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Effective Purification of *Cerrena unicolor* Laccase Using Microfiltration, Ultrafiltration and Acetone Precipitation

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Abstract Microfiltration followed by concentration and diafiltration on ultrafiltration membranes (Biomax-100, Biomax-10 and Ultracel-10) was used to recover extracellular laccase (EC 1.10.3.2) from culture broth of wood-rotting fungus *Cerrena unicolor*. Feed, permeate, retentate and membrane wash-out solutions were analysed for the presence of laccase, proteases, protein and brown impurities. An easy, cheap and short-term procedure was proposed to obtain retentates with low yields of total proteins (less than 14%), proteases activity (less than 15%) and brown impurities (from 2% to 29%) with a simultaneous laccase recovery above 73%. The degree of laccase purification varied from 6.7 to 11.0 and depended on the type of membrane used and content of brown pigments in the feed. Subsequent protein precipitation with cold acetone increased the degree of purification about twice and reduced proteases and brown impurities to some extent. Ultracel-10 membrane was recommended as the best solution to prevent fouling of membranes and to obtain laccase-enriched fraction with a very low content of proteases and brown pigments.

Keywords Laccase · Membrane fractionation · Microfiltration · Ultrafiltration · Diafiltration · Precipitation

Introduction

Laccase (EC 1.10.3.2, systematic name: benzenediol:oxygen oxidoreductases) belongs to a multi-copper containing oxidases that are capable of oxidising various forms of organic substrates at the expense of molecular oxygen [1]. The enzyme occurs in many species of fungi, in higher plants and bacteria [1, 2]. Laccase substrate specificity is fairly wide and ranges from polyphenols through aromatic amines to aminophenols [3]. These properties make laccase a potentially attractive catalyst for many applications. Since 2000, all the

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available information about laccase sources, methods of immobilisation and potential industrial applications has been reviewed extensively. The enzyme has been shown useful for the bioremediation of environmental pollutants such as organic substances (specifically industrial dyes) or chlorophenols [1, 4, 5]. In food industry, laccases are used to eliminate undesirable phenolic components [6], but more evident application of laccase is seen in nanobiotechnology [7]. Many researchers have investigated this enzyme for biosensors, organic and/or polymer syntheses [8, 9]. The number of laccase applications has been increasing lately; thus, one should expect the growing demand for the enzyme production and purification.

Literature survey has revealed several procedures for laccase purification. Generally, each preparation method consists of three main steps: (1) cell debris removal, (2) concentration of culture broth and (3) removal of undesired low and high molecular weight compounds. Separation of the culture broth from cell debris can be done by passing fluid through a filter paper [10–18], glass fibre filters [19] and Miracloth [20, 21], by microfiltration (MF) [18] or centrifugation [13, 14, 22, 23]. Taking into account protein concentration methods, salting out [10, 12, 14, 23–25] and dead-end ultrafiltration (UF) [10, 12–14, 17–21, 24] are used predominantly, whereas evaporation [16], acetone precipitation [13] and lyophilisation [12, 20] are not common procedures. Chromatography (ion exchange [10–14, 17–25], affinity [16, 17, 22, 23] and hydrophobic interaction [15]) seems to be an obvious technique of laccase separation. In the next group of separation processes, aimed at removing undesired small molecules, there is dialysis [10, 12, 14, 15, 17, 20, 23–25], gel filtration [11–13, 16, 18, 20, 22, 25] and diafiltration [14, 19, 21, 24]. These steps are often combined and repeated which made the isolation process complicated and expensive. Sometimes, for example, three successive chromatographic [13, 17, 21–23], dead-end filtration [18, 21], dialysis [12, 23] or diafiltration [21] runs satisfied the protein purity requirements. A deeper inspection has revealed that, on average, membrane processes were used for enzyme concentration after chromatography and for desalting purposes. Moreover, nominal cut-off of membranes varied from 5 to 30 kDa, and there were no systematic studies either on the selection of membrane material or their other properties. The main benefits of membrane separation such as easiness of the process control, its flexibility and integrity were not used effectively.

Membrane processes are widely used in biotechnology. The operation conditions are gentle enough so that sensitive biomolecules cannot change their properties. Low temperatures and pressures that are commonly applied, the lack of phase change during the process or minimal, if any, usage of chemicals, the reduced time of contact, a species with high/low energy surfaces and low extent of mechanical forces can limit the processes of molecule decomposition or inactivation [26]. Now, it is a common practice to turn membrane separation to the production line for plenty of biotechnological processes. UF and MF are the most frequently used techniques [27, 28], especially when proteins are separated and fractionated from the culture broth. Although a lot of recent studies have demonstrated the possibility of proteins fractionation by UF, some of them dealt with simulated binary or ternary mixtures [29, 30]. Experimental studies of real biological streams are not very often presented [31–35].

The main goal of this study was to select UF membrane that can be used for separation and purification of laccase from the real cultivation broth when feed solution is not entirely defined and coloured impurities are in low or high concentrations. For that reason, MF was used as the first step, followed by membrane concentration (volume reduction) and diafiltration (low molecular species removal) in a single unit operation. Possible complex interactions between the components in the feed suggested the use of three UF membranes

with different chemistries and pore sizes. The quality of separation was routinely monitored by total protein content and the enzyme activity. However, in the cultivation broth, undefined yellow to brown impurities, probably originating from polymerisation of phenolic by-products by laccase action, were observed. Thus, special attention was paid to remove these species from laccase-enriched solution. As stability is essential for practical enzyme applications, consideration was also given to removing proteases. Finally, acetone precipitation was applied for subsequent volume reduction. Taking into account that some species in the feed are potent stabilisers/destabilisers of the enzyme, thermal tolerance of laccase preparations at different purification stages was measured to evidence its conformational stability.

Materials and Methods

Chemicals

Bovine serum albumin, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) sodium salt (ABTS) and Lowry reagent were from Sigma (USA). Other chemicals, analytical grade, were purchased from POCh (Poland).

Analytical Methods

All analyses were done at least in duplicate.

Laccase activity was determined from the change of optical density in time and calculated from initial reaction rate region. In 0.1 M citrate-phosphate buffer, pH 5.3, 207 μmol L^{-1} ABTS was used as a substrate [36]. The enzyme activity unit (U) was defined as the amount of the enzyme in 1 mL that oxidises ABTS to coloured products (420 nm, spectrophotometer Helios-α, Unicam) causing absorbance increase by 1.0 per min at 30 °C. Mean analytical error was less than 3%.

Proteolytic activity was determined by the caseinolytic test as specified previously [37]. The absorbencies of digested products in control and test tubes were measured spectrophotometrically at 280 nm. One unit of proteolytic activity (U) was defined as the amount of enzyme that induced absorbance increase of 1.0 per 10 min at 37 °C. Mean analytical error was less than 5%.

Protein concentration was determined spectrophotometrically according to Lowry's method (modified Sigma procedure P 5656) using bovine serum albumin as a standard. Mean analytical error was less than 3%.

The absorbance of all feed, retentate, permeate, wash-out solutions and precipitate samples was scanned from 200 to 900 nm. To track brown impurities, the absorbance of 420 nm was chosen for a linear relationship between absorbance and colour intensity.

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, University of Lublin (Poland). Microorganism cultivation and extracellular laccase production was performed according to a method described earlier [38]. Laccase containing the culture fluid was separated from mycelium by filtration on a steel sinter and was frozen. Depending on the cultivation method, specific activity of laccase in the fluid ranged from 100 to 400 U mg^{-1} of protein,

while its colour changed from yellow to light brown due to the presence of polymerised phenolic by-products. For experiments, collected fluids were defrosted and mixed to obtain 2,200 mL of the medium with small or big amount of impurities (yellow or brown in colour).

Microfiltration

Prefiltration was carried out using a stirred cell with up to 400 mL working volume (Amicon, USA) and MF disc membrane filter made of mixed cellulose esters with pore diameter of 0.22 μm (GSWP 09000, Millipore, Bedford, MA, USA). The effective membrane filtration area was 41.8 cm^2. The system was operated at 200 rpm, room temperature, and constant pressure was applied from an air-pressurised reservoir. Before experiments, the MF unit with the membrane was rinsed intensively several times with deionised water. The feed (2,200 mL of the culture fluid) was filtered in the dead-end mode up to practical depletion of the feed. After prefiltration, 20 mL of 0.1 mol L^{-1} citrate-phosphate buffer, pH 5.3, was added to the reservoir, and the stirrer was turned on for 15 min to wash proteins loosely bound to the membrane. The wash-out, feed and permeate samples were analysed for laccase, protease and protein presence. To track the coloured impurities, the samples were UV–VIS scanned. The permeate was used as a feed for subsequent concentration on UF membranes.

Membrane Concentration and Diafiltration

Experiments were conducted at room temperature on a Labscale™ TFF System (Millipore, Bedford, MA, USA) consisting of a 500-mL reservoir, stir base, retentate valve, pressure gauges and a diaphragm pump. Three Pellicon® XL devices (Millipore, Bedford, MA, USA) that are plate-and-frame style cassettes with 50 cm^2 of filtration area were tested: Biomax-100 and Biomax-10 with membranes made of polyethersulphone and nominal molecular weight cut-off (NMWCO) 100 and 10 kDa, respectively, and Ultracel-10 PLCGC with a membrane from composite regenerated cellulose and NMWCO 10 kDa. Before the experiments, the UF unit with the Pellicon membrane was rinsed according to the supplier's procedure. Pure water flux was measured prior to processing.

UF was carried out at room temperature. The permeate after prefiltration was divided into three parts (675–700 mL), each being a feed for subsequent concentration (26–28 times) and diafiltration (8–15 exchange of the volume of the retentate) on one of the tested membranes. The diafiltration was started just after retentate concentration up to 22–25 mL, maintaining a constant reservoir level with 0.1 mol L^{-1} citrate-phosphate buffer, pH 5.3. The permeate volumes (100 mL, concentration; 50 mL, diafiltration) were time collected and expressed as flux (L h^{-1} m^{-2}). The transmembrane pressure was set at 0.137 MPa. After the diafiltration and retentate drainage, 25 mL of 0.1 mol L^{-1} citrate-phosphate buffer, pH 5.3, was added to the system. Permeate and retentate were recirculated without transmembrane pressure for 15 min to wash proteins loosely bound to the membrane. Retentate from this step was named wash-out solution. Feed, wash-out, retentate and permeate samples were analysed for laccase, protease, protein and brown impurities concentrations, and their overall recovery was estimated with reference to the crude culture broth.

After completion of the experiments, cleaning of the system was conducted according to the manufacturer's specifications. The reservoir was filled with 500 mL of 0.3 mol L^{-1} NaOH at 45 °C (Biomax membranes) or 0.1 mol L^{-1} at 30 °C (Ultracel membrane), and

cleaning solution was pumped (feed pressure gauge about 0.137 MPa) through retentate and permeate ports to a waste collection vessel until the level in the reservoir dropped to 250 mL. Then permeate and retentate were recirculated for about 45 min (0.137 MPa transmembrane pressure), and then the system was drained. The procedure was repeated three times replacing cleaning solution by deionised water, and the pure water flux was monitored. Cleaning of the whole system was repeated when the flux was lower than 95% of the virgin membrane's initial value.

Laccase Precipitation with Cold Acetone

Laccase preparation (retentate and membrane wash-out separately) was precipitated with cold acetone (fluid:acetone, 1.0:1.5 (*v*/*v*), centrifuged (10,000 rpm, −2 °C, 20 min, Hettich 32R), and the precipitate was dissolved in 2–7 mL of the buffer.

Thermal Inactivation Experiments

Thermal inactivation was made in a thermostated stirred glass reactor (70 °C, 250 rpm). The buffer (0.1 M, pH 5.3, 50 mL) was preheated, and then 0.5 mL of laccase from the culture broth was added. After 15 s of vigorous mixing, the first sample was taken and cooled rapidly to 0 °C in an iced-water bath. In certain time intervals, consecutive aliquots were taken, cooled and stored in ice water prior the activity measurement, which was conducted after 1 h storage. A similar procedure was applied for the enzyme solution after MF, UF (concentration and diafiltration) or acetone precipitation. In all cases, protein concentration of the enzyme solutions was 1 mg mL^{-1}.

Results and Discussion

To obtain the preparation enriched with laccase, the broth was subjected to the three steps of purification: pre-treatment on the MF membrane, concentration and diafiltration with the UF membrane and protein precipitation with cold acetone. It was observed previously that some culture broths had brownish colour, whereas some were light yellow. Coloured by-products were considered to be impurities composed of polymerized phenolic compounds that interfered with subsequent purification steps and had to be separated by anion exchange chromatography [10, 14, 23]. Thus, special attention was given to this problem, and selection of MF and UF membrane was checked for culture broths with low or high level of brown impurities.

Microfiltration

On average, culture broth contains cell debris and other unspecified particles, and for this reason, the prefiltration on MF membrane was investigated as a necessary step before UF. Among several MF membranes, hydrophilic material made of mixed cellulose esters with pore diameter of 0.22 μm was selected in independent experiments (data not shown). It was observed that during filtration, the flux declined, especially when the solution with a higher amount of brown impurities was used. This phenomenon was partly due to a very large fluid volume (2,100 mL) passed through 41.8 cm^2 of the MF membrane. Four elements of the feed and permeate were balanced: total protein amount, activities of laccase and proteases and the presence of brown pigments. The obtained results (Fig. 1a) indicated that

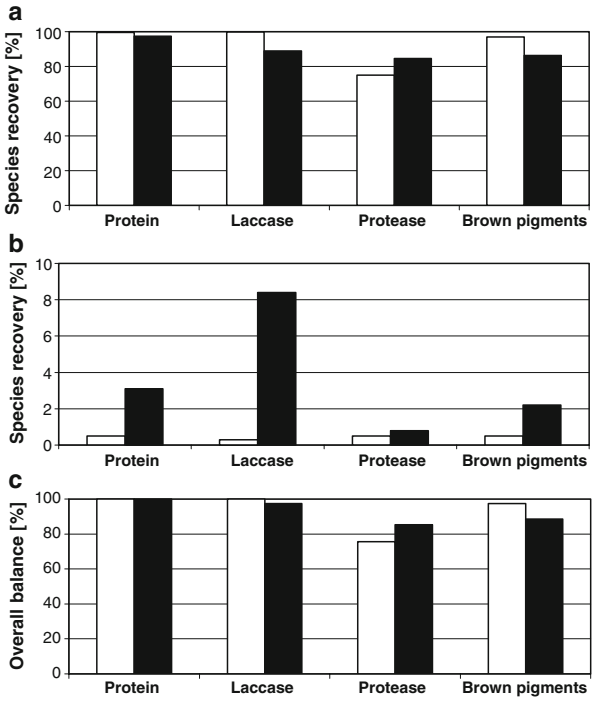

Fig. 1 Relative amounts of protein, laccase, proteases and brown impurities in the permeate after microfiltration (MF; **a**), solution wash-out from the membrane surface (**b**) and overall balance (**c**) for culture broth with small (*white bars*) or big (*black bars*) amount of brown impurities. The values before MF were set as 100%

the membrane allowed all the species to pass into the permeate. In the case of the broth with large amount of coloured by-products, proteins, the enzyme and brown pigments were partly retained. The situation turned over for proteases filtration, especially when less coloured feed was applied. The state in which part of proteolytic enzymes was adsorbed on the membrane and/or self-digested was profitable from the preoperational point of view, so at the next purification steps, laccase will be subjected to attack by proteases to a lesser degree.

After gentle washing of the membrane after MF, one can remove loosely bound species (Fig. 2b). It may be concluded that laccase is reversibly adsorbed on the membrane surface. In the case of proteases, they were subjected to self-digestion rather (activity balance below 100%, Fig. 2c), and the amount of freed enzyme was marginal. The removal of brown pigments from the MF membrane surface was also observed, but decolourisation of the permeate was insignificant.

Ultrafiltration

The solution obtained after MF was concentrated up to 22–25 mL (26–28 times reduced volume of the retentate), following by diafiltration with constant retentate volume. Diafiltration was conducted till only traces of the coloured by-products were found in the permeate. Usually, the exchange of retentate volume reached the values of 8–14 and was higher for solutions with a bigger amount of brown impurities. Three kinds of the UF

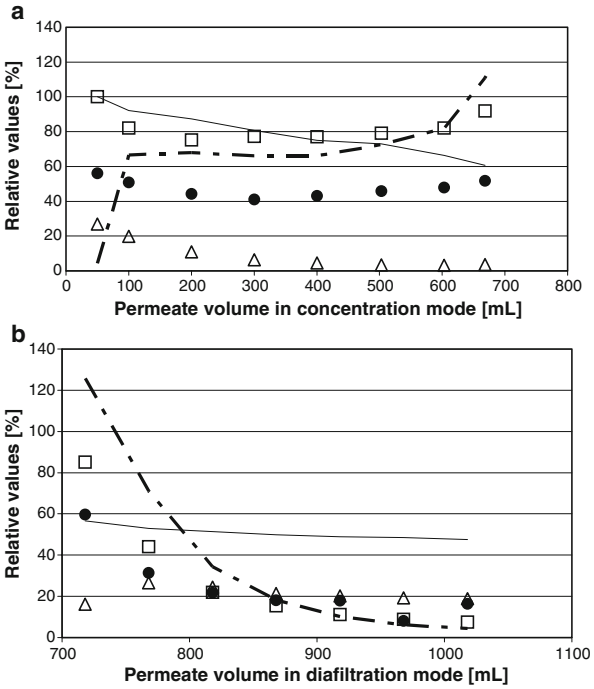

Fig. 2 Relative amounts of protein (*squares*), laccase (*triangles*), proteases (*circles*) and brown impurities (*dashed line*) in the permeate during concentration (**a**) and diafiltration (**b**) of culture broth with small amount of brown impurities on Biomax-100. The values before ultrafiltration were set as 100%. *Solid line*: relative permeate flux, respective to the initial flux (first 20 mL of the permeate)

membrane were tested: Biomax-100, Biomax-10 and Ultracel-10. Feed, permeate, retentate and membrane wash-out solution samples were analysed for laccase, protease, protein and the presence of brown impurities. A representative example of the concentration of analysed compounds and permeate flux variations in the permeate are shown in Fig. 2. As it is seen in the concentration part (Fig. 2a), protein content in the permeate was relatively high so that with concomitant low laccase activity, the enzyme in retentate was partly purified from the ballast proteins. It is worth to point that both proteases and brown pigments were observed in the permeate as well. Taking into account the permeate flux, its gradual decrease was observed in both, concentration and diafiltration modes. Data presented in Fig. 2b for diafiltration step shows that complete decolourisation of the retentate is practically difficult to reach even after 14 exchanges of the retentate volume. Such intensive diafiltration is tantamount to the higher laccase loss, and it must be a user's decision which procedure is better in a particular case: a higher laccase yield or lower content of brown impurities in the retentate.

More detailed data on laccase and proteases activities in the permeate, retentate and wash-out solutions obtained during concentration and diafiltration with three different membranes and two kinds of broth are listed in Tables 1 and 2. Generally, it was observed that most of the proteins passed the membranes in the concentration step, and their amount in the permeate was significantly lower during intensive diafiltration. Surprisingly, differences in the membrane cut-offs were only slightly evidenced. Total protein elimination in the permeate from concentration and diafiltration steps was almost the same

Table 1 Purification of culture broth with small amount of brown impurities by concentration and diafiltration on three ultrafiltration membranes.

Membrane		Total protein (mg)	Protein recovery (%)	Total activity (U)		Specific activity (U mg^{-1})		Purification fold (–)	
				Lac	Prot	Lac	Prot	Lac	Prot
Biomax-100	Feed	208.1	–	93,870	42.0	451	0.20	1.00	1.00
	P_{conc}	162.5	78.1	7,293	17.5	45	0.11	0.10	0.39
	$P_{diafiltr}$	28.9	13.9	9,855	5.2	342	0.18	0.74	0.64
	R	15.0	7.2	72,651	4.0	4,860	0.27	10.46	0.96
	W-O	1.5	0.7	5,953	0.4	4,049	0.29	8.72	1.04
Biomax-10	Feed	208.1	–	93,870	42.0	451	0.20	1.00	1.00
	P_{conc}	136.4	65.5	305	10.5	2	0.08	0.01	0.29
	$P_{diafiltr}$	26.2	12.6	149	5.7	5.7	0.22	0.01	0.79
	R	25.3	12.1	85,034	7.8	3,358	0.31	7.23	1.11
	W-O	2.16	1.0	7,088	0.7	3,282	0.34	7.07	1.21
Ultracel-10	Feed	208.1	–	93,870	42.0	451	0.20	1.00	1.00
	P_{conc}	169.6	81.5	2,744	12.9	16	0.08	0.03	0.29
	$P_{diafiltr}$	26.8	12.9	11,679	6.3	473	0.24	0.94	0.86
	R	13.9	6.7	70,834	7.1	5,111	0.52	11.00	1.85
	W-O	1.26	0.6	5,256	0.3	4,171	0.22	8.98	0.79

P_{conc} permeate from concentration phase, *$P_{diafiltr}$* permeate from diafiltration phase, *R* retentate, *W-O* wash-out solution, *Lac* laccase, *Prot* proteolytic enzymes.

in the case of Biomax-100 and Ultracel-10 membranes, although they differed in the cut-off by the order of magnitude (100 and 10 kDa, respectively). Moreover, the influence of a higher level of brown impurities decreased proteins in the permeates by a few percent only. In the case of Biomax-10 membrane, there were 78% of proteins in the permeate when the broth with lower content of brown impurities was filtered and 66% in the case of brownish culture. Thus, phenolic by-products affected protein separation, and it depended on the membrane cut-off and material from which the membrane was made. Similar behaviour of Biomax-100 and Ultracel-10 membranes can be due to the fact that these commercially available membranes show a pore size distribution, and so a sharp cut-off cannot be expected. Although they differed significantly in water flux (see Fig. 6), the chemical affinity of culture growth medium species could effectively change the ability of material to separate proteins. It is believed that phenolic by-products could be adsorbed on the polyethersulphone (Biomax) membrane rather than on the material made of hydrophilic cellulose, changing effective pore diameter.

Molecular weights of *C. unicolor* laccase isoforms were estimated to be 64 and 57 kDa [19], and thus they should not be eliminated from the retentate when membranes with the cut-off of 10 kDa are used. It was true only for the Biomax-10 membrane, whereas about 16% of laccase units passed Ultracel-10 membrane (Fig. 3). The influence of brown impurities on membrane selectivity was visible only in the case of Biomax-100. Using this membrane, 18% of laccase was observed in the permeate from culture broth with a small amount of brown impurities and around 8% if the darker broth was ultrafiltered. Though Biomax-100 and Ultracel-10 membranes were not completely impermeable for laccase, the very big amount of other proteins in the permeates enabled purification of main part of the

Table 2 Purification of culture broth with big amount of brown impurities by concentration and diafiltration on three ultrafiltration membranes.

Membrane		Total protein (mg)	Protein recovery (%)	Total activity (U)		Specific activity (U mg^{-1})		Purification fold (–)	
				Lac	Prot	Lac	Prot	Lac	Prot
Biomax-100	Feed	252.7	–	103,642	47.1	410	0.19	1.00	1.00
	P_{conc}	188.8	74.7	4,598	26.7	24	0.14	1.05	0.64
	$P_{diafiltr}$	39.1	15.5	4,153	6.0	106	0.15	0.22	0.68
	R	25.2	10.0	81,881	5.0	3,249	0.20	6.71	0.91
	W-O	2.6	1.0	7,346	0.5	2,817	0.18	5.82	0.82
Biomax-10	Feed	252.7	–	103,642	47.1	410	0.19	1.00	1.00
	P_{conc}	138.8	54.9	127	18.5	1	0.13	0.00	0.59
	$P_{diafiltr}$	27.3	10.8	88	8.6	3	0.20	0.01	0.91
	R	25.7	10.2	91,031	7.0	3,542	0.27	7.31	1.23
	W-O	1.7	0.7	5,826	0.5	3,368	0.31	6.95	1.41
Ultracel-10	Feed	256.6	–	116,620	42.6	455	0.17	1.00	1.00
	P_{conc}	208.4	81.2	2,418	22.6	11	0.10	0.02	0.46
	$P_{diafiltr}$	35.0	13.6	17,034	10.6	487	0.30	1.05	1.36
	R	17.2	6.7	80,399	5.2	4,669	0.30	10.06	1.36
	W-O	1.4	0.5	6,533	0.3	4,666	0.21	11.38	1.11

P_{conc} permeate from concentration phase, *$P_{diafiltr}$* permeate from diafiltration phase, *R* retentate, *W-O* wash-out solution, *Lac* laccase, *Prot* proteolytic enzymes.

enzyme in the retentates. Purification factor for three membranes tested varied from 6.7 to 11.0 (Tables 1 and 2), depending on the membrane and amount of brown impurities in the feed that, in fact, was a complex effect of membrane selectivity towards the enzyme molecules and ballast proteins.

A routine check-up for the balance of analysed species revealed the high quality of membrane wash-out solutions. Purification factors, calculated for laccase loosely bound to

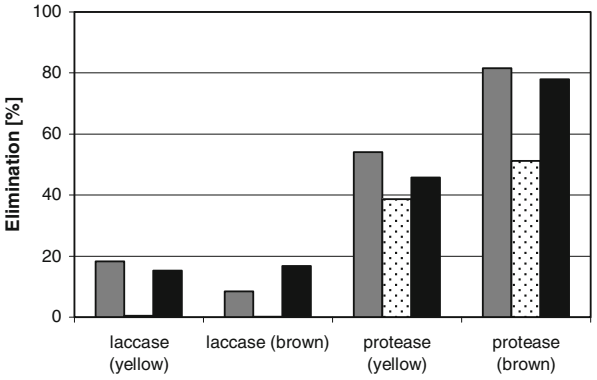

Fig. 3 Elimination of laccase and proteases in the permeate during ultrafiltration of culture broth with low (yellow) or high (brown) level of coloured by-products on Biomax-100 (*grey bars*), Biomax-10 (*dotted bars*) or Ultracel-10 (*black bars*) membranes

the membranes, varied from 5.9 to 11.1 and were comparable to respective retentates. As these solutions contained 5.6% to 7.6% of total laccase activity, they were subjected to further purification (see next paragraph).

Taking into account proteolytic enzymes, the benefit is seen in removing these enzymes from the retentate. Data from Tables 1 and 2 clearly demonstrated the influence of material of the membrane, its cut-off and amount of brown impurities in the feed although selectivity of the Biomax-100 and Ultracel-10 membranes was similar. In the case of Biomax-100, considerably more proteases passed through the membrane when the feed with a higher level of impurities was ultrafiltered (Fig. 3). A similar observation was made in the case of the Ultracel-10 membrane, but Biomax-10 was evidently less permeable. As some proteases remained in the retentate and other ballast proteins (in this case, laccase is a ballast protein as well) got through the membrane, the purification factor of proteases varied from 0.91 to 1.85 (Tables 1 and 2). Taking into account this parameter, the lowest values and the best properties were obtained using the Biomax-100 membrane, then Biomax-10 and finally Ultracel-10. This statement was in conflict with the percentage of proteases in the retentates (Fig. 4). After systematisation of the membranes, based on protease recovery in the retentate, the following series was obtained: Biomax-100<Ultracel-10<Biomax-10. The contradiction could be reconciled by data presented in Fig. 5 that depicted a complete balance (sum of amounts in the permeate, retentate and wash-out solution versus the feed) of all the analysed species and clearly showed low balance of proteolytic activity, especially in the case of the feed with a small amount of brown impurities but with the protein balance above 88%. It indicated that proteases lost large part of their activity, probably due to inactivation caused by shear forces, auto-digestion or air/liquid/solid interface contact. Interestingly enough, the higher concentration of brown pigments stabilised proteolytic

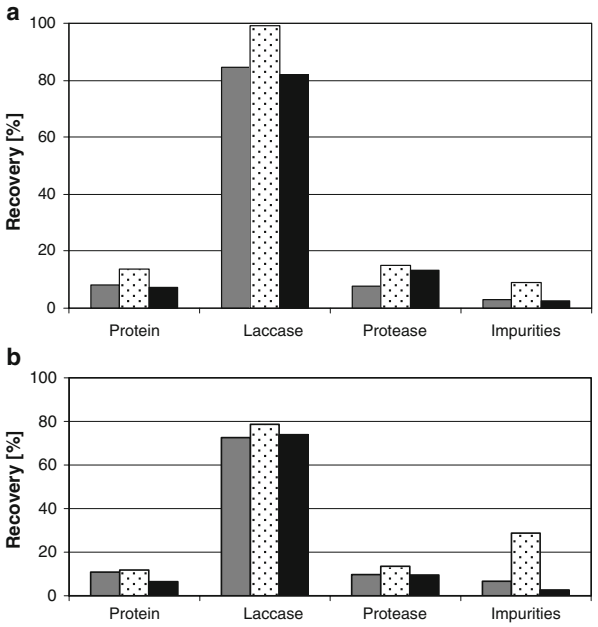

Fig. 4 Recovery of protein, laccase, proteases and brown impurities in the retentates after concentration and diafiltration of culture broth with low (**a**) or high (**b**) level of coloured by-products on Biomax-100 (*grey bars*), Biomax-10 (*dotted bars*) or Ultracel-10 (*black bars*) membranes

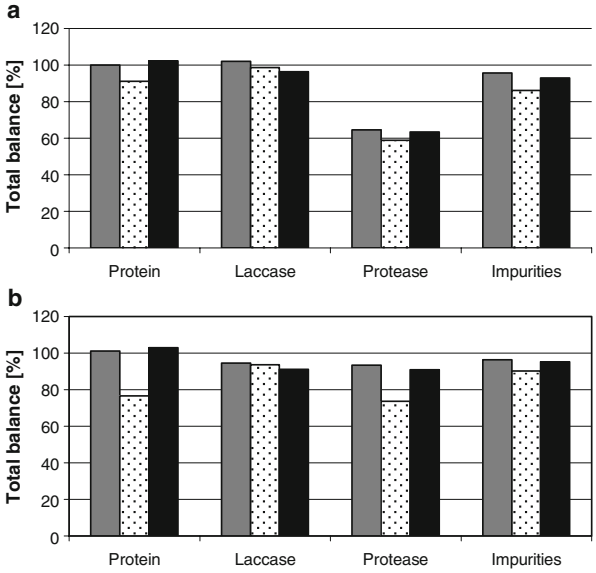

Fig. 5 Total balance of protein, laccase, proteases and brown impurities in the retentates after concentration and diafiltration of culture broth with low (**a**) or high (**b**) level of coloured by-products on Biomax-100 (*grey bars*), Biomax-10 (*dotted bars*) or Ultracel-10 (*black bars*) membranes

activity, giving the balance above 70%. The observations are in good agreement with data presented in Fig. 1; the permeate after MF of dark broth had a higher proteolytic activity than the light broth. On the contrary, laccase was more inactivated in the darker culture fluid. Thus, in the case of enzymes, elimination factor has less practical meaning than enzyme recovery in the retentate. It is worth pointing that complete balancing of all species analysed during the filtration allowed us to evaluate inactivation of the enzymes and to indicate possible species that fouled the membrane. As seen in Fig. 4, Biomax-10 can be fouled by proteins (protein balance below 10% and 20%, depending on the culture fluid) and by brown impurities, whereas two other membranes reversibly bound proteins, and they were fouled probably mainly by brown by-products.

As it was mentioned, the recovery of analysed species in the retentate was more informative than elimination factor. Taking into account low yields of total proteins, proteases and brown impurities with simultaneous high laccase recovery (Fig. 4), a general advantage of the membrane concentration and subsequent diafiltration of culture broth was evident. All the membranes tested were suitable for this process: (1) using Biomax-10 the highest laccase recovery was obtained with the highest, but not profitable, amount of proteolytic activity and brown impurities in the retentates; (2) Biomax-100 and Ultracel-10 were merely comparable, but Biomax-100 was more effective in proteases elimination in the feed with smaller amount of brown impurities, whereas Ultracel-10 was better in the elimination of brown by-products with darker feed. Thus, there was no evident recommendation to point at the membrane with the best property.

Until this stage, there were no serious arguments to exclude any tested membrane for laccase purification. A disadvantage of the Biomax-10 membrane was searched out in the biggest amount of proteases and brown impurities in the retentates, irrespective of the kind of culture broth. An additional and very important factor was derived from the flux

decrease analysis (Figs. 2 and 6). As it is seen in Fig. 2a, the permeate flux declined gradually at the concentration step and, to a smaller extent, during diafiltration (Fig. 2b). It was expected that such phenomena as fouling and secondary layer formation would be responsible for lowering of the flux. A closer inspection of Fig. 6 revealed that the flux measured with water (dotted bars) was the highest for Biomax-100 membrane, then Ultracel-10 and the lowest for Biomax-10. These fluxes were substantially lowered even at the very early stage of the UF process when Biomax-100 (by 44% and 52% of water flux for the broth with low and high level of impurities, respectively) and Biomax-10 (57% and 64%) membranes were used. On the contrary, Ultracel-10 membrane was less susceptible to the flux decrease, and it was reduced by 12% and 32% for two kinds of broth. It indicated possible sorption of filtered species on a more hydrophobic material (Biomax; polyethersulphone) and then pore plugging. As a result, the flux measured in the last fraction of permeate using the Biomax membranes was low (20–27% in reference to water flux), whereas Ultracel-10 provided flux of 53–78% measured with water. It was translated into comparable time of UF processes using the Ultracel-10 membrane and Biomax-100 (from 1 h to 1 h 15 min), although Biomax-100 was characterised by twice as high flux of water. On the other hand, the Biomax-10 membrane was prone to pore plugging, especially by proteins and brown impurities, that prolonged the process duration even more than three times comparing to other membranes tested (3 h 30 min and 4 h 45 min). Thus, lower susceptibility of the Ultracel-10 membrane to flux decrease puts this material on the top of the interest in laccase isolation and purification from cultures broth containing big and small amounts of brown impurities.

The quality of obtained solutions can be easily controlled spectrophotometrically in the visible region as laccase is a blue-copper oxidase with a typical peak at 608 nm [19, 25]. Apart from the membrane fractionation of dark feed on Biomax-10, all the retentates were less or more green in colour (weak peak in the range 400–420 nm in Fig. 7), as yellow to brown colour of phenolic impurities overlapped the deep blue colour of pure laccase. In all cases, a broad peak in the wavelengths from 550 to 700 nm was observed, and the presented case (Fig. 7), obtained after UF of the broth with low level of impurities on the Ultracel-10 membrane, was very similar to that presented by other authors [19, 25]. This peak can be used for monitoring of the process and evaluation of laccase purity in the retentate.

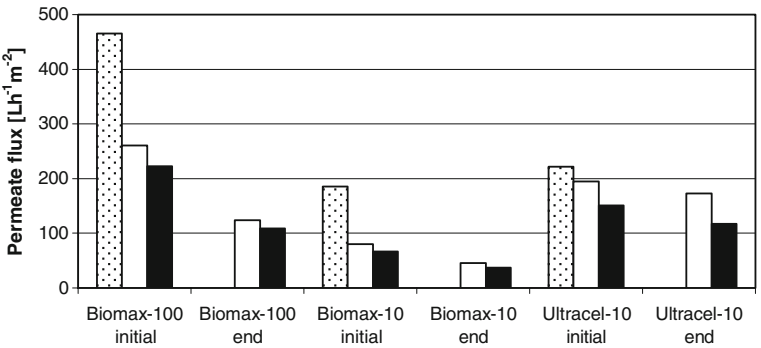

Fig. 6 Initial permeate flux (first 20 mL) during concentration of the feed with low (*white bars*) or high (*black bars*) amount of brown impurities on tested membranes and permeate flux in last fraction of permeate (end) after consecutive diafiltration. *Dotted bars* represent reference flux with deionised water

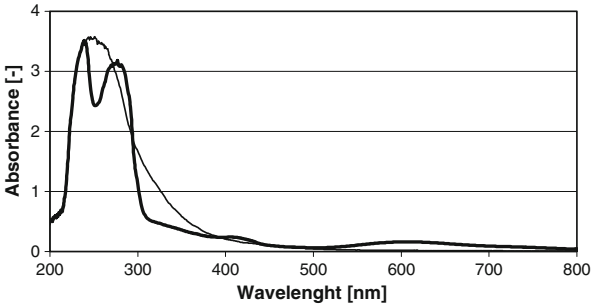

Fig. 7 Absorbance versus wavelength measured for the feed with small amount of brown impurities (*dashed line*) and the retentate after concentration and diafiltration on the Ultracel-10 (*thick solid line*) membrane

Acetone Precipitation

The obtained retentates and membrane wash-out solutions after UF were subjected to cold acetone treatment, and after centrifugation, the precipitated proteins were suspended in several millilitres of the buffer, giving 100–120-fold concentration of the initial broth volume. The data obtained for laccase purification with the Ultracel-10 membrane are shown in Table 3. As was predicted, acetone precipitation of proteins resulted in a subsequent improvement of laccase purification factor. One more profitable effect was observed—a reduction of proteases content in all evaluated retentates (the data in Table 3 refer to Ultracel-10 only but others were similar). In the case presented in Table 3, acetone precipitation allowed us to obtain a solution slightly enriched with laccase from the dark culture broth but with an almost 50% light improvement in the colour of culture broth. In both cases, parallel to laccase enrichment, the amount of proteases was reduced by 30–40% to the value noted in the retentate. Even better preparations can be obtained from the wash-out solutions; Ultracel-10 wash-out solution contained laccase with as high as 20-fold purification for the light broth and above 17-fold for the dark broth. As these solutions contained 4.8% and 4.9% of total laccase activity, respectively, it is recommended to mix washing-out and retentate solutions before acetone precipitation.

To compare experimental results with those presented in the literature, typical purification procedures were summarised in Table 4. The yield of the preparation and also enzyme purification degree were calculated and shown in last two columns of the table. As it is seen, the listed procedures contain several preparative steps that make the

Table 3 Acetone precipitation of retentates and membrane wash-out solutions obtained after ultrafiltration of feed with low or high brown impurities level on Ultracel-10 membrane.

Solution/colour	Total protein (mg)	Specific activity (U mg^{-1})		Purification fold (–)		Total yield (%)		
		Lac	Prot	Lac	Prot	Protein	Lac	Prot
R/brown	12.3	5,263	0.20	11.34	0.91	4.8	55.5	5.8
W-O/brown	0.7	8,060	0.31	17.36	1.41	0.3	4.8	0.5
R/yellow	10.1	7,083	0.32	15.25	1.14	4.9	76.2	7.7
W-O/yellow	0.5	9,233	0.31	19.88	1.11	0.2	4.9	0.4

P_{conc} permeate from concentration phase, $P_{diafiltr}$ permeate from diafiltration phase, *R* retentate, *W-O* wash-out solution, *Lac* laccase, *Prot* proteolytic enzymes.

Table 4 Examples of laccase purification steps.

Chromatography	Concentration	Removal of small molecules	Yield (%)	Purification fold (–)	Reference
I	S-O, DEF	D	26	3	[10]
I		*GF, GF*	*18*	*7*	[11]
I	S-O, DEF, L, L	GF, D, D, D	53	10	[12]
I	*DEF, DEF*	*DiaF, DiaF,*	*13/9*	*7/3*	[19]
I, I, I, I	*DEF, DEF, DEF*	*DiaF, DiaF, DiaF*	*23–67*	*9–10*	[21]
I, I	S-O, DEF, DEF	D, DiaF, DiaF,	28	9/12	[24]
I	*DEF, L*	*GF, D*	*15.8*	*13*	[20]
I, I, I	Ac, DEF	GF, GF	32	15	[13]
	DEF	*DiaF, Ac*	*56–76*	*11–15*	*here*
I, I	S-O, DEF	D, DiaF	15	24	[14]
I, A, A		GF	0.4	31	[22]
H, H		D, D	24	25/54	[15]
A	E	GF	14/21	37/98	[16]
I, I, A	*S-O, DEF, DEF*	*D*	*9*	*120*	[17]
I, A	Ac	D, D	0.4	152	[26]
I	DEF, DEF, DEF	GF, GF, D	12	182	[18]
I, I, I	*S-O*	*GF, D, D*	*NA*	*200/310*	[25]
I, I, A	S-O	D, D, D	16	967	[23]

Details concerning laccase from *Cerrena unicolor* are given in italic.

Number of abbreviations shows the repeatability of the process, e.g. for the last line: step I was repeated twice; A, once; S-O, once; and *D*, three times.

I ion exchange chromatography, *A* affinity chromatography, *Ac* acetone precipitation, *D* dialysis, *DEF* dead-end filtration, *DiaF* diafiltration, *E* evaporation, *GF* gel filtration, *H* hydrophobic interaction chromatography, *L* lyophilization, *S-O* salting out, *NA* not available.

isolation process complex and expensive. However, in most cited references, the main aim was to characterise a new enzyme, and for this, the biocatalysts had to be purified up to homogeneity and, hence, very low yields of processes had little importance. The goal of our research was to obtain not a homogeneous enzyme but the enzyme-enriched preparation for practical applications, therefore, inexpensive and active as only possible at the same time. This preparation can directly be used in most biotechnological processes (polymerisation of phenolic substrates, immobilisation and dyes decolourisation [39, 40]) or can serve as a feed for further purification. Interestingly, MF followed by UF fractionation and acetone precipitation offered the laccase preparations with purity degree in the middle of common values and with high yield. Taking into account *C. unicolor* laccase, it can be seen from Table 4 (examples in italic) that our simple and short procedure offers comparable degree of purification at considerably higher [11, 19, 20] or at least similar [21] yields. It is noteworthy that only three operational steps enable to achieve high laccase recovery at very low loss in activity probably due to avoiding harsh conditions such as high ionic strength, denaturants, and long contact with gas/liquid or liquid/solid interface. On the other hand, if needed, a higher degree of homogeneity can be obtained after further purification (e.g. by chromatography) to achieve 10–20 times higher purification factor reported by other authors [17, 25].

Influence of Purification on Laccase Thermal Stability

The enzyme stability, especially thermal tolerance, is one of the most important factors from the processing point of view. Thus, assuming that some species in the real broth can be potential stabilisers or destabilisers of the enzyme and their removal can affect its thermal tolerance, special attention was given to inactivation experiments. As it is seen in Fig. 8, *C. unicolor* laccase from the cultivation broth preserved above 20% of the initial activity after 2 h incubation at 70 °C, but its high thermal tolerance decreased gradually at consecutive steps of membrane purification. As protein concentration in the enzyme solutions was chosen so that it was always the same (1 mg mL^{-1}), the loss of stability could be attributed to the removal of potential stabilisers or to protein defolding, caused by the contact with the membrane surface and/or air–liquid interface. Interestingly, although acetone is a well-known denaturant of proteins, laccase after acetone precipitation showed as high thermal tolerance as the enzyme from the cultivation broth. It is suspected that acetone denatured the enzyme molecules which were partly defolded, removing molecules inactivated more easily at elevated temperature.

Conclusions

A membrane-based process for laccase purification from post-cultivation broth was successfully demonstrated. The experiments presented in this study indicated that the MF → UF → acetone precipitation procedure resulted in an acceptable resolution and recovery of laccase with a reduction of the number of operational steps. It is worth pointing that not only laccase activity was monitored during the whole process. Special attention has been paid to assessing the presence of brown pigments and proteolytic enzymes. According to our best knowledge, such approach to the subject is rarely presented. On the basis of the obtained results, several conclusions have been drawn:

1. MF partly reduced the amount of proteases and brown pigments in the permeate with an almost complete protein recovery and very low laccase activity loss;
2. UF concentration and diafiltration offer laccase preparations with higher purity and high yield. Moreover, one unit operational step enables minimisation of laccase activity loss. This preparation can be used directly in most biotechnological processes or can serve as the feed for further enzyme purification;

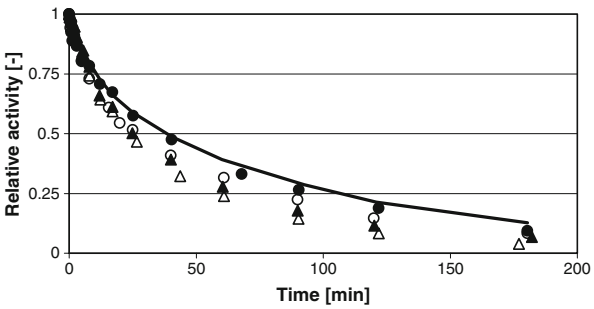

Fig. 8 Thermal inactivation (70 °C) of laccase in time: post-cultivation broth (*solid line*) and after microfiltration (*open circles*), membrane concentration (*closed triangles*), diafiltration (*open triangles*) or acetone precipitation (*closed circles*)

3. It is recommended to use the Ultracel-10 membrane for concentration and diafiltration of microfiltered broth. In that case, broths from light yellow up to dark brown colour can be treated using this membrane which offers a reasonable purification level, low permeate flux decay and a possibility to eliminate most of brown impurities and proteolytic enzymes;
4. Acetone precipitation is an optional treatment which reduces the retentate volume, the content of colour impurities to some extent and removes additional part of proteases in both kinds of cultivation broth. Moreover, laccase thermal stability is as high as that observed in the culture broth.

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