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A biocatalytic cascade reaction sensitive to the gas–liquid interface: Modeling and upscaling in a dynamic membrane aeration reactor

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ABSTRACT

The oxidation of lactose to lactobionic acid by a bi-enzymatic system in a membrane-aerated reactor was scaled up 100-fold from a miniaturized membrane contactor to a 20-L dynamic membrane aeration reactor. The conversion was catalyzed by an enzyme cascade consisting of cellobiose dehydrogenase as synthesizing enzyme and laccase as regenerating enzyme coupled by a redox mediator. A model of the process, combining mass-transfer and enzyme kinetics, was developed to predict optimal conversion conditions. The dynamic membrane aeration reactor was successfully operated in discontinuous and CSTR mode to achieve maximum productivity at very low power input ($27.7\,W\,m^{-3}$) and also greatly reduced enzyme inactivation by eliminating the high gas/liquid interfacial area of conventionally aerated stirred reactors or bubble columns. The reaction product, lactobionic acid was obtained with a space–time yield of $74.4\,g\,L^{-1}\,d^{-1}$ and a degree of conversion higher than 97%. The dynamic membrane aeration reactor is well suited for the bubbleless oxygenation of laccase-regenerated dehydrogenase reactions and other oxidase-catalyzed reactions on large scale to perform sustainable enzymatic oxidation reactions employing enzymes sensitive to shear or the gas/liquid interface.

1. Introduction

Manufacturing of fine chemicals traditionally involves oxidation reactions depending on stoichiometric amounts of oxidants such as hypochlorite, dichromate, bromine, permanganate etc., leading to large amounts of salt-containing effluents with a huge environmental impact. In contrast, molecular oxygen is the most inexpensive and safest oxidant available. The enzymatic activation of molecular oxygen is therefore highly interesting for the conversion of a wide range of substrates into valuable oxidized products. Industrial bio-oxidations are mostly based on whole-cell processes [1,2]. In this way, purification of the enzymes is avoided and cofactors for oxidoreductases can be regenerated *in vivo*. However, processes catalyzed by isolated enzymes generally result in higher volumetric productivities and easier down-stream processing. In addition, the production of enzymes is decoupled from the conversion process and allows separate optimization of both, catalyst production and the biocatalytic process. This is advantageous since the optimal conditions for cell growth, enzyme expression and biotransformation can vary vastly [3]. Nevertheless, one of the major challenges for process developers is the economic application of isolated catalysts. Enhanced stability, high turnover numbers, separation or recycling methods govern the specific catalyst costs and have to be painstakingly optimized before a successful transfer to production scale [4].

The beneficial effect of membrane facilitated, diffusive (bubbleless) oxygenation on enzyme stability through prevention of enzyme inactivation at the gas/liquid interface has been observed by several authors for various enzymatic oxidation reactions [5–7]. The experiments in all of these studies were performed in minireactors ranging from 20 to 200 mL volume. In an industrial context, however, reactor volumes are easily three orders of magnitude larger, and hence the question arises whether the results obtained with the minireactors are still applicable. In order to identify pos-

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Nomenclature

a	ratio of outer membrane surface to liquid volume ($m^2\ m^{-3}$)
C°	initial concentration ($mol\ m^{-3}$)
$C^{0.999}$	concentration after 99.9% conversion
C^*	equilibrium concentration of dissolved ABTS (μM)
C_{assay}	concentration of chemicals used in assay (M)
C_{O_2}	oxygen concentration in liquid phase ($mol\ m^{-3}$)
$C_{O_2,t}$	oxygen concentration in liquid phase at time t ($mol\ m^{-3}$)
$C_{O_2,t=0}$	oxygen concentration in liquid phase at time $t=0$ ($mol\ m^{-3}$)
$C^*_{O_2}$	oxygen saturation concentration ($mol\ m^{-3}$)
P	permeate flow rate ($m^3\ s^{-1}$)
F	feed flow rate ($m^3\ s^{-1}$)
K_I	inhibition constant (M)
K_L	overall mass-transfer coefficient based on liquid concentrations ($m\ s^{-1}$)
k_La	overall volumetric mass-transfer coefficient (s^{-1})
k_{hyd}	rate constant, hydrolysis of lactobionolactone to lactobionic acid (s^{-1})
K_M	Michaelis constant (M)
r	reaction rate ($mol\ m^{-3}\ s^{-1}$)
t	time (h)
ν	limiting rate ($U\ mL^{-1}$)
V_R	reactor volume (m^3)

Greek symbols

ε	absorption coefficient ($M^{-1}\ cm^{-1}$)

Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ABTS$^{+\bullet}$	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical
CDH	cellobiose dehydrogenase
DCIP	2,6-dichloroindophenol
DMA	dynamic membrane aeration
DO	dissolved oxygen (%); 100% corresponding to air-saturation
EMR	enzyme membrane reactor
LAC	laccase
LBA	lactobionic acid
LBL	lactobionolactone
MWCO	molecular weight cut-off
RM	oxidized redox mediator
RMH$_2$	reduced redox mediator
RT	retention time
SSE	sum of squared errors
STY	space–time yield

sible bottlenecks and eventually open the possibility to use these processes and reactors on an industrial scale, such an interface-sensitive oxidation reaction was scaled up to pilot scale (20 L) using bubbleless oxygenation based on a novel reactor design.

The dynamic membrane aeration (DMA) bioreactor uses an innovative and attractive concept for bubbleless aeration and was originally developed by Bayer Technology Services for the oxygenation of sensitive animal cell cultures at low shear stress and low power input [8]. The dynamic/moving membrane concept consists of a centrally arranged, oscillating rotor wrapped with silicone tubing in a star-like arrangement (Fig. 1) and has been successfully developed up to the 200-L scale. Oxygenation occurs by diffusion of oxygen through the silicone tubing material from the gaseous phase inside the tubes directly into the aqueous phase of the reactor, and hence air bubbles are avoided and gentle agitation can be used for stirring. Furthermore, the DMA reactor, enabling enhanced enzyme stability because of reduced shear stress, can be conveniently combined with the concept of the enzyme membrane reactor (EMR). The EMR concept was introduced in 1981 and enables continuous process operation or recycling of the biocatalysts for subsequent batches through containment of free, non-immobilized enzymes. Originally used for the production of enantiomerically pure amino acids via the acylase route by Degussa (now Evonik) [9], it is nowadays used for various enzyme systems on a technical scale [4].

For the scale-up of a membrane facilitated, bubbleless oxygenation and implementation of continuous operation we chose a well characterized model reaction employing a bi-enzymatic system for the production of aldonic acids, e.g. lactobionic acid. The process is based on the regeneration of the synthesizing flavoheme enzyme cellobiose dehydrogenase (CDH), which oxidizes lactose to lactobionic acid (the initial reaction product is lactobionolactone, which hydrolyses spontaneously in aqueous solutions) by laccase and an electron mediating redox compound (Fig. 2) [10,11]. Lactobionic acid is used in the pharmaceutical industry to enhance the solubility of macrolide antibiotics such as erythromycin (Erythrocin, Abbott) and clarithromycin (Klaricid, Abbott) for intravenous use, in organ preservation solutions (Viaspan, Bristol-Myers Squibb) to maintain osmotic pressure, and in the cosmetics industry for skin-care products [12]. Furthermore, its fatty acid derivatives can be used as biosurfactants [13]. The use of laccase for the mediated regeneration of flavin- or pyrroloquinoline quinone-containing enzymes enables a broad range of oxidation reactions depending on the catalytic activity of the synthesizing enzyme employed.

In order to identify important parameters both for biocatalyst activity and stability, a minireactor of 200 mL working volume was applied with different reaction regimes, and kinetic modeling of the batch conversion reactions provided a thorough understanding of this biocatalytic cascade oxidation [7,14]. In this present report, the results thus obtained in the minireactor were used to model and study this process at 20-L scale using the DMA reactor coupled to an ultrafiltration module for discontinuous and continuous operation.

2. Materials and methods

2.1. Experimental set-up

The discontinuous and continuous conversion experiments were carried out using a 20-L dynamic membrane aeration (DMA) reactor (glass version applied for visual control of gas bubble formation; Bayer Technology Services, Leverkusen, Germany) powered by a servo motor with a static torque of 0.9 N m (Jenaer Antriebstechnik, Jena, Germany). The servo motor was controlled via an associated control cabinet and the ECO2WIN software. The rotor was set to move 180° in one direction before reversing the direction and moving 180° back. Within one oscillation cycle (forth- and back-movement) the rotor thus covered an overall angle of 360° as does a conventional stirrer in one revolution. The membranes wrapped around the eight arms of the oscillating rotor (Fig. 1) were silicone tubes with an outer diameter of 3.18 mm and an inner diameter of 1.98 mm (Silastic RX 50 Medical Grade Tubing Special; Dow Corning, Midland, MI, USA). The rotor carried a total of 106 m of vertically wrapped silicone tubing on its eight rotor arms. The tubing material was divided into eight separate segments (one per rotor arm) into which the gas flow was split. Further information on the DMA reactor used in this study is given in Frahm et al. [8].

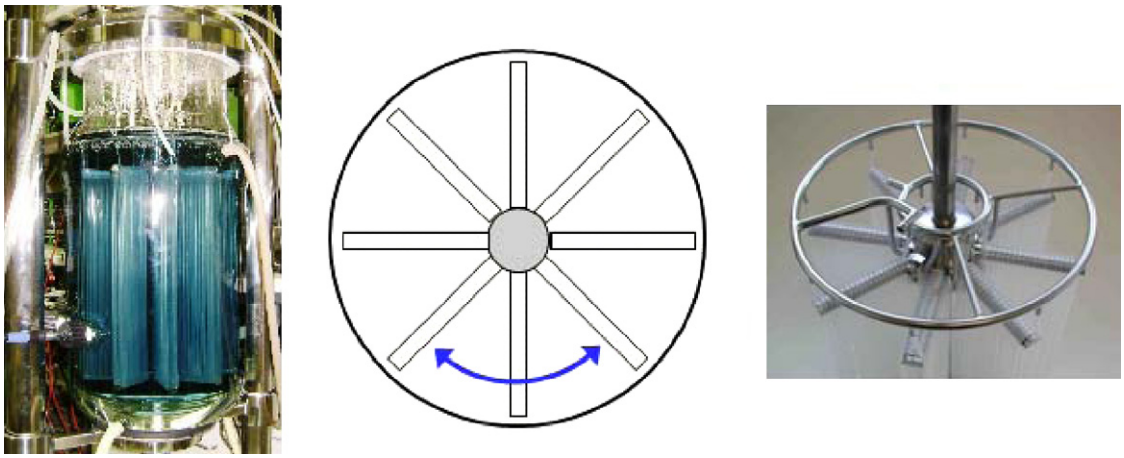

Fig. 1. Dynamic membrane aeration reactor (20 L working volume). The operating system (left); top view sketch of the reactor showing the setup of the star-like tubing with indicated oscillating movement (center); top view of the oscillating rotor with mounted tubing (right).

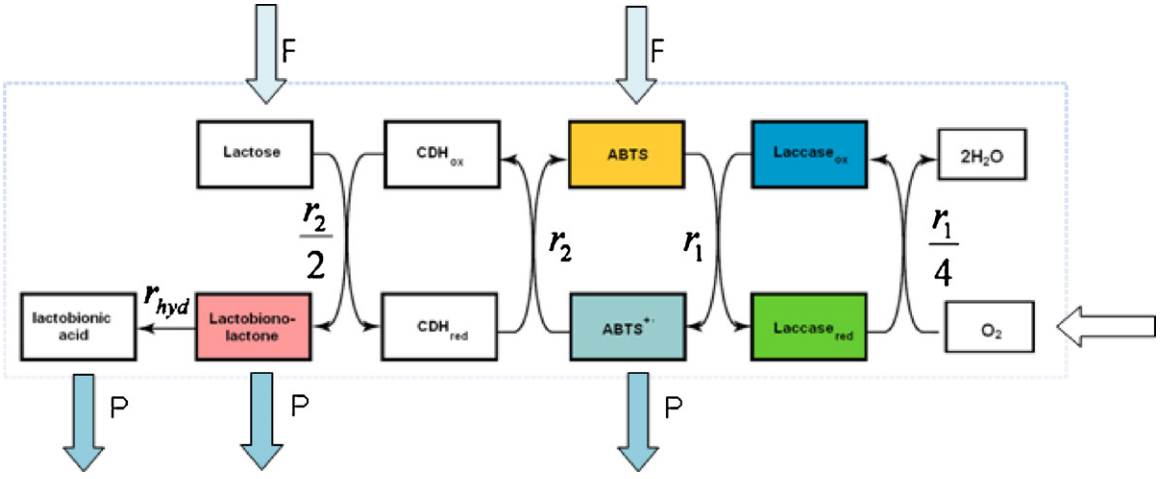

Fig. 2. Process scheme of the continuous conversion experiment in the DMA reactor (indicated by a dotted line). The arrows indicate substrates entering the reactor with the feed stream (F) and products leaving the system in the permeate stream (P). The redox mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or, when oxidized the ABTS cation radical ($ABTS^{+\bullet}$) is a one-electron donor/acceptor. Two molecules of $ABTS^{+\bullet}$ have to be reduced for the oxidation of one molecule of lactose, and one molecule of oxygen, which is reduced to water, oxidizes four molecules of ABTS.

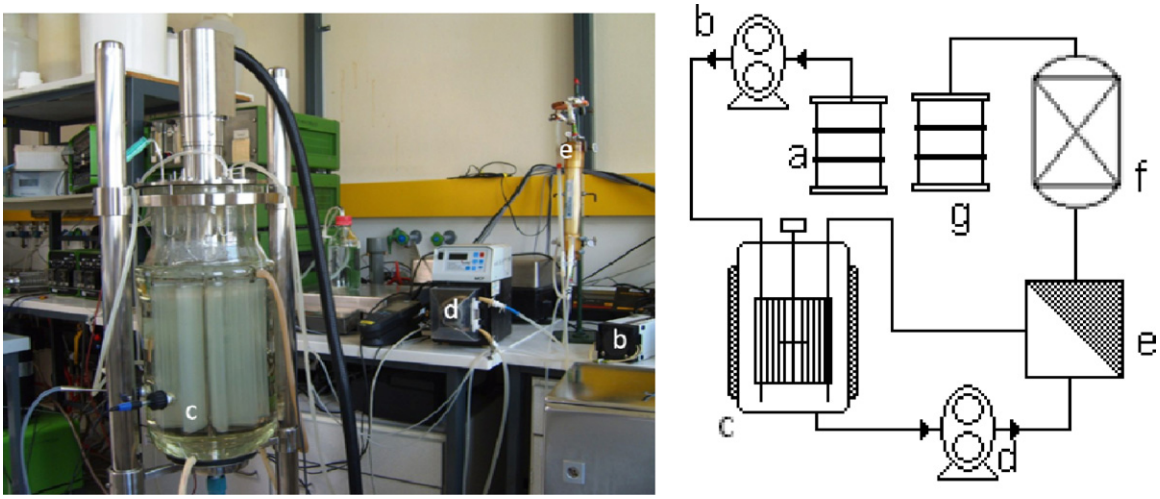

Fig. 3. The dynamic membrane aeration reactor and auxiliary equipment for the continuous production of lactobionic acid (left), and schematic drawing (right) showing lactose reservoir (a), peristaltic feeding pump (b), DMA reactor (c), peristaltic cross-flow pump (d), ultrafiltration module (e), activated charcoal adsorption step (f) and product tank (g).

The dissolved oxygen concentration (DOT) was measured with a luminiscent dissolved oxygen sensor (Hach-Lange; Düsseldorf, Germany), which was connected to a HQ20 analyzer (Hach-Lange). The analyzer was interfaced to a computer, which acquired dissolved oxygen data as a function of time. The reactor was double-jacketed to allow temperature control. The working temperature was set to 30 °C for all the experiments described here using the Biostat MD process control system (B. Braun Biotech International GmbH, Melsungen, Germany). This system also controlled the pH value by adding potassium carbonate buffer (400 mM) to maintain a pH of 4.0. During continuous operation a level sensor was used to maintain the liquid volume in the reactor by activating a peristaltic pump (Fig. 3, left side) connected to a Microza ultrafiltration module (10 kDa cut-off; Pall, East Hills, NY, USA). The redox mediator ABTS used is too small to be retained by the ultrafiltration membrane and hence was added continuously to the system at a concentration of 18 μM together with the lactose feed (395 mM) at the bottom of the reactor using a diptube and a peristaltic pump (Fig. 3, right side) when the reactor was operated continuously. ABTS present in the permeate stream was removed by adsorption to activated charcoal prior to storage and further analysis.

2.2. Mass-transfer coefficient measurements

A dynamic method was used for the determination of the overall volumetric mass-transfer coefficient k_La at different volume-specific power inputs. The overall mass-transfer rate for gases is the sum of the mass-transfer rates via the membrane surface and via the free liquid surface at the headspace of the reactor. The contribution of the latter is decreasing with increasing scale of the reactor. The mass-transfer resistance exerted by the membrane is the sum of the resistance to mass-transfer in the membrane itself and of the liquid phase boundary layer. Both the membrane component and the boundary layer component of the overall mass-transfer resistance are temperature dependent, whereas the influence on mass transfer of the thermophysical properties of the liquid itself is considered low. The overall mass transfer coefficient K_L is the resultant of the resistance to mass transfer in the gas phase boundary layer, the membrane and the liquid phase boundary layer. More information on the resistance-in-series model can be found in the relevant literature [15–17]. It was not the aim of this work to determine these different resistances separately, since the overall mass-transfer coefficient will determine the oxygen transfer rate.

The procedure was as follows: the reactor was filled with 20 L distilled water and heated to 30 °C, the temperature that was also applied in the bioconversion experiments. Then, oxygen was stripped by purging the reactor with pure nitrogen. When the dissolved oxygen (DO) was lower than 30%, air was flown through the membranes at a flow-rate of 3 L min^{-1} (inlet pressure not measured, outlet pressure equal to atmospheric pressure). The reactor headspace was held at atmospheric pressure and not flushed since this did not show significant improvements of the overall mass-transfer coefficient. The DO was then continuously measured until it reached 95%.

The probe response time (90% response within 30 s) was not taken into account, given that the rate of change of the dissolved oxygen concentration was much lower than the probe response time. The driving force ($C^*_{O_2} - C_{O_2}$) can, depending on the experimental conditions, slightly vary over the length of the silicone tubes at a given time, since the oxygen concentration inside the tubes may decrease, and thus change $C^*_{O_2}$. An average value of $C^*_{O_2}$ can be calculated using the Henry coefficient for oxygen as well as an average total pressure and an average oxygen content inside the tubing (based on data at the tubing entrance and end). Measurements of DO until its steady-state final concentration was reached also gives $C^*_{O_2}$.

Under these conditions, the increase of the oxygen concentration in the liquid, C_{O_2}, over time can be described as:

$$\frac{dC_{O_2}}{dt} = K_La(C^*_{O_2} - C_{O_2}) \quad (1)$$

Integration of Eq. (1) gives:

$$C_{O_2,t} = C^*_{O_2} - \left(C^*_{O_2} - C_{O_2,t=0}\right) e^{-K_La*t} \quad (2)$$

$C_{O_2,t=0}$ is a known parameter. $C^*_{O_2}$ and k_La were estimated by fitting the experimental data between 30% and 95% dissolved oxygen concentration to this model. To this end, the generalized reduced gradient algorithm (GRG2) for nonlinear programming [18] was used with a tolerance level set at 5% and convergence at 0.001.

2.3. Chemicals

Sodium fluoride, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,6-dichloroindophenol/DCIP), lactobionic acid, sodium hydroxide, sodium chloride and calcium chloride were obtained from Sigma-Aldrich (Schnelldorf, Germany) and lactose and acetic acid were from Fluka (Buchs, Switzerland) with purities ranging from 99 to 99.8%.

2.4. Enzymes

Cellobiose dehydrogenase (EC 1.1.99.18) from *Sclerotium rolfsii* (*Sr*CDH) was produced in accordance with a published procedure [19]. The basidiomycete *S. rolfsii* CBS 191.62 was cultivated at 30 °C in a fermentor with a working volume of 30 L using a medium based on 30 g L^{-1} cellulose. After 9 days, 7900 U L^{-1} of CDH activity was produced, harvested and partially purified by a single anion exchange chromatography step on a DEAE-Sepharose fast flow column (GE Healthcare) using a 50-mM sodium acetate buffer (pH 5.5) for binding and a linear NaCl gradient (0–0.5 M) for elution. The partially purified *Sr*CDH preparation had a specific activity of 15 U mg^{-1} (homogeneous enzyme: 63 U mg^{-1} [20]) and excerted no hydrolytic nor any other adverse activities on lactose.

Laccase (EC 1.10.3.2) was produced by the white-rot fungus *Trametes pubescens* (*Tp*LAC) based on described procedures [21]. To induce enzyme production by the fungus 1 mM $CuSO_4$ was added to the growing culture of *T. pubescens* MB 89, which was cultivated at 30 °C in a 30-L fermenter using a medium based on 48 g L^{-1} glucose. After 16 days 45,000 U L^{-1} of laccase activity were harvested and partially purified by anion exchange chromatography using a column packed with Q-Sepharose fast flow (GE Healthcare). The column was equilibrated with 50 mM sodium acetate buffer pH 5.5 to ensure good binding conditions. The laccase isoenzyme of interest (LAP2) was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The specific activity of this partially purified and lyophilized enzyme preparation was 17 U mg^{-1} (homogeneous enzyme: 350 U mg^{-1} at pH 4.5 [22]), and no adverse enzymatic activities were present in this preparation.

2.5. Enzyme activity assays

Cellobiose dehydrogenase (CDH) activity was assayed by following the decrease in absorbance of 2,6-dichloroindophenol (DCIP) with a UV–vis spectrophotometer at 520 nm (ε_{520} = 6800 M^{-1} cm^{-1}) at pH 4.0 and 30 °C, which is the same temperature as used in the bioconversion experiments. The reaction mixture contained DCIP (0.15 mM) and lactose (30 mM) in 81 mM sodium acetate buffer. One unit of CDH activity is defined as the amount of enzyme reducing 1 μmol of DCIP per minute under the

Table 1
Kinetic parameters (Michaelis constant K_M, inhibition constant K_I) for cellobiose dehydrogenase from *S. rolfsii* (*Sr*CDH) and laccase from *T. pubescens* (*Tp*LAC). Data are from Baminger et al. [20] and Galhaup et al. [22].

Constant	*Sr*CDH		*Tp*LAC	
	Component	Value (mM)	Component	Value (mM)
K_M	Lactose[a]	2.4	Oxygen	0.41
K_M	$ABTS^{+\bullet}$[b]	0.0004	ABTS[c]	0.033
K_I	Lactobiono-1,5-lactone	31		

[a] Kinetics were measured using the standard 2,6-dichloroindophenol assay at pH 4.0 and 25 °C.
[b] Constants were determined at 25 °C and pH 4.0 using 2 mM cellobiose as the substrate.
[c] Determined using oxygen (air-saturated solution) as electron acceptor at pH 3.5.

above reaction conditions. To avoid interference of laccase activity with the determination of CDH activity by rapidly reoxidizing the reduced form of DCIP, sodium fluoride (4 mM final concentration) was added to the assay to selectively inhibit laccase activity [23]. The reaction mixture for assaying laccase activity contained 1 mM ABTS in 20 mM acetate buffer, pH 4.0 [22], and the oxidation of ABTS to the bluish-green cation radical $ABTS^{+\bullet}$ was followed spectrophotometrically at 420 nm (ε_{436} = 43,200 $M^{-1}\,cm^{-1}$) and 30 °C. To avoid CDH interference with the determination of laccase activity by rapidly reducing the ABTS cation radical in the presence of lactose, the assay mixture (enzyme sample plus buffer) was incubated with 1.8 U mL^{-1} (final activity) of β-galactosidase from *Aspergillus oryzae* (Sigma G 5160) for 10 min at 30 °C prior to the laccase assay. The reaction was then started by the addition of the ABTS reagent. One unit of laccase activity is defined as the amount of enzyme oxidizing 1 μmol of ABTS per minute under the above reaction conditions.

2.6. HPLC Analysis

Lactose (retention time of 7.7 min) and lactobionic acid (retention time of 17.1 min) were separated and quantified by ion-moderated partition chromatography using the resin-based Aminex HPX 87C column (Bio-Rad, Hercules, CA, USA) at 85 °C with 5 mM $CaCl_2$ as eluent (0.7 mL min^{-1}) and refractive index detection.

2.7. Model development

A model that was previously developed for batch conversions [14] was modified for continuous conversion by including the feed flow F containing lactose, the redox mediator ABTS and oxygen at ambient concentration as well as the permeate flow P (Fig. 2) with concentrations identical to those prevailing in the DMA reactor into the model.

$$r_1 = v_{LAC,ABTS}\frac{C_{O_2,L}C_{ABTS}}{K_{M,ABTS}C_{O_2,L} + K_{M,O_2}C_{ABTS} + C_{O_2,L}C_{ABTS}} \quad (3)$$

$$r_2 = v_{CDH,ABTS^{+\cdot}}\frac{C_{lactose}C_{ABTS^{+\cdot}}}{K_{M,lactose}(1+(C_{LBL}/K_{I,LBL}))C_{ABTS^{+\cdot}} + K_{M,ABTS^{+\cdot}}C_{lactose} + C_{lactose}C_{ABTS^{+\cdot}}} \quad (4)$$

$$\frac{dC_{O_2,L}}{dt} = k_L a(C^*_{O_2,L} - C_{O_2,L}) - \frac{r_1}{4} + \frac{F}{V_R}C_{O_2,F} - \frac{P}{V_R}C_{O_2,L} \quad (5)$$

$$\frac{dC_{ABTS^{\cdot+}}}{dt} = r_1 - r_2 - \frac{P}{V_R}C_{ABTS^{+\cdot}} \quad (6)$$

$$\frac{dC_{ABTS}}{dt} = -r_1 + r_2 - \frac{P}{V_R}C_{ABTS} + \frac{F}{V_R}C_{ABTS,F} \quad (7)$$

$$\frac{dC_{lactose}}{dt} = -\frac{r_2}{2} - \frac{P}{V_R}C_{lactose} + \frac{F}{V_R}C_{lactose,F} \quad (8)$$

$$\frac{dC_{lactobionolactone}}{dt} = \frac{r_2}{2} - r_{hyd} - \frac{P}{V_R}C_{lactobionolactone} \quad (9)$$

$$\frac{dC_{lactobionic\ \ acid}}{dt} = k_{hyd}C_{lactobionolactone} - \frac{P}{V_R}C_{lactobionic\ \ acid} \quad (10)$$

To solve this set of ordinary differential equations the ODE15s subroutine in the MATLAB R2007B software (The MathWorks, Natick, MA, USA) was used, which is designed specifically to deal with stiff differential systems of equations. The kinetic constants of the enzymes employed and used to solve these equations were taken from the literature (Table 1).

3. Results and discussion

3.1. Oxygen mass-transfer characteristics

The overall volumetric mass-transfer coefficient for the DMA reactor was determined for three different values of the volume-specific power input (27.7 W m^{-3}, 48.6 W m^{-3} and 64.4 W m^{-3}, presenting averaged values for the oscillating movement). The determined values for k_La were 3.71 h^{-1}, 4.32 h^{-1} and 4.98 h^{-1}, respectively. The procedure of power input measurements has been described previously [8]. As expected, an increased power input results in a higher mass-transfer coefficient; however, the improvements in the mass-transfer coefficient were only moderate. For all subsequent conversions and simulations a low volume-specific power input of 27.7 W m^{-3} was applied. For this power input the rotor oscillated at a frequency of 0.33 s^{-1} (corresponding to a rotor movement of 180° in one direction and a subsequent movement of 180° back in 3 s).

3.2. Discontinuous conversion experiment

In order to evaluate the model previously developed for the batch conversion process [14] at larger scale in the 20-L DMA reactor with respect to conversion rates and optimum enzyme stability, a discontinuous conversion experiment employing 100 mM lactose was conducted. The model was used to select a suitable combination of activities for both enzymes together with a redox mediator concentration for a given overall volumetric mass transfer coefficient. The experiment was performed in the oxygen diffusion-limited regime (to achieve a maximal productivity) at 30 °C using a flow of 3 L min^{-1} pure oxygen in the silicone tubing at ambient pressure. The applied enzymatic activities were 420 U L^{-1} of *Sr*CDH and 2030 U L^{-1} of *Tp*LAC and the ABTS concentration was 18 μM as calculated by the model. The measured oxygen concentration and the activities of *Sr*CDH and *Tp*LAC are plotted as a function of time over the entire conversion experiment. As can be seen in Fig. 4, the measured values of the oxygen and lactose concentration fit the modeled values well.

The DMA reactor is well suited for the proposed reaction, which is exemplified by the following performance criteria: the biocatalyst stability is very high and comparable with the results from the 200 mL static membrane contactor [7]. The enzyme consump-

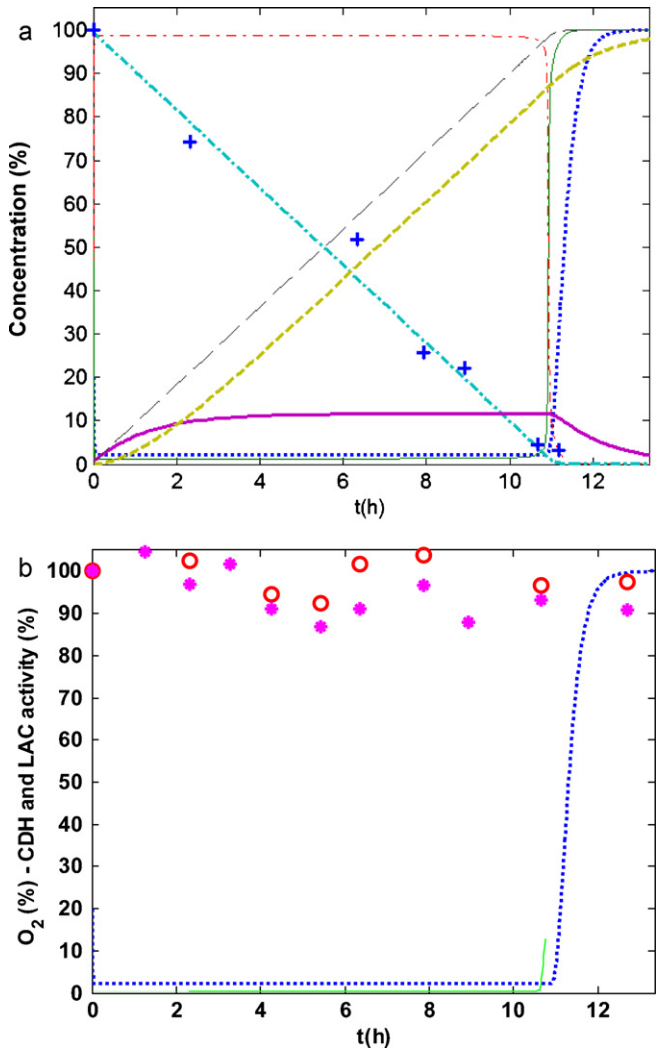

Fig. 4. (a) Modeling and experimental data of the discontinuous conversion of 100 mM lactose to lactobionic acid in a 20 L DMA reactor using the following experimental conditions: 420 U L^{-1} CDH; 2030 U L^{-1} LAC; 18 μM ABTS; $k_La = 3.71\ h^{-1}$. Modeled data: oxygen concentration, dotted line; lactose concentration, dash-dotted line; lactobiono-δ-lacton, full line; lactobionic acid, short dashes; sum of conversion products (lactobiono-δ-lacton and lactobionic acid) long dashes; ABTS cation radical, thin full line; ABTS, thin dash-dotted line. Measured lactose concentration: crosses. (b) The measured enzymatic activity of cellobiose dehydrogenase (stars) and laccase (circles) is given as percentage of the initial activity, the measured dissolved oxygen (dotted line) as percent air saturation.

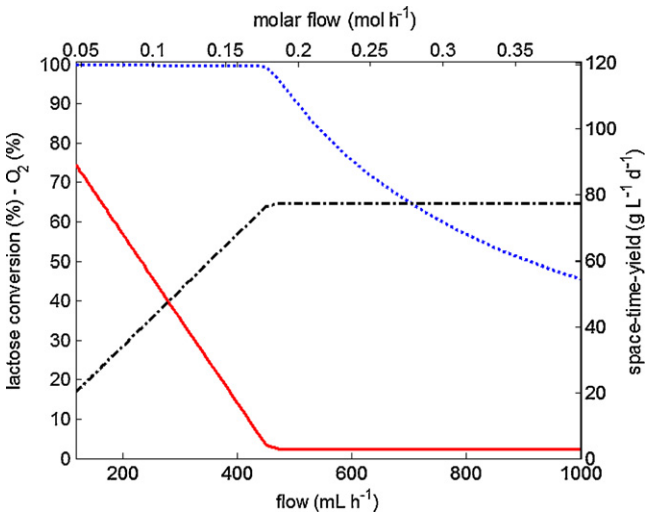

Fig. 5. Modeling of continuous lactose conversion (dotted line), steady-state oxygen concentration (full line) and space–time yield (dash-dotted line) as a function of volumetric and molar flow using pure oxygen in the gas phase in the tubes and the DMA reactor with a working volume of 20 L (experimental conditions: 420 U L^{-1} CDH; 2030 U L^{-1} LAC; 18 μM ABTS in feed; $k_La = 3.71\ h^{-1}$).

tion number of pure CDH is 19 mg per kg of lactobionic acid (partially purified CDH preparation: 78 mg kg^{-1}) and for laccase 8 mg kg^{-1} (impure laccase preparation: 165 mg kg^{-1}). The total turnover number (TTN) for CDH is 1.5×10^6 with 90% of enzymatic activity still present at the end of the conversion, the TTN for laccase is 1.1×10^6 and 95% residual activity. The turnover frequency of CDH for lactose was 36 s^{-1}; for laccase, oxidising ABTS, a value of 54 s^{-1} was calculated. The reactor productivity (space–time yield 74.4 g L^{-1} d^{-1}) was similar to that obtained in the static membrane reactor (72 g L^{-1} d^{-1} [7]). This is due to the similarity of the overall volumetric mass-transfer coefficients for the DMA reactor (1.08×10^{-3} s^{-1}) and the static membrane contactor (1.01×10^{-3} s^{-1}). The STY can be improved by increasing the factor by e.g. the ratio of membrane/tubing surface to liquid volume. The same enzymatic reaction performed under similar conditions (500 U L^{-1} *Sr*CDH, 30 °C) in a conventionally sparged and aerated bioreactor at different *DO* values (10–60%) [11] gave STYs ranging from 134 g L^{-1} d^{-1} (10% *DO*) to 245 g L^{-1} d^{-1} (60% *DO*). The 1.8–3.3-fold higher STYs are solely due to the higher overall volumetric mass-transfer coefficient of the conventional system. However, the increased productivity is heavily outweighted by the high activity loss of the gas/liquid interface sensitive catalysts and a high energy dissipation to reach the high *DO*. Another enzymatic process for the production of lactobionic acid, which uses carbohydrate oxidase from *Microdochium nivale* [24] reached space–time yields of 36–162 g L^{-1} d^{-1} (values are calculated from published data), depending on the *DO* (20–60%) and a 98% conversion. Here, a comparison of the specific power input is possible and shows also a far lower energy consumption for the DMA reactor (stirring power: 27.7 W m^{-3}) than for the rotary jet head system used in the *M. nivale* carbohydrate oxidase process (pumping power: 382–590 W m^{-3}, gas compression power: 19–556 W m^{-3}). The lactose conversion by *Sr*CDH was shown to be quantitative [11], but when the discontinuous process reached a degree of conversion of 97%, the experiment was switched to the continuous mode.

3.3. Continuous conversion experiment

3.3.1. Optimization of the feed rate

When operating the dynamic membrane aeration reactor in continuous mode, lactose and ABTS have to be added continuously to the system to ensure an adequate supply of these substrates for the reaction and reach a steady-state. It is generally known that the reduction in residence time results in a decrease of steady-state conversion. Yet the existence of an optimal feed rate where the degree of conversion starts to decrease sharply with a further increase in feed can be assumed. If the steady-state oxygen concentration during the conversion is higher than zero (with the enzymatic activities and k_La as during the batch conversion, i.e. in diffusion limited regimes), then the conversion of the added lactose has to be near to completion. When the oxygen concentration is approaching zero, then the degree of conversion can still be near completion, but will then decrease with an increasing feed rate. It is not attractive to operate the system at a point lower than this critical flow, because then the (almost complete) conversion is slower than necessary. Above this point downstream processing costs will increase due to separation efforts to remove the unreacted substrate.

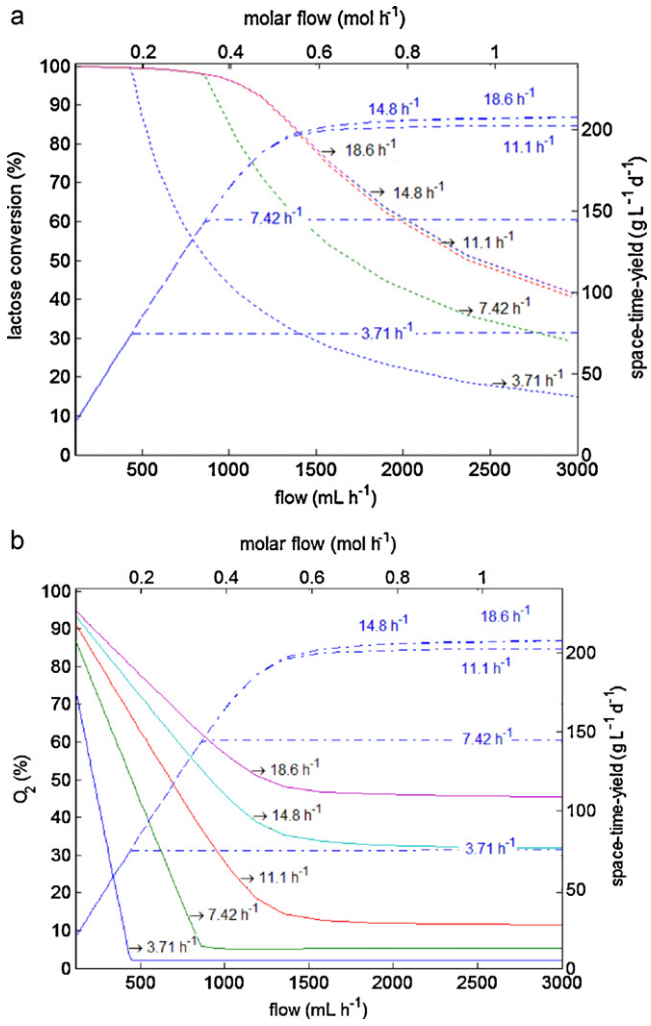

Fig. 6. (a) Modeling of lactose conversion (dotted line) and space–time yield (dash-dotted line) and (b) the steady-state oxygen concentration (full line) and space–time yield (dash-dotted line) as a function of flow for different mass-transfer coefficients using pure oxygen in the gas phase for oxygenation of the 20-L DMA reactor (experimental conditions: 420 U mL^{-1} CDH; 2030 U L^{-1} LAC; 18 μM ABTS in feed). Results are shown for k_La values of 0.00103, 0.00206, 0.00309, 0.00412 and 0.00515 s^{-1} (corresponding to 3.71, 7.42, 11.12, 14.83 and 18.54 h^{-1}).

To determine this optimal feed rate, the model was iterated for increasing substrate feed (lactose concentration of 395 mM and ABTS concentration of 18 μM). For every iteration, space–time yield, steady-state oxygen concentration and degree of conversion were plotted (Fig. 5). As judged from this figure, the assumptions made are confirmed, and the selection of a suitable feed rate is straightforward. To give an example, a feed rate of 474 mL h^{-1} results in a (modeled) lactose conversion of 95.7%. This flow was subsequently used during the continuous conversion experiment in the DMA reactor. The lactose concentration at this flow-rate is 4.3 mM, which is 1.8-times higher than the apparent K_M of CDH for lactose.

3.3.2. *Influence of k_La*

Next we modeled the effect of the overall mass-transfer coefficient on lactose conversion for the same catalytic activities as used during the batch conversion (Fig. 6). When increasing the overall mass-transfer coefficient, the maximal space–time yield increases as now the same degree of conversion can be reached at higher substrate feed rates. However, the effect of a higher k_La on the space–time yield is negligible above a certain value of the feed rate. The conversion then proceeds completely under reaction-limited conditions, i.e. the reaction rate is limited by the biocatalytic system itself. In order to improve the space–time yield of lactobionic acid production under these conditions, the activities of the enzymes employed in the biocatalytic reaction have to be increased.

For process economics, it would be desirable to replace the use of pure oxygen by air. This could be achieved without compromises for the productivity of the system by increasing the mass-transfer coefficient (e.g. by increasing the membrane surface, decreasing the membrane thickness or using polymers with a higher oxygen permeability). A DMA reactor using membrane tubing with reduced wall thickness, provided in pre-assembled modules for ease of handling, has already demonstrated considerably higher k_La-values, but this system has not yet been applied to biocatalytic oxidation reactions [25].

3.3.3. *Continuous conversion experiment*

To corroborate the predictions made by the model, a continuous conversion experiment was conducted. When 97% of the lactose was converted in the discontinuous conversion experiment (after 11 h of conversion), the DMA reactor was switched to continuous mode of operation by coupling the reactor with the ultrafiltration module as described in Materials and Methods. A feed solution containing 395 mM lactose and 18 μM ABTS was added continuously to the reactor at a rate of 474 mL h^{-1}. The reactor was operated in continuous mode for three days with a high degree of conversion and very good enzyme stability (the activity of both enzymes after 3 days was ~80% of the initial activity). As predicted by the model (Fig. 5) the experimentally obtained lactose conversion was in the range of 95–98% throughout the entire experiment. Overall, a STY for lactobionic acid production of 74.4 g L^{-1} d^{-1} was obtained as predicted by the model. The reaction ran successfully for three days, until microbial growth was observed, which was to be expected under non-sterile conditions and plenty of lactose as nutrient. However, when properly addressing this problem by autoclaving the reactor prior to the experiment and sterile filtration of substrates and enzymes, a much longer operating time can be predicted.

4. Conclusions

The upscaling of a membrane-aerated biocatalytic cascade oxidation for lactobionic acid production from 0.2 to 20 L scale was successfully performed using a dynamic membrane aeration reactor for discontinuous and continuous conversion. The developed model allowed the calculation of important, interconnected process parameters such as enzymatic activity, redox mediator concentration, oxygen transfer-rate and feed rate to ensure a high substrate conversion rate during discontinuous and continuous operation. The selected conditions – specific power input governing the k_La, temperature, CDH activity, activity ratio, mediator concentration and substrate feed – resulted in a high turnover stability of both enzymes. Further improvements of the productivity can be made by increasing the membrane surface or by using membranes with higher oxygen permeability. The developed methodology and the established biocatalytic system of cellobiose dehydrogenase and laccase can also be used on an industrial scale for a sustainable oxidation of other aldoses to aldonic acids e.g. cellobionic or maltobionic acid. From an economic point of view the combination of a DMA reactor (to increase enzyme TTN), a cross-flow ultrafiltration module (to retain enzymes) and an adsorption unit (to extract and recover the redox mediator) in a continuous process demands a substantially higher initial investment than a sparged stirred tank reactor. However, four features favour the introduced system and make it economically viable for products manufactured in quantities from 1 to 30 tons per year: (i) the high degree

of conversion (95–100%), (ii) the partially integrated downstream processing, (iii) high catalyst stability and high volumetric and specific reaction rates of the non-immobilized enzymes and (iv) a low energy consumption and greatly reduced enzyme costs. The scale-up of the process to the existing 200-L DMA technology will be straightforward when using the developed methodology, showing an attractive example of bubbleless biocatalytic cascade oxidations based on molecular oxygen as oxidant.

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