Characterisation of Synthetic Dye Decolourising Genes and their Enzymes Produced by an Endophytic Fungus *Marasmius cladothyllum* UMAS MS8

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Characterisation of Synthetic Dye Decolourising Genes and their Enzymes
Produced by an Endophytic Fungus *Marasmius cladophyllus*
UMAS MS8

Ngieng Ngui Sing

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DECLARATION

The thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

(Ngieng Ngui Sing)

Date:
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ABSTRACT

Wide spectrum usage of synthetic dyes particularly by the textile and dyestuff industry has caused serious pollution of rivers and lake due to insufficient treatment of dye containing wastewater by current known wastewater treatment methods. Endophytic fungi were therefore isolated from *Melastoma malabathricum* (local name: Senduduk) to test for their ability to decolourise synthetic dyes. Screening on agar plate for synthetic dye decolourisation has enabled the identification of an isolate MS8 that is capable of decolourising all the synthetic dye tested. Synthetic dye decolourisation in liquid medium by MS8 further confirmed the ability of the fungus to decolourise both azo and anthraquinone dyes. Comparing the ITS sequence with NCBI GenBank identified the fungus to be *Marasmius cladophyllus*. Study on the dye decolourising mechanism of *M. cladophyllus* in liquid medium using RBBR dye showed the fungus degradative capability in decolourising the dye with no dye adsorption on the fungal mycelium. Further ligninolytic enzyme assay revealed that the presence of RBBR dye induced a 70 folds increase in laccase activity by *M. cladophyllus*. This laccase enzyme after precipitation by ammonium sulphate can also decolourise 90.2% of RBBR dye, 80.0% of Methyl red, 59.1% of Congo red and 56.1% of Orange G in just 24 hours thus indicating that laccase is the dye decolourising enzyme. Further examination by PAGE revealed that the observed laccase activity was contributed by two laccase isoforms designated as nLCC1 and nLCC2. Further purification by anion exchange chromatography has allowed the successful purification of laccase isoform nLCC1. Purification of nLCC2 however was unsuccessful when using similar method. Further characterisation of nLCC1 using ABTS as the substrate revealed that the molecular mass of this isoform was 50 kDa and showed optimal activity at pH 4 and temperature of 70°C. The isoform also showed high affinity towards ABTS with a $K_m$ of 17.5
μM. Further screening for laccase gene by using degenerate primers designed based on the highly conserved sequences in copper-binding regions I and IV of laccase gene revealed the presence of at least 2 different laccase genes in *M. cladophyllus*. RT-PCR using degenerate primer have allowed the successful isolation of the first ever full laccase gene (*Lcc1*) from *Marasmius* species. Further gene characterisation shows that the deduced protein sequence of this gene contains the 2 protein fragments identified from the purified nLCC1 of *M. cladophyllus*. This therefore indicates that the isolated *Lcc1* gene is the laccase gene that encodes for the purified nLCC1 laccase isoform. Further heterologous expression in the methylotrophic yeast *Pichia pastoris*, has also enabled the successful production of a fully functional recombinant laccase enzyme. Characterisation using ABTS as the substrate shows that the recombinant laccase shared similar pH, temperature and kinetic properties with the native laccase enzyme. The recombinant laccase also exert comparable RBBR dye decolourisation ability with the native laccase isoform nLCC1. The success in expressing a functional recombinant laccase enzyme using *P. pastoris* thus indicates the potential for large scale continuous production of this dye decolourising enzyme for the treatment of dye containing wastewater.

**Keywords**: Synthetic dyes, *M. cladophyllus*, laccase enzyme, heterologous expression.
Pengajian Pencirian Gen Penyahwarnaan Perwarna Sintetik dan Enzim yang Dihasilkan oleh Kulat Endofitik Marasmius cladophyllus UMAS MS8

ABSTRAK

Penggunaan pewarna sintetik secara berleluasa terutamanya oleh industri tekstil telah menyebabkan pencemaran sungai dan tasik yang serius akibat daripada rawatan air kumbahan berwarna yang tidak mencukupi oleh kaedah perawatan yang sedia ada. Oleh itu kulat-kulat endofitik telah dipencilkan daripada tanaman Senduduk Melastoma malabathricum bagi menguji keupayaan kulat-kulat ini menyahwarnakan perwarna sintetik. Penyaringan atas piring agar berdasarkan penyahwarnaan pewarna sintetik telah membolehkan pengenalpastian satu pencilan MS8 yang mampu menyahwarnakan semua pewarna sintetik yang diuji. Penyahwarnaan pewarna sintetik dalam media cecair oleh MS8 telah memastikan keupayaan kulat tersebut untuk melunturkan pewarna azo dan antraquinon. Perbandingan jujukan ITS dalam NCBI GenBank telah mengenal pasti kutat tersebut sebagai Marasmius cladophyllus. Kajian mengenai mekanisme penyahwarnaan M. cladophyllus dalam media cecair menggunakan pewarna RBBR menunjukkan bahawa kulat tersebut menyahwarnakan pewarna secara degradasi tanpa penjerapan pewarna pada miselium kulat. Assai enzim ligninolitik seterusnya menunjukkan bahawa kehadiran pewarna RBBR menyebabkan peningkatan 70 kali ganda dalam aktiviti lakase oleh M. cladophyllus. Enzim lakase ini selepas dimendakkan dengan ammonium sulfat juga boleh menyahwarnakan 90.2% pewarna RBBR, 80% metil merah, 59.1% kongo merah dan 56.1% oren G dalam masa 24 jam dan itu menunjukkan bahawa lakase adalah enzim penyahwarna. Pemeriksaan seterusnya dengan elektroforesis gel poliakrilamid menunjukkan bahawa aktiviti lakase yang dipaparkan adalah disebabkan oleh dua isoform lakase yang dikenalpasti sebagai nLCC1 dan nLCC2. Seterusnya penulenan
dengan menggunakan kromatografi pertukaran anion telah membolehkan penulenan isoform lakase nLCC1. Namun, penulenan nLCC2 menggunakan kaedah yang sama adalah tidak berjaya. Pencirian nLCC1 menggunakan ABTS sebagai substrat menunjukkan bahawa jisim molekul isoform ini adalah 50 kDa dan menunjukkan aktiviti optimum pada pH 4 dan suhu 70°C. Isoform ini juga menunjukkan pertalian yang tinggi terhadap ABTS dengan $K_m$ 17.5 µM. Seterusnya, penyaringan gen lakase dengan menggunakan primer degenerasi yang direka berdasarkan jujukan yang sangat dipelihara di kawasan-kawasan ikatan tembaga I dan IV gen lakase menunjukkan kehadiran sekurang-kurangnya 2 gen lakase berbeza dalam M. cladophyllus. RT-PCR menggunakan primer degenerasi telah membolehkan pemencilan buat kali pertama jujukan gen lakase (Lcc1) yang penuh dari spesies Marasmius. Pencirian gen ini seterusnya menunjukkan bahawa urutan protein yang disimpulkan daripada gen ini mengandungi 2 jujukan protein yang dikenal pasti daripada nLCC1 tulen M. cladophyllus. Ini menunjukkan bahawa gen Lcc1 yang dipencilkan ini adalah gen lakase yang mengekodkan isoform lakase nLCC1 yang telah ditulenkan itu. Pengekspresan heterologus dalam metilotrofik yis Pichia pastoris, juga telah membolehkan penghasilan enzim lakase rekombinan yang aktif. Pencirian menggunakan ABTS sebagai substrat menunjukkan bahawa lakase rekombinan ini mempunyai pH, suhu dan sifat kinetik yang sama dengan enzim lakase yang asli. Lakase rekombinan juga menunjukkan keupayaan dalam menyahwarnakan pewarna RBBR yang setanding dengan keupayaan penyahwarnaan menggunakan isoform lakase asli nLCC1. Kejayaan dalam mengekspreskan enzim lakase rekombinan yang berfungsi menggunakan P. pastoris menunjukkan potensi untuk penghasilan berterusan enzim penyahwarna ini dalam skala besar untuk rawatan air kumbahan berwarna.

**Kata kunci:** Pewarna sintetik, M. cladophyllus, enzim lakase, pengekspresan heterologus
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>i</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td><strong>ABSTRAK</strong></td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xxiv</td>
</tr>
</tbody>
</table>

### CHAPTER 1: INTRODUCTION

### CHAPTER 2: LITERATURE REVIEW

2.1 Synthetic Dyes  
2.2 Water Body Pollution by Synthetic Dyes  
2.3 Difficulties in the Treatment of Dye Containing Effluents  
2.4 Bioremediation  
2.5 Dye Decolourisation by White Rot Fungi

### CHAPTER 3: ENDOPHYTIC FUNGAL FLORA FROM SENDUDUK  

*Melastoma malabathricum*: ISOLATION, CHARACTERISATION AND EVALUATION ON DECOLOURISATION OF SYNTHETIC DYES

3.1 Introduction  
3.2 Experimental Procedure  
3.3 Results and Discussion  
3.4 Conclusion
<table>
<thead>
<tr>
<th>Section</th>
<th>Subsection</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>3.2.1</td>
<td>Isolation of Endophytic Fungi</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3.2.2</td>
<td>Dye Decolourisation on Agar Plates Amended with Dye</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3.2.3</td>
<td>Dye Decolourisation in Liquid Medium Amended with Dye</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>3.2.4</td>
<td>Fungal Characterisation and Identification</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>3.2.4.1</td>
<td>Morphological Characterisation</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>3.2.4.2</td>
<td>Molecular Identification of Fungus</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3.2.4.2.1</td>
<td>DNA Isolation</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3.2.4.2.2</td>
<td>PCR Amplification</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>3.2.4.3</td>
<td>DNA Sequence and Phylogenetic Analysis</td>
<td>22</td>
</tr>
<tr>
<td>3.3</td>
<td>3.3.1</td>
<td>Isolation of Endophytic Fungi</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>3.3.2</td>
<td>Dye Decolourisation on Agar Plates Amended with Dye</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>3.3.3</td>
<td>Dye Decolourisation in Liquid Medium Amended with Dye</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>3.3.4</td>
<td>Fungal Characterisation and Identification</td>
<td>27</td>
</tr>
<tr>
<td>3.4</td>
<td>Conclusion</td>
<td></td>
<td>30</td>
</tr>
</tbody>
</table>
CHAPTER 4 : REMAZOL BRILLIANT BLUE R (RBBR) DYE

DECOLOURISATION BY AN ENDOPHYTE Marasmius cladophyllus AND ITS INDUCED ENZYMES

CAPABLE OF DECOLOURISING AZO DYE

<table>
<thead>
<tr>
<th>Section</th>
<th>Heading</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>4.2</td>
<td>Materials and Methods</td>
<td>33</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Fungus and Culture Maintenance</td>
<td>33</td>
</tr>
<tr>
<td>4.2.2</td>
<td>RBBR Dye Decolourisation in Liquid Medium Amended with the Dye</td>
<td>34</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Ligninolytic Enzymes Assay</td>
<td>34</td>
</tr>
<tr>
<td>4.2.4</td>
<td>In vitro RBBR Dye Decolourisation Using Fungal Crude Enzyme</td>
<td>36</td>
</tr>
<tr>
<td>4.2.5</td>
<td>Successive RBBR Dye Addition and Decolourisation</td>
<td>37</td>
</tr>
<tr>
<td>4.2.6</td>
<td>In vitro Azo Dye Decolourisation Using Ammonium Sulphate Precipitated Enzyme</td>
<td>37</td>
</tr>
<tr>
<td>4.3</td>
<td>Results and Discussion</td>
<td>38</td>
</tr>
<tr>
<td>4.3.1</td>
<td>RBBR Dye Decolourisation in Liquid Medium</td>
<td>38</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Production of Ligninolytic Enzymes</td>
<td>40</td>
</tr>
<tr>
<td>4.3.3</td>
<td>In vitro RBBR Dye Decolourisation</td>
<td>42</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Successive RBBR Dye Addition and Decolourisation</td>
<td>43</td>
</tr>
<tr>
<td>4.3.5</td>
<td>In vitro Azo Dye Decolourisation Using Ammonium Sulphate Precipitated Enzyme</td>
<td>44</td>
</tr>
<tr>
<td>4.4</td>
<td>Conclusion</td>
<td>50</td>
</tr>
</tbody>
</table>
CHAPTER 5 : PURIFICATION AND CHARACTERISATION OF DYE

DECOLOURISING LACCASE ENZYMES OF Marasmius cladophyllus UMAS MS8

5.1 Introduction 51

5.2 Materials and Methods 54

5.2.1 Culture Conditions and Laccase Enzyme Production 54

5.2.2 Laccase Enzyme Activity and Protein Content 54

5.2.3 Purification of the Dye Decolourising Laccase Enzyme 55

5.2.4 Polyacrylamide Gel Electrophoresis 55

5.2.5 Laccase Identification by MALDI-TOF/TOF-MS 56

5.2.6 Laccase Enzyme Characterisation 56

5.2.6.1 Effect of pH and Temperature on nLCC1 Activity and Its Stability 56

5.2.6.2 Enzyme Kinetics 57

5.3 Results and Discussion 57

5.3.1 Laccase Enzyme Purification 57

5.3.2 Laccase Characterisation 61

5.3.2.1 Effect of pH and Temperature on nLCC1 Activity and Its Stability 64

5.3.2.2 Enzyme Kinetics 66

5.4 Conclusion 67
CHAPTER 6 : MOLECULAR CLONING AND CHARACTERISATION OF
THE DYE DECOLOURISING GENES OF *Marasmius cladophyllus* UMAS MS8

6.1 Introduction 68

6.2 Materials and Methods 70

6.2.1 Organisms and Culture Conditions 70

6.2.2 Genomic DNA Isolation 70

6.2.3 Total RNA Isolation 71

6.2.4 Degenerate Primers for Screening of Laccase Genes 72

6.2.4.1 Primer design 72

6.2.4.2 PCR and Purification of PCR products 73

6.2.4.3 Preparation of Competent Cells and Cloning of PCR Product 74

6.2.5 Cloning of the Full Dye Decolourising Laccase Genomic DNA 76

6.2.6 Cloning of the Full Dye Decolourising Laccase cDNA 76

6.2.7 Bioinformatic Analysis of Laccase Gene sequence 77

6.3 Results and Discussion 78

6.3.1 Screening of *M. cladophyllus* Laccase Gene 78

6.3.2 Isolation of Full Laccase gDNA and cDNA 81

6.3.3 Analysis of *M. cladophyllus* Laccase Gene Sequence and Its Deduced Protein 82

6.4 Conclusion 89
CHAPTER 7 : HETEROLOGOUS EXPRESSION OF LACCASE (Lcc1) OF Marasmius cladothyllus UMAS MS8 IN YEAST Pichia pastoris

7.1 Introduction 90
7.2 Materials and Methods 92
7.2.1 Vectors and Microbial Strains 92
7.2.2 Construction of Recombinant Expression Vectors 92
7.2.3 Transformation and Selection of Yeast Strains 96
7.2.4 Expression of the Recombinant Laccase Enzyme in Liquid Media 98
7.2.5 Optimisation of Recombinant Laccase Enzyme Expression 99
7.2.6 Characterisation of the Recombinant Laccase Enzyme 100
7.2.7 In vitro RBBR Dye Decolourisation Using Native and Recombinant Laccase Enzyme 100

7.3 Results and Discussion 101
7.3.1 Construction of the Expression Vectors 101
7.3.2 Transformation and Selection of Transformants 102
7.3.3 Expression of the Recombinant Laccase Enzyme (rLCC) 105
7.3.4 Optimisation of Recombinant Laccase Enzyme Expression 107
7.3.5 Characterisation of the Recombinant Laccase Enzymes 111
7.3.6 \textit{In vitro} RBBR Dye Decolourisation Using

Native and Recombinant Laccase Enzyme 114

7.4 Conclusion 117

CHAPTER 8: GENERAL DISCUSSION AND RECOMMENDATION 118

CHAPTER 9: CONCLUSIONS 121

REFERENCES 122

APPENDICES 144
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Decolourisation of RBBR, Orange G, Congo red and Methyl red on agar plate by the 20 isolated endophytic fungi after 16 days of cultivation</td>
<td>23</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Fungal biomass produced by isolate MS8 in glucose minimal liquid medium containing RBBR, Orange G, Congo red and Methyl red within a period of 16 days. Liquid medium with no dye was used as a control for fungal biomass</td>
<td>27</td>
</tr>
<tr>
<td>Table 4.1</td>
<td><em>In vitro</em> synthetic dye decolourisation using ammonium sulphate precipitated crude enzyme</td>
<td>46</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Steps in purification to homogeneity of nLCC1 enzyme of <em>M. cladophyllus</em> UMAS MS8</td>
<td>61</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>BLASTN and BLASTX search of <em>M. chladophyllus</em> Lee1 and Lee2 gene fragment showing percentage query coverage and sequence similarity</td>
<td>80</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>Chemical structures of sulfonated azo type dyes (a) Orange G, (b) Congo Red and anthraquinonic dye (c) Remazol Brilliant Blue R (RBBR).</td>
<td>5</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Dye decolourisation by isolate MS8 resulting in the formation of halo (arrow) on agar plate medium containing (a) RBBR, (b) Orange G, (c) Methyl red and (d) Congo red after four days.</td>
<td>24</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Percentage decolourisation of RBBR, Orange G, Congo red and Methyl red by isolate MS8 in glucose minimal liquid medium amended with the respective dyes within a period of 16 days.</td>
<td>25</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Endophytic fungal isolate MS8. (a) Fungal colony cultured on malt extract agar for 7 days (b) microscopic view of the isolate showing fungal hyphae with the production of a clamp connection (arrow), magnification 1000x.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Agarose gel electrophoresis of purified PCR products for isolate MS8 amplified using universal primer ITS1 and ITS4. Lane L: 100 bp DNA ladder (Promega).</td>
<td>29</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Neighbour-joining tree from ITS sequences showing the relationship between isolate MS8 and other closely related <em>Marasmius</em> species retrieved from the GenBank (accession number). Bootstrap value &gt;70% (1000 replicates) are shown at the branches. Bar = 5 nucleotide substitutions per 100 nucleotides.</td>
<td>29</td>
</tr>
</tbody>
</table>
Figure 4.1  Decolourisation of RBBR dye (200 mg L\(^{-1}\)) by *M. cladophyllus* in glucose minimal medium. (a) Abiotic control with no fungal inoculum. (b) Decolourised RBBR dye after 15 days by *M. cladophyllus* with no visible dye adsorbed onto the fungal mycelium (arrow).

Figure 4.2  Absorbance ratio showing the degradation of RBBR dye (200 mg/L) in glucose minimal medium by *M. cladophyllus* within a period of 15 days. Bar indicates percentage of dye adsorption on fungal mycelium.

Figure 4.3  Lignin peroxidase and laccase activities produced by *M. cladophyllus* within 15 days in culture medium containing RBBR dye (blue line) and in medium without the dye (black line). Lines with open marker uses the second y-axis.

Figure 4.4  *In vitro* RBBR dye decolourisation using crude enzyme harvested from culture medium containing RBBR dye and culture medium without RBBR dye within a period of 15 days.

Figure 4.5  RBBR dye decolourisation by *M. cladophyllus* with 2 times of fresh RBBR supplementation (final concentration approximately 200 mg/L). The arrows indicate the addition of fresh RBBR dye into the fungal culture.
Figure 4.6 Absorbance spectrum of (a) RBBR dye, (b) Orange G and (c) Congo red after 24 hours of degradative decolourisation using ammonium sulphate precipitated crude enzyme. Control was prepared with heat inactivated enzyme. (arrow indicates the decrease of the spectrum at the maximum absorption wavelength of the respective dye).

Figure 5.1 SDS PAGE and zymogram of ammonium sulphate precipitated crude laccase enzyme preparation with (a) ABTS for laccase activity staining, (b) RBBR dye for laccase dye decolourisation and (c) Coomassie brilliant blue R-250 to visualize all proteins present. (L- EZ-Run Prestained Rec Protein Ladder).

Figure 5.2 Purification of *M. cladophyllus* laccase isoforms by anion exchange chromatography on HiTrap™ DEAE FF column with (a) blue line corresponding to optical density at 280 nm and red line representing NaCl concentration during gradient elution of the 2 laccase isoforms. The fractions collected were analysed by PAGE with (b) staining by ABTS and (c) staining by Coomassie Brilliant Blue R250. Sample in the first lane with C represent crude enzyme.

Figure 5.3 SDS PAGE and zymogram of the purified nLCC1 of *M. cladophyllus* with (a) staining using Coomassie brilliant blue R-250 (L- Amersham low molecular weight marker, GE Healthcare); (b) staining with ABTS for laccase activity and (c) staining with RBBR dye for decolourisation.
Figure 5.4  UV-Vis absorbance spectrum of the purified nLCC1 of *M. cladophyllus*. Red arrow indicating a shoulder at 320 nm and black arrow indicating an absorption peak at 600 nm.

Figure 5.5  Effect of pH and temperature on the purified nLCC1 activity. (a) and (c) identify the optimal pH and temperature, respectively for the enzyme while (b) and (d) illustrate the residual activity of the enzyme after 1 hour of incubation at different pH and temperature, respectively. Bars represent standard deviation.

Figure 6.1  Design of degenerate primers, LAC-N1 and LAC-C1a for the screening of laccase gene based on laccase protein sequence of *Lentinus* sp. WR2.

Figure 6.2  Agarose gel electrophoresis of (a) PCR amplified laccase gene fragment (~1700 bp) and (b) restriction analysis of positive clone carrying the desired laccase gene fragment (~1700 bp) in pGEM-T Easy Vector (3015 bp). Lane L: 1 kb DNA ladder (Vivantis).

Figure 6.3  Agarose gel electrophoresis of the PCR amplified *M. cladophyllus* full laccase gene (a) *Lcc1* gDNA (2118 bp) and (b) *Lcc1* cDNA (1566 bp). Lane L: 1 kb DNA ladder (Vivantis).
Figure 6.4  Genomic and deduced amino acid sequence of \textit{Lcc1} gene. Introns are represented in shaded lower case letters. The putative signal peptide sequence (the first 21 amino acid) is indicated in bold. The multicopper oxidase signature sequence of LCC1 is represented in blue. The four conserved copper-binding region, [H-X-H], are boxed. Seven possible N-glycosylation sites (Asn-X-Ser/Thr) were underlined and peptide fragments of LCC1 identified by MALDI-TOF/TOF-MS are represented in red.

Figure 6.5  Alignment of \textit{M. cladophyllus Lcc1} and \textit{Lcc2} gDNA with other fungal laccase gene sequences encoding copper binding regions I, II, III and IV (red box) . Number in parentheses are the number of clones for laccase gene of \textit{M. cladophyllus}.

Figure 6.6  Alignment of \textit{M. cladophyllus Lcc1} and \textit{Lcc2} deduced amino acid sequences with other fungal laccase protein. His and Cys residues involved in the in copper binding are indicated in black boxes. Cys residues involved in disulfide bridges are indicated in black boxes with asterisks (*). N-terminal amino acid sequences are bold.

Figure 6.7  Phylogenetic tree showing the relationship of laccase from \textit{M. cladophyllus} UMAS MS8 (LCC1) with laccase from other Basidiomycetes, (I); Ascomycetes, (II) and insect (III). Bootstrap values from 1000 replicates are shown on branches. Numbering in bracket is the accession number for laccase of the corresponding organism from GeneBank.
Figure 7.1  Expression vectors constructed for the expression of *M. cladophyllus* laccase LCC1 enzyme by *P. pastoris* strain GS115. *M. cladophyllus* Lcc1 cDNA was cloned without its native signal peptide coding sequence into pPICZαB expression vector to express extracellular recombinant laccase with (a) native N-terminal sequence and (b) an alanine preceding the native N-terminal sequence using *S. cerevisiae* α-factor secretion signal. (c) *M. cladophyllus* Lcc1 cDNA was cloned into pPICZB expression vector with its native signal sequence. Primers used for PCR screening were represented in purple colours.

Figure 7.2  Agarose gel electrophoresis of *Bst*XI restriction enzyme linearized (a) empty expression vectors and (b) the three different expression vectors constructed to carry the Lcc1 cDNA of *M. cladophyllus*. Lane 1: pPICZαB, lane 2: pPICZB, lane 3: pPICZαB/xLcc1, lane 4: pPICZαB/pLcc1 and lane 5: pPICZB/eLcc1. Lane L: 1 kb DNA ladder (Fermentas).

Figure 7.3  Agarose gel electrophoresis of *P. pastoris* transformants’ colony PCR product using the primers 5’AOX1 and 3’AOX1. Lane1: PCR product of wild type untransformed *P. pastoris* strain GS115; lane 2 to lane 6: PCR product of CaB (lane 2), CB (lane 3), X (lane 4), P (lane 5) and E (lane 6) transformants; lane 7 and lane 8: PCR products of X and P amplified with an additional primer, LCC1MSpf; lane L: 1 kb DNA ladder (Fermentas).
Figure 7.4  Assay of recombinant laccase activity expressed by *P. pastoris* on BMM agar medium containing ABTS after (a) two days and (b) four days of incubation at 30°C. Formation of green coloured zone around the streaking of yeast cells indicates the secretion of active and functional recombinant laccase enzyme.

Figure 7.5  Comparison of recombinant laccase (rLCC) expression in BMMH liquid medium between *P. pastoris* GS115 transformants with P1 using α-factor secretion signal peptide and E1 using native signal peptide. Both CαB and CB were control strains transformed without *Lcc1* cDNA. Bars represent standard deviation of triplicates.

Figure 7.6  Recombinant laccase (rLCC) activity produced by E1 transformant induced with 0.5 % methanol in BMMH liquid medium containing different copper concentration within a period of 8 days. Bars represent standard deviation of triplicates.

Figure 7.7  Recombinant laccase (rLCC) activity produced by E1 transformant induced with different concentration of methanol in BMMH liquid medium containing 0.1 mM of copper within a period of 8 days. Bars represent standard deviation of triplicates.
Figure 7.8  Recombinant laccase (rLCC) activity produced by E1 transformant in BMMH liquid medium under optimised copper and methanol concentrations. Culture medium was harvested every eighth days of incubation and cell biomass was reused for 2 additional cycle of enzyme expression in the original culture vessel. Bars represent standard deviation of triplicates.

Figure 7.9  SDS PAGE and zymogram of crude enzyme containing recombinant laccase (rLCC) expressed by E1 transformant with staining using (a) Coomassie brilliant blue R-250 and (b) ABTS substrate for laccase activity as compared with purified native laccase (nLCC1) of *M. cladophyllus* in lane 1. Lane L: Amersham low molecular weight marker, GE Healthcare.

Figure 7.10  Effect of pH and temperature on the recombinant laccase (rLCC) activity compared with the purified nLCC1 of *M. cladophyllus*. (a) and (c) identify the optimal pH and temperature respectively of the enzyme while (b) and (d) illustrate the residual activity of the enzyme after 1 hour of incubation at different pH and temperature respectively. Bars represent standard deviation of triplicates.

Figure 7.11  Absorbance spectrum of RBBR dye before (control) and after 1 hour of decolourisation using recombinant laccase (rLCC-orange line) and the purified native laccase LCC1 (nLCC1- blue line) enzyme of *M. cladophyllus*.