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Laccase-Based Biosensors: Advancements and Applications in Environmental, Biochemical and Biomedical Sensing

Angel Peshkov[1,2], Ilia Iliev[1,2], Nina Dimcheva[2,3]*

[1]Plovdiv University, Department of Biochemistry & Microbiology, 21 Kostaki Peev Str., Plovdiv, BULGARIA
[2]Plovdiv University, Centre of Technologies, 21 Kostaki Peev Str., Plovdiv, BULGARIA
[3]Plovdiv University, Department of Physical Chemistry, 24 Tsar Asen Str., Plovdiv, BULGARIA
*Corresponding author: peshkov@uni-plovdiv.bg

Abstract. Laccase-based biosensors represent a promising and innovative area of biotechnology that harnesses the natural enzymatic properties of laccase, a copper-containing oxidative enzyme, for detecting various aromatic compounds. These biosensors have garnered attention due to their high specificity, sensitivity, and eco-friendly nature, making them suitable for multiple applications, including environmental monitoring, biomedical diagnostics, and industrial process control. This overview provides insights into the fundamental principles, advantages, and key applications of laccase-based biosensors, along with emerging trends and challenges in this rapidly advancing field.

Key words: Laccase, biosensors, biotechnology, eco-friendly, enzyme immobilization.

Introduction

Aerobic organisms have undergone evolutionary adaptations to combat oxidative stress, integrating different metal ions into protein structures. A key example is copper-containing proteins like laccase, which are vital in this process (Janusz et al., 2020). Laccase (EC 1.10.3.2) is a multicopper oxidase (MCO) found across diverse biological kingdoms, including plants, fungi, insects, and bacteria. It exhibits the ability to oxidize a wide range of phenolic substrates. The enzymatic properties of laccase were first described in 1883 when the enzyme was isolated from the Japanese lacquer tree *Rhus vernicifera* and several fungal species. The laccase enzyme is a complex glycoprotein that typically contains up to 30% carbohydrates by mass and predominantly exists as a monomer with a molecular weight ranging from 50 to 90 kDa (Ansari et al., 2021; Bento et al., 2010) It is noteworthy that laccases have garnered significant attention as promising biocatalysts due to their potential applications in various industrial sectors, including pharmaceuticals, textiles, and food processing. In the scientific literature, laccases are frequently considered superior to conventional chemical methods, primarily due to their specificity and the absence of undesired side reactions during catalysis (Su et al., 2018; Fathali et al., 2019).

Laccases have been identified in over 60 fungal strains, positioning them as one of the most extensively studied and biologically significant multicopper oxidases (Giardina et al., 2010). The catalytic mechanism of laccase is well-documented, involving the formation of radical intermediates that serve as mediators in the oxidation of non-phenolic compounds. These radicals subsequently participate in various reaction pathways, forming dimers, polymers, and other oxidation products. Additionally, laccases are implicated in molecular reorganization processes that yield a diverse range of end products (Pezzella et al., 2015).

From a physiological perspective, laccases catalyze both polymerization and depolymerization reactions. In fungi, laccases are particularly well-characterized for their role in lignin degradation (delignification), whereas in insects, they contribute to cuticle sclerotization (Binning-ton & Barrett, 1988). Another well-documented function of fungal laccases is their involvement in the biosynthesis of melanin pigments. Moreover, laccases catalyze the cleavage of aromatic rings in phenolic compounds, a reaction with significant implications for environmental monitoring, food safety, and medical diagnostics, particularly concerning the control and assessment of phenolic compound levels (Mogharabi & Faramarzi, 2014).

In summary, the versatility of laccases as biocatalysts, along with their diverse physiological and catalytic roles, highlights their potential as valuable tools in both basic and applied biosciences.

Structure, properties and differences

Laccase is a member of the multicopper oxidase (MCO) superfamily and contains four copper ions, distributed across three cupredoxin domains designated as T1, T2, and T3 (T3α and T3β), within its protein structure. Based on its enzymatic activity toward tyrosine, laccases can be classified into two distinct types: true laccases and false laccases. False laccases exhibit enzymatic activity toward tyrosine (tyrosinase-like function), whereas true laccases do not exhibit this activity (Chandra & Chowdhary, 2015). Laccases can also be categorized based on spectral characteristics, the need for a mediator to facilitate catalytic activity toward non-phenolic compounds, and the source organism (Mot et al., 2012). These distinctions give rise to two primary groups: blue and yellow/white laccases. Blue laccases are more thoroughly studied in the literature, in contrast to the less-characterized yellow/white laccases (Chaurasia et al., 2013).

Further research has led to the identification of two-domain and three-domain laccases. The two-domain laccase, such as the one isolated from *Streptomyces coelicolor*, lacks the second domain present in three-domain laccases, which is essential for forming the trinuclear copper cluster. The second domain in three-domain laccases serves as a linker between the first and third domains, thereby enabling the formation of the trinuclear catalytic cluster (Machczynski et al., 2004). In the absence of this domain, the trinuclear cluster cannot form in a single laccase molecule, and oligomerization is required for catalytic activity.

A critical distinction in the application of laccases is their redox potential (Gunne et al., 2014), which varies significantly between different types. Laccases with high redox potential are commonly found in fungi, whereas those with low redox potential are more prevalent in bacteria, insects, and plants (Munk et al., 2015). This variation in redox potential is a key factor in selecting the appropriate laccase for industrial applications.

Industrial application

Laccase-based biosensors have garnered significant attention in industrial applications due to their ability to catalyze the oxidation of a wide range of substrates, making them versatile tools for environmental monitoring, food quality control, and industrial process optimization. These biosensors, which typically involve the immobilization of laccase on various electrode platforms, offer several advantages, including high specificity, sensitivity, and the potential for real-time monitoring. Laccases, primarily derived from fungi such as *Trametes versicolor*, *Cerrena unicolor*, and *Aspergillus oryzae*, are known for their ability to oxidize phenolic compounds, aromatic amines, and other substrates relevant to industrial processes. Recent advancements have led to the development of laccase-based biosensors that incorporate advanced materials such as carbon nanotubes (CNTs), gold nanoparticles (AuNPs) and graphene, which enhance the electrochemical properties, stability, and overall performance of the biosensors. These modified sensors have been successfully applied in the food industry for the detection of antioxidants, in wastewater treatment for monitoring phenolic pollutants, and in biosensing applications for pharmaceutical analysis (Tarasov et al., 2023; Mohit et al., 2020; Kadam et al., 2022).

Moreover, the integration of laccase-based biosensors in bioremediation processes and as a tool for industrial quality control highlights their growing importance. Despite their potential, challenges such as enzyme stability, reusability, and sensitivity remain, prompting ongoing research into the optimization of immobilization techniques and the development of more robust biosensor platforms (Maghraby et al., 2023). In conclusion,

laccase-based biosensors represent a promising technology for a wide range of industrial applications, with continued innovation poised to overcome current limitations and broaden their use in diverse sectors.

Enzyme immobilization techniques

Enzyme immobilization is a critical step in the development of bio-electrochemical devices, enhancing enzyme stability, industrial applicability, and reusability. Several well-established immobilization techniques, including adsorption, covalent binding, encapsulation, cross-linking, and entrapment, have been extensively studied, with laccase serving as a prime example of successful immobilization (Gonzalez-Coronel et al., 2017; Bilal et al., 2017).

Entrapment and adsorption are the simplest methods, preserving the enzyme's native structure. However, entrapment requires careful selection of an appropriate carrier with minimal toxicity and favorable electrochemical properties. A notable challenge with this method is enzyme leakage, which can reduce catalytic efficiency (Sun et al., 2015). In contrast, covalent binding and cross-linking may induce minor conformational changes in the enzyme, leading to a slight reduction in activity. Nevertheless, covalent immobilization is widely preferred due to its strong interactions between the enzyme and the electrode, ensuring robust catalytic performance (Bilal & Iqbal, 2019).

While these classic methods remain prevalent, recent advancements in enzyme immobilization have introduced high-tech approaches, such as electrospinning, 3D printing, and inkjet printing. These innovations offer enhanced efficiency and simplify the immobilization process. Additionally, novel techniques like matrix-assisted pulsed laser evaporation (MAPLE) and soft plasma polymerization (SPP) have been explored. MAPLE utilizes a laser to vaporize frozen target molecules, offering a cost-effective, simple, and versatile method for biofilm deposition. SPP, on the other hand, involves the application of non-toxic coatings at room temperature and atmospheric pressure, making it ideal for enzyme immobilization through cross-linking of thin films on solid supports (Wang et al., 2024).

Overall, the development of diverse and innovative enzyme immobilization techniques continues to advance the field of bio-electrochemical devices, offering improved efficiency, stability, and functionality.

Laccase-based biosensors – mechanism of functioning

Laccase is a bi-substrate enzyme – it oxidizes substituted phenols to corresponding quinones via one-electron pathway with concomitant reduction of molecular oxygen to two water molecules with the uptake of 4 protons and 4 electrons. When properly oriented on electrode surface, immobilized laccase is one of the few enzymes capable to exchange electrons with underlying conductive material, which is termed "direct electron transfer" (DET). However, this capability is highly dependent not only on enzyme orientation, but also on the distance between its active site and the electrode surface that shall not exceed 20 Å (Yamashita et al., 2018).

The ability of immobilized on electrodes laccase to work in DET mode is manifested by a large reductive wave when dissolved molecular oxygen is present in the operating medium. This phenomenon is also known as bioelectrocatalysis (Chen et al., 2020).

In the presence of aromatic compounds, such as substituted phenols, amines, etc., the reductive current of oxygen reduction is enhanced proportionally to the concentration of the second substrate. The latter serves as a shuttle of electrons (redox mediator) and eliminates the need for proper laccase orientation. This mode of laccase operation is also known as "mediated electron transfer" (MET) and is widely used for fabrication of laccase-based biosensors with electrochemical detection (Pimpilova et al., 2022).

Laccase-based biosensors for dopamine

Dopamine (DA), also known as 3,4-dihydroxy-β-phenylethylamine, is a catecholamine neurotransmitter that functions both as a hormone and a critical regulator in the human body. It plays a pivotal role in numerous physiological processes and is of significant medical relevance. Low levels of dopamine are associated with a variety of neurodegenerative and psychiatric disorders, including Parkinson's disease, Huntington's disease, and schizophrenia, making the monitoring of DA levels essential for both diagnosis and therapeutic management (Schindler & Bechtold, 2019).

Given its importance, accurate measurement of dopamine is critical in clinical settings. Traditional analytical methods such as chemiluminescence, spectrophotometry (Vuorensola et al., 2003), high-performance liquid chromatography (HPLC) (Tsunoda et al., 2010), and capillary electrophoresis are commonly used for quantification. However, recent advancements in electrochemical and biochemical (Bagheri et al., 2017) sensing technologies have led to the development of more efficient and rapid devices for DA detection, offering advantages such as simplicity and speed compared to conventional techniques.

For instance, a biosensor based on laccase from *Trametes pubescens* immobilized on a glassy carbon (GC) electrode, modified with porous gold, was developed by Pimpilova et al. (2022). The porous gold electrodeposited layer used in this system enhanced the effective surface area of the electrode. The gold-modified GC electrode was further pre-modified with self-assembled cystamine molecules, and the laccase was covalently bond to it via a bi-functional reagent glutaraldehyde. The resulting biosensor demonstrated excellent reproducibility and a detection limit (LOD) for DA close to the physiological levels (Pimpilova et al., 2022).

In addition, halloysite nanotubes (HNTs) have been explored as an alternative to MWCNTs for constructing dopamine biosensors. HNTs are highly biocompatible, easy to modify, non-toxic, and possess excellent thermal stability, making them ideal candidates for use in electrochemical sensors. A biosensor incorporating HNTs, imidazolium zwitterionic surfactants, graphite, and laccase from *Aspergillus oryzae* was developed for DA detection. This biosensor exhibited exceptional selectivity and was effectively utilized for DA analysis in pharmaceutical formulations (Decarli et al., 2022).

These diverse strategies demonstrate the growing potential of laccase-based biosensors in the rapid, sensitive, and selective detection of dopamine, highlighting their relevance for both medical diagnostics and pharmaceutical applications.

Laccase-based biosensors for rutin

Rutin is a flavonoid glycoside obtained primarily from *Ruta graveolens*, composed of the flavonoid quercetin bound to the disaccharide rutinose (Ganeshpurkar & Saluja, 2017). Known for its therapeutic benefits, rutin is commonly utilized in medicine due to its anti-inflammatory, antibacterial, anti-aging, and antioxidant effects. It exerts its antioxidant (Sun et al., 2013) activity by neutralizing reactive oxygen species, such as superoxide anions, hydroxyl radicals, and peroxyl radicals (Franzoi et al., 2008).

Innovative approach for rutin biosensing in pharmaceuticals involves the use of soft plasma polymerization (SPP). In this method, microdroplets of a *Cerrena unicolor C-139* laccase solution are polymerized and deposited on a glassy-carbon (GC) electrode. The suspension was applied as microdrops onto the GC electrode, producing a biosensor successfully employed for rutin detection in pharmaceutical products (Malinowski et al., 2018).

Several bioelectrochemical and electrochemical devices have been developed for the electrochemical detection of flavonoids, with particular focus on devices incorporating laccase from *Trametes versicolor*. This enzyme has been utilized in the fabrication of a glassy carbon-based biosensor for the detection of rutin. The biosensor is enhanced with multiwalled carbon nanotubes (MWCNTs), cetyltrimethylammonium bromide (CTAB), and hydroxyfullerenes (HFs) as modifying materials. CTAB aids in the dispersion of MWCNTs, while laccase facilitates the oxidation of catechol groups within the rutin structure. The resulting nanocomposite biosensor demonstrates high selectivity and sensitivity, making it suitable for the detection of rutin in serum samples (Song et al., 2022).

The incorporation of alternative materials and enzymes from diverse sources for the development of rutin-detecting biosensors has been reported in the literature. One such approach involves the use of gold nanoparticles (AuNPs) stabilized in β-cyclodextrin (CD) as a nanocomposite matrix. This composite serves as the platform for immobilizing laccase from *Aspergillus oryzae*. The biosensor construction is noteworthy, as the AuNPs-CD-laccase nanocomposite is combined with graphite mineral oil to form a homogeneous paste, which is then securely applied to a plastic cylinder and connected to a copper wire immersed in the paste. The resulting biosensor demonstrated successful application in detecting rutin in pharmaceutical samples (Brugnerotto et al., 2016).

Overall, several advanced biosensing strategies for rutin detection have been explored, particularly in pharmaceutical applications. One such innovation involves soft plasma polymerrization (SPP) is applied effectively for detection of rutin in pharmaceutical products. Additionally, laccase from *Trametes versicolor* has been integrated into glassy carbon-based biosensors, enhanced with multiwalled carbon nanotubes, CTAB, and hydroxyfullerenes to improve selectivity and sensitivity for rutin detection, particularly in serum samples. Another approach uses gold nanoparticles stabilized in β-cyclodextrin for laccase immobilization, resulting in a highly effective biosensor for rutin in pharmaceutical applications. These diverse techniques highlight the growing versatility and potential of laccase-based biosensors for rutin detection in various settings.

Laccase-based biosensors for catechol

Catechol, also known as 1,2-benzenediol, is a naturally occurring phenolic compound that plays a significant role in various biochemical processes. It is commonly found in higher plants, including tea, fruits, vegetables, and tobacco, and serves as an intermediate in the biosynthesis of other important compounds. Catechol is widely used as a raw material in several industrial applications, including the production of plastics, pharmaceuticals, and agrochemicals (Anku et al., 2017; Kulys & Bratkovskaja, 2007).

Despite its utility, catechol can be toxic to humans and other organisms. Even at low concentrations, it has been shown to cause severe damage to vital organs such as the kidneys and liver, highlighting the importance of monitoring its levels in environmental and biological samples. This toxicity is primarily due to the compound's ability to generate reactive oxygen species (ROS), which can lead to oxidative stress and subsequent cellular damage (Flickinger, 1976). Therefore, accurate detection and quantification of catechol are crucial for ensuring public health and environmental safety.

Interesting approach for catechol determination in wastewater and green tea extract has shown by Demkiv et al. (2022), where laccase from *Trametes zonatus* is incorporated with electroactive nanoparticles for development of amperometric biosensor (ABS). Authors report that the wastewater solution containing 1 mM catechol, the ABS accurately measured an average catechol concentration of 1.01 mM, showing only a 1% difference from the expected value. In green tea extract, catechol concentrations ranged from 3.4 to 3.8 mM, with an average of 3.55 mM, demonstrating a variation of less than 10%. The catechol content in the tea extract is calculated at 390 mg/L (7.8 mg/g dry weight). Authors report that these findings validate the suitability of the ABS for catechol quantification in both wastewater and green tea samples (Demkiv et al., 2022).

A highly sensitive and selective laccase biosensor is reported and developed for catechol (CC) detection, utilizing a laccase from *Trametes versicolor* immobilized onto GR-CMF modified screen-printed carbon electrode (SPCE). This is the first time such a biosensor is created. The electrochemical behavior of laccase is evaluated using different modified SPCEs, with cyclic voltammetry showing that the GR-CMF modified SPCE provided superior electrochemical performance. The high conductivity of graphene (GR) and the excellent biocompatibility of carbon microfibers (CMF) ensured stable laccase attachment on the composite-modified electrode. The biosensor demonstrates a low limit of detection (85 nM), high sensitivity (0.932 $\mu A \mu M^{-1}$ cm^{-2}), rapid response time (2 s), and a wide linear detection range (up to 209.7 μM) for CC. Additionally, the bioelectronic device exhibits excellent reproducibility and long-term storage stability. The biosensor is successfully applied in various water samples suggests its potential for environmental monitoring of CC. Looking forward, the GR-CMF composite may serve as an effective immobilization matrix for other redox-active proteins (Palanisamy et al., 2017).

According to the research, these developments underscore the growing potential of biosensors for catechol detection in various contexts.

Conclusions

Laccase-based biosensors represent a rapidly advancing field with significant potential for applications across diverse sectors, from environmental monitoring to healthcare and food safety. With their high specificity, sensitivity, and ecofriendly nature, laccase-based biosensors offer advantages over traditional chemical sensors. Although challenges such as enzyme stability and interference from complex samples remain,

ongoing research is addressing these issues, and the future of laccase-based biosensors looks increasingly promising. As technological advancements continue, laccase-based biosensors will likely become integral components of future monitoring and diagnostic systems

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