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Oxidation of indole with CPO and GOx immobilized on mesoporous molecular sieves

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ABSTRACT

Green chemistry and environmentally benign reaction engineering play an important role for future industrial processes. It is expected that the number of chemical reactions carried out via enzymatic catalysis will increase strongly. To achieve this aim, stable (viz. leaching and deactivation is prevented) heterogeneous biocatalysts are required. In this study, cross-linked enzyme aggregates of chloroperoxidase were grown in large-pore mesocellular foams (MCF). By changing the various synthesis parameters, the specific activity and the effective activity (viz. the enzyme activity units per mmol of adsorbed enzyme) are improved. The resulting biocatalysts composed of cross-linked chloroperoxidase and cross-linked glucose oxidase were tested in the oxidation of indole. The catalytic test under continuous operation conditions in a fixed-bed reactor confirmed that the cross-linked enzymes are less prone to leaching compared to the physically adsorbed enzymes in the pores of MCF or SBA-15.

1. Introduction

The non-enzymatic oxidation of indole to 2-oxindole (2-indolinone) requires harsh conditions or complex inorganic catalysts to be successful, since oxidation of the electron-rich 3-position is typically favored. Fujita et al. synthesized 2-oxindole from 3-(2-aminophenyl)-1-propanol [1] and Crestini and Saladino used a 2,3-dioxoindole derivative [2]. Corbett and Chipko found in 1979 that indole can be oxidized in the presence of chloroperoxidase with hydrogen peroxide to give 2-oxindole as the major product. Sheldon and co-workers investigated the formation of oxindole as a prominent example for the ability of haem chloroperoxidase (CPO, E.C. 1.11.1.10) from *Caldariomyces fumago* to oxidize activated C–H bonds [3,4]. Furthermore, they have chosen the CPO catalyzed oxidation of indole to oxindole as a model reaction to investigate different ways of adding the oxidant, various reactor types, and the use of a hydrogen peroxide-stat for controlling the hydrogen peroxide concentration [5]. The authors compared different types of reactors like batch, fed-batch or continuously operated membrane reactor combined with the use of a H_2O_2-stat [6]. Nevertheless, the industrial use of this enzyme is still lacking because of the expenses for the production of CPO and the difficulties in recovering the native enzyme from the reaction mixture. It has been suggested that permanent immobilization and encapsulation of enzymes enables their industrial use [7]. Furthermore, enzyme immobilization allows continuous operation in flow-type fixed-bed reactors. The various immobilization techniques like binding onto mesoporous silica supports, entrapment in organic or inorganic polymer matrices, and cross-linking of enzymes were recently highlighted in reviews by Hartmann [8] and Sheldon [9]. To circumvent leaching effectively, Kim et al. reported the formation of cross-linked enzyme aggregates (CLEAs) of lipase and α-chymotrypsin into hierarchically ordered mesoporous silica [10]. In our previous publication, we reported the formation of CLEAs from chloroperoxidase in the pore cages of MCF and their characterization by small angle neutron scattering (SANS) [11]. Besides permanent and stable immobilization of CPO, its susceptibility to oxidative deactivation by hydrogen peroxide, which is often applied as oxygen donor, is a frequently encountered problem. We have shown previously that deactivation can be circumvented by in situ generation of H_2O_2 by glucose oxidation with glucose oxidase (GOx, E.C. 1.1.3.4) from *Aspergillus niger* [12]. As Sheldon pointed out in his review, it is difficult compare different methodologies of enzyme immobilization. In papers published in literature, most authors compare the performance of the immobilized enzyme prepared using a particular technique with that of the free enzyme [9].

Herein, we report the enzymatic catalysis of indole to 2-oxindole using in situ generated hydrogen peroxide with immobilized haem chloroperoxidase and glucose oxidase. The enzymes have been

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physisorbed onto the ordered mesoporous silicate SBA-15, covalently anchored onto functionalized SBA-15 or cross-linked in the pore cages of mesocellular foams (MCF). For the evaluation of the effectivity of the biocatalyst used, the space-time yields and the turnover numbers are compared.

2. Material and methods

2.1. Synthesis of SBA-15

SBA-15 was synthesized according to procedures published in our previous publications [12,13]. A typical synthesis was performed as follows: In a polypropylene bottle (V = 500 ml), 4 g of the amphiphilic triblock copolymer P123 were dispersed in 30 g of water and stirred for 4 h until the copolymer was completely dissolved. Thereafter, 120 g of a 2 M aqueous HCl solution were added and the mixture was stirred for another 2 h. Then 9.5 g (45.6 mmol) of tetraethyl orthosilicate (TEOS) were slowly added under stirring. The resulting gel was aged in a water bath at 40 °C for 24 h and finally heated in an oven for 48 h at 130 °C. The resulting product was washed with 100 ml of ethanol and the as-synthesized SBA-15 was recovered by vacuum suction filtration over black ribbon filter paper. The material was then washed two times with 400 ml of a water-ethanol mixture (3:1, v:v) and dried at 100 °C for 24 h. Finally, the template was removed by calcination at 540 °C in air.

2.2. Functionalization of SBA-15

Prior to the grafting step, 1.0 g of SBA-15 was activated at 150 °C for 24 h under vacuum. The material was then transferred to a round bottom flask with stopcock and condenser. 50 ml of dry toluene and 2.0 ml (1.892 g, 8.55 mmol) of 3-aminopropyl trimethoxysilane (ATS) were added under nitrogen. The reaction mixture was refluxed under magnetic stirring for 24 h. After cooling, the solid product was recovered by suction filtration. Washing with toluene (100 ml) and acetone (100 ml) yielded a white solid that was dried under vacuum for 3 h and stored under argon. In a subsequent step, 500 mg of ATS-SBA-15 were mixed with 30 ml of an aqueous 1 wt.% glutaraldehyde (GA) solution and stirred for 4 h. Immediately after mixing, the suspension turned yellow, later orange and finally red. After 4 h, the product was recovered by suction filtration over a filter funnel equipped with a D3 frit. Washing with 500 ml water yielded a red solid that was dried under vacuum. The recovered material is referred to as GA-ATS-SBA-15.

The catalysts were characterized by X-ray powder diffraction (Bruker AXS D5005) and nitrogen sorption at 77 K using a Micromeritics ASAP 2010 instrument. The functionalized SBA-15 material was also characterized by solid-state ^{13}C CP magic-angle spinning (MAS) nuclear magnetic resonance (NMR) spectroscopy. The spectra were recorded at a resonance frequency of 100.6 MHz on a Varian AS 400 NMR spectrometer.

2.3. Synthesis of mesocellular foam

The synthesis of the mesocellular foam materials has been reported elsewhere [14]. For this study, all samples were prepared by dissolving 2 g of Pluronic P123 triblock copolymer in 75 ml of HCl (1.6 M) at 40 °C. Than 2 g of TMB (mesitylene) were added to the polymer solution, and the resulting mixture was stirred for 1 h. Thereafter, 4.25 g of tetraethyl orthosilicate (TEOS) were added dropwise as the silica source. The mixture was stirred for 24 h at 40 °C and finally heated in an oven for 24 h at 120 °C. The resulting product was recovered by vacuum suction filtration over black ribbon filter paper. The material was then washed two times with 400 ml of a water–ethanol mixture (3:1, v:v) and dried in air at room temperature. Finally, the template was removed by calcination at 540 °C in air. The obtained materials were characterized by nitrogen sorption at −196 °C.

2.4. Immobilization of CPO and GOx onto SBA-15 and GA-ATS-SBA-15

10 mg of SBA-15 (functionalized SBA-15) and an aliquot of CPO (GOx) were suspended in 5 ml of a 50 mM aqueous citrate buffer at pH 3.8 (4.0). The obtained solid materials were referred to as CPO (GOx)-(GA-ATS)-SBA-15.

2.5. Preparation of cross-linked CPO and GOx in the mesoporous material

The formation of the cross-linked enzymes in the pore cages of MCF is described in more detail in our previous publications [11,15]. 20 mg of MCF and an aliquot of CPO (GOx) as well as 0.51 mmol of glutardialdehyde as cross-linker were suspended in 2 ml of a 0.1 mM aqueous phosphate buffer solution (pH 5.5). The solid was separated from the supernatant liquid by filtration and washed with a buffered solution. The obtained solid material is referred to as CPO-CLEA-MCF and GOx-CLEA-MCF, respectively.

The activity of the CPO loaded material was tested employing the monochlorodimedone activity assay [16,17]. The activity test of the GOx loaded materials is described in [12].

2.6. Indole oxidation

In the batch reactor, an aliquot of the heterogeneous enzymatic catalyst was suspended in 5 ml of a saturated aqueous buffered indole solution (3.5 mM, 18 μmol of indole) and allowed to equilibrate for 5 min. For the continuous-flow experiments, a fixed-bed reactor was filled with the solid biocatalyst. A buffered indole (3.5 mM) glucose (50 mM) solution (pH 5.5) was pumped through the reactor at a flow rate of 0.2 ml min^{-1}.

3. Results and discussion

The XRD pattern of SBA-15 indicates that the material possesses a well-ordered hexagonal array of pores [18]. The pore size distribution calculated from the desorption branch of the nitrogen isotherm using the BJH formalism results in a pore diameter of 8.2 nm. The successful grafting of the mesoporous SBA-15 material give rise to the following ^{13}C NMR resonances (not shown) with chemical shifts of δ = 8, 19 and 40 ppm, which are assigned to different carbons in the 3-aminopropyl trimethoxysilane moiety (Si–CH_2–, –CH_2– and –CH_2–N). The signals at δ = 140 and 215 ppm are assigned to C=N and C=O, respectively. The nitrogen isotherm of the used MCF material provides evidence for its ink-bottle-type pores [14]. The MCF material possesses a BJH pore diameter for the adsorption branch of d_{cage} = 37 nm and one for the desorption branch of $d_{entrance}$ = 18 nm. The specific surface area amounts to about 470 m^2/g.

Fig. 1 shows the reaction network of GOx and CPO. The reductive half reaction of the catalytic cycle of GOx leads to the oxidation of β-D-glucose to δ-gluconolactone, while in the oxidative half reaction the enzyme is oxidized by molecular oxygen yielding hydrogen peroxide. The H_2O_2 is used for the oxidation of the CPO to form a high-valent iron oxo intermediate. The oxidation proceeds via an oxygen transfer reaction between this enzyme intermediate and the substrate.

In the first set of experiments, the catalysts prepared by physisorption of CPO and GOx separately on SBA-15 were tested

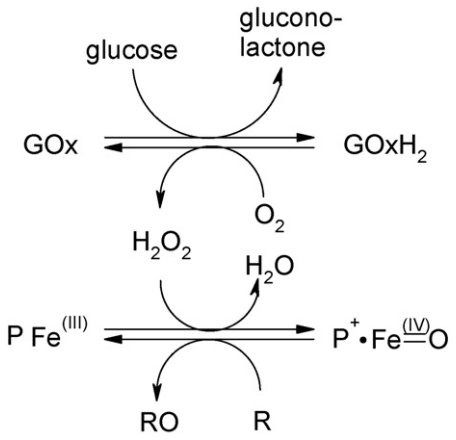

Fig. 1. The reaction network of glucose oxidase [19] and chloroperoxidase [4] involving the generation and the consumption of hydrogen peroxide (P, protoporphyrin).

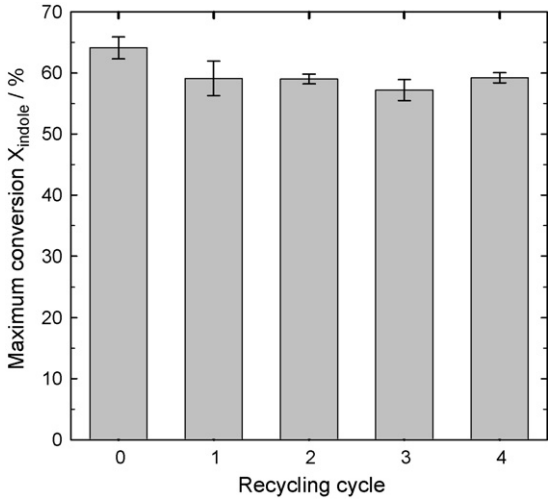

Fig. 2. Repeated use of 2.5 U CPO-SBA-15 and 1.5 U GOx-SBA-15; T_R = 20 °C, substrate: indole (5 ml, 3.5 mM), glucose (5 ml, 45 mM).

with respect to recyclability in a batch reactor. Upon adsorption of the enzymes, the pore volume decreases from 1.14 to 0.82 $cm^3\ g^{-1}$. However, we have shown in our previous publication that the remaining pore volume and the pore size, respectively, which is the more important value in this context, is large enough to allow the diffusion of the reactants within the porous network [11,12]. A detailed discussion on the physisorption of CPO onto SBA-15 and its immobilization onto grafted SBA-15 is given in [20].

Fig. 2 displays the maximum conversion of indole as a function of the recycling cycle employing the tandem system (CPO-SBA-15 and GOx-SBA-15). The oxidation was conducted at room temperature and aerial oxygen was used as the oxidant for glucose oxidation. Samples were withdrawn and analyzed by HPLC. After the final maximum indole conversion was reached (after about 7 h), the experiment was stopped, the heterogeneous biocatalysts were removed by filtration, washed and reused in the subsequent catalytic experiment. The limitation of the indole conversion to about 60% under the prevailing reaction conditions may be due to reversible enzyme inhibition. We assume that the product, 2-oxindole, interacts with the active site of the CPO (or GOx). After each recycling step, the heterogeneous biocatalyst is washed with buffer solution, thus, the 2-oxindole is easily removed by dilution and the catalyst is prepared for the next catalytic run. The recycling experiment shows convincingly that the maximum yield is constant within experimental error. Thus, the heterogeneous tandem system circumvents CPO deactivation by limiting the H_2O_2 concentration in the reaction mixture.

In order to investigate the operational stability of the immobilized enzymes employing various techniques like physisorption, covalent anchoring and cross-linking, the performance of the heterogeneous biocatalysts (immobilized CPO and immobilized GOx) was tested under continuous-flow conditions. Therefore, a fixed-bed reactor was filled with a physical mixture of immobilized CPO and immobilized GOx. A buffered solution (pH 5.5) of indole (3.5 mM) and glucose (50 mM) was pumped through the reactor at a flow rate of 0.2 ml min^{-1}. The buffered indole-glucose solution in a storage tank is continuously stirred leading to a high dissolved amount of oxygen. In this context, we have also tested whether piping an additional air stream through the reactor has a positive influence in our catalytic runs, which was, however, not the case.

The fixed-bed reactor performance of covalently immobilized and of physisorbed chloroperoxidase and glucose oxidase is compared in Fig. 3 (left). For CPO and GOx physisorbed onto SBA-15, a strong decrease of the oxindole yield with time-on-stream is detected. The initial oxindole yield for the catalyst bed consisting of 150 U CPO-SBA-15 and 7 U GOx-SBA-15 amounts to 16%. After 48 h time-on-stream, the oxindole yield is reduced to 3.5%. In contrast, the catalyst prepared by covalent immobilization of the enzymes exhibits a different behavior. In the first 2 h, the oxin-

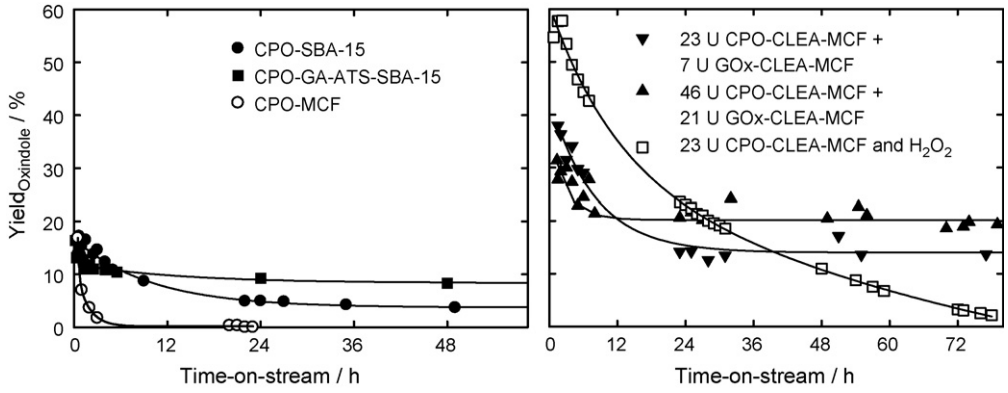

Fig. 3. Oxidation of indole over 150 U CPO-GA-ATS-SBA-15 and 7 U GOx-GA-ATS-SBA-15 (filled square), 150 U CPO-SBA-15 and 7 U GOx-SBA-15 (filled circle) and 23 U CPO-MCF and 7 U GOx-MCF (–) (left); 23 U CPO-CLEA-MCF and 7 U GOx-CLEA-MCF (triangle down) and 47 U CPO-CLEA-MCF and 21 U GOx-CLEA-MCF (triangle up) and 23 U CPO-CLEA-MCF with an amount of hydrogen peroxide corresponding to that generated by 7 U GOx-CLEA-MCF (open square) (right); T_R = 20 °C; flow rate 0.2 ml min^{-1}; substrate: indole (3.5 mM), glucose (45 mM).

Table 1
Steady-state oxindole concentration at the reactor outlet and the calculated space-time-yield (STY) for different catalysts.

sample	Activity (U)	$c_{const.,\ oxindole}$ (mmol L^{-1})	STY ($mmol_{oxindole}\ h^{-1}\ g_{catalyst}{}^{-1}$)
CPO-SBA-15	150	0.16	0.007
CPO-GA-ATS-SBA-15	150	0.27	0.010
CPO-CLEA-MCF	23	0.55	0.346
CPO-CLEA-MCF	46	0.78	0.243

dole yield is significantly reduced from 23% to about 11.5% over the mixture of 150 U CPO-GA-ATS-SBA-15 and 7.5 U GOx-GA-ATS-SBA-15. After more than 48 h a final yield of 8.3% is obtained. The roughly constant 2-oxindole yield is reached earlier as in case of the adsorbed enzymes. Thus, we have to conclude that even functionalized SBA-15 materials contain a certain amount of only physisorbed enzymes, which were not removed by the washing procedure employed during the catalyst preparation. This weak interaction between the mesoporous host and the enzyme results in rapid leaching of the enzyme during the first few hours of time-on-stream, which is indicated by a strong decrease of the oxindole yield.

Here, we discuss only leaching of the enzyme as the major cause of the observed decrease in 2-oxindole concentration in the product stream. In our opinion, enzyme deactivation plays only a minor role. In the later case, deactivation will continue with time-on-stream and a constant product flux is not achievable. This is indeed observed for external hydrogen peroxide addition as shown in Fig. 3 (right). Leaching of CPO and its deactivation with time leads to a steady decrease of 2-oxindole yield down to zero within 75 h. Furthermore, in our previous publication, we have demonstrated employing SANS experiments that leaching of the enzymes from the mesoporous host is the main cause for the reduction of the oxindole yield [11].

However, it appears that based on units, that immobilized CPO has to be filled into the reactor in excess compared to immobilized GOx. According to Fig. 1, one catalytic cycle of GOx generates one molecule of H_2O_2, while one molecule of indole is oxidized per catalytic cycle of CPO. The key to an optimum process is the maximum utilization of H_2O_2 generated for the catalytic oxidation of CPO. Because the accessibility of the immobilized CPO for H_2O_2 is reduced due to the immobilization onto SBA-15 exhibiting an ordered structure consisting of pore channels, immobilized CPO is added in excess. Using mesocellular foams as enzyme support, the excess of immobilized CPO may be reduced in the reactor, presumably because the pore structure of MCF materials has various pore entrances at the outer surface of the catalyst particle and the diffusion of the reactants to the active sites is facilitated.

In contrast to the performance of enzymes physisorbed onto SBA-15, the initial oxindole yield of the biocatalyst prepared by physisorption on MCF amounts to only 17% and drops to almost zero after 12 h. Due to the large-pore cages and entrances of the MCF material, the enzymes cannot be partially stabilized as in the case of SBA-15. The diameter of the SBA-15 pore channels has been adjusted to a diameter closed to the enzyme size, which fits the enzymes ideally. Therefore, the indole conversion using CPO-SBA-15 results in a final constant oxindole yield of 3.5 %, while the yield decreases to zero over CPO-MCF.

The oxindole yield over catalysts containing cross-linked enzymes in the mesopores of MCFs is shown in Fig. 3 (right). The initial activity of both runs amounts to about 35% and drops to constant yield within the first 24 h. For a catalyst mixture of 23 U CPO-CLEA-MCF and 7 U GOx-CLEA-MCF, a final yield of 2-oxindole of 14% is reached, while for the mixture of 46 U CPO-CLEA-MCF and 21 U GOx-CLEA-MCF a yield of 20% is achieved. As demonstrated before, an enhancement in enzyme activity due to an increase of the amount of biocatalyst used results in a rise of the 2-oxindole yield. Nevertheless, an increase in activity of immobilized CPO used by a factor of two (from 23 to 46 U) and a three-fold increase in activity of immobilized GOx (from 7 to 21 U) results in an enhanced final oxindole yield by a factor of 1.4 (from 14% to 20%). It appears that in the first 10 h the oxindole yield is higher for the smaller amount of catalyst (23 U CPO-CLEA-MCF + 7 U GOx-CLEA-MCF). Both enhanced enzyme leaching as well as faster deactivation due to a higher local H_2O_2 concentration for 46 U CPO-CLEA-MCF + 21 U GOx-CLEA-MCF may contribute to the observed behavior. Moreover, the use of a larger amount of catalyst results in a noticeable increase in the length of the packed bed of the heterogeneous biocatalysts in the reactor. Furthermore, a gradient in the indole–2-oxindole ratio from the top to the bottom of the fixed-bed reactor is the consequence of pumping the indole solution over the packed bed. The concentration of indole is reduced from the top to the bottom of the reactor. However, internal and external mass transfer limitations also have to be considered.

Table 1 compares the different catalysts with respect to the resulting final oxindole concentration at the reactor outlet and the corresponding space-time-yield (STY). The space-time-yields of physisorbed CPO and covalently anchored CPO on SBA-15 amount to 0.007 mmol h^{-1} g^{-1} and to 0.010 mmol h^{-1} g^{-1}, respectively. A significant increase of the space-time-yield is realized by using the cross-linked CPO in the mesoporous cages of MCFs. Here, 0.346 and 0.243 mmol h^{-1} g^{-1} are obtained for 23 U CPO-CLEA-MCF and 46 U CPO-CLEA-MCF, respectively. These results clearly confirm that the CLEA-MCF biocatalysts show higher product yields and better space-time-yields compared to physisorbed CPO on SBA-15. That is because the MCF support (i) allows a higher enzyme loading resulting in higher activity and (ii) offers an increased availability of the enzyme for the substrate due to its three-dimensional pore structure. Furthermore, as shown in Table 1, an increase in the amount of catalyst results in higher product yields but in reduced space-time-yields.

In principle, the turnover number (TON) in enzyme catalysis denotes the maximum value of the rate per catalytic site at saturation of the enzyme by the reacting substrate as defined by the Michaelis–Menten kinetics. Subsequently, the rate referred to the number of catalytic sites is known as turnover rate or turnover frequency (TOF) which is defined as the number of repetitions of the catalytic cycle per time. The difficulty in determining the TOF involves not only the measurement of the reaction rate, but also the counting of the active sites, which is a sophisticated problem.

The turnover frequency TOF_{total} (Table 2) is determined using the total amount of enzymes immobilized, which was accurately quantified by chemical analysis. We have to point out that the TOF calculated here is related to the amount of CPO used in the fixed-bed reactor at the start of the catalytic run and not to the amount of CPO that remained on the support after leaching, when a constant 2-oxindole flux is achieved. The effective activity, that is the activity per amount of enzyme immobilized, was calculated to determine the amount of enzymes which are active and available. The quotient of the effective activity of the immobilized CPO and the native CPO estimates the amount of accessible and active enzymes. Nevertheless, since the activity is determined employing the MCD activity assay, it has to be assumed that the number of immobilized CPO enzymes which are accessible and active do not

Table 2
Molar flux of 2-oxindole at the reactor outlet over physically mixed immobilized GOx and CPO.

Sample	Activity (U)	$\dot{n}_{2-oxindole}$ (mmol min^{-1})	TOF_{total} (h^{-1})	$TOF_{corrected}$ (h^{-1})
CPO-SBA-15	150	0.01	42	1044
CPO-GA-ATS-SBA-15	150	0.04	149	1726
CPO-CLEA-MCF	23	1.29	4661	9279
CPO-CLEA-MCF (pH change)	23	1.68	6042	9674

change with changing the substrate from MCD to indole. Employing the number of active enzymes as determined by the MCD activity assay, a corrected turnover frequency ($TOF_{corrected}$) is calculated. Table 2 summarizes the turnover frequencies for the oxindole production in the fixed-bed reactor of CPO immobilized on SBA-15 and MCF employing different techniques. Physisorbed CPO onto SBA-15 results in a $TOF_{corrected}$ of 1044 h^{-1}, while for the covalent anchored CPO onto SBA-15 a $TOF_{corrected}$ of 1726 h^{-1} is calculated. The enormous benefit in using cross-linked enzymes supported on MCF instead of physisorbed or covalently linked CPO onto SBA-15 is evident when the turnover frequencies are compared. The $TOF_{corrected}$ obtained for CPO-CLEA-MCF amounts to 9279 h^{-1}, which is an increase by a factor of 8.9 and 5.4 compared to CPO-SBA-15 and CPO-GA-ATS-SBA-15, respectively. In one of our previous publications we have shown that an increase in activity from 6944 to 9073 U g^{-1} is achieved after optimization of the preparation method [11]. After the adsorption of CPO on MCF for 1 h at pH 3.8, the pH was increased to 5 for optimum CLEA formation and thereafter, the cross-linker (GA) was added. Using this material in the fixed-bed reactor, a further increase in TOF upto 9674 h^{-1} is noticed, which is due to an increase in product flow from 1.295 to 1.678 mmol L^{-1}.

In order to display the direct influence of the pH change during the enzyme immobilization process, the fixed-bed reactor was filled with co-immobilized CPO and GOx onto the same MCF support. Biocatalyst A was prepared by changing the pH level from 3.8 to 5.0 before adding the cross-linker. Therefore, to a suspension of 50 mg of MCF in 5 ml of a buffered solution (pH 3.8), 2000 U of CPO and 2000 U GOx were added. After 1 h shaking at 5 °C, the pH level was changed to 5.0 and 2 mmol of GA were added, thereafter the suspension was shaken for further 24 h. Biocatalyst B was prepared in a similar manner: the support, the enzymes and the cross-linker were added simultaneously to the buffer solution at pH 3.8. Because the activity and the amount of enzymes immobilized cannot be determined separately, the two biocatalysts, with and without changing the pH level, were compared with respect to their performance in indole conversion in the fixed-bed reactor.

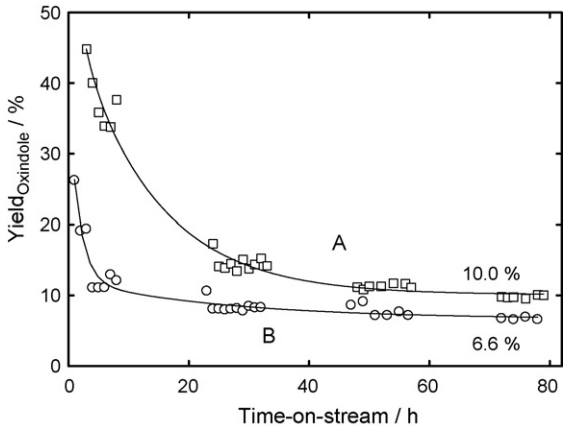

Fig. 4. Tandem reaction with co-immobilized CPO/GOx-CLEA-MCF prepared by simultaneous addition of enzymes and cross-linker (A) and by pH change before the cross-linker is added (B); T_R = 20 °C; flow rate 0.2 ml min^{-1}; substrate: indole (3.5 mM), glucose (45 mM).

Fig. 4 depicts the yield of oxindole as a function of time-on-stream. The final constant yield of oxindole obtained for the biocatalyst CPO/GOx-CLEA-MCF prepared by simultaneous addition of enzyme and cross-linker and prepared by the pH change method amounts to 6.6% and 10.0%, respectively, while the initial yield of oxindole amounts to about 28% and 45%.

We assume that for simultaneous addition of enzymes and cross-linker, the non-permanent cross-linked enzymes are washed out to a large extent by the washing procedure after the catalyst preparation. Thus, the initial leaching of enzymes in the fixed-bed reactor, noticeable by the decrease in 2-oxindole yield, is reduced compared to the biocatalyst prepared by the pH change method. In this case, it is known from the adsorption kinetics that the maximum adsorption capacity is reached after 1 h (not shown). This implies that the enzymes, which are not cross-linked in the pore cages, are dispersed in the porous network. Hence, they overcome the washing procedure after the immobilization process and therefore, for the pH change method no difference in the amount of enzyme adsorbed before and after washing is detectable [21]. Thus, the catalyst prepared by changing the pH level, exhibits a higher leaching rate in the fixed-bed reactor, because the dispersed enzymes adsorbed in the porous network do not overcome the harsh reaction condition in the fixed-bed reactor [11]. Thus, the leaching rate viz. the decrease in oxindole yield at the beginning of the experiment of the CPO/GOx-CLEA-MCF material prepared by pH change method is higher compared to the material prepared by simultaneous addition of enzyme and cross-linker.

4. Conclusions

The oxidation of indole to oxindole using immobilized chloroperoxidase and glucose oxidase in batch as well as in fixed-bed reactors was investigated. Two different supports (SBA-15 and MCF) and various immobilization techniques (i.e. physical adsorption, covalent binding, cross-linking) were compared. It was shown that the bi-enzymatic catalyst consisting of a physical mixture of adsorbed CPO and GOx onto SBA-15 can be recycled several times without significant loss of enzyme activity. It was furthermore demonstrated that the operational stability of the immobilized enzymes with respect to leaching increases in the order physical adsorption < covalent binding < cross-linking. The second encountered problem besides leaching, deactivation of chloroperoxidase is circumvented employing immobilized GOx for H_2O_2 generation. In addition, the space-time yield (STY) increases significantly from 0.007 to 0.010 and finally to 0.346 $mmol_{oxindole}$ h^{-1} $g_{catalyst}^{-1}$ for CPO-SBA-15, CPO-GA-ATS-SBA-15 and for CPO-CLEA-MCF, respectively. It was also demonstrated that an increase in the amount of biocatalyst used in the fixed-bed reactor may not enhance the efficiency because the product flow does not increase proportionally. Thus, for 46 U CPO-CLEA-MCF only a STY of 0.346 $mmol_{oxindole}$ h^{-1} $g_{catalyst}^{-1}$ is obtained compared to 0.243 $mmol_{oxindole}$ h^{-1} $g_{catalyst}^{-1}$ found for 23 U CPO-CLEA-MCF. Furthermore, the STY increases by a factor of almost 35 for CPO-CLEA-MCF compared to CPO-SBA-15. Moreover, the turnover frequencies increase in the order of CPO-SBA-15, CPO-GA-ATS-SBA-15 and CPO-CLEA-MCF. These results demonstrate that the

immobilization of chloroperoxidase in mesoporous silica for enzymatic oxidations performed in continuously operating fixed-bed reactors is a promising strategy for further evaluation with respect to envisaged industrial applications.

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