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Significance of membrane bioreactor design on the biocatalytic performance of glucose oxidase and catalase: Free vs. immobilized enzyme systems

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A B S T R A C T

Membrane separation of xylose and glucose can be accomplished via oxidation of glucose to gluconic acid by enzymatic glucose oxidase catalysis. Oxygen for this reaction can be supplied via decomposition of hydrogen peroxide by enzymatic catalase catalysis. In order to maximize the biocatalytic productivity of glucose oxidase and catalase (gluconic acid yield per total amount of enzyme) the following system set-ups were compared: immobilization of glucose oxidase alone; co-immobilization of glucose oxidase and catalase; glucose oxidase and catalase free in the membrane bioreactor. Fouling-induced enzyme immobilization in the porous support of an ultrafiltration membrane was used as strategy for entrapment of glucose oxidase and catalase. The biocatalytic productivity of the membrane reactor was found to be highly related to the oxygen availability, which in turn depended on the reactor configuration, hydrogen peroxide concentration and catalase origin. When glucose oxidase and catalase (from *Aspergillus niger*) were free in the membrane bioreactor a total biocatalytic productivity of 122 mg gluconic acid/mg enzyme was obtained after five consecutive reaction cycles. The free enzymes showed superior performance compared to the immobilized systems as a result of limited substrate and product diffusion in the latter case.

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1. Introduction

Industrial separation of monosaccharides is challenging due to their similar structure, size and charge. In a previous study [1], we demonstrated that significant improvement in xylose-glucose separation could be achieved via enzyme-assisted nanofiltration (NF) to yield xylose streams of >99% purity. High purity streams of xylose are the basis for chemical or enzymatic production of high value industrial products like for example xylitol. In this process, glucose was first oxidized to gluconic acid – a value-added chemical building block [2] – through two coupled enzymatic Reactions (1) and (2) with glucose oxidase (GOD) and catalase (CAT), respectively. Oxygen for (1) was provided via (2) by initially adding hydrogen peroxide in stoichiometric amounts to the xylose-glucose solution. Subsequently, xylose was separated from gluconic acid by NF via electrostatic repulsion.

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H_2O_2 \tag{1}$$

$$H_2O_2 \xrightarrow{\text{CAT}} H_2O + \frac{1}{2} O_2 \tag{2}$$

In order to ensure high throughputs of xylose and gluconic acid, the biocatalytic productivity (mass of product/mass of enzyme) must be maximized. In general, the re-use of enzymes and hence the biocatalytic productivity in enzymatic membrane reactors can be improved by recycling the enzymes or by immobilizing them in appropriate supports [3]. Although some enzyme activity is often lost upon immobilization, improved stability is often gained by confining the enzymes in support materials [4,5]. Membranes are particularly favorable among the potential supports for enzyme immobilization because reaction and separation can be combined [6,7]. Several studies have already employed membranes as immobilization supports for GOD. However, these studies often used rather complicated techniques for synthesizing and functionalizing the membranes as suitable enzyme carriers. For example, Smuleac et al. [8] immobilized GOD inside multilayer assemblies of poly-

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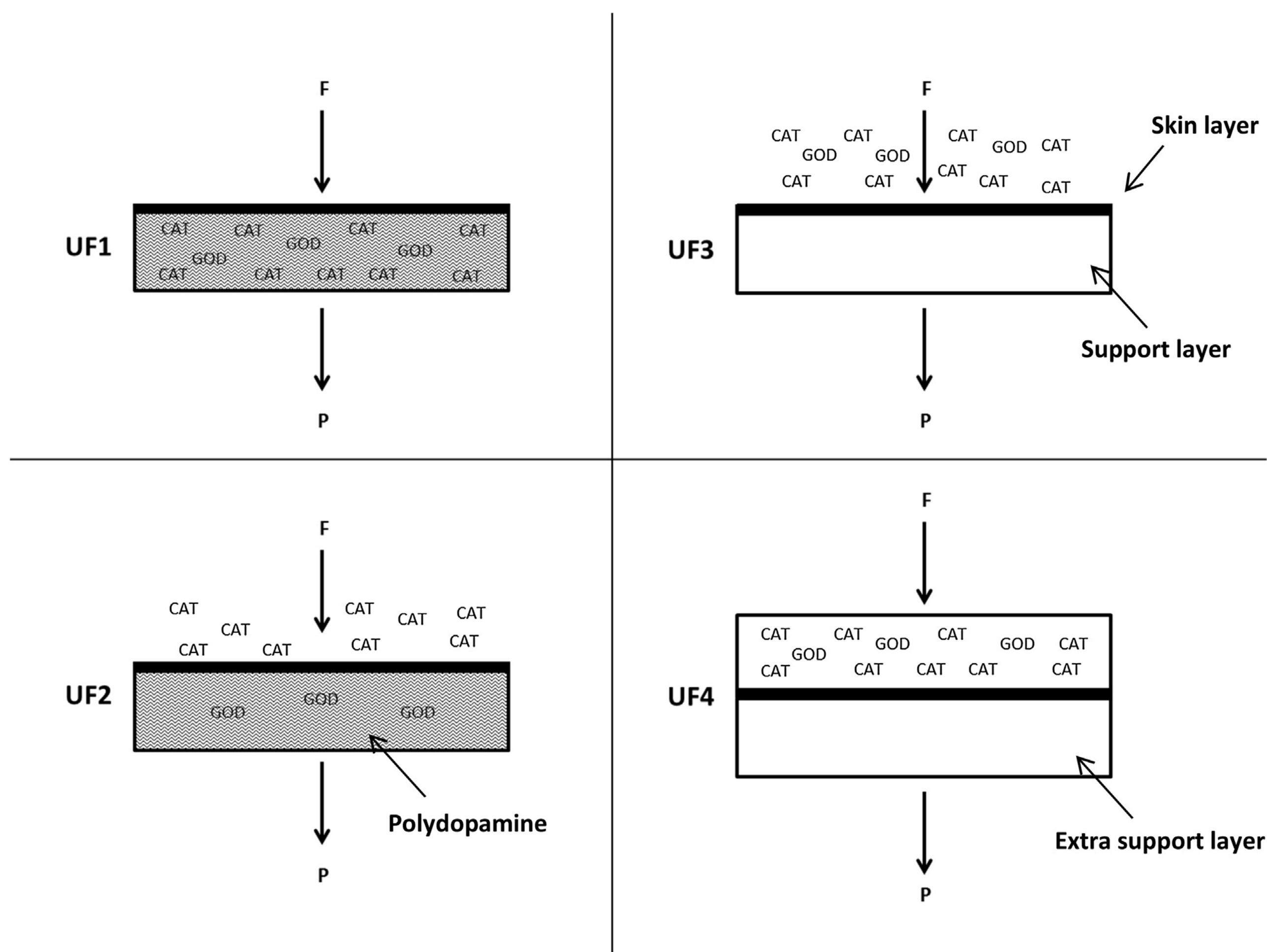

Fig. 1. Four reactor configurations (UF1-UF4). UF1: GOD and CAT immobilized by entrapment (membrane in normal mode) and polydopamine coating; UF2: GOD immobilized by entrapment (membrane in normal mode) and polydopamine coating and CAT free in solution; UF3: GOD and CAT free in solution (membrane in normal mode); UF4: GOD and CAT immobilized by entrapment (membrane in reverse mode). The polydopamine coating applied in UF1 and UF2 is indicated by the dark layer. The GOD to CAT activity ratio is 1:3 (the number of molecules in the illustration is only qualitative). GOD = glucose oxidase; CAT = catalase. F: Feed (xylose, glucose, hydrogen peroxide). P: Products (xylose, gluconic acid).

electrolytes, while Bora et al. [9] used a photoreactive cellulose membrane and applied UV irradiation to covalently attach GOD. Vasileva et al. [10] and Ying et al. [11] immobilized GOD by direct covalent attachment to self-fabricated membranes, while Rauf et al. [12] used glutaraldehyde crosslinking. Immobilization of GOD on photoreactive cellulose membranes resulted in improved enzyme activity as well as improved pH and thermal stability [9]. Immobilization via covalent binding led to an activity loss of 28–32% [10] and 60–70% [11], respectively, while pH and thermal stability increased in both cases. Lastly, immobilization of GOD via glutaraldehyde crosslinking also caused reduced activity of the immobilized enzyme but improved the pH and thermal stability [12]. A conceptually simpler and gentler enzyme immobilization strategy was recently developed by our group [13]. This method relies on entrapping the enzymes inside the membrane support layer by reverse-membrane filtration and thus allows high enzyme loadings in the membrane support. Following enzyme entrapment, the support layer was coated with polydopamine, a very hydrophilic "bio-glue", to reduce enzyme leakage and enhance membrane permeability and anti-fouling performance. Inspired by this study, the present work was undertaken to investigate the influence of four different reactor configurations (Fig. 1) on the biocatalytic productivity of the coupled GOD/CAT reaction system (1 and 2). In the first configuration (UF1), both enzymes were entrapped in the support layer of an UF membrane coated with polydopamine and operated in normal filtration mode. In the second configuration (UF2), only GOD was entrapped in the membrane coated with polydopamine and operated in normal filtration mode, while CAT was free in solution. In the third configuration (UF3), both enzymes were free in solution so that the membrane in normal filtration mode merely served as a barrier for the enzymes. In the fourth configuration (UF4), both enzymes were entrapped and the membrane operated in reverse filtration mode with an extra polypropylene support underneath. Luo et al. [14] refer to UF4 as the "sandwich mode" which achieved very high biocatalytic conversion efficacies for immobilized alcohol dehydrogenase. When a coupled enzyme reaction like the GOD/CAT is performed with immobilized enzymes, there are specific consequences that can have direct impact on the final biocatalytic productivity as compared to the free enzyme system. Different reactor configurations of the immobilized system can result in e.g. lower substrate availability for the enzymes, different disposition of the enzymes in the immobilization space (leading to particular 'shielding' phenomena), localized pH effects, etc.; phenomena which could be compensated by the enhanced enzyme stability associated with immobilization. The purpose of this work was then to evaluate if such expected enhanced stability could be used to increase the biocatalytic productivity on an extended use of enzymes (in repeated cycles) as compared to the free enzyme system. At the same time,

it was also the purpose of this study to understand the impact of different factors and phenomena on the performance of the system.

2. Materials and methods

2.1. Chemicals and membranes

D(+)-Glucose was purchased from EMD Millipore (Darmstadt, Germany). (D)-xylose (≥99%), hydrogen peroxide (H_2O_2) solution (30 wt.% in H_2O), dopamine chloride, *o*-dianisidine dihydrochloride tablets, peroxidase type II ("POD", EC 1.11.1.7, ~44 kDa, 181 U/mg) from horseradish, glucose oxidase ("GOD", EC 1.1.3.4, 160 kDa, 136 U/mg) from *Aspergillus niger*, catalase ("CAT", EC 1.11.1.6, 250 kDa) from bovine liver (4540 U/mg) and *Aspergillus niger* (7741 U/mg), respectively, were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrafiltration RC70PP membranes (MWCO: 10 kDa) with a skin layer of regenerated cellulose on polypropylene support material were kindly provided by Alfa Laval (Nakskov, Denmark).

2.2. Substrate solutions for biocatalytic reactions

Substrate solutions (20 ml) of xylose, glucose and H_2O_2 were prepared in 0.1 M sodium acetate buffer at pH 5.4. The H_2O_2 concentration (0.03 M), total sugar concentration (0.15 M) and the xylose to glucose molar ratio (9:1) were those determined in [1]. Substrate solutions without hydrogen peroxide were prepared in an identical way.

2.3. Preparation of biocatalytic membranes

The UF1-UF2 biocatalytic membranes were prepared according to the procedure outlined in [13] using a polydopamine coating period of 3 h. The UF4 biocatalytic membranes were prepared in a similar manner but without polydopamine coating. Though the procedure involves enzyme immobilization in the membrane support, in this paper it is referred to simply as immobilization in the membrane. The UF3 membranes were soaked in ethanol (50%) for 5 min and subsequently washed with sodium hydroxide (pH 10) for 1 h and water for 20 min before each experiment. Enzyme solutions (GOD or GOD-CAT) were prepared in 0.1 M sodium acetate buffer at pH 5.4. Enzymes were loaded at 300 U GOD and 900 U CAT, which corresponded to an enzyme activity ratio of 1:3 as determined in [1]. CAT from bovine liver and *Aspergillus niger*, respectively, was used in UF1, UF3 and UF4, while only bovine liver CAT was used in UF2.

2.4. Operation of biocatalytic membranes

The UF1-UF4 membranes were prepared and operated in a stirred dead-end cell (Amicon 8050, Millipore, USA). The working volume of the cell was 50 ml and the effective membrane area was 13.4 cm^2. During the reactions the temperature in the cell was kept constant at 35 °C (the temperature optimum of the free GOD [15]) by circulating hot water through an insulated tube wrapped around the cell. The UF1-UF3 membranes were operated in normal filtration mode (skin layer facing feed), while the UF4 membrane was operated in reverse filtration mode (to minimize enzyme leakage during the reactions) and with an extra polypropylene support underneath (to protect the skin layer). Five consecutive 30-min cycles were conducted with UF1, UF2 and UF4 by pressurizing the cell with atmospheric air at ~1 bar (equivalent to ~2 bar in the cell), while with UF3 five consecutive 40-min cycles were conducted without pressure followed by filtration at 2 bar to remove the resulting products. Between each reaction cycle, fresh substrate solutions were added while the enzymes were either entrapped in the membrane support (UF1-UF2, UF4) or retained by the membrane (UF3).

2.5. Stability studies

The pH stability of each of the free enzymes at 35 °C was tested by incubating aliquots of the enzyme at pH 2.5, 5.4 and 8.5, respectively, at relevant time periods up to 200 min with and without hydrogen peroxide (0.03 M). When the enzyme was incubated with hydrogen peroxide, the ratio of hydrogen peroxide to the enzyme was the same as in the UF membrane reactor. During incubation of CAT with hydrogen peroxide, fresh hydrogen peroxide was added every 40 min (corresponding to one reaction cycle in UF3) in order to imitate the conditions in the UF membrane reactor. After incubation, the CAT activity was measured with a microplate reader (Infinite 200, Tecan, Austria) at 240 nm in a standard CAT activity assay [16] with 0.03 M hydrogen peroxide in 100 mM sodium acetate buffer at pH 5.4 and 35 °C. GOD activities were measured at 500 nm in a standard GOD activity assay [17] with 0.164 mM *o*-dianisidine dihydrochloride, 92.8 mM glucose and 2 POD units/ml in 50 mM sodium acetate buffer at pH 5.4 and 35 °C.

2.6. Analytical methods

2.6.1. Determination of enzyme loading in the membrane

The concentration of enzyme in the samples was measured as protein using the Bradford assay [18]. The mass of immobilized enzyme was determined from a mass balance based on the known initial enzyme dosage and the concentration of leaked enzymes in the permeate samples collected during preparation of the biocatalytic membranes [13]. The enzyme loading in the membrane was calculated as mg enzyme/cm^2 membrane.

2.6.2. Sugar quantification

The concentration of sugars and gluconic acid in the permeate samples were determined by HPAEC-PAD as described previously in [1].

2.7. Statistical analysis

One-way ANOVA for determination of statistical significance was performed in RStudio (RStudio, Inc., Boston, MA, USA) using Tukey's test. Statistical significance was established at the $p < 0.05$ level.

3. Results and discussion

3.1. Comparing configurations UF1–UF4 with bovine liver catalase

The biocatalytic productivity of each of the four different reactor configurations was first compared with catalase from bovine liver (BL CAT). In all cases, the GOD was only specific towards glucose so xylose could pass through the membrane bioreactor unconverted. The total enzyme loading in the three immobilized configurations were similar, namely 0.15 ± 0.01 (UF1), 0.14 ± 0.01 (UF2) and 0.13 ± 0.01 mg/cm^2 (UF4) (Table 1). These loadings were higher than those obtained by Vasileva et al. [10] when immobilizing GOD by direct covalent bonding on a polymeric UF membrane (0.089 mg/cm^2), but lower than those reported by Ying et al. [11] when immobilizing GOD on a polymeric MF membrane (0.27 mg/cm^2). The enzyme loading in the free system (UF3) was significantly higher (corresponding to 0.18 mg/cm^2) than in any of the other configurations due to zero loss of enzymes during preparation and operation of the membrane. In all the immobilized configurations, enzyme leakage occurred mainly during the

Table 1
Enzyme loading (mg/cm^2) and enzyme leakage (%) during preparation and operation of the membranes (UF1-UF4). Enzyme leakage was calculated as percentage of initial loading of glucose oxidase and catalase. Membrane operation refers to the five reaction cycles. GOD = glucose oxidase; CAT = catalase; BL = bovine liver; AN = *Aspergillus niger*. The data are displayed as means ± standard deviations (data for UF1 AN CAT were not obtained). Configurations that have different letters (a, b) are significantly different ($p < 0.05$) both with respect to enzyme loading and enzyme leakage.

	GOD and BL CAT (2.41 mg initial loading)				GOD and AN CAT (2.33 mg initial loading)		
	UF1[a]	UF2[a]	UF3[b]	UF4[a]	UF1	UF3[b]	UF4[a]
Enzyme loading (mg/cm^2)	0.15 ± 0.01	0.14 ± 0.01	0.18 ± 0	0.13 ± 0.01	–	0.18 ± 0	0.12 ± 0
Enzyme leakage (%)	23.4 ± 4.8	25.0 ± 3.1	0	25.1 ± 3.8	–	0	31.0 ± 0.6
– Preparation (%)	22.2 ± 4.1	24.7 ± 2.7	0	24.4 ± 3.7	–	0	30.5 ± 0.1
– Operation (%)	1.2 ± 0.9	0.3 ± 0.3	0	0.7 ± 0.1	–	0	0.5 ± 0.4

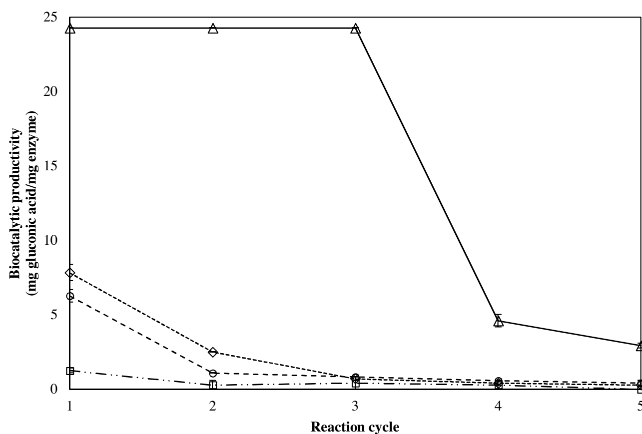

Fig. 2. Biocatalytic productivity during five consecutive reaction cycles for the four different reactor configurations (UF1-UF4). UF1 (diamonds): GOD and CAT immobilized (cycle time: 30 min) with polydopamine coating; UF2 (squares): GOD immobilized with polydopamine coating and CAT free in solution (cycle time: 30 min); UF3 (triangles): GOD and CAT free in solution (cycle time: 40 min); UF4 (circles): GOD and CAT immobilized (cycle time: 30 min) without polydopamine coating. In all configurations CAT from bovine liver was applied. Between each reaction cycle, fresh substrate solutions with xylose, glucose and hydrogen peroxide were added. The data are displayed as means ± standard deviations.

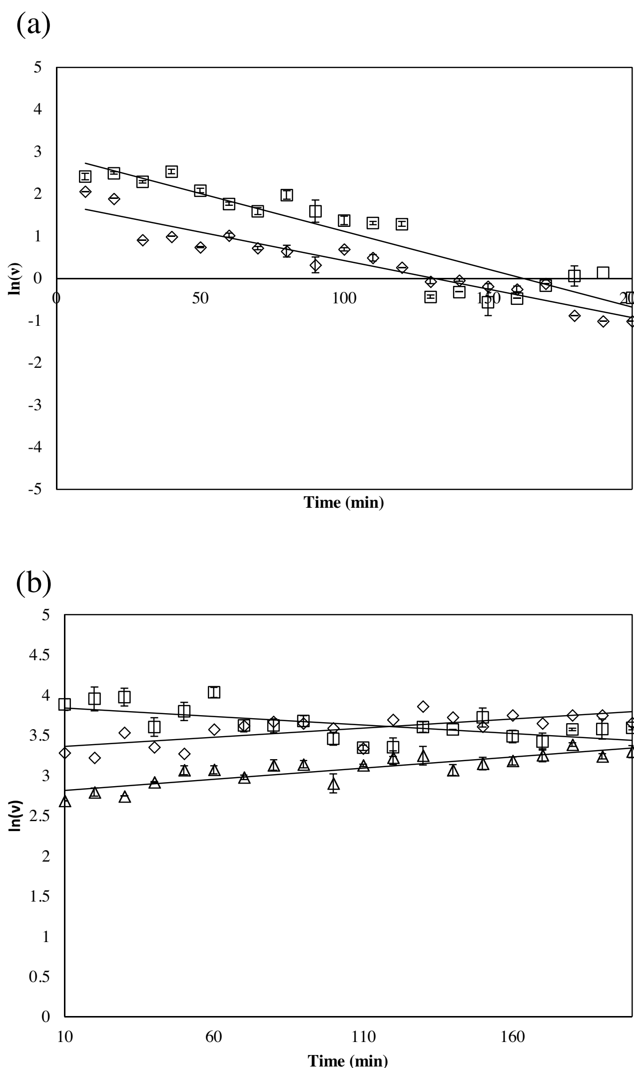

Fig. 3. pH stability of free catalase from bovine liver (a) and *Aspergillus niger* (b). Inactivation constants, k_D, are determined from the linear regression slopes in the ln(ν) vs. incubation time plots, where ν is given as $\mu mol\ min^{-1}\ ml^{-1}$: a) pH 8.5 (diamonds, $k_D = 0.0136$) and pH 5.4 with H_2O_2 (squares, $k_D = 0.018$). Since BL CAT did not have any activity at pH 2.5, data for pH 2.5 are not shown; b) pH 8.5 (diamonds, $k_D = 0$), pH 5.4 with H_2O_2 (squares, $k_D = 0$) and pH 2.5 (triangles, $k_D = 0$). All data are displayed as means ± standard deviations.

preparation stages (>20%) while enzyme loss during the reaction cycles was most often negligible (Table 1).

The first three configurations, UF1-UF3, all showed significantly different cycle patterns and total biocatalytic productivities (Fig. 2). After five consecutive reaction cycles the total productivities were 12 ± 1.1, 2.3 ± 0 and 80 ± 0.59 mg gluconic acid/mg enzyme for UF1, UF2 and UF3, respectively. When the membrane was used only as a separation barrier (UF3), a productivity of 24.4 mg gluconic acid/mg enzyme was obtained during each of the first three cycles, after which the productivity dropped steeply. When the membrane was used as immobilization support for GOD and CAT (UF1) or GOD alone (UF2), the productivity in the first cycle was significantly lower, namely 7.8 and 1.2 mg gluconic acid/mg enzyme, respectively. The productivity in UF1 continued to decrease with increasing number of cycles, while the productivity in UF2 was already so low at the start that very little further decrease was possible (Fig. 2). The drop in productivity after the third cycle with UF3 could be explained by inactivation of catalase by hydrogen peroxide, which was verified by BL CAT stability studies at pH 5.4 (Fig. 3a). Based on the inactivation constant (k_D) obtained from the ln(ν) vs. time plot for BL CAT at pH 5.4, a half-life of ~40 min was calculated, which indicated that 50% loss of the initial enzyme activity had already occurred after the first reaction cycle in UF3. The inactivation of catalase by hydrogen peroxide agrees well with results reported in other studies [19–21]. Since hydrogen peroxide could also inactivate GOD during extended exposure [22–24], further GOD stability studies were performed at pH 5.4 in the presence of hydrogen peroxide (Fig. 4). Since the GOD activity remained constant during the entire time of incubation (corresponding to five reaction cycles of UF3), it was concluded that the productivity drop in UF3 after the third cycle only resulted from insufficient supply of oxygen due to catalase inactivation by hydrogen peroxide. When comparing the biocatalytic productivities of UF1 and UF2, there seemed to be an advantage of co-immobilizing the enzymes in the membrane rather than immobilizing only GOD (Fig. 2). When CAT

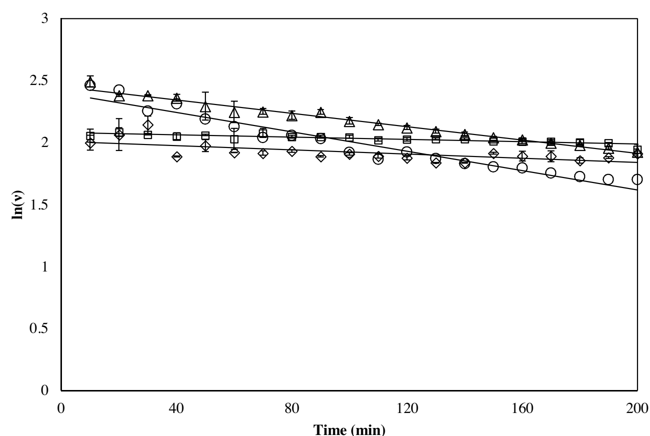

Fig. 4. pH stability of free glucose oxidase. Inactivation constants, k_D, are determined from the linear regression slopes at pH 8.5 (squares, $k_D = 0$), pH 5.4 with H_2O_2 (diamonds, $k_D = 0$), pH 2.5 (triangles, $k_D = 0.0027$, $R^2 = 0.978$) and pH 2.5 with H_2O_2 (circles, $k_D = 0,0039$, $R^2 = 0.938$) in the ln(ν) vs. incubation time plot, where ν is given as μmol min^{-1} ml^{-1}. All data are displayed as means ± standard deviations.

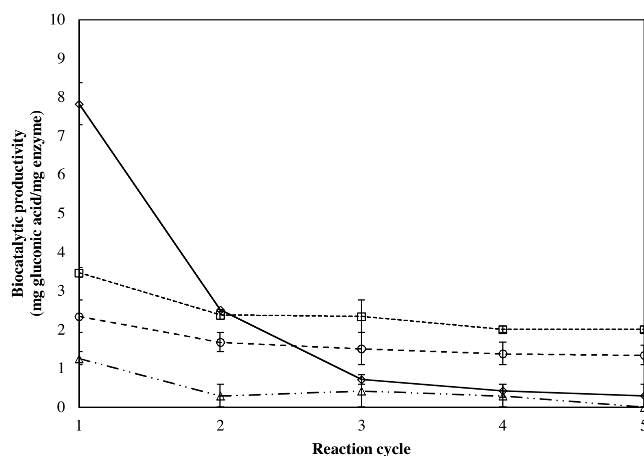

Fig. 5. Biocatalytic productivity during five consecutive 30-min reaction cycles for two different reactor configurations (UF1-UF2) with and without H_2O_2 in the substrate solutions: UF1 with H_2O_2 (diamonds), UF1 without H_2O_2 (squares), UF2 with H_2O_2 (triangles) and UF2 without H_2O_2 (circles). In all configurations CAT from bovine liver was applied. Between each reaction cycle, fresh substrate solutions were added. The data are displayed as means ± standard deviations.

was immobilized together with GOD (UF1), it was assumed that the oxygen produced in (2) could readily oxidize glucose via (1) rather than escape to the gas phase, which was the risk of having CAT free in solution (UF2). Furthermore, the catalase confined in the membrane was able to decompose the hydrogen peroxide formed in (1) that could be re-used as oxygen in the next (1). Considering that the stoichiometric amount of oxygen required for complete oxidation of glucose was 0.015 M (~480 mg/l) and that the solubility of oxygen in water is only <15 mg/l at 35 °C and 2 bar [25], the low productivity observed in UF2 was most likely explained by the poor oxygen availability in this configuration.

3.1.1. Impact of H_2O_2 addition

Additional reactions without hydrogen peroxide in the substrate solutions were investigated to verify that the lower productivity of UF2 compared to UF1 was caused by limited oxygen diffusion into the membrane pores (Fig. 5). There was no affect of not adding hydrogen peroxide to the substrate solutions in UF1 on the total productivity after five cycles (12 ± 0.84 mg gluconic acid/mg enzyme), but the cycle pattern was changed significantly as indicated by the fact that the productivity of each cycle remained almost constant after the second cycle. The productivity in UF2 also remained rather constant throughout the five cycles regardless of hydrogen peroxide addition. However, when hydrogen peroxide was not added to the substrate solutions, the total productivity of UF2 after five cycles increased to 8.2 ± 1.6 mg gluconic acid/mg enzyme, which was more than three times as much as when hydrogen peroxide was added. When hydrogen peroxide was not added, the performance of UF1 and UF2 was very similar. This result proved that diffusion of oxygen from the solution to the membrane was the main reason for the reduced performance of UF2 compared to UF1 when hydrogen peroxide was added to the substrate solutions. The slightly higher total productivity of UF1 (12 ± 0.84 mg gluconic acid/mg enzyme) compared to UF2 (8.2 ± 1.6 mg gluconic acid/mg enzyme) could probably be explained by the fact that the immobilized CAT in UF1 was still able to decompose the hydrogen peroxide formed in (1). One may speculate as to whether the poor performance of the immobilized configurations compared to the result obtained in UF3 were due to the application of polydopamine. When hydrogen peroxide was added to the substrate solutions, the oxygen produced in (2) could have contributed to further polymerization of dopamine rather than to glucose to gluconic acid conversion. If oxygen was consumed for dopamine polymerization, this would have a double negative impact on the biocatalytic productivity because: i) the oxygen used for dopamine polymerization would simply not be available for glucose oxidation and the gluconic acid production would therefore decrease; ii) if the polymerization of dopamine increased with each consecutive reaction cycle, the oxygen diffusion inside the membrane would be decreased for every reaction cycle. Although this effect would be most prominent during the first cycle, it could explain the continuous decline in productivity from the first to the fifth cycle of UF1 when hydrogen peroxide was added and likewise explain why the productivity remained constant when hydrogen peroxide was not added (Fig. 5). Similarly for UF2, if the decomposition of hydrogen peroxide to oxygen in the free solution contributed to polydopamine formation on the membrane surface and increased the mass transfer impediments, such diffusion limitations could explain why the biocatalytic productivity decreased compared to when hydrogen peroxide was not added. In order to assess whether or not polydopamine was disturbing the system, a fourth configuration was proposed without polydopamine coating (UF4) and with the membrane in reverse filtration mode to minimize enzyme leakage during operation (Fig. 1). However, the biocatalytic performance of UF4 was not significantly different from UF1, which indicated that dopamine polymerization did not proceed during the reactions. This result is also well in line with previous studies which show that dopamine polymerization normally does not take place at acidic pH [26–28]. The mechanism of polydopamine deposition during preparation of the membrane is, however, not entirely clear. Since polydopamine coating was conducted without pressure, the polydopamine should in principle not be able to penetrate the membrane pores but instead should be deposited as a layer on top of the membrane support. Alternatively, since the UF membrane is very permeable (>30 $L m^{-2} h^{-1} bar^{-1}$), the polydopamine may be able to penetrate the pores and form a network; this phenomenon was also suggested by Luo et al., 2014 [13] for laccase immobilization. Although the polydopamine mechanism still remains an open question, the results obtained (Fig. 2) support the hypothesis that polydopamine deposits only as a layer because the diffusion inside the membrane seemed similar for UF1 and UF4.

3.2. Comparing configurations UF1, UF3 and UF4 with *Aspergillus niger* catalase

Since the biocatalytic performance of UF3 was shown to be totally dependent on the oxygen availability which in turn

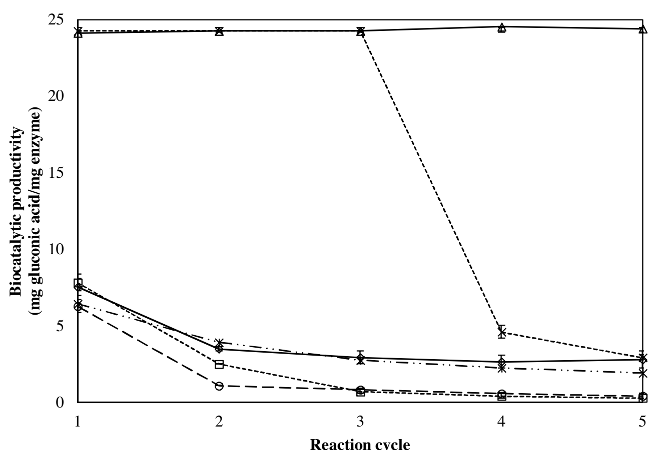

Fig. 6. Biocatalytic productivity during five consecutive reaction cycles for three different reactor configurations (UF1, UF3-UF4) with CAT from bovine liver and *Aspergillus niger*, respectively. UF1 AN CAT 30 min (diamonds), UF1 BL CAT 30 min (squares), UF3 AN CAT 40 min (triangles), UF3 BL CAT 40 min (crosses), UF4 AN CAT 30 min (stars), UF4 BL CAT 30 min (circles). Between each reaction cycle, fresh substrate solutions with xylose, glucose and hydrogen peroxide were added. CAT = catalase; BL = bovine liver; AN = *Aspergillus niger*. The data are displayed as means ± standard deviations.

depended on the catalase activity, the influence of catalase origin was investigated in UF1, UF3 and UF4 to see how the oxygen availability would be affected. Changing the CAT origin in UF2 was not considered important, because the limited oxygen availability in this configuration was not explained by the rate of (2) but rather by the transfer of oxygen into the membrane pores which in turn was related to the oxygen solubility. The enzyme loading and enzyme leakage in the different configurations were the same irrespective of the catalase origin (Table 1). When the catalase origin was changed from bovine liver to A*spergillus niger* (Fig. 6), the biocatalytic productivity in UF3 was the same in all cycles (24.4 mg gluconic acid/mg enzyme) and thus the total productivity increased to 122 ± 0 mg gluconic acid/mg enzyme. The improved performance of A*spergillus niger* catalase (AN CAT) compared to BL CAT was verified by stability studies at pH 5.4 in the presence of hydrogen peroxide (Fig. 3b). The AN CAT activity remained constant during 200 min of incubation (corresponding to five reaction cycles of UF3). This result again supported the previous conclusion that low concentration of oxygen was the sole reason for the sudden drop in productivity after the third cycle with BL CAT (Fig. 2). Using AN CAT also increased the total productivity of UF1 and UF4. After five cycles, the total productivities of UF1 and UF4 were similar at 19.4 ± 1.0 and 17.3 ± 0 mg gluconic acid/mg enzyme, respectively. The similar performance of UF1 and UF4 for BL and AN CAT further indicated that the reduced performance of the immobilized configurations compared to the free enzymes in UF3 was not caused by pH effects resulting from the polydopamine coating applied at pH 8.5 after immobilization of the enzymes. The enzyme stability studies at pH 8.5, which showed constant activity of AN CAT (Fig. 3b) and GOD (Fig. 4) during the entire time of incubation (Fig. 3), verified that alkaline pH did not affect the system. Stability studies of BL CAT (Fig. 3a) at pH 8.5 even revealed a slightly improved half-life (51 min) compared to the half-life at pH 5.4 (40 min). The reduced biocatalytic performance of the immobilized configurations (UF1, UF4) compared to the free enzymes in the first cycle, could very well result from suppression of the enzymes. Suppression could be due to poor biocompatibility of the membrane material with the enzymes [29] or due to unfavorable packing of the enzymes inside the membrane. According to Sheldon & Pelt 2013 [4] and Ganapathi-Desai et al. [30], molecular crowding as a result of high enzyme loadings in the membrane (as obtained with our fouling-induced immobilization strategy) can lead to activity losses due to reduced accessibility of the active sites when the enzyme molecules are situated deep within the carrier pores. In order to allow easy contact between the substrates and the enzymes, the enzyme molecules should therefore optimally be immobilized in monolayers [4]. With a coupled reaction system like the GOD/CAT where one reaction depends on the other, this shielding effect would be exceptionally strong. An impact of shielding could explain the apparent productivity maximum in the first cycle of UF1 and UF4 compared to UF3 (Fig. 6). Although the enzyme distribution inside the membrane is not known, it seems likely that CAT could be shielded by GOD because the molecular ratio (not activity ratio) of CAT to GOD was 1:17 (BL CAT) and 1:30 (AN CAT). Shielding of CAT by GOD would reduce the oxygen production in (1) and thus lower the gluconic acid production in (2). Furthermore, the advantage of having the enzymes in close proximity in terms of mass transfer (substrate-active site of the enzymes) could have been outweighed by the difficulty of the substrates to reach the active site of the enzymes (in particular H_2O_2 to CAT) within the stagnant layer formed in the membrane upon immobilization. The continuous decline in productivity during the five cycles cannot indeed be explained by the shielding effect only because this is not assumed to change during the reactions. Since the decline was only observed when the substrate solutions contained hydrogen peroxide (Fig. 5), and since (2) (when there was no other oxygen supply) proceeded only when hydrogen peroxide was added [1], a further hypothesis was that the enzymes were exposed to very low pH when gluconic acid was formed in the first cycle. Local pH effects could result from product diffusion limitations due to lack of agitation inside the membrane in contrast to when enzymes were free in solution. The local pH effect was thus most likely to occur in the stagnant layer around the glucose oxidase because this was where the enzyme was exposed to the highest concentration of gluconic acid. In order to verify this hypothesis, enzyme stability studies were performed at pH 2.5, which is below the pKa of gluconic acid (pKa = 3.4). While the AN CAT activity was not significantly affected at pH 2.5 (Fig. 3b), the BL CAT did not have any activity at this low pH (hence data are not shown). Although the GOD activity decreased slightly with time (Fig. 4), the GOD half-life at pH 2.5 was still 257 min. Therefore pH effects alone do not seem to be a likely explanation of severe decline in productivity after the first cycle in the immobilized configurations. Since stoichiometric amounts of hydrogen peroxide were formed together with the gluconic acid in (2), the possible additional negative impact of presence of hydrogen peroxide at pH 2.5 on the GOD activity was investigated (Fig. 4). The presence of hydrogen peroxide at pH 2.5 reduced the half-life to 178 min, which showed that accumulation of hydrogen peroxide in the stagnant layer at low pH is critical to the biocatalytic productivity. The gradual decline in biocatalytic productivity may thus be a result of several phenomena, but the contribution of other effects cannot yet be excluded, e.g. the orientation and flexibility of the enzymes.

4. Conclusions

A number of experiments were conducted to elucidate the impact of different parameters on the biocatalytic productivity of glucose oxidase and catalase in a membrane bioreactor. While productivity was found to depend on the oxygen availability which in turn depended on reactor configuration, hydrogen peroxide concentration and catalase origin, the contribution of other factors requires further evaluation in the particular enzyme systems that they influence. Robustness of the immobilized enzymes against local pH effects, hydrogen peroxide and other factors could possibly play a larger role than revealed in the stability studies of the

free enzymes. The study contributes to explaining the differences in performance of free versus immobilized systems for glucose oxidase and catalase and other coupled enzyme reactions with similar characteristics.

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