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Immobilized Laccase for Sustainable Technological Processes

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Enzyme-catalyzed processes are currently a powerful tool in the chemical industry due to the reduction of process time, specificity, eco-friendly characteristics, intake of low energy input, lower cost and nontoxic properties. Laccases are versatile enzymes, which catalyze oxidation reactions coupled to four-electron reduction of molecular oxygen to water. Laccases are used for decolorization and detoxification of industrial effluents and are widely used in wastewater treatment. For improving enzyme utilization in biotechnological processes, the process cost has to be reduced, the enzyme stability during industrial processes should be enhanced and the recycle and reuse step should be favorable. The immobilization of the enzyme is an important step for enhancing enzyme catalytic properties and operational stability. In our study, two different types of immobilization were used for laccase: cross-linked enzyme aggregates (CLEA) and magnetic cross-linked enzyme aggregates (mCLEA). The enzyme activity of synthesized CLEA was 0.237 U/mg laccase and mCLEA 0.637 U/mg laccase, respectively. In the next step, immobilized laccase in the form of CLEA and mCLEA was treated under supercritical conditions. Supercritical fluids (SCFs) comprise a unique class of nonaqueous media for biocatalysis and bioseparation. It is very difficult (almost impossible) to predict the stability and activity of an enzyme in any SCF at a certain pressure. Therefore, the stability of both laccase immobilized forms in supercritical carbon dioxide (SC CO_2) at different pressures was studied. The highest residual activity for both laccase immobilized forms were obtained after exposure to SC CO_2 at 20 MPa, 35 °C for 5 h. The obtained residual activities were 145 ± 3 % for mCLEA and 128 ± 3 % for CLEA. Improved stability of immobilized laccase in the form of CLEA and mCLEA after SC CO_2 treatment indicates the possibility of using laccase in supercritical media. Further application of CLEA laccase and mCLEA laccase could be reflected in the field of hospital wastewater treatment for removal of drugs.

1. Introduction

The removal of emerging organic contaminants (EOC) from municipal wastewater poses a major challenge and could be carried out through various wastewater treatment processes (Gasser et al., 2014). Different methods for EOC transformation, such as ozonation and photocatalytic oxidation are currently in use, but the cost of the processes is high since the addition of catalysts and oxidants are required. During these processes, the formation of even more toxic by-products may occur. An environmentally friendly wastewater treatment process is an enzyme-catalyzed process (Leow et al., 2018). Especially, laccases have gained interest as a biocatalyst for EOC removal in wastewater treatment because of their broad substrate range and since they only need molecular oxygen as a cosubstrate. Laccases have the potential to be implemented as a tertiary wastewater treatment process in sewage treatment plants (Gasser et al., 2014). Laccase belongs to the group of blue multicopper oxidases, having the potential ability of oxidation. Laccases can be produced by different organisms like bacteria, fungi and plants (Upadhyay et al., 2016) and are capable of degrading lignin. Because of their ability to detoxify a range of pollutants, they have been used for several purposes in many industries including the paper, pulp, textile, wastewater treatment and petrochemical industries. Since they can act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants, laccase can also be used for xenobiotic degradation, for bioremediation and for removal of endocrine disruptors. Other applications of laccase can be found in the food processing industry, medical and health care as well as in the

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design of biosensors and nanotechnology (Upadhyay et al., 2016). Laccases can be also successfully used to remove pharmaceuticals from wastewater (Lloret et al., 2013).
The current demands of the world's biotechnological industries are enhancement in enzyme productivity and development of novel techniques for increasing their stability (Datta et al., 2013). Enzyme stability and their retention in the process (the possibility to perform continuous processes) and reuse could be improved by immobilization of enzymes on a carrier or in a form without a carrier. Apart from facilitating industrial processes, immobilization provides us with an easy biocatalyst control and high stability for storage and operational conditions. In addition, it is a crucial point to consider that by using immobilized enzymes, compared to pure enzymes, a simple and more efficient separation step is obtained, and therefore purity of the product is highly improved. The immobilization of the enzyme laccase is an important step for enhancing enzyme catalytic properties and operational stability. The immobilization of enzymes as cross-linked enzyme aggregates (CLEA) and their applications in industrially relevant biotransformations are well documented (Sheldon, 2011). The technique is simple and broadly applicable. CLEA have several benefits, including enhanced shelf life and operational stability; they are easy to recover and reuse, are stable towards leaching in aqueous media and have high volumetric and catalyst productivities (kg of product per kg of biocatalyst) as a result of the absence of noncatalytic ballast-like carrier (Sheldon, 2011). Immobilization of an enzyme in the form of CLEA is a relatively cheap method, since no highly pure enzyme is required, and the costs of carriers are omitted. Since precipitation is a commonly used method for enzyme purification, CLEA formation using precipitation step combines purification and immobilization into a single unit operation. The most often used cross-linking agent is glutaraldehyde, since it is inexpensive. The clumping of CLEA, which results in increased size clusters as a result of the separation of CLEA from the reaction mixture by centrifugation or filtration, causes internal mass-transfer limitations (Wang et al., 2011), particularly in the case of enzymes acting on macromolecular substrates (Talekar et al., 2012). In order to overcome this limitation of CLEA technology, magnetic cross-linked enzyme aggregates (mCLEA) can be used. The mCLEA includes functionalized magnetite nanoparticles (MNPs) as an additive into the enzyme solution. Precipitation of the enzyme to form aggregates and then cross-linking of enzyme aggregates and nanoparticles are the main steps in the preparation of mCLEA. MNPs have high surface area and can be coated with a large number of amino groups on their surface (Hu et al., 2009). mCLEA can be easily controlled and separated from the reaction mixture by the application of a magnetic field, eliminating the need for centrifugation and filtration processes. In our study, laccase from *Trametes versicolor* was immobilized in the form of CLEA and mCLEA. Water, which is an *in vivo* solvent for enzymes, was replaced with supercritical carbon dioxide (SC CO_2). Because no theoretical prediction whether an enzyme should be active in SC CO_2 or not can be done, an experimental study of the system behaviour of laccase/SC CO_2 was performed. Incubation of laccase in SC CO_2 under extreme conditions may modify the three-dimensional structure of the enzyme, leading to denaturation with loss of activity. If the treatment is carried out under less harsh conditions, the protein advanced structure can be maintained or a minor structural change may be possible to induce an alternative active protein state in order to change or even improve enzyme activity (Knez et al., 2005) and stability (Habulin et al., 2007). SC CO_2 as a green solvent was used safely because of its non-toxic and non-flammable nature and the favorable critical conditions (31.1 °C and 7.38 MPa) which are suitable for treatment of bioactive substances, i.e. enzymes. The residual activity study of exposed laccase in the immobilized and free form in SC CO_2 was done. The aim of the study was to find out whether laccase in the immobilized form can be activated in SC CO_2 for future use as an agent for decomposition of some contaminants

2. Material and methods

2.1 Preparation of CLEA laccase and mCLEA laccase

Preparation of CLEA laccase and mCLEA laccase is a process that consists of two steps: precipitation and cross-linking.

- Precipitation: solvent used for precipitation of CLEA was ethanol and for mCLEA was 2-propanol. Enzyme solution with an initial concentration of 20 mg/mL was slowly added dropwise into chosen solvent for precipitation to reach the final concentration of laccase 2 mg/mL. When the mCLEA was prepared the enzyme was mixed with 100 mg of aminosilane MNPs and was added dropwise and slowly into 2-propanol for precipitation to reach the final concentration of laccase 2 mg/mL. The solution was stirred.
- Cross-linking: the cross-linking agent was glutaraldehyde. Glutaraldehyde was added to the precipitation solution and the suspension was mixed. Then, the glutaraldehyde was reduced with sodium cyanoborohydride ($NaBH_3CN$) and mixed. The solution was centrifuged at 11,000 rpm. CLEA or mCLEA aggregates were rinsed with buffer at least twice until no protein concentration in the supernatant was determined.

2.2 Determination of protein concentration

The protein concentration in supernatants after rinsing of CLEA and mCLEA aggregates with acetate buffer was assayed using the Bradford method (Bradford, 1976). 1 mL of Bradford reagent was pipetted into microcentrifuges and 20 µL of the sample was added. For a blank sample, 20 µL milliQ of water or suitable buffer were added instead of the sample. The samples were vortexed and incubated for 15 min at room temperature. The sample absorbance was measured at a wavelength of 595 nm. The concentration of protein in a selected sample was determined from the calibration curve. Bovine serum albumin (BSA) in a concentration range of 0 to 1 mg/mL was used to prepare the calibration curve.

2.3 Determination of immobilization efficiency

The immobilization efficiency is a criterion for the degree of binding of the enzyme during the immobilization process. It was calculated using Eq(1), Eq(2) and Eq(3):

$$c = \frac{A}{k} \tag{1}$$

Where:
c- the protein concentration in the sample is expressed in mg/mL,
A- measured absorbance of the sample,
k- inclination of the calibration curve for protein concetration determination using Bradford method.

$$c_p = c_e - c_s \tag{2}$$

Where:
c_p- concentration of residual proteins in pellets (mg/mL),
c_e- initial enzyme concentration (mg/mL),
c_s- concentration of proteins in supernatant after synthesis of CLEAs and mCLEAs and in supernatants after rinsing.

$$\rho\ (\%) = \frac{c_p}{c_e} \cdot 100 \tag{3}$$

Where:
ρ- immobilization efficiency, given in %,
c_p- concentration of residual proteins in pellets (mg/mL),
c_e- initial enzyme concentration (mg/mL).

2.4 Laccase activity assay

Laccase activity was spectrophotometrically determined by measuring oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) at 420 nm. For the assay, 1mM ABTS and 100 mM sodium acetate buffer were used. The activity test was performed by adding 1000 µL of acetate buffer to the free or immobilized enzyme. Just before the measurement, 200 µL of ABTS was added, the content was stirred for 1 min using vortex, and after exactly 1 min, the absorbance of the sample was measured at 420 nm on the UV/Vis spectrophotometer. The blank sample contained 1 mL of acetate buffer and 200 µL ABTS. Figure 1 shows the coloring of the samples after the performance of activity assay for laccase in dark green.

Figure 1: Coloring of the samples after performance of activity assay for laccase in dark green.

2.5 Calculation of laccase activity

The laccase activity (U/mg_{enzyme}) was calculated using Eq(4):

$$\frac{U}{mg_{enzyme}} = \frac{\left(\frac{A_{sample}}{t}\right) \cdot 1.2 \cdot df}{3.6 \cdot m_{enzyme}} \quad (4)$$

Where:
A_{sample} - absorbance of sample,
t - reaction time (1 min),
1.2 – total volume of reaction mixture (mL),
df - dilution factor,
3.6 - extinction coefficient,
m_{enzyme} – mass of enzyme in the reaction mixture (mg).

2.6 Residual activity of immobilized laccase in the form of CLEA and mCLEA

The residual activity of immobilized enzyme laccase in the form of CLEA or mCLEA (A (%)) was calculated using Eq(5).

$$A\,(\%) = \frac{\frac{U}{mg_{enzyme}}(immobilizedlaccase)}{\frac{U}{mg_{enzyme}}(nonimmobilizedlaccase)} \cdot 100 \quad (5)$$

2.7 Exposure of enzyme preparations to SC CO_2

The stability of free laccase and immobilized laccase in the form of CLEA and mCLEA in SC CO_2 at 35 °C and various pressures (10, 20 and 30 MPa) was determined. The enzyme preparations were weighed into vials and inserted into a high-pressure stirred batch reactor. The reactor was thermostated at 35 °C and filled with SC CO_2 to the desired pressure. The exposure lasted for 5 h. At the end of the exposure, slow expansion of CO_2 was carried out. All three enzyme preparations were also incubated for 5 h at 35 °C under atmospheric pressure.

3. Results and discussion

Immobilization of CLEA laccase and immobilization of laccase on MNPs by a crosslinking process for the purpose of obtaining mCLEA were successfully performed. The immobilization efficiency for different form of immobilized laccase (CLEA and mCLEA) was determined using equation 3 described in section 2.3 Determination of immobilization efficiency. Very high immobilization efficiency was detected for CLEA, while immobilization efficiency for mCLEA was lower (Table 1).

Table 1: Immobilization efficiency for CLEA and mCLEA

	Immobilization efficiency (%)
CLEA laccase	98.29 ± 2.95
mCLEA laccase	47.54 ± 1.42

The reason for lower immobilization efficiency for mCLEA was most likely in less intensive mixing due to inability to mix with a magnetic stirrer, because of magnetic properties of MNPs. Therefore, the contact between MNPs, glutaraldehyde and enzyme were weak and there was no intensive binding of the enzyme to the surface of MNPs. The enzyme activity of synthesized CLEA was 0.237± 0.007 U/mg laccase and mCLEA 0.637 ± 0.019 U/mg laccase. The reason for lower activity of CLEA in the comparison to activity of mCLEA can be the due to the fact that when the laccase was cross-linked into aggregates without a carrier (CLEA), the active sites of enzyme were turned into the inside of the aggregates, and therefore the access for the substrate to the active site of the enzyme was difficult. In the case of mCLEA preparation, a spacer arm between enzyme and carriers are formed by means of a bifunctional reagent glutaraldehyde. With the introduction of a flexible spacer arm onto the supports, the enzyme can be allowed to stretch flexibly and catch the substrate more easily. Many studies indicated that the spacer arm between enzyme and carriers could remove the enzyme away from the surface of carriers and prevent undesirable side attachment between enzyme molecules and support. This immobilization method also favours the activity retention of immobilized enzyme (Zhang et al., 2013). The influence of pressure on the stability of free laccase and immobilized laccase in the form of CLEA and mCLEA in SC CO_2 was studied. The samples were exposed to SC CO_2 for 5 h at 35 °C and different pressures (10, 20 and 30 MPa). After exposure, a slow CO_2 expansion was carried out, and then the activities of free and immobilized laccase in the form of CLEA and mCLEA were determined according to the procedure for laccase activity assay. For the comparison, all three samples (free, CLEA and mCLEA laccase) were exposed for 5 h to

35 °C at atmospheric pressure. The obtained activities were compared with the activities of the original enzyme samples to obtain residual activities. Figure 2 shows the residual activity of free laccase and immobilized laccase in the form of CLEA and mCLEA in % before and after exposure to 35 °C at 10, 20 and 30 MPa and to 35 °C at atmospheric pressure. The activities of free laccase and immobilized in the form of CLEA and mCLEA before exposure were defined as 100 %. The experiments were repeated three times. On the basis of obtained measurements, residual activity of free enzyme, CLEA and mCLEA at selected conditions was calculated.

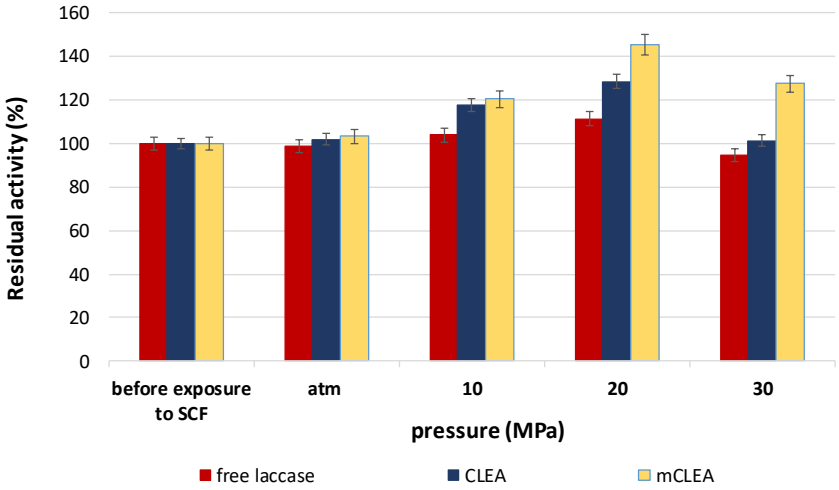

Figure 2: The influence of pressure on the stability of free laccase, CLEA laccase and mCLEA laccase in SC CO_2 at 35 °C and exposure time of 5 h. For the comparison, the stability of free laccase, CLEA laccase and mCLEA laccase was studied at atmospheric pressure and 35 °C. Standard deviations were ± 3 %.

The stability of the enzyme regardless of its shape (free or immobilized) was not affected after exposure to 35 °C at atmospheric pressure (Figure 2.). No significant differences in the residual activities in comparison to untreated enzyme samples were detected. The residual activity of free laccase increased with increasing pressure from 10 MPa to 20 MPa, and with further increase in pressure to 30 MPa, the residual activity of free laccase decreased. It is known that, in case of elevated pressures, the enzyme molecular structure can be changed and consequently its activity decreased. At 20 MPa, the activity of the free laccase increased, which can be attributed to the fact that SC CO_2 can dissolve non-polar impurities present in the basic enzyme preparation - free laccase. Thus, active sites on the enzyme after exposure to SC CO_2 were more accessible and therefore the activity of exposed free laccase was higher. At 30 MPa, however, deactivation of the enzyme occured and, consequently, lower residual activity of free laccase was detected. The activity of CLEA also increased with a pressure increase from 10 MPa (118 ± 3 %) to 20 MPa (128 ± 3 %) and with a further increase in pressure to 30 MPa, the residual activity of CLEA dropped to 101 ± 3 %. At elevated pressure, changes in the pore size of the enzyme clusters may occur; consequently, the access of the substrate to the active site on the enzyme was facilitated. At 30 MPa, deactivation of the CLEA laccase was detected and thus lower residual activity was determined. A similar trend as for free laccase and CLEA laccase was also found for mCLEA. Activity of mCLEA with an increase in pressure from 10 MPa (120 ± 3 %) to 20 MPa (145 ± 3 %) significantly increased and with further increase in pressure to 30 MPa, the residual activity of mCLEA decreased to 127 ± 3 %. However, in the case of mCLEA, the residual activity after exposure to SC CO_2 at 30 MPa is still higher than before exposure. The high residual activity of mCLEA after exposure to SC CO_2 at selected pressures could be due to the favorable influence of the pressure on the immobilized enzyme structure and the more openness and accessibility to the active site of the enzyme for the substrate.

4. Conclusions

CLEA laccase (immobilization without a carrier) was successfully synthesized and immobilization of laccase on MNPs by a crosslinking process for the purpose of obtaining mCLEA was performed. The effect of SC CO_2 on the activity of free laccase and immobilized laccase in the form of CLEA and mCLEA was studied. After exposure of all three enzyme preparations to SC CO_2, higher residual activities were achieved, which suggests that the enzyme preparations are stable in SC CO_2 under selected conditions. The highest residual activity for CLEA (128 ± 3 %) and for mCLEA (145 ± 3 %) was obtained after exposure to SC CO_2 at 20 MPa, 35 °C for 5 h. However, mCLEA showed the highest residual activity of all studied forms of laccase (free laccase, CLEA and

mCLEA) at all tested pressures. Exposure of CLEA and mCLEA in SC CO_2 greatly improved their residual activity, suggesting the possibility of using immobilized laccase in the form of CLEA and mCLEA also for enzymatic reactions that could be performed in SC CO_2 as a reaction medium. The advantage of such a system is shown in a simple separation of the enzyme from the reaction mixture and the implementation of an enzyme reaction in a medium that can be completely removed after the reaction is completed. In the case of using mCLEA as an immobilized enzyme, separation could be very simple, based on the magnetic field. Using immobilized enzymes that can be used repeatedly, process costs are greatly reduced. Such activated immobilized laccase in the form of CLEA and mCLEA could be used for various applications such as hospital wastewater treatment for removal of drugs.

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