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Application of laccase in aflatoxin B1 degradation: a review

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Abstract-
The second component of the sustainable development goals, which is focused on zero hunger can only be actualized when safety issues that affect food security are tackled. In addressing food safety issues, the decontamination of aflatoxin b1 is a priority. Aflatoxin b1 is a class of mycotoxin that predominantly contaminates crops and their derivative processed foods. It has been reported to trigger the formation of cancer cells as well as pose a serious health challenge in humans that consume the contaminated food. The effective use of laccase enzyme (a ligninolytic enzyme) as an agent of bioremediation for wastewater and industrial effluent treatment initiated its use in aflatoxin degradation. This review highlights the potentials of laccase enzyme as a useful tool in aflatoxin b1 degradation. Also considered is the proposed mechanisms by which laccase enzyme catalyzes aflatoxin b1 degradation, and the factors affecting laccase activity such as temperature, ph, mediators and inhibitors. Studies have shown that due to the non-phenolic characteristic of aflatoxin b1, laccase-catalyzed aflatoxin degradation requires a long time, and was more effective in the presence of a mediator. Various reports observed different degradation time from 55 mins to 72 hours. Such degradation could be > 50 % after 48 hours of exposure to laccase. Furthermore, the use of laccase prevents the presence of chemical residue after treatment giving it advantage over the application of the existing chemical methods. This method provides a safer means of degrading aflatoxin b1 with minimal loss of the nutritional quality of treated food sample.

Keyword: Laccase, Aflatoxin B1, degradation

1.Introduction

In introducing laccase enzymes, it is essential to note that laccase enzymes are not just ligninolytic enzymes or oxidoreductase enzymes, as this definition is found among other enzymes such as the manganese peroxidase enzyme (MnP). Laccase enzymes (L.C.) (benzene-diol: oxygen oxidoreductase, EC 1.10.3.2) are ligninolytic oxidoreductase enzyme that possesses the ability to oxidize their substrate by transferring electrons accepted at the mononuclear center to a trinuclear copper center with the reduction of O_2 to water; hence, they are classified as multi-copper oxidases [1,2].
Chemically represented as;

$$XH_2 + 1/2O_2 \xrightarrow{\text{laccase}} X + H_2O \ldots\ldots\ldots\ldots \text{ equation 1}$$

White-rot basidiomycetes, among other lignocellulolytic enzyme-producing fungi, are the most studied fungi-laccase producers [1]. Due to their massive production of laccase enzyme, lignin substrate has been observed to be the best substrate for their growth. Hence, they are considered

as ligninolytic enzymes [1]. Laccase is known to possess low specificity for the substrate; thus, it can oxidize both aromatic and non- aromatic compounds [3], although L.C. ability to catalyze non-phenolic compounds are enhanced by the use of mediators [3].

Laccase has been found to exist in about four different forms, namely; monomeric, homotetrameric, heterodimeric, and multimeric. Laccase exists in four separate copper catalytic forms per protein unit; and spectroscopically divided into three types T1, T2, and T3 [4], one of these types gives laccase a blue coloration while those without the Cu atom of the blue laccase are considered yellow or white since they are not blue in color [5].

1.1 Aflatoxin B1

Aflatoxin is a class of mycotoxin produced by *Aspergillus* fungi. They are chemically represented as a difuranocoumarin structure and biologically harmful to vertebrates, including man [6]. Generally, Crops grown on the soil and stored after harvesting are often attacked by fungi[7]. At the same time, human exposure to aflatoxin is mostly a result of the consumption of contaminated nuts, grains, and its derived product [8]. *Aspergillus flavus, Aspergillus parasticus*, and *Aspergillus nomius* are the majorly recognized aflatoxigenic fungi [6]. The international agency for cancer research has long classified aflatoxin as a carcinogen to man [9], with over 17 different aflatoxins identified. *A*flatoxin B1 (AFB1) is considered the most toxic and chronic[8,10].

With WHO reports of over 25% annual destruction of food crops by aflatoxin globally [8]; and the investigation of aflatoxin-contaminated chili pepper in Nigeria [11], aflatoxin contamination is considered a threat. This mycotoxin is resistant to most physical and chemical treatment; hence, the rationale behind the use of an enzyme. The double bond in the 8,9 position of the furan ring and the lactone ring in the coumarin structure are the main sites in AFB1 structure responsible for its toxic activity[12,13]. This review is intended to highlight the mechanism of laccase-**c**atalyzed aflatoxin B1 degradation, showing how certain factors such as the sources of laccase enzyme play an effective role on aflatoxin degradation.

1.2 Applications of laccase

Figure 1 depicts the application of laccase enzyme for industrial and bioremediation purposes. Laccase enzyme is used in the food industry for beer and wine stabilization, fruit juice processing. It is also added to the dough in bread making to strengthen the gluten structure, which in turn enhances the flavor, freshness, volume, and texture of bread and baked product [14]. It is worth noting that rapid industrialization has resulted in increasing environmental pollution arising from industrial effluents and use of pesticides. Thus, research has suggested the use of enzymes in the removal of pollutants and toxicant as an active, cheaper, and eco-friendly bioremediation approach [15–17]. Presently, laccase is used in the bioremediation of polycyclic aromatic hydrocarbons, organophosphorus compounds, chlorophenols, azo dyes, and lignin-related structures [5,15]. For the active production of biofuel, lignocellulosic materials are promising feedstock. This is because laccase plays a role in biofuel production by degrading

lignin in the feedstock, thus exposing polysaccharides present in the lignocellulosic material to hydrolysis [14].

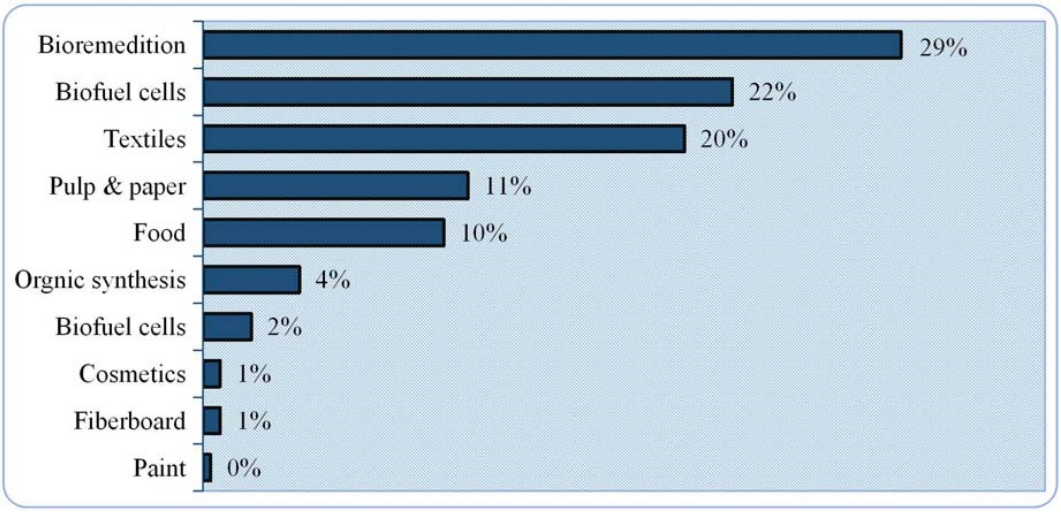

Figure 1: Laccase application. Source: Mate and Alcalde [14].

2. Sources of laccase enzyme

The sources of laccase enzymes including the strain and species of the organism directly influence laccase yield [13]. However, optimization of the culture condition for natural laccase producers constitute an effective and viable method of obtaining tremendous laccase production from laccase producers [18,19]. Laccase producers include plants, fungi, bacteria, and insects. Higher fungi producing laccase are isolated from deuteromycetes, basidiomycetes, and ascomycetes fungi. White rot fungi are the most common laccase producing fungi; they include; *Trametes ochracea, Trametes versicolor*, *Trametes gallica*, *Trametes hirsuta*, and *Trametes villosa*, [20].

Few bacterial species such as *Bacillus subtilis, Marinomonas mediterranea*, *Azospirillum lipoferum*, and *Streptomyces griseus* have reported laccase activity. Laccase-producing insects include; *Bombyx, Schistocerca, Rhodnius, Musca and* Sarcophaga [13]. In plants, some vegetables have been reported to contain laccase enzymes; examples are apples, cabbages, potatoes, turnip, and pears, [21]. Plant laccase family is reportedly a lot larger than fungal laccase family [18,22]. Edible mushrooms such as *Pleurotus ostreatus* and *Pleurotus pulmonarius* have been reported to extracellularly produce laccase enzymes [4,23].

2.1 Difference between fungal and bacterial laccase

Prior research reveals that laccase production is higher in fungal laccase than in bacterial laccase; other distinctive differences can be seen in Table 1. A study conducted by [24] on the potential of fungal and bacterial laccase in micro-pollution degradation reveals that fungal laccase is the best option in micro-pollution degradation. This conclusion was drawn after results showed that laccase production was higher in fungi than in bacterial source. More so, it was observed that the

rate of degradation was faster in fungal laccase; and under acidic condition (pH 3 -5), fungal laccase activity was high and could withstand a wide temperature and pH range unlike bacterial laccase [20].

Table 1: Distinctive difference between fungal and bacterial laccase.

Elements	Bacterial laccase	Fungi laccase
Localization	intracellular	extracellular
Level of redox potential	low	high
	high	low
Level of thermal stability	melanin formation, endospore coat, protein synthesis	pigmentation, delignification, body formation
Use of laccase		

Source: Chandra and Chowdhary [25].

3. Degradation of aflatoxin B1 with laccase enzyme

There have been current findings on the application of laccase enzyme in aflatoxin B1 degradation. The duration for aflatoxin degradation ranges from minutes to hours depending on the laccase source, the optimum condition provided for its activity, and the mediator used [13].

Table 2 depicts a summary of various researches on laccase treatment on aflatoxin degradation. From the table below, it is essential to point out that the temperature range was within 25 ˚C – 35 ˚C and pH (4.0 - 5.0); however, most reports lack information on pH level. In addition to the report summarized in table 1, Alberts et al. [26]also observed a 55 % reduction of Aflatoxin b1 concentration while investigating the degradation effects of a recombinant laccase produced by *Aspergillus niger*. The report suggests that fungal laccase resulted in the loss of mutagenicity and a decrease in fluorescent property of aflatoxin B1. This was attributed to the changes that occurred as laccase enzymes targeted the double bond of the furofuran ring of AFB1.

Table 2: A summary of the application of laccase on aflatoxin B1 degradation.

Source of laccase	Level of degradation	Duration	pH	Temperature	Reference
T. versicolour	67 %	48 hours	4.5	35 ˚C	[27]
	87.34 %	72 hours	4.0-	30 ˚C	[26]
T. versicolour	32.8 %	55 mins	5.0	25 ˚C	[13]
	50 %	60 mins			[23]
C. hiritus					
P. ostreatus					
Peniophora sp	40.45 %	72 hours		30 ˚C	[21]
	35.90 %	72 hours		30 ˚C	[21]
P. ostreatus	70 %	48 hours		30 ˚C	[28]
T. versicolor					
P. pulmonarius	23%	72 hours		25 ˚C	[4]

Laccase treatment on aflatoxin degradation is majorly enhanced by the addition of a mediator, and this is as a result of the ionization level of non-phenolic compounds. The combination of laccase enzyme and its mediator has widened its use on the various substrate as well as its application in various biotechnological areas

3.1 Degradation mechanism

Laccase catalyzed reaction can be of two types: direct oxidation and indirect oxidation. In the direct oxidation, copper clusters present in laccase enzyme directly oxidize substrate through the radicals generated. In contrast, indirect oxidation requires a mediator or an activator for laccase to oxidize a substrate since some compound possesses ionization potentials that exceed the redox potential present in T1 copper ion of laccase. In-direct oxidation follows a two-step process where laccase enzyme first oxidizes the mediator, after which the substrate is oxidized by the oxidized mediator[5]. Direct oxidation of AFB1 by lac2 pure enzyme from edible mushroom (*Pleurotus pulmonatius*) resulted in 23 % degradation, while after the introduction of a naturally occurring mediator (10 mM acetosyringone), aflatoxin B1 was degraded by 90% [4].

The mechanism for aflatoxin detoxification using laccase enzyme possibly involves two different pathways. Figure 2 illustrates that the first pathway is the elimination of the double bond located at the terminal furan ring and secondly, the opening of the lactone ring found in the g- group aflatoxin [12]. A pathway was proposed by Liu *et al*. [29] that describes the oxidation of AFB1 to form aflatoxin epoxide and, subsequently, the conversion of aflatoxin epoxide to AFBI -8,9-dihydrodol via hydrolysis. This triggers the opening of the difuran ring in further hydrolysis step [30]; also the opening of the lactone group enhances new reaction to terminate the binding of the terminal furan ring to DNA. and proteins that could eventually cause mutations [12]. The quantitative level of laccase enzyme increases the extent of aflatoxin B1 degradation [13]. The use of laccase enzyme in aflatoxin degrading is considered safe because it uses oxygen as its only co-substrate [26].

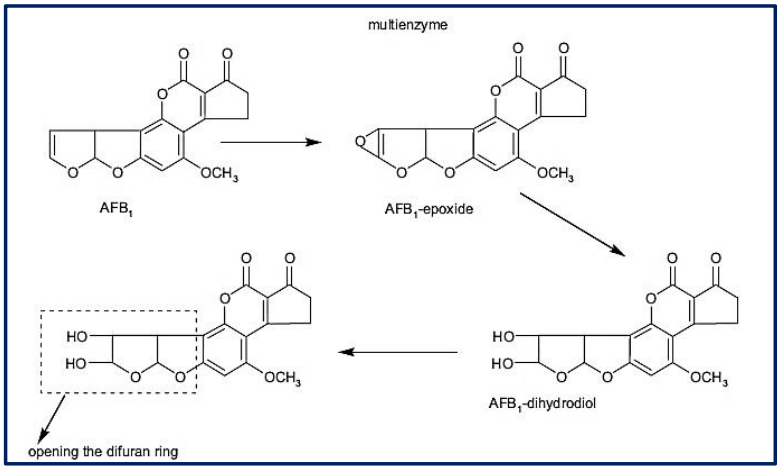

Figure 2: A proposed pathway for aflatoxin degradation. [30].

4. Factors affecting laccase activity

4.1 Effect of Temperature

The optimum reaction temperature for laccase activity using aflatoxin as the substrate was assayed with a wide range of temperatures from 25 ^{0}C – 65 ^{0}C [13]. The experiment reported that laccase from *C. histrus* obtained its highest laccase specific activity at 57.5 ^{0}C. The result also showed that gradual inhibition in laccase activity was observed when the temperature was further increased or decreased. This is following the report by Baldrian [2], that at 35 ^{0}C -75 ^{0}C the optimum reaction temperature of most fungal laccase was attained. Nevertheless, Table 2 shows that *T. versicolor* which is reported to be the highest fungal laccase producing organism, achieved a high optimum temperature at 30 ^{0}C; with 80 μgml^{-1} of AFB1 and 30 Uml^{-1} of *T. versicolor* laccase enzyme. When a range of temperature (5 ^{0}C – 65 ^{0}C) was studied, 35 ^{0}C was identified as the optimum temperature for laccase activity [27].

These reports suggest that laccase enzyme function may also be substrate-dependent, since it is generally believed that temperature affects substrate accessibility, enzyme activity, and molecular motion.

4.2 Effect of pH

The optimum pH of fungal laccase varies depending on the laccase producing organism, the pH of the culture medium [15] and the substrate. However, based on prior literature review, optimum pH of fungal laccase on aflatoxin b1 degradation has been observed from 3 to 7 [13,15]. Without an effective and optimum pH, enzyme stability may be altered, subsequently making enzymatic elimination of xenobiotics unachievable. According to Zeinvand-Lorestaniet al. [27], a range of pH values (2.5 – 7) on *T. versicolor* laccase was studied and optimum pH for AFB1 degradation was observed at 4.5. An investigation on *C. histrus* laccase showed that the optimum pH for laccase catalyzed reaction was achieved at 6.0, and a potent enzyme inhibition was observed at pH 8.0 [13]. The laccase enzyme generally has a bell-shaped pH profile [31].

4.3 Effect of activators/mediators

Laccase catalytic ability has been observed to drastically increase in the presence of activators/mediators. These mediators are small molecules and substrate of laccase that produce free radicals when reacting with laccase [16]. Laccase enzyme readily catalyzes phenols and aromatic amines, but when used on compounds that possess high molecular weight or high ionization energy, a high redox potential is required [4]. Hence, a mediator is used to enhance laccase activity when catalyzing compounds with ionization energy higher than the redox potential of laccase T1 copper ion [32]. 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is a chemical commonly used as laccase mediator and substrate. Compounds like 1-hydroxy-benzotriazole [HBT], acetosyringone (AS), p-coumaric acid (p-CA), syringaldehyde (S.A.) [33], and natural phenolic compounds such as ferulic acid and vanillin can all act as mediators and expand the use of laccase on various substrates. According to Zang et al. [34] the impact of structurally defined chemicals and complex natural mediators on *B. subtilis* CotA laccase catalyzed degradation of AFB1 and Zearalenone was investigated. Among the structural chemical analyzed, methyl syringate enhanced the highest degradation of 94.2 % AFB1 after 10 hours, followed by syringaldehyde. 1-hydroxy-benzotriazole (HBT) was the least effective mediator observed in the experiment. They also reported that the presence of metal ions such as

Mn^{2+}, Al^{3+}, Cr^{3+}, Cu^{2+} and Co^{2+} combined with laccase enzyme achieved aflatoxin degradation. The study also explored the potential of plant extract *(E. brevicornu* extract, *S. tenuifolia, L. angustifolia* extract, and *Cucumis sativus L*. (cucumber) extract) as natural mediators. *N*-hydroxyphthalimide (HPI) was also reported to enhanced laccase activity by 88.2 % [13,35].

4.4 Effect of inhibitors

The activity of most enzymes is inactivated in the presence of an inhibitor, including laccase enzyme. The activity of laccase enzyme has been reported to be hindered or inactivated in the presence of small anions like dithiothreitol [DTT], sodium azide, fluoride, cyanide, halide, hydroxide, thiocyanide, and halide [5,13,36,37]. The effect of a certain inhibitor on laccase enzyme was investigated and it showed 78.2 % of laccase inactivity in the presence of 1 mM of DTT – when aflatoxin was used as the substrate; the rate of inhibition increased with an increasing amount of DTT [13]. Other chemicals reported to inhibit laccase activity from that experiment include CuSO4, CuCl2, L- cysteine, etc. Prior research suggests that the inhibitors hinder laccase activity by modifying the active site of laccase enzyme, through the formation of complexes with laccase structural copper ions[37] .

5. Significance and challenges

5.1 Significance of laccase-catalyzed aflatoxin degradation to food security

Aflatoxin B1 has generally affected the availability of healthy crops, this in turn has led to the rise of food contamination in grains, nuts and pepper. Aflatoxin B1 is a food safety issue which has defied many food decontaminations method. The use of laccase enzyme is a biological method that has provided a means of degrading aflatoxin B1. Laccase-catalyzed aflatoxin degradation has been reported to be effective as well as provide solution to the limitations of previous methods. Such limitations include the presence of chemical residue after treatment; an unpleasant change in the organoleptic property of treated food sample; and formation of less toxic metabolites. It is, therefore, a positive step in eliminating aflatoxin food contamination.

5.2 Challenges of laccase treatment of aflatoxin degradation

A highly effective laccase-catalyzed aflatoxin degradation is mainly observed in the presence of a mediator. While natural mediators may be safe for food sample, synthetic mediators may be limited by high cost, toxicity, and the highly needed mediator-substrate ratio [4,38].

Also considered is the long degradation time required for maximum aflatoxin degradation, followed by the seeming unavailability of high caliber equipment used in assessing the by-product of aflatoxin degradation in developing countries.

6. Future Prospects

With the demand for laccase treatments on aflatoxin degradation expected to further increase in the future, on one hand, more research is needed to confirm the absence of by-products with any residual toxicity [39]. On the other hand, besides analysis of the degradation products, molecular technology is expected to be a tool for elucidating the reasons underlying the formation of degraded products [40]. Additional knowledge would also be needed on the identification, quantity and toxicity of degradation metabolites like aflatoxicol or aflatoxin-8,9-epoxide prior to applications in food matrices [41].

Nevertheless, scientists have put forward some insights for future studies on Aflatoxin B1 degradation. This is expected to involve the complete unravelling of the mechanism of Aflatoxin B1 degradation induced by a laccase from white-rot fungus [42]; selection of microorganisms using coumarin as carbon source; validation of Aflatoxin B1 degradation effectiveness; and characterization of the intra and extracellular extracts. More so, *in-vitro* and *in-vivo* toxicity tests should be done to estimate the laccase detoxification efficacy on the major commodities affected by Aflatoxin B1. The development and formulation of such cocktails of Aflatoxin B1 degradation protocols using laccase would optimize efficacy and provide solutions to the Aflatoxin B1 burden on the global agro-food chain [43].

7 Conclusion

Huge economic losses are recorded along the food and feed supply chain worldwide due to Aflatoxin B1 contamination. The resultant loss of crops and animals constitutes a significant global food security and public health concern considering the strong correlation in Aflatoxin B1 contamination with cancer occurrence in parts of Africa, China and South East Asia. Due to climate change, these risks may be more severe as Aflatoxin B1 occurrence is expected to also increase in Europe and third-world countries.

Various physical and chemical detoxification techniques have been evaluated. Unfortunately, few conform with FAO requirements regarding reduction of Aflatoxin B1 without residual toxicity; and in completely fulfilling the required efficacy, specificity and nutrient retention. As such, biological degradation involving treatments with enzyme preparations and application of genetically engineered microbial strains present an Aflatoxin B1 detoxification alternative. This is because it provides a possible means of transforming Aflatoxin B1 into non-toxic or less toxic metabolites under mild conditions, while also retaining the sensory quality, safety, acceptability and nutritional value of agricultural commodities.

Based on the foregoing, research on biological detoxification with laccases produced by white-rot fungi continues to gain attention. The treatment of Aflatoxin B1 with laccase enzymes targets and changes the double bond of the furfuran ring of the Aflatoxin B1 molecule and as a result influences its fluorescence and mutagenicity properties. The uniqueness of this approach relies on the evidence that the mechanism of an effective degradation occurs via the mediation of natural phenolic compounds. Finally, by application of new technologies involving the use of extremophile enzymes, micro-encapsulation or enzyme immobilization and rDNA technology, laccase can be used as a decontamination and protective agent for Aflatoxin B1 contaminated food and feedstuff.

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