Construction of an oxygen detection-based optic laccase biosensor for polyphenolic compound detection

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Abstract: A fiber optic biosensor was constructed from Pleurotus ostreatus laccase for the detection of polyphenolic compounds. Laccase was immobilized on the surface of the commercially available fiber optic oxygen sensor spots by using 3-aminopropylsilanetriol, glutaraldehyde, and amino-modified carboxycellulose. A diffusion layer containing tetramethyl orthosilicate (TMOS), trimethoxymethylsilane (Tri-MOS), and polyvinyl alcohol (PVA) was added to the immobilized laccase layer. The consumption of oxygen as a result of laccase activity was monitored using a fiber optical measuring setup with catechol as a model substrate. The optimal enzyme amount was determined as 1.5 mg per 50 µL of enzyme layer mixture, and with one diffusion layer and at pH 6.9, optimum detection conditions were attained. The biosensors have high reproducibility, stability (at least 85 days if stored in PBS at 4 °C), and convenient measurement duration (ca. 25 min between two successive measurements). The biosensor was found to have a broad linear working range for catechol (40–600 µM) and to be applicable to a flow-through system. In summary, an easy-to-produce, reproducible, and stable laccase sensor with a broad linear working range was produced. The sensor has potential in the food industry as well as in environmental monitoring for the detection of phenolic compounds.

Key words: Laccase biosensor, phenolic compounds, catechol, oxygen-based optic sensor

1. Introduction

Detection of polyphenolic compounds has been drawing attention both in the environmental control of toxic contaminants (side products of industrial and agricultural processes) and in food quality analysis (Moccelini et al., 2008; Barroso et al., 2011; Prehn et al., 2012; Singh et al., 2012; Neoh et al., 2013). Detection could be done by various methods involving spectrophotometry, gas and liquid chromatography, electrochemical methods, and capillary electrophoresis (Karim and Fakhruddin, 2012; Alshahrani et al., 2014). Both spectrophotometry and chromatography, however, need time-consuming pretreatment steps. In addition, spectrophotometry is prone to interference and chromatography requires expensive instruments and is not suitable for on-site detection (Karim and Fakhruddin, 2012; Alshahrani et al., 2014). These limitations make the design of a portable device that does not require pretreatment steps inevitable for rapid on-site monitoring of phenolic compounds and biosensors are attractive candidates for this purpose.

In food analysis, laccase (Rahman et al., 2008; DiFusco et al., 2010), tyrosinase (Kim et al., 2009; Cortina-Puig et al., 2010), and peroxidase (Mello et al., 2005) enzymes have been used to detect different phenolic compound of natural origin such as chlorogenic acid (Moccelini et al., 2008), catechin (Rahman et al., 2008), caffeic acid (Cortina-Puig et al., 2010), or food additives like propyl gallate (Morales et al., 2005). The majority of currently available sensors for phenolic compound monitoring are based on electrochemical principles (Barroso et al., 2011). One disadvantage of these sensors is their limited electrochemical selectivity because of the electrooxidation of species other than the target phenolic compound. In contrast to electrochemical biosensors, fiber optic sensors have extremely selective transducers whose signals are not disturbed by diverse interferences. They also do not have direct interference from surrounding electric or magnetic fields because their signal is optic (Mazhorova et al., 2012; Narsaiah et al., 2012). Fiber optics are self-sufficient and do not require an external reference signal as electrochemical
biosensors do (Shaikh and Patil, 2012). Laccases belong to
the blue-copper family of oxidases and have been widely
used in biotechnological applications ranging from textile
and food industries to fuel cell applications (Roman-
Gusetu et al., 2009; Arora and Sharma, 2010; Tastan et al.,
2011; Andreu-Navarro et al., 2012; Betancor et al., 2013).
They could oxidize different substrates with the reduction
of oxygen to water, without hydrogen peroxide generation.

In this study, the aim was to construct a reliable and
easy-to-use laccase biosensor based on a fiber optic system
for on-site detection of phenolic compounds. The principle
of detection was based on the emission of luminescence
from oxygen-sensitive dyes contained in sensor spots.
After excitation, when the luminescent dyes encounter an
oxygen molecule, the excess energy is transferred to that
molecule through a process called dynamic quenching
(Figure 1). The light emission intensity of the sensor spot
is decreased (fewer excited dyes emit photons) as well as
the average excitation lifetime (many dyes reach ground
level rapidly). The relationship between the excitation
lifetime and the oxygen concentration is quantified by the
Stern–Volmer relationship (Carraway et al., 1991). For
precise referencing, the phase modulation technique is
applied (Trettnak et al., 1996). The sensor spot is excited
with sinusoidally modulated light from a light-emitting
diode and therefore the emitted light is also sinusoidally
modulated. The average excitation lifetime is then detected
as the phase angle between these two light emissions. To
construct a biosensor for polyphenol detection, laccase
from _Pleurotus ostreatus_ was immobilized on planar oxygen
sensor spots (SP-PSt3) using 3-aminopropylsilanetriol
(APST) for surface activation, glutaraldehyde (GA) as
a cross-linker, and amino-modified carboxycellulose
(AMC) to form a mechanically stable matrix. Thereafter,
optimum construction and working conditions, e.g.,
reproducibility, measurement duration, response time,
repeatability of response, dynamic working range,
storage stability, and applicability, were investigated.
Flow-through oxygen minisensors (FTC-PSt3) were also
prepared to develop a stable, reproducible laccase sensor
for continuous detection of phenolic compounds. The
construction method is easy and rapid and the resulting
easy-to-use biosensor could allow on-site detection
of phenolic compounds in the food industry and the
environment. The fiber optic system offers flexibility to
the design, which allows for multiple sample detection
or adaptation to flow-through systems and so could be
explored for different applications. Optic measurements,
especially fluorescence measurements, generally require
nonturbid, clear solutions and simple matrices; if not,
the signal intensity is affected. The proposed detection
method, however, involves the use of oxygen-sensitive
dye attached to a fiber optic cable. In this system, the optic
signal is formed on the biosensor surface depending on
oxygen concentration and is transferred directly to the
detector through fiber optics so the fluorescent signal will
not be affected by the turbidity of the solution.

2. Materials and methods

2.1. Materials

The fiber-optic oxygen sensors and transmitters were
purchased from PreSens Precision Sensing GmbH
(Regensburg, Germany). Laccase (EC 1.10.3.2 from
_Pleurotus ostreatus_ , ≥4.0 U/mg) and freshly prepared
catechol (on a weekly basis) were obtained from Sigma-
Aldrich. 3-Aminopropylsilanetriol was purchased from
Degussa AG Aerosil & Silanes, glutaraldehyde from Carl
Roth, and tetramethyl orthosilicate (TMOS), 2-propanol,
and trimethoxymethylsilane (Tri-MOS) from Fluka.

Figure 1. Detection of mechanism of the oxygen detection-based optic system: the emission of luminescence from oxygen-sensitive dyes contained in sensor spots depends on the amount of oxygen in the environment.
Mowiol was obtained from Kuraray and glycerol from Carl Roth.

2.2. Methods

2.2.1. Activity determination of laccase
The immobilized laccase activity was determined spectrophotometrically in 0.1 M sodium acetate buffer (pH 5) containing catechol (50 mM) or 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (5 mM) as the substrate at 404 nm (for catechol) or 414 nm (for ABTS) at room temperature. Laccase-immobilized sensor spots were fixed into cuvettes with silicone glue. All measurements were run in parallel with a negative control containing no enzyme. The value of the extinction coefficient for the oxidized products of catechol is 740 M⁻¹ cm⁻¹ and for ABTS it is 36,800 M⁻¹ cm⁻¹.

2.2.2. Construction of the laccase-immobilized SP-PSt3 sensor spots

2.2.2.1. Preparation of amino-modified cellulose
Ten grams of carboxycellulose (CC) was washed for 1 h with 200 mL of Millipore filtered deionized water containing 5 g of sodium chloride. After that, CC was vacuum filtered and a second wash was carried out with 200 mL of deionized water (Millipore) for another hour. After the vacuum filtration, a last wash was done for 1 h using 70% ethanol. The filtered CC was then resuspended in 200 mL of deionized water. To obtain amino-modified CC, 4 g of ethylenediamine-dihydrochloride (EDD) was added into CC solution in small portions under continuous stirring. Ten minutes later, 350 mg of carbodiimide (EDC) was added and the pH was set to 4.6 by the addition of 0.1 M hydrochloride acid. The solution was stirred for 4 h and the cellulose was filtered off. The cellulose was then resuspended in 200 mL of water, stirred for 15 min, and vacuum filtered. This step was repeated five times. In the next step, the cellulose was successively washed with NaOH solution (100 mL, 1 M) and water. Finally, the powder was washed with 100 mL of ether and dried over silica gel in a desiccator at room temperature for at least 2 days.

2.2.2.2. Immobilization of laccase
Planar oxygen sensor SP-PSt3 (Figure 2A), a matrix of polymers with an oxygen-quenchable luminescent dye and an additional overcoat of medical grade silicone, was used for the construction of the biosensor. These matrices were first cut into small round pieces (Ø 3 mm) and washed with 75% (v/v) 2-propanol (1 mL) and distilled water twice. The sensor’s nonshiny side was then laid on the 3-aminopropylsilanetriol (APST, 20%) overnight at room temperature under light protection for activation. The next day, the sensors were washed twice in water. In order to immobilize laccase on the sensor spots, three different solutions were prepared by dissolving enzyme (1.0, 1.5, and 3.0 mg) in sodium phosphate buffer (0.1 M, pH 6.9, 22 µL). The cross-linker (glutaraldehyde, 25%, 3 µL) and a matrix stabilizer (amino-modified carboxycellulose, AMC, 25 µL) were then added and the well-mixed suspension (50 µL per sensor) was transferred onto the upper (nonshiny) surface of the sensor. The enzyme layer dried overnight at room temperature under light protection.

2.2.2.3. Addition of diffusion layer
The diffusion layer was prepared by using TMOS:PVA:HCl:Tri-MOS in a volumetric ratio of 2:8:5:1. After preparation, 40 µL of this mixture was pipetted onto the laccase layer and left to dry overnight at room temperature under light protection. To evaluate the effect of the diffusion layer on sensor performance, the absence and presence of the diffusion layer (single or double layers) were tested. To prepare the double diffusion layer containing sensor spots, the first layer was left to dry for 2 h before pipetting the second layer.

Figure 2. a) Planar oxygen sensor SP-PSt3 and b) fiber-optic chemical sensor that is integrated in a T-shaped flow-through cell (FTC).
2.2.3.4. Measurement
After the diffusion layer dried, sensor spots were glued (RS 692-542, silicone glue) into vials that were rinsed prior to use with acetone to remove all fatty residues and sensors were left to dry for 24 h at room temperature under light protection. Phosphate buffer saline (PBS, 20 mL, pH 6.9) was added to each vial after the glue dried and vials were connected to an OXY-4 transmitter with fiber optic cables. The measurements were done under continuous stirring with the help of a magnetic stirrer with measurement intervals of 15 s. When the background signal was stabilized, an increasing concentration of catechol solution was added into vials step by step and the phase angles for different catechol concentrations were plotted.

2.2.3. Characterization of the laccase-immobilized SP-PSt3 sensor spots

2.2.3.1. Optimization of enzyme and diffusion layer amount
In order to determine the laccase amount needed for optimum response, three different enzyme amounts (1.0, 1.5, and 3.0 mg/sensor) were tested. The effect of diffusion layer presence and its quantity on sensor response was evaluated by preparing sensors with (one or two layers) or without a diffusion layer. For all cases, 1.5 mg/sensor laccase concentration was used.

2.2.3.2. Determination of optimum pH
The performances of the sensors prepared with optimum enzyme amount and diffusion layer quantity were evaluated at three different pH conditions that corresponded to acidic, neutral, and basic pH, namely 5, 6.9, and 9, using PBS as a buffering system. Since the immobilization of the enzyme and its storage were carried out at pH 6.9, this value was also used for activity assays.

2.2.3.3. Determination of dynamic working range
The maximum and minimum phenol detection limit of the biosensor was investigated in PBS buffer (pH 6.9) by using optimum sensor conditions. For the highest detection limit, catechol concentrations between 0.1 and 5.0 mM were used and were added up to 0.6 mM final concentration step by step (0.1 mM at each step), and then the buffer was refreshed. This procedure was performed before each measurement for 1 mM, 2 mM, and 5 mM catechol. After that, catechol samples with concentrations between 0.01 and 0.1 mM were used to find the lowest limit of detection and the linear working range of the biosensor.

2.2.3.4. Determination of reproducibility and hysteresis behavior
To determine the reproducibility of sensor spots, five spots were produced simultaneously and the responses of each sensor were compared using catechol as an analyte. To determine the hysteresis of the biosensors, first different concentrations of catechol were added gradually to the same sensor spot, and then the buffer was refreshed. When the signal reached equilibrium, measurements were done in the opposite order, gradually decreasing the catechol concentration, and the results were compared.

2.2.3.5. Storage stability of the biosensor
The sensor spots were stored in PBS (pH 6.9) at 4 °C, and at different time points (1, 30, and 85 days), three sensor spots were randomly taken out and activity measurements were done using catechol (5 mM).

2.2.3.6. Measurement duration of the biosensor
To determine the measurement duration of the biosensor, the time necessary for the sensor to complete detection, i.e. the time between the addition of sample and the equilibration of the signal, was measured by using samples of different catechol concentrations, where the final concentrations ranged between 0.1 and 1.5 mM.

2.2.4. Construction of the laccase-immobilized FTC-PSt3 sensor spots

2.2.4.1. Activation and immobilization
For the flow-through system, an oxygen minisensor (FTC-PSt3) was used. This is a miniaturized fiber-optic chemical sensor that is integrated in a T-shaped flow-through cell (FTC) (Figure 2B). One arm of the T-shape serves as a connection point to the FIBOX oxygen meter via a polymer optical fiber (Ø 2 mm) as the light guide. The activation was done as explained in Section 2.2.2.2. Laccase (10 mg), bovine serum albumin (BSA, 10 mg), and glycerol (6 mg) were then mixed thoroughly in 100 µL of dH2O and glutaraldehyde (25 µL, 2.5%) was added to this solution. For the immobilization, 2.6 µL of this mixture was pipetted onto the surface of each sensor and left to dry overnight at room temperature in the dark.

2.2.4.2. Addition of the diffusion layer
The diffusion layer was prepared using ethylcellulose:D4:ethanol solution (90%) with a mass ratio of 2:1:27. The prepared mixture (1.6 µL) was pipetted once onto each sensor surface and left to dry overnight at room temperature in the dark.

2.2.4.3. Measurement
Sensor spots were glued to the head of a plastic tube attached to the fiber optic cable with the help of silicone glue and were left to dry for 24 h at room temperature in the dark. Plastic tubes containing sensor spots were incubated in PBS (0.1 M, pH 6.9) overnight in order to humidify the matrix of the sensors. Measurements were done the following day with the FIBOX 3 transmitter, introducing catechol solution with a known concentration via a peristaltic pump after the background signal was stabilized.
3. Results and discussion

3.1. Optimization of the SP-PSt3 fiber optic biosensor

3.1.1. Effect of enzyme amount

Sensors with different laccase amounts (1.0, 1.5, and 3.0 mg/sensor) and without a diffusion layer were used. The change in the phase angles based on catechol concentration was plotted (Figure 3). It could be seen that sensors constructed with 1 mg of laccase could not respond effectively to the changes in catechol concentration. When the laccase amount was increased to 1.5 mg, there was a dramatic increase in the sensor response. A further increase in the laccase amount (3 mg), however, led to a decrease in the phase angle values. This could be due to the excessive amount of enzyme on the surface (an increased enzyme layer thickness), which could limit the free movement of the enzyme needed for enzymatic activity and slow the mass transport rate of the analyte. This type of behavior has been reported in the literature for different enzyme sensors (Mulchandani et al., 1999; Kushwah et al., 2011). The optimum laccase amount was therefore determined as 1.5 mg per sensor and used in further studies.

3.1.2. Effect of diffusion layer quantity

Sensors (1.5 mg of laccase) with (one or two layer) or without a diffusion layer were prepared and the results were plotted according to the changes in phase angle values (Figure 4). The presence of the diffusion layer affected the maximum phase angle reached by the system; biosensors with two diffusion layers reached a lower phase angle (ca. 48°), while biosensors with one diffusion layer reached the highest one (ca. 55°). Without a diffusion layer, the reusability of the sensors was limited because of external factors that led to the easy loss of enzyme layer from the sensor surface. This could be the reason for the lower phase angle values obtained in the absence of the diffusion layer. A biosensor with one diffusion layer was chosen for further studies.

3.2. Characterization of the biosensor

3.2.1. Effect of pH

The effect of pH was evaluated with the optimized sensor (1.5 mg of enzyme and 1 diffusion layer) and the measurements were done in PBS buffer with different pH values (5, 6.9, and 9) (Figure 5). Optimum pH was found to be shifted to pH 6.9 upon immobilization when compared with that of the free form (pH 4.5). This shift in optimum pH is generally caused by the proton exchange properties of the immobilization matrix (Cho et al., 2008). The microenvironment surrounding the immobilized enzymes affects their apparent optimum values and anionic polymers, for example, were shown to shift the pH optimum of the enzyme to more basic values (Tastan et al., 2011). The biosensor still had considerable activity at pH 5, but its activity was almost completely hindered at pH 9. It was seen that the loss of activity in alkaline conditions (e.g., pH 9.0) was not a permanent one and activity could be restored when the optimum pH value (pH 6.9) was reached again (data not shown). Laccases generally tolerate acidic conditions well (4.0–5.5), but their activity drops abruptly at more basic pH values (>6.0) (Li et al., 2014). The constructed biosensor therefore has the advantage of being used in the measurement of samples with pH values near neutral as opposed to other laccase sensors and it seemed to keep its activity considerably in mildly acidic conditions.

3.2.2. Linear working range and limit of detection (LOD) of the biosensor

The lowest detection phase value (LDPV) was calculated as 27.76 by the formula LDPV = 3 × (standard deviation...
BİLİR et al. / Turk J Biol

value of 0 mM) + (phase angle value of 0 mM). A catechol concentration of 0.04 mM yielded a phase number of 27.88 (±0.04), so it was noted as the LOD of the biosensor for catechol. When a wide range of catechol concentrations (0.1–0.6 mM) was used to determine the linear working range, a linear regression analysis result with a good $R^2$ value (0.9876) was obtained (Figure 6). When the concentration range was narrowed down to lower concentrations, however, a different linear relation with a better $R^2$ value (0.9985) was attained. This clearly showed that the biosensor could be used for the prediction of catechol concentrations over a broad range of values (0.1–0.6 mM), but the calibration curve obtained for this concentration range could fail to determine the lower concentrations with high accuracy, so one should be careful in the interpretation of the signal at low concentrations. For the optical laccase sensor developed by Abdullah et al. (2007), the LOD was determined as 0.33 mM. A similar LOD (0.3 mM) was found by Singh et al. (2013) using tyrosinase for amperometric detection of catechol with a linear working range of 1.0–6.0 mM. To obtain better LOD values, incorporation of nanoparticles was generally proposed by different groups. The usage of gold nanoparticles, for example, has been found to decrease the LOD down to 0.03 µM (Brondani et al., 2013). It could therefore be said that a better LOD value was obtained when compared with similar electrochemical biosensors without any nanoparticle incorporation.

3.2.3. Reproducibility of the biosensor

Five sensor spots were produced simultaneously according to protocol and their responses were tested with different concentrations of catechol. The slopes of phase angle vs. catechol concentration graphs were found to be similar with an acceptable standard deviation (46.8 ± 0.6), indicating that the reproducibility of the biosensor was high.

3.2.4. Hysteresis behavior of biosensor response

Different concentrations of catechol were added gradually up to 0.6 mM (1st measurement). The buffer was then refreshed and measurements were done in the opposite order by gradually decreasing the catechol concentration. During this step, buffer was changed and a specific buffer + catechol mixture was added before each measurement (2nd measurement). Results of sensor response according to phase angle values were compared (Figure 7). The biosensor showed almost the same response against the same catechol concentrations whether the catechol’s concentration was decreased or increased gradually, so no detectable hysteresis on biosensor response was observed.

3.2.5. Storage stability of the biosensor

Activity measurements of the immobilized system were done at different time intervals (1st, 30th, and 85th days after production) and phase angle vs. catechol

**Figure 5.** Optimum pH determination of the fiber optic biosensor. pH (▲) 5.0, (●) 6.9, and (■) 9.0

**Figure 6.** Linear working range of the biosensor (insert shows the behavior of the sensor at low catechol concentrations).

**Figure 7.** Comparison of 1st (■) and 2nd (●) measurements’ phase angles to determine hysteresis behavior.
concentration graphs were compared. The slope of these curves was found to be 44.0, 43.5, and 41.7, respectively, meaning that no significant change could be detected after a month and ca. 95% of the biosensor’s initial activity was retained after 85 days. This result was found to be better than that of the laccase biosensor constructed by Gomes and Rebelo (2003), who reported a 62% decrease in activity after 38 days. Abdullah et al. (2007), on the other hand, reported a comparable result with almost 90%–98% activity after 2 months of storage at 4 °C.

3.2.6. Measurement duration
When the measurements were carried out by gradually increasing the catechol concentration from 0.1 mM to 1.5 mM, a measurement period of around 13 min was needed for the stabilization of the signal. When the signal recovery after the washing step, etc. was taken into account, 25–30 min were needed between two successive sample measurements. It could therefore be concluded that the biosensor has a short measurement duration (ca. 25 min between two successive measurements) at optimum conditions, i.e. with one diffusion layer and at pH 6.9.

3.3. Applicability to flow-through system
FTC-PSt3 sensor spots were prepared considering the optimum parameters of the previous sensor (SP-PSt3) and measurements were done with randomly chosen catechol concentrations to determine the response and reliability of the sensor. Figure 8 shows that the signal could be reproduced at each attempt, showing that FTC-PSt3 sensors work properly in a flow-through system.

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References


