

Chapter 6

Summary

Ph.D. Thesis



The oceans are the last frontier on Earth that has yet to be fully explored. Marine life holds great promise for the isolation of novel and useful compounds and metabolites. Marine sponges have been touted as the single most important source of novel marine metabolites. Obligately marine and marine-derived fungi too hold great potential as a source of such compounds or at least similar metabolites but with novel properties that give them a commercial edge, their enzymes which are salt tolerant especially hold great promise.

The object of the present study was to isolate and identify obligate marine or marine-derived fungi that were hyper-laccase producers. The laccase from the selected marine-derived fungus was to be characterized and its application in bioremediation and biobleaching, studied. These objectives were fulfilled in the thesis.

Seventy-five fungi were isolated from decaying lignocellulosics from the mangrove swamps of Choraõ Island in Goa, using techniques such as particle-plate, hyphal and single spore isolation methods. Among these, fifteen isolates were found to be laccase producers. Out of these, three anamorphic fungi proved to be the most efficient dye decolourizers and laccase producers and were designated NIOCC #2a, NIOCC #7a and NIOCC #Xa. Among these, NIOCC #2a was the laccase hyper-producing strain selected for this study, since it was superior in terms of both laccase production and dye decolourization. It was deposited at the Microbial Type Culture Collection (MTCC) under accession no. 5159 and identified using rDNA homology to be a *Cerrena unicolor* strain. The fungus NIOCC #2a is therefore referred to as *Cerrena unicolor* MTCC 5159. Its marine origin was confirmed by achieving maximum growth in full strength (34 ppt) seawater and maximum laccase production in 25 ppt seawater.

Crude laccase, contained a consortium of laccase isozymes with differing surface charges and molecular masses. It collectively showed optimum activity at pH 3 and 60°C. Out of these isozymes, an isozyme, 'Lac IId' was purified to homogeneity with a final yield of 17 %. Its UV-visible spectrum showed a shoulder at 330 nm and peak at 610 nm which is characteristic of blue laccases. The N-terminal and an internal peptide sequence, both deposited under the accession no. P85430 in the 'Uniprot' database confirms Lac IId to be a blue laccase. Lac IId has a molecular mass of 59 kDa and a pI of 5.3 with 17 % N-linked glycosylation. This glycosylation besides playing a structural role

also confers upon Lac IId, resistance to proteases, which are also produced by *Cerrena unicolor* MTCC 5159 in copious amounts (data not shown).

Lac IId showed optimum activity at pH 3 and 70°C and a half life of 90 min at 70°C. It retained more than 60 % activity up to 180 min at 60°C and pH 9. Even after 1 year of storage at -20°C, no loss in activity was observed. This coupled with its extremely low energy of activation (2.5 kJ mol⁻¹) at pH 3 recorded between 60° to 70°C, attests to its long shelf life and thermostability. Lac IId could catalyze the oxidation of a wide range of substrates and its substrate range increased in the presence of certain mediators. Of all the inhibitors tested, only azide was able to effectively inhibit it up to 95 % of its original activity. Pure sodium chloride up to 0.3 M, did not inhibit it above which, the laccase was reversibly inhibited. From the wide range of metal ions tested, Lac IId was maximally inhibited by 56 % with chromium whereas the other metals showed lesser inhibition.

After having confirmed the commercial potential of laccases from this fungus, *Cerrena unicolor* MTCC 5159 was subjected to medium optimization to maximize laccase production. It was found that carbon and nitrogen act antagonistically with each other with respect to laccase production, when nitrogen is used in an inorganic form. A combination of low inorganic nitrogen and high carbon concentrations favour good biomass and laccase production. When nitrogen was supplied in the inorganic form but in low concentrations, laccase titer was maximal. However, organic nitrogen at much higher concentrations also supported good laccase production. Buffering action of citrate-phosphate buffer was required to decrease the temporal variation in laccase titer. Inducible laccases are produced in the idiophase only after addition of an appropriate inducer. Known inducers like CuSO₄ induced maximum laccase activity (~84,000 U L⁻¹ by 21 days of growth). However comparable activity had been obtained with raw textile effluent B when used at 1 % (~86,000 U L⁻¹ by 12 days of growth). This is economically lucrative. Only a single addition of inducer effectively aided in inducing laccase, it is especially effective if added after sufficient growth had occurred. It was observed that pure sodium chloride cannot replace seawater for growth of the fungus and that oxygenation enhances laccase production. Addition of a surfactant such as Tween 80 at 0.25 % after sufficient growth had already occurred positively impacted both biomass and laccase production. At higher concentrations however, the specific activity of laccase

decreased. The medium is designed to almost exclusively promote and maximize laccase production with a high specific activity. The surface to volume ratio of culturing flasks is also a critical factor for biomass and laccase production and requires to be taken into consideration.

The medium had been optimized to almost exclusively support laccase production, cellulase was not induced under the present medium conditions, which makes this especially suitable for use in the biopulping processes. When applied in biobleaching of sugarcane bagasse, it was found that these laccases are quite self sufficient even at low concentrations and do not require the presence of mediators. However, the timely addition of the right kind of mediators, as soon as repolymerization begins not only reverses this process but also reduces the time required to attain the same level of biobleaching.

The whole culture of *Cerrena unicolor* MTCC 5159 and its lignin-degrading enzymes especially laccases along with the exopolymeric substance (EPS) work together in the decolourization processes. The fungal mycelia and the EPS decolourize using processes such as active and passive adsorption and absorption whereas the lignin-degrading enzymes actively degrade the colourants.

Triarylmethane and triphenylmethane dyes are not only good substrates of these laccases but also good inducers of them. Diazo dyes are preferentially degraded over monoazo dyes and also act as laccase inducers. However, the decolourization of some of the diazo dyes may not be proportional to the laccase activity. The simple diazo dyes such as trypan blue are good laccase inducers and their decolourization is proportional to laccase activity. Whereas although congo red, a secondary diazo dye gets decolourized to a similar extent as trypan blue, its decolourization is not proportional to the laccase activity it induces. An anthroquinone dye such as remazol brilliant blue R gets decolourized by 50 % within 6 days, no laccase activity is detected in its presence. Its decolourization is possibly due to the presence of enzymes other than laccase.

Cerrena unicolor MTCC 5159 has a great potential in biobleaching processes as well as decolourizing dyes and industrial effluents and possesses at least one laccase which is of commercial importance.

Chapter 7

Future Prospects

Ph.D. Thesis



Production of metabolites and enzymes in fungi can be increased by several methods. The most common and easiest way is to vary the nutritional and physicochemical parameters which influence product synthesis (Mundra *et al.*, 2007; Soni *et al.*, 2007). Directed evolution is a technique wherein a gene that codes for a particular product is expressed in another host which has a high doubling rate and capacity for successfully translating the inserted gene as seen in the expression of a fully functional laccase from *Myceliophthora thermophila* in *Saccharomyces cerevisiae* (Bulter *et al.*, 2003).

Genetic transformation to increase the output of the original producer organism and make it a hyper producer has also been achieved. In *Aspergillus terreus*, the production of itaconic acid was elevated by the introduction of a gene from *Vitreoscilla stereocoraria* that codes for *Vitreoscilla* hemoglobin (Vhb). The Vhb aided in scavenging oxygen in periods of lowered oxygen tension thus maintaining the production level of itaconic acid, even under oxygen limitation (Lin *et al.*, 2004).

The present study emphasizes the industrial applications of *Cerrena unicolor* MTCC 5159 and its enzymes such as a high quality laccase. This fungus has maximum homology with marine sponge-derived basidiomycetes, suggesting that it has probably been associated with the marine environment long enough for secondary adaptations to occur which are reflected in the ITS rDNA region, making these adaptations stable and capable of being vertically transferred. Fungi which have undergone such adaptations in the marine environment are lucrative for identifying novel marine metabolites and marine-sponge-derived fungi are one of the most important sources for discovering novel marine metabolites (Jensen & Fenical, 2002). This fungus also produces large amounts of exopolymeric substance which is capable of extensive dye decolourization (D'Souza *et al.*, 2006) which merits further investigation. There is an immense scope for further study with regards to this fungus and marine fungi in general.

To determine the type of laccases produced by *Cerrena unicolor* MTCC 5159, the following work was carried out. Degenerate primers which amplify a 200 bps region between the conserved copper binding regions I and II of the laccase gene from white rot and brown rot terrestrial fungi (D'Souza *et al.*, 1996) were used on MTCC 5159. The sequenced region was found to be different from terrestrial laccases (data not included in the thesis). Since this region corresponds, to roughly only 50 AAs, less than

1/10th the actual size of laccase. Finding the laccase from the plethora of isozymes produced by this fungus to which this region corresponds, is a daunting task, especially keeping in mind the low expression level of several of the isozymes.

To first identify a laccase which has desirable properties such as thermotolerance, salt and metal tolerance and then sequence it is a more practical approach. One such a laccase isozyme 'Lac IId' from *Cerrena unicolor* MTCC 5159 has been successfully identified and characterized. Two peptide sequences namely the N-terminal peptide and an internal peptide which is situated near the C-terminal end, have been identified. Using the sequences of these two peptides, primers can be designed which scan the entire length of the gene by various modifications of the PCR technique.

Another alternative is by using the two sets of DNA primers I have designed (data not included in the thesis) which target laccases from terrestrial and marine basidiomyceteous fungi. One set of primers contain a forward and reverse primer which target the conserved copper binding region I and IV and the other set targets the conserved copper binding region II and IV. Since the conserved copper binding region, IV is at the distal end of laccase, more than 4/5th the laccase gene can be sequenced using the traditional PCR technique. This region will also include introns, which can be spliced *in-silico*. Properties such as thermostability and substrate range can be tested for *in-silico* and the most favourable sequence can then be isolated from the laccase gene pool of *Cerrena unicolor* MTCC 5159 by designing specific primers.

Once the sequence is obtained, the entire laccase gene can be transformed into a suitable host which has a short replication time and suitable post translational machinery required for glycosylation of laccase. Fully functional laccase can then be produced on a large scale for industrial and bioremediation purposes.

Transformation of the laccase gene to another host does not utilize the potential of *Cerrena unicolor* MTCC 5159. This fungus also produces lignin-degrading enzymes other than laccase such as MnP and LiP. Their titer was low in the medium used since the entire machinery of the fungus was routed to produce laccase via medium modification. The titer of these enzymes (MnP and LiP) may be enhanced by simply including manganese and veratryl alcohol in the medium and reducing the level of copper to a bare minimum. Medium modification with the intention to increase the MnP or LiP titer requires further investigation. Besides laccase, MnP and LiP, it is worth while to

screen for the production of other commercially important enzymes and metabolites from this fungus.

The decolourization and delignification capability of MTCC 5159 has been well documented in this thesis (Chapter 5). However, the toxicity and other effects of the resulting degradation products requires further investigation before the fungus can be used for bioremediation purposes.

Oxygenation has been known to enhance production of many secondary metabolites (Zhu *et al.*, 2006) in fungi. It has been observed that oxygenation greatly increases laccase production, an enzyme generally produced in the idiophase. Oxygenation of the culture is an expensive and laborious process. If the culture itself had the innate capability to scavenge available oxygen, external oxygenation could be avoided or reduced. This can be accomplished by transforming *Cerrena unicolor* MTCC 5159 with an oxygen scavenging gene such as *Vitreoscilla* hemoglobin gene (Vgb) to make it self sufficient. The effects of *Vitreoscilla* hemoglobin (Vhb) have been well documented. In the wild, *Vitreoscilla stereocoraria* produces Vhb under oxygen limiting conditions (Boerman & Webster, 1982) which captures oxygen and feeds it to the terminal oxidase (Wakabayashi *et al.*, 1986).

It has been successfully transformed into filamentous fungi (Lin *et al.*, 2004) as well as in basidiomyceteous yeast to increase cell density and to enhance metabolite and enzyme production by enhancing oxygen uptake (Zhu *et al.*, 2006). However, *Vitreoscilla stereocoraria* the source of the gene is a prokaryote if this prokaryotic gene is introduced into eukaryotic *Cerrena unicolor* MTCC 5159, it may experience a codon bias. Codon bias is the unequal use of synonymous codons for encoding amino acids and has been found across kingdoms, this bias varies among organisms and even within the genes of the same organism. It is thought that optimal codons help to achieve faster translation rates and high accuracy. As a result of these factors, translational selection is often stronger in highly expressed genes. Whereas in organisms that do not show high growing rates or genes that are minimally expressed, codon usage optimization is normally absent. Codon usage differs greatly between prokaryotes and eukaryotes, this difference is more pronounced between a basidiomycete, which use GC rich codons instead of the AT rich codons used by prokaryotes (Codon Usage Database;

<http://www.kazusa.or.jp/codon>). This could result in low Vgb translation which may not meet the critical concentration required for effective oxygen scavenging.

To overcome this, *Vitreoscilla stereocoraria* strain C1 was used as the source of Vhb with superior oxygen scavenging capabilities. The Vgb sequence of this organism was corrected for codon bias such that this gene should be optimally expressed in basidiomycetes, more specifically with *Phanerochaete chrysosporium* (most well documented basidiomycete). Once transformed, growth and secondary metabolite production of the basidiomycete should be enhanced. This gene has already been synthesized by recursive PCR and deposited at National Center for Biotechnology Information (NCBI) under accession number DQ787436. It has been transformed into p416 vector containing a fungal glyceraldehyde-3-phosphate dehydrogenase, (GPD) expression promoter. This promoter-gene cassette requires to be tagged with a reporter gene for easy identification of clones with the fully functional Vhb. After which it can be transformed into *Cerrena unicolor* MTCC 5159. This should result in enhanced growth or normal growth under lowered oxygen tensions as well as up-regulation of several secondary metabolite / enzyme-coding genes.

It would be worth while to complete these studies to obtained high quality and greater yields of metabolites and enzymes from this fungus.

Ph.D. Thesis

Appendix



8.1 MEDIA

8.1.1 Low nitrogen (LN) (Tien & Kirk, 1988) modified medium: Glucose and glycine at 3.75 % and 0.5 % concentration respectively were added to a mineral salts base which contained the following components; 0.0001 %, 7 % & 10 % final concentration of thiamine, trace elements solution and basal salts solution respectively. The pH was adjusted to 4.5 using citrate phosphate buffer. The volume was brought up to 100 mL using either DW or SW, as per the requirement.

8.1.1.1 Basal salts solution: KH_2PO_4 , MgSO_4 and CaCl_2 at 2 %, 5 % & 0.1% respectively, the final volume was made with DW.

8.1.1.2 Trace elements solution: 1.5 g nitric triacetate was dissolved in 800 mL distilled water and the pH was adjusted to 6.5 using 1 N KOH to which, the following components were added:

MgSO_4	3 g
MnSO_4	0.5 g
NaCl	1 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	0.1 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1 g
CdCl_2	0.08 g
$\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$	0.01 g
H_3BO_3	0.01 g
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.01 g

The solution was made up to 1 L with DW and stored in the dark.

8.1.2 Boyd and Kohlmeyer (B&K) medium (Kohlmeyer & Kohlmeyer, 1979): contained 1 % glucose, 0.2 % peptone and 0.1 % yeast extract. The volume was made up with either DW or SW or half strength (50 %) SW, as per the requirement. In case of solid medium, 1.8 % agar was used.

8.1.3 Malt extract broth (MEB): 2 % (w/v) of ready made malt extract broth powder (HiMedia, Mumbai) dissolved in either DW or SW or half strength (50 %) SW, as per the requirement. In case of solid medium, malt extract agar (MEA), 1.8 % agar was used.

8.1.4 Carrot juice modified agar (Hasan *et al.*, 1995): 10 % (v/v) filtered carrot juice and 0.5 % (w/v) yeast extract was added to LN medium base to which 1.8 % agar was added.

Ready-made media were from Hi-media (Mumbai, India) and other chemicals were from either Sigma or Merck and were of molecular biology or analytical grade as per the requirement.

8.2.1 ESTIMATION METHODS

8.2.1.1 Dissolved Oxygen Estimation (Wrinklers Method) (Eaton *et al.*, 2005)

A 300-mL glass Biological Oxygen Demand (BOD) stoppered bottle was placed below the water surface and filled up to the brim. Immediately without the introduction of air bubbles, 2 mL of MnSO_4 followed by 2 mL of KI-azide reagent was added. The bottle was then stoppered with care to ensure no air was introduced. The sample was mixed by inversion several times. A brownish-orange cloud of precipitate indicated the presence of dissolved oxygen. It was allowed to precipitate after mixing several times by inversion. Just above the surface, 2 mL of concentrated H_2SO_4 was added and the bottle was then stoppered and inverted several times to allow the precipitate to get dissolved by the acid. An aliquot of this treated sample (201 mL) was titrated with $\text{Na}_2\text{S}_2\text{O}_3$ using starch as indicator. The concentration of dissolved oxygen (1 mg L^{-1} dissolved oxygen) in the sample is equivalent to the amount of titerant (mL) used.

8.2.1.2 Chloride Content Estimation (Argentometric Method) (Eaton *et al.*, 2005)

The chloride content of the water sample was estimated via titration with AgNO_3 . The AgNO_3 precipitates out the chlorides in the water sample due to the reaction of Ag^+ with the Cl^- present in the water sample to form a white precipitate of AgCl . Once all the Cl^- ions are used up, the Ag^+ then reacts with the CrO_4^{2-} of the indicator to form Ag_2CrO_4 which is a reddish brown precipitate. The end-point of the reaction is reached when a stable reddish brown precipitate is obtained.

To 5 mL of water sample, 5 mL of (0.35 %) K_2CrO_4 indicator solution was added. This was titrated against 0.2184 M AgNO_3 with constant stirring until a presence of a persistent orange colour occurred. The volume of AgNO_3 required for the colour change to occur was recorded.

CALCULATIONS:

$$0.2184 \text{ M AgNO}_3 * \text{vol of AgNO}_3 (\text{L}) = 'x' \text{ mol AgNO}_3 = 'x' \text{ mol Ag}^+ = 'x' \text{ mol Cl}^-$$

$$('x') * (35.45 \text{ g Cl}^- \div 1 \text{ mol Cl}^-) = \text{mass Cl}^- = 'y' \text{ g of Cl}^-$$

$$('y' \div \text{mass of sample}^\dagger) * (1000) = z \text{ chlorinity (ppt)}$$

$$^\dagger \text{mass of sample} = \text{volume of sample (mL)} * \text{density of sample}^\#$$

$$^\# \text{when the sample is sea water, the density of sea water is } 1.05 \text{ g mL}^{-1}$$

NOTE: 1.80655 Cl (ppt) = *Salinity (ppt)*

8.2.1.3 Lignin Estimation (Kappa Number Method) (Anonymous, 1988)

STANDARDIZATION OF POTASSIUM PERMANGANATE (KMnO₄) SOLUTION

The burette was filled with KMnO₄ whose normality had to be determined. 20 mL of 0.1 N ferrous ammonium sulphate containing 0.2 % H₂SO₄ was taken in a conical flask to which, 10 - 15 mL of 1 M H₂SO₄ was added. This solution of ferrous ammonium sulphate was titrated by slowly adding small amounts of KMnO₄ from the burette with continuous mixing. The pink colour obtained upon addition of KMnO₄ solution disappears on mixing. The titration is continued until a permanent pale pink colour appears.

STANDARDIZATION OF SODIUM THIOSULPHATE (Na₂S₂O₃) SOLUTION

10 mL of 0.1N K₂Cr₂O₇ solution was taken in a conical flask to which, 20 mL of 1N HCl was added. To this mixture, 2 g of KI was added and mixed well. The mixture was kept in the dark for 10 min after which, it was titrated against Na₂S₂O₃ solution present in the burette. After adding about 3 - 4 mL of Na₂S₂O₃ or till the solution in the conical flask turned pale yellow, 1 mL of starch was added. The titration was continued till the end-point, which was bluish green.

PROCEDURE

To 0.1g of moisture-free sugarcane bagasse, 79.5 mL distilled water was added and mixed until the bagasse was completely wet. To this, a mixture of 10 mL (0.1N) KMnO₄ & 10 mL (4N) H₂SO₄ was added and mixed well. The solution was gently mixed for 10 min and the temperature of the solution was recorded mid way of the reaction. The reaction was stopped with 2 mL of (1 N) KI. This too was well mixed and then the free I₂ released as a result was titrated against (0.2 N) Na₂S₂O₃ using starch (0.2 %) as an indicator. The volume of Na₂S₂O₃ was recorded. The blank determination was carried out using exactly the same method but omitting the pulp.

CALCULATIONS:

$$p = [(b - a) * N] \div 0.1$$

Where; p = amount of KMnO₄ (0.1N) actually consumed by the test (mL).

b = amount of Na₂S₂O₃ consumed by the blank (mL).
 a = amount of Na₂S₂O₃ solution consumed by the test (mL).
 N = normality of Na₂S₂O₃.

Using the value of 'p', the value of 'f' is determined from the table below:

Factor 'f' correction for different percentages of permanganate used

<i>f</i>	0	1	2	3	4	5	6	7	8	9
30	0.958	0.960	0.962	0.964	0.966	0.968	0.970	0.973	0.975	0.977
40	0.979	0.984	0.983	0.985	0.987	0.989	0.991	0.994	0.996	0.998
50	1.000	1.002	1.004	1.006	1.009	1.011	1.013	1.015	1.017	1.019
60	1.002	1.024	1.026	1.028	1.030	1.033	1.035	1.037	1.039	1.042
70	1.044									

When,

When a constant temperature bath was not available, the temperature was determined after 5 min of reaction period. This was assumed the average reaction temperature throughout the test. If the temperature was not higher than 30°C or lower than 20°C, the correction for the kappa number is as follows:

$$K = [p * f * (1 + 0.013 (25 - t))] \div w$$

Where;

K = kappa number

f = correction factor to a 50 % KMnO₄ consumption, dependent on the value of 'p'

w = weight of the moisture free bagasse (g)

p = amount of 0.1N KMnO₄ actually consumed by the test (mL).

t = temperature in °C of the reaction mixture midway during incubation period.

Relationship with lignin:

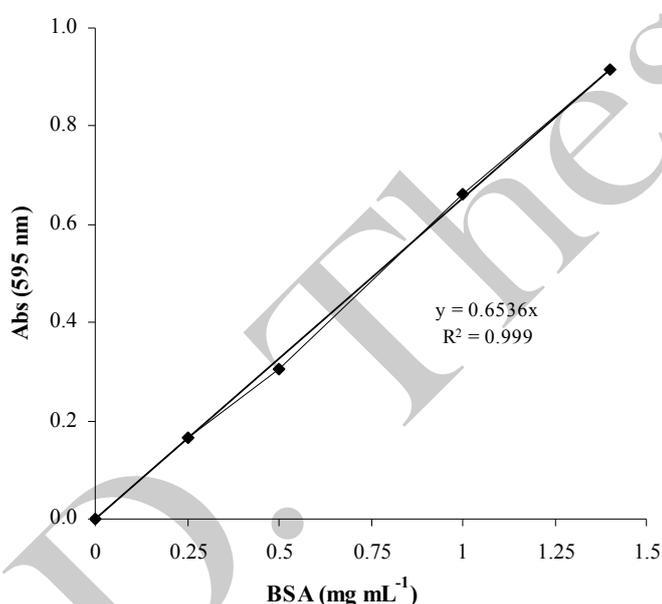
The kappa number gives essentially a straight line relationship with both Klason lignin and chlorine number for pulp below 70 % total pulp yield.

$$\text{Klason lignin (\%)} \approx K \times 0.1$$

8.2.1.4 Protein Estimation (Bradford's Method) (Bradford, 1976; Stoscheck, 1990)

Bovine serum albumin (BSA); 2 g was dissolved in 1 mL distilled water and used as stock for the preparation of protein standards for protein estimation by Bradford's method. The various dilutions were prepared in distilled water.

To 30 μL of each protein dilution, 900 μL of Bradford's reagent was added and incubated at RT for 30 min. The absorbance was recorded spectrophotometrically at 595 nm and a standard graph for the same was plotted.



CALCULATIONS:

$$\text{Protein (mg mL}^{-1}\text{)} = [A_{595} / 0.6536]$$

Where;

A_{595} = Absorbance at 595 nm

Protein was expressed as mg mL^{-1} or g L^{-1} .

8.2.1.5 Biomass Estimation

The fungal biomass was estimated as its dry weight. The culture broth was filtered through pre-weighed Whatman No.1 filter paper. The paper was dried at 60°C, until a constant weight was achieved. This was then conditioned at room temperature and then reweighed. The difference in weight was considered as the dry weight and expressed as g L^{-1} of culture medium.

8.2.2 ENZYME ESTIMATION METHODS

8.2.2.1 Laccase Assay

8.2.2.1.1 Using ABTS (Childs & Bardsley, 1975; Niku-Paavola *et al.*, 1988)

Laccase activity in the sample was spectrophotometrically determined by monitoring the rate of product (dark green colour) formation due to the enzymatic oxidation of ABTS.

In a 1 mL of cuvette, the following components were added;

0.5 mL of 2 mM ABTS prepared in buffer of desired pH range + 0.05 to 0.5 mL of enzyme (to be tested) was added, the same buffer in which the ABTS was prepared in, was used to make up the total volume to 1 mL

The kinetic reaction was spectrophotometrically measured at 405 nm for 1 min at the desired temperature, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

$$\text{Laccase (U L}^{-1}\text{)} = [\Delta A_{405} * \text{total vol} * \text{dilution factor} * 10^6] \div [E_{\text{ABTS}} * \text{sample vol}]$$

Where;

ΔA_{405} = rate of reaction i.e. final abs - initial abs \div time (min)

E_{ABTS} = molar extinction coefficient of the radical-cation ABTS (35000)

Total vol = total volume of reaction mixture (mL)

Sample vol = volume of enzyme used (mL)

Laccase was expressed as enzyme units per liter i.e. U L^{-1} or $(\mu\text{mol min}^{-1}) \text{L}^{-1}$, where one enzyme unit is expressed as μmol of the product formed per minute.

8.2.2.1.2 Using Syringaldazine (Harkin & Obst, 1973)

Laccase activity in the sample was spectrophotometrically determined by monitoring the rate of product (dark pink colour) formation.

In a 1 mL of cuvette, the following components were added;

0.1 mL of 10 mM syringaldazine prepared in ethanol + 0.4 mL of buffer of desired pH range + 0.05 to 0.5 mL of enzyme (to be tested) was added, buffer, was used to make up the total volume to 1 mL

The kinetic reaction was spectrophotometrically measured at 525 nm for 1 min at 30°C, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

$$\text{Laccase (U L}^{-1}\text{)} = [\Delta A_{525} * \text{total vol} * \text{dilution factor} * 10^6] \div [C_{\text{Syringaldazine}} * \text{sample vol}]$$

Where;

ΔA_{525} = rate of reaction i.e. final abs - initial abs \div time (min)

$C_{\text{Syringaldazine}}$ = molar extinction coefficient of the product (65000)

Total vol = total volume of reaction mixture in (mL)

Sample vol = volume of enzyme used in (mL)

Laccase was expressed as enzyme units per liter i.e. U L^{-1} or $(\mu\text{mol min}^{-1}) \text{L}^{-1}$, where one enzyme unit is expressed as μmol of the product formed per minute.

NOTE: Both ABTS and syringaldazine also act as substrates for peroxidases in the presence of hydrogen peroxide. To exclude the inclusion of peroxidase in laccase estimation, endogenous peroxide is required to be removed by pre-incubating the enzyme sample with catalase ($10 \mu\text{g mL}^{-1}$) with stirring prior to assay for laccase activity.

8.2.2.1.3 Using Guaiacol (modified) (Setti *et al.*, 1998; Xiao *et al.*, 2001)

Laccase activity in the sample was spectrophotometrically determined by monitoring the rate of product (dark brown colour) formation.

In a 1 mL of cuvette, the following components are added;

0.1 mL of 10 mM guaiacol prepared in ethanol + 0.4 mL of buffer of desired pH range + 0.05 to 0.5 mL of enzyme (to be tested) was added, buffer, is used to make up the total volume to 1 mL.

The kinetic reaction was spectrophotometrically recorded at 460 nm for 3 min after 1 min delay at 30°C, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

$$\text{Laccase (U L}^{-1}\text{)} = [\Delta A_{460} * \text{total vol} * \text{dilution factor} * 10^6] \div [C_{\text{Guaiacol}} * \text{sample vol}]$$

Where;

ΔA_{460} = rate of reaction i.e. final abs - initial abs \div time (min)

C_{Guaiacol} = molar extinction coefficient of the product (27.75)

Total vol = total volume of reaction mixture in (mL)

Sample vol = volume of enzyme used in (mL)

Laccase was expressed as enzyme units per liter i.e. U L^{-1} or $(\mu\text{mol min}^{-1}) \text{L}^{-1}$, where one enzyme unit is expressed as μmol of the product formed per minute.

8.2.2.2 Lignin Peroxidase (LiP) Assay (Paszczynski *et al.*, 1988)

LiP catalyses the oxidation of veratryl alcohol to veratraldehyde by H₂O₂. Only the aldehyde shows absorbance at 310 nm.

In a 1 mL cuvette, the following components are added;
 0.5 mL of reaction mixture #
 + 0.5 mL of enzyme solution to be tested

Reaction mixture (25 mL) = 20 mL of 125 mM d-tartaric acid buffer (pH 2.5)
 + 2.5 mL of 40 mM veratryl alcohol + 2.5 mL of 8 mM H₂O₂.

The kinetic reaction was spectrophotometrically measured at 310 nm for 1 min at the desired temperature. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme.

CALCULATIONS:

$$\text{LiP (U L}^{-1}\text{)} = [\Delta A_{310} * \text{total vol} * \text{dilution factor} * 10^6] \div [\epsilon_{\text{Veratrylaldehyde}} * \text{sample vol}]$$

Where;

ΔA_{310} = rate of reaction i.e. final abs - initial abs \div time (min)

$\epsilon_{\text{Veratrylaldehyde}}$ = molar extinction coefficient of veratrylaldehyde (9300)

Total vol = total volume of reaction mixture (mL)

Sample vol = volume of enzyme used (mL)

Lignin peroxidase was expressed as enzyme units per liter i.e. U L⁻¹ or ($\mu\text{mol min}^{-1}$) L⁻¹, where one enzyme unit is expressed as μmol of the product formed per minute.

8.2.2.3 Manganese-dependant Peroxidase (MnP) Assay (Paszczynski *et al.*, 1988)

MnP catalyses the oxidation of Mn^{+2} to Mn^{+3} by H_2O_2 the product Mn^{+3} , forms a transient stable complex with tartaric acid, showing a characteristic absorbance at 238 nm.

In a 1 mL cuvette, the following components are added;
 0.8875 mL reaction mixture [#] + 0.0125 mL of 8 mM H_2O_2
 + 0.1 mL enzyme solution to be tested

[#] **Reaction mixture** (17.75 mL) = 4 mL of 500 mM sodium tartarate buffer (pH 5) + 0.2 mL of 10 mM MnSO_4 + 13.55 mL DW.

The kinetic reaction was spectrophotometrically measured at 238 nm for 1 min at the desired temperature, as an increase in absorbance. The blank contained all the assay constituents except the active enzyme, buffer or heat inactivated enzyme was used in its place.

CALCULATIONS:

$$\text{MnP (U L}^{-1}\text{)} = [\Delta A_{238} * \text{total vol} * \text{dilution factor} * 10^6] \div [C_{\text{Mn}^{+3}} * \text{sample vol}]$$

Where;

ΔA_{238} = rate of reaction i.e. final abs - initial abs \div time (min)

$C_{\text{Mn}^{+3}}$ = molar extinction coefficient of Mn^{+3} -tartarate complex (6500)

Total vol = total volume of reaction mixture (mL)

Sample vol = volume of enzyme used (mL)

Manganese dependant peroxidase was expressed as enzyme units per liter i.e. U L^{-1} or $(\mu\text{mol min}^{-1}) \text{L}^{-1}$, where one enzyme unit is expressed as μmol of the product formed per minute.

8.2.2.4 Estimation of Cellulase (modified) (Miller, 1959; Trigiano *et al.*, 2003)

Paper is the source of cellulose, the reducing sugars released as a result of cellulase action on cellulose is estimated by the dinitro salicylic acid (DNSA) method using D-glucose as the sugar standard.

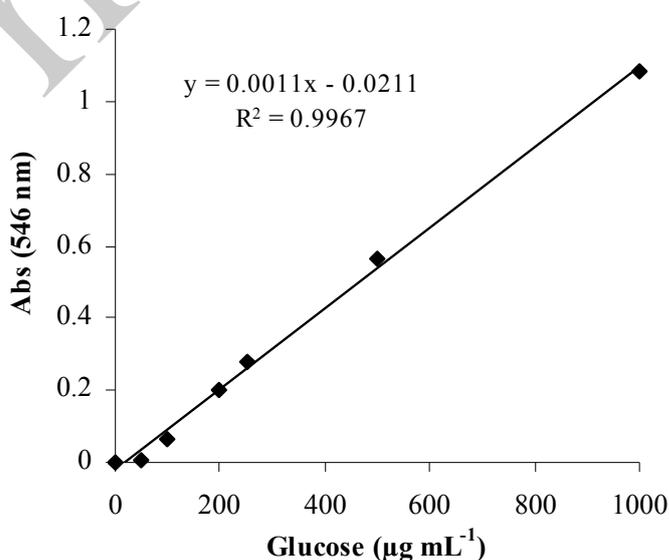
Finely cut-pieces of Whatman No. 1 paper, 0.01 g was incubated with the enzyme sample dissolved in 0.2 M Na-acetate buffer, pH 4.5 at 30°C for 1 h. One mL of this sample (without the paper) + 1 mL of DNSA reagent[#] were incubated in a boiling water bath for 10 min. It was then cooled to RT, the total volume was brought to 10 mL with DW and the absorbance was spectrophotometrically recorded at 546 nm. The blank was enzyme free.

#DNSA Reagent (to be prepared without heating):

Solution A – Sodium potassium tartarate (Rochelle salt), 150 g dissolved in 100 mL of distilled water was added to DNSA, 5 g dissolved in 20 mL of 10 % NaOH. The total volume was made to 250 mL using distilled water.

Solution B – Sodium bisulphate, 0.25 g was dissolved in a solution containing 1 g of phenol prepared in 30 mL of 10 % NaOH. The total volume was made to 250 mL using distilled water.

Solution A & B are mixed and stored in the dark at 4°C.



CALCULATIONS:**AMOUNT OF REDUCING SUGAR**

The concentration of reducing sugar was determined as mg mL^{-1} , from the following equation obtained from the above Std. graph;

$$Y = 0.0011X - 0.0211$$

Where X = 'A' $\text{mg reducing sugar mL}^{-1}$

Y = Absorbance of sample at 546 nm

Total Reducing sugar:

A* total vol of reaction mixture (mL) = '**B**' **mg total reducing sugars**

Amount of mg moles of D-glucose:

Molecular weight of D-glucose = 180.2 g i.e. $180.2 \text{ g mole}^{-1}$

Converting g to mg,

$$180.2 * 10^3 = 180200 \text{ mg D-glucose mole}^{-1} \text{----- (1)}$$

AMOUNT OF PROTEIN

Using the Bradfords method protein concentration was determined, '**C**' mg mL^{-1} .

Total Protein:

Total protein = C * vol of sample used in assay (mL) = '**E**' **mg total protein**

Since the sample was incubated for 1 h,

$$= (\text{B mg total reducing sugars}) \div (\text{E mg total protein}) \div 60$$

$$= \text{'F'} (\text{mg reducing sugar mg}^{-1} \text{ total protein}) \text{ min}^{-1} \text{----- (2)}$$

AMOUNT OF CELLULASE

Dividing equation (2) by (1),

$$F \div 180200 = \text{'G'} (\text{moles of reducing sugar mg}^{-1} \text{ total protein}) \text{ min}^{-1}$$

$$= G * 10^6 = \text{'H'} (\mu\text{moles of reducing sugar mg}^{-1} \text{ total protein}) \text{ min}^{-1}$$

Cellulase activity = 'H' ($\mu\text{moles of reducing sugar mg}^{-1} \text{ total protein}) \text{ min}^{-1}$

One unit of cellulase was defined as the amount of enzyme which produced 1 μmole glucose equivalent per minute under the assay conditions and expressed as **($\mu\text{moles mg}^{-1} \text{ total protein}) \text{ min}^{-1}$.**

8.2.3 LACCASE CHARACTERIZATION

8.2.3.1 Molecular mass of laccase using size exclusion chromatography

(Superdex 75 column)

$$K_{av} = (\text{Elution vol} - \text{Void vol}) \div (\text{Total vol} - \text{Void vol})$$

Where Total vol = 120.637 mL ;

Void vol = 39.810001 mL

This value is substituted in the following equation which was previously determined in the laboratory using the std proteins (BSA, chicken egg albumin, carbonic anhydrase, α -lacto albumin) for Superdex 75 column at the same flow rate (1 mL min⁻¹) which was used to separate the protein. $K_{av} = -0.2287 * \ln (\text{Mol wt}) + 2.6367$.

By solving the equation, the molecular mass is obtained in **Daltons (Da)**.

8.2.3.2 LACCASE KINETIC PARAMETERS (Nelson & Cox, 2004)

8.2.3.2.1 V_{max} (maximum velocity): A reciprocal plot of velocity of reaction (y-axis) and substrate concentration (x-axis) [Line Weaver burke Plot] is plotted. Upon extrapolating the line, the reciprocal of the intercept on the positive Y-axis is the V_{max} ($\mu\text{mol min}^{-1}$).

8.2.3.2.2 K_m (Michaelis constant): From the above plot, the reciprocal of the intercept on the negative X-axis is the K_m (μM) [the -ve sign of the value is ignored].

8.2.3.2.3 K_{cat} (Catalytic constant): $K_{cat} = V_{max} \div E_t$ (μmol). Where E_t = Total amount of enzyme (in terms of total protein in μg) \div Mol mass of laccase (kDa). The unit for K_{cat} is either min^{-1} or sec^{-1} .

8.2.3.2.4 Specificity Constant = $K_{cat} \div K_m$.

The unit for specificity constant is $\mu\text{M min}^{-1}$.

8.2.3.2.5 Q_{10} (temperature coefficient) http://www.csupomona.edu/~seskandari/physiology/physiological_calculators/Q10.html: This represents the factor by which the rate (R) of a reaction increases for every 10 degree rise in the temperature (T) in degrees Celsius or Kelvin (K).

$$Q_{10} = [R_2 \div R_1]^{[10 \div (T_2 - T_1)]}$$

Q_{10} is a unitless quantity.

8.2.3.2.6 E_{act} (Energy of activation) (<http://www.rod.beavon.clara.net/Q10.html>): This has been determined in terms of Q_{10} value.

$$E_{act} = [R * \ln (k_2 \div k_1)] \div [(1 \div T_1) - (1 \div T_2)]$$

Where ; k_1 and k_2 are the rate constants of the reaction at temperatures T_1 and T_2 in Kelvin (K).

R (gas constant) = 8.314472 J K⁻¹ mol⁻¹ or 1.987 C K⁻¹ mol⁻¹.

The units of E_{act} is either **J mol⁻¹** or **Cal mol⁻¹** depending of the units of 'R'. It is converted in k J mol⁻¹ or Cal mol⁻¹ by multiplying the result with 10⁻³.

8.2.3.3 PURIFICATION TABLE (Nelson & Cox, 2004)

8.2.3.3.1 Total Specific Activity:

= Total laccase (U) in step x \div Total protein (mg) in step x .

The unit for total specific activity is U mg⁻¹.

8.2.3.3.2 Yield:

= Total laccase (U) in step x \div Total original laccase (U).

The unit for yield is % relative considering original as 100%.

8.2.3.3.3 Purification Fold:

= Specific activity in step x \div Original specific activity

8.2.3.4 Specific Activity (U mg⁻¹): = Laccase (U mL⁻¹) \div protein (mg mL⁻¹).

The unit for specific activity is U mg⁻¹.

8.3 PROCEDURES & PROTOCOLS

8.3.1 Fungal DNA Extraction (van Burik *et al.*, 1998)

The fresh fungal biomass was rinsed in sterile DW twice, lyophilized and then ground with a mortar and pestle. This was re-suspended in extraction buffer and 1 % β -mercaptoethanol preheated to 65°C. The mixture was then subjected to a deproteination step which consisted of extraction in a mixture of Tris-saturated phenol (pH 8), chloroform and isoamyl alcohol in the ratio of 25:24:1, after vigorous mixing, the solution was subjected to centrifugation (13,000 g / 15 min / 4°C). The supernatant was subjected to RNase (10 % of 2 mg mL⁻¹ stock) treatment at 37°C and immediately followed by an extraction in a mixture of chloroform and isoamyl alcohol in the ratio of 24:1. The mixture was subjected to centrifugation (13,000 g / 15 min / 4°C), 2.5 vols of isopropanol was added to the supernatant and stored at -20°C for at least 1 h. This caused the DNA to precipitate which was then rinsed in 70 % ethanol, air-dried and then resuspended in high salt Tris EDTA (TE) buffer.

The quality of the DNA was estimated via agarose gel electrophoresis at ~ 60 V using 1X Tris-Borate-EDTA (TBE) running buffer. The gel contained 0.8 % (w/v) agarose in 1X TBE buffer, to which ethidium bromide was added. After electrophoresis, the gel was visualized under UV light, DNA appears as fluorescent orange bands due to the intercalation of ethidium bromide with the DNA double helix. The image was then documented using a gel documentation system (Alpha Imager, Alpha Innotech, C.A, USA).

Extraction buffer: 100 mM Tris-HCl, 20 mM Na-EDTA, 2 % CTAB, 1.4 M NaCl & 1 % poly vinyl pyrrolidone in DW

High Salt TE Buffer: 10 mM Tris-HCl, 1 mM Na-EDTA & 1 M NaCl in DW

10X TBE Buffer: 900 mM Tris-base, 0.5 M Na-EDTA & 900 mM boric acid in DW

DNA Loading Dye: 0.25 % bromophenol blue, 0.25 % xylene cyanol & 0.25 % ficoll in DW.

8.3.2.1 SDS PAGE Composition & Reagents (Sambrook & Russell, 2001)

COMPONENTS	12 % Resolving gel (mL)	5 % Stacking gel (mL)
<i>DW autoclaved</i>	3.4	5.7
<i>Monomer solution</i>	4.0	1.7
<i>Gel buffer</i>	2.5 [†]	2.5 [#]
<i>SDS (10 %)</i>	0.1	0.1
<i>APS (10 %) freshly prepared</i>	0.05	0.05
<i>TEMED</i>	0.02	0.01

[†]**Resolving buffer:** 1.5 M Tris-HCl, pH 8.8 adjusted with 6 N HCl.

[#]**Stacking buffer:** 0.5 M Tris-HCl, pH 6.8 adjusted with 6 N HCl.

Monomer solution (acrylamide-bisacrylamide solution, 30 %): 29.2 g of acrylamide and 0.8 g of N'N'-bis-methylene-acrylamide was made to a final volume of 100 mL using DW. The stock solution was filter sterilized through 0.22 µm filter and stored at 4°C in dark.

SDS-PAGE sample solubilizing buffer (6×): contained 300 mM Tris-HCl (pH 6.8), 7.5 % β-mercaptoethanol, 10 % SDS, 60 % Glycerol, 0.6 % bromophenol blue prepared in autoclaved DW.

SDS-PAGE running buffer (pH 8.3, 10×): 30.2 g Tris base, 144 g glycine & 100 mL of 10 % SDS, the final volume is made to 1 L using DW.

8.3.2.2 Electro-Blotting (<http://www.oardc.ohio-state.edu/stockingerlab/Protocols/ProteinElectroblotting.pdf>)

After SDS PAGE was over, the gel was washed in milli Q water to remove the SDS and then soaked in 1X CAPS buffer for 5 min. The packing (Whatman sheets) were also similarly soaked. The PVDF membrane was soaked in 100% methanol for 5 min. The gel was then placed onto the membrane and the air

bubbles, if any were removed. On either side, CAPS buffer-soaked Whatman sheets were placed. Together they were placed in the electro blotting chamber with the gel facing the negative electrode and the PVDF membrane, the positive electrode. Electrophoresis was carried out overnight in the cold.

The PVDF membrane was stained in Coomassie stain for 2 - 3 min and then destained using 50 % methanol with 2 - 3 intermittent changes. After the background was removed the membrane was dried between clean dry Whatman sheets.

10X CAPS Buffer: 5.535 g CAPS was dissolved in 200 mL milli Q water. The pH was adjusted to 11 with 2 N NaOH. The total volume was made up to 250 mL with milli Q water.

1X CAPS Buffer: 100 mL of 10 X CAPS Buffer was added to 800 mL milli Q water and then chilled. 100 mL methanol was added just before use to the chilled mixture.

8.3.2.3 Polyacrylamide gel / PVDF Membrane Staining Procedures

8.3.2.3.1 ACTIVITY STAINING FOR LACCASE (Xiao *et al.*, 2004)

After electrophoresis was over, the polyacrylamide gel was placed in a citrate phosphate buffer (pH 6) solution containing 10 % guaiacol till staining occurs. Protein bands corresponding to laccase oxidize guaiacol to a deep brown colour and these bands retain the colour. The excess guaiacol was removed with repeated rinses in distilled water. The gel was placed in fixative (same as used in silver staining of proteins). This removes the background and previously invisible laccase bands begin to appear. The gel was then placed in water for complete rehydration and the image was then documented using the gel documentation system.

NOTE: All incubation steps are carried out on a gel rocker.

8.3.2.3.2 PROTEIN STAINING

8.3.2.3.2.1 Silver Staining (Heukeshoven & Dernick, 1985): After electrophoresis was over, the gel was placed in fixative for at least 1 h. After which it was transferred to methanol (for dehydration) and kept for at least 20 min. This was repeated at least 2 - 3 times using fresh methanol each time. The gel was then rehydrated in distilled water till complete rehydration occurred. It was then placed in 0.02 % $\text{Na}_2\text{S}_2\text{O}_3$ for 2-3 min and then subjected to 30 min incubation in 0.2 % AgNO_3 in the dark. The AgNO_3 was rinsed off with distilled water and then the gel was placed in developer and shaken very gently till protein bands appeared. The reaction was stopped by placing the gel in fixative for 5 - 10 min. The gel was then placed in water for complete rehydration and the image was then documented using the gel documentation system.

Fixative: 250 mL methanol, 62.5 mL acetic acid & 500 μL formalin in 187 mL DW.

Developer: 6 g Na_2CO_3 , 40 μL of 10 % $\text{Na}_2\text{S}_2\text{O}_3$ & 75 μL formalin in 100 mL DW.

8.3.2.3.2.2 Coomassie Blue Staining (<http://www.ohsu.edu/proteomics/sampleprep/coomassie.pdf>): After electrophoresis was over, the gel was placed in Coomassie Blue stain for 2 h and then destained overnight using Coomassie Blue destain solution with several intermittent changes, until the background was cleared and only the protein bands stained blue.

Coomassie Blue staining solution: 40 % methanol, 10 % acetic acid, 0.25 % Coomassie brilliant blue R-250 prepared in milli Q water.

Coomassie Blue destaining solution: 40 % methanol, 10 % acetic acid, prepared in milli Q water.

8.3.2.3.3 GLYCOPROTEIN STAINING (Schiffs Method) (Zacharius *et al.*, 1969):

After electrophoresis was over, the polyacrylamide gel was placed in fixative for at least 30 min. This step was repeated at least 4 times using fresh fixative solution each time. The fixation overnight was continued in fresh fixative and then the gel was subjected to another two washes for 30 min each in fresh fixative. The glycoprotein bands were oxidized by placing them in 1 % periodic acid – 3 % acetic acid mixture for 60 min. The oxidized gel was washed with water at least 10 times, with 10 min incubation each time, to remove traces of periodic acid. The gel was then incubated in Schiff's reagent (Sigma, USA) for 60 min in the dark. Red-purple glycoprotein bands began to appear within the first 20 min itself. To remove the background (for image capture), the gel was washed at least thrice, with a 10 min incubation each time in 0.58 % potassium metabisulphite - 3 % acetic acid prepared in water. Washing with this solution at 30 min intervals using fresh solution each time, was continued till all the background was eliminated.

NOTE: For all staining procedures, the incubation steps were carried out on a gel rocker.

8.3.3 Exopolymeric Substance (EPS) Staining (Long & Azam, 1996)

A 21 day-old fungal culture grown in liquid medium was placed on a slide and teased to separate the fungal strands. A drop of 0.22 μ m filtered Alcian blue stain was placed onto the preparation, over which a cover slip was placed. The Alcian blue stains the EPS blue whereas the fungal hyphae remain transparent.

Alcian blue stain: 0.02 % Alcian blue in 0.06 % acetic acid (pH 3.3)

8.4 DETAILS OF THE INDUSTRIAL EFFLUENTS

8.4.1 Textile Effluent A (TeA) was obtained from Atul Ltd., Gujarat, India and contained mainly Azo dye - 20 with 0.34 % carbonate. The effluent was deep purple and had a pH of 8.9.

8.4.2 Textile Effluent B (TeB) was obtained from from Atul Ltd., Gujarat, India and contained a mixture of dyes with 21700 colour units, total solids 4.2 %, Na^{+1} 2440 ppm, Ca^{+2} 31 ppm, SO_4^{-2} 0.6 %, Cl^{-1} 3.2 % and PO_4^{-3} 0.5 %. The dyes in this mixture were reactive blue 140 base, reactive blue 140, reactive blue 160 base, reactive blue 163, reactive red 11, reactive yellow 145, reactive green 19 and reactive blue 4. The effluent was dark green in colour and had a pH of 2.5.

8.4.3 Molasses Spent Wash (MSW) in the raw untreated form was obtained from Rhea Distilleries Ltd., Goa, India. It had a BOD of 42,000 mg L^{-1} and COD of 80,000 mg L^{-1} . It was dark brown in colour and had a pH of 4.3.

8.4.4 Black Liquor (BL) was obtained from Seshasayee Paper Mills, a bagasse and wood chip-based newsprint manufacturing unit in Tamil Nadu, India. The effluent was black in colour and had a COD of 416 mg L^{-1} and BOD of 190 mg L^{-1} .

Bibliography

Ph.D. Thesis



Abdel-Wahab, M. A. & El-Sharouny, H. M. (2002) Ecology of subtropical mangrove fungi with emphasis on *Kandelia candel* mycota. In: *Fungi in Marine Environments*, (Hyde, K. D., Ed.), Fungal Diversity Research Series 7. Fungal Diversity Press, Hong Kong, 247-265.

Ali, M. & Sreekrishnan, T. R. (2001) Aquatic toxicity from pulp and paper mill effluents: A review. *Advances in Environmental Research*, **5**, 175-196.

Allen, A. C. & Walker, J. R. L. (1988) The selective inhibition of catechol oxidase by salicylhydroxamic acid. *Phytochemistry*, **27**, 3075–3076.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389-3402.

Ander, P. & Eriksson, K.-E. (1978) Lignin degradation and utilisation by microorganisms. *Progress in Industrial Microbiology*, **14**, 1-58.

Anonymous (1988) Kappa number of pulp-T 236-cm-85. In: *TAPPI test methods*. TAPPI Press, Atlanta, U.S.A., **1**, 1-3.

Anonymous (2001) Lignin and its Properties: Glossary of Lignin Nomenclature. The Lignin Institute, **9**. <http://www.lignin.org/01augdialogue.html>

Arora, D. S. & Gill, P. K. (2000) Laccase production by some white rot fungi under different nutritional conditions. *Bioresource Technology*, **73**, 283-285.

Baldrian, P. (2003) Interactions of heavy metals with white-rot fungi. *Enzyme and Microbial Technology*, **32**, 78-91.

Baldrian, P. (2006) Fungal laccases - occurrence and properties. *FEMS Microbiology Reviews*, **30**, 215-242.

Bandaranayake, W. M. (1998) Traditional and medicinal uses of mangroves. *Mangroves and Salt Marshes*, **2**, 133-148.

Bauer, C. G., Kuehn, A., Gajovic, N., Skorobogatko, O., Holt, P. J., Bruce, N. C., Makower, A., Lowe, C. R. & Scheller, F. W. (1999) New enzyme sensors for morphine and codeine based on morphine dehydrogenase and laccase. *Fresenius' Journal of Analytical Chemistry*, **364**, 179–183.

Begerow, D., Bauer, R. & Oberwinkler, F. (1997) Phylogenetic studies on nuclear large subunit ribosomal DNA sequences of smut fungi and related taxa. *Canadian Journal of Botany*, **75**, 2045-2056.

Benito, B., Garcíadeblás, B. & Rodríguez-Navarro, A. (2002) Potassium-or sodium-efflux ATPase, a key enzyme in the evolution of fungi. *Microbiology*, **148**, 933-941.

Benner, R. & Hodson, R. E. (1985) Microbial degradation of the leachable and lignocellulosic components of leaves and wood from *Rhizophora mangle* in a tropical mangrove swamp. In: *Marine Ecology Progress Series*, **23**, 221-230.

Berbee, M. L. & Taylor, J. W. (1992) Detecting the morphological convergence in true fungi using 18S RNA sequence data. *BioSystems*, **28**, 117-125.

Bernier, R. L., Kluepfel, D., Morosoli, R. & Shareck, F. (May 1992) Cellulase-free endo-xylanase enzyme of use in pulp delignification. **Patent No. 5116746**, Canada.

Bertrand, M. G. (1895) Sur la laccase et sur le pouvoir oxydant de cette diastase. *Les Comptes rendus de l'Académie des Sciences (Paris)*, **120**, 266–269.

Bes, B., Petterson, B., Lenholm, H., Iverson, T. & Eriksson, K. E. (1987) Synthesis, structure and enzyme degradation of an extracellular glucan produced in nitrogen-starved culture of the white-rot fungus *Phanerochaete chrysosporium*. *Biotechnology and Applied Biochemistry*, **9**, 310-318.

Besitulo, A. D., Sarma, V. V. & Hyde, K. D. (2002) Mangrove fungi from Siargio Island, Philippines. In: *Fungi in Marine Environments*, (Hyde, K. D., Ed.), Fungal Diversity Research Series 7. Fungal Diversity Press, Hong Kong, 267-283.

Bhatt, N., Patel, K. C., Keharia, H. & Madamwar, D. (2005) Decolorization of diazo-dye Reactive Blue 172 by *Pseudomonas aeruginosa* NBAR12. *Journal of Basic Microbiology*, **45**, 407-418.

Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Görg, A., Westermeier, R. & Postel, W. (1982) Isoelectric focusing in immobilized pH gradients: Principle, methodology and some applications. *Journal of Biochemical and Biophysical Methods*, **6**, 317-339.

Blackwell, M. (1993) Phylogenetic systematics and ascomycetes. In: *The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics*, (Reynolds, D. R. & Taylor, J. W., Eds.). CAB International, Wallingford, U.K., 93-103.

Blanz, P. A. & Unsel, M. (1987) Ribosomal RNA as a taxonomic tool in mycology. In: *The expanding realm of yeast-like fungi*, (de Hoog, G. S., Smith, M. T. & Weijman, A. C., Eds.). Elsevier, Amsterdam, The Netherlands, 247-258.

Blomberg, A. & Adler, L. (1992) Physiology of osmotolerance in fungi. *Advances in Microbial Physiology*, **33**, 145-212.

Boekhout, T. (1991) *A revision of ballistoconidia-forming yeasts and fungi*, Studies in Mycology, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, 194 pgs.

Boerjan, W., Ralph, J. & Baucher, M. (2003) Lignin biosynthesis. *Annual Review of Plant Biology*, **54**, 519-546.

Boerman, S. J. & Webster, D. A. (1982) Control of heme content in *Vitreoscilla* by oxygen. *Journal of General and Applied Microbiology*, **28**, 35-43.

Bollag, J.-M. & Leonowicz, A. (1984) Comparative studies of extracellular fungal laccases. *Applied and Environmental Microbiology*, **48**, 849-854.

Bonomo, R. P., Cennamo, G., Purrello, R., Santoro, A. M. & Zappala, R. (2001) Comparison of three fungal laccases from *Rigidoporus lignosus* and *Pleurotus ostreatus*: correlation between conformational changes and catalytic activity. *Journal of Inorganic Chemistry*, **83**, 67-73.

Boominathan, K. & Reddy, C. A. (1992) Fungal degradation of lignin. In: *Handbook of Applied Mycology*, (Arora, D. K., Elander, R. P. & Mukerji, K. G., Eds.), Fungal Biotechnology. Marcel Dekker, New York, U.S.A., **4**, 763-822.

Bourbonnais, R., Paice, M. G., Reid, I. D., Lanthier, P. & Yaguchi, M. (1995) Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2'-azino bis (3-ethyl benzthiazoline-6-sulfonate) in kraft lignin depolymerization. *Applied and Environmental Microbiology*, **61**, 1876-1880.

Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248-254.

Brown, M. A., Zhao, Z. W. & Mauk, A. G. (2002) Expression and characterization of a recombinant multi-copper oxidase: laccase IV from *Trametes versicolor*. *Inorganica Chimica Acta*, **331**, 232-238.

Bucher, V. V. C., Hyde, K. D., Pointing, S. B. & Reddy, C. A. (2004) Production of wood decay enzymes, mass loss and lignin solubilization in wood by marine ascomycetes and their anamorphs. *Fungal Diversity*, **15**, 1-14.

Bugni, T. S. & Ireland, C. M. (2004) Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Natural Products Report*, **21**, 143-163.

Bulter, T., Alcalde, M., Sieber, V., Meinhold, P., Schlachtbauer, C. & Arnold, F. H. (2003) Functional expression of a fungal laccase in *Saccharomyces cerevisiae* by directed evolution. *Applied and Environmental Microbiology*, **69**, 987-995.

Buswell, J. A., Cai, Y. & Chang, S.-T. (1995) Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Lentinula (Lentinus) edodes*. *FEMS Microbiology Letters*, **128**, 81-87.

Camarero, S., Ruiz-Duenas, F. J., Sarkar, S., Martinez, M. J. & Martinez, A. T. (2000) The cloning of a new peroxidase found in lignocellulose cultures of *Pleurotus eryngii* and sequence comparison with other fungal peroxidases. *FEMS Microbiology Letters*, **191**, 37-43.

Camarero, S., García, O., Vidal, T., Colom, J., del Río, J. C., Gutiérrez, A., Gras, J. M., Monje, R., Martínez, M. J. & Martínez, Á. T. (2004) Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system. *Enzyme and Microbial Technology*, **35**, 113–120.

Camarero, S., Ibarra, D., Martínez, M. J. & Martínez, A. T. (2005) Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Applied and Environmental Microbiology*, **71**, 1775-1784.

Camarero, S., Ibarra, D., Martínez, A. T., Romero, J., Gutiérrez, A. & Río, J. C. D. (2007) Paper pulp delignification using laccase and natural mediators. *Enzyme and Microbial Technology*, **40**, 1264-1271.

Carrol, G. C. (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology*, **69**, 2-9.

Chabannes, M., Ruel, K., Yoshinaga, A., Chabbert, B., Jauneau, A., Joseleau, J.-P. & Boudet, A.-M. (2001) In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. *The Plant Journal*, **28**, 271-282.

Chagas, E. P. & Durrant, L. R. (2001) Decolorization of azo dyes by *Phanerochaete chrysosporium* and *Pleurotus sojae*. *Enzyme and Microbial Technology*, **29**, 473-477.

Childs, R. E. & Bardsley, W. G. (1975) The steady state kinetics of peroxidase with 2,2'-azino bis (3-ethyl benzthiazoline-6-sulphonate) as chromogen. *Biochemical Journal*, **145**, 93-105.

Claus, H. & Filip, Z. (1997) The evidence for a laccase-like enzyme activity in a *Bacillus sphaericus* strain. *Microbiological Research*, **152**, 209-216.

Claus, H. (2004) Laccases: structure, reactions, distribution. *Micron*, **35**, 93-96.

Coggins, C. R. (1977) PhD Thesis "Aspects of the growth of *Serpula lacrymans* the dry rot fungus", University of Liverpool, Liverpool, U.K.

Coumo, V., Vazanella, F., Fresi, E., Cinelli, F. & Mazella, L. (1985) Fungal flora of *Posidonia oceanica* and its ecological significance. *Transactions of the British Mycological Society*, **84**, 35-40.

- Coumo, V., Palomba, I., Perretti, A., Guerriero, A., D'Ambrosio, M. & Pietra, F.** (1995) Antimicrobial activities from marine fungi. *Journal of Marine Biotechnology*, **2**, 199-204.
- Couto, S. R. & Herrera, J. L. T.** (2006) Industrial and biotechnological applications of laccases: A review. *Biotechnology Advances*, **24**, 500-513.
- Damare, S., Raghukumar, C., Muraleedharan, U. D. & Raghukumar, S.** (2006) Deep-sea fungi as a source of alkaline and cold-tolerant proteases. *Enzyme and Microbial Technology*, **39**, 172-181.
- Das, N., Chakraborty, T. K. & Mukherjee, M.** (2001) Purification and characterization of a growth-regulating laccase from *Pleurotus florida*. *Journal of Basic Microbiology*, **41**, 261-267.
- Dawley, R. M. & Flurkey, W. H.** (1993) Differentiation of tyrosinase and laccase using 4-hexyl-resorcinol, a tyrosinase inhibitor. *Phytochemistry*, **33**, 281-284.
- de Hoog, G. S., Uijthof, J. M. J., Gerrits van den Ende, A. H. G., Figge, M. J. & Weenink, X. O.** (1997) Comparative rDNA diversity in medically significant fungi. *Microbiology and Culture Collections*, **13**, 39-48.
- Dehorter, B. & Blondeau, R.** (1993) Isolation of an extracellular Mn-dependent enzyme mineralizing melanoidins from the white-rot fungus *Trametes versicolor*. *FEMS Microbiology Letters*, **109**, 117-122.
- Demain, A. L., Phaff, H. J. & Kurtzman, C. P.** (1998) *The industrial and agricultural significance of yeasts* (Kurtzman, C. P. & Fell, J. W., Eds.), The yeasts, A Taxonomic Study, 4th Edition, Elsevier Science Publishers, Amsterdam, The Netherlands, 13-19.

Doebbeling, B. N., Lehmann, P. H., Hollis, R. J., Wu, L. C., Widmer, A. F., Voss, A. & Pfaller, M. A. (1993) Comparison of pulsed-field gel electrophoresis with isoenzyme profiles as a typing system for *Candida albicans*. *Clinical Infectious Diseases*, **16**, 377-383.

D'Souza, D. T., Tiwari, R., Sah, A. K. & Raghukumar, C. (2006) Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. *Enzyme and Microbial Technology*, **38**, 504-511.

D'Souza, T. M., Boominathan, K. & Reddy, C. A. (1996) Isolation of laccase gene-specific sequences from white rot and brown rot fungi by PCR. *Applied and Environmental Microbiology*, **62**, 3739-3744.

D'Souza-Ticlo, D., Verma, A. K., Mathew, M. & Raghukumar, C. (2006) Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by a fungus isolated from mangrove wood. *Indian Journal of Marine Science*, **35**, 364-372.

Du, P., Collins, J. R. & Loew, G. H. (1992) Homology modeling of a heme protein, lignin peroxidase, from the crystal structure of cytochrome c peroxidase. *Protein Engineering*, **5**, 679-691.

Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Ostergaard, P., Schneider, P., Yaver, D. S., Pedersen, A. H. & Davies, G. I. (1998) Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2°Å resolution. *Nature Structural Biology*, **5**, 310-316.

Eaton, A. D., Clefceri, L. S., Rice, E. W. & Greenberg, A. E. (2005) *Standard Methods for the Examination of Water and Wastewater*, 21st Edition, American Public Health Association (APHA), American Water Works Association (AWWA) & Water Environment Federation (WEF), U.S.A., 1368 pgs.

Edens, W. A., Goins, T. Q., Dooley, D. & Henson, J. M. (1999) Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. *tritici*. *Applied and Environmental Microbiology*, **65**, 3071-3074.

Eggert, C., Temp, U. & Eriksson, K.-E. (1996a) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied and Environmental Microbiology*, **62**, 1151-1158.

Eggert, C., Temp, U., Jefferey, F. D. D. & Eriksson, K. E. L. (1996b) A fungal metabolite mediates degradation of non-penolic lignin structures and synthetic lignin by laccase. *FEBS Letters*, **391**, 144-148.

Eriksson, K.-E. (1981) Fungal degradation of wood components. *Pure and Applied Chemistry*, **53**, 33-43.

Fåhraeus, G. & Reinhammar, B. (1967) Large scale production and purification of laccase from cultures of the fungus *Polyporus* and some properties of Laccase A. *Acta Chemica Scandinavica*, **21**, 2367-2378.

Faraco, V., Giardina, P. & Sannia, G. (2003) Metal-responsive elements in *Pleurotus ostreatus* laccase gene promoters. *Microbiology*, **149**, 2155-2162.

Farnet, A.-M., Tagger, S. & Le Petit, J. (1999) Effects of copper and aromatic inducers on the laccases of the white-rot fungus *Marasmius quercophilus*. *Life Sciences*, **322**, 499-503.

Farver, O. & Pecht, I. (1984) The reactivity of copper sites in the "blue" copper proteins. In: *Copper Proteins and Copper Enzymes*, (Lontie, R., Ed.). CRC Press, Boca Raton, Florida, U.S.A., **1**, 183-214.

Fell, J. W. & Newell, S. Y. (1981) Role of fungi in carbon flow and nitrogen immobilization in coastal marine plant litter systems. *Mycology Series*, **2**, 665-678.

Ferrari, R. P., Laurenti, E., Ghibaudi, R. M. & Casella, L. (1997) Tyrosinase-catecholic substrates in vitro model: kinetic studies on the *o*-quinone /*o*-semiquinone radical formation. *Journal of Inorganic Biochemistry*, **68**, 61-69.

Froehner, S. T. & Eriksson, K.-E. (1974) Induction of *Neurospora crassa* laccase with protein synthesis inhibitors. *Journal of Bacteriology*, **120**, 450-457.

Fu, Y. & Viraraghavan, T. (2001) Fungal decolorization of dye wastewaters: A review. *Bioresource Technology*, **79**, 251-262.

Galhaup, C. & Haltrich, D. (2001) Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Applied Microbiology and Biotechnology*, **56**, 225-232.

Gardiol, A. E., Hernandez, R. J., Reinhammar, B. & Harte, B. R. (1996) Development of a gas-phase oxygen biosensor using a blue copper-containing oxidase. *Enzyme and Microbial Technology*, **18**, 347-352.

Gargas, A. & DePriest, P. T. (1996) A nomenclature for fungal PCR primers with examples from intron-containing SSU rDNA. *Mycologia*, **88**, 745-748.

Gené, J., Guillamón, J. M., Guarro, J., Pujol, J. & Ulfig, K. (1996) Molecular characterization, relatedness and antifungal susceptibility of the basidiomycetous *Hormographiella* species and *Coprinus cinereus* from clinical and environmental sources. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology*, **70**, 49-57.

Ghindilis, A. L., Skorobogat'ko, O. V., Gavrilova, V. P. & Yaropolov, A. I. (1992) A new approach to the construction of potentiometric immunosensors. *Biosensors & Bioelectronics*, **7**, 301-304.

Gianfreda, L., Sannino, F., Filazzola, M. T. & Leonowicz, A. (1998) Catalytic behavior and detoxifying ability of a laccase from the fungal strain *Cerrena unicolor*. *Journal of Molecular Catalysis B-Enzymatic*, **4**, 13-23.

Gianfreda, L., Xu, F. & Bollag, J. M. (1999) Laccases: A Useful Group of Oxidoreductive Enzymes. *Bioremediation Journal*, **3**, 1-25.

Golubic, S., Radke, G. & Le-Campion-Alsumard, T. (2005) Endolithic fungi in marine ecosystem. *Trends in Microbiology*, **13**, 229-235.

Görg, A., Boguth, G., Obermaier, C. & Weiss, W. (1998) Two-dimensional electrophoresis of proteins in an immobilized pH 4-12 gradient. *Electrophoresis*, **19**, 1516-1519.

Gräser, Y., El Fari, M., Vilgalys, R., Kuijpers, A. F. A., de Hoog, G. S., Presber, W. & Tietz, H.-J. (1999) Phylogeny and taxonomy of the family Arthrodermataceae (dermatophytes) using sequence analysis of the ribosomal ITS region. *Medical Mycology*, **37**, 105-114.

Guarro, J., Gene, J. & Stchigel, A. M. (1999) Developments in Fungal Taxonomy. *Clinical Microbiology Reviews*, **12**, 454-500.

Guého, E., de Hoog, G. S. & Smith, M. T. (1992) Typification of the genus *Trichosporon*. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology*, **61**, 285-288.

- Guého, E., Improvisi, L., Christen, R. & de Hoog, G. S.** (1993) Phylogenetic relationships of *Cryptococcus neoformans* and some related basidiomycetous yeasts determined from partial large subunit rRNA sequences. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology*, **63**, 175-189.
- Guého, E., Leclerc, M. C., de Hoog, G. S. & Dupont, B.** (1997) Molecular taxonomy and epidemiology of *Blastomyces* and *Histoplasma* species. *Mycoses*, **40**, 69-81.
- Haase, G., Sonntag, L., van de Peer, Y., Uijthof, J. M. J., Podbielski, A. & Melzer-Krick, B.** (1995) Phylogenetic analysis of ten black yeast species using nuclear small subunit rRNA gene sequences. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology*, **68**, 19-33.
- Hadrys, H., Balick, M. & Schierwater, B.** (1992) Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Molecular Ecology*, **1**, 55-63.
- Hakulinen, N., Kiiskinen, L.-L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A. & Rouvinen, J.** (2002) Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. *Nature Structural Biology*, **9**, 601-605.
- Harkin, J. M. & Obst, J. R.** (1973) Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia*, **29**, 381-387.
- Hasan, S., Jourdan, M., Brun, L. & Sheppard, A. W.** (1995) Axenic culture and influence of wetness period and inoculum concentration on infection and development of *Cercospora* blight of *Heliotropium europaeum*. *European Journal of Plant Pathology*, **101**, 491-496.
- Hassett, R. F., Yuan, D. S. & Kosman, D. J.** (1998) Spectral and kinetic properties of the Fet3 protein from *Saccharomyces cerevisiae*, a multicopper ferroxidase enzyme. *Journal of Biological Chemistry*, **273**, 23274-23282.

Hassouna, N., Michot, B. & Bachelire, J. (1984) The complete nucleotide sequence of mouse 28S rRNA gene. Implications for the process of size increase of the large subunit rRNA in higher eukaryotes. *Nucleic Acids Research*, **8**, 3563-3583.

Hatakka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. *FEMS Microbiology Reviews*, **13**, 125-135.

Heinfling, A., Ruiz-Duenas, F. J., Martinez, M. J., Bergbauer, M., Szewzyk, U. & Martinez, A. T. (1998) A study on reducing substrates of manganese-oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkandera adusta*. *FEBS Letters*, **428**, 141-146.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P. & Anke, T. (1998) Characterization of laccase and peroxidase from wood-rotting fungi (family Coprinaceae). *Applied Environmental Microbiology*, **64**, 1601-1606.

Hennebert, G. L. & Sutton, B. C. (1994) Unitary parameters in conidiogenesis. In: *Ascomycete systematics: problems and perspectives in the nineties*, (Hawksworth, D. L., Ed.). Plenum Press, New York, U.S.A., 65-76.

Heukeshoven, J. & Dernick, R. (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis*, **6**, 103-112.

Hibbett, D. S. (1992) Ribosomal RNA and fungal systematics. *Transactions of the Mycological Society of Japan*, **33**, 533-556.

Hillis, D. M., Huelsenbeck, J. P. & Cunningham, C. (1994) Application and accuracy of molecular phylogenies. *Science*, **264**, 671-677.

Hou, H., Zhou, J., Wang, J., Du, C. & Yan, B. (2004) Enhancement of laccase production by *Pleurotus ostreatus* and its use for the decolorization of anthraquinone dye. *Process Biochemistry*, **39**, 1415-1419.

Hullo, M. F., Moszer, I., Danchin, A. & Martin-Verstraete, I. (2001) Cot A of *Bacillus subtilis* is a copper-dependent laccase. *Journal of Bacteriology*, **183**, 5426-5430.

Hyde, K. D. & Jones, E. B. G. (1988) Marine Mangrove Fungi. *Marine Ecology*, **9**, 15-33.

Hyde, K. D., Sarma, V. V. & Jones, E. B. G. (2000) Morphology and taxonomy of higher marine fungi. In: *Marine Mycology - A Practical Approach*, (Hyde, K. D. & Pointing, S. B., Eds.), Fungal Diversity Research Series 1. Fungal Diversity Press, Hong Kong, 172-204.

Jennings, D. H. (1983) Some aspects of the physiology and biochemistry of marine fungi. *Biological Review*, **58**, 423-459.

Jennings, D. H. (1986) Fungal growth in the sea. In: *The Biology of Marine Fungi*, (Moss, S. T., Ed.). Cambridge University Press, London, U.K., 1-10.

Jensen, P. R. & Fenical, W. (2000) Marine microorganisms and drug discovery; current status and future potential. In: *Drugs from the Sea*, (Fusetani, N., Ed.), Karger, Basel, Switzerland, 6-29.

Jensen, P. R. & Fenical, W. (2002) Secondary metabolites from marine fungi. In: *Fungi in Marine Environments*, (Hyde, K. D., Ed.), Fungal Diversity Research Series 7. Fungal Diversity Press, Hong Kong, 293-315.

Johannes, C. & Majcherczyk, A. (2000) Laccase activity tests and laccase inhibitors. *Journal of Biotechnology*, **78**, 193-199.

Johansson, M., Denekamp, M. & Asiegbu, F. O. (1999) Production and isozyme pattern of extracellular laccase in the S and P intersterility groups of the root pathogen *Heterobasidion annosum*. *Mycological Research*, **103**, 365-371.

Jordaan, J. & Leukes, W. D. (2003) Isolation of a thermostable laccase with DMAB and MBTH oxidative coupling activity from a mesophilic white-rot fungus. *Enzyme and Microbial Technology*, **33**, 212-219.

Kaarik, A. A. (1974) Decomposition of wood. In: *Biology of Plant Litter Decomposition*, (Dickinson, C. H. & Pugh, G. J. F., Eds.). Academic Press, London, U.K., 129-174.

Karas, M., Bachmann, D., Bahr, U. & Hillenkamp, F. (1987) Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *International Journal of Mass Spectrometry and Ion Processes*, **78**, 53-68.

Keyser, P., Kirk, T. K. & Zeikus, J. G. (1978) Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. *Journal of Bacteriology*, **135**, 790-797.

Ko, E. M., Leem, Y. E. & Choi, H. T. (2001) Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. *Applied Microbiology and Biotechnology*, **57**, 98-102.

Kobayashi, J. & Ishibashi, M. (1993) Bioactive metabolites of symbiotic marine microorganisms. *Chemical Review*, **93**, 1753-1769.

Kohlmeyer, J. & Kohlmeyer, E. (1979) *Marine Mycology; The Higher Fungi*, Academic Press, New York, U.S.A., 690 pgs.

Kohlmeyer, J. & Volkmann-Kohlmeyer, B. (1991) Illustrated key to the filamentous higher marine fungi. *Botanica Marina*, **34**, 1-61.

Kohlmeyer, J. & Volkmann-Kohlmeyer, B. (1993) Biogeographic observations on Pacific marine fungi. *Mycologia*, **85**, 337-346.

Kohn, L. M. (1992) Developing new characters for fungal systematics: an experimental approach for determining the rank of resolution. *Mycologia*, **84**, 139-153.

Kramer, K. J., Kanost, M. R., Hopkins, T. L., Jing, H., Zhu, Y. C., Xu, R., Kerwin, J. L. & Turecek, F. (2001) Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron*, **57**, 385–392.

Kukor, J. J. & Martin, M. M. (1983) Acquisition of Digestive Enzymes by Siricid Woodwasps from Their Fungal Symbiont. *Science*, **220**, 1161-1163.

Kumar, S. V., Phale, P. S., Durani, S. & Wangikar, P. P. (2003) Combined sequence and structure analysis of the fungal laccase family. *Biotechnology and Bioengineering*, **83**, 386-394.

Kumaresan, V. & Suryanarayanan, T. S. (2002) Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. *Fungal Diversity*, **9**, 81-91.

Kumaresan, V., Suryanarayanan, T. S. & Johnson, J. A. (2002) Ecology of mangrove endophytes. In: *Fungi in Marine Environments*, (Hyde, K. D., Ed.), Fungal Diversity Research Series 7. Fungal Diversity Press, Hong Kong, 145-166.

Kurtzman, C. P. (1994) Molecular taxonomy of the yeasts. *Yeast*, **10**, 1727-1740.

Kyhse-Andersen, J. (1984) Electroblothing from multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins to nitrocellulose membranes. *Journal of Biochemical and Biophysical Methods*, **10**, 203-209.

Lang, E., Gonser, A. & Zadrzil, F. (2000) Influence of incubation temperature on activity of ligninolytic enzymes in sterile soil by *Pleurotus* sp. and *Dichomitus squalens*. *Journal of Basic Microbiology*, **40**, 33-39.

Larsson, S., Cassland, P. & Jonsson, L. J. (2001) Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase. *Applied and Environmental Microbiology*, **67**, 1163–1170.

Lathe, G. H. & Ruthven, C. R. (1956) The separation of substances and estimation of their relative molecular sizes by the use of columns of starch in water. *Biochemical Journal*, **62**, 665-674.

Leatham, G. F. & Kirk, T. K. (1983) Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. *FEMS Microbiology Letters*, **16**, 65-67.

Lebo, S. E. J., Gargulak, J. D. & McNally, T. J. (2001) *Lignin*, Kirk-Othmer Encyclopedia of Chemical Technology, John Wiley & Sons Inc., Hoboken, New Jersey, U.S.A, 10-14.

Lee, I.-Y., Jung, K.-H., Lee, C.-H. & Park, Y.-H. (1999) Enhanced production of laccase in *Trametes versicolor* by the addition of ethanol. *Biotechnology Letters*, **21**, 965-968.

Lin, Y.-H., Li, Y.-F., Huang, M.-C. & Tsai, Y.-C. (2004) Intracellular expression of *Vitreoscilla* hemoglobin in *Aspergillus terreus* to alleviate the effect of a short break in aeration during culture. *Biotechnology Letters*, **26**, 1067–1072.

Litthauer, D., van Vuuren, M. J., van Tonder, A. & Wolfaardt, F. W. (2007) Purification and kinetics of a thermostable laccase from *Pycnoporus sanguineus* (SCC 108). *Enzyme and Microbial Technology*, **40**, 563-568.

Liu, L., Tewari, R. P. & Williamson, P. R. (1999) Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infection*, **67**, 6034–6039.

- Lo, S. C., Ho, Y. S. & Buswell, J. A.** (2001) Effect of Phenolic Monomers on the Production of Laccases by the Edible Mushroom *Pleurotus sajor-caju* and Partial Characterization of a Major Laccase Component. *Mycologia*, **93**, 413-421.
- Lomascolo, A., Cayol, J.-L., Roche, M., Guo, L., Robert, J.-L., Record, E., Lesage-Meessen, L., Ollivier, B., Sigoillot, J.-C. & Asther, M.** (2002) Molecular clustering of *Pycnoporus* strains from various geographic origins and isolation of monokaryotic strains for laccase hyperproduction. *Mycological Research*, **106**, 1193-1203.
- Long, R. A. & Azam, F.** (1996) Abundant protein containing particles in the sea. *Aquatic Microbial Ecology*, **10**, 213-221.
- Longato, S. & Bonfante, P.** (1997) Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. *Mycological Research*, **101**, 425-432.
- Lorenz, R. & Molitoris, H. P.** (1992) Combined influence of salinity and temperature (Phoma-pattern) on growth of the marine fungi. *Canadian Journal of Botany*, **70**, 2111-2115.
- Luo, W., Vrijmoed, L. L. P. & Jones, E. B. G.** (2005) Screening of marine fungi for lignocellulose-degrading enzyme activities. *Botanica Marina*, **48**, 379 -386.
- MacArthur, R. & Wilson, E. O.** (1967) *The Theory of Island Biogeography*, Princeton University Press, Princeton, New Jersey, U.S.A., 203 pgs.
- Maldonado, M., Cortadella, N., Trillas, M. I. & Rützler, K.** (2005) Endosymbiotic yeast maternally transmitted in a marine sponge. *Biological Bulletin*, **209**, 94-106.
- Maloy, O. C.** (1974) Benomyl-malt agar for the purification of cultures of wood decay fungi. *Plant Disease Reporter*, **58**, 902-904.

Mann, K. H. (1988) Production and use of detritus in various freshwater, estuarine and coastal marine ecosystems. *Limnology and Oceanography*, **33**, 910-933.

Mantyla, A. W. & Reid, J. L. (1983) Abyssal characteristics of the world ocean waters. *Deep-Sea Research*, **30**, 805-833.

Manzanares, P., Fajardo, S. & Martín, C. (1995) Production of ligninolytic activities when treating paper pulp effluents by *Trametes versicolor*. *Journal of Biotechnology*, **43**, 125-132.

Marmagne, O. & Coste, C. (1996) Color removal from textile plant effluents. *American Dyestuff Reporter*, 15-21.

Martínez, A. T. (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme and Microbial Technology*, **30**, 425-444.

Martinez, D., Larrondo, L. F., Putnam, N., Gelpke, M. D., Huang, K., Chapman, J., Helfenbein, K. G., Ramaiya, P., Detter, J. C., Larimer, F., Coutinho, P. M., Henrissat, B., Berka, R., Cullen, D. & Rokhsar, D. (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nature Biotechnology*, **22**, 695-700.

Martins, L. O., Soares, C. M., Pereira, M. M., Teixeira, M., Costa, T., Jones, G. H. & Henriques, A. O. (2002) Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *Journal of Biological Chemistry*, **277**, 18849-18859.

Masclaux, F., Guého, E., de Hoog, G. S. & Christen, R. (1995) Phylogenetic relationships of human-pathogenic *Cladosporium* (*Xylohypha*) species inferred from partial LS rRNA sequences. *Journal of Medical and Veterinary Mycology*, **33**, 327-338.

Mayer, A. M. & Staples, R. C. (2002) Laccase: new functions for an old enzyme. *Phytochemistry*, **60**, 551-565.

Mayer, A. M. S. & Hamann, M. T. (2004) Marine pharmacology in 2000: marine compounds with antibacterial, anticoagulant, antifungal, antiinflammatory, antimalarial, antiplatelet, antituberculosis and antiviral activities; affecting the cardiovascular, immune and nervous systems and other miscellaneous mechanisms of action. *Marine Biotechnology*, **6**, 37-52.

Michniewicz, A., Ullrich, R., Ledakowicz, S. & Hofrichter, M. (2006) The white-rot fungus *Cerrena unicolor* strain 137 produces two laccase isoforms with different physico-chemical and catalytic properties. *Applied Microbiology and Biotechnology*, **69**, 682-688.

Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, **31**, 426-428.

Mills, D. & McCluskey, K. (1990) Electrophoretic karyotypes of fungi: the new cytology. *Molecular Plant-Microbe Interactions*, **3**, 351-357.

Mochizuki, T., Takada, K., Watanabe, S., Kawasaki, M. & Ishizaki, H. (1990) Taxonomy of *Trichophyton interdigitale* (*Trichophyton mentagrophytes* var. *interdigitale*) by restriction enzyme analysis of mitochondrial DNA. *Journal of Medical and Veterinary Mycology*, **28**, 191-196.

Monteiro, M. & De Carvalho, M. (1998) Pulp bleaching using laccase from *Trametes versicolor* under high temperature and alkaline conditions. *Applied Biochemistry and Biotechnology*, **70-72**, 983-993.

Mougin, C., Boyer, F. D., Caminade, E. & Rama, R. (2000) Cleavage of the diketonitrile derivative of the herbicide isoxaflutole by extracellular fungal oxidases. *Journal of Agricultural and Food Chemistry*, **48**, 4529-4534.

Mougin, C., Jolival, C., Briozzo, P. & Madzak, C. (2003) Fungal laccases: from structure-activity studies to environmental applications. *Environmental Chemistry Letters*, **1**, 145-148.

Mundra, P., Desai, K. & Lele, S. S. (2007) Application of response surface methodology to cell immobilization for the production of palatinose. *Bioresource Technology*, **98**, 2892-2896.

Munoz, C., Guillen, F., Martinez, A. T. & Martinez, M. J. (1997) Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties, and participation in activation of molecular oxygen and Mn²⁺ oxidation. *Applied and Environmental Microbiology*, **63**, 2166-2174.

Murao, S., Arai, M., Tanaka, N., Ishikawa, H., Matsumoto, K. & Watanabe, S. (1985) A method for assaying of α -glucosidase and α -amylase using laccase. *Agricultural and Biological Chemistry*, **49**, 981-985.

Mustafa, R., Muniglia, L., Rovel, B. & Girardin, M. (2005) Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydroorganic biphasic system. *Food Research International*, **38**, 995-1000.

Myers, R. H. & Carter Jr, W. H. (1973) Response Surface Techniques for Dual Response Systems. *Technometrics*, **15**, 301-317.

Nakagiri, A. (2002) *Halophytophthora* species from tropical and subtropical mangroves: A review of their characteristics. In: *Fungi in Marine Environments*, (Hyde, K. D., Ed.). Fungal Diversity Press, Hong Kong, 1-14.

Namikoshi, M., Akano, K., Kobayashi, H., Koike, Y., Kitazawa, A., Rondonuwu, A. B. & Pratasik, S. (2002) Distribution of marine filamentous fungi associated with marine

sponges in coral reefs of Palau and Bunaken Island, Indonesia. *Journal of Tokyo University and Fisheries*, **88**, 15-20.

Nelson, D. L. & Cox, M. M. (eds.) (2004) *Lehninger Principles of Biochemistry*. 4th Edition, Freeman, W.H. & Co., New York, U.S.A., 1100 pgs.

Niesters, H. G. M., Goessens, W. H. F., Meis, J. F. M. G. & Quint, W. G. V. (1993) Rapid polymerase chain reaction-based identification assays for *Candida* species. *Journal of Clinical Microbiology*, **31**, 904-910.

Niku-Paavola, M. L., Karhuner, E., Salola, P. & Raunio, V. (1988) Lignolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochemical Journal*, **254**, 877-884.

Niku-Paavola, M.-L., Fagerström, R., Kruus, K. & Viikari, L. (2004) Thermostable laccases produced by a white-rot fungus from *Peniophora* species. *Enzyme and Microbial Technology*, **35**, 100-102.

Nyanhongo, G. S., Gomes, J., Gübitz, G. M., Zvauya, R., Read, J. & Steiner, W. (2002) Decolorization of textile dyes by laccases from a newly isolated strain of *Trametes modesta*. *Water Research*, **36**, 1449-1456.

O' Farrell, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry*, **250**, 4007-4021.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B. & Sannia, G. (2000) Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. *Applied and Environmental Microbiology*, **66**, 920-924.

Palonen, H., Saloheimo, M., Viikari, L. & Kruus, K. (2003) Purification, characterization and sequence analysis of a laccase from the ascomycete *Mauginiella* sp. *Enzyme and Microbial Technology*, **33**, 854-862.

Paszczynski, A., Crawford, R. L. & Huynh, V.-B. (1988) Manganese peroxidase of *Phanerochaete chrysosporium*. *Methods in Enzymology*, **161**, 264-271.

Pazoutova, S. & Srutka, P. (2007) A record of symbiotic relationship between *Cerrena unicolor* and horntail *Tremex fuscicornis* in the Czech Republic. *Czech Mycology*, **59**, 83-90.

Peterson, S. W. & Kurtzman, C. P. (1991) Ribosomal RNA sequence divergence among sibling species of yeasts. *Systematic and Applied Microbiology*, **14**, 124-129.

Petrini, O. (1991) Fungal endophytes of tree leaves. In: *Microbial ecology of leaves*, (Andrews, J. H. & Hirano, S. S., Eds.). Springer Verlag, Germany, 179-197.

Petrini, O., Sieber, T. N., Toti, L. & Viret, O. (1992) Ecology, metabolite production and substrate utilization in endophytic fungi. *Natural Toxins*, **1**, 185-196.

Petroski, R. J., Peczynska-Czoch, W. & Rosazza, J. P. (1980) Analysis, production and isolation of an extracellular laccase from *Polyporus anceps*. *Applied and Environmental Microbiology*, **40**, 1003–1006.

Pezet, R., Pont, V. & Hoang-Van, K. (1992) Enzymatic detoxication of stilbenes by *Botrytis cinerea* and inhibition by grape berries proanthocyanidins. In: *Recent Advances in Botrytis Research*, (Verhoeff, K., Malathrakis, N. E. & Williamson, B., Eds.). Pudoc Scientific, Wageningen, The Netherlands, 87–92.

Piel, J., Hui, D., Wen, G., Butzke, D., Platzer & Matsunaga, S. (2004) Anti-tumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 16222-16227.

- Piontek, K., Antorini, M. & Choinowski, T.** (2002) Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90°A resolution containing a full complement of coppers. *Journal of Biological Chemistry*, **277**, 37663-37669.
- Pivkin, M. V.** (2000) Filamentous fungi associated with holothurians from the Sea of Japan, off the Primorye Coast of Russia. *Biological Bulletin*, **198**, 101-109.
- Plackett, R. L. & Burman, J. P.** (1946) The Design of Optimum Multifactorial Experiments. *Biometrika*, **33**, 305-325.
- Poch, G. K. & Gloer, J. B.** (1989) Obionin A; a new polyketide metabolite from the marine fungus *Leptosphaeria obiones*. *Tetrahedron Letters*, **30**, 3483-3486.
- Pointing, S. B., Vrijmoed, L. L. P. & Jones, E. B. G.** (1998) A qualitative assessment of lignocellulose degrading enzyme activity in marine fungi. *Botanica Marina*, **41**, 293-298.
- Pointing, S. B. & Hyde, K. D.** (2000) Lignocellulose-degrading marine fungi. *Biofouling*, **15**, 221-229.
- Pointing, S. B.** (2001) Feasibility of bioremediation by white-rot fungi. *Applied Microbiology and Biotechnology*, **57**, 20-33.
- Pritchard, P. H., Lin, J. E. & Mueller, J. G.** (2006) Bioremediation research in EPA: An overview of needs, directions and potentials. In: *Biotechnology in Industrial Waste Treatment and Bioremediation*, (Hickey, R. F. & Smith, G., Eds.). CRC Press, Florida, U.S.A, 3-26.
- Proksch, P., Ebel, R., Edrada, R. A., Scupp, P., Lin, W. H., Sudarsono, Wray, V. & Steube, K.** (2003) Detection of pharmacologically active natural products using ecology. Selected examples from Indopacific marine invertebrates and sponge-derived fungi. *Pure and Applied Chemistry*, **75**, 343-352.

Raghukumar, C. & D'Souza, D. T. (2 December 2004) A process for decolorization of colored effluents using a marine fungus, its enzyme and extracellular polymeric substance. **Indian Patent No: 2420 DEL 2004**, India and world patent no. **WO 2006/059348**.

Raghukumar, C., Raghukumar, S., Sharma, S. & Chandramohan, D. (1992) Endolithic fungi from deep-sea calcareous substrata: isolation and laboratory studies. In: *Oceanography of the Indian Ocean*, (Desai, B. N., Ed.). Oxford IBH Publ, New Delhi, India, 3-9.

Raghukumar, C., Raghukumar, S., Chinnaraj, A., Chandramohan, D. D., D'Souza, T. M. & Reddy, C. A. (1994) Laccase and other lignocellulose modifying enzymes of marine fungi isolated from the coast of India. *Botanica Marina*, **37**, 515-523.

Raghukumar, C., D'Souza, T. M., Thorn, R. G. & Reddy, C. A. (1999) Lignin-modifying enzymes of *Flavodon flavus*, a basidiomycete isolated from a coastal marine environment. *Applied and Environmental Microbiology*, **65**, 2103-2111.

Raghukumar, C. (2002) Bioremediation of colored pollutants by terrestrial *versus* facultative marine fungi. In: *Fungi in Marine Environments*, (Hyde, K. D., Ed.), Fungal Diversity Research Series 7. Fungal Diversity Press, Hong Kong, 317-344.

Raghukumar, C., Muraleedharan, U., Gaud, V. R. & Mishra, R. (2004) Xylanases of marine fungi of potential use for biobleaching of paper pulp. *Journal of Industrial Microbiology & Biotechnology*, **31**, 433-441.

Raghukumar, C. (2005) Diversity and adaptations of deep-sea microorganisms. In: *Microbial diversity: Current perspectives and potential applications* (Satyanarayana, T. and Joshi, B. N., Eds.) I. K. International Pvt. Ltd., New Delhi, 53-70.

Raghukumar, C. (2006) Algal-fungal interactions in the marine ecosystem: symbiosis to parasitism. In: *Recent Advances on Applied Aspects of Indian Marine Algae with Reference to Global Scenario*, (Tewari, A., Ed.). Central Salt and Marine Chemicals Research Institute, Bhavnagar, Gujarat, India, **1**, 366-385.

Raghukumar, C., D'Souza-Ticlo, D. & Verma, A. K. (2008) Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. *Critical Reviews in Microbiology*, **34**, 189-206

Raghukumar, S. (2004) The Role of Fungi in Marine Detrital Processes. In: *Marine Microbiology: facets & opportunities*, (Ramaiah, N., Ed.). National Institute of Oceanography, Dona Paula, Goa, India, 125-140.

Ralph, J., Lapierre, C., Marita, J. M., Kim, H., Lu, F., Hatfield, R. D., Ralph, S., Chapple, C., Franke, R., Hemm, M. R., Doersselaere, J. V., Sederoff, R. R., O'Malley, D. M., Scott, J. T., MacKay, J. J., Yahiaou, N., Boudet, A.-M., Pean, M., Pilate, G., Jouanin, L. & Boerjanf, W. (2001) Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochemistry*, **57**, 993-1003.

Ramsay, J. A. & Nguyen, T. (2002) Decoloration of textile dyes by *Trametes versicolor* and its effect on dye toxicity. *Biotechnology Letters*, **24**, 1757-1761.

Reddy, C. A. (1995) The potential for white-rot fungi in the treatment of pollutants. *Current Opinion in Biotechnology*, **6**, 320-328.

Rinkevich, B. (1999) Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. *Journal of Biotechnology*, **70**, 133-153.

Robene-Soustrade, I. & Lung-Escarmant, B. (1997) Laccase isoenzyme patterns of European *Armillaria* species from culture filtrates and infected woody plant tissues. *European Journal of Forest Pathology*, **27**, 105-114.

Robinson, T., Chandran, B. & Nigam, P. (2001a) Studies on the production of enzymes by white-rot fungi for the decolorization of textile dyes. *Enzyme and Microbial Technology*, **29**, 575-579.

Robinson, T., McMullan, G., Marchant, R. & Nigam, P. (2001b) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresource Technology*, **77**, 247-255.

Roy, B. P. & Archibald, F. S. (1994) An indirect free radical based assay for the enzyme cellobiose: Quinone oxidoreductase. *Analytical Biochemistry*, **216**, 291-298.

Saccardo, P. A. (1880) Conspectus genera fungorum Italiae inferiorum nempe ad Sphaeropsideas, Melanconieas et Hyphomyceteas pertinentium systemate sporologico dispositurum. *Michelia*, **2**, 1-38.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406-425.

Sambrook, J. & Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd Edition, Cold Spring Harbor Laboratory Press, New York, U.S.A., 2100 pgs.

Saparrat, M. C. N., Guillen, F., Arambarri, A. M., Martinez, A. T. & Martinez, M. J. (2002) Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*. *Applied and Environmental Microbiology*, **68**, 1534-1540.

Sarkanen, K. V. & Ludwig, C. H. (eds.) (1971) *Lignins: Occurrence, formation, structure and reactions*. John Wiley & Sons, Inc., New York, U.S.A., 916 pgs.

Selinheimo, E., Kruus, K., Buchert, J., Hopia, A. & Autio, K. (2006) Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. *Journal of Cereal Science*, **43**, 152–159.

Selvam, K., Swaminathan, K. & Chae, K.-S. (2003) Microbial decolorization of azo dyes and dye industry effluent by *Fomes lividus*. *World Journal of Microbiology and Biotechnology*, **19**, 591-593.

Servili, M., De Stefano, G., Piacquadio, P. & Sciancalepore, V. (2000) A novel method for removing phenols from grape must. *American Journal of Enology and Viticulture*, **51**, 357–361.

Setti, L., Scali, S., Angeli, D. I. & Pifferi, P. G. (1998) Horseradish peroxidase-catalyzed oxidative coupling of 3-methyl 2-benzothiazolinone hydrazone and methoxyphenols. *Enzyme and Microbial Technology*, **22**, 656-661.

Shin, K. S. & Lee, Y. J. (2000) Purification and characterization of a new member of the laccase family from the white-rot basidiomycete *Coriolus hirsutus*. *Archives of Biochemistry and Biophysics*, **384**, 109–115.

Sjöström, E. (ed.) (1993) *Wood Chemistry: Fundamentals and Applications*. Academic Press, Oxford, U.K., 293 pgs.

Smith, M. T., de Cock, A. W. A. M., Poot, G. A. & Steensma, H. Y. (1995) Genome comparisons in the yeast like fungal genus *Galactomyces Redhead* et Malloch. *International Journal of Systematic Bacteriology*, **45**, 826-831.

Soares, G. M. B., Pessoa de Amorim, M. T. & Costa-Ferreira, M. (2001) Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. *Journal of Biotechnology*, **89**, 123-129.

Soni, P., Singh, M., Kamble, A. L. & Banerjee, U. C. (2007) Response surface optimization of the critical medium components for carbonyl reductase production by *Candida viswanathii* MTCC 5158. *Bioresource Technology*, **98**, 829–833.

Stasz, T., Nixon, K., Harman, G. E., Weeden, N. F. & Kuter, G. A. (1989) Evaluation of phenetic species and phylogenetic relationships in the genus *Trichoderma* in the absence of parasexuality. *Experimental Mycology*, **14**, 145-159.

Stepanova, E. V., Pegasova, T. V., Gavrilova, V. P., Landesman, E. O. & Koroleva, O. V. (2002) Extracellular Laccases from *Cerrena unicolor* 059, *Cerrena unicolor* 0784, and *Pleurotus ostreatus* 0432: A Comparative Assay. *Applied Biochemistry and Microbiology*, **39**, 375-381.

Stoeck, T., Taylor, G. T. & Epstein, S. (2003) Novel eukaryotes from the permanently anoxic Cariaco Basin (Caribbean Sea). *Applied and Environmental Microbiology*, **69**, 5656-5663.

Stoscheck, C. M. (1990) Quantitation of Protein. *Methods in Enzymology*, **182**, 50-69.

Strongman, D. B., Miller, J. D., Calhoun, L., Findlay, J. A. & Whitney, N. J. (1987) The biochemical basis for interference competition among some lignicolous marine fungi. *Botanica Marina*, **30**, 21-36.

Sugita, T. & Nishikawa, A. (2003) Fungal Identification Method Based on DNA Sequence Analysis: Reassessment of the Methods of the Pharmaceutical Society of Japan and the Japanese Pharmacopoeia. *Journal of Health Science*, **49**, 531-533.

Svobodová, K., Senholdt, M., Novotný, C. & Rehorek, A. (2007) Mechanism of Reactive Orange 16 degradation with the white rot fungus *Irpex lacteus*. *Process Biochemistry*, **42**, 1279-1284.

Tabata, M. & Abe, Y. (1995) *Cerrena unicolor* isolated from the mycangia of a horntail, *Tremex longicollis*, in Kochi prefecture, Japan. *Mycoscience*, **36**, 447-450.

Tamura, K., Nei, M. & Kumar, S. (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 11030-11035.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596-1599.

Tateishi, T., Murayama, S. Y., Otsuka, F. & Yamaguchi, H. (1996) Karyotyping by PFGE of clinical isolates of *Sporothrix schenckii*. *FEMS Immunology and Medical Microbiology*, **13**, 147-154.

Tatusova, T. A. & Madden, T. L. (1999) Blast 2 sequences - a new tool for comparing protein and nucleotide sequences. *FEMS Microbiology Letters*, **174**, 247-250.

Tautz, D. (1993) Notes on the definition and nomenclature of tandemly repetitive DNA sequences. In: *DNA fingerprinting: state of science*, (Pena, S. D. J., Chakrabarty, R., Eppelen, J. T. & Jeffreys, A. J., Eds.). Birkhauser, Basel, Switzerland, 21-28.

ten Have, R. T. & Teunissen, P. J. M. (2001) Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chemical Review*, **101**, 3397-3414.

Terrón, M. C., López-Fernández, M., Carbajo, J. M., Junca, H., Téllez, A., Yagüe, S., Arana-Cuenca, A., González, T. & González, A. E. (2004) Tannic acid interferes with the commonly used laccase-detection assay based on ABTS as the substrate. *Biochimie*, **86**, 519-522.

- Thurston, C. F.** (1994) The structure and function of fungal laccases. *Microbiology*, **140**, 19–26.
- Tien, M. & Kirk, T. K.** (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods in Enzymology*, **161**, 238-249.
- Tomlinson, P. B.** (1986) *The Botany of Mangroves*, Cambridge University Press, Cambridge, UK, 440 pgs.
- Tortella, G. R., Diez, M. C. & Durán, N.** (2005) Fungal diversity and use in decomposition of environmental pollutants. *Critical Reviews in Microbiology*, **31**, 197-212.
- Trigiano, R. N., Windham, M. T. & Windham, A. S.** (2003) *Plant Pathology: Concepts and Laboratory Exercises*, 1st Edition, CRC Press LLC, Boca Raton, Florida, U.S.A., 413 pgs.
- Uijthof, J. M. J., de Hoog, G. S., de Cock, A. W. A. M., Takeo, K. & Nishimura, K.** (1994) Pathogenicity of strains of the black yeast *Exophiala (Wangiella) dermatitidis*: an evaluation based on polymerase chain reaction. *Mycoses*, **37**, 235-242.
- Uijthof, J. M. J., van Belkum, A., de Hoog, G. S. & Haase, G.** (1998) *Exophiala dermatitidis* and *Sarcinomyces phaeomuriformis*: ITS1-sequencing and nutritional physiology. *Medical Mycology*, **36**, 143-151.
- Urzua, U., Fernando, L. L., Lobos, S., Larrain, J. & Vicuna, R.** (1995) Oxidation reactions catalyzed by manganese peroxidase isoenzymes from *Ceriporiopsis subvermispora*. *FEBS Letters*, **371**, 132-136.

van Burik, J. A., Schreckhise, R. W., White, T. C., Bowden, R. A. & Myerson, D. (1998) Comparison of six extraction techniques for isolation of DNA from filamentous fungi. *Medical Mycology*, **36**, 299-303.

van de Peer, Y., Jansen, J., De Rijk, J. & De Wachter, R. (1997) Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research*, **25**, 111-116.

Vanechoutte, M., Rossau, R., DeVos, P., Gillis, M., Janssens, D., Paepe, N., DeRouck, A., Fiers, T., Claeys, G. & Kersters, K. (1992) Rapid identification of bacteria in the Comamonadaceae with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiology Letters*, **93**, 227-234.

Vasconcelos, A. F., Barbosa, A. M., Dekker, R. F. H., Scarminio, I. S. & Rezende, M. I. (2000) Optimization of laccase production by *Botryosphaeria* sp. in the presence of veratryl alcohol by the response-surface method. *Process Biochemistry*, **35**, 1131-1138.

Vilgalys, R. (1988) Genetic relatedness among anastomosis groups in *Rhizoctonia* as measured by DNA/DNA hybridization. *Phytopathology*, **78**, 698-702.

Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*, **172**, 4238-4246.

Vrijmoed, L. L. P. (2000) Isolation and culture of higher filamentous fungi. In: *Marine Mycology - A Practical Approach*, (Hyde, K. D. & Pointing, S. B., Eds.), Fungal Diversity Research Series 1. Fungal Diversity Press, Hong Kong, 1-18.

Vyas, B. R. & Molitoris, H. P. (1995) Involvement of an extracellular H₂O₂-dependent ligninolytic activity of the white rot fungus *Pleurotus ostreatus* in the decolorization of Remazol brilliant blue R. *Applied and Environmental Microbiology*, **61**, 3919-39127.

- Wafar, S., Untawale, A. G. & Wafar, M. V. M.** (1997) Litter fall and energy flow in a mangrove ecosystem. *Estuarine, Coastal and Shelf Science*, **44**, 111-124.
- Wakabayashi, S., Matsubara, H. & Webster, D. A.** (1986) Primary sequence of a dimeric bacterial hemoglobin from *Vitreoscilla*. *Nature*, **322**, 481-483.
- Wang, G.-Y.-S., Abrell, L. M., Avelar, A., Borgeson, B. M. & Crews, P.** (1998) New hirsutane based sesquiterpenes from salt water cultures of a marine sponge derived fungus and the terrestrial fungus *Coriolus consors*. *Tetrahedron*, **54**, 7335-7342.
- Wesenberg, D., Buchon, F. & Agathos, S. N.** (2002) Degradation of dye-containing textile effluent by the agaric white-rot fungus *Clitocybula Dusenii*. *Biotechnology Letters*, **24**, 989-993.
- Wesenberg, D., Kyriakides, I. & Agathos, S. N.** (2003) White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances*, **22**, 161-187.
- White, T. J., Bruns, T. D., Lee, S. B. & Taylor, J. W.** (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: *PCR - Protocols and Applications - A Laboratory Manual*, (Innis, N., Gelfand, D., Sninsky, J. & White, T., Eds.). Academic Press, New York, U.S.A., 315-322.
- Wilcox, W. W.** (1970) Anatomical changes in wood cell walls attacked by fungi and bacteria. *Botanical Review*, **36**, 1-28.
- Wilm, M., Shevchenko, A., Houthave, T., Breit, S., Schweigerer, L., Fotsis, T. & Mann, M.** (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano electrospray mass spectrometry. *Nature*, **379**, 466-469.

Wilmotte, A., van de Peer, Y., Goris, A., Chapelle, S., De Baere, R., Nelissen, B., Neefs, J. M., Hennebert, G. L. & de Wachter, R. (1993) Evolutionary relationships among higher fungi inferred from small ribosomal subunit RNA sequence analysis. *Systematic and Applied Microbiology*, **16**, 436-444.

Wilson, K. & Walker, J. (eds.) (2000) *Principles and techniques of practical biochemistry*. 5th Edition, Cambridge University Press, Cambridge, U.K., 784 pgs.

Wingender, J., Neu, T. R. & Flemming, H.-C. (eds.) (1999) *Microbial Extracellular Polymeric Substances: Characterization, Structure, and Function*. Springer, Germany, 258 pgs.

Woese, C. R., Kandler, O. & Wheelis, M. L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 4576-4579.

Wong, Y. & Yu, J. (1999) Laccase-catalyzed decolorization of synthetic dyes. *Water Research*, **33**, 3512-3520.

Worrall, J. J., Chet, I. & Hüttermann, A. (1986) Association of rhizomorph formation with laccase activity in *Armillaria* spp. *Journal of General Microbiology*, **132**, 2527-2534.

Wu, J., Xiao, Y.-Z. & Yu, H.-Q. (2005) Degradation of lignin in pulp mill wastewaters by white-rot fungi on biofilm. *Bioresource Technology*, **96**, 1357-1363.

Xavier, A. M. R. B., Evtuguin, D. V., Ferreira, R. M. P. & Amado, F. L. (4th - 8th June 2001) Laccase production for lignin oxidative activity. *Proceedings of the 8th International Conference on Biotechnology in the Pulp and Paper Industry*, Helsinki, Finland.

Xiao, Y., Tu, X., Wang, J., Zhang, M., Cheng, Q., Zeng, W. & Shi, Y. (2003) Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. *Applied Microbiology and Biotechnology*, **60**, 700-707.

Xiao, Y. Z., Zhang, M., Wu, J., Wang, Y. P., Hang, J., Zeng, W. Y. & Shi, Y. Y. (2001) Factors of laccase producing and fermentation conditions by a new white-rot fungus AH28-2. *Sheng Wu Gong Cheng Xue Bao*, **17**, 579-583.

Xiao, Y. Z., Chen, Q., Hang, J., Shi, Y. Y., Xiao, Y. Z., Wu, J., Hong, Y. Z. & Wang, Y. P. (2004) Selective induction, purification and characterization of a laccase isozyme from the basidiomycete *Trametes* sp. AH28-2. *Mycologia*, **96**, 26-35.

Xu, F. (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition. *Biochemistry*, **35**, 7608-7614.

Xu, F. (1997) Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. *Journal of Biological Chemistry*, **272**, 924-928.

Yan, Z. H., Rogers, S. O. & Wang, C. J. K. (1995) Assessment of *Phialophora* species based on ribosomal DNA internal transcribed spacers and morphology. *Mycologia*, **87**, 72-83.

Yoon, S. K. & Singh, A. P. (2000) Micromorphological characteristics of wood biodegradation in wet environments: A review. *International Association of Wood Anatomists Journal*, **21**, 135-155.

Yoshida, H. (1883) Chemistry of lacquer (urushi). *Journal of the Chemical Society*, **43**, 472-486.

Yoshitake, A., Katayama, Y., Nakamura, M., Iimura, Y., Kawai, S. & Morohoshi, N. (1993) N-linked carbohydrate chains protect laccase III from proteolysis in *Coriolus versicolor*. *Journal of General Microbiology*, **139**, 179-185.

Zacharius, R. M., Zell, T. E., Morrison, J. H. & Woodlock, J. J. (1969) Glycoprotein staining following electrophoresis on acrylamide gels. *Analytical Biochemistry*, **30**, 148-152.

Zak, B. & Bryan, W. C. (1963) Isolation of Fungal Symbionts from Pine Mycorrhizae. *Forest Science*, **3**, 270-278.

Zhao, X., Lu, Y. & Hardin, I. (2005) Determination of biodegradation products from sulfonated dyes by *Pleurotus ostreatus* using capillary electrophoresis coupled with mass spectrometry. *Biotechnology Letters*, **27**, 69-72.

Zhu, H., Wang, T.-W., Sun, S.-J., Shen, Y.-L. & Wei, D.-Z. (2006) Chromosomal integration of the *Vitreoscilla* hemoglobin gene and its physiological actions in *Tremella fuciformis*. *Applied Microbiology and Biotechnology*, **72**, 770-776.

Zille, A., Gornacka, B., Rehorek, A. & Cavaco-Paulo, A. (2005) Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. *Applied and Environmental Microbiology*, **71**, 6711-6718.

Publications

Ph.D. Thesis



Publications

1. **D'Souza-Ticlo, D.**, Garg, S. and Raghukumar, C. (2009) Effects and Interactions of Medium Components on Laccase from a Marine-Derived Fungus Using Response Surface Methodology. *Marine Drugs*, **7**, 672 – 688.
2. **D'Souza-Ticlo, D.**, Sharma, D. and Raghukumar, C. (2009) A Thermostable Metal-Tolerant Laccase with Bioremediation Potential from a Marine-Derived Fungus. *Marine Biotechnology*, **7** (4), 672-688.
3. Raghukumar, C., **D'Souza-Ticlo, D.** and Verma, A. K., (2008) Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. *Critical Reviews in Microbiology*, **34**, 189 - 206.
4. **D'Souza-Ticlo, D.**, Verma, A. K., Mathew, M. and Raghukumar, C. (2006) Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by a fungus isolated from mangrove wood. *Indian Journal of Marine Science*, **35**, 364 - 372.
5. **D'Souza, D.**, Tiwari, R., Sah, A. K. and Raghukumar, C. (2006) Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. *Enzyme and Microbial Technology*, **38**, 504 - 511.

Patent

- Raghukumar, C. & **D'Souza, D. T.** (2 December 2004) A process for decolorization of colored effluents using a marine fungus, its enzyme and extracellular polymeric substance. **Indian Patent No: 2420 DEL 2004**, India, applied for international patent.

Presentations at Symposia & Conferences

1. **D'Souza-Ticlo, D** and Raghukumar, C. (2009) Bioremediation of high saline colored waste waters with a marine-derived fungus *Cerrena unicolor* MTCC 5159. *FEMS 2009 – 3rd Congress of European Microbiologists-Microbes and Man Interdependence and Future Challenges*, Sweden, 166 (Poster presentation).
2. **D'Souza-Ticlo, D.**, and Raghukumar, C. (2007) Characteristics and applications of laccase from an estuarine fungus NIOCC #2a. *New Trends in Biotechnology*, India, 13 (oral presentation).
3. D'Souza-Ticlo, D., Verma, A. K[#], Matthew, M. and Raghukumar, C. (2006) Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by a fungus (NIOCC # 2a) isolated from mangrove wood. *7th Asia Pacific Marine Biotechnology Conference*, India, 88 (oral presentation).
4. **D'Souza-Ticlo, D.**, and Raghukumar, C. (2006) Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes. *Recent trends in Mycological Research and 33rd Annual Meeting of the Mycological Society of India*, India, 79 (oral presentation).

-- Presenting author