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Continuous decomposition of Acid Blue 74 in a membrane reactor with soluble laccase

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ABSTRACT

The discussed investigation is focused on decolorization of Acid Blue 74 (AB74) carried out in enzymatic membrane reactor (EMR) at presence of laccase from *Cerrena unicolor*. A range of assumptions were held for EMR, that is: (i) total amount of laccase present in feed was rejected by membrane and remained in EMR; (ii) the enzyme was sufficiently stable; (iii) substrate and by-products of its decomposition were not adsorbed on membrane; and (iv) additional aeration was not required. Modeling of the process was made using selected kinetic equation, parameters of which were firstly evaluated and next applied to process planning protocol and experimental verification. The residence time of ca. 9 min and 70% substrate conversion at pH 5.3 were assumed, while actual results showed conversion of 70.2% for τ = 9.36 min (calculated: 70% and 9.2 min). It allowed to conclude that AB74 decolorization process performed at constant concentration of laccase in a given volume of EMR could be fully predictable. In the long-term process, the enzyme inactivation was noted, but still it was four times lower than the one obtained in the fed-batch reactor. However, in the EMR system, the activity level could be easily refreshed.

Keywords: Membrane reactor; Laccase; Continuous process; Dye decolorization

1. Introduction

Nowadays, synthetic dyes play an important role in various industrial processes, among which textile industry is found to cause serious surface waters contamination. Toxic materials, hazardous ions and dyes present in wastewaters have a great impact on environment and human health. Furthermore, highly colored water impedes sunlight penetration, what can result in inhibition of aquatic organisms' growth. So far, many efficient and techniques of textile wastewater treatment, including physicochemical, biochemical, and combined treatment processes, have been developed. Unfortunately, physicochemical treatment methods are mostly energy-consuming, require use of additional chemicals, and generate secondary contaminants [1]. In contrary, decolorization processes, which are carried out with the use of microorganisms and enzymes, are regarded as an attractive and environmentally safe alternative to physicochemical processes [2–5].

Most efforts given to enzymatic decomposition of fabric dyes have been focused on laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2.) [6,7]. It is a group of oxidases that reveals wide spectrum of action to phenolic substrates, with no use of expensive, additional, co-factors or H_2O_2. Hence, oxidases are favorable among other enzymes (oxidoreductases or peroxidases) [8]. Laccases have been tested in removal of many dyes via cross-coupling reactions, polymerization or degradation, very often at presence of mediators [6].

Indigoid dyes are a group of dyes commonly used for dying fabrics. A main representative of this group is indigo carmine (5.5'indigo disulfonic acid disodium salt; C.I.73015; Acid Blue 74 [AB74]). AB74 is a dark blue, water-soluble dye,

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mainly used in the textile industry for coloration of cotton cloths and other denims. Besides its use as a textile dye, AB74 is also applied to medical diagnostic purposes and in food and cosmetic industries [9]. Thus, growing interest in decolorization of industrial effluents is noted, but only several articles focused on application of native or immobilized laccases for successful removal of indigo carmine without addition of harmful mediators can be found in the literature [8,10–14].

In the study, which precedes the discussed investigation, we noted that laccase from *Cerrena unicolor*, immobilized on silica carriers, is able to completely decompose indigo carmine without any use of external additives [12]. However, a calculated value of adsorption capacity of immobilized enzyme prepared toward AB74 was very high (about 80 mg g^{-1}), what strongly interfered with obtained results. Moreover, application of expensive immobilized enzyme seemed to be less attractive than the use of its native form. Thus, our attention was focused on the investigation of continuous system equipped with a semipermeable membrane to separate the native enzyme and reaction products from a treated mixture.

An enzymatic membrane reactor (EMR) with an enzyme freely circulated in a reactor volume (retentate side) equipped with a membrane completely impermeable to enzyme was regarded as an integrated set-up, ideal for conduction of continuous processes, during which both reaction and separation units were located in one device [15,16]. In such a system, the most important issue was to select a proper membrane, which would be able to completely reject the enzyme in order to prevent its stepwise activity loss. Moreover, membrane material and pore size were responsible for both substrates and products rejection. One should know that complete retention of enzyme in an EMR volume enables the performance of unique homogenic catalysis, devoid of diffusional resistances. Many assumed advantages of EMR have not yet been reflected in practice, mainly due to both biocatalyst instability in reaction conditions and occurrence of membrane fouling and concentration polarization layer formation. However, an enzyme activity loss in EMR caused by pH, temperature or reagents adsorption at a membrane wall is rather rarely observed.

The use EMR with laccase seems to be an interesting alternative for decolorization of the textile effluents [17–19]. However, process modeling with enzymatic reaction kinetics was sparsely applied [20–22]. In our previous investigations, the complete retention of laccase from *Cerrena unicolor* by polyethersulfone membrane of molecular weight cutoff of 5 kDa was demonstrated [23]. Next, successful continuous removal of Acid Blue 62 (AB62) in EMR was showed, although a very strong sorption of the substrate onto the membrane was noted [22]. In our opinion *Cerrena unicolor* laccase is an enzyme of unique properties, which makes it suitable to be used in EMR for dyes containing effluents decolorization, that is, the enzyme is extracellular and can be produced without any inducers; its purification is cost effective; and it is thermostable [12,24].

The main aim of our presented work is to show the applicability of laccase-based EMR for AB74 decolorization. Acid Blue 74 was selected due to two main reasons: its laccase oxidation products are well known [25], and it is less toxic than AB62 [14]. Moreover, according to our best knowledge, there are no papers on AB74 removal in the EMR. In most of the experiments, AB74 concentration was set at 100 μM, that is, 20% higher than in industrial effluents [13]. Our work was focused on evaluation of enzyme stability and on sorption of reagents onto membrane. We also investigated reaction kinetics, performed process modeling and validated it in long-term process of AB74 removal in the EMR.

2. Materials and methods

2.1. Materials

Lowry's reagent by Sigma-Aldrich and indigo carmine (AB74) and other reagents of analytical grade by POCH (Poland) were used in experiments.

Laccase production and purification were done according to methods described in the preceding study [24].

The dye conversion was evaluated spectrophotometrically (GBC Spectrophotometer, Australia) on the basis of UV-VIS spectra in the range from 240 to 800 nm for multiple time points. For determination of concentration of dye and its biotransformation products, absorbance at 612 nm was chosen for AB74 and at 530 nm products, due to insignificant absorbance of counterparts (Fig. 1). The wavelength found for the substrate corresponded to the data obtained by Younes et al. [14] and was in a contradiction to other authors, who noted values 640 and 650 nm [13,25]. The feed solution was obtained after 12 h of AB74 incubation with laccase at 30°C (no change in UV-VIS spectra observed for reaction mixture after 12 h). Substrate and products concentrations were calculated using linear region of calibration curves (612 nm – R^2 = 0.999; 530 nm – R^2 = 0.991).

Laccase activity toward AB74 was measured as an absorbance (612 nm) decrease at initial reaction conditions (linear dependence of absorbance vs. time). Unit activity (U_{AB74}) was defined as the amount of protein that was needed to decompose 1.0 μM AB74 concentration in 1 min under reaction conditions (pH 5.3, 0.1 M phosphate-citrate buffer, 30°C, initial substrate concentration 100 μM). The prepared enzyme had a specific activity of 640 U mg^{-1} protein. The enzyme concentration was measured by Lowry's method, according to Sigma procedure, using bovine serum albumin as a standard. All measurements were repeated three times (±2.6% and ±2.7% for activity and protein concentration, respectively).

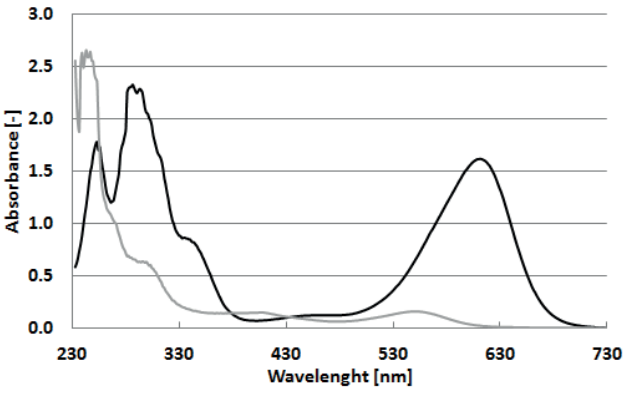

Fig. 1. AB74 spectra (95 μM) before (black) and after (grey) decolorization with laccase.

Effect of pH on laccase activity and stability was tested as in previous study [22]. In the preceding research, it was stated that substrate spectrum had not been changed with change of pH of the buffer. All measurements were repeated three times (±2.5%). Activity values obtained at pH 5.3 were taken as ones corresponding to 100% activity.

The influence of oxygen consumption on the enzyme activity was checked by monitoring the oxygen concentration using a luminescent dissolved oxygen probe (LDO10101, Hach Lange, Poland).

AB74 decomposition in a thermostated and well-mixed batch reactor was done at following process conditions: final dye concentration 100 μM, 30°C, 120 rpm, 30 mL 0.1 M phosphate-citrate buffer (pH 5.3), 8 U_{AB74} mL^{-1}. The decolorization process was monitored in a continuous mode (a cell through cuvette; 612 nm; 6 s intervals) up to the complete substrate depletion. Laccase activity was calculated on the basis of initial linear absorbance depletion vs. time. The value obtained in the first process was set as 100%. Next, 19 consecutive portions of the substrate were added after substrate depletion was noted.

Membrane set-up was a Labscale™ TFF System (Millipore, Bedford, USA), provided with a reservoir, stir base, retentate valve, pressure gauges and a diaphragm pump. Membrane made of polyethersulfone and molecular weight cutoff 5 kDa (Pellicon® XL module; Biomax-5; 50 cm^2 of filtration area; Millipore) was used. All studies were performed at 30°C.

The membrane separation/sorption test was performed at the presence of substrate and decolorization products as discussed in [22]. The system was used in diafiltration mode, and the transmembrane pressure (TMP) was set at 0.137 MPa. Permeate samples were collected at various time intervals. In the experiments, AB74 solution (100 μM) or decolorized solution were continuously fed into the membrane reactor filled with 50 mL of AB74 or reaction products, and the absorbance in 612 or 530 nm in all collected samples was determined.

Enzyme activity and stability in a range of pH values was determined according to methods described in [22].

Kinetic studies of AB74 removal were done in a batch mode, at 30°C and pH 5.3 or 6.3, substrate concentration ranging from 20 to 80 μM and using monitoring of the absorbance decrease at 612 nm with 1.45 mL of buffered dye solution (pH 5.3 and 6.3) of initial AB74 concentration of 20, 40, 60 and 80. Final protein concentration was 40, 20, 10 and 5 μg L^{-1}. All measurements were repeated at least three times (±2.4%). Parameters of kinetic equation were estimated by linear or nonlinear regression by means of OriginPro 9 software.

Continuous decolorization of AB74 containing solution was performed at 30°C, using buffered 100 μM solution (pH 5.3 or 6.3) of AB74 as feed. The reactor volume was firstly filled with 50 mL of laccase solution (10.3 and 20.8 U_{AB74} mL^{-1}), and next the feed was pumped into the reactor operated in diafiltration mode (the reaction mixture volume was constant through the whole experiment). Decolorization was performed using selected permeate fluxes, which were controlled by modification of TMP. The permeate samples (50 mL) were periodically collected, and the absorbance at 612 nm was monitored. The steady-state conditions (five reactor volume exchanges) were determined by specifying the same absorbance values in permeate in three consecutive analysis in 0.25 h intervals.

3. Results and discussion

3.1. Laccase stability in a reaction mixture

It is obvious that the most strong requirement to use a native enzyme in EMR is its satisfactory stability at reaction conditions, especially at presence of substrate(s) and product(s). Moreover, laccase oxidizes substrates in presence of molecular oxygen, and a depletion of this co-substrate in reaction mixture stops the reaction progress. Hence, *Cerrena unicolor* laccase stability was tested in a fed-batch reactor equipped with online monitoring of oxygen concentration. In Fig. 2(a), it can be seen that laccase was satisfactory stable in 20 successive runs with fresh substrate portions supply and with products accumulation. The observed loss of activity by 10% could be regarded as exceptionally low. Cho et al. [10] reported decolorization elongation from 3 min up to 13 min in the fifth run, whereas Liu et al. [26] noted 23% reduction of laccase activity in the sixth cycle. However, in the long-term continuous process, laccase activity loss should be included in a kinetic model. As laccase inactivation at pH 5.3 could be described by first-order kinetics (data not shown), inactivation rate constant was calculated by nonlinear regression (k_{inact} = 4.07 ± 0.13 × 10^{-4} min^{-1}).

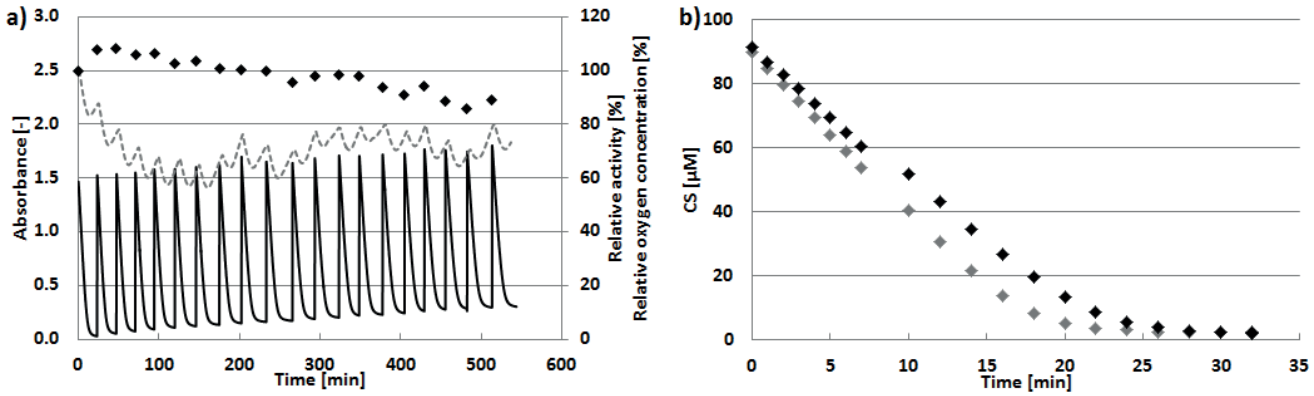

Fig. 2. (a) Repeated fed-batch decomposition of AB74 (90 μM) with laccase (4 U mL^{-1}) at pH 5.3 and 30°C. Solid line – absorbance at 612 nm; dashed line – relative oxygen concentration; diamonds – relative laccase activity. (b) AB74 (90 μM) decomposition progress with laccase (4 U mL^{-1}) at pH 5.3 and 30°C in batch reactors with (grey) and without (black) constant aeration.

Taking into account the necessity of oxygen for the reaction occurrence, its consumption seemed to be moderate (40% decrease in oxygen concentration), although it was greater than in the case of AB62 oxidation (20%) [22]. In order to prove that oxygen concentration in reaction mixture was sufficient to oxidize AB74, two parallel processes were run, and a continuous aeration was performed in one of them. Data presented in Fig. 2(b) clearly shows that at tested conditions, oxygen consumption was too slow to significantly disturb the reaction progress.

3.2. Sorption of AB74 and its oxidation products onto the membrane

Strong sorption of reagents onto a UF membrane was regaded to be a serious problem in EMR as it could be responsible for lower substrate conversion (enzyme sorption), and it could disable a steady-state operation. It was already shown [22] that about 18% of laccase could be adsorbed in the EMR device without change in the general enzyme reactivity. However, AB62 and products of its degradation adsorbed strongly on membrane causing elongation in reaching steady-state condition (16 or 29 times of the residence time, respectively). In presented study, sorption of reagents (Fig. 3) was relatively low and steady-state conditions were noted after fourth–fifth exchange of the reactor volume.

3.3. Kinetics of AB74 decomposition by laccase

In order to predict the dye conversion in EMR, parameters of kinetic equation should be calculated. As EMR provides homogeneous reaction as total amount of enzyme is kept in reactor volume, the use of Michaelis–Menten approach is obvious, and the reaction rate can be described by a well-known Eq. (1):

$$r = \frac{k_{\text{cat}} \cdot C_E \cdot C_S}{K_m + C_S} \tag{1}$$

where k_{cat} is the rate constant, C_E is the enzyme concentration, K_m is the Michaelis constant, and C_S is the substrate concentration.

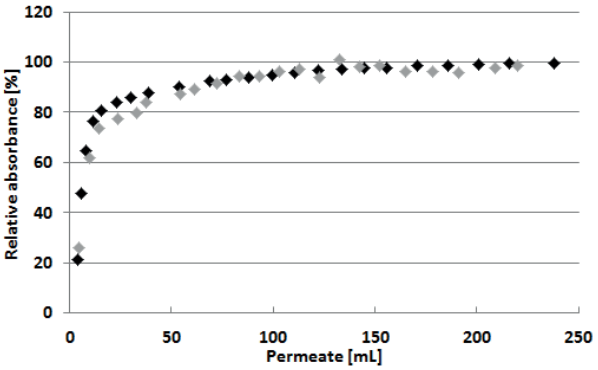

Fig. 3. Relative (in reference to the feed solution) absorbance in permeate vs. permeate volume obtained during diafiltration of substrate (black diamonds; 612 nm) or products (gray diamonds; 530 nm) in the system with Pellicon UF membraneat pH 5.3, 30°C, transmembrane pressure 0.137 MPa, τ = 12.1 min.

Estimated parameters of Eq. (1) were as follows: k_{cat} = 5.21 ± 0.15 min^{-1} and K_m = 6.88 ± 0.37 10^{-2} μM (experimental data not shown). Comparison of K_m value with AB74 concentration used in this study (100 μM) showed that Michaelis constant is above 10 times higher. It allowed us to use simplified Eq. (2):

$$r = (K_{\text{obs}} \cdot C_E \cdot C_{SO}) \tag{2}$$

where k_{obs} is the observed rate constant for the pseudo-first-order kinetics.

The value of k_{obs} for laccase decomposition of AB74 at pH 5.3, calculated by a non-linear regression, was 8.47 ± 0.15 10^{-3} min^{-1} μM^{-1}.

On the other hand, the overall component balance for a well-mixed continuous tank reactor, with constant volume (Vr) due to equal inlet and outlet flow rates (V), predicts that the reaction rate r, at steady state, can be obtained from Eq. (3) [27]:

$$r = -\frac{C_{SO} - C_S}{\tau} \tag{3}$$

where C_{S0} and C_S are the initial substrate concentration and substrate concentration in a reactor at steady-state, respectively; $\tau = \frac{Vr}{V}$ is the residence time.

Assuming that a membrane in EMR acts only as a physical barrier, at the steady-state reaction rate, Eqs. (2) and (3) could be equivalent, and they were used for an experiment planning and the model verification.

In the next step, the initial reaction rates were measured at a constant substrate concentration and reaction products concentration varied from 0 to 270 μM in order to examine eventual inhibition effect of reaction products. It was found that laccase activity remained unchanged (data not shown), and it let us to conclude that there was no inhibition effect of reaction products toward the enzyme and that Eq. (2) could be applied to predict the enzyme behavior in a continuous-mode EMR.

3.4. Validation of the kinetic model in continuous AB74 decomposition in EMR

The kinetic parameters were used to plan processes and to verify its applicability to long-term AB74 decomposition. In planning of experiment, the residence time that would allow to reach desired conversion rate (α) for specified initial substrate and enzyme concentrations was calculated from Eq. (4):

$$\tau = \frac{a.C_{SO}}{r} = \frac{C_{SO} - C_S}{K_{\text{obs}}.C_E.C_{SO}} \tag{4}$$

It was assumed that a very low laccase activity and moderate residence time (Table 1) would allow reaching the substrate conversion not higher than 70%. One has to notice that Eq. (4) does not take into account deactivation of the enzyme and/or its behavior after deposition on the membrane surface.

Experimental results (Table 1) showed a very good correlation between predicted and measured values, what allowed us to conclude that AB74 decomposition with laccase kept in the volume of membrane reactor was fully predictable.

In long-term process, however, laccase inactivation rate should be taken into account. It was already shown [22] that inactivation followed first-order kinetics. In order to examine the enzyme activity loss in EMR, a constant conversion rate of 70% was set and kept constant, while the residence time was changed. It allowed to follow the change in reaction rate (all measurements were done after reaching steady-state conditions) (Fig. 4(a)) and to compare experimental results on the basis of inactivation rate constant (Fig. 4(b)).

One can find interesting that predicted activity decrease was above twofold more intense than the experimental activity loss (observed k_{inact} = 1.79 ± 0.11 10^{-4} min^{-1}). It could be explained by stabilization of the protein conformation after adsorption in polarization layer formed on the membrane. The dual nature of enzyme appearance (in the volume of EMR and on the membrane) improved the decomposition of AB74 in longer time. Moreover, the loss of the enzyme activity during the process in the EMR could be easily refreshed.

In order to enhance laccase stability, pH of reaction mixture was increased up to 6.3. At such pH, laccase has already been found to be more stable (60% of residual activity at pH 6.3, whereas at pH 5.3 laccase only 30% of activity was

Table 1
Verification of laccase-assisted decomposition of AB74 in EMR

Enzyme concentration (mg L^{-1})	Parameter	Assumed	Experimental
29	α (%)	70	7.20 ± 0.52
	τ (min)	9.2	9.36 ± 0.23
61	a (%)	65	67.0 ± 0.314
	τ (min)	9.0	8.47 ± 0.18

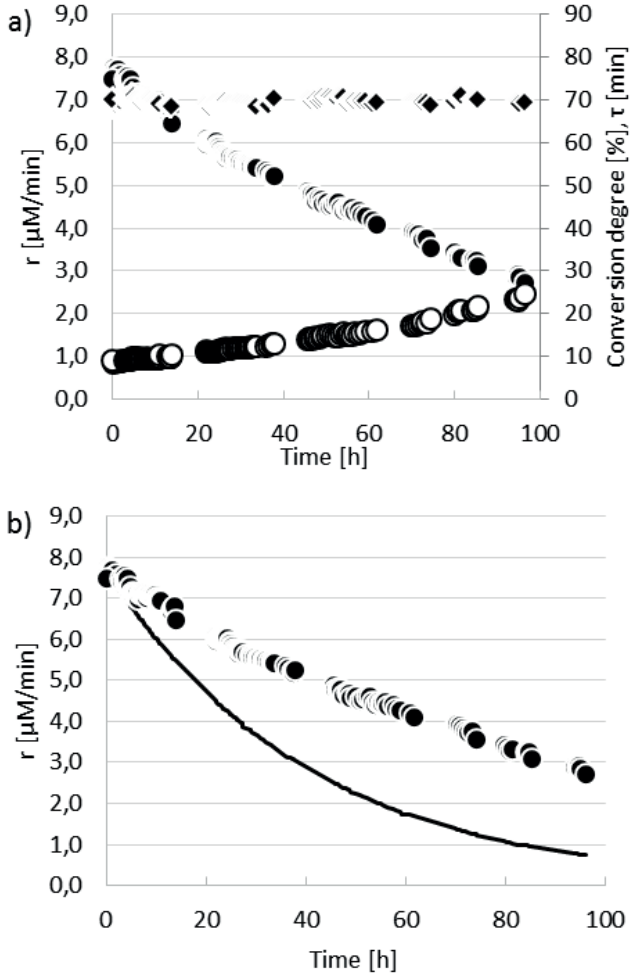

Fig. 4. (a) Decomposition of AB74 (95 μM) with laccase (10.3 U_{AB74} mL^{-1}) completely kept in the EMR volume (pH 5.3; 30°C). Symbols: (o) – residence time, (♦) – conversion rate, (●) – reaction rate. (b) Experimental (symbols as in Fig. 4(a)) and simulated (solid line) reaction rates in long-term process of dye decomposition using inactivation rate constant obtained from the fed-batch processes: k_{inact} = 4.07 10^{-4} min^{-1}.

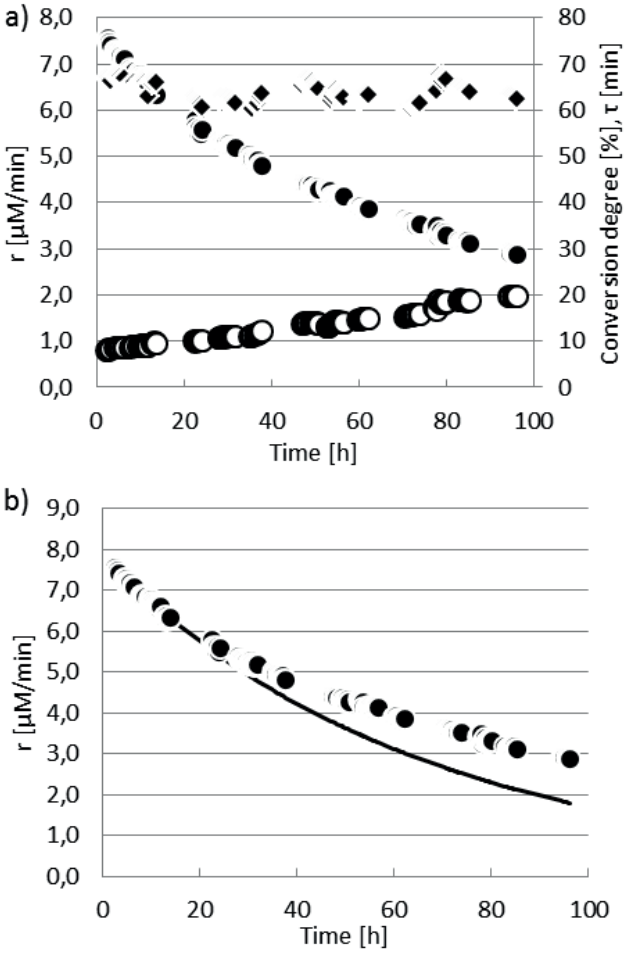

Fig. 5. (a) Decomposition of AB74 (95 μM) with laccase (10.3 U_{AB74} mL^{-1}) (pH 6.3; 30°C). Symbols: (o) – residence time, (♦) – conversion rate, (●) – reaction rate. (b) Experimental (symbols as in Fig. 5(a)) and simulated (solid line) reaction rates in long-term process using inactivation rate constant obtained from the fed-batch processes: k_{inact} = 2.44 10^{-4} min^{-1}.

preserved after incubation in the buffer solution for 4 d), but simultaneously it resulted in 50% reactivity reduction. At higher pH, k_{obs} was 3.58 ± 0.47 10^{-3} min^{-1} μM^{-1} that forced us to double the enzyme concentration to obtain comparable activities in both experiments (Table 1). It could be observed that predictability of assumed conversion was very similar to one obtained at pH 5.3, but expected higher stability was not observed (Figs. 5(a) and (b)). Surprisingly, k_{inact} = 1.96 ± 0.16 10^{-4} min^{-1} observed in EMR was only 25% lower than the value obtained in the fed-batch reactor (fed-batch k_{inact} = 2.44 ± 0.17 10^{-4} min^{-1}). In fact, k_{inact} at pH 6.3 was only 8% lower than the value obtained at pH 5.3. It meant that stability of laccase in EMR was slightly dependent on pH. Thus, pH 5.3 was more feasible for decomposition of AB74 by laccase in EMR.

4. Conclusions

The EMR reactor completed the following points: (i) polyethersulfone membrane with 5 kDa cutoff completely rejected laccase; (ii) enzyme was stable in a feed-batch reactor fed with 20 substrate portions; (iii) the used membrane did not adsorb neither substrate AB74 nor products of its oxidation, and it was fully permeable to both of them; (iv) no additional aeration was needed; and (v) laccase was more stable at pH 6.3 than at 5.3, but at simultaneous 50% activity reduction. The use of simplified Michaelis–Menten equation to model the process was sufficient for process planning protocol. In experiments, the residence time around 9 min and 70% substrate conversion were assumed for processes run at pH 5.3 and 6.3, and an excellent correlation between the predicted and the measured data was noted. It allowed to conclude that continuous process of AB74 decomposition with laccase kept in the volume of membrane reactor was fully predictable. In the long-term processes, the enzyme inactivation was observed, but it was about four times lower than one obtained in the fed-batch reactor. It is probably related to the enzyme stabilization caused by its additional immobilization on membrane.

Acknowledgment

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