LACCASE AND MANGANESE PEROXIDASE DIVERSITY IN *Cyathus bulleri* AND MECHANISMS EMPLOYED TOWARDS DYE DEGRADATION/ADAPTATION

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by

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I would like to dedicate this thesis to my beloved parents who have been constant source of my strength and encouragement throughout my life.

CERTIFICATE

This is to certify that the thesis entitled "Evaluation of laccases produced by "Laccase and Manganese Peroxidase diversity in *Cyathus bulleri* and mechanisms employed towards dye degradation/adaptation" being submitted by **Ms. Aakanksha Ahlawat** to the Indian Institute of Technology Delhi, for the award of the degree of '**Doctor of Philosophy**', is a record of the bona fide research work carried out by her, which has been prepared under my supervision and guidance in conformity with the rules and regulations of the Indian Institute of Technology, Delhi. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

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Abstract

Due to the depleting fossil fuel resources, price of gasoline and environmental concern, research on alternative sources of energy, such as bioethanol, has accelerated in the last few decades. Coupled with this is to look for environmentally friendly technologies in the area of bioremediation. Production of bioethanol from the lignocellulosic wastes has gained significant attention in the last many decades and removal of lignin is an essential pre-requisite for availability of cellulose for hydrolysis to glucose. In this study, work was carried out to identify the lignin degrading enzymes from the white-rot fungus *Cyathus bulleri*, which is an avid lignin degrader. A total of 13 laccase (*Lcc*) encoding and 6 manganese peroxidase or MnPs (*MnP*) encoding genes were identified in the draft genome of *C. bulleri*. The *Lcc* genes could be sub-divided in to 3 classes based on their structural organization whereas no common structures were identified in the *MnP*s. Similarity at the genomic level for all laccases (except *Lcc12*) indicated their evolution as a result of gene duplication and these appeared to be very closely related based on the phylogenetic relationship. All the copper binding domains and the heme signature sequences were conserved in the laccases and the MnPs respectively. Various online tools were used to predict basic physico-chemical properties of these isoforms. A temporal analysis of the transcription of the *Lcc* and the *MnP* genes was carried out on wheat bran (WB) and the data indicated *Lcc1* to be transcribed the most on Day 4 while Lcc12 was transcribed during later stages of growth. Importantly, the crude culture filtrate obtained from WB-grown fungus released reducing sugars from rice husk (~18.5 mg/g of rice husk) and wheat straw (~9.5 mg/g of WS) without any prior pre-treatment of these materials. This indicated effective delignification as well as saccharification of these materials by the enzymes produced by this fungus. Due to production of effective ligno-cellulases by *C. bulleri* and textile dyes sharing structural similarities with the lignin molecule, the effectiveness of these enzymes was also investigated on dyes and effluent. Several dyes and highly derivatized crystal violet (in the

combined effluent from denim dyeing units) were acted upon and decolorized by the crude enzyme produced on WB which was accompanied by degradation as well as detoxification. Treatment of the combined effluent resulted in nearly 90% decolorization with concomitant removal of dimethyl-amino units and accumulation of *o*-dimethyl amino dimethyl aniline (m/z 164) indicating an oxidative breakdown pathway which is reverse of the chemical synthetic route of these dyes.

It was observed that *C. bulleri* was able to grow well in presence of a number of dyes and decolorize/degrade them. The stimulation of growth by the addition of indigo carmine (5, 5 indigo disulfonic acid sodium salt, IC) was studied on solid media (at 5000 mg/L level) as well as in the liquid medium. IC was degraded into anthranilic acid by oxidoreductases, particularly the laccases, present in the culture broth of the fungus grown on WB which was confirmed by treatment of IC with purified laccase. Anthranilic acid was proposed to further enter into the tryptophan (TRP) biosynthetic pathway, as demonstrated by up-regulation of newly identified *trpD, trpC* and *trp1* genes from this fungus. These genes encode anthranilate phosphoribosyl transferase, a multi-functional enzyme with phosphoribosyl anthranilate isomerase/other activities and tryptophan synthase respectively. No such increased transcription of these genes was observed in the absence of IC. In order to identify the mechanisms used by the fungus to resist/degrade the dyes, the general transcriptome response of this fungus to two azo dyes was studied. Transcriptome analysis was carried out followed by validation of the expression of differentially expressed genes by real time (RT) -qPCR. The data indicated that genes responsible for protection against oxidative stress, oxidoreductases, several channels, transporters, efflux pump, chaperones and other genes were mostly up-regulated in presence of RO16, indicating this dye to be posing more oxidative stress for the fungus, while most of the genes were down-regulated and some did not show much effect in presence of RV5. This indicated that RV5 is less toxic for the fungus and is being co-metabolized by the fungus

leading to enhancement in fungal growth. The data provided a deeper insight into the mechanisms employed by the fungus to resist the dye toxicity.

.

अमूर्त

घटते जीवाश्म ईंधन संसाधनों, गैसोलीन की कीमत और पर्यावरणीय चिंता के कारण, पिछले कुछ दशकों में बायोएथेनॉल जैसे ऊर्जा के वैकल्पिक स्रोतों पर शोध में तेजी आई है। इसके साथ-साथ बायोरेमेडिएशन के क्षेत्र में पर्यावरण के अनुकूल प्रौद्योगिकियों की तलाश करना भी शामिल है। लिग्नोसेल्यूलोसिक अपशिष्टों से बायोएथेनॉल के उत्पादन ने पिछले कई दशकों में महत्वपूर्ण ध्यान आकर्षित किया है और ग्लूकोज को हाइड्रोलिसिस के लिए सेलूलोज़ की उपलब्धता के लिए लिग्निन को हटाना एक आवश्यक पूर्व-आवश्यकता है। इस अध्ययन में, सफेद-सड़न कवक साइथस बुलेरी से लिग्निन डिग्रेडिंग एंजाइमों की पहचान करने के लिए काम किया गया था, जो एक उग्र लिग्निन डिग्रेडर है। सी. बुलेरी के ड्राफ्ट जीनोम में कुल 13 लैकेकेस (एलसीसी) एन्कोडिंग और 6 मैंगनीज पेरोक्सीडेज या एमएनपी (एमएनपी) एन्कोडिंग जीन की पहचान की गई थी। एलसीसी जीन को उनके संरचनात्मक संगठन के आधार पर 3 वर्गों में उप-विभाजित किया जा सकता है जबकि एमएनपी में कोई सामान्य संरचना की पहचान नहीं की गई थी। सभी लैकेकेस (Lcc12 को छोड़कर) के लिए जीनोमिक स्तर पर समानता ने जीन दोहराव के परिणामस्वरूप उनके विकास का संकेत दिया और ये फ़ाइलोजेनेटिक संबंध के आधार पर बहुत निकटता से संबंधित प्रतीत हुए। सभी कॉपर बाइंडिंग डोमेन और हेम हस्ताक्षर अनुक्रम क्रमशः लैकेकेस और एमएनपी में संरक्षित किए गए थे। इन आइसोफॉर्मों के बुनियादी भौतिक-रासायनिक गुणों की भविष्यवाणी करने के लिए विभिन्न ऑनलाइन उपकरणों का उपयोग किया गया था। गेहूं की भूसी (डब्ल्यूबी) पर एलसीसी और एमएनपी जीन के प्रतिलेखन का एक अस्थायी विश्लेषण किया गया था और डेटा से संकेत मिलता है कि एलसीसी1 को चौथे दिन सबसे अधिक प्रतिलेखित किया गया था जबकि एलसीसी12 को विकास के बाद के चरणों के दौरान प्रतिलेखित किया गया था। महत्वपूर्ण बात यह है कि डब्ल्यूबी-विकसित कवक से प्राप्त क्रूड कल्चर फिल्ट्रेट इन सामग्रियों के किसी पूर्व-उपचार के बिना चावल की भूसी (~ 18.5 मिलीग्राम/ग्राम चावल की भूसी) और गेहूं के भूसे (~9.5 मिलीग्राम/ग्राम डब्ल्यूएस) से कम करने वाली

शर्करा जारी करता है। इससे इस कवक द्वारा उत्पादित एंजाइमों द्वारा इन सामग्रियों के प्रभावी परिशोधन के साथ-साथ पवित्रीकरण का संकेत मिला। सी. बुलेरी द्वारा प्रभावी लिग्नो-सेल्यूलस के उत्पादन और लिग्निन अणु के साथ संरचनात्मक समानताएं साझा करने वाले कपड़ा रंगों के कारण, इन एंजाइमों की प्रभावशीलता की रंगों और अपशिष्टों पर भी जांच की गई थी। कई रंगों और अत्यधिक व्युत्पन्न क्रिस्टल वायलेट (डेनिम रंगाई इकाइयों से संयुक्त प्रवाह में) पर डब्ल्यूबी पर उत्पादित कच्चे एंजाइम द्वारा कार्रवाई की गई और रंगहीन कर दिया गया, जिसके साथ-साथ गिरावट के साथ-साथ विषहरण भी हुआ। संयुक्त प्रवाह के उपचार के परिणामस्वरूप डाइमिथाइल-एमिनो इकाइयों के सहवर्ती निष्कासन और ओ-डाइमिथाइल एमिनो डाइमिथाइल एनिलिन (एम/जेड 164) के संचय के साथ लगभग 90% रंग फीका पड़ गया, जो एक ऑक्सीडेटिव ब्रेकडाउन मार्ग का संकेत देता है जो इन रंगों के रासायनिक सिंथेटिक मार्ग के विपरीत है।

यह देखा गया कि सी. बुलेरी कई रंगों की उपस्थिति में अच्छी तरह से विकसित होने और उन्हें रंगहीन/क्षीण करने में सक्षम था। इंडिगो कारमाइन (5, 5 इंडिगो डिसल्फोनिक एसिड सोडियम नमक, आईसी) के अतिरिक्त विकास की उत्तेजना का अध्ययन ठोस मीडिया (5000 मिलीग्राम/एल स्तर पर) के साथ-साथ तरल माध्यम में भी किया गया था। डब्ल्यूबी पर उगाए गए कवक के कल्चर शोरबा में मौजूद ऑक्सीडोरडक्टेस, विशेष रूप से लैकेकेस द्वारा आईसी को एन्थ्रानिलिक एसिड में बदल दिया गया था, जिसकी पुष्टि शुद्ध लैकेस के साथ आईसी के उपचार से हुई थी। एंथ्रानिलिक एसिड को आगे ट्रिप्टोफैन (टीआरपी) बायोसिंथेटिक मार्ग में प्रवेश करने का प्रस्ताव दिया गया था, जैसा कि इस कवक से नए पहचाने गए टीआरपीडी, टीआरपीसी और टीआरपी1 जीन के अप-विनियमन द्वारा प्रदर्शित किया गया था। ये जीन क्रमशः फॉस्फोरिबोसिल एंथ्रानिलेट आइसोमेरेज़/अन्य गतिविधियों और ट्रिप्टोफैन सिंथेज़ के साथ एक बहु-कार्यात्मक एंजाइम एन्थ्रानिलेट फॉस्फोरिबोसिल ट्रांसफरेज़ को एनकोड करते हैं। आईसी की अनुपस्थिति में इन जीनों का ऐसा कोई बढ़ा हुआ प्रतिलेखन नहीं देखा गया। रंगों का विरोध/निम्नीकरण करने के लिए कवक द्वारा उपयोग किए जाने वाले तंत्र की पहचान करने के लिए, दो एज़ो रंगों के प्रति इस

कवक की सामान्य ट्रांसक्रिप्टोम प्रतिक्रिया का अध्ययन किया गया था। वास्तविक समय (आरटी) - क्यूपीसीआर द्वारा विभेदित रूप से व्यक्त जीन की अभिव्यक्ति के सत्यापन के बाद ट्रांसक्रिप्टोम विश्लेषण किया गया। आंकड़ों से संकेत मिलता है कि ऑक्सीडेटिव तनाव, ऑक्सीडोरडक्टेस, कई चैनल, ट्रांसपोर्टर, इफ्लक्स पंप, चैपेरोन और अन्य जीनों के खिलाफ सुरक्षा के लिए जिम्मेदार जीन को ज्यादातर आरओ 16 की उपस्थिति में विनियमित किया गया था, जो इस डाई को कवक के लिए अधिक ऑक्सीडेटिव तनाव उत्पन्न करने का संकेत देता है, जबकि अधिकांश जीनों को डाउन-रेगुलेट किया गया और कुछ ने आरवी5 की उपस्थिति में ज्यादा प्रभाव नहीं दिखाया। इससे संकेत मिलता है कि आरवी5 कवक के लिए कम विषाक्त है और कवक द्वारा सह-चयापचय किया जा रहा है जिससे कवक के विकास में वृद्धि हो रही है। डेटा ने डाई विषाक्तता का विरोध करने के लिए कवक द्वारा नियोजित तंत्र में गहरी अंतर्दृष्टि प्रदान की।

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LIST OF SYMBOLS

Symbols	Meanings
%	Percent
~	Approximately
bp	Base pair
^{o}C	Degree celsius
(µ/m) M	(Micro/ milli) Molar
(µ/m/n) g	(Micro/ milli/ nano) Gram
(m) l	(Milli) Litre
ε	Molar extinction coefficient
E^{o}	Redox potential
kDa	Kilo daltons
λ_{max}	Wavelength at which there is maximum absorption
OD	Optical density
ppm	Parts per million
rpm	Revolutions per minute
U	Enzyme activity unit
v/v	Volume/ volume
w/v	Weight/ volume