A High Throughput Screening and Characterization of Laccase-Producing Bacterium *Serratia quinivorans* AORB19 that exhibits Lignin degradation traits, Dye decolorization efficiency, and Enhanced Laccase Production in Biomass

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Abstract

Laccase (EC 1.10.3.2) assumes a vital role in the degradation of lignin by utilizing oxygen as an oxidant and catalyzing bond cleavage in both phenolic and nonphenolic lignin model compounds. However, their industrial application is limited due to reduced enzymatic activity and lower tolerance to extreme conditions in most laccases isolated from microorganisms. To address these challenges, bioprospecting for strains harboring novel laccases with enhanced activity and versatile properties is paramount. Natural biodegradation processes offer promises for lignin degradation, and rapid screening methods aid in isolating microorganisms secreting extracellular lignin-degrading enzymes, including laccases. In our study, we developed a novel high-throughput screening process to isolate a promising lignin-degrading bacterium from decomposed wood samples. Whole-genome analysis and LC-UV (Liquid Chromatography-Ultraviolet Detection) analysis was employed to identify the species and assess its lignin-degrading traits. This bacterium exhibited significant extracellular laccase production and was biochemically characterized. Moreover, the potential of this bacterium in bioremediation, specifically in the decolorization of two major dyes, was probed. Additionally, the laccase secretion capabilities of the bacterial strain were assessed by utilizing low-cost industrial wastes from the Canadian agro-industries.

Bacterial strains from various genera, including *Serratia*, *Enterobacter*, *Raoultella*, and *Bacillus*, along with fungal counterparts including *Mucor*, *Trametes*, *Conifera*, and *Aspergillus* having unique characteristics were screened by developing and employing a novel high throughput screening (HTP) method using laccase production as the marker to isolate lignin-degrading strains. Notably, *Aspergillus sydowii* (AORF21), *Mucor* sp. (AORF43), *Trametes versicolor* (AORF3), and *Enterobacter sp*. (AORB55) isolates exhibited xylanase and β- glucanase activities in addition to laccase production, isolated through the HTP process.

Based on the ability to naturally produce significant amount of extracellular laccase, the culture conditions of *Serratia* AORB19 were characterized and optimized employing a one-factor-at-a-time

(OFAT) methodology. Significant cultural parameters for laccase production under submerged conditions were identified: temperature 30°C, pH 9, yeast extract (2 g/l), Li^{+}, Cu^{2+}, Ca^{2+}, and Mn^{2+} (0.5 mM), and acetone (5%). Under the selected conditions, a 6-fold increase (73.30 U/L) in laccase enzyme production was achieved when compared with the initial culturing conditions (12.18 U/L). Furthermore, laccase production of *Serratia* AORB19 was enhanced under alkaline and mesophilic growth conditions in the presence of metal ions and organic solvents.

To validate the lignin-degrading potential of the strain and determine its taxonomic classification, strain AORB19 underwent whole genome analysis and core genome phylogeny. The analysis revealed that the strain belongs to the species *Serratia quinivorans* AORB19. Furthermore, the bacterial strain exhibited the secretion of various carbohydrate-active enzymes (CAZymes) and lignin-degrading enzymes, including aromatic compound-degrading and detoxifying enzymes. A comprehensive analysis identified a total of 123 annotated CAZyme genes, including ten cellulases, four hemicellulases, five predicted carbohydrate esterase genes, and eight lignin-degrading enzyme genes. LC–UV analysis of the culture media confirmed the presence of p-hydroxybenzaldehyde and vanillin, providing further evidence of the strain's capability to degrade lignin.

Furthermore, an extracellular laccase *Serratia quinivorans* AORB19 is partially purified using anion exchange chromatography and characterized. The partially purified protein showed two distinct bands on SDS PAGE at 75 kDa and 10 kDa, respectively. The partially purified laccase showed an optimum pH 3 with ABTS, pH 4.5 with 2,6 DMP substrate and a temperature of 45°C -55°C. The V_{max} and K_m of partially purified laccase towards ABTS and 2,6 DMP were determined as 0.7082 μM s^{-1} and 1.161 μM s^{-1}; 610 mM and 17 mM, respectively. Laccase activity was enhanced in the presence of Cu^{2+} and Ca^{2+} and retained in the presence of Mg^{2+} and Mn^{2+}. Fe^{2+} highly inhibited laccase activity at 5mM. DTT, sodium azide, methanol, ethanol, and isopropyl alcohol also inhibited laccase activity, so did the conventional inhibitors like L-cysteine and EDTA that mildly inhibited laccase activity.

To test the efficacy of the strain in bioremediation, the isolated strain *Serratia quinivorans* AORB19 was subjected to dye-decolorization of Remazol Brilliant Blue R(RBBR) and Malachite Green (MG) dyes. *Serratia quinivorans* AORB19 biodegraded 250 mg/l MG and RBBR at 94.31% and 76.35%, respectively.

The efficacy of *Serratia quinivorans* AORB19 to secrete laccase was further assessed by applying them to various low-cost agro-industrial residues, including pea hull, flax seed meal, canola meal, okara and barley malt sprouts sourced from Canadian agro-industries. Among the tested biomasses, flax seed meal exhibited maximum laccase activity of 257.71 U/L, which was three-fold higher than the laccase activity observed when alkali lignin (Sigma Aldrich) was utilized as the carbon source (83.65 U/L). This was followed by pea hull at 230.11 U/L, canola meal at 209.56 U/L, okara at 187.67 U/L, and barley malt sprouts at 169.27 U/L. The results confirmed the ability of the strain *Serratia quinivorans* AORB19 to grow and produce laccase in the presence of low-cost agro-industrial residues.

In its entirety, this study introduces a novel high-throughput screening method in successfully isolating lignin-degrading microbes and unveils novel findings highlighting the lignin-degrading characteristics of the bacterial strain *Serratia quinivorans AORB19*, including its remarkable ability to produce extracellular laccase. Additionally, this study presents the first report on the dye-decolorization capabilities of *Serratia quinivorans* AORB19. Moreover, the study showcases the enhanced laccase production of this strain when utilizing low-cost agricultural residues, demonstrating its potential application in Canadian agro-based industries.

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Introduction

Lignocellulosic biomass, encompassing both forest and nonwoody sources, represents an abundant renewable resource that holds great potential for the transition from traditional petrochemistry to a sustainable biobased economy. Amidst this transformative shift, one of the significant challenges in the efficient utilization of lignocellulosic biomass lies in the breakdown of lignin, an inherently recalcitrant polymer that populates the gaps in plant cell walls to provide structural protection and mechanical strength (Ragauskas et al., 2014). Lignin encapsulates the plant cell walls and act as a barrier to degradation. Proficient lignin degradation requires multiple enzymes working in cascade reactions. However, the existing repertoire of enzymes reported thus far has demonstrated only limited efficacy in the degradation or modification of monomeric or polymeric lignin. So far, enzymes have been reported to degrade/modify monomeric or polymeric lignin with only low efficiency. The identification of novel enzymes with novel functionalities has been hindered by both technical reasons, stemming from lignin's recalcitrance, and economic reasons, as lignin valorization has traditionally been deemed less profitable compared to cellulose and hemicellulose, the other components of plant lignocellulose that are more amenable to conversion to usable materials.

Although there has been some progress over the last few decades, the establishment of a biocatalytic process for lignin depolymerization has still not fully reached its potential (Tonin et al., 2017). However, as the demand for sustainable alternatives to fossil fuels and petrochemicals increases, there is a growing recognition of the value and potential profitability of lignin-based products with new technologies based on integrated biorefinery processes (Patel & Shah, 2021). At this time point, complete lignin depolymerization to monomers is a fascinating research frontier that could lead to the production of valuable aromatic or aliphatic chemicals that are currently produced from fossil fuel resources (Gall

et al., 2017). One of the key elements to reach this goal is the availability of low-cost novel enzymes that can selectively modify/degrade natural lignin.

Lignin degradation in nature is carried out by ligninolytic microorganisms including bacteria and fungi, that utilize their enzymatic machinery to break down lignin into smaller assimilable compounds. These lignolytic enzymes, particularly laccases, have attracted significant interest due to their ability to oxidize lignin including a wide range of recalcitrant pollutants and xenobiotics (Upadhyay et al., 2016). Screening natural microorganisms is a promising approach for quick discovery of novel enzymes and an increasing number of enzymes with unique functionalities have been identified from natural microorganisms (Mei et al., 2020). The vast majority of microbial diversity remains still unexplored and by leveraging desired microbial communities by screening methods, efficient microorganisms and enzymes could be possibly identified. Additionally, it has the potential for addressing concerns with high enzyme cost, yield, and product specificity, thereby contributing to the development of improved and profitable processes in the biorefinery industry.

As the next-generation identification and characterization of novel enzymes for lignin degradation continue to grow in complexity, traditional screening approaches may be suboptimal or completely inadequate, creating a need for novel and efficient screening approaches. To overcome these challenges, lignin bioprocessing researchers have been experimenting with innovative screening approaches to capture and polish steps of traditional microbial isolation. High throughput screening processes have emerged as one of the most rapidly growing methods of isolation of known and previously unknown natural microorganisms or microbial consortiums; their metabolites and enzymes, revolutionizing ongoing lignin reduction and conversion initiatives. Furthermore, optimized screening and culture conditions are critical to ensure the quality and efficacy of potent microbial candidates increasing their overall yield and purity, as well as their widescale use of them in lignin degradation and conversion.

As mentioned before, prioritization of identifying and characterizing cost-effective, novel enzymes capable of selectively modifying or degrading lignin is essential for the successful pursuit of lignocellulose degradation and robust biomass conversion for sustainable biorefineries. Nature has incredible ways to degrade lignin by unleashing diverse lignin-degrading enzymes. Microorganisms produce enzymes either extracellularly or intracellularly and their activity falls in a wide range of temperature and pH, and their characterization promises in terms of high specificity, and exceptional stability allowing repeated preparative application. These potent enzymes not only increase the economic viability of biorefinery in the pretreatment of the lignocellulose biomasses but can also convert lignin into high-value compounds. Additionally, these enzymes have a wide range of applications in the food industry, pharmaceutical industry, and bioremediation due to their unique capability of degrading polymers. Hence it is crucial to characterize these enzymes for their application to the specific industry it appeals to.

The process of identifying and characterizing cost-effective, novel enzymes capable of selectively modifying or degrading lignin is essential for the successful pursuit of lignocellulose degradation and efficient biomass conversion in sustainable biorefineries. Nature offers a diverse array of lignin-degrading enzymes with remarkable abilities. These enzymes are produced by microorganisms, either extracellularly or intracellularly, and exhibit a wide range of temperature and pH optima. Thus, the characterization of such enzymes holds great promise in terms of high specificity and exceptional stability, enabling their repeated preparative application. These potent characterized enzymes not only enhance the economic viability of biorefineries by facilitating the pretreatment of lignocellulosic biomass, but they also have the ability to convert lignin into valuable compounds. Moreover, their unique capability to degrade polymers makes them highly versatile for applications in various industries such as food, pharmaceuticals, and bioremediation. Therefore, it is crucial to thoroughly characterize these enzymes to tailor their application to specific industries it appeals.

Additionally, there are a plethora of bio-analytical methods described for the identification of lignin-derived compounds and metabolites in order to gain insights into the target microorganism and its degradative potential. However, the use of a specific bio-analytical method in isolation to detect lignin-derived compounds and their metabolites supply inconsistent or inconclusive depictions of the degradation potential of a target microorganism and hence the understanding of their functionality remains elusive. Correspondingly, Whole Genome Sequencing (WGS) in tandem with Liquid Chromatography with UV detection (LC-UV) analyses presents a conjugation strategy and findings stemming from them, for instance, help to provide additional evidence supporting the involvement of microbial strains in the process of lignin degradation in nature and upending our understanding of lignin degradation mechanisms of microorganisms, a pivotal process in the global carbon cycle, that provides a framework for further development in lignin research.

Currently, investigations are persistently focusing on the isolation and characterization of promising microbes and consortia capable of degrading lignin and phenolic substances in wastewater. Consequently, it becomes imperative to direct attention towards the fundamental processes and metabolic pathways involved in the decolorization procedures. This involves the identification of relevant genes and metabolites, with the aim of developing more efficient strains capable of effectively eliminating a broad spectrum of synthetic dyes from dye-contaminated effluents. The treatment of waste materials before discharge has often emphasized the utilization of biological dye decolorization as a highly suitable method (Stolz, 2001). The ever-increasing demand for efficient laccases and enzyme cocktails for bio-delignification, industrial oxidative processes and environmental bioremediation requires the production of large quantities of enzymes at low cost; a concern which may be addressed through the discovery of novel enzymes from microbiome.

Finally, the utilization of enzymes or microorganisms for the biological pretreatment of lignocellulosic materials, aimed at producing biofuels, represents a highly promising technology owing

to its eco-friendly nature and cost-effectiveness (Wei, 2016). However, fungal enzymes have predominantly been employed for pretreating lignocellulosic biomass, while enzymes derived from bacterial sources are less commonly explored for their potential in biological degradation. Thus, characterizing potent enzymes from bacterial sources from nature with broad range of substrate specificity is paramount for efficient lignocellulosic biomass exploitation.

This thesis, thus explore and demonstrate 'invitro' these new frontiers in lignin research and investigates four distinct objectives in the ensuing sections of the paper. This study hypothesized that:

(1) Innovative screening of decomposed wood samples has the potential to identify ligninolytic microorganisms.

(2) The optimization of culture parameters may demonstrate ideal conditions for significant extracellular laccase production in the strain AORB19.

(3) The extracellular laccase enzyme derived from the strain AORB19 may be purified and functionally characterized.

(4) The whole genome analyses of strain AORB19 may reveal the existence of genes and metabolic pathways linked to laccase synthesis and lignin degradation.

(5) The strain AORB19 may significantly reduce the color intensity of azo and anthraquinone dyes during the dye decolorization process, indicating its potential application in bioremediation.

(6) The strain AORB19 may be utilized in industrial byproducts from local Canadian industries as a growth medium for increased laccase production providing an avenue for economically viable and sustainable bioprocessing.

Together, the overarching goal of the study is to develop an innovative screening method and to identify and characterize novel lignin degrading microorganisms and enzymes with desired characteristics. The specific objectives include:

Objective 1: Conception, design and implementation of a high throughput screening process that helps quick isolation of novel lignin-degrading microbes from large numbers of natural biomasses.

Objective 2: Identification and biochemical characterization of the strain AORB19 and its optimization of culture conditions for maximum laccase production.

Objective 3: Purification and functional characterization of the strain AORB19, probing its capabilities to decolorize synthetics dyes, and enzyme enhancement using industrial byproducts from local Canadian industries.

Objective 4: To derive fundamental insights on the lignin degradative capabilities of strain AORB19 using genomic analyses.

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CHAPTER 1

Literature Review

1. Lignocellulosic Biomass – Opportunities for a bio-based economy

The world is transitioning towards a sustainable bio-based economy as a response to the increasing concern over the negative impacts of the traditional petrochemistry on the environment. This is crucial as production of petrochemicals is energy-intensive causing global temperature rise leading to greenhouse gas emissions, a significant contributor to climate change. This shift has also been driven by economic factors such as high oil prices, increasing demand for sustainable and eco-friendly products, and the growth of new bio-based industries (Popp et al., 2021; Vanholme et al., 2013). Lignocellulosic or plant biomass is a promising feedstock and is getting increased popularity for implementing innovative strategies to establish circular bioeconomy and is projected to play a key role in meeting global climate targets. Using lignocellulosic materials from forestry and agricultural and sectors could help reduce the over-dependence on petrochemical resources and act as sustainable alternatives as feedstock in the production of biofuels and biomaterials while providing a sustainable waste management alternative (Stegmann et al., 2020; Okolie et al., 2020). Additionally, it offers several advantages, including the potential to create new economic opportunities in the form of new products and markets, while reducing environmental impact.

To use biomass in an innovative and cost-efficient fashion, the idea of "biorefinery" has been accepted where integrated sustainable processes and technologies are applied for the complete utilization of lignocellulosic biomasses including lignin valorization (Patel & Shah, 2021). However, compared to cellulose and hemicellulose, the other two major components, valorization of lignin has received relatively little attention, due to its insoluble nature, structural complexity, and limited hydrolysable linkages (Feng et al., 2022; Ayeronfe et al., 2018). Currently, lignin generated is mostly burned or used

in low-value material applications, which accounts for about 50-70 million tons from the pulping and biorefinery industries alone (Ruwoldt et al., 2023). Complete biomass utilization incorporating effective lignin valorization is essential for an economically viable and environmentally sustainable biomass-based industry.

1.1 Lignin structure

Lignocellulose constitutes of lignin, hemicellulose, and cellulose with lignin accounts for about 10% – 40% of total weight (Sun & Cheng, 2002). Lignin has a highly heterogeneous structure and is primarily a polymer of three different monolignols: sinapyl alcohol, *p*-coumaryl alcohol and coniferyl alcohol (Kirk & Farrell, 1987, see Fig.1). These monomeric units of lignin, is integrated into the lignin molecule to form the building blocks which are known as syringyl unit (S), *p*- hydroxyphenyl unit (H) and guaiacyl unit (G) (Vanholme et al., 2010). The proportion of G:S:H units varies widely within plant kingdom. Softwoods are predominantly G type lignin, comprising of mainly guaiacyl (G) units. Hardwoods largely have GS-type lignins, composed of combined guaiacyl and syringyl units. On the other hand, grass lignins belong to G type lignins and found to contain a higher ratio of p-hydroxyphenyl (H) units (Faix, 1991).

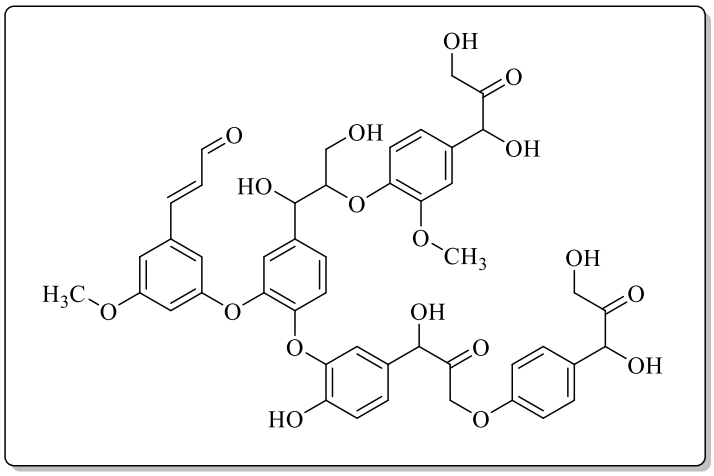

Figure 1. (a) Chemical structure of lignin (b) Monolignols: The Three Primary Monomers of Lignin Biopolymer - Sinapyl Alcohol, Coniferyl Alcohol, and p-Coumaryl Alcohol

Lignin forms a matrix that is tightly associated with the cellulose filaments and covalently attached to hemicelluloses, serving the biological functions of providing mechanical strength and protecting cellulose and hemicellulose from enzymatic hydrolysis. (Zakzeski et al., 2010; Kirk & Farrell, 1987). The predominant linkages observed during lignification are β-O-4 bonds, accounting for 40-50% of the

total linkages between monolignol building blocks in both hardwood and softwood lignin. Additional linkages include α-O-4, β-5, β-β, β-1, and 5-5 (Freudenberg, 1965; See Fig.2).

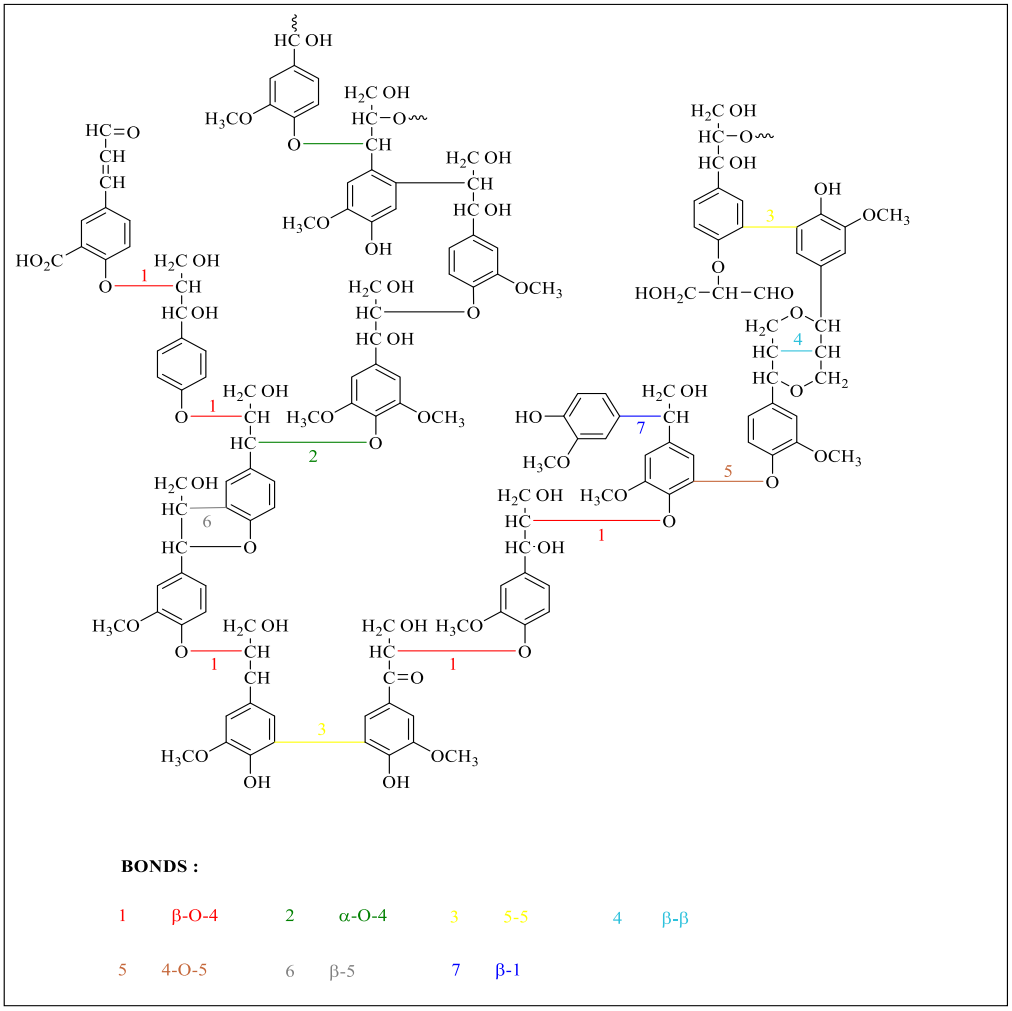

Figure 2. Lignin model structure with colored annotations for the major inter unit linkages.

1.2 Lignin as sustainable energy source

Lignin is an abundant and the second largest renewable source of carbon on earth after cellulose (Kirk & Farrell, 1987). The United States Department of Energy has its goal to replace 30% of the transportation fuel supply with biofuels by the year 2030 (Riley et al., 2007). One of the upsides of lignin is its availability as a raw material, either as an industrial waste product or from the plants by natural lignification (Ning et al., 2021). Chemical pulping operations generate over 50 million tons of technical lignin as by-products, a substantial portion of which are unfortunately burned as fuel (Demuner et al.,

2019) to be unrecognizable. Utilization of lignin and its conversion to value-added products enhances the profitability, competitiveness, and carbon efficiency of the entire biorefinery process (Demuner et al., 2019; Yu et al., 2023).

2. Biological lignin degradation

Biodegradation of lignin occurs in nature despite it is a highly recalcitrant molecule as otherwise it would be trapped in the biomass and accumulate in nature (de Gonzalo et al., 2016). Microorganisms and enzymes are the key players which bring about lignin degradation in nature. Microorganisms utilize their metabolic machinery by employing various enzymes such as lignin modifying and lignin degrading auxiliary enzymes, to degrade lignin (Tan et al., 2021). The microbial flora in specific environments have evolved specialized pathways for lignin decomposition, showcasing significant potential for the bioconversion of lignin into value added chemicals.

2.1 Overview of biological lignin conversion in microorganisms

Biological lignin valorization can be categorized in three stages; a) Lignin depolymerization b) aromatic cleavage and c) compound biosynthesis. These three stages take place in two different environments viz; 1) open environment (Extra cellular) and 2) inside the microbes (Intracellular) (Liu et al., 2018). The three stages can be further divided in two categories based on nature of compounds and referred to as "*peripheral or* Upper pathway" (where lignin depolymerization leads to the formation of central intermediate compounds*)* and "*Central* or Lower pathway" (where aromatic ring opening occurs for biosynthesis of precursor molecules and compounds), see Fig. 3. The depolymerization of lignin is the initial step in lignin degradation and results in the formation of a mixture of smaller aromatic compounds by several extracellular enzymes such as laccases, Lignin peroxidases (LiP), Manganese peroxidases (MnP), aryl alcohol oxidases (AAO), versatile peroxidases (VP) and many other unknown enzymes.

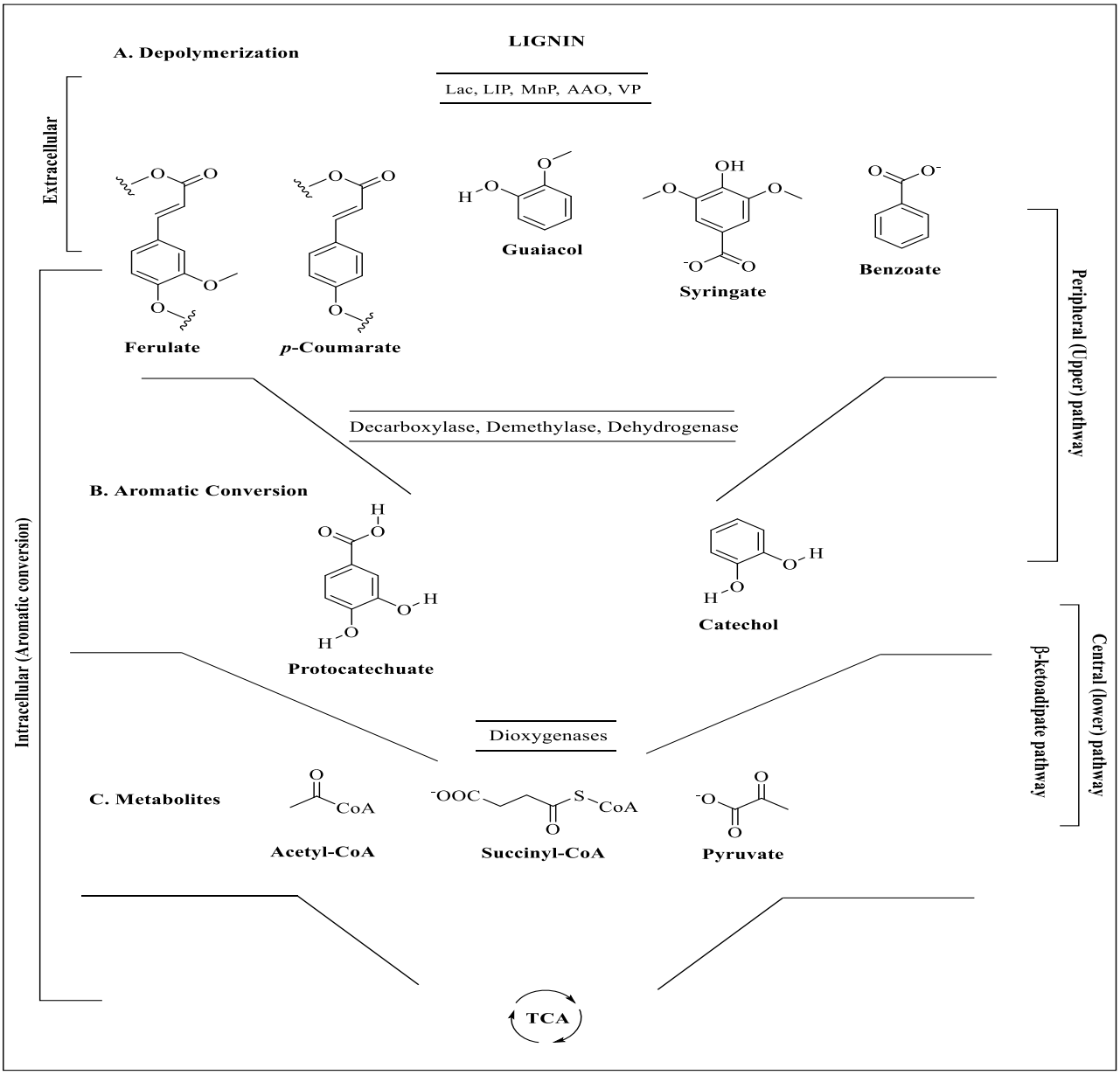

Figure 3. **A snapshot of biological degradation of lignin by microbes.** Extracellular enzymes such as laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) can cleave major lignin linkages. This results in lignin depolymerization and the production of various lignin-derived aromatics, including Ferulate, p-coumarate, guaiacol, syringate, benzoate etc. The depolymerized products are then metabolized through the upper pathway, leading to the formation of key intermediates such as catechol and protocatechuate; These intermediates undergo further transformation via central pathways, ultimately generating important metabolites such as acetyl-CoA, succinyl-CoA, and pyruvate, which are then directed into the tricarboxylic acid (TCA) cycle.

Further, these oligomers are converted to aromatic intermediates subsequently to central intermediate compounds (Protocatechuate and catechol) by different pathways and enzymes. These both stages collectively referred as upper pathway (Linger et al., 2014). Protocatechuate & catechol undergo aromatic ring-cleavage to central metabolites such as acetyl-CoA succinyl CoA and pyruvate to TCA cycle, and this stage is referred as central or lower pathway (Li et al., 2022). The central pathways responsible for lignin-derived aromatics involve metabolic processes that utilize the central intermediates obtained from the upper pathways. These intermediates are converted into central metabolites, which subsequently direct the carbon into the tricarboxylic acid (TCA) cycle, providing energy for microorganisms. Various central pathways, such as β-ketoadipate, phenylacetate, homogentisate, and 2-hydroxypentadienoate hydroxyquinol pathways, have been identified in different microbial species. Furthermore, Acetyl Co-A and Succinyl Co-A acts as a precursor molecule for several biosynthetic pathways (Lipid biosynthesis, Amino-acid biosynthesis, MEP pathway, etc.) and central energy generation pathway through Krebs cycle. Acetyl Co-A enters to pathways for biosynthesis of lipid biosynthesis, amino acid biosynthesis, fatty acid biosynthesis, and polyhydroxy acids (PHA). It is a precursor molecule for MEP pathway where terpene biosynthesis occurs (Vardon et al., 2015; Lin et al., 2016). With the help of synthetic biology monoterpene biosynthesis could be utilized for the production of advanced biofuels.

2.2 Microbiology of lignin degradation

Microorganisms, including fungi and bacteria, play a key role in the natural depolymerization of lignin through a relay of metabolic processes and enzymatic activities (Liu et al., 2019; Yang et al., 2014). Saprophytic fungi including white rot, brown rot and soft rot fungi are considered the primary agents of lignin degradation in most ecosystems. These fungi produce a vast and efficient repertoire of extracellular enzymes such as peroxidases and laccases (Su et al., 2018; Janusz et al., 2017). These are oxidoreductase group of enzymes and generate reactive intermediates leading to oxidation which

eventually result in lignin degradation. Even though all these fungal types are reported to decompose lignin only white rot fungi degrade it fully to CO_2 and H_2O. They are adept at lignin degradation possessing a unique set of enzymes including versatile peroxidases and DyP-type peroxidases and are known for their ability to mineralize lignin (Blanchette, 1995; Kirk & Farrell, 1987). White-rot fungi, a specialized group of basidiomycetes, are particularly adept at lignin degradation. They possess a unique set of enzymes that can efficiently depolymerize lignin, including laccases and peroxidases. White-rot fungi are known for their ability to mineralize lignin, converting it into carbon dioxide and water. Some well-studied white-rot fungi include *Phanerochaete chrysosporium, Pleurotus ostreatus, Fomes fomentarius, Lentinula edodes, Xylaria, Libertella, Hypoxylon, Trametes versicolor* and *Phanerochaete chrysosporium.* (Kirk & Farrell, 1987).

While, fungi are the primary decomposers of lignin, bacteria also contribute significantly to lignin degradation, by employing diverse enzymatic strategies and metabolic pathways. The dominant bacterial phyla associated with lignin degradation includes Actinobacteria, Proteobacteria, and Firmicutes (Bugg et al., 2011). Strains including *Rhodococcus, Streptomyces, Sphingomonas* and *Pseudomonas* are well-known lignin-degrading bacteria that produce extracellular lignin-modifying enzymes, like laccases and peroxidases, enhancing the breakdown of lignin (Huang et al., 2013). Additionally, some bacteria like *Thermobifida busca* possess additional enzymes, such as DyP-type peroxidase capable of breaking down lignin (Rahmanpour et al., 2016).

3. Screening of ligninolytic microorganisms

Novel and innovative screening approaches are uncovering the existence of a wide range of previously unknown enzyme activities from active microbial communities distributed in nature. Biomass conversion utilizes microorganisms and their enzymes, such as cellulases, xylanases, and ligninases, to transform lignocellulose into platform chemicals that can then be converted to various products, including biofuels, chemicals, and materials. Microorganisms that can effectively break down biomass

in high yields are vital for achieving sustainable and economically viable production of high value products from lignocellulosic feedstock (Yang et al., 2014; Vu et al., 2020).

Lignin is a natural polymer which is abundant and has immense potential to be valorized into high-value bioproducts after degradation into short oligomers or monomeric subunits. Biological and enzymatic lignin degradations hold great potential for the development of green biotechnology, a very promising method that offers sustainable alternatives to conventional fossil fuel-based production and is extremely selective and environmentally benign (Jäger & Büchs, 2012; Adewale et al., 2021; Ali et al., 2017). However, the structural complexity of lignin and expensive enzymes are the two main obstacles that reduce the process' efficiency and make it unprofitable (Tarasov et al., 2018; Ali et al., 2022).

Microbes that can break down lignin can produce a range of enzyme combinations comprising several types of lignin-degrading enzymes, such as peroxidases and laccases (Dashtban et al., 2009; Chio et al., 2019). Proficient lignin degradation can be achieved by the concerted action of multiple enzymes that work in cascade reactions which enhance the saccharification of polysaccharides of lignocellulosic biomass. Screening of microorganisms with significant lignin degradation ability allows the opportunity to discover and characterize novel enzymes that potentially allow low-cost biological processes. However, discovering lignin-degrading microorganisms with high performance largely depends on the screening strategy. A variety of microbial screening approaches and strategies were employed and adapted to identify novel enzymes for specific functionalities and targets (Chandra et al., 2007; Choolaei et al., 2021; Maruthamuthu et al., 2016; Chandra et al., 2008). Culture-dependent screening strategies mainly utilize a culture enrichment method which involves growing microorganisms in a cultivating medium with lignin as the carbon source, and the screening is solely activity based without relying on the prior knowledge of the enzyme. After the culture enrichment process, the most common practice is to perform a qualitative screening using an agar plate containing dyes resembling lignin fragments, followed by a quantitative screening of selected microbes based on activity (Xu et al., 2018; Raj et al.,

2007; Zhou et al., 2017). Zhang et al. (2021) obtained four lignin degrading microbial consortia from 40 antique wooden samples in a tedious way. Each sample was grown up in a separate flask, and selections were based on subjective, visual color change. Even though the main fungal and bacterial compositions of the four consortia were identified by genomic sequencing, no specific strain was isolated. Similarly, from twelve samples of decayed tree trunks, stumps, and surrounding soil samples. Fang et al. (2018) isolated a microbial consortium that demonstrated the ability to selectively break down lignin from tree trimmings. In which case, a chromogenic substrate was incorporated in the culture media that produced a color change when laccase is produced. The reported process is arduous and takes months of culture with many generations of subcultures.

Xiong et al. (2020) identified four bacterial strains from the soil, silage, and straw samples by first flask extraction of microorganism mixtures, followed by growth on ligninolytic selection agar media. Colonies that demonstrated aniline blue decolorization were chosen individually, and the strains were identified through 16S rDNA sequencing. There is no selection and screening of the starting biomass before single colony selection, and the total numbers of biomass samples tested were not reported. Recently, Elframawy et al (2022) screened 23 samples (14 soil samples and 9 old black liquor samples) to identify lignin degrading Actinobacteria (Streptomyces strains). Soil samples were first pretreated for 10 days, followed by the soil dilution plate technique (AGA plates with nystatin to reduce fungal growth), while black liquor samples were directly spread onto agar plates after dilution. Colonies were selected after 21 days of incubation, transferred to new AGA plates, and further cultured for 14 days to purify the isolates.

The agar plate screening method is laborious, time-consuming, has low throughput, and lacks precision as they may not always accurately detect the target enzyme. Submerged screening methods, on the other hand, offer better control over the culture conditions, which can lead to higher enzyme production. They also allow the possibility for high throughput (HTP) screening of a vast number of

samples simultaneously. Additionally, through the use of specific assay conditions or detection methods, the target enzymes can be identified with better specificity and sensitivity compared to agar plate screening methods. Moreover, screening in liquid media also mimics industrial submerged culture conditions, thus allowing the identification of potential microbes and enzymes for industrial applications (Arnthong et al., 2020).

Metagenomic screening involves the analyses of collective DNA from environmental microbial samples without prior culturing and examines microbiome at two levels. The first level encompasses the structure and interactions of microorganisms present in a sample. The next level is their function, which focuses on the genetic diversity and biochemical pathways within the bacterial community (Schmeisser et al., 2007; Batista-García et al., 2016). By employing Illumina seq 2500, the metagenomic examination of humus samples uncovered a significant presence of lignocellulose-degrading genes and domains, demonstrating both high diversity and abundance. The identified taxa involved in lignocellulose degradation included Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Acidobacteria, with Proteobacteria being the most prevalent. Moreover, the investigation also identified several previously unknown lignocellulose-degrading genes, expanding our knowledge in this field (Le et al., 2022). The study by (Wilhelm et al., 2018) reveals the diversity of bacterial and fungal populations that degrade lignocellulose in coniferous forest soils across North America, using stable isotope probing coupled with metagenomics. The paper identifies Caulobacteraceae as a major bacterial group that can degrade all three lignocellulosic polymers, and uncovers novel lignin-degrading taxa from mineral soils. Jose et al.2017 utilized high-throughput sequencing and bioinformatic analysis to infer major bacterial contributors of CAZymes in the cattle rumen including *Prevotella, Bacteroides, Clostridium, Fibrobacter, and Ruminococcus*. A new strain of *Burkholderia cepacia* UJ_SKK_1.2 was discovered based on metagenome assembled sequence from the gut of a termite species that can digest lignocellulose (Lenka et al., 2023). Furthermore, 454 pyrosequencing of 16S rRNA gene amplicons revealed a wide

range of gut microbiomes within termite guts, showcasing their functional significance in lignocellulose degradation (Su et al., 2016).

4. Ligninolytic enzymes

Nature has incredible ways to destruct or decompose lignin by deploying an ensemble of ligninolytic enzymes systems. It is often referred as enzymatic combustion and the enzymes involved in the process are nonspecific with high oxidation potential (Kirk & Farrell, 1987). Recently, there is a surge of interest on lignin valorization among the researchers worldwide as it is crucial for the efficient biomass utilization and achieving economically viable biorefinery. However, the heterogeneity of lignin and its recalcitrance is a major hurdle for efficient lignin valorization initiatives (Liu et al., 2019). In biorefineries, enzymes are used for extensive pre-treatment of diverse lignocellulosic biomass. This involves selective oxidation of lignin molecules by converting lignin to low molecular weight compounds, resulting in improved efficiency of downstream cellulose and hemicellulose hydrolyses efficiency and lower production cost. Moreover, the unique catalytic properties of ligninolytic enzymes enable the extraction of high-value chemicals such as aromatics and phenolic compounds. These valuable substances can be utilized in the production of fine chemicals, thereby contributing to the establishment of a circular bioeconomy. Therefore, the discovery of robust and new ligninolytic enzymes and related metabolic pathways may accelerate and open up several new routes to improve lignin conversion to aromatic platform chemicals leading to better economic prospects and sustainable biorefineries.

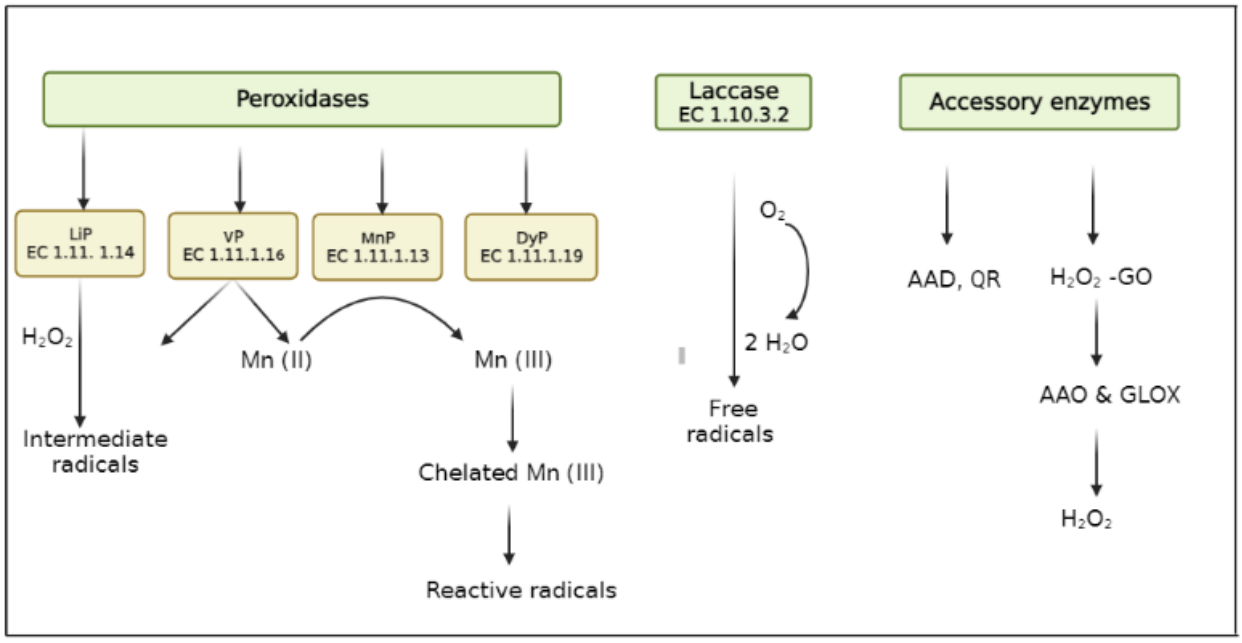

Figure 4. Enzymes involved in lignin depolymerization (adapted from Dashtban et al., 2010)

Lignin degrading enzymes (LDE) can be classified into two broad groups or domains: (a) the core depolymerizing enzymes involved in breaking the linkages in lignin, i.e., Lignin Modifying enzymes (LME), and (b) lignin-degrading auxiliary (LDA) enzymes that usually not participate directly in lignin degradation but can carry out accessory functions to complete lignin degradation (Silva Coelho-Moreira et al., 2013), See Fig. 4. Lignin modifying enzymes include the first line enzymes for lignin depolymerization including peroxidases, namely, lignin peroxidases (LiP), manganese peroxidases (MnP) and versatile peroxidases (VP), and Laccases (Zámocký et al., 2015; Moilanen et al., 2011; Hatakka, 1994). Based on occurrence, catalytic properties and amino acid sequences of peroxides can be categorized into class 1 (prokaryotes or in the eukaryote organelles), class 2 (fungal extracellular peroxidases) and class 3 (peroxidases in plants) (Janusz et al., 2017).

On the contrary, LDA enzymes are the group of enzymes that work simultaneously with lignin modifying enzymes, to facilitate lignin degradation. The members of auxiliary enzymes include, aryl alcohol oxidases (AAO; EC 1.1.3.7), cellobiose dehydrogenase (CDH; EC 1.1.99.18), pyranose 2-

oxidase (POX; EC 1.1.3.10), glucose oxidase (EC 1.1.3.4), and glyoxal oxidase (GLOX; EC 1.2.3.5). These enzymes promote lignin degradation by allowing electron transfer which might include oxidative release of H_2O_2 (Levasseur et al., 2008; Tian et al., 2014).

4.1 Peroxidases

Peroxidases are diverse group of enzymes distributed widely among different life forms including animals, plants and microorganisms and is involved in various biological processes. Peroxidases are heme-containing enzymes with a conserved active site which can catalyze oxidation reactions employing hydrogen peroxide as a co-substrate (Bugg et al., 2012; Vlasova, 2018). Peroxidases aids cell wall lignification, metabolism of reactive oxygen species and defense responses in plants (Kawano, 2003). In animals, peroxidases are involved in wound healing, immunological responses and detoxification functions (Kurahashi & Fujii, 2015). Additionally, microbial peroxidases play a crucial role in the breakdown of aromatic compounds and the detoxification of environmental contaminants (Bansal & Kanwar, 2013).

4.1.1 Lignin peroxidases (LiP; E.C. 1.11.1.14)

Lignin peroxidases (LiPs) were first discovered from *Phanerochaete chrysosporium*, a white-rot fungus (Tien & Kirk, 1983). The enzyme can catalyze oxidative degradation of lignin employing hydrogen peroxide as the co substrate. It belongs to peroxidase catalase superfamily and are heme-containing enzymes with a protoporphyrin IX prosthetic group (Zámocký & Obinger, 2010). LiP is a globular protein and its folding motif comprises of three antiparallel β sheets, eight minor helices and eight major α-helices (Choinowski et al., 1999). Additionally, the enzyme contains glycosylation sites, disulfide bridges and Ca^{2+} binding sites which helps to help stabilizing the 3-D structure of this enzyme. Moreover, the isoelectric point range of lignin peroxidase falls between 3.3 and 4.7, while its optimal pH is extremely low at approximately 3.0 when veratryl alcohol is used as the substrate with molecular weight

varying from 38 kDa to 43 kDa (Glumoff et al., 1990; Kirk et al., 1986; Furukawa et al., 2014; Tien & Kirk, 1988).

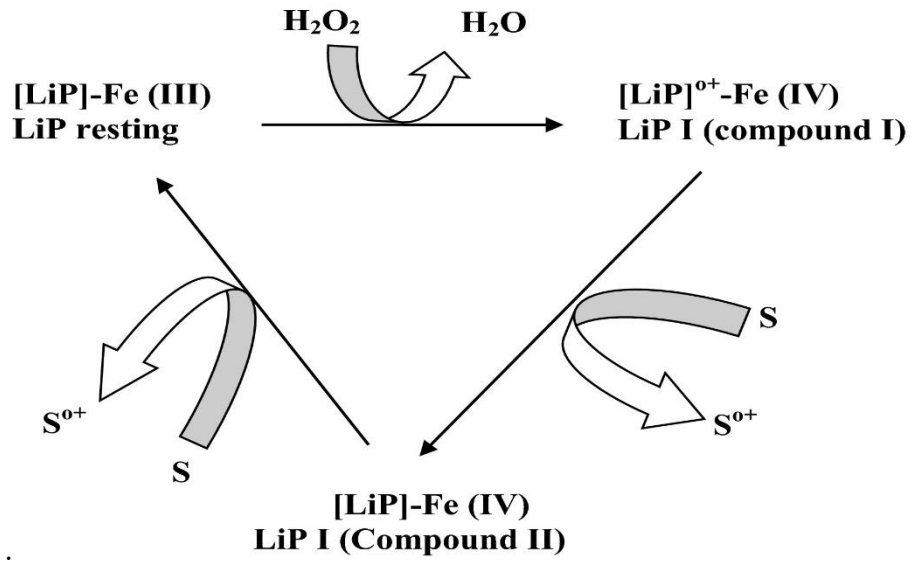

Figure 5. Catalytic cycle of Lignin peroxidase (adopted from Abdel-Hamid et al., 2013)

There are three major steps present in the catalytic cycle of lignin peroxidase (Fig. 5). Initial step involves the binding of the enzyme to hydrogen peroxide leading to its oxidation of the heme group in the enzyme leading to the formation of oxo ferryl intermediate (compound I). Ferryl intermediate undergoes reduction by the substrates which in turn donates an electron which subsequently results in the formation of a cation radical intermediate referred to as compound II. The reduced substrate then contributes an electron to compound II in the final step, restoring LiP to its resting ferric oxidation state (Abdel-Hamid et al., 2013).

LiP production can be induced by growing microbes in minimal media using technical substrates and natural biomasses (Falade et al., 2017; Lai et al., 2017). Fungal candidates reported to produce LiP so far includes *P. chrysosporium* (Farrell et al., 1989), *Phlebia radiata* (Moilanen et al., 1996), *Phanerochaete sordida* (Sugiura et al., 2009), *Tramates versicolor* (Johansson et al., 1993), *Bjerkandera*

sp., and *Phlebia tremellosa,* (Aarti et al., 2015). Lignin peroxidase produced by bacterial counterparts include proteobacterial strains, actinomycetes, *Pseudomonas* spp., *Escherichia coli*, *Enterobacter* spp, *Acinetobacter calcoaceticus* and *Streptomyces viridosporus* (Dube et al., 2023; Bugg et al., 2011; Paliwal et al., 2012; Dashtban et al., 2010).

4.1.2 Manganese peroxidase (MnP; E.C.1.11.1.13)

One of the most significant extracellular ligninolytic enzymes secreted by microbes include MnP (EC 1.11.1.13) and was first discovered in *P. chrysosporium* approximately three decades ago (Glenn & Gold, 1985; Janusz et al., 2017). MnP shares a similar molecular structure to LiP, consisting of two α-helices domains with the heme group sandwiched between them. The active enzyme structure of MnP is maintained by five disulfide bridges and two Ca^{2+} ions (Sutherland et al., 1997). Microorganisms which have been reported to produce MnP include *P. chrysosporium, P. sordida, P. radiata, P. rivulosu, C. subvermispora, Dichomeris squalens, Fusarium sp., C. unicolor BBP6, A. praecox, Bjerkandera adusta, F. fomentarius, Cerrena and Trametes genera, Irpex lacteus and Cupriavidus basilensis B-8, A. baumanii* and *Meyerozyma caribbica* (Chowdhary et al., 2018).

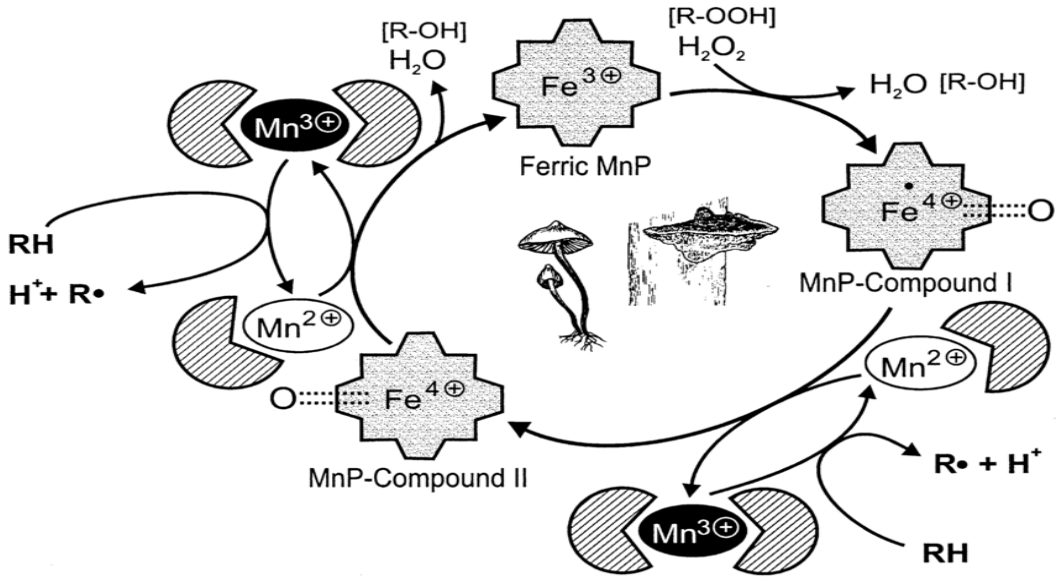

Figure 6. Catalytic cycle of manganese peroxidases (adopted from Hofrichter, 2002).

Manganese peroxidases (MnPs) exhibit a molecular weight that falls within the range of 38 to 62.5 kDa, while their isoelectric point (pI) spans from 2.9 to 7.1 (Sigoillot et al., 2012). LiPs and MnPs have very similar catalytic cycles. In a typical catalytic cycle, hydrogen peroxide binds to the active site of the enzyme when the oxygen in H_2O_2 bonds to a Fe (III) ion in the heme cofactor compound I which is a Fe4-oxo-porphyrin-radical complex and water. Compound I then catalyze the oxidation of substrate compounds, generating free radicals and Compound II (Fe4-oxo-porphyrin complex). Compound II oxidizes Mn^{2+} to form Mn^{3+}, and is responsible for oxidation of compounds containing aromatic rings (see Fig.6). The final step involves reduction of Compound II by Mn (II), regenerating the native enzyme and releasing water (Hofrichter, 2002). Mn-dependent peroxidases differ from LiPs in that they employ Mn (II) as their reducing substrate, producing Mn (III), which diffuses from the enzymes into the lignocellulose structure. Additionally, in contrast to LiP, MnP lacks the ability to oxidize non-phenolic compounds, which constitute up to 90% of the lignin polymer. This indicates that LiP is involved in further transforming lignin fragments released by MnP (Wesenberg, 2003).

4.1.3 Versatile peroxidases (VP; EC 1.11.1.16)

Versatile peroxidases were first reported in *P.chrysosporium* and it exhibits a combination of molecular configuration of lignin peroxidase and manganese peroxidase and is able to react with their typical substrates (Ruiz-Dueñas & Martínez, 2009; Hofrichter et al., 2010; Garcia-Ruiz et al., 2014). In terms of structure, VPs have 11–12 helices, 4 disulphide bridges, 2 structural Ca^{2+} sites, a heme pocket, and a Mn^{2+}-binding site that resembles MnP. These VPs are released as various isoenzymes with pI values between 3.4 and 3.9 and molecular masses between 40 and 45 kDa (Pérez-Boada et al., 2005). VPs has the similar catalytic cycle as other peroxidases, using two intermediary molecules, compound I and compound II. However, due to their ability to process variety of substrates, the cycle of VPs is more complicated (Sigoillot et al., 2012).

The ferric group of VP interacts with hydrogen peroxide forming Compound I, a Fe^{4+}-oxo-porphyrin^{+} complex, which undergoes apparent spectral modifications (Ruiz-Dueñas et al., 2009; (Camarero et al., 1999). Compound II (Fe^{4+}-oxo) is produced when Compound I oxidize veratryl alcohol. Compound II has the ability to oxidize more molecules of veratryl alcohol, returning the enzyme to its resting state (Pérez-Boada et al., 2005). In terms of oxidizing compounds, VP demonstrated that it can also oxidize high redox potential azo dyes such as reactive black 5 in addition to veratryl alcohol (Garcia-Ruiz et al., 2014).

4.1.4 Dye decolorizing peroxidase (DyP; EC 1.11.1.19)

Dye-decolorizing peroxidases (DyP) represents a novel class of heme peroxidase and they derived their name due to its capability of not only degrading non-phenolic methoxylated aromatics but also high redox dyes like azo and anthraquinone dyes. Their structure is composed of two domains characterized by helices and anti-parallel sheets, with a cavity between the two domains containing a heme cofactor (Janusz et al., 2017). DyPs are divided into four categories (A, B, C, and D) based on phylogenetic study of genomic sequences. While type D DyPs are generally grouped to fungal species, type A and C DyPs are primarily bacterial enzymes (Chen & Li, 2016). Although there is mounting evidence that some of these bacterial variations are efficient lignin degraders and/or involved in oxidative stress defense systems, the physiological functions of these organisms are still unknown (Janusz et al., 2017).

4.2 Laccases (EC 1.10.3.2)

Laccase, an enzyme that has been extensively researched since the 19th century, holds the position of being one of the earliest enzymes to be identified (Rodríguez-Couto, 2018) and is found in various life forms, including fungi, plants, bacteria, lichen, and insects (Arregui et al., 2019). Its initial discovery traces back to the lacquer of the Japanese tree *Rhus vernifera*, which inspired the name "laccase" (Yoshmaxida, 1883). Subsequently, research revealed that laccase enzymes are more far reaching or widespread in nature, being present in fungi, plants, bacteria (Hattori et al., 2005), as well as

insects (Thomas et al., 1989). Following its initial discovery in *Azospirillum lipoferum*, a bacterium commonly found jointly with plant roots (Givaudan et al., 1993), bacterial laccases was later identified in *Marinomonas mediterranea*, as well as in the endospores' coat protein A (cotA) of *Bacillus* species.

Alternatively, the discovery of the fungal laccase was first reported by Bertrand, who closely observed its role in the color change of mushrooms belonging to the *Boletus* genus upon exposure to air (Magnin, 1896; Desai & Nityanand, 2011). More so, numerous fungi have since been explored as producers of laccase, with white rot fungi being particularly notable. Among them, the white-rot fungus *Trametes versicolor* (also known as *Coriolus versicolor*) has been extensively studied for laccase production (Bourbonnais et al., 1995). Other well-studied laccase-producing fungi within the Trametes genus include *Trametes pubescens*, *Trametes hirsuta*, and *Trametes gallica* (Zhang et al., 2019). Additionally, various laccase isoforms are produced by basidiomycetes, specifically *Agaricus bisporus*, *Pleurotus ostreatus*, *Trametes versicolor*, *Phanerochaete chrysosporium*, and *Coprinus cinereus* (Arregui et al., 2019).

The physiological functions of laccase are as diverse as the sources from where they originate. Other than its role in lignin degradation, laccase is also involved in various processes such as morphogenesis and differentiation of sporulation in basidiomycetes, improves cell-to-cell adhesion, pigmentation in mycelia and fruiting bodies and helps in rhizomorph formation (Thurston, 1994). The unique biological properties of laccases have earned the name “green catalysts,” as they oxidize various substrates, solely require oxygen molecules as reactants, and producing only water molecules as byproducts (Mate & Alcalde, 2015). Essentially, laccases reside in a variety of structures; with most being monomeric, although some exist in homodimeric, heterodimeric, and multimeric forms. Their molecular mass varies between 50 to 140 kDa, contingent upon the organism. Notably, fungal laccases typically fall within the 60 to 70 kDa range, with an isoelectric point around pH 4.0 (Baldrian, 2006; Arregui et al., 2019).

4.2.1. Catalytic cycle of laccases

Typically, most laccases have a molecular structure comprising of three cupredoxin domains organized in a greek barrel orientation (Fig. 7). However, two-domain or Small Laccases have been found, that are laccases with only two of those domains (Gabdulkhakov et al., 2019). The active centre of a typical laccase structure is made up of three different types of copper atoms, each of which has unique electron paramagnetic resonance characteristics (Morpurgo et al., 1980). Purified laccase is a deep blue colour due to type 1 copper (T1Cu), which also exhibits a significant absorption peak at 600 nm. Type 3 copper (T3Cu) displays an absorption peak at 330 nm, while type 2 copper (T2Cu) is colorless and exhibits mild absorption in the visible region of the electromagnetic spectrum. T2Cu and T3Cu combine to form a copper cluster called the trinuclear copper-binding site (TNC) (Madhavi and Lele, 2009).

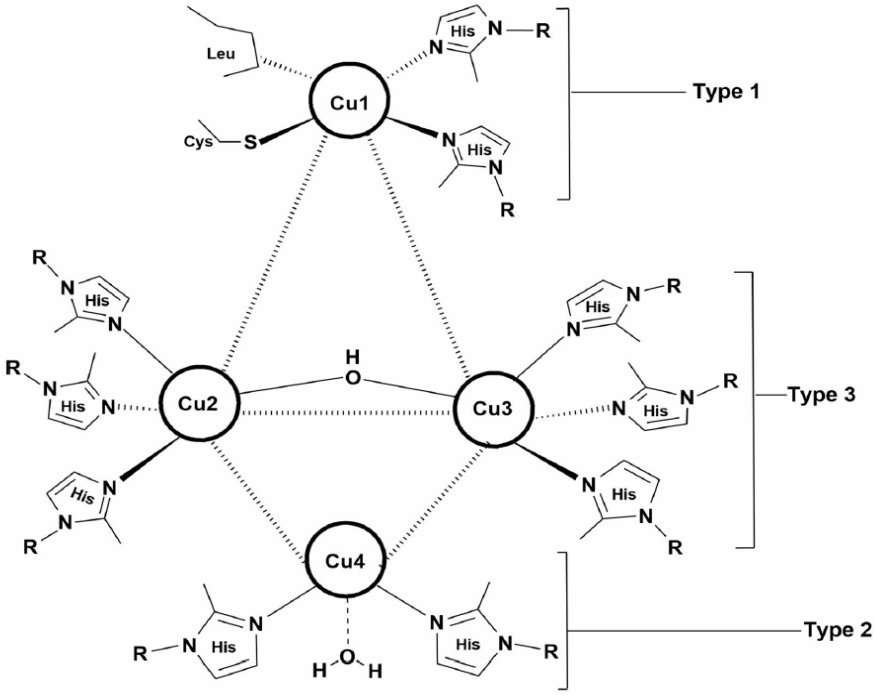

Figure 7. Active site of laccase depicting relative orientation of Type 1, Type 2, and Type 3 copper centers (adopted from Agrawal et al., 2018)

While the TNC aids in the four-electron reduction of O_2 to H_2O, the T1 copper site is engaged in the substrate's one-electron oxidation. A laccase molecule's several copper atoms must be distinguished

using spectroscopic data. The coordination of two histidine (His) residues and one cysteine (Cys) residue in a trigonal configuration with the copper atom determines the topology of the T1 copper-binding site. Two more weakly coordinating or non-coordinating residues are located in an axial location (Sitarz et al., 2015). In bacterial and plant laccases, one of these axial ligands is typically methionine (Met), which possesses a low redox potential (E0) of 0.3-0.5 V compared to the normal hydrogen electrode (NHE). This Met coordinates to the T1 copper center, whereas most fungal laccases contain leucine (Leu) or phenylalanine (Phe) in this position (Reiss et al., 2013; Hakulinen & Rouvinen, 2015). The presence of methionine as the fourth axial ligand at the T1 Cu center, or its substitution by phenylalanine or leucine, may have a potential influence on the E0 of the enzyme.

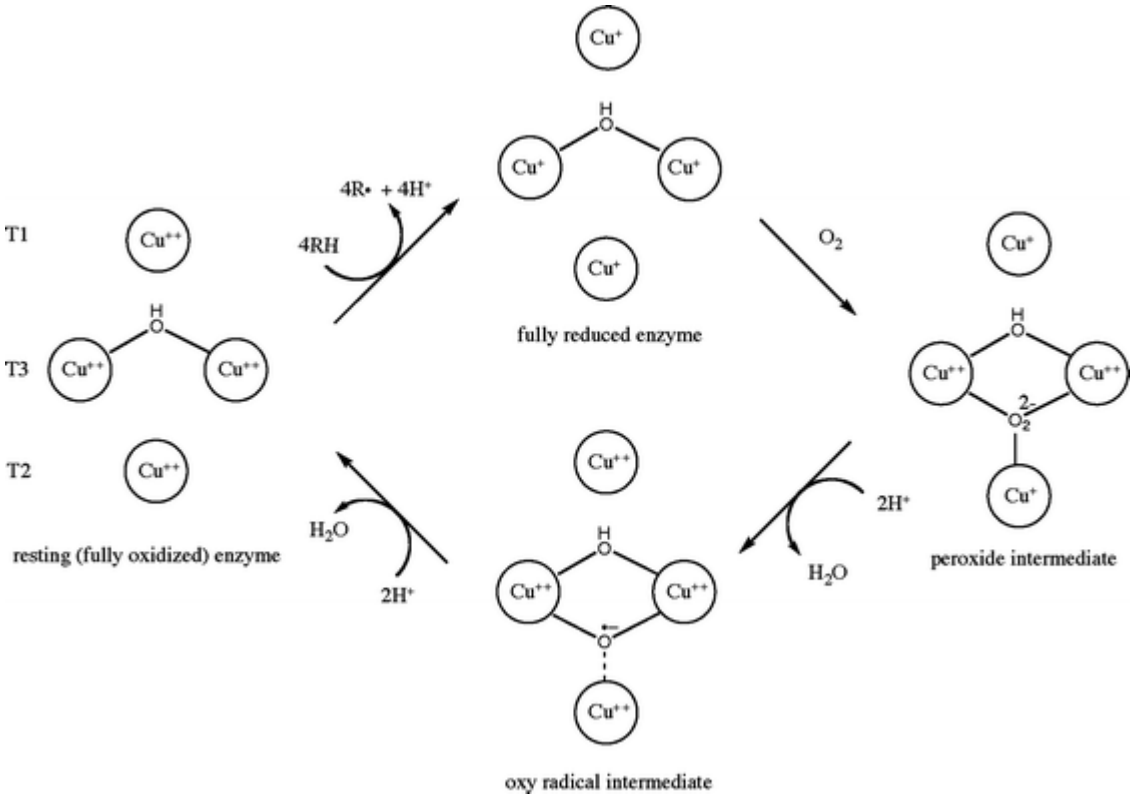

Figure 8. Catalytic cycle of laccases (adopted from Wong, 2008)

In a typical catalytic reaction, substrate is oxidized to its reactive radical (see Fig.8). The enzymatic reaction is completed by the participation of different copper centers found in laccase. In contrast to

peroxidases, laccases can oxidize substrates without the requirement of hydrogen peroxide. The three primary phases of enzyme catalysis include the initial reduction of the type-I copper ion using the reducing substrate, subsequent internal electron transfer between the type-I, type-II, and type-III Cu clusters, and ultimately the production of water through the reduction of oxygen at the type II and type III Copper clusters (Su et al., 2017).

4.2.2 Role of laccase in lignin degradation

Laccase oxidize the phenolic lignin by producing cationic radicals in the substrate, leading to cleavage in aromatic or aliphatic bonds resulting in lignin depolymerization. Specifically, laccase act as a catalyst for the cleavage of the Cα – Cβ bonds within phenolic subunits particularly β-1 and β-O-4 dimers. This involves the breaking of aryl-alkyl bonds (Wong, 2008), as illustrated in Figure 9. Subsequently, Cα oxidation occurs, leading to the formation of phenoxy radicals which undergo various rapid reactions, resulting in the cleavage of multiple aromatic rings and other bonds. Additionally, polymerization reactions take place resulting in the formation of quinone compounds (Wong, 2008; Thurston, 1994).

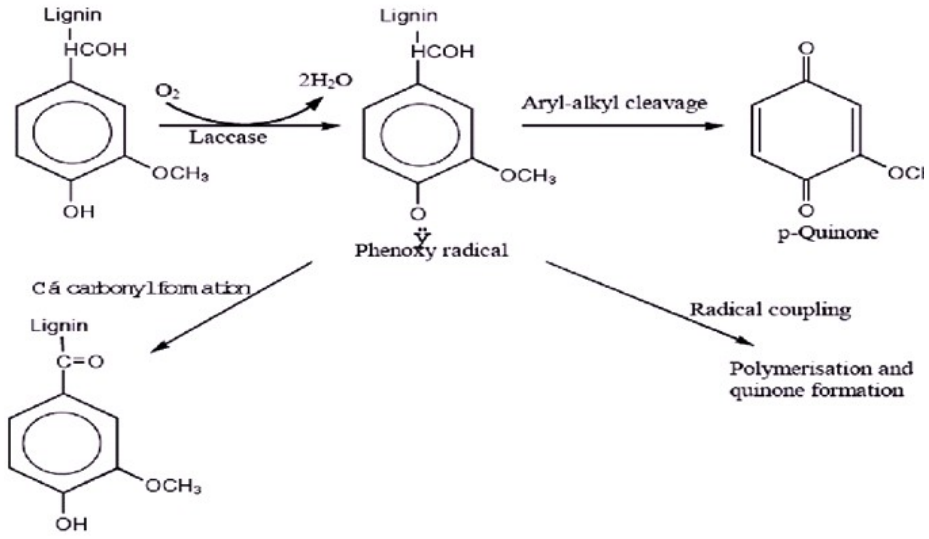

Figure 9. Laccase-mediated oxidation of phenolic lignin subunits (adopted from Archibald et al., 1997)

However, due to the low redox potential, laccase could not directly oxidize non phenolic units of lignin. In this case, small molecules called mediators like as 2,2' – azinobis-(3-ethylbenthiazoline-6-sulfonate can function as intermediary substrate for laccase there by increasing the low redox potential and result in an expanded range of substrates for the conversion of lignin (see Fig.10). Examples of natural mediators include oxalate or malonate, while ABTS, 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO) and 1-hydroxybenzotriazole (HBT) include synthetic mediators which are initially converted to radicals that further reacts with the substrates to cause its degradation (Hahn, 2023).

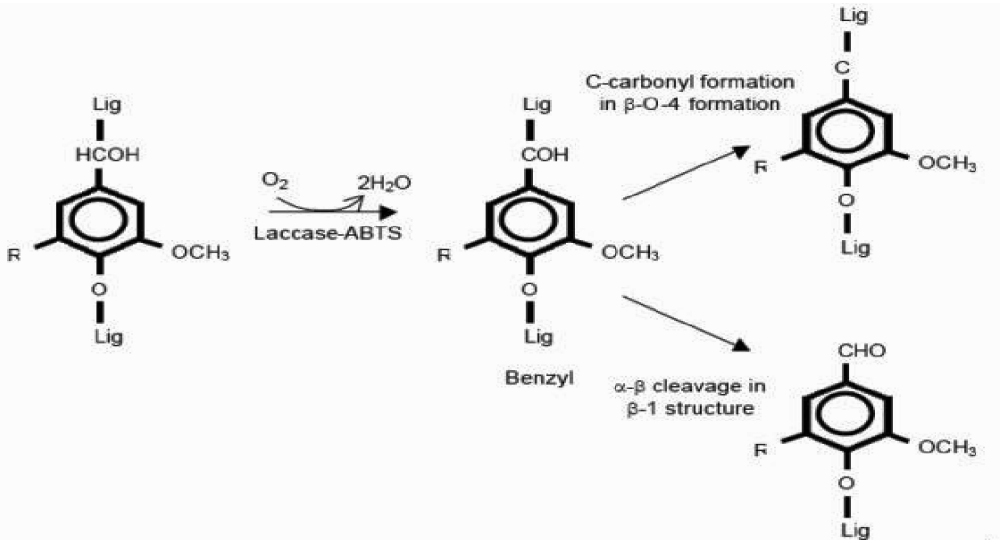

Figure 10. ABTS-mediated oxidation of non-phenolic lignin subunits by laccase (adopted from Archibald et al., 1997)

4.2.3 Laccases of bacterial origin

Laccases have wide applications as green biocatalysts as it is capable of oxidizing wide range of substrates including phenolic and non-phenolic compounds (Brugnari et al., 2021). This oxidation reaction leads to the formation of reactive intermediates which can be used for the biosynthesis of fine chemicals (Witayakran & Ragauskas, 2009). Furthermore, this versatility of laccase enzyme enables its

application in the field of bioremediation, detoxification of environmental pollutants including waste water treatment (Rodríguez Couto & Toca Herrera, 2006; Zerva et al., 2019; Becker & Wittmann, 2019).

However, fungal laccases have certain limitations due to their inherent physiological and biochemical properties, when it comes to industrial applications. These limitations include slower growth rates, low tolerance in the presence of inhibitors, long fermentation periods, low pH optima and difficulties in expression in heterologous host organisms (Upadhyay et al., 2016; Kim et al., 2010).

On the other hand, bacterial laccases have reported to exhibit several advantages which tolerance to a broad range of pH levels and temperatures, ease to genetic manipulations, broad substrate specificity, and exceptional stability in the presence of inhibitors which makes them appealing and suitable for industrial applications (Du et al., 2015; Chauhan et al., 2017). Other laccase producing bacteria identified from several research studies have been compiled and presented in Table 1 below:

Table 1: Laccase producing bacterial strains identified from literature reports

Gram nature	Phyla	Bacterial strains	References
Gram-negative	Proteobacteria	*Azospirillum lipoferum*	(Chauhan et al., 2012)
		Pseudomonas putida	(Mandic et al., 2019)
		Stenotrophomonas maltophilia	(Galai et al., 2008)
		Xanthomonas campesteris	(Lee et al., 1994)
		Bhargavaea beijingensis	(Chaudhary et al., 2023)
		Enterobacter asburiae ES1	(Edoamodu & Nwodo, 2021)
		Escherichia coli (yacK)	(Ma et al., 2017)
		Klebsiella pneumoniae	(Gaur et al., 2018)
		Acinetobacter baumannii	(Pooalai et al., 2022)
		Serratia marcescens	(Kaira et al., 2015)
		Marinomonas mediterranea	(Tonin et al., 2016)
		Pandoraea sp. ISTKB	(Kumar et al., 2017)
Gram-positive	Firmicutes	*Aquisalibacillus elongatus*	(Deepa et al., 2020)
		Bacillus Subtilis MTCC1039	(Mishra & Srivastava, 2016; Chauhan et al., 2017)
		Bacillus Subtilis MTCC 2414	(Muthukumarasamy et al., 2015)

		Bacillus cereus TSS1	(Niladevi et al., 2009)
		Bacillus tequilensis SN4 MTCC 11828	(Sondhi et al., 2014)
		Bacillus safenis DSKK5	(Singh et al., 2014; Chandra & Singh, 2012)
		Bacillus subtilis WPI	(Sheikhi et al., 2012)
		Bacillus licheniformis LS04	(Lu et al., 2012)
		Streptomyces cyaneus	(Arias et al., 2003)
		Geobacillus thermocatenulatus	(Li & Zhang, 2005)
		Bacillus aquimaris AKRC02	(Kumar et al., 2021)
Gram-positive	Actinobacteria	*Streptomycetes species*	(Machczynski et al., 2004)
		Streptomycetes cyaneus	(Ece et al., 2017)

5. Value-added products from lignin

5.1 Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the most widely produced aroma compound globally. Its annual production reaches 20,000 tons, with only 15% derived from lignin and approximately 40 to 50 tons originating from natural vanilla extract and the major share 85%, is produced from petro-based intermediates like guaiacol (Huang et al., 2012; Costa et al., 2021). The natural source of vanillin is obtained from the bean or pod of the Vanilla plant species, mainly *Vanilla planifolia* (Gallage et al., 2017). The food and cosmetic industries use vanillin extensively as a flavouring and scenting ingredient and its application in pharmaceuticals has also recently gained attention (Iram et al., 2021). Notably, vanillin serves as a crucial intermediate for synthesizing fine chemicals like L-DOPA (L-3,4 dihydroxyphenylalanine) (Rodrigues Pinto et al., 2012). However, the natural production of vanillin can barely fulfill lower than 1% of the total market demand. An alternative approach to produce vanillin involves the bioconversion of ferulic acid or eugenol, utilizing the metabolic abilities of various fungi and bacteria to convert these compounds into vanillin or vanillic acid (Converti et al., 2010). Nevertheless, the natural source accounts for only 0.25% of the over 16,000 metric tonnes of vanillin available in the market each year. Although vanillin is typically manufactured through chemical

synthesis, recent advancements in the organic production of vanillin utilizing lignin as a renewable substrate have gained considerable interest.

Vanillin can be produced by microbes utilizing lignin and two methods are predominantly involved in vanillin production. Firstly, they can be extracted as an intermediate product during the biodegradation of lignin. Alternatively, it can also be produced utilizing the ferulic acid transformation pathway. Researchers are employing various genetic engineering techniques to harness the microbial capability of producing vanillin from lignin (Sainsbury et al., 2013). The productivity of vanillin production from lignin relies on the inter-unit linkages present in lignin monomers. A higher content of β-O-4 linkages leads to increased vanillin yields (Tilay et al., 2010). Vanillin can be obtained either as an intermediate during the breakdown of lignin or through the conversion pathways of ferulic acid, eugenol, or isoeugenol (Kundu, 2017). Numerous studies have been conducted to produce vanillin using both native and engineered microorganisms. For instance, *Bacillus* sp. ZB1 demonstrates the potential to convert guaiacyl monomeric lignins, with 61.1% conversion of isoeugenol and eugenol in 10 g/L of pyrolyzed masson pine bio-oil resulting in 56.09 mg/L of vanillin (Zuo et al., 2022). *Phanerochaete chrysosporium* NCIM 1197 can produce 55 µg/mL of vanillin from groundnut shells after 72 h (Jiang et al., 2023). Additionally, *Staphylococcus lentus* bacteria can produce 72.55 mg/L of vanillin from 2000 mg/L Kraft lignin after 6 days at 35°C (Baghel & Anandkumar, 2019).

Vanillin production can also be achieved using natural bacterial consortia isolated from bamboo, with a consortium of natural microbial strains capable of producing 0.9 mg/mL of vanillin through the degradation of lignin found in bamboo while preserving cellulose (Harshvardhan et al., 2017). Using *Escherichia coli* JM109/pBB1, Torres et al. (2009) produced 239 mg/L of vanillin through alkaline hydrolysis of maize cob, with a yield of 0.66 mol of vanillin per mol of ferulic acid. *Pediococcus acidilactici* utilized rice bran as a substrate and produced nearly 1.1 g/L of vanillin (Chakraborty et al., 2017).

The liquors obtained from alkaline hydrolysis of corn cob were neutralized, diluted, and used as a substrate for whole-cell bioconversion (Torres et al., 2009). Similarly, Zheng et al. (2007) achieved the production of 2.8 g/L of vanillin with a yield of 61.9% through the alkaline hydrolysis of rice bran oil using *Aspergillus niger* and *Pycnoporus cinnabarinus.* According to Torres et al. (2009), the liquors obtained from the alkaline hydrolysis of corn cob were neutralized, diluted, and employed as a substrate for whole-cell bioconversion. Similarly, Zheng et al. (2007) used *Aspergillus niger* and *Pycnoporus cinnabarinus* to produce 2.8 g/L of vanillin with a yield of 61.9% from alkaline hydrolyze rice bran oil. *Amycolatopsis* sp., *P. cinnabarinus*, and *Pseudomonas fluorescens* BF13 were able to transform ferulic acid into vanillin by deleting the vanillin dehydrogenase encoding gene (vdh) (Fleige et al., 2013; Tilay et al., 2010; Di Gioia et al., 2011).

Optimization of growth conditions and bioconversion parameters can significantly enhance vanillin production. Under optimized conditions, *P. fluorescens* BF13 produced 1.28 g/L of vanillin (Di Gioia et al., 2011). *R. jostii* RHA1 with vdh deletion utilized the lignin present in wheat straw lignocellulosic biomass to produce 96 mg/L of vanillin (Sainsbury et al., 2013). Approximately 0.9 g/L of vanillin was produced during the fermentation of *Bacillus subtilis* in the presence of ferulic acid (Chen et al., 2017). Vanillin was produced by E. coli JM109 possessing the genes for ferulic acid degradation. Under optimized growth conditions, the recombinant *E. coli* produced the highest recorded output of vanillin (2.52 g/L) (Barghini et al., 2007). According to Rana et al. (2013), Bacillus subtilis has also shown the capability to transform ferulic acid into vanillin. Deletion of the Lap ABC operon in *Pseudomonas putida* strain led to an increase in vanillin yield, quickly reaching approximately 83% (Graf & Altenbuchner, 2013). Furthermore, recombinant *Pseudomonas putida* KT2440 in whole-cell bioconversion produced 0.70 ± 0.20 mM and 0.92 ± 0.30 mM of vanillyl amine (Manfrão-Netto et al., 2021).

5.2 Lipids

Microbial lipids produced by oleaginous species are one of the primary products derived from lignin conversion. Oleaginous microorganisms are characterized by having a lipid content exceeding 20% of their dry weight (Ratledge & Wynn, 2002). Fatty acid biosynthesis pathways are crucial in the buildup of lipids within these microorganisms. These lipids can serve as a promising feedstock for biofuel production and can be obtained through the aromatic catabolism of lignin by oleaginous species. When it comes to phenolic compounds, the acetyl-CoA derived from the β-ketoadipate pathway can be integrated into the fatty acid biosynthesis cycle, harnessing the energy produced by incorporating succinyl-CoA into the tricarboxylic acid cycle. The resulting lipids can be trans esterified into methyl or ethyl esters of fatty acids for biofuel applications (Kosa & Ragauskas, 2013).

Various microbial species possess significant lipid-producing capabilities. They include *Acinetobacter calcoaceticus* (27-37%), *Rhodococcus opacus* (23-25%), *Bacillus alcalophilus* (20-24%), *Chlorella vulgaris* (20-32%), and other oleaginous species with lipid contents exceeding 20% (Iram et al., 2021; Reshmy et al., 2022). *Rhodococcus* species possess the β-ketoadipate pathway, enabling them to enzymatically open the oxidative ring of catechol compounds and convert them into lipids. Specifically, the strain *Rhodococcus jostii* utilizes the carbon derived from aromatic acids to produce acetyl-CoA, a critical precursor for fatty acid and lipid synthesis (Li et al., 2019). In a study, it was demonstrated that the solubility of kraft lignin can be increased, while its molecular weight can be decreased through biological pretreatment using the enzyme laccase. *R. opacus* PD630 can then utilize this modified lignin to accumulate lipids (Zhao et al., 2016).

In addition to other applications, yeasts have been utilized for the production of lipids from lignin and lignin-related aromatic compounds. Moreover, researchers employed Aspergillus fumigatus WSligRF to generate short-chain fatty acids using a lignin-rich fraction obtained from wheat straw (Baltierra-Trejo et al., 2015). A newly isolated oleaginous fungus, *Mucor circinelloides* Q531, can

directly convert mulberry branches into lipids by solid-state fermentation without pretreatment or exogenous enzymes. The fungus can produce up to 42.4 mg of lipids per gram of dry substrate with a high content of unsaturated fatty acids, especially γ-linolenic acid, which has potential applications for biodiesel production and human health (Qiao et al., 2018). Fungal species such as *Aspergillus oryzae* (57%), *Humicola lanuginose* (75%), and *Mortierella isabelline* (86%) have demonstrated the ability to produce significant quantities of lipids, as indicated by their dry weight percentages.

5.3 Polyhydroxyalkanoate (PHA)

PHA (Polyhydroxyalkanoates) are becoming increasingly recognized as a sustainable alternative to petroleum-based plastics due to their biodegradability and desirable physical properties (Raza et al., 2018). The process of converting lignin monomers to PHA involves the biological breakdown of lignin, which is a crucial step that decides the rate of bioconversion. The specific routes through which lignin is broken down affect the types of PHAs generated (Reshmy et al., 2022).

Various types of bacterial strains have demonstrated the ability to degrade lignin and produce PHAs. Among them, *Pseudomonas putida* has been researched extensively for its capacity to both break down lignin and synthesize PHAs (Chen and Lee, 2018). Recently, a new strain of Pseudomonas putida, namely NX-1, has been developed for PHA production (Xu et al., 2021). Another bacterium, *Cupriavidus basilensis*, has demonstrated efficient lignin degradation and PHA synthesis using lignin derived from pretreated rice straw, achieving a PHA concentration of 450 mg/L (Si et al., 2018).

In addition to lignocellulosic biomass, agricultural feedstocks such as mango peel have been explored as substrates for PHA production. Bacillus thuringiensis was found to utilize pretreated mango peel as the sole substrate, resulting in an improved PHA yield of 4 g/L (Gowda & Shivakumar, 2014). The utilization of lignin as a feedstock for PHA production by bacteria holds great promise for sustainable plastics. By harnessing the capabilities of lignin-degrading bacteria, we can transform lignin,

a byproduct of various industries, into valuable PHAs. This not only reduces the reliance on petroleum-based resources but also addresses the environmental issues associated with plastic waste.

6. Whole genome sequencing of lignin degrading bacteria

Whole genome sequencing of bacteria provides valuable insights into the genetic basis of lignin breakdown and facilitate the manipulation of metabolic pathways to convert lignin towards desired end products. Whole genome analysis using Next-generation sequencing platforms (NGS) has revolutionized the field of genomics, enabling researchers to obtain vast amounts of DNA sequence data quickly in a cost-effective manner. Next generation sequencing incorporates several techniques, including Illumina sequencing, Roche, Ion Torrent sequencing, and Pacific Biosciences (PacBio) sequencing with its unique strengths and applications (Kameshwar & Qin, 2016). Among them, Illumina sequencing has the advantages of high through put, scalability and cost effectiveness which makes it the most widely used NGS platform (Metzker, 2009)

Compared to fungal counterparts, the whole genome sequencing of bacteria involved in lignin degradation is less characterized and explored. Table 2 summarizes lignin-degrading bacterial strains from diverse phyla and classes, including Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes. These strains have undergone whole genome sequencing and were obtained from different environments, such as compost, soil, rainforest soil, and gut environments. The sequencing platforms employed in this study comprise Illumina and PacBio, with certain strains having been sequenced using multiple platforms. Additionally, the table includes information on the GC content, genome sizes, and the submission/reference IDs for each strain.

6.1 Proteobacteria

The phylum Proteobacteria is a group of gram-negative bacteria found in the domain Bacteria with an outer membrane that mainly consists of lipopolysaccharides. They are classified into the classes: ∞, β,

γ, δ and ε proteobacteria, with each class having separate orders, families, genus, and species. Among these classes, the complete genome sequence of the γ-proteobacterial strain *Serratia quinivorans* 124R, isolated from forest soil was sequenced using PacBio RS II platform and has shown a GC content of 52.85% and a genome size of 5 Mb. The strain was able to utilize organosolv lignin as a sole carbon source and grow under anoxic conditions and putative aromatic pathways were analyzed using the MetaCyc and KEGG databases (Chaput et al., 2019). A β-proteobacterial strain *Pandoraea* sp. ISTKB has been reported to degrade lignin and produce a bioplastic, polyhydroxyalkanoate (PHA). The genomic and proteomic analysis of this strain was performed to reveal its a GC content of 62.05% and a genome size of 6.37 Mb. Illumina MiSeq platform was used for sequencing and novel gene clusters for lignin degradation and PHA production were discovered (Kumar et al., 2016). The genome of an α-proteobacterial strain *Bradyrhizobium* sp. S23321, an oligotrophic bacterium recovered from paddy field soil underwent whole-genome shotgun sequencing. It showed a GC content of 64.10% and a genome size of 7231841 bp and genes encoding lignin monomer catabolism was found in the S23321 genome (Okubo et al., 2012). *Tolumonas lignolytica* BRL6-1 T, a novel γ-proteobacterial species, was reported to degrade lignin anaerobically. The genome sequence and annotation of this strain was performed using the Illumina HiSeq 2000 and PacBio RS platforms and revealed several genes and pathways involved in lignin breakdown and utilization. It has a GC content of 48% and a genome size of 3600000 bp (Billings et al., 2015). A novel bacterium, *Burkholderia* sp. ISTR5, exhibited growth and enzyme activities on 12 lignin-derived compounds and reduced the total organic carbon of the substrates. The degradation intermediates and pathways were identified by UV–Vis spectral scan and GC–MS analysis, revealing the bio-funneling potential of the strain. The genome analysis of the strain was performed using the Illumina MiSeq platform. It has a GC content of 68.11% and a genome size of 8160000 bp and revealed the presence of genes and pathways involved in the metabolism of lignin and aromatic compounds (Morya et al., 2019).

6.2 Firmicutes

Bacillus velezensis LC1, a symbiotic bacterium, demonstrates the potential to break down bamboo lignocellulose components and convert them into ethanol. A comprehensive analysis of carbohydrate-active enzymes revealed the presence of 136 genes associated with CAZy families, encompassing GH, GTs, CEs, PLs, AAs, and CBMs. Notably, several genes involved in lignin degradation were identified, including oxidoreductases, reductases, dehydrogenases, esterases, thioesterases, transferases, and hydrolases (Li et al., 2020). Additionally, the genome of *Bacillus ligninesis* L1, an alkaliphilic bacterial strain isolated from seafloor sediment, was sequenced using the Illumina-Solexa HiSeq 2000 platform. With a genome length of 3.8 Mbp and a GC content of 40.76%, strain L1 exhibits the ability to thrive on medium containing lignin as the sole carbon source. Analysis of its genome uncovered specific genes associated with lignin degradation, such as two aryl-alcohol dehydrogenase genes, 19 NADH dehydrogenase genes, two glycolate oxidase genes, and one Mn-superoxide dismutase gene (Zhu et al., 2013).

6.3 Actinobacteria

Streptomyces sp. strain S6 is derived from biomass from palm oil mill and this Actinobacterial strain was sequenced using the Ion X5 XL Sequencer platform. It has a GC content of 71.23% and a genome size of 6420514 bp (Riyadi et al., 2020). The strain can produce different ligninolytic enzymes, including lignin peroxidase, laccase, dye-decolorizing peroxidase, and aryl-alcohol oxidase activity, and grow when Kraft lignin is used as the only carbon source. The draft genome analysis of the strain reveals the presence of genes encoding ligninolytic enzymes, oxidative stress response proteins, and central aromatic intermediate metabolic pathways, confirming its lignin degradation capability. Couger et al., 2020 sequenced and annotated the genome of *Arthrobacter* sp. strain RT-1, a bacterium derived from termite gut and rumen fluid that can degrade lignin and lignin-derived aromatic compounds. The genome sequencing was performed using the Illumina HiSeq 4000 platform and has a GC content of 65.69% and

a genome size of 282801 bp. The genome analysis revealed the presence of genes involved in the catabolism of lignin and its derivatives, indicating the potential role of strain RT-1 in the conversion of lignocellulosic biomass.

Table 2. Whole genome sequencing studies of different lignin-degrading bacterial strains

Phylum	Bacterial Strain	Source	Sequencing Platform	GC content	Genome Size (Mb)	Accession No.	References
Actinobacteria	*Microbacterium sp. RG1*	Compost	PacBio RS II	69.50%	3.2 Mbp	CP034121	Ravi, K et al; 2019
Actinobacteria	*Arthrobacter sp. Strain RT-1*	Termite gut and rumen fluid	Illumina HiSeq 4000	65.69%,	282801bp	QRGQ00000000.	Couger M B et al., 2020
Actinobacteria	*Streptomyces sp. strain S6.*	Biomass from palm oil Mill	Ion X5 XL Sequencer	71.23%	6420514bp	CP040654	Riyadi, F. A et al., 2020
Firmicutes	*Bacillus pumilus S-1*	Rainforest Soil	Illumina Genome analyzer IIx	41.26	3,692,744 bp	AGBY00000000.	Su F et al.,2011
Firmicutes	*Bacillus velezensis LC1*	Beetle gut	PacBio	46.50%	3929782 bp	CP044349	Li, Y et al., 2020
Firmicutes	*Bacillus ligninesis L1*	Sea floor sediment	Illumina- Solexa HiSeq 2000	40.76%	3.8 Mbp	ANNK00000000	Zhu D et al., 2013
Proteobacteria	*Pantoea ananatis Sd-1*	Rice endophyte	Illumina Hiseq 2000	53%	4927500bp	AZTE00000000.	Ma J et al., 2016
Proteobacteria	*Burkholderia sp. Strain LIG30*	Tropical forest soil	Illumina MiSeq	66.40%	5.5 Mb	JGVW00000000.	Woo, H.L et al., 2014
Proteobacteria	*Pandoraea sp. ISTKB*	Rhizospheric soil	Illumina MiSeq	62.05%	6.37 Mb	MAOS00000000	Kumar M et al., 2016
Proteobacteria	*Pseudomonas sp. Strain LLC-1 (NBRC 111237)*	Soil	Illumina MiSeq, Roche 454 sequencing	62.40%	5,946,122 bp	LUVY00000000.	Hirose J et al., 2018
Proteobacteria	*Tolumonas lignolytica sp*	Rainforest soil	Illumina HiSeq 2000, PacBio RS	48%	3.6 Mbp	AZUK00000000.1	Billings A F et al., 2015
Proteobacteria	*Burkholderia sp. ISTR5*	Soil	Illumina MiSeq	68.11%	8.16 Mb	MK106102.1	Morya et al., 2019
Proteobacteria	*Serratia quinivorans*	Forest soil	PacBio RS II	52.85%	5 Mb	NZ_SHMO00000000	Chaput G et al., 2019
Proteobacteria	*Cedecia lapagei*	Camel cricket gut	PacBio RS II	55%	4.6 Mb	PIQM00000000	Mathews S L et al., 2019
Proteobacteria	*Klebsiella sp. strain BRL6-2*	Tropical forest soil	llumina HiSeq 2000, PacBio	55.24%	5.8 Mb	ARVT00000000	Woo H L et al., 2014
Proteobacteria	*Bradyrhizobium sp. S23321*	Soil	Wholegenome shortgun sequencing	64.10%	7,231,841 bp	BA000040	Okubo et al., 2012

7. Conclusion:

In essence, lignin valorization is critical for a sustainable biobased industry. This literature review has thoroughly examined the entire lignin valorization process, from understanding the intricate structure of lignin to harnessing the potential of microbial enzymes, with an emphasis on bacterial laccases. Understanding the intricacies of lignin and its enzymatic breakdown is important for developing novel and innovative strategies for transforming lignin into high value products, including biofuels and platform chemicals. The integration of innovative screening methods and whole genome sequencing techniques substantially advances the understanding of the discovery of novel lignin-degrading bacteria, providing insights into their genetic repertoire and metabolic pathways. This literature review highlights the immense potential of lignin as a sustainable resource and sets the stage for future research and technological advancements in the field of lignin biorefinery.

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CHAPTER 2

A high throughput screening process and quick isolation of novel lignin-degrading microbes from large number of natural biomasses

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Abstract

High throughput screening approaches can significantly speed up the identification of novel enzymes from natural microbial consortiums. A two-step high throughput screening process was proposed and explored to screen lignin-degrading microorganisms. By employing this modified culture enrichment method and screening based on enzyme activity, a total of 82 bacterial and 46 fungal strains were isolated from fifty decayed wood samples (100 liquid cultures) collected from the banks of the Ottawa River in Canada. Among them, ten bacterial and five fungal strains were selected and identified based on their high laccase activities by 16S rDNA and ITS gene sequencing, respectively. The study identified bacterial strains from various genera including *Serratia*, *Enterobacter*, *Raoultella*, and *Bacillus*, along with fungal counterparts including *Mucor*, *Trametes*, *Conifera*, and *Aspergillus*. Moreover, *Aspergillus sydowii* (AORF21), *Mucor sp*. (AORF43), *Trametes versicolor* (AORF3), and *Enterobacter sp.* (AORB55) exhibited xylanase and β- glucanase activities in addition to laccase production. The proposed approach allowed for the quick identification of promising consortia and enhanced the chance of isolating desired strains based on desired enzyme activities. This method is not limited to

lignocellulose and lignin-degrading microorganisms but can be applied to identify novel enzymes from different natural samples.

Keywords: β-glucanase, High throughput screening, Laccase, Lignin degradation, Xylanase

1. INTRODUCTION

Novel and innovative screening approaches are uncovering the existence of a wide range of previously unknown enzyme activities from active microbial communities distributed in nature. Biomass conversion utilizes microorganisms and their enzymes, such as cellulases, xylanases, and ligninases, to transform lignocellulose into platform chemicals which can then be converted to various products, including biofuels, chemicals, and materials. Microorganisms that can effectively break down biomass in high yields are vital for achieving sustainable and economically viable production of value-added products from lignocellulosic feedstock (Yang et al., 2014, Vu et al., 2020).

Lignin is an abundant natural phenolic polymer with immense potential to be valorized into high-value bioproducts after degradation into short oligomers or monomeric subunits. More so, biological and enzymatic degradation of lignin has the potential to advance sustainable green biotechnology as a highly selective and environmentally benign substitute for conventional fossil-based fuel production (Jäger & Büchs, 2012, Adewale et al., 2021, Ali et al., 2017). Indeed, adequate removal of lignin from green waste, a vital byproduct in the paper industry and biofuel manufacture, occupies the center stage in lignin biodegradation and bioremediation initiatives (Fang et al., 2018). In plant cell walls, cellulose, hemicellulose, and lignin are united in a tight embrace, and denuding them of lignin provides microbial consortia with larger accessibility for cellulose bioprocessing. The lignin separated from woody biomass is converted into target compounds like vanillin, polyhydroxyalkanoates, lipids, and cis-muconic acid, rendering it a promising feedstock. However, the structural complexity of lignin and expensive enzymes

are the two main obstacles that reduce the process' efficiency and make it unprofitable (Tarasov et al., 2018).

Microbes that can break down lignin can produce a range of enzyme combinations comprising several types of lignin-degrading enzymes, such as laccases, manganese peroxidases, and lignin peroxidases (Chio et al., 2019). Lignin degradation can be achieved by the concerted action of multiple enzymes that work in cascade reactions which enhance the saccharification of polysaccharides of lignocellulosic biomass. Screening of microorganisms with significant lignin degradation ability allows the opportunity to discover and characterize novel enzymes that potentially allow low-cost biological processes. However, discovering lignin-degrading microorganisms with a high performance largely depends on the screening strategy. A variety of microbial screening approaches and strategies were employed and adapted to identify novel enzymes for specific functionalities and targets (Chandra et al., 2007; Choolaei et al., 2021; Maruthamuthu et al., 2016; Chandra et al., 2008). Culture-dependent screening strategies mainly utilize a culture enrichment method which involves growing microorganisms in a cultivating medium with lignin as the carbon source, and the screening is solely activity based without relying on the prior knowledge of the enzyme. After the culture enrichment process, the most common practice is to perform a qualitative screening using an agar plate containing dyes resembling lignin fragments, followed by a quantitative screening of selected microbes based on activity (Xu et al., 2018; Raj et al., 2007; Zhou et al., 2017; Tian et al., 2016). Zhang et al. (2021) obtained four lignin-degrading microbial consortia from 40 antique wooden samples in a tedious way. Each sample was grown up in a separate flask, and selections were based on subjective, visual color change. Even though the main fungal and bacterial compositions of the four consortia were identified by genomic sequencing, no specific strain was isolated. Similarly, from twelve samples of decayed tree trunks, stumps, and surrounding soil samples, Fang et al. (2018) isolated a microbial consortium that demonstrated the ability to selectively break down lignin from tree trimmings where a chromogenic substrate was incorporated

in the culture media that produced a color change when laccase is produced. The reported process is laborious and time-consuming as it takes months of culture and many generations of subcultures.

Xiong et al. (2020) identified four bacterial strains from the soil, silage, and straw sam- ples by first flask extraction of microorganism mixtures, followed by growth on ligninolytic selection agar media. Colonies that demonstrated aniline blue decolorization were chosen individually, and the strains were identified through 16S rDNA sequencing. There is no selection and screening of the starting biomass before single colony selection, and the total numbers of biomass samples tested were not reported. Recently, Elframawy et al. (2022) screened 23 samples (14 soil samples and 9 old black liquor samples) to identify lignin degrading Actinobacteria (*Streptomyces* strains). Soil samples were first pretreated for 10 days, followed by the soil dilution plate technique (AGA plates with nystatin to reduce fungal growth), while black liquor samples were directly spread onto agar plates after dilution. Colonies were selected after 21 days of incubation, transferred to new AGA plates, and further cultured for 14 days to purify the isolates.

The agar plate screening method is laborious, time-consuming, has low throughput, and lacks precision as it may not accurately detect the target enzyme. Submerged screening methods, on the other hand, offer better control over the culture conditions, which can lead to higher enzyme production. They also allow the possibility for high throughput (HTP) screening of a vast number of samples simultaneously. Additionally, through the use of specific assay conditions or detection methods, the target enzymes can be identified with better specificity and sensitivity compared to agar plate screening methods. Moreover, screening in liquid media also mimics industrial submerged culture conditions, thus allowing the identification of potential microbes and enzymes for industrial applications (Arnthong et al., 2020).

There are no studies reported thus far on the HTP screening process of a large number of natural biomasses based on liquid culture and specific enzyme activities. This study aimed to 1) develop an HTP

process that speeds up the identification of efficient lignin-degrading consortia, requiring fewer iterations and labor and facilitating the quick isolation of a large number of efficient microbial strains; 2) application of the developed process in practical isolation of new ligninolytic strains from natural biomasses.

In the preliminary screening step, decomposed wood samples were obtained from the Ottawa River banks. Each sample was separately incubated using an enriched culture approach, and the total laccase activities of each culture were quantitatively screened on 96-well plates. This initial step allows quick HTP screening of a vast number of samples. For secondary screening, the majority of samples were rejected due to low or negative laccase activities, and only a small number of samples with high laccase activities were selected for the identification of individual strains with high enzyme activities. This technique significantly reduced the time and labor to go through a large number of samples, meanwhile increasing the success rate of identifying new bacterial and fungal strains with superior lignin-degrading ability. More interestingly, some lignin-consuming strains also exhibited significant xylanase and β-glucanase activities, suggesting that this technique may enhance the opportunity to identify super strains that express multiple enzymes simultaneously. In addition, the proposed strategy can be easily modified and adapted to isolate new strains based on different enzyme activities.

2.MATERIALS AND METHODS

2.1. Chemicals

Czapek dox broth (CDB), 2,6 Di methoxy phenol (DMP), Kraft lignin, Dinitrosalicylic acid, Glucose, Xylose, Xylan (Birchwood, EC. No: 232-760-6, Sigma), β- glucan (Barley, Cat. No: P-BGBL, Megazyme).

2.2. Sampling

The samples used in the study were long-decomposed logs (n=50) randomly collected from a forested area near Ottawa river, Canada (geographical coordinates: 45° 26′ 59.7″N, 75° 41′ 23.3″W) for screening of potential lignin-degrading microorganisms. The samples were obtained in sealed sterile sampling containers and stored in the refrigerator at 4◦C.

2.3. Primary screening by culture enrichment method

Primary screening is carried out to identify wood samples with high laccase activity. Briefly, 0.5 g of each wood sample was inoculated onto two sets of 50 ml Czapek dox medium with 0.1% kraft lignin (referred to as L-CDB), (Kraft 98%, Sigma Aldrich, MW>28000) pH7.3 ± 0.3, for bacterial and fungal isolation, separately. The fungal culture medium was made selective by adding 50 ppm chloramphenicol to suppress bacterial overgrowth and incubated for two weeks. Similarly, with bacterial cultures, 100 ppm cycloheximide was incorporated in the medium to inhibit fungal overgrowth for 7 days of incubation (Bonnet et al., 2020). Cultures were incubated at 28°C with 125 rpm shaking under aerobic conditions. After incubation, broth cultures were centrifuged at 13,000 rpm at 4°C for 5 min, and the supernatant was then treated as the crude enzyme, and laccase activity was measured spectrophotometrically.

2.4. Secondary screening for potential isolates

Based on the primary screening, the samples which showed high laccase activity was selected to validate potential laccase-producing microorganisms. In all cases, 1 ml broth culture of each bacterial samples was serially diluted up to 10^{-6} and plated on Luria Bertani (LB) agar plates (containing g L−1 of distilled water in: 10.0 g peptone, 5.0 g sodium chloride, 5.0 g yeast extract and 15.0 g agar). Similarly, fungal

samples were plated on Czapek Dox Agar (CDA) plates (containing g L^{-1} of distilled water in: 30.0 Sodium Nitrate, 3.0 Magnesium Sulfate, 0.5 Potassium Chloride, 0.5 Potassium Phosphate Dibasic, 1.0 Ferrous Sulfate and 15.0 g agar) to obtain morphologically distinct pure colonies (see Appendix 1). This included 82 pure bacterial cultures and 46 fungal isolates, which were further inoculated individually on 50 ml L-CDB under the previously mentioned culture conditions to measure laccase activity quantitatively at 72 h for bacterial strains as the optimum laccase production of most bacterium ranges 24 h – 96 h (Neifar et al., 2016, Sondhi et al., 2014) and sixth day for fungal strains (Zhu et al., 2016, Senthivelan et al., 2019). The colonies which grew well in culture media and showed the highest laccase activity (≥ 20 U/L for bacteria; ≥ 30 U/L for fungi) were selected for molecular identification and preserved at 4°C for further studies. The total protein amount was measured using the Bio-Rad protein assay reagent, USA, and bovine serum albumin (BSA) as the standard protein, following Bradford's method. Time course analysis was also performed concurrently, to determine the optimal incubation period for laccase production by the selected isolates.

2.5. Sequence-based identification of ligninolytic microorganisms

To identify bacterial strains, genomic DNA was obtained from the samples using bacterial DNA isolation kit (Bio Basic, Canada). Universal eubacterial primers, 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′), as described by (Narde et al., 2004), were used to amplify the 16S rRNA gene. The PCR reactions were conducted in a Bio-Rad T-100 Thermal cycler, with 35 cycles typically used to amplify the 16S rRNA gene after the initial denaturation at 95°C for 2 min, followed by denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 2 min, and final extension at 72°C for 15 min.

To identify fungal strains, pure mycelium was collected from each strain and fungal genomic DNA was isolated using a fungal DNA isolation kit (Bio Basic, Canada) with liquid nitrogen. The

internal transcribed spacer (ITS) gene sequencing analysis was used to analyze the ITS1-5.8S-ITS2 genomic region of each fungal isolate. The for- ward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS region of the genomic DNA, as described by (Mukhtar et al., 2019). For the fungal PCR, the amplification process involved an initial denaturation step of 5 min at 95°C, followed by 35 cycles of amplification at 95°C for 1 min, 55°C for 1 minute, and 72°C for 1 min, and then a final extension of 10 min at 72°C.

The PCR products were then resolved on a 1% agarose gel and purified using a Qiagen quick gel extraction kit. The purified PCR product was sequenced by Eurofins Genomics, Canada for 16S rDNA and ITS gene sequencing analysis for bacteria and fungi respectively. Multiple sequence alignment analysis of 10 type strains that showed the highest identity and similarity to the bacterial isolate's sequence was performed using MEGA X program. The evolutionary history was inferred using the neighbor-joining (NJ) criterion, and a bootstrap analysis was performed using 1,000 pseudo-replicates.

2.6. Laccase enzyme activity assay

Laccase activity assay was performed spectrophotometrically using 2,6 Di methoxy phenol as a substrate according to the method of Ali et al. 2022). The centrifuged culture supernatant from bacterial and fungal cultures after 13,000 rpm for 5 min was used as the crude enzyme. The assay mixture contained sodium acetate buffer (0.1 M, pH 5), 160 µL, 2,6 DMP (2 mM), and 50 µL enzyme solution with a total reaction volume of 210 µL. The change in absorbance due to the oxidation of DMP was monitored at 469 nm for 20 min at an interval of 1 min. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of 2,6- DMP within a one-minute interval.

2.7. Xylanase and β-glucanase activity assays by DNS method

The selected strains were also examined for their ability to produce xylanase and β- glucanase following the method by Shrestha et al. (2015). This is performed by growing the selected high laccase positive

strains from the study in the Czapek Dox medium with xylan, β-glucan, and lignin as the sole carbon source separately and checking the activity of cell-free supernatant after appropriate incubation. Xylanase activity was determined by incubating the crude enzyme with a suspension of 1% birchwood xylan in 0.1 M sodium acetate buffer solution at pH 4.0 and 30°C for 5 min. The amount of xylose released during this time was then measured using the 3,5-dinitrosalicylic acid method (DNS). The reaction was halted by adding DNS, the samples were boiled for 10 min, cooled in water, and the optical density was measured at 540 nm. The xylanase activity was described as the amount of enzyme required to release 1 µmol of xylose per minute. The concentration of xylose was determined by creating a standard xylose curve using xylose standards with concentrations ranging from 100 to 800 µM.

Similarly, the β-glucanase activity was assessed by incubating the crude enzyme with 0.5% β-glucan at 30°C for 5 min and then measuring the amount of reducing sugars released, using the 3,5-dinitrosalicylic acid method. Two controls were put up, lacking crude enzyme and substrate separately. One unit of β-glucanase activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute under these conditions. Two controls were put up, lacking crude enzyme and substrate separately. The amount of glucose present was determined by comparing it to a standard curve which was created using glucose standards with concentrations ranging from 100 to 800 µM.

Furthermore, xylanase and β-glucanase activities in the selected strains were evaluated by cultivating them in CDB with lignin as the carbon source. Additionally, the strains were also grown in CDB media without any carbon source, and the activities of all three enzymes, xylanase, β-glucanase and laccase, were assessed.

2.8. Statistical Methods

All the above experiments were performed in triplicate, and the average data were reported. Pearson's coefficient was used to evaluate correlation using GraphPad Prism 9.0.0. and p-value less than 0.05 was considered as the criterion for statistical significance.

3. RESULTS AND DISCUSSION

3.1. Establishing a high throughput screening process

Traditionally, lignin-degrading microorganisms have been selected based on qualitative methods such as direct screening using culture plates from environmental samples or by first enriching the environmental samples and then performing a plate-based screening (Rahman et al., 2013). The conventional screening process followed in previous studies is illustrated in the steps (left) shown in Fig.1. To improve the reliability and efficiency of selected strains that highly express laccase enzymes using an HTP process, the methodology was changed by including a quantitative detection of laccase activity in the primary screening step as illustrated in the steps (right) shown in Fig.1.

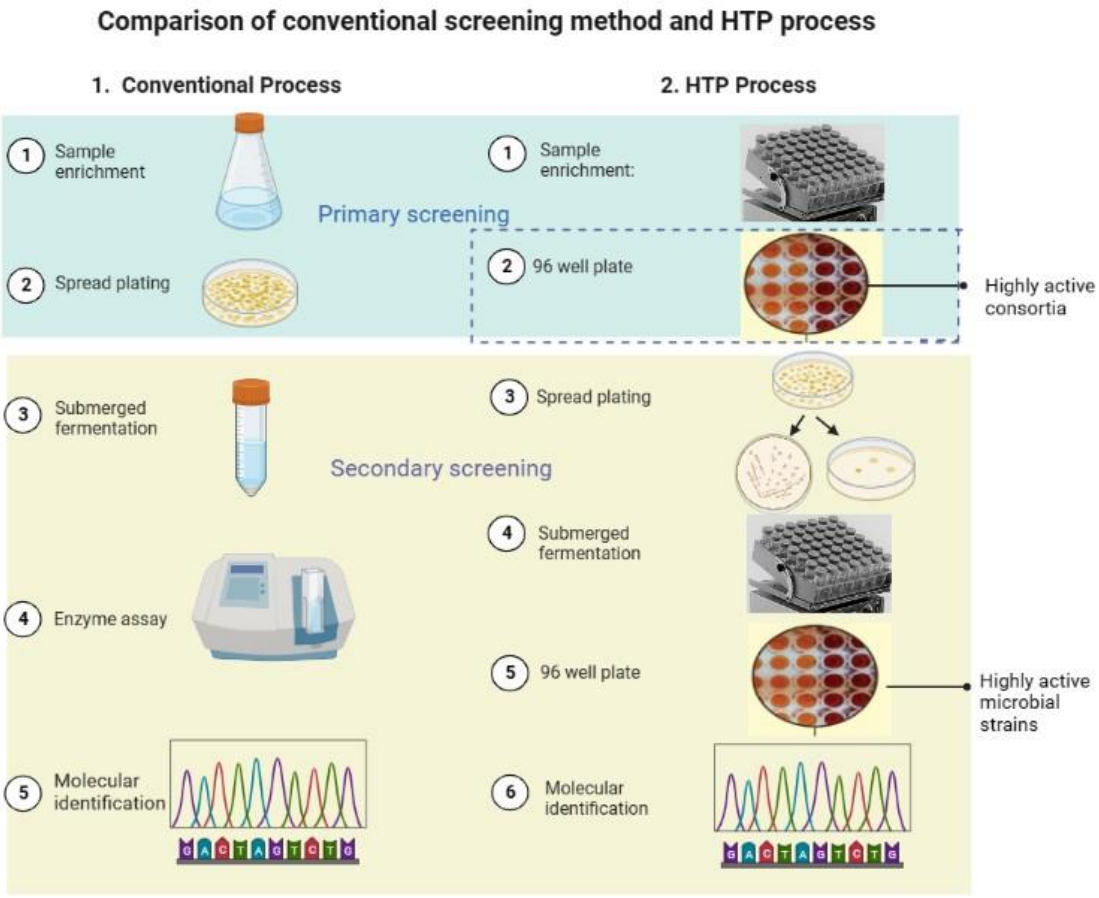


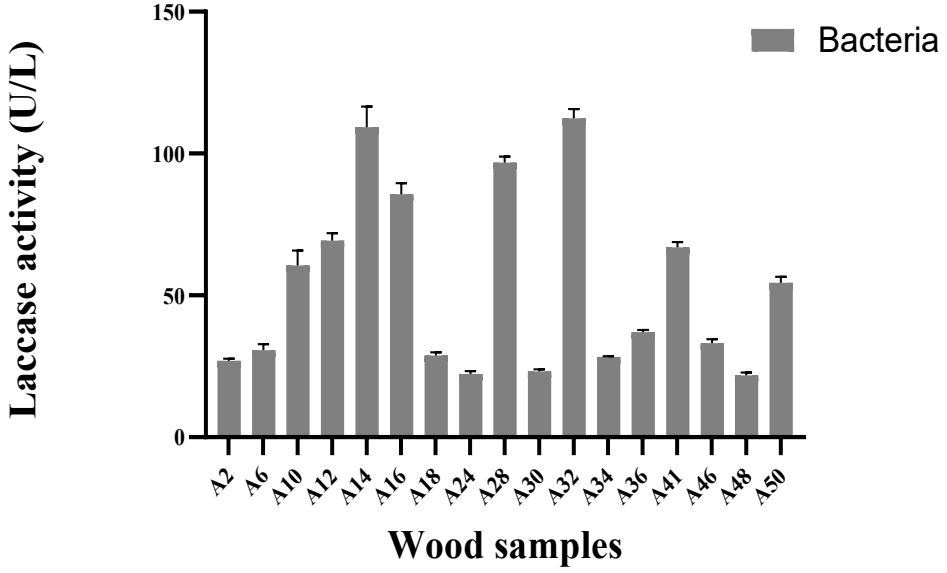
Figure 1. Schematic of the screening workflow for ligninolytic microorganisms using the conventional method (Fang et al., 2018) and the HTP technique in this study.

The process applied in this study allows for the screening of large numbers of natural biomass samples (50 samples, 100 cultures), allowing quick identification of promising consortia. The HTP process was further applied in secondary screening by growing multiple colonies in a small volume that allowed quick isolation of high-activity strains. The identity of the selected strains was confirmed by sequencing, and the reliability of the screening process was further confirmed by growing up some of the selected strains in larger volumes and testing expressed enzyme activities.

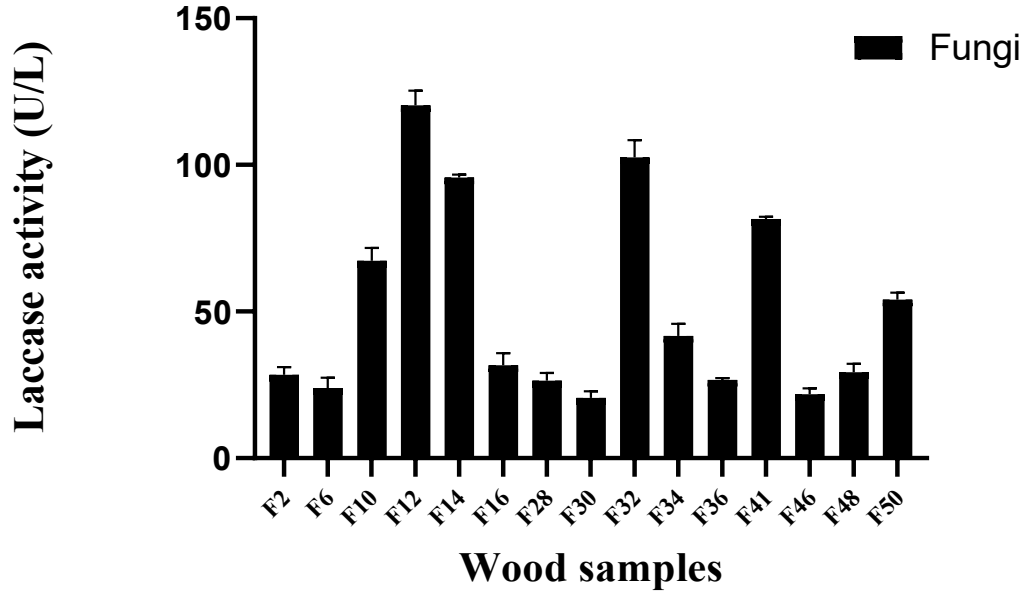
3.2. Isolation and Preliminary Screening of Laccase-Producing Bacterial and Fungal Strains

This study aimed to apply the HTP process to isolate potential ligninolytic microorganisms from the decayed wood samples collected near the Ottawa River in Ontario, Canada. A total of fifty wood samples were incubated by submerged fermentation and checked for total laccase activity quantitatively. Antimicrobials were added in the culturing medium separately for bacterial and fungal cultures, thus separating mixed microbial communities into each individual monoculture.

Out of 50 decomposed wood samples, eight bacterial cultures (A10, A12, A14, A16, A28, A32, A41, A50) and six fungal cultures (F10, F12, F14, F32, F41, F50) demonstrated high laccase activities (≥ 50 U/L), with six samples overlapping in both groups and the other two only showed high activities in bacterial cultures and not in fungal cultures. Additionally, nine bacterial cultures (A2, A6, A18, A24, A30, A34, A36, A46, A48) and nine fungal cultures (F2, F6, F16, F28, F30, F34, F36, F46, F48) showed moderate laccase activity (≥ 20 U/L), with seven overlapping samples (Fig.2 A, B). The remaining 19 bacterial cultures and 21 fungal cultures showed low activity (< 20 U/L), and 14 wood samples showed no laccase activity under either culturing condition (Table 1). As a result, only a small percentage of the biomass samples - 16% for bacterial cultures and 12% for fungal cultures - underwent secondary screening, with the majority of samples (84% for bacterial cultures and 88% for fungal cultures) being excluded. Yet, a good number of both bacterial and fungal strains with high laccase activities were isolated in secondary screening as presented in section 3.3. Therefore, this methodology led to a significant reduction in labor and a considerable increase in the probability of identifying highly positive isolates.



(A)



(B)

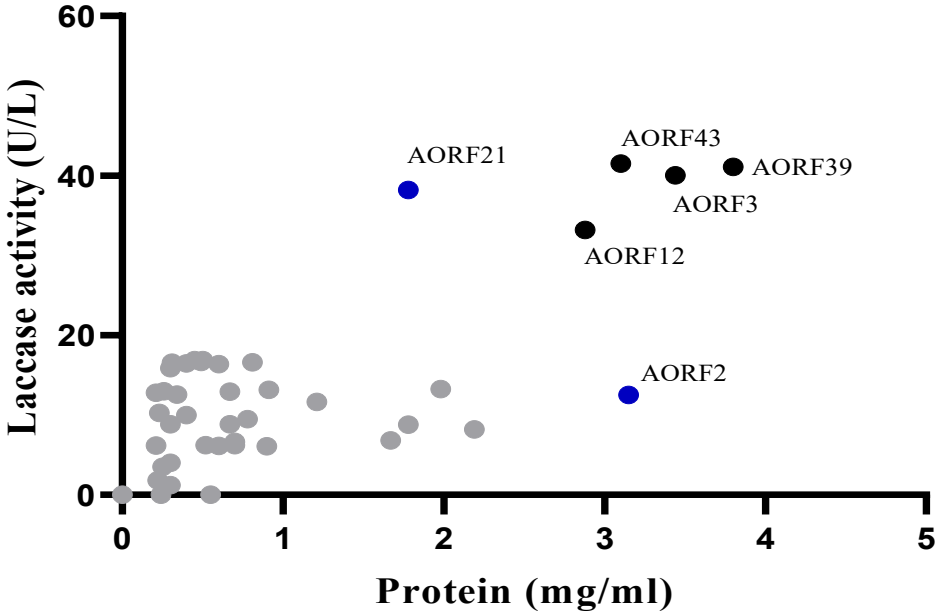
Figure 2. Bar graph representing laccase activity of decomposed wood samples under bacterial (A) and fungal (B) culture conditions with high and moderate laccase activities. Wood samples with laccase activity ≥ 50 U/L were selected for secondary screening.

Table 1. Distribution of wood samples based on laccase activity in primary screening.

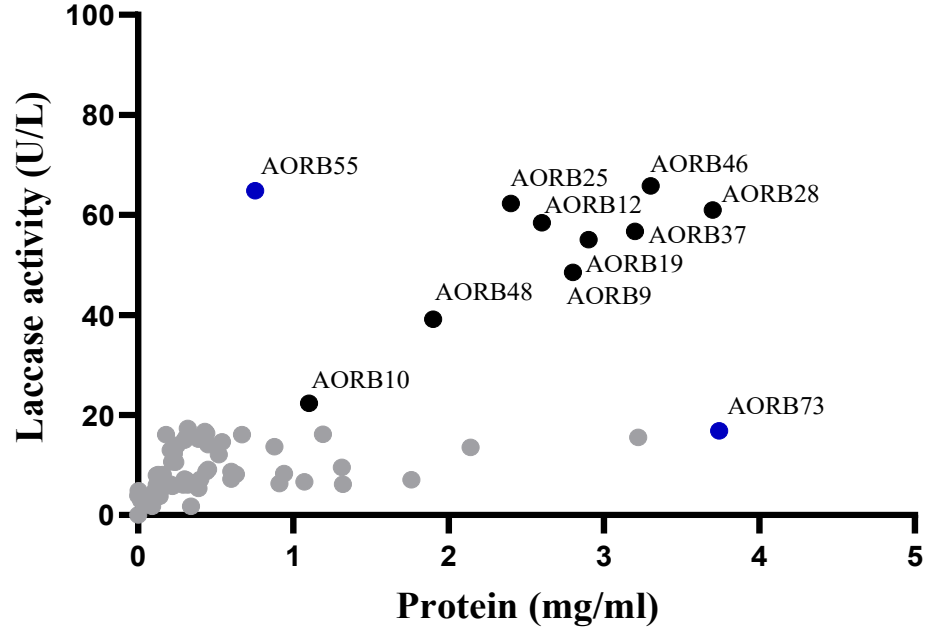
Laccase activity of wood samples in primary screening	Samples under bacterial growth conditions	Samples under fungal growth conditions
Low active wood samples (<20 U/L)	**19**	**21**
Moderately active wood sample (≥ 20 U/L)	**9**	**9**
High active wood samples (≥ 50 U/L)	**8**	**6**
Wood samples with no activity	**14**	**14**
Total wood samples	**50**	**50**

3.3. Secondary Screening for High Laccase-Producing strains

In the secondary screening, the wood samples from primary screening that exhibited high levels of total laccase activities were selected for isolating individual bacterial and fungal strains that express at least 20 U/L laccase activity. Subsequently, ten out of 82 bacterial isolates were chosen after isolating axenic cultures from primary screened wood samples. The potential bacterial strains selected were designated as AORB9, AORB10, AORB12, AORB28, AORB37, AORB46, AORB55, AORB25, AORB19, and AORB48 with laccase activity ranging from 22.31 – 65.77 U/L (Fig. 3A). Similarly, in the case of fungal strains, five out of 46 strains were selected that included AORF12, AORF21, AORF39, AORF43, and AORF39, as they revealed laccase activities ranging from 33.19 – 41.51 U/L (Fig. 3B).



(A)



(B)

Figure 3. (A) Scatter plot representing the total protein content and laccase activity on secondary screening of bacterial isolates. Bacterial strains which showed laccase activity ≥ 20 U/L were selected for molecular

identification (B) Scatter plot representing the total protein content and laccase activity on secondary screening of fungal isolates. Fungal strains which showed laccase activity ≥ 30 U/L were selected for molecular identification. Black dots represent selected strains and grey dots represent non-selected strains. Blue dots showed representative strains with different enzyme activities and protein concentrations.

Total protein concentration in the microbial cultures was also examined in addition to the enzyme activity, and the majority of the strains ($P<0.001$) with low enzyme activity exhibited low protein concentrations. It has been reported that, generally, high protein concentrations might be associated with higher enzyme activity (Veerana et al., 2022). However, it should be noted that protein concentration is not specific to a particular enzyme and can be affected by other factors, such as the growth conditions and the presence of other enzymes and proteins. For example, in the case of a few strains, high enzyme activity was noticed despite low protein concentration (AORB55, AORF21), and high protein concentrations with low enzyme activities were also observed (AORB73, AORF2) as depicted in blue dots in Fig.3 A, B. Assuming the majority of the expressed protein is composed of enzymes, then the laccase-specific activity of AORB55 would be much higher than AORB46, which showed similar activity but a much higher protein content. Future detailed identification and characterization of the specific enzymes would address this question.

3.4. Time course profile of laccase production by selected microbes

The selected microbial isolates were assayed to determine the time course of laccase production. To this end, time course studies were carried out for ten bacterial strains over a 120-h period with 24-h time intervals and five fungal strains over a 240-h period with 48-h time intervals. The results of the laccase activity of selected fungal and bacterial strains are shown in the heat map plot, Fig.4 A and B.

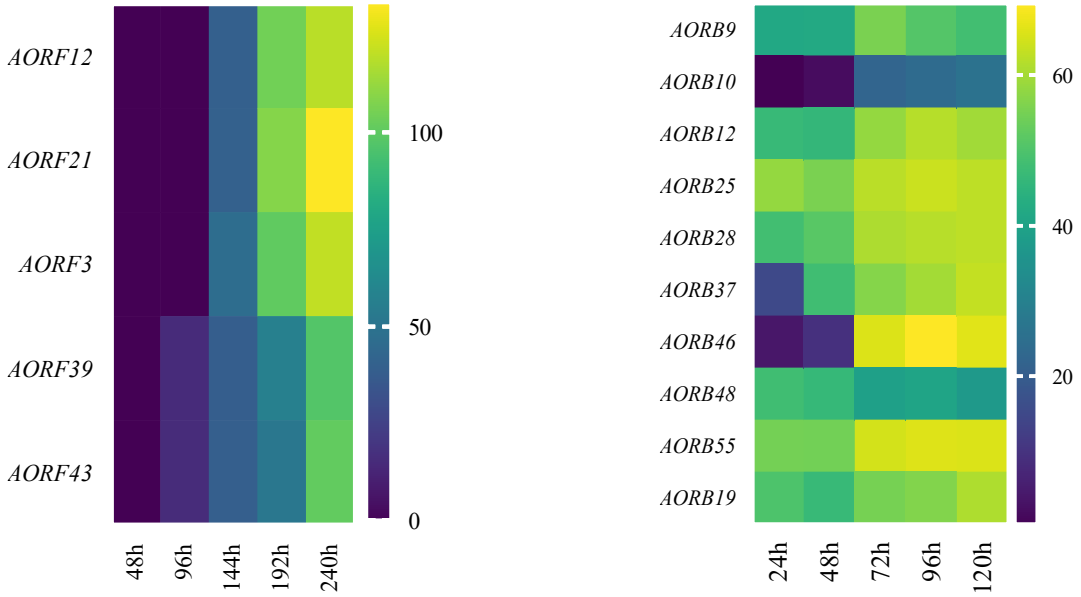


Figure 4. Heatmap of the time course of laccase activity profiles of (A) fungal strains (B) bacterial strains. The purple-yellow color scheme was used to represent the laccase activity in the culture media of individual strains.

The onset of laccase production occurred at different times and was not the same for all bacterial strains. Most tested bacterial strains showed significant laccase activities at 24 h and increased significantly at later hours, with AORB46 showing the highest activity at 96 h (69.42 U/L), followed by AORB55 (66.03 U/L). Further extended cultures after 96 h led to decreased activities in most strains except AORB10, AORB19, AORB28, and AORB37. In the case of fungal isolates, laccase production started at 96 h of incubation for AORF39 and AORF43, the other three stains started at 144 h, and enzyme activities kept increasing during the culturing period up to 240 h. Strain AORF21 (133.44 U/L) showed the highest activity, followed by AORF3 (121.10 U/L) and AORF12(119.35 U/L), respectively. AORF21 from the fungal isolates and AORB46 from the bacterial isolates demonstrated the highest laccase activity among each group.

3.5. Molecular Identification of Selected Laccase-Producing Fungal Strains

After the secondary screening, five potential fungal isolates were selected for genotypic identification based on internal transcribed spacer (ITS) gene sequencing analyses (Figure. 5). BLAST search results of five ligninolytic fungal strains were distributed among four genera in 3 different taxa. This includes an ascomycete *Aspergillus sydowii* 49G11 (AORF21), two basidiomycetes: *Trametes versicolor* ANT213 QFB286 (AORF39) and *Coniferaporia weirii* JV0309 (AORF12) and two zygomycetes: *Mucor circenelloides* Pub005 (AORF39) and *Mucor sp.* CMRP 3219 (AORF43). Phylogenetic tree of fungal strains was constructed using the neighbor joining criteria of MEGA-X software (see Appendix 2).

Many species of the genus *Trametes sp*. including *T. versicolor* are known for their laccase production and its applications in denim bleaching (Bourbonnais et al., 1995). Among the identified fungal strains (see Appendix 4), the two Basidiomycetes - AORF39 and AORF12 are typical white rot fungi that have been reported as the most efficient lignin degraders in nature by the production of extracellular lignin-degrading enzymes (Niku-Paavola et al., 2002). Moreover, the white rot fungus *Coniferaporia weirri* (AORF12), formerly *Phellinus weirii*, is a destructive root pathogen of conifers, and its laccase production has been studied previously (Li, 1981).

Ascomycetes also play an integral role in the recycling of lignin in nature. The strain *Aspergillus sydowii* NYKA 510 has been isolated from agricultural soil and has been re- ported as a powerful laccase producer, and has been successfully employed in a microbial fuel cell (Abdallah et al., 2019). There are few studies on the involvement of lower fungi in laccase production, as Zygomycota do not utilize lignin and cellulose in general. However, few members of this phylum are confined to the outer layers of decomposing plant tissue and can break down lignin (Ingold, 1978, Janusz et al., 2017). Moreover, there are very few studies on the role of lower fungi, zygomycetes, in lignin degradation. Among them, an indigenous litter-dwelling fungus *Mucor circinelloides* GL1 is reported as a promising laccase producer (Geethanjali et al., 2020).

3.6. Molecular Identification of Selected Laccase-Producing bacterial Strains

The BLAST search results of 16S rRNA gene sequence of ten chosen bacterial strains revealed gammaproteobacterial strains under three genera and one strain under phylum Firmicutes, class Bacilli as follows: *Serratia* (*Serratia sp*. CT197(AORB9), *Serratia proteamaculans* 336X(AORB19), *Serratia marcescens* AS09 (AORB28), *Enterobacter* (*Enterobacter sp.* XBGRY7(AORB10), *Enterobacter ludwigii* JUQ409 (AORB25), *Enterobacter hormaechei* EN 336X (AORB55), and *Raoutella* (*Raoultella ornitholytica* 4625 (AORB12), *Raoultella ornitholytica* Sch7(AORB37), *Raoutella ornitholytica* RT 1902(AORB9) and *Bacillus sp.* Ti1 (AORB48) (Figure. 6). Phylogenetic tree of bacterial strains was constructed using the neighbor joining criteria of MEGA-X software (see Appendix 3).

All the ten potential laccase-producing bacterial species identified belonged to the phylum Gammaproteobacteria and class Bacilli (see Appendix 5). Gammaproteobacteria is a large and diverse category of bacteria with a wide range of phenotypes and metabolic capabilities. Their degradative traits are displayed in various nutrient-restrictive and xenobiotic habitats (Unuofin et al., 2019). They have been isolated as one of the predominant bacterial phyla, which have been associated with lignin degradation from tropical forest soil (Pold et al., 2015, Deangelis et al., 2011). Among the genus *Serratia*, *S. marcescens* isolated from a glacial site in India has been shown to produce laccase, which can tolerate wide pH and temperature range (Kaira et al., 2015). The ability of *Serratia proteamaculans* sp. to secrete the laccase enzyme has been studied, and its cultural conditions were optimized (Ali et al., 2022). In addition, *Enterobacter ludwigii,* isolated from decayed wood samples, has been shown to produce laccase and dye-decolorizing properties (Odeniyi et al., 2017). The bacterial species *Raoultella ornithinolytica* OKOH-1, isolated from sediment samples using culture enrichment, showed ligninolytic properties and held significant promise for use in various industrial applications, including the treatment of dye-contaminated wastewater management processes (Falade et al., 2017). More so, there are several

reports of the ability of bacterial strains under the genus *Bacillus* on lignin degradation (Yang et al., 2021, Mei et al., 2020).

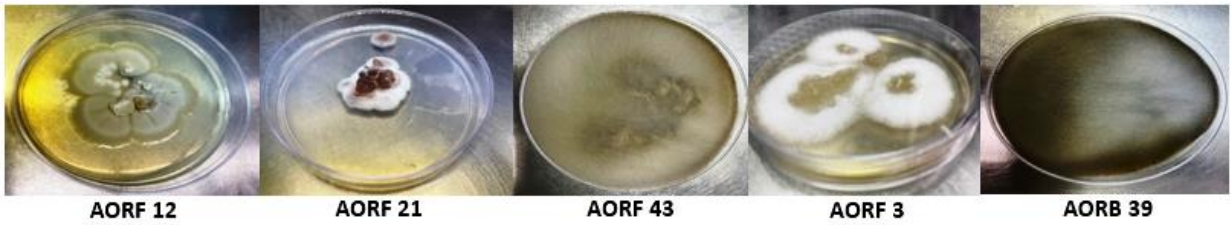


Figure 5. Pure cultures of fungal isolates selected from secondary screening.

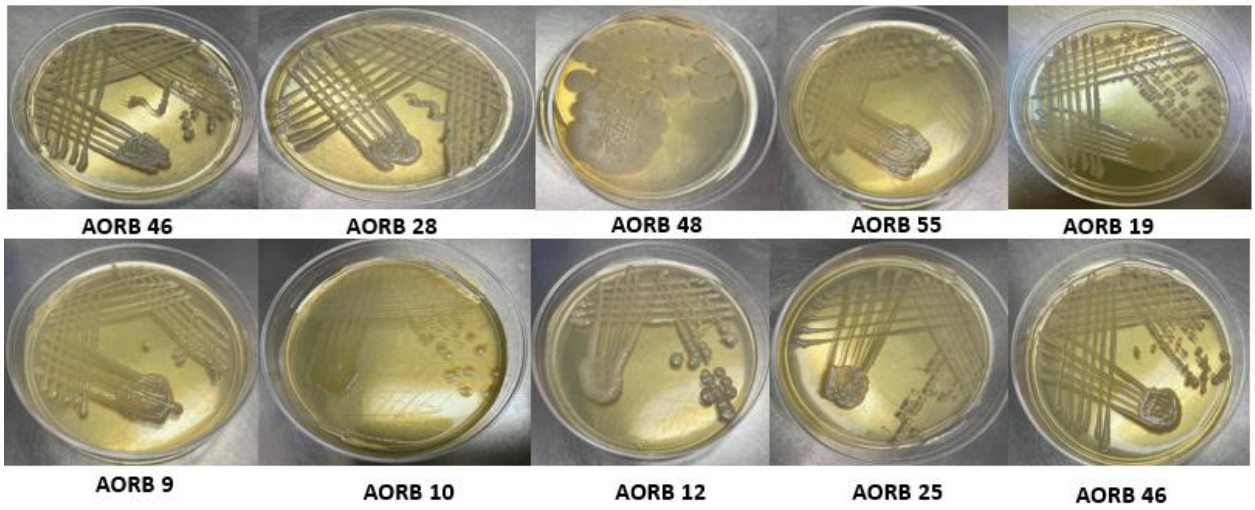


Figure 6. Pure cultures of bacterial isolates selected from secondary screening.

Together, among the identified microbial strains, bacterial candidates AORB10, AORB12, AORB28 and fungal isolates AORF3 showed a sequence identity of 98%. Furthermore, fungal strains AORF43 and AORF39 along with bacterial strain AORB48 displayed sequence identities of 97%, 96% and 95%, respectively. Additionally, bacterial strains AORB55 and AORB37 showed sequence identities of 94% and 91%. Microbial isolates with sequence identity less than 99% compared to reference sequences may potentially represent novel species, and further studies are warranted for their classification. Moreover, a sequence identity below 95% identity is an indication of the possibility of a novel genus (Schoenherr et al., 2018). Thus, this methodology translates into an efficient and successful

HTP screening process and isolation of unique lignin-degrading microorganisms. Although high throughput screening may expeditiously isolate an ensemble of microbes, they essentially might not unlock all entourage of microbes in an environmental sample due to the need for the preparation of varied culture media types based on nutritional requirements that promote growth to enhance their detection and isolation. Thus, the preparation of specific culture media takes precedence allowing for more efficient HTP screening of microbes, including unique isolates from the target samples. Furthermore, while biological lignin degradation is achieved by the combined action of multiple enzymes through diverse degradation pathways, this study was constrained to using laccase enzyme as the exclusive marker for evaluating the lignin-degrading traits of the isolates, consequently, may miss strains producing other enzymes instead of detected ones given the high-throughput nature of the study.

3.7. Xylanase and β-glucanase activity profiles of selected microbial strains

The selected microbial strains were also assessed for their ability to produce other biomass hydrolyzing enzymes, such as β-glucanase and xylanase, on different culture media. The β-glucanase activity was determined in separate media using β-glucan and lignin as carbon sources. In the presence of β-glucan as the carbon source, *Aspergillus sydowii* 49G11 (AORF21) and *Trametes versicolor* ANT213QFB286 (AORF3) displayed β-glucanase activity of 3.13 U/ml and 2.92 U/ml, respectively. On the other hand, *Mucor circinellodes* Pub005 (AORF39) exhibited β-glucanase activity of 3.5 U/ml when lignin was used as the carbon source, while no enzyme activity was detected when β-glucan was the carbon source (Figure 7).

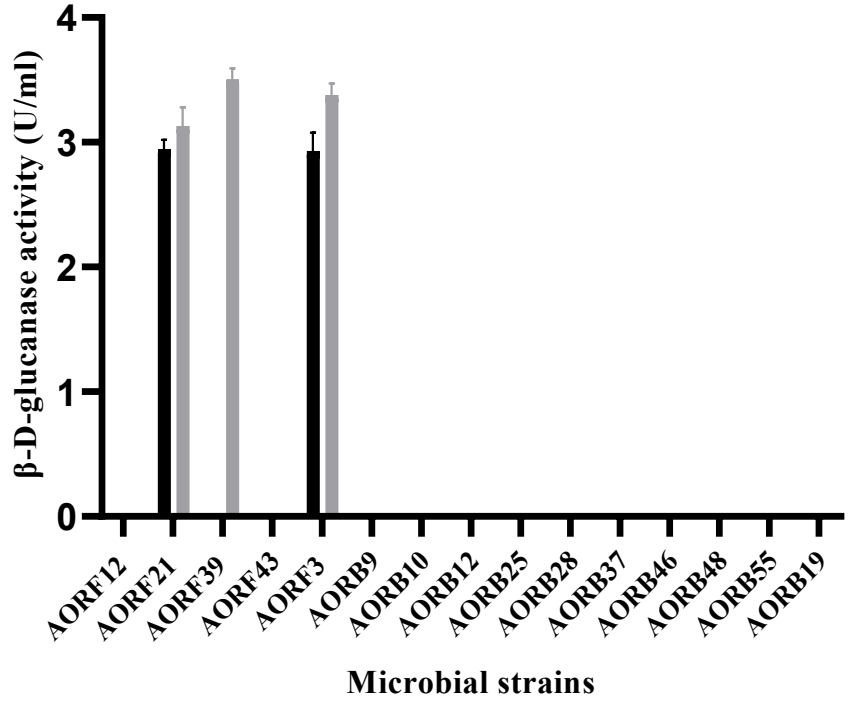


Figure 7. Bar graph representing β-glucanase activity by screened microbial strains using β -glucan as carbon source (Black) and lignin (Grey) as carbon source. Error bars indicate the standard error of the mean of three replicates.

Following this approach, Xylanase activity was measured in separate media using xylan and lignin as carbon sources. When xylan was the carbon source, *Aspergillus sydowii* 49G11 (AORF21) and *Trametes versicolor* ANT213 QFB286 (AORF3) exhibited xylanase activity of 4.09 U/ml and 3.22 U/ml, respectively. In contrast, *Mucor sp*. CMRP 3219 (AORF43) showed xylanase activity of 1.16 U/ml when lignin was the carbon source, with no enzyme activity detected when xylan was used. Furthermore, the bacterial strain *Enterobacter hormaechei* EN 314T (AORB55) demonstrated xylanase activity of 0.66 U/ml and 0.69 U/ml when xylan and lignin were used as the carbon sources, respectively (Figure 8).

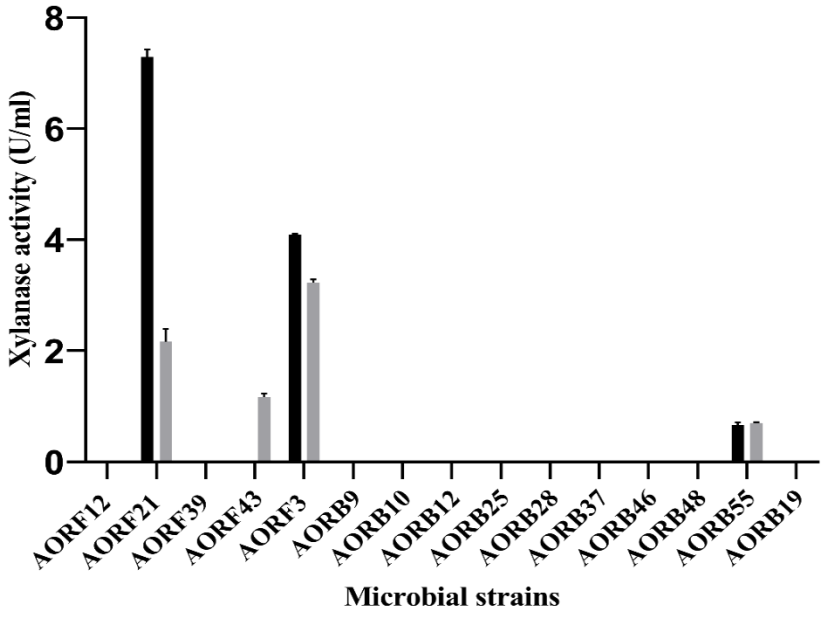


Figure 8. Bar graph representing xylanase activity by screened microbial strains using xylan as carbon source (Black) and lignin (Grey) as carbon source. Error bars indicate the standard error of the mean of three replicates.

Aspergillus sydowii proved to be one of the best xylanase-producing strains isolated from shrimp shells (Brandt et al., 2020). *Trametes versicolor* has been extensively studied and is considered a highly promising strain due to its ability to produce numerous biodegrading enzyme (Tišma et al., 2021). *Mucor Circinellodes* Pub005 and *Mucor sp*. CMRP 3219 exhibited β-glucanase and xylanase while using lignin as a carbon source. *Mucor circinelloides* is a fungus species that are dimorphic and extensively mentioned as a model producer of different enzymes that are useful for various industrial applications (Reis et al., 2019). Additionally, the bacterial strain *Enterobacter sp*. exhibited xylanase activity while using both xylan and lignin separately. A heat and alkali-tolerant *Enterobacter* sp. was obtained from a sediment sample gathered from the Mandovi estuary located on the west coast of India and was determined to be an efficient xylanase-producing strain (Khandeparkar and Bhosle, 2006).

Biological lignin valorization is a complex multi-enzymatic process (Schoenherr et al., 2018), including laccase, which is known to oxidize lignin employing molecular oxygen as the exclusive

cofactor which sets laccase apart from other lignin-degrading enzymes - lignin peroxidases, manganese peroxidases and versatile peroxidase; that depend on H_2O_2 (Munk et al., 2015). Laccase can cause the oxidative breakdown of phenolic units within lignin, resulting in the oxidation of the Cα position, cleavage of the Cα-Cβ bond, and cleavage of aryl-alkyl bonds. It can also attack the non-phenolic subunits of lignin through synthetic and natural mediators (Archibald et al., 1997; Agustin et al., 2021). Several earlier studies have implicated laccase in the biodegradation of lignin, such as in *Sporotrichum pulverulentum*, *Coriolus versicolor*, and *Trametes versicolor*, where laccase constitutes part of medley of enzymes causing lignin degradation in different types of lignin substrates such as model compounds and treated lignin (Ander & Eriksson, 1976; Kawai et al., 1988; Bourbonnais & Paice, 1990). Additionally, *Pycnoporus cinnabarinus*, a fungal mutant strain devoid of laccase failed to secrete manganese peroxidase and lignin peroxidase, thus deteriorating its ability to metabolize synthetic lignin (Bermek et al., 1998; Eggert et al., 1997). Furthermore, it was observed that a mutant strain of fungus, *Pleurotus,* lacking laccase enzyme activity exhibited inefficient degradation of lignin (Kim et al.,1986). In line with these observations and findings, laccase was selected as a marker where microbial isolates are screened to identify the targeted lignin degrading microorganisms using a high throughput screening process.

In the primary screening, lignin was used as the sole carbon source that acts as a selective pressure to limit the growth of microorganisms that cannot use lignin as a single carbon source. This step focused the screening process towards strains that secreted high level ligninolytic enzymes needed to break down lignin polymer into smaller, more easily metabolizable compounds. Unexpectedly, a few, including both bacterial and fungal strains, also showed high-level cellulase and hemicellulase activities. Due to the fact that all enzymes are highly expressed under a single condition, these few strains may have significant valuable applications to be used for their combined or selective delignification ability in lignocellulosic biomass degradation. These results also suggest that this HTP approach may allow the quick

identification of some particularly interesting strains that can produce multiple, synergistic, and cohesive enzymes for efficient lignocellulose hydrolysis.

4. CONCLUSION

Innovative screening techniques allow the isolation of microbes and enzymes that have previously eluded detection and extend the capability to discover novel enzymes. Essential to this endeavor, an HTP screening technique using a modified culture enrichment method for screening and selection of microbial strains from a large number of natural biomasses based on laccase activities was proposed and demonstrated in the study. This two-step strategy quickly identified promising consortia and isolated individual strains for efficient lignin and lignocellulose valorization, resulting in the identification of ten new bacterial and five new fungal strains with high lignin-degrading potential, including *Aspergillus sydowii* and *Raoutella ornitholytica* with the highest laccase activities. Moreover, the study evaluated the capability of the selected strains to produce other hydrolyzing enzymes, showing promising results for reducing enzyme costs in lignocellulosic biorefinery processes and discovering new enzymes with novel activities for the valorization of not only woody biomass but also biological waste, crops, and grains (Murphy et al., 2023).

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CONFLICT OF INTEREST

The authors have no financial conflicts of interest to declare.

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CHAPTER 3

Identification and characterization of a new *Serratia proteamaculans* strain that naturally produces significant amount of extracellular laccase

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Abstract

Natural biodegradation processes hold promises for the conversion of agro-industrial lignocellulosic biomaterials into biofuels and fine chemicals through lignin-degrading enzymes. The high cost and low stability of these enzymes remain a significant challenge to economic lignocellulosic biomass conversion. Wood degrading microorganisms are great source for novel enzyme discoveries. In this study, decomposed wood samples were screened, and a promising γ - proteobacterial strain that naturally secreted significant amount of extracellular laccase enzyme was isolated and identified as *Serratia proteamaculans* AORB19 based on its phenotypic and genotypic characteristics. The laccase activities in culture medium of strain AORB19 was confirmed both qualitatively and quantitatively. Significant cultural parameters for laccase production under submerged conditions were identified following a one-factor-at-a-time methodology (OFAT): temperature 30°C, pH 9, yeast extract (2g/l), Li^{+}, Cu^{2+}, Ca^{2+}, Mn^{2+} (0.5mM) and acetone 5%. Under optimal conditions, a 6-fold increase (73.30 U/L) in laccase production was achieved when compared to the initial culturing conditions (12.18 U/L). Furthermore, laccase production was enhanced at alkaline and mesophilic growth conditions in the presence of metal

ions and organic solvents. The results of the study suggest the promising potential of the identified strain and its enzymes in the valorization of lignocellulosic wastes. Further optimization of culturing conditions to enhance the AORB19 strain laccase secretion, identification and characterization of the purified enzyme and heterologous expression of the specific enzyme may lead to practical industrial applications.

Keywords: Bacterial laccase, Lignin degradation, Lignocellulose, Screening, *Serratia proteamaculans*, Submerged culture

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are copper-containing oxidoreductases that can oxidize a wide range of phenolic and nonphenolic substrates, including ortho, meta, and para diphenols, specifically lignin, a lignocellulose component, and is employed as an attractive tool for the pre-treatment of biomass and its valorization. They are versatile oxidative biocatalysts that contains copper atoms in their active site and oxidize diverse substrates using only molecular oxygen as the known co-substrate instead of hydrogen peroxide, as in peroxidases (Agrawal et al., 2018; Agrawal & Verma, 2020). As green biocatalysts, laccases have been exploited for potential application in broad biotechnological areas viz. environmental remediation processes, biosensor design, synthesis of fine chemicals, food, cosmetic, pharmaceutical industries and synthetic dye decolorization capacity to detoxify a range of noxious and recalcitrant environmental pollutants (Zerva et al., 2019; Yang et al., 2017; Becker and Wittmann, 2019). However, most currently known laccases, are difficult to overproduce in heterologous hosts (Kim et al., 2010). Abundant and inexpensive laccases are needed if the microbiological treatments are to compete with chemical treatments in industries to which it appeals.

Identification and engineering for more efficient and tolerant laccases for industrial applications are ongoing effort to date. Laccase genes dwell in numerous biologically important taxa including plants, insects, lichen, bacteria and fungi, with basidiomycetes class of fungi being the most important source

(Arregui et al., 2019; Hoegger et al., 2006). It has been reported that microbial laccases are primarily involved in wood decay, lignin decomposition, detoxification and linked to resistance to different environmental stresses (Janusz et al., 2020; Arregui et al., 2019). Fungal laccases have been studied in a variety of biotechnological applications due to their high redox potential (Upadhyay et al., 2016; Abdel-Hamid et al., 2013). However, its application is usually hampered due to the long fermentation periods, acidic pH optima, intolerance to extreme conditions and difficulty to overproduce in heterologous hosts (Du et al., 2015; Baldrian, 2006; Kim et al., 2010). Meanwhile, small laccases, both three and two domain laccases, from bacterial sources gained attention recently, due to their exceptional attributes like ability to withstand wide temperature and pH ranges, ease in genetic manipulation and tremendous stability even when inhibitory agents are present. (Chauhan et al., 2017; Sharma & Leung, 2021; Mukhopadhyay et al., 2013; Arregui et al., 2019; Gianolini et al., 2020). In addition, short generation time of bacteria makes it easier to scale up laccase production processes on a commercial scale (Akram et al., 2022; Brugnari et al., 2021). Major bacterial genera which have been reported to produce laccase-like multicopper oxidases include *Streptomyces, Bacillus, Meiothermus, Gramella, Geobacillus, Aquisalibacillus, Lysinibacillus, Azospirillum, Rhodococcus, Ochrobactrum*, *Amycolatopsis, Pseudomonas, Stenotrophomonas, Iodidimonas, Alteromonas* and *Nitrosomonas* (Chauhan et al., 2017; Yang et al., 2021; Arregui et al., 2019; Granja-Travez et al., 2018).

Bacterial species under the genus *Serratia*, belonging to the family Enterobacteriaceae, has been identified for its numerous applications in biodegradation (Dabrowska et al., 2021; Majumdar et al., 2020; An et al., 2021). *Serratia proteamaculans*, a gram-negative, non-pigmented strain under this genus, was first reported to cause a leaf spot disease and is the only identified phytopathogen under the genus *Serratia* (Mahlen, 2011; Paine & Stansfield., 1919). It is widely distributed in nature and is frequently isolated from the gut microbiota of insects, including spiders and bark beetles (Bersanetti et al., 2005; Mikhailova et al., 2014). S*erratia proteamaculans* has been recently recognized for its

capability to produce bio-degradative enzymes (Cano-Ramírez et al., 2016; Madhuprakash et al., 2015; Mehmood et al., 2009) and possess remarkable antagonistic traits against plant pathogens (Wang et al., 2014).

Even though its ability to synthesize extracellular enzymes like chitinase, endoglucanase and protease has been reported (Madhuprakash et al., 2015; Mikhailova et al., 2014; Cano-Ramírez et al., 2016), no research regarding its growth and extracellular laccase production has been reported so far. During an investigation of the cultivable microbial community from decomposed wood on the Ottawa River bank, a large number of bacterial and fungal strains were isolated. Among them, a new strain of *Serratia proteamaculans,* AORB19, has been isolated and characterized that showed significant laccase activities during screening process. The present study characterized the identified strain AORB19; investigated its laccase production potential and the important culture parameters for enhanced laccase production in submerged culture conditions. The characterization and delineation of significant culture parameters of the strain AORB19 in this study might allow for the secretion of further increased amount of laccase thus expanding the possibilities of directly using them in fermentation process or for laccase enzyme production.

2. MATERIALS AND METHODS

2.1 Chemicals

ABTS (2-2'-Azino-bis- [3-ethyl benzthiazoline-6-sulfonic acid]), 2,6 Di methoxy phenol (DMP), Syringaldazine (SGZ), Kraft lignin, Guaiacol, and Czapek dox (CDA). All of the compounds were analytical grade and purchased from Sigma-Aldrich in Canada.

2.2 Organism screening and growth conditions

The decomposed wood samples were collected near the Ottawa river bank (Geographical coordinates: 45◦26’59.7"N, 75◦41’23.3"W). The microorganisms were isolated from the decomposed wood samples by enrichment culture method using Czapek dox broth (L-CDB) with 0.1% lignin alkali (Kraft 98%, Sigma Aldrich, MW>28000). The best strain was selected on the basis of copious and stable laccase activity for a prolonged time period in the culture medium. The pure culture of the organism was maintained in glycerol stocks and fresh cultures from overnight incubation were used in all experiments. The bacterium isolated from wood samples was phenotypically characterized based on the physiological, morphological, and biochemical tests. Besides, bacterium was also further characterized and determined based on 16S rDNA sequencing.

2.3 Phenotypic characterization of the bacterial strain

Bacterial isolate was phenotypically analyzed for presumptive identification, and categorized based on cell morphology, gram’s reaction, colony morphology, growth at various pH and temperatures.

2.3.1 Biochemical characterization of the bacterial strain

The bacterial strain was identified using Microscan conventional biochemical detection system for gram negative bacilli (NID2) panel, Beckman Coulter (Brea, CA, USA) with dehydrated substrates. pH variations, substrate utilization, and growth in the presence of antimicrobials were used to identify the bacterial strain. The panels were inoculated with bacterial culture and incubated following manufacturer’s instructions (Ombelet et al., 2021). The bacterial strain with probability score was identified based on the Beckman Coulter program.

2.4 Molecular identification of bacteria

2.4.1 DNA preparation, PCR amplification and 16S rRNA gene sequencing analysis.

The genomic DNA was extracted using bacterial DNA isolation kit (Bio Basic, Canada). The universal eubacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'- 103 CGGTTACCTTGTTACGACTT-3'; Narde et al., 2004) were used to amplify the 16S rRNA gene. The reaction mixture contained in a final reaction volume of 50 µL with 5 µL DNA template, 200 µM of dNTPs, 1X Phusion buffer, 0.5µM of primer and 1 unit of Phusion DNA polymerase (Thermo Fisher Scientific). The PCR reactions were carried out on a Bio-Rad T-100 Thermal Cycler. 35 cycles were performed to amplify the 16S rRNA gene following the initial denaturation at 95°C for 2 mins, subsequent denaturation at 95°C for 30 sec followed by annealing at 52°C for 30 sec, extension at 72°C for 2 mins and final extension at 72°C for 15 mins. The PCR products were then resolved on a 1% agarose gel and purified using a Qiagen quick gel extraction kit. The purified PCR product was sequenced by Eurofins genomics in Canada for 16S rRNA gene sequencing analysis.

Chromas Lite 2.0 software (https://technelysium.com.au/wp/chromas) was used to assess the quality of the sequences acquired. After aligning the forward and reverse sequences with Bio edit software, a conserved sequence (1404bp) was created. Blast analyses of the conserved sequence was performed (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the sequences that shows maximum identity to those in GenBank. Multiple Sequence Alignment analysis of 18 type strains that showed the highest identity and similarity to the bacterial isolate's sequence was performed using the Clustal W program. MEGA X program was used to determine the evolutionary relationships between closely related sequences using multiple alignment files. The evolutionary history was inferred using the Neighbor-Joining (NJ) criterion and a bootstrap analysis was performed using 1,000 pseudo-replicates.

2.5 Laccase activity measurement

2.5.1 Qualitative laccase assay

The ABTS and guaiacol plate assay method were used to perform a qualitative laccase activity assay (Senthivelan et al., 2019). ABTS and guaiacol were used as substrates to screen the isolated bacterium for laccase enzyme. Individually, the bacteria were inoculated on CDA agar plates with 3 mM ABTS and 4 mM guaiacol and incubated at 27°C. The culture plates were visually observed for color change and formations in the media. Laccase production is indicated by a green or brown halo around the bacterial colony on ABTS and guaiacol agar, respectively.

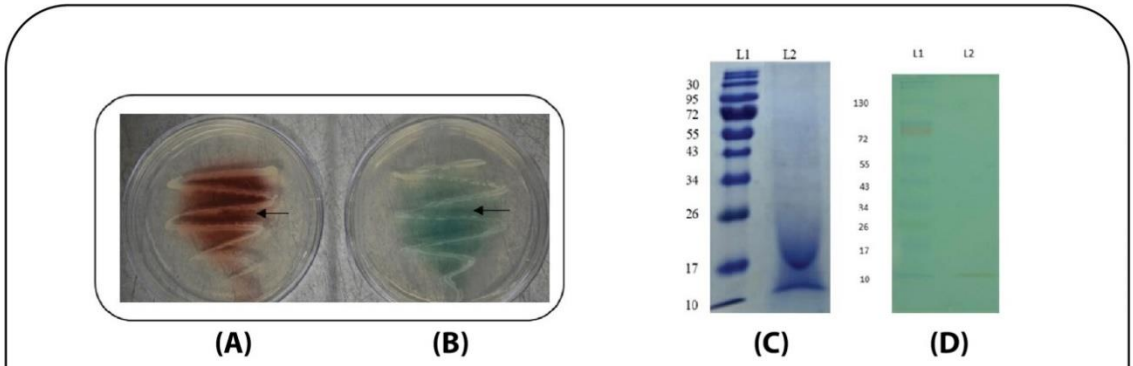

Figure 1 | Confirmation of laccase production by strain AORB19 **(A)** Guaiacol plate assay showing reddish brown oxidation zone around bacterial colony; and **(B)** ABTS plate assay showing green zone oxidation zone around bacterial colony. **(C)** SDS-PAGE analyses of culture supernatant of strain AORB19. L1: Molecular mass marker, L2: culture supernatant; **(D)** NATIVE- PAGE analyses of culture supernatant of strain AORB19. L1: Molecular size marker L2: Laccase activity staining of culture supernatant with ABTS as substrate (green band).

Figure 1. Confirmation of laccase production by strain AORB19

2.5.2 Quantitative laccase assay

A 24 h old freshly grown bacterial colony (O.D.600nm ± 0.05) is inoculated aseptically onto 50ml Czapek dox broth (pH 7) with 0.1% lignin alkali (Kraft 98%, Sigma Aldrich, MW>28000) at 27°C with

125 rpm shaking. The broth culture was centrifuged for 5 minutes at 13000 rpm at 4°C after the incubation m period, and the supernatant was used as the enzyme source.

Laccase activity was determined using 2,6-dimethoxyphenol (DMP) in sodium acetate buffer (0.1 M) pH 5 and quantified spectrophotometrically (SpectraMax M5, Molecular Devices, San Jose, CA, USA) at 469 nm ε469 = 14,800 M^{-1} cm^{-1} (Agrawal & Verma, 2019). A total reaction volume of 210µL was used for the standard assay, which included 160µL 2,6 DMP (10 mM) in sodium acetate buffer (0.1 M, pH 5), and 50 µL crude enzyme. One unit of laccase activity (U) was defined as the amount of the enzyme required to oxidize 1 µmol of 2,6 DMP per minute. A control was run in parallel by replacing the enzyme with buffer under standard conditions. With minor modifications, laccase activity was determined based on the formula described by (Leonowicz and Grzywnowicz, 1981).

$$(U/L) = (\Delta Abs \times V \times 106) / (\varepsilon \times Ve \times d \times \Delta t)$$

Where ΔAbs is the difference in absorbance values, ε is the extinction coefficient; V is the total volume of the sample (mL); Ve is the volume of the enzyme (mL); Δt is the time (min); and d is the path length of microplate.

Additionally, the laccase activity was also determined using the following substrates: (a) ABTS (ε420 = 36000 M^{-1} cm^{-1} ; Johannes & Majcherczyk, 2000) in 100 mM sodium acetate buffer pH 5.0 with 5 mM final concentration of the substrate; (b) Syringaldazine (ε 530 = 65000 M^{-1} cm^{-1}; Holm et al., 1998)in 100 mM phosphate buffer pH 6.0 with 0.22 mM final concentration of the substrate; and (c) L-DOPA (ε 530 = 3600 M^{-1} cm^{-1}; Jara et al., 1988) in 10 mM sodium phosphate buffer pH 6.8 with 7.6 mM final concentration of the substrate.

2.6 Optimal culture conditions for laccase production

To investigate the optimal conditions for laccase production, one factor at a time (OFAT) strategy was employed on *Serratia proteamaculans* AORB19 strain. Growth conditions of the bacterial strain were

evaluated to identify the best parameters for maximum laccase production. The tested variables include: physicochemical conditions (temperature and pH), nutritional conditions (Carbon sources, nitrogen sources), the addition of organic solvents (acetone, chloroform, formaldehyde), and metal ions ($Mn^{2+,}$ Cu^{2+}, Ca^{2+} and Li^{+}). In addition, the OD values of liquid culture were measured and tabulated to compare bacterial cell growth and laccase production. Optical density of bacterial growth was determined spectrophotometrically at λ = 600 nm. The results were analyzed and illustrated graphically by Graph Pad Prism, version 9.0.0 software.

2.7 Confirmation of *Serratia proteamaculans* Strain AORB19 laccase activities

2.7.1 Crude enzyme preparation

The supernatant was collected by centrifugation at 13000 rpm for 5 minutes after bacterial culture was induced with 0.1mM ABTS. The supernatant was filtered using a Millex syringe filter unit with a 0.22 m pore size (Millipore, Sigma) to remove intense brownish color of the lignin degradation products from Kraft lignin present in the culture medium. The filtrate was concentrated using centrifugal concentrator (Vacufuge plus, Canada) under vacuum conditions.

2.7.2 SDS-PAGE and Zymogram analysis

For sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), a 12 percent polyacrylamide slab gel was employed (Laemmli, 1970). Native gel electrophoresis was performed using tris-glycine as running buffer (pH 8.3), native loading dye (Bio Rad) under non-reducing and non-denaturing conditions on 10 % resolving gel omitting SDS and heating step to restore the native state of protein. After that, samples were electrophoresed at 50 V on the stacking gel (6%) and 100 V on the resolving gel. After electrophoresis, the gel was immersed for 30 minutes at room temperature in 100 mM sodium acetate buffer (pH 4) which is then submerged in 2 mM ABTS in the same buffer. Laccase

activity bands was visualized and confirmed by the appearance of green colored bands on the gel due to oxidation of ABTS.

2.8 Statistical analysis

All experiments were carried out in triplicates (n=3), and the results were expressed as mean ± standard error in Graph Pad Prism Software. One-way ANOVA with Dunnett's multiple tests were carried out for post-hoc comparisons using Graph Pad Prism 9.0.0. The Dunnett's test range test values ($P<0.0001$) are significant and ($P>0.05$) are non-significant respectively.

3. RESULTS

3.1 Phenotypic and biochemical characterization of laccase-producing bacterial strain

The bacterial strain developed whitish, mucoid opaque, convex colonies on Czapek Dox agar after 24 h incubation at 27°C. Microscopic morphology revealed gram-negative bacilli. The bacterium was facultative anaerobic in nature, able to thrive at temperatures ranging from 20°C to 37°C (opt. 30°C) and pH levels ranging from 6 to 11 (opt.7-9 pH). The strain was able to utilize five out of nine sugars in carbohydrate fermentation experiments. Furthermore, the strain was positive for lysine, ornithine, galactosidase, citrate and voges-poskauer tests. Negative results were observed with arginine, tryptophan deaminase, indole, malonate, acetamide, tartarate, H2S and esculin hydrolysis. The bacterial strain was found resistant to cephalothin (8g/mL), among all other antibiotics used in the gram-negative ID Type 2 (NID2) panel (see Appendix 8).

Following the phenotypical characterization, the bacterial strain was subjected to molecular identification using 16SrRNA gene sequencing. The size of PCR amplified 16S rRNA gene fragment was approximately 1,500 bp and was indicated by an intact band (see Appendix 6). Purified PCR product

was sequenced and showed 99% homology to the 16S rRNA sequence of *Serratia proteamaculans* 336X. A total of 18 deposited 16S rRNA sequences with the highest identities were selected and aligned with the sequenced 16S rRNA sequence. Phylogenetic tree confirmed that the isolated bacterial strain belongs to *Serratia proteamaculans* and named *Serratia proteamaculans* AORB19 (see Appendix 7).

3.2 Confirmation of *Serratia proteamaculans* AORB19 laccase activity

3.2.1 Qualitative laccase assay

After 48h of incubation, the isolate's ability to produce laccase enzyme was qualitatively assessed by the formation of a reddish-brown colored zone around colonies on guaiacol agar plates and a green-colored zone around colonies on ABTS agar plates (Fig.1 A & B).

3.2.2 Quantitative laccase assay

The strain AORB19 was incubated on L-CDB medium for 24h and supernatant was used as the crude enzyme to analyze laccase activity. The laccase activity was measured spectrophotometrically using phenolic substrate 2,6 DMP as a substrate and its oxidation in the presence of laccase enzyme results in the formation of a stable dimeric form, 3,3P,5,5P tetra-methoxy-diphenyl-quinone with the appearance of a bright orange color (Solano et al., 2001). Laccase activity was also determined by measuring the rate at which syringaldazine was oxidized to generate tetra-methoxy-azo-bis-methylene quinone, which developed a pink to purple tint (Holm et al., 1998). Furthermore, the oxidation of ABTS to the cation radical $ABTS^{+}$ was catalyzed by laccase, as demonstrated by the green-blue color (Johannes & Majcherczyk, 2000). Three distinct substrates, DMP, SGZ, and ABTS, each leading to specific products observable at 469nms, 530nms, and 420nms respectively, were observed yielding laccase activity of 0.0384 µmol/mg/min, 0.0062 µmol/mg/min, and 0.0088 µmol/mg/min, respectively.

The culture supernatant of *Serratia proteamaculans* AORB19 was further analyzed for its ability to oxidize ABTS, syringaldazine and tyrosine spectrophotometrically. Both syringaldazine and ABTS were oxidized, but no positive reaction for tyrosine oxidation was detected (data not shown). The most common substrates used for laccase are 2,6 DMP and guaiacol that can also be oxidized by other enzymes. Enzymes exhibiting oxidation of ABTS and SGZ but not tyrosine are regarded as laccase specifically (Sondhi & Saini, 2019). Our assay results revealed that the bacterial isolate could oxidize ABTS and SGZ but not tyrosine, indicating that strain AORB19 mostly secreted laccase in L-CDB medium. Since L-CDB medium contains lignin as the sole carbon source, it is commendable to suggest that strain AORB19 and its secreted laccases may be applicable for lignin degradation and valorization.

3.2.3 In-gel assay of strain AORB19 laccase activity

Cell culture supernatant of strain AORB19 was analyzed by SDS-PAGE (Fig. 1 C) and multiple bands were observed suggesting that mixture of proteins was secreted in the collected samples. As our assay detects total laccase activities, it is not sure if the detected activities were from different enzymes or single enzyme. The supernatant was also analyzed by native PAGE followed by In-gel enzyme activity assay using ABTS s substrate (Fig. 1 D). The green band towards the lower molecular weight end of the gel indicated that our detected laccase activities may be due to a single enzyme. However, native gel cannot determine the size of the enzyme.

3.3 Time course study

Growth and laccase production by strain AORB19 was studied in L-CDB medium up to 144 h. Laccase production in the L-CDB media was easily detectable after 12 h of incubation, increased significantly during the exponential phase (12-36 h), and peaked at the early stationary phase ~ 48 h of incubation. Afterwards, the laccase activity showed slight decrease but more or less maintained stable throughout the rest of the incubation time. The cell growth and density curve based on OD600 readings corresponds

well to the enzymatic activity curve (Fig. 2A) suggesting that cell growth stopped and entered stationary phase at ~48 hour.

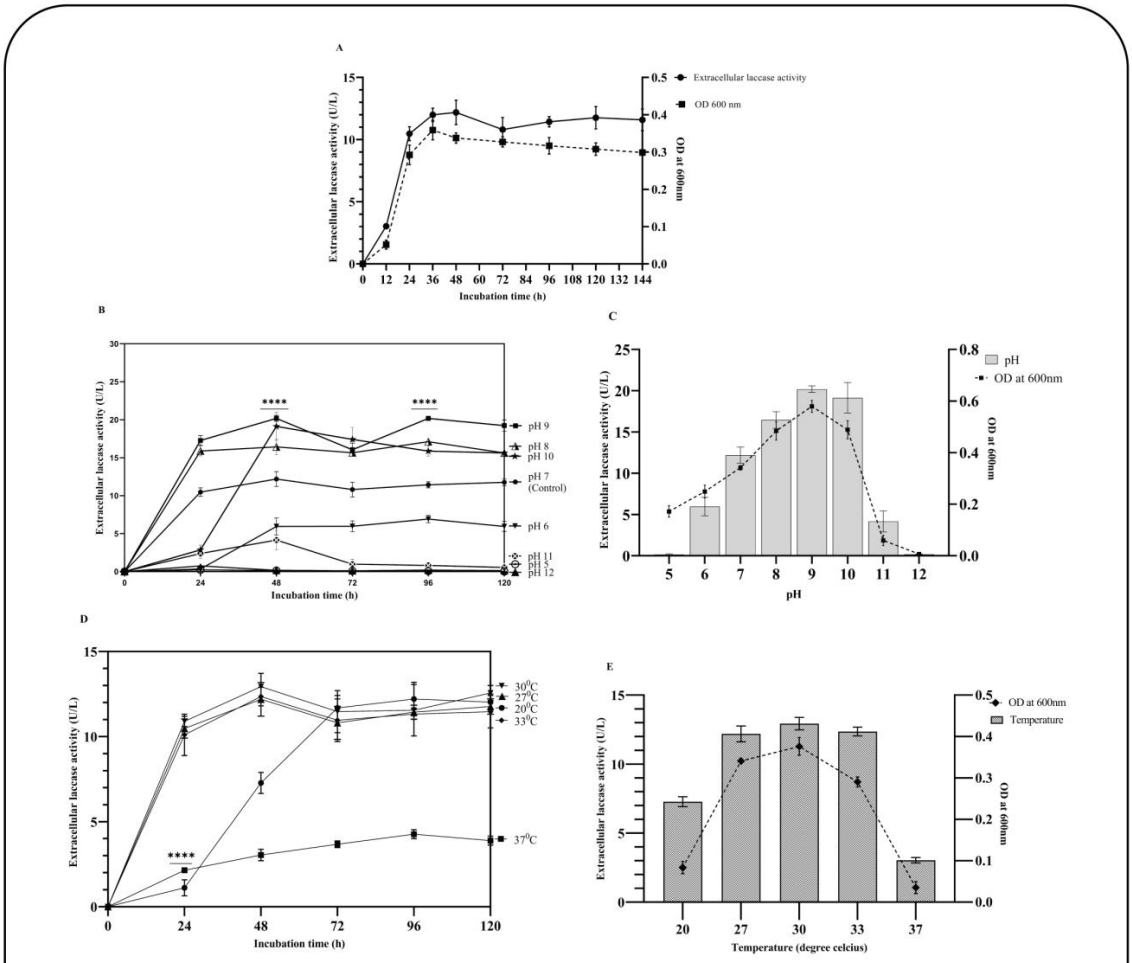

Figure 2 | (A) Growth and laccase activity profiles of strain AORB19. **(B)** Effect of initial pH values on laccase production; **(C)** Effect of pH on growth and laccase production at 48h; **(D)** Effect of temperature values on laccase production; and **(E)** Effect of temperature on growth and laccase production at 48h. The four stars (****) denoted the statistical significance according to the Dunnett's multiple comparison test at P< 0.0001.

Figure 2. Growth and activity profiles of strain AORB19.

3.4 Effect of pH and temperature

The effect of pH on strain AORB19 extracellular laccase activity was explored by adjusting the initial cultural media pH from 5-12 and culturing the cells for 120 h. (Fig. 2B). Maximum activities were achieved at different pH and higher laccase activities were detected with neutral to weak alkaline media (pH 7-10). To explore if the impact of media pH on laccase activity was due to impact on cell growth, laccase activities and cell densities of 48h cultures were compared and indeed higher cell density corresponds well to higher laccase activity (Fig. 2C).

The growth and enzyme yield were both adversely affected by an acidic pH (5.0). Compared to control pH 7 (12.18 U/L), laccase production reached its maximum at pH 9 ($P<0.0001$, 20.18 U/L) with a 65% increase, followed by 56% increase (19.13 U/L) at pH 10, and 34% increase (16.44 U/L) at pH 8. The maximum bacterial biomass at pH9 indicated the alkaliphilic nature of strain AORB19. However, higher pH (11) inhibited cell growth as much as lower pH (5) and at pH 12, cell growth was totally inhibited.

Strain AORB19 was cultivated for 120 h at various temperatures ranging from 20°C to 37°C to determine the optimal temperature for laccase production. Similarly, higher laccase activities were detected between temperatures of 27°C - 33°C and maximum activities were reached at 48h incubation. Lower temperature (20°C) showed lower activity at 48 h and caught up at 72 h. However, at higher temperature (37°C), laccase activities were consistently low over the whole culturing period (Fig. 2D).

To explore if the impact of laccase activities by different temperatures is related to cell growth, cells were grown for 48 h and the corresponding cell densities and enzyme activities were monitored (Fig. 2E). The highest laccase activity was recorded at 30°C (12.93 U/L) followed by a slight reduction at 33°C (12.35 U/L) and 27 °C (12.18 U/L). Much lower activities were observed at 20°C, and the lowest ($P<0.0001$) at 37°C (Fig. 2E). Correspondingly, similar pattern of cell densities was observed suggesting that the enhanced laccase activity was due to increased cell growth.

3.5 Influence of Nitrogen and Carbon Sources

Nitrogen sources like ammonium sulphate, yeast extract and Sodium nitrate were added exogenously to determine their effects on laccase production. Addition of yeast extract to the culture media yielded 19% increase in laccase production (14.50 U/L) at 48h, when compared to control medium. When compared to other nitrogen substrates, yeast extract was shown to be the best nitrogen source in the study ($P<0.001$), resulting in higher laccase yields (Fig. 3A). Ammonium sulphate resulted in a 34% decrease (8.05 U/L) in laccase production at 48h. Different nitrogen sources were rated in terms of their impact on laccase production: yeast extract > sodium nitrate > ammonium sulphate.

Carbon source of the original medium (Sucrose, control) was replaced with fructose, maltose, xylose and cellulose, each of which was tested individually for impacts on laccase activities. Among the tested carbon sources, none performed better than the control with the following rank: sucrose (12.18 U/L) > glucose (5.96 U/L) > maltose (5.52 U/L) > fructose (1.18 U/L) > Cellulose (0.76 U/L). Particularly, the latter two substitutions significantly lowered ($p< 0.0001$) laccase production (Fig. 3B).

3.6 Influence of metal ions and organic solvents on laccase production

The low and high molecular weight organic solvents, acetone, formaldehyde and chloroform were added to the medium in two concentrations (5%, 10%), separately. It was noted that acetone (5% and 10%) enhanced laccase production by 15% and 21% at 48h (14.03 U/L and 14.86 U/L). During the initial phase of incubation (24 h), the presence of chloroform (5% and 10%) lowered laccase production (7.65 U/L and 4.73 U/L, respectively), but this was gradually increased by 48 h. On the other hand, laccase production was significantly inhibited ($p< 0.0001$) when formaldehyde was present in the culture media (Fig. 3C).

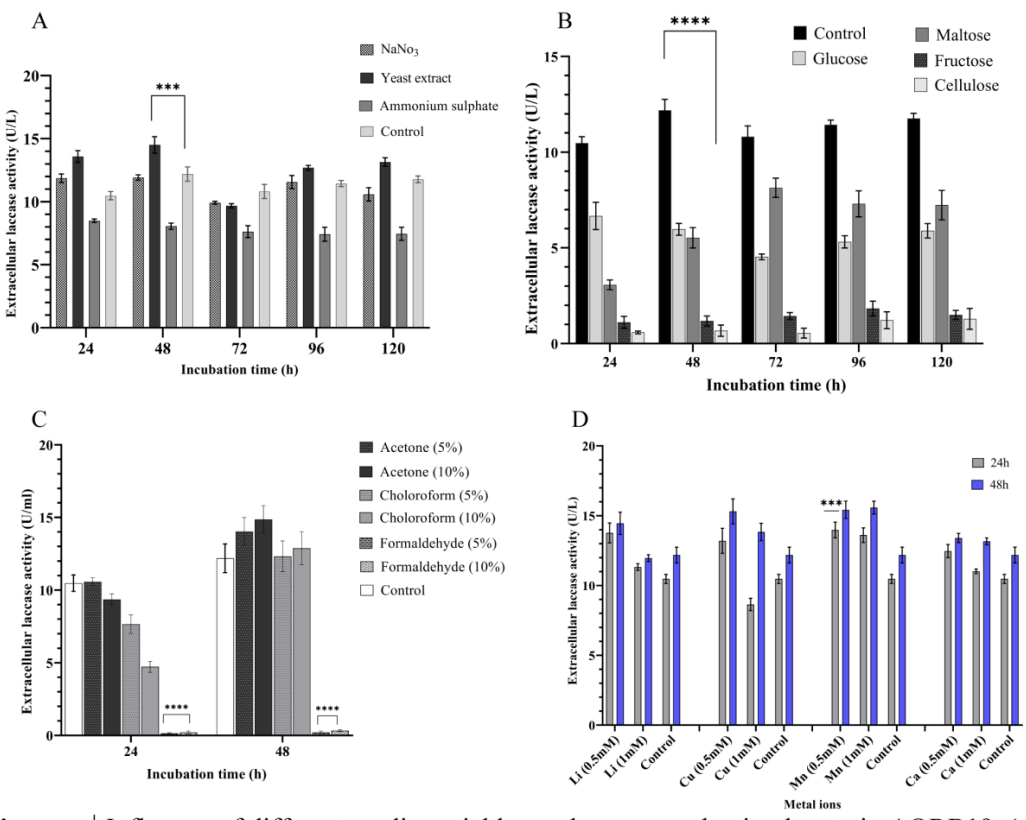

Figure 3 | Influence of different media variables on laccase production by strain AORB19. **(A)** nitrogen source; **(B)** carbon source; **(C)** organic solvents; and **(D)** metal ions. The four stars (****) and three stars (***) denoted the statistical significance according to the Dunnett's multiple comparison test at P< 0.0001 and P< 0.001 respectively.

Figure 3. Influence of media variables on laccase production by strain AORB19.

Cations Mn^{2+}, Cu^{2+}, Ca^{2+} and Li^{+} (0.5mM and 1mM) was separately supplemented to the basal medium to determine their effects on laccase production by *Serratia proteamaculans*. All metal ions used in the study stimulated laccase yield positively at 0.5mM concentration. Addition of Mn^{2+} (0.5mM and 1mM) increased laccase production by 33% (P<0.001) and 29% at 24 h (13.98; 13.60 U/L) and 25% and 10% at 48 h (15.57; 15.35 U/L) respectively. Furthermore, the addition of Li^{+} and Ca^{2+} (0.5mM) improved laccase production by 31% and 18% at 24 h (13.77 U/L; 12.47 U/L). Cu^{2+} (0.5mM) enhanced laccase production by 25% at 24h and 48h (13.20 U/L) consistently. However, at 1mM concentration,

laccase production showed a decline in the presence of Cu^{2+} at 24 h (8.64 U/L) which was followed by an increase of 13% (13.83 U/L) at 48 h (Fig. 3D).

3.7 Influence of Media Variables

To reach and hold the points of optimum levels, experiments were performed on culture media by varying one media variable at a time to monitor cell growth and laccase production. Different media variables affecting laccase production was tested under submerged fermentation by inoculating 1% v/v (O.D.600nm, 0.1) of freshly grown overnight culture of AORB19 strain on 50 ml L-CDB. The culture suspension was withdrawn periodically as per the factor analyzed and the supernatant was measured for laccase activity with 2,6 DMP assay. A control was also included with all the factors present except the one being studied. Subsequently, submerged fermentation with all the factors at optimum condition were carried out and compared to compute the fold increase in laccase production.

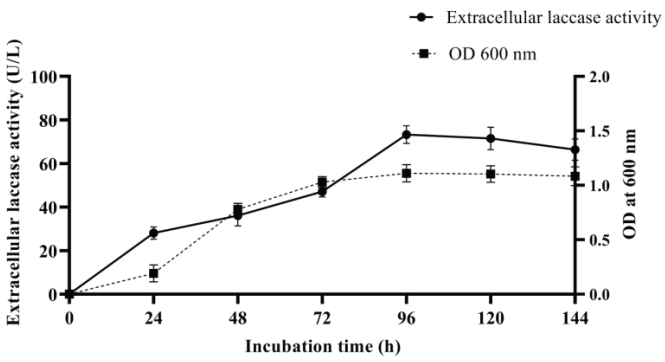

Figure 4| Extracellular laccase production by strain AORB19 using optimized media.

Figure 4. Extracellular laccase production by strain AORB19 using optimized media.

The variables enhancing laccase production were: temperature 30°C, pH 9, yeast extract (2g/l), Li^{+}, Cu^{2+}, Ca^{2+}, Mn^{2+}(0.5mM) and acetone 5%. Under optimal condition, a 6-fold increase (73.30 U/L)

in laccase production (Fig. 4) was achieved when compared to the initial pre-optimized media condition (12.18 U/L).

4. DISCUSSION

As the quest for economic, novel and robust enzymes becomes increasingly important for biotechnological applications, more attention has been paid to systematic exploitation of microorganisms through natural biodiversity screenings and optimization of cultural conditions to produce abundant enzymes that are affordable as well as stable and active under desired operational conditions. By screening microorganisms of decayed wood stumps in this study, we have isolated a bacterial strain that naturally secreted significant amount of laccase enzyme activities.

The selected strain was characterized phenotypically based on its morphological, physiological and biochemical characteristics. Unlike many other species like species in the genus *Serratia* (Grimont et al., 1978), the bacterial strain produced non-pigmented colonies with a characteristic mucoid texture on nutrient agar. The biochemical reactions like l-arabinose, ornithine and lysine decarboxylase, dulcitol, adonitol, d-arabitol and d-sorbitol fermentation can aid to diversify different biogroups or species with in the genus *Serratia* (Rafii, 2014). The bacterial strain was identified with a high probability score of 99.9% from the MicroScan version 4.4 x databases as *Serratia liquefaciens* complex which included *S. proteamaculans*, *S. grimesii*, and *S. liquefaciens*. As species specific identification of *Serratia liquefaciens* complex was not included in the database, the results were further validated using 16S rRNA gene sequencing. The results of 16S rRNA gene sequencing were comparable with the biochemical characterization and the species showed 99.9% homology to *Serratia proteamaculans*.

Aromatic compounds formed during the lignin degradation process are lethal to many microorganisms and can negatively affect productivities in the fermentation of lignocellulosic hydrolysates (Henson et al., 2018). However, while investigating the lignin degrading potential, it was

found that the isolated strain AORB19 was able to grow well with lignin as a sole carbon source in the culture media and produced copious laccase. In the study by Wang et al., (2021), it was reported that cell growth in the lignin-based culture medium is linked to lignin degradation by extracellular enzymes. Thus, *Serratia proteamaculans* AORB19 possesses an important phenotype of lignin degrading microorganism in terms of bacterial cell proliferation and laccase production in lignin-based media. Beyond this indication, the bacterial strain also exhibited exceptionally stable laccase activity in the culture medium for a prolonged period suggesting an inherent tolerance of the strain and its enzymes to end-product accumulation during lignin degradation and metabolism.

Microbes produce extracellular enzymes to breakdown complicated organic materials into usable compounds that can be transported through the cell membrane (Ramin & Allison, 2019). The time course analysis of strain AORB19 revealed extracellular laccase production throughout the growth phase following a short lag phase. During the exponential phase (12- 36h), there was a marked increase in laccase production in the growth medium and peaked at the early stationary phase, reaching the maximum by 48 h of incubation. The time for optimum laccase production vary depending on the microorganism. For *Psuedomonas extremorientalis* BU118 and *Bacillus subtilis* MTCC 2414, 24h and 96h were reported (Neifar et al., 2016; Muthukumarasamy et al., 2015).

The temperature and pH of the culture medium have a substantial impact on the strain's cell growth and metabolite synthesis. Although the production of laccase was estimated between pH 5 and 12, the optimal range of pH was about 7-10, which corresponded to optimal cell growth in the study. It was noticed that the laccase production remained steady throughout the incubation period except minor ups and downs during the stationary phase of incubation. Laccase production from other species of *Serratia* has been reported around a neutral to alkaline pH range and in mesophilic temperature (Chandra et al., 2012). For strain AORB19, the optimal laccase production and cell growth occurred between 27-33°C similar to *Bacillus sp*., 30°C (Sondhi & Saini, 2019).

As nitrogen is a fundamental component of proteins and nucleic acids, it is widely known that it is one of the most important nutrients for microbial metabolism (Hernández et al., 2015). For strain AORB19, yeast extract was the most effective nitrogenous source and was consistent with a previous discovery regarding *Bacillus subtilis* DS (Kumar et al., 2018). In this study, maximum laccase production was noticed while using sucrose as the carbon source consistent with a study by (Muthukumarasamy et al., 2015), for *Bacillus subtilis* MTCC 2414 strain. Cellulose and fructose significantly inhibited laccase production. While fructose is readily assimilable, cellulose may increase medium viscosity thus lower oxygen supply and interfere with cell division and metabolic rate (Othman et al., 2018)

Laccase production of strain AORB19 was enhanced by the organic solvents acetone and chloroform with acetone exhibited higher impact. Such inductions by organic solvents have also been reported with *Serratia marcescens* and fungus GBPI-CDF-03 and results were in concurrence with prior studies (Kaira et al., 2015; Dhakar & Pandey, 2013; Wu et al., 2019). Our study showed that several metal cations improved strain AORB19 laccase production. Copper was found to induce laccase production in MTCC2414 (Muthukumarasamy et al., 2015). At 0.5 mM, Cu^{2+} increased laccase production, but at 1 mM, Cu^{2+} initially lowered laccase production at 24h, and then increased enzyme production on further incubation. This reversal can be explained by the considerable toxicity of copper at greater concentrations, as well as the restriction of normal metabolic pathways caused by redox cycling of ions. As a result, laccase production could be viewed as a regulating system used by bacteria to survive the toxicity of inorganics like metal ions by changing their oxidation status under aerobic conditions (Kaur et al., 2019).

Laccase synthesis by microorganisms can be influenced by many factors like chemical and nutritional composition of the media; optimization approach used may be strain specific. In this study, factors (temperature 30°C, pH 9, yeast extract (2g/l), Li^{+}, Cu^{2+}, Ca^{2+}, Mn^{2+}(0.5mM) and acetone) were used to enhance laccase synthesis using one factor at a time strategy, resulting in a 6.0-fold overall

increase (73.30 U/L) in laccase yield. However, by employing a statistical approach of culture media optimization using five factors by response surface methodology, a 5.5-fold increase in laccase yield (58 U/L) was reported for Pseudomonas putida LUA15.1 (Verma et al., 2015). Similarly, *Streptomyces psammoticus* MTCC 7334 produced three-fold increase in laccase using response surface methods (Niladevi et al., 2009). Furthermore, when compared to the unoptimized media (2.05 U/ml), a four-fold increase in laccase production in *Bacillus cereus* TSS1 (9.03 U/ ml) was attained after altering the media variables using response surface approach (Rajeswari et al., 2015).

5. CONCLUSION

Taken together, a new bacterial strain *Serratia proteamaculans* AORB19 has been characterized that stably secreted high level laccase activity after 24-hour culture over an extended culture time. The combination of selected culture conditions resulted in a 6-fold increase in laccase production without the addition of any toxic inducers. This is, to our knowledge, the first report on the natural production and parameter selection for the expression of extracellular laccase by a *Serratia proteamaculans* strain. The isolated bacterial strain preferred weak alkaline pH and mesophilic culture conditions that are desired traits for many industrial applications. This first phase of research focused on identification and characterization of a new bacterial strain and screening of important factors that influence laccase production using the OFAT strategy. Future experiments will be designed to explore the impact of other important factors such as the amount of oxygen in the culture medium on enzyme activity; the interactions among different nutritional and conditional parameters; and to identify the optimized conditions by the statistical method — Design of Experiment and Response Surface Methodology. The final optimized process should allow secretion of further increased amount of laccase that confer strain

AORB19 greater potential to be either directly used in fermentation process or for laccase enzyme production.

In addition, high level enzyme expression would expedite the biochemical purification and characterization of the specific AORB19 laccase. A greater understanding of the functionality of this bacterial enzyme will likely lead to its practical application in lignin valorization and other biotechnologies that focus on cost-effective biomass conversion.

CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

NA carried out majority of experiments and drafted the manuscript. FH set up laccase assay conditions and assisted in experiments. TY and WQ conceived the concept, designed the experiments, reviewed the manuscript and coordinated the entire study. The final version of the manuscript was read and approved by all authors.

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CHAPTER 4

Uncovering the lignin-degrading potential of *Serratia quinivorans* AORB19: Insights from genomic analyses and alkaline lignin degradation

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Abstract

Lignin is an intricate phenolic polymer found in plant cell walls that has tremendous potential for being converted into value-added products with the possibility of significantly increasing the economics of bio-refineries. In an effort to discover microorganisms capable of lignin degradation, we isolated and characterized *Serratia* sp. AORB19 from long decomposed logs in a forested area near the Ottawa river in Canada. *Serratia* species were targeted because of their ability to grow and produce stable ligninolytic activity in culture medium, with lignin as the sole carbon source, and its excellent ability to survive in extreme conditions. Strain AORB19 was identified based on whole genome sequencing and core genome phylogeny. A total of 123 annotated CAZyme genes were identified with ten cellulases, four hemicellulases, five predicted carbohydrate esterase genes and eight lignin-degrading enzyme genes. Strain AORB19 was also found to possess genes associated with metabolic pathways such as the β-ketoadipate, gentisate, anthranilate, homogentisic, and phenylacetate CoA pathways. All of these genes

and pathways are known to contribute to the break down and utilization of aromatic compounds as a carbon and energy source and could be effective targets for experimental studies of microbial lignin degradation and valorization in the future. LC–UV analysis demonstrated the presence of *p*-hydroxybenzaldehyde and vanillin in the culture media which revealed further evidence of strain's capability to degrade lignin.

Keywords: Lignin degradation, CAZymes, *Serratia quinivorans* AORB19, LC–UV, Whole genome analyses

1. INTRODUCTION

Next to cellulose, lignin is the most generic renewable material available on earth, and the primary by-product of lignocellulosic bio-refineries such as pulp and paper, bioethanol and biogas industries. It is a major component found in plant cell walls in association with cellulose and hemicellulose and act as a physical and chemical deterrent to biodegradative systems (Yang et al., 2014). Lignin is a crosslinked aromatic polymer comprised of various hydroxy phenylpropanoid units, namely syringyl (S), guaiacyl (G) or *p*-hydroxyphenyl (H) propanoid units, linked together through ether and carbon–carbon bonds (Kamimura et al., 2019). Due to its high relative abundance and its high content of phenolic units, lignin is seen as the substrate of choice for the production of value-added aromatic biochemicals. Microbial and enzymatic lignin degradations hold great potential for the development of bioprocesses which offers sustainable and selective alternatives to conventional thermochemical production (Jäger & Büchs, 2012; Ali et al., 2017; Adewale et al., 2021). However, despite this theoretical potential, lignin valorization into aromatic monomers *via* thermochemical or biological processes remains limited (Zakzeski et al., 2010; Monteil-Rivera, 2016; Kamimura et al., 2019). One of the major challenges in biological lignin conversion is the complexity of lignin and the fact that proficient lignin degradation requires a range of

multiple enzymes working in cascade reactions. So far, enzymes that degrade monomeric phenolic units have been reported to degrade/modify lignin with only low efficiency. Identifying microbial catalysts with enhanced lignin-degrading capabilities is crucial for breaking down lignin into usable fragments and achieving high productivity in bioconversion of lignin.

Serratia is a gram-negative bacterial genus belonging to the large and diverse *Yersiniaceae* family. There are currently more than 20 recognized species within the genus *Serratia*, which are differentiated based on different characteristics, including morphology, physiology, and biochemical properties. Due to its relevance as an opportunistic human pathogen, much of the research on this genus has focused on the *Serratia marcescens*. Other related species including *S. rubidaea, S. odorifera*, and *S. liquefaciens* have been also reported to be associated with nosocomial infections.

Serratia quinivorans was previously classified as a subspecies of *S. proteamaculans* and designated as *S. proteamaculans subsp. quinivorans* (Grimont et al., 1982). It was recently included as a separate species in *Serratia liquefaciens* complex, which contains other species like *S. liquefaciens, S. grimesii* and *S. proteamaculans* (Williams et al., 2022), based on average nucleotide identity (ANI) phylogroup analysis. The subspecific epithet quinovora in *Serratia quinivorans* is derived from the ability of the strain to utilize quinate (Brenner & Staley, 2005), a compound found in the bark of plants. They are non-spore-forming rods, facultative anaerobic, motile by means of peritrichous flagella and have been mostly isolated from plants, wild rodents, insects, and water (Grimont et al., 1982).

The research conducted by Kumar et al. (2021) presents compelling evidence of the remarkable cold-adaptation and biocontrol abilities exhibited by *Serratia quinivorans* PKL:12. In addition, the discovery of a psychrotolerant strain of *Serratia quinivorans*, which secretes β-D-galactosidase that remains active in cold environments, highlights the potential of this species for future biotechnological applications (Kumar et al., 2015). Furthermore, Chlebek et al. (2022) identified a novel endophytic strain of *Serratia quinivorans*, KP32, which has the ability to produce a variety of lytic and antioxidant

enzymes, highlighting its immense potential as a biocontrol agent with promising applications in several fields. Notably, a *Serratia quinivorans strain* 124R was found associated with aromatic metabolism while using organosolv lignin as the sole carbon source under anoxic conditions, demonstrating the potential of *S. quinivorans* strains for lignin biorefinery applications (Chaput et al., 2019).

Whole genome sequencing can provide insights into the metabolic pathways and regulatory mechanisms involved in the process and is important for advancing the understanding of lignin degradation and its potential applications in the production of biochemicals. For example, in a recent study of the lignin-degrading bacteria *Streptomyces thermocarboxydus* DF3-3, sequencing of the whole genome allowed for the identification of key regulatory genes and pathways involved in the degradation of lignin (Tan et al., 2022). Recently, we isolated from decomposed wood samples a *Serratia* sp. (AORB19) able to grow on lignin-based medium and produce laccase enzyme (Ali et al., 2022). The goal of the present study was to investigate the ligninolytic machinery of strain AORB19 by performing whole genome sequencing and complementing the gathered genetic information with identification of degradation products.

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

The lignin used in this study, *i.e.*, Lignin, alkali (CAS8068-05-1, Catalog number 370959), was purchased from Sigma Aldrich (Oakville, ON, Canada). Based on the generic structure provided by Sigma-Aldrich (primarily G units, and an -SH group on the benzylic carbon of the propanoid chain), Lignin, alkali can be assimilated to a partially ionized Kraft lignin obtained from softwood; The growth medium used was Czapek dox broth containing g L^{-1} of distilled water in: 30.0 Sodium Nitrate, 3.0 Magnesium Sulfate, 0.5 Potassium Chloride, 0.5 Potassium Phosphate Dibasic, 1.0 Ferrous Sulfate; and phenolic substrate 2,6 Di methoxy phenol was used in laccase assays.

2.2 Microorganism and laccase assays

In this study, a bacterial strain AORB19 isolated using a high throughput screening (HTP) method and characterized from decomposed wood samples (Ali et al., 2022) was used. The lignin degrading potential of this strain were previously evaluated by its growth in culture media with alkali lignin as the carbon source and laccase activity assays employing quantitative and qualitative methods with different substrates such as 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,6-Dimethoxyphenol (2,6 DMP), syringaldazine, and guaiacol.

2.3 Genome assembly and annotation

The genomic DNA of strain AORB19 was extracted using DNA isolation kit (Biobasic Inc. Canada) and the next-generation sequencing libraries were sequenced based on the Illumina NovaSeq sequencing platform. FastQC was used to evaluate the quality of the raw reads, and Trimmomatic (v.0.39) (Bolger et al., 2014) was employed to perform trimming of low-quality bases and adapters. Low quality nucleotides and reads that were less than 50 bp were clipped. *De novo* assembly of genome sequences was performed with SPAdes (v.3.15.4) (Bankevich et al., 2012). The assembled genome contiguity was evaluated with QUAST (Gurevich et al., 2013) and genome completeness was assessed with BUSCO (Simão et al., 2015). The assembled genome was annotated utilizing RASTk under Patric (3.6.12) (https://www.patricbrc.org). The functional annotation was completed utilizing eggNOG (http://eggnog.embl.de) for COG prediction, whereas, metabolic pathways prediction was completed using KEGG Automatic Annotation Server (KAAS) (https://www.genome.jp/kegg/kaas/). The gene ontology (GO) annotation was carried out using InterProScan 5 (Jones et al., 2014).

2.4 Whole genome sequence analysis

Average nucleotide identity (ANI) was determined for *Serratia quinivorans* AORB19 genome along with 14 other type strain genomes of *Serratia* using the ANI calculator of EzBiocloud (Yoon et al., 2017). The ANI values were presented in a matrix-like manner. The ANI cutoff value to circumscribe prokaryotic species boundaries was approximately 95%. In silico DNA–DNA hybridization (DDH) was also performed using the Genome-to-Genome Distance Calculator (GGDC) (http://ggdc.dsmz.de/home.php) (Meier-Kolthoff et al., 2013). The estimation of *is*DDH values involves utilizing formula 2 from GGDC, which calculates the sum of identities found in HSPs (High scoring Segment Pairs) divided by the total length of all HSPs. The results of formula 2 were adopted and a cutoff value in *is*DDH was chosen to be 70% for delimitation of species.

2.5 Phylogenetic analysis

16S rRNA phylogeny was constructed using the Neighbor-Joining algorithm in MEGA11 Software (Tamura et al., 2021). Bacterial core genome-based phylogeny was also constructed along with 20 other strains of *Serratia* utilizing the UBCG2 pipeline (Kim et al., 2021). The UBCG2 pipeline employs the prevailing approach for constructing phylogenetic trees based on core genes. Core genes are defined as: 1) Genes that are present in a majority of species and 2) Genes that are present as a single copy (likely orthologous but not paralogous).

In another approach, a whole genome phylogenetic tree for 74 *Serratia* genomes was generated using the codon tree method within BV-BRC 3.28.22 (https://www.bv-brc.org) (Olson et al., 2022). This method utilizes BV-BRC PGFams as homology groups and analyzes aligned proteins and coding DNA from single-copy genes using the tree building program RAxML (Stamatakis, 2014). The Codon Tree option was selected with gene number 100. The generated tree was visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

2.6 Analysis of CAZymes and mining of lignocellulose degrading genes

The integrated dbCAN3 meta server was used with default settings to classify the carbohydrate active enzyme (CAZyme) encoding genes in the genome of strain AORB19. This classification was performed using the dbCAN database (with HMMER), the CAZy database (with DIAMOND), and the dbCAN-Sub database (with HMMER). (Zhang et al., 2018). The resulting data was imported into R studio and organized. Manual screening was conducted to further analyze CAZyme genes, and confirmation was based on the requirement that the sequence must be recognized by at least two of the aforementioned databases. The lignocellulose degrading genes were further revealed by cross-checking with the annotations available in CAZy database (Lombard et al., 2014).

2.7 Analytical method for lignin degradation products

The Strain AORB19 was grown in 50 mL of culture medium with 0.1 % Lignin, alkali from Sigma Aldrich as the carbon source and cultured at 27°C on a shaker for 8 d. Every 48 h, a sample was sacrificed and centrifuged at 15,000 rpm for 10 min to remove the bacteria, and the culture supernatant was separated for further analysis. A control sample (culture media devoid of bacterial strain) was also prepared and subjected to the same treatment and analytical path. Phenolic compounds typical of lignin degradation (*i.e.*, phenol, *o*-catechol, 3-methylcatechol, guaiacol, syringol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, vanillin, vanillic acid, syringaldehyde, syringic acid, acetovanillone, acetosyringone, ferulic acid) were qualitatively monitored in the supernatant using an Agilent 1260 Infinity II HPLC system equipped with a diode array detector (Agilent Technologies, Inc., Santa Clara, CA, USA). Separation of analytes was carried out with a Gemini® NX-C18 column (3 µm, 110 Å, 150 mm × 4.60 mm, Phenomenex, Torrance, CA, USA), at a temperature of 40°C and a flow rate of 0.5

mL/min. A gradient of 5 to 95 % MeOH in 0.044 N H_3PO_4 was used and detection was conducted at 280 nm. The injection volume was 100 µL.

3. RESULTS AND DISCUSSION

3.1 General Genomic Features of *Serratia A*ORB19

The final *Serratia* AORB19 genome assembly contained 42 contigs sequences longer than 500 bp with a total consensus genome size of 5587018 bp (~5.5 Mb) and GC content of 54.99 %, which was comparable to the genome sizes of previously sequenced *Serratia* genomes. The largest contig size was 1236132 bp, and the N50 size was 370852 bp. The BUSCO evaluation showed 99.7% completeness of this genome, indicating a high-quality genome assembly. All annotation statistics are listed in Table 1.

Table 1 General features of strain AORB19 genome

Features	
Length in bp	5587018
GC content (%)	54.99
Number of Contigs (>500 bp)	42
N50 Values	370852
N90 Values	86520
L50 Values	4
L90 Values	13
Largest contig length (bp)	1236132
Number of protein coding genes	5655
Number of tRNA	99
Protein-Encoding Genes with Functional Assignment	4002
Protein-Encoding Genes without Functional Assignment	1653
% Features that are in Local protein families	86.37
% Features that are Hypothetical	29.23

The genome had 5655 protein-coding genes, of which 4002 genes were functionally assigned, while the remaining genes were annotated as hypothetical proteins (29.23%). In addition, 108 non-coding RNAs, including tRNAs, rRNAs, and snRNA were identified in the *Serratia* genome. The circular genome plot of the genome is represented in Figure 1.

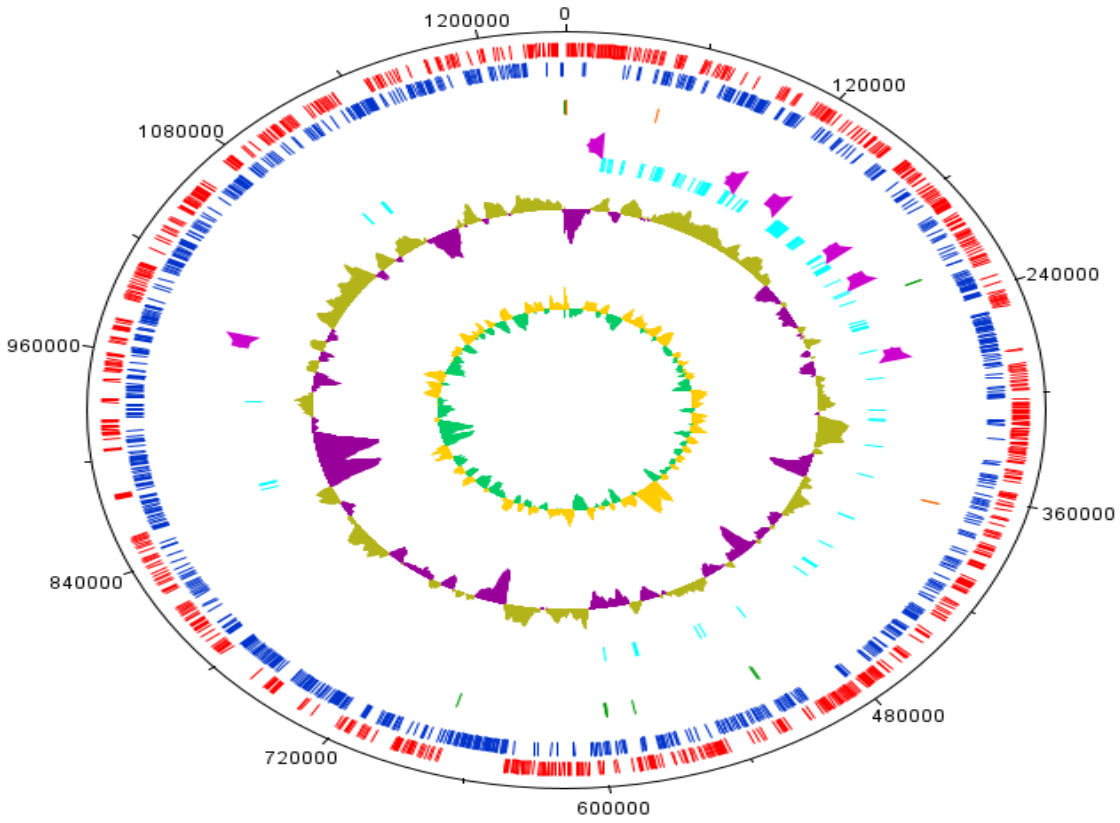

Figure 1. Overview of *S. quinivorans AORB19* genome. A: Circular plot generated by DNA plotter. Circles indicate, from inside outwards: GC skew; GC content; putative lignocelluloses enzymes (cyan); putative laccase –like enzyme genes (purple); tRNA coding genes (green) and rRNA (red); protein-coding genes on reverse strand (blue); and protein-coding genes on forward strand (red).

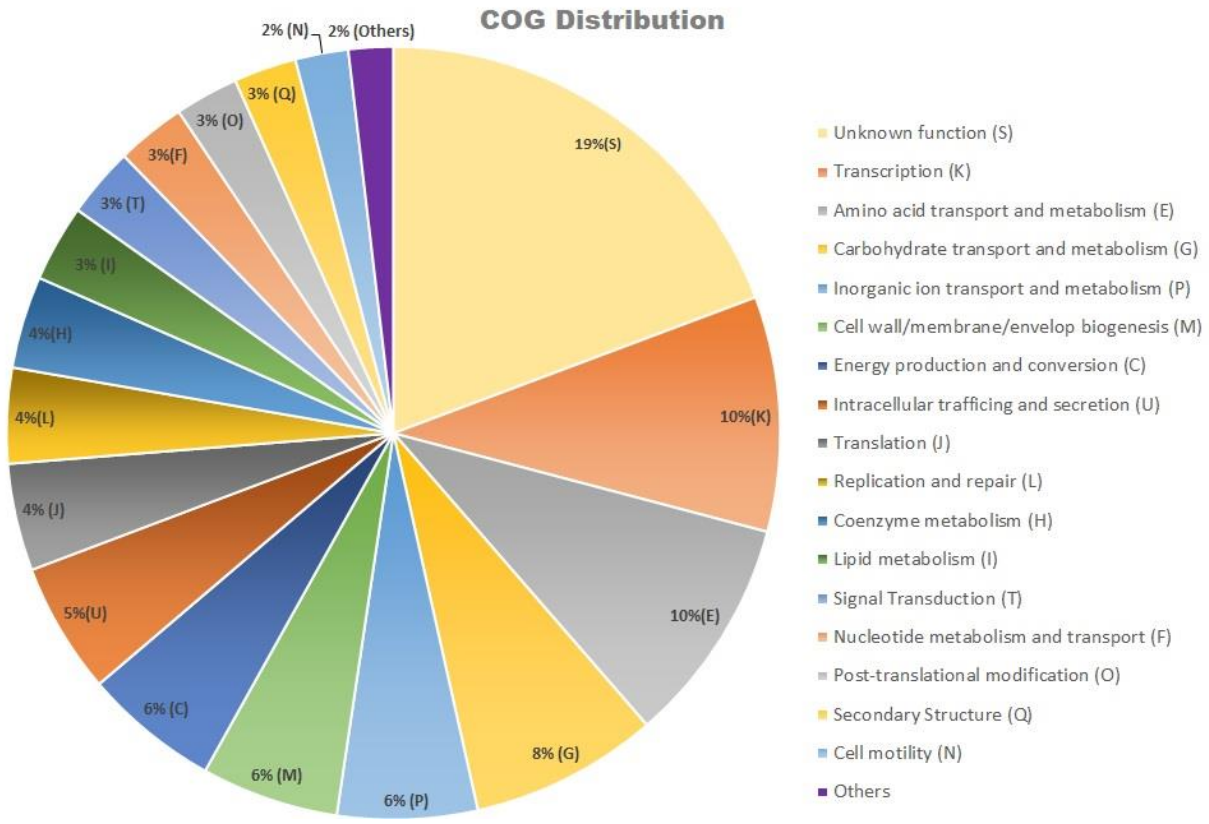

Figure 2. Distribution of COG functional categories in the complete genome sequence of S. *quinivorans* AORB19 genome.

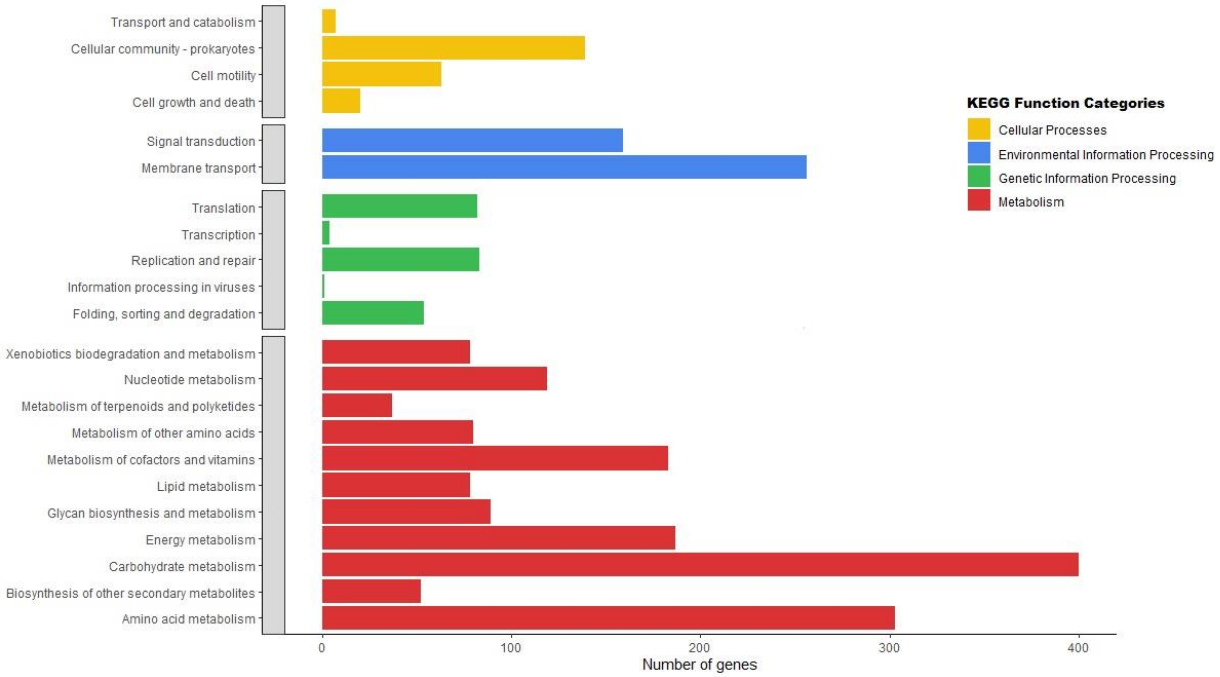

Figure 3. KO functional annotation was performed using KEGG Automatic Annotation Server (KAAS). Percentage of gene sequences assigned to each sub-category of the four top KO categories, namely metabolism (red), genetic information processing (green), environmental information processing (blue), cellular processes (yellow) were calculated and displayed.

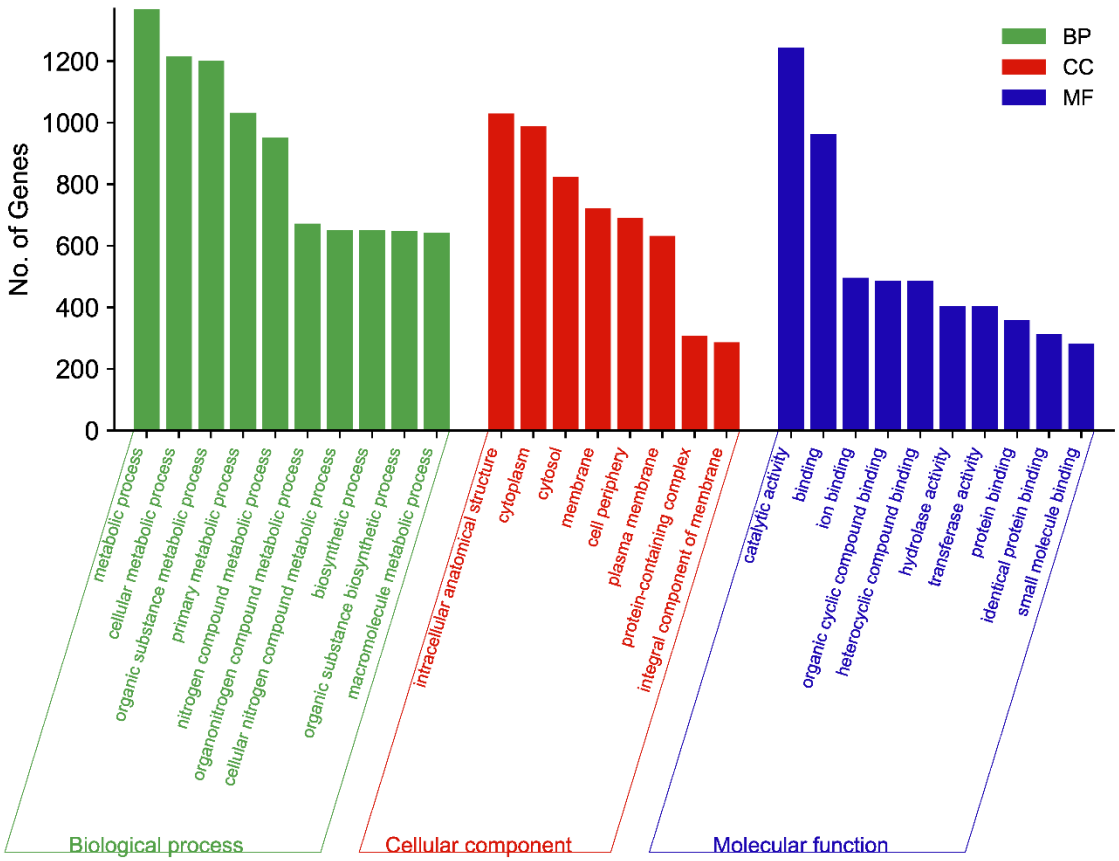

Figure 4. GO classification of bacterial gene function annotation. MF stands for molecular function; BP for biological process and CC for cellular components.

The functional annotation reveals that out of the total protein-coding genes, 4955 genes that have been assigned were annotated with COG terms and grouped into 21 classes, generally covering the essential functions of cellular processes, metabolism, information storage and general function. The top five functional COG terms were transcription, K (10 %), amino acid transport and metabolism, E (10%), carbohydrate transport and metabolism, G (8 %), inorganic ion transport and metabolism, P (6%), and cell wall/membrane/envelop biogenesis, M (6%) (Figure 2). In the KEGG annotation of the genome, 3320 genes were successfully annotated with 2333 KEGG Orthologous (KO) terms. Detailed analysis of the KEGG pathways (Figure 3) reveals that the genes were enriched in 23 pathways and four major

functions of metabolism, including genetic information processing, environmental information processing and cell process. In the category of metabolic processes, 11 pathways were annotated among which 400 and 303 genes were enriched in the carbohydrate metabolism and amino acid metabolism pathways, respectively. Five pathways in the of genetic information processing category were annotated, with the translation pathway having the most annotations with 82 genes. Under the environmental information processing category, 256 and 159 genes were enriched in the membrane transport and signal transduction pathways, respectively. A total of 2366 genes from AORB19 were annotated in the GO database under the four broad categories of biological process (BP), cellular component (CC), and molecular function (MF). As seen in the Figure 4, the top three annotated BPs were the metabolic processes, cellular metabolic process and organic substance metabolic process, in which 1370, 1216, and 1202 genes were enriched, respectively. There were 1030, 988, and 824 genes enriched in the top three CCs, including intracellular anatomical structure, cytoplasm and cytosol respectively. The top annotated MFs includes catalytic activity, binding and ion-binding with 1244,963 and 496 genes.

3.1.1 Whole genome comparison

The GenBank now contains numerous whole-genome sequences due to advancements in next-generation sequencing, enabling the utilization of whole-genome sequencing as a novel approach for species differentiation. ANI and *in-silico* DNA-DNA hybridization (DDH) analysis are the most commonly used parameters for taxonomic assignments. In the context of genome-based species delineation, *in-silico* DNA-DNA hybridization (DDH) is widely regarded as a reliable alternative to traditional DDH methods.

	Serratia quinivorans AORB19	*Serratia proteamaculans* CCUG 14510^T	*Serratia liquefaciens* ATCC 27592 ^{T}	*Serratia grimesii* NBRC 13537^T	*Serratia quinivorans* NCTC11544 ^{T}	*Serratia fonticola* DSM 4576^T	*Serratia plymuthica* AS9^T	*Serratia inhiben* s PRI-2c^T	*Serratia ficaria* NCTC 12148^T	*Serratia entomophila* A1^T	*Serratia marcescens* ATCC 13880^T	*Serratia odorifera* NCTC 11214^T	*Serratia rubidaea* NCTC 12971^T	*Serratia surfactantfaciens* YD25^T	*Serratia symbiotica* CWBI-2.3^T
Serratia quinivorans AORB19		91.81	88.02	87.69	98.74	81.05	86.97	86.79	85.04	84.91	83.58	81.1	81.23	83.42	81.74
Serratia proteamaculans CCUG 14510^T	69.8		87.91	87.45	91.82	80.98	87.01	86.97	84.82	84.75	83.39	80.95	80.9	83.38	81.78
Serratia liquefaciens ATCC 27592 ^{T}	65.7	74.7		86.12	88.05	81.07	86.64	86.78	85.05	84.89	83.65	81.32	81.14	83.45	81.84
Serratia grimesii NBRC 13537^T	67.3	76	20.4		87.71	80.1	85.14	85.12	82.91	82.89	81.76	79.75	79.32	81.65	80.9
Serratia quinivorans NCTC11544^T	81.1	72.7	20	26.7		81.16	86.84	86.94	85.03	85.07	85.03	85.07	81.02	83.61	81.02
Serratia fonticola DSM 4576^T	27.8	28.8	20.1	30.8	27.2		81.35	81.29	81.61	81.67	81.15	80.36	79.92	80.98	79.82
Serratia plymuthica AS9^T	56.2	63.9	17.7	24.3	55.6	27		94.04	85.89	85.67	84.32	81.5	81.55	84.15	82.26
Serratia inhiben s PRI-2c^T	55	62.1	20.3	32.7	54.6	26.8	75.5		85.99	85.7	84.55	81.72	81.81	84.39	82.43
Serratia ficaria NCTC 12148^T	52.9	56.9	20.4	32.2	51.7	27.8	55.1	54.2		91.61	87.94	82.54	83.27	87.8	84.51
Serratia entomophila A1^T	49.1	55	22.9	41.3	49	28.1	52.3	51.5	69.5		87.47	82.42	83.1	87.37	84.64
Serratia marcescens ATCC 13880^T	47.5	53.2	23	38.9	45.3	27.8	49.5	46.8	61.3	63.4		82.06	82.89	94.27	82.54
Serratia odorifera NCTC 11214^T	33.7	36.1	21.2	39	32.6	25	33.7	33.8	39.2	37.5	36.7		82.48	81.9	79.71
Serratia rubidaea NCTC 12971^T	31.5	33.5	18.6	37.4	30.8	24.3	32.7	32.2	41.3	38.9	37.4	39		82.81	79.96
Serratia surfactantfaciens YD25^T	45.7	51.4	18.4	38	43.9	27.8	47.7	44.5	58.6	60.9	36.7	83.9	38		82.81
Serratia symbiotica CWBI-2.3^T	20.1	20.4	21	18.4	20.1	17.7	20.3	20.4	22.9	23	18.6	21.2	18.4	21	

Figure 5. Matrix showing the values of ANI and in-silico DDH comparison of *S. quinivorans* AORB19 with 14 other type strain of *Serratia*. The upper half of the matrix (highlighted in yellow) shows the results of ANI analysis whereas the lower half of the matrix (highlighted in blue) shows the results of in-silico DDH analysis.

Average Nucleotide Identity (ANI) is a measure of nucleotide-level genomic similarity between the coding regions of two genomes. The ANI matrix (Figure 5) reveals that the genome of AORB19 showed an ANI < 95 % with most of the other type strains in the genus *Serratia* including the *S. proteamaculans* CCUG 14510. A 95 % ANI cutoff is the most frequently used standard for species demarcation (Konstantinidis and Tiedje, 2005). However, AORB19 shows a high ANI value (>95 %) with *S. quinivorans* NCTC11544 and therefore this genome is very close to the *S. quinivorans genome.* GGDC analyses among the 15 *Serratia* strains (Figure 5) yielded *in-silico* DDH estimates greater than 81 % between strain AORB19 and *S. quinivorans* NCTC11544 indicating that they potentially belong to

the same species. All other 13 pairwise comparisons with AORB19 indicated that they were different species (based on 70% species delimitation threshold proposed by Meier-Kolthoff et al., 2013).

To further infer the phylogenomic relationship of this genome, 16S phylogenetic neighbour joining type tree was constructed. The 16S rRNA tree (Appendix 9) reveals that *S. quinivora* AORB19 is placed in a clade along with *S. proteamaculans, S. liquefaciens, S. grimesii* and *S. quinivorans* CP6a but quite distinctly placed from *S. quinivorans* NCTC 11544 strain. Classical 16S rRNA gene-based trees do not possess sufficient resolution to differentiate between closely related species like *S. liquefaciens, S. grimesii, S. proteamaculans* and *S. quinivorans.* This issue has been previously discussed in the work of Begrem et al. (2021). Core genome-based phylogeny provides a better resolution and is more robust than conventional marker gene-based phylogeny. Thus, we investigated the genetic diversity within the *Serratia* genus by inferring the phylogenomic relationship based on the core genome. For this purpose, we used the up-to-date bacterial core gene set (UBCG2) consisting of 81 core genes from 3,508 bacterial species covering 43 phyla (Kim et al, 2021). Phylogenetic tree analysis inferred by the maximum-likelihood method based on core genes defined by UBCG2 (Figure 6) clearly indicated that AORB19 belongs to the same clade as S. *proteamaculans, S quinivorans, S. liquefaciens* and *S. grimesii* strains. But AORB19 is closer to *S. quinivorans* strains than to *S. proteamaculans* strains in this analysis. The maximum likelihood-based whole genome-based phylogenetic tree (Figure 7) constructed on 74 publicly available *Serratia* genomes was consistent with the ANI and *in-silico*- DDH results. It also corroborated the fact that this genome is closest to the *Serratia quinivorans* species although it had close relationships with *S. proteamaculans* and *S. liquefaciens* strains. Despite this strain having been previously reported as *S. proteamaculans* AORB19 (Ali et al., 2022) based on its 16S rRNA sequence, comparative genomic analyses of the entire genome suggests that the strain should be designated as *Serratia quinivorans* AORB19.

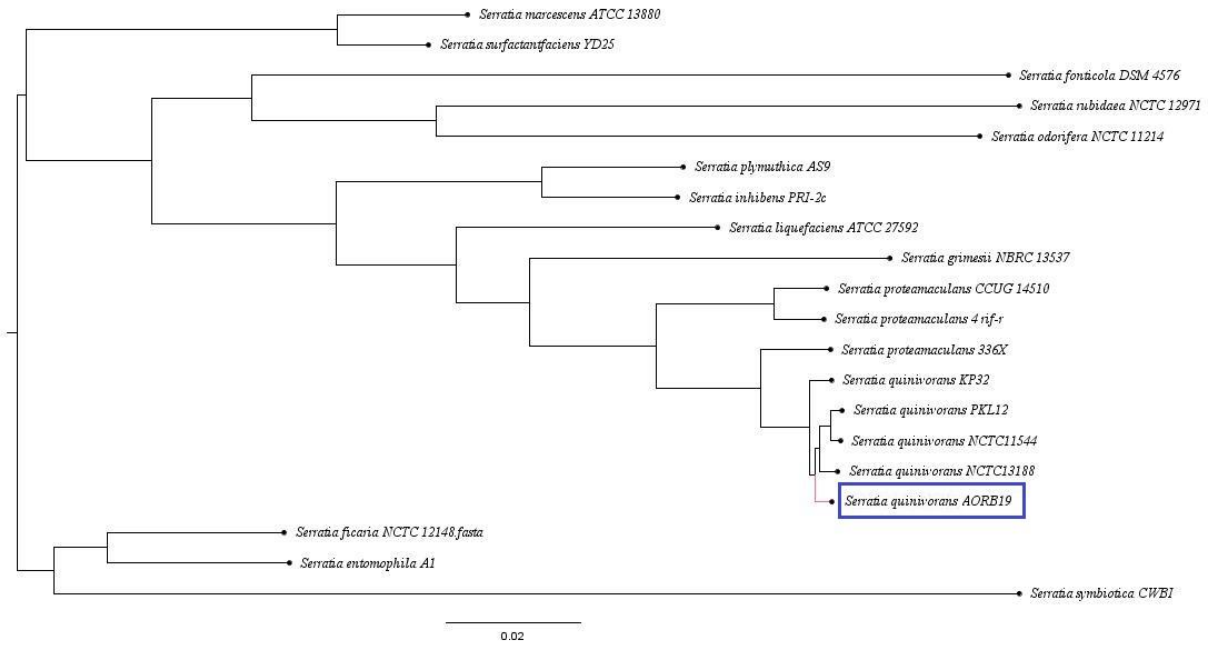

Figure 6. Core genome-based phylogenetic tree constructed by UBCG pipeline.

3.1.2 Lignocellulose degrading enzymes of strain AORB19

CAZyme (carbohydrate-active enzyme) annotation for the AORB19 genome was completed utilizing the dbCAN 3 meta server (https://bcb.unl.edu/dbCAN2/). The different classes of CAZymes, including glycosyltransferase (GT), glycoside hydrolase (GH), polysaccharide lyase (PL), carbohydrate esterase (CE), auxiliary activity (AA), and carbohydrate-binding modules (CBMs), play crucial roles in sugar metabolism, specifically in the synthesis, binding, and breakdown of carbohydrates. The total annotated CAZymes gene number in AORB19 genome was 123, in which 47 GTs, 58 GHs, 5 CEs, 8 AAs and 13 CBMs were found in the genome (Appendix 10). No genes were assigned to PLs. A few genes were assigned to more than one class of CAZymes. For example, SQAORB19_5091 was assigned to both CBM32 and GH144 classes.

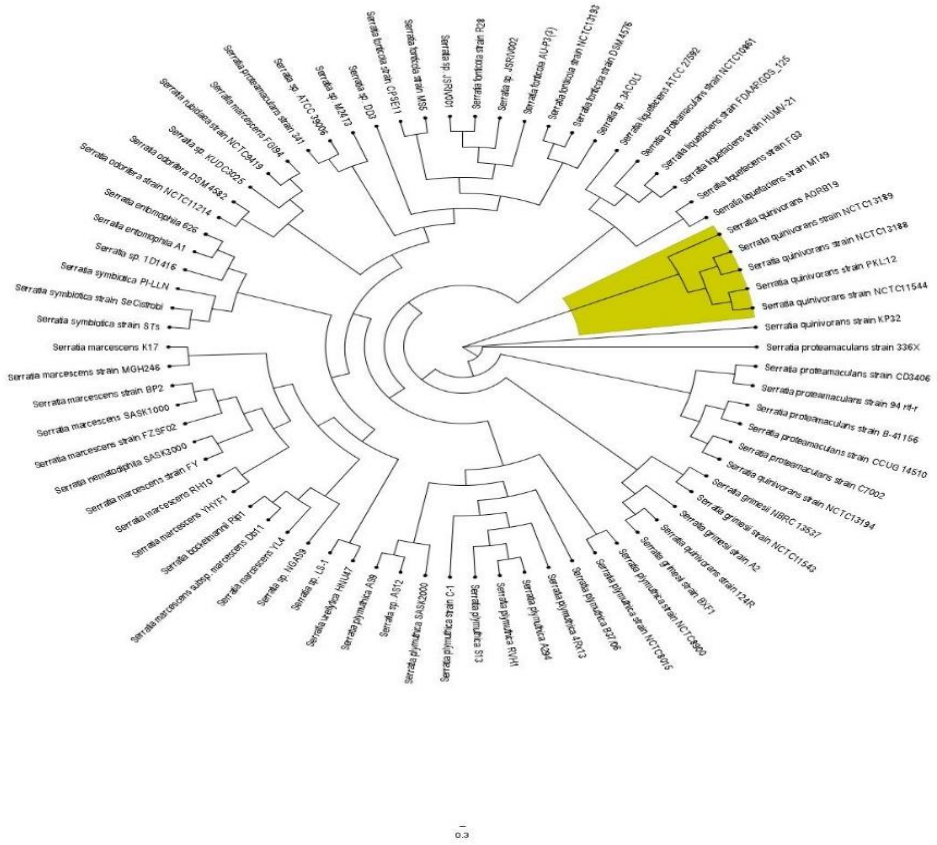

Figure 7. Whole genome phylogeny constructed for S. quinivorans AORB19 and 74 other publicly available *Serratia* genomes. Tree was constructed using the BV-BRC (https://www.bv-brc.org).

The glycoside hydrolases in the GH23 family were widely represented in this genome, including 8 predicted encoding genes involved in the deconstruction of peptidoglycan along with chitinase activity. The chitin degrading ability of *Serratia marcescens* has been previously described (Owen et al., 2018). The genome of strain AORB19 contains 5 genes encoding GH1, and 5 GH4s. GH1 members are a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates. The GH1 family comprises the majority of bacterial β-glucosidases used in cellulose hydrolysis. (Cantarel et al., 2009). Members of GH4 are also involved in cleavage of glycosidic bonds, but exhibit unusual cofactor requirements for activity involving NAD+ (Yip et al., 2004). A total of 26 encoded

proteins in the AORB19 genome were found to be involved in lignocellulose degradation including chitinase activity (Table 2). These included 10 cellulases, which are key enzymes involved in the decomposition of cellulose into glucose; they are members of the GH1, GH3, GH8, GH43, GH45, GH144 families. A variety of annotated GHs and CEs in AROB19 are predicted to participate in hemicellulose degradation. A total of 4 hemicellulases, including xylanases and mannanases were found in the genome belonging to GH43, CE4 and GH2 families.

Table 2. List of potential lignocellulose degrading enzymes found in the genome of strain AORB19 genome

Category	CAZy family	Activities in the family	Gene_id
Cellulase	GH1	β-glucosidase	SQAORB19_2281
			SQAORB19_2395
			SQAORB19_3422
			SQAORB19_1980
			SQAORB19_71
	GH3	β-glucosidase	SQAORB19_518
			SQAORB19_1077
	GH8	polyspecific with cellulase activity	SQAORB19_4140
	GH144	endo-β-1,2-glucanase	SQAORB19_5091
	GH45	Endoglucanase	SQAORB19_591
Xylanase	GH43	β-xylosidase	SQAORB19_2940
	CE4	acetyl xylan esterase	SQAORB19_2919
Mannanase	GH2	β-mannosidase	SQAORB19_418
			SQAORB19_1230
Chitinase	GH18	Chitinase	SQAORB19_4153
			SQAORB19_4595
			SQAORB19_1708
			SQAORB19_3187
	GH23	lysozyme (type G)	SQAORB19_4718
			SQAORB19_2967
			SQAORB19_3012
			SQAORB19_2616
			SQAORB19_3825

			SQAORB19_2092
			SQAORB19_2407
			SQAORB19_270

The second most frequent enzyme family contained in this genome was the glycosyltransferases GT family (47 encoding genes). The transfer of sugar residues from activated donor molecules to saccharide or non-saccharide acceptor molecules to form glycosidic linkages is facilitated by GTs. The most abundant GTs belong to the GT2 family including cellulose synthase, chitin synthase, mannosyltransferase, glucosyltransferase, galactosyltransferase. The output from dbCAN2 also included multiple hits corresponding to carbohydrate esterases (CEs) represented with 5 predicted genes attributed to CE11, CE14, CE4 and CE9 families. CEs accelerate the degradation of polysaccharides by acting on ester bonds in carbohydrates, thereby facilitating the access of glycoside hydrolases. The most abundant CEs in the genome belonged to the CE4 family acting on acetylated xylan and chitin. The genome also had 13 genes predicted to encode carbohydrate-binding modules (CBMs). The majority of predicted CBMs belonged to CBM50 and CBM48. CBM50 proteins play a crucial role in binding of enzymes with cleavage activity of chitin or peptidoglycan, whereas CBM48 encodes specific modules with glycogen-binding function and are appended to GH13 modules.

The bacterial strain AORB19 secreted an array of CAZymes and lignin-degrading enzymes (including aromatic compound-degrading and detoxifying enzymes) for the degradation of lignocellulose. CAZymes database (www.cazy.org) includes a class AA (Auxiliary Activities) which houses a wide range of catalytic modules related to lignocellulose conversion and involved in plant cell wall degradation. The AA class works together with PL, CE and GH enzymes to get access to the carbohydrates within the cell wall of plants and facilitate lignin degradation. Within the AA classes, lignin oxidizing enzymes (LO) are classified into three subclasses including AA1, AA2, and AA3; and

lignin degrading enzymes are classified into four: AA4, AA5, AA6, and AA8 (Liu, et al 2019). Currently, the AA class encompasses 9 families of ligninolytic enzymes and 7 families of lytic polysaccharide mono-oxygenases. Our study revealed the presence of eight genes encoding ligninolytic enzymes under the AA class in the AORB19 genome. The genes among the AA class were: one gene in AA1 class which encodes the laccase enzymes, one gene from AA2 class, two genes from AA3 class, which comprises enzymes like aryl alcohol oxidase and glucose-1-oxidase and one gene is classified under the AA6 class and three genes belonging to the AA10 class, known as copper-dependent lytic polysaccharide monooxygenases (LPMOs, Table 3). LPMOs are copper-dependent enzymes with multiple functionalities in plant biomass degradation and play a crucial role in lignin breakdown (Rani Singhania et al., 2021). The presence of specific genes for different classes of lignin degrading enzymes indicates the strain's ability for efficient break down of lignin and provide further evidence of the potential of AORB19 to be further genetically modified on specific metabolic pathways and regulatory mechanisms based on practical applications.

Table 3. Category and number of annotated open reading frames (ORFs) of auxiliary activities family in AORB19

Auxiliary Activities Enzymes	Number of ORFs	Annotated Genes IDs	Annotation
AA10	3	SQAORB19_1298, SQAORB19_2413, SQAORB19_3185	Lytic polysaccharide mono-oxygenase, Chitin-binding protein
AA3	2	SQAORB19_928, SQAORB19_1920	Choline dehydrogenase, Glucose-Methanol-Choline (GMC oxidoreductase)
AA6	1	SQAORB19_635	NADPH: quinone oxidoreductase
AA1	1	SQAORB19_2924	multicopper oxidase
AA2	1	SQAORB19_1212	peroxidase/catalase

3.2 Analyses of lignin degradation pathways of *Serratia quinivorans* AORB19

Microbial lignin degradation is a complex process involving multiple enzymes and intermediate products. The specific pathway and products produced may vary depending on the specific microorganisms, conditions involved in the degradation process, as well as source or type of lignin. Degradation process can be broadly categorised into two stages based on conditions and nature of compounds: i) the peripheral, or upper pathway that includes lignin depolymerization to central intermediate products such as catechol and protocatechuate and ii) the central, or lower pathway that includes aromatic ring opening to precursor molecules and compounds such as muconic acid derivatives, acetyl-CoA, succinyl-CoA and pyruvate (Fuchs et al., 2011; Linger et al., 2014; Liu et al., 2019; Kamimura et al., 2019).

Liquid chromatography with UV detection (LC-UV) was employed to identify (using UV spectra) and monitor potential intermediates produced during the degradation of the alkali lignin by *Serratia quinivorans* AORB19 over an 8-d period (Figure 8; Appendix 13). Alkali lignin medium without bacterial inoculation was used as a negative control. In the negative control, a number of lignin-derived phenolic compounds were detected including *p*-hydroxybenzaldehyde, vanillic acid, and vanillin. The presence of compounds detected in the negative control is due to the residual by-products of the alkali treatment in the manufacturing process (Duan et al., 2016).

In the treated samples, three phenolic compounds were observed with concentrations varying as follows: *p*-hydroxybenzaldehyde, a typical phenolic compound derived from the degradation of lignin, was found to gradually increase over time; vanillin, which experienced a significant decrease after 48 h compared to the control, then gradually rose over the remaining time period of the study; and vanillic acid which was completely consumed after 48 h, remained undetectable until the end of the experiment (Figure 8; Appendix 13). The preponderance of vanillin and vanillic acid in the supernatants is in

agreement with the origin of alkali lignin used in this study, *i.e*., softwood. The increasing concentration of *p*-hydroxybenzaldehyde and vanillin in the samples is indicative of lignin degradation by the *Serratia quinivorans* AORB19 bacterium. Both chemicals result from the cleaving oxidation of the benzyl carbon adjacent to the *p*-hydroxyphenyl (H) and guaiacyl (G) units, respectively (Takada et al., 2018; Zulkarnain et al., 2016; Behr et al., 2018). The *Serratia quinivorans* AORB19 genome harbored several oxidative lignin degrading enzymes including a few potential laccase-like genes - multi multicopper oxidases (SQAORB19_2332; SQAORB19_1606; SQAORB19_2924), DyP-peroxidases (SQAORB19_3208; SQAORB19_3467; SQAORB19_3468) and dehydrogenases (SQAORB19_610; SQAORB19_635) contributing to the formation of the two difunctionalized aldehydes. In similar lines, a thermoalkaliphilic laccase from *Caldalkalibacillus thermarum* TA2.A1 has also been reported to depolymerize kraft lignin to *p*-hydroxybenzaldehyde as a metabolic intermediate (Yang et al., 2019). Once formed, the aldehydes can be oxidized into *p*-hydroxybenzoic acid or vanillic acid by the action of dehydrogenase enzymes, as previously reported by others (Nishimura et al., 2018; Fleige et al., 2013). In agreement, two aldehyde dehydrogenases genes (SQAORB19_5141; SQAORB19_610) were identified during the genome analyses of strain AORB19. Similarly, Kamimura et al. (2017) identified an aldehyde dehydrogenase gene responsible for the conversion of syringaldehyde to syringic acid.

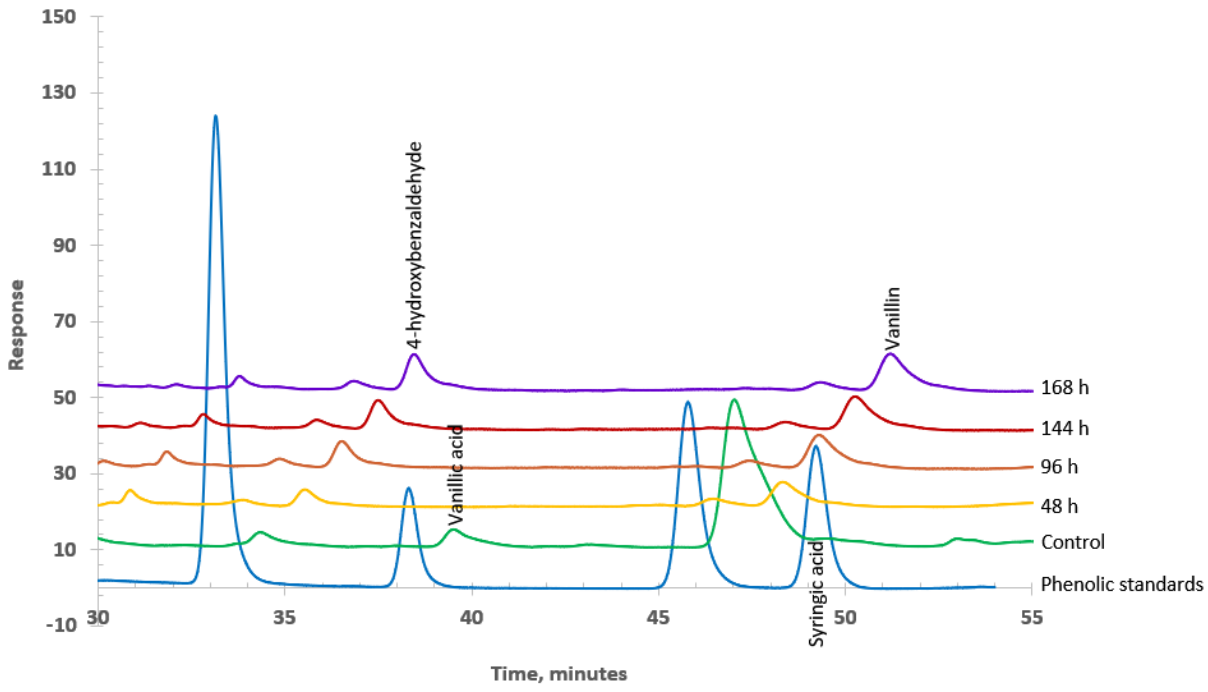

Figure 8. Zoomed LC-UV (λ=280 nm) chromatograms of Alkali Lignin (Sigma Aldrich) incubated for 168 h without strain (Control) or for increasing times (48-168h) with *Serratia quinivorans* AORB19.

The fact that vanillic acid, present in the controls appeared completely depleted in the presence of *S. quinivorans* AORB19 suggests that the latter has the capability to transform the acid. The genome of strain AORB19 possessed the candidate genes of vanillate O-demethylase oxygenase (SQAORB19_2890; SQAORB19_5632) that is known to convert vanillic acid into protocatechuic acid (Jiang et al., 2022). The *o*-catechol and protocatechuate obtained from the depolymerization of lignin monomers can then be converted to *cis,cis*-muconate and 3-carboxy-*cis,cis*-muconate, respectively, by the ring opening dioxygenases (SQAORB19_3419; SQAORB19_3420) identified in the genome of strain AORB19. The β-ketoadipate pathway plays a vital role in the degradation of aromatic compounds, including lignin, by enabling microorganisms to utilize carbon from complex polymers, and derives its name from the characteristic intermediate, β-ketoadipate, which serves as a branching point for further metabolism (Stainer & Ornston, 1973; Harwood & Parales, 1996; Li and Zheng, 2019; Weng et al., 2021). The genome of strain AORB19 also possessed numerous key genes for the β-ketoadipate pathway

that could be involved in the degradation of aromatic compounds. SQAORB19_989 and SQAORB19_3417 encode CMD γ γ -carboxymuconolactone decarboxylase; SQAORB19_1365 encodes ELH, enol lactone hydrolase; SQAORB19_616, SQAORB19_1321, SQAORB19_1366 encode TH, β-ketoadipyl-CoA thiolase; SQAORB19_1367 (subunit B), SQAORB19_1368 (subunit A) encode TR, β-ketoadipate:succinyl-CoA transferase; SQAORB19_3416 encodes CMH, β-carboxymuconolactone hydrolase and CMLE, β-carboxy-cis,cis-muconate lactonizing enzyme; SQAORB19_3419 (alpha chain) and SQAORB19_3420 (beta chain) encode P3,4O, protocatechuate 3,4-dioxygenase.

Notably, genes for additional pathways linked with lignin degradation such as the gentisate, anthranilate, homogentisic and phenylacetate–CoA pathways were also detected (Appendix 11 and 12) in the genome analyses. These degradation pathways allow prokaryotes to break down and utilize aromatic compounds as a carbon and energy source. Taken together, the results of *Serratia quinivorans* AORB19 genome analysis and the formation of detectable trails of transient aromatic compounds in LC-UV analysis validated each other and confirmed the lignin-degradative traits of the strain *Serratia quinivorans* AORB19. More so, with the genetic pathways characterized, the strain *Serratia quinivorans* AORB19 has the potential to undergo genetic modification and optimization in order to enhance the cost-effectiveness and sustainability of lignocellulosic biorefineries, addressing the urgent need for efficient techniques to convert lignin into bioproducts.

4. CONCLUSION

Analysis of the genome data is a way to enhance our understanding of bacterial degradation, a pivotal process in the global carbon cycle, and provides a framework for further development. The complete genome sequence of *Serratia quinivorans* AORB19 strain has revealed that it harbors a multitude of genes for carbohydrate-active enzymes, which facilitates growth of this bacterium on lignocellulosic

biomasses. More so, eight key lignin-degrading enzyme genes in class AA, including lignin oxidizing and lignin degrading genes, were also identified along with an array of enzymes responsible for the degradation of lignin-derived aromatic compounds. The utilization of whole genome sequencing analyses, coupled with LC-UV analyses provided additional evidence supporting the involvement of *Serratia quinivorans* AORB19 in the process of lignin degradation in nature.

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CHAPTER 5

Purification and biochemical characterization of extracellular laccase from *Serratia quinivorans* AORB19 and its application in degradation of synthetic dyes and biomass

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Abstract

Laccase is a vital lignin degrading enzyme found in multiple organisms including eukaryots, prokaryots and plants. The purification and biochemical characterization of more versatile laccases in natural state from varied sources and pH sensitive modalities is crucial to the degradation of lignin and phenolic compounds. In the study, an extracellular laccase derived from *Serratia quinivorans* AORB19 is reported for the first time, which is partially purified employing anion exchange chromatography and characterized. The partially purified protein showed two distinct bands on SDS PAGE at 75 kDa and 10 kDa, respectively. The partially purified laccase also showed an optimum pH 3 with ABTS, pH 4.5 with 2,6 DMP substrate, and temperature of 45-55°C. The V_{max} and K_m of partially purified laccase towards ABTS and 2,6 DMP were determined as 0.7082 μM s^{-1} and 1.161 μM; 610 mM s^{-1} and 17 mM respectively. Laccase activity was enhanced in the presence of Cu^{2+} and Ca^{2+} and laccase activity was retained in the presence of Mg^{2+} and Mn^{2+} and Fe^{2+} highly inhibited laccase activity at 5mM. DTT, sodium azide, methanol, ethanol, and isopropyl alcohol was found to be inhibit laccase activity. Laccase activity was mildly inhibited in the presence of conventional inhibitors like L-cysteine and EDTA. Furthermore, the efficacy of the strain in the decolorization of two major dyes were investigated. *Serratia*

quinivorans AORB19 biodegraded 250 mg/l Malachite Green and Remazol Brilliant Blue R at 94.31% and 76.35% respectively and presents the first report of its dye-decolorization capabilities. Finally, the study evaluated the laccase activity of *Serratia* AORB19 against various industrial lignocellulosic residues, with the highest activity detected on flax seed meal (257.71 U/L) followed by pea hull (230.11 U/L), canola meal (209.56 U/L), okara (187.67 U/L), and barley malt sprouts (169.27U/L). Overall, these findings highlight the strain's broad substrate promiscuity and its potential to accelerate lignocellulosic residue valorization processes and bioremediation applications.

Keywords: Barley malt sprouts, biochemical characterization, canola meal, decolorization, flax seed meal, laccase, malachite green, okara, pea hull, Remazol brilliant blue R, *Serratia quinivorans* AORB19

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are copper containing oxidoreductases with the ability to oxidize a broad range of phenolic and nonphenolic compounds (Janusz et al., 2020; Arregui et al., 2019). Laccases are produced by bacteria fungi, plants and insects (Forootanfar et al., 2011; Jin et al., 2018; Madhavi and Lele, 2009; Chauhan et al., 2017). Mostly, laccase is present as monomeric subunits, but the dimeric and other multimeric combinations of the monomeric subunits also have been reported (Flurkey, 2003). Compared to fungal laccases, only a few bacterial laccases have been characterized till date. *Azospirillum lipoferum*, the first bacterium reported to produce laccase was obtained from the rhizosphere of rice (Givaudan et al., 1993). The structure of a bacterial laccase was first elucidated in Cot A laccase from *Bacillus subtilis* (Enguita et al., 2003). Fungal laccases are unstable under harsh conditions such as extreme temperatures, pH levels, and salinity which renders them unsuitable for industrial purposes (Gałązka et al., 2023; Kaira et al., 2015) where high yields per cycle are required. Moreover, the large-scale production of fungal laccases poses challenges due to their slow

growth rate and substantial accumulation of fungal biomass. Nevertheless, bacterial laccases demonstrate stability at high temperature and pH, salt tolerance and cost effectiveness (Zhang et al., 2013; (Ruijssenaars & Hartmans, 2004; Mishra & Srivastava, 2016; Margot et al., 2013) making their efficacy and economics attractive for industries in delignification, lignin valorization and green chemistry initiatives. Hence, there is a need for more extracellular low-cost prokaryotic laccases as their biochemical and catalytical properties are ideal for industrial applications.

Laccases are widespread in nature and represents an immense promise although a number of concerns limits industrial application resulting in its residual attention; reduced yield of enzymatic activity and lower tolerance to extreme conditions in most laccases isolated from microorganisms (Si et al., 2013). Only few strains of narrow specificity are commercially available, so the obvious solution is the bioprospecting of strains that harbors novel laccases that offer enhanced activity yields and exhibit versatile properties. Based on its capacity to screen and isolate a plurality of microbial strains with unique characteristics, high throughput screening (HTP) processes have emerged as one of the most efficient and rapid methods for the isolation of known and previously unknown natural microorganisms or microbial consortiums (Ali et al., in press). In our previous study, a new strain of *Serratia quinivorans* that naturally produces extracellular laccase was isolated employing a high throughput screening process from decomposed wood samples (Ali et al., in press). Followingly, in the present study, the extracellular laccase derived from the new *Serratia quinivorans* AORB19 strain is partially purified and characterized. To our knowledge, this is the initial study to report a natural extracellular laccase enzyme in *Serratia quinivorans* species, although other lytic enzymes such as amylazes, proteases and chitinases were previously reported (Chlebek et al., 2022).

Laccase, operating as an oxidoreductive enzyme employs environmentally benign processes in its catalysis thus exemplifying its intrinsic facet as a sustainable green catalyst. Beyond lignin degradation, the utilization of laccase as a ‘green catalyst’ for the dye decolorization process has been

extensively researched; either as a principal catalyst (Shanmugam et al., 2017) or co-catalyst (Du et al., 2013) in dye removal particularly in the context of industrial dye effluents degradation and their treatment. The broad substrate range of laccases makes them highly efficient in the degradation of synthetic dyes with diverse chemical structures (Dhaarani et al., 2012). This has piqued interest in the textile industry that actively search for enzymes that are "benign by nature" for effective wastewater remediation technologies to comply with stringent environmental regulations. Hence, utilizing laccase enzymes in these processes holds great promise as it provides an environmentally friendly mechanisms and potentially address the dye-related pollution concerns (Madhavi and Lele, 2009).

The textile industry holds a significant position as one of the largest manufacturing sectors globally and plays a vital role in the global economy. Nevertheless, it is also recognized as one of the most environmentally polluting industries, as untreated disposal of textile dyes into water bodies pose risk to human and aquatic life (Desore & Narula, 2017). The persistent dye contamination of the environment can result in its accumulation within the food chain, posing a threat to higher-level organisms including humans (Al-Tohamy et al., 2022). Additionally, textile dyes and pollutants are highly toxic, carcinogenic, and mutagenic leading to significant risks to human health (Lellis et al., 2019). The textile industry employs various classes of dyes and among them the most commonly used dyes include azo, anthraquinone, and triphenylmethane dyes. These dyes are extremely stable due to their aromatic nature and their degradation is challenging. Over the past few years there has been a growing interest in the utilization of microbial sources to treat dye effluents as it is economic and ecofriendly (Pinheiro et al., 2022; Desore and Narula, 2017). Microorganisms like algae, fungi and bacteria have been employed for dye contaminated wastewater treatment. Among them, bacterial species has gained increased attention due to their shorter growth cycles, superior capability to degrade and mineralize dyes (Jamee and Siddique, 2019).

Driven by the hazardous nature and extensive use in textile industry there had been several approaches reported where bacterial strains having demonstrated effective decolorization and degradation and of dye-containing textile waste water containing triphenylmethane dyes (Cheriaa et al., 2012; Ayed et al., 2010) and anthraquinone dyes (Pan et al., 2017; Cerboneschi et al., 2015). Microorganisms facilitate the biodegradation of dyes by utilizing enzymes within their cell structures. Enzymes including laccases, lignin peroxidases, and azo reductases have been identified with significant potential for the degradation of textile dyes from microorganisms. Bacterial isolates that produce laccases and peroxidases have shown effective degradation of synthetic dyes (Legerská et al., 2016; Falade et al., 2019; Solís et al., 2012). These bacteria are mostly isolated from soil samples surrounding textile manufacturing plants (Khan and Malik, 2017), dye-contaminated textile wastewater (El Bouraie and El Din, 2016) and textile effluent sludge waste (Meerbergen et al., 2018). Here, we investigate a bacterial strain from the genus *Serratia* isolated from decomposed wood sourced from the banks of Ottawa river that was found to naturally produce significant amount of laccase.

Although many research exists on the degradation of bacterial dye decolorization, the studies on the dye-degrading capabilities of genus *Serratia* are not well exploited. In a previous study by Verma and Madamwar (2003), *Serratia marcescens* was tested for the decolorization of Ranocid Fast Blue and Procion Brilliant Blue-H-GR dyes. However, their study overlooked other major textile dyes including Remazol Brillilant Blue R(RBBR).and Malachite Green (MG). Indeed, the strain *Serratia quinivorans* is widely recognized for its role as a biological control agent and exhibits antagonistic activity against fungal phytopathogens (Chlebek et al., 2022; Nascimento et al., 2016). However, limited attention has been given to the utilization and application of *Serratia quinivorans* strain in bioremediation processes targeting environmental pollutants. Thus far, there have been no studies on the decolorization of synthetic dyes including triphenylmethane and anthraquinone classes of dyes by this species. This present study

represents the first report of the dye decolorizing capabilities of *Serratia quinivorans* strain; efficiently decolorizing MG and RBBR dyes.

The utilization of industrial residues for cost-effective laccase production and lignocellulosic waste valorization presents a promising avenue for sustainable biotechnology. Industrial processes generate significant amounts of lignocellulosic waste, such as agricultural residues, food processing byproducts, and forestry waste (Mujtaba et al., 2023). By employing microbial strains capable of converting lignocellulosic materials, the environmental challenges associated with waste disposal may be effectively addressed (Pothiraj et al., 2006; Ravindran & Jaiswal, 2016). Therefore, besides establishing the potential for decolorization, the bacterial strain AORB19 was further tested for its ability to utilize agro-industrial residues to evaluate the growth and adaptability to cultures conditions in a broad range of substrates for potential use in lignocellulosic waste valorization.

The objectives of the present study are, therefore, threefold: (1) to purify and partially characterize extracellular laccase derived from *Serratia quinivorans* AORB19 strain; (2) to determine the decolorization potential and efficiency of the strain towards two major classes of dyes including triphenylmethane (Malachite Green) and Anthraquinone (Remazol Brilliant Blue R) dye which may possibly be used for the bioremediation of these dye contaminated environments; and (3) to evaluate the efficacy of the strain to produce laccase in various industrial lignocellulosic waste residues to identify economically viable strains to accelerate lignocellulosic waste valorization.

2. MATERIALS AND METHODS

2.1 Chemicals and Dyes

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,6-dimethoxyphenol (2,6-DMP), Ethylenediaminetetraacetic acid (EDTA), L-Cysteine, Dithiothreitol (DTT), Sodium azide

(NaN_3), Kraft lignin, Malachite Green (MG), Remazol Brilliant Blue R (RBBR). All the chemicals used in the study are analytical grade and sourced from Sigma Aldrich, Canada.

2.2 Bacterial strain and growth conditions

The rotten wood samples were collected in proximity to the Ottawa River bank (geographical coordinates: 45° 26′ 59.7″N, 75° 41′ 23.3″W). Microorganisms were isolated from these samples by a high-throughput screening approach using lignin amended Czapek-Dox broth based on the production of extracellular laccase enzyme and was identified. Among the many isolated strains, *Serratia quinivorans* was selected for the purification and characterization of laccase enzyme and its role in decolorization of synthetic dyes.

2.3 Recovery of crude enzyme extract

The strain AORB19 was freshly grown in nutrient agar for 48 h at 27°C after which bacterial colony (O.D.600 nm ± 1) was aseptically inoculated on 50 ml production media (L-CDB). The L-CDB media used in the study (containing g L^{-1} of distilled water: 30.0 Sodium Nitrate, 3.0 Magnesium Sulfate, 0.5 Potassium Chloride, 0.5 Potassium Phosphate Dibasic, 1.0 Ferrous Sulfate and with 0.1% alkali lignin with pH 7.3+/- 0.2). The bacterial cultivation was performed in a dark chamber at 125 rpm shaking at 27°C. After 48h of incubation, the broth culture was centrifuged for 10 min at 13,000 rpm to remove bacterial cells and coarse lignin particles in the medium and the supernatant was used as the source of crude enzyme.

2.4 Protein estimation

Protein concentration was assessed by a BCA Protein Assay Kit (Thermo Scientific Pierce) using bovine serum albumin as standard for calibration.

2.5 Laccase Activity Assays

Laccase activity was assessed using 2,6-dimethoxyphenol (DMP; ε469 = 14,800 M−1 cm−1) in sodium acetate buffer (0.1 M) at pH 5, using 10mM final concentration of the substrate (Agrawal and Verma, 2019). Additionally, laccase activity was also determined using the substrate ABTS (ε420 = 36,000 M−1 cm−1; Johannes and Majcherczyk, 2000) in 0.3 mM sodium acetate buffer at pH 4 with 2 mM final concentration of the substrate. Controls were run by substituting substrate and enzyme with buffer under standard conditions.

2.6 Purification of Laccase

Laccase enzyme was isolated from the fermentation broth using a combination of purification techniques. Initially, the crude enzyme is concentrated by ultra-centrifugation using Amicon centrifugal filters (3KDa) which enabled the separation of kraft lignin from the culture medium. The extract was then applied to a strong fast flow anionic-exchanger Hi prep QFF, (20 ml bed volume, 10ml /min flow rate) pre-equilibrated with 50 mM potassium phosphate buffer, at pH 6. The bound proteins were eluted with a linear 0 –1M NaCl gradient using the same buffer. Laccase rich fractions were combined, concentrated and then subjected to size exclusion chromatography using an AKTA fast protein liquid chromatography (FPLC) system (Pharmacia Amersham Biotech) with a Hiload 16/60 Superdex 200 gel filtration column (GE Healthcare Life Sciences). The column was pre-equilibrated with 50 mM potassium phosphate buffer – 0.15M NaCl, pH 6 at a flow rate of 1.0 mL/min in room temperature. The eluted peaks were monitored at 280nm and laccase activity of the collected fractions were measured. The active fractions were applied to Tricine SDS-PAGE (Laemmli,1970). The protein bands were visualized by staining with Coomassie Brilliant Blue (R350; Pharmacia) and compared with pre-stained molecular weight markers.

2.7 Determination of optimum pH

Effect of pH on laccase activity was determined at 25°C in 0.1 M Sodium acetate buffer (pH 3.0–5.5) 50 mM phosphate buffer (pH 6.0–7.5) and 50 mM Tris- HCl buffer (pH 8.0–9.5) for the 2,6 DMP substrate at a pH interval of 0.5. Additionally, the effect of pH on laccase activity was also examined using 2 mM ABTS substrate in 0.3M citrate phosphate buffer (pH 1.5–3) and 0.1 M Sodium acetate buffer (pH 3.5–5).

2.8 Determination of temperature optimum

The temperature optimum was determined at varying temperatures ranging from 25°C to 60°C by measuring 2,6 DMP oxidation at 5°C interval at the optimum pH.

2.9 Determination of the effect of metal ions

The effect of several metal ions including $Mg^{2+}(MgSO_4)$, $Cu^{2+}(CuSO_4)$, $Ca^{2+}(CaCl_2)$, Mn^{2+} $(MnCl_2)$ and $Fe^{2+}(FeSO_4)$ on laccase activity was investigated at two different concentrations (1mM and 5mM) using 2,6 DMP in sodium acetate buffer (0.1 M, pH 4.5). A control experiment was carried out without metal ions, and all the experiments were repeated three times.

2.10 Determination of effects of inhibitors

The effect of conventional inhibitors on purified laccase were assessed by separately adding two concentrations (1 and 0.1 mM) of each into the reaction mixture. The relative activity of purified laccase was measured using the substrate 2,6 DMP at 25°C. A control test was conducted without inhibitors and all the experiments were repeated three times**.**

2.11 Determination of influence of organic solvents

The influence of organic solvents on the purified laccase was evaluated by introducing varying amounts of organic solvents (25% and 50%) into the reaction mixture. The relative activity of the purified laccase was assessed at 25°C using the substrate 2,6 DMP. Additionally, a control experiment was performed in the absence of organic solvents, and all tests were performed three times.

2.12 Determination of Kinetic Characteristics

Laccase activities were assessed using two commonly employed substrates, namely 2,6 DMP and ABTS. For the ABTS substrate, the reaction mixture was composed of 0.3 M citrate buffer at pH 3, with a laccase enzyme concentration of 34 µg/mL Various concentrations of ABTS (0.6 µM to 10 µM) were added to initiate the reactions. Similarly, for the 2,6 DMP substrate, the reaction mixture composed of 0.1 M sodium acetate buffer at pH 4.5, with a laccase enzyme concentration of 101 µg/mL. Various concentrations of 2,6 DMP (ranging from 10 mM to 130 mM) were added to initiate the reactions.

Laccase enzyme activity was assessed by measuring the increase in product formation at regular one-minute intervals for a total of 40 minutes. The reactions were conducted in a 96-well UV-Star microplate (Greiner Bio-one) using a spectrophotometer. The absorbance was measured at wavelengths of 469 nm and 420 nm for 2,6 DMP and ABTS substrates respectively. The initial linear slopes of the absorbance measurements were used to determine the kinetic parameters of laccase. The obtained experimental data were analyzed using a nonlinear regression model and GraphPad Prism 9 software. The parameters V_{max} and K_m were determined by fitting the data from triplicate experiments, and the results were presented as the mean and standard deviation.

3. Determination of dye decolorization

The Strain AORB19 was cultured separately in the CDB medium supplemented with either Malachite Green (MG, 250 mg L^{-1} and 500 mg L^{-1}) or Remazol Brilliant Blue R (RBBR, 250 mg L^{-1} and 500 mg L^{-1}). The cultures were then incubated at 27°C with 125 rpm shaking. At regular intervals of 24 h for a total of 144 h, the culture broth was collected and centrifuged (Mini spin, Eppendorf) at 10,000 rpm for 5 minutes. The supernatant was obtained, and the absorbance was measured at 620 nm for MG and 595 nm for RBBR using a UV–Vis spectrophotometer. A control was run parallelly without inoculating the bacterial strain for both MG and RBBR. The measurement was performed up to 144 h, and maximum dye degradation (%) was calculated at each time interval as:

$$\% D = \text{Initial absorbance} - \text{Observed absorbance} / \text{Initial absorbance} \times 100.$$

4. Determination of laccase production using industrial biomasses

Lignocellulosic residues were sourced from various Canadian companies including pea hull (NutriPea), flax seed meal (Pilling Foods), okara (Sunrise Soya Foods), canola meal (Richardson), and barley malt sprouts (Mad Barn). After oven dried overnight at 60°C, the residues were grinded in a coffee grinder and stored at 4°C. Culture media was prepared using 0.1% biomass waste as carbon source separately, and the strain AORB19 was cultivated using submerged fermentation conditions. The production of laccase was measured periodically through spectophotometry and compared to the control media prepared using alkali lignin as the carbon source.

5. Statistical analyses

All the experiments were performed in triplicate, and the results were expressed as mean ± standard error in GraphPad Prism software. Post-hoc comparisons were conducted using GraphPad Prism 9.0.0 by applying one-way ANOVA with Dunnett's multiple tests.

6. RESULTS AND DISCUSSION

6.1 Laccase activity assays

2,6 DMP and ABTS are the conventional laccase substrates, exhibiting structural similarity to phenolic and non-phenolic compounds respectively. The laccase derived from strain AORB19 exhibited the ability to oxidize both and 2,6-DMP and ABTS.

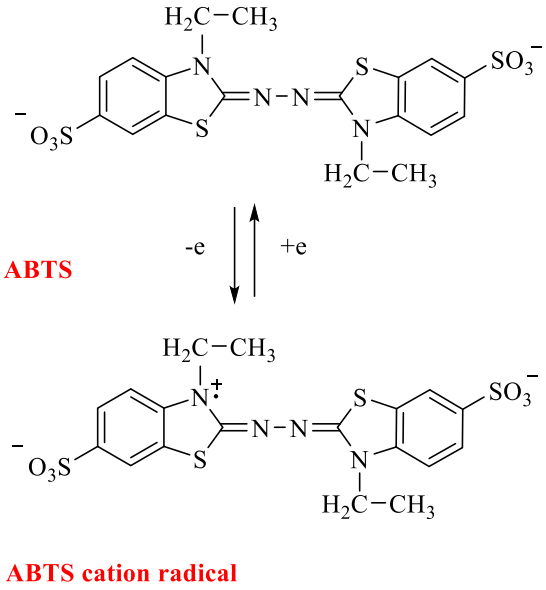


Figure 1. Oxidation of ABTS by laccase.

ABTS is a widely used non-phenolic synthetic substrate molecule for laccase that can also act as a mediator. For instance, wood delignification is one of the most known mediated laccase reactions. Laccase causes the oxidation of ABTS resulting in the formation of cation radical $ABTS^{+\cdot}$ (Fig.1), producing a dark green color. The intensity of the cation radical in the reaction mixture can be correlated to the laccase activity (Pankaj et al., 2016).

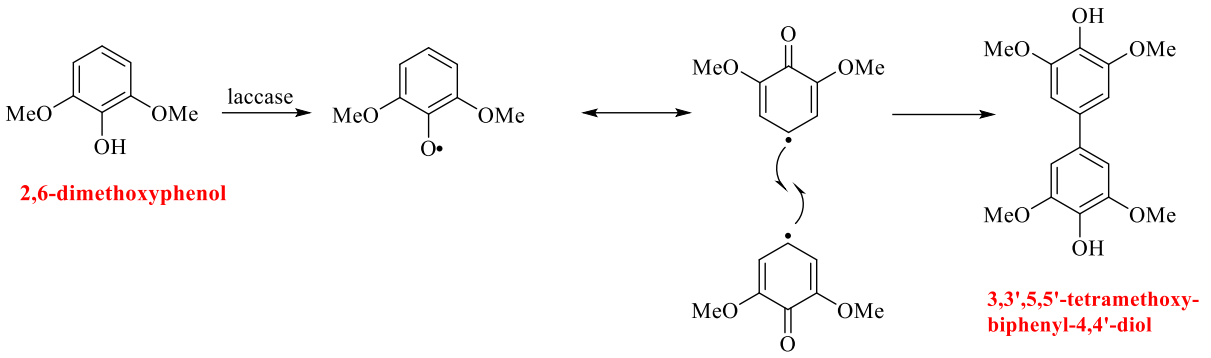


Figure 2. Laccase-catalyzed oxidation of 2,6-dimethoxyphenol.

Correspondingly, 2,6-DMP, along with its derivatives, belongs to the group of plant phenols and constitutes a significant portion of methoxyphenols found in birchwood smoke. These compounds are highly valued for their contribution to smoke flavor, preservation, and antioxidant properties (Kjällstrand and Petersson, 2001). The use of 2,6-DMP as a substrate for assessing laccase activity is common due to its ability to perform one-electron oxidation of different substrates (Kudanga et al., 2011), including methoxy-substituted monophenols, diphenols, aliphatic and aromatic amines. When laccase oxidizes 2,6-DMP, it generates phenoxy radical species, that are further stabilized through resonance to form para-radical species. Subsequently, the combination of two para-radical species results in the formation of dimer (Fig 2; Adelakun et al., 2012).

6.2 Purification of laccase

The anion exchange chromatography profile revealed a relatively sharp distinct peak (A2) as shown in Fig. 3. Laccase activity was evaluated within this peak, and all fractions showed laccase activity. Consequently, these active fractions were combined and subjected to size exclusion chromatography. A large peak is visible towards the end of the chromatographic run as depicted in Fig.4. However, not all the fractions eluted under this peak exhibited laccase activity, indicating inadequate separation of proteins during size exclusion chromatography. HiLoad Superdex 200 column is known for its high selectivity in separating proteins within the molecular weight range of 30,000 to 250,000

kDa, although its separation range extends from 10,000 to 600,000 KDa. The relatively small size of the protein could be a reason for the inadequate resolution observed in the size exclusion chromatographic separation. To confirm this, it may be necessary to use columns with smaller pore sizes specifically designed for separating small proteins and peptides.

The specific activity of the protein was increased from 0.04863 to 0.09699 umol/min/mg in the anion exchange chromatography step which resulted in a 1.99-times increase in purification fold (Table 1). The pooled fractions containing laccase activity from the anion exchange were analyzed using a 16.5% Tricine-SDS-PAGE gel, revealing the presence of two bands at approximately 75 kDa and 10 kDa, respectively (Figure. 5). Further purification steps will allow the complete purification and isolation of extracellular laccase from *Serratia* AORB19.

Bacterial laccase studies are relatively less when compared to fungal counterparts. Most of the bacterial laccases reported thus far have a molecular mass of 50 to 140 kDa (Arregui et al., 2019). The observed molecular weight of the partially purified laccase was inconsistent with the previously reported bacterial laccases. However, there are exceptions where small laccases have been isolated and biochemically characterized, like the extracellular laccase LacAn from *Anoxybacillus ayderensis* SK3-4 having a molecular weight of about 29.8 kDa, and the laccase SN4LAC *from B. tequilensis* with a molecular weight of 32 kDa. Additionally, (Sonica et al., 2014) reported that the difference in molecular weight makes the small laccases interesting for further investigations on its structure-function relationship.

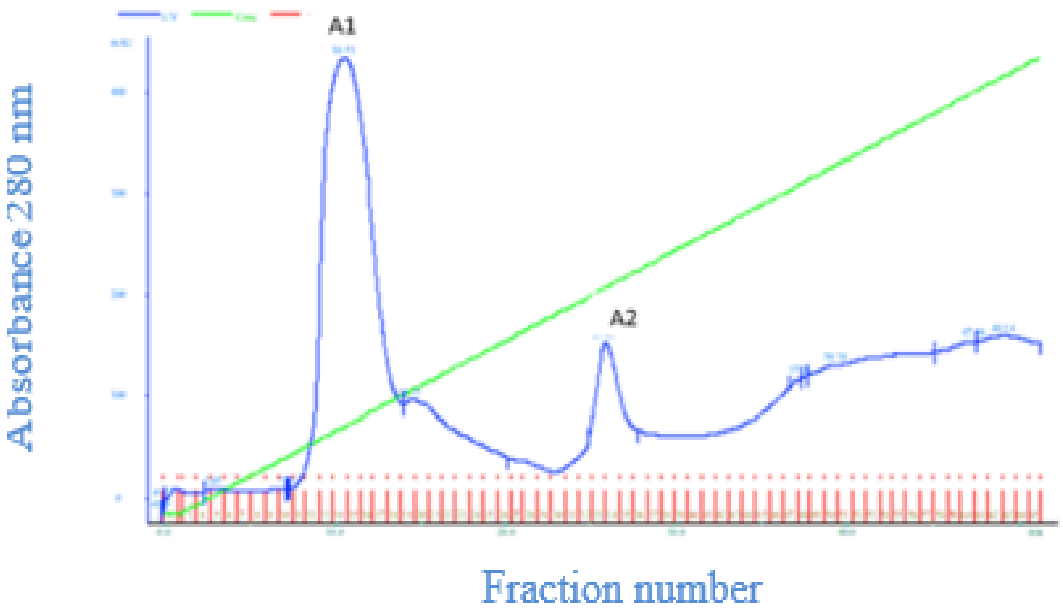


Figure 3. Hi Prep QFF Anion exchange chromatography profile of strain AORB19.

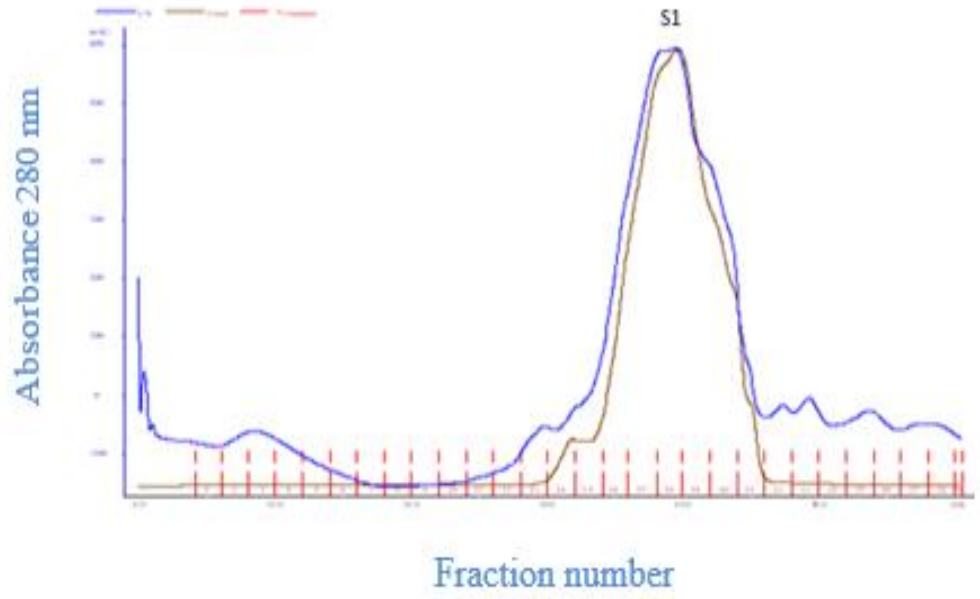


Figure 4. Superdex 200 size exclusion chromatography of strain AORB19 laccase.

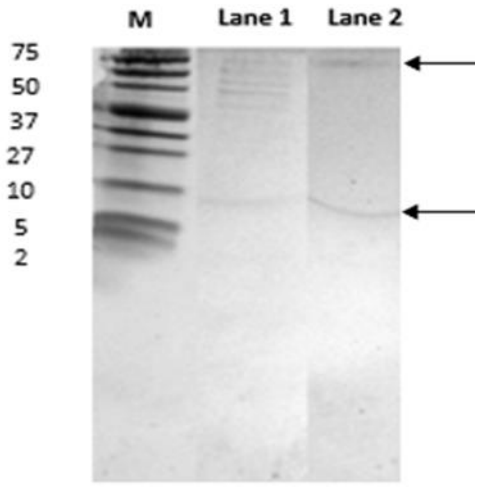


Figure 5. SDS-PAGE gel stained with coomassie blue showing the purification steps. Molecular weight standard (M); Culture supernatant (Lane 1); and Anion exchange partially purified fraction (Lane 2) showing two distinct bands at 75 kDa and 10 kDa respectively.

Table 1. Summary of the partial purification of laccase from *Serratia quinivorans* AORB19

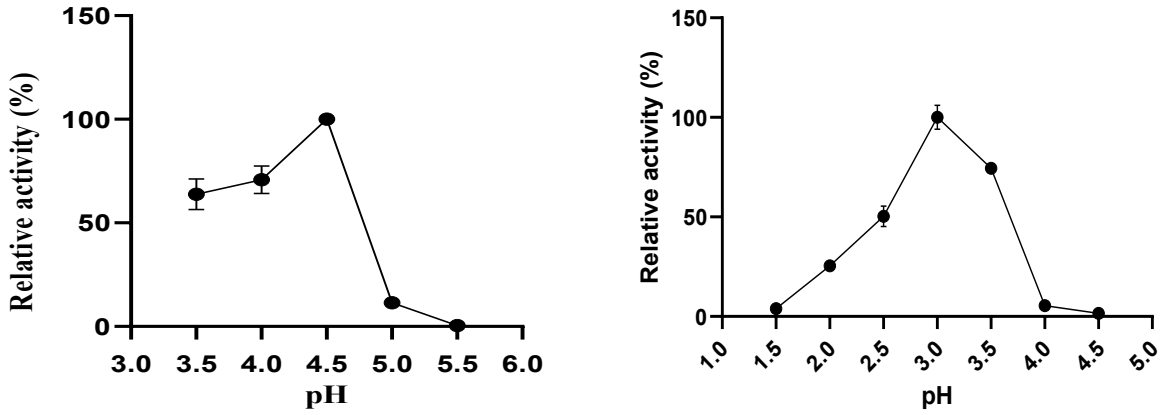
Purification step	Protein Concentration(mg/ml)	Specific Activity(umol/min/mg)	Purification level
Crude extract	0.02988	0.04863	1
Hi Prep QFF	0.00604	0.09699	1.99

7. Characterization of purified enzyme

7.1 Effect of pH

With the substrate 2,6 DMP, the enzyme exhibited activity in an acidic pH range of 3.0 –5.5, reaching its maximum activity at pH 4.5 (Fig.6a). Laccase activity was not detected at pH values higher than 5.5. Laccase activity did not occur at pH values higher than 5.5 (Fig. 6b). On the other hand, when the ABTS substrate was used, the enzyme exhibited activity at a highly acidic pH and reached its peak at pH 3,

which was the optimal pH. Beyond pH 3, the enzyme's activity decreased, and at pH 4.5, it was completely flattened.



(a) **(b)**

Figure 6. Effect of pH on relative activity of purified laccase using (a) 2,6 DMP and (b) ABTS as the substrate.

As prior studies suggest, wild fungal laccases have been shown to exhibit their highest activities at pH 2 to 3 when using ABTS and at pH 3 to 5 with phenolic substrates (Xu, 1996; Baldrian, 2006; Rodríguez-Escribano et al., 2022). For example, the wild fungal laccase PM1L demonstrated optimum activity at pH 2 with substrate ABTS and pH 3-4 when utilizing DMP as the substrate (Coll et al., 1993). Similarly, laccase 7A12 reached its peak activity at pH 3 with substrate ABTS and at pH 4 during the oxidation of DMP (Pardo et al., 2012).

In contrast, laccases originated from bacterial sources functions in a wide pH range (3.0 – 9.0) (Chauhan et al., 2017). The laccase derived from strain AORB19 exhibited optimum activity at pH 3 with substrate ABTS, while no activity was detected at a pH 5.5. Its optimal pH with 2,6 DMP was recorded as 4.5. Laccase isolated from *Pantoea ananatis* Sd-1 demonstrated maximum activity at pH 2.5

with substrate ABTS and pH 4.5 with guaiacol as substrates (Shi et al., 2015). Additionally, *Aeromonas hydrophila* isolated from activated sludge showed an optimum pH of 2.6 with substrate ABTS (Wu et al., 2010).

7.2 Effect of temperature

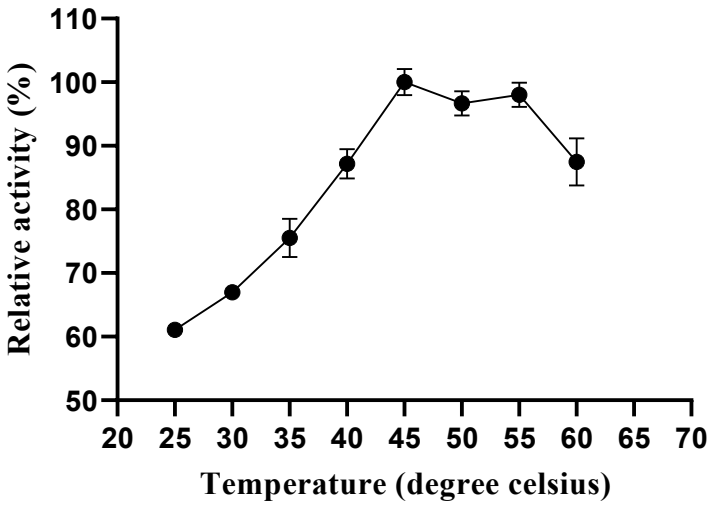


Figure 7. Effect of temperature on relative activity of purified laccase.

The enzyme was active in a broad range of temperatures from 25°C to 60°C with optimum temperatures ranging from 45°C to 55°C for the strain AORB19 (Fig. 7). One of the remarkable characteristics of bacterial laccase is its ability to survive in its broad range of temperature ranging from 30°C to 85°C (Chauhan et al., 2017). Indeed, the result of the current study is consistent with the activity of bacterial laccases previously reported as having temperature optima between 45°C and 60°C (Dalfard et al., 2006; Chauhan et al., 2017; Guan et al., 2014). Furthermore, bacterial strains *Bacillus* HR03 and γ-*Proteobacterium* JB reportedly have an optimal temperature range of 55°C (Singh, 2010).

7.3 Effect of metal ions

Metal ions typically have a limiting effect on the laccase activity during catalytic reactions, which can hinder their practical applications (Murugesan et al., 2009). The influence of metal ions on laccase enzyme activity was investigated using substrate 2,6 DMP at different concentrations (1mM and 5mM).

As shown in (Fig. 8), the laccase activity was not altered by the addition of Mg^{2+} and Mn^{2+} in the reaction mixture. Moreover, Ca^{2+} ion at 5mM demonstrated a slight stimulating effect on laccase activity. Additionally, laccase enzyme activity was improved by the addition of $Cu^{2+,}$ resulting in a 15% increase at 1mM and a 30% increase at 5mM respectively.

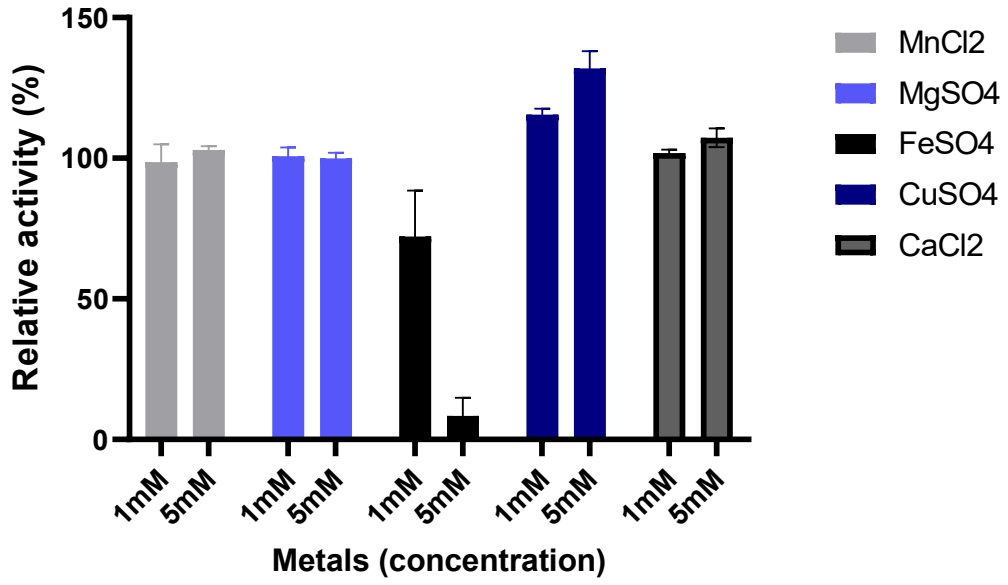


Figure 8. Effect of metal ions on relative activity of purified laccase

Earlier studies have reported that high concentrations of metal ions, including Ca^{2+}, Mn^{2+} and Cu^{2+} (up to 5 mM), can enhance laccase activity by modifying the substrate, thereby directly improving the reaction rate (Wiyada Mongkolthanaruk, 2012; Sondhi et al., 2014; Muthukumarasamy et al., 2015;). On the other hand, in the study, the addition of Fe^{2+} at 5 mM resulted in significant inhibition of over 90% of laccase activity ($P \leq 0.0001$). In other studies (Forootanfar et al., 2011; Ezike et al., 2020), similar findings were reported, demonstrating that the presence of Fe^{2+} led to a significant inhibition of laccase activity, resulting in a reduction of over 90% in laccase activity. Similar observations were seen in other studies where Fe^{2+} inhibited laccase activity causing more than 90% reduction of laccase activity. However, when Fe^{2+} was added at 1 mM, approximately 72% of laccase activity was retained. Conversely, several studies have shown that Mg^{2+} exerts inhibitory effects on laccase enzyme activity

(Muthukumarasamy et al., 2015; Sondhi et al., 2014). Notably, the AORB19 laccase exhibited stability in the presence of Mg^{2+}. This stability makes the AORB19 laccase suitable for applications in pulp and paper industries or the treatment of wastewater containing heavy metal contaminants.

7.4 Effect of Inhibitors

The inhibitors used in the study demonstrated inhibition of the laccase enzyme, and the degree of inhibition varied depending on the concentration and type of inhibitor (Table. 2). Specifically, the laccase enzyme was strongly inhibited by 1mM DTT, a well-known laccase inhibitor. As noted in prior studies, both the CotA laccase and spore laccase from *B. subtilis* WD23 showed significant inhibition when exposed to 1mM dithiothreitol and EDTA (Wang et al., 2015). Moreover, sodium azide at 1mM inhibited over 50% of the laccase activity. Similary, spore laccase was also inhibited by sodium azide and it prevents substrate oxidation by the enzyme to inhibit the reaction (Johannes and Majcherczyk, 2000). In contrast, AORB19 laccase and laccase from *Alcaligens faecalis* experienced only slight inhibition with EDTA, displaying a similar pattern of response (Mehandia et al., 2020).

Table 2. Effect of inhibitors on relative activity of purified laccase

Compound	Concentration (mM)	Relative activity (% of control)
DTT	**1 mM**	**5 ± 0.1**
	0.1 mM	**49 ± 0.3**
NaN3	**1 mM**	**34 ± 0.3**
	0.1mM	**76 ± 0.1**
EDTA	**1 mM**	**79 ± 0.4**
	0.1mM	**88 ± 0.1**
L-Cysteine	**1 mM**	**76 ± 6.0**

	0.1mM	**97 ± 0.1**

7.5 Effect of organic solvents

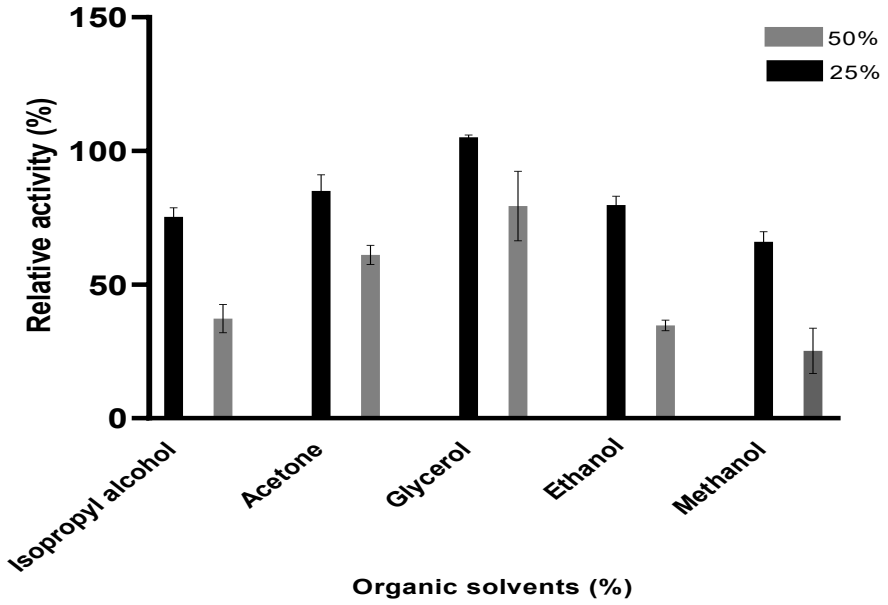


Figure 9. Effect of organic solvents on relative activity of purified laccase.

The laccase activity was significantly inhibited when exposed to methanol, ethanol, and isopropyl alcohol at a concentration of 50% (P>0.000). The laccase retained approximately 61% of its activity in the presence of 50% acetone, while about 79% activity was maintained with 50% glycerol (Fig.9). In comparison, *Bacillus subtilis* WD23 retained more than 80% of its laccase enzyme activity under similar conditions (Wang et al., 2011). Of note, the presence of 25% glycerol mildly enhanced laccase activity. Laccase retained around 80% activity with 25% acetone and ethanol, while isopropyl alcohol retained 75% activity, followed by methanol which retained 65% activity. Similarly, Lu et al. (2012) demonstrated that in *Bacillus licheniformis* LS04, methanol, ethanol (up to 30%), acetone, acetonitrile, and DMSO (up to 20%) may enhance laccase activity. However, these solvents start inhibiting laccase activity beyond these concentrations.

7.6 Kinetic parameter determination of partially purified laccase

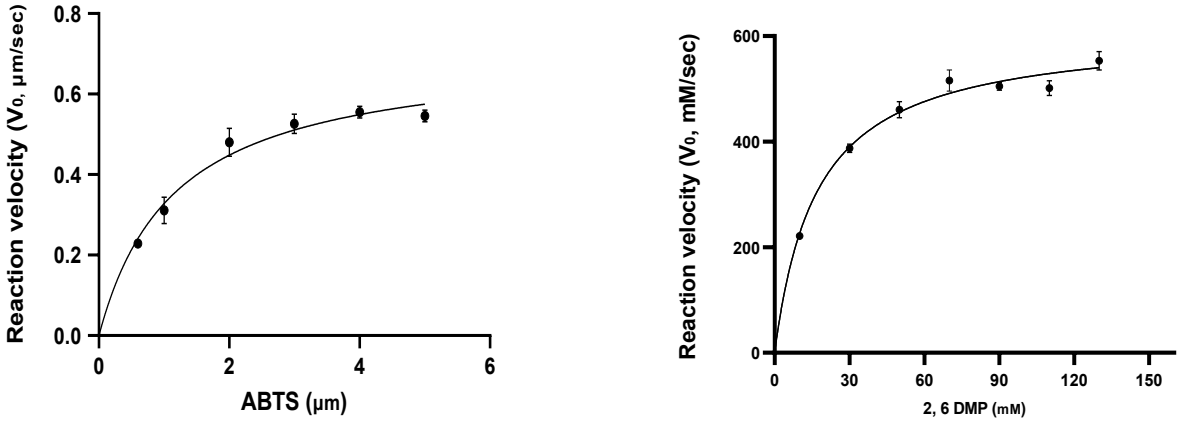


Figure 10. Michaelis–Menten plot for substrate oxidation by strain AORB19 (a) ABTS; (b) 2,6 DMP. A substrate range of 0.6 µM to 10 µM for ABTS and 10 mM to 130 mM was chosen for 2,6 DMP for the kinetic assay.

The reaction rate of AORB19 laccase was dependent concentration of substrate and its behavior followed the Michaelis-Menten kinetics. The partially purified laccase exhibited V_{max} and K_m values for ABTS and 2,6 DMP as follows: 0.7082 µM s−1 and 1.161 µM, and 610 mM s−1 and 17 mM, respectively (Fig. 10a and b). The laccase exhibited a stronger affinity for ABTS, as indicated by its lower K_m value, whereas it showed a lower affinity for 2,6 DMP, as reflected in its higher K_m value. Similarly, laccase derived from the same genus, *Serratia marcescens*, had a V_{max} of 0.83 µM s−1 (Kaira et al., 2015). However, the tested genus had a lower K_m value (1.161 µM) compared to *Serratia marcescens* (100 µM). It is worth noting that the K_m value obtained for 2,6 DMP was high for all reported laccases so far. A similar observation was reported by Adewale et al. (2021) regarding catechol 2,3-dioxygenase, where both the K_m and catalytic efficiency value (k_{cat}/K_m) fell outside the range of other reported enzymes. Additionally, other bacterial laccases with relatively higher K_m values include *Streptomyces viridochromogenes*, which has a small (two-domain) laccase with a K_m value of 4.5 ± 0.3 mM (Trubitsina et al., 2015). Furthermore, another two-domain laccase from *Streptomyces ipomoea* CECT 3341 SilA was characterized and found to have a K_m value of 4.27 mM (Molina et al., 2015).

8. Decolorization of synthetic dyes

Bacterial strain *Serratia quinivorans* AORB19 isolated from decomposed wood samples was tested for its efficacy to decolorize RBBR and MG dyes (Fig: 11a and b) by inoculating in CDB broth amended with 250 mg/ml and 500 mg/ml of both dyes under standard conditions. The laccase production profiles of the strain were also evaluated for both dyes simultaneously. Firstly, the laccase production profiles of *Serratia quinivorans* in CDB media with both 250 mg/L and 500 mg/L concentrations of RBBR dye were evaluated (Fig. 13a and b). In both specified concentrations, extracellular laccase production commenced after 24 h of incubation and exhibited a positive correlation with the decolorization of the respective concentrations by the bacteria. For the CDB media with 250 mg/L RBBR, the maximum laccase production (185.56U/L) was observed at 120 h of incubation; and with 500 mg/L RBBR the maximum laccase production (172.29 U/L) at 120 h of incubation, beyond which the laccase activity reached a plateau, indicating a leveling off of enzyme production in both the scenarios.

Secondly, the laccase production profiles of *Serratia quinivorans* in CDB media containing 250 mg/L and 500 mg/L of Malachite Green (MG) were quantified (Fig. 14 a and b). In both cases, extracellular laccase production initiated after 24 h of incubation and exhibited a positive correlation with the decolorization of MG by the bacterial strain. For the CDB media with 250 mg/L MG, the maximum laccase production (166 U/L) was achieved at 96 h of incubation, with a slight decrease in laccase production observed beyond 96 h up to 144 h. On the other hand, in the CDB media with 500 mg/L MG, the maximum laccase production (172.29 U/L) was attained at 120 h of incubation, after which the laccase activity levelled off, indicating a plateau in enzyme production. Consistent with the observation, a study by Du et al. (2013) reports that laccase activity showed an increasing trend with increase in MG dye concentration and time in dye degradation by bacteria *Micrococcus* indicating the potential involvement of laccase in the dye degradation process. Whereas NADH-DCIP reductase in the study showed a reverse trend in enzymatic activity with increasing MG concentration and time.

Among the two selected dyes, the strain aced the efficacy test for both the dyes with RBBR (Fig. 12 a) exceeded 76.35% and 52.03% decolorization by 144h of incubation for 250 mg/L and 500 mg/L respectively. Similarly, 94.31% and 77.61% decolorization was achieved with MG (Fig. 12 b) by 144h of incubation for 250 mg/L and 500 mg/L respectively, attesting to efficient decolorization. Weighing together, the strain demonstrated much higher efficiency for decolorizing MG rather than RBBR. Thus, the results suggests that *Serratia quinivorans* AORB19 entails a broad specificity for triphenylmethane and anthraquinone dye degradation and holds considerable potential for decolorizing these dyes in the environment.

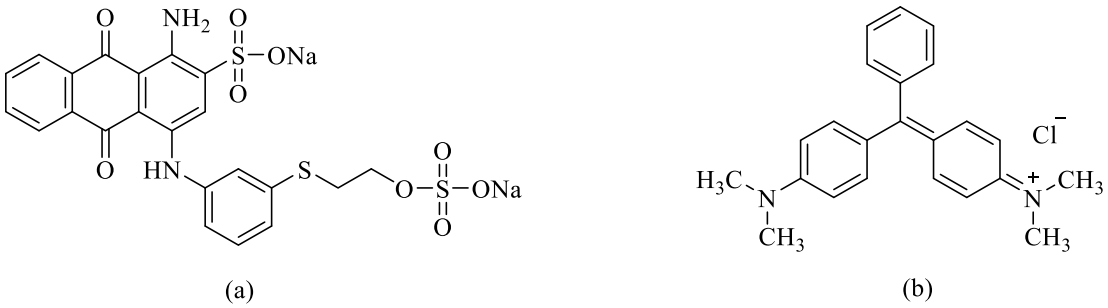
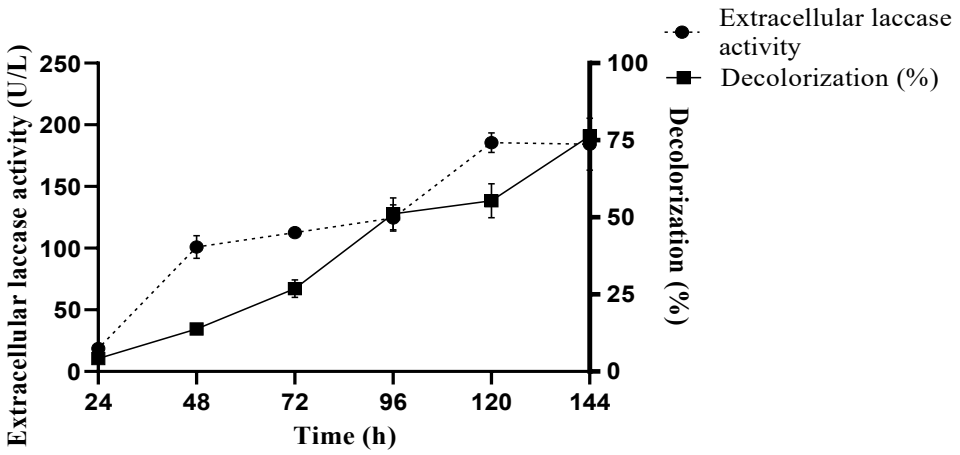


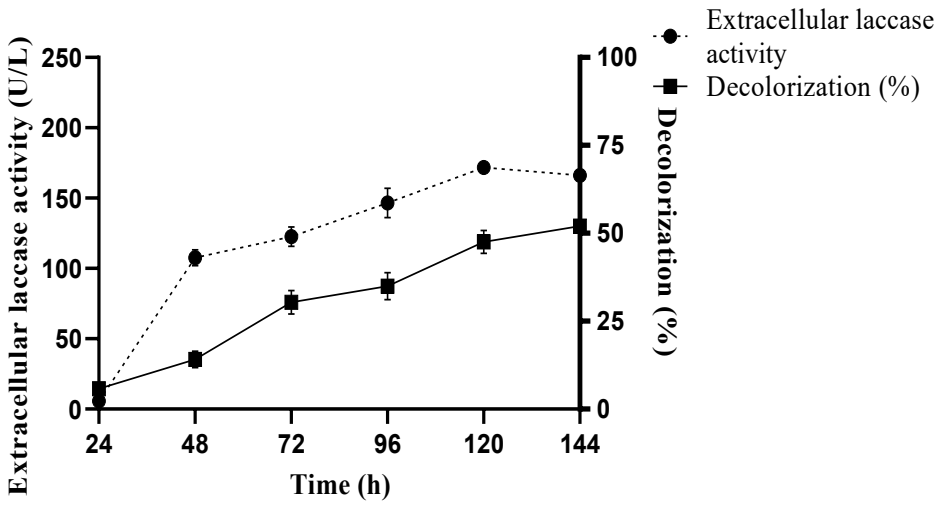
Figure 11. Molecular structure of (a) RBBR and (b) MG.

(a) (b)

Figure 12. Decolorization of synthetic dyes by *Serratia quinivorans* AORB19 (a) 76% decolorization of RBBR dye at 250mg/L, and; (b) 94% decolorization of MG dye at 250mg/L.



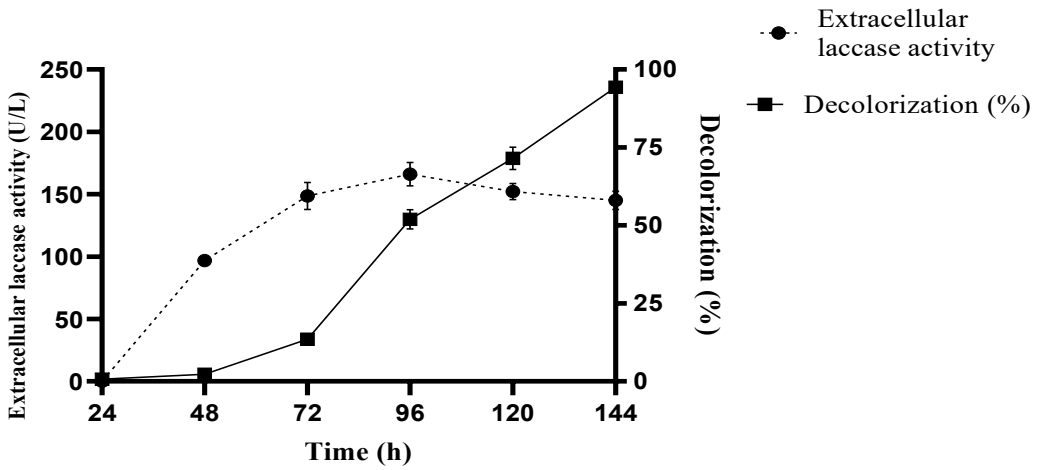
(a)



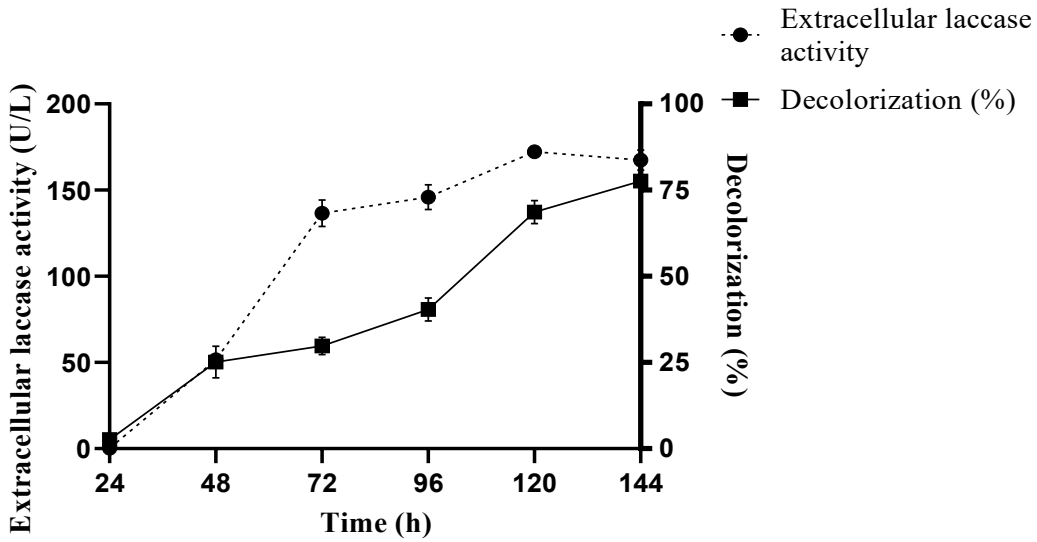
(b)

Figure 13. The dye-decolorization of RBBR by *Serratia quinivorans* AORB19 at (a) 250mg/L (b) 500 mg/L. Solid line indicates decolorization rate (%) and dotted line indicates laccase activity.

In a previous study conducted by Khudhair et al. (2015), it was reported that the bacterial strain *Enterococcus faecalis* ZL achieved a degradation rate of 58% for 50 mg/L of RBBR after 4 days of incubation. Additionally, Niladevi and Prema (2008) found that the strain *S. psammoticus* exhibited decolorization capabilities, achieving 80% decolorization of RBBR after 10 days of incubation in a dye-based medium. The degradation of RBBR is attributed to lignin-degrading enzymes, such as laccases and peroxidases, which can catalyze the asymmetric cleavage of RBBR into less toxic byproducts (Prasad et al., 2011; Liu et al., 2009). Furthermore, Afreen et al. (2018) reported that a partially purified laccase derived from the cyanobacterial strain *Arthrospira maxima* achieved a decolorization rate of 49% for RBBR.



(a)



(b)

Figure 14. The dye-decolorization of MG by *Serratia quinivorans* AORB19 at (a) 250mg/L (b) 500 mg/L. Solid line indicates decolorization rate (%) and dotted line indicates laccase activity.

In terms of decolorization of MG, two gram-negative bacterial isolates, *Enterobacter* sp. CV-S1 and *Enterobacter* sp. CM-S1, completely decolorized MG dye at a concentration of 15 mg/L after 78 h and 144 h, respectively (Roy et al., 2020). Similarly, a high decolorizing capability of 90.3% to 97.2% of MG was demonstrated by *Pseudomonas* sp. strain DY1 within 24h (Du et al., 2011). Additionally,

laccase from *Ganoderma lucidum* decolorized 40.7% MG at 25 mg/L in 24h with an increased decolorization of 96% after the addition of a mediator. Further, CotA-laccase exhibited up to 90% to 94% decolorization of MG in fresh and saline waters. It was documented that N-demethylation of MG was the main mechanism for decolorization (Murugesan et al., 2009; Cheng et al., 2021) by CotA-laccase as elucidated in *Bacillus subtilis* ISW1214 strain.

The MG dye degradation is a complex process due to the presence of multiple sites vulnerable to hydroxyl attack and its byproducts in solution and the formation of a carbon centered radical is followed by the breakdown of chromophore dye structures (Oturan et al., 2008; Chen et al., 2007). In the study of biodegradation of MG in *Micrococcus sp*. strain BD15 by Du et al., (2013), it was reported that the first step of biodegradation is initiated by hydroxylation of the central carbon of MG with subsequent cleavage of different C-C bonds. Furthermore, there are many studies which indicated that hydroxyl radical attack could result in N-demethylation during the initial degradation of MG (Saha et al., 2012; Chen et al., 2007).

Furthermore, laccases can oxidize the methyl carbon attached in Triphenylmethane dyes (TPM) structure, giving stable products which are affected by p-substituted phenyl. N-demethylation was the key factor of TPM degradation (Bibi et al., 2011). Two simultaneous degradation pathways have been proposed for the action of MG by laccase from *Cerrena* sp. (Yang et al., 2015). In the initial pathway, MG is demethylated initially followed by its polymerization or degradation for chromophore destruction. The second pathway involves the hydroxylation of MG to form its carbinol form, which is then rapidly broken down (Fischer et al., 2011). The presence of both pathways contributes to fast and efficient degradation of MG by laccases and the pathways are dependent on the type of enzyme and reaction conditions.

Additionally, laccases have demonstrated a higher efficiency in decolorizing anthraquinone dyes compared to other classes of dyes (Zeng et al., 2011). Multiple experimental studies have investigated

the decolorization of anthraquinone dyes by laccases (Afreen et al., 2018; Yang et al., 2009). During the degradation of RBBR by laccase, reduction, hydroxylation, deamination, and oxidation reactions have been observed. For instance, the laccase from *Polyporus* sp. S133 degraded RBBR, resulting in products that lacked an amino group due to the absence of analogous deamination reactions (Hadibarata et al., 2011).

Intriguingly, the results of the study suggest that the mechanism through which these synthetic dyes are decolorized are conserved in the strain *Serratia quinovarans* AORB19 and further studies are warranted to probe their key degradation pathways and phytotoxicity to plants and other life forms.

9. Determination of laccase Production using industrial biomasses

Considering the strain's inherent capacity for natural extracellular laccase production and its lignin-degradative traits identified through genomic analysis, the strain was tested for its ability to enhance laccase production in various agro-industrial biomasses sourced from Canadian industries. Various industrial waste biomasses including pea hull, flax seed meal, canola meal, okara and barley malt sprouts were used separately as a carbon source to assess their impacts on the strain's laccase production. It was observed that for all substrates, the enzyme activity maximized at 48 hours. At this time, flax seed meal exhibited maximum laccase activity of 257.71 U/L, which was three-fold higher than the laccase activity observed when alkali lignin (Sigma Aldrich) was utilized as the carbon source (83.65 U/L). It was followed by pea hull of 230.11 U/L, canola meal of 209.56 U/L, okara of 187.67 U/L and barley malt sprouts of 169.27 U/L (Figure.15). These results indicated the excellent adaptability of the strain to grow well on a broad range of substrates, particularly on flax seed meal leading to enhanced laccase production. Of note, the strain *Serratia quinivorans* AORB19 efficiently produced laccase enzymes using these heterogeneous biomasses without requiring further substrate optimization.

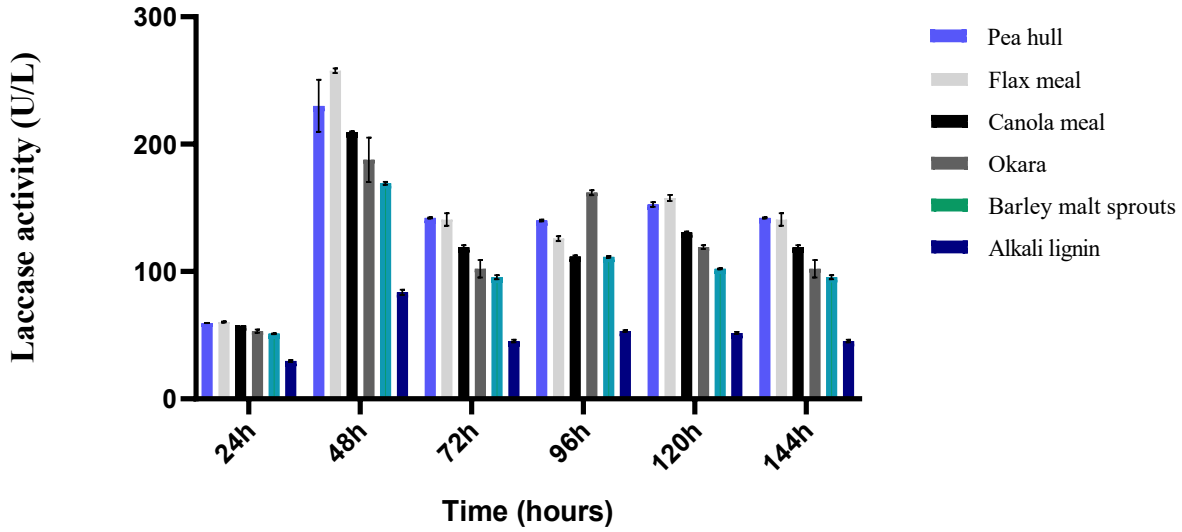


Figure 15. Laccase production by *Serratia quinivorans* AORB19 using industrial lignocellulosic waste residues as carbon source.

Agro-industrial residues such as pea hull, flax seed meal, canola meal, okara, and barley malt sprouts are the most common feed ingredients for poultry and livestock in Canada (Government of Canada, 2023). Multiple cohesive enzymes, including laccases are required to facilitate the degradation of intact cell walls of these agro-industrial residues. Okara or tofu dregs are the fibrous waste derived from soybeans that remain after filtering pureed soybeans during the production process of soy milk and tofu. In the study, a 2.24-fold increase in laccase production was observed when 0.1% okara was used as a carbon source in submerged fermentation. Similarly, a 2.11-fold increase in laccase production was observed when 0.1% tofu dreg was used in submerged fermentation with gram negative *Rheinheimera* sp. (Sharma et al., 2017). Pan et al. (2004) observed that a two-fold increase in laccase production was achieved while utilizing a combination of okara and wheat (8:2) in a mixed culture comprising of *Candida utilis, T. viride, S. cerevisiae* and *Aspergillus niger.*

Canola meal is the second largest protein meal produced in the world after soybean meal and is the left over after oil extraction from canola seeds. It is abundant in protein and phenolic compounds and its high fiber content can hinder its digestibility in livestock feed (Aachary et al., 2015; Ramadan, 2020). The Canadian production of canola meal in 2021-2022 year was 5, 700 369 metric tonnes (Government of Canada, 2022). The cultivation of strain AORB19 in 0.1% canola meal resulted in a 2.5-fold increase in laccase production. Similarly, the fungi *Pleurotus ostreatus* DAOM 197961 was able to produce laccase when grown in canola meal and found to decrease the meal's phenolic content upon solid state fermentation (Hu and Duvnjak, 2004).

Next to flax seed meal, pea hull exhibited maximum laccase activity of 230.11 U/L. In the study of Kumar et al (2021), different agro-industrial waste residues were screened for laccase production with *Bacillus aquimaris* AKRC02 and found that pea peels were shown to induce laccase production (0.85 U/mL) in submerged fermentation. Similarly, pea hulls were used as a novel low-cost feedstock to produce cellulase enzyme using *Trichoderma reesei* under submerged fermentation, and the fermentation parameters were optimized for its enhanced production (Sirohi et al., 2018). Additionally, in the study, the utilization of flax seed meal and barley malt sprouts by strain AORB19 led to an increase in laccase production of 3.08-fold and 2.02-fold respectively. However, at present, there are few detailed reports on the influence of flax seed meal and barley malt sprouts on laccase production. Notably, all biomasses used in the study exhibited significantly higher potential for laccase production when compared to technical alkali lignin (Sigma Aldrich). All the different substrates used in this study were the agro-wastes generated from local industries in Canada that were abundant and easily available. Multiple cohesive enzymes, including laccases are required to facilitate the degradation of intact cell walls of these agro-industrial residues.

Microorganisms that encompass broad substrate utilization including lignocellulose-rich plant or agricultural residues are deemed essential for robust enzymatic degradation of lignocellulose (Almarsdottir et al., 2011; Thomas et al., 2016). Given that the strain induced laccase production under the same cultivable conditions in different substrates shows its remarkable adaptability to these agro-substrates rendering them appealing for lignocellulose waste valorization initiatives. Bacterial laccases are exceptional environmentally friendly catalysts known for their wide substrate specificity, offering numerous potential applications in areas such as bioremediation, lignocellulose processing, waste valorization and beyond. In the study of bioprocessing using mixed cellulosic feedstocks for ethanol production (Althuri et al., 2017), the increased release of reducing sugars was attributed to the synergistic action of cellulases and ligninolytic laccase, which potentially enhances the accessibility of holocellulolytic enzymes to holocelluloses, consequently leading to improved production of fermentable sugars. Similarly, strain AORB19's ability to thrive and produce laccase using low-cost raw substrates as carbon sources suggested it may, at the same time, produce holocellulolytic enzymes that synergistically work together with laccase to efficiently hydrolyze lignocellulose. Thus, it offers great potential for applications in low-cost enzyme production, biomass pretreatment and valorization. Overall, the strain and enzymes offer a novel avenue for future explorations in lignocellulosic biomass conversion and residue valorization which may further enhance the economics of biorefineries.

10. CONCLUSION

In the study, an extracellular laccase derived from the strain *Serratia quinivorans* AORB19 isolated by means of high throughput screening was partially purified and characterized. The partially purified laccase exhibited two separate bands on SDS-PAGE, measuring 75 kDa and 10 kDa, respectively. Detailed characterization revealed its stability in the presence of metal ions and organic solvents, as well as its ability to function at high temperatures of up to 55°C and under acidic pH conditions. Additionally,

the study investigated the dye decolorization potential of the bacterial strain, demonstrating impressive decolorization rates of 94% for Malachite green and 76% for Remazol Brilliant Blue R. Furthermore, all the agro-industrial residues used in the study demonstrated significantly higher potential for laccase production compared to technical alkali lignin, with flax seed meal exhibiting the highest laccase activity of 257.71 U/L, followed by pea hull (230.11 U/L), canola meal (209.56 U/L), okara (187.67 U/L), and barley malt sprouts (169.27 U/L), indicating its adaptability and potential use in residue valorization processes. Thus, this newly characterized strain possesses dye-decolorizing and unique enzyme characteristics serving as a valued offering to further embolden and accelerate the ongoing green initiatives and bioremediation efforts, a high priority area in the environmental sustainability agenda. Furthermore, the distinct characteristics and advantages make the strain and its green enzyme a suitable candidate for industries that demands such conditions.

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CHAPTER 6

General discussion, summary of major contributions and recommendations for future research

The identification and application of selective biocatalysts for valorizing lignocellulose are essential for the economic viability of future biorefineries (Ho et al., 2023). With the perennial demand for versatile strains with higher specificity in bioprocessing and industrial applications, isolating microorganisms from natural microbial populations or complex environmental samples becomes a crucial and inevitable preparative step or process. This process is mainly turned to practice using various screening approaches, including conventional methods that involve qualitative detection using aromatic dyes in agar plates (Xu et al., 2018; Gonçalves et al., 2020) while various screening techniques have also been employed such as spectrometric assay of lignin degradation products (Zamzuri et al., 2013), metagenomics screening and analysis (Kroeger et al., 2018), and the application of biosensors (Ho et al., 2023).

The high throughput screening (HTP) process designed and implemented in the present study is a rapid and efficient screening method that can be used on a large number of samples since it extends from the separation of a promising microbial consortium to a single microbial strain for targeted studies and development. This HTP method involved using lignin as a carbon source and quantitatively detected laccase enzyme activities. The specificity of the HTP method is played at the level of the selectivity of the 'marker' to enable uniform screening and detecting the presence or absence of a desired activity or trait for effective isolation and microbial classification based on the throughput requirements of a study. When a very specific activity or interaction is desired, the substrate against the target enzyme is naturally used. Thus, HTP screening is a selective screening method involving the usage of a specific substrate

that can exclusively interact with target microorganisms to be isolated from complex environmental samples. The pre-defining of a marker is an important step in the HTP process, thus saving working time compared to conventional screening procedures. Here the notion of 'marker' is very distinct; and not intended to be a target microorganism but a desired enzyme activity capable of being detected selectively being secreted by complex microorganisms.

This novel HTP approach was successfully implemented in the present study facilitating the isolation of unique lignin-degrading microorganisms in large numbers from decomposed wood samples that naturally secrete laccase enzyme with desired characteristics. Lignin degradation is accomplished by a complex mechanism involving multiple enzymes, including laccase, which was selected as a marker in the present high-throughput screening. The isolated microorganisms included bacterial strains from various genera, including *Serratia, Enterobacter*, *Raoultella*, and *Bacillus*, and fungal counterparts, including *Mucor*, *Trametes*, *Conifera*, and *Aspergillus*. Moreover, *Aspergillus sydowii* (AORF21), *Mucor* sp. (AORF43), *Trametes versicolor* (AORF3), and *Enterobacter* sp. (AORB55) exhibited xylanase and β- glucanase activities in addition to laccase production.

As an alternative to the traditional culture-based screening, this study recommends an efficient high throughput screening process for the rapid isolation of lignin-degrading microbes from a large number of biomasses, a great addition to the repertoire of innovative screening approaches, empowering lignin degradation, conversion, and valorization efforts. Due to its selectivity and specificity, HTP screening approaches represent an immense promise even if, in the current study, certain modalities were not experimented with, such as the application of yet another marker to isolate microbes for comparison. However, given the large number of isolated microbes, carrying out an extended screening was overwhelming in this study. Future studies on these lines are proposed to further address this comparison of isolated microbes based on the chosen purpose of the study. Nevertheless, a more rational view of screening target microorganisms using the HTP method is to construct rational combinatorial

microorganisms called libraries to expand the franchise of lignin-degrading microbial communities, strains, and enzymes which are being proposed as a future direction.

Natural biodegradation processes may hold promise for the conversion of agro-industrial lignocellulosic biomaterials into biofuels and fine chemicals through lignin-degrading enzymes. The high cost and low stability of these enzymes remain a significant challenge to economic lignocellulosic biomass conversion. Wood-degrading microorganisms are a great source of novel enzyme discoveries. Among the potential microbial strains isolated using a high throughput screening process from decomposed wood samples, a promising γ -proteobacterial strain that naturally secreted a significant amount of laccase enzyme was chosen. The strain was biochemically characterized and identified as *Serratia proteamaculans* AORB19 based on its genotypic and phenotypic characteristics.

Laccase synthesis by microorganisms can be influenced by many factors, such as the chemical and nutritional composition of the media; nonetheless, the optimization approach used may be strain specific. *Serratia proteamaculans* AORB19 was found to secrete laccase under alkaline pH conditions and mesophilic culture conditions and was seen as tolerant to many metal ions and organic solvents in the study. The results point towards the greater potential of the strain to be directly used in the fermentation process or for laccase enzyme production. The one-factor-at-a-time methodology resulted in a 6-fold increase (73.30 U/L) in laccase production when compared with the initial culturing conditions (12.18 U/L). The results suggest that the extracellular laccase may be rationally optimized to enhance the capacity for lignin depolymerization/degradation. Moreover, the *Serratia proteamaculans* AORB19 may be further optimized using statistical optimization techniques such as Response Surface Methodology (RSM) to enhance the extracellular secretion efficiency and thus expand the repertoire of optimal laccase-producing strains for lignin bioconversion and degradation processes.

Limited knowledge exists regarding the microbial metabolism of aromatic compounds derived from lignin, with only a few bacterial species like *P. putida* (Lin et al., 2016) and *S. paucimobilis* (Masai

et al., 2007) currently studied. With the growing inclusion of various microorganisms in lignin degradation research, greater emphasis should be placed on comprehending the functional characteristics of lignin-degrading organisms, particularly at the genomic level. Analysis of the genome data is a way to enhance our understanding of bacterial degradation, a pivotal process in the global carbon cycle, and provides a framework for further development. Genomic analysis can provide insights into the potential genes associated with lignin metabolism, the pathways they may be involved in, and the enzymes responsible for lignin degradation, particularly on new strains with limited research on lignin degradation. The screening of complex microbial samples requires analytical techniques with a high resolving power in order to facilitate high throughput screening from complex microbial populations.

Genome analysis with high resolving power is necessary to confirm the identity of microbes isolated from complex microbial samples. The whole genome of the strain AORB19 was analyzed to validate the species identification, the putative genes, and its enzymes related to lignin degradation. It is observed that the classical 16S rRNA gene-based trees do not possess sufficient resolution to differentiate between closely related species; however, core genome-based phylogeny provides a better resolution and is more robust than conventional marker gene-based phylogeny. Despite the strain being earlier reported as *S. proteamaculans* AORB19 (Ali et al., 2022) based on its 16S rRNA sequence, comparative genomic analyses of the entire genome suggest that the strain should be designated as *Serratia quinivorans* AORB19. The ANI and in-silico DNA-DNA hybridization (DDH) analysis were the parameters used for taxonomic assignments, which was subsequently confirmed by the core genome-based phylogenetic tree constructed by the UBCG pipeline.

A robust gene annotation process is essential for identifying functional genes, enabling comprehensive studies on how genetic variation influences enzyme activity. The gene annotation of the newly designated *Serratia quinivorans* AORB19 strain confirmed that it harbored a multitude of genes for carbohydrate-active enzymes, which likely facilitates growth on lignocellulosic biomass. More so,

eight lignin-degrading enzyme genes in class AA, including lignin oxidizing and lignin-degrading genes, enriched this strain which is crucial for lignin degradation. To confirm these hits from whole genome analysis, Liquid chromatography with UV detection (LC-UV) analyses were performed in search for active metabolites linked to lignin degradation. LC-UV analysis confirmed the presence of compounds such as p-hydroxybenzaldehyde and vanillin in the culture media. While low concentrations of vanillic acid were eluted in the beginning, it was completely depleted over time during the incubation period, thus indicating that the strain possesses enzymes capable of metabolizing vanillic acid.

Moreover, few potential laccase-like genes, including multi multicopper oxidases (SQAORB19_2332; SQAORB19_1606; SQAORB19_2924), DyP-peroxidases (SQAORB19_3208; SQAORB19_3467; SQAORB19_3468) and dehydrogenases (SQAORB19_610; SQAORB19_635) were identified in the bacterial genome pointing towards the strain's potential to degrade lignin. Furthermore, the genome of strain AORB19 also possesses numerous key genes for the β-ketoadipate pathway that may be potentially involved in the degradation of aromatic compounds. Thus, the results of whole genome sequencing analyses coupled with LC-UV analyses results correlated well, supporting the involvement of *Serratia quinivorans* AORB19 in the process of lignin degradation in nature. The LC-UV analysis successfully delineated active compounds involved in lignin degradation, which is an essential prerequisite for future metabolic engineering studies and for the derivatization of value-added products. Essentially, this study unveiled unique features of the lignin-degrading bacterial strain *Serratia quinivorans* AORB19, and comprehensive explorations are suggested to elucidate the lignin degradation mechanisms and clearly define their pathways.

Laccase enzymes have a wide range of applications as green biocatalysts due to their ability to oxidize a wide range of substrates, including phenolic and non-phenolic compounds (Rodríguez et al., 2006; Zerva et al., 2019; Becker & Wittmann, 2019). Fungal laccases have certain limitations due to their inherent physiological and biochemical properties when it comes to industrial applications. These

limitations include slower growth rates, low tolerance in the presence of inhibitors, long fermentation periods, low pH optima, and difficulties in expression in heterologous host organisms (Upadhyay et al., 2016; Kim et al., 2010). On the other hand, bacterial laccases have been reported to exhibit several advantages, which are tolerant to a wide range of temperatures and pH levels, broad substrate specificity, ease to genetic manipulations, rapid enzyme production and exceptional stability in the presence of inhibitors which makes them appealing and suitable for industrial applications (Du et al., 2015; Chauhan et al., 2017). However, compared to fungal laccases, prokaryotic laccases characterized are fewer, and hence there is a need for more extracellular low-cost prokaryotic laccases as their biochemical and catalytical properties are suitable for industrial applications.

In industrial settings, bacterial laccases are reported to be used for effective bioremediation of dyes and recalcitrant xenobiotics in industrial textile waste water (Guan et al., 2015), denim bleaching (Panwar et al., 2020), treatment of distillery effluents (Yadav et al., 2012), and pulp and paper mill waste water (Chandra et al., 2011). Bacterial laccases are also used in waste valorization involving lignocellulosic biomasses (Moreno et al., 2016). For instance, in the utilization of bacterial laccase in lignocellulosic waste valorization, a novel thermoalkaliphilic bacterial laccase, CtLac derived from *Caldalkalibacillus thermarum* strain TA2.A1, was found to depolymerize lignin and produce two value-added chemicals including vanillin and p-hydroxybenzaldehyde using different lignocellulosic biomasses (Yang et al., 2019**).** Moreover, there are many applications of bacterial laccases with patents, which include the utilization of thermophilic laccase from *Brevibacillus* in compositions intended for the biodegradation of plastics (Zerva et al., 2019).

As mentioned above, the isolated *Serratia quinivorans* AORB19 strain naturally produced significant extracellular laccase enzymes in the culture media with alkali lignin as the sole carbon source. Additionally, the genome analyses of *Serratia quinivorans* AORB19 revealed the presence of three laccase-like genes; multi-multicopper oxidases (SQAORB19_2332; SQAORB19_1606;

SQAORB19_2924). Therefore, we partially purified and characterized the extracellular laccase derived from the new strain of *Serratia quinivorans* as the next step of our study. In the study, the crude enzyme was partially purified using anion exchange chromatography, resulting in a 1.99 times increase in the purification fold. The partially purified protein revealed the presence of two bands at approximately 75 kDa and 10 kDa, respectively, 16.5% Tricine-SDS-PAGE gel. Most bacterial laccases reported thus far have a molecular mass of 50 to 140 kDa (Arregui et al., 2019). However, there are exceptions where small laccases have been isolated and biochemically characterized, like the extracellular laccase LacAn from *Anoxybacillus ayderensis* SK3-4 having a molecular weight of about 29.8 kDa, and the laccase SN4LAC from *B. tequilensis* with a molecular weight of 32 kDa. The partially purified laccase showed an optimum pH 3 with ABTS, pH 4.5 with 2,6 DMP substrate and a temperature of 45°C -55°C. The V_{max} and K_m of partially purified laccase towards ABTS and 2,6 DMP were determined as 0.7082 µM s−1 and 1.161 µM; 610 mM s−1 and 17 mM, respectively. Laccase activity was enhanced in the presence of Cu^{2+} and $Ca^{2+,}$ and laccase activity was retained in the presence of Mg^{2+} and Mn^{2+}, and Fe^{2+} highly inhibited laccase activity at 5mM. DTT, sodium azide, methanol, ethanol, and isopropyl alcohol was found to inhibit laccase activity. Laccase activity was mildly inhibited in the presence of conventional inhibitors like L-cysteine and EDTA. These distinct characteristics and advantages of this partially purified laccase make it suitable for industries that demand such conditions.

Textile dyes and pollutants are highly toxic, carcinogenic, and mutagenic, and untreated disposal of textile dyes into water bodies poses a risk to human and aquatic life (Desore & Narula, 2017; Lellis et al., 2019). *Serratia quinivorans* strain has not been studied for its bioremediation capabilities, particularly in its ability to decolorize synthetic textile dyes. Hence, for the first time, we explored the decolorizing dye capabilities of *Serratia quinivorans* strain for two widely used textile dyes such as Malachite green and Remazol brilliant blue R belonging to triphenylmethane and anthraquinone classes of dyes, respectively. The strain exhibited remarkable efficacy in decolorizing both MG and RBBR dyes.

The decolorization of MG at concentrations of 250 mg/L and 500 mg/L reached over 94.31% and 77.61%, respectively, after 144 h of incubation, indicating highly efficient decolorization. Similarly, RBBR achieved decolorization rates of 76.35% and 52.03% at concentrations of 250 mg/L and 500 mg/L, respectively, after 144 h of incubation. Overall, the strain displayed significantly higher efficiency in decolorizing MG compared to RBBR. For both dyes, the strain demonstrated a concurrent rise in laccase activity simultaneously with an increase in dye decolorization. These findings suggest that *Serratia quinivorans* AORB19 possesses broad specificity for degrading triphenylmethane and anthraquinone dyes, highlighting its potential to be used as a suitable candidate for effectively decolorizing these dyes in the environment.

Agro-industrial residues such as pea hull, flax seed meal, canola meal, okara, and barley malt sprouts are the most common feed ingredients for poultry and livestock in Canada. Multiple cohesive enzymes are required to facilitate the degradation of intact cell walls of these agro-industrial residues. To obtain efficient laccases for lignocellulose bioprocessing, various fiber biomass wastes, including pea hull, flax seed meal, canola meal, okara, and barley malt sprouts, were used as carbon sources separately to induce *Serratia quinivorans* AORB19 for extracellular laccase production. Among the tested biomasses, flax seed meal exhibited maximum laccase activity of 257.71 U/L, which was three-fold higher than the laccase activity observed when alkali lignin (Sigma Aldrich) was utilized as the carbon source (83.65 U/L). This indicates the potential of flax seed meal to be used as an excellent low-cost raw substrate for enhanced laccase production with *Serratia quinivorans* AORB19.

High laccase production in the presence of flax seed meal was followed by pea hull of 230.11 U/L, canola meal of 209.56 U/L, okara of 187.67 U/L, and barley malt sprouts of 169.27 U/L. Pee peels were shown to induce laccase production (0.85 U/mL) with *Bacillus aquimaris* AKRC02 upon submerged fermentation (Kumar et al., 2021). Similarly, the fungi *Pleurotus ostreatus* DAOM 197961 was able to produce laccase when grown in canola meal and was found to decrease the meal's phenolic

content in solid-state fermentation (Hu & Duvnjak, 2004). Furthermore, a 2.11-fold increase in laccase production was observed, while 0.1% okara was used in submerged fermentation by gram-negative *Rheinheimera sp.* (Sharma et al., 2017).

The study revealed that all the biomasses utilized demonstrated a significantly higher capacity for laccase production, implying that the strain *Serratia quinivorans* AORB19 holds a plausible promise as a potential enzyme source for lignocellulosic biomass waste valorization and conversion. In the study of bioprocessing using mixed cellulosic feedstocks for ethanol production (Althuri et al.,2017), it was observed that the increased release of reducing sugars could be attributed to the synergistic action of cellulases and ligninolytic laccase, which potentially enhanced the accessibility of holocellulolytic enzymes to holocelluloses, consequently leading to improved production of fermentable sugars. Therefore, the strain's ability to thrive and produce laccase when exposed to these low-cost multiple raw substrates suggests its potential application in biomass pretreatment for the efficient release of sugars in biofuel production.

In conclusion, based on the overall study findings, the following recommendations highlight areas for future research and investigation:

1. Construction and exploitation of rational combinatorial microorganism libraries: Based on the outcomes of the study, the construction of a rational combinatorial library of lignin-degrading microorganisms using an HTP screening process is proposed. The HTP process employed in this study specifically focused on screening microorganisms that secreted laccase. In this way, a combinatorial library of organisms of other lignin-degrading enzymes using an HTP process may be constructed as these libraries present an excellent opportunity for identifying novel enzymes and functionalities and to discover highly effective combinations of ligninolytic enzymes, which can work synergistically for the pretreatment and conversion of lignocellulosic biomass.

2. To undertake the complete purification of the laccase enzyme and identification of its amino acid sequence using Edman sequencing.

3. To optimize dye decolorization conditions and phytotoxicity assessments for the strain *Serratia quinivorans* AORB19 for its application in bioremediation.

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Publications list

(1) **Ali, N. S.,** Huang, F., Qin, W., & Yang, T. C. (2022). Identification and characterization of a new *Serratia proteamaculans* strain that naturally produces significant amount of extracellular laccase. *Frontiers in Microbiology*, 13. https://doi.org/10.3389/fmicb.2022.878360

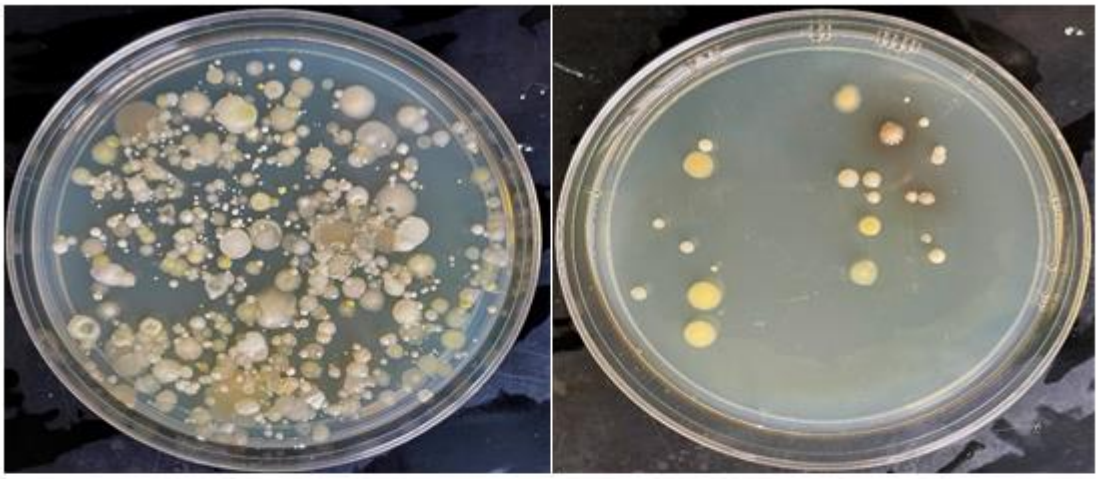
(2) **Ali, N.S**., Huang, F., Qin, W., & Yang, T. C. (2023). A High Throughput Screening Process and Quick Isolation of Novel Lignin-Degrading Microbes from Large Number of Natural Biomasses. *Biotechnology Reports*, 39. https://doi.org/10.2139/ssrn.4392139

(3) **Ali, N.S**., Thakur, S., Ye, M, Monteil-Rivera, F., Pan, Y., Qin, W., & Yang, T. (under review). Uncovering the lignin-degrading potential of *Serratia quinivorans* AORB19: Insights from genomic analyses and alkaline lignin degradation

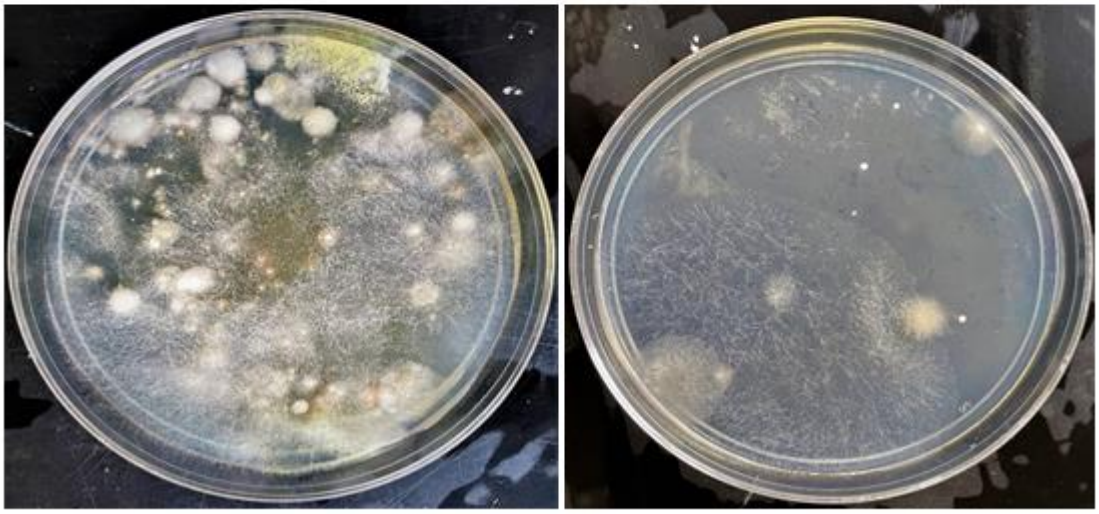
(4) **Ali, N.S**., Huang, F., Qin, W., & Yang, T. (under review). Purification and biochemical characterization of extracellular laccase from *Serratia quinivorans* AORB19 and its application in degradation of synthetic dyes

Appendices

Appendix 1. Secondary screening of microbial isolates (a) bacterial samples without serial dilution (b) bacterial samples after serial dilution (10^{-6}); and (c) fungal samples without serial dilution (d) fungal samples after serial dilution (10^{-6}).



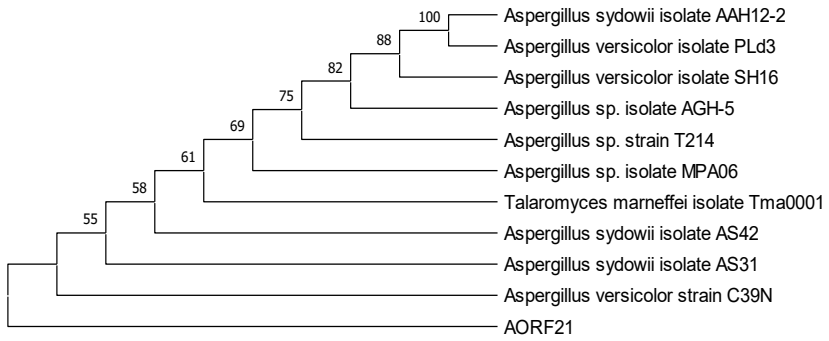
(a) (b)



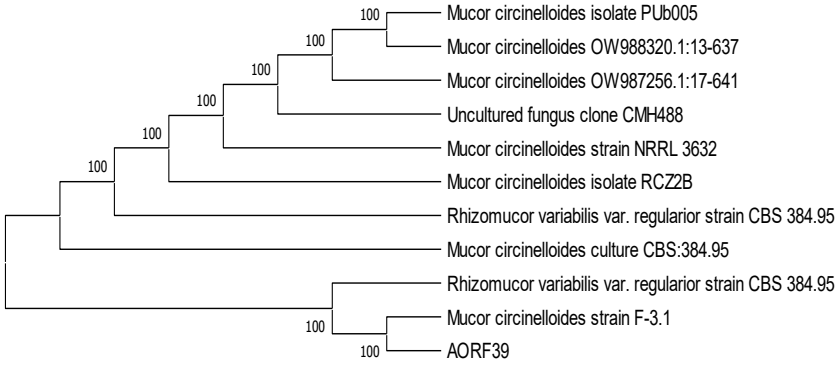
(c) (d)

Appendix 2. (a) to (e): Phylogenetic tree of five selected lignin degrading fungal strains based on neighbor joining criteria using MEGA-X. The percentage of replicate trees associated taxa are clustered together using the bootstrap test (1000 replicates) are shown next to the branches.

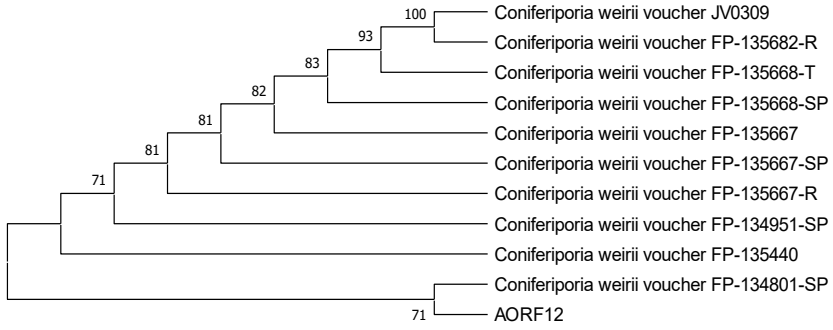
a) AORF21



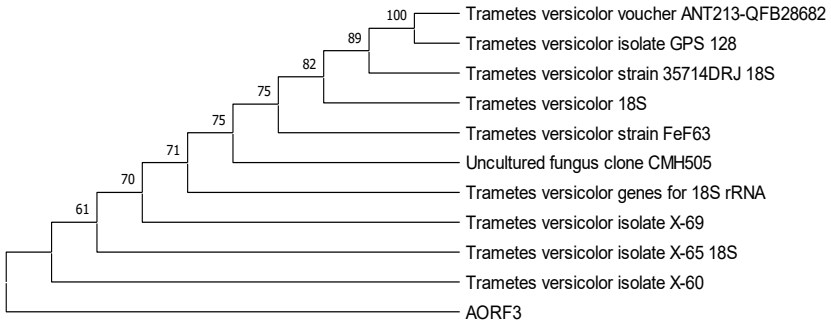
b) AORF39



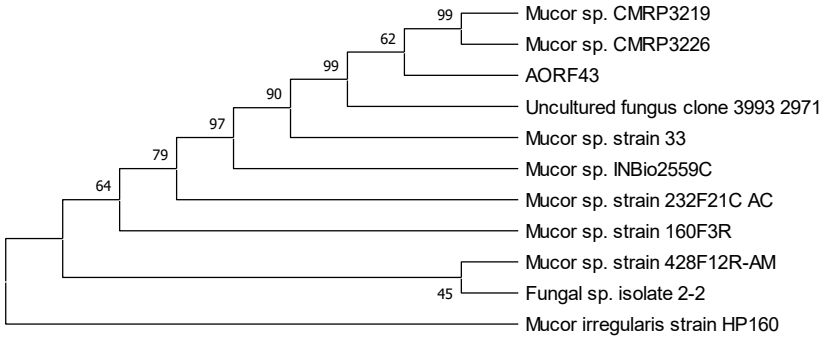
c) AORF12



d) AORF3

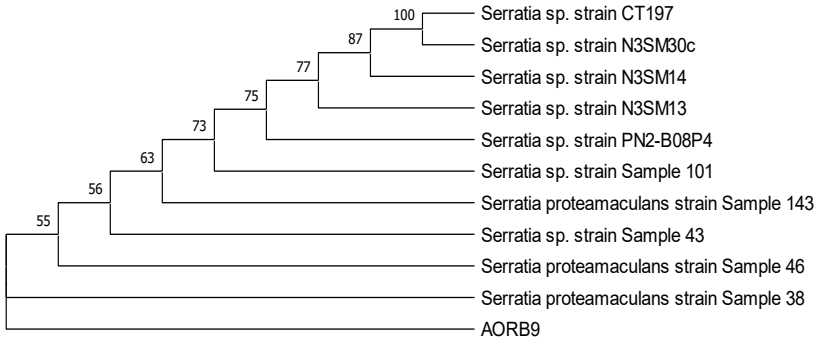


e) AORF43

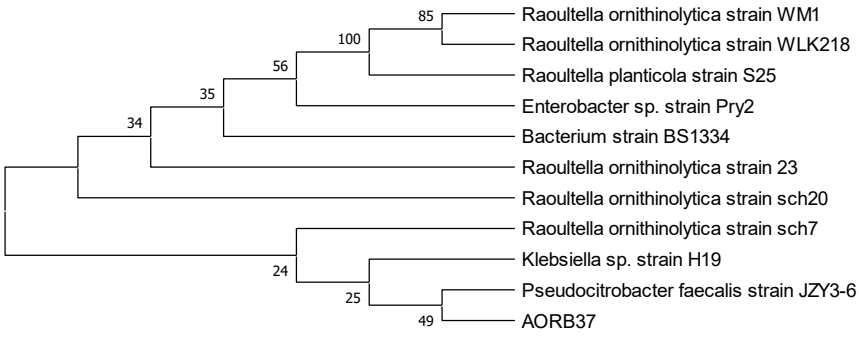


Appendix 3. (a) to (j): Phylogenetic tree of ten selected lignin degrading bacterial strains based on neighbor joining criteria using MEGA-X. The percentage of replicate trees associated taxa are clustered together using the bootstrap test (1000 replicates) are shown next to the branches.

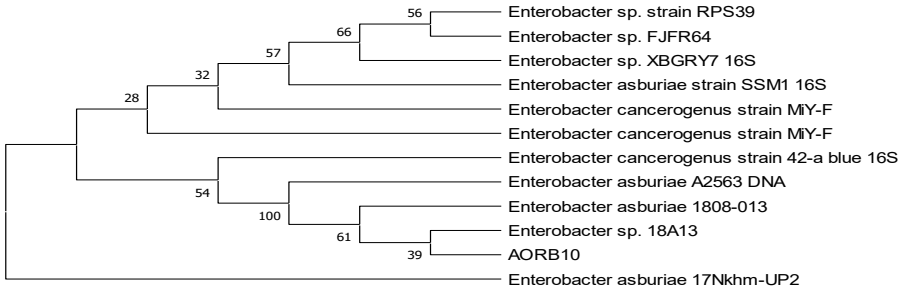
a) AORB9



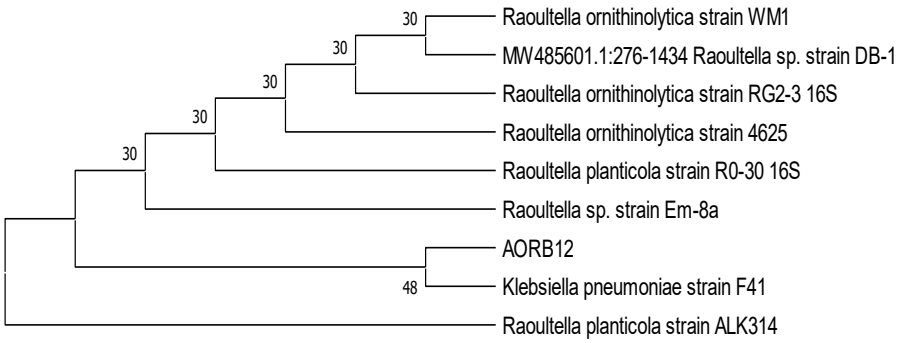
b) AORB37



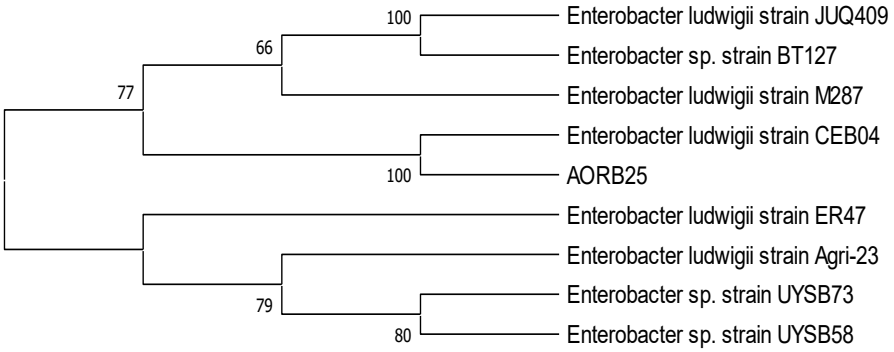
c) AORB10



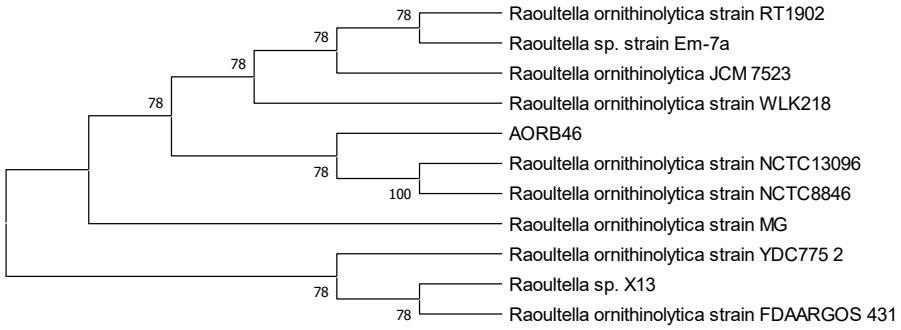
d) AORB12



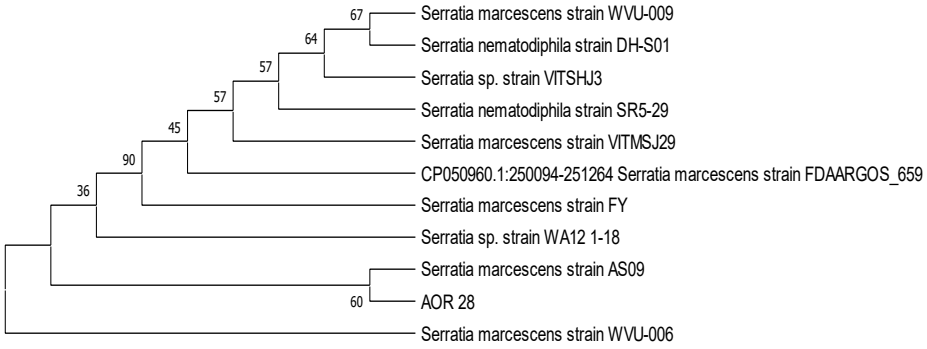
e) AORB25



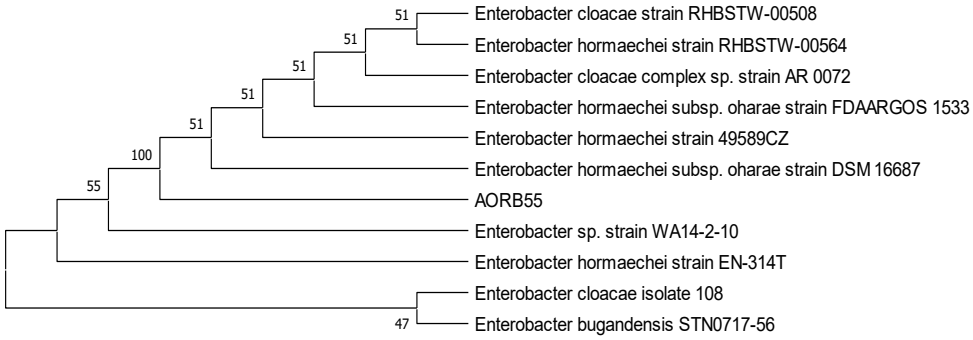
f) AORB46



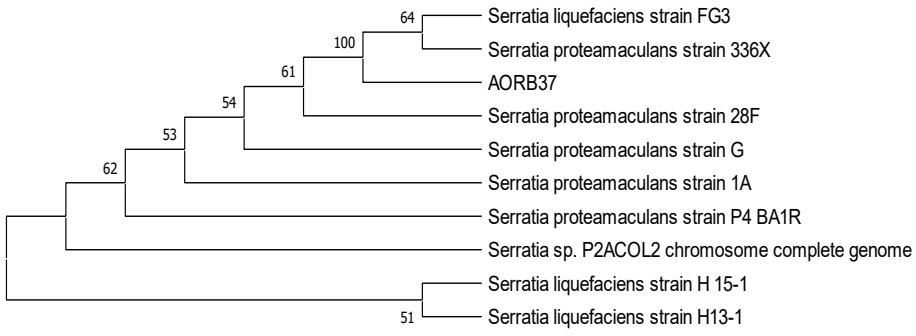
g) AORB28



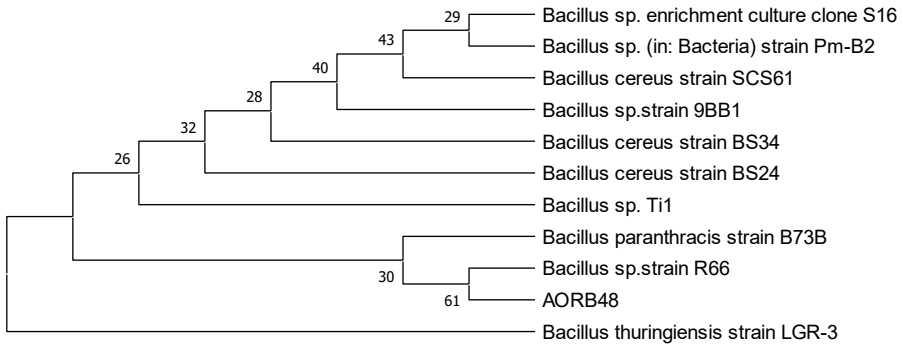
h) AORB55



i) AORB19



j) AORB48



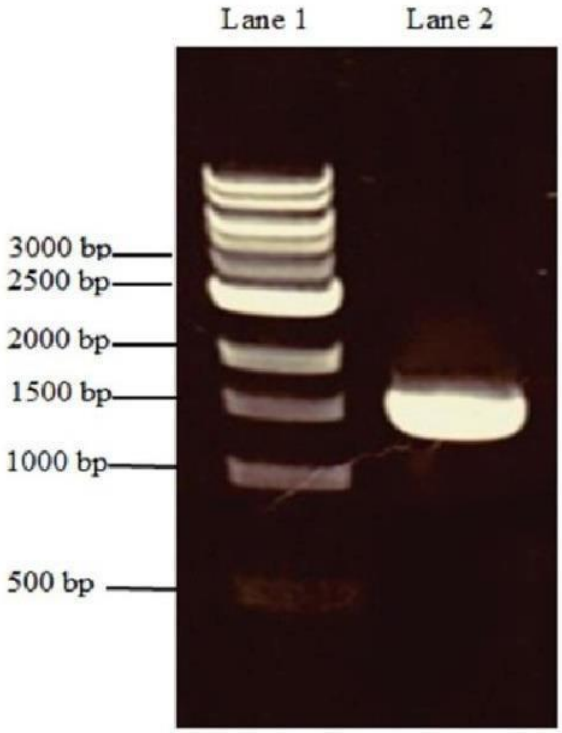
Appendix 4. Species and isolate information of five selected fungal strains based on laccase enzyme production

Sample No	Similarity %	Strain	Division	Accession No.
AORF12	99%	*Conifera poria weirii* JV0309	Basidiomycota	KR350568.1
AORF21	100%	*Aspergillus sydowii* 49G11	Ascomycota	MT594361.1
AORF39	96%	*Mucor circinellodes* Pub005	Zygomycota	MT279285.1
AORF43	97%	*Mucor* sp. CMRP 3219	Zygomycota	MH424492.1
AORF3	98%	*Trametes versicolor* ANT213 QFB286	Basidiomycota	MN992530.1

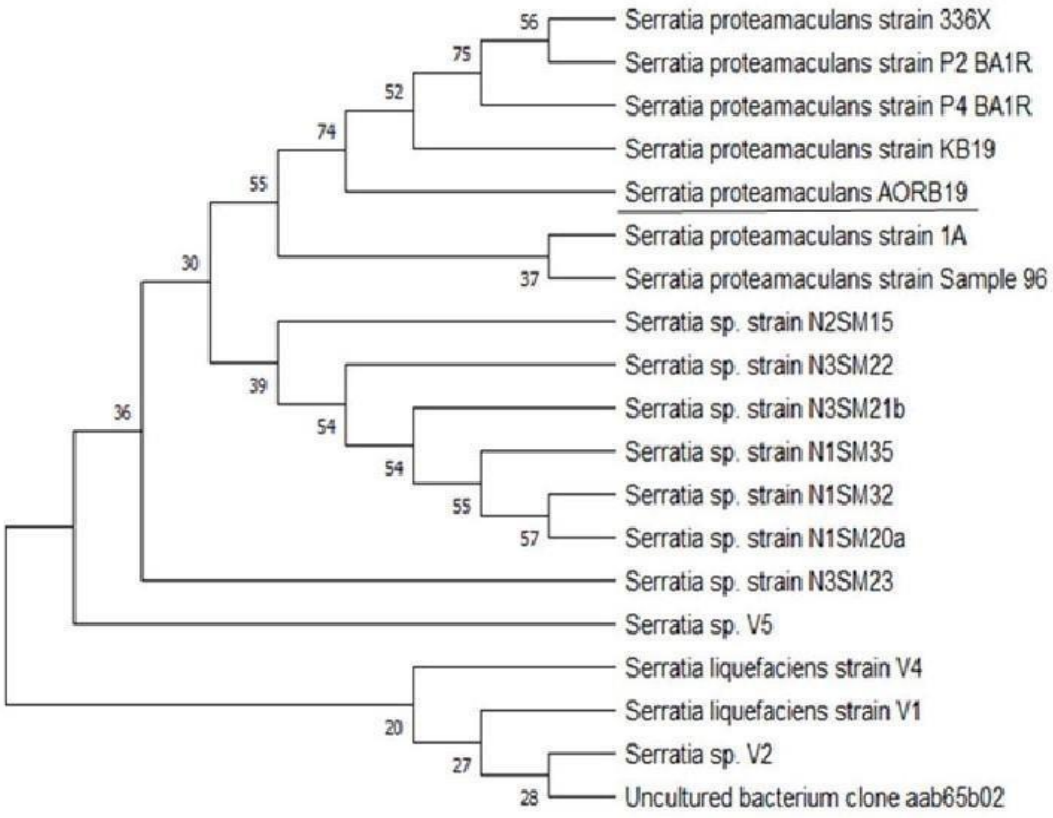
Appendix 5. Species and isolate information of ten selected bacterial strains based on laccase enzyme production

Sample No	Similarity %	Strain	Class	Gram nature	Accession No.
AORB9	99%	*Serratia* sp. CT197	Gammaproteobacteria	Gram negative	MT561436.1
AORB10	98%	*Enterobacter* sp. XBGRY7	Gammaproteobacteria	Gram negative	KJ1849721.1
AORB12	98%	*Raoultella ornitholytica* 4625	Gammaproteobacteria	Gram negative	MT545114.1
AORB25	99%	*Enterobacter ludwigii* JUQ409	Gammaproteobacteria	Gram negative	MN826154.1
AORB 28	98%	*Serratia marcescens* AS09	Gammaproteobacteria	Gram negative	MT598027.1
AORB37	91%	*Raoultella ornitholytica* Sch7	Gammaproteobacteria	Gram negative	JX294891.1
AORB48	95%	*Bacillus* sp. Ti1	Bacilli	Gram positive	MZ707571.1
AORB55	94%	*Enterobacter hormaechei* EN 314T	Gammaproteobacteria	Gram negative	MT539386.1
AORB19	99%	*Serratia proteamaculans* 336X	Gammaproteobacteria	Gram negative	MT598027.1
AORB46	99%	*Raoutella ornitholytica* RT 1902	Gammaproteobacteria	Gram negative	MT568560.1

Appendix 6. Agarose gel electrophoresis of the amplified 16SrRNA gene of bacterial strain AORB19. (Lane 1: 1 Kb DNA ladder, Lane 2: amplified DNAof strain AORB19).



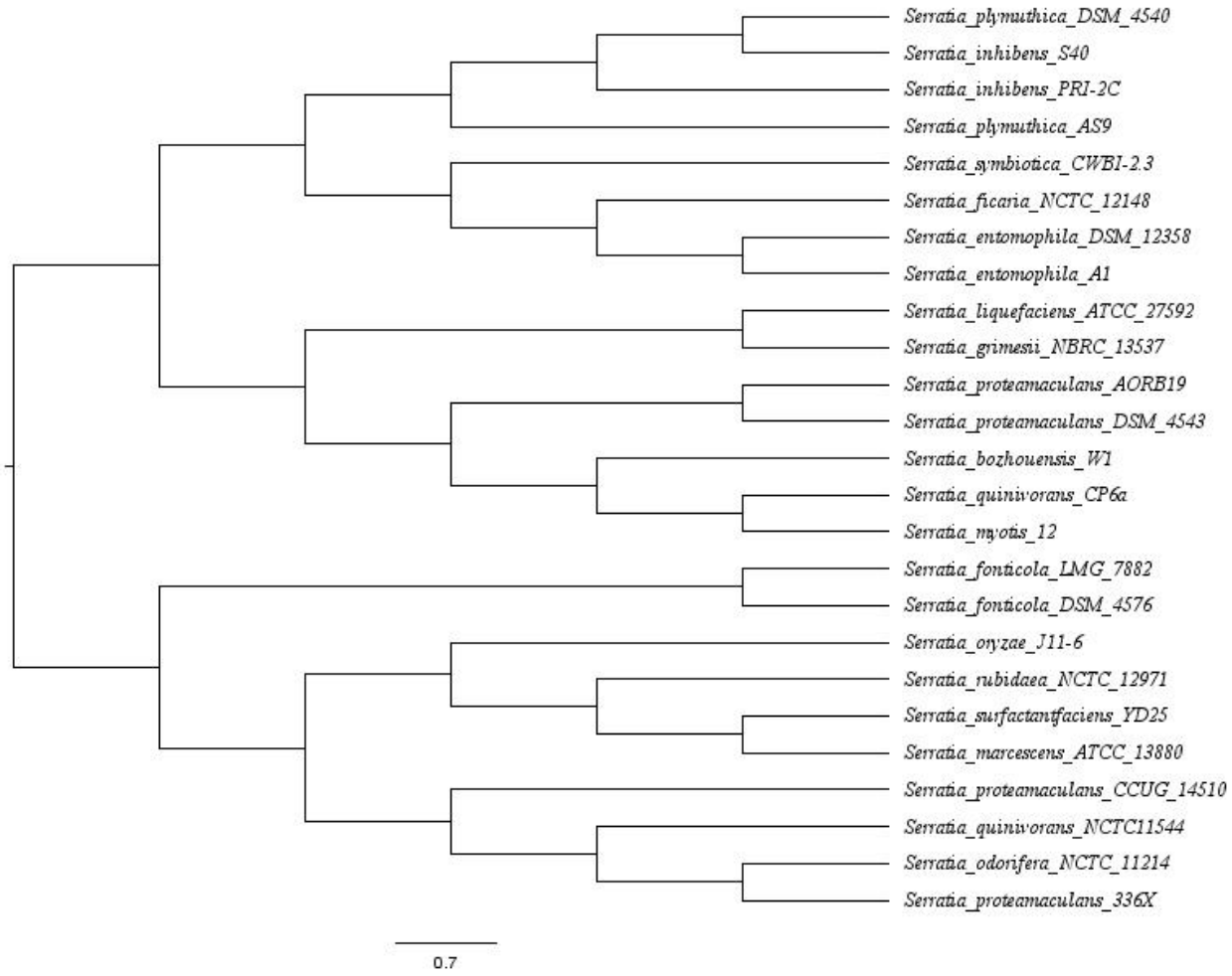
Appendix 7. Phylogenetic tree of strain AORB19 showing the relationship with 18 nucleotide sequences of closely related neighboring species**.**



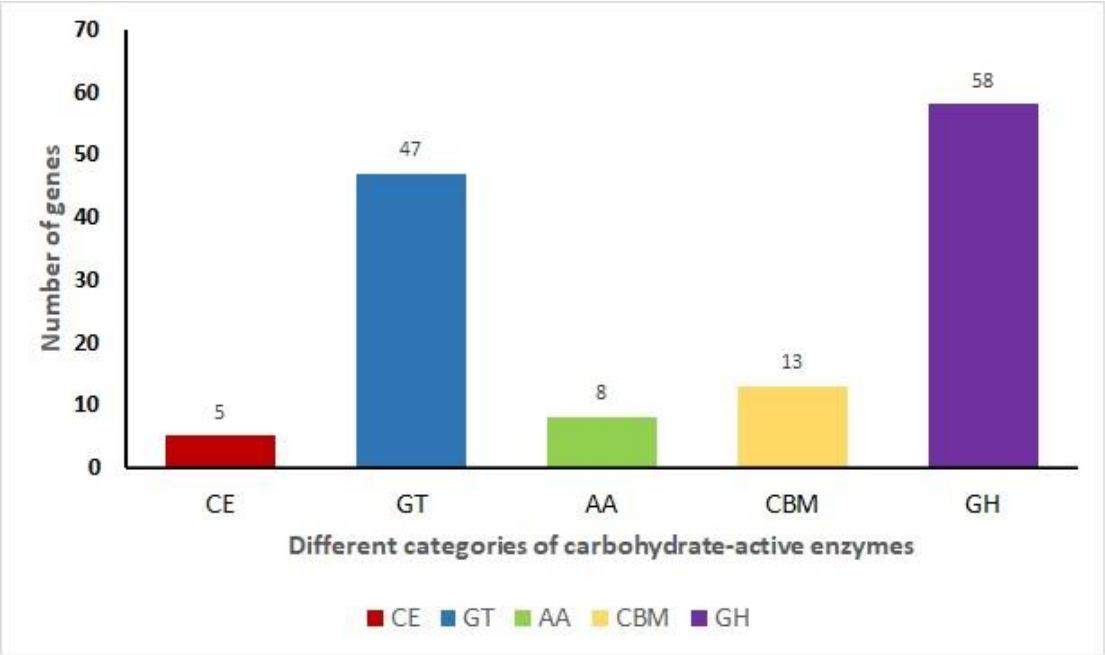
Appendix 8. Biochemical characterization of AORB19 in gram negative ID Type 2(NID2) panel

Characteristic features	AORB19
Carbohydrate fermentation	
Glucose	+
Sucrose	+
Inositol	+
Raffinose	+
Adonitol	-
Rhamnose	-
Arabinose	+
Sorbitol	-
Melibiose	-
Substrate utilization	
Oxidase	-
Cetrimide	-
Arginine	-
Lysine	+
Ornithine	+
Tryptophan deaminase	-
Esculin	-
o-Nitrophenyl-D-Galactopyranoside	+
Citrate	+
Malonate	-
Acetamide	-
Tartrate	-
Hydrogen sulfide	-
Indole	-
Nitrate	+
Voges-proskauer	+
Urea	-
Growth in the presence of antibiotics	
Penicillin G 4 g/mL	-
Kanamycin 4 g/mL	-
Colistin 4 g/mL	-
Cephalothin 8 g/mL	+
Nitrofurantoin 64 g/mL	-
Tobramycin 4 g/mL	-

Appendix 9. 16S rRNA phylogenic tree constructed using 25 different strains of *Serratia* using the NJ algorithm in MEGA Software.



Appendix 10. Number of CAZymes domains (CEs, GTs, AAs, CBMs and GHs) encoded in the genome of AORB19, annotated using dbCAN 2 meta server.



Appendix 11. Main genes responsible for lignin degradation in AORB19

Main genes responsible for lignin degradation in AORB19					
Type	**Encoded Protein**	**Gene ID**	**START**	**END**	**Strand**
Enzymes	Vanillate O-demethylase oxygenase subunit (EC 1.14.13.82)	SQAORB19_2890	225320	224277	-
	Vanillate O-demethylase oxygenase subunit (EC 1.14.13.82)	SQAORB19_5632	228	1	-
	Predicted dye-decolorizing peroxidase (DyP), YfeX-like subgroup	SQAORB19_3208	259475	260374	+
	Predicted dye-decolorizing peroxidase (DyP), encapsulated subgroup	SQAORB19_3467	242908	242510	-
	Predicted dye-decolorizing peroxidase (DyP), encapsulated subgroup	SQAORB19_3468	243483	242905	-
	Aldehyde dehydrogenase	SQAORB19_610	609690	611300	+
	Catalase-peroxidase KatG (EC 1.11.1.21)	SQAORB19_1212	15113	12915	-
	Catalase KatE (EC 1.11.1.6)	SQAORB19_4451	160525	159089	-
	FIG00003370: Multicopper polyphenol oxidase	SQAORB19_2332	573367	574098	+
	FIG00003370: Multicopper polyphenol oxidase	SQAORB19_1606	433410	432694	-
	Blue copper oxidase CueO precursor	SQAORB19_2924	263537	261918	+
	Flavin prenyltransferase UbiX	SQAORB19_4400	97921	98493	+
	NAD(P)H dehydrogenase (quinone), Type IV (EC 1.6.5.2)	SQAORB19_635	633962	634561	+
	Glutathione S-transferase, unnamed subgroup (EC 2.5.1.18)	SQAORB19_1310	128059	127442	-
	Glutathione S-transferase (EC 2.5.1.18)	SQAORB19_2611	283869	283201	-
	Glutathione S-transferase (EC 2.5.1.18)	SQAORB19_172	185305	185910	+
	Glutathione S-transferase (EC 2.5.1.18)	SQAORB19_914	950639	951346	+
	Probable glutathione S-transferase (EC 2.5.1.18), YfcF homolog	SQAORB19_2481	149089	149724	+
	Probable glutathione S-transferase (EC 2.5.1.18), YfcG homolog	SQAORB19_4407	103931	103302	-
	Probable glutathione s-transferase protein (EC 2.5.1.18)	SQAORB19_5251	289	2	-
	Glutathione S-transferase, omega (EC 2.5.1.18)	SQAORB19_2510	177888	178874	+
	Glutathione S-transferase, unnamed subgroup 2 (EC 2.5.1.18)	SQAORB19__4043	243457	244065	+
	Alcohol dehydrogenase (EC 1.1.1.1)	SQAORB19_5134	10866	11879	+
	Phenylacetaldehyde dehydrogenase (EC 1.2.1.39)	SQAORB19_4047	247169	248668	+
	Acetaldehyde dehydrogenase (EC 1.2.1.10) / Alcohol dehydrogenase (EC 1.1.1.1)	SQAORB19_1731	557246	554574	-
	Cinnamyl alcohol dehydrogenase/reductase (EC 1.1.1.195) @ Alcohol dehydrogenase (EC 1.1.1.1)	SQAORB19_2619	292403	293455	+
	Alcohol dehydrogenase (EC 1.1.1.1)	SQAORB19_2027	251827	250700	-
	L-galactonate-5-dehydrogenase	SQAORB19_1042	1083722	1082709	-
	Probable Zinc-binding dehydrogenase	SQAORB19_1347	161678	160671	-
	Succinate-semialdehyde dehydrogenase [NAD(P)+] (EC 1.2.1.16)	SQAORB19_1307	125964	124489	-
	NADPH-dependent broad range aldehyde dehydrogenase YqhD	SQAORB192393	56121	57284	+
	Succinate-semialdehyde dehydrogenase [NAD(P)+] (EC 1.2.1.16)	SQAORB19_2483	152553	151099	-
	Gamma-glutamyl-aminobutyraldehyde dehydrogenase (EC 1.2.1.-)	SQAORB19_344	356199	357695	+
	Aldehyde dehydrogenase (EC 1.2.1.3)	SQAORB19_5141	19676	18156	-
	Alcohol dehydrogenase class III (EC 1.1.1.1)	SQAORB19_4538	2757	3851	+
	Alcohol dehydrogenase	SQAORB19_5355	269	15	-
	Zinc-type alcohol dehydrogenase-like protein	SQAORB19_1221	24356	25375	+
	Alcohol dehydrogenase (EC 1.1.1.1)	SQAORB19_3009	35917	34766	-
	Zinc-type alcohol dehydrogenase-like protein	SQAORB19_3300	56356	57306	+
	Superoxide dismutase [Cu-Zn] precursor (EC 1.15.1.1)	SQAORB19_187	202689	202168	-
	Superoxide dismutase [Fe] (EC 1.15.1.1)	SQAORB19_202	215461	216039	+
	Superoxide dismutase [Mn] (EC 1.15.1.1)	SQAORB19_4210	163166	163786	+
	Choline dehydrogenase (EC 1.1.99.1)	SQAORB19_928	968344	966677	-
	Oxidoreductase, GMC family	SQAORB19_1920	143242	141635	-

Appendix 12. Degradative pathways for lignin-derived aromatic compounds

Degradative Pathways for Lignin-Derived Aromatic Compounds						
	RAST Subsystem	**Encoded Protein**	**Gene ID**	**start**	**stop**	**strand**
Peripheral pathways for catabolism of aromatic compounds	Quinate degradation	3-dehydroquinate dehydratase II (EC 4.2.1.10)	SQAORB19_313	325319	325747	+
			SQAORB19_2595	268065	268517	+
	Benzoate degradation	Benzoate transport protein	SQAORB19_162	178218	179411	+
	Biphenyl Degradation	Acetaldehyde dehydrogenase, acetylating, (EC 1.2.1.10) in gene cluster for degradation of phenols, cresols, catechol	SQAORB19_1381	197955	198842	+
	Biphenyl Degradation	4-hydroxy-2-oxovalerate aldolase (EC 4.1.3.39)	SQAORB19_1382	198826	199827	+
	Biphenyl Degradation	Acetaldehyde dehydrogenase (EC 1.2.1.10)	SQAORB19_1731	557246	554574	-
	Biphenyl Degradation	Biphenyl-2,3-diol 1,2-dioxygenase (EC 1.13.11.39)	SQAORB19_4553	16499	17008	+
	p-Hydroxybenzoate degradation	4-hydroxybenzoate transporter	SQAORB19_2247	496432	495095	-
	p-Hydroxybenzoate degradation	P-hydroxybenzoate hydroxylase (EC 1.14.13.2)	SQAORB19_5600	2	232	+
			SQAORB19_2862	194919	196268	+
			SQAORB19_5237	3	341	+
			SQAORB19_3408	175141	176499	+
			SQAORB19_3414	180537	181892	+
			SQAORB19_3415	181903	183081	+
Metabolism of central aromatic intermediates	Catechol branch of beta-ketoadipate pathway	Beta-ketoadipate enol-lactone hydrolase (EC 3.1.1.24)	SQAORB19_1365	179426	178665	-
	Catechol branch of beta-ketoadipate pathway	3-oxoadipate CoA-transferase subunit B (EC 2.8.3.6)	SQAORB19_1367	181301	180621	-
	Catechol branch of beta-ketoadipate pathway	3-oxoadipate CoA-transferase subunit A (EC 2.8.3.6)	SQAORB19_1368	181999	181313	-

	Catechol branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A (EC 2.8.3.5)	SQAORB19_2005	228909	229607	+
	Catechol branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B (EC 2.8.3.5)	SQAORB19_2006	229618	230271	+
	Salicylate and gentisate catabolism	Maleate cis-trans isomerase (EC 5.2.1.1)	SQAORB19_87	98212	98964	+
	Salicylate and gentisate catabolism	Fumarylacetoacetate hydrolase family protein	SQAORB19_93	105184	106188	+
	Salicylate and gentisate catabolism	4-hydroxybenzoate transporter	SQAORB19_2247	496432	495095	-
			SQAORB19_2862	194919	196268	+
			SQAORB19_3408	175141	176499	+
			SQAORB19_3414	180537	181892	+
	Salicylate and gentisate catabolism	Gentisate 1,2-dioxygenase (EC 1.13.11.4)	SQAORB19_3657	176529	175495	-
	Salicylate and gentisate catabolism	Maleylacetoacetate isomerase (EC 5.2.1.2)	SQAORB19_5358	46	270	+
	Protocatechuate branch of beta-ketoadipate pathway	4-carboxymuconolactone decarboxylase (EC 4.1.1.44)	SQAORB19_989	1027933	1027616	-
	Protocatechuate branch of beta-ketoadipate pathway	Beta-ketoadipate enol-lactone hydrolase (EC 3.1.1.24)	SQAORB19_1365	179426	178665	-
	Protocatechuate branch of beta-ketoadipate pathway	3-oxoadipate CoA-transferase subunit B (EC 2.8.3.6)	SQAORB19_1367	181301	180621	-
	Protocatechuate branch of beta-ketoadipate pathway	3-oxoadipate CoA-transferase subunit A (EC 2.8.3.6)	SQAORB19_1368	181999	181313	-
	Protocatechuate branch of beta-ketoadipate pathway	Pca regulon regulatory protein PcaR	SQAORB19_1369	182179	183012	+
	Protocatechuate branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A (EC 2.8.3.5)	SQAORB19_2005	228909	229607	+
	Protocatechuate branch of beta-ketoadipate pathway	Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B (EC 2.8.3.5)	SQAORB19_2006	229618	230271	+
			SQAORB19_3405	174043	173210	-
	Protocatechuate branch of beta-ketoadipate pathway	3-carboxy-cis,cis-muconate cycloisomerase (EC 5.5.1.2)	SQAORB19_3416	183215	184573	+

			SQAORB19_3417	184566	184955	+
	Protocatechuate branch of beta-ketoadipate pathway	Protocatechuate 3,4-dioxygenase alpha chain (EC 1.13.11.3)	SQAORB19_3419	186610	185984	-
	Protocatechuate branch of beta-ketoadipate pathway	Protocatechuate 3,4-dioxygenase beta chain (EC 1.13.11.3)	SQAORB19_3420	187335	186607	-
	4-Hydroxyphenylacetic acid catabolic pathway	Homoprotocatechuate degradative operon repressor	SQAORB19_2040	264357	263902	-
	4-Hydroxyphenylacetic acid catabolic pathway	5-carboxymethyl-2-oxo-hex-3- ene-1,7-dioate decarboxylase (EC 4.1.1.68)	SQAORB19_2041	264683	265315	+
	4-Hydroxyphenylacetic acid catabolic pathway	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase (EC 5.3.3.-)	SQAORB19_2042	265312	266076	+
	4-Hydroxyphenylacetic acid catabolic pathway	5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60)	SQAORB19_2043	266073	267527	+
	4-Hydroxyphenylacetic acid catabolic pathway	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	SQAORB19_2044	267537	268394	+
	4-Hydroxyphenylacetic acid catabolic pathway	5-carboxymethyl-2-hydroxymuconate delta-isomerase (EC 5.3.3.10)	SQAORB19_2045	268404	268790	+
	4-Hydroxyphenylacetic acid catabolic pathway	2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.-.-)	SQAORB19_2046	268801	269604	+
	4-Hydroxyphenylacetic acid catabolic pathway	4-hydroxyphenylacetate symporter, major facilitator superfamily (MFS)	SQAORB19_2048	270543	271913	+
	4-Hydroxyphenylacetic acid catabolic pathway	Transcriptional activator of 4-hydroxyphenylacetate 3-monooxygenase operon, XylS/AraC family	SQAORB19_2049	271973	272869	+
	4-Hydroxyphenylacetic acid catabolic pathway	4-hydroxyphenylacetate 3-monooxygenase, reductase component (EC 1.6.8.-)	SQAORB19_2051	274629	275144	+
	N-heterocyclic aromatic compound degradation	1H-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase	SQAORB19_1776	599632	600429	+
	Central meta-cleavage pathway of aromatic compound degradation	Acetaldehyde dehydrogenase, acetylating, (EC 1.2.1.10) in gene cluster for degradation of phenols, cresols, catechol	SQAORB19_1381	197955	198842	+

	Central meta-cleavage pathway of aromatic compound degradation	5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (EC 1.2.1.60)	SQAORB19_2043	266073	267527	+
	Central meta-cleavage pathway of aromatic compound degradation	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	SQAORB19_2044	267537	268394	+
	Central meta-cleavage pathway of aromatic compound degradation	5-carboxymethyl-2-hydroxymuconate delta-isomerase (EC 5.3.3.10)	SQAORB19_2045	268404	268790	+
	Central meta-cleavage pathway of aromatic compound degradation	2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.-.-)	SQAORB19_2046	268801	269604	+
	Central meta-cleavage pathway of aromatic compound degradation	Protocatechuate 4,5-dioxygenase beta chain (EC 1.13.11.8)	SQAORB19_5222	438	1	-
Metabolism of Aromatic Compounds - no subcategory	Aromatic Amin Catabolism	3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15)	SQAORB19_2044	267537	268394	+
	Aromatic Amin Catabolism	4-hydroxyphenylacetate 3-monooxygenase, reductase component (EC 1.6.8.-)	SQAORB19_2051	274629	275144	+
	Aromatic Amin Catabolism	Phenylacetaldehyde dehydrogenase (EC 1.2.1.39)	SQAORB19_4047	247169	248668	+
	Gentisate degradation	Fumarylacetoacetate hydrolase family protein	SQAORB19_93	105184	106188	+
	Gentisate degradation	4-hydroxybenzoate transporter	SQAORB19_2247	496432	495095	-
			SQAORB19_2862	194919	196268	+
			SQAORB19_3408	175141	176499	+
			SQAORB19_3414	180537	181892	+
	Gentisate degradation	Gentisate 1,2-dioxygenase (EC 1.13.11.4)	SQAORB19_3657	176529	175495	-
	Gentisate degradation	Maleylacetoacetate isomerase (EC 5.2.1.2)	SQAORB19_5358	46	270	+
Other Peripheral Pathways	anthranilate pathway	Indole-3-glycerol phosphate synthase (EC 4.1.1.48) / Phosphoribosylanthranilate isomerase (EC 5.3.1.24)	SQAORB19_1769	593917	592556	-
	anthranilate pathway	Anthranilate synthase, aminase component (EC 4.1.3.27)	SQAORB19_1772	597078	595516	-

	anthranilate pathway	Para-aminobenzoate synthase, amidotransferase component (EC 2.6.1.85)	SQAORB19_3698	220497	221072	+
	anthranilate pathway	Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	SQAORB19_1770	594919	593921	-
	anthranilate pathway	Anthranilate synthase, amidotransferase component (EC 4.1.3.27)	SQAORB19_1771	595516	594935	-
	anthranilate pathway	Aromatic-amino-acid aminotransferase (EC 2.6.1.57)	SQAORB19_1314	130448	131635	+
	anthranilate pathway	Tryptophan 2,3-dioxygenase (EC 1.13.11.11)	SQAORB19_1386	201487	202329	+
	homogentisic pathway	Phenylalanyl-tRNA synthetase beta chain (EC 6.1.1.20)	SQAORB19_254	270624	268237	-
	homogentisic pathway	Phenylalanyl-tRNA synthetase alpha chain (EC 6.1.1.20)	SQAORB19_255	271622	270639	-
	homogentisic pathway	ShikiF5-dehydrogenase I alpha (EC 1.1.1.25)	SQAORB19_3776	277216	278034	+
	phenylacetate-CoA pathway	Phenylacetate-coenzyme A ligase (EC 6.2.1.30)	SQAORB19_1320	138605	137295	-
	phenylacetate-CoA pathway	3-hydroxyadipyl-CoA dehydrogenase	SQAORB19_1323	141779	140259	-
	phenylacetate-CoA pathway	1,2-epoxyphenylacetyl-CoA isomerase (EC 5.3.3.18)	SQAORB19_1324	142573	141782	-
	phenylacetate-CoA pathway	2,3-dehydroadipyl-CoA hydratase (EC 4.2.1.17)	SQAORB19_1325	143350	142577	-
	phenylacetate-CoA pathway	1,2-phenylacetyl-CoA epoxidase, subunit E (EC 1.14.13.149)	SQAORB19_1326	144418	143360	-
	phenylacetate-CoA pathway	1,2-phenylacetyl-CoA epoxidase, subunit D (EC 1.14.13.149)	SQAORB19_1327	144924	144427	-
	phenylacetate-CoA pathway	1,2-phenylacetyl-CoA epoxidase, subunit C (EC 1.14.13.149)	SQAORB19_1328	145695	144934	-
	phenylacetate-CoA pathway	1,2-phenylacetyl-CoA epoxidase, subunit B (EC 1.14.13.149)	SQAORB19_1329	145991	145704	-
	phenylacetate-CoA pathway	1,2-phenylacetyl-CoA epoxidase, subunit A (EC 1.14.13.149)	SQAORB19_1330	146940	146002	-
	resorcinol pathway	4-hydroxythreonine-4-phosphate dehydrogenase (EC 1.1.1.262)	SQAORB19_2147	376509	375517	-
	resorcinol pathway	2-polyprenyl-6-methoxyphenol hydroxylase	SQAORB19_2838	172475	171297	-

Appendix 13. HPLC-UV qualitative monitoring of phenolic compounds identified in the culture supernatant using Alkali Lignin (Sigma Aldrich) (Control: no strain; time: incubation time with *Serratia quinivorans* AORB19)

Phenolic Compounds	**RT (min)**	**Area (control)**	**Area (48 h)**	**Area (96 h)**	**Area (144 h)**	**Area (168 h)**
4-Hydroxybenzaldehyde	33.3	186.2022	168.2848	255.4662	279.6373	350.1594
Vanillin	46.1	2756.4584	367.5246	481.5995	478.3785	527.6389
Vanillinic acid	38.5	249.3493	Nd	nd	nd	nd