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Comparative studies on immobilized laccase behaviour in packed-bed and batch reactors

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ABSTRACT

Extracellular laccase produced by the wood-rotting fungus *Cerrena unicolor* was immobilized covalently via glutaraldehyde to cellulose-based carrier Granocel. Laccase was partially purified by membrane concentration and diafiltration followed by precipitation with acetone. Five-fold increase in the measured activity of immobilized enzyme was obtained when six times purer laccase was used for immobilization. For the best preparation, with very high activity of 2053 U per 1 mL of the carrier, thermal- and pH-stability, and activity profiles were determined. Experiments carried out in a batch reactor showed that k_{cat}/K_m for immobilized enzyme (0.65) is three times lower than the value obtained for the native laccase (2.19) whereas k_{cat}/K_m estimated from continuous reactor (1.50) is notably closer to that for the native enzyme. Continuous process probably reflects more precisely kinetics of the reaction accompanied by simultaneous product precipitation on the carrier's surface. Operational stability of immobilized laccase was tested in continuous mode operation with ABTS, guaiacol and trichlorophenol as substrates and showed that packed-bed reactor is unprofitable system for laccase immobilized on Granocel carrier due to the high bed compaction. However, excellent stability of the preparation was noted under 20 successive runs in the well mixed tank reactor and better ability towards trichlorophenol biotransformation was observed in the case of immobilized laccase.

1. Introduction

Currently, many enzymes have attracted attention as potential catalysts in industrial applications, especially in processes where mild chemical conditions and high specificity are required. The unique advantage of the enzymatic catalysis is their high enantio-, chemo- and regioselectivity, which lead to high purity of products. Moreover, enzyme catalysts are very effective even at low concentrations and are biodegradable, thus they perfectly meet the targets set for Green Chemistry. The essential drawbacks of many important enzymes used in process applications are their low stability and productivity and high production costs [1]. Different methods, such as the use of stabilizing additives, derivatization, chemical modification of protein structure, immobilization and medium engineering are applied to improve enzymes stability [2]. The most frequently used stabilization method is immobilization, which provides many other process benefits besides increasing operational stability, i.e. reduction of enzyme replacement, facilitation of separation and reuse of the catalyst, and assistance of reaction control [3,4]. Moreover, this is well known [3–5] that immobilization shifts the enzyme properties like: optimum values of pH and temperature, kinetics parameters and strengthens protein structure. Especially higher thermostability of the enzyme allows conducting the processes in higher temperature and so it reduces reaction time. A lot of recent research on enzymes immobilization has been focused on laccase.

Laccase (EC 1.10.3.2) is a multicopper oxidase that catalyzes the oxidation of wide range of aromatic and inorganic substrates with simultaneous reduction of oxygen to water. Laccases are produced by many plant, bacteria and fungi species as extra- and intracellular enzymes and can be used in many industrial applications, as was recently summarized in a few reviews [6–12]. Many researchers, for example, have investigated this enzyme for organic [7,13] and polymer synthesis [14,15], dye decolorization [16], oxidation of the steroid hormone, 17β-estradiol [17], the self-coupling of anthralinic acid derivatives to give actinomycin-like compounds [18] or cross-coupling of p-hydroquinones and aromatic amines [19,20] offering a mixture of derivatives in which some of them are of pharmaceutical interest. It is generally accepted that in order to increase the potential use of laccase, its immobilization is a requisite for enzyme stabilization and recovery.

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In the previous work [21] we compared methods of laccase immobilization on Granocel carriers with different pore sizes, using various anchor groups with different surface density and found that NH_2-Granocel with exclusion limit 2×10^6 Da activated by glutaraldehyde showed the highest activity and stability. Then we optimized laccase immobilization conditions and received both stable and very active preparation. In this work we aimed to investigate the influence of laccase purity on the efficiency of its immobilization on selected carrier as well as characterization of the enzyme–carrier preparation and its properties in a batch and packed-bed reactors.

2. Materials and methods

2.1. Materials

Trichlorophenol (TCP), glutaraldehyde, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) sodium salt (ABTS) and 4-hydroxy-3,5-dimethoxy-hydroxybenzaldehyde (syringaldazine) were purchased from Sigma (USA) whereas 2-methoxyphenol (guaiacol) from Riedel-Deltaen AG (Germany). Other reagents, analytical grade, were supplied by POCh (Poland).

2.2. Production of laccase

The wood-rotting fungus *Cerrena unicolor* (Bull.ex.Fr.) Murr, No. 139, was obtained from the culture collection of the Department of Biochemistry, Maria Curie Sklodowska University of Lublin (Poland). Microorganism cultivation and laccase production was performed according to a method described earlier [22] with modification showed in [23].

2.3. Activity assays

Laccase activity was determined from the change in optical density in time and calculated from initial reaction rate region. Syringaldazine (31.3 μM) [24] or ABTS (207.3 μM) [25] in 0.1 M citrate–phosphate buffer, pH 5.3, were used as substrates. The enzyme activity was expressed in U L^{-1}, where *U* was defined as the amount of enzyme required to oxidize 1 μmol of ABTS to color products (ABTS 420 nm, $\varepsilon = 36\,000$ M^{-1} cm^{-1} [26]) per min at 30 °C. The mean analytical error was less than ±2%.

Proteolytic activity was determined by mixing 1 mL of 1.0% casein in 0.2 M Tris–HCl buffer, pH 7.8, and 1 mL of enzyme solution. Samples were preincubated and incubated at 37 °C for 5 and 20 min (modified Kunitz's method [27]). Reaction was stopped by addition of 3 mL solution of trichloroacetic acid (0.11 M), acetic acid (0.33 M) and sodium acetate (0.2 M). After standing at room temperature for 20 min, the solution was centrifuged (10 000 rpm, 15 min). The absorbance of digested products, which are soluble in the stopping reagent, was measured spectrophotometrically at 280 nm against water. In the control samples, the enzyme was added after stopping reagent. One unit of proteolytic activity (*U*) was defined as the amount of the enzyme that gave a rise in absorbance of 1.0 min^{-1}. The mean analytical error was less than ±3%.

Protein concentration was determined spectrophotometrically at λ = 280 nm (spectrophotometer Helios α, Unicam) and/or by Lowry's method (Sigma procedure P 5656) using bovine serum albumin as a standard. The mean analytical error was less than ±3%.

2.4. Laccase concentration and purification

The laccase containing culture fluid was separated from the mycelium by filtration on a steel sinter and was frozen, as microfiltration step was considerably slower for fresh culture fluid. Microfiltration was done using an Amicon cell with MF-Millipore Membrane Filters (GSWP 09000). Obtained permeate was feed for subsequent concentration 25 times and diafiltration (10 volume exchange) on the Labscale TFF System with Pellicon XL Device (PLC-10, Millipore). Finally, laccase preparation (retentate) was precipitated with cold acetone (fluid:acetone—1.0:1.5, v/v), centrifuged (10 000 rpm, −2 °C, 20 min, Hettich 32R) and precipitate was dissolved in 15 mL of the buffer. For immobilization purpose, laccase solutions after microfiltration, diafiltration and precipitation with acetone were used. In these solutions the protein concentration, laccase and proteolytic activity were measured.

2.5. Immobilization of laccase

Cellulose-based Granocel-4000 with exclusion limit 10^6 Da, modified with primary amine groups, was used for laccase immobilization. The carrier characteristics and method of immobilization via glutaraldehyde was reported elsewhere [21].

2.6. Activity of immobilized preparations

The immobilized enzyme (0.1–0.5 mL of the settled preparation), suspended in 12–36 mL of the citrate–phosphate buffer, was placed in a thermostated reactor (30 °C). Then 10–30 mL of ABTS (456.2 μM) was added to reach the final substrate concentration of 207.3 μM. After 15 s of mixing 1 mL sample was taken off, initial absorbance (λ = 420 nm) was measured and then it was turned back to the reactor. The procedure was repeated at 1-min intervals for 5–10 min. In syringaldazine conversion, the settled preparation was suspended in 30 or 60 mL of buffer and reaction was started by adding 2 or 4 mL of syringaldazine (the final substrate concentration reached 31.3 μM). The absorbance (λ = 525 nm) was measured as before. The mean analytical error was less than ±6%.

After activity measurements, the immobilized preparations were washed and stored at 4 °C, then washed several times with the buffer shortly prior to other experiments.

2.7. Properties of immobilized enzyme

In all cases, control experiments using the native enzyme were carried out. The activity was measured with ABTS as the substrate. All the experiments were carried out at least in duplicate.

The effect of temperature on the activity of the immobilized enzymes was determined by incubating the preparation at 12–80 °C. Enzymatic activity at 50 °C (native enzyme) or 65 °C (immobilized enzyme) was arbitrarily set as reference value of 100%. Thermal stability of the enzyme preparations was determined by incubating the samples at 12–75 °C for 1 h. After incubation the preparation was left at room temperature for 1 h and then its activity was measured (irreversible inactivation). The activity at 12 °C was considered as a reference value (100%).

For thermal inactivation experiments a stirred glass reactor of 100 mL capacity was used. The temperature of 80 °C of the 0.1 M phosphate–citrate buffer (pH 5.3) in the reactor was maintained constant by a circulating water bath. The enzyme solution (1 mL) was then added and after 15 s of vigorous mixing the first sample was withdrawn and cooled rapidly to 0 °C in an ice-water bath. In certain time intervals consecutive aliquots were taken, cooled and stored in ice water prior to activity measurement, which was conducted after 1 h storage. In case of the immobilized preparation, the procedure was similar but 0.2 mL of the settled preparation in buffer was introduced into a thin-layer probe and the excess buffer was removed; then the probe was tightly closed and inserted into the water bath for a certain time.

The optimum pH was evaluated in the range of 2.5–8.5. The activity value obtained for the native enzyme at pH 5.7 was taken as 100%. In the case of immobilized laccase the value of 100% was determined at pH 5.7 or 6.5, for ABTS or syringaldazine, respectively. The pH-stability was determined by incubating the sample at the given pH (ranging from 2.5 to 9.0) for 1 h. The pH of the solution was then adjusted to 5.3 and the preparation was left for 1 h to equilibrate. The value of activity obtained at pH 5.3 was taken as a reference activity (100%).

2.8. Properties of immobilized laccase in a batch reactor

All experiments were carried out in a well mixed and thermostated batch reactor using either 0.1 or 0.2 mL of settled immobilized preparation.

The effects of temperature and mixing rate on the initial value of reaction rate were measured at temperatures ranging from 12 to 50 °C and mixing rate of 200 rpm or at 30 °C and mixing rates of 135, 200, 350 and 500 rpm.

To compare reaction runs, immobilized laccase (0.1–1.0 mL of settled preparation) was added to 30 mL of preheated substrate (thermostated batch reactor, 200 rpm, 30 °C). At predetermined times, 2 mL sample was taken out, centrifuged (6000 rpm, 5 min) and subjected for absorbance measurement. After that the sample was returned to the reactor. The same procedure was applied to the native enzyme for ABTS (207 μM), syringaldazine (31.3 μM) and TCP (506 μM) as the substrates. To compare the performance of free and immobilized enzyme in these processes, the absorbencies (420 nm ABTS; 525 nm syringaldazine, 350 nm TCP) were plotted against time for the same initial enzyme activities, measured previously with ABTS as the substrate. The initial reaction rates were 0.315 (native enzyme) and $0.319A_{420}$ min^{-1} (immobilized) for ABTS; 0.154 and 0.168 for syringaldazine; 1.49 and 1.42 for TCP, respectively.

Kinetic parameters of the Michaelis–Menten equation (K_m and k_{cat}) for free and immobilized enzyme were calculated from reaction rates determined in batch experiments using initial ABTS concentrations ranging from 22.73 to 1818.18 μM. For each run the absorbance (420 nm) was recorded in time and initial reaction rates were calculated for substrate conversions lower than 7%. Kinetic parameters were estimated by fitting the data to the Michaelis–Menten equation using a nonlinear regression code (Origin Pro 7.5).

Operational stability of the preparations in a batch reactor was assessed by incubating 0.2 mL of the immobilized laccase with 21.8 mL of ABTS (final concentration 207.3 μM) in citrate–phosphate buffer at 30 °C and under moderate mixing (250 rpm). At the beginning of each cycle, five consecutive samples were withdrawn in 1-min intervals, absorbance was measured and then they were returned to reactor (initial reaction rate measurements). Afterwards, the reaction was run up to the substrate depletion. Then the immobilized enzyme was collected by filtration, washed twice with the buffer and resuspended in a fresh substrate solution to begin the next cycle. The same procedure was applied to immobilized laccase earlier treaded with sodium borohydride in order to reduce the Schiff's bases.

2.9. Properties of immobilized laccase in a packed-bed reactor

5 mL of the settled preparation was packed in a jacketed glass column (10 cm high, 0.7 cm of internal diameter) with the initial catalyst bed height of 8.8 cm. Due to the high activity of immobilized preparation towards ABTS, 0.5 mL of preparation was mixed with 4.5 mL of an inert carrier. The substrate solution (ABTS: 456.2 μM, TCP: 506 μM, guaiacol: 500.5 μM) in 0.1 M citrate–phosphate buffer (pH 5.3), both earlier held at 30 °C, were fed continuously to the top of the reactor with a peristaltic pump until no more settling occurred. Volume of voids in the reactor was evaluated as proposed in [28]. The reactive volume of the reactor (V_r) was calculated by subtracting that value from the total volume of the reactor. Residence time (τ) of the substrate in the column was calculated from the ratio V_r/Q, where Q was the flow rate, and the rate of product formation (r) from $r = Cp/\tau$, where Cp was the product concentration. In the operational stability studies, the substrate was fed continuously over prolonged time and the fixed residence time equal to 0.14 min for ABTS, 1.79 min for guaiacol and 0.36 min for TCP.

Kinetic parameters of Michaelis–Menten equation were determined for ABTS inlet concentration equal to 472 μM and the flow rates from the range of 2–7 mL min^{-1}. The experiment was repeated after 24 h of continuous operation.

3. Results and discussion

3.1. Influence of laccase purity on its immobilization on Granocel

The aim of this study was to achieve higher activity of the immobilized laccase preparation than reported previously. After optimization of the Granocel superstructure as well as pH-value and protein concentration in a coupling mixture it was found that maximum activity varied from 417 to 694 U per 1 mL of freely settled bead, depending on actual specific activity of crude culture fluid [21]. Hence, the subsequent increase of activity could be achieved only by purification of the crude enzyme preparation. In order to reduce high costs typical for the most traditional purification procedures we decided to apply the cheap and quick method only. Laccase was purified in three steps: (i) microfiltration that allowed to remove larger particles and cells debris; (ii) ultrafiltration in which the proteins were concentrated and diafiltered in order to lower ballast protein amount (e.g. proteolytic enzymes) and brown in color products of *C. unicolor* metabolism; (iii) precipitation of proteins with cold acetone for subsequent removing of brown particles and ballast proteins. The results obtained are summarized in Table 1. Above six-fold purification of the crude laccase was observed in the final product. Moreover, the purified preparation showed almost twelve times lower proteolytic activity. Additionally, the precipitation with acetone removed half of the ballast proteins from retentate whereas laccase and proteolytic enzyme(s) retained almost all initial activities.

For immobilization purpose, Granocel carrier and laccase solutions after prefiltration, ultrafiltration and precipitation with acetone were used. From the results collected in Table 2 it can be seen, that five-fold increase in the measured activity could be obtained when six times purer enzyme preparation was used for immobilization. It was also found that measured activity and expressed activity in bound units are almost proportional to the specific activity used for immobilization. It clearly indicates that even higher activities of laccase on Granocel carrier can be obtained if purer enzyme preparation is used. However, the costs of further purification should not prevail over the profit of higher activity. As long as the increase in activity of immobilized enzyme is proportional to the specific activity increase, the additional purification steps are economically reasonable. In the case discussed here, the values of expressed activity in bound activity units (based on activity balance) show that only 1% of laccase was fully active when enzyme with the highest specific activity was used for immobilization. On the other hand, when crude preparation was used even up to 12% of the bound laccase appeared to be active. This seems to indicate that further purification of the enzyme, beyond the level

Table 1
Purification of laccase.

Purification step	Total protein [mg]	Total laccase activity [U]	Specific activity [U mg^{-1}]	Purification (fold)	Total protease [U]	Yield [%]	
						Protein	Activity
Microfiltration	578.0	6 444 444	11 150	1.0	17.9	100	100
Ultrafiltration and diafiltration	55.5	2 165 028	39 010	3.5	3.79	21.1	33.6
Precipitation	28.6	2 014 722	70 445	6.3	3.08	17.2	31.3

Table 2
Covalent immobilization of laccase of different purity on Granocel.

Specific activity/protein in coupling mixture [U mg^{-1}]/[mg]	Bound protein[a] [mg mL^{-1}]	Expected activity[a] [U mL^{-1}]	Measured activity [U mL^{-1}]	Expressed activity in bound units[b] [%]
11 139/7.88	1.02	3 389	406	12.0
39 000/9.23	1.16	43 806	1344	3.1
70 472/9.83	1.30	97 778	2053	2.1

[a] The amount of bound protein and expected activity were calculated by subtracting the washed off amount from the amount used for immobilization (mass/activity balance) and recalculated per 1 cm^3 of the carrier.
[b] Expressed activity in bound units was calculated as the ratio of measured activity and expected activity.

achieved, can only lead to a moderate increase of activity, with notably lower profit of activity expression.

Both immobilized preparations, obtained with pre-purified laccase, can be assessed as highly active. Due to different substrates, temperatures and recalculations used by other researchers, comparison of our results to reported is rather limited. Lu et al. [29] reported laccase activity immobilized in alginate–chitosan microcapsules to be 6.84 U per 1.5 g of the preparation (room temperature). Cabana et al. [30] obtained cross-linked aggregates with activity of 0.148 U per 1 mg of protein (30 °C) whereas Roy and Abraham [31] reported activity of laccase cross-linked crystals as high as 2300 U per 1 mg of protein. This short literary review clearly indicates that laccase immobilized on Granocel carrier is very active and that the preparation is even comparable with cross-linked crystals.

3.2. Properties of immobilized laccase

To characterise the properties of immobilized laccase the effects of temperature and pH values on the enzyme activity and stability were examined. Fig. 1a reveals that native laccase shows the highest activity at 50 °C but the enzyme is relatively unstable at this temperature (cf. Fig. 1b, 84%). It appeared that temperature of long-term processes with native laccase should not exceed 20 °C. In the case of immobilized enzyme, the maximum activity temperature was notably higher (65 °C), yet this resulted in rather poor stability (73%). Fortunately, immobilized laccase was stable up to 55 °C. Hence, a substantial increase in the enzyme activity and stability, compared to the native preparation, could be achieved if the process was carried out at this temperature. For the immobilized enzyme the shift of optimal temperature range and thermal stability into higher temperatures could be caused by stiffening of the enzyme tertiary structure.

Temperature is a critical variable in any enzymatic process, producing opposite effects by simultaneously increasing enzyme reactivity and inactivation rate. From the practical point of view, the enzyme should be active and stable under processing conditions, especially at elevated temperatures, thus special attention of researchers is paid to obtain highly stable immobilized enzymes. Thermal inactivation of enzymes in the absence of reagents and at elevated temperatures gives some information on usefulness of the preparation used. The data presented in Fig. 2 show that native enzyme was fully inactive after storage at 80 °C for more than 25 min, whereas the immobilized preparation preserved a few percent of activity after 120 min of heating. Fig. 2 gives another information. In comparison to the native enzyme, the inactivation

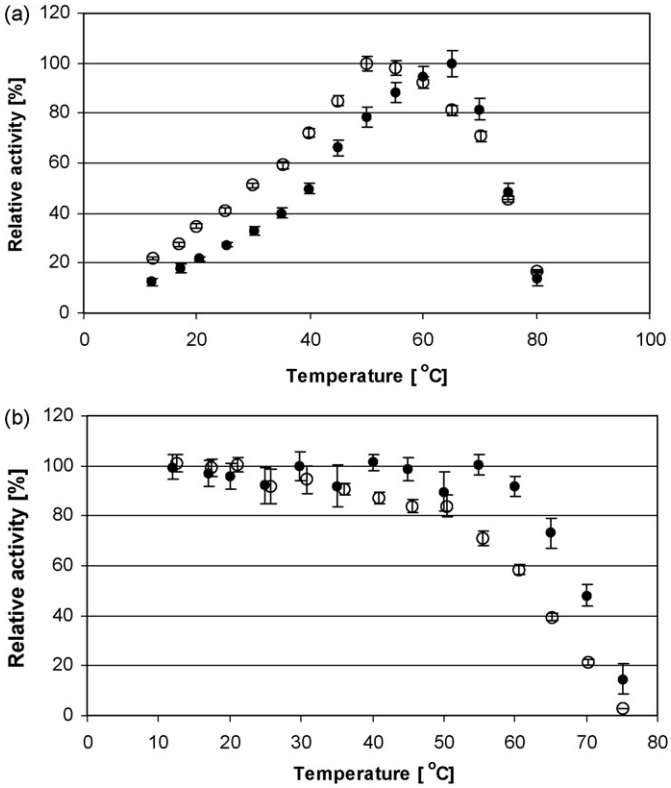

Fig. 1. Effect of temperature on the relative activity (a) and stability (b) of the enzyme preparations: native (open circles) and immobilized (closed circles).

of which at 80 °C could be seen as a pseudo first order kinetics, the immobilized enzyme evidently showed a biphasic inactivation. The change of inactivation kinetics is probably caused by the creation of two – less and more stable – fractions of immobilized enzyme and that is in good agreement with the earlier observation and with evaluations of laccase immobilized on acrylic carrier [32].

The pH value at the liquid–matrix interfaces plays essential role in the performance of enzyme preparations. This effect originates mainly from the buffering properties of the bound proteins, the matrix, and Schiff's bases formed by the reaction between glutaraldehyde, protein and carrier. Fig. 3a shows the pH-activity profile of immobilized laccase that is slightly shifted into more acidic region and is broader than for unbound enzyme when ABTS

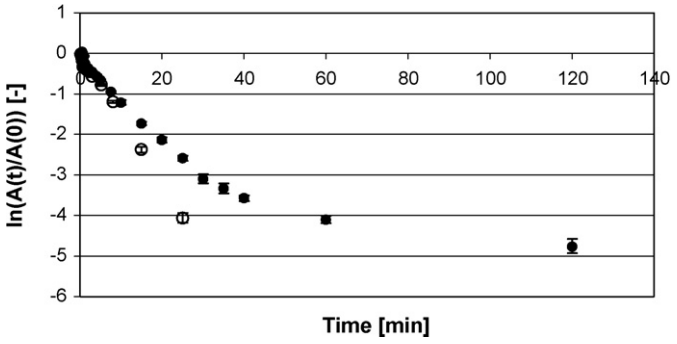

Fig. 2. Irreversible inactivation of native (○) and immobilized (●) laccase as residual activity in semilogarithm form. *Reaction conditions*: 0.1 M phosphate–citrate buffer, pH 5.3, 80 °C. $A(t)$: activity measured after time; $A(0)$: initial activity.

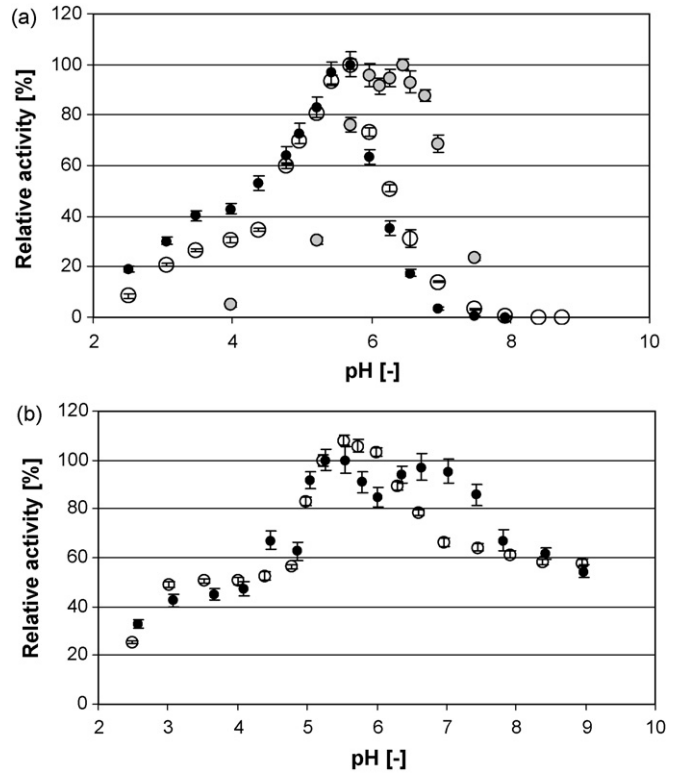

Fig. 3. Effect of pH on relative activity (a) and stability (b) of the enzyme preparations: native (open circles) and immobilized (closed circles: black, ABTS; grey; syringaldazine as substrates).

is used as substrate. For syringaldazine, immobilized preparation shows two pH maxims and the profile is shifted into more basic region. The markedly different behaviour of the immobilized laccase for two different substrates may result from the buffering effect (shifting), diffusion resistances for the substrate (broadening) [5] and/or may reflect the difference in oxidation mechanism with different substrates [33]. Two pH maxims for syringaldazine can be explained by the fact that *C. unicolor* laccase occurs in two isoforms with different pH optima for Lac I (4.5) and Lac II (5.5) [34]. After immobilization the values were shifted to 5.9 and 6.5, respectively. As it is seen, it is necessary to find pH_{opt} for each potential substrate for laccase separately. The pH-stabilities of both immobilized and native enzymes are illustrated in Fig. 3b. Both enzyme preparations show pH-stability maxims but immobilized preparation has two peaks that can be explained by the formation of two enzyme subpopulations with different stability and/or

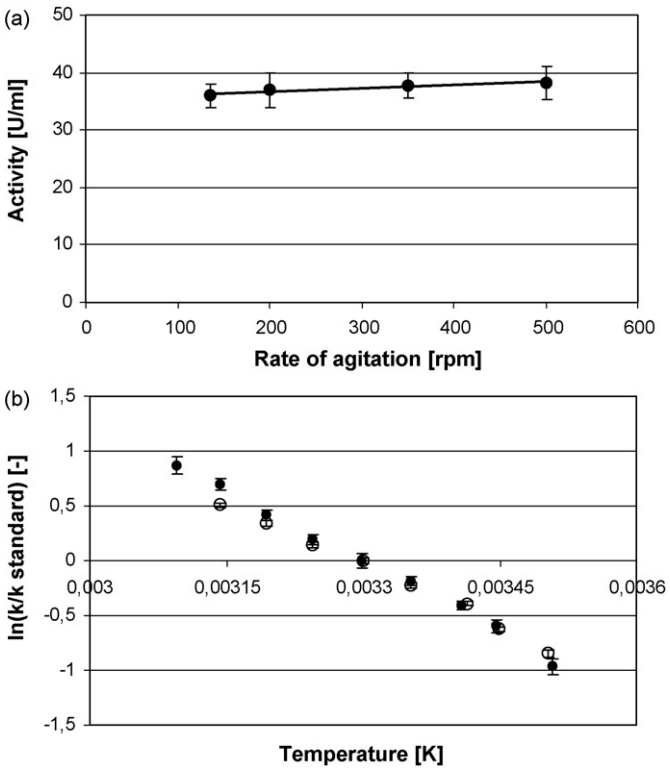

Fig. 4. (a) Effect of agitation rate on immobilized laccase activity. (b) Influence of temperature on the reaction rate of native (open circles) and immobilized laccase (closed circles).

by a different shift of pH-stability maxims for both isoforms of laccase.

3.3. Properties of immobilized laccase in a batch reactor

For a reactor design it is essential to know the kinetic properties of a bound enzyme catalyst. The observed catalytic properties depend on substrate transport to and from an active site and the intrinsic catalytic activity of the enzyme. Our previous studies indicated that Granocel-4000, features wide pores which after modification with pentaethylenehexamine (19-atom spacer) keeps the steric limitations close to minimum [21]. As glutaraldehyde activation affects mainly amino groups located on the carrier's surface, one can assume that the rate of the studied reaction is kinetically controlled. In order to verify this assumption, the influence of agitation rate (Fig. 4a) and temperature (Fig. 4b) on the reaction rate was determined. As it is seen, increased rates of agitation of reaction mixture produced insignificantly higher rates of ABTS oxidation. Moreover, results summarized in Fig. 4b clearly demonstrate that the temperature effect on reaction rate is almost identical when both native and immobilized laccase were used. Thus, the mass transport limitations could be effectively eliminated by a choice of suitable conditions. Finally, to compare the performance of native enzyme and the Granocel-based preparation in more real situation of ABTS, syringaldazine and TCP oxidation, tests were carried out in a batch regime for similar values of initial reaction rates. For both forms of catalyst the variations of absorbance with time are shown in Fig. 5. For the case of ABTS conversion (Fig. 5a), the performance of native and immobilized laccase was quite similar during the whole period. This demonstrates and evidences that no deactivation of immobilized enzyme preparation due to pore blockage or other cause occurred during

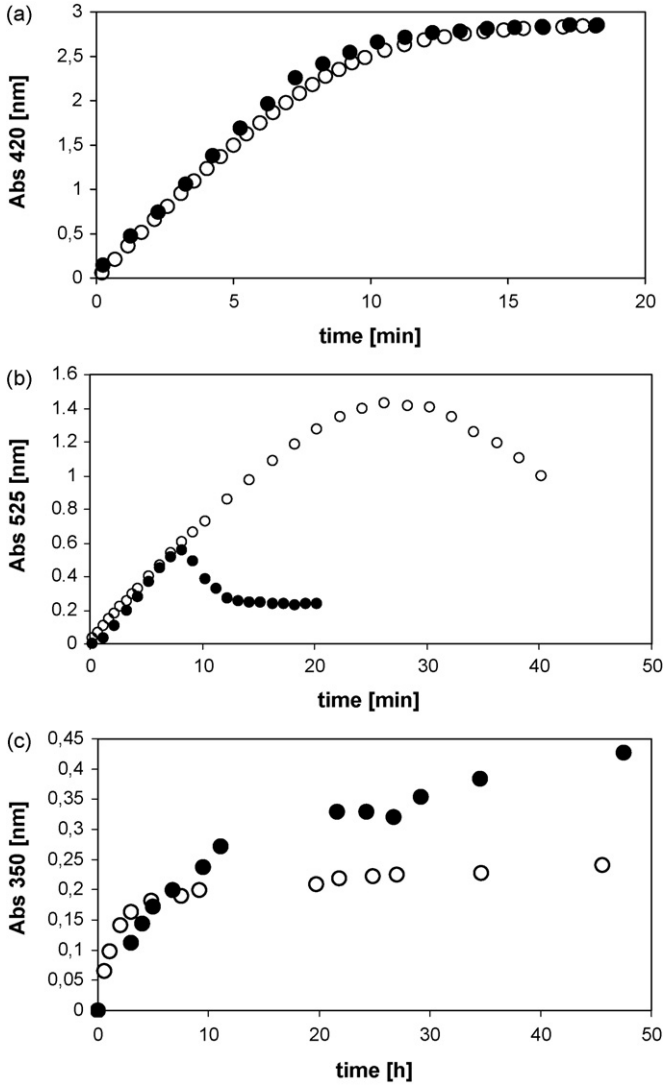

Fig. 5. Progress curves of oxidation of ABTS (a) syringaldazine (b) or TCP (c) by native (open circles) and immobilized (closed circles) laccase. *Reaction conditions*: 30 °C, 250 rpm, 207.3 μM ABTS, 31.3 μM syringaldazine or 506 μM TCP in 0.1 M citrate–phosphate buffer, pH 5.3.

the run and that mass transport limitations could be effectively eliminated. Unlike in the former case, for syringaldazine conversion (Fig. 5b), the significant difference in reaction runs was observed. Although both enzyme preparations caused the increase in product absorbance followed by its decrease (product polymerization) the intensity of this phenomenon was different. Probably, the large amount of the enzyme present in boundary layer of the carrier stimulated the accumulation of product radicals and their massive polymerization and agglomeration in/on the carrier's surface. Taking into account oxidation of TCP (Fig. 5c) unexpected higher ability to produce colored products was observed in the case of immobilized enzyme. During the first hours of the process pink products accumulated on the carrier's surface but the absorbance in solution was still higher than in the case of the native enzyme. It suggests the potential of immobilized laccase towards phenolic substrates biotransformations.

Traditionally, enzyme kinetic studies have focused on evaluation of the kinetic parameters from initial velocity data. In Fig. 6a examples of experimental and simulated data are presented, whereas estimated values of V_{max} and K_m from the native and immobilized laccase are collected in Table 3. It is seen that on the base of evaluated parameters one is able to predict the dependence of the substrate concentration on reaction rate very well. Additionally, immobilized enzyme has five-fold higher K_m value than native enzyme that means lower "apparent" affinity of the substrate to the bound enzyme, and may also be linked to the mass transport constraint. On the other hand, for immobilized enzyme the reaction rate constant is about twice as high and as a sum, k_{cat}/K_m is only three times lower than the value obtained for the native enzyme. This behaviour is probably caused by accumulation of product radicals in/on carriers' surface. Thus, evaluated kinetic parameters must be carefully interpreted, even more so that insignificant differences in diffusional resistance were postulated previously. In our opinion, the problem lies in the sorption of products that lowers initial reaction rate in different extent, depending on substrate concentration and actual rate of reaction.

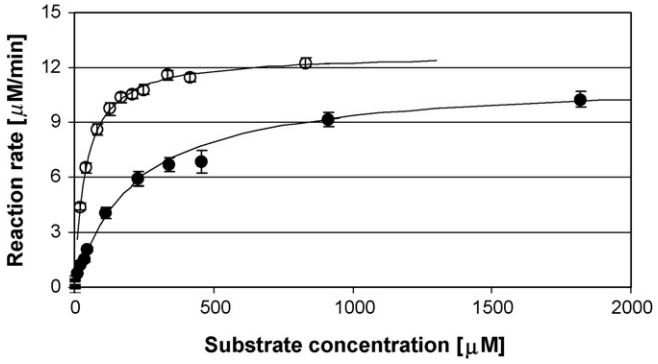

Fig. 6. Dependence of the initial reaction rate of native (○) and immobilized (●) laccase on the initial substrate concentration. Initial reaction rate in standard condition: 10.57 (native) and 4.06 (immobilized) μM min^{-1}. Solid lines: modelled reaction rates with the use of kinetic parameters from Table 3.

Table 3
Kinetic constants for ABTS oxidation by free and immobilized laccase.

Kinetic parameter	Native laccase	Immobilized laccase
V_{max} [μM min^{-1}]	12.73 ± 0.1	11.37 ± 0.2
K_m [μM]	39.4 ± 1.4	214.9 ± 10.2
k_{cat} [min^{-1}]	86.1	140.3
k_{cat}/K_m	2.188	0.653

The stability of an enzyme–carrier preparation in a stirred batch reactor is the valuable information about mechanical resistance of obtained preparation. As can be seen from Fig. 7, immobilized laccase is well suited for repeated use in a batch system, as after

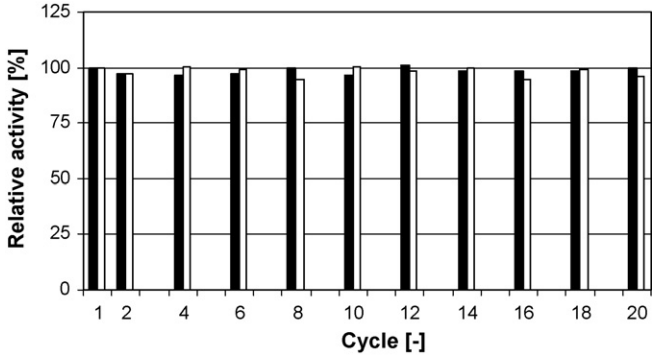

Fig. 7. Relative activity of immobilized laccase (white) and after reduction by sodium borohydride (black) in subsequent processes in the batch reactor. *Reaction conditions*: 30 °C, 250 rpm, ABTS (207.3 μM) in 0.1 M citrate–phosphate buffer, pH 5.3.

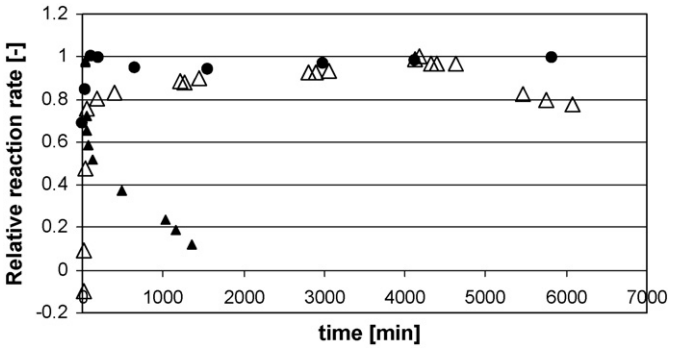

Fig. 8. Operational stability of immobilized laccase in the packed-bed reactor. *Reaction conditions*: 30 °C; pH 5.3; (●) 456.2 μM ABTS and τ = 0.14 min; (Δ) 253.2 μM TCP and τ = 0.36 min; (▲) 500.5 μM guaiacol and τ = 1.79 min. In the experiments the highest activity was set as 1.0.

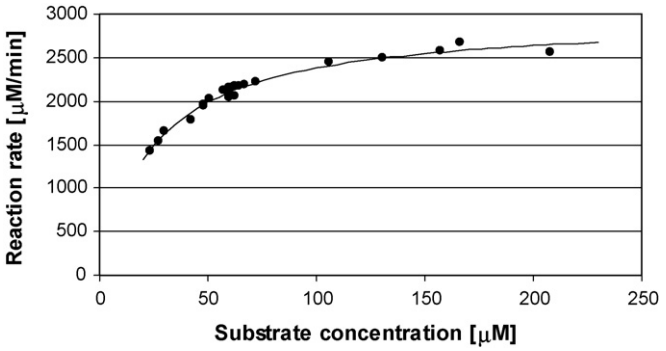

Fig. 9. Example of dependence of the reaction rate on the substrate concentration in the packed-bed reactor with immobilized laccase. *Reaction conditions*: ABTS concentration in inlet 472 μM, 0.5 mL of immobilized preparation mixed with 4.5 mL of inert carrier, volume of the reactor 0.7 mL, residence time (τ) from 0.10 to 0.32 min. Solid line: modelled reaction rates, closed circles: experimental data (two independent runs).

20 successive runs no activity loss is observed and that is most probably caused by an elastic structure of the carrier. The same behaviour is observed for immobilized laccase treated with sodium borohydride that shows no need to reduce Schiff's bases in order to improve the immobilized enzyme stability. Long-term experiments with laccase were accompanied by deposition of product on the carrier. Although no inactivation of immobilized preparation was observed during 2 days of successive processes, the trial of removing dark green products from the carrier's surface was done. It was found that repeated washing of the enzyme–carrier preparation with distilled water, 0.5 M and 1 M NaCl solutions removes almost all colored product without any loss in activity (data not shown).

3.4. Properties of immobilized laccase in a packed-bed reactor

In spite of excellent stability of the immobilized laccase in the batch mode system the experiments in the packed-bed reactor were also performed. Advantages of continuous process over batch mode are: (i) simultaneous reaction and product/unreacted substrate separation; (ii) low labour cost; (iii); lack of batch-to-batch variations; (iv) higher efficiency due to minimized start-up and shut-down operations; (v) easy process control by the change of residence time (flow rate). On the other hand additional special properties of the carrier are required, as the high pressure drop in the bed can provoke its compaction and hence biocatalyst damage. In fact, laccase immobilized on elastic cellulose-based Granocel is not well suited to the use in the packed-bed reactor operation. Initially the bed was equilibrated with buffer for 5 h as the bed height decreased gradually. Finally, outlet flow rate decreased three-fold, overall bed volume two-fold and the reactive volume of the reactor from 1.1 to 0.7 mL (data not shown). Despite these drawbacks a series of experiments was performed. For the enzyme stability studies ABTS, guaiacol or trichlorophenol was continuously pumped through the bed for a fixed residence time (Fig. 8). The results presented show excellent stability of immobilized laccase when synthetic substrate (ABTS) is used. Generally, chlorophenols (e.g. TCP) are known recalcitrant substrates of laccases [35] that together with reaction products can modulate enzyme stability/inactivation rate. As it is seen, activity of the immobilized laccase slightly increases during first 3 days and then it decrease. That supports the earlier information on the lower laccase stability in the presence of chlorophenols. However, when quaiacol is used as substrate, immobilized laccase activity decreases almost completely in 24 h time. To find the reason for this behaviour, the bed of reactor was withdrawn, washed off several times and its activity was measured in standard conditions. As the immobilized laccase preserved 68% of initial activity it was concluded that the loss of activity in the packed-bed reactor was caused partly by the enzyme inactivation and/or by the pore blockage with polymerized products of quaiacol oxidation. Thus, the operational stability of immobilized laccase depends strongly on the substrate used and the affinity of products to the carrier's surface.

It was shown that immobilized laccase is stable in the packed-bed reactor for not less than 100 h when ABTS is the substrate. Thus, we could easily perform experiments to determine kinetic parameters of the system studied. This was done by changing the flow rate to achieve a new steady state. The latter was checked after a passage of 5 reactive volumes of the reactor. Once the new steady state had been reached it was kept over a prolonged period time during which the sample taken from the effluent was analysed. The values of K_m and k_{cat} determined from the presented data (Fig. 9) were found to be equal 57.18 ± 5.51 μM and 85.37 μmol min^{-1} mg^{-1}, respectively, and they could be used for mathematical modelling studies. As it can be seen, the kinetic constants are comparable to those obtained for the native laccase (Table 3) and the ratio k_{cat}/K_m = 1.5 is notably closer to the corresponding value for the native enzyme than that calculated from the batch experiments. In our opinion, it is probably caused by sorption of the reaction products on all the sites available on the carrier surface in long-term continuous process that can reflect more precisely immobilized enzyme kinetics.

4. Summary

It was demonstrated that low-cost purification of laccase by ultrafiltration, followed by precipitation with cold acetone, affords 6 times purer enzyme than original native preparation, and, this gives five-fold increase in immobilized enzyme activity. As the preparation obtained can be regarded as highly active and stable, its activity properties were compared with the native form. Regarding the influence of pH on the laccase activity and stability it was found that it is necessary to find pH_{opt} for each potential substrate, as markedly different behaviour was observed in the conversions of syringaldazine and ABTS.

Laccase immobilized on Granocel carrier is stable up to 55 °C whereas temperature for the native enzyme should not exceed 20 °C. This finding was supported by the comparison of inactivation kinetics of both enzyme catalysts at 80 °C. Moreover, the influence of agitation rate and temperature on the initial reaction rate indicates that the studied reactions can proceed under kinetically controlled conditions.

To examine the kinetic properties of bound laccase, two experimental methods were applied. The first one based on the initial reaction rate method and carried out in the batch reactor showed that k_{cat}/K_m is three times lower than the value obtained for the native laccase. The experiments carried out in the packed-bed reactor showed that the ratio k_{cat}/K_m is notably closer to that obtained for native laccase and this probably reflects more precisely kinetics of reaction in the case of immobilized enzyme accompanied by simultaneous product precipitation on the carrier's surface.

Finally, comparison of the immobilized enzyme reactivity and stability in the batch and continuous modes led us to the conclusion that the preparation is very well suited to the batch mode operation.

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