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Diffusion-based reverse membrane bioreactor for simultaneous bioconversion of high-inhibitor xylose-glucose media

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ABSTRACT

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 encasement. 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 cm^2). 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).

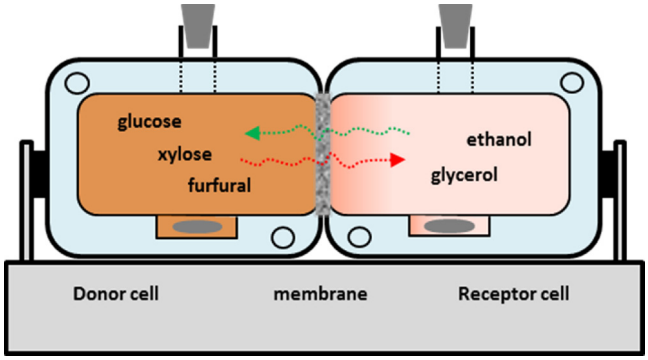


Fig. 1. The schematic of the side-by-side diffusion cell used in this study.

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]:

$$Q = \left\{ C_n V + \sum_{i=1}^{n-1} C_i S \right\} / A \tag{1}$$

Where Q is the cumulative amount of a compound passed through the surface area of the membrane (g/cm^2), 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 cm^2), C_n is the receptor cell concentration (g/ml) at the nth sampling and $\sum_{i=1}^{n-1} C_i S$ is the total amount of a compound released from the 1 st 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 (K_p) of compounds through the membrane layer was estimated using Eq. (2) [30]:

$$K_p = \left(\frac{1}{AC_0}\right)\left(\frac{dM}{dt}\right) \tag{2}$$

Where A is the membrane surface area, C_0 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 KH_2PO_4, 3 g/l $(NH_4)_2SO_4$ 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 600 ppm of hops acid were added to the reactor, respectively. 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 ~650 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 foulants 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 cm^2. 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.m^2.bar of clean water permeability.

The rMBR used in this study consisted of two such customized 2nd-generation IPC membrane panels immersed in a 3 l 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%, H_3PO_4 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 rec006Fvered 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 H_2SO_4 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

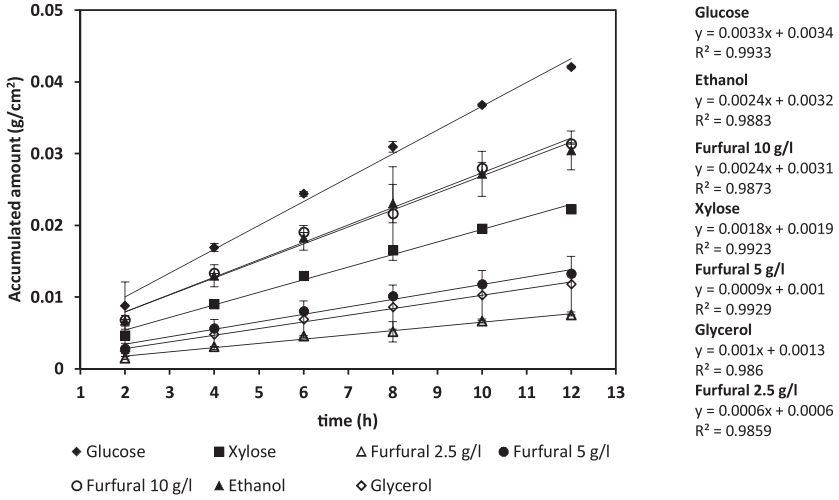


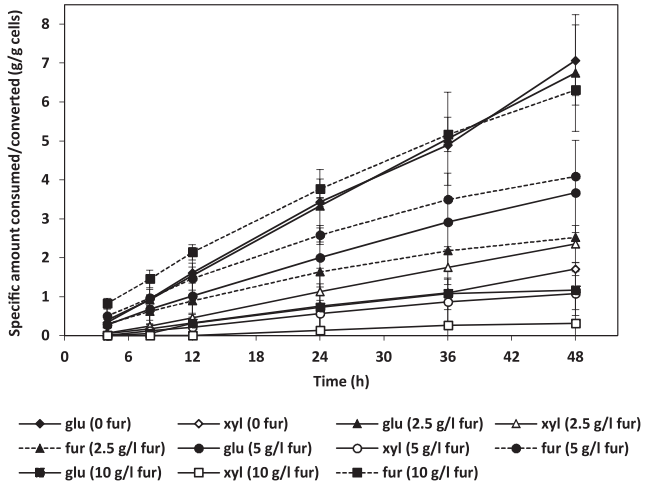
Fig. 2. Changes in the accumulated amount of different compounds (g/cm^2) by time in the diffusion cell. The slope of the regression line represents the diffusion rate (g/cm^2.h) of compounds through a single membrane layer of an IPC membrane.

glucose (0.0033 ± 0.0003 g/cm^2.h) than xylose (0.0018 ± 0.0001 g/cm^2.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 ± 0.0001 and 0.0009 ± 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 ± 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 ± 0.0004 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-



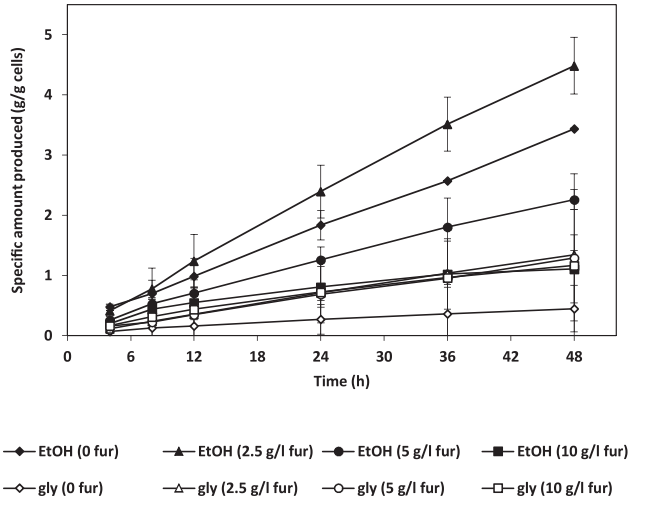


Fig. 3. Changes in the specific amount (g/g cells) of different substrates and metabolites released or converted over time during rMBR fermentation.

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

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

Medium		Flux[a]	Flux coefficient of determination[b]	Apparent permeability coefficient[c]	rMBR/diffusion cell flux	Specific rate of conversion[d]
No furfural	Glucose	0.0017 ± 0.0004	0.996	0.00007	0.52	0.1473 ± 0.0190
	Xylose	0.0004 ± 0.0001	0.989	0.00003	0.22	0.0355 ± 0.0035
	Ethanol	0.0008 ± 0.0000	0.997	NA	0.33	0.0716 ± 0.0025
	Glycerol	0.0001 ± 0.0000	0.984	NA	0.11	0.0093 ± 0.0005
2.5 g/l furfural	Glucose	0.0017 ± 0.0002	0.999	0.00007	0.50	0.1405 ± 0.0311
	Xylose	0.0006 ± 0.0000	0.999	0.00005	0.32	0.0490 ± 0.0099
	Ethanol	0.0011 ± 0.0000	0.998	NA	0.46	0.0934 ± 0.0001
	Glycerol	0.0003 ± 0.0000	0.999	NA	0.33	0.0280 ± 0.0020
	Furfural	0.0006 ± 0.0000	0.978	0.00022	1.00	0.0527 ± 0.0025
5 g/l furfural	Glucose	0.0010 ± 0.0003	0.996	0.00004	0.30	0.0765 ± 0.0280
	Xylose	0.0003 ± 0.0002	0.990	0.00002	0.16	0.0118 ± 0.0269
	Ethanol	0.0005 ± 0.0003	0.997	NA	0.21	0.0471 ± 0.0233
	Glycerol	0.0003 ± 0.0000	0.998	NA	0.33	0.0269 ± 0.0033
	Furfural	0.0010 ± 0.0000	0.982	0.00018	1.00	0.0853 ± 0.0002
10 g/l furfural	Glucose	0.0003 ± 0.0002	0.942	0.00001	0.09	0.0244 ± 0.0106
	Xylose	0.0001 ± 0.0000	0.962	0.00001	0.06	0.0066 ± 0.0193
	Ethanol	0.0002 ± 0.0001	0.965	NA	0.08	0.0231 ± 0.0039
	Glycerol	0.0003 ± 0.0000	0.993	NA	0.33	0.0244 ± 0.0020
	Furfural	0.0015 ± 0.0001	0.993	0.00014	0.63	0.1314 ± 0.0081

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.cm^2), [b] = a representation of the compatibility of the regression line to the sample readings (R^2), [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).

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/cm^2.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

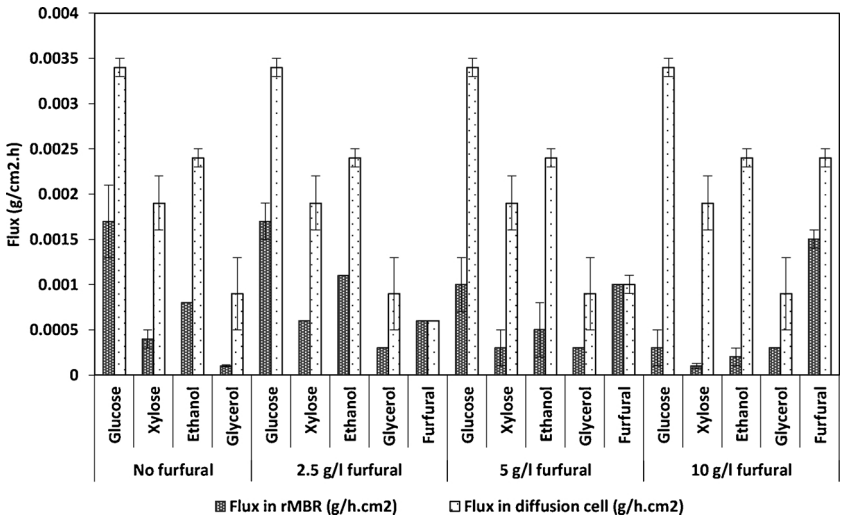


Fig. 4. The comparison of the flux of different compounds through the single membrane layers of the IPC membrane in the diffusion cell with their conversion/release rate in rMBR at different furfural concentrations.

(0.0003 ± 0.0002 g/cm^2.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 plunges 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-hydroxyl 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 readily 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

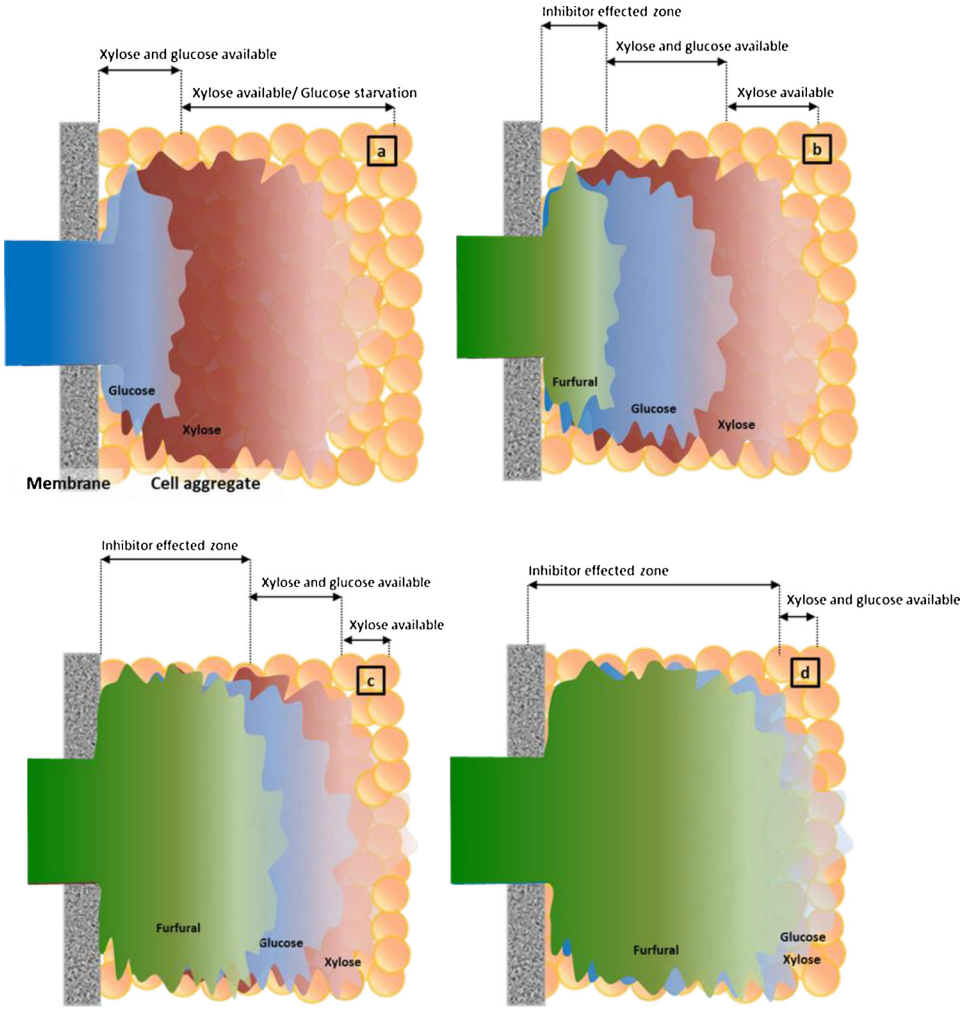


Fig. 5. Schematic of the four different conditions built up in the cell aggregate due to concentration gradient of different medium components in rMBR fermentation: (a) zero, (b) 2.5 g/l, (c) 5 g/l and (d) 10 g/l furfural.

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

[1] P. Le-Clech, V. Chen, T.A.G. Fane, Fouling in membrane bioreactors used in wastewater treatment, J. Membr. Sci. 284 (2006) 17–53.
[2] S. Judd, The MBR Book, second edition, Butterworth-Heinemann, Oxford, 2011.
[3] S.H. Yoon, Membrane bioreactor processes: principles and applications, Advances in Water and Wastewater Transport and Treatment, CRC press, 2015, p. 452.
[4] P.S. Nigam, A. Singh, Production of liquid biofuels from renewable resources, Prog. Energy Combust. Sci. 37 (2011) 52–68.
[5] P. Ylitervo, J. Akinbomi, M.J. Taherzadeh, Membrane bioreactors potential for ethanol and biogas production: a review, Environ. Technol. (UK) 34 (2013) 1711–1723.
[6] A. Mahboubi, P. Ylitervo, W. Doyen, H. De Wever, M.J. Taherzadeh, Reverse membrane bioreactor: introduction to a new technology for biofuel production, Biotechnol. Adv. 34 (2016) 954–975.
[7] T.R. Brown, R.C. Brown, A review of cellulosic biofuel commercial-scale projects in the United States, Biofuels Bioprod. Biorefin. 7 (2013) 235–245.
[8] A. Limayem, S.C. Ricke, Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects, Prog. Energy Combust. Sci. 38 (2012) 449–467.
[9] D.P. Maurya, A. Singla, S. Negi, An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol, 3 Biotech 5 (2015) 597–609.
[10] P. Börjesson, S. Ahlgren, G. Berndes, The climate benefit of Swedish ethanol: present and prospective performance, Wiley Interdiscip. Rev. Energy Environ. 1 (2012) 81–97.
[11] E. Gnansounou, Production and use of lignocellulosic bioethanol in Europe: current situation and perspectives, Bioresour. Technol. 101 (2010) 4842–4850.
[12] S.N. Naik, V.V. Goud, P.K. Rout, A.K. Dalai, Production of first and second generation biofuels: a comprehensive review, Renew. Sustain. Energy Rev. 14 (2010) 578–597.
[13] M.J. Taherzadeh, K. Karimi, Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review, Int. J. Mol. Sci. 9 (2008) 1621–1651.
[14] F. Monlau, C. Sambusiti, A. Barakat, M. Quéméneur, E. Trably, J.P. Steyer, H. Carrère, Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review, Biotechnol. Adv. 32 (2014) 934–951.
[15] A. Matsushika, H. Inoue, T. Kodaki, S. Sawayama, Ethanol production from xylose in engineered Saccharomyces cerevisiae strains: current state and perspectives, Appl. Microbiol. Biotechnol. 84 (2009) 37–53.
[16] V. Sànchez Nogué, K. Karhumaa, Xylose fermentation as a challenge for

commercialization of lignocellulosic fuels and chemicals, Biotechnol. Lett 37 (2015) 761–772.
[17] F. Li, S. Tian, Y. Yu, G. Zhou, X. Yang, Ethanol production from dilute-acid hydrolysate in situ detoxification by co-culture of yeasts, Taiyangneng Xuebao/Acta Energiae Solaris Sinica 30 (2009) 1582–1586.
[18] Z.L. Liu, Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates, Appl. Microbiol. Biotechnol. 90 (2011) 809–825.
[19] J.R.M. Almeida, T. Modig, A. Petersson, B. Hähn-Hägerdal, G. Lidén, M.F. Gorwa-Grauslund, Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by Saccharomyces cerevisiae, J. Chem. Technol. Biotechnol. 82 (2007) 340–349.
[20] J.O. Westman, R.B. Manikondu, C.J. Franzén, M.J. Taherzadeh, Encapsulation-induced stress helps Saccharomyces cerevisiae resist convertible lignocellulose derived inhibitors, Int. J. Mol. Sci. 13 (2012) 11881–11894.
[21] J.O. Westman, V. Mapelli, M.J. Taherzadeh, C.J. Franzén, Flocculation causes inhibitor tolerance in Saccharomyces cerevisiae for second-generation bioethanol production, Appl. Environ. Microbiol. 80 (2014) 6908–6918.
[22] P. Ylitervo, C.J. Franzén, M.J. Taherzadeh, Ethanol production at elevated temperatures using encapsulation of yeast, J. Biotechnol. 156 (2011) 22–29.
[23] J.O. Westman, N. Bonander, M.J. Taherzadeh, C.J. Franzén, Improved sugar co-utilisation by encapsulation of a recombinant Saccharomyces cerevisiae strain in alginate-chitosan capsules, Biotechnol. Biofuels 7 (2014) 102.
[24] M. Ishola, P. Ylitervo, M. Taherzadeh, Co-Utilization of glucose and xylose for enhanced lignocellulosic ethanol production with reverse membrane bioreactors, Membranes 5 (2015) 844.
[25] S. Youngsukkasem, J. Akinbomi, S.K. Rakshit, M.J. Taherzadeh, Biogas production by encased bacteria in synthetic membranes: protective effects in toxic media and high loading rates, Environ. Technol. (UK) 34 (2013) 2077–2084.
[26] S.-F. Ng, J.J. Rouse, F.D. Sanderson, V. Meidan, G.M. Eccleston, Validation of a static Franz diffusion cell system for in vitro permeation studies, AAPS PharmSciTech 11 (2010) 1432–1441.
[27] A. Mahboubi, P. Ylitervo, W. Doyen, H. De Wever, B. Molenberghs, M.J. Taherzadeh, Continuous bioethanol fermentation from wheat straw hydrolysate with high suspended solid content using an immersed flat sheet membrane bioreactor, Bioresour. Technol. 241 (2017) 296–308.
[28] K.D. Thakker, W.H. Chern, Development and validation of in vitro release tests for semisolid dosage forms-case study, Dissolut. Technol. 10 (2003) 10–15.
[29] S.A. Raina, G.G.Z. Zhang, D.E. Alonzo, J. Wu, D. Zhu, N.D. Catron, Y. Gao, L.S. Taylor, Enhancements and limits in drug membrane transport using supersaturated solutions of poorly water soluble drugs, J. Pharm. Sci. 103 (2014) 2736–2748.
[30] R. Opatrilova, A. Cernikova, L. Coufalova, J. Dohnal, J. Jampilek, In vitro permeation of micronized and nanonized alaptide from semisolid formulations, Sci. World J. 2013 (2013).
[31] W. Doyen, W. Mues, B. Molenberghs, B. Cobben, Spacer fabric supported flat-sheet membranes: a new era of flat-sheet membrane technology, Desalination 250 (2010) 1078–1082.
[32] M. Ask, M. Bettiga, V.R. Duraiswamy, L. Olsson, Pulsed addition of HMF and furfural to batch-grown xylose-utilizing Saccharomyces cerevisiae results in different physiological responses in glucose and xylose consumption phase, Biotechnol. Biofuels 6 (2013) 181.
[33] E. Bellissimi, J.P. van Dijken, J.T. Pronk, A.J.A. van Maris, Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based Saccharomyces cerevisiae strain, FEMS Yeast Res. 9 (2009) 358–364.
[34] M.E. Diaz De Villegas, P. Villa, M. Guerra, E. Rodriguez, D. Redondo, A. Martinez, Conversion of furfural into furfuryl alcohol by saccharomyces cervisiae 354, Acta Biotechnol. 12 (1992) 351–354.
[35] P. Ylitervo, C.J. Franzén, M.J. Taherzadeh, Impact of furfural on rapid ethanol production using a membrane bioreactor, Energies 6 (2013) 1604–1617.