Biotechnology Advances 28 (2010) 407–425

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis
II. Quantification of inhibition and suitability of membrane reactors

Pavle Andrić, Anne S. Meyer *, Peter A. Jensen, Kim Dam-Johansen

Department of Chemical and Biochemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

ARTICLE INFO

Article history:
Received 14 October 2009
Received in revised form 26 January 2010
Accepted 13 February 2010
Available online 19 February 2010

Keywords:
Membrane reactors
Glucose removal
Enzyme inhibition
Hydrolysis kinetics

ABSTRACT

Product inhibition of cellulolytic enzymes affects the efficiency of the biocatalytic conversion of lignocellulosic biomass to ethanol and other valuable products. New strategies that focus on reactor designs encompassing product removal, notably glucose removal, during enzymatic cellulose conversion are required for alleviation of glucose product inhibition. Supported by numerous calculations this review assesses the quantitative aspects of glucose product inhibition on enzyme-catalyzed cellulose degradation rates. The significance of glucose product inhibition on dimensioning of different ideal reactor types, i.e. batch, continuous stirred, and plug-flow, is illustrated quantitatively by modeling different extents of cellulose conversion at different reaction conditions. The main operational challenges of membrane reactors for lignocellulose conversion are highlighted. Key membrane reactor features, including system set-up, dilution rate, glucose output profile, and the problem of cellobiose are examined to illustrate the quantitative significance of the glucose product inhibition and the total glucose concentration on the cellulolytic conversion rate. Comprehensive overviews of the available literature data for glucose removal by membranes and for cellulose enzyme stability in membrane reactors are given. The treatise clearly shows that membrane reactors allowing continuous, complete, glucose removal during enzymatic cellulose hydrolysis, can provide for both higher cellulose hydrolysis rates and higher enzyme usage efficiency ($kg_{product}/kg_{enzyme}$). Current membrane reactor designs are however not feasible for large scale operations. The report emphasizes that the industrial realization of cellulosic ethanol requires more focus on the operational feasibility within the different hydrolysis reactor designs, notably for membrane reactors, to achieve efficient enzyme-catalyzed cellulose degradation.

Contents

* Corresponding author. Tel.: +45 4525 2800.
E-mail address: am@kt.dtu.dk (A.S. Meyer).

0734-9750/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.biotechadv.2010.02.005

1. Introduction

Product inhibition of cellulases by cellobiose and glucose has long been known to significantly retard the rates of enzyme-catalyzed cellulose hydrolysis (Gan et al., 2003; Gusakov et al., 1987). This inhibition constitutes a main obstacle for achieving efficient enzymatic degradation of cellulose and high glucose yields in current lignocellulose-to-ethanol processing schemes (Andrić et al., 2010a; Bélafi-Bakó et al., 2006; Xiao et al., 2004). The product inhibition of cellulolytic enzymes also affects the efficiency of other processes involving conversion of lignocellulosic biomass to valuable products. Alleviation of this product inhibition, notably the inhibition by the hydrolysis end-product glucose, is therefore a key prerequisite for achieving cost-efficient conversion of lignocellulosic biomass to biofuels — notably bioethanol and biobutanol — and other valuable products such as platform biochemicals. A number of glucose tolerant fungal β-glucosidases, produced by various *Aspergillus* spp. and e.g. *Humicola insolens*, have been identified relatively recently (Decker et al., 2001; Sonia et al., 2008), but the prospects of developing and using glucose tolerant enzymes seem to receive surprisingly limited attention in the commercial enzyme development for biomass utilization. Rather, the industrial focus has mainly been on reducing the enzyme costs by improving the efficiency of known enzymes, identifying new, more active enzymes, creating optimal enzyme mixtures for selected pre-treated substrates, and on minimizing the enzyme production costs (Merino and Cherry, 2007; Rosgaard et al., 2007b). A careful analysis of the mechanisms and kinetics of the product inhibition induced by glucose and cellobiose on microbial cellulases and β-glucosidase has substantiated that reactor designs which involve continuous or semi-continuous product removal — notably glucose removal — must be at the core of future-directed design strategies for lignocellulose-to-ethanol processes (Andrić et al., 2010b).

Simultaneous saccharifaction and fermentation (SSF), with or without separate fermentation of pentose monosaccharides, is considered a main technology scenario in current biomass-to-ethanol processes (Hahn-Hägerdal et al., 2006; Lynd et al., 2008). Although alleviation of product inhibition is a rationale for SSF, it seems to have been overlooked that the efficiency of this technology is restricted by the inhibition that the ethanol exerts on the cellulolytic enzymes (Bezerra and Dias, 2005). Hence, a certain degree of separate enzymatic hydrolysis of the cellulosic biomass appears to be the most feasible approach for accomplishing the enzymatic degradation of cellulose to glucose in future large scale cellulose-to-ethanol processes and in other lignocellulosic biomass upgrading processes as well.

The purpose of this review is to examine the quantitative effects of product removal on lignocellulose hydrolysis efficiency, i.e. the influence of glucose removal on the rates and extents of conversion in enzymatic cellulose hydrolysis, and to discuss the key reactor design issues, operational features, and the overall advantages and disadvantages of membrane reactors for glucose product removal during cellulolytic enzyme hydrolysis. By highlighting the immense potential as well as the challenges that lie ahead in the development of reactor systems that reduce the product inhibition of cellulases, our objective is to provide an improved knowledge-base for rationally designing reactor systems for efficient enzymatic cellulose hydrolysis. The present review is tightly connected to another report which examines the reaction mechanisms and product inhibition kinetics on enzymatic cellulose hydrolysis in relation to the particular complexities of enzyme-catalyzed cellulose hydrolysis (Andrić et al., 2010b).

1.1. Influence of product inhibition on enzyme-catalyzed rates

The effects of inhibitors — especially their influence on the initial reaction rate — have been extensively studied in classical enzyme kinetics and enzymology. The evaluation of enzyme inhibition has for example for a long time been one of the major methods used in pharmacological research to analyze and quantify the action of drugs and in drugs development (Levenspiel, 1993). It is of course also well known that product inhibition can hinder the obtainment of high yields and high converison rates in industrial enzyme technology (Riebel and Bommarius, 2004; Frieden and Walter, 1963; Fullbrook, 1996). However, apart from a few important cases (e.g. lactose hydrolysis), the negative effects of product inhibition has surprisingly rarely led to drastic changes in processing regimes and reactor design in large scale industrial enzyme reactions. If product inhibition had been more in focus it is our presumption that significantly fewer simple batch reactors and batch reactions would be in place in industrial enzyme technology.

The particular inhibition effect that product inhibition exerts can be assessed by comparing the quantitative influence of a product inhibitor (P) and a "classic" (non-product) inhibitor (I) on biocatalytic product formation rates in a batch reactor. Based on a previous analysis (Andrić et al., 2010a) we employed non-competitive inhibition kinetics to quantify the product inhibition in multi-enzymatic lignocellulose hydrolysis (Fig. 1). In this case, where two inhibited enzymatic reactions have the same kinetic properties (K_M, k_{cat}) — and (hypothetically) have the same reactants and enzyme concentrations, and are analyzed under the same conditions, the presence of I affects the (uninhibited) rates through the constant quantity $1+I/K_I$ while P acts through the term $1+P/K_{IP}$ (the same inhibition strength, $K_{IP}=K_I$, both non-competitive). Although the two terms are essentially similar, the important difference is that $1+P/K_{IP}$ increases as the reaction progresses because P increases, while the term $1+I/K_I$ remains constant throughout the enzymatic reaction. Thus, the presence of I in the reaction medium reduces the rates virtually instantaneously, followed by a reduction of an equal portion of the reaction rate throughout the reaction (Fig. 1). P on the other hand diminishes the enzyme-catalyzed rates to a lesser extent during the initial stages of the reaction, namely where P is low and from where it follows that also P/K_{IP} and hence $1+P/K_{IP}$ are low. However, the factor $1+P/K_{IP}$ then increasingly affects the reaction rate as the reaction progresses because of the product concentration increment (Fig. 1). It is a poor consolation that the only curb on this is that the fraction of free, soluble enzymes (f^{cl}) that may be inhibited by "classical" non-product inhibitors, is constant during the course of reaction, whereas the fraction of theoretically available free enzyme that is product-inhibited (f^{pr}) will increase as the reaction progresses:

$$f^{cl} = 1-\frac{E^{cl}}{E'} = 1-\frac{1}{\left(1+\frac{I}{K_I}\right)}$$

$$f^{pr} = 1-\frac{E^{pr}}{E'} = 1-\frac{1}{\left(1+\frac{P}{K_{IP}}\right)}$$

In these equations E^{cl} and E^{pr} represent the concentrations of free enzyme when "classical" non-product and "product" inhibitors are present, respectively, E_0 is the total initial enzyme concentration, and

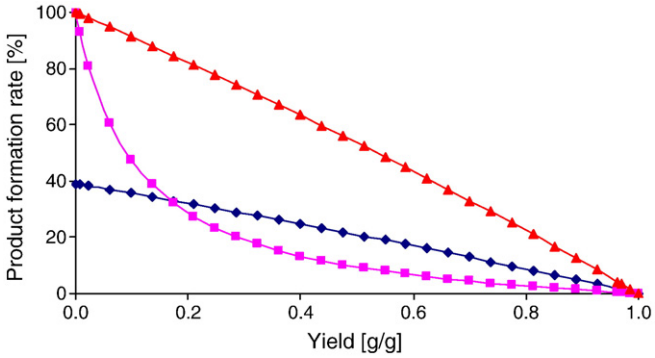

Fig. 1. The effect of the non-competitive 'classical' $\left(\frac{dG}{dt} = \frac{n\cdot k_{cat}\cdot E_0\cdot S}{(K_M+S)\cdot\left(1+\frac{I}{K_I}\right)}\right)$ and non-competitive 'product' inhibitor $\left(\frac{dG}{dt} = \frac{n\cdot k_{cat}\cdot E_0\cdot S}{(K_M+S)\cdot\left(1+\frac{P}{K_{IP}}\right)}\right)$ on the enzymatic reaction — model simulation of a batch reaction. The product formation rates are given as the percentage of the initial uninhibited rate ($t=0$; I, P (yield) $=0$). Model parameters and constants: $k_{cat}=12\ h^{-1}$, $K_M=0.9$ mM, $K_{IP}=K_I=6.4$ mM; $S_0=0.14$ mM, $E_0=0.01$ mM, $M_S=73566$ g/mol, $M_P=M_I=180$ g/mol; $I=0.01$ M. Figure legend: I — classical inhibitor P — product inhibitor No inhibition.

E' is the concentration of the theoretically available free enzyme in the uninhibited state:

$$E^{cl} = \frac{E_0}{\left(1+\frac{S}{K_M}\right)\cdot\left(1+\frac{I}{K_I}\right)} = \frac{E'}{\left(1+\frac{I}{K_I}\right)}$$

$$E^{pr} = \frac{E_0}{\left(1+\frac{S}{K_M}\right)\cdot\left(1+\frac{P}{K_{IP}}\right)} = \frac{E'}{\left(1+\frac{P}{K_{IP}}\right)}$$

These two types of inhibitors will exhibit the same effect on the catalysis rate at the point where $P=I$ (in Fig. 1 this point is 0.17 g/g or 0.01 M). Since the product concentration increases during the enzyme-catalyzed reaction — starting from virtually 0 and then gradually approaching a maximal concentration at the maximum extent of conversion — the enzyme will experience a range of product concentrations in succession that are (usually) higher than the concentration of I. The efficiencies of enzyme-catalyzed reactions at high extents of substrate conversion are thus significantly affected in both batch and continuous processes when sensitive to product inhibition. Since high product concentrations are required in the prospected large scale lignocellulosic conversion processes it must be anticipated that the product inhibition will significantly retard the hydrolysis reaction rates.

It is important to note that the progressive feature of product inhibition is the reason why it is often neglected in initial rate enzyme kinetics inhibition studies. Nevertheless, the significant influence of the product concentration increment in product inhibition is exactly the reason why design of reactors that involve continuous or semi-continuous removal of the products from the enzyme-catalyzed reaction *during the reaction* must be considered in industrial-scale biomass processing demanding high conversion degrees.

Cellobiose exerts the strongest inhibition effect on cellulase activity with typical K_I ranges between 0.01 and 6 g/L (Andrić et al., 2010b). However, this inhibition is usually alleviated by adjusting the dosing of β-glucosidase (EC 3.2.1.21) so that the cellobiose is rapidly hydrolysed to glucose. Unfortunately, glucose also inhibits cellulase activities, with reported overall K_I ranges varying widely from 0.1 to 70 g/L — the variation depending mainly on the experimental conditions. Glucose also exerts a strong inhibition on the activity of β-glucosidases with reported K_I values typically ranging from ~0.1 to 0.8 g/L (Andrić et al., 2010b).

The effect that glucose exerts as a product of cellulose degradation on the lignocellulose enzymatic conversion may formally be classified as medium when the molar ratio $K_M/K_{IP}=0.14$, or very strong, with mass ratio $K_M/K_{IP}=58$ — where IP indicates glucose as product inhibitor. These effect estimates are based on parameters published previously (Andrić et al., 2010a) and a classification given by Riebel and Bommarius (2004). Even for the medium effect (molar $K_M/K_{IP}=0.14$) the effect of the inhibition on the hydrolysis rate and the glucose yield is considerable (Fig. 2). When $K_M/K_{IP}=10$, the reaction is almost halted, requiring a massively extended reaction time to increase the yields (Fig. 2).

The physical meaning of the inhibition constant K_I or K_{IP} may be interpreted in a similar fashion as the Michaelis constant K_M.

The K_M designates the initial substrate concentration (S_0) at which the initial reaction rate (v_0) is exactly equal to ½ of the maximal rate V_{max}:

$$v_0 = \frac{k_{cat}\cdot E_0\cdot S_0}{(K_M+S_0)} = \frac{k_{cat}\cdot E_0\cdot S_0}{(S_0+S_0)} = \frac{V_{max}}{2}$$

Correspondingly, the K_I or K_{IP} can be defined as the concentration of inhibitor (present initially), e.g. glucose concentration, which

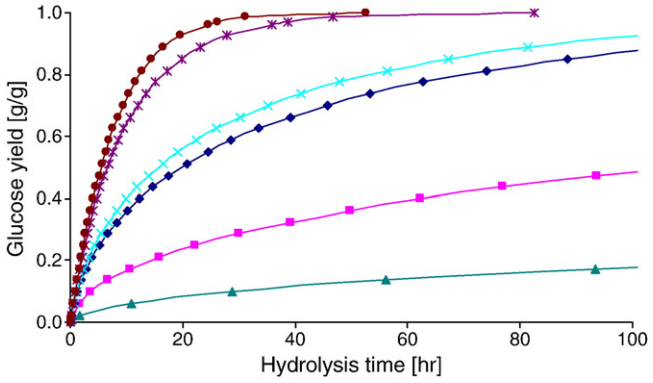

Fig. 2. Effect of supposed glucose inhibition power on glucose yield. Model simulation. Model parameters and constants are given in Fig. 1, except K_I which is varied according to a desired molar K_M/K_I ratio. The real molar K_M/K_I ratio is equal to 0.14 (Andrić et al., 2010a). Figure legend: $K_M/K_I=10$ $K_M/K_I=1$ $K_M/K_I=0.14$ $K_M/K_I=0.1$ $K_M/K_I=0.01$ $K_M/K_I=0$.

reduces the initial catalyzed rate of *uninhibited* enzyme ($v_{P,0}$) to one half meaning that $K_I=I$ or $K_{IP}=P_0$:

$$v_0 = \left(\frac{dP}{dt}\right)_0 = \frac{k_{cat}\cdot E_0\cdot S_0}{(K_M + S_0)\cdot\left(1+\frac{P_0}{K_{IP}}\right)} = \frac{k_{cat}\cdot E_0\cdot S_0}{(K_M + S_0)\cdot\left(1+\frac{P_0}{P_0}\right)} = \frac{v_{P,0}}{2}$$

Because $v_{P,0}$ is given as:

$$v_{P,0} = \frac{k_{cat}\cdot E_0\cdot S_0}{(K_M + S_0)}$$

The numerical value of the inhibition constant K_I or K_{IP} may thus be regarded as fundamental since it provides the quantitative information about the effect of the inhibitor on the initial catalyzed rate (at a certain initial substrate concentration S_0 and initial total enzyme concentration E_0). In turn, the value is also fundamental for the extended rates which are moreover of industrial relevance. Herein, the inhibition constants for the "classical"-non-product and product inhibitor might not be directly compared due to the progressive nature of the product inhibition which particularly diminishes the extended rates. Furthermore, provided that the reported inhibition constants for glucose on cellulases and β-glucosidases are roughly <10 g/L and in many cases <1 g/L (Andrić et al., 2010b), it is indeed clear that the glucose as a product displays a profound effect on the enzyme-catalyzed rate of cellulose hydrolysis. The values of these inhibition constants are particularly significant considering that current cellulose hydrolysate goals are with glucose at least >100 g/L (Andrić et al., 2010b).

1.2. Dimensioning of ideal continuous reactors for enzymatic degradation of (ligno)cellulose

Classical chemical conversion of large quantities of raw material is almost always more feasible and economical when done in continuous reactor regimes rather than in batch reactors (Levenspiel, 1999). The advantages of continuous reactor systems are also expected to become apparent in the near-future large-scale production of lignocellulose-based biofuels. For continuous biocatalytic reactions in general, the continuous stirred-tank reactor (CSTR) type is a suitable reactor configuration for reactions subject to substrate inhibition, since the design allows minimization of the substrate concentration. In contrast, plug-flow reactors (PFR) are considered as more advantageous for reactions subject to product inhibition since they allow for minimization of the reactor volume for high extents of conversions as compared to CSTRs (Riebel and Bommarius, 2004). Enzyme-catalyzed degradation of lignocellulosic biomass is however complicated by:

a) The required action of multiple enzymatic activities for the hydrolysis,
b) The dynamically changing heterogeneous reaction system consisting of a solid substrate, i.e. the (ligno)cellulose, and a liquid phase of soluble intermediate and final products,
c) The product inhibition on the enzymatic reactions,
d) That the current complicated kinetic models of the reactions only partially describe the events, and that significant confusion regarding the inhibition kinetics exists (Andrić et al., 2010b).

We have recently reported that simplified Michaelis–Menten based inhibition models actually reasonably well describe the glucose inhibition of enzyme-catalyzed (*Trichoderma reesei* cellulases (Celluclast® 1.5L) + *A. niger* β-glucosidase (Novozym 188)) degradation of hot-water pre-treated wheat straw at 2% (w/w) DM in a batch reactor lab-scale system (Andrić et al., 2010a). The supplementation of β-glucosidase was done because of the low β-glucosidase activity in the *T. reesei* cellulase product, which is due to the Celluclast® 1.5L production process (Rosgaard et al., 2006). However, the β-glucosidase supplementation also served to prevent cellobiose build-up during the conversion. The progress curves of experiments with different levels of glucose added to the enzymatic cellulose hydrolysis reactions were modeled best with the non-competitive Michaelis–Menten inhibition model (Andrić et al., 2010a). This model can be used to compare the required dimensions, i.e. volume and/or length, of ideal "hypothetical" continuous reactors of the CSTR and PFR types, respectively, to obtain a given conversion of (ligno)cellulose, e.g. 30% conversion.

For a given conversion (we have chosen 30% conversion as a goal), the required reaction time in a batch reactor ($t_{BR}=6$ h) and residence time in continuous reactor ($\tau_{CSTR}=15.6$ h, $\tau_{PFR}=6$ h), can be obtained from design equations (Table 1). To demonstrate the correlation between cellulosic conversion requirements and reactor dimensions, the appropriate ideal reactor dimensions and productivities were calculated from 3 different scales of flow rates corresponding to typical lab, intermediate and pilot/larger scale, respectively: Continuous conversion of lignocellulose requires ideal reactors of large sizes even to obtain low yields, e.g. only 0.3 $g_{glucose}/g_{glucose\ potential}$, and resulting low glucose concentrations, 3.6 g/L (Table 1). For instance, at the largest scale, the required CSTR volume of 15.6 m^3 or a PFR of 2 m with L/D = 1 (and a related linear velocity $\sim 10^{-6}$ m/s) give an impression of the effect of the reaction rate being <1 g/(L h) (Table 1).

All other things being equal, the size of the equipment will obviously increase profoundly with increased desired product concentrations as the higher product concentration will demand for higher conversion degrees (Table 2). In cellulosic processing this must however be achieved at very low rates (Fig. 1). For instance, an increase in the desired conversion degree from 15 to 80% will require an increase in reactor dimensions of 100 times for a CSTR and 40 times for a PFR. In a hypothetical case where no inhibition by glucose is occurring, and with the same desired conversion degree increase from 15 to 80%, the required increase in reactor dimensions would "only" be 26 times for a CSTR and 10 times for a PFR. Under the kinetic conditions employed, the PFR volume will always be lower than the volume of a CSTR. For lignocellulose conversion this volume is 30–75% lower and 10–60% lower with glucose inhibition and (hypothetically) without glucose inhibition, respectively. The direct influence of inhibition on reactor dimensioning is seen from Table 2. The presence of inhibition requires hydrolysis reactors that have a 2–10 times (CSTR) or 1.5–6 times (PFR) higher volume, for cellulose conversion degrees of 15 and 80%, respectively, than in the absence of inhibition. Thus, the product inhibition by glucose directly increases the capital costs of the hydrolysis reactors in the envisaged large-scale production of bioethanol or biochemicals from cellulosic biomass.

Table 1
Comparison of ideal reactor sizes for conversion of lignocelluloses (hydrothermally pre-treated wheat straw) based on experimental results in batch reactor: 30% cellulose conversion, 3.6 g/L glucose outlet/final concentration, $r = 1.1v = 0.23$ g/(L h) (Andrić et al., 2010a). The τ_{CSTR} and τ_{PFR} were calculated from the design equations based on the experimentally determined r from the batch reactor (at $t = 6$ h). The predetermined ratio of the reactors' height to diameter (H/D) for BR/CSTR and reactor's length to diameter (L/D) for PFR is equal to 1. Reaction conditions: pH 5, 50 °C, enzyme dosage 8 FPU/g_{DM} and 13 CBU/g_{DM}; substrate 2% DM content (48% cellulose), $S_0 = 10$ g/L.

	Ideal reactor type		
	Batch (BR)	Continuous stirred (CSTR)	Plug-flow (PFR)
Design equation and reaction/residence time [h]	$t_{BR} = -\int_{S0}^{S} \frac{dS}{v} = 6\,\text{h}$	$\tau_{CSTR} = \frac{S_0 - S}{v} = 15.6\,\text{h}$	$\tau_{PFR} = -\int_{S0}^{S} \frac{dS}{v} = 6\ hr$
Flow rate[a] [L/h]	– – –	$1 \cdot 10^{-2}$ 1 1000	$1 \cdot 10^{-2}$ 1 1000
Volume[b] [L]	0.06 [c] 6 6000	0.16 15.6 15600	0.06 6 6000
Glucose productivity [g/(L h)]	0.6 [d]	0.23	0.6
Diameter ($D_{BR/CSTR}$) or length (L_{PFR}) [m]	0.043 0.2 2	0.058 0.27 2.7	0.043 0.2 2
Suspension velocity [m/s]	– – –	– – –	$1.2 \cdot 10^{-6}$ $9.1 \cdot 10^{-6}$ $9.1 \cdot 10^{-5}$

[a] Fixed.
[b] From reactors design equation.
[c] Approximate size of BR used in inhibition study (Andrić et al., 2010a).
[d] Productivity in batch mode.

1.3. Glucose formation rates at different lignocellulose dry matter contents (DM%) in a batch reactor

When using the Michaelis–Menten model incorporating non-competitive product inhibition (Andrić et al., 2010a) to predict the glucose concentration levels and the glucose formation rates on a broad range of lignocellulose dry matter contents, ranging from 2 to 40 DM%, it becomes apparent that the glucose formation rate decreases rapidly as the reaction progresses (Fig. 3). It also becomes evident that this decrease is pronounced at all substrate dry matter levels (Fig. 3). Apart from being related to the rate decrease due to the substrate consumption, because the reaction rate is a function of [S], the main part of the rate decrease is caused by the inhibitory effect exerted by the glucose formed. For example, when more than 20 g/L of glucose have been released as a result of the enzymatic hydrolysis of cellulose to glucose, the estimated reaction rate fall-off for a 10% w/w DM substrate reaction will be similar to that of a 40% w/w DM substrate reaction, namely, ~95% (Fig. 3). These values correspond to a volumetric productivity decrease to 0.2–0.45 $kg_{glucose}/(m^3_{reactor\ volume}\,h)$. However, the extent of conversion obtained for the 40% DM reaction is significantly smaller than that obtained for the 10% DM substrate: 6% vs. 35%, respectively. Hence, all other things being equal, it appears that the absolute level of glucose — or related glucose yield — plays a key role in decreasing the hydrolysis rate. Since it is the relative ratio of inhibitor: enzyme(s) that is decisive for the inhibition and not the absolute glucose concentration, the quantitative data are in effect a consequence of the increase in the glucose:enzyme ratio, as in the given model simulation the enzyme concentration per L mixture was kept constant. Since the glucose:enzyme ratio then constantly increases during regular enzymatic degradation of cellulose to glucose, the continuous product removal is obviously a main prerequisite to keep conversion rates high. With the currently employed enzyme dosage levels in (experimental) lignocellulosic conversion processes — that do vary widely, but which are generally in the enzyme:substrate range of 5–10% by weight — a

Table 2
Comparison of ideal continuous reactor sizes for conversion of lignocelluloses at different conversion degrees and influence of glucose inhibition on reactor dimension, for the given flow rate of 1000 L/h. Model simulation. Model parameters and constants are given in Fig. 1. For a special case where inhibition is excluded from simulation, K_{IP} (for glucose) = 0. The reaction conditions and other data are given in Table 1. r_{calc} — glucose formation rate obtained from non-competitive kinetic model (Andrić et al., 2010a).

Conversion [%]	Glucose [g/L]	r_{calc} [g/(L h)]	Reactor volume [m^3]	
			Continuous stirred (CSTR)	Plug-flow (PFR)
With glucose inhibition				
14	1.5	0.51	2.9	1.9
29	3.1	0.27	11.5	6.5
81	8.8	0.03	293	75
Without glucose inhibition				
14	1.5	1.17	1.3	1.2
29	3.1	0.98	3.2	2.7
81	8.8	0.28	31	12.8

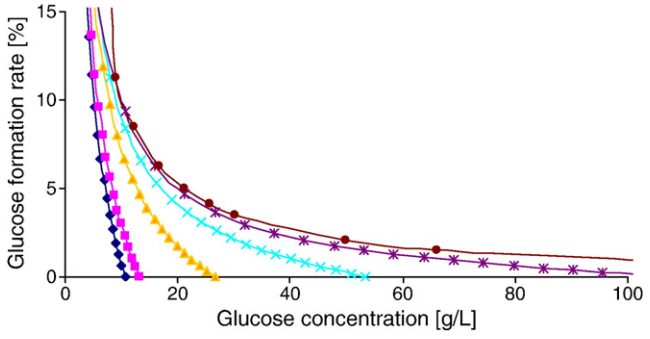

Fig. 3. Influence of actual glucose concentration on glucose formation rate at different DM % levels — model simulation of batch reaction. Model parameters and constants are given in Fig. 1, except S_0 which was varied according to the DM % level. Glucose formation rate is given in % of initial value and initial enzyme concentration, $E_0 = 0.01$ mM is assumed constant. Figure legend: 2%DM 2.5%DM 5% DM 10%DM 20%DM 40%DM.

rough rule of thumb is, therefore, that the glucose should be removed to at least below 10 g/L in order to drastically regain a heavily inhibited glucose production rate. It is important to note that this extent of removal will be independent of the employed substrate concentration. If the remaining glucose concentration is much above a glucose level of 10 g/L even with glucose removal, the relative ratio of glucose:enzyme will usually be so high that the positive effect of the glucose removal will be insignificant (Fig. 3). These relations may explain why many product removal studies have failed to obtain prominent effects on the cellulolytic hydrolysis rates (see discussion below).

2. Design of membrane reactors for hydrolysis products removal

In-situ product removal by integration of the biocatalysis reactor with a separation unit (reaction–separation hybrids) has shown promising results with product inhibited or equilibrium limited enzyme-catalyzed conversions (Ahmed et al., 2001; Gan et al., 2002). On this background, the introduction of membrane (bio)reactors seems to be one of the obvious approaches to accomplish simultaneous *in-situ* removal of glucose during enzymatic hydrolysis of lignocellulose.

2.1. Membrane bioreactors

A membrane (bio-)reactor is a multifunction reactor that combines the reaction with a separation, namely in this case product removal by membrane separation, in one integrated unit, i.e. *in-situ* removal, or alternatively in two or more separate units. In practice, the hitherto used membrane bioreactors in enzyme technology have mainly employed ultra- and nanofiltration for the separation (Drioli, 2004; Pinelo et al., 2009).

Ultrafiltration membrane reactors were first used in conjunction with development of novel enzyme immobilization techniques. However, immobilized enzymes are not suitable for insoluble, polymeric substrates, and this will include lignocellulose, due to the necessary enzyme adsorption on the macromoleculer substrate particles that becomes severely mass transfer limited with immobilized enzymes (Alfani et al., 1983). The use of free, un-immobilized, enzymes confined in membrane reactors avoids some of these problems, and still allows continuous product removal (Hahn-Hägerdal et al., 1981). Membrane reactors have been investigated for use in inorganic catalytic reactions for a very long time (Sun and Khang, 1988), but have also been employed for a range of very different biocatalysis based reactions. These applications include e.g. classical production of citric acid by fermentation in which the product has to be removed to maintain high production rates (Chekhova et al., 2000); selective production of physiologically active chitosan oligosaccharides by continuous hydrolysis of chitosan (Kuroiwa et al., 2009); production of whey hydrolysates with low contents of phenylalanine (Cabrera-Padilla et al., 2009), and continuous production of pure and sterile glucose solutions from tapioca starch powder (Sarbatly et al., 2007). Membrane reactors also find use in the pharmaceutical industry, e.g. for production of S-ibuprofen (Cauwenberg et al., 1999) as well as in waste water treatment (Meng et al., 2009). However, apart from a few seminal studies discussed below, there is a surprising scarcity of data on membrane reactor performance for enzymatic conversion of lignocellulose in potential lignocellulose-to-ethanol processes.

2.2. Membrane bioreactors for lignocellulose hydrolysis: key issues

The molecular weight of glucose is 180 g/mol while the molecular weight of most of the currently used fungal cellulases used for lignocellulose hydrolysis range from ~35,000 to 65,000 g/mol (Cantarel et al., 2009). Several studies using various fungal cellulase systems and different cellulose substrates have confirmed that it is possible, via membrane technology, to retain the enzymes present in the system while allowing the transfer of low-molecular weight reaction products such as glucose through a membrane (Alfani et al., 1983; Bélafi-Bakó et al., 2006; Ghose and Kostick, 1970). Due to the possibility of complete rejection of the long polysaccharide chain (or rather the solid lignocellulosic substrate particles) and the biocatalyst, and zero rejection of the main reaction products passing through the membrane, it can be assumed that the concentration of the products, i.e. glucose, in the reactor is equal to the concentration of the products in the permeate. Although some differences may exist due to concentration polarization affecting the flux (Pinelo et al., 2009), employment of a membrane reactor principally enables a design configuration involving continuous feeding of substrate and removal of product without enzyme loss (Bélafi-Bakó et al., 2006; Gan et al., 2002; Hong et al., 1981; Yang et al., 2006). The major advantages of using membrane reactors encompassing product removal during enzymatic hydrolysis of lignocellulosic materials include:

(a) the use of the cellulolytic enzymes for long periods of time, via retention in the system;
(b) the obtainment of a higher degree of conversion due to the reduced product inhibition;
(c) the obtainment of pure hydrolysis products, i.e. free of contaminants such as enzymes, unconverted substrate or other high-molecular weight substances that can harm the processing steps downstream from the hydrolysis; and
(d) the possibility of maintaining a stream with constant product concentration — without supplying additional enzymes — during extended hydrolysis (i.e. fed-batch or continuous hydrolysis).

On the other hand, a major drawback of using membrane reactors for glucose removal during bioconversion of lignocellulose is the relatively low concentration of the product glucose obtained in the permeate, and the possible leaching of cellobiose. However, the latter will only take place in case there is not sufficient β-glucosidase relative to cellulases in the enzyme mixture employed. In case of lignocellulose-to-ethanol the low glucose concentration in the permeate will result in a low final percentage of ethanol in the fermentation mixture and related high distillation costs for the ethanol recovery (Andrić et al., 2010b). Moreover, in a membrane reactor, simultaneous permeation and dilution is required to keep the reaction volume constant. A key issue with respect to membrane reactors is that their operational feasibility is currently not fit for high solids biomass loadings; this is mainly due to the unresolved problems of membrane fouling in current membrane reactor designs, lack of robustness of the membranes, mixing problems etc. Moreover, during extended enzymatic reaction, some loss of enzyme activity may result from thermal or other inactivation, and this in effect may decrease the final product concentration in membrane reactor operations unless the dilution rate is accordingly controlled (Ishihara et al., 1991). In addition, there may be problems with enzyme concentration polarization — i.e. the build-up of an enzyme boundary layer near the membrane. If this phenomenon occurs it may reduce both the flux through the membrane and deplete the enzyme in the bulk solution (Hong et al., 1981). The enzyme concentration polarization may be pronounced at elevated feed flow rates and pressures (Hong et al., 1981), but may thus be controlled via optimization of the particular reaction. Just as in other hydrolysis reactor types, the high viscosity of the lignocellulosic biomass mixture is a particular challenge, especially at high solids loadings. The high viscosity is an obstacle for obtaining favorable mixing and mass transfer conditions to promote the enzymatic reactions and the product removal. Another main issue in relation to lignocellulose conversion is obviously the build-up of unreacted lignocellulose in the reactor. This unreacted substrate may notably include lignin and particularly recalcitrant cellulose (see discussion further below). The problem of unreacted lignocellulose substrate build-up may in fact be the most significant problem to overcome in practical large-scale and/or continuous lignocellulose processing encompassing membrane bioreactors for the enzymatic hydrolysis step.

2.3. Quantitative effects of product removal on the cellulolytic hydrolysis rates and extent of cellulose conversion

The positive effect of the product removal on the enzyme-catalyzed hydrolysis rate, on the extent of hydrolysis (degree of conversion), as well as on the hydrolysis yields is well documented (Table 3). In general, the extents of conversion of cellulose achieved by using membrane reactors encompassing product removal have been up to 40% higher than comparable conversions achieved in batch processes — in many cases the final degree of conversion has been between 70 and 90%, which is higher than the results typically obtained in batch reactions (Table 3). Conversion degrees >90% have been reported only in a few cases, and this high conversion has mainly been obtained with: (a) operation at very low substrate concentrations (Alfani et al., 1982; Henley et al., 1980); (b) relatively high enzyme dosage and reaction time (Kinoshita et al., 1986) or, (c) with a

Table 3
Overview of the studies of glucose removal by UF membranes.

Enzyme feeding	Substrate feeding	MF unit/buffer replacement	Removal method	Dilution rate [h^{-1}]	Cut-off [kDa]	$V_{reactor}$ [L]	G_{max} [g/L]	Source
Start	Continuous[1]	No/yes	*In-situ*, continuous	0.3[8]	30	0.2	75[31]	Ghose and Kostick (1970)
Fed-batch	Fed-batch[2]		*In-situ*, intermittent	N/A		5.5[18]	63[32]	
Start	Continuous	No/yes	Separate, continuous	N/A[9]	50	0.22 + 0.1[19] 0.22 + 0.03[20]	N/A	Henley et al. (1980)
Start	Continuous[3]	No/yes	*In situ*, continuous	0.6–1.9	10	0.26–0.33[21,24]	1.9[33]	Hong et al. (1981)
Start	Start/continuous	No/yes	Separate/continuous	0.3[10]	30	7	3.5	Klei et al. (1981)
Start	Start	No/yes	*In situ*, continuous	0.2–0.5	10	0.065	0.08	Alfani et al. (1982)
Start	Start/fed-batch	No/yes	*In situ*, continuous	0.6–1.3	10	0.25–0.3[25]	9.5[34]	Ohlson et al. (1984)
Start	Fed-batch	No/yes	*In-situ*/continuous	0.06–0.6	20	0.05	18	Kinoshita et al. (1986)
Start	Fed-batch	Yes/yes[6]	Separate, intermittent	N/A[11]	4/5	10[26]	N/A[35]	Ishihara et al. (1991)
Start	Start/continuous	No/yes	*In situ*, continuous	0.06–0.3[12] 0–2[13]	10	0.1	11[12,34] 25[13]	Lee and Kim (1993)
Start	Start	No/yes	*In situ*, intermittent and continuous	0.12–0.16[14]	10	1[27]	9.5 [34]	Gan et al. (2002)
Start	Start/fed-batch	Yes/yes	Separate, intermittent	N/A	50[16]	0.05[22,28]	27[36] –60	Knutsen and Davis (2004)
Start	Start[4]	No/yes	Separate, continuous	0.06–0.25	10	0.06[29]	35[34]	Yang et al. (2006)
Start	Continuous[5]	No/yes[7]	*In situ*, continuous	0.44[15]	N/A[17]	0.27[30]	N/A	Bélafi-Bakó et al. (2006)
Enzyme source	**Substrate type**	**pH**	***T*** **[°C]**	**DM_{max} [% w/v]**	**E_0 [% w/v]**	**$t_{reaction}$ [h]**	**X_{max} [%]**	
T. viride[37]	Solca Floc[50]	4.2–5.2[60]	50	10	90	8[1]	77	Ghose and Kostick (1970)
	Solca Floc[51]	4.85		10[63]		243	71	
T. viride[38]	Solca Floc[52]	4.8	50	0.045[64]	0.077[73]	0.2–0.5[84]	92	Henley et al. (1980)
T. viride[39]	Cellobiose	4.8	50	0.2[65]	0.0002[74]	20[33]	91[33]	Hong et al. (1981)
T. reesei[38,40l] *A. phoenicis*[41]	Solca Floc[53]	4.8	50	1	0.022[75,76]	200	80	Klei et al. (1981)
T. viride[42] Sweet almonds[43]	Avicel	4.8	45	0.11[66]	0.0033[77]	25	5.2	Alfani et al. (1982)
T. reesei[38] *A. niger*[44]	Sallow[54]	4.8	40	10[67]	1[78]	20	94[85]	Ohlson et al. (1984)
Sporotrichum cellulophilum[45]	KC Floc	5–5.5[61]	37	10[67]	2[79]	120	>90	Kinoshita et al. (1986)
T. viride[46]	Steamed hardwood[55]	4.7	45	5	0.5	192	49	Ishihara et al. (1991)
	Hardwood kraft pulp					240	82	
T. viride	α-cellulose	4.8[62]	50[62]	5[68]	0.1[80]	48	72[86]	Lee and Kim (1993)
T. reesei[47]	α-cellulose[56]	4.7	40	2.5[69]	0.01[81]	48	53	Gan et al. (2002)
N/A[48]	Corn stover[57]	4.7	45	15[70]	3.2[82]	168[36]–408	>60[70]	Knutsen and Davis (2004)
T. reesei[45]	Rice straw[58]	4.8	50	18.5[71]	3.4[83]	25	44	Yang et al. (2006)
T. reesei[49]	Solca Floc[53]	4.8	50	2.5	5[88]	25	50	Bélafi-Bakó et al. (2006)
	Mavicell[59]						20–70[59]	

[1]46 h batch, 20% w/v.
[2]48 h batch.
[3]Cellobiose.
[4]0.5 h batch.
[5]9 h batch, circulation.
[6]Lignin removal.
[7]Not clear.
[8]1 mL/min.
[9]2–5, t_{res} based.
[10]37 mL/min.
[11]max 2.5, 4 L/h.
[12]Experimental.
[13]Modeling.
[14]Membrane 0.0177 m^2, 1 L.
[15]6.6 L/(m^2h), 0.018 m^2.
[16]Vaccum filter 20–25 μm.
[17]0.1 μm = 1000 kDa.
[18]4 L CSTR, 1.5 L UF cell.
[19]CSTR + UF cell.
[20]CSTR + hollow-fiber cartridge.
[21]Without UF cell.
[22]50 g slurry.
[23]250–300 rpm.
[24]810 rpm, magnetic bar.
[25]500 rpm, 4-blade propeller.
[26]Flat-blade impeller.
[27]50–90 rpm.
[28]Shaking.
[29]No stirring.
[30]Recirculation.
[31]7.5 w/v%.
[32]6.3 w/v%.
[33]3-stage reactor.
[34]Reducing sugar.
[35]3.5%, 19 h.
[36]No fed-batch.
[37]QM 9123.
[38]SP122.
[39]Isolated cellobiase, Miles labs.
[40]Powder.
[41]QM329 alumina immobilized.
[42]B.D.H. Italia.
[43]Cellobiase, BBR.
[44]Novozym 188.
[45]Enzyme powder extracted.
[46]Meicelase.
[47]Extracted Sigma.
[48]Iogen.
[49]Celluclast 1.5 L.
[50]SW 40A, <37 μm.
[51]SW 40A, <25 μm.
[52]BW200, 30–35 μm.
[53]BW 200.
[54]Salix Q082 alkali pre-treated.
[55]Shirakamba.
[56]180 mm length.
[57]Sulfuric acid pre-treated.
[58]Steam exploded.
[59]Untreated and heat treated.
[60]CSTR studies.
[61]Enzyme optimum.
[62]From (Lee and Fan, 1983).
[63]Total − 30% initial.
[64]0.45 g/L.
[65]2 g/L.
[66]1.1 g/L, kinetic study max 2.9.
[67]100 g/L dry wt.
[68]50 g/L.
[69]25 g/L.
[70]w/w %.
[71]185 g/L dry wt.
[72]10% suspension in cellulases.
[73]0.77 g/L.
[74]2 mg/L.
[75]0.22 g/L, 1115 IUC_x/L, 5000 IUC_x/g.
[76]112 IUC_1/L, 500 IUC_1/g.
[77]33 mg/L.
[78]10 g/L both enzymes.
[79]20 g/L.
[80]1 g/L.
[81]0.1 g/L.
[82]20 FPU/$g_{cellulose}$.
[83]20 FPU/g_{straw}.
[84]t_{res}.
[85]1 day operation.
[86]Optimal D profile.
[87]60 in BR control.
[88]v/w %.

3-stage hydrolysis of cellobiose (Hong et al., 1981). In several instances, however, the conversion was lower <70%, and reaching the limit has typically been explained to be a result of the build-up of recalcitrant substrate (Bélafi-Bakó et al., 2006).

Ohlson et al., (1984) reported that when the product was continuously removed the initial rate of enzymatic hydrolysis of unwashed and washed lignocellulose substrate increased by 4 to 7 times, respectively, as compared to what was obtained in comparable batch processes. The corresponding extents of conversion on the unwashed and washed substrate were 70% and 95% as compared to ~40% conversion obtained in a batch mode (Ohlson et al., 1984). Henley et al. (1980) achieved high conversion of cellulosic material as a result of the diminishing influence of inhibitory end-products as these were immediately removed when combined CSTR-HFC (hollow-fiber cartridge) or CSTR-UF (ultrafiltration unit) systems were used. The amounts removed were 89–91% (residence time 14–17 min) and 87.5–91.5% (23–27 min), respectively. The comparable reactions in a batch reactor (BR) had 50% conversion in 20 min or 62–66% in a continuous stirred reactor (CSTR) (12.3–14.5 min). Klei et al., (1981) found that the operation of a reactor with immobilized β-glucosidases in continuous mode gave 60% higher enzyme efficiency ($g_{product}/g_{enzyme}$) than in a comparable batch hydrolysis, and ascribed the effect to be a result of the reduced product inhibition. Similarly, Kinoshita et al. (1986) reported that membrane reactor operation in a semi-continuous system was five times more effective per unit weight of enzyme than the batch reaction. Hahn-Hägerdal et al. (1981) described a batch hydrolysis (cellulase SP122, 40 °C) with product removal at a dilution rate of 0.09–0.28 h^{-1} which gave 4 times higher reaction degree than that obtained in a comparable classical batch reactor run. Ghose and Kostick (1970) found that the continuous membrane reactor was very effective in separation of sugars from a digest, composed of a dense suspension of unhydrolyzed cellulosic material in cellulolytic enzymes, reaching 77% conversion, compared to 60% in a CSTR. Gan et al. (2002) thus found that the semi-continuous and continuous removal of inhibitory reaction products — i.e. glucose — markedly increased the extents of conversion (51 and 53%, respectively) compared to what was obtained in a batch reaction (35%).

3. Membrane reactors operation strategies

The operation of integrated membrane reactors for the simultaneous cellulose hydrolysis and removal of inhibitory product can be performed using several different configurations (Figs. 4 and 5). The membrane reactor is the central feature of every set-up; in some instances there is a stirred reactor (STR) which provides an auxiliary mixing unit, additional volume for reaction, and/or a possibility to discharge the unconverted material (Fig. 4). The separation units most frequently encountered are the common ultrafiltration modules, including tubular and flat-sheet membrane modules, while special types of ultrafiltration, e.g. incorporating adsorption surface or with immobilized β-glucosidases in the shell, and microfiltration units have been used less often. The application of widely available cellulose-based membranes is infrequently seen due to suspected cellulose-degrading effect of the cellulases.

3.1. Product removal strategies

The inhibitory product can be intermittently, i.e. semi-continuously, or continuously removed from the reaction system primarily in the form of a permeate. Principally, the product can be removed via pressure by means of simple dead-end filtration, which, however, also partially removes the aqueous medium necessary for the continuous glucose release by the enzyme-catalyzed reaction, and in this way concentrates the reactants, including the unreacted substrate, in the retained reaction medium. If removal of the product inhibitor from the reaction environment is accomplished by membrane separation in this way, it is necessary to adequately and semi-continuously supplement new liquid to maintain a constant reaction volume. With time this supplementation will dilute the glucose level in the permeate stream.

In one of the earliest works, Ghose and Kostick (1970) used a continuous saccharification membrane reactor system consisting of an agitated reservoir, in fact a substrate slurry supply vessel, which contained an aqueous suspension of the predigested (46 h) substrate, the membrane cell reactor, and a permeate reservoir. The units were connected to pressurized cylinders for maintenance of desired pressures (Ghose and Kostick, 1970). The equilibrium conditions for the flow and reaction rate were attained soon after the flow of the substrate slurry into the membrane reactor was at the same rate as the aqueous solution of the reaction products was removed from the membrane cell (Fig. 5, 2a and 2b).

3.2. System set-up

In membrane reactors the enzymes are usually added in the beginning of the reaction and re-used during its course, but the substrate can be added at the start of the reaction, i.e. fed semi-continuously as a

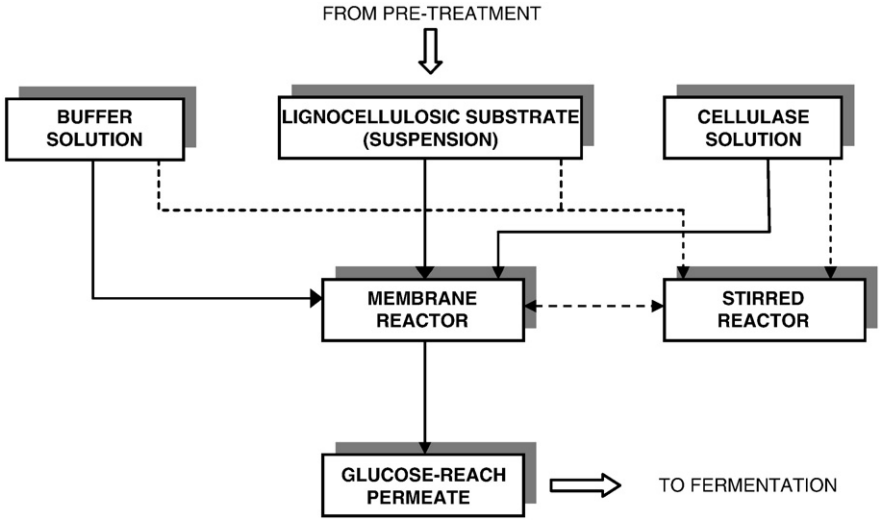

Fig. 4. Schematic representation of the process flow sheet for simultaneous lignocellulose hydrolysis and removal of produced glucose. STR (stirred reactor) is needed for achieving additional conversion due to available volume and/or for unconvertible fraction discharge.

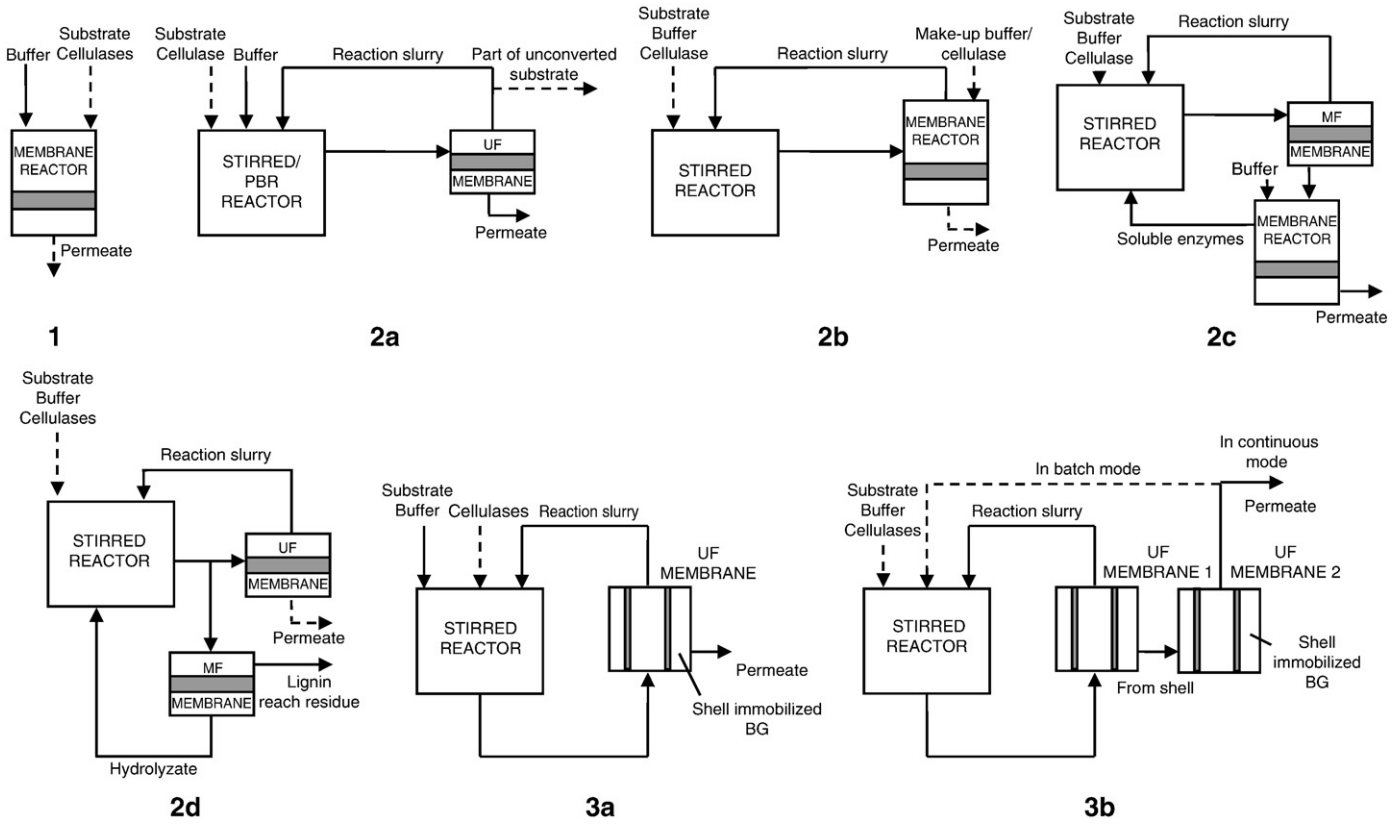

Fig. 5. Strategies for operation of membrane reactors: 1. Integrated reaction vessel (stirred reactor) with UF membrane (Alfani et al., 1982; Alfani et al., 1983; Gan et al., 2002; Ghose and Kostick, 1970; Hong et al., 1981; Kinoshita et al., 1986; Lee and Kim, 1993; Ohlson et al., 1984); 2. Separate reaction vessel with: (2a) UF membrane (Henley et al., 1980; model system 2 from Ghose and Kostick (1970); combined with PBR in Yang et al. (2006)) , (2b) ordinary (model system 1 from Ghose and Kostick (1970) and special membrane reactor (with adsorption of substrate and enzymes from Bélafi-Bakó et al., 2006), (2c) MF and UF for recovery of bounded and soluble enzymes, respectively (Knutsen and Davis, 2004), (2d) UF and MF membrane for recovery of enzymes and removal of lignin reach fraction, respectively (Ishihara et al., 1991); 3. Separate reaction vessel with UF membrane with shell immobilized β-glucosidases (BG), in (3a) 1-stage, and (3b) 2-stages (Klei et al., 1981). PBR — packed bed reactor.

fed-batch — or rarely continuously. Lee and Kim (1993) used a cellulose hydrolysis reactor system which consisted of a water jacketed stirred tank connected to a cellulose and buffer feed tank for maintenance of a constant reaction volume. The enzymatic hydrolysis reactor was equipped with a polysulfone membrane for the ultrafiltration of the reaction mixture which was collected in a fraction collector (Fig. 5, 1). In the work by Kinoshita et al., 1986 air was supplied from the compressor to the reservoir with the buffer solution which was sent to the intermittently substrate-fed ultrafiltration membrane reactor in which thermotolerant cellulases were retained, while the filtrate was collected (Fig. 5, 1). Hong et al., 1981 used a system where the reacting volume inside a membrane reactor for cellobiose hydrolysis to glucose was replaced by a substrate solution continuously fed from a pressurized reservoir, while the permeate was automatically collected (Fig. 5, 1). Alternatively, the reaction may be accomplished in the same vessel as the separation (*in situ*) or separately, i.e. in the stirred vessel or unstirred bed, packed with substrate. In the case of separate units, the reaction mixture (stirred reactor) or the filtrate (packed bed reactor) have to be transported to the separation unit and then recycled back.

The cellulose hydrolysis-reducing sugars removal system designed by Yang et al. (2006) consisted of a tubular reactor, in which the substrate was retained with a porous filter at the bottom and buffer entered at the top through a distributor, and the separate hollow-fiber module ultrafiltration polysulfone membrane, through which the permeate was transported and removed (Fig. 5, 2a). To keep the volume constant in the tubular reactor, all remaining buffer was recycled back from the UF membrane and the make-up buffer was continuously supplied from the reservoir (Yang et al., 2006). To improve the efficiency of the batch and continuous hydrolysis, Henley et al. (1980) incorporated an UF membrane stirred cell (UF) or hollow-fiber cartridge (HFC) into the CSTR-UF and CSTR-HFC system, respectively (Henley et al., 1980) (Fig. 5, 2a).

In some of the few reports describing lignocellulose hydrolysis in membrane reactors, an additional microfiltration unit has exceptionally been used to retain the unconverted lignin-rich solid fraction due to the present tightly bound enzymes (Knutsen and Davis, 2004) or has been employed to remove the unconverted substrate from the reactor. These set-ups result in slightly complex process layouts for the hydrolysis (Fig. 5, 2c and 2d).

Ishihara et al. (1991) accomplished a semi-continuous hydrolysis reaction by using a continuously stirred reservoir tank, connected to a suction filter unit for the removal of the lignin-rich residue and an ultrafiltration membrane unit (tubular module), through which the filtrate was pumped in order to separate the hydrolysis products from the filtrate containing cellulases (Figs. 5, 2d). The concentration of the lignocellullose substrate in the reactor was maintained almost constant by addition of fresh substrate at appropriate intervals; the filter and ultrafiltration units were operated intermittently, while the enzymes were added at the start, recovered in the UF module and recycled back into the reactor (Ishihara et al., 1991).

3.3. Enzyme retention

The retention of enzymes in the reaction system, either *in situ* or in separate units, is as a rule accomplished by the membrane which is at the same time permeable for the products. In order to retain the enzymes in the reacting system, the combination of MF and UF units may be used to recover the enzymes that are not firmly bound to the substrate. Knutsen and Davis (2004) did a batch saccharification using

shaked flasks and simple, intermittent solid–liquid large-pore vacuum filtration and ultrafiltration after 4, 12, 24 and 96 h, with replenishment of buffer removed as permeate after each separation. The permeate removal was done to eliminate inhibitory products such as glucose and cellobiose, while retaining the corn stover and the cellulose enzymes in the reactor. In a semi-batch hydrolysis, vacuum was used to remove the hydrolysis products as filtrate while the soluble enzymes were recovered by ultrafiltration (both filtrations after 4 and 8 days) and added together with the fresh pre-treated corn stover slurry to the residual solids and tightly bound enzymes (Knutsen and Davis, 2004)(Fig. 5, 2c). The cellulosic material was continuously added and the soluble hydrolysis products were continuously withdrawn from the system with immobilized β-glucosidase on the shell side of the hollow-fiber reactor (Klei et al., 1981) (Fig. 5, 3a). The cellulose slurry from the reservoir was converted by Klei et al. (1981) to soluble low-molecular weight mono and oligosaccharides that permeated the shell side of the fiber walls of the hollow-fiber cartridge (Romicon), that retained the enzymes and unreacted cellulose within its lumens to be recycled back to the reservoir. In a different set-up, the soluble reducing sugars were transferred to the shell of second hollow-fiber cartridge (Amicon), which contained immobilized β-glucosidase (Fig. 5, 3b) (Klei et al., 1981). In this set-up, the saccharified product could cross the lumens of the second cartridge and could be led back to the reservoir (batch) or be withdrawn separately (continuous) (Klei et al., 1981) (Fig. 5, 3b).

3.4. Specific design features

The specific mechanical design features in connection to the membranes are usually employed in order to reduce the negative effect that the hydrolysis reaction mixture has on membrane flux. Ohlson et al., 1984 investigated batch and semi-continuous hydrolysis with continuous product removal and replacement of permeate with buffer, in a stirred polyamide membrane reactor with 2 mm margin between membrane and propeller to prevent fouling (Fig. 5, 1). A stirred reactor integrated with a flat-sheet polysulfone membrane and supplied with *in-situ* electrokinetic membrane cleaning to prevent continuous accumulation of enzyme molecules and substrate particles at the surface, was used by Gan et al. (2002) in a batch reaction and in operation with intermittent and continuous removal of products, with replenishment of buffer lost in the permeate (Fig. 5, 1). An electrical backpulse drastically increased the flux level immediately after the impulse, but the elevated flux could not be sustained (Gan et al., 2002). A batch hydrolysis, followed by operation in a continuous mode with simultaneous reducing sugars removal, was performed by Bélafi-Bakó et al. (2006): They used a tubular membrane module consisting of a stainless steel tube covered by a non-woven textile layer, providing a hairy surface for immobilization of cellulose particles and cellulases by adsorption to reduce membrane fouling and diffusion resistances, and improve membrane selectivity (Fig. 5, 2b). In a batch mode, the reaction mixture containing the substrate and the enzymes was first circulated (80 mL/min) between the agitated vessel and the membrane module to allow fractional adsorption on the membrane surface, while in continuous mode the permeate outlet was opened and the permeate containing glucose was collected (Bélafi-Bakó et al., 2006).

4. Key factors influencing membrane reactor performance for enzymatic cellulose hydrolysis

The performance of membrane biocatalytic reactors for conversion of lignocellulose may in general, depend on the following factors:

- the rate of product formation;
- cellulases product inhibition rate;
- the rate of product removal;
- lignocelluloses, cellulases and buffer feeding rates;
- cellulases deactivation rate;
- concentration polarization and fouling.

As the above may be general for the enzymatic reaction with product inhibition, with lignocellulose enzymatic conversion there might be several specific and distinguishing issues — such as the inherently slow reaction rate, relatively fast inhibition rate and pronounced membrane fouling with sticky cellulose–cellulase reaction mixture. Furthermore, as opposite to classical membrane bioreactor systems in which the macromolecular substrates have much more different sizes than its products, the intermediate product of cellulose hydrolysis — cellobiose — has similar molecular dimensions as the product glucose and is typically withdrawn together with it as the permeate without being converted to glucose.

The change in the cellulose (S), cellobiose (C) and glucose (G) concentration, respectively, in the membrane reactor can be represented as follows:

$$\frac{dS}{dt} = \frac{F_S}{V_R} - \left(\frac{dS}{dt}\right)_{\text{consumed in reaction 1}}$$

$$\frac{dC}{dt} = -D\cdot C_{\text{out}} + 1.11\left(\frac{dC}{dt}\right)_{\text{produced in reaction 1}} - \left(\frac{dC}{dt}\right)_{\text{consumed in reaction 2}}$$

$$\frac{dG}{dt} = -D\cdot G_{\text{out}} + 1.05\left(\frac{dG}{dt}\right)_{\text{produced in reaction 2}}$$

Reaction 1 denotes cellulose hydrolysis to cellobiose which is further converted to glucose in reaction 2, as may be illustrated by the following model, adopted from Philippidis et al. (1993) and Wyman (1996), a model which was originally developed for SSF:

$$\left(\frac{dC}{dt}\right)_{\text{produced in reaction 1}} = \frac{k_1\cdot E_c\cdot S_S\cdot e^{-\lambda t}}{1 + C/K_{1C} + G/K_{1G}}\cdot\frac{(1-K_{1L}L)}{K_E + E_C}$$

$$\left(\frac{dG}{dt}\right)_{\text{produced in reaction 2}} = \frac{k_2\cdot E_{\beta G}\cdot E_g^*\cdot C}{K_M(1 + G/K_{2G}) + C}\cdot(1-K_{2L}L)$$

F_S designates the substrate feeding rate (kg s^{-1}), V_R is the reaction volume (m^3), D is the dilution rate (h^{-1}), S_s is the concentration of the available cellulose surface ($m^2\ m^{-3}$) which is related to the cellulose concentration S (kg m^{-3}) by the specific area (m^2 kg^{-1}), C is the cellobiose concentration (kg m^{-3}), G is the glucose concentration (kg m^{-3}), E_C and $E_{\beta G}$ are the concentration of the cellulases and β-glucosidases in the solution, respectively (kg m^{-3}). The k_1 (kg m^{-2} h^{-1}) and k_2 (kg CBU^{-1} h^{-1}) are specific rates of reactions 1 and 2, respectively, K_E is the equilibrium constant for cellulose adsorption to cellulose (kg m^{-3}), K_{1L} and K_{2L} are constants for the cellulase and β-glucosidase adsorption to lignin (m^3 kg^{-1}), respectively, L is the concentration of lignin (kg m^{-3}), K_{1C}, K_{1G} and K_{2G} are cellobiose (C) and glucose ($1G$ and $2G$) inhibition constants for cellulases and β-glucosidases (kg m^{-3}), E_g^* is β-glucosidase specific activity (CBU kg^{-1}), K_M is the Michaelis–Menten constant for cellobiose (kg m^{-3}), and λ is the specific rate of decrease of effective cellulose specific surface area (h^{-1}) which defines the quality of the cellulosic substrate, but can as well — in general — designate the exponential decrease of the enzymatic activity with time. The above models highlight the significance of the product inhibition constants for the rate of the cellobiose and glucose formation, respectively, and are some of the few models, that include the unproductive adsorption of the enzymes to the lignin.

4.1. Glucose output profile

Depending on the operational mode, the glucose output profile (Fig. 6, 1–2) from the membrane bioreactor typically demonstrates a soft peak in both batch and fed-batch after some time of reaction — usually this glucose output peak appears in the order of hours into the reaction (Kinoshita et al., 1986; Knutsen and Davis, 2004; Lee and Kim, 1993; Ohlson et al., 1984.; Yang et al., 2006) (Fig. 6, 1a–c). In the case of continuous operation, the glucose output level reaches a plateau after initial dynamic phases (Alfani et al., 1982; Ghose and Kostick, 1970; Hong et al., 1981; Klei et al., 1981) (Fig. 6, 2a–b). The different cases from Fig. 6 are explained later in the paper. In several instances, the permeate glucose concentration is given as the level of total sugars produced, and this level typically increases steadily during the reaction (Gan et al., 2002; Ishihara et al., 1991). The glucose concentration profile depends on the *E/S* ratio, the ratio of β-glucosidases to cellulases and the kind of pre-treatment employed (Frenneson et al., 1985). However, the glucose concentration profile first and foremost depend on the reaction rate and the flux through the membrane. The glucose maximum will be achieved when the glucose production rate is equal to the removal rate (Alfani et al., 1982) (Fig. 6, 1a). The maximal value will be directly proportional to the reaction rate and inversely to *D* (Frenneson et al., 1985., Alfani et al., 1982) (Fig. 6, 3). The time it takes to reach the glucose peak will be shorter if the residence time ($\tau = V/Q$) increases or the dilution rate ($D = Q/V$) decreases. Clearly, higher dilution rates leads to lower glucose output concentrations. As an example, Yang et al., (2006) obtained 2–20 g/L of product at the end of hydrolysis (5–27 g/L of the total permeate, 280–440 g per g of dry biomass) when converting 125–185 g/L of substrate, and the product curves showed typical peak after 2–4 h, reaching 15–35 g/L. However, the end glucose concentrations were lower than the corresponding glucose concentration achieved at the end of the batch hydrolysis (20–31 g/L) (Yang et al., 2006).

4.2. The effect of dilution rate

The operation of enzymatic degradation of lignocellulose at high glucose concentration will cause a decrease in the hydrolytic activity of the enzyme system due to the product inhibition, and thus, it is typically seen that the increase in dilution (removal) rate results in an increased conversion rate — up to a certain level. Ohlson et al. (1984) showed that the reducing sugar concentration profile had a typical peak after 2–4 h for all dilutions rate examined (Table 3). They also showed that an increased dilution rate, in their case up to 1 h^{-1}, led to an increased conversion rate, owing to the elevated rate of the continuous removal of inhibitory products, i.e. both sugars and low-molecular byproducts resulting from the biomass pre-treatment. Yang et al., (2006) observed an increase in the hydrolysis rate and glucose concentration at the low dilution rates, 0.057–0.075 h^{-1}, but obtained higher conversion rates and lower glucose concentrations at the higher dilution rates, namely 0.075–0.25 h^{-1}. Kinoshita et al. (1986) also noticed that the conversion rate increased with dilution rate from 0.06 to 0.3 h^{-1}, but also found that the trade-off was a decrease in the end-product concentration. Obviously, the exact "break-even" point for the conversion rate and the dilution rate will depend on the reaction features, including substrate and enzyme concentrations in the reactor unit.

4.3. The problem of cellobiose

When both the substrate and product have similar molecular size and if they both pass the membrane, it is necessary to match the membrane transport rate with the reaction rate to ensure that as the enzyme reaches the substrate, it is converted, and the product is transported to the other side (Giorno and Drioli, 2000). An increase in dilution rates over a critical point may however result in a decrease in the lignocellulose hydrolysis conversion rate — even when the inhibition is reduced. This phenomenon is due to the simultaneous removal of soluble oligosaccharides, mainly cellobiose, i.e. β-glucosidase substrates.

In a modeling study with a membrane reactor Lee and Kim (1993) have demonstrated the importance of retaining the cellobiose at a certain level in order to increase the glucose production rate, especially when having a cellulase mixture with low BG activity. Based on hydrolysis kinetic models proposed by (Lee and Fan, 1983), Lee and Kim, (1993) also determined the optimal profile of the dilution rates to maximize the glucose production from a membrane bioreactor system (Fig. 6, 1c). The bioreactor system consisted of: *1.* a first batch operation, *2.* operation at maximum dilution rate, *3.* a second batch operation, and *4.* operation at approximately constant dilution rate. The optimal profile was dependent on the balance between increase in glucose formation from the cellobiose and a reduction due to the cellulase inhibition by cellobiose and glucose (Lee and Kim, 1993). Mainly, when the latter became higher than former reactor operation mode was switched from batch to maximum dilution rate in order to reduce the inhibition and maximal dilution was maintained until the gain from higher cellobiose concentration became larger. Thus, the cellulose conversion to glucose was increased by roughly 7% and the glucose concentration from the collected permeate was up to 2 times higher when the membrane reactors were operated with the described optimal profile of dilution rates, than with the constant dilution rate (Lee and Kim, 1993). When inspecting Table 3, it is interesting to note, that the product concentration in many cases has been given as the amount of reducing sugars and that no β-glucosidase was added. This raises the suspicion that a significant amount of the product obtained could be cellobiose.

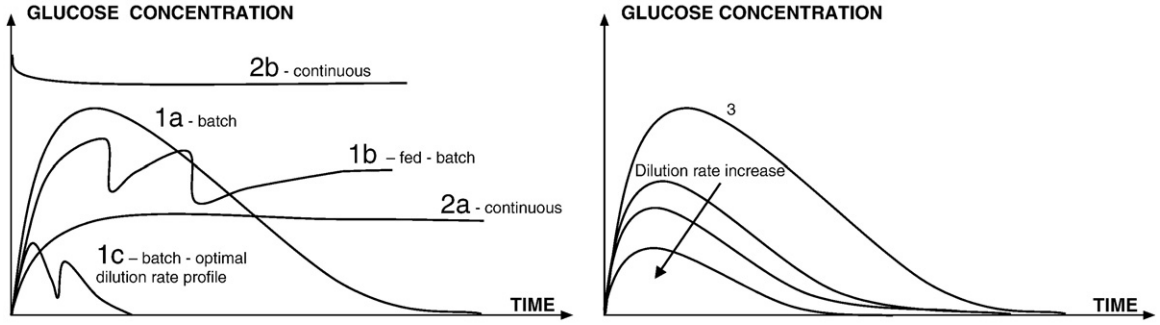

Fig. 6. Typical glucose concentration profile in the permeate from membrane bioreactor in a: (1a) batch (Lee and Kim, 1993; Ohlson et al, 1984, Yang et al., 2006); (1b) fed-batch (Knutsen and Davis, 2004; Kinoshita et al., 1986); (1c) batch with optimal dilution rate profile (Lee and Kim, 1993); (2a), (2b) continuous operation mode (Alfani et al., 1982; Ghose and Kostick, 1970; Hong et al., 1981; Klei et al., 1981); and (3) the typical change of glucose permeate profile with increase in dilution rate in the batch mode (Kinoshita et al., 1986; Lee and Kim, 1993; Ohlson et al., 1984; Yang et al., 2006). The relative position of the curves is due only to the overview and it is not related to the absolute value of the glucose concentration.

5. Factors affecting the membrane flux

Each of the separate compounds from the hydrolysis reaction mixture — lignocelluloses particles, macromolecular cellulases and product glucose — can cause a drastic reduction of flux through the membrane compared to the flux of pure water. This flux reduction is due to the membrane fouling, enzyme concentration polarization and/or high permeate viscosity. Furthermore, when the cellulosic substrate and enzymes are mixed together, the flux through the membrane can be more reduced than with the individual components.

5.1. Fouling

With dead-end filtration and crossflow microfiltration, Mores et al. (2001) found that the water flux (~14 000 L/(m^2 h)) through a polysulfone 0.2 μm (ΔP = 0.7 bar) membrane was appreciably reduced by pre-treated yellow poplar (5% (w/v), 1500 L/(m^2 h); 0.2% (w/v), 1300 L/(m^2 h), respectively), cellulases (0.3% (w/v), 300 and 400 L/(m^2 h) , respectively) and their mixture (100 and 360 L/(m^2 h) , respectively). The authors speculated that the cellulase–lignocellulose mixture showed increased fouling due to the stickiness of enzyme-bounded particles, especially since the flux was dramatically recovered by backflushing of the membrane (11 200–11 300 L/(m^2 h)).

5.2. Reaction slurry properties

The permeation rate during the ultrafiltration is affected principally by solids concentration of the reaction mixture, but also by apparent viscosity and density of the slurry system, and the total soluble sugars (Ghose and Kostick, 1970). Within 20 h of reaction time, Ohlson et al. (1984) found that the membrane fouling was responsible for the 30% and 55% initial UF membrane flux decline, for the washed and unwashed substrate slurry, respectively. Mores et al., 2001 found a 30% reduction of the permeate flux through the UF membrane (ΔP = 3.7 bar) in the presence of 8% (w/v) solution of glucose, owing to its high viscosity. When increasing the glucose concentration from 5 to 10% (w/v), Ghose and Kostick, 1970 saw roughly 15% (425–360 L/(m^2 h)) and 30% (35–25 L/(m^2 h)) reduction in the UF membrane flux, for 30 and 10 kDa membranes, respectively.

5.3. Molecular cut-off

The selection of the membrane with respect to molecular cut-off can have a profound influence on flux and glucose removal rates (dilution), the permeation of the cellulases and the response to trans-membrane pressure change. In general, the highest UF flux rates and increased glucose removal rates were seen with the higher cut-off values, in this case 10–30 kDa, 5–17 L/(m^2 h) (Ghose and Kostick, 1970). On the other hand, increasing the cut-off to more than 30–50 kDa, can result in leaking of the cellulases through the membrane (Kinoshita et al., 1986; Mores et al., 2001). Furthermore, in order to increase the permeate velocity, it is usually necessary to increase the trans-membrane pressure and the response will be dependant on the membrane fouling. However, the permeate flow rate may be influenced by the history of the applied pressure, e.g. due to membrane compaction (Alfani et al., 1983). This problem poses a significant operational challenge, because a consistent relationship velocity–pressure is then difficult to obtain (Hong et al., 1981). Kinoshita et al. (1986) have shown that with 0.5–50 kDa membranes, the smaller the membrane pore size, the higher is the pressure required to maintain a suitable flow rate. Table 3 highlights that most studies were conducted using membrane sizes from 10 to 30 kDa, and that lower (4 kDa) or higher (50 kDa) cut-off values were only employed occasionally.

5.4. Concentration polarization

The lignocellulose and enzyme particles present in the substrate solution can cause strong concentration polarization in the membrane bioreactor resulting in a notable flux decline.

In one study, the permeate flow rate through a UF membrane (= the flux) declined as a result of the presence of agitated cellulases (0.04% (w/v)) or soluble cellulose (0.1% (w/v) ZMC), and this decline was ascribed to be most likely due to concentration polarization (Alfani et al., 1983). In contrast, in the same study, the flux did not decrease when crystalline cellulose (0.3% (w/v) Avicel) was added, most likely because the added cellulose remained suspended, i.e. it was not soluble, in the bulk solution (Alfani et al., 1983).

The phenomenon of this concentration (c) build-up of a solute, e.g. in the case of lignocellulose notably the enzyme(s), the soluble cellulose, or the soluble products cellobiose and glucose, in the boundary layer may be represented by the following general non-steady-state differential mass balance:

$$\frac{\partial c}{\partial t} = D_S \cdot \frac{\partial^2 c}{\partial x^2} - J_v \cdot \frac{\partial c}{\partial x}$$

in which J_v is the solvent flux ($m^3\ m^{-2}\ h^{-1}$) and D_S is the solute diffusion coefficient ($m^2\ h^{-1}$).

If the solute molecules are completely retained by the membrane, the convective flow of the solute molecules towards the membrane surface will be equal to the diffusive transport back to the bulk phase, at steady-state conditions. The concentration polarization will be more pronounced at higher dilution rates, i.e. at higher product removal rates or at higher permeate flux. Unfortunately, the negative effect of increased dilution rate is not counterbalanced by the benefit of the corresponding reduction in the concentration of the reaction product in the reactor (Alfani et al., 1982). An illustration of this was problem was reported in a modeling study with a fixed boundary polarization layer thickness of 25 μm: In this case a doubling of the dilution rate from 0.8 to 1.6 h^{-1} decreased the fractional bulk concentration of enzyme from 0.97 to 0.12 (Hong et al., 1981). Since the cellulose is insoluble and requires the enzymes to be adsorbed in order for the reaction to take place, concentration polarization will decrease the effective concentration of enzyme and correspondingly the conversion rate.

The enzyme concentration polarization level mainly depends on solute accumulation due to membrane rejection, which is very strong for enzymes, and back diffusion, which is slow for enzymes, into the bulk solution. It can therefore be reduced and partially avoided by vigorous stirring in the region next to the membrane surface to decrease the polarization layer or by increasing the trans-membrane pressure for a higher permeate flux. However, the high local shear rate can deactivate the enzymes that are rejected and accumulated in a polarization film region immediately next to the membrane surface. This deactivation is accelerated by the exchange of the deactivated enzyme in the polarization layer where shear from stirring is high with the enzyme in the bulk solution through the diffusion and convection (Alfani et al., 1982; Hong et al., 1981). Thus, the conversion rate is affected both by the decrease of bulk concentration of enzyme and at the same time by the shear deactivation of the accumulated enzyme. Due to simultaneous effect of 2 distinct phenomena (product inhibition and concentration polarization), Alfani et al. (1982) found that the cellulose conversion rate was increased for small and medium values of the dilution rate (0.17–0.33 h^{-1}) and reached a maximum (0.38 h^{-1}). This increase in the conversion rate for the relatively small dilution rates was mainly due to the lower product accumulation in the reactor, at which there is a negligible enzyme inactivation by accumulation in the laminar sub-layer on the membrane surface that can be avoided by agitation of the reactor contents. Hong et al. (1981) determined that, when operating CSTR membrane cell for cellobiose

hydrolysis at a low dilution rate ($0.58\ h^{-1}$) the initially loaded enzyme participates 100% to the overall conversion and only 25% when dilution rate is increased to critical, $1.3\ h^{-1}$, after 10 h.

6. Membrane reactors for glucose removal during (ligno)cellulose hydrolysis: operational challenges

6.1. The glucose concentration

The overall benefit of removing the enzyme inhibiting product (here glucose) can be heavily diminished if its concentration is not greatly reduced. It can be seen that, on the contrary to conversion, the final outlet concentration of glucose is in general ranges between 0.1 and 75 g/L and typically bellow 35 g/L (Table 3). Knutsen and Davis (2004) showed that the resulting glucose concentrations (7–27 g/L) after corn stover hydrolysis with intermittent removal were lower than in the control batch experiment (maximal 70 g/L) but did not result in an increased final cellulose conversion (~60%). This may be due to the reduced concentration still being too high to significantly influence the reaction rate (see Fig. 3). The authors speculated that the lower product concentration and thus enzyme inhibition could possibly be obtained by more frequent filtration or washing. This would however result in a too dilute product stream. Yang et al. (2006) presented a several-fold increase in the hydrolysis rate when increasing the substrate concentration, but each of the individual hydrolysis rates declined drastically during the first several hours. This was suggested to be a result of a gradual increase in the non-productively adsorbed enzymes, transformation of cellulose into a less digestible form and incomplete elimination of the product inhibition. In another study, by Gan et al. (2002), the reaction rate improvement obtained immediately after product removal was equivalent to an increase in the conversion rate of 4–10 times, but the average reaction rate after 20 h was not significantly affected. This led the authors to conclude that relief of the product inhibition through the continuous product removal is limited due to the inherently slow cellulose hydrolysis kinetics (Gan et al., 2002). We disagree, with this conclusion, and would rather suggest that the overall result may be a consequence of insufficient — or delayed — product removal (re Fig. 3). A relatively small concentration of glucose strongly inhibited the activity of β-glucosidase such that a two-fold increase in glucose concentration, from 0.27 to 0.66 g/L, caused a specific cellobiose hydrolysis rate reduction of almost 40% (Alfani et al., 1983). Therefore, glucose concentration in the reaction medium has to be kept as low as possible to limit the extent of inhibition. This can be achieved only in a reactor working at relatively high dilution rates and encompassing continuous removal of glucose. This however, yields low glucose concentration in the permeate and requires drastic concentration (up to $\approx$100 g/L) in order to perform the subsequent fermentation to ethanol economically (Alfani et al., 1983).

6.2. Fed-batch operation

It appears that the fed-batch feeding of the cellulosic substrate can have a positive effect on the conversion rate and especially, on the specific enzyme efficiency or consumption allowing more substrate to be converted with the same loading of enzymes, while at the same time the viscosity of the reaction slurry may be reduced. Ishihara et al. (1991) reduced the specific enzyme consumption from 32 FPase IU per g reducing sugars, produced by fed-batch substrate–enzyme hydrolysis of steamed hardwood and hardwood kraft pulp to 27 and 7.4 FPU per gram of reducing sugar produced for steamed hardwood and hardwood kraft pulp, respectively. The fed-batch feeding was accomplished by a semi-continuous feeding of substrate with intermittent product removal. When Ohlson et al. (1984) ran the membrane reactor semi-continuously for 20 h, the fresh substrate was intermittently fed while the dry matter concentration was kept constant based on estimation of products in the permeate. This operation, which was repeated each day for 3 days with the same batch of enzymes, gave a final conversion of 80% (94%, 81% and 60% after each day), but also very high cellulase efficiency of roughly 25 g reducing sugars per g of enzyme used compared to classical batch hydrolysis, which by a rough estimate gives 5 g reducing sugars/g enzyme (Ohlson et al., 1984). In the work of Knutsen and Davis (2004), the hydrolysis was promoted by the fed-batch addition of fresh substrate and an increase in the hydrolysis rate was seen after each filtration and substrate addition (Fig. 6, 1b). This effect was explained by presence of fresh amorphous cellulose, rather than by reduced inhibition due to the removal or increased substrate concentration. Furthermore, the semi-batch hydrolysis with thorough washing of material with water or extra addition of enzymes along with fresh substrate to replace deactivated enzyme, after each filtration step, resulted in a marked increase in conversion (Knutsen and Davis, 2004). On the contrary, Kinoshita et al. (1986) found that the concentration in the membrane bioreactor gradually decreased during the reaction although with transient increase due to the fed-batch feeding of substrate, following the first-order decay kinetics. Finally, unless very dry, pre-treated substrate is added, a negative 'dilution effect' which lowers the actual glucose concentration, will result because of the simultaneous addition of water with the pre-treated substrate. This phenomenon has been observed in practice in fed-batch reactions with pre-treated straw (Rosgaard et al., 2007a).

6.3. Continuous operation

The continuous mode of operation assumes continuous feeding of reactants and continuous removal of the products (glucose) and unconverted biomass. In the related literature, the term 'continuous hydrolysis' is slightly ambiguous, since, sometimes, it really refers to continuous product removal, and not to the overall mode of reactor's operation. By operating around 13 h in the continuous mode, Bélafi-Bakó et al. (2006) found that conversion was practically maintained at the same level as achieved after 9 h batch hydrolysis, but the authors did not state explicitly whether the continuous mode assumed continuous feeding of the fresh substrate and buffer into the reservoir vessel.

The reported dry matter levels of cellulosic material in exploratory enzyme-catalyzed lignocellulose hydrolysis reactions have generally been <10%, and has only been higher, e.g. 15–19% in exceptional cases (Table 3). These levels are considerably lower than what is required for a glucose-rich hydrolysate for subsequent fermentation to produce bioethanol (Andrić et al., 2010b). The advantages of continuous operation of biocatalytic reactors for hydrolysis of (ligno)cellulose has been advocated already 40 years ago (Ghose, 1969), but has still only been little studied, and only exceptionally with substrate levels higher than 2.5–5 DM% (Table 3).

The inherently slow reaction of the enzyme-catalyzed cellulolytic hydrolysis requires relatively large volumes for the given conversion or in practice low flow rates of the reaction mixture. Another main problem related to continuous operation at high DM% is the inefficient mixing of the viscous slurry. Other problems include the fouling of the membranes and the difficulty in discharging the unconverted residue that accumulates in the system. Ghose and Kostick (1970) converted a membrane cell into a continuous reactor which maintained steady hydrolysis of 10% (w/w) cellulose and simultaneous removal of products at a high conversion level, namely 77%, mainly because of the rapid transport of the reaction products through the membrane film (0.074 g/min). This resulted in a relatively high average glucose content, 7.5%, in the effluent.

The membrane reactors in the experimental set-ups reported until now (Table 3) have chiefly been in mL scale, from 50 to 300 mL, and have rarely included larger reactors of e.g. 5.5–10 L. Thus, to date there is a surprising scarcity of reports on continuous operation of

membrane reactors for lignocellulose hydrolysis. Considering the significant importance of operational features to overcome the possible restrictions outlined above, the design of large scale membrane reactors for lignocellulose hydrolysis still deserves significant research efforts.

6.4. Enzyme activity retainment

Maintenance of a high level of cellulase activity is essential for the efficient operation of the membrane reactor, due to the possible reuse of the enzymes in several successive reaction cycles. Although — within the membrane reactors with product removal — the enzyme activity loss as a result of the product inhibition is significantly reduced, the other well known reasons for the enzyme deactivation, i.e. mainly unproductive adsorption of enzymes to lignocellulose and heat inactivation in these reactors also can arise. Table 4 gives an overview of the activity studies performed in relation to the operation of the biocatalytic membrane reactors. In general, it appears that cellulolytic enzymes can relatively long maintain a reasonably high level of activity. The presence of substrate generally stabilizes the activities of CBH and EG more than the activity of β-glucosidases, which act in the aqueous phase. The (ir)reversible adsorption of enzymes to the non-convertible fraction seems to be one of the major causes of the activity reduction, especially if the substrate in question is lignocellulose, except in (Knutsen and Davis, 2004), where the tightly bound enzymes were actually capable of efficiently hydrolyzing the freshly added ligocellulosic material. The shear deactivation may also, albeit to a lower extent, be responsible for activity decay due to the passage through the hollow fibers (Klei et al., 1981) or tubes (Ishihara et al., 1991; Yang et al., 2006) or in the stirred cells because of localization of enzymes in the thin layer due to the concentration polarization, especially in the case β-glucosidases (Hong et al., 1981).

Table 4
Overview of enzyme deactivation studies in membrane reactors. CBH is cellobiohydrolase; EG is endoglucanase; BG is β-glucosidase.

Activity type	Activity loss [%]	Period [day]	Substrate presence	Comment	Source
CBH[1] EG[4] BG	56–67 44–50 –	0.2	No	None of the enzyme found in effluent; maybe inadequacies in enzyme assay procedure; some other inactivation	Ghose and Kostick (1970)
CBH EG BG	– – 0–35	10–60	No	Thermal deactivation negligible; shear–stress and concentration polarization related deactivation in membrane reactor	Hong et al. (1981)
CBH EG BG	 29	9	Yes[10]	Deactivation due to passage through hollow fibers or adsorption onto substrate; soluble BG easily shear-deactivated; 40% of BG activity lost on immobilization, but stability better than in the soluble form	Klei et al. (1981)
CBH EG BG	– 10 10	6	No	Low thermal deactivation; remarkable mechanical stability at 500 rpm	Ohlson et al. (1984)
CBH EG BG	– 90[6,7] 60[6,7]	3	Yes[10]	Reversible/non-reversible adsorption and denaturation	Ohlson et al. (1984)
CBH[3] EG[2] BG	20–50[8] 5–15[8] 45–80[8]	1	Yes	Thermotolerant; at 30 °C negligible deactivation; inactivation in the reactor at temperatures 30–60 °C follows 1. order decay— the activity of proteases from enzyme preparation probable reason	Kinoshita et al. (1986)
CBH[1] EG[2] BG	85 75 65	15	No	Significant loss after 6–7 days; proteolytic modification $-T$, pH; in reactor irreversible adsorption on insoluble residue and UF tubular unit physical deactivation	Ishihara et al. (1991)
CBH[1] EG[2] BG[5]	55 10 30	8	No	Thermal deactivation	Lee and Kim (1993)
CBH EG BG	 Small[9]	2	Yes[10]	Shear deactivation seems insignificant	Gan et al. (2002)
CBH[1] EG BG	50 – 30	1	Yes[10]	Adsorption to non-convertible biomass fraction; shear–stress deactivation negligible in tubular reactor; no thermal deactivation in 1 day without substrate	Yang et al. (2006)
CBH EG BG	 N/A	0.9	Yes[10]	Slow permeation of enzymes though the membrane	Bélafi-Bakó et al. (2006)

[1]Filter paper activity.
[2]Activity on CMC.
[3]Activity on KC Floc.
[4]C_x activity.
[5]Activity on pNPG.
[6]Soluble activity.
[7]Semi-continous operation.
[8]50–60 °C.
[9]90 rpm.
[10]In the reactor.

The thermal inactivation does not emerge as the particularly important factor (Hong et al., 1981; Ohlson et al.; 1984, Yang et al., 2006), although quite a few studies were performed at temperatures lower than 50 °C (Alfani et al., 1982; Gan et al., 2002; Ishihara et al., 1991; Kinoshita et al., 1986; Knutsen and Davis, 2004; Ohlson et al., 1984), including one case, as explicitly stated, with thermotolerant enzymes (Kinoshita et al., 1986).

7. Membrane reactors and hydrolysis kinetic studies

Apart from representing a promising concept of potential realization in real industrial processes, it appears that integrated reactors with product removal such as membrane bioreactors can be valuable tools for understanding mechanisms and performing various kinetic studies of enzymatic reactions, including lignocellulose enzymatic hydrolysis. Specifically, membrane reactors can be used for obtaining kinetic information on reaction order towards substrates, the rate of enzyme thermal inactivation and the extent of product inhibition (Alfani et al., 1983; Frenneson et al., 1985).

7.1. General kinetic studies using membrane reactors

With respect to thermal inactivation of cellulases and hydrolysis reaction orders, the advantage lies in eliminating the strong product inhibitory effect which can disguise the true results under the studied conditions. Further, it is known that the enzyme stability is increased by the substrate — presumably because the cellulases remain bound to the residual solids (Knutsen and Davis, 2004) — especially when the substrate is present in high levels. The batch reactors seem inappropriate for this purpose because *S* changes with the course of the reaction making it necessary to extrapolate the measured activity at zero time (leading to errors). In membrane reactors it is possible to perform studies on cellulase activity due to the substrate feeding option and the selectivity of the membrane. These options allow for keeping the substrate and enzyme concentrations constant at the same time and thus determination of enzyme activity or stability at prolonged times and different temperatures (Alfani et al., 1983). As equally important is that the activity/stability studies can be performed at operationally encountered substrate levels and not just at typical assay conditions which are the most likely performed without the substrate presence. Although not kinetically modeled, comparisons of special membrane reactor designs and membrane constructions have also allowed the experimental assessment of the consequences of e.g. simultaneous immobilization of the enzymes and the substrate onto the membrane surface — with continuous product removal (Bélafi-Bakó et al., 2006).

In order to obtain more information on cellulose hydrolysis kinetics during continuous product removal Alfani et al. (1982) used an ultrafiltration cell for removal of glucose, continuously fed with buffer solution, for studies on microcrystalline cellulose. To maintain a constant substrate concentration in the reactor, the conversion was kept at a low level using very low substrate and enzyme concentrations. With longer reaction times a negative derivative in the glucose kinetic curve could be due to the enzyme deactivation and/or substrate consumption by cellulose hydrolysis.

7.2. Product inhibition studies

In the case of product inhibition, the reaction rate and inhibitor concentration are related. Experiments with constant product inhibitor concentration(s) can obviously not be accomplished in batch reactors. In membrane reactors, inhibitor levels can be selected and the extents of product inhibition can be tested at a wide range of concentrations and reaction times — by varying the dilution rate and enzyme concentration — all at a constant substrate concentration. Theoretically, even a completely uninhibited reaction could be accomplished if the product removal was initiated immediately and if it was then run continuously and securing a complete product removal.

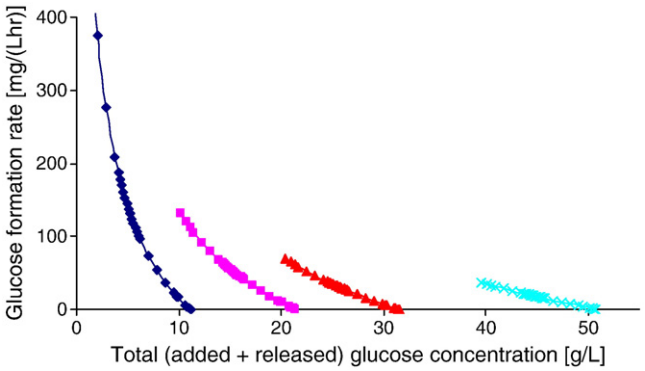

Fig. 7. Influence of added glucose on glucose formation rate in a study where glucose is externally added (0–40 g/L) prior to reaction (2%DM); the formation rates are calculated from the non-competitive inhibition model (Andrić et al., 2010a). Figure legend: 0 g/L 10 g/L 20 g/L 40 g/L.

Because the reaction mixtures of enzymatic lignocellulose hydrolysis experiments are viscous and heterogeneous, most of the inhibition studies have assessed the inhibition in batch reactions with addition of the inhibitor (e.g. glucose) at concentrations ranging from high to relatively low substrate levels, i.e. low *S*/*G* and *E*/*G* ratios (Figs. 7 and 8). During genuine operations, however, without *added* glucose, the *S*/*G* ratio (as well as the *E*/*G* ratio) will be quite high for most of the reaction time, due to lower level of present glucose. Hence, the initial addition of glucose in inhibition studies in effect exaggerates the product inhibition (Fig. 7).

Inhibition can be studied in membrane reactors in the operational range of *S*/*G* and *E*/*G* ratios by varying the dilution rate. The removal rather than addition of inhibitor is very helpful with respect to avoiding the unwanted reversible reaction (transglycosylation) which occurs at low *E*/*G* ratios and which may give false information on the real inhibitory influence of the studied products. As the enzymes are in practice dosed per weight of substrate (cellulose or dry matter), low *E*/*G* will mean usually low *S*/*G* ratios. We have recently observed the transglycosylation effect at $S/G<0.1\%$ (w/v)/(g/L) and $E/G<0.25$ g/g (Andrić et al., 2010b). Many of the studies on inhibition of cellulolytic enzymes — and especially on β-glucosidases — have been performed at very low *S*/*G* ratios (and related *E*/*G*) (Fig. 8) which in turn might have induced transglycosylation effect. The attention should furthermore be paid to reactions with genuine lignocellulosic substrates, as the *S*/*G* in reality may be diminished due to the presence of a non-convertible substrate fraction (Fig. 8).

8. Other techniques for glucose removal

Although the ultrafiltration has extensively been used as the most convenient glucose removal technique, a few other techniques must be mentioned: (*a*) two-phase systems; (*b*) addition of another enzyme (glucose-oxidase) which oxidizes glucose to gluconic acid; (*c*) simultaneous saccharification and fermentation (SSF), where the glucose is instantly fermented to ethanol; or (*d*) glucose crystallization. Among these, the SSF and aqueous two-phase system, reported by Tjerneld et al. (1985), are the most commonly encountered.

8.1. Two-phase systems

This technique is based on partitioning of the enzyme and substrate to the bottom phase while the product can be extracted in the top phase. The enzymes can then be recycled and process run in a semi-continuous mode. The phases with extractants are biocompatible in order not to denature or inhibit the enzymes. The reduction of the specific enzyme

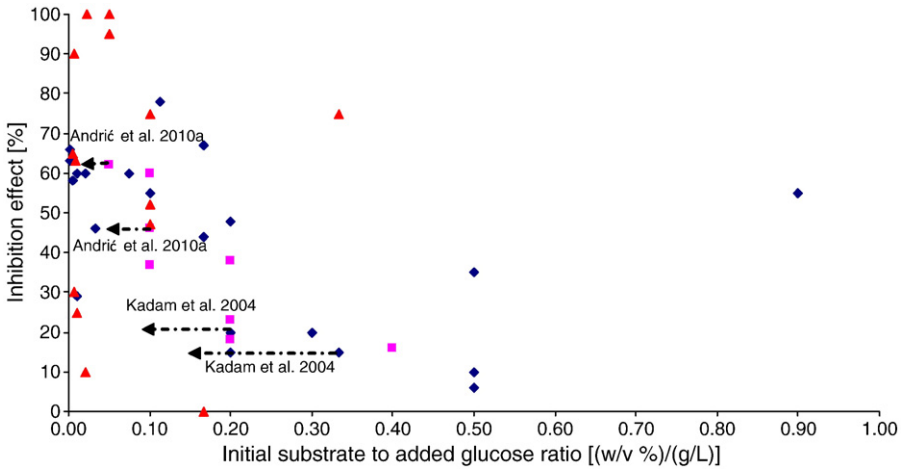

Fig. 8. Summary of inhibition studies from Andrić et al., 2010a. The dashed lines designate the beginning of transglycosylsation effect observed in Andrić et al., 2010a. The marked studies have employed lignocellulosic substrates — the dashed-dotted arrows point at real 'cellulosic' substrate-to-added glucose ratio. Frenneson et al., 1985 have used a membrane reactor. Figure legend: Cellulases Cellulases + β-glucosidases β-glucosidases.

consumption is reported (17 FPU per gram reducing sugar) in these systems as compared to the batch hydrolysis (Tjerneld et al., 1985).

8.2. Dialysis

The dialysis separation technique is based on different diffusion coefficients of glucose and cellulases, so the membrane is permeable only for the low-molecular weight compound while the larger ones (enzymes and substrate) are retained within the membrane. Only the glucose thus diffuses through the thin dialysis membrane (Fig. 9) and there is no need for the liquid (buffer) replacement which lowers the glucose concentration, as seen with the MF or UF membranes. However, the glucose must diffuse into the surrounding dialysate buffer whose large volume ensures sufficient driving force for the separation and — unfavorably — lowers the final glucose concentration (Fig. 10). In addition, due to the differences in the chemical potential across the dialysis membrane, caused by the presence of the biomass substrate and osmotic pressure, the dialysate buffer penetrated into the dialysis membrane reactor, lowering the reactants and products concentration inside the reaction mixture (Fig. 11).

We have recently used dialysis to remove the glucose from the reaction mixture consisting of hydrothermally pre-treated wheat straw and commercial enzyme mixture (Andrić et al., 2010a). When glucose was removed by *in-situ* dialysis during the enzymatic hydrolysis, the rate of enzyme-catalyzed glucose release during 48–72 h of reaction recovered from 20 to 40% to become ≈70% of the rate recorded during 6–24 h of reaction (Table 5). This indicated the importance of the alleviation of the product inhibition, and the potential workability of *in-situ* dialysis as a principle for product removal during enzymatic lignocellullose hydrolysis.

Apart from the obvious problems, i.e. mechanical stability, mixing in the membrane, employment of high DM%, the future dialytic process for removal of glucose from lignocelluloses mixture with cellulase, will need to address: *(a)* prevention of the dialysate in-flux to the membrane (for example by applying pressure from the internal side); *(b)* reduction of the amount of dialysate buffer needed for the separation, in order to obtain higher glucose concentrations, without drastically reducing the driving force and thus membrane flux, and/or *(c)* energy efficient post-concentration step.

8.3. Simultaneous hydrolysis and fermentation (SSF) and removal of ethanol

The concept of *in-situ* removal of ethanol during the SSF reaction may encompass simultaneous removal of cellobiose, glucose and

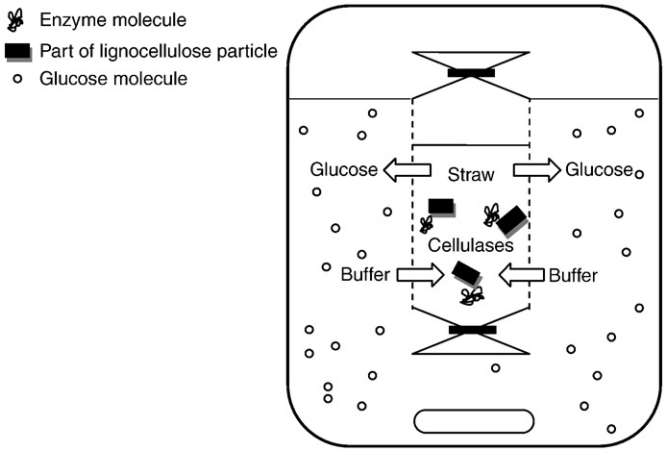

Fig. 9. The principle of hydrolysis of lignocelluloses with *in-situ* removal of glucose by dialysis.

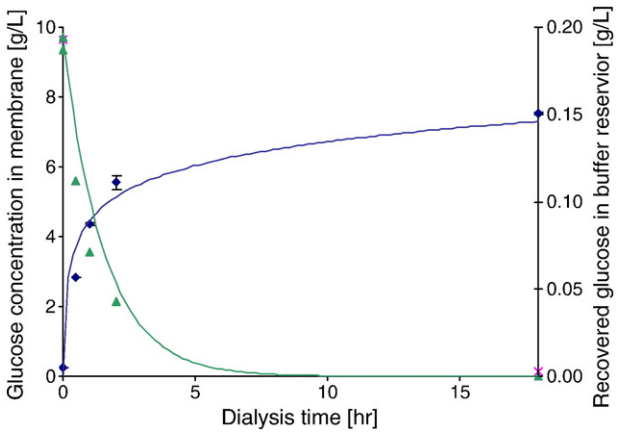

Fig. 10. Dialysis of model solutions. The performance of membrane with 10 g/L glucose solution. The glucose is collected in a buffer reservoir surrounding the membrane; the concentration is measured and used to calculate the glucose concentration in a dialysis membrane, which was furthermore measured at the start and end of dialysis. Starting volume was 25 mL. The green and dark blue solid lines are the fits of the calculated glucose concentration in dialysis membrane and in the reservoir, respectively. Figure legend: × glucose in dialysis membrane_exp ▲ glucose in dialysis membrane_calc ◆ glucose in reservoir_exp.

Table 5
The effect of glucose removal by *in-situ* dialysis on glucose yield (g/g) from a reaction mixture with pre-treated wheat straw during treatment with a commercial cellulase mixture (Andrić et al., 2010a). The dialysis was applied from 24 to 48 h.

	24 h	48 h	72 h	96 h
Control hydrolysis	0.54	0.65	0.70	0.76
In-situ glucose removal	0.54	0.71	0.93	0.94

ethanol, all of which are inhibitory for the enzymes. Therefore, this concept from the theoretical point of view represents the most desired processing option, as minimizes all inhibitory effects on the rate of the enzyme- and yeast-cell-catalyzed reactions. The concept is further based on producing fermentations broths with concentrated ethanol that is less energy demanding to further purify by distillation to the product grade than the ordinary distillation, which is in turn typically done after the fermentation of the hydrolyzate (Cardona and Sanchez, 2007). Furthermore, the ethanol may increase volatility when found in the fermentation broth containing also the enzymes (Roychoudhury et al., 1986). One attractive option within this concept is to evaporate the water–ethanol mixture from the SSF vessel by rapid vacuum shortly applied in cycles while the substrate can be semi-continuously fed (Roychoudhury et al., 1992). Another interesting solution is to remove the ethanol by the membranes; in a SSF process coupled with the pervaporation membranes or the membrane distillation modules (Cardona and Sanchez, 2007). It is our impression that this potentially attractive subject has not received a major attention in the literature. In general, the search for the feasible integrated reaction–separation technology for the lignocellulose degradation is currently ongoing and the summary of these concepts e.g. vacuum fermentation, fermentation with gas stripping, fermentation coupled with pervaporation, extractive fermentation, etc, is presented in Cardona and Sanchez (2007).

The SSF coupled with ethanol removal is conceptually advantageous configuration to SHF due to the reduced cellobiose, glucose and ethanol inhibition, but on the other hand, represents a heavy compromise of two optimal reaction conditions (pH and temperature) and includes another (vulnerable) biocatalyst i.e. fermenting microorganism like yeast cells, in the reaction vessel containing highly heterogeneous and viscous mixture (lignocellulose, cellulases, yeasts, nutrients, buffers, sugars, etc.). Furthermore, if it is desired to manufacture some other products from glucose hydrolyzate such as biochemicals, biobutanol or all together in a biorefinery, the SSF concept may become inappropriate. We highly acknowledge the SSF and connected separation technologies, but give an advantage to the SHF concept due to the larger variety of products that is possible to generate from glucose and uncompromised optimal conditions, as well as due to the simpler reactor operation and interpretation of the experimental data.

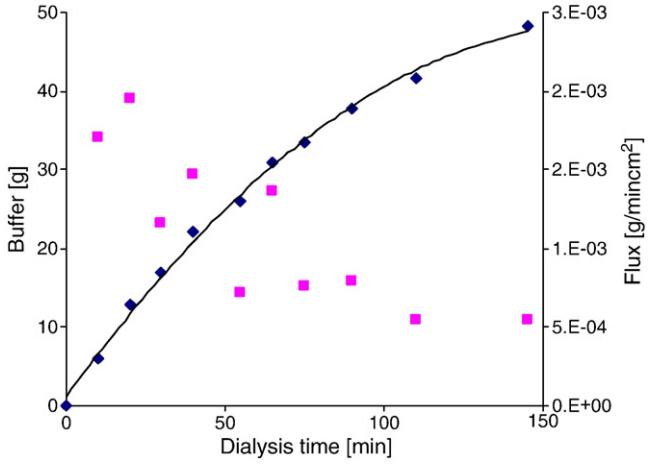

Fig. 11. Dialysis of model solutions, Buffer back-flux into the membrane with 2% DM real lignocellulosic substrate (pre-treated wheat straw). Starting volume was 25 mL. The solid line is the fit of the measured amount of buffer which penetrated into the dialysis membrane. Figure legend: ◆ weight of buffer penetrated [g] ■ buffer back-flux [g/(min cm^2)].

9. Membrane bioreactor design for glucose removal: conclusions and recommendations

9.1. Summary

A number of reactor improvements for achieving optimal membrane reactor performance for lignocellulose product removal reactions have been suggested in the literature. For the production of concentrated glucose syrups from lignocellulose, Ghose and Kostick (1970) already advised the use of a relatively large reactor volume and a comparatively small separation system to attain a high initial saccharification rate with a dense reaction slurry. This approach is of course only justifiable if it is possible to remove the products as fast as they are formed, so that the product removal results in a glucose concentration below at least 10 g/L (Fig. 3). This will also allow re-use of the enzymes. A model system was proposed with an STR for finely milled cellulose with a concentrated cellulase preparation. The inputs consisted of substrate and small amounts of dilute cellulases combined with a thin-channel membrane separation cell (Ghose and Kostick, 1970). Essentially, the idea is to have a high initial rate to build-up glucose fast, since the 'controlling factor' of the model to operate successfully is the initial rate of the product formation and not the flux of product removal.

For the prevention of concentration polarization and the deactivation by shear forces, Hong et al. (1981) proposed operation of membrane reactors for enzymatic cellulose hydrolysis to be done at dilution rates lower than critical. Similarly, Kinoshita et al. (1986) recommended adjustment of the flow rate from high initial level to lower, in accordance with the decrease in the activity in reactor, in order to obtain a constant concentration of products.

For partial recovery of enzymes Knutsen and Davis (2004) reasoned that use of ultrafiltration appeared unnecessary suggesting that sufficient (even β-glucosidase) activity was adsorbed onto lignocellulosic particles to convert the fresh added material, and concluding that the use of more than 10 FPU/$g_{cellulose}$ is unnecessary if long-term conversion is the goal. Finally, Bélafi-Bakó et al. (2006) have suggested to immobilize β-glucosidases in the lumen (permeate) side of the module for the improvement of the conversion in the membrane reactor, with respect to expected considerable amount of cellobiose in the product solution.

9.2. Advantages and challenges of membrane reactors

Contemplation of the available data lead us to recommend the following: although the operational feasibility of membrane reactors is currently questionable at high solids loadings, membrane reactors principally seem advantageous for large-scale enzyme-catalyzed production of hydrolysates for bioethanol manufacture or for platform biochemicals based on lignocellulosic material.

Advantages:

- Membrane reactors can provide for increased cellulolytic hydrolysis reaction rates because glucose product removal, and thus reduced inhibition, can be accomplished.
- Membrane reactors allow the continuous re-use of enzymes.
- The possibility of cellulases re-utilization paves the way for higher enzyme usage efficiency ($kg_{product}/kg_{enzyme}$) and, thus, allows

(economically) viable employment of enzyme dosages that are higher than in the conventional batch or continuous reactors (enzyme single-usage) which in turn further increases the conversion rates.
- Fed-batch feeding to secure low viscosity and high reaction rates may in principle be accomplished without altering the reaction volume as long as the dilution rate for product removal is adjusted accordingly, while maintaining a high cellulase partitioning tendency (insoluble vs. liquid phase) as the reaction is progressing.
- Membrane reactors with glucose product removal enable simultaneous removal of other inhibitory lower-molecular compounds, e.g. resulting from substrate pre-treatment that can eventually reduce the biocatalyst performance.

However, a number of important challenges must be recognized to accomplish successful operation.

These challenges are:

- The outlet (permeate) glucose concentrations is generally too low. To fully alleviate product inhibition by glucose product removal, the glucose levels in the reactor — and hence in the permeate, must be very low, typically <10 g/L to have an effect.
- Membrane fouling is pronounced a high substrate (dry matter) levels, and currently only low to medium dry matter levels are possible to process.
- Discharge of the non-convertible fraction which accumulates during extended reaction, especially with fed-batch operation is difficult. Removal of the lignin fraction prior to hydrolysis might be helpful.
- Scale-up of the membranes as well as the membrane reactors.

The final selection of the appropriate system, i.e. integrated/separate units; conventional reactors without removal, etc., depends on the capital and operational cost of the filtration units, cost of slurry transport, and biocatalyst and raw material and the market price of the final product. However, it seems certain that a serious focus on reactor design for biocatalytic conversion of cellulose to glucose is required for successful industrial realization of cellulosic ethanol.

References

Ahmed F, Stein A, Lye GJ. In-situ product removal to enhance the yield of biocatalytic reactions with competing equilibria: α-glucosidase catalysed synthesis of disaccharides. J Chem Technol Biotechnol 2001;76:971–7.

Alfani F, Albanesi D, Cantarella M, Scardi V, Vetromile A. Kinetics of enzymatic saccharification of cellulose in a flat-membrane reactor. Biomass 1982;2:245–53.

Alfani F, Cantarella M, Scardi V. Use of a membrane reactor for studying enzymatic hydrolysis of cellulose. J Membr Sci 1983;16:407–16.

Andrić P, Meyer AS, Jensen PA, Dam-Johansen K. Effect and modeling of glucose inhibition and in situ glucose removal during enzymatic hydrolysis of pretreated wheat straw. Appl Biochem Biotechnol 2010a;160:280–97.

Andrić P, Jensen PA, Meyer AS, Dam-Johansen K. Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of glucose inhibition on cellulolytic enzymes; 2010b. doi:10.1016/j.biotechadv.2010.01.003.

Bélafi-Bakó K, Koutinas A, Nemestóthy N, Gubicza L, Webb C. Continuous enzymatic cellulose hydrolysis in a tubular membrane bioreactor. Enzyme Microb Technol 2006;38:155–61.

Bezerra RMF, Dias AA. Enzymatic kinetic of cellulose hydrolysis. Inhibition by ethanol and cellobiose. Appl Biochem Biotechol 2005;126:49–59.

Cabrera-Padilla RY, Pinto GA, Giordano RLC, Giordano RC. A new conception of enzymatic membrane reactor for the production of whey hydrolysates with low contents of phenylalanine. Process Biochem 2009;44:269–76.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 2009;37:D233–8.

Cardona CA, Sanchez OJ. Fuel ethanol production: process design trends and integration opportunities. Bioresour Technol 2007;98:2415–57.

Cauwenberg V, Vergossen P, Stankiewicz A, Kierkels H. Integration of reaction and separation in manufacturing of pharmaceuticals: membrane-mediated production of S-Ibuprofen. Chem Eng Sci 1999;54:1473–7.

Chekhova E, Barton P-I, Gorak A. Optimal operation processes of discrete-continuous biochemical processes. Comput Chem Eng 2000;24:1167–73.

Decker CH, Visser J, Schreier P. β-Glucosidase multiplicity from *Aspergillus tubingensis* CBS 643.92: purification and characterization of four β-glucosidases and their differentiation with respect to substrate specificity, glucose inhibitin and acid tolerance. Appl Microbiol Biotechnol 2001;55:157–63.

Drioli E. Membrane reactors. Chem Eng Process 2004;43:1101–2.

Frenneson I, Trägårdh G, Hahn-Hägerdal B. An ultrafiltration membrane reactor for obtaining experimental reaction rates at defined concentrations of inhibiting sugars during enzymatic saccharification of alkali-pretreated sallow: formulation of a simple empirical rate equation. Biotechnol Bioeng 1985;27:1328–34.

Frieden E, Walter C. Prevalence and significance of the product inhibition of enzymes. Nature 1963;198:834–7.

Fullbrook PD. Practical limits and prospects (kinetics). In: Godfrey T, West S, editors. Industrial Enzymology 2nd Ed. London UK: MacMillan Press Ltd; 1996. p. 505–40.

Gan Q, Allen SJ, Taylor G. Design and operation of an integrated membrane reactor for enzymatic cellulose hydrolysis. Biochem Eng J 2002;12:223–9.

Gan Q, Allen SJ, Taylor G. Kinetic dynamics in heterogeneous enzymatic hydrolysis of cellulose: an overview, an experimental study and mathematical modeling. Process Biochem 2003;38:1003–18.

Ghose TK. Continuous enzymatic saccharification of cellulose with culture filtrates of *Trichoderma viride* QM 6a. Biotechnol Bioeng 1969;11:239–61.

Ghose TK, Kostick JA. A model for continuous enzymatic saccharification of cellulose with simultaneous removal of glucose syrup. Biotechnol Bioeng 1970;12:921–46.

Giorno L, Drioli E. Biocatalytic membrane reactors: applications and perspectives. Trends Biotechnol 2000;18:339–49.

Gusakov AV, Sinitsyn AP, Klyosov AA. Factors affecting the enzymatic hydrolysis of cellulose in batch and continuous reactors: computer simulation and experiment. Biotechnol Bioeng 1987;40:663–71.

Hahn-Hägerdal B, Andersson E, López-Leiva M, Mattiasson B. Membrane biotechnology, co-immobilization, and aqueous two-phase systems: alternatives in bioconversion of cellulose. Biotechnol Bioeng Symp 1981;11:651–61.

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G. Bio-ethanol — the fuel of tomorrow from the residues of today. Trends Biotechnol 2006;24:549–56.

Henley RG, Yang RYK, Greenfield PF. Enzymatic saccharification of cellulose in membrane reactors. Enzyme Microb Technol 1980;2:206–8.

Hong J, Tsao GT, Wankat PC. Membrane reactor for enzymatic hydrolysis of cellobiose. Biotechnol Bioeng 1981;23:1501–6.

Ishihara M, Uemura S, Hayashi N, Shimizu K. Semicontinuous enzymatic hydrolysis of lignocelluloses. Biotechnol Bioeng 1991;37:948–54.

Kinoshita S, Chua JW, Kato N, Yoshida T, Taguchi H. Hydrolysis of cellulose by cellulases of *Sporotrichum cellulophilum* in an ultrafilter membrane reactor. Enzyme Microb Technol 1986;8:691–5.

Klei HE, Sundstrom DW, Coughlin RW, Ziolkowski K. Hollow-fiber enzyme reactors in cellulose hydrolysis. Biotechnology and Bioengineering Symposium 1981;11:593–601.

Knutsen JS, Davis RH. Cellulase retention and sugar removal by membrane ultrafiltration during lignocellulosic biomass hydrolysis. Appl Biochem Biotechnol 2004;113–116:585–99.

Kuroiwa T, Izuta H, Nabetani H, Nakajima M, Sato S, Mukataka S, et al. Selective and stable production of physiologically active chitosan oligosaccharides using an enzymatic membrane bioreactor. Process Biochem 2009;44:283–7.

Lee Y-H, Fan LT. Kinetic studies of enzymatic hydrolysis of insoluble cellulose: (II). Analysis of extended hydrolysis times. Biotechnol Bioeng 1983;25:939–66.

Lee S-G, Kim H-S. Optimal operating policy of the ultrafiltration membrane bioreactor for enzymatic hydrolysis of cellulose. Biotechnol Bioeng 1993;42:737–46.

Levenspiel O. The chemical reactor omnibook. Cortvallis, Oregon: OSU Book Stores, Inc.; 1993.

Levenspiel O. Chemical reaction engineering. Third edition. John Wiley & Sons Inc; 1999.

Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, et al. How biotech can transform biofuels. Nat Biotechnol 2008;26:169–72.

Meng F, Chae S-R, Drews A, Kraume M, Shin H-S, Yang F. Recent advances in membrane bioreactors (MBR): membrane fouling and membrane material. Water Res 2009;43:1489–512.

Merino ST, Cherry J. Progress and challenges in enzyme development for biomass utilization. Adv Biochem Engin/Biotechnol 2007;108:95-120.

Mores WD, Knutsen JS, Davis RH. Cellulase recovery via membrane filtration. Appl Biochem Biotechnol 2001;91–93:297–309.

Ohlson I, Trägårdh G, Hahn-Hägerdal B. Enzymatic hydrolysis of sodium-hydroxide-pretreated sallow in an ultrafiltration membrane reactor. Biotechnol Bioeng 1984;26:647–53.

Philippidis GP, Smith TK, Wyman CE. Study of the enzymatic hydrolysis for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnol Bioeng 1993;41:846–53.

Pinelo M, Jonsson G, Meyer AS. Review: Membrane technology for purification of enzymatically produced oligosaccharides: molecular features affecting performance. Sep Pur 2009;70:1-11.

Riebel BR, Bommarius AS. Biocatalysis, enzyme reaction engineering. Weinheim: Wiley-VCH; 2004. p. 91-134.

Rosgaard L, Pedersen S, Cherry JR, Harris P, Meyer AS. Efficiency of new fungal cellulase systems in boosting enzymatic degradation of barley straw lignocellulose. Biotechnol Progr 2006;22:493–8.

Rosgaard L, Andrić P, Dam-Johansen K, Pedersen S, Meyer AS. Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. Appl Biochem Biotechnol 2007a;143:27–40.

Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS. Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrate. Biotechnol Progr 2007b;23:1270–6.

Roychoudhury PK, Ghose TK, Ghosh P, Chotani GK. Vapor liquid equilibrium behaviour of aqueous ethanol solution during vacuum coupled simultaneous saccharification and fermentation. Biotechnol Bioeng 1986;23.

Roychoudhury PK, Ghose TK, Ghosh P. Operational strategies in vacuum-coupled SSF for conversion of lignocellulose to ethanol. Enzyme Microb Technol 1992;14: 581–5.

Sarbatly R, Krishnaiah D, England R. Continuous recycle enzymatic membrane reactor system for *in situ* production of pure and sterile glucose solution. J Appl Sci 2007;7: 1063–8.

Sonia KG, Chadha BS, Badhan AK, Saini HS, Bhat MK. Identification of glucose tolerant acid active β-glucosidases from thermophilic and thermotolerant fungi. World Microbiol Biotechnol 2008;24:599–604.

Sun Y-M, Khang S-J. Catalytic membrane for simultaneous chemical reaction and separation applied to a dehydrogenation reaction. Ind Eng Chem Res 1988;27: 1136–42.

Tjerneld F, Persson I, Albertsson P-Å. Enzymatic hydrolysis of cellulose in aqueous two-phase systems. II. Semicontinous conversion of a model substrate, Solca Floc BW 200. Biotechnol Bioeng 1985;27:1044–50.

Wyman C. Cellulose bioconversion technology: the simultaneous saccharification and fermentation process. Model formulation. Handbook on bioethanol: production and utilization. Taylor & Francis; 1996. p. 265–6.

Xiao Z, Zhang X, Gregg DJ, Saddler JN. Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechol 2004;113–116:1115–26.

Yang S, Ding W, Chen H. Enzymatic hydrolysis of rice straw in a tubular reactor coupled with UF membrane. Process Biochem 2006;41:721–5.