Immobilised-laccase bioreactors for wastewater treatment

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Abstract

Laccases have shown to be efficient biocatalysts for the removal of recalcitrant pollutants from wastewater. Thus, they catalyse the oxidation of a wide variety of organic compounds by reducing molecular oxygen to water. However, the use of free laccases holds several drawbacks such as poor reusability, high cost, low stability and sensibility to different denaturing agents that may occur in wastewater. Such drawbacks can be circumvented by immobilising laccase enzymes in/on solid carriers. Hence, during the last decades different approaches considering various techniques and solid carriers to immobilise laccase enzymes have been developed and tested for the removal of pollutants from wastewater. To scale up wastewater treatment bioprocesses, the immobilised laccases are placed in different reactor configurations.

1. Introduction

Urbanization, growing population and industrialization have led to a considerable release of manmade pollutants that due to their synthetic origin are recalcitrant to biodegradation, causing their persistence and accumulation in the environment. In addition, most of them are highly toxic posing wildlife and human health at risk. Therefore, the removal of the aforementioned pollutants from wastewater before being discharged in the environment is an urgent need. However, conventional wastewater treatments are inefficient in the removal of such type of pollutants and emerging technologies are either costly, non-environmentally friendly or non-feasible on a large scale. This has driven forward the search for new efficient, cost-effective and ecological wastewater treatment technologies. In this sense, the use of ligninolytic enzymes produced by certain microorganisms are particularly interesting due their broad substrate specificity and natural origin. Among the different existing ligninolytic enzymes, laccases (E.C. 1.10.3.2; p-bezenediol: dioxygen oxidoreductases) have attracted increasing interest during the last decades on account of they only need environmental molecular oxygen to exert their catalytic action producing water as the only by-product. Their catalytic site consists of four copper atoms that are classified, according to their electron paramagnetic resonance and UV-vis spectroscopy characteristics, in three types and located at different sites.[1] The type 1 copper (T1) is involved in the oxidation of the substrate. The type 2 copper (T2) and the two type 3 coppers (T3) form a trinuclear cluster where the molecular oxygen is reduced to water. The connection between the two sites (i.e., the T1 site and the T2-T3 cluster site) is guaranteed by a tripeptide coupling of amino acids (His-Cys-His) (Figure 1).[1]

Laccases are widely distributed in Nature and, thus, they have been found in bacteria, fungi, higher plants and insects.[2] Among them, laccases from white-rot fungi are particularly interesting since they have the highest redox potential. In addition, fungal laccases are often glycosylated what gives them conformational stability and protection from inactivation by radicals and proteolysis.[3,4] Also, recently the mechanism of molecular oxygen reduction for high-redox potential laccases has been described and has been found that contrary to the low-redox potential laccases, the T1 copper reduction is very fast[5] and, hence, their high appeal for biotechnological processes. Consequently, laccase production and application as a biocatalyst have been increasingly reported in the literature.[4,6,7] However, despite laccase production cost is an important issue for its industrial exploitation[8], techno-economic analyses have been scarcely reported

On the other hand, regarding laccase applications, the use of free enzymes for wastewater treatment holds different drawbacks such as non-reusability, high cost, lack of long-term stability and sensibility to different denaturing agents (e.g., pH, temperature, mechanical stress and inhibiting compounds that may occur in wastewater). In fact, there is no recorded industrial application of free laccase.[9] The above-mentioned drawbacks can be solved by immobilising laccase enzymes in/on solid carriers. The selection of a suitable solid carrier for laccase immobilisation is essential for the efficiency of the developed bioprocess. An ideal carrier should protect both laccase structure and activity under different operational and environmental conditions while keeping its own physical integrity. In addition, it should have high affinity for laccase enzyme, be inert, easily available, low-cost and environmentally friendly. Recently, Kyomuhimbo and Brink (2023) have reviewed the different supports used for laccase immobilisation.[10]

Different immobilisation techniques than can be categorised in physical (adsorption, encapsulation, entrapment) and chemical (covalent binding, crosslinking) and different solid carriers have been tested to make laccase enzymes reusable and increase their stability as recently reviewed by Alvarado-Ramírez et al. (2021).[11]Also, Zhou et al. (2021) reviewed the different immobilisation methods and carriers used to immobilise laccase as well as the application of the immobilised laccases in water purification.[12] Each immobilisation protocol presents its advantages and disadvantages (Table 1). So, there is not a preferred method for laccase immobilisation and will depend on the laccase source, the carrier, the intended application and the operating conditions.[13] Therefore, the design of new laccase immobilisation protocols is still worth of investigation. Current trends are oriented towards the use of a low quantity of laccase enzyme, minimum chemical reagents and biodegradable and low-cost materials, such as agro-industrial wastes, as solid carriers.[9] To scale up the wastewater treatment process, the immobilised laccases are placed in different reactor configurations. This review explores the reported use of immobilised-laccase reactors for pollutant removal and wastewater treatment from 2020 up to date.

2. Immobilised-laccase bioreactors for wastewater treatment

There are several bioreactor configurations that can be used with immobilised laccase enzymes for the removal of pollutants from wastewater (e.g., stirred tank, fixed bed, fluidised bed and membrane). The selection of a determined configuration and the operation strategy will depend on the reaction kinetics and the properties of the immobilisation carrier.[14] In Figure 2 different reactor configurations usually utilised for immobilised-laccase bioprocesses are schematically depicted. Likewise, in Table 2 the advantages and drawbacks of each reactor configuration are presented. Despite the numerous publications about laccase immobilisation and its applications in the removal of pollutants, there are few papers reporting the removal of pollutants by immobilised laccases in bioreactors. In Table 3 recent published research on immobilised-laccase bioreactors for wastewater treatment and removal of pollutants from aqueous solutions is gathered.

Ahmad et al. (2020) studied the degradation of tetracycline (20 mg/L) by laccase from *T. versicolor* immobilised by covalent grafting on silica monoliths in a plug tubular reactor operating at a flow rate of 1 mL/min with continuous recycling.[15] They found that tetracycline was degraded by 40-50% in 5 h. Additionally, the silica monolith immobilised laccase presented high operational stability during 75 h which, according to the authors, indicated the applicability of the developed reactors on a large scale. However, the scalability of such minireactors is dubious.

Ladole et al. (2020) immobilised laccase enzymes in peroxidase mimicking magnetic metal organic frameworks (MMOFs).[16] The immobilised biocatalysts (laccase@MMOFs), with a particle size below 100 nm, were placed in a fixed-bed reactor (working volume 50 mL) and tested for the degradation of the industrial dyes Methylene Blue (MB) and Crystal Violet (CV) in continuous mode. The former was degraded by 96% and the later by 98% in 15 min and from there onwards degradation was kept steady. However, a considerable amount of dye removal (47% for MB and 56% for CV, in 15 min) was due to MMFOs. Therefore, laccase was only responsible for the additional dye removal (about a half) which make the developed bioprocess questionable. In addition, the authors indicated neither the dye concentrations nor the hydraulic retention time used.

López-Barbosa et al. (2020) immobilised crude laccase from *Pycnoporus sanguineus* on silanised silica nanoparticles, synthesised in the presence of either water or acetone, by covalent binding with glutaraldehyde.[17] The immobilised laccase was accommodated in a flow reactor configuration and tested for the continuous decolouration of the dye Congo Red (CR). They found that CR (7 g/L) was removed by 39% with the nanoparticles synthesised in the presence of acetone. However, it was not indicated whether some amount of dye was adsorbed on the nanoparticles. Likewise, neither the working volume nor the hydraulic residence time (HRT) of the reactors were provided which makes difficult to assess the scale-up feasibility of the developed system.

Yuan et al. (2020) compared the efficiency of a horizontal rotating reactor (HRR) with laccase immobilised on strips of bacterial nanocellulose and a vertical mixing reactor (VMR) with laccase immobilised on wafers of bacterial nanocellulose for the decolouration of the textile dye Reactive Blue 19 (RB19).[18]The total volume of both reactors was 600 mL. The HRR showed a much better performance for the RB19 decolouration than the VMR. Thus, it led to about a 2-fold higher RB19 decolouration over a wider temperature range together with higher reusability and detoxification than those obtained by the VMR. This was likely due to the HRR provided higher oxygen availability and larger contact area than the VMR. However, in both cases the addition of the synthetic mediator 1-hydroxibenzotriazole was required which is neither economic nor ecological.

Zdarta et al. (2020) used a packed-bed reactor with laccase immobilised by adsorption on 3D chitin scaffolds for the continuous removal of the antibiotic tetracycline (1 mg/L). However, neither the volume of the reactor nor the HRT were indicated. Thus, it is difficult to assess the real potential of the developed approach.[19]

Girelli et al. (2021) utilised a packed-bed reactor with laccase immobilised by covalent binding on silica-chitosan carriers for the removal of phenol and a mixture of phenolics (4-methylcatechol, catechol, caffeic acid, syringic acid, vanillic acid, p-coumaric acid, and tyrosol) in continuous mode at a flow rate of 0.7 mL/min.[20] They found that phenol (525 mg/L) was degraded by 33% in 8 h and by 49% in 14 h and the phenolic mixture (525 mg/L) by 90% in 21 h. However, the volume of the reactor used was very small (about 16 mL) which makes the feasibility of the developed approach for large scale applications questionable.

Masjoudi et al. (2021) investigated mini-membrane reactors (working volume 50 mL) with laccase immobilised by covalent binding on polyvinylidene fluoride (PVDF) membranes modified with multi-walled carbon nanotubes (MWCNTs) for the removal of carbamazepine (5 mg/L) and diclofenac (5 mg/L).[21] They observed degradation efficiencies of 27% in 48 h for the former and 95% in 4 h for the latter and suggested that the developed system had potential for large scale water treatment. However, without scaling up and economic studies, such assertion is debatable.

Xia et al. (2021) tested a fixed-bed reactor with laccase immobilised on polyethylenimine functionalised magnetic nanoparticles for the removal of phenol in continuous mode.[22] They found that the degradation rate was kept over 70.3% in 48 h when operated under optimal conditions (15 mg laccase nanoparticles, 50 μg/mL phenol and 25 μL/min flow rate). Nonetheless, the volume of the solution treated was very small (27 mL) making difficult to evaluate the viability of the system for large scale applications.

Yamaguchi and Miyazaki (2021) studied the removal of the endocrine disruptor BPA (100 μM) by laccase immobilised by cross-linking on polyethylene glycol acrylamide (PEGA) resin in batch and flow reactors.[23] They found a BPA removal of 144 μM/h at 30^{o}C in the former and of 2880 μM/h at 50^{o}C in the latter. However, the volume of the batch reactor was not mentioned and that of the flow reactor was very tiny (a polytetrafluoroethylene tube of 39.25 μL). Therefore, the feasibility of the developed system for large scale applications is very uncertain.

George et al. (2022) investigated the removal of trace organic contaminants (TrOCs) from the secondary effluent of municipal wastewater by cross-linked laccase aggregates (CLEAs) in a 2-L perfusion reactor that operated in continuous mode for 500 h.[24]They reported that 60 min of operation was required to obtain the steady state for the maximum degradation (93%). The obtained results were promising but issues related to the decrease of laccase activity along time need to be solved.

Jankowska et al. (2022) built a novel enzymatic membrane reactor consisting of a nanofiltration or ultrafiltration membrane combined with laccase encapsulated in sodium alginate beads and entrapped between polystyrene electrospun fibers.[25] The developed approaches were tested for the decolouration of the azo dyes Acid Yellow 23, Direct Blue 71 and Reactive Black 5 at a concentration of 5 mg/L each and a working volume of 20 mL. They found a decolouration of almost 100% of all tested dyes during 3 successive cycles which was due to a synergistic action between the membrane and the biocatalyst. In addition, a toxicity (*Artemia salina* test) decrease of about 70% was shown. However, ABTS (0.5 mM) was used as a redox mediator which is toxic and expensive. Also, the treated volume (20 mL) was very small which makes difficult to assess the practicability of the developed biosystem to treat industrial wastewater volumes.

Lassouane et al. (2022) applied cross-linked crude laccase from *Trametes pubescens* entrapped in calcium alginate beads to remove BPA in a 2-L fluidised-bed reactor during 3 successive batches of 10 h each with increasing BPA concentrations (60, 80 and 100 mg/L).[26] A BPA degradation higher than 75% after the third successive cycle was attained, indicating the operational stability of the developed biocatalyst. Also, the addition of redox mediators was not necessary. Moreover, BPA removal was only due to laccase action since BPA adsorption onto the carrier was negligible. Therefore, the developed approach seems very promising for the removal of xenobiotics in continuous mode on a large scale.

Mehandia et al. (2022) co-immobilised a partially purified bacterial laccase and the natural mediator acetosyringone by entrapment in chitosan-clay composite beads.[27] The immobilised laccase mediator system was placed in a packed-bed reactor and applied to treat a real textile effluent operating in continuous mode. However, the volume of the reactor used was very small (about 35 mL), so more studies on a larger scale are required to test the real potential of the developed approach.

Shen et al. (2022) developed a directional microreactor with laccase immobilised by covalent binding in internal channels made of delignified wood treated with dimethylacetamide/ lithium chloride (DMAc/LiCl) which showed its feasibility for the removal of 4-nitrophenol (0.1 mM; 4-NP).[28] Thus, they found a removal rate of 94.4% of 4-NP in only 30 min and an efficiency of 86.9% was kept after 25 cycles with no evidence of laccase inactivation. The authors stated that the developed reactor was simple to prepare and easy to scale up showing great commercial application. Nevertheless, they only used 5 mL of 4-NP solution which makes their asserted suitability for industrial volumes difficult to envisage.

Sotelo et al. (2022) immobilised laccase from *Pycnoporus sanguineus* by encapsulation in alginate microbeads and by covalent binding on alumina pellets.[29] The immobilised laccases were accommodated in flow microreactors (160 µL) and assessed for the continuous removal of acetaminophen from an artificial wastewater. They found that acetaminophen (18 mg/L) was removed by 72% and 15% by the alginate and the alumina immobilised laccase, respectively, for an HRT of 30 min and a flow rate of 2 mL/h. The authors attributed the better catalytic performance of laccase immobilised into alginate microbeads to their higher porosity. However, the feasibility of scaling up the developed system is open to question.

Trivedi and Chhaya (2022) prepared a laccase nanoemulsion with a commercial laccase from *T. versicolor* , the surfactant sodium bis(2-ethylhexyl) sulfosuccinate and the organic solvent 2,2,4-trimethylpentane (isooctane).[30] Then, this laccase nanoemulsion was encapsulated in alginate beads and the produced biocatalysts were tested for the removal of the endocrine disruptor BPA. They found that the immobilised laccase was able to remove 94% of BPA in a packed-bed reactor operating a flow rate of 15 mL/h and a HRT of 2 h. In addition, the developed biocatalysts were able to remove 60% and 67% of BPA (200 mg/L) from a real industrial effluent in a packed-bed reactor (150 mg of beads and 50 mL of BPA solution) operating in batch and continuous mode, respectively, in 4 h of reaction time.

3. Conclusions

Laccase enzymes are outstanding green versatile sustainable biocatalysts that can be applied in numerous biotechnological and industrial processes. However, laccase research has mostly been carried out on laboratory scale and under controlled conditions which does not reflect the conditions encountered in industrial settings. Low productivity, low stability and limited reusability are the main issues that challenge the

production and application of laccase enzymes on an industrial scale. Past research focused on increasing laccase production by different methods and enhancing its stability, resilience and reusability by immobilisation. However, there still exist concerns about the cost-effectiveness of the developed approaches what makes their industrial feasibility questionable. Briefly, the following gaps in laccase research have to be considered in future studies: techno-economic analyses, large scale studies and laccase application under industrial conditions. It is expected that laccase technology can compete economically with the existing ones and provide a green and sustainable wastewater treatment.

CONFLICT OF INTEREST STATEMENT

The author declares that she has no conflict of interest for the publication of the manuscript.

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TABLE 1. Laccase immobilisation methods with their advantages and drawbacks.

		Advantages	**Drawbacks**
PHYSICAL METHODS	**Adsorption**	-Simple -No reagents are required -Low cost -No modification of the enzyme -Support reusability	-Enzyme desorption -Low stability -Low efficiency
	Encapsulation	-Simple -Low cost -Enzymes are stable for long time -Native conformation of the enzyme is kept	-Mass transfer limitations of the active site -Enzyme leakage -High enzyme concentration required -Pore size limitations
	Entrapment	-Simple and fast -Low cost -High stability	-Enzyme leakage -Difficult to implement at industrial level -Pore diffusion restraint
CHEMICAL METHODS	**Covalent binding**	-Prevents leaking -High heat stability -Strong binding -Facilitates the contact between enzyme and substrate	-Expensive -Complex method -Loss of activity -Chemical enzyme modification
	Crosslinking	-Strong binding -No carrier needed -Increases stability	-Might cause alteration in the active site of the enzyme -Crosslinking reagent is needed -Loss of activity -Diffusion limitations

TABLE 2. Advantages and drawbacks of the different types of reactor configurations used with immobilised laccases.

Reactor type	Advantages	Drawbacks
Stirred tank	Good homogenisation Simple operation	Difficult to scale-up Laccase inactivation due to shear forces
Fixed bed	Decrease of shear forces Easy to scale up Low operational cost	Charge losses Preferential channels Diffusion limitations Risk of bed clogging
Fluidised bed	Decrease of charge losses Good homogenization	Diffusion limitations Higher shear forces than fixed-bed Energy expenditure for bed fluidisation
Membrane	Stabilization of the enzyme if immobilised on/in the membrane Good contact between the enzyme and the substrate Washing loss of enzymes is reduced Easy to scale up	Costly Membrane fouling

TABLE 3. Removal of pollutants from wastewater in immobilised-laccase reactors from 2020 onwards.

Laccase source	Immobilisation	Reactor	Reactor	Pollutant	Reference
Trametes versicolor (Sigma-Aldrich)	Covalent grafting on silica monoliths	Continuous plug flow with recycling	Continuous plug flow with recycling	Tetracycline	[15]
n.i* (India)	Magnetic metal organic frameworks	Continuous packed bed	Continuous packed bed	Industrial dyes	[16]
Pycnoporus sanguineus CS43	Covalent binding on SiO_2 nanoparticles	Continuous flow	Continuous flow	Congo Red	[17]
Corioulus versicolor (Bio-Technology Co., Ltd.)	Adsorption on bacterial nanocellulose	Vertical mixing Horizontal rotating	Vertical mixing Horizontal rotating	Reactive Blue 19	[18]
T. versicolor (Sigma-Aldrich)	Adsorption on 3D chitin scaffolds	Packed bed	Packed bed	Tetracycline	[19]
T. versicolor (Sigma-Aldrich)	Covalent binding on silica-chitosan	Packed bed	Packed bed	Phenol Phenolic mixture	[20]
Trametes hirsuta (Novozyme)	Covalent binding on PVDF/MWCNT	Minimembrane	Minimembrane	Carbamazepine Diclofenac	[21]
T. versicolor	Magnetic nanoparticles	Fixed bed	Fixed bed	Phenol	[22]
T. versicolor (Sigma-Aldrich)	Amino PEGA resin	Batch Continuous flow	Batch Continuous flow	Bisphenol A	[23]

Laccase source	Immobilisation	Reactor	Reactor	Pollutant	Reference
T. versicolor (Sigma-Aldrich)	CLEAs	Perfusion basket	Perfusion basket	Trace organic contaminants from municipal wastewater	[24]
T. versicolor (Sigma-Aldrich)	Entrapped between polystyrene electrospun fibers	Membrane	Membrane	Azo dyes	[25]
Trametes pubescens	Crosslink with gluaraldehyde plus entrapment in alginate beads	Fluidised bed	Bisphenol A	Bisphenol A	[26]
Alcaligenes faecalis XF1	Entrapped in chitosan-clay composite beads	Packed bed	Textile effluent	Textile effluent	[27]
n.i.*	Covalent binding in microchannels of delignified wood treated with DMAc/LiCl	Microfluidic	4-nitrophenol	4-nitrophenol	[28]
P. sanguineus	Encapsulation in alginate Covalent binding on alumina pellets	Continuous flow	Acetaminoiphen	Acetaminoiphen	[29]
T. versicolor (commercial)	Nanoemulsion encapsulated in alginate beads	Packed bed	Bisphenol A Industrial wastewater	Bisphenol A Industrial wastewater	[30]

n.i.*: not indicated

FIGURE LEGENDS

FIGURE 1. Catalytic cycle of laccase.[1]

FIGURE 2. Different reactor configurations usually utilised for immobilised laccases bioprocesses.

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