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Review Article

Production of Fungal Laccase from Beer Industry Waste and Its Application in Biodegradation of Pesticides

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Abstract

Persistence of xenobiotic residues in environment due to exaggerated application of pesticides in agricultural fields has disturbed the balance in ecosystem. Many bacteria and fungi have been reported to produce laccases capable of degrading toxic and structurally complex chemicals. The issue has been focused by emphasizing on the biological remediation methods using fungal enzyme as compared to non-affordable physicochemical treatments. Considering this scenario, the present work has been carried out using fungal laccase in degradation of pesticides. Laccase enzyme production was carried out using *Aspergillus nidulans* with substrate as spent grains. Partially purified enzyme was subjected to pesticide degradation studies. In this work, fourteen commercial grade pesticides were subjected to *Aspergillus nidulans* mediated degradation. Plates showing growth of *Aspergillus nidulans* in presence of pesticides were selected for further degradation. Nine pesticides were screened for degradation studies. Maximum Degradation was observed for Spinosad, followed by Dichlorovos, and Captra, with 52.51%, 29.78% and 24.04% degradation respectively using UV Spectrophotometeric analysis. Considerable reduction of pesticides from agricultural samples i.e. cabbage was 81.5% and for grapes was found to be 43.1%. On the basis of present findings *Aspergillus nidulans* strain can be exploited as a potential agent to protect the environment from hazardous pesticides and deleterious effects of the residue.

Keywords: *Aspergillus nidulans*; Bioremediation Laccase; Pesticides; Spent grains

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Introduction

Laccase is member of multimeric glycoprotein enzyme belonging to the multicopper blue oxidoreductase family. Laccases are the enzymes which are secreted out in medium extracellularly by several fungi by secondary metabolism. Laccase structure contains 4 types of copper ions [1]. The type I copper catalyses primary binding to substrate, where the O_2 is activated and chemical bond is synthesized on tri nuclear copper centre and is completely reduced to water. Laccases are widely distributed enzyme in higher plants and fungi. Fungi like basidiomycetes, ascomycetes and deuteromycetes are potential producers of laccase. Among all the white rot fungi from basidiomycetes like *Trametes versicolor, Aspergillus nidulans, Pleurotus ostreatus* are predominant. These fungi can utilize several compounds like poly phenols, methoxy-substituted mono phenols, aromatic amines, di phenols, benzene triazole etc for laccase synthesis. It includes guaiacol, syringaldazine, vanillin, catechol, α naphthol [2].

Laccase producing fungi are one of the interesting subjects in current Era. Researchers in Environmental biotechnology are going to their ability to degrade recalcitrant pollutants like pesticides, dyes, toxic compounds. The production methods include solid state fermentation [3], submerged state fermentation [4]. Laccases can also be produced using straw [5]. Brewery Spent Grain (BSG) is an important by-product of the brewing industry. Spent grains serves as low cost and complete nutrient medium for enzyme production because of the high protein content, complex phenolic components and rich carbohydrate composition.

Laccase have widespread applications in the fields of Pharmaceuticals for Biotransformation [6]; Detoxification [7]; in food industry [8], Pulp and paper industry [9], Dye Decolorization [10], nanoparticle synthesis [11], as biosensors and bio remediating agents [12, 13].

In India, the agriculture sector dominates primarily in economic perspective. Variety of crops and plants are cultivated at large scale worldwide using traditional methods. The crop yield and quality are influenced by soil characteristics, seasonal changes and biological interference. The health of plants is maintained by application of chemical compounds which deter insects, birds, animals and microorganisms that influence the plant yield by feeding upon the grains, leaves and parts of crops. These compounds include insecticides, fungicides, Molluscicide, herbicides, Rodenticides, nematicides, plant growth regulators. Being unaware of toxic effects of pesticides, farmers apply them in excess, which results into persistence on different parts

of the crops, thus it constrains the health of consumers. Despite the restrictions and regulations on usage of pesticide, India accounts for one-third of pesticide poisoning cases in the world. According to reports, in leafy vegetables like cabbage, spinach, dill and fruits like grapes, strawberries, apples, the concentration of the pesticide residue was higher [14].

Hence, in addition to the conventional physicochemical methods, the researchers are emphasizing on the biological methods for degradation of these toxic compounds from environment using microorganisms as key factors. The research history has implicated the microbial enzymes for bioremediation purpose, for instance, laccases have been used in degradation of pesticides and toxic compounds owing to their oxidative action against phenolic aromatic compounds and nontoxic by product formation. Study of Xie, et. al. [15], showed that, recombinant laccase from *Pichia pastoris* strain together with vanillin as mediator displayed 98% degradation of organo phosphorus compounds.

Considering the consequences of pesticide exposure to the environment, the development and implication of efficient, reproducible yet cost effective enzymatic strategies for bioremediation is essential. One of them is the immobilization of enzyme on the cheaply available matrix such as the used grains from Beer industry, which has functional group provisions for successful and strong binding along with environment friendly properties that can potentially be recovered from the reaction mixture after enzymatic degradation treatment.

According to earlier bioremediation studies, pesticide removal using laccase has shown considerable results, which has bestowed the foundation for present work. Main objective of our study is the laccase catalysed detoxification of pesticide residues from fruits and vegetables, which are strong victims of pesticide bombardment. Moreover, the present study has also executed *in vitro* degradation of few commonly used pesticides by a fungus, which is a potential source of laccase, which catalyse the actual decomposition. Based on these experiments, the potential application of laccase in pesticide removal from fruits and vegetables has been assessed.

Considering these endowed properties of laccase, the present study has been focused on application of laccase producing fungus in pesticide degradation that can render the methodology ecologically and economically sound.

Materials and Methods

The standard culture of *Aspergillus nidulans* MTCC-344 was obtained from MTCC Chandigarh, India and was maintained on Sabourauds dextrose agar (Lab M Limited, UK). Guaiacol (Hi Media Laboratories Pvt. Ltd., India was taken as substrate for laccase production. Ethyl acetate was procured from (Avantor performance materials India Ltd.) Ammonium sulfate was procured from Thermofisher Scientific India Pvt. Ltd Sodium sulphate from Hi Media was used for pesticide extraction were also obtained and primary secondary amine were made available by NHRDF, Nashik. Spent grains were collected from Beer industry in Aurangabad.

Sample collection

The standard pesticide samples viz., Mandipropamid (23.4%), Spinosad (45%), Dinocap (48%), Captra (50%), Zineb (75%), Dichlorovos (76%), Chlorpyrifos (20%), Lambdacyhalothrin (5%), Propineb (70%), Fluopyra (17.7%), Defenoconazole (25%), Folicular tebuconazole (25.9%), Myclobutanil (10%), Mendoze P (45%) were collected from Dinde farm, Nandur Naka, Nashik for degradation analysis. Agricultural samples i.e. grapes and cabbages were collected from same farm using Agritech portal, AGRISNET and National Research Centre for Grapes (Indian Council of Agricultural Research) protocol.

Confirmation, production and purification of laccase

The pure culture of *Aspergillus nidulans* was spot inoculated onto SDA plate containing 0.05% Guaiacol and incubated at 27°C for 2days. The plate was examined for development of reddish brown color in the medium. For production medium, Nutrient Salt Medium (Glucose -10g, $(NH4)_2PO_4$ - 0.2g, KH_2PO_4 -1g, $MgSO_4.7H_2O$ - 0.5g, KCl - 0.5g, $FeSO_4.7H_2O$ - 0.005g, Thiamine hydrochloride - 0.001g, distilled water - 1000ml, pH 5.2) and washed Spent grain (Spent grains-5%) were used. Fungal discs were inoculated in the media and incubated at 27°C for 3 days under shaking conditions (150 rpm). The culture was then supplemented with Guaiacol (50 µl/100 ml) to promote laccase synthesis. The enzyme was purified using ammonium sulphate salt precipitation method with 70% cut off.

Degradation of pesticides

Screening for pesticide degradation

To check whether the fungus can grow in presence of pesticides, SDA media were spiked with 50ppm each of 14 pesticide samples followed by inoculation with *Aspergillus nidulans*. The plates were incubated at 27°C for 7 days.

Degradation of standard pesticide and residues from agricultural samples

The pesticides containing plates showing growth of *Aspergillus nidulans* were subjected to degradation analysis. For degradation study, Nutrient salt medium were spiked with pesticides for degradation analysis using *Aspergillus nidulans* culture. The pesticide containing media without culture was taken as control. The cabbage sample was diced into uniform pieces and the grapes were peeled off. The pieces of cabbage and peels of grapes were subjected to partially purified laccase with Phosphate buffer (pH 6.8, 0.1M) for 1h at 27°C.

Extraction of pesticides

The degradation was evaluated after extraction of pesticides from treated samples, wherein 10ml of the treated NSM broths were withdrawn and centrifuged at 6000 rpm for 5min. Similarly, the treated grape and cabbage samples were added into 10ml of extraction solvent (ethyl acetate), vortexed for proper mixing of solvent and sample components to obtain two distinct layers. Centrifugation was performed at 6000 rpm for 5 min. 10gm anhydrous sodium sulphate was incorporated for dehydration. Mixture was vortexed for 5 min and the centrifugation was repeated at 6000 rpm for 5min. The upper solvent layer was transferred into new test tube and was treated with 30 mg/ml of Primary Secondary Amine (PSA) as adsorbent for removal of polar compounds and fatty acids as they impart impurities to the sample. The tubes were vortexed for 30sec and centrifuged at 6000 rpm for 5min [16]. The solvent extract (supernatant) was evaporated; the residue was recovered with 0.2 ml methanol and was suspended into 3 ml of distilled water.

Pesticide residue Analysis using UV-VIS Spectrophotometer

The pesticide content was evaluated in terms of UV absorbance viz. Mandipropamid at 223nm, Spinosad at 250 nm, Dinocap at 265nm, Captra at 220nm, Zineb at 284nm, Dichlorvos at 500nm, Chlorpyrifos at 290nm, Lambdacyhalothrin at 218nm, Propineb at 272nm.wavelength.

$$\text{Degree of degradation (\%)} = (C\text{-}T)/C \times 100$$

Where C-Absorbance of uninoculated pesticide broth T- Absorbance of pesticide broth inoculated with *Aspergillus nidulans* after 7 days

Results

Confirmation and production of laccase

Intense Brown colour was observed on plates containing Guaiacol as shown in figure 1. Successful induction was symbolized by development of brown color in the broth due to oxidation of Guaiacol by laccase. *Aspergillus nidulans* was cultivated in productive media including SDB, NSM as well as Spent grain medium, whose induction was resulted into laccase synthesis characterized by color change of broth. The spent grains are rich source of carbon and nitrogen, as well as are rich in phenolic compounds, that promote the growth of the fungus content and the synthesis of laccase at expense of these phenolic substrates.

Figure 1: Oxidation of Guaiacol.

Degradation of pesticides

Plates showing Growth of *A.nidulans* when subjected to submerged fermentation figure 2 showed results as per table 1. Two samples that did not showed reduction of absorbance, viz. Lambdacyhalothrin and Zineb. The maximum degradation was observed for Spinosad (52.56%) as compared to Captra (43.07%), followed by Dinocap (30.9%), Dichlorvos (29.78%), Mandipropamid (24.04%), Chlorpyrifos (19.29%), Propineb (11.42%), indicating that given fungus can degrade Spinosad more efficiently as per graph 1. The results signify the ability of *A.nidulans* in degradation of toxic pollutants of varying structural and chemical properties.

Degradation studies on agricultural samples

The treatment of solvent extract from cabbage and grapes showed maximum degradation of 81.552 % and 43.11% respectively of pesticide residues as shown in graph 2 through the action of laccase enzyme.

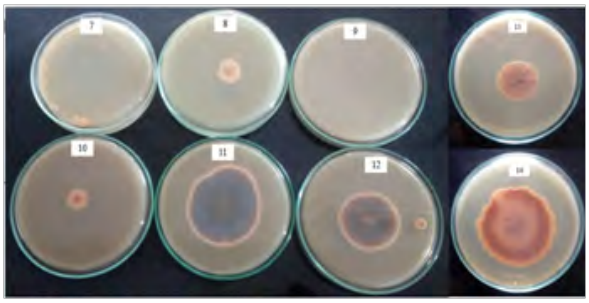
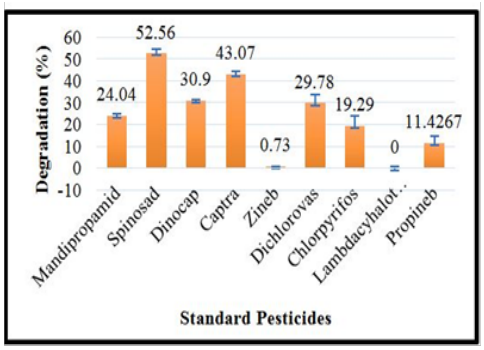


Figure 2: Growth of Fungus on Pesticide (50ppm) Containing Media.

Sr. No.	Pesticides names	Growth	Diameter of growth (cm)
1)	Mandipropamid	+	7.5
2)	Spinosad	+	7
3)	Fluopyra	-	0
4)	Defenoconazole	-	0
5)	Tubuconazole	-	0
6)	Dinocap	+	5
7)	Myclobutanil	-	0
8)	Captra	+	3.5
9)	Zineb	-	0
10)	Mendoze P	+	3
11)	Dichlorovas	+	8
12)	Chlorpyrifos	+	5.5
13)	Lambdacyhalothrin	+	3.4
14)	Propineb	+	3

Table 1: Growth of *Aspergillus Nidulans* on Pesticide Containing Media.

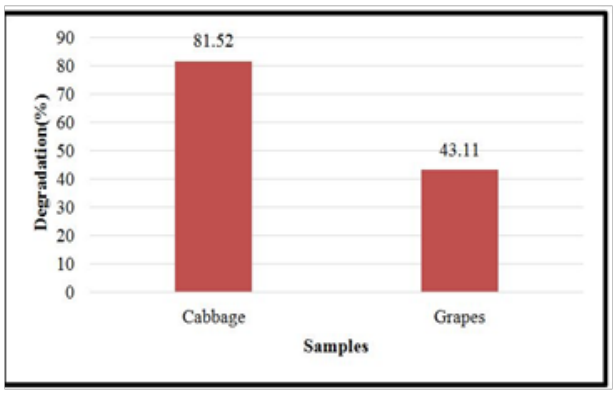


Graph 1: Degradation analysis in submerged fermentation using laccase produced by *Aspergillus nidulans.*

Discussion

Intensifying demand for Laccase has necessitated the research on optimization studies in order to exploit best suitable culture media and laccase producing microbial strains. *Aspergillus nidulans, Trametes versicolor* have served as potent producers of laccase in previous

studies [17-19], notably former has given appreciable laccase production in variable culture media comprising of simple to complex carbon sources in the present study. Several reports are in line with use of Organic wastes, such as Wheat bran, Banana stalks for laccase production [3]. Research also reports laccase production using semi-synthetic media [20]. The present study has utilized spent grains as one of the medium for laccase production.



Graph 2: Degree of degradation of pesticides from agricultural samples.

In present study, Brewer's Spent grains have established an economic source for induction as well as synthesis of laccase over the conventional and modern synthetic media formulations owing to its negligible cost, strong phenolic composition having enhanced stimulation for laccase synthesis and nutrient composition which makes it self-sufficient source from growth and metabolism of fungus [21]. The enzyme produced by the fungus must confer its ability to tolerate and grow at the expense of pesticides. In the present study, phenolic, non-phenolic, as well as heterocyclic pesticides was degraded through the action of *A. nidulans* laccase, with profound effect on organo halogenated pesticides without use of a mediator oxidative compound, which had not been fruitful in previous studies [22].

The study evaluated the degradation of various pesticides using *Aspergillus nidulans*. The growth of fungal biomass was observed in 9 pesticides using solid state fermentation viz. Mandipropamid (23.4%), Spinosad (45%), Dinocap (48%), Captra (50%), Zineb (75%), Dichlorovos (76%), Chlorpyrifos (20%), Lambdacyhalothrin (5%), Propineb (70%) amongst which, 6 were organo halogenated members, remaining two were Spinosad member having amino sugar rings and the Zn- dithiocarbamate complex. Thus, they were selected for degradation analysis.

We have come across the data regarding the use of direct fungi and bacteria for bioremediation application *ex vivo,* [23,24] instead of employing the purified enzyme, that has endowed us for accomplishing 'Enzymatic Bioremediation' of pesticide residues from the agricultural food samples. We have chosen Cabbage and wine grapes as the subjects since they are amongst those crops that are most exposed to wide variety of pesticides like in Nasik region of Maharashtra. Although pesticide residues are partially removed by washing and cooking procedures, the raw consumption of cabbage salads introduces the residues directly into consumer's body, whereas the risk of pesticide ingestion is higher in case of fruits because of direct consumption except for juices, since washing procedure is insufficient in removal of residues due variability in their partition coefficient [14]. Partially purified laccase was capable of minimizing the pesticide content from the surface of grapes as well as cabbage. Since scanty research is available for the same, our work can direct research on *ex vivo* as well as *in vivo* bioremediation enzymatically in an atypical tactic, which shall contribute in environmental detoxification and restore ecosystem.

Conclusion

The findings of present study have paved the way for further development of green synthetic and bioremediation chemistry depending on a single biocatalyst. It will address the upcoming pollution issues of agriculture, industry and clinical circles of the society. The mere use of beer industry waste without supply of a single external nutrient additive can meritoriously reduce the cost of enzyme production. Additionally, the strong denaturing impact of laccase on otherwise inaccessible complex pesticides can increase the value of Laccase as a biological reclamating agent together with the conventional physicochemical methods. The healthy environmental circumstance can be restored by detoxification of colorants and pesticides.

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