Improving the catalytic performance of laccase using a novel continuous-flow microreactor

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Abstract

A novel, inexpensive and efficient method for the preparation of laccase-immobilized microreactors has been proposed, based on the formation of an enzyme-polymeric membrane on the inner wall of microtubes (500 μm inner diameter) as a result of the cross-linking polymerization reaction between the laccase and bifunctional cross-linkers agents (paraformaldehyde and glutaraldehyde). Under the optimum conditions, an immobilization yield of 72% and an activity of 45 μM/min, determined using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) as substrate in a continuous-flow assay, were detected. The biochemical characterization of the laccase-immobilized microreactors demonstrated their enhanced characteristics: they exhibited a broader range of optimum pH and temperature and excellent stability under different conditions of pH, temperature, chemical inactivating agent, storage and long-term operation. The laccase-immobilized microreactors were applied for the biotransformation of model

compounds to demonstrate their efficiency and performance. Important reaction yields were obtained, even at lower residence times compared with conventional bioreactors. Furthermore, it was designed a two-stage bioreactor for the application on laccase-mediated reactions, preventing the biocatalyst from inactivation. The great performance of the microreactor system, possibly due to the more rapid mass transfer and the larger area to volume ratio, makes the presented technologies excellent platforms for increasing the laccase uses and improving their catalytic action in several fields such as biotransformations or bioanalyses.

Keywords: Laccase; Immobilization; Microreactor; Cross-linking; Stability; Biotransformation

1. Introduction

Microreactor systems are a novel and promising technology in the fields of chemistry, chemical engineering and biotechnology due their several advantages. These reactors can be assembled by microfabrication techniques or by the modification of microcapillaries and use reaction apparatus with dimensions in the range of micrometers (μm) with handling volumetric capacities in the range of microliter (μL) [1].

These systems take the advantages of micro- or nano-fluidics to enable the use of drastically reduced volumes of reactant solutions and offer high efficiency and repeatability, better selectivity and flexible production [2-4]. Furthermore, they present important benefits in the performance of chemical reactions in comparison with traditional methods: increased heat exchange and mass transfer, process intensification, relatively large surface and interfacial areas, and moreover, the streams in microfluidics mainly form a laminar flow which allows a strict control of the reaction conditions [5-6]. Additionally, rapid screening and low material requirements are also potential advantages of miniaturized systems [7-8].

Moreover, the use of microreactors favors the scale-out of the system by the parallel operation of several reaction devices and enables the extension of reaction conditions optimized in a single reactor thus eliminating scale-up problems arising from the conventional process [5,7].

These features make the microreactor technology suitable for its application on catalytic reactions such as the biotransformation of a wide range of compounds, biosynthesis and bioanalysis. Hickey et al. [9] carried out the conversion of benzamide to benzoic acid by γ-lactamase using capillary tubes packed with cross-linked enzyme, while Pohar et al. [10] considered packed-bed microreactors for the synthesis of butyl butyrate by lipase. Another interesting example is the work reported by Matsuura et al. [11], who

developed a microreactor containing lipase-nanoporous material composites for the hydrolysis reaction of a triglyceride. Regarding the use of microreactors for analytical techniques, Heijnis et al. [12] performed the in-line quantification of peroxidase-catalyzed cross-linking of α-lactalbumin in a Y-shaped microreactor. Interestingly, Yamaguchi et al. [13] applied protease-immobilized microreactors for the analysis of the protein sequence with improved results in terms of promptness and reliability, probably due to large area-to-volume ratio and the reduced diffusional constraints in the microsystems.

The microstructured flow reactor also constitutes a potent scale-down system in which a range of process conditions can be investigated in a relatively short time. Thus, microreactors are also useful in screening of substrates, enzymes, reaction conditions as well as for the determination of kinetic parameters. For example, Matosevic et al. [14] prepared microreactors based on the attachment of His_6-tagged enzymes via Ni-NTA linkage to the surface of capillaries for the screening of multi-step conversions and the determination of kinetic parameters in the synthesis of chiral amino alcohols.

Laccases (EC 1.10.3.2, benzenediol: oxygen oxidoreductases) are enzymes widespread in nature, which are produced by a wide variety of plants, fungi and bacteria. Laccases are able to oxidize a wide variety of substrates and are potentially powerful biocatalysts for their application in several biotechnological processes such as detoxification of

industrial effluents, production of cosmetics, synthesis of anti-cancer drugs, biosensors, etc. [15]. However, there are only few studies that consider microreaction systems for the application of this type of enzymes. For instance, Roman-Gusetu et al. [16] prepared a capillary-size microreactor packed with encapsulated laccase by interfacial cross-linking for its coupling off-line to capillary electrophoresis for measurement of oxidation reactions. Lin et al. [17] prepared magnetic microreactors with laccase immobilized on magnetite nanoparticles, which were adhered on the inner wall of the microreactor due to external magnetic field forces.

These methods require complicated multi-step procedures, which imply high costs of performance. Additionally, the use of a support in a packed-bed microreactor may lead to significant pressure drop, not suitable for long operational periods. Thus, the reduction of costs, effort and time in the manufacture of laccase-immobilized microreactors as well as the high performance and stability of the microreactors, are the main challenges for their implementation. In this study, a straightforward method to immobilize laccases on microchannels is presented with the objective of improving the efficiency of laccase-catalyzed microreactions, as well as broadening their range of applications.

The proposed immobilization method relies on the formation of an enzyme-immobilized membrane on the inner wall of microtubes as a result of the cross-linking

polymerization reaction between the enzyme and bifunctional cross-linkers agents. This procedure, previously assayed for the immobilization of acylase and chymotrypsin [6,13,18], has been adapted here for the immobilization of laccase. During this process, the internal surface of the microchannel is covered by a cylindrical substrate membrane, composed of the cross-linked polymerized enzyme product formed during the reaction. The structure of this type of microreactor prevents high pressure in comparison with packed-bed microreactors, and this carrier-free immobilization method would avoid the interactions between the enzyme and the carrier [19].

To sum up, the main goal of this work was to enhance the applicability of laccases and extend their use to those fields where the microreactors are used as efficient and potent tools, by developing a simple, versatile and inexpensive method to prepare laccase-immobilized microreactors. The immobilized microreactors were characterized with respect to pH, temperature and stability of the biocatalyst under different conditions, and also a kinetic study under continuous flow conditions was performed to elucidate the kinetic behavior during operation. Moreover, the microreactors were applied for the continuous biotransformation of different model compounds and compared with the efficiency attained by conventional reactor alternatives.

2. Materials and methods

2.1. Materials

Glutaraldehyde (GA), paraformaldehyde (PA), triethylamine (TEA) and phenyl isothiocyanate (PITC) were obtained from Wako Pure Chemical (Osaka, Japan). Poly(L)-lysine hydrobromide (4200 Da), the anti-inflammatories: diclofenac (DCF) and naproxen (NPX), the estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) and the mediators: 1-hydroxibenzotriazole (HBT) and syringaldehyde (SA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the target compounds were prepared in methanol (HPLC grade, 99.8%, Wako, Osaka, Japan). 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was purchased from KPL (Gaihersburg, MD, USA). Laccase from *Trametes versicolor* was also purchased from Sigma-Aldrich. All the other chemicals were of analytical grade.

Poly(tetrafluoroethylene) (PTFE) microtubes (500 μm inner diameter (i.d.), 1.59 mm outer diameter (o.d.)), PTFE adapter, and heat-shrink tubing were purchased from Flon Chemical (Osaka, Japan). Silica-fused microcapillaries (100 μm i.d., 350 μm o.d.) and stainless-steel T-shaped connectors (Union Tee, SUS-316) were provided by GL Science Co., Ltd. (Tokyo, Japan).

2.2. Preparation of laccase-immobilized microreactors

Laccase-immobilized microreactors were prepared by adapting the procedure described for the immobilization of chymotrypsin and acylase [6,18]. The method was based on the formation of a cylindrical polymeric membrane on the inner wall of microtubes. A scheme for microreactor preparation is shown in Figure 1.

A mixed solution of laccase and poly(L)-lysine at concentrations of 0.5 and 1 mg/mL, respectively, was prepared in a 50 mM phosphate buffer solution (pH 8). On the other hand, stock solutions of the cross-linkers, GA (25%, v/v) and PA (20%, v/v), were mixed in the same buffer at a ratio of 1/16 (v/v), which was previously optimized [6,20].

For a simple and inexpensive preparation, a commercially available PTFE microtube (500 µm i.d. and 13 cm length, 26 µL of total volume) was used for the microreactor preparation. The PTFE tube was supplied with the solutions of laccase-poly(L)-lysine and cross-linkers for 3 h at 4ºC at flow rates of 0.50 and 0.75 µL/min, respectively, by means of Pico Plus 1 mL syringe pumps (Harvard Apparatus, Holliston, MA, USA) and through respective PTFE tubes (500 µm i.d. and 5 cm length) which were connected to the system by means of a T-shape and female luer connectors. Moreover, the injection of the cross-linkers solutions was performed through the central region of the microtube by means of a fused silica microcapillary (100 µm i.d., 5 cm length) which was set in

the T-shape connector and positioned at the concentric position of the PTFE microtube (Figure 1A), aiming to form a concentric laminar flow.

After 3 h of polymerization, the injection of both solutions was stopped and the microtube, containing now the immobilized-laccase membrane, was disconnected from the laccase-poly(L)-lysine and cross-linkers inlets. Then, the obtained microreactor was rinsed with 1 M Tris-HCl (pH 8), which simultaneously quenched active aldehyde groups remaining on the membrane, by passing that solution at 20 µL/min for 10 min. Afterwards, the microreactor was treated with 50 mM $NaBH_4$ in borate buffer (pH 9) at 20 µL/min for 10 min, in order to reduce the resulting Schiff base. Thereafter, the microreactor was extensively washed by circulating phosphate buffer (50 mM, pH 8) at a flow rate of 20 µL/min for 10 min. Finally, the laccase-immobilized microreactor was filled with phosphate buffer (50 mM, pH 8) and stored at 4°C until its use.

2.2.1. Effect of the cross-linkers concentration on the laccase immobilization

The effect of the cross-linkers concentration on the immobilization was studied by preparing the microreactors as previously described in section 2.2 with a GA/PA ratio of 1/16 (v/v). The concentrations tested for GA and PA were: 0.125:2, 0.25:4, 0.376:6, 0.5:8, 0.562:9 and 0.625:10 (in percentage ratio v/v).

The immobilization efficiency was calculated as the percentage of immobilized protein related to the total protein used for the preparation of the laccase-immobilized microreactor, determined by amino acid analyses. In addition, the catalytic efficiency of each microreactor was determined by its continuous performance with ABTS as substrate to measure its laccase activity. The relative activity was calculated as the ratio between the laccase activity detected in each microreactor prepared at the corresponding cross-linkers concentrations and the maximum activity attained**.**

2.3. Protein estimation by amino acid analysis

The amount of immobilized protein was determined by quantitative amino acid analysis, following the procedure previously described [18] and according to the amino acid composition of the laccase standard.

2.4. Continuous laccase activity assay

The enzymatic activity of the laccase-immobilized microreactors was evaluated by the oxidation of 50 μM ABTS to its cation radical ($ABTS^{\cdot+}$) under standard conditions (50 mM potassium acetate buffer pH 5 and 30ºC) in a continuous flow.

The microreactor was washed with 50 mM potassium acetate buffer (pH 5) at a flow rate of 20 μL/min for 2 min. Then, the solution of ABTS prepared in the same buffer

was injected at a flow rate of 10 μL/min and a HRT of 2.6 min. At steady-state conditions, a sample from the outlet was withdrawn and the absorbance was measured at 405 nm (ε = 36,800 1/(M·cm)). The enzymatic activity of each microreactor was determined as the concentration of substrate oxidized per residence time (μM/min). The microreactor was washed prior to reuse with 50 mM phosphate buffer (pH 8) at a flow rate of 20 μL/min for 10 min and stored at 4°C.

3. Results and discussion

3.1. Preparation of laccase-immobilized microreactors

The preparation of the laccase-immobilized microreactors (Figure 1A) consisted basically on the injection of two different solutions into a microtube, one containing laccase and poly(L)-lysine, and the other containing the cross-linker agents. This procedure enabled the subsequent formation of an enzyme-polymeric membrane on the inner wall of the microchannel by means of cross-linking polymerization in a concentric laminar flow. The immobilization mechanism is based on the formation of cross-linked enzyme aggregates (CLEAs) through the reaction of the aldehyde groups of the cross-linkers with the amine groups of the laccase-poly(L)-lysine mixture. Although this procedure has been previously applied to different enzymes in batch experiments [21-24], in this work the reaction took place inside a microchannel and the solutions were

continuously added in laminar flow. As a result, the cylindrical membrane, composed of the cross-linked polymerized enzyme, covered the internal surface of the microchannel. The formation of the membrane is deemed to be attributed to the parabolic velocity profile in the microtube due to the laminar flow regime (Figure 1B). According to that profile, the flow velocity in the central region of the microtube reachs its maximum and thus, when the complex begins to polymerize would be unable to have sufficient reaction time to mature into insoluble aggregates due to the shorter residence time in such area. However, the velocity in the region close to the inner wall decreases markedly, being difficult for the products polymerizing in such area to drift forward in the direction of the flow. These factors would lead to enzyme aggregates growth on the inner wall as compared to that in the central region of the microchannel, which would be also favoured by the fact that the enzyme-poly(L)-lysine solution is injected through the outer area whereas the cross-linkers are introduced through the central region by means of the microcapillary (Figure 1A). Consequently, CLEAs of laccase are formed, covering the inner wall of the microchannel and resulting in a polymeric cylindrical membrane. This phenomenon was verified by confocal microcopy, depicting a cylindrical membrane with a thickness of 40-60 μm (Figure 1C).

When a suspension of the reagents was prepared in batchwise mode for the subsequent injection through the PTFE tube, no membrane was observed in the inner wall. Thus, it

was concluded that the polymerization in the microfluid allowed the formation of a membrane structure on the inner wall of the microtube. Furthermore, when a PTFE tube was flowed with the laccase-poly(L)-lysine solution without the cross-linkers, no enzymatic activity was detected. This indicated that laccase activity of the microreactors was due to immobilized enzyme in the CLEA-based membrane.

Although there are slight differences between the batchwise formation of CLEAs and the CLEA-based microreactor, the preparation and operation of the microreactor is easier. For instance, the batchwise procedure requires a series of operations or washing procedures, such as mechanical agitation, centrifugation and removal of the supernatant; while in the microreactor system such steps are simply substituted by pumping, leading to simpler, more efficient and accurate procedure.

The method presented for the preparation and performance of a laccase-immobilized microreactor is simple and inexpensive, and high pressure is not required. Moreover, the enzymatic immobilization takes place on site, which means a reduction in time and effort, as well as prevention from possible losses of enzymatic activity related to multi-step procedures. Furthermore, this carrier-free method avoids the interactions between the enzyme and the support materials, avoiding the reduction of specific and volumetric activity of the biocatalyst [25].

Additionally, the system presents the benefits generally associated to the immobilization of enzymes: i.e. reduction of enzyme dosage, potential reuse of the biocatalyst and improved stability in the presence of inhibitors, higher range for pH and temperature [26-27]; as well as those advantages associated to the microreaction such as enhanced heat and mass transfer, high surface/interface area, better controlled chemical transformation and process intensification [2,4-5,7,28-29]. The following experiments of characterization, kinetic study and application of the laccase microreactors aim to demonstrate the improved characteristics of the enzyme-immobilized microreactors.

3.1.1. Optimum conditions for the immobilization

The efficient formation of enzyme aggregates is essential for the successful preparation of the microreactor in which laccase is immobilized by cross-linking polymerization. The poor immobilization of electronegative enzymes had been already reported due to the relatively low content of lysine residues, leading to inefficient aggregation [6]. Thus, the use of a cationic polymer was recommended, aiming the formation of an enzyme-enzyme complex prior to the cross-linking reaction [18-19]. In the current work, poly(L)-lysine was selected as the coupling agent at a concentration of 1 mg/mL, which was selected according to previous results (data not shown). Some authors have previously reported the use of albumin to improve the formation of co-aggregates of

enzymes with low lysine residues content; however, this method could be inefficient to enhance stability [23].

In preliminary experiments, the possibility of using a single cross-linker for the microreactor preparation was investigated (data not shown). The use of high concentrations of GA, required for significant cross-linking yields, led to the obstruction of the tube. In the case of using only PA, the resulting membrane formed was very fragile and gradually detached when washing the microreactor. Hence, the combination of GA and PA was concluded to be a key factor in the successful formation of a stable CLEA-based membrane.

The effect of the cross-linkers concentration on the immobilization efficiency and laccase activity has also been studied. Different GA:PA ratios: 0.125:2, 0.25:4, 0.376:6, 0.5:8, 0.562:9 and 0.625:10 (v/v) were evaluated. It is well known that enzymes may be inactivated by chemical modifiers, including cross-linking agents. On the other hand, the degree of cross-linkage is dependent on the amount of cross-linkers used. These effects were demonstrated by the results achieved, as can be seen in Figure 2. As expected, progressively increasing the concentrations of GA and PA from 0.125 and 2% until 0.5 and 8%, resulted in a significant improvement of immobilization yields and laccase activities. However, relative enzymatic activities decreased to 84% (37.8 μM/min) and 62% (27.9 μM/min) when higher concentrations were used, while

immobilization efficiencies remained at 68%. It means that the maximum values for both immobilization yield (72%) and laccase activity (45 μM/min of activity) were achieved at GA and PA concentrations of 0.5 and 8%, respectively. Thus, these conditions were selected for the preparation of the microreactors for the following experiments. The immobilization efficiencies of laccase attained here are similar to those recently obtained in Eupergit supports (44-99%) and by encapsulation (59-83%) [30-31] and they are significantly superior than other reports based on covalent bonding to epoxy carriers such as Sepabeads EC-EP3 and Dilbeads NK with yields between 18 and 33% [26].

3.2. Biochemical characterization of laccase-immobilized microreactors

In order to investigate the effect of the immobilization on the behavior of the biocatalyst and examine the optimum operational conditions of the microreactors, the effects of pH, temperature and the stability of the laccase-immobilized microreactors under a wide range of conditions were investigated. The experiments were also performed with free enzyme following the procedure described elsewhere for free laccase from *Myceliophthora thermophila* [31]. The incubation and determination of free laccase activity were conducted batchwise, while the microreactors were operated in continuous mode.

The effect of pH in the range of 2 to 8 in citrate-phosphate-borate buffer (50 mM) was investigated for free and immobilized laccase at 30ºC, while the effect of temperature was tested at 20-70ºC and pH 7 (50 mM, phosphate buffer). It was observed that the relative activity of the laccase-immobilized microreactor was significantly higher than that of free laccase in the pH range of 4 to 7 (Figure 3A). For example, the immobilized laccase retained 73% of the maximum activity at pH 7, while the free enzyme only exhibited 48% of relative activity. Similar results were obtained by other immobilization methods such as bonding to supports [26,31], entrapment in semi-interpenetrating polymer networks [32] and microencapsulation [16], although in these cases the enhancement was minimal, around 10%. The improvement was not so significant when evaluating the effect of the temperature, although the relative activity of the immobilized laccase was higher under all the conditions tested (Figure 3B). The broader range of optimum activity against pH and temperature after immobilization might be caused by the modification of amino groups in the laccase [18] and/or the buffering action of poly(L)-lysine matrix in the microenvironment of the enzyme as previously reported [33], besides the restricted mobility of the molecules [30].

Thus, it was demonstrated that microreactors present a wider profile of optimum pH and temperature, which could make the technology suitable for a wider range of applications. Nevertheless, the efficiency of an enzyme-catalyzed process depends on its

tolerance to inactivation over time under certain environmental conditions, commonly pH and temperature. Since the characteristics of ionizable site-chains and thus, the tertiary structure of the enzyme depend on pH, the enzyme might be denaturated at extreme values of pH. Furthermore, thermal inactivation can be caused by denaturation of tertiary structure as a result of protein unfolding [34]. In the current work, the effects of pH and temperature on laccase stability were investigated by incubating free laccase and the microreactors for 24 h at different conditions in the absence of substrate: pH 2 to 8 at 30ºC and 20 to 70ºC at pH 7. For this purpose, the corresponding buffer solution was continuously fed at a flow rate of 1 μL/min through the microreactors, and at different incubation times the operation was stopped and the microreactors were transferred to standard conditions to determine the residual laccase activity (see Section 2.4). The results after 4 h of operation are shown in Figure 4A and B, respectively. It was demonstrated the improved stability of the laccase-immobilized microreactors in comparison with free enzyme. For instance, free laccase only retained 5-30% after incubation at pH 2-4 and it was completely inactivated after only 4 h of incubation at high temperatures (60 and 70ºC), while the microreactors exhibited 25-70% and 20-40% of their initial activities under identical conditions. Furthermore, free laccase activity dropped more rapidly than that of immobilized laccase within the 24 h of incubation (data not shown). It could be explained by the fact that cross-linking

prevented the unfolding of laccase [35]. These findings agree those of Cabana et al. [21] for the CLEAs of *Coriolopsis polyzona* laccase as well as Honda et al. [18] and Yamaguchi et al. [13] for the immobilization of acylase and protease in microreactors.

The stability against different chemical inactivating agents was assessed by circulating the buffer solution (50 mM phopshte buffer, pH 7) containing the different compounds through the microreactors at 1 μL/min and determining the residual activities at different incubation times under standard conditions; the results are shown in Table 1. Microreactors retained between 73 and 79% of the initial activity after 30 min of continuous flow of a solution containing chlorides of calcium and cobalt, whereas free laccase presented only 45-57% activity after incubation under similar conditions. As expected, the azide greatly affected laccase activity with slightly enhanced stability for the microreactor. Besides, laccase-immobilized microreactors presented an improved stability against the organic solvents tested, methanol and acetone, probably due to the immobilization-based conformational rigidity which allows laccase to avoid the conformational collapse, responsible for inactivation not only by pH and heat but also organic solvents [35]. Chymotrypsin-immobilized microreactors, prepared by a similar procedure, showed higher resistance to urea and dimethyl sulfoxide (DMSO) [6], and microreactors with immobilized acylase were also demonstrated to be efficient in the presence of N,N-dimethylformamide (DMF) [18].

Aiming to check the stability in long-term operation, the microreactor was operated under continuous flow of 500 μM ABTS at pH 7 and 30ºC for 12 days. Under all the flow rates tested (0.5, 2.5 and 10 μL/min), the residual activity was approximately 92% after the experiment. In addition, a microreactor was subjected to high pressure with a flow rate of 5 mL/min of phosphate buffer. Neither the destruction of the membrane nor enzyme detachment from the microtube was observed, which indicated that the membrane has sufficient mechanical strength for microfluidic system applications. It was also demonstrated that the microreactors retained their initial activity completely after 3 months of storage at 4ºC.

3.3 Continuous flow kinetics of laccase-immobilized microreactors

In continuous flow kinetics, the investigation seeks to determine the variability of kinetic parameters ($K_{m(app)}$) with the flow rate [14,36]. For this purpose, enzyme kinetics in continuous flow reaction systems are usually investigated using the Lilly-Hornby model [37]. This model enables the estimation of kinetic parameters for immobilized enzyme reactors and more specifically, the quantification of any diffusional or mass transfer limitation which may be masking the true kinetics of the immobilized enzyme. The model is an adaption of the standard Michaelis-Menten model for enzyme kinetics and is described by the Equation (1):

$$f \cdot [A]_0 = \frac{C}{Q} + K_{m(app)} \cdot \ln(1 - f) \quad (1)$$

where f is the fraction of substrate converted during the reaction, Q is the flow rate, $[A]_0$ is the initial concentration of substrate, C is the reaction capacity of the microreactor and $K_{m(app)}$ is the apparent Michaelis constant.

Thus, a kinetic study under continuous flow conditions was conducted in the current work in order to evaluate the effect of flow rate on the value of $K_{m(app)}$ of the laccase-immobilized microreactors at 30ºC. ABTS was used as substrate in the range 25-500 μM in phosphate buffer (50 mM, pH 7), and the inlet flow rate was varied in the range 2.5-20 μL/min (HRTs 10.4-1.3 min). The results obtained are shown in Figure 5A. As expected, the increase in the flow rate implied lower conversion of ABTS. Moreover, the highest levels of initial ABTS concentration were shown to be more affected by the flow rate. From these data, linear plots of $f \cdot [A]_0$ versus -ln(1-f) were obtained by fitting the data to the Equation (1) (Figure 5B). The slope of the straight-lines increased with the flow rate, which suggests that the apparent kinetics of laccase immobilized microreactor is significantly affected by mass transfer [14].

The values of $K_{m(app)}$ and the corresponding correlation coefficient (R^2) are shown in Table 2. The increase of the $K_{m(app)}$ with the flow rate indicates the presence of a mass transfer effect, commonly found in fast enzymatic reactions, although it was not

excessively marked: when increasing the flow rate from 2.5 to 20 μL/min, $K_{m(app)}$ was only 3.25-fold higher. It has been reported that a catalyzed-reaction in microchannel might be affected by the transfer of the substrate through a difussional layer surrounding the immobilized enzyme, which is dependent on the flow rate, also determining the conversion rate of the immobilized enzyme-catalyzed reaction [14].

3.4. Application of laccase-immobilized microreactors

In order to demonstrate the efficiency as well as the operational stability of laccase-immobilized microreactors, this technology was applied for the continuous biotransformation of five model compounds. Three estrogens (E1, E2 and EE2) and two anti-inflammatories (NPX and DCF) were selected as model substrates of laccase since their biotransformation by laccases had been previously investigated [30-31,38-40]. The target compounds were quantified by HPLC as described elsewhere [40], and residual activities were evaluated under standard conditions after 24 h of operation.

3.4.1. Continuous transformation of estrogenic compounds

The elimination of E1, E2 and EE2 (18 μM each) from the reaction medium by immobilized laccase was proved by operating the microreactors at different flow rates (0.5 to 5 μL/min) to study the effect of HRT (52 to 5.2 min) and therefore, the effect of

the feed addition rate (0.35 to 3.5 μmol/(L·min)), as schematized in Figure 6A. The reaction was conducted at pH 7 and 30ºC since these conditions were demonstrated to be the optimal considering the stability of the biocatalyst. The results are shown in Table 3.

As observed, high transformation percentages were found for the highest values of HRT considered. For example, nearly complete eliminations of the three compounds were achieved when working at HRTs of 52 (removal rates 0.35 μmol/(L·min)) and E1, E2 and EE2 were removed by 0.66, 0.70 and 0.67 μmol/(L·min), respectively, for a HRT of 26 min. As expected, the removal efficiency decreased with the HRT; however, removal yields between 43 and 74% were attained at HRTs of only 17.3 and 10.4 min. E1 was the compound whose removal was mostly influenced by the flow rate, as previously reported with free laccase [38] and immobilized laccase on epoxy supports [39]. With regard to the residual laccase activity of the microreactors, the biocatalyst retained almost the total initial activity after 24 h of operation under all the conditions investigated.

Auriol et al. [41] reported the need of using large doses of enzymatic activity in order to attain complete removal of E1, E2 and EE2 after 1 h of batch operation. Suzuki et al. [42] removed E2 and EE2 by only 80% after 1 h and the use of HBT as mediator was required. Moreover, Sei et al. [43] achieved the biotransformation of estrogens under

acid pH conditions. However, great removal yields were found in the present work with considerably reduced residence times and mild conditions. Additionally, the results presented were also improved when comparing with those found with free or immobilized laccases in larger-scale continuous reactors. For instance, a HRT of 4 h was needed to completely oxidize E1 and E2 with free enzyme in a continuous enzymatic membrane reactor [38], and 150 min when using immobilized laccase on Eupergit supports in a fluidized bed reactor [39], even with the supply of oxygen to the reaction medium. Also, only removal percentages between 55 and 75% were attained by immobilized laccase in packed bed reactors [30-31]. Nevertheless, the implementation of the biotransformation in microchannels allowed a noticeable intensification of the reaction: e.g., for HRTs of only 10.4 min, approximately half of the initial concentration of the substrates was transformed. Hence, the microreactor design was demonstrated to be capable of outperforming the conventional designs.

These results are in agreement with those previously reported by Miyazaki et al. [44], who reported a 15 times faster process with cucumisin immobilized in a microreactor than the batch reaction. Marques et al. [29] reported a 100-fold decrease in the residence time required to attain similar yields of enzyme-catalyzed conversion of cholesterol in microchannels in comparison with the traditional stirred tank and plug-flow reactors.

That great performance of the microreaction system may be due to the more rapid mass transfer and the larger area to volume ratio, and makes the prepared laccase-immobilized microreactors an excellent technology to improve and favor the uses of laccases, such as biotransformations and bioanalysis [16-17] by means of process intensification and reduction of the system volume. Besides, the developed system could have significant potential as a platform technology for laccase-immobilized microreactors to be used in the exploration of new applications.

3.4.2. Continuous transformation of anti-inflammatory compounds

The removal of NPX and DCF was investigated by the design of two different configurations. First, the laccase immobilized microreactors were applied for the continuous elimination of these compounds (18 μM each) from the reaction medium at pH 7 and 30ºC (Figure 6A). The flow rate selected was 0.5 μL/min to maintain a HRT of 52 min, which is equivalent to a feed addition rate of 0.35 μmol/(L·min). Moreover, the effect of using laccase mediators was investigated by the addition of 500 μM of HBT, SA or ABTS in the inlet flow. The results are shown in Table 4.

It was demonstrated that the use of a mediator is required to attain significant reaction efficiencies, since only DCF was oxidized by 25% when no mediator was used. The same conclusion was reported when using free laccase [40]. HBT and SA (500 μM)

provided a slight enhancement of the results, but these compounds are likely to cause biocatalyst inactivation since the residual activity after 24 h of operation with SA was 88% while HBT caused a 25% of inactivation. This inactivation of the immobilized laccase by HBT and SA was verified through stability assays by incubating the microreactors at 30ºC and continuous flow rate of phosphate buffer (50 mM, pH 7) containing SA or HBT (data not shown). The best reaction efficiency results were found for ABTS: eliminations of NPX and DCF of 50 and 75%, respectively, were attained with removal rates of 0.18 and 0.26 μmol/(L·min). Additionally, the biocatalyst retained 90% of its initial activity.

Although we have previously demonstrated the suitability of laccase from *Myceliophthora thermophila* to degrade NPX and DCF in batch operation [40], NPX was only transformed by 60% after 8 h and in the presence of 1 mM and DCF was completely oxidized after 1 or 2 h of operation using SA or HBT, respectively. Moreover, those assays were conducted at pH 4, with the consequent enzyme inactivation. Marco-Urrea et al. [45-46] also reported important elimination of NPX and DCF by *Trametes versicolor* laccase after larger reaction times than that tested in the current work. Hence, our results present further evidence of the noticeable improvement on the reaction progress attained with the microtechnology designed in this investigation.

On the other hand, laccases usually present the maximum activities at acid pHs. In fact, previous investigations demonstrated the requirement of reaction media at pH 4 aiming to achieve significant oxidation of recalcitrant compounds such as NPX and DCF [40]. Nevertheless, it was demonstrated that acid pHs led to an important enzyme deactivation of the laccase-immobilized microreactors (Figure 4A). However, acid pHs should facilitate the biotransformation of these compounds due to their acidic character since the pKa values for these compounds are between 4 and 4.5 [47]. Also, the action of the mediator would be improved by acid pH since higher stability and reactivity at low pH values of the radical cation $ABTS^{\cdot+}$ formed by the laccase-oxidation of ABTS has been demonstrated [48].

Thus, in the current work a novel two-stage microreactor configuration for the biotransformation of target compounds was designed: the first step consisted of a prepared laccase-immobilized microreactor (26 μL) for the oxidation of the mediator at pH 7; and the second stage was based on a tubular microreactor (PTFE tube of 500 μm i.d. and 26 cm length, 52 μL) coupled to the enzymatic microreactor by means of a T-connector, to perform the chemical transformation of the substrates at pH 4 (Figure 6B). The laccase-immobilized microreactor was fed with ABTS (500 μM in 50 mM phosphate buffer, pH 7) at 0.5 μL/min (HRT 52 min) for its oxidation to $ABTS^{\cdot+}$, responsible specie of the transformation of the substrates in laccase-mediated reactions

[49]. The second reactor was fed with both ABTS oxidized solution (0.5 μL/min, HRT 104 min) and a solution of NPX and DCF prepared in potassium acetate buffer (250 mM, pH 4) at a flow rate of 1 μL/min (HRT 52 min). Due to the change of scale from the first to the second reactor, the feed addition ate of ABTS varied from 9.6 to 4.8 μmol/(L·min) in the tubular microreactor. Thus, the concentration of NPX and DCF was 9 μM (feed addition rate 0.17 μmol/(L·min)) with the aim of maintaining the same ratio between addition rates of ABTS and the target compounds used in the first configuration.

With that system, improved removal yields of both compounds were found: NPX and DCF were oxidized by 71 and 90%, respectively (removal rates 0.12 and 0.15 μmol/(L·min)) (Table 4). In addition, the inactivation of the biocatalyst by pH was completely avoided. The effect of the ABTS initial concentration was also investigated (data not shown) and it was observed that the concentration could be decreased to 200 μM and still high removal percentages of NPX and DCF could be achieved (55 and 80%, respectively). Solís-Oba et al. [50] reported the feasibility of $ABTS^{\cdot+}$ produced by immobilized laccase and separated by filtration to eliminate dyes, although both radical cation production and removal processes were only tested in batchwise mode.

Although the presented system should be further studied for its optimum implementation, this novel configuration is a potential tool to expand the applicability

of laccases to those fields where adverse pH conditions make this enzyme unsuitable. In addition, this two-stage microreactor technology would present some other benefits: e.g. the inactivation of the immobilized laccase not only by pH but also by the presence of inactivating agents and/or products formed during reaction would be prevented; both reactors could operate under different conditions (pH, temperature, etc.) to adapt the technology to the requirements of the specific application; the activity of the laccase-immobilized microreactor could be monitored by using an in-line UV detector, instead of the time-consuming off-line analysis required to determine laccase activity of the microreactor when other compounds besides ABTS are present; easy replacement in case of deactivation or microreactor damage; easy scale-up; and potential mediator reuse.

4. Conclusions

A novel and efficient method for the preparation of laccase-immobilized microreactors has been proposed and conducted by the formation of an enzyme-polymeric membrane on the inner wall of microchannels by a cross-linking polymerization method. The resulted microreactors showed excellent performance and stability under a wide range of conditions. Moreover, it has been successfully demonstrated their improved efficiency during biotransformation reactions in comparison to conventional reactor

configurations. Furthermore, it was designed for the first time a two-stage continuous bioreactor for the application of laccase-mediated reactions, preventing the biocatalyst from inactivation and thus, allowing to expand the uses of laccases to those applications which require adverse operational conditions. The great efficiencies of the designed technologies make them suitable as a platform to enhance and increase the catalytic action of laccases in several fields.

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CAPTIONS TO FIGURES

Figure 1. Preparation of laccase-immobilized membrane on the inner wall of a PTFE microtube (A), parabolic velocity profile characteristic of the laminar flow inside the microtube (B), and confocal acquisition of the sectional view of the laccase-immobilized microreactor (dry state) (C).

Figure 2. Effects of cross-linkers (GA and PA) concentration on laccase activity (black bars) and immobilization efficiency (white bars) of the microreactors. Data show the mean value of two independent experiments with standard deviations.

Figure 3. Effects of pH (A) and temperature (B) on the activity of free laccase and laccase-immobilized microreactors. pH in the range 2-8 and 30ºC and temperatures in the range 20-70ºC and pH 7 were tested using 50 μM ABTS. Data show the mean value of two independent experiments with standard deviations.

Figure 4. pH (A) and thermal (B) stability of free laccase (black bars) and laccase-immobilized microrreactors (white bars). Residual activities after 4 h of incubation at different values of pH (2-8) and 30ºC or temperature (20-70ºC) and pH 7 are shown; laccase activities were determined under standard conditions using 50 μM ABTS. Data show the mean value of two independent experiments with standard deviations.

Figure 5. Flow rate and concentration effects on the oxidation of ABTS in laccase-immobilized microreactors under continuous flow conditions (A), and analysis of the kinetic data collected using Equation (1) model (B). Solid lines fitted by linear regression.

Figure 6. Schematic representation of the laccase-immobilized microreactor used for the elimination of estrogens and anti-inflammatory compounds (A), and two-stage microreactor designed for the transformation of anti-inflammatory compounds using ABTS as mediator (B).

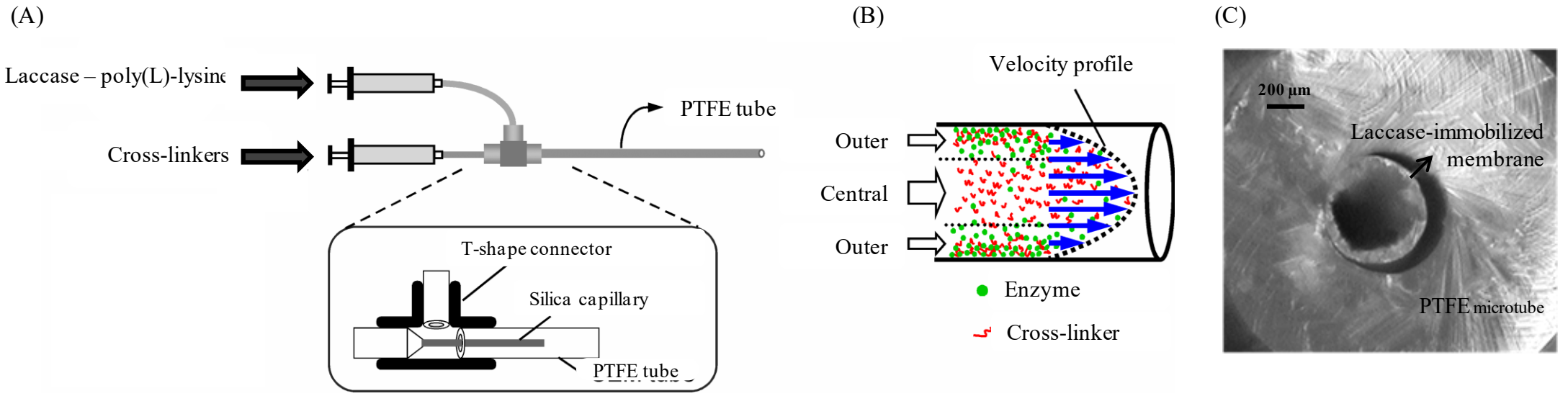

(A)
Laccase – poly(L)-lysine
Cross-linkers
PTFE tube
T-shape connector
Silica capillary
PTFE tube
(B)
Velocity profile
Outer
Central
Outer
Enzyme
Cross-linker
(C)
200 µm
Laccase-immobilized membrane
PTFE microtube

FIGURE 1

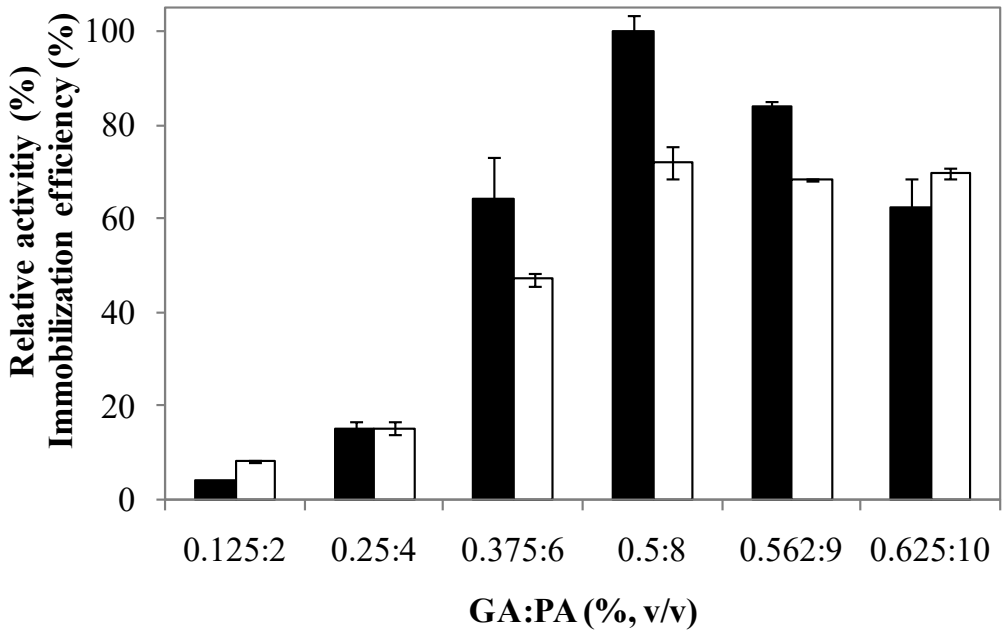

Relative activitiy (%)
Immobilization efficiency (%)
100
80
60
40
20
0
0.125:2
0.25:4
0.375:6
0.5:8
0.562:9
0.625:10
GA:PA (%, v/v)

FIGURE 2

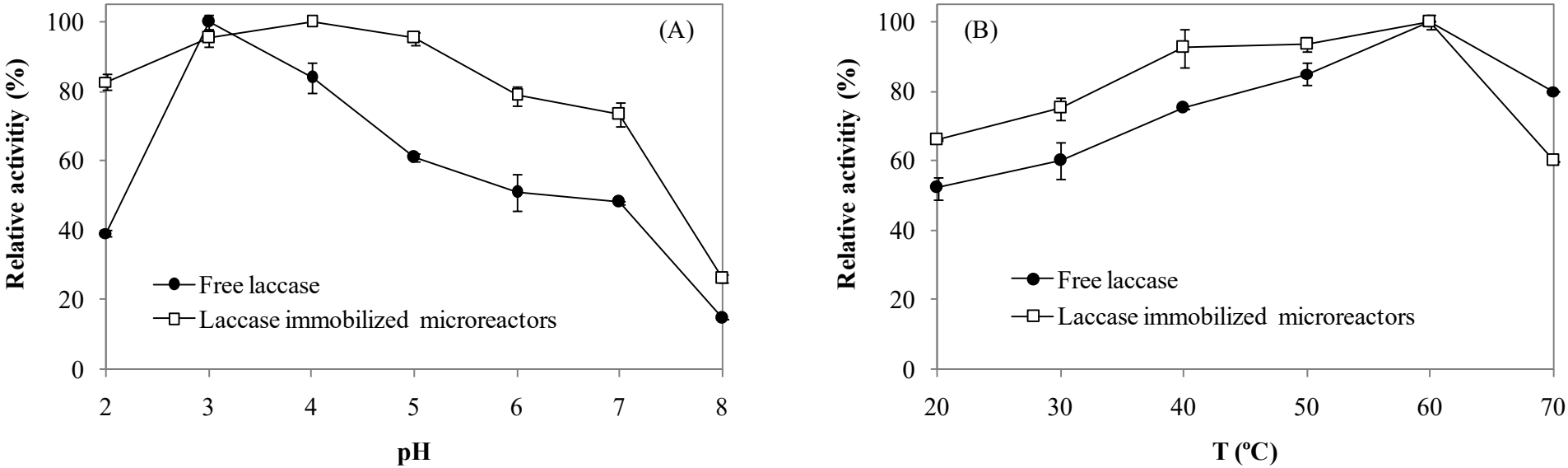
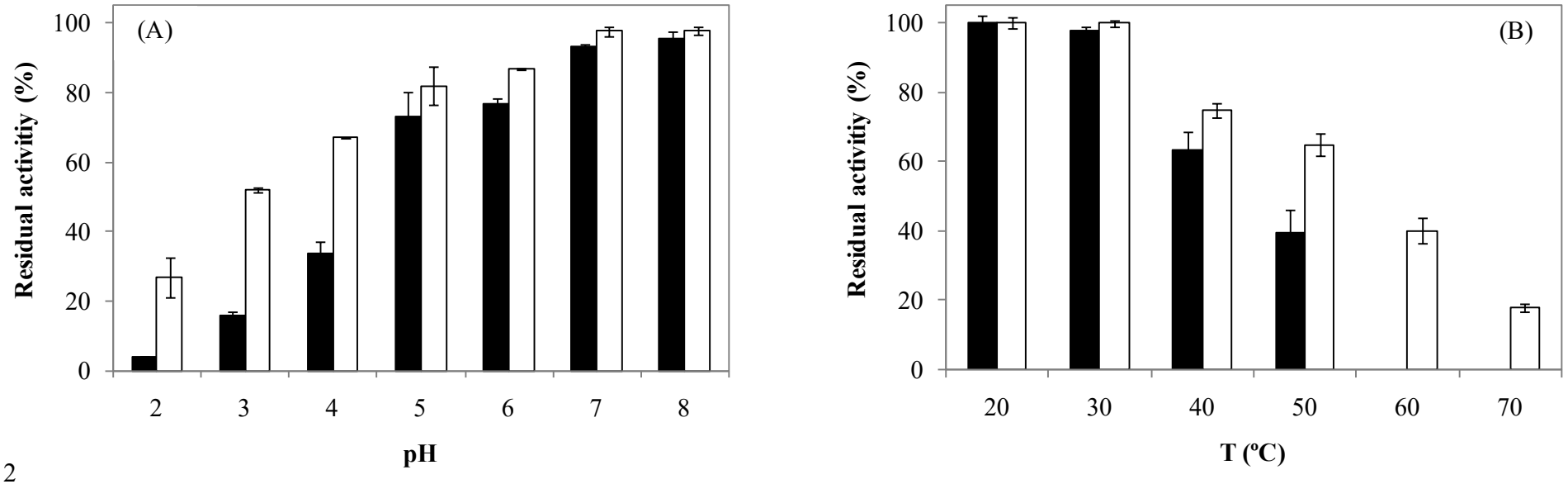

(A)
Relative activitiy (%)
100
80
60
40
20
0
2
3
4
5
6
7
8
pH
Free laccase
Laccase immobilized microreactors
(B)
Relative activitiy (%)
100
80
60
40
20
0
20
30
40
50
60
70
T (ºC)
Free laccase
Laccase immobilized microreactors

FIGURE 3

1



2
3
4 **FIGURE 4**
5
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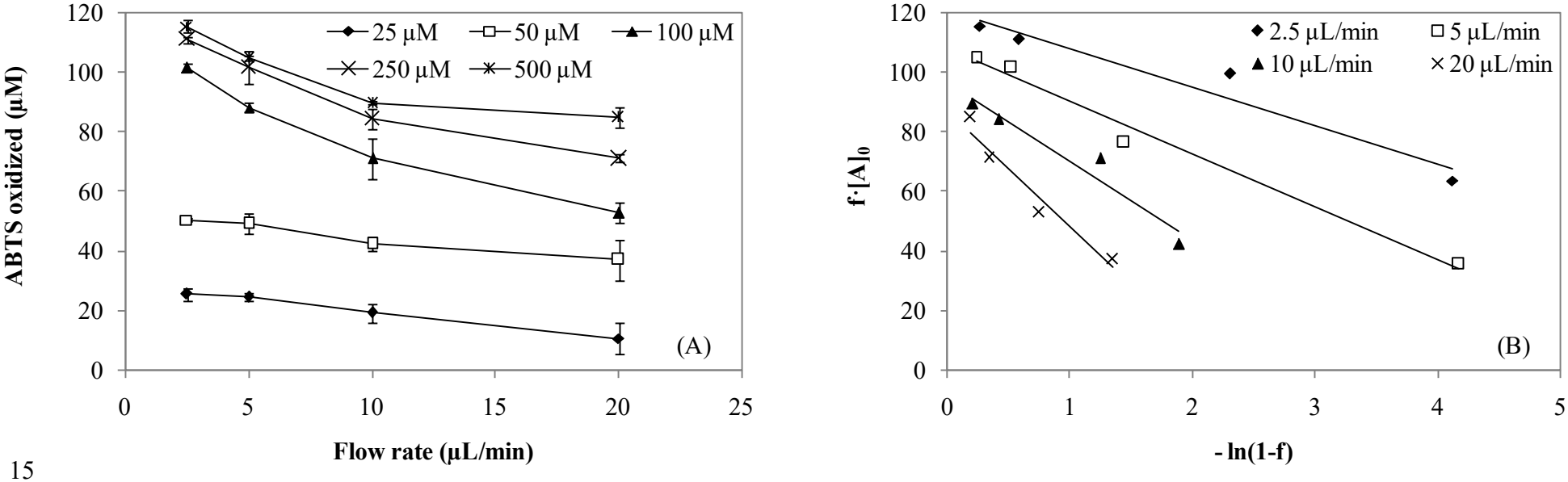
ABTS oxidized (μM)
Flow rate (μL/min)
25 μM
50 μM
100 μM
250 μM
500 μM
(A)
f·[A]0
- ln(1-f)
2.5 μL/min
5 μL/min
10 μL/min
20 μL/min
(B)

FIGURE 5

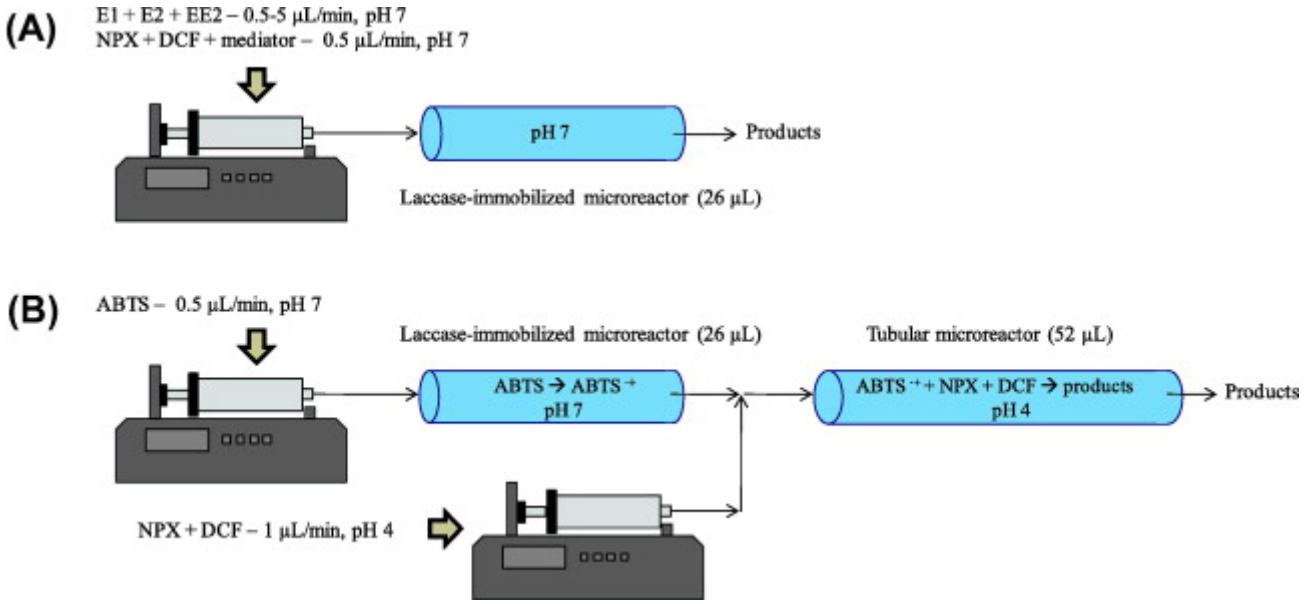
(A)
E1 + E2 + EE2 – 0.5-5 µL/min, pH 7
NPX + DCF + mediator – 0.5 µL/min, pH 7
pH 7
Products
Laccase-immobilized microreactor (26 µL)
(B)
ABTS – 0.5 µL/min, pH 7
Laccase-immobilized microreactor (26 µL)
Tubular microreactor (52 µL)
ABTS → ABTS +
pH 7
ABTS +• + NPX + DCF → products
pH 4
Products
NPX + DCF – 1 µL/min, pH 4

FIGURE 6

Table 1. Residual activity (%) of free laccase and laccase- immobilized after incubation during 30 min with different denaturants in 0.1 M phosphate buffer (pH 7) at 30ºC. Data show the mean value of two independent experiments with standard deviations.

Denaturant	Free laccase	Laccase-immobilized microreactor
NaN_3 (30 µM)	30.2 ± 1.2	47.8 ± 0.8
$CoCl_2$ (10 µM)	44.5 ± 2.2	79.3 ± 5.3
$CaCl_2$ (10 µM)	56.6 ± 1.5	72.7 ± 0.9
Methanol (25%, v/v)	58.4 ± 4.2	63.5 ± 1.3
Acetone (25%, v/v)	44.3 ± 1.1	63.6 ± 0.7

Table 2. Apparent Michaelis constants ($K_{m(app)}$) and correlation coefficients from the Lilly–Hornby model (Eq. (1)).

Flow rate (μL/min)	$K_{m(app)}$ (μM)	R^2
2.5	12.8 ± 1.2	0.971
5	17.7 ± 2.2	0.992
10	26.4 ± 4.2	0.970
20	39.1 ± 1.1	0.977

Table 3. Elimination of estrogens (E1, E2 and EE2) by laccase-immobilized microreactors (Figure 6A) operated under different flow rate conditions. Standard deviation of duplicates assays <5% were found.

Flow rate (μL/min)	HRT (min)	Removal percentage (%)			Removal rate (μmol/(L·min))			Residual activity (%)
		E1	E2	EE2	E1	E2	EE2	
0.5	52.0	>99*	>99	>99	0.35	0.35	0.35	98
1.0	26.0	95	>99	98	0.66	0.70	0.67	97
1.5	17.3	56	74	74	0.58	0.77	0.77	97
2.5	10.4	43	52	50	0.74	0.90	0.87	95
5.0	5.2	18	28	25	0.62	0.97	0.87	95

*Concentrations below detection limit

Table 4. Elimination of anti-inflammatories (NPX and DCF) in the laccase-immobilized microreactor (Fig. 6A) without and with mediators (SA, HBT and ABTS), and elimination in the two-stage microreactor (Fig. 6B) using ABTS as mediator. Standard deviation of duplicates assays < 5% were found.

Configuration	Mediator	Removal percentage (%)		Removal rate (μmol/(L·min))		Residual activity (%)
		NPX	DCF	NPX	DCF	
Single step	—	0	25	0.00	0.09	92
	SA	15	35	0.05	0.12	88
	HBT	30	65	0.11	0.23	75
	ABTS	50	75	0.18	0.26	90
Two steps	ABTS	71	90	0.12	0.15	99