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Membrane chromatography for the purification of laccase from the supernatant of *Pleurotus sapidus*

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ABSTRACT

Extracellular laccase from the fermentation broth of *Pleurotus sapidus* was purified using membrane chromatography. Five laccase iso-enzymes produced in submerged cultures were purified; the enzymes had isoelectric points (p*I*) ranging from 3.3 to 4.7 and a calculated molecular weight of 57.4 kDa. The fermentation broth had a low product titre of 80 μg/l. Hydrophobic interaction (HIC) and ion exchange (IEX) membranes chromatography modules were used in an ÄKTApurifier 100 liquid chromatography system for the dynamic experiments. The pre-treatment of the fermentation broth was not required. The enzyme activity was retained, and enrichment up to 15-fold was observed via activity assays and SDS-Page analysis.

1. Introduction

Laccase is a copper-containing oxidase that catalyses the reduction of oxygen to water while simultaneously oxidising various aromatic components. It belongs to the group of polyphenol oxidases, but it can also oxidise methoxy-substituted phenols, aromatic di-amines and some other substrates (but not tyrosine). Thus, it has a broad substrate range, which varies depending on the origin of the laccase [1]. It can be used to oxidise xenobiotics, synthetic dyes and pesticides. Laccase can also be applied to detoxify agricultural by-products, e.g., olive mill wastes, coffee pulp or bleach kraft pulp [2,3].

In terms of eco-friendly processing, laccase offers the possibility to harness crude materials from renewable sources by effectively digesting natural materials such as lignocellulose and making them available as carbon sources for the biotechnological production of fine chemicals [4].

The supernatant of the white-red basidiomycete *Pleurotus sapidus* from a submerged culture was used to harvest laccase in this study. *P. sapidus* is an edible fungus of the oyster mushroom family that secretes many extracellular enzymes, of which laccase is the most commonly investigated enzyme [5].

Laccase is often obtained from the supernatant of submerged fungi cultures at a very low protein concentration. Common methods for its purification combine precipitation and ultrafiltration to reduce the liquid volume and different chromatography techniques to isolate and capture the enzyme [6]. These procedures are time-consuming, require pre-treatments and are also disadvantageous in terms of product purity, recovery and stability [7]. Column chromatography offers some benefits regarding high resolution, recovery and capacity and is thus mainly used for therapeutic proteins. To overcome some of the aforementioned drawbacks, alternative methods for laccase purification have been widely investigated [7]. Membrane chromatography has been used as an alternative unit operation in this study because the fermentation volume to be processed is very high and the total protein content (80 μg/ml) in the fermentation broth is very low.

The bed for membrane chromatography is essentially identical to the bed for normal flow filtration membranes. However, the membrane pore surface is functionalised with a ligand, allowing this unit operation to bind the product of interest from a process solution, which is pumped through the membrane [8]. The immobilised ligands, which allow for the selective interaction of the target molecules with the membrane, are the same as those utilised in column chromatography. Consequently, the operation of both technologies is performed in a similar way using similar equipment. Because of the high porosity, large cross sectional area and small thickness of the membrane, this process can be operated at low pressure drops. The open pore structure of the membrane favours the convective transport of the solute to the binding sites within the pores. Therefore, the rate-limiting pore diffusion observed in column chromatography can be eliminated. This leads to the reduction of both the process time and the recovery liquid volume. Furthermore, very high flow rates can be applied because the binding

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Table 1
Composition of the standard medium.

Component	Concentration (mg/ml)
D(+)-Glucose monohydrate	30.0
L-Asparagine monohydrate	4.5
KH_2PO_4	1.5
Magnesium sulphate	0.5
Yeast extract	3.0
Trace element solution	1 (ml l^{-1})

efficiency is independent from the feed flow rate, enhancing the productivity of the overall process. The rate limiting steps are not the binding kinetics but the solute transportation to the binding site. Because no diffusional limitations exist, convective transport is the dominating transport mechanism and can be controlled via the applied flow rate. Membrane chromatography has therefore found various applications not only for the removal of large biomolecules in flow-through mode for polishing but also in bind and elute mode for capturing molecules [9–13]. Coupling the functionality of a chromatography resin with the hydrodynamic characteristics of normal flow filtration provides a unique and reliable unit operation. Membrane chromatography modules are easy to scale up and cheaper to mass-produce, enabling their use as disposables and eliminating the requirement for cleaning and revalidating equipment [14]. Therefore, the aim of this work is to develop a procedure for the purification of extracellular laccase from *P. Sapidus* that employs membrane chromatography while maintaining the individual physicochemical and catalytic properties of these enzymes.

2. Materials and methods

2.1. Laccase

The cultivation and fermentation of laccase was performed as proposed by Linke et al. [15] at the Institute of Food Chemistry and Food Biotechnology at the Justus Liebig University of Giessen, with some modifications. The mycelia (1 cm^2) of *P. sapidus* (8266 DSM) were excised from 1.5% agar plates and used as the inoculum in five 300 ml Erlenmeyer flasks containing 100 ml of standard medium.

The standard medium (Tables 1 and 2) was adjusted to a pH of 6.0 with NaOH and autoclaved before use (121 °C, 20 min). The homogenised solution was then incubated for 7 days at 150 rpm and 24 °C. After transferring the inoculum for production into a 7.5 l fermenter with a fermentation volume of five litres (standard medium), the secretion of laccase into the medium was induced by adding 0.1% (w/v) lignin, 300 μM $CuSO_4$, 1% ethanol, and 0.25% Tween 80 to the media. The main culture was incubated under the same conditions as the preculture. At the time of maximum laccase activity (day 4), the supernatant was separated from the mycelium by filtration using cellulose filter paper (12–15 μm).

The supernatant of *P. sapidus* was kept at pH 6.2 and frozen at −20 °C until use. Due to the freezing and thawing process, coagulation occurred and had to be removed before starting the membrane adsorption, as the tubing and membrane are prone to fouling. This was performed by centrifuging the supernatant for 5 min at 700 g using a Hettich Universal centrifuge.

Laccase from *Trametes versicolor* was purchased from Sigma Aldrich (product number: 51639).

Table 2
Composition of the trace element solution.

Component	Concentration (mg/ml)
Iron (III) chloride hexahydrate	0.08
Zinc sulphate heptahydrate	0.09
Manganese sulphate hydrate	0.03
Copper sulphate pentahydrate	0.005
EDTA	0.4
Iron (III) chloride hexahydrate	0.08

Table 3
Characteristics of the membrane modules [16,17].

Technical data	Nano 1 ml	Nano 3 ml
Bed volume (ml)	1	3
Bed height (mm)	4	12
Maximum pressure (bar)	4	4
Surface area (cm^2)	36.4	110
Pore size (μm)	>3	>3

2.2. Membrane adsorbers

All membrane adsorbers were obtained from Sartorius Stedim Biotech GmbH. A hydrophobic interaction membrane (HIC; Sartobind Phenyl nano 3 ml), an anion exchange membrane (AIEX; Sartobind Q Singlesep nano 1 ml) and a cation exchange membrane (CIEX; Sartobind S Singlesep nano 1 ml) were used. All of the membranes were made of stabilised reinforced cellulose with a housing material of polypropylene and a long-term pH stability of 3–14.

The membranes have different types of ligands, depending on the adsorption mechanisms: the AIEX membrane adsorber uses quaternary ammonium, the CIEX uses sulphonic acid and the HIC uses phenyl ligands for interacting with the target molecules. The main membrane module characteristics are depicted in Table 3.

2.3. Experimental setup and procedure

All membrane adsorption experiments were carried out in triplicate with a ÄKTApurifier 100, an automated liquid chromatography system connected to a Frac-950 fraction collector. UV absorption was determined at a wavelength of 280 nm, and the pH and the conductivity were monitored online with the UNICORN control system on a personal computer.

The buffers used for the experiments can be found in Table 4. A volume of 50 ml per step was used for equilibration, loading, washing and elution, and the buffer was pumped through the membrane at a flow rate of 10 ml/min.

2.4. Analytical methods

The 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay and the Bradford assay were carried out to determine the laccase activity and the total protein concentration,

Table 4
The buffer system used for the enzyme purification.

Step	Buffer	pH
Equilibration/ washing	25 mM acetate buffer	4.5
AIEX	25 mM NaH_2PO_4/K_2HPO_4 buffer	6.2
CIEX	20 mM citric acid buffer	3.5
HIC	25 mM NaH_2PO_4/K_2HPO_4 buffer + 1 M Na_2SO_4	6.2
Loading	Supernatant/100 mM acetate buffer (3:1)	4.5
AIEX	Supernatant	6.2
CIEX	Supernatant/20 mM citric acid buffer (3:1)	3.5
HIC	Supernatant + 1 M Na_2SO_4	6.2
Elution	25 mM acetate buffer + 1 M NaCl	4.5
AIEX	25 mM NaH_2PO_4/K_2HPO_4 buffer + 1 M NaCl	6.2
CIEX	20 mM citric acid buffer + 1 M K_2HPO_4	3.5
HIC	25 mM NaH_2PO_4/K_2HPO_4 buffer	6.2
Regeneration	1 M NaOH	12