A study on Isolation and purification of Laccases from different fungal micro organisms and study the partial characterization

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Abstract : Laccase is a copper-containing polyphenol oxidase that acts on a wide range of substrates. This enzyme is found in many plant species and is widely distributed in fungi including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both. Laccases catalyze the polymerization of several phenolic substances to polymeric products. In addition they transform lignin and lignin -related compounds, showing very broad substrate specificity. Laccases are used in a variety of applications, such as bioremediation, bioleaching of denims in processing of beverages, as bio analytical tool in biosensors to estimate the quantity of phenols in natural juices or the presence of other enzymes. Recent success in fungal molecular and cellular engineering technology has contributed to significantly increase the industrial production of recombinant laccase. Kinetic (Michaelis -Menten parameters, optimum pH, k cat) and stability properties of Laccases vary according to the source of the enzymes. Exploration of microbial diversity for novel Laccases can yield improved Laccases for varied application. This work presents primary investigations on the isolation and Purification of Laccases from different fungal strains

IndexTerms **- Polymerization, lignin, Recombinant laccase.**

I. INTRODUCTION

Biological agents such as microorganisms and enzymes have received great attention because of their potential to remove pollutants from the environment without harsh side effects .Among the biological agents, Laccases represent an interesting group of ubiquitous, oxido reductive enzymes that show promise of offering great potential for biotechnological and environmental applications: especially removal of phenol and its derivative from the environment [1].

Laccases are multi- copper containing enzymes that catalyze the oxidation of various aromatic amino compounds, specially phenols and anilines, while concomitantly reducing molecular oxygen to water. They form a group of enzymes with broad specificity and catalyze the oxidation of a wide range of both o-and p- quinols, and also amino phenols and phenylene diamine. Although the specificity for the electron donor is low, the specificity for the acceptor (oxygen) is essential.

According to capability to polymerize or de polymerize aromatic substrates, laccase has a number of environmental or biotechnological applications that include waste water treatment, bio pulping bioleaching, and also soil bioremediation. Basic studies have examined the roles of Laccases in the formation of humid substances in soil and in lignification or delignification processes. The use of these enzymes detoxifies waste waters or soils polluted with xenobiotic compounds of aromatic nature have received special attention [2,3].

Future perspectives include suggestions for using these enzymes as immobilized catalysts for in situ remediation of polluted sites and to treat polluted drinking waters, waste waters originating from various manufacturing processes, pulping industry wastes, and other wastes. This enzyme is marketed for use in the brewing of beer to prevent the formation of off-flavor compounds, such as trans-2-noneal.laccase scavenges oxygen which otherwise would react with fatty acids, amino acids, proteins and alcohols to from off -flavor precursors.

To utilize laccase for biotechnological and environmental applications as well as to better understand the properties of the enzyme at the molecular and kinetic levels, large amounts of readily available crude and purified Laccases are required many studies have been devoted to identifying the most efficient laccase producing source, to selecting the most suitable culture medium ; to developing appropriate reproducible, and in expensive isolation procedures and mainly to optimizing the enzyme production [4]. Studies in this field are done to discover new laccase with new substrates specificities (both broad and narrow range) and kinetic parameters. The main aim of the present work is to study the production of laccase enzyme from different fungal strains using combination of common isolation and purification procedures and its further characterization.

II. MATERIALS AND METHODS

Organisms and culture conditions

Total of Four different strains were used for Laccase production.

The four strains were Fennelia nivea, Aspergillus niger, Tricoderma viride, Aspergillus flavus. The master cultures were maintained on following medium by monthly subculture.

Composition of medium includes (g/l)

Peptone…….……0.5%
Yeast extract ……..0.3%
Glucose……...…… 1.0%
Agar ……...1.5%
KH2PO4 ……...…...0.2%
Distilled water ……..... 100 ml

All the ingredients were weighed precisely and transformed into 250 ml flask. This 250ml flask was then autoclaved at 121°C for 150minutes .Then transformed into laminar airflow chamber. The process of inoculation was performed by inoculating the four strains into different Petri plates.

Selection for laccase producing organisms was done on plates containing following media:

Composition of medium included (g/l)

Glucose...……...…...10.0
KH2PO4 …….…….0.6
ZnSO4 …….0.001
K2HPO4……..…..0.4
FeSO4 …… 0.0005
MnSO.....40.05
MgSO…..…….……40.5
Agar……..……..20.5
Guaiacol…….0.02%
Distilled water:……1000ml

Laccase activity was visualized on plates containing 0.02% guaiacol since laccase catalyses the oxidative polymerization of guaiacol to form reddish brown zones in the medium.

Isolation of laccase producing organisms:

To obtain Laccase supernatant fungal colonies were maintained on CMC medium and PD medium.

CMC Production Media contained

Carboxy Methyl Cellulose ……..…. 0.1%
Peptone …….0.25%
Yeast extract…….…….0.2%
Potassium Dihydrogen Phosphate……..…...0.2%

A total of 400ml of the CMC medium was prepared and poured into two 1000ml flasks equally.

Potato dextrose prodution medium contained

Dextrose……2%
Potato extract……20%

A total of 400ml medium was prepared and poured into two 1000ml flasks equally. Cultures were incubated at 300C and 200rpm for 15 days or until laccase activity had reached maximum. Cell mass was removed from the broth by filtration using whatman filter Paper No.1.The obtained filtrate from the broth by using whatman filter paper was allowed to dry at 600C in hot air oven until it reaches constant dry weight. The dry weight was measured and recorded. For obtaining filtrate, 10ml of sample was used in intervals of 24hours.

Every day the cultures were checked and samples of 10ml were taken. They were filtered by using whatman paper filter No.1.Then the filter paper was subjected to drying to yield dry weight.

The dry weight was determined by subtracting paperweight from the combined weight of filtrate and the paper.

Assay For laccase activity

Enzyme activity was assayed at 30 c by using 10mM guaiacol in 100 mM acetate buffer containing 10 % (v/v) acetone. The changes in absorbance of the reaction mixtures containing guaiacol was monitored at 470 nm (_= 6,740 M-1cm-1) for 5min of incubation. The enzyme activities were calculated using an extinction coefficient of 6,740 M-1cm-1 and expressed as Katals (1mol of substrate conversion/s) [3].

Protein Determination

Protein concentration was estimated according to lowry method using BSA as standard [6].

Laccase Purification

The culture was monitored for laccase activity from second day of inoculation. It showed maximum activity from 13th -15th day and then a broad Decline. The 15-day culture was filtered through whatman filter paper No.1 to separate the biomass.

Ultra Filtration

The supernatant was then concentrated by Ultra filtrations 30KD filter .Ultra filtered concentrate was dia filtered twice with 10mM Phosphate buffer (pH 8).

III. Results & Discussion

Laccase is enzyme with great environmental and non-environmental applications. To utilize laccase for Biotechnological and environmental applications as well as to do better understand the properties of enzymes at the molecular and kinetic levels, large amounts of readily available crude and purified Laccases are required.

Many studies have been devoted to identifying the most efficient laccase producing source; to selecting the most suitable culture medium; for developing appropriate, reproducible, and inexpensive isolation procedures; and mainly to optimizing the enzyme production. Its further characterization will be helpful for its more effective biotechnological application.

Presently Laccases are neither inexpensive nor easily available enzymes. A full-scale laccase application to decontaminate polluted systems (aquatic or terrestrial) could require huge amounts of enzymes. Even the use of crude enzyme preparations could be expensive. The characteristics of available Laccases (ex: specificity, catalytic efficiency, stability) indicate that before these enzymes can be used for bioremediation, improvements in the methodology must be made. Successful application of DNA Recombinant Technology will result in both increased laccase and improved catalytic performance. Tapping on the high secretion capacity combined with the presence of low toxin levels of selected filamentous fungal hosts,(such as Aspergillus oryzae), laccase

could be produced by industrial fermentation on a large scale by economical and environment-friendly means. In addition to laccase production, DNA recombinant technology can affect the structure-function relationship of laccase and its substrates by generating Laccases whose performance is tailored to specific applications [7].

In the present study, a total of four different strains were taken and they were assayed for the selection. The results reported here shows that only one fungal strain (Trichoderma viride) produces extracellular Laccase. Laccase activity was not found in Aspergillus niger, Finnelia nivea and Aspergillus flavus.

The presence of Laccase producing strain was identified by the appearance of reddish brown zones. Laccase enzyme catalyzes the process of oxidative polymerization of Guaiacol which resulted in the appearance of reddish brown coloured zones.

Table 1. Screening of Laccase-producing fungi by plate tests using the indicator compound Guaiacol.

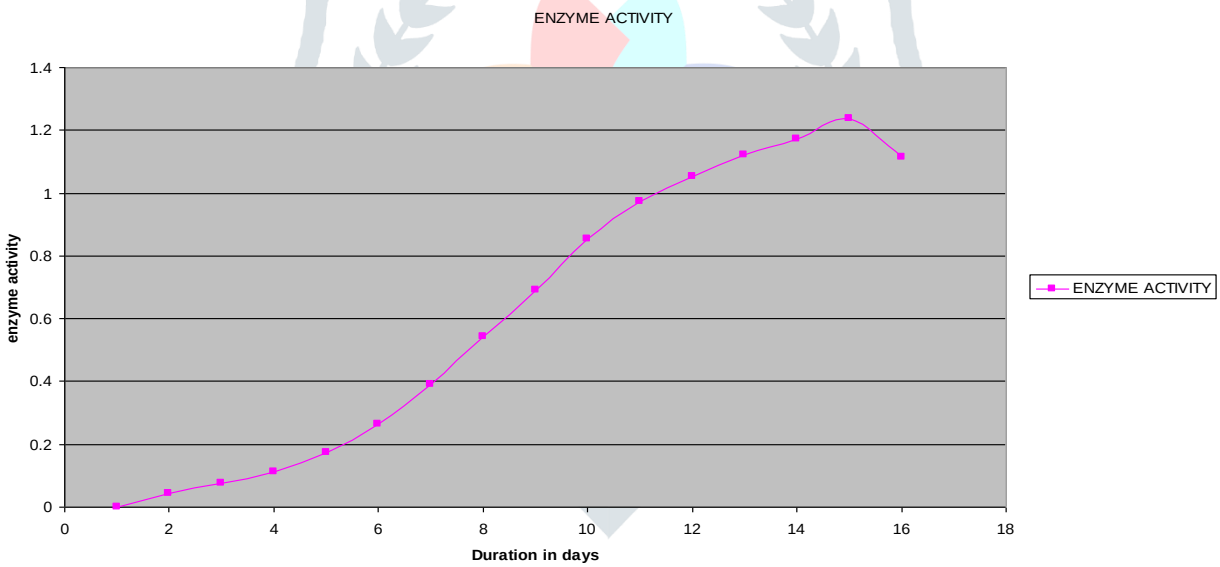
Name of the strain	**Result**
Aspergillus niger	-
Trichoderma viride	+
Finnelia nivea	-
Aspergillus flavus	-

Isolation

When novel fungal strain was cultivated in 0.1%CMC media, Laccase activity in the media reached to its maximum on 15th day. That time profile of the enzyme indicates that activity increase from 4th day to 15th day. After 15 days there is broad decline in enzyme activity.

Enzyme concentration was found to be 1.24 U/ml and protein concentration was130 µg/ml that was the average maximum. The culture supernatant was therefore harvested after 15 days to obtain maximum yield.

Fig 1. Enzyme activity of Laccase producing strain in CMC medium



The results of enzyme activity of the strain in the CMC medium have showed a maximum value at 15th day culture.

Table 2. The results of enzyme activity of the strain in the CMC medium have showed a maximum value at 15th day culture

Day	1	2	3	4	5	6	7
Activity	0	0.042	0.076	0.113	0.173	0.264	0.392

8	9	10	11	12	13	14	15	16
0.544	0.692	0.854	0.973	1.051	1.121	1.172	1.237	1.116

The enzyme activity in the potato dextrose medium has shown maximum in 13th day culture.

Fig 2. Enzyme activity of laccase producing strain in PD medium

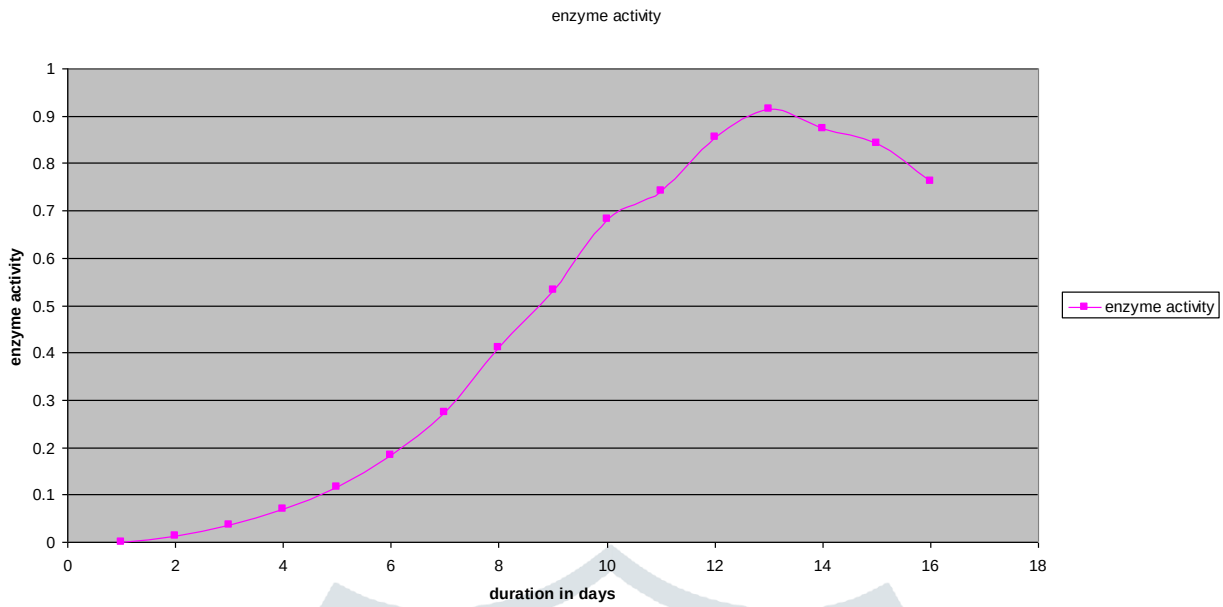


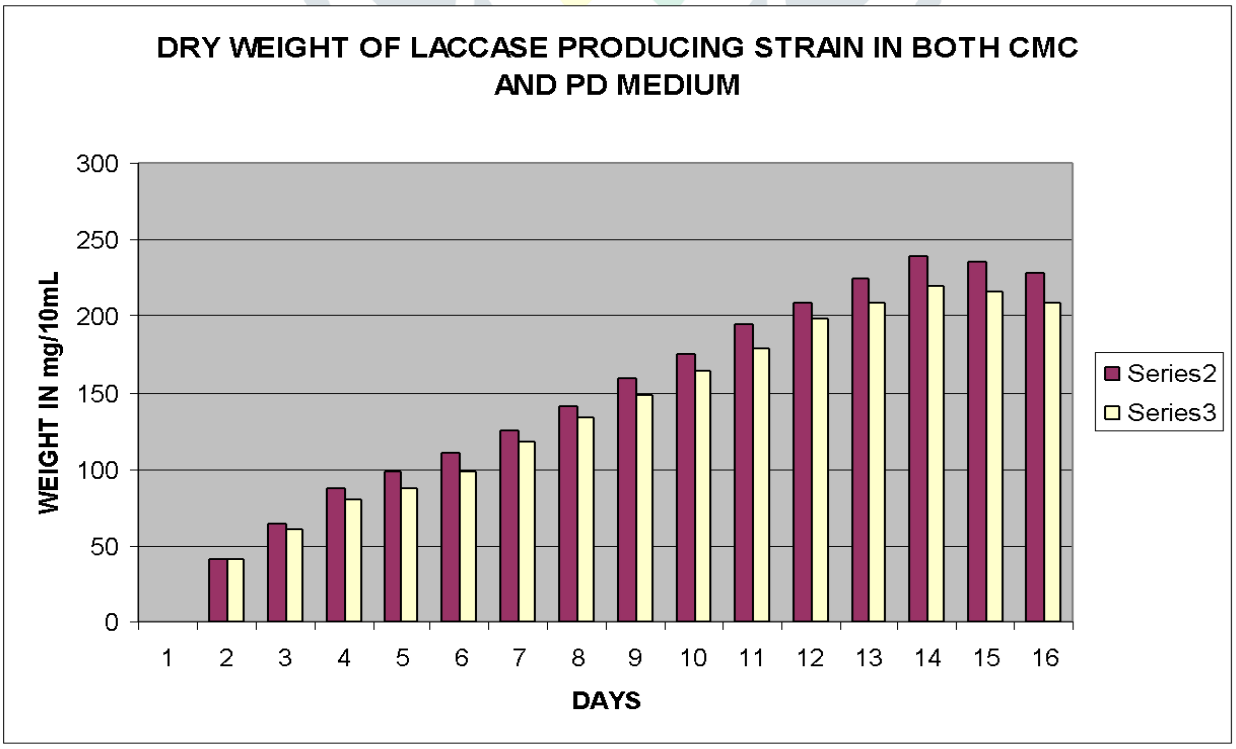
Table 3. The results of enzyme activity of the strain in the PD medium have showed a maximum value at 13th day culture

Day	1	2	3	4	5	6	7
Activity	0	0.014	0.036	0.069	0.115	0.184	0.273

8	9	10	11	12	13	14	15	16
0.411	0.533	0.682	0.741	0.856	0.914	0.873	0.842	0.762

In the present study, the results have showed that the laccase activity of trichoderma viride has maximum value at 15 th day culture in CMC medium and at 13th culture in PD medium [10].

Fig 3. Dry weight of laccase producing strain in both CMC and PD medium



Every day the cultures were checked and samples of 10 ml were taken. They were filtered by using whatman paper filter No. 1. Then the filter paper was subjected to drying to yield dry weight. The relative concentration of the dry weight has increased continuously till the 13th day of culture and then small decrease in weight was observed.

In both CMC and PD medium the maximum amount of yield were observed during 13th day culture. The amounts were 238 mg/10ml and 220 mg/ 10 ml respectively [8].

The dry weight was determined by subtracting paper weight from the combined weight of filtrate and the paper [13,14].

0.1 % CMC was used as a carbon source and inducer. Higher concentration of CMC can cause binding of enzyme to CMC and can hinder the purification process. Hence lower concentration of CMC was used. Highest enzyme activity was observed during 13 -15th day cultures. Then decrease in the activity was observed which may be due to declination of concentration [8, 9]. The maximum activity produced in the CMC medium was found to be more than the maximum activity produced in the PD medium. Even the amount of dry weight of the strain per fixed volume of the media also differs. The dry weight was higher in the sample present in CMC medium than the PD medium. The dry weight also happened to be more during 13-15 th day of culturing [9,10].

Purification

The culture was filtered to remove the cell mass and ultra filtration and diafilteration was done for concentrating and purifying the enzyme.225 µg/mL protein and 5.21 U/ml enzyme activities was detected in the ultra-filtered concentrate [11,12, 15].

Table 4. Showed concentration of protein and specific activity of Laccase enzyme

Sample	Quantity (ml)	Protein conc. (µg/ml)	Total protein conc.	Enzyme activity (U/ml)	Total enzyme activity	Specific activity (Umg^{-1})	Fold purification	Efficiency (%)
Broth	600	130	78000	1.24	744	9.5	1	100
U.F conc.	120	225	27000	5.21	625	23.14	2.43	84.05

Further characterization of this enzyme is required to understand this enzyme more clearly it could be a suitable candidate for further characterization and investigation for application.

Conclusion

Further studies are needed to improve the methodologies of immobilization, the efficiency of immobilized enzymes, and the design of appropriate reactors. If research satisfies these requirements in the future, the use of Laccases will represent a valid means for the recovery and Restoration of polluted systems.

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