Stability improvement of laccase for micropollutant removal of pharmaceutical origins from municipal wastewater

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Abstract
Micropollutants are persistent and hazardous materials in low concentrations (ng L−1–μg L−1), including substances such as pharmaceuticals, personal care products and industrial chemicals. The advancement of analytical chemistry has allowed for the detection of micropollutants; however, an efficient and economical treatment solution is yet to be installed. Fungal laccase has been a successful biocatalyst of these compounds. However, large-scale application of free enzyme is currently not feasible for removing water-borne micropollutants, partly due to relatively rapid loss in enzyme stability. In this paper, three types of cyclodextrin, α, β and γCD, were chosen to immobilise the laccase under various conditions with the aim to improve the stability of the enzyme. Laccase activity was chosen as a response parameter, and laccase-cyclodextrin binding was evaluated by Fourier-transform infrared spectroscopy (FTIR). Results showed an optimum using α-cyclodextrin immobilisation. At that level, α-cyclodextrin increased the half-life of laccase and slightly improved its activity in all tested pH by physically bonding to laccase. By protecting the enzyme structure, activity was maintained under a range of circumstances (acidic conditions, from 10 to 50 °C). Under room temperature and at pH 5, α-cyclodextrin-laccase nanocomposite had a better removal efficiency of diclofenac compared to free laccase of the same concentration.
Keywords Biocatalytic wastewater treatment · Micropollutant · Laccase · Immobilised enzyme · α-cyclodextrin · Enzyme stabilisation

List of symbols

\( A \) Laccase activity \([U \; L^{-1}]\)
\( A_0 \) Activity at the initial time \([U \; L^{-1}]\)
\( \text{ABTS} \) 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammomium salt
\( A_t \) Activity at a given time \([U \; L^{-1}]\)
\( \text{ATR} \) Attenuated Total Reflectance
\( \text{CD} \) Cyclodextrin
\( \alpha\text{CD} \) α-Cyclodextrin
\( \beta\text{CD} \) β-Cyclodextrin
\( \gamma\text{CD} \) γ-Cyclodextrin
\( \text{DCF} \) Diclofenac
\( \varepsilon \) The change of absorbance at 420 nm over 1 min \([\text{min}^{-1}]\)
\( \text{FTIR} \) Fourier-transform infrared spectroscopy
\( \text{HPLC} \) High-performance liquid chromatography
\( l \) Pathlength of the beam of light \([\text{cm}]\)
\( \text{L-CD} \) Laccase-cyclodextrin
\( \text{MP} \) Micropollutant
\( \text{MQ} \) Milli-Q ultrapure water
\( \text{SD} \) Standard deviation
\( t \) Time \([\text{d, min, s}]\)
\( t_{1/2} \) Half-life \([\text{d}]\)

\( U \) Unit of activity defined as the change of ABTS radical concentration in 1 min \([\mu\text{mol}\cdot\text{min}^{-1}]\)
\( V_{\text{sample}} \) Amount of the investigated sample (that contained the free or immobilised enzyme) \([\text{mL}]\)
\( V_{\text{total}} \) Total volume used \([\text{mL}]\)
\( E \) Extinction coefficient of the ABTS radical \([36000 \; \text{M}^{-1} \; \text{cm}^{-1}]\)
\( \lambda \) Decay constant \([\text{d}^{-1}]\)

Introduction

Micropollutants (MPs) come from agriculture (Heberer, 2002), industry (Barbosa et al., 2016), as well as households and municipal facilities (Jiang et al., 2013) and have been proven to have harmful effects on living organisms despite their concentration being between ng \( L^{-1} \)–µg \( L^{-1} \) (Kasprzyk-Hordern et al., 2009). The recipients of these pollutants are mostly the municipal wastewater plants, which can only withhold a fraction of the MPs (Spina et al., 2020). Subsequently, MPs appear in natural water bodies (Szymańska et al., 2019) and downstream water resources, thus risking the contamination of drinking water (Kondor et al., 2021).
The production of drinking water of sufficient purity is essential, and it is our social responsibility to keep our environment and our waters clean. Thus, developing more efficient and more environmentally friendly wastewater treatment technologies is indispensable for treating the complex and increasing quantities of hazardous pollutants of all quantities (Jain, 2012). Numerous analytical studies have pointed out weaknesses (Grandclément et al., 2017) of municipal wastewater treatment technologies regarding the removal of persistent and poorly biodegradable materials that are present in small quantities, such as anti-inflammatory drugs (e.g. diclofenac (DCF)) (Vieno and Sillanpää, 2014), various types of antibiotics (Langbehn et al., 2021), personal care products (Luo et al., 2014) and hormones (Carballa et al., 2004). Conventional wastewater treatment solutions are not able to eliminate these contaminants; the amount that is retained is enriched in the wastewater sludge (Martín et al., 2012). Other treatment technologies, such as adsorption (Younis and Mustafa, 2017), advanced oxidation processes (Yacoub et al., 2021), photocatalytic treatment (Majumdar and Pal, 2020) (Eskandarian et al., 2016), provide 40–100% removal efficiency for micropollutants. However, enzyme-enhanced membrane filtration had the least negative environmental effect (Manda et al., 2014). Of the enzymes used in wastewater treatment, such as lyases and hydrolases (Demarche et al., 2012), the most effective enzymes are the oxidoreductases (Medina et al., 2017).

In this study, laccase from Trametes versicolor was used, which can oxidise a wide range of aromatic and non-aromatic compounds (Hautphenne et al., 2016) to treat pollutants containing phenolic groups utilising oxygen as substrate (Mishra and Maiti 2019). Owing to its high activity, good stability and low toxicity, laccase can be used directly in wastewater treatment. Therefore, the activity of laccase can be increased by immobilising it on carrier materials, which determine the enzyme’s stability against environmental conditions. Immobilised laccase was used in wastewater treatment, such as for improved biological wastewater treatment (Martín et al., 2012). Other treatment technologies, such as adsorption (Younis and Mustafa, 2017), advanced oxidation processes (Yacoub et al., 2021), photocatalytic treatment (Yacouba et al., 2021), adsorption (Younis and Mustafa, 2017), and even enzymatic conversion processes (Morin-Crini et al., 2021), was chosen for the current study.

Cyclodextrins, due to their ability to form inclusion complexes (Dodziuk, 2006), are known carriers for pharmaceutical (Mura, 2020), cosmetic (Hwang et al., 2020) and food additives (Astray et al., 2009). Using this property, several attempts have been made to trap micropollutants with encouraging results; 87–99% of estrogenic compounds were removed with CD bead-polymer (Nagy et al., 2014), and CD-grafted cellulose filter retained 25 mg DCF per 1 g filter as opposed to <0.2 mg·g⁻¹ when cellulose filter without functionalisation was used (Ares et al., 2019).

In order to mitigate the activity loss of the laccase that can occur during immobilisation, instead of wrapping or grafting, the inclusion properties of CD were selected for use. This way, the enzyme is free to "move" as it is not locked into an entwined shape. Thus, the activity can be preserved while providing protection to the structure of the enzyme so that it can retain its functionality under unfavourable conditions or for a longer time. To achieve this, different immobilisation conditions using three native types of CD (αCD, βCD, γCD) were tested. The structural differences between α-, β- and γ-cyclodextrins are due to the different amounts of (1→4)-linked α-D-glucopyranosyl units that make up their backbone. The αCD ring consists of six, the βCD seven and the γCD eight (1→4)-linked α-D-glucopyranosyl units which make up their backbone. The αCD ring consists of six, the βCD seven and the γCD eight (1→4)-linked α-D-glucopyranosyl units (Hedges, 2009). The characteristics of the examined types are shown in Table 1.

| αCD | βCD | γCD | References |
|-----|-----|-----|------------|
| Solubility in water at 25 °C (g/100 mL) | 12.8 | 1.8 | 25.6 | Hedges (2009) |
| MW (Da) | 972 | 1135 | 1297 | Hedges (2009) |
| Half-life (days) | 17.5 | 17.5 | 20 | Sidler et al. (2019) |
| Outer diameter (nm) | 1.46 | 1.54 | 1.75 | Ho et al. (2017) |
| Cavity diameter (nm) | 0.47 | 0.60 | 0.75 | Ho et al. (2017) |

In addition to the immobilisation technique, the selection of the carrier material is also an important consideration; it has to be biocompatible, inert and chemically stable besides its production being straightforward and inexpensive (Fathi et al., 2019). Considering all of these requirements, a biodegradable nanoparticle, cyclodextrin (CD), which is produced from starch by enzymatic conversion (Morin-Crini et al., 2021), was chosen for the current study.

Table 1. Characteristics of the different types of cyclodextrins
Materials and methods

The citric acid and Na$_2$HPO$_4$ required to prepare the McIlvaine citrate-phosphate solution for the enzyme nanocomposite were purchased from AVANTOR. The laccase enzyme and all three cyclodextrins, which form the basis of the enzyme nanocomposite, were supplied by Merck KGaA. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) used for enzyme activity measurements were purchased from Roche Diagnostics GmbH. DCF, used for the degradation assay and analytical grade methanol and trifluoroacetic acid, used for its high-performance liquid chromatography (HPLC) analysis, were purchased from Merck KGaA. Ultra-pure water (from here MQ) was obtained from Millipore DirectQ 5 UV purification system (resistivity ≥ 18.2 MΩ cm).

Preparation of enzyme-nanocomposite

The laccase-cyclodextrin (L-CD) nanocomposites were prepared based on the work of Tarasi et al. (2018). Briefly, the enzyme nanocomposite was prepared through mixing laccase (2 mg mL$^{-1}$) and CD (2–13 mmol L$^{-1}$) solution, dissolved in McIlvaine buffer (pH 4, pH 5, pH 6). Citric acid and sodium phosphate were used individually for pH 2 and 8, respectively. The reaction mixtures were stirred at 100 rpm, at 25 °C for 90 min. Three types of native CD (αCD, βCD and γCD) were used. Three parallel measurements were taken from each sample. After the reaction time, the initial activity of the formed nanocomposites and the free laccase solutions were measured using Macherey-Nagel Nanocolor UV–Vis spectrophotometer. The steps of the experiments are displayed in Fig. 1.

Activity measurement

Measurement and calculation of free and immobilised laccase activities were carried out by oxidising ABTS (Varga, et al., 2019). The assay mixture contained free or immobilised enzyme, and ABTS in McIlvaine buffer and the change of the absorbance was detected at 420 nm. The enzyme activity value was derived from Eq. (1) in U L$^{-1}$, based on Beer–Lambert law (Swinehart, 1962) where $A$ means the activity (U L$^{-1}$), $E$ the change of absorbance at 420 nm over 1 min (min$^{-1}$), $V_{\text{sample}}$ is the amount of the investigated sample (that contained the free or immobilised enzyme) (mL), $l$ is the pathlength of the beam of light (cm), $\epsilon$ is the extinction coefficient of the ABTS radical (36,000 M$^{-1}$ cm$^{-1}$ (Childs et al., 1975)) and $V_{\text{total}}$ is the total volume used (mL). Room temperature and pH 5 were used as the base of comparison. To assess the changes of the storage stability experiments, the activity measurements were modified according to the circumstances, i.e. between 10 and 50 °C with 10 °C increments at pH 5, and at room temperature at pH 2, pH 4, pH 5, pH 6 and pH 8.

\[
A = \frac{E \times V_{\text{sample}}}{l \times \epsilon \times V_{\text{total}}}
\]  

(1)

Study of laccase-cyclodextrin binding

The Fourier-transform infrared spectroscopy (FTIR) spectra were recorded with a Varian Scimitar FTS2000 spectrometer (64 scans, 4 cm$^{-1}$ resolution) equipped with liquid nitrogen cooled MCT detector and Pike GladiATR (with diamond micro-ATR element) accessory. The water solution samples

![Fig. 1 Steps of the experimental setup](image)
were dried onto the diamond micro-ATR element under mild airflow.

**Stability study of laccase-cyclodextrin nanocomposite**

The efficiencies of the produced L-CD nanocomposites compared to the free enzyme can be verified by examining the change in enzyme activity under different extreme conditions. First, the effect of different immobilisation pHs (4, 5, 6 and MQ) and different CD concentrations (2, 4, 9, 13 mmol L⁻¹) on the enzyme shelf life was tested. During the study, the extent of activity decrease was examined for two weeks at room temperature with stirring at 100 rpm. The exponential decay constant was calculated by fitting Eq. 2 to the measured activities, where \( A_0 \) and \( A_t \) indicate the activities [U L⁻¹] at the initial and sampling time, respectively, and \( \lambda \) denotes the decay constant [d⁻¹] and \( t \) stands for the time of sampling [d]. The half-life of the enzyme (\( t_{1/2} \), [d]) was then calculated by Eq. 3.

\[
A_t = A_0 e^{-\lambda t}
\]  
\[
t_{1/2} = \frac{\ln(2)}{\lambda}
\]

From this point, the sample that ensured the best stability in storage was investigated further. The activity assay was performed at room temperature for five different pHs (2, 4, 5, 6, 8) as well as at pH 5 at different temperatures (10, 20, 30, 40 and 50 °C).

**Study of reducing the amount of diclofenac**

The efficiency of the produced L-CD nanocomposite is mainly manifested in the reduction of the micropollutant quantity. For this study, the change in the DCF concentration was examined by HPLC. Free and immobilised enzymes were added in equivalent concentration to 100 µg mL⁻¹ DCF solution in the mixed tank reactor. The reaction was incubated at 100 rpm at room temperature. After sampling, the reaction was stopped adding 50 V/V% methanol and the change in DCF concentration was measured by YL 9100 HPLC instrument (YL Instruments Co., Ltd., Gyeonggi-do, Korea) at 35 °C using UV detection at 276 nm. The DCF was separated by a Zorbax SB-Aq column (150 mm × 4.6 mm, 5 µm; Agilent, Santa Clara, CA, USA) with gradient elution. The mobile phase consisted of 0.1 V/V% trifluoroacetic acid in MQ-water as eluent A and methanol as eluent B. The gradient program consists of 0–1 min 30% mobile phase B and 1–5 min gradient up to 100% mobile phase B. This was continued for up to 8 min. After this, the gradient was returned to the initial stage. The flow rate was adjusted to 1 mL min⁻¹. Under this condition, the calibration was linear between 5 and 100 µg mL⁻¹ DCF concentrations.

**Results**

First, the effect of mixing 2 mg mL⁻¹ laccase at different pHs with different concentrations of CD was tested (Table S1). It has to be mentioned that for the experiments of βCD, a different batch of laccase was used hence the lower activity values. To eliminate this, blank measurements were taken separately for all batches of laccase; the changes were evaluated accordingly.

In the case of βCD and γCD, the higher the amount of CD was used, the lower the initial activity was measured at each pH. Regarding αCD, the initial activity of the enzyme was not affected by the concentration. The lowest initial activities were observed at pH 4 for all three types. Over time, the activity started to decline, following an exponential decay curve. The extent of change depended on the amount of CD as well as the pH. Apart from pH 6, the activity diminished after day 7. By day 7, the residual activities were between 0.4 and 5.2% for pH 4, 4.6–36.9% for pH5 and 0.7–27.9% in the MQ solution compared to the initial values. On the contrary, in pH6 buffer, the results were 55.8–85.8%, the activity values being consistently higher using 2–9 mM αCD.

The half-life of the enzyme was calculated (Fig. 2) by Eqs. 2 and 3 for each case. At pH 4, the half-life is between 1.5 and 2.7 days, for pH 5, it increases slightly to 2.8–4.5 days, and in MQ, it is 1.5–4.2 days. The greatest improvement was achieved by adding 9 mM αCD in pH 6.
buffer. The half-life, in this case, increased from 14.8 (in the blank) to 16.9 days.

The type of bonding in the solution was tested under FTIR. Based on the evaluation of the recorded infrared spectra, there was no covalent bond present between the CD support and the laccase enzyme, so the immobilisation was achieved by physical adsorption. On the infrared spectrum of the L-CD sample, no additional band appeared, or band shifts were observed compared to the sample containing only cyclodextrin or laccase.

Since the best results were achieved by applying 9 mM αCD in pH 6 buffer as an immobilising agent, this method was chosen to be used for further experiments. The effect of temperature was tested at pH 5 with and without αCD between 10 and 50 °C (Fig. 3). In this case, the relative activity was used to compare the results to one another (the highest value being 100%). While in municipal wastewater treatment, these temperatures are considered too high for the microbial processes, for industrial wastewater, it is not uncommon to have higher temperatures. Therefore, the treatment method should be suitable under those conditions, too.

As it is expected, the laccase activity marginally increased with the temperature up to 40 °C and decreased at 50 °C showing the optimal operation range of the enzyme. The positive effect of the inclusion can be detected at 10, 30 and 50 °C but only to a modest extent.

Additionally, to test the effect of pH on the catalytic reactions of the free and immobilised enzyme, ABTS was added to the solution at various pH values at room temperature (Fig. 4). The absorbance of the solution was measured the same way as for the activity measurements, only this time, the pH was adjusted to certain values. The enzyme performance after immobilisation was between 98 and 118% compared to its respective values of the free enzyme experiments.

Figures 5 and 6 show the results of treating DCF solution of 100 µg mL\(^{-1}\) at room temperature and pH 6. The reaction time was set to 24 h, and samples were taken at 15, 30, 60, 240 min and after 24 h. The decrease in the blank sample is attributed to the photolysis of the DCF.

The lowest removal percentage (20% under 24 h) was achieved with αCD. This can be attributed to its ability to form inclusion complexes with phenolic compounds such as DCF and protect them from photolysis. L-CD complex was able to transform 94% of the DCF, leaving a yellowish reaction product behind similarly to the free laccase. This was confirmed by UV–Vis spectrometry as well (Fig. 7).
Analysing the reaction mixture with HPLC-UV revealed several reaction products with similar retention times. Even without identifying the reaction products, it can be said that the different components were formed in different amounts based on the area under the detector peaks.

Depending on the circumstances, the DCF has a half-life of 0.008–21 days; higher values are measured in the dark and/or in environmental samples (Bu et al., 2016). In the conditions used, the half-life of DCF was determined as 0.73 day, 0.29 day and 0.25 day for blank, free laccase and L-CD, respectively.

**Discussion**

In this study, three different types of cyclodextrin (α, β and γ) were mixed with laccase with the aim to immobilise the enzyme to increase its stability under various circumstances while maintaining its performance.

The activity of the enzyme did not change substantially for α and γ after immobilisation. The highest value increase in reaction rate was observed in MQ using 4 and 9 mM αCD (110% at room temperature compared to free laccase). As a reference, the lowest value for 2 mM αCD was 90% at pH 6. For γCD, the lowest obtained initial activity after immobilisation was at pH 4 using 13 mM (83%) and the highest in MQ with 2 mM immobiliser (104%). On the other hand, βCD decreased the activity of the enzyme (45–96%), the lowest result being at pH 4 with 13 mM and the highest at pH 6 with 2 mM βCD. This is in correspondence with the findings of others. While in some cases, the activity can increase after the immobilisation (Ba et al., 2014), the decrease in activity is more commonly observed. For example, 16–68% of activity recovery was noted when cross-linked enzyme aggregates were prepared using various types of laccases and glutaraldehyde (Yang et al., 2016). Compared to that, the 90–110% activity recovery obtained in the present study for αCD can be considered a success.

Regarding the storability of the laccase, αCD at pH 6 passed with flying colours. At pH 6, the laccase is stable. According to Kurniawati et al. (2008), laccase is very stable at pH 6 but less so at pH 5 and pH 8. Therefore, it is understandable that the half-life values for all CD types were high at pH 6 but substantially lower for the other pHs. A positive linear correlation between the amount of CD and the half-life cannot be drawn clearly considering the results. In the case of αCD at pH 6, there is an increase between 0 and 9 mM, and the half-life drops to 14.4 days, the optimum being 16.9 days at 9 mM αCD. Considering that the half-life of the free enzyme under the same circumstances was calculated to be 14.8, this is a small improvement. If laccase has to be used at pH 6, utilising the inclusion complex formation ability of CD can be beneficial as the life-span of the enzyme can be increased by 14%. Contrary to that, the laccase from *Trametes versicolor* both in immobilised and free form seemed to be inactive in alkaline conditions. Similar results were obtained by Arca-Ramos et al. (2016). It follows that the laccase can only be used to treat acidic wastewater streams.

The temperature profiles of the free and immobilised enzyme showed that the optimal range of the laccase is between 20 and 40 °C. The initial activity compared to optimal temperature decreased by 18.3% and 14.8% at 10 °C in the case of free and immobilised enzymes. In both cases, the enzyme remained active at a higher temperature. Improvement in enzyme temperature tolerance was observed at 50 °C compared to the free laccase, similarly to the methods using covalent bonding or solid carriers (Wang et al., 2010). It is assumed that the CD slightly restricted the conformational mobility of the laccase (Zhang et al., 2012), thus increasing its stability.

The modest improvements in the stability can be explained with the FTIR results showing only weak interaction between the laccase and CD as stated by others as well (Tarasi et al., 2018). This allows the laccase to move more freely as it would be able in the case of covalent bonding, and the CD preserved its adsorption capacity, too. On the other hand, because both the laccase and DCF can form inclusion complexes with the CD, laccase might be prone to release from the immobilised form, making it more vulnerable to environmental effects.

The native CDs are able to form inclusion complexes with other compounds, mainly through van der Waals forces, hydrogen bonds and hydrophobic interactions (Li et al., 2020). Regarding micropollutants, the connection is realised via their phenolic group (Huang et al., 1997). Laccase catalyses the oxidation of phenolic compounds among other hydrogen-donating substances (Naghdi et al., 2018).
Therefore, competition may arise between the laccase and the CD for the binding of DCF, and the mechanism of DCF removal can be influenced by the sorption properties of the CD. In the case of higher CD concentration, the adsorption process is assumed to be more dominant than the enzymatic reaction. Similar effects were observed in other studies. In the case of bonding anticancer drugs to CD in a non-covalent manner, higher than optimal CD concentration increased the absorption of the drug, thereby reducing its availability (Tian et al., 2020). Molecular docking analysis of CD and pullulanase proved that CD competed with the starch substrate to bind the enzyme (Li et al., 2020). This phenomenon might have affected the ABTS reaction as well. The reduced absorbance values did not occur because of the decrease of enzyme activity but the shift in the reaction mechanism. This occurs in processes involving physical adsorption; for instance, in alginate beads, the reaction rate is modified by the diffusion of the substrate into the beads (Tanaka et al., 1984). The same yellow product appeared when using L-CD nanocomposites as observed in the free laccase solution, suggesting that the reaction mechanism of laccase and DCF was not altered by CD (Pype et al., 2019). The inclusion properties of the CD may also apply to the yellow product, which can be advantageous regarding the toxicity of the effluent. The level and mechanism of the sorption of that compound in the carrier require further study.

**Conclusion**

This article studied nanocomposites resulting from the interaction in different conditions between the three native types of cyclodextrin and laccase. Considering the residual activity of the enzyme after immobilisation, αCD showed the best results. The advantage of the chosen immobilisation method is that it could preserve the enzyme activity as opposed to literature results. Due to the weak bonding forces between the cyclodextrin and laccase, the stability improved slightly between pH 2–5 and at extremes of the examined temperature ranges (10 and 50 °C). Nevertheless, the adsorption capacity of the CD remained, which is beneficial for the micropollutant removal. This was highlighted by...
comparing the HPLC measurements of DCF removal experiments for free and immobilised laccase. The immobilisation agent did not modify the reaction mechanism of the enzyme. Therefore, besides immobilising laccase, CD may be used to concentrate the micropollutant near the enzyme and retain the substance and its reaction product from the effluent. Further experiments are planned to identify the type of bond between the intermediate products of the enzymatic reaction and the CD.

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**Data Availability** Enquiries about data availability should be directed to the authors.

**Declarations**

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

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