

**Isolation and characterization of bacterial strains and  
their enzymes with lignolytic potential**

**SUPREET KAUR**



**CENTRE FOR RURAL DEVELOPMENT AND TECHNOLOGY**

**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**AUGUST 2020**

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# **Isolation and characterization of bacterial strains and their enzymes with lignolytic potential**

by

**SUPREET KAUR**

**Centre for Rural Development and Technology**

**Submitted**

**In fulfilment of the requirements of the degree of Doctor of Philosophy**

**to the**



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**AUGUST 2020**

# *Certificate*

This is to certify that the thesis entitled “**Isolation and characterization of bacterial strains and their enzymes with lignolytic potential**” submitted by **Ms. Supreet Kaur** to the Centre for Rural Development and Technology, Indian Institute of Technology Delhi for the award of **DOCTOR OF PHILOSOPHY** is a record of the bonafide research work carried out by her. Ms. Supreet Kaur has worked under our supervision for submission of this thesis which to our knowledge has reached requisite standard. To the best of our knowledge the results in this thesis have not been submitted for any degree or diploma in any other Institute or University. She has fulfilled all the requirements for submission of her thesis.

**Dr. Hariprasad P.**

Centre For Rural Development  
and Technology

Indian Institute of Technology Delhi  
New Delhi

**Prof. V. K. Vijay**

Centre For Rural Development  
and Technology

Indian Institute of Technology Delhi  
New Delhi

## Acknowledgements

It is an honor for me to be in the academic system of Indian Institute of Technology Delhi and choosing the area of research “Environmental Biotechnology”. This subject of research is becoming more interdisciplinary and challenging for many environmentalists as well as biologists. From the beginning of my research, I have been in a continuous learning process and have successfully carried out the planned experiments from which significant results have been incorporated in this thesis, “**Isolation and Characterization of Bacteria and Their Enzymes with Lignolytic Potential**”.

I would like to thank **DST-SERB** for providing funding for DST Young Scientist Project entitled “**Isolation and characterization of bacterial strains and their enzymes with lignolytic potential**”. I sincerely thank the **Indian Institute of Technology Delhi** for financial support and providing necessary research space and facilities for the chosen work. I am grateful to my PhD supervisors **Dr. Hariprasad P.** and **Prof. V. K. Vijay** for their constant encouragement and guidance to think beyond conventional experimentation. I sincerely thank the **Head of the Center, all faculty members and Prof. S. N. Naik, Prof. Anushree Malik, Prof. Zia Sheikh** for their timely suggestions and moral support. It has been a valuable experience at Centre for Rural Development and Technology where I got constant support and co-operation of **all my lab mates, and Pratibha and Hridayaram** and all other students of the center. I wish them all success in their life and I wish the center to achieve its glory through modern scientific temperaments. All of them have given me a platform to enhance my knowledge and cope with growing scientific arena.

I would also like to acknowledge **Nano-Research Facility**, Indian Institute of Technology Delhi and **SAIF**, Indian Institute of Technology Bombay and **SAIF**, Centre for Drug Research Institute, Lucknow for processing GC-MS/MS samples and LC-MS/MS samples.

I am grateful to **my family** and **Almighty** for their blessings and support throughout my research for this duration.

**Supreet Kaur**

## Abstract

The Western Ghats region of Karnataka, India, is an unhabitated area where native micro-organisms have been known to degrade lignin. Among such micro-organisms, white-rot fungi are well reported for their ability to degrade lignin by the production of extracellular laccases and peroxidases. Brown rot fungi partially modify the lignin. Also, bacteria are well reported to degrade lignin. In the current study, the ability of the “native bacteria” has been explored to degrade lignin via the production of extracellular enzymes and to explore the genomics of the best-known lignin degrader.

Lignin, a complex heteropolymer, is one of the renewable organic sources present on the earth that plays a vital role for the growth and survival of the plants. On the other hand, when it comes to the application of the lignocellulosic biomass, the whole fraction of lignin is considered as waste. In most of the cases, lignin which is obtained as a by-product via various industrial processes is burnt as a source of energy or released into water bodies creating an unfavorable condition to aquatic life. Though several researchers had made efforts to valorize the lignin to value-added chemicals but its adoption at the industrial level is awaited. In nature, microbial depolymerization and degradation of lignin is a common process through which they convert lignin to various intermediates, finally to acetyl CoA as a feeding molecule of the Tri Carboxylic Acid cycle. The most common lignin-degrading microbes are white-rot basidiomycetes, brown-rot basidiomycetes, and bacteria. During the processes of lignin depolymerization, degradation and assimilation, several intermediates are produced which are of economic importance such as vanillin, catechol, and propionic acid, etc. Though the fungal biodegradation of lignin is extensively studied, but only a small population of lignin-degrading bacteria are explored. There is always a scope to determine new pathways of lignin degradation and intermediate compounds of high value. Hence, the ambition of the authors through this

study is to focus on the bacterial lignin depolymerization and degradation pathways and further possible route of high-value chemicals.

In chapter 3, 24 soil samples were collected from the Western Ghats, Karnataka region, and brought to the Environmental biotechnology laboratory for further processing. 112 suspected lignolytic bacteria were isolated from those 24 samples based on their ability to grow on Kraft Lignin (KL). These bacteria were also checked for their growth on different carbon sources where they showed good growth on paddy straw powder followed by popular wood powder and KL (with/without glucose and peptone). Bacteria were able to decolorize or adsorb lignolytic indicator dyes to various extents showing their ability to break different bonds of lignin. Lignin degradation studies also revealed that 24 bacteria were potential lignin degraders, capable of degrading KL > 50 %. Morphological, biochemical characterization along with 16S rRNA sequencing of these isolates revealed the presence of *Bacillus* sp. majorly, with four actinomycetes viz, *Streptomyces mangrovi* CRDT-EB-18.4, *Streptomyces* sp. CRDT-EB-19.7, *Streptomyces griseorubens* CRDT-EB-21.6, and *Streptomyces* sp. CRDT-EB-21.12. The bacteria were also found to be non-pathogenic.

In chapter 4, all the 24 selected bacteria were found to be significant lignin degraders, as they recorded growth in media amended with 200 to 800 ppm KL and follow a particular pattern of lignin degradation as analyzed by high performance liquid chromatography (HPLC) and gel permeation chromatography (GPC). The decrease in the absorbance and shift in the major peak towards later retention time and appearance of additional minor peaks indicated the degradation of high molecular weight lignin and the formation of lower molecular weight lignin. Analysis of bacterial degraded products of lignin through gas chromatography-mass spectrometry (GC-MS/MS) and liquid chromatography-mass spectrometry (LC-MS/MS) in culture supernatant revealed that they majorly follow ortho cleavage pathway in addition to meta cleavage pathway, which was deduced using the compounds identified such as vanillic

acid, protocatechuic acid, gentisic acid, coniferyl alcohol, and cinnamic acid, etc. Further, the above results were supported by aromatic monomer utilization experiments which also indicated that these lignin-degrading bacteria have the ability to assimilate these monomers as a carbon source by up taking and degrading them by using the intercellular enzymes. The dominant bacterial enzyme responsible for lignin degradation was identified as a peroxidase in most efficient lignin-degrading bacteria *Streptomyces griseorubens* CRDT-EB-21.6, which was further purified and characterized.

In chapter 5, it was found that the isolate *Streptomyces griseorubens* CRDT-EB-21.6 was showing the highest enzyme activity in the presence of H<sub>2</sub>O<sub>2</sub>, thereby showing the possible involvement of a peroxidase in lignin degradation. The enzyme was further purified to nine fold using the size exclusion chromatography and the activity was increased to 3.09 U from 0.34 U. The purified enzyme was stable over a wide range of temperatures. However, the enzyme was stable only for a pH of 3.0. The enzyme activity was found to be inhibited by Cu<sup>2+</sup>, and Hg<sup>+</sup>, but increased by K<sup>+</sup>, Mg<sup>2+</sup>, Li<sup>+</sup>, and Ca<sup>2+</sup>. Also, the enzyme was found to be stable over a greater range of salt concentration with maximum activity around 60-80 mM NaCl concentration. This clearly shows the stability of the peroxidase enzyme from the isolate CRDT-EB-21.6 over a large spectrum of environmental conditions and thereby proves its capability to match the needs of industrial applications.

In chapter 6, in order to establish the lignin degradation ability of *Streptomyces griseorubens* CRDT-EB-21.6, whole-genome sequencing was performed and analyzed. A total of ~2.11 Gb HQ data of the sample used for de-novo assembly resulting in 343 scaffolds having an assembly size of ~7.2 Mb with an N50 of 36,329 bp. A total of 6471 CDS were predicted from 343 scaffolds. In total, 6441 CDS in NR, 3903 in UniProt, 1471 in KOG, and 3224 CDS in Pfam database were annotated. It was found that the majority of the hits were against the species *Streptomyces griseorubens* followed by *Streptomyces* sp. 4F in the sample. A total of

2410 GO terms were assigned to the CDS wherein, 1634, 999, and 948 GO terms, were assigned to Biological Process, Cellular Component, and Molecular Function, respectively. The pathway analysis was carried out using KAAS server (KEGG Automatic Annotation Server). The CDS were enriched in different functional pathway categories which were predominantly categorized into Metabolism, Genetic Information Processing, Environmental Information Processing, and Cellular Processes. Presence of CDS encoding the xenobiotic biodegradation terpenoids and polyketides metabolic pathways, along with the presence of genes coding for  $\beta$ -ketoacid, Phenol catabolism, and Gentisate pathway, clearly indicated that the bacteria, *Streptomyces griseorubens* CRDT-EB-21.6 is a potential lignin degrader, and a dynamic research material for future studies as well.

## आब्सट्रैक्ट

पश्चिमी घाट क्षेत्र, कर्नाटक, भारत का एक अप्रभावित क्षेत्र है, जो लिग्निन को तोड़ने के लिए देशी सूक्ष्म जीवों के लिए जाना जाता है। ऐसे सूक्ष्म जीवों में, सफेद-सड़न कवक बाह्य कोशिकीय लैकेसिस और पेरोक्सीडेस के उत्पादन द्वारा लिग्निन को तोड़ने की उनकी क्षमता के लिए रिपोर्ट किया गया है। ब्राउन रोट कवक आंशिक रूप से लिग्निन को संशोधित करता है। इसके अलावा, बैक्टीरिया अच्छी तरह से लिग्निन को तोड़ने के लिए रिपोर्ट किया गया है। इस अध्ययन में, "देशी जीवाणुओं" की क्षमता का पता लगाने के लिए बाह्य कोशिकीय एंजाइमों के उत्पादन के माध्यम से लिग्निन को कम करने और सबसे प्रसिद्ध लिग्निन डीजेंडर के जीनोमिक्स की तलाश की गई है।

लिग्निन, एक जटिल हेटरोपॉलीमर, पृथ्वी पर मौजूद अक्षय कार्बनिक स्रोतों में से एक है जो पौधों की वृद्धि और अस्तित्व के लिए महत्वपूर्ण भूमिका निभाता है। दूसरी ओर, जब लिग्निनोसेल्युलॉसिक बायोमास के आवेदन की बात आती है, लिग्निन के पूरे अंश को बेकार माना जाता है। अधिकांश मामलों में, लिग्निन जिसे विभिन्न औद्योगिक प्रक्रियाओं के माध्यम से उप-उत्पाद के रूप में प्राप्त किया जाता है, ऊर्जा के स्रोत के रूप में जलाया जाता है या जलीय जीवन के लिए प्रतिकूल स्थिति पैदा करने वाले जल निकायों में जारी किया जाता है। हालांकि कई शोधकर्ताओं ने लिग्निन को मूल्य वर्धित रसायनों के लिए वैध बनाने के प्रयास किए थे, लेकिन औद्योगिक स्तर पर इसके अपनाने का इंतजार किया जा रहा है। प्रकृति में, लिग्निन के माइक्रोबियल डेपोलाइमरिज़ेशन और डिग्रेडेशन एक सामान्य प्रक्रिया है जिसके माध्यम से वे लिग्निन को विभिन्न इंटरमीडिएट में परिवर्तित करते हैं, अंत में एसिटाइल सीओए को ट्राई कार्बोक्जिलिक एसिड चक्र के एक खिला अणु के रूप में। सबसे आम लिग्निन-डिग्रेडिंग रोगाणु सफेद-सड़ांध वाले बेसिडिओमाइसेट्स, ब्राउन-रोट बेसिडिओमाइसीस और बैक्टीरिया होते हैं। लिग्निन डेपोलाइमरिज़ेशन, डिग्रेडेशन और अस्मिताकरण की प्रक्रियाओं के दौरान, कई मध्यवर्ती उत्पन्न होते हैं जो आर्थिक महत्व के होते हैं जैसे कि वैनिलिन, कैटेचोल और प्रोपियोनिक एसिड, आदि। हालांकि लिग्निन के फंगल बायोडिग्रेडेशन का बड़े पैमाने पर अध्ययन किया गया है, लेकिन लिग्निन-डिग्रेडिंग बैक्टीरिया की केवल एक छोटी आबादी का पता लगाया गया है। लिग्निन क्षरण और उच्च मूल्य के मध्यवर्ती यौगिकों के नए मार्गों को निर्धारित करने के लिए हमेशा एक गुंजाइश है। इसलिए, इस अध्ययन के माध्यम से लेखकों की महत्वाकांक्षा बैक्टीरिया लिग्निन डेपोलाइमरिज़ेशन और डिग्रेडेशन मार्ग और उच्च मूल्य वाले रसायनों के आगे संभावित मार्ग पर ध्यान केंद्रित करना है।

अध्याय 3 में, पश्चिमी घाट, कर्नाटक क्षेत्र से 24 मिट्टी के नमूने एकत्र किए गए, और आगे की प्रक्रिया के लिए पर्यावरण जैव प्रौद्योगिकी प्रयोगशाला में लाया गया। क्राफ्ट लिग्निन (केएल) पर

बढ़ने की उनकी क्षमता के आधार पर उन 24 नमूनों में से 112 सस्पेक्टेड लिग्नोलिटिक बैक्टीरिया को अलग किया गया था। इन जीवाणुओं को विभिन्न कार्बन स्रोतों पर उनकी वृद्धि के लिए भी जांचा गया जहां उन्होंने धान के पुआल पाउडर पर अच्छी वृद्धि दिखाई और इसके बाद लोकप्रिय लकड़ी के पाउडर और केएल (ग्लूकोज और पेप्टोन के साथ) के साथ। बैक्टीरिया लिग्निन के विभिन्न बंधनों को तोड़ने की क्षमता दिखाते हुए विभिन्न विलुप्त होने वाले रंगों की नकल करने वाले लिग्नोलिटिक इंडिकेटर डाइज़ को हटाने या विज्ञापन करने में सक्षम थे। लिग्निन की डिग्रेडेशन के अध्ययन से यह भी पता चला है कि 24 बैक्टीरिया संभावित लिग्निन डिग्रेडर्स थे, जो केएल > 50% को क्षीण करने में सक्षम थे। इन आइसोलेट्स के 16S rRNA अनुक्रमण के साथ रूपात्मक, जैव रासायनिक लक्षण वर्णन बेसिलस स्पीशीस की उपस्थिति का पता चला। प्रमुख रूप से, चार एक्टिनोमाइसेट्स अर्थात् स्ट्रेप्टोमाइसेस मेंगोवी CRDT-EB-18.4, स्ट्रेप्टोमी स्पीशीस CRDT-EB-19.7, स्ट्रेप्टोमीस ग्रिसेओरुबेन्स CRDT-EB-21.6, और स्ट्रेप्टोमी स्पीशीस CRDT-EB-21.12। बैक्टीरिया गैर-रोगजनक भी पाए गए। अध्याय 4 में, सभी 24 चयनित बैक्टीरिया महत्वपूर्ण लिग्निन डिग्रेडर्स पाए गए, क्योंकि उन्होंने 200 से 800 पीपीएम केएल के साथ मीडिया में वृद्धि दर्ज की और हाइड्रॉफॉर्मिन्स लिक्विड क्रोमेटोग्राफी (एचपीएलसी) और जेल पमपेशन क्रोमेटोग्राफी (GPC) द्वारा विश्लेषण के रूप में लिग्निन डिग्रेडेशन के एक विशेष पैटर्न का पालन किया। बाद की अवधारण समय और अतिरिक्त मामूली चोटियों की उपस्थिति के लिए प्रमुख शिखर में अवशोषण और बदलाव में कमी ने उच्च आणविक भार लिग्निन के क्षरण और कम आणविक भार लिग्निन के गठन का संकेत दिया। गैस क्रोमेटोग्राफी-मास स्पेक्ट्रोमेट्री (जीसी-एमएस / एमएस) और तरल क्रोमेटोग्राफी-मास स्पेक्ट्रोमेट्री (एलसी-एमएस / एमएस) के माध्यम से बैक्टीरियल कल्चर सुपरनेटेंट के विश्लेषण से पता चला कि मेटा क्लीवेज पाथवे के अलावा ऑर्थो क्लीवेज पाथवे का प्रमुख रूप से पालन किया जाता है, जो कि वैनिलिक एसिड, प्रोटोकैटेचिक एसिड, जेंटिसिक एसिड, कॉन्फेरील अल्कोहल और सिन्नमीक एसिड, आदि जैसे यौगिकों का उपयोग के माध्यम से ज्ञात किया गया था। इसके अलावा, उपरोक्त परिणामों को सुगंधित मोनोमर उपयोग द्वारा समर्थित किया गया था, जो यह भी संकेत देता था कि लिग्निन-डिग्रेडिंग बैक्टीरिया इन मोनोमर्स को एक कार्बन स्रोत के रूप में आत्मसात करने और अंतरकोशिकीय एंजाइमों का उपयोग करके उन्हें डिग्रेड करने की क्षमता रखते हैं। लिग्निन डिग्रेडेशन के लिए जिम्मेदार प्रमुख बैक्टीरियल एंजाइम की पहचान सबसे कुशल लिग्निन-डिग्रेडिंग बैक्टीरिया स्ट्रेप्टोमीस ग्रिसेओरुबेन्स CRDT-EB-21.6 में पेरोक्सीडेस के रूप में की गई थी, जिसे और अधिक शुद्ध और विशेषता बनाया गया था।

अध्याय 5 में, यह पाया गया कि पृथक स्ट्रेप्टोमीस ग्रिसोरूबेन्स CRDT-EB-21.6 H<sub>2</sub>O<sub>2</sub> की उपस्थिति में उच्चतम एंजाइम गतिविधि दिखा रहा था, जिससे लिग्निन डिग्रेडेशन में पेरोक्सीडेज की

संभावित भागीदारी दिखाई दे रही थी। आकार अपवर्जन क्रोमैटोग्राफी का उपयोग करके एंजाइम को नौ गुना तक शुद्ध किया गया था और गतिविधि को 0.34 यू से 3.09 यू तक बढ़ाया गया था। शुद्ध एंजाइम तापमान की एक विस्तृत श्रृंखला पर स्थिर था। हालांकि, एंजाइम केवल 3.0 के पीएच के लिए स्थिर था। एंजाइम गतिविधि  $\text{Cu}^{2+}$ , और  $\text{Hg}^{+}$  द्वारा बाधित पाई गई, लेकिन  $\text{K}^{+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Li}^{+}$  और  $\text{Ca}^{2+}$  द्वारा बढ़ी गई। इसके अलावा, एंजाइम को 60-80 मिमी NaCl एकाग्रता के साथ अधिकतम गतिविधि के साथ नमक एकाग्रता की एक बड़ी रेंज पर स्थिर पाया गया था। यह स्पष्ट रूप से पर्यावरण की स्थिति के एक बड़े स्पेक्ट्रम पर अलग CRDT-EB-21.6 से पेरॉक्सीडेज एंजाइम की स्थिरता को दर्शाता है और जिससे औद्योगिक अनुप्रयोगों की जरूरतों को पूरा करने की क्षमता साबित होती है।

अध्याय 6 में, स्ट्रेप्टोमाइसेस ग्रिसोरूबेस CRDT-EB-21.6 की लिग्निन डिग्रेडेशन क्षमता को स्थापित करने के लिए, पूरे जीनोम अनुक्रमण का प्रदर्शन और विश्लेषण किया गया था। डी-नोवो असेंबली के लिए उपयोग किए गए नमूने के कुल ~ 2.11 जीबी मुख्यालय डेटा के परिणामस्वरूप 36332 बीपी के एन 50 के साथ 343 स्कैफोल्ड का एक विधानसभा आकार ~ 7.2 एमबी है। 343 मचानों से कुल 6471 सीडीएस की भविष्यवाणी की गई थी। कुल मिलाकर NR में 6441 CDS, UniProt में 3903, KOG में 1471 और Pfam डेटाबेस में 3224 CDS एनोटेट किए गए। नमूने में यह पाया गया कि हिट्स की अधिकांश प्रजातियाँ स्ट्रेप्टोमीज़ ग्रिज़ोरूबेस थीं, इसके बाद स्ट्रेप्टोमी स्पीशीस 4F। कुल 2410 GO की शर्तें CDS को सौंपी गई थीं, जिसमें 1634, 999 और 948 GO की शर्तें क्रमशः बायोलॉजिकल प्रोसेस, सेलुलर कंपोनेंट और मॉलिक्यूलर फंक्शन को सौंपी गई थीं। पाथवे विश्लेषण KAAS सर्वर (KEGG स्वचालित एनोटेशन सर्वर) का उपयोग करके किया गया था। सीडीएस को विभिन्न कार्यात्मक मार्ग श्रेणियों में समृद्ध किया गया था, जिन्हें मुख्य रूप से चयापचय, आनुवंशिक सूचना प्रसंस्करण, पर्यावरण सूचना प्रसंस्करण और सेलुलर प्रक्रियाओं में वर्गीकृत किया गया था। सीडीएस की उपस्थिति जीनोबायोटिक बायोडिग्रेडेशन टैरेपीनोइड्स और पॉलीकेटाइड्स मेटाबॉलिक पथ एन्कोडिंग की उपस्थिति के साथ-साथ बीटा-केटआदिपते, फिनोल अपचय और जेंटेट मार्ग के लिए जीन कोडिंग की उपस्थिति से स्पष्ट रूप से संकेत मिलता है कि बैक्टीरिया, स्ट्रेप्टोमाइसेज ग्रिसेओरूबेस CRDT-EB-21.6 एक पोटैन्शियल लिग्निन एक पोटैन्शियल लिग्निनडेग्राडर है, और भविष्य के अध्ययन के लिए एक गतिशील अनुसंधान सामग्री भी।

## Table of contents

S. No.	Title	Page No.
	<i>Acknowledgment</i>	<i>i</i>
	<i>Abstract</i>	<i>ii</i>
	<i>Table of contents</i>	<i>vi</i>
	<i>List of figures</i>	<i>xi</i>
	<i>List of tables</i>	<i>xv</i>
	<i>List of abbreviations</i>	<i>xvii</i>
<b>Chapter 1.</b>	<b>Introduction</b>	<b>1-14</b>
	1.1 General	1
	1.2 Problem identification	13
<b>Chapter 2.</b>	<b>Literature Review</b>	<b>15-34</b>
	2.1 Bacterial biodegradation of lignin	15
	2.2 Lignin degrading enzymes	24
	2.3 Degradation of lignin monomers and their assimilation in bacteria	29
	2.4 Objectives of the thesis	34
<b>Chapter 3.</b>	<b>Isolation and characterization of lignin degrading bacteria</b>	<b>35-70</b>
	3.1 Introduction	35
	3.2 Materials and methods	
	3.2.1 Soil sample collection	37
	3.2.2 Chemicals used	37
	3.2.3 Isolation of lignin degrading bacteria	38
	3.2.4 Bacterial growth on different lignin sources	39
	3.2.5 Dye decolorization assay	40
	3.2.6 Quantitative analysis of bacterial lignin degradation	42
	3.2.7 Identification of bacterial isolates	44
	3.2.8 Safety analysis	46
	3.3 Results and Discussion	
	3.3.1 Soil sample collection	46

3.3.2	Isolation of lignin degrading bacteria	49
3.3.3	Growth on different lignin sources	50
3.3.4	Dye decolorization assay	52
3.3.5	Bacterial Kraft lignin degradation studies	57
3.3.6	Characterization and identification of bacterial isolates	60
3.3.7	Safety analysis	66
3.4	Conclusion	70
<b>Chapter 4.</b>	<b>Elucidating the mechanism of lignin degradation by selected bacteria</b>	<b>71-102</b>
4.1	Introduction	71
4.2	Material and methods	
4.2.1	Chemicals used	73
4.2.2	Lignin tolerance assay	73
4.2.3	Analytical methods	73
4.2.3.1	HPLC analysis	74
4.2.3.2	Gel filtration chromatography	74
4.2.3.3	Gas chromatography-Mass spectroscopy	74
4.2.3.4	Ultra-performance liquid chromatography-tandem mass spectrometer	75
4.2.4	Utilization of aromatic monomers	76
4.2.5	Enzyme activities	77
4.3	Results and discussion	
4.3.1	Lignin tolerance assay	79
4.3.2	Lignin degrading pattern analysis	
4.3.2.1	HPLC analysis	80
4.3.2.2	Gel filtration chromatography	81
4.3.2.3	Analysis of lignin degraded products	85
4.3.3	Utilization of aromatic monomers	90
4.3.4	Enzyme activities	97
4.4	Conclusion	101
<b>Chapter 5.</b>	<b>Purification and characterization of lignin</b>	<b>103-128</b>

<b>degrading enzyme</b>	
5.1 Introduction	103
5.2 Material and methods	
5.2.1 Chemicals used	104
5.2.2 Selection of best bacteria for enzyme purification	105
5.2.3 Enzyme purification	106
5.2.3.1 Cultivation of isolate CRDT-EB-21.6	106
5.2.3.2 Ammonium sulfate precipitation and dialysis	108
5.2.3.3 Anion exchange chromatography	108
5.2.3.4 Size exclusion chromatography	109
5.2.3.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	109
5.2.4 Characterization of lignin-degrading peroxidase	110
5.2.4.1 Enzyme kinetics	110
5.2.4.2 Substrate specificity	111
5.2.4.3 Effect of pH and temperature on the enzyme activity	111
5.2.4.4 Effect of metal salts and salinity on the enzyme activity	112
5.2.4.5 Total protein estimation	112
5.2.5 Statistical analysis	113
5.3 Results and discussion	
5.3.1 Selection of the best lignin degrading bacteria for the enzyme purification	113
5.3.2 Enzyme purification and characterization	115
5.3.3 Characterization of the lignin-degrading peroxidase	119
(i) Enzyme kinetics	119
(ii) Substrate specificity	121
(iii) Effect of pH and temperature on the enzyme activity	122

(iv) Effect of metals salts and salinity on the enzyme activity	125
5.4 Conclusion	127
<b>Chapter 6. Genomic and metabolomic analysis of best lignin degrading bacteria</b>	<b>129-160</b>
6.1 Introduction	129
6.2 Material and methods	
6.2.1 Total genomic DNA extraction, library preparation, and sequencing	130
6.2.1.1 Isolation, qualitative and quantitative analysis of DNA	130
6.2.1.2 Preparation of library and analysis	131
6.2.1.3 Cluster generation and sequencing	131
6.2.2 Bioinformatics analysis	132
6.2.2.1 Denovo analysis	133
6.2.2.2 Non-coding RNA prediction	133
6.2.2.3 SSR (Simple Sequence Repeats) Identification	134
6.2.2.4 Protein coding CDS prediction and functional annotation	134
6.2.2.5 Gene ontology sequence distribution	135
6.2.2.6 Pathway analysis	135
6.2.3 Bacterial culture and Nucleotide sequence deposition	136
6.3 Results and discussion	
6.3.1 Characteristics of <i>Streptomyces griseorubens</i> CRDT-EB-21.6	136
6.3.2 Total genomic DNA extraction, library preparation and sequencing	137
6.3.3 Bioinformatics analysis	
6.3.3.1 Data statistics	138
6.3.3.2 Denovo assembly	138
6.3.3.3 Non-coding RNA prediction	138

6.3.3.4 SSR (Simple Sequence Repeats)	140
Identification	
6.3.3.5 Protein coding CDS prediction	141
6.3.3.6 Functional annotation of predicted CDS	142
6.3.3.7 Gene ontology sequence distribution	149
6.3.3.8 Pathway analysis	150
6.4 Conclusion	159
<i>Future prospects</i>	<i>161-162</i>
<i>References</i>	<i>163-192</i>
<i>Annexure</i>	<i>193-226</i>
<i>About the author</i>	<i>227-228</i>

---

## List of figures

Figure	Title	Page No.
Figure 1.1	Schematic representation of the plant cell wall	1
Figure 1.2	Molecular structure of (a) Cellulose, (b) Hemicellulose, and (c) Five major bonding patterns in a lignin matrix structure	2
Figure 1.3	Monolignols involved in lignin biosynthesis	4
Figure 1.4	Building blocks of lignin <i>p</i> -hydroxyphenyl (H), guaiacyl (G) and syringyl (S) residues	5
Figure 1.5	Industrial ways of obtaining different types of lignin as a by-product	8
Figure 1.6	Potential market value for lignin-based products and corresponding value-added chemicals	9
Figure 2.1	Ligninolytic indicator dyes containing aromatic rings similar to that present in lignin	20
Figure 2.2	The enzymatic reaction catalyzed by the lignin-degrading enzymes	26
Figure 2.3	The bacterial lignin degradation pathway representing various intermediate high value chemicals	33
Figure 3.1	Molecular structure of the dyes used in the present study	41
Figure 3.2	Flowchart representing different steps involved in quantitative analysis of Kraft lignin in minimal media.	44
Figure 3.3	A) The geographical location of the Western Ghats, Karnataka, (B) Enlarged map of India showing the soil sample collection areas (Circled) of the Western Ghats, Karnataka	47
Figure 3.4	Some of the selected areas of the Western Ghats region showing soil sample collection sites	48
Figure 3.5	Isolation of lignin-degrading bacteria by enrichment culture technique	49

Figure 3.6 a	Bacterial isolates showing growth on minimal medium amended with different lignin sources	51
Figure 3.6 b	Percentage of the bacteria showing growth on different lignin sources	51
Figure 3.7 a.	Solid-phase dye decolorization assay, showing the various degrees of the decolorization by the bacteria	53
Figure 3.7 b.	Percentage of the bacteria showing the degradation of different dyes on the solid-phase assay	54
Figure 3.8 a	Aqueous-phase dye decolorization assay, showing the various degrees of the selected dye decolorization by the selected lignin-degrading bacteria	55
Figure 3.8 b	Pie chart representing percentage of bacteria showing varied degree of dye decolorization in liquid phase assay	56
Figure 3.9	Calibration curves of the Kraft lignin	58
Figure 3.10	Liquid phase Kraft lignin degradation assay	59
Figure 3.11	Morphology of bacterial isolates grown on Actinomycetes isolation agar	63
Figure 3.12	Separation of PCR amplified products of 16s rRNA gene on agarose gel showing amplified band $\approx$ at 1500 bp for the selected bacteria	66
Figure 3.13	DNase activity and Hemolytic activity of the selected bacteria on DNase and Blood sheep agar media	68
Figure 4.1	List of aromatic monomers used for the study	77
Figure 4.2	Lignin toleranace assay: IC <sub>50</sub> values of Kraft lignin (concentration in ppm) for the selected bacteria	80
Figure 4.3	HPLC chromatogram of the few lignin degrading bacteria. Circles show the degradation after incubation period as compared to control	82
Figure 4.4	Gel permeation chromatograms of the bacteria showing molecular weight distribution of uninoculated and inoculated Kraft lignin	84

Figure 4.5	Standard graphs of the selected aromatic monomers, showing their concentration range used for standardization and their respective $\lambda_{\max}$ values	92
Figure 4.6	Qualitative analysis of the utilization of the lignin-derived monomers by the selected bacteria	94
Figure 4.7	Heat map of the quantitative analysis of the lignin-derived monomers degradation or utilization by the selected bacteria	95
Figure 4.8	Heat map for the extracellular laccase (Lac) and Lignin peroxidase (LiP) like activity recorded with 24 bacteria	99
Figure 4.9	Heat map for the extracellular Manganese peroxidase (MnP) and Dye-decolorising peroxidase (DyP) like activity recorded with 24 bacteria	100
Figure 5.1	Enzyme activity of the culture filtrate of CRDT-EB-21.6, in the presence and absence of $H_2O_2$ as against Veratryl alcohol	116
Figure 5.2	Fractions of Anion exchange chromatography showing peroxidase activity and protein content	117
Figure 5.3	SDS-PAGE showing protein bands at various steps of purification of peroxidase with silver nitrate staining	119
Figure 5.4	The graph depicting the relationship between the rate of the reaction, V, and the substrate concentration, [S]	120
Figure 5.5	Line-Weaver Burk plot depicting the slope ( $K_M/V_{\max}$ ) and the intercept ( $1/V_{\max}$ )	120
Figure 5.6	Substrate specificities of the purified enzyme	122
Figure 5.7	pH stability of the purified enzyme	124
Figure 5.8	Temperature stability of the purified enzyme	124
Figure 5.9	The percentage effect of the metal salts on the purified peroxidase enzyme	126
Figure 5.10	Effect of NaCl on the purified peroxidase enzyme	126
Figure 6.1	Bioinformatics workflow	132
Figure 6.2	Genomic DNA analysis on 0.8% agarose gel	137
Figure 6.3	Bioanalyzer profiles of library loaded in Agilent DNA HS chip	137

Figure 6.4	Gene length distribution for the sample	142
Figure 6.5	Enzyme code distribution for the sample	143
Figure 6.6	Top-hit species distribution for the sample	144
Figure 6.7	KOG functional classification of sample	146
Figure 6.8	Venn diagram for annotated proteins in different databases	147
Figure 6.9	Annotation and functional classification of <i>Streptomyces griseorubens</i> CRDT-EB-21.6 using RAST online tool	148
Figure 6.10	GO distribution (i) Biological Process, (ii) Molecular function, (iii) Cellular Component for the sample	149
Figure 6.11	Distribution of CDS assigned to different pathways for the sample	151
Figure A	Applications of lignin derived monomers	161

---

## List of tables

Table	Title	Page No.
Table 1.1	Various physico-chemical methods to degrade lignin	10-12
Table 2.1	Some industrially relevant compounds produced during bacterial lignin degradation	21-22
Table 3.1	Location of soil sample collection at different regions of Western Ghats	48
Table 3.2	Morphological and differential characterization of the selected bacteria	61-62
Table 3.3	Biochemical characterization of the selected bacteria using the Hi media biochemical test kit	64-65
Table 3.4	Identification of selected bacterial isolates based on morphological, biochemical, and molecular characterization	67
Table 4.1	Compounds as detected by GC-MS/MS and LC-MS/MS for 24 selected bacteria	87-89
Table 4.2	Monomers with their $\lambda_{\max}$ and stock concentration as obtained after spectrophotometric analysis	93
Table 5.1	Peroxidase activity of the culture filtrate of the isolates at different incubation time	115
Table 5.2	Summary of the enzyme activity and protein concentration at various stages of purification	118
Table 6.1	Detail of software used for analysis	133
Table 6.2	Characteristics of <i>Streptomyces griseorubens</i> CRDT-EB-21.6	136
Table 6.3	Reads Statistics	138
Table 6.4	Statistics of gap filled assembly	138
Table 6.5	tRNAs statistics	139
Table 6.6	Overview of tRNA identified in the sample	139
Table 6.7	Overview of rRNA identified in the sample	140
Table 6.8	Overview of ncRNA identified in the sample	140

Table 6.9	Identified SSR Statistics in the sample	141
Table 6.10	Statistics of predicted CDS	141
Table 6.11	Blast statistics against Uniprot, KOG and Pfam database.	145
Table 6.12	Overview of CD1S assigned to Pfam in the sample	145
Table 6.13	Peroxidase genes identified in the genome of the isolate <i>Streptomyces griseorubens</i> CRDT-EB-21.6	152-155
Table 6.14	Genes encoding $\beta$ -keto adipate pathway and related reactions	156
Table 6.15	Genes encoding catabolic pathways for phenols and its derivatives	157
Table 6.16	Genes encoding Gentisate pathways and related reactions	158

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### List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DMP	2,6-Dimethoxyphenol
ANOVA	Analysis of Variance
approx.	Approximately
AB	Azure B
BSA	Bovine Serum Albumin
cm	Centimetre
CR	Congo Red
Da	Dalton
°C	Degree Celsius
DEAE	Diethylaminoethyl
DyP	Dye-decolourising Peroxidase
eV	Electron Volt
GC-MS/MS	Gas Chromatography coupled with Mass Spectroscopy/ Mass Spectroscopy
GPC	Gel Permeation Chromatography
g	Gram
GU	Guaiacol
HSD	Highest Significant Difference
HPLC	High-Performance Liquid Chromatography
h	Hour
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
kD	Kilo Dalton

KL	Kraft lignin
Lac	Laccase
LiP	Lignin Peroxidase
LC-MS/MS	Liquid Chromatography coupled with Mass Spectroscopy/ Mass Spectroscopy
L	Litre
MnP	Manganese Peroxidase
m/z	Mass by charge ratio
$\lambda_{\max}$	Maximum wavelength
MHz	Megahertz
MeOH-MeCN	Methanol-acetonitrile
MB	Methylene Blue
$\mu\text{L}$	Microlitre
$\mu\text{m}$	Micrometre
$\mu$	Micron
Mg	Milligram
mL	Millilitre
Mm	Millimetre
mM	Millimolar
Min	Minutes
M	Molar
MW	Molecular weight
MWCO	Molecular weight cut off
BSTFA	N, O-bis(trimethylsilyl)trifluoroacetamide
Ng	Nanogram

Nm	Nanometre
N	Normal
OD	Optical Density
Ppm	Parts per million
%	Percentage
PBS	Phosphate Buffer Saline
Pmol	Picomolar
RBBR	Remazol Brilliant Blue R
rpm	Revolutions per minute
S	Seconds
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TB	Toluidine Blue O
TCA	Tri-Carboxylic Acid
TMCS	Trimethylchlorosilane
TE	Tris-EDTA
VA	Veratryl Alcohol
VP	Versatile Peroxidase
v/v	Volume by volume
H <sub>2</sub> O	Water
w/v	Weight by volume