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Inducers for increasing the production of fungal ligninolytic enzymes for applications in bioremediation.

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1. Abstract

White rot fungi are able to produce ligninolytic enzymes in little amounts. These enzymes have potential interest in industrial applications such as bioremediation, due to their pollutant transformation capability. That is why an increase in their enzymatic activities, and their consequent higher production, is pursued.

Several inducers' sources have been tested for that goal. They can be classified in nitrogen compounds, metal ions, aromatic compounds, and other inducers, which comprise some substances being explored to have a second use as inducers of these enzymatic activities. For example, wheat straw water effluents from industries, herbicides, surfactants, or drugs. Among the list of compounds studied, copper in its Cu^{2+} form synergically added with ar nitrogen-containing compound (yeast extract) gave the most promising results: an 80-fold increase in the laccase activity compared with a *Pleurotus ostreatus* control culture. What is more, this opens the way to further investigation of inducers added not only individually but simultaneously with compounds of different nature.

However, it is necessary to come up with the compound or method able to produce even better yields, since for the moment, no universal inducing molecules nor set of standard induction conditions have been elucidated.

Even so, a good inducer must be nontoxic in high concentrations, must be applied in mild ranges of temperature and must produce great increases, if possible, in the enzymatic activities of the three principal ligninolytic enzymes: laccases, manganese peroxidases and lignin peroxidases.

2. Introduction to the topic. Antecedents

We, the human beings, have been living for years beyond our means and resources. Nature, forests and oceans have been exploited to maximum levels in recent decades. It is inconceivable that our way of life does not have any price or consequence, because soil contamination, biodiversity loss, deserts erosion and climate change in general are concepts that, at this point, are universally acknowledged.

Although most of these problems are anthropogenic in origin, so are the solutions that are being applied to try to get rid of them. An example is the development and application of biotechnological techniques. Among these, bioremediation is one of the most promising, which consists of "the microorganism mediated transformation, degradation or detoxification of pollutants into non or less hazardous forms". (1)

The intrinsic nature- and "environment-friendliness" of bioremediation makes it appealing for application but could imply other problems. According to recent research, there is a need to use microorganisms cautiously as the presence of potential pathogenic microorganisms in the environment could be enhanced. Also, it exists a possibility to contribute to the global issue of "antibiotics and multidrug resistance". (2)

Considering the foregoing, a sustainable alternative must be chosen: the role of enzymes in pollutant transformation can be a feasible option (3). Degradative enzymes can originate from plants, bacteria or fungi and their potential is the result of complex metabolic pathways in which the compound in point is progressively transformed in simpler products able to enter the common metabolism. (4)

Among them, microbial oxidative enzymes including laccases (Lac, lacc) and three heme-binding peroxidases (manganese peroxidases (MnP), lignin peroxidases (LiP) and versatile peroxidases (VP)) have demonstrated vast potential for the pollutant remediation (5). Concretely, the ligninolytic enzymes produced by white rot fungi, which is a heterogeneous group of fungi belonging to the basidiomycetes family, are capable of decomposing lignin and degrade recalcitrant pollutants as for example: polycyclic aromatic hydrocarbons (PAHs) (6), synthetic dyes, pesticides, herbicides and other xenobiotics. They achieve these goals because they have very low substrate specificity.

However, ligninolytic enzymes from white rot fungi show a drawback: they are only produced in little amounts so they cannot be applied in industrial applications as this is costly. (7)

Even so, further research is being done and the use of natural or synthetic inducers could be an option to increase the enzymatic activity of white rot fungi. That would guarantee not only a higher and faster conversion of the target substrate, but also an

improvement in the applicability of enzyme-catalyzed processes (4). This is sometimes not easy to achieve because the enzymatic activity can be affected by inducers as well as many other factors; carbon and nitrogen concentrations are among the most critical ones. (7)

3. Aims and objectives

This bibliographic review aims to review the studies on the main compounds which have been demonstrated to induce the production of ligninolytic enzymes in fungi with particular interest on their use in bioremediation.

For this purpose, the objectives to be achieved are the following:

1. Summarize the characteristics, applications and reactions catalysed by the principal fungal ligninolytic enzymes.
2. Categorize the main groups of potential inducers by analysing and interpreting the results obtained by a selection of different authors.
3. Compare the conditions employed to apply inducers and highlight the presumable advantages and drawbacks of each kind of inducer trough the data collected.

4. Literature review

4.1. Ligninolytic enzymes

Plant cell walls are composed by a network of different compounds, mainly cellulose, hemicellulose, pectin and lignin. Regarding lignin, it is the principal non-carbohydrate structural component of the vegetal cell wall. It creates a hard, hydrophobic and insoluble barrier thanks to its network of phenolic compounds and the ester bonds through which it is attached to hemicellulose. Although these components could be independently studied, they are broken synergically by the general mechanisms of lignocellulolysis. (8)

The cited structural complexity renders its enzymatic degradation challenging. Nevertheless, several microbial enzymatic strategies have been evidenced to achieve the modification or degradation of the recalcitrant lignin matrix. (8)

Regarding its main mechanism of action, it can be classified as an "enzymatic combustion" involving several unspecific oxidative species. Though, this non-specific oxidative action might not be limited to lignin digestion alone and all the other plant cell wall components are, as cited below, prone to undergoing oxidation. (8)

A big number of ligninolytic enzymes have been identified in all kingdoms of life but this review is going to focus on the ones released by fungi. Belonging to this group we can find laccases and three heme-binding peroxidases (EC 1.11.1.-) (9): manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP). The latter ones can be classified within the heme-peroxidase superfamily associated with bacteria, fungi and plants, which is divided in three classes (I, II and III). These enzymes concretely belong to class II: secretory fungal peroxidases. (9)

4.1.1. Laccases (EC 1.10.3.2)

Laccases (Lac or lacc, EC 1.10.3.2), also named as p-diphenol oxidases, are a group of multi-copper proteins of low specificity (10). Being precise, they have four Cu atoms per active protein unit. They could exist in mono-, di- or tetramer isoforms and their pH range is quite big (2.0-8.5) (11). Many microorganisms and plants secrete laccases but so do *Pleurotus ostreatus*, *Trametes versicolor* and other white rot fungi (5).

Considering the structure, the primary one is formed by 500 amino acid residues approximately, forming three domains of a β-barrel topology. This structure is stabilized by two disulfide bridges which are placed between the domains (12). The four atoms of copper of each monomer are distributed in three redox active sites (13) called types 1, 2 and 3 (T1, T2 and T3). The catalysis of the one-electron transfer reaction occurs in these sites (14) and molecular oxygen is reduced into two molecules of water, which is advantageous with respect to the use of H_2O_2 by peroxidases. (15)

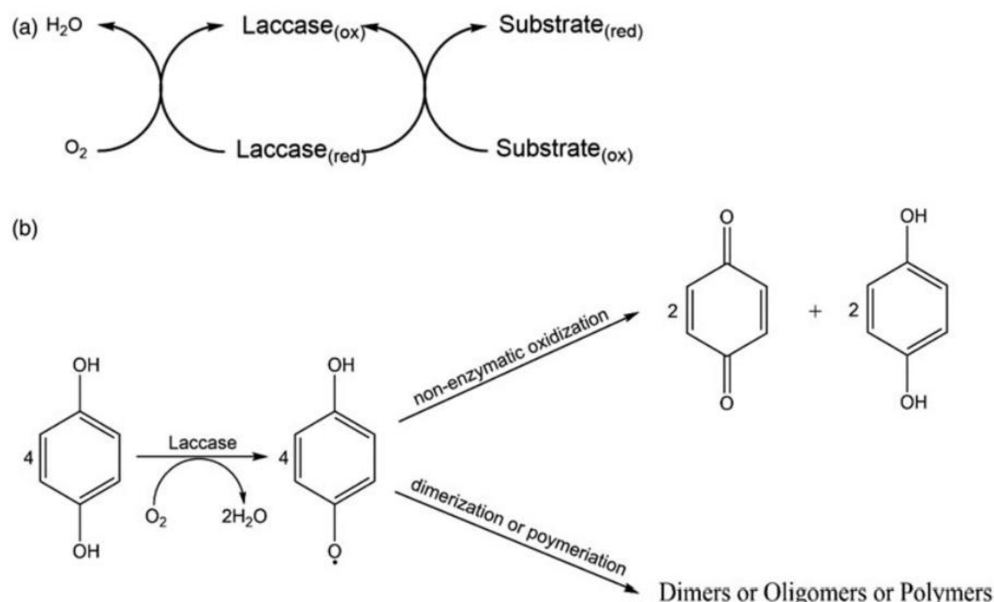

Fig. 1. (a) Scheme of laccase-catalyzed redox cycles for substrate oxidation. (b) Oxidation of hydroquinone by laccase. Source: Su J et al. Laccase: a green catalyst for the biosynthesis of poly-phenols. Crit Rev Biotechnol. 2018 Feb 17;38(2):294–307.

They have a great interest because apart from being extracellular and with low specificity, they do not require cofactors (5). What is more, laccase production has been demonstrated to be intensified by the addition of several molecules (16), as it will be discussed later in the review.

4.1.2. Manganese peroxidase (EC 1.11.1.13)

Manganese peroxidase (MnP, EC 1.11.1.13), also known as Mn-dependent peroxidase is a monomeric glycoprotein involved in the degradation of lignin. It is principally produced by a broad range of species of white-rot fungi basidiomycetes such as *Phanerochaete chrysosporium*. (17)

MnP has a molecular weight ranging from 37 to 62.5 kDa, with an average isoelectric point (pI) near 4.5. Its molecular structure is similar (43%) to LiP, and it has a mean number of 377 amino acid residues. It comprises two domains with the "heme sandwiched in-between". (5,17)

Regarding the Mn (II)-binding site, it can be stated that it is vastly flexible to hold the joining of a diversity of metal ions. MnP, apart from its five disulfide bonds, has an additional bond Cys341-Cys348 that helps forming the structure of the cited site (5). Two

calcium ions “heptacoordinate” with both proximal and distal sides of the enzyme’s cofactor (heme group) and have been elucidated to be crucial for thermally stabilizing the active site. (18)

The catalytic cycle starts in the presence of hydrogen peroxide. The enzyme oxidizes then a bound Mn^{2+} ion to Mn^{3+}, which is the main product of the reaction. Mn^{3+} is dissociated from the active site of the enzyme and stabilized against disproportionation to Mn^{2+} and insoluble Mn^{4+} by some chelators, mostly oxalate and malate. (5,17)

Usually, the enzyme oxidizes phenolic compounds as those present in the lignified cell wall. It is inactive with veratryl alcohol or non-phenolic substrates (17) but considering the last ones, MnP can only oxidize them with a second mediator (thiols such as glutathione). (15)

4.1.3. Lignin peroxidase (EC 1.11.1.14)

Lignin peroxidase (LiP, EC 1.11.1.14) is another glycoprotein, monomeric in structure, that can act on a wide range of aromatic compounds (19). Its pH range goes from 2.0 to 5.0 and possesses a high redox potential. This is of outmost importance when degrading non-phenolic aromatic substrates and organic compounds presenting high redox potentials (up to 1.4 V versus the known as standard hydrogen electrode (SHE)) when hydrogen peroxide is present, a fact that is fairly unusual among peroxidases. (20)

The enzyme is composed of about 373 amino acid residues, distributed into two main domains: proximal (C-terminal) and distal (N-terminal) (19). A heme group, its cofactor, is embedded in the active site between these domains (5,21). In both domains there are two calcium-binding sites and eight cysteine (Cys) residues, all forming four disulfide bridges. These specific links are in charge of stabilizing and maintaining the active site’s topology (3D-shape structures). (5)

The reaction catalyzed involves an initial oxidation of the heme iron by hydrogen peroxide, forming the known as “LiP compound I” at the active site. A single one-electron reduction of compound I performed by an electron derived from a substrate molecule yields “LiP compound II”. Then, a second one-electron transfer occurs and returns the enzyme to the initial ferric oxidation state. The electron transfer events allow the substrate molecule to be converted into a transient cation radical intermediate which fragments

spontaneously (19). One possible substrate could be veratryl alcohol as it not only stimulates the oxidation by preventing the enzyme to inactivate, but also acts as an electron transfer mediator in the catalytic reaction explained. (5)

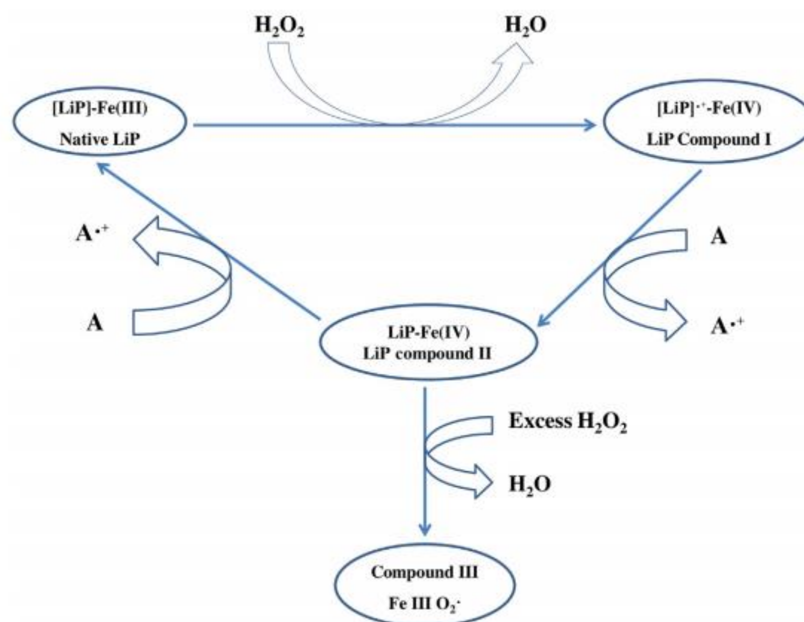

Fig.2. Catalytic structure of lignin peroxidase enzyme (LiP). Source:(5)

4.1.4. Versatile peroxidase (EC 1.11.1.16)

Versatile peroxidase (VP, EC 1.11.1.16) is a hemoprotein (22) unique in its substrate-specificity because it combines the catalytic properties of both LiP (EC 1.11.1.13) and MnP (EC 1.11.1.14) and is consequently capable of oxidizing Mn (II), phenolic and non-phenolic substrates (23). This characteristic is due to its hybrid molecular architecture, where several binding sites are available for the corresponding substrates (22), such as Mn^{2+}.

VP is also glycosylated, existing in the form of various isoforms (5) and having a molecular weight between 35 to 45 kDA with a pI range from 3.5 to 4.2. (22)

One of its main advantages is that the enzyme has been found to oxidize lignin compounds with high-redox potential in the absence of mediators. (11,24)

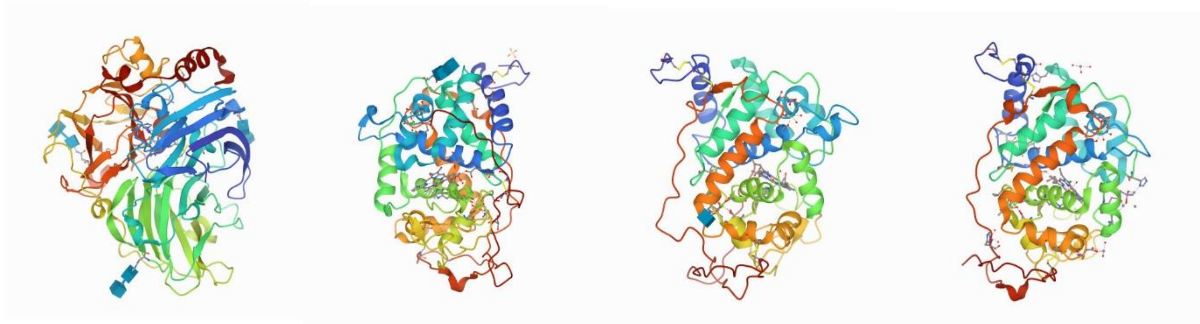

Fig. 3. (A) A laccase from T. versicolor; Protein Data Bank (PDB) ID: 1GYC. Source: (25)
(B) A manganese peroxidase from P. chrysosporium; PDB ID: 1YYD. Source: (26)
(C) A lignin peroxidase from P. chrysosporium; PDB ID: 1LGA. Source: (27)
(D) A versatile peroxidase from P. eryngii; PDB ID: 2BOQ. Source: (28)

These four examples of ligninolytic enzymes are not the only ones produced by fungi or other microorganisms. Also, other peroxidases identified not long ago are able to degrade lignin, as it is the case of dye-decolorizing peroxidases (DyPs, EC 1.11.1.19). (11)

Apart from being able to degrade lignin, the role of ligninolytic enzymes for industrial applications is extensive. Laccases may be feasible for nanotechnology and synthetic chemistry applications. Lignin and manganese peroxidases could have an application in the pharmaceutical industry and regarding versatile peroxidases, they could act as industrial biocatalysts. The three first also can be applied in the paper and pulp, food and textile industries but the unique application shared by the four of them is bioremediation.

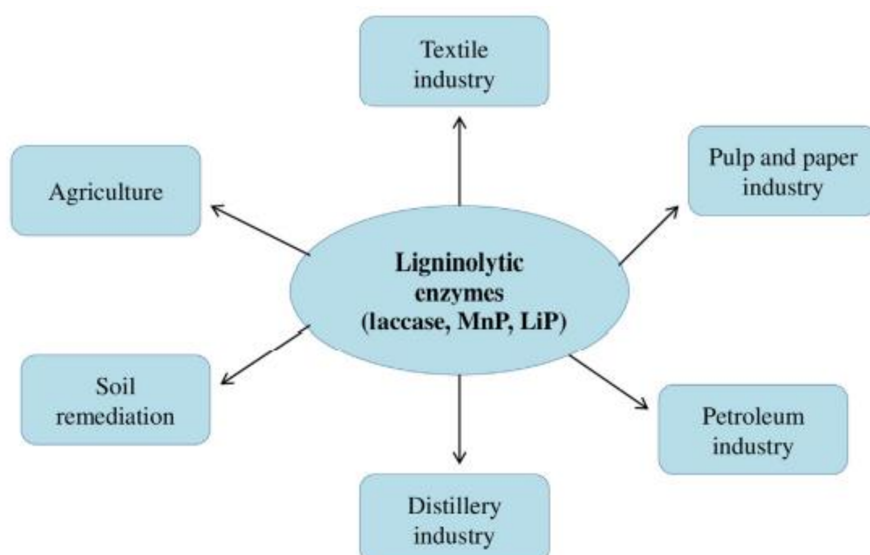

Fig. 4. Ligninolytic enzymes and their application in bioremediation of industrial pollutants and wastewaters. Source: (5)

Although these enzymes have broad range of industrial uses, its large-scale production is yet limited because of the lack of production (29). That is why supplementation of the culture medium with a suitable inducer could be an efficient method to multiply the ligninolytic enzyme's yield.

4.2. Inducers

First of all, an inducer could be defined as a specific molecule which aims to induce the synthesis of the pertinent inducible enzyme. What is more, it could play the role of substrate for the given enzyme. They can be both natural or synthetic compounds (e.g. guaiacol for inducing laccase activity). (30)

The effect of an extensive range of inducers at the level of enzyme gene transcription have been revealed for the particular case of laccases, the most studied ligninolytic enzymes. However, the mechanism behind laccase transcriptional regulation is not completely elucidated; it seems that it is a complex phenomenon involving various "putative *cis* acting elements" (31). Therefore, further research is needed to come up with the clues underlying the ligninolytic enzymes transcriptional regulation.

So many parameters and variables can be tested to prove the effect of these inducers. One of the most used is the measure of the enzyme activity (U=[μmol/min/ml]). This measurement varies a lot with the time, so it is crucial to state properly the moment of sampling. Once achieved, enzyme activities could be accurately compared, which is the next step in testing the efficiency of these molecules.

4.2.1. Nitrogen compounds

Some nitrogen substances are known to be not only a nutrition factor, but also an inducer for the ligninolytic enzyme secretion.

Levin, L. et al. tested the effect of amino acids and complex nitrogen sources (such as peptones) in the production of laccases and manganese peroxidases. The authors

incorporated them in the corresponding liquid growth medium of three different white-rot fungi strains. Concretely, fourteen amino acids, peptone, yeast extract and casamino acids were evaluated. The enzyme production results were very satisfactory especially with the effect of glutamic acid and also with peptone, which rendered the biggest amounts of both enzymes. They also got good results with aspartic acid and asparagine. Nevertheless, other amino acids suppressed MnP and laccase production. This could be due to how $-CH_3$ and $-NH_2$ groups are positioned on the linear carbon or aromatic chain (at least in the case of laccase production). (32)

Corn steep liquor (CSL), another excellent source of organic nitrogen, was explored by Wang, F. et al. not only as a nitrogen source but also as a laccase inducer. An almost 2-fold increase in enzyme activity was reported compared to control medium without CSL added, which resulted in 96,3% more laccase production relative to the control. (33)

In 2016, Zhu C. and co-workers also studied some nitrogen sources as potential inducers, including $(NH_4)_2SO_4$, NH_4NO_3, urea, yeast extract, peptone and malt extract. Among these ones, the last three of the list were the ones that increased laccase activities considerably. The exact values reported were, correspondingly, 353.33 ± 20.5 U/ml, 198.53 ± 13.58 U/ml and 165.67 ± 19.74 U/ml after eight days under cultivation with PDB medium and a final concentration of 1% for each nitrogen-containing source. If these values are compared with the control, the increases reach 5.5-, 3- and 2.6- fold, respectively (see *Figure 5*). (34)

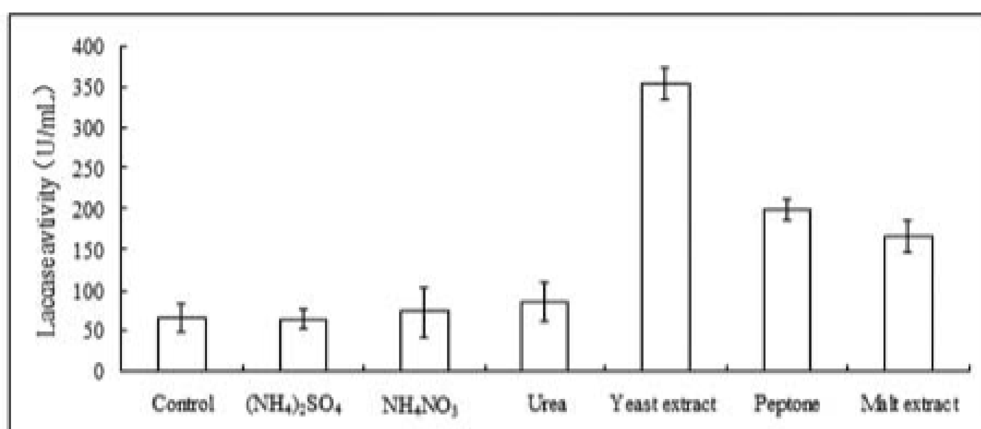

Fig. 5. Effects of potential inducers (nitrogen resources) of laccase activity in Pleurotus ostreatus (ACCC 52857). Source: (34)

4.2.2. Metal ions

Another interesting group of potential inducers are metal ions.

Tinoco R. et al. investigated in 2011 about the effect of media supplementation with copper and/or lignin on laccase production by a strain of *Pleurotus ostreatus* (*P. ostreatus CP-50*). Both substances had a strong positive impact in laccase production, but more interesting results were obtained when these compounds were added at the same time. A volumetric activity of 12 U ml^{-1} was found, which represents a higher value than the sum of activities obtained with the individual induction. So, as it can be seen in *Figure 6*, the specific productivity of this strain of *Pleurotus ostreatus* was boosted by the simultaneous use of $CuSO_4$ (Cu^{2+}) and lignin (and without obvious toxic effects, too). (35)

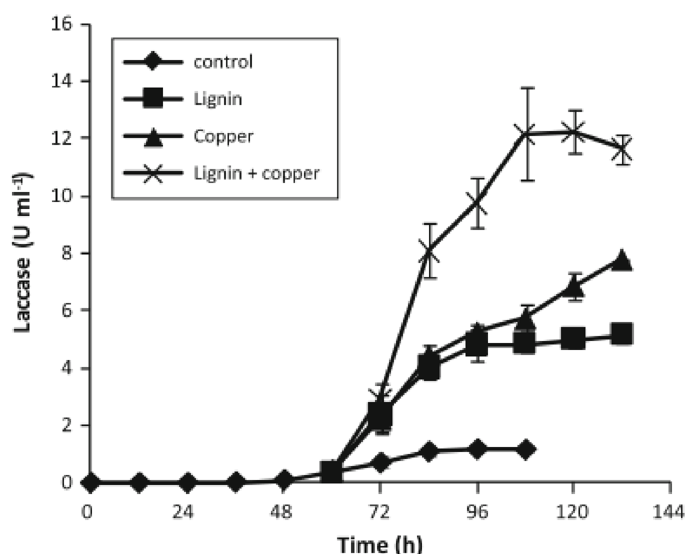

Fig. 6. Laccase production during the culture of P. ostreatus CP-50, where Cu^{2+} and/or lignin were added as inducers at the middle of the growth phase (60 h). Source: (35)

This research was one of the first to study and report better induction results due to the synergistic action of two inducers of different nature but compatible. More examples will be discussed in the following paragraphs.

Kuhar F. and Papinutti L. evaluated enzyme induction with heavy metals and phenolic compounds in two strains of *Ganoderma lucidum*. Within the metals assayed were $CuSO_4$, $MnCl_2$, $Cd(NO_3)_2$, and $Cr_2(SO_4)_3$, all in a concentration of 0.25 mM. In both strain cases, copper 0.25 mM seemed to be the best inducer, followed by manganese

0.25 mM. What is remarkable is that Cu^{2+} effects turned to be more marked in the presence of ferulic acid in high concentration. (36)

Citing again Zhu C. and colleagues, the following list of metal ions were also screened: KCl, NaCl, $MgSO_4$, $ZnSO_4$, $MnSO_4$, $CaCl_2$, $FeSO_4$, $Fe_2(SO_4)_3$, $CuSO_4$, $CdCl_2$ and $Pb(NO_3)_2$. Among these, K^+, Na^+, Mg^{+2}, Zn^{+2}, Mn^{+2}, Ca^{+2}, Fe^{+2} and Fe^{+3} did not show evident effect on laccase production. However, Cd^{+2} and Pb^{+2} generated an almost 3-fold increase in enzyme activity (189.98 ± 28.07 U/ml and 205.54 ± 13.05 U/ml, correspondingly) on the 8th day of cultivation. (34)

But undoubtedly, Cu^{+2}, which is the laccase's cofactor, showed the strongest induction, leading the activity to a peak of 412.50 ± 46.24 U/ml (6-fold increase regarding the control) with a concentration of 1 mmol/l. Further research was done then, and an even higher laccase activity was obtained after the addition of 2 mmol/l Cu^{+2} to the medium (eleven days of cultivation after): 1595.83 ± 358.57 U/ml. (34)

As it has been specified before (see *point 4.2.1*), yeast extract is the nitrogen source that, according to this research, is the most powerful inducer. Both copper and yeast extract were together investigated and effectively, there was a positive synergistic effect on laccase production in this *Pleurotus ostreatus* strain. The enzyme activity intensely raised up to a maximum of 8533.33 ± 1228.94 U/ml. This value represents a more than 80-fold expansion from the original level (see *Figure 7*). (34)

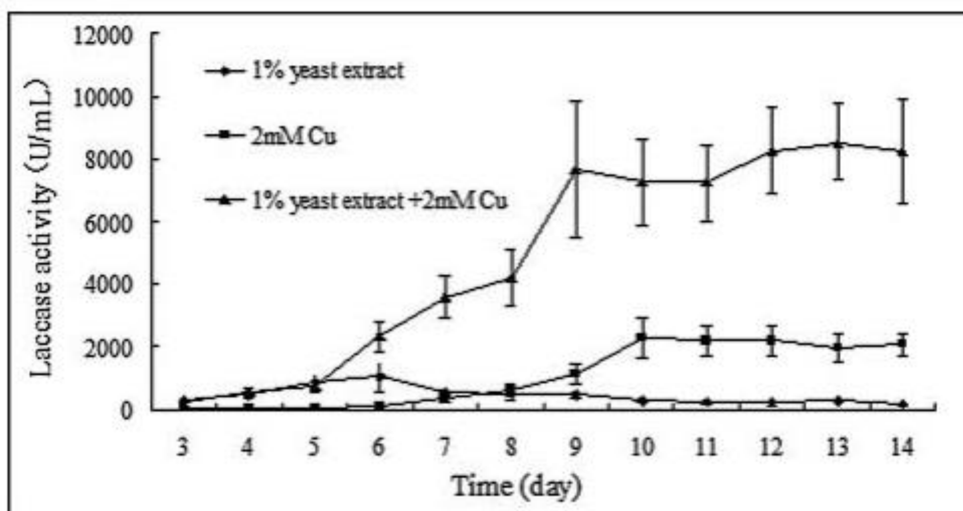

Fig. 7. Synergistic effect of yeast extract and Cu^{+2} on laccase production in Pleurotus ostreatus (ACCC 52857). Source: (34)

The role of metallic compounds as potential laccase inducers, in combination with another kind of molecule, had recently been reinforced by the study carried out by Santana TT., et al. (37). *Lentinus crinitus* was the basidiomycete selected and once again, the results were promising. The supplementation consisted of copper sulfate at different concentrations, added alone or combined with aromatic compounds such as veratryl alcohol or 2,5-xylidine. Although copper demonstrated to increase the laccase activity all alone, adding copper (Cu^{+2}, 250 μM) simultaneously with two of the substances tested (guaiacol and veratryl alcohol) achieved to increase the activity in a 151% and 138%, respectively.

Even if the induction of laccase activity is the most broadly investigated among all the ligninolytic enzymes, the induction of manganese and lignin peroxidases was also studied by Vrsanska M. et al. (38) in 2016. The inducers selected were copper sulfate ($CuSO_4$) and three copper (II)-containing complexes. Regarding the effect of copper on the activity of the LiP enzyme, the only background that Vrsanska and co-workers had was the relative to Mäkelä MR. et al. (39), but they found that the effect was variable depending on the type of inductor and strain used. However, MnP activity did not change significantly after the addition of copper inducers. This was anticipated since this activity was negligible and there were previous results in the study of Shah et al., where the production of MnP neither experienced an influence after the aggregation of copper nanoparticles (40). In view of this data, the research concluded that both copper ions and also the type of copper compounds play a relevant task in Lac and LiP activities. (38)

4.2.3. Aromatic compounds

One of the first studies about the effect of aromatic compounds as potential inducers was carried out in 2010, by Elisashvili V. et al. His colleagues and him chose different white-rot fungi and came into the conclusion that aromatic compounds may have a role as either an inducer or a repressor, depending on the fungus strain and enzyme selected. In other words, they found that there is no aromatic/phenolic compound that serves as a universal enzyme synthesis inducer. (41)

Regarding the MnP production by the first fungus, *Cerrena unicolor*, grown in synthetic mannitol-containing medium, veratric acid and pyrogallol stimulated an increase of 1.6- and 2-fold in it with respect to control. On the other hand, laccase activity was increased between 1.6- and 2.5-fold by pyrogallol, 2,6-DMP (dimethoxyphenol), catechol and veratric acid. In contrast, vanillin and vanillic acid considerably repressed laccase synthesis. Then, the same fungus but grown in medium containing EPR, was supplemented too and MnP activity increased by more than 50% thanks to the action of vanillic acid, ferulic acid, pyrogallol and veratric acid too. Laccase yield was also raised a bit by vanillin, TNT, vanillic acid and pyrogallol. (41)

Trametes versicolor, the second fungus, was grown in EPR medium too. The most important fact revealed was that all the tested aromatic compounds reduced MnP levels. Considering laccase yield, the compound which most increased it was hydroquinone. (41)

The reason why aromatic compounds stimulate laccase formation is because they are substrates for laccases, and they can send recognizable signals to fungi to activate the mechanism by which laccases are produced. This was stated by Feng X. et al. in 2013, during a study carried with an oceanic fungus: *Pestalotiopsis* sp. J63. They tested some substrates of laccases, aromatic in nature (ABTS, guaiacol and phenol) and a well-known secondary metabolite (veratryl alcohol). They all had a positive effect on laccase formation but especially phenol, which promoted mycelium growth and accelerated two isoenzymes' laccases. What is more, it demonstrated its stimulating laccase production power even when the nitrogen sources were poor in nitrogen (see *Figure 8*). (42)

Nitrogen source	Laccase activity/U·L^{-1}		Increase fold
	Without phenol	0.09 mmol·L^{-1} phenol	
wheat bran	319.5 ± 27.5	5791.7 ± 192.5	18.1
water hyacinth powder	172.5 ± 16.1	4755.6 ± 117.9	27.6
beanpod powder	169.5 ± 11.8	4119.5 ± 200.4	24.3

Fig. 8. Laccase activity induction by the addition of phenol (0.09 mmol/l) to three poor nitrogen-containing media: wheat bran, water hyacinth powder and beanpod powder. Source: (42)

Different physiological responses of laccase production depending on the fungal strain selected were also shown in the work done by Kuhar F. and co-workers with *Ganoderma lucidum*, already cited in this review. Concretely, it was found that the two

groups of inducers tested (phenolic and also metallic substances) produced diverse electrophoretic patterns of laccase activity. While in previous studies veratryl alcohol was reported to be a good inducer for a strain of *Ganoderma lucidum* and ferulic acid was supposed to have no effect on it, Kuhar F. et al found that the latter one was the best substance to increase the laccase activity, especially when combined with metals. (36)

Zhu C. and co-workers, two years later, go further with the use of aromatic compounds as inducers but decided not to use them in their following experiments because the raise up of the laccase activity in *P. ostreatus* ACCC 52857 was only of 2.5-fold in the presence of phenol, ferulic acid and ABTS. These results were less pronounced than the reported by other kind of inducers. (34)

4.2.4. Other inducers

In addition to the most explored inducers (nitrogen sources, metals or aromatic, compounds), there is a need to search for new sources of inducers. Not only to try to improve the effects they produce on enzyme activities, but also to take advantage of other substances that even if they were not thought to be potentially useful for this purpose, they end up giving good or promising induction results. Some examples are herbicides, wheat straw water extracts, surfactants, or drugs.

The first description (to their knowledge) of laccase induction by using two herbicides such as diuron and bentazon in white-rot fungi was performed by da Silva Coelho J. et al. Both compounds seemed to be a novel stimulation pathway of laccase activity. They used the fungus *Ganoderma lucidum* for their experiments. In a control medium cultured for seven days, they found a laccase activity of 20 $\mathrm{U\,g^{-1}}$. After adding bentazon and diuron, this value raised up to correspondingly 170 and 207 $\mathrm{U\,g^{-1}}$ (see *Figure 9*). Regarding MnP production in the conditions chosen, this fungus was merely able to produce this kind of enzyme. Even so, diuron herbicide achieved at least a 3-fold augmentation of the MnP activity (it went from a 0.7 $\mathrm{U\,g^{-1}}$ value in absence of the herbicides to 8.6 $\mathrm{U\,g^{-1}}$, see *Figure 9*). No lignin peroxidase was observed in the cultures filtrates. (43)

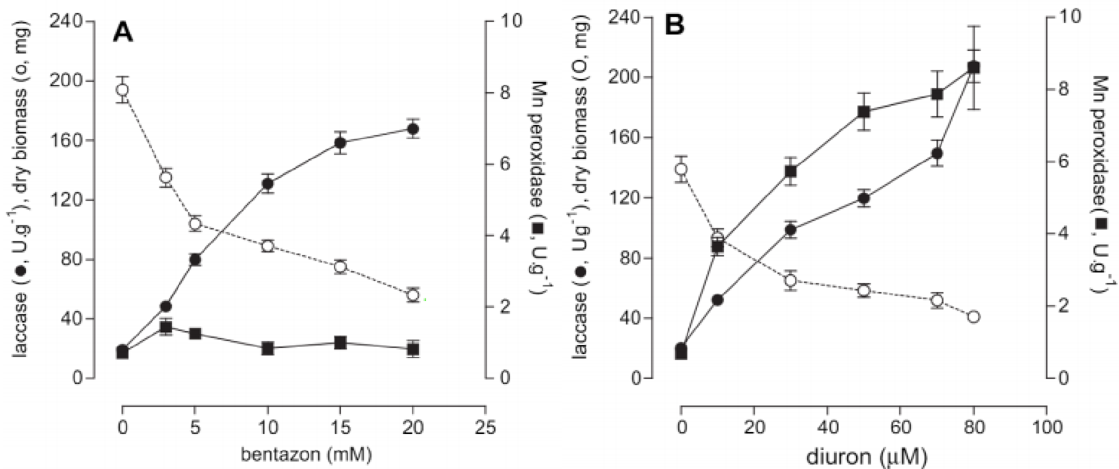

Fig. 9. Effects of bentazon and diuron concentration on growth and production of ligninolytic enzymes by G. lucidum. Various concentrations of bentazon (0 to 20 mM) and diuron (0 to 80 μM) were added at time zero of cultures. The cultures were then developed under static conditions for 7 days, at 28ºC. Source: (43)

In 2013, Parenti A. and colleagues investigated the effect of a wheat straw water extract (mainly composed by gallic acid) as inducer for the ligninolytic enzyme activities produced by two strains of the white rot fungus *Pleurotus ostreatus*. Even if the levels of phenol and manganese peroxidases were very low in the control cultures, the addition of the inducer had a vast effect on the laccase production since it was followed by a robust induction of the laccase activity recovered from the culture supernatant. Additionally, they conclude that the induction effect seemed to be cumulative and dependent on the culture and medium used. Regarding MnP activities values, they all were lower in the induced cultures than in the uninduced cultures. (44)

Surfactants (or surface-active agents) are one of the most versatile products of the chemical industry. They are amphiphilic molecules that have hydrophilic and hydrophobic parts and are therefore absorbed in the air-water interface (45). They are thought to augment the bioavailability of the less soluble substrates for the fungus so consequently, they would be ideal for increasing the production of enzymes (46). For this reason, Usha K. et al. decided to study the influence of non-ionic surfactants like Tween 20, Tween 80 and Triton X-100 on the enzyme production by *Stereum ostrea*. They all stimulated the production of Lac and MnP in the order of Tween 80, Tween 20 and Triton X-100. However, LiP activity was only enhanced with the incorporation of Tween 80 to

the medium (47). To reinforce these results, other authors have obtained improvements in enzyme excretion with the addition of surfactants such as Tween 80 in cultures of *Phanerochaete chrysosporium*. (48,49)

A recent study carried out in 2020 by Bankole PO. et al. evaluated the induction of the three principal ligninolytic enzymes (Lac, LiP and MnP) by a fungal consortium composed by *Ganoderma applanatum* and *Laetiporus sulphureus*. Concretely, they tested the induction during the degradation of some drugs (diclofenac, ibuprofen and celecoxib) and a drug mixture of Non-Steroidal Anti-Inflammatory Drugs, also known as NSAIDs. Between all these, the highest induction increase was achieved between the range of days 0 to 10, when the mixture of NSAIDs was added. The values were 201, 180 and 135% higher for the Lac, LiP and MnP secretion, respectively (see *Figure 10*). This stimulation (especially the one of laccases), could have an origin in the cyclic aromatic compounds which are part of the chemical structures of the NSAIDs used (50). Anyway, it should be pointed out that the estimation of the inducers effect in this case is not fully accurate as they are applied in a culture where two different fungal strains coexist and influence each other.

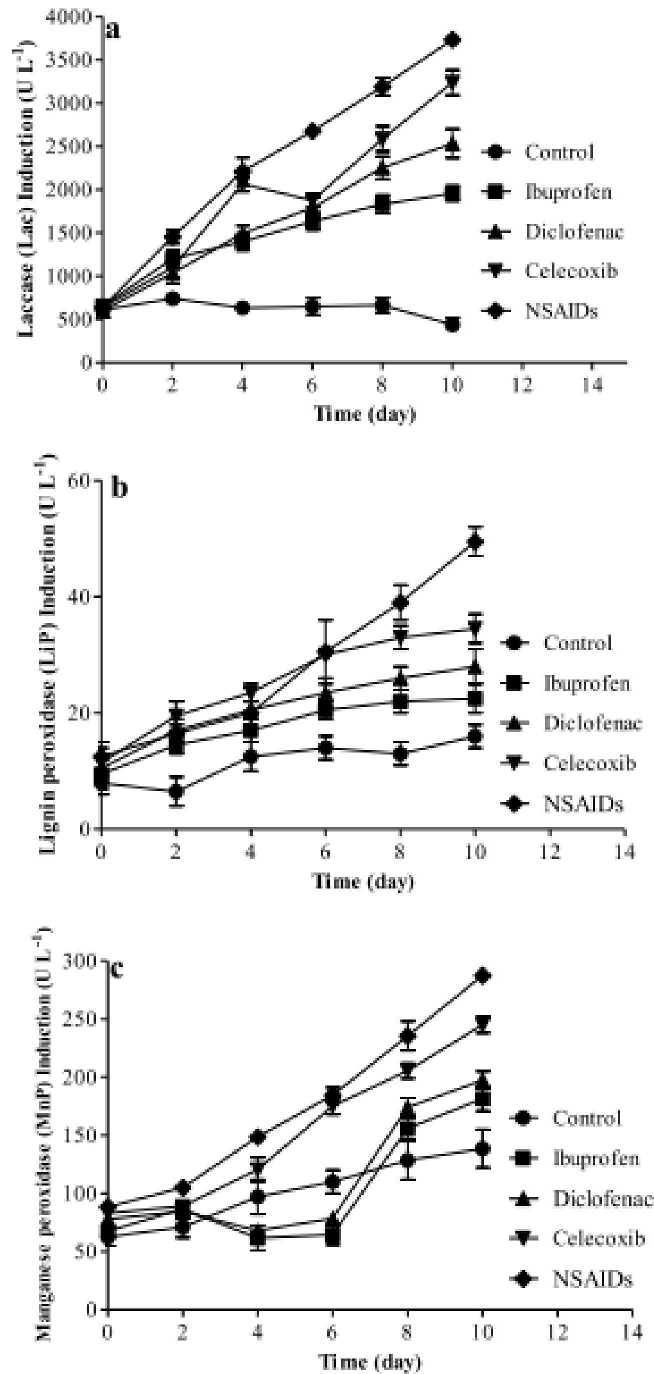

Fig. 10. Induction of extracellular enzymes of (a) laccase, (b) lignin peroxidase, (c) manganese peroxidase during the degradation of pharmaceuticals by the fungal consortium (150 rpm, pH 4.5. and Temp. 30ºC). Source: (50)

4.3. Conditions to apply inducers

The induction of the ligninolytic enzymes (Lac, LiP and MnP) is mainly dependent on various physicochemical factors such as pH, salinity or temperature (50).

Considering all the studies analysed in this review, the following chart gathers the parameters chosen in each case:

Table 1. Temperature, pH and culture days selected by the authors mentioned.

Temperature (ºC)	pH	Culture days	References
28 ± 1	6.5$^{(1)}$	28	***Levin L. et al.***
26	4.0	7	***Wang F. et al.***
25	4.5$^{(2)}$	14	***Zhu C. et al.***
30	6.0 (during culture) and 3.6$^{(2)}$	6-day old culture + 4	***Tinoco R. et al.***
Till 70	3, 4, 5, 6	20, 40	***Kuhar F. et al.***
28 ± 1	4.5	30	***Santana TT. et al.***
Not provided	4.5$^{(2)}$ (for Lac and MnP) and 3.0$^{(2)}$ (for LiP)	15	***Vrsanska M. et al.***
27	6.0$^{(1)}$	14	***Elisashvili V. et al.***
28	5.0	10	***Feng X. et al.***
28	4.5$^{(2)}$	7 days or 3 before the inducer addition	***da Silva Coelho J. et al.***
25	6.3	24	***Parenti A. et al.***
30	7.0$^{(3)}$	19	***Usha K. et al.***
30	4.5	10	***Bankole PO. et al.***

(1) Initial value of pH (adjusted), (2) pH for the enzyme assay, (3) pH of the enzyme extraction.

Temperature ranges are mild, between 25-30 ºC. Only in the case of the authors Kuhar F. et al. (36) the temperature value reach 70 ºC because they studied the thermostability of laccases. They found that the thermostability of laccases produced in the tested culture media was higher than reported in the previous literature (60 ºC).

Regarding pH, in many investigations it is only specified the pH at which the enzyme assays are performed. Even though, the tendency to apply inducers seems to be mainly acidic. For laccases, that would be in accordance with the results obtained in 2014

by Fernández-Fueyo et al. (51), who reported high induction of laccases achieved at acidic pH. What is more, this fact was corroborated years later by Bankole and co-workers (50).

Undoubtedly, the most variable parameter is the culture time. Each author chooses a major or minor period depending on the results or in their purposes. For example, Wang F and colleagues (33) choose a 7-day period of culture after adding the corresponding nitrogen source, and the highest level of laccase production was achieved in day five.

4.4. Advantages and drawbacks of inducers

The groups of inducers mentioned in this review are different in nature, but they are all applied for the same purpose: increasing the production of ligninolytic enzymes for a later use in applications such as bioremediation.

Firstly, all of them share a feature. Inducers can be applied in mild ranges of temperature (see *point 4.3*), which is advantageous for several reasons. On the one hand, these values are close to ambient temperature thus they would not suppose a control or monitoring problem. On the other hand, the costs associated would not be as high as if extreme temperatures were required.

Then, each inducer is added in different concentrations. And not all the concentrations provide the same enzyme activities; authors must test and evaluate which of them give the highest yields compared with uninduced cultures. The main results collected by the researchers mentioned in this review are available in *Annex 1*. To have a good comprehension of these results, it must be pointed out that studies do not refer to the same fungi strains nor the same culture conditions. Since fungi are complex systems, each parameter chosen somehow has influence on the others. What is more, each research group selects different methods and equipments to estimate the enzymatic activity, so the values obtained are not easily comparable. Even so, this recompilation of results reflects a few well-known facts.

Nitrogen sources, which are the first group of potential inducers, have been demonstrated to be effectively able to induce laccase and manganese peroxidase activities of several fungal strains. Among all, the addition of yeast extract to a culture of *Pleutorus ostreatus ACCC 52857* gave the highest laccase yield: 353.33 ± 20.5 U/ml, which

corresponds to a 5.5-fold increase with respect to control (34). Regarding MnP induction, Levin et al. also reported promising results, especially with the addition of glutamic acid to a culture of *Trametes villosa*. Even if the production of manganese peroxidase from the fungus was low compared with the production of laccase, a 4.9-fold increase with respect to control was reached. Finally, any of the fungal strains selected was able to secrete lignin peroxidase nor the inducers were able to increase its activity. (32)

Metal ions are the next in the list of possible inducers. Even if the results of Tinoco et al. (35) and Kuhar F. et al. (36) were not very revelatory (there is little data about enzymatic activity of control cultures, so n-fold increase cannot be calculated), other three research groups got evidence of their potential.

As Zhu and co-workers obtained a laccase activity of 412.50 ± 46.24 U/ml (6-fold increase compared to control) when adding 1 mmol/l of Cu^{2+} (34), they went further and tried with higher copper concentrations. Indeed, a peak of laccase activity was reached (1595.83 ± 358.57 U/ml) with 2 mmol/l Cu^{2+}, but the addition of 2.5 mmol/l Cu^{2+} to the medium triggered a lower laccase activity in comparison with the control group. This may seem that a high copper concentration could inhibit the mycelial growth (34).

What is more, they demonstrated that copper in its Cu^{2+} ion nature can act synergically as an inducer with other compounds, for example, yeast extract (nitrogen-containing compound). This is very advantageous because the n-fold increase was about 80-fold higher compared to control (34), which represented the maximum expansion stated and could also open the door to further investigation with other compounds.

Santana and his group (37) reinforced the fact that Cu^{2+} could give higher enzyme activities when added to the culture medium in the presence of another compound. Concretely, they obtained greater n-fold increases in the laccase activity of another fungal strain (*Lentinus crinitus*) when mixing Cu^{2+} with guaiacol or veratryl alcohol, two aromatic compounds.

Finally, the study carried out by Vrsanska and colleagues (38) contributed to the use of copper (II) and copper (II)-complexes as inducers. They obtained not only higher laccase activities but also improvements in the lignin peroxidase activities of two different fungal

species: *Trametes suaveolens* and *Daedaleopsis confragosa*. Even if these two were not great producers of lignin peroxidase in control cultures, an increase was reached with the use of the copper (II)-compounds synthesized by the group: M1 0.1 mM ($[Cu(im)_4(H_2O)_2](btc) \cdot 3H_2O$, where im=imidazole) and M3 0.3 mM ($[Cu_3(mdpta)_4(btc)](ClO_4)_3 \cdot 4H_2O$, where mdpta = *N,N*-bis-(3-aminopropyl) methylamine). The production of manganese peroxidase by these fungal strains was insignificant.

Next, aromatic compounds have also been studied to induce the ligninolytic enzyme activity for a long time. Among all the aromatic sources tested by different investigation groups, hydroquinone (41) and phenol (34,42) provided the most satisfactory results in the role of inducers. However, the n-fold increases did not exceed the 3-fold, so this fact may reveal that they do not have the greatest inducer potential if compared with other groups of compounds. Regarding which enzyme activities were demonstrated to enhance, any of the four fungal strains was not able to produce lignin peroxidase. Consequently, aromatic compounds could not be tested as inducers for this type of ligninolytic enzyme.

In addition to these well-known compounds, some researchers innovated by testing new possible inducers. To give an example, da Silva Coelho et al. (43) used two herbicides (diuron and bentazon) and Parenti A. and co-workers (44) tried with a wheat straw water extract (which was mainly composed by gallic acid). This suppose an advantage since they gave a “second life” to these products who were not principally thought to have a role as inducers of ligninolytic enzymes.

Next, Usha et al. (47) took advantage of the intrinsic versatility of surfactants and tested three of them as potential inducers. Only Tween 80 was able to increase the production of the three principal ligninolytic enzymes (Lac, MnP and LiP) in a culture of *S. ostrea.* The other two, Tween 20 and Triton X-100, proved to increase at least Lac and MnP activity. The most relevant fact revealed was that all of them were not toxic and did not affect the growth of the fungi (47), which may be the chief benefit of these compounds.

Finally, Bankole PO. and his group (50) used a consortium of two fungal strains (*G. applanatum and L. sulphureus*) to explore different drugs in the enzyme induction. Both together were able to produce Lac, MnP and LiP and regarding the compounds explored, NSAIDs gave a 6-, 5.8- and 5-fold increase, respectively (50). Again, researchers gave a second chance to these compounds and opened the door to further investigation of the use of fungi consortia, since this fact might give important advantages in the ligninolytic enzyme production.

5. Conclusions

The scientific information included in this bibliographic review has been obtained from various databases (including ScienceDirect, PubMed, Google Scholar, BRENDA and ExPASy) and from books and web pages available on the Internet. There is agreement in the information provided, especially in not only the need but also the feasibility of using compounds that serve as inducers to increase the production of fungal ligninolytic enzymes.

1. The characteristics of the four main ligninolytic enzymes were found in specific databases: BRENDA, ExPASy and the Protein Data Bank (PDB). The reactions catalysed by them were also outlined accessing these sources and other general reviews. Regarding the applicability, a vast number of articles and reviews showed that they can be applied for tasks of outmost importance such as bioremediation. Among all, laccases are the most explored by researchers.
2. A big list of studies served to classify the inducers in four groups: nitrogen sources, metal ions, aromatic compounds, and other possible inducers. The most revealing results were stated, being the comparison of enzymatic activities and the n-fold increases crucial to confirm their potential role.
3. Even if a unification of conditions to apply inducers was expected, the reality is that there is little data about the pH at which inducers are added (authors only state the pH at which the enzyme assay is performed), culture days are very variable depending on the fungal strain used and so is the culture nutrient composition. What seems to be general is that inducers are commonly applied in mild ranges of temperature. Although the data available in literature is not easily comparable, because fungi are complex systems, some compounds are probably more advantageous than others:

- Nitrogen sources apparently induce the production of both laccases and manganese peroxidases, a feature not shared by all the inducers as many of them only induce the first one.

- Copper in its Cu^{2+} form gives high laccase yields and is thought to give even better ones when synergically added with other different compounds. However, it may be toxic when present in high concentrations.

- Aromatic compounds are also able to raise the ligninolytic enzyme activity but not in a very noticeable way.

- There is a need to keep testing other possible inducers that were not principally thought to have this role but are given a second life in that way.

6. References

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7. Annexes

Annex 1. *Summary of the conditions chosen and results obtained in the studies cited in this bibliographic review (see point 6: References). Studies marked in green refer to the use of nitrogen compounds, those marked in pink refer to metal ions, the ones marked in yellow refer to aromatic compounds and finally, the studies coloured in blue refer to "other inducers" group.*

Investigation	Fungal strain	Inducer source	Inducer concentration	Lac/MnP/LiP activity (CONTROL)	Lac activity (induced)	MnP activity (induced)	LiP activity (induced)	n-fold increase or % increase
Levin L. et al. (2010)	*Trametes trogii*	Glutamic acid	0.75 g N/l	148.6 U/ml 1.67 U/ml	188.3 U/ml	4.5 U/ml	-	1.27-fold 2.69-fold
		Peptone		148.6 U/ml 1.67 U/ml	124.1 U/ml	3.1 U/ml	-	< 1.9-fold
	Trametes villosa	Glutamic acid		31.15 U/ml 0.61 U/ml	73.8 U/ml	3.0 U/ml	-	2.4-fold 4.9-fold
		Peptone		31.15 U/ml 0.61 U/ml	36.1 U/ml	0.6 U/ml	-	1.2-fold =
	Coriolus versicolor	Glutamic acid		8.10 U/ml 0.15 U/ml	9.1 U/ml	0.3 U/ml	-	1.1-fold 2-fold
		Peptone		8.10 U/ml 0.15 U/ml	25 U/ml	0.5 U/ml	-	3.1-fold 3.3-fold
Wang F. et al. (2014)	*Trametes versicolor*	Corn steep liquor	5-40 g/l	322.6 U/ml	633.3 U/ml	-	-	1.96-fold
Zhu C. et al. (2016)	*Pleurotus ostreatus ACCC 52857*	Yeast extract	1%	≈ 64 U/ml	353.33 ± 20.5 U/ml	-	-	5.5-fold
		Peptone		≈ 64 U/ml	198.53 ± 13.58 U/ml	-	-	3-fold
		Malt extract		≈ 64 U/ml	165.67 ± 19.74 U/ml	-	-	2.6-fold
Tinoco et al. (2011)	*Pleurotus ostreatus CP-50*	Cu^{2+}	-	-	≈ 7 U/ml	-	-	-
		Cu^{2+} + lignin	-	-	12.2 U/ml	-	-	30 %
Kuhar F. et al. (2014)	*Ganoderma lucidum E47*	Cu^{2+}	0.25 mM	-	1.0 U/g	-	-	-
		Mn^{2+}	0.25 mM	-	0.75 U/g	-	-	-

Zhu C. et al. (2016)	*Pleurotus ostreatus ACCC 52857*	Cu^{2+}	1 mmol/l	≈ 64 U/ml	412.50 ± 46.24 U/ml	-	-	6-fold
		Cu^{2+}	2 mmol/l	≈ 64 U/ml	1595.83 ± 358.57 U/ml	-	-	25-fold
			2.5 mmol/l	≈ 64 U/ml	<	-	-	<
		Cu^{2+} + Yeast extract	2mM/L + 1%	106.62 ± 13.18 U/ml	8533.33 ± 1228.94 U/ml	-	-	> 80-fold
		Cd^{2+}	100 µmol/l	≈ 64 U/ml	189.98 ± 28.07 U/ml	-	-	Almost 3-fold
		Pb^{2+}	400 µmol/l	≈ 64 U/ml	205.54 ± 13.05 U/ml	-	-	Almost 3-fold
Santana TT. et al. (2018)	*Lentinus crinitius*	$Cu^{2+}(CuSO_4)$	150 µM	4280 U/l	49000 U/l	-	-	14.5%
			250 µM	4280 U/l	55000 U/l	-	-	28.5%
		Veratryl alcohol	-	4280 U/l	46140 U/l	-	-	8%
		Xylidine	-	4280 U/l	75000 U/l	-	-	76%
		$(CuSO_4)$ + guaiacol	250 µM	4280 U/l	107408 ± 394 U/l	-	-	151%
		$(CuSO_4)$ + veratryl alcohol	250 µM	4280 U/l	109751 ± 1257 U/l	-	-	156%
Vrsanska M. et al. (2016)	*Trametes versicolor*	Cu^{2+} $(CuSO_4)$	1 mM	≈ 42 U/l	1291.70 U/l	-	-	≈ 30 fold
		M1	0.1 mM	≈ 8 U/l	-	Negligible	333.30 U/l	≈ 40 fold
	Trametes suaveolens	$Cu^{2+}(CuSO_4)$	1 mM	≈ 33 U/l	2108.30 U/l	-	-	≈ 63 fold
			0.5 mM	≈ 33 U/l	2000 to 3700 U/l	-	-	118-fold
		M1	0.5 mM	≈ 12-14 U/l	-	-	≈ 180 U/ml	Nearly 20-fold
	Daedaleopsis confragosa	M3	0.3 mM	≈ 15 U/l	-	Negligible	460.10 U/	≈ 30 fold

	Fomes fomentarius	M1	0.3 mM	≈ 75 U/l	1700 U/ml	Negligible	-	≈ 23 fold
Elisashvili V. et al. (2010)	*Cerrena unicolor (Mannitol-containing medium)*	Pyrogallol	-	15.0 ± 1.4 U/ml 2.0 ± 0.2 U/ml	37.2 ± 3.4 U/ml	3.9 ± 0.3 U/ml	-	2.5-fold 2-fold
	Trametes versicolor (EPR medium)	Hydroquinone	-	7.1 ± 0.5 U/ml 0.6 ± 0.08 U/ml	19.7 ± 2.3 U/ml	0.3 ± 0.02 U/ml	-	2.8-fold <
Feng et al. (2013)	*Pestalotiopsis sp. J63*	ABTS	0.25 mmol/l	1838 ± 100 U/l	>	-	-	>
		Guaiacol	0.5 mmol/l	1838 ± 100 U/l	≈ 3250 U/l	-	-	1.8-fold
		Veratryl alcohol	-	1838 ± 100 U/l	>	-	-	
		Phenol	0.05 mmol/l	1838 ± 100 U/l	3925 ± 25.7 U/l	-	-	2.1-fold
			0.09 mmol/l	1838 ±100 U/l	5089 ± 15 U/l	-	-	2.7-fold
Zhu C. et al. (2016)	*Pleurotus ostreatus ACCC 52857*	Phenol, ferulic acid, ABTS	-	≈ 64 U/ml	≈ 180 U/ml ≈ 160 U/ml ≈ 160 U/ml	-	-	2.5-fold
da Silva Coelho et. al. (2010)	*Ganoderma lucidum*	Diuron	80 µM	20 U/g 0.7 U/g	207 U/g	8.6 U/g	-	10.3-fold 3-fold
		Bentazon	20 mM	20 U/g 0.7 U/g	170 U/g	-	-	8.5-fold -
Parenti et al. (2013)	*Pleurotus ostreatus (shaking SMY medium)*	Wheat straw water extract (phenolic nature mainly)	50 ml of inducer extract	Very low, 0-10 U/l	≈ 600 (after a 2nd induction time)	<	-	> <
Usha K. et al. (2014)	*Stereum ostrea*	Tween 80	1%, 1 ml	17189 U/g 3631 U/g 40.3 U/g	25109 U/g	6303 U/g	252.5 U/g	1.46-fold 1.74-fold 6.3-fold

		Tween 20	1%, 1ml	17189 U/g 3631 U/g 40.3 U/g	19667 U/g	5692 U/g	Not enhanced	1.15-fold 1.6-fold <
		Triton X-100	-	17189 U/g 3631 U/g 40.3 U/g	20786 U/g	5115 U/g	Not enhanced	1.2-fold 1.4-fold <
Bankole PO. et al. (2020)	*Consortium: Ganoderma applanatum and Laetiporus sulphureus*	Non- Steroidal Anti-inflammatory drugs (NSAIDs)	-	0.5-0.7 U/ml ≈ 0.05 U/ml ≈ 0.01 U/ml	3734 U/g	288 U/g	50 U/g	201 % 135 % 180 %

Clarification 1*: Control enzymatic activity values (and consequently, n-fold increases) expressed with an "≈" before are not exact values because they are obtained approximately from graphics available in each resource.*

Clarification 2*: Enzymatic activities values and concentration values are expressed in the same units that authors chose to express in their research.*

Clarification 3*: Articles or reviews not always specified the concentration or quantities in which the inducers were added.*

Clarification 4: *n-fold increases or % increases are referred to increases in the enzymatic activities of the three principal ligninolytic enzymes (induced cultures) compared to control values (uninduced cultures).*

Clarification 5: *Enzymatic activities values displayed in red or the symbol "<" are referred to values lower than control ones.*