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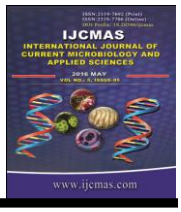

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Screening of Potent Laccase Producing Organisms Based on the Oxidation Pattern of Different Phenolic Substrates

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A B S T R A C T

Soil samples were collected from different forest areas of Kerala and screened for laccase activity using guaiacol plate assay technique. An increased concentration of guaiacol was used for the isolation of resistant strains. Substrate oxidation studies were carried out and the potent organisms were taken for enzyme production studies. The organisms, isolated by the screening strategies, were found efficient laccase producers. The potent organisms were identified as *Arthrographis sp.* and *Enterobacter cloacae.* The enzyme production rate of *Arthrographis* was found to increase logarithmically and a maximum quantity of 53 U/ml was obtained and the bacterial strain, *Enterobacter cloacae*, gave a maximum laccase activity of 8U/ml. The difference in the pattern of substrate oxidation by laccase from different organisms is paid attention in the study.

Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) catalyze the oxidation of various aromatic, particularly phenolic substrates (eg. hydroquinone, guaiacol, 2,6-dimethoxyphenol or phenylene diamine), coupled to the reduction of molecular oxygen to water. Laccases as well as ascorbate oxidases (EC 1.10.3.3) and ceruloplasmins/ferroxidase (EC 1.16.3.1) usually contain several copper atoms in the catalytic centre. They belong to the enzyme superfamily of multicopper oxidases, which is a widely distributed protein family among prokaryotes and eukaryotes. However, laccases or laccase-like multicopper oxidases (LMCO) were predominantly described in fungi and plants, where they occur as multigene family with sometimes more than 10 different laccase genes. The basidiomycete *Coprinopsis cinerea* and the plant *Arabidopsis thaliana* have both 17 different genes (Hoegger *et al.*, 2006).

Properties of Laccases

Current knowledge about the structure and physico-chemical properties of fungal proteins is based on the study of purified proteins. Up to now, more than 100 laccases have been purified from fungi and been more or less characterized. The laccase

molecule, as an active holoenzyme form, is a dimeric or tetrameric glycoprotein, usually containing four copper per monomer (Cu) atoms bound to three redox sites (Type 1, Type 2 and Type 3 Cu pair). The molecular mass of the monomer ranges from about 50 to 100 kDa with acidic isoelectric point around pH 4.0. An important feature is the high level of glycosylation (with covalently linked carbohydrate moieties ranging from 10– 50% of the total weight, depending on the species or the heterologous host), which may contribute to the high stability of the enzyme (Duran *et al.,* 2002). Several laccase isoenzymes have been detected in many fungal species. More than one isoenzyme is produced in most white-rot fungi.

In contrast to most enzymes, which are generally very substrate specific, laccases act on a surprisingly broad range of substrates, including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines and benzenethiols. Furthermore, laccases with unusual and potentially useful properties have been isolated from ascomycetes. Laccase from *Monocillium indicum* was the first laccase to be characterized from as ascomycetes showing peroxidative activity (Thakker *et al.,* 1992). Examples include the higher stability and activity at alkaline pH together with the high thermostability of the laccase produced by *Melanocarpus albomyces* (Kiiskinen *et al.,* 2002), the high acid tolerance of intracellular laccase from *Hortaea acidophila* (Tetsch *et al.,* 2006), and the high activity of laccase from *Xylaria polymorpha* at elevated concentrations of NaCl (Liers *et al.,* 2007). It was postulated that laccase acts as a defense mechanism against oxidative stress (Fernandez-Larrea and Stahl, 1996). This protective function was partly attributed to the chelation of copper ions during synthesis of the laccase enzyme.

Substrates and Products formed by Laccase

Laccase has broad substrate specificity as well as the advantage of not requiring an addition of harmful hydrogen peroxide to the oxidative reaction. Because of complex structure of lignin, its biodegradation system is considered highly nonspecific. Lignolytic enzymes can degrade environmental pollutants that differ structurally (Dec and Bollag, 1994). The substrate oxidation pattern of laccase is tabulated in Table 1.

Materials and Methods

Soil samples were collected from different forest areas of Kerala and screened for laccase activity. The serially diluted soil samples were inoculated on Vogel's Mineral Media (VMM) agar plates (pH 5.6) containing guaiacol (Di- methoxy phenol) (Sigma) as substrate (Vogel, 1956; *Coll et al.,* 1993). The positive strains were purified and modified plate assay for selecting high tolerance laccase producing strains was conducted using an increased concentration of guaiacol.

Substrate Oxidation Studies by Laccase

The substrate tolerance was studied by incorporating 0.02% of guaiacol, p-cresol, p-aminophenol, p-phenylene diamine, hydroquinone and tropolone in VMM agar plates. The fungal plates were incubated at 27±1°C for 5 days and the bacterial plate was incubated for 48 h at 37°C. The growth and oxidation pattern were noted.

Organisms and Culture Conditions

Preparation of Pre-inoculum

Pre inoculum was prepared by inoculating VMM agar plates for fungi and VMM broth

for bacteria. Agar plugs from the outer circumference of a fungal colony stored on a SDA plate was used as the inoculum. The fungal strains were inoculated to VMM plates and were incubated for 6 days at 27±1°C. The bacterial strain was inoculated from NA slant to VMM broth and was incubated for 24 h at 120 rpm, 37°C.

Preparation of Inoculum

Agar plugs (5 mm diameter) from the outer circumference of fungal colonies growing on VMM plate (8 days) was used as inoculum for fungal production media and 1% bacterial culture with 0.7 Abs at 600nm was used for inoculating the bacterial production media.

Activity Based Secondary Screening of Organisms

Laccase production was carried out in VMM broth, pH 5.6. The fungal cultures were incubated at 27±1°C and the bacterial culture at 37°C. The samples were collected in every 24 h and centrifuged at 10,000 rpm for 15 min. The supernatant was taken for enzyme assay. The enzyme assay was conducted using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Biogene, USA) as substrate.

The assay mixture contained 2mM ABTS in 0.1M sodium citrate buffer, pH 3.0. Oxidation of ABTS was monitored by determining the absorbance increase at 420 nm ($\varepsilon = 3.6x10^4\ M^{-1}cm^{-1}$) (Jia Li Dong, 2004). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min. Assay was conducted at regular intervals. The laccase production rate and the optimum day of enzyme production were noted for each of the selected organisms. Statistical model was constructed based on enzyme activity and day of incubation.

Tolerance Assay

The KSF_2 strain was subjected to substrate tolerance assay. 1mM concentration of the phenolic substrates (*p*-cresol, *p*-aminophenol and *p*-phenylene diamine, guaiacol, hydroquinone and tropolone) were added to VMM broth, KSF_2 culture was inoculated and incubated at 27±1ºC at 120 rpm. The oxidation of the substrate and the tolerance pattern was noted in broth cultures. The laccase assay was conducted at regular intervals using ABTS as substrate

The potent strains selected were sent to IMTECH, Chandigarh, India for identification.

Results and Discussion

Pure Culture Isolation

Soil samples were plated on to guaiacol containing medium. Oxidation polymerization of guaiacol to bisphenoquinone was visualized as reddish brown zones on VMM agar plates (Fig. 1). Efficient and potent organisms screened by this method were purified and inoculated to the respective agar plates to study the colony morphology (Fig. 2). Of the 41 isolated strains isolated by this technique, 4 strains were selected after the primary screening studies. Out of the 4 strains, 3 were fungi (KSF_1, KSF_2 and KSF_3) and the other one was bacteria, KSB_4.

Modified Plate Assay for High Tolerance Laccase Producing Strains

Potent DMP tolerant laccase producing organisms were isolated by this technique. The concentration of guaiacol in the plates was increased to 0.04% to find the tolerance of the organisms. All the organisms could grow in the increased concentration of

guaiacol and oxidize the substrate. The fungal colonies were incubated for 5 days at 27°C and the bacterial colony was incubated for 48 h at 37°C. The bacterium was found to be less tolerant to the increased concentration of the substrate as after incubation period the viability of the organism decreased to considerable extends (Fig.3).

Substrate Oxidation Pattern of the Isolated Strains

Out of the six compounds selected for the study guaiacol was oxidized by all the selected strains. KSF_1 could grow in the presence of p-cresol, p-aminophenol and hydroquinone but could not oxidize the compounds. KSF_2 could grow in the presence of all substrates except tropolone and could oxidize p-phenylene diamine. KSF_3 could not grow in the presence of any of the substrates. KSB4 and KSF_2 could grow on all substrates except tropolone and could oxidize p-phenylene diamine and hydroquinone. Results are tabulated in Table 2. The oxidation reaction is presented in Fig. 4. The level of tolerance was directly proportional to the amount of laccase produced by the organisms.

The potent strain, *KSF_2* was inoculated to the VMM agar plates supplemented with the assay substrates (guaiacol, catechol and ABTS) to confirm the best substrate for laccase assay. The plates are incubated at 27±1ºC for 5 days. It was observed that the laccase of *KSF_2* was more reactive to ABTS (Fig. 5).

Activity Based Secondary Screening of Organisms

The organisms were inoculated into VMM broth for laccase production and ABTS assay was conducted every 24 h to find the optimum day of enzyme production. The quantity of enzyme produced by the strains, enzyme production pattern and the optimum day of enzyme production by each organism also differed.

The results are tabulated in Table 3 and Fig. 6. The enzyme production rate of KSF_2 was found to increase logarithmically till 102nd day of inoculation. From this secondary screening method KSF_2 and KSB_4 were screened as the most potent strains for laccase production.

Results indicate that the laccase production is high in the exponential growth phase; the activity appears to be closely correlated with biomass production. Different strains can produce different laccases, each with its own unique features. Environmental factors influence the ability of fungi to produce high titers of laccase, and different strains react differently to these conditions. Screening of strains capable of producing high concentrations of enzyme and then to optimize the conditions for laccase production is important in the industrial production of the enzymes.

Statistical Model Construction Based on Enzyme Activity and Day of Incubation

Different statistical models were tried to find the correct fitted model to explain the enzyme activity when day was set as the variable (Fig. 7). The model for which the coefficient of determination (R2) is maximum, was selected as the best suited model. It was observed that the cubic model was best suited to explain the enzyme activity by the isolated strains.

The tolerance of the organism in SmF is tried for its application studies. In 1mM concentration of the substrates *KSF_2* was able to grow in broths supplemented with *p*-aminophenol and *p*-phenylene diamine and

was also able to oxidize them. *p*-aminophenol was not oxidized by the organism in plate assay method. The enhanced production of laccase in shaking condition attributed to the oxidation of *p*-aminophenol.

All the other substrates inhibited the growth of the organism in broth culture. The oxidation of the two substrates was visualized in broths and the laccase assay was conducted to study the effect of the presence of these compounds on enzyme production. The ABTS assay was conducted for the media with *p*-phenolic compounds. The culture was inoculated to modified VMM minimal media and was kept as control. There was a decrease in laccase activity observed in the presence of phenolic compounds.

Morphological and Biochemical Identification of Organisms

The Ascomycetes strain KSF_2 was identified as *Arthrographis sp*. by IMTECH, Chandigarh, India and was deposited with reference MTCC 8880. It has branched, hyaline conidiophores, thallic and arthric conidiogenesis and hyaline single celled conidia in dry chains (Fig 8). The bacterial strain KSB_4 was identified as *Enterobacter cloacae* by IMTECH, Chandigarh, India and was deposited with reference MTCC 9145.

Table.1 Substrate oxidation pattern of laccase

Substrate	**Product**	**Colour**	**λmax (nm)**	**ε_{max} ($M^{-1}cm^{-1}$)**
Catechol	*o*-benzoquinone	Yellow	450	2,211
Hydroquinone	*o*-benzoquinone	Yellow	248	17,252
Pyrogallol	Purpurogallin	Yellow	450	4,400
2,6 dimethoxy phenol	3,5,3',5' tetramethoxy diphenoquinone	Yellow	468	35,645
Guaiacol	Biphenoquinone	Brown	470	26,600
ABTS	$ABTS^+$	Blue	420	36,000
Syringaldazine	Quinones	Purple	525	65,000
Catechin	*o*-quinone	Yellow	390	4,019

Table.2 Oxidation Pattern of Phenolic Compounds by the Screened Organisms

Substrates	Growth/ Oxidation Pattern			
	KSF_1	KSF_2	KSF_3	KSB_4
guaiacol	+/+	+/+	+/+	+/+
***p*-cresol**	+/-	+/-	-/-	+/-
***p*-aminophenol**	+/-	+/-	-/-	+/-
***p*-phenylene diamine**	-/-	+/+	-/-	+/+
hydroquinone	+/-	+/-	-/-	+/+
tropolone	-/-	-/-	-/-	-/-

Table.3 Enzyme Activity of the Isolated Strains

Organism	Optimum day	Enzyme activity (U/L)
KSF_1	31	155
KSF_2	102	52,764
KSF_3	15	15
KSB_4	5	8,317

Fig.1 Plate Assay of Laccase

Fig.2 Cultural Characteristics of Isolated Strains on Agar Media

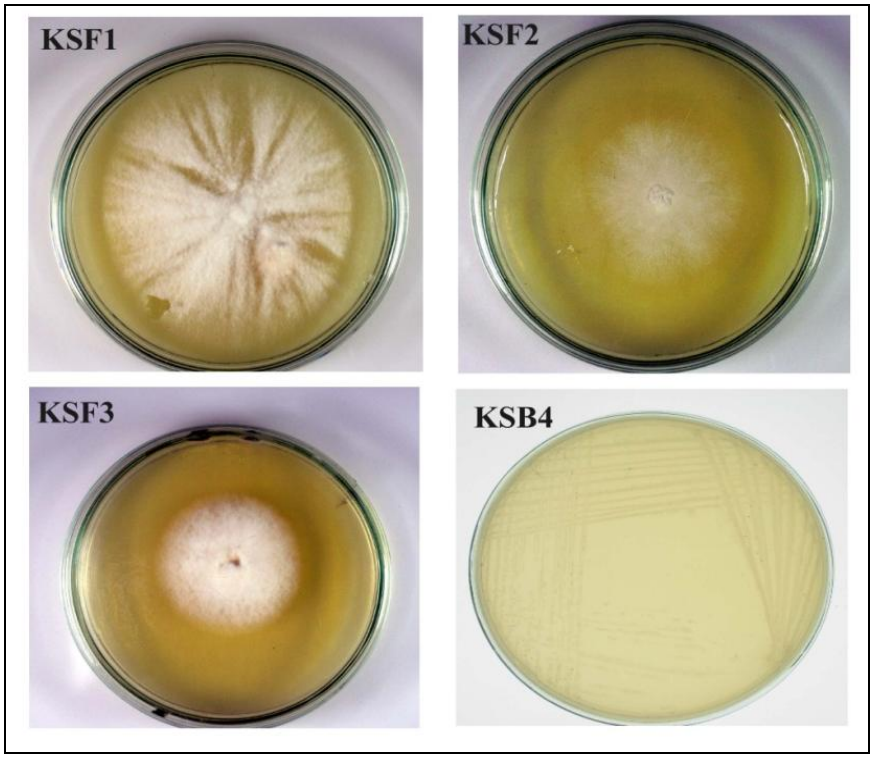


Fig.3 Modified Plate Assay for Laccase

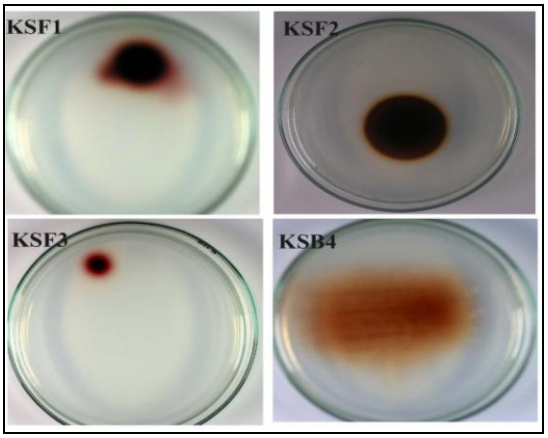


Fig.4 Oxidation of the Phenolic Compounds by the Potent Strains

Fig.5 Oxidation of the Assay Substrates by KSF_2

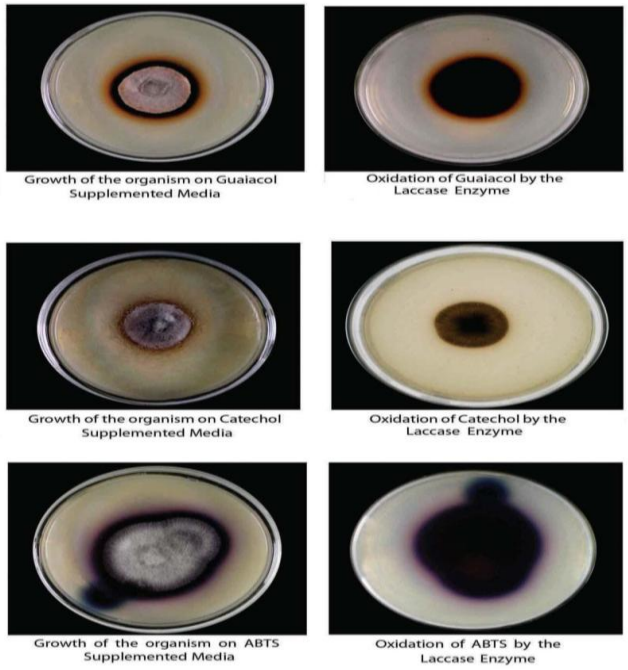


Fig.6 Laccase Production by the Strains

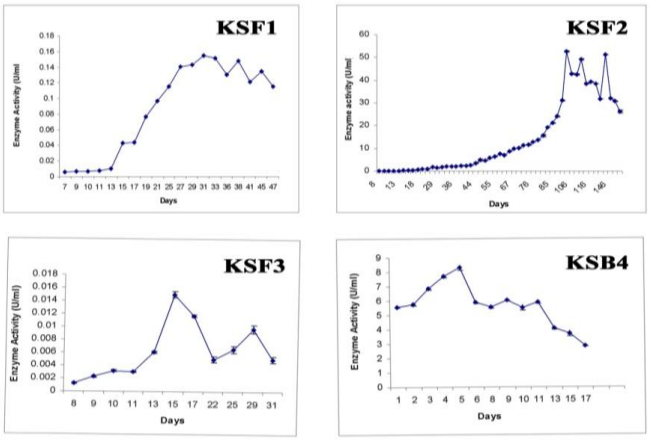


Fig.7 Statistical Models Based on Enzyme Production Pattern

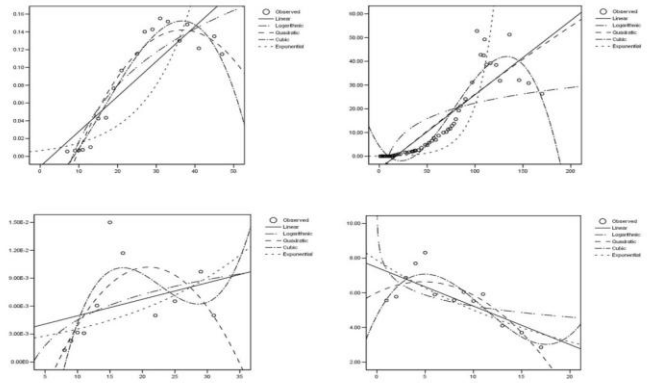
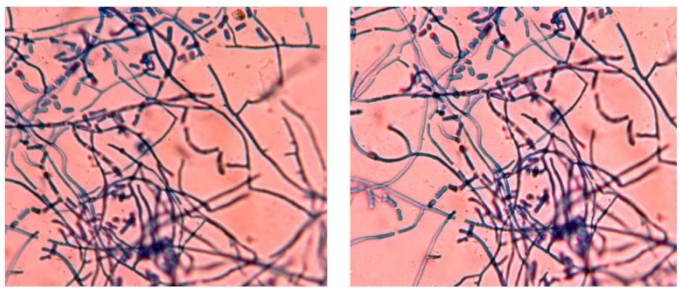


Fig.8 Lactophenol Cotton Blue Staining of Slide Culture



The selected potent strains can grow in the presence of guaiacol, p-cresol, p-aminophenol, p-phenylene diamine and hydroquinone explains their remarkable potential for application in bioremediation and wastewater treatment, especially in detoxification of phenolic wastes.

The organisms, isolated by the screening strategies, were found efficient laccase producers. The enzyme production rate of *Arthrographis KSF*$_2$ was found to increase

logarithmically and a maximum quantity of 53U/ml was obtained on the 102nd day of inoculation. The bacterial strain, *Enterobacter cloacae KSB*$_4$, was also found promising with a maximum laccase production of 8U/ml. Laccases from ascomycetes and bacteria has been paid only less attention, although they have unusual and potential biotechnological use, hence from the primary and secondary screening studies conducted the ascomycetes, *Arthrographis KSF*$_2$ and the proteobacteria, *Enterobacter cloacae KSB*$_4$ were selected for further studies.

The lignolytic enzymes: lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase completely biodegrade lignin polymers. The biological treatment of industrial wastewaters usually depends upon the oxidative activities of microorganisms. These enzymes do environmental clean up by oxidative degradation (Tanaka *et al.*, 2001). These are extracellular enzymes that may be usefully engineered to improve the efficiency of particular bioremediation processes. These enzymes can reduce the concentration of selected phenolic compounds in refinery wastewater. Laccases from fungi have been shown to be useful for the degradation of a variety of persistent environmental pollutants.

Discovery of novel laccases with different substrate specificities and improved stabilities is important for industrial applications. Microbes that produce laccases are screened on solid media containing coloured indicator compounds that enable the visual detection of laccase production (Nishida *et al.,* 1988; De Jong *et al.,* 1992; Barbosa *et al.,* 1996) or in liquid cultivations monitored with enzyme activity measurements (Luterek *et al.,* 1997). The use of coloured indicators is generally simpler as no sample handling and measurement is required. As laccases oxidize various types of substrates, several different compounds have been used as indicators for laccase production. The traditional screening reagents tannic and gallic acid (Harkin and Obst, 1973) have nowadays mostly been replaced with synthetic phenolic reagents, such as guaiacol and syringaldazine (Nishida *et al.,* 1988; De Jong *et al.,* 1992) or with the polymeric dyes Remazol Brilliant Blue R (RBBR) and Poly R-478 (Barbosa *et al.,* 1996; D'Souza *et al.,* 1999; Raghukumar *et al.,* 1999). RBBR and Poly R-478 are decolourized by lignin-degrading fungi (Gold *et al.,* 1988; Barbosa *et al.,* 1996), and the production of ligninolytic enzymes is observed as a colourless halo around microbial growth. With guaiacol a positive reaction is indicated by the formation of a reddish-brown halo (Nishida *et al.,* 1988), while with tannic and gallic acid the positive reaction is a dark-brown coloured zone (Harkin and Obst, 1973). These studies show that novel laccase producers can be discovered from environmental samples by very simple plate-test screening methods.

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