h has been reported in an rMBR using a xylose-glucose semi-synthetic medium with a starting xylose concentration of 21 g/l [24]. However, as expected, as the inhibitor content of the media increases, the xylose consumption drops (Fig. 3a) due to high concentrations of unutilized glucose (glucose suppression) plus the direct effect of furfural on cell metabolic and physiologic activity. One reason can be that as there is lower metabolic energy flux (less ATP production) for xylose than glucose, inhibitor-stricken cells have low energy levels to invest in cell maintenance [33]. Furthermore, xylose conversion by yeast is extremely co-factor-dependent (NADH and NADPH) and when furfural (as a reactive aldehyde) acts as an electron accepter (electron sink) the level of intercellular co-factors drops [32]. In addition, the enzyme alcohol dehydrogenase (ADH) that is involved in the conversion of furfural beneﬁts from the same co-factors, contributing to an even less xylose conversion [34]. The flux achieved for xylose with a starting concentration of 12.5 g/l and furfural content of 5 g/l

<table>
<thead>
<tr>
<th>Medium</th>
<th>Fluxa</th>
<th>Flux coefficient of determinationb</th>
<th>Apparent permeability coefficientc</th>
<th>rMBR/diffusion cell flux</th>
<th>Specific rate of conversiond</th>
</tr>
</thead>
<tbody>
<tr>
<td>No furfural</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.0017 ± 0.0004</td>
<td>0.996</td>
<td>0.000007</td>
<td>0.52</td>
<td>0.1473 ± 0.0190</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.0004 ± 0.0001</td>
<td>0.989</td>
<td>0.00003</td>
<td>0.22</td>
<td>0.0355 ± 0.0035</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.0008 ± 0.0000</td>
<td>0.997</td>
<td>NA</td>
<td>0.33</td>
<td>0.0714 ± 0.0025</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.0001 ± 0.0000</td>
<td>0.984</td>
<td>NA</td>
<td>0.11</td>
<td>0.0093 ± 0.0005</td>
</tr>
<tr>
<td>2.5 g/l furfural</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Glucose</td>
<td>0.0017 ± 0.0002</td>
<td>0.999</td>
<td>0.00007</td>
<td>0.50</td>
<td>0.1405 ± 0.0311</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.0006 ± 0.0000</td>
<td>0.999</td>
<td>0.00005</td>
<td>0.32</td>
<td>0.0490 ± 0.0099</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.0011 ± 0.0000</td>
<td>0.998</td>
<td>NA</td>
<td>0.46</td>
<td>0.0934 ± 0.0001</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.0003 ± 0.0000</td>
<td>0.999</td>
<td>NA</td>
<td>0.33</td>
<td>0.0280 ± 0.0020</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.0006 ± 0.0000</td>
<td>0.978</td>
<td>0.00022</td>
<td>1.00</td>
<td>0.0527 ± 0.0025</td>
</tr>
<tr>
<td>5 g/l furfural</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.0010 ± 0.0003</td>
<td>0.996</td>
<td>0.00004</td>
<td>0.30</td>
<td>0.0765 ± 0.0280</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.0003 ± 0.0002</td>
<td>0.990</td>
<td>0.00002</td>
<td>0.16</td>
<td>0.0118 ± 0.0269</td>
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<tr>
<td>Ethanol</td>
<td>0.0005 ± 0.0003</td>
<td>0.997</td>
<td>NA</td>
<td>0.21</td>
<td>0.0471 ± 0.0233</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.0003 ± 0.0000</td>
<td>0.998</td>
<td>NA</td>
<td>0.33</td>
<td>0.0269 ± 0.0033</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.0010 ± 0.0000</td>
<td>0.982</td>
<td>0.00018</td>
<td>1.00</td>
<td>0.0853 ± 0.0002</td>
</tr>
<tr>
<td>10 g/l furfural</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Glucose</td>
<td>0.0003 ± 0.0002</td>
<td>0.942</td>
<td>0.00001</td>
<td>0.09</td>
<td>0.0244 ± 0.0106</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.0001 ± 0.0000</td>
<td>0.962</td>
<td>0.00001</td>
<td>0.06</td>
<td>0.0666 ± 0.0193</td>
</tr>
<tr>
<td>Ethanol</td>
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<td>0.965</td>
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<td>0.08</td>
<td>0.0231 ± 0.0039</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.0003 ± 0.0000</td>
<td>0.993</td>
<td>NA</td>
<td>0.33</td>
<td>0.0244 ± 0.0020</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.0015 ± 0.0001</td>
<td>0.993</td>
<td>0.00014</td>
<td>0.65</td>
<td>0.1314 ± 0.0081</td>
</tr>
</tbody>
</table>

The errors represent twice the sample standard deviation (95% confidence interval). NA = not applicable (as the exact concentration of metabolites such as ethanol and glycerol inside the panel is unknown the permeability coefficient cannot be measured), a = the flux, diffusion or release rate of compounds (g/h.cm²), b = a representation of the compatibility of the regression line to the sample readings (R²), c = coefficient relative to the ease at which compounds permeate through the membrane layer (cm/h), d = gram of compounds utilized, converted or produced per initial gram cells per hour (g/g.h).

Table 1
The flux, permeability and specific rate of conversion of different compounds in rMBR cultivation.

![Fig. 4. The comparison of the flux of different compounds through the single membrane layers of the IPC membrane in the diffusion cell with their conversion/release rate in rMBR at different furfural concentrations.](image-url)
(0.0003 ± 0.0002 g/cm².h) was comparable (p-value = 0.423) to that of wheat straw hydrolysate with 4.45 g/l and 21 g/l of furfural and xylose, respectively [24]. This drop in xylose utilization can be as high as 95% in presence of 1% furfural in the rMBR cultivation medium (Fig. 3a). More or less the same trend was observed in ethanol production and release rate (Figs. 4 and 3b).

The diffusion behavior of furfural as the cell inhibitory compound and the detoxification capabilities of the cells in rMBR system are of great importance to the robustness of a fermentation process. In order to prevent long lag phases and cell metabolic and physiologic damage during fermentation [14,19], conditions should be provided that cells can readily convert the toxic inhibitor at exposure. Surprisingly, as an outcome of rMBR application, the diffusion rate of furfural recorded for feed media with 2.5 and 5 g/l of furfural (Table 1) is about the same as the detoxification rate by yeast cells (Fig. 4). Having the same conversion (rMBR) to diffusion rate (diffusion cell) ratio (about 1:1) for 2.5 and 5 g/l initial furfural preparations while benefitting from around the same 0.82 ± 0.04 g/l starting cell concentration, shows that the conversion rate is not the limiting factor for furfural as it increases by the increase in furfural content. In the case of 2.5 g/l initial furfural, it can be concluded that although all the furfural passing through the membrane is being converted by cells to less inhibitory furfuryl alcohol, there seems to be no interference with the utilization of glucose and xylose as compared to non-inhibitory conditions. In this rMBR set-up, the specific rate of detoxification for initial furfural of 5 g/l (Table 1) was considerably higher than that reported (0.0051 g/g cells.h) for rMBR with wheat straw hydrolysate containing around 4.5 g/l of furfural by M. Ishola et al. [24]. However, it should be considered that in the actual wheat straw hydrolysate other inhibitory compounds such as HMF and weak acids are present that synergistically increase the toxicity of the medium. While doubling the initial furfural content to 10 g/l is only followed by 50% increase in the conversion rate, the conversion rate (rMBR) to diffusion rate (DC) ratio is about 0.62:1 (Fig. 4). This low detoxification rate to diffusion rate ratio describes the dramatic drop in the consumption of substrates and production of metabolites due to cell redox imbalance and cell metabolic and physiologic disturbance. However, the rMBR system provides substantially higher inhibitor tolerance levels compared to cultures with freely suspended cells that experience this dramatic shift in metabolic performance with furfural content of less than 2 g/l [23]. This is attributed to induced high local cell density that helps sustaining yeast activity up in inhibitory media.

The changes in the concentration of glycerol in the cultivation medium during anaerobic fermentation signal alternations in yeast cell’s redox balance [35]. The trend of changes in the amount of glycerol during rMBR fermentation is presented in Fig. 3b. In the non-inhibitory rMBR fermentation condition the lowest glycerol production rate was observed, although having a totally anaerobic fermentation. However, addition of a low amount of furfural leads to a 12-fold jump in the rate of glycerol production. The low concentration of furfural is adequate enough to disturb the redox balance and lead to increase in glycerol production [32,35]. However, as high concentrations of furfural can take the role of electron sink and oxidize the excess co-factors such as NADH that have been produced during anaerobic bioreactions, the concentration of glycerol plunes at 5 and 10 g/l of furfural. In addition, the bioconversion of furfural by yeast to less inhibitory compounds requires the help of oxidoreductases that benefit from the reducing power of NADH and NADPH. Therefore, when high concentration of furfural or 5-hydroxy methylfurfural is present, all other conversions including glycolysis chain reactions are affected by low co-factor levels [32].

3.3. Diffusion patterns of compounds through the cell-aggregate

Based on the results obtained during rMBR fermentation, four scenarios are visualized in Fig. 5. These four conditions (Fig. 5a–d) are built based on diffusion (concentration gradient) and conversion patterns of substrates and metabolites to ease the understanding of the phenomena described in details in Section 3.2. These hypothesized scenarios relatively vary depending on initial cell inoculum size, yeast strain, initial concentration of compound in the medium, membrane layers (quality, pore size etc.), membrane module (space between membrane layers etc.) and other factors.

As illustrated in Fig. 5a, in the non-inhibitory medium, glucose apparent permeability coefficient was higher than xylose (Table 1). Considering that when there is no inhibition, yeast rapidly and preferentially metabolizes glucose [6,24]. The cell layers in the frontier of the cell aggregate get involved in glucose utilization. The cells in the inner (close to center) of the cell aggregate that are glucose-starved tend to utilize the xylose. As a consequence there seems to be no lag in xylose consumption due to catabolite repression. The condition built based on glucose and xylose diffusion rate and conversion rate leads to simultaneous sugar consumption. This trend may differ for different recombinant xylose consuming yeast depending on whether they have separate xylose transporter proteins in their cell membrane or glucose transporters are to be used for both purposes.

At 2.5 g/l of initial furfural, furfural is being converted at the same rate as xylose is consumed (Fig. 4). Although in conditions with less furfural than practiced in this study, the inhibitor imposes a long lag phase on yeast activity, here detoxification of furfural readilys happens without metabolic disturbance as in cell encapsulation [23]. As in the projected image (Fig. 5b), this exposure to the inhibitory compound can divide the cell aggregate into three distinct regions: high-inhibitor/detoxification region, low-inhibitor/glucose consumption region and low-inhibitor and glucose/xylose consumption region. The initial 2:1 ratio of glucose to xylose was chosen based on the concentrations reported for wheat straw hydrolysate [27]. However, it can be foreseen that with choosing a ratio closer to 1:1 same conversion rate of glucose, xylose and detoxification of furfural can be achieved.

In contrast to the fermentation condition with 2.5 g/l furfural, the metabolic balance is dramatically disturbed as the concentrations of furfural rise to 5 and 10 g/l (Fig. 3). As the results of diffusion rate measurement in the diffusion cell show (Fig. 2), the diffusion rate of furfural through the membrane jumped 1.7 and 4 times from the condition with 2.5 g/l furfural to those with 5 and 10 g/l, respectively. This increase in flux of furfural through the membrane layers causes deeper infiltration of furfural (Fig. 5d) at the early stages of fermentation (Table 1) and causes cell toxicity in the cell aggregate confined to membrane layers. This change is well-projected in the drop of specific productivity of ethanol (g ethanol/g cells. h) from 0.093 to about half and one-third by doubling the initial furfural content to 5 and 10 g/l, respectively (Table 1). As cited in Table 1, more or less the same specific consumption (g substrate/g cells. h) trend applies to xylose and glucose with 86% plunge for both from the lowest to highest inhibitor content (Fig. 4). This could be expected as the presence of furfural and HMF during fermentation has a more noticeable impact on xylose consumption rate than glucose as these inhibitors sink the reduction potential (the NADH and NADPH content) and energy level (ATP, ADP and AMP) of the xylose-consuming yeast cells. However, these results cannot be fully compared with the results acquired from experiments with stepwise glucose and xylose consumption [32], as in this work the emphasis has been to prepare a condition for co-consumption of substrates. Considering that the cross-sectional distance (hollow space) between the two membrane layers in the IPC is 2 mm and the average diameter of the yeast used is ∼2.5 μm, increasing the distance between membrane layers and cell aggregate width, relative to increasing inhibitors level can be a further practice that may lead to enhanced co-consumption of sugars and detoxification of considerably high levels of furfural.

4. Conclusions

The results from rMBR fermentation experiments showed that
confinement of xylose-consuming yeast in between membrane layers can tackle the confronted issues with second generation bioethanol production such as prioritized fermentation of pentose and hexose sugars and high inhibitory content. The rMBR set up provided a desirable diffusion-based mass transfer conditions over the membrane layers of the used IPC membrane and cell aggregated that led to enhanced co-utilization xylose and glucose. In addition, the concentration gradient built over the high local concentration of cells boosted furfural tolerance and in situ detoxification capability of the cell aggregate in highly inhibitory media. The rMBR proved that it can cover the limitations confronted with cell encapsulation and replace it to be further applied for industrial production of second generation bioethanol.

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References

[16] V. Sánchez Nogué, K. Kachumaa, Xylose fermentation as a challenge for


Concentration-driven Reverse Membrane Bioreactor for the Fermentation of Highly Inhibitory Lignocellulosic Hydrolysate

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Abstract

Optimal production of lignocellulosic bioethanol is hindered due to commonly faced issues with the presence of inhibitory compounds and sequentially consumed sugars in the lignocellulosic hydrolysate. Therefore, in order to find a robust fermentation approach, this study aimed at enhancing simultaneous co-assimilation of sugars, and inhibitor tolerance and detoxification. Therefore, ethanol fermentation of toxic wheat straw hydrolysate containing up to 20 g/l furfural, using the concentration-driven cell encasement technique of reverse membrane bioreactor (rMBR) was studied. The rMBR fermentation of the hydrolysate led to complete furfural (1.7 g/l) detoxification, along with the conversion of 87% of sugars into ethanol at a yield of 0.48 g/g. Moreover, when the toxicity level of the hydrolysate was increased to 9 g/l of initial furfural, the system responded exceptionally by reducing 89% of the inhibitor while only experiencing about 25% drop in the ethanol yield. In addition, using this diffusion-based set-up in extremely inhibitory conditions (16 g/l furfural), cells could detoxify 40% of the furfural at a high initial furfural to cell ratio of 9.5:1. The diffusion-based rMBR set-up applied in this study proved that by properly synchronizing the medium condition, the membrane area, and the inhibitor to cell ratio, some of the shortcomings with conventional lignocellulosic fermentation can be tackled, guaranteeing a robust fermentation.

Keywords: reverse membrane bioreactor, wheat straw hydrolysate, diffusion rate, fermentation, inhibitor detoxification
1 Introduction

In order to mitigate dramatic climate changes and to meet the ever-growing demand for energy and fuel, reliable renewable energy and fuel sources should replace the over exploited fossil-based sources. Transport biofuels such as bioethanol are a part of this transition toward a low carbon economy [1, 2]. Concerns with the application of first generation bioethanol produced from sugar- and starch-based feedstocks have motivated the shift toward production of lignocellulosic bioethanol, which has the advantage of relatively cheap and abundant lignocellulosic biomasses such as agricultural (e.g., wheat straw) and forest residues that do not compete with food or feed [2]. However, the processing stages of lignocellulosic bioethanol production, mainly comprised of pretreatment, hydrolysis, fermentation, and ethanol recovery and purification, suffer from techno-economical complexities that hinder full capacity commercialization [3]. These issues originate from the recalcitrant nature of lignocellulosic material, composed of an intertwined matrix of cellulose, hemicellulose, and lignin [4]. Therefore, in order to produce ethanol, firstly, the lignocellulosic matrix should be disintegrated, structural porosity should be increased, and structural crystallinity should be reduced through pretreatment so that the effectivity of the enzymatic hydrolysis step is enhanced [4]. Thereafter, cellulose and/or hemicelluloses should be enzymatically hydrolyzed to fermentable sugars [5].

Although maximal sugar release is the main goal sought in consecutive pretreatment and hydrolysis of lignocelluloses, depending on the type of lignocellulosic biomass and the technique and intensity of the pretreatment and hydrolysis, other unwanted by-products are generated along with fermentable sugars [4, 6]. These fermentation inhibitory by-products can range from weak acids such as acetic acid (mainly generated from deacetylation of hemicellulose) and furans (furfural and 5-hydroxymethyl furfural (HMF) generated from the degradation of five and six carbon sugars, respectively) to phenolic compounds (products of lignin degradation) [7, 8]. Figure 1 presents the origin of some of the inhibitors generated during the lignocellulosic biomass pretreatment and hydrolysis. These inhibitors impose metabolic and physiological stress on yeast, resulting in an increased lag phase, reduction in the cell viability, drop in the intracellular pH, disturbance in the cell membrane integrity, negatively affecting some catabolic enzymes (e.g., pyruvate dehydrogenase, acetaldehyde dehydrogenase, etc.), consequently, hindering the fermentation yield and productivity [9-12]. In specific fermentation conditions, even as low as 1 g/l of furfural can hinder cell activity [9, 11]. The concomitant presence of these inhibitors bring along even higher synergistic toxicity, paralyzing the fermentation process [13].

The yeast *Saccharomyces cerevisiae*, with its high ethanol tolerance and production capacity, is the main microorganism of choice when it comes to industrial ethanol fermentation [14, 15]. Some of the above-mentioned inhibitors such as furfural and HMF can be detoxified to less inhibitory compounds such as
furfuryl alcohol and 2,5-bis-hydroxymethylfuran, respectively, by *S. cerevisiae*, whereas some others such as acetic acid are not biotransformed under anaerobic conditions [16-19]. However, direct exposure of yeast cells to a combination of these inhibitory compounds even at low concentrations can bring a batch fermentation to a halt [13, 20]. In this regard, it has been reported that if high local concentrations of yeast cells are provided naturally (e.g., flocculation) or by different immobilization techniques such as cell encapsulation, the cells’ inhibitor tolerance and detoxification capacity increases [21-23]. Another benefit of providing such cell-dense microenvironments as in cell encapsulation is the ability to have simultaneous consumption of different prioritized substrates [21, 22]. This is a functional remedy for the fermentation of lignocellulosic hydrolysate that contains sequentially consumed hexose (e.g., glucose) and pentose (e.g., xylose) sugars originating from the hydrolysis of cellulose and hemicellulose. Therefore, through such cell concentration, confinement and/or immobilization approaches, higher ethanol yields and productivity, and enhanced process robustness can be expected [21-23]. The key to enhanced tolerance and detoxification of inhibitors, and co-utilization of substrates in encapsulated cell cultures compared to freely suspended cells, is the type of mass transfer [21, 24, 25]. Although all cells are concomitantly exposed to highly inhibitory medium and a range of different substrates in suspended cell cultures, cells in the aggregates confined by the capsule membrane, experience a gradient of different substrates based on their positioning in the cell cluster (i.e., the location of the cells from the aggregate surface to the core) [21, 24, 25]. In this regard, as the cells closer to the cell aggregate and capsule membrane surface are exposed to high toxicity and substrate levels, the cells deeper in the core (away from the surface) experience sub-inhibitory concentrations and lower sugar availability. This phenomenon occurs due to the diffusional mass transfer mechanism built in the capsules in the absence of bulk mixing [24]. However, issues affiliated with capsule production and application limit their practical application in larger scales. These issues include a laborious and time consuming production process and capsule breakage due to production defects, severe medium agitation, and gas production [26-28].

The above-mentioned issues have been tackled using reverse membrane bioreactors (rMBRs) that benefit from synthetic membranes for cell encasement/confine ment [24]. Different rMBR configurations have successfully been used for the production of biogas and bioethanol [29-32]. The rMBR systems are formed from merging the cell encapsulation and immersed membrane bioreactors, and they function based on the concentration differences across the membrane as the main separation driving force [24]. The rMBR provides better processing flexibility, depending on the feed type and composition, and the type and scale of the bioconversion process. Although pressure-driven MBRs have been used at different stages of lignocellulosic bioethanol production proving substantial process intensification possibilities [3], technical challenges remain, concerning the conditioning of the pretreated lignocellulosic hydrolysate prior to
fermentation. Conventionally, in a post pretreatment and hydrolysis treatment stage, the pretreated slurry or hydrolysate is detoxified (e.g., alkaline treatment or ion exchange) from the inhibitors hindering fermentation [12], and C5- and C6-rich sugar streams generated from the hydrolysis of hemicelluloses and cellulose, respectively, are separated for fermentation. However, in order to maximize the process yield and feasibility, simultaneous bioconversion (detoxification) of highly inhibitory hydrolysate, and ethanol fermentation from both C5 and C6 sugars, should be targeted. This issue has been focused on, to some extent, in our previous work using a modeled xylose-glucose semi-synthetic medium containing different levels of furfural [32]; however, the robustness and potential of the application of rMBRs for bioconversion of actual lignocellulosic hydrolysate at different extreme inhibitory levels remain to be investigated.

The aim of this work was to apply the concentration-driven rMBR set-up in robust fermentation of wheat straw hydrolysate, containing up to 20 g/l of furfural to enhance the diffusion-based simultaneous pentose and hexose co-utilization, inhibitor tolerance and detoxification, and ethanol fermentation. In this regard, the rMBR using xylose-consuming recombinant yeast was exposed to high concentrations of inhibitory compounds to investigate the effect of substrates and inhibitors’ diffusion rates on bioconversion rates and overall process robustness. The study included the determination of the diffusion behavior of different hydrolysate constituents and metabolites using a diffusion cell, followed by a comparison with the recorded consumption, production, and detoxification rates of compounds by the stress-effected cell during rMBR fermentations. The achieved results present the advantages plus the application threshold of the rMBR set-up from an interesting and unconventional viewpoint.

2 Materials and methods

2.1. Diffusion rate measurement

In order to simulate the diffusion rate and flux of different compounds (substrates and metabolites) involved in the fermentation, a diffusion cell (Side-Bi-Side, PermeGear Inc., Hellertown, PA., U.S.A.) consisting of a donor and a receptor chamber (each 60 ml in volume) connected through an orifice of 7.07 cm² was applied. The chambers were separated by a single membrane layer isolated from a 2nd generation Integrated Permeate Channel (IPC) membrane panel used in rMBR fermentation (section 2.4) [33]. The donor compartment contained wheat straw hydrolysate with four different furfural concentrations (1.7±0.3, 9.0±0.5, 16.0±0.5, and 20.0±1.0 g/l), while the receptor chamber contained ethanol (15 g/l) and glycerol (5 g/l). The schematic of the applied diffusion cell is presented in our previous study [32]. In the current study, each diffusion cycle lasted 10 h with 2 h sampling intervals when 1 ml of aliquot was withdrawn from the
receptor cell and was replaced with the same amount of fresh receptor medium. Each diffusion experiment was conducted in duplicate.

Fick’s first law of diffusion (equation (1)) was considered as the basis for determining the diffusive flux of compounds through the membrane in the diffusion cell. The law relates diffusive flux \( J \) of compounds to their concentration gradient \( \frac{dc}{dx} \) at an assumed steady state:

\[
J = -D \frac{dc}{dx}
\]  
(1)

Where \( D \) is the diffusivity of compound, \( x \) is the diffusion distance, and \( c \) is the concentration.

Considering Fick’s first law of diffusion, the diffusive flux was defined as the amount of compound (g) passing per unit of membrane area (cm\(^2\)) per unit of time (h). In this regard, the cumulative amount of each compound that passed per unit area of the membrane was calculated according to the Thakker et al. [34] (equation (2)):

\[
Q = \left\{ C_n V + \sum_{i=1}^{n-1} C_i S \right\} / A
\]  
(2)

Where \( V \) is the volume of receptor chamber, \( A \) is the membrane surface area (cm\(^2\)), \( S \) is the sample volume (ml), \( C_n \) is the receptor chamber concentration (g/ml) at the nth sampling, and \( Q \) is the cumulative amount of a compound passed through a unit area of the membrane (g/cm\(^2\)).

Consequently, the slope of the regression line on the plotted graphs regarding the cumulative amount \( Q \) of each compound versus time (h) was considered as the diffusive flux (g/h.cm\(^2\)) of that compound [35].

2.2. Lignocellulosic biomass and enzymatic hydrolysis

Wheat straw, a xylose-rich agricultural residue provided in Sweden, was used as the model lignocellulosic substrate. The as-received dilute-acid pretreated (H\(_2\)SO\(_4\) (0.3–0.5%) at 185°C for 8 min) wheat straw slurry was provided by SEKAB (Örnsköldsvik, Sweden) and was kept at 4–5°C until use. The treated biomass was diluted to half its original concentration with Milli-Q water to reduce the amount of suspended solids (SS) to facilitate mixing during enzymatic hydrolysis. In order to increase the available fermentable monomeric sugars, further enzymatic hydrolysis was applied using cellulase Celli\(^{\circledast}\) Ctec2 enzyme (Novozymes, Denmark) with an activity of 130 FPU/ml, determined according to the NREL method [36]. Pre-hydrolysis, pH of the slurry was increased to 5.0 using 10 M NaOH. The enzymatic hydrolysis was conducted for 24 h at 50°C using an enzyme loading of 15 FPU/g SS. Following the hydrolysis, the liquid fraction of the hydrolysate was separated through decantation, autoclaved, and used for the rMBR fermentation.

2.3. Pre-culture and reactor medium preparation

A recombinant xylose-utilizing strain of \textit{Saccharomyces cerevisiae} [37] was used as the ethanologenic microorganism in this work. The yeast was loop inoculated on yeast extract peptone dextrose (YPD) plates
and incubated at 30°C for three days, followed by storage at 4°C prior to use. The cell pre-cultures were prepared in 2 L Erlenmeyer flasks containing 500 ml of broth consisting of 20 g/l glucose, 10 g/l xylose, 5 g/l peptone, and 5 g/l yeast extract. The flasks were loop inoculated and incubated in a shaking water bath (115 rpm) (Grant OLS 200, Grant Instrument Ltd., U.K.) at 30°C for 48 h. In order to achieve high cell concentrations for further membrane panel inoculation, the 2000 ml preculture medium was then concentrated 20-times by centrifugation (3000×g for 2 min). The concentrated inoculum contained 64 ± 5 g/l yeast. The concentrated inoculum was aseptically syringe injected into the membrane-confined hollow space of four IPC membrane panels, resulting in a total cell concentration of 1.9 ± 0.2 g/l.

Batch rMBR experiments were conducted using a hydrolysate containing 41.3 ± 2.8 g/l glucose, 22.2 ± 1.7 g/l xylose, and different furfural concentrations (1.7–20.0 g/l). Batch fermentation cycles were carried out for 72 h, and samples were taken from the reactor medium at different time intervals to monitor the substrate consumption (glucose and xylose), furfural conversion, and metabolites production (ethanol and glycerol).

2.4. Membrane module and reverse membrane bioreactor set-up

In this study, four 2nd generation IPC membrane panels developed by the Flemish Institute for Technological Research (VITO NV, Mol, Belgium) were integrated into the reactor. The membrane panels consist of double membrane layers of polyethersulfone (PES)/Polyvinylpyrrolidone (PVP), with a pore size of 0.3 μm casted onto a weft-type spacer-fabric support (membrane layer thickness ~650 μm). The inter-membrane hollow space of the IPC panels, provided by the 2 mm distance between the two adjacent membrane layers with 85% open volume, is used for housing high a concentration of yeast cells. Each membrane panel had a total membrane area of 68.6 cm².

The schematic configuration of the rMBR used in the current research is shown in Figure 2. The membrane panels were immersed in a 2 L water-jacketed bioreactor (Biostat B plus, Sartorius BBI Systems GmbH, Germany) with a working volume of 1.7 L. Before each experiment, the bioreactor and tubings were autoclaved at 121°C for 20 min. As the membrane panels cannot be sterilized by autoclaving, they were chemically cleaned and disinfected before each fermentation experiment, according to Mahboubi et al. [38]. After the complete sterilization of the rMBR and prior to the addition of the hydrolysate, 25 ml of concentrated inoculum was injected to each membrane panel. In order to facilitate degassing of the membrane envelope from the CO₂ produced during the fermentation, air filters were attached to the ports used primarily for cell inoculation (Figure 2). Afterwards, to have anaerobic fermentation condition and a homogenized medium, N₂ gas was sparged into the reactor for 10 min. A tube with inner and outer diameters of 8.0 mm 12.6 mm, respectively, was used for recirculating the reactor medium using a 403U/R1 peristaltic pump (Watson Marlow, Falmouth, England). The temperature and pH of the medium were maintained at 30°C and 5.0, respectively, through the Biostat B plus fermentation-controlling unit.
2.5. Analytical methods
The concentration of different substrates and metabolites in the rMBR and diffusion cell were determined using high performance liquid chromatography (HPLC) (Walters 2695, Walters Corporation, Milford, U.S.A.), with a hydrogen-based column (Aminex HPX-87H, Bio-Rad, Hercules, U.S.A.) working at 60°C with 0.6 ml/min eluent (5 mM H₂SO₄) flow rate. The samples that were taken at different time intervals were centrifuged at 5000×g for 3 min, and the supernatant was used for the HPLC analysis. The inoculum cell dry weight was measured by sampling 5 ml of the medium in duplicate, centrifuging at 3000×g for 5 min, replacing the supernatant with Milli-Q water (repeated three-times), and drying the cell pellet in 70°C oven for 24 h. The error bars presented on the graphs represent the standard deviation of duplicate experiments and samples.

3 Results and discussion
In order to have a better understanding of the phenomena observed during fermentation using an rMBR system, an estimate of the diffusion rate of the different compounds involved in fermentation through the applied membrane layer is essential. In this regard, first, the counter diffusion rate of the main compounds in the wheat straw hydrolysate and fermentation products was measured using a side-by-side diffusion cell having an isolated membrane layer-spacer fabric (Figure 3). Thereafter, rMBR fermentation of the wheat straw hydrolysate was performed with and without different levels of added furfural.

3.1 Diffusive flux of compounds through the membrane
The diffusion rates were measured by placing the liquid fraction of the wheat straw hydrolysate against a receptor solution containing ethanol and glycerol (representing fermentation metabolites), separated by a single membrane layer. Figure 3 presents the cumulative amount (g) of an individual compound that diffused through the membrane plotted versus time (h). As described in section 2.1, based on equations (1) and (2), the slope of the regression line presents the diffusive fluxes [24].

Conform Fick’s first law of diffusion (section 2.1), the diffusion of a compound through the membrane is directly related to its concentration in the wheat straw hydrolysate. The dominant sugars in the hydrolysate are glucose and xylose, with the former having about double the concentration of the latter. Accordingly, the glucose diffusion flux was 1.8-times faster (0.0060 g/h.cm²) compared to xylose (0.0033 g/h.cm²) (Figure 3). This complies with the results achieved for the diffusion rates measured using a xylose-glucose semi-synthetic medium [32]. The higher diffusion rate of glucose compared to xylose becomes problematic.
when a xylose-consuming yeast that prioritizes hexose consumption is used as the ethanologenic microorganism. In such condition, glucose suppresses xylose utilization until glucose is highly depleted, which hinders reaching similar sugar uptake rates [24, 39, 40].

Wheat straw hydrolysate contains a combination of lignocellulosic inhibitors. Considering the low initial concentration of HMF (0.16 g/l) in the hydrolysate used in this study, its lower inhibitory effect on cells, and slower bioconversion rate compared to furfural [41], the focus was on the other inhibitory compounds acetic acid and furfural. Furfural can be converted into the less inhibitor compound furfuryl alcohol by yeast, while acetic acid is not biotransformed during anaerobic fermentation [16-18]. Therefore, different furfural concentrations of up to 20 g/l were used in the diffusion rate measurement, while acetic acid was kept at its initial levels (3.8 g/l). As expected, the diffusion rate of furfural changed proportionally to the initial concentration, resulting in fluxes of 0.0004, 0.0017, 0.0028, and 0.0041 g/h.cm² corresponding to 1.7 ± 0.3 g/l (hydrolysate), 9.0 ± 0.5 g/l, 16.0 ± 0.5 g/l, and 20.0 ± 1.0 g/l of the initial furfural, respectively. The acetate diffusive flux in the as-received hydrolysate was 0.0007 g/h.cm². Moreover, the fermentation metabolites, ethanol (15.0 ± 2.2 g/l) and glycerol (5.0 ± 1.0 g/l), had mass fluxes of 0.0029 and 0.0008 g/h.cm², respectively, from the donor to the receptor cell.

3.2 Conversion, consumption, and production of different compounds during rMBR fermentation

In a next step rMBR fermentation was performed on the liquid fraction of the wheat straw hydrolysate. The initial hydrolysate contained about 64 g/l of glucose and xylose, with a glucose to xylose ratio of approximately 1.8. As observed during the diffusion rate measurement (Figure 3), glucose reaches the cell envelope 1.8-times faster than xylose; consequently, it is consumed 2.7-times faster than xylose (0.0014 g/h.cm²) (Table 1). Interestingly, both sugars were simultaneously consumed by cells due to the concentration gradient built through the cell aggregate [24]. However, glucose and xylose consumption rates were about 0.63- and 0.42-times slower than their measured diffusion rates in the diffusion cell, respectively (Table 1). This is probably due to the fact that the diffusion rates determined in the diffusion cell only present the fluxes through the membrane in well-mixed receptor and donor compartments, disregarding the resistance imposed by the packed cell aggregate or rather stagnant shell-side bulk medium in an rMBR. After 72 h of fermentation about 87% (Figure 4) of initial sugars were consumed leading to a specific sugar consumption rate of 0.43 g sugars consumed/g cells.h (Table 1).

As presented in Figure 3, for the as-received hydrolysate the diffusion rates of the main inhibitors, acetic acid (0.0007 g/h.cm² at initial concentration of 4.0 ± 0.5 g/l) and furfural (0.0004 g/h.cm² at initial
concentration of 1.7± 0.4 g/l), are much lower than those of sugars. In this rMBR fermentation condition, benefitting from an initial furfural to cell ratio of 0.93:1, a complete furfural bioconversion was achieved within 40 h of fermentation. Although these concentrations of lignocellulosic degradation products have been reported to considerably disturb the fermentation [11, 12], such effects were not observed in this rMBR set-up. According to the hypothetical scenarios presented previously [24], this represents sub-inhibitory conditions in which the cells’ physiological and metabolic state are not severely affected as the low diffusion rate of furfural only affects a fraction of cells located in the surface of the cell aggregate, while cells placed in the deeper layers continue to thrive on the nutrients provided in a sub-inhibitory microenvironment. As reported in the literature, lignocellulosic inhibitors primarily affect cell reproduction and biosynthesis activities, and may even increase the ethanol yield on glucose and xylose [7, 12, 42]. Moreover, at low furfural levels (up to 2.8 g/l), furfural acts as an electron sink regenerating the required co-factor, NAD⁺, from surplus NADH, carbon consumption required for glycerol production drops, and the ethanol yield on substrates increases [43]. Also in our tests, the sub-inhibitory levels resulted in the production of 27.4 ± 0.6 g/l of ethanol (Figure 4) presenting a high yield of 0.48 g ethanol/g sugars (94% of the theoretical yield) corresponding to a specific ethanol productivity of 0.2 g/g.h and an ethanol release rate of 0.0025 g/h.cm² back to the reactor bulk media. This flux of ethanol away from the cell aggregate toward the bulk medium is about 86% of the diffusion rate in the diffusion cell with an initial ethanol concentration of 15 g/l.

Increasing the initial furfural content of the hydrolysate to about 9 g/l, resulted in a furfural to cell ratio of 5:1, and reduced the overall sugar consumption to nearly 42% (Figure 4). The flux of glucose and xylose utilization dropped about 2.2- and 3.5-times, respectively, compared to that of the original hydrolysate (Table 1), corresponding to 28% and 12% of the diffusion rate estimated from the diffusion cell measurements. This indicates that as the cells experience a higher initial inhibitor concentration and diffusion rate (0.0017 g/h.cm² in diffusion cell), more layers of cells in the cell aggregate are involved in furfural detoxification and less total sugar is utilized. Such a scenario is visualized in our previous work considering rMBR fermentation of xylose-glucose semi-synthetic media containing different concentrations of furfural [32]. Considering that all other conditions were kept the same, the changes in the sugar uptake can only be an effect of the higher inhibitor concentration on cell viability and catabolic activity. As the amount of furfural increases, the cell requires more maintenance energy for cellular damage made to the cell chromatin, cytoskeleton, membrane etc. [12]. Therefore, the reason for the higher reduction in xylose consumption compared to glucose may be glucose repression and lower energy generation (ATP production) by xylose metabolism compared to glucose [44]. Another reason can be that xylose conversion by yeast is more co-factor (NAD(P)H) dependent and that in the presence of furfural as an electron acceptor, the level of these co-factors plunges [45]. As has been comprehensively reviewed by Almeida et al. [12],
A considerably high ethanol yield of 0.36 g/g (70.5% of the theoretical yield) and specific productivity of 0.07 g/g.h (10.0 ± 1.1 g/l after 72 h) was obtained (Figure 4). Although the initial concentration and hence the diffusion rate (assumed based on the diffusion cell measurements) of furfural increased nearly 5.2- and 4.2-times, respectively, the confined cell aggregate incased between the membrane layers performed exceptionally by detoxifying about 89% of the added furfural in 72 h (Figure 4).

Considering the concomitant presence of different lignocellulosic inhibitors in the hydrolysate and their synergistic effect on cell activity [13], this rMBR system had a robust performance regarding inhibitor tolerance and detoxification, and simultaneous sugar consumption when compared to fermentation experiments conducted in our previous work using semi-synthetic media containing 1 to 10 g/l of furfural [32]. The aforementioned semi-synthetic rMBR fermentation experiments had half the initial sugar content (same glycoce to xylose ratio), membrane area (two IPC panels), and inoculum size compared to the current study. In the semi-synthetic media containing 10 g/l of furfural, as the only inhibitor, just around 3% of the total sugars were consumed in 48 h [32], whereas in this study, cells could utilize 32% of the initial sugars in 48 h in a hydrolysate containing about 9 g/l of furfural plus other cell toxic compounds. In addition to the membrane area provided for in the diffusive transfer of compounds in and out of the membrane encased cell space, proper dispersion of cells throughout the panel is of great importance. The 2nd generation IPC panels used in this study are not specifically tailored for rMBR cell encasement; therefore, the smaller the panels, the better the control over cell dispersion. Ishola et al. [28] benefited from 2.8-times and 4.5-times more membrane area (three membrane panels, each having about 3-times the membrane area as the panels used in this research work), and initial inoculum size, respectively, while treating wheat straw hydrolysate containing around the same sugar content compared to this study. However, during the rMBR fermentation, despite having the same furfural detoxification flux, Ishola et al. [28] experienced a glucose consumption flux of 0.0025 g/h.cm², which is 36% less than what was obtained in the current research work, resulting in 15% less ethanol production in 48 h.

Although the high concentration of cells confined between the membrane layers experiences a gradual exposure to the inhibitors, for every rMBR system there is an inhibitor threshold based on the inoculum size and the cell aggregate thickness (also compactness), above which, the diffusion rate surpasses the detoxification rate, and the fermentation comes to a halt. For the conditions used in our tests, this threshold is reached at 16 g/l, because the furfural bioconversion rate only increased 50% to 0.0009 g/h.cm² (Table 1).
when increasing the furfural levels from 9 to 16 g/l (furfural to cell ration of 9.5:1). Surprisingly, even in this condition, cells were capable of sustaining their viability up to 40 h, reaching a furfural conversion rate of 3.5 g/gcells. However, after 40 h, a drop in the specific conversion rate occurs (Table 1), indicating that the metabolic activities such as detoxification and sugar utilization came to a halt. This can be due to an extreme cell redox imbalance, inhibition of enzymes such as alcohol dehydrogenase, hexokinase, and glyceraldehyde-3-phosphate dehydrogenase, and other cell structural damages caused by long-term exposure to high furfural content [7, 12]. In this condition, the furfural conversion rate plunged to less than 5.5% of the conversion rate experienced prior to 40 h (Table 1). In the presence of the surplus of inhibitor, only 11% of the sugars were consumed in 72 h (Figure 4). Furthermore, ethanol yield dropped to 0.28 g/g, and specific ethanol productivity to 0.013 g/g.h, which is only 6% of that achieved with the original hydrolysate (Table 1). At an even higher initial furfural concentration of around 20 g/l, the cell’s detoxification capacity was clearly exceeded, resulting in only 8% of sugar utilization and 0.8 ± 0.1 g/l of ethanol production (ethanol yield of 16%) (Figure 4). In this extreme condition, the whole cell aggregate is inhibitor-stricken. Comparing the results obtained in our rMBR tests, to those of Mahboubi et al. [32] and Ishola et al. [28] that applied a rather similar submerged rMBR set-up, shows that the inoculum size, furfural to inoculum ratio, membrane area and membrane panel size play a critical role in an rMBR. However, the extent at which each of these factors affect the performance of an rMBR process requires further investigation.

4 Conclusions
Diffusion-based rMBR fermentations were run on dilute-acid pretreated wheat straw hydrolysate containing low to extreme levels of inhibitory compounds. The results showed the benefits of this membrane-assisted cell encasement approach. High local concentrations of xylose-consuming yeast and concentration-driven mass transfer of substrates and inhibitors to the cell aggregate led to enhanced simultaneous consumption of xylose and glucose, and in situ detoxification in a hydrolysate containing up to 16 g/l furfural (furfural to cell ratio of 9.5:1). The applied rMBR set-up in this study proved that by properly adjusting factors such as membrane area and inhibitor to cell ratio, a robust diffusion-based fermentation can be obtained.
5 Acknowledgements

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References


Table 1. The flux and specific conversion, utilization, or production rate of different compounds in the rMBR fermentation.

<table>
<thead>
<tr>
<th>Medium</th>
<th>rMBR Flux* (g/h.cm²)</th>
<th>rMBR/diffusion cellb</th>
<th>Specific conversion ratec (g/gcells.h)</th>
<th>Specific conversion rate per unit membrane area (g/gcells.h.cm²)×10⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysate 1.7±0.3 g/l furfural</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.00380 ± 0.00000</td>
<td>0.633</td>
<td>0.3121 ± 0.0012</td>
<td>0.110 ± 0.000</td>
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<tr>
<td>Xylose</td>
<td>0.00140 ± 0.00000</td>
<td>0.424</td>
<td>0.1156 ± 0.0011</td>
<td>0.040 ± 0.000</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.00030 ± 0.00000</td>
<td>0.375</td>
<td>0.0236 ± 0.0005</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.00250 ± 0.00000</td>
<td>0.862</td>
<td>0.2036 ± 0.0002</td>
<td>0.070 ± 0.000</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.00010 ± 0.00000</td>
<td>0.250</td>
<td>0.0111 ± 0.0005</td>
<td>0.004 ± 0.000</td>
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<td>Hydrolysate 9.0±0.5 g/l furfural</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
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<td>0.283</td>
<td>0.1561 ± 0.0073</td>
<td>0.060 ± 0.010</td>
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<td>Xylose</td>
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<td>0.121</td>
<td>0.0350 ± 0.0055</td>
<td>0.010 ± 0.000</td>
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<tr>
<td>Glycerol</td>
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<td>0.0385 ± 0.0009</td>
<td>0.010 ± 0.000</td>
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<td>0.275</td>
<td>0.0720 ± 0.0038</td>
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<tr>
<td>Furfural</td>
<td>0.00060 ± 0.00000</td>
<td>0.352</td>
<td>0.0545 ± 0.0009</td>
<td>0.020 ± 0.000</td>
</tr>
<tr>
<td>Hydrolysate 16.0±0.5 g/l furfural</td>
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<td></td>
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<tr>
<td>Glucose</td>
<td>0.00080 ± 0.00010</td>
<td>0.133</td>
<td>0.0782 ± 0.0058</td>
<td>0.030 ± 0.000</td>
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<tr>
<td>Xylose</td>
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<td>0.0102 ± 0.0009</td>
<td>0.004 ± 0.001</td>
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<td>Glycerol</td>
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<td>0.0062 ± 0.0009</td>
<td>0.002 ± 0.001</td>
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<tr>
<td>Ethanol</td>
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<td>0.034</td>
<td>0.0131 ± 0.0005</td>
<td>0.005 ± 0.000</td>
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<tr>
<td>Furfural</td>
<td>0.00090 ± 0.00010</td>
<td>0.321</td>
<td>0.0884 ± 0.0055</td>
<td>0.030 ± 0.000</td>
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<td></td>
<td>0.00005 ± 0.00002</td>
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<td>0.0052 ± 0.0019</td>
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<tr>
<td>Hydrolysate 20.0±1.0 g/l furfural</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Glucose</td>
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<td>0.033</td>
<td>0.0179 ± 0.0013</td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td>Xylose</td>
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<td>0.030</td>
<td>0.0083 ± 0.0002</td>
<td>0.003 ± 0.000</td>
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<tr>
<td>Glycerol</td>
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<td>0.0078 ± 0.0001</td>
<td>0.003 ± 0.000</td>
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<tr>
<td>Ethanol</td>
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<td>0.020</td>
<td>0.0043 ± 0.0002</td>
<td>0.002 ± 0.001</td>
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<tr>
<td>Furfural</td>
<td>0.00040 ± 0.00010</td>
<td>0.097</td>
<td>0.0275 ± 0.0077</td>
<td>0.010 ± 0.003</td>
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</table>

* = the flux presenting the rate of conversion, utilization, or production of compounds in rMBR. b = the ratio of rMBR flux to the flux of compounds in the diffusion cell. c = gram of compounds utilized, converted or produced per initial gram cells per hour. d = results for furfural conversion and glucose consumption before and after 40 h are presented separately.
Figure 1. The origin of the main inhibitory compounds (dashed lines) generated during the pretreatment and hydrolysis of lignocellulosic biomass.
Figure 2. Schematic of the concentration-driven rMBR set-up used in the current study.
Figure 3. Changes in the amount (g) of compounds involved in the fermentation of the wheat straw hydrolysate passing through the membrane per unit area (cm²) by time (h) in the diffusion cell.

- Glucose: $y = 0.006 X + 0.0045$  
  $R^2 = 0.996$
- Furfural 20 g/l: $y = 0.0041X + 0.004$  
  $R^2 = 0.9937$
- Xylose: $y = 0.0033 X + 0.0026$  
  $R^2 = 0.996$
- Ethanol: $y = 0.0029 X + 0.0029$  
  $R^2 = 0.9956$
- Furfural 16 g/l: $y = 0.0028 X + 0.0033$  
  $R^2 = 0.994$
- Furfural 9 g/l: $y = 0.0017 X + 0.0007$  
  $R^2 = 0.9854$
- Glycerol: $y = 0.0008 X + 0.0009$  
  $R^2 = 0.9929$
- Acetic acid: $y = 0.0007 X + 0.0013$  
  $R^2 = 0.9863$
- Furfural 1.7 g/l: $y = 0.0004 X + 0.0002$  
  $R^2 = 0.9638$

'n
Figure 4. Changes in the amounts glucose and xylose consumed, ethanol produced, and furfural converted in the rMBR fermentation of wheat straw hydrolysate containing 1.7, 9, 16, and 20 g/l of furfural.