Enzymatic Degradation of Lignin by Bacteria Isolated from Pulp and Paper Mill Effluent

By

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A thesis submitted in partial fulfillment of the requirements for the Degree of

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In

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In the name of Allah, the Most Gracious,
the Most Merciful

Dedicated To

My Beloved Father

My Sweet Mother

&

My Brother and Sisters

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Sanam Islam Khan

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List of Acronym/abbreviations

MSM	Minimal Salt Medium
SL	Sludge
BL	Black Liqour
NCBI	National Centre for Biotechnology Information
BLAST-N	Basic Local Alignment Statistical Tool – Nucleotide
BLAST-P	Basic Local Alignment Statistical Tool – Protein
Kan^R	Kanamycin Resistance
Amp^R	Ampicillin Resistance
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
EPA	Environmental Protection Agency
Kan^R	Kanamycin Resistance
NEB	New England Biolabs
OD	Optical Density
PCR	Polymerase Chain Reaction
Ppm	Parts per million
Rpm	Rotation per minute
rRNA	ribosomal Ribose Nucleic Acid
RT	Retention Time
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscope
FT-IR	Fourier-transform infrared spectroscopy
GC-MS	Gas chromatography–mass spectrometry
TBE	Tris Borate EDTA
TE	Tris EDTA
TSI	Triple Sugar Iron
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid)
AhR	Aryl Hydrocarbon Receptor

COD	Chemical Oxygen Demand
EA	Enzyme activity
Lac	Laccase
LDP	Lignin Degrading Peroxidase

ABSTRACT

Lignin is one of the key by-product of the paper industries, which is polymeric in nature and shows resistance to depolymerization. The ligninolytic enzymes can be apply to degrade lignin. The present study was designed to evaluate the lignin degradation by lignin degrading peroxidase (LDP) and laccase (Lac) from bacteria isolated from pulp and paper mill effluent. The total 55 bacterial strains were isolated from effluent and investigated for lignin degradation efficiency and ligninolytic enzymes production, strain BL5 and SL7 showed maximum lignin degradation efficiency 35% and 44%, respectively, and were selected for further experiments. On the basis of morphological depictions, biochemical characterization and 16S rRNA sequencing, strain BL5 and SL7 were identified as *Bacillus* sp. and *Bacillus altitudinis*, respectively. Effects of physiochemical factors were investigated for production of LDP from *Bacillus* sp. BL5 and Lac from *Bacillus altitudinis* SL7. The optimum production of LDP and Lac was attained at 30°C and pH 8.0 and 7.0, respectively, in lignin amended minimal salt medium (L-MSM). The best carbon and nitrogen source for production of LDP and Lac were found to be (glucose, peptone) and (starch, yeast extract), respectively. The concentration of nutritional factors was optimized by Central Composite Design, and highest LDP and Lac production under optimized condition were found to be 56.9 U/mg and 29.5 U/mg, respectively, after 72 h of fermentation. The native LDP and Lac was purified by acetone and gel filtration chromatography, and SDS-PAGE analysis revealed their molecular weights as 45 kDa and 55 kDa, respectively.

The Efeb gene of 1251bp encoding DyP-type lignin degrading peroxidase from *Bacillus* sp. BL5 (DyPBL5) and laccase gene of 1500 bp encoding laccase from *Bacillus altitudinis* SL7 was amplified in the current study and subsequently cloned into a pET-28a (+) vector and expressed in *E. coli* BL21 (DE3) cells. Expressed DyPBL5 and LacSL7 were purified though ion-exchange chromatography and molecular weight was determined as 46 kDa and 56 kDa, respectively, which was almost same as native enzymes. High level of enzyme activity produced by recombinant *Bacillus* sp. BL5 and *Bacillus altitudinis* SL7 enzymes. The specific activity of purified DyPBL5 and LacSL7 was up to 1319 and 2530 IU/mg with 37.9 and 33 fold purity, respectively. The kinetic parameters of DyPBL5 and LacSL7 were determined and *Km*, *Vmax*, and *Kcat* values were (1.06, 0.28 mM,), (519.75, 2073 μmol/min/mg) and (395, 3878 $S^{-}1$), respectively. DyPBL5 showed the highest sequence similarity with DyP-type peroxidase of *Bacillus subtilis* (WP148982369). While

LacSL7 showed the highest sequence similarity with the laccase of *Bacillus altitudinis* (APP17947).

Purified DyPBL5 was active at wide temperature (25-50°C) and pH (3.0-8.0) range with optimum activity at 35°C and pH 4.0. While LacSL7 is thermostable enzyme and was active at high temperature, ranges from 25-80°C and pH 3.0-8.0 with optimum activity at 55°C and pH 5.0. DyPBL5 and LacSL7 were stable in the presence of low concentration of metals and higher concentration caused inhibitory effect on it. DDT and β-mercaptoethanol caused strong inhibition of both the enzymes, whereas SDS inhibited DyPBL5 only, and LacSL7 showed resistance to SDS. DyPBL5 and LacSL7 activity was enhanced in the presence of organic solvents such as methanol and ethanol. The recombinant DyPBL5 and LacSL7 resulted in the reduction of lignin contents up to 27.04% and 31.2%, respectively, and combination of both the enzymes degraded lignin up to 63.14%. The lignin degradation was further confirmed by SEM and FT-IR analysis of enzymatically treated samples. The smooth surface of lignin was completely eroded, deformed and particle size was reduced during the course of treatment. FT-IR analysis indicated significant changes in composition of functional groups of degraded lignin such as carboxyl (-COOH), carbonyl (C=O), amines ($-NH_2$), alkene (C=C) and sulfonic ($-SO_3$). Further, various low molecular weight compounds were detected such as vanillin, 2-methyoxyhenol, 3-methyl phenol, oxalic acid, and ferulic acid, suggested the degradation of coniferyl and sinapyl groups of lignin. Lignin degradation rate of DyPBL5 and LacSL7 suggested that both enzymes can be ideal bio-catalysts for remediation of lignin containing effluent.

INTRODUCTION

Ligniocellulosic biomass is an attractive renewable feedstock for different industries which includes pulp and paper, biofuels and biocomposites. Pulp and paper industries are among the largest and fast-growing portions of the world's economy (Buzz, 2021), and manufacturing over 390 million metric tons of paper every year in-order to meet the consumption of paper around the world. It is estimated that per person consumes about 60 kg of paper per year but its consumption in the US is at an extreme level i.e., 265 kg of paper per US citizen. Whereas in Asian countries, only 40 kg of paper per person is being consumed but this value is estimated to increase in upcoming years (Lichpen, 2021). In 2020 worldwide consumption of paper and paperboard was approximately 399 million metric tons and estimated to increase in future upto 461 million metric tons in 2030 (Statista, 2021).

The Paper industry is among the important industries in Pakistan and primarily serves the internal or domestic market. Pakistan have more than 100 paper manufacturing units located in all parts of the country. These industries produces different types of papers which includes, packaging board, corrugate medium, liner board, banknote paper, writing paper, and tissues. Consumption of paper in Pakistan is 1.299 million metric tons per annum and domestic production is 0.7 million metric tons. So the excess demand of paper is being met through import, total import of Pakistan was approximately 0.611 million metric tons in 2018 (https://paperonweb.com /FAO2018 .Paper.pdf).

Pulp and paper (P &P) industries utilizes different types of lignocellulosic raw materials as a feed stock, which are mostly wood, agriculture residues and recycled papers. In many countries, wood is not available in enough quantity to overcome the needs of pulp and paper industries. In the past, researches have been conducted on the use of other raw material such as non-woody for production of paper. In the developing countries, approximately 60% of the cellulose fibers are obtained from non-wood raw materials for example sugarcane bagasse, oat straw, sisal and flax etc (Britannica, 2020). Paper production is a sequential process, in first step raw material or biomass in converted into pulp and thne pulp is converted into fine paper (Bajpai, 2011). During pulping lignocellulosic biomass is disintegrated into its fibers, cellulose, hemicellulose and lignin.

Cellulosic fibres are utilized for production of paper and lignin is released in effluent as a waste product.

The P & P industries are among the largest industrial network regarding production of paper and generation of waste product, these industries have major influence on environment and stands sixth in generation of large amount of waste water following metal and chemical industries (Gupta et al., 2019). It is estimated that one ton of paper production generates 150-200 m^3 of wastewater (Taha et al., 2016). A large proportion of lignin is extracted from cellulose and hemicelluloses during pulping and discharged as trash in the effluent. Effluent from pulp industries is reffered to as black liquor, which is highly turbid due to the presence of high concentration of lignin (Chaudhry and Paliwal 2018). Because of lignin and polysaccharide leftovers, the liquid waste contains highly dissolved solids, COD, BOD and chlorine. The reaction of chlorine with the lignin present in wastewater generates organic halides. Lignin (high-molecular-weight) and its derivatives are the primary factors that contributes to the toxicity and color of wastewater. The amount of lignin produced every year is approximately 50 million tons in a pulping process and the amount of lignin that is used for industrial application is only 1 million tons. (Mazumder at al., 2020). Disposing of untreated or partially treated effluent accounts for undesirable coloration of aquatic resources along with the deterioration of aquatic flora and fauna by obstructing the passage of sunlight. Lignin derivatives such as, chlorolignins affects the reproductive system in fishes by causing delayed maturity, lower sex hormone, and reduction in gonad size. In the terrestrial ecosystem, these contaminants enter the food chain and have carcinogenic and genotoxic effects on humans and other animals (Singh and Chandra, 2012).

Lignin is a complex polymers and composed of different phenylpropane units, these monomers includes, *p*-coumaryl alcohol, guaiacyl alcohol and syringyl alcohol. These monomers are linked together through C=C and β-O-4-ether linkages (Cho et al., 2020; Shin et al., 2019). Lignin is not an easy polymer to degrade due to the presence of heterogeneous and irregular arrangement of phenylpropanoid monomers, therefore it persist in the surroundings for longer duration of time (Bugg et al., 2011a).

The P & P industry is now facing challenges to comply with stringent environmental

regulations. The extensive environmental contamination have attracted increasing social attention, especially in developing countries like Pakistan. However, effective monitoring and remediation of effluent from pulp and paper mills is lacking due to high cost of remediation strategies. This has geared the attention towards investigation of efficient, low cost and environment friendly lignin remediation strategies. Different types of physicochemical methods such as, sedimentation, floatation, filtration, chemical oxidation and ozonization have been reported, although these are well-established methods but there are some disadvantages associated with these methods i.e., rise in the overall cost of treatment, formation of chemical sludge, fouling of membrane, increase in the chemical and energy consumption. Lignin does not undergo a complete degradation during physicochemical treatment rather it transforms from one form to other and persist in the environment in different forms even after treatment (Haq et al., 2020).

Biological treatment is a well-known process in reducing toxic organic load. Intensive research has been carried out to decontaminate the effluent from pulp and paper industries using fungi, bacteria, and their enzymes (Ebanyenle et al., 2016; Schmidt et al., 2016). Most of the research on detoxification of ligninocellusic waste involved brown rot and white rot fungi (Karp et al., 2012; Karim et al., 2016; Mathews et al., 2016), but the usage of fungal species for effluent detoxification have limitations of growth conditions. The fungus requires acidic pH to produce ligninolytic enzymes; usually, the pH of pulp and paper mills effluent tends to be alkaline in nature, so the practice of a fungal system for effluent treatment requires pH adjustment, which adds extra cost to the process. In contrast, bacteria are gaining interest because of environmental adaptability and biochemical versatility compared to fungi (Mathews et al., 2016).

Several studies have reported soil bacteria for degradation of lignin and lignin model compounds like, *Serratia marcescens, Citrobacter freundii*, *Streptomyces viridosporus* T7A, *Pseudomonas* species and *Bacillus* species (Chandra et al., 2007; Abd-Elsalam, 2009; Xu et al., 2018). These microorganisms break down lignin through the action of enzymes. Ligninolytic enzymes including peroxidases and laccases has been exploited to oxidize aromatic units of lignin by using molecular oxygen and H_2O_2 as an electron acceptor (Bugg and Rahmanpour, 2015; Zhang et al., 2020). Low energy consumption, mild reaction

conditions and low production of toxic products makes enzymatic depolymerization as an ideal route for lignin degradation (Shin et al., 2019).

Laccases (benzenediol: oxygen oxidoreductases) belongs to multicopper oxidase family and catalyzes the oxidation of lignin and other phenolic compounds with the reduction of molecular oxygen to water and generates 2, 6-dimethoxybenzene-1, 4-diol as a major product (Kumar et al., 2011). *Streptomyces lividans* TK24, *Streptomyces coelicolor* A3, *Streptomyces viridosporus* T7A, and *Amycolatopsis sp*. laccases have been characterized, and were found to oxidize the lignin model compounds (Singh et al., 2013; Majumdar et al., 2014).

Peroxidases are iron containing enzymes and have been classied into six groups by peroxidase database PeroxiBase.These groups are; catalases, di-heme cytochrome c peroxidases, haloperoxidases, animal peroxidases, plant peroxidases and DyP-type peroxidases (Fawal et al., 2013). The bacterial dye-decolorizing peroxidase (DyP) family represents an interesting bio-catalysts for lignin depolymerization which has been discovered nearly a decade ago. Dye-decolorizing peroxidase (DyPB) from *Rhodococcus* sp. T1, *Rhodococcus jostii* RHA1, *Thermobifida fusca* and *Pseudomonas fluorescens* Pf-5 has been identified and utilized for degradation of phenolic compounds (Sahinkaya et al., 2019; Brown et al., 2012; Bharagava et al., 2019). Dye decolorizing YfeX and EfeB from *Escherichia coli* has been identified and showed modest guaiacol peroxidase activity (Bloois et al., 2010).

The production of ligninolytic enzymes have been reported from different organisms, however, the successful use of ligninolytic enzymes on industrial level have limitations. These limitation includes their low production yields, high production cost, instability of enzymes at low pH and high temperature, specificity towards specific substrates and solubility in organic solvents. There is need to discover novel enzymes and enhance stability of already available enzymes, which can work in extreme conditions. Different techniques can used to enhance the production of enzymes. The conventional methods for enhanced production of enzymes are laborious, time consuming having problems in enzymes purification and characterization from native hosts. Recombinant DNA technology plays a vital role in production of novel proteins and development of bacterial

expression systems for over production of proteins (Wiltschi et al., 2020).

To make the successful application on industrial level, properties of enzymes should be clearly understood. Characterization of enzymes helps to generate data which can be used to understand the nature of enzymes. The key properties which an enzyme must have to be utilized for industrial proposes are; stability at elevated pH and temperature. The production and downstream processing can be achieved at low cost and use of expression systems which can be cultivated easily to reduce production cost.

In the current study, we have isolated several lignin-degrading bacteria from pulp and paper mill effluent of which, two bacterial strains were showing good degradation efficiency. *Bacillus altitudinis* strain SL7 and *Bacillus subtilis* strain BL5 were selected for production of laccase and DyP-type peroxidase, respectively. Enzymes were cloned, over-expressed and purified in microbial hosts as well as characterized to determine their degradative activity, thermodynamic and kinetic stability. Recombinant laccase and DyP-type peroxidase were applied for degradation of lignin and the degradation was analyzed by reduction in lignin contents, FT-IR and SEM. The degradation products were detected by GC-MS. Ligninolytic enzymes are mandatory for the degradation of lignin, thus the choice of bacteria producing large amount of laccase and DyP-type peroxidase would be needed.

Problem Statement:

Lignin is one of the most recalcitrant polymer that continuously releases in effluent from pulp and paper mill. It possess serious human and environmental hazards.

Research question:

Are the microorganisms living in pulp and paper mills effluent are capable of lignin degradation? Can we increase the rate of lignin degradation by the use of lignin degrading enzymes?

Aim and Objectives

The aim of the current study was to investigate the bioremediation of lignin by bacteria and its enzymes from pulp and paper mill effluent.

Objectives

The aim was achieved through the following objectives**:**

1. Isolation and characterization of lignin degrading bacteria from pulp and paper mill effluent;
2. Optimization of culture conditions for the enhanced production of laccase and lignin degrading DyP-type peroxidase from selected bacteria;
3. Cloning and expression of laccase and DyP-type peroxidase;
4. Purification and functional characterization of recombinant laccase and DyP-type peroxidase;
5. Degradation of lignin by recombinant enzymes and identification of lignin degradation products by GC-MS.

2. LITERATURE REVIEW

2.1 Background

Pulp and paper (P & P) manufacturing are the major producer and user of biomass-based energy and materials (Bonhivers et al., 2014). Pulp and paper are generally mass-produced from raw resources, which comprises of cellulose fibers commonly agriculture residues, recycled papers and wood. About 60% of the cellulose fibers in developing countries are obtained from non-wood raw constituents such as sisal, fax, jute, esparto grass, reeds, bamboo, cereal straw and bagasse (Saini et al., 2015). Plant cell wall contains cellulose, hemicellulose and lignin. Lignin is an aromatic complex polymer of monolignols such as syringyl alcohol, coumaryl alcohol and, coniferyl alcohol. It is highly resistant to deterioration, having a strong chemical bond that holds it together. It is bonded to hemicelluloses (carbohydrate) in wood in a complex and variety of ways. The proportion of lignin and cellulose in raw plant material determines paper strength in pulp and paper manufacturing (Tarasov et al., 2018). Lignin is an undesirable polymer, and it is removed during the pulping process because its presence contributes to the yellowness of paper and also decreases its durability, but it takes a lot of energy and chemicals to remove it during pulping and released as a by product. The production phase that converts wood from the trees to sleek uniform papers stuff needs huge volume of synthetic chemical compounds and water energy. A single ton of paper production requires water in thousands of gallons. P & P industries are the world's largest waste generator and energy user amongst the manufacturing industries (Sadh et al., 2018).

2.2 Paper Production

2.2.1 Paper Production Globally

The growth of the economy and the development of social civilization are inextricably linked with such activities. The pulp and paper sector encompasses a wide range of disciplines, including chemicals, forestry, biology, agriculture, transportation, distribution, and have major role global economy. Lives of people, the world economy, and industrial output have affected by Covid 19 all of which have ultimately impacted the P & P industry (Liu et al., 2020). Due to the closure of colleges and institutions, demand for copier paper and printing paper has decreased significantly. On the other hand, Covid 19 has resulted in an increase in the demand for particular paper products such as tissues, food packaging products and corrugated packaging materials. Annual global paper

production is 400 million metric tons and requirement has reached to 423.3 million metric tons (Statista, 2019).

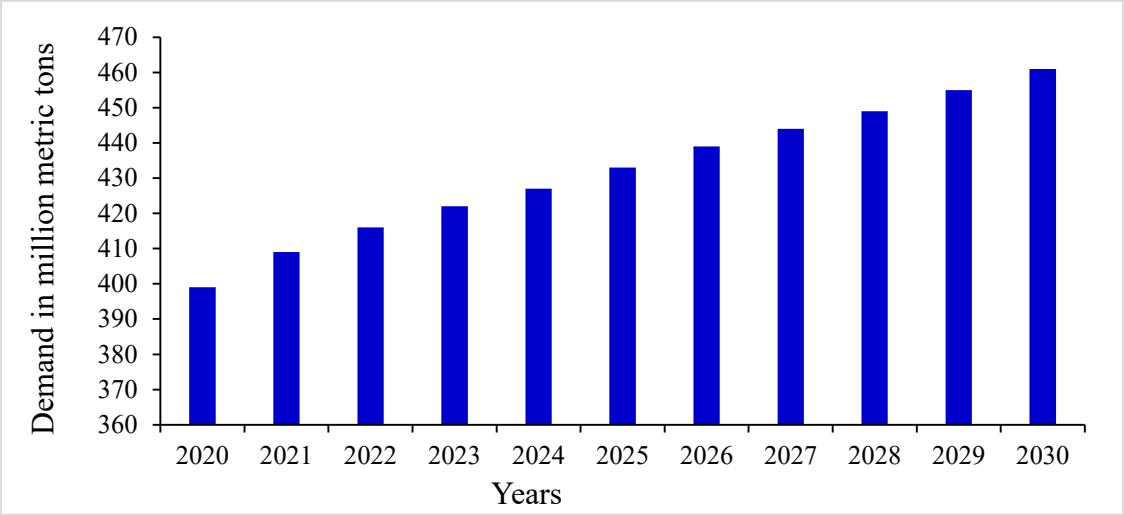


Figure 2. 1. Predicted annual Global production of paper and paperboard in the years 2020-2030

2.2.2. Paper Production in Pakistan

The paper sector occupies a significant role in the economic framework of Pakistan because Pakistan is predominantly an agricultural economy offering a suitable source of raw materials and also the market size of Pakistan is larger with a population size about 180 million. Pakistan have more than 100 paper manufacturing units in organized and unorganized sectors, major paper production industries includes Premier Paper Mill Ltd, Packages Ltd, Century Paper & Board Mills Ltd and Flying Group of industries (Shahid et al., 2021).

Consumption of paper in Pakistan is 1.299 million metric tons per annum and domestic production is 0.7 million metric tons. So the excess demand of paper is being met through import, total import of Pakistan was about to 0.611 million metric tons in 2018 (https://paperonweb.com/FAO2018.Paper.pdf). Pakistan is also exporting some types of papers but paper industry suffered a lot due to Covid 19. The maximum export of paper and paperboard was about US$100 million in 2017 which declined to US$90 in 2019 and US$46.17 in 2020 because of pandemic (Icma, 2020)

Table 2. 1 The installed capacity of paper production in Different Paper and Board Mills

Paper industries /mills	**Installed Capacity (Metric Tons per Annum)**
Century Paper and Board Mills Ltd	230,000
Packages Limited	302,000
Premier Paper Mill Ltd	25,000
Flying Board and Paper Products Limited	30,000

2.3. Paper Making Process

Paper manufacturing includes 5 basic steps and each step can be achieved by various methods. Then, the ultimate effluent is a blend of wastewaters coming from each and every of the 5 diverse unit procedures and the approaches used.

2.3.1. Debarking

Debarking eliminates the bark and alters plant fiber to reduced pieces called chips. In this stage, the raw materials used, i.e. agro residues, softwood wood and hard wood. Debarking and chipping are the main part of the pulp manufacturing process. The logs are debarked to begin the pulping process because bark cannot be used in papermaking, it must be peeled from the logs (Bajpai, 2015). In chemical debarking, chemicals such as sodium arsenite are applied to the wood of girdled trees. After three months, the chemical causes the wood to die and the bark to loosen. This is done to prepare the wood for pulping. The debarking process yields two main products: chips, which are the primary product, and bark, which is a by-product.

2.3.2. Pulping

The pulping process includes the separation of cellulose fibers from the wood chips via the removal of lignin. There are two main types of pulping i.e., chemical pulping and mechanical pulping.

Chemical pulping involves the “cooking” of lignocellulosic biomass using alkaline chemical such as NaOH, and Na_2S under elevated pressure and temperature to disintegrate cellulosic fibres. Lignin generates sodium ligate when it interacts with sodium hydroxide. Then sodium ligate causes the fibers to separate (Britannica, 2015).

In Mechanical pulping, fibers are separated without the use of chemicals. A high yield of pulp is obtained via this technique i.e., about 92 to 96% but that pulp is rich in lignin contents (Haggar, 2007). In the traditional process wood chips are fed to a spinning stone, which rubs or grinds them into pulp. The stone is constantly sprayed with water to remove fibres from the pulping platform and to prevent fibre damage caused by friction on the stone, which causes heat.

2.3.3. Bleaching

Bleaching is a process in which pulp is chemically modified to increase their brightness. The paper that is made up of bleached pulps is softer, whiter, and brighter as compared to the paper made up of unbleached pulps. In bleaching, pulp is treated with the bleaching agents such as peroxide, chlorine, chlorine dioxide, oxygen, and hypochlorite. During bleaching, lignin reacts with a bleaching agents and forms water soluble compounds or modified to colorless lignin products. In this phase resin acids, phenols, lignin are chlorinated and converted to very toxic xenobiotic (Sharma et al., 2020)

2.3.4. Washing

In washing process, the pulp is cleansed from bleaching agents. In this stage, the bleached pulp is treated with caustic soda that dissolves the chlorinated chemicals and then rinsed off. This step is also known as alkaline extraction. The final stage comprises a neutralization treatment with a very alkaline hypochlorite, followed by a final wash (Sharma et al, 2020).

2.3.5. Paper Making

Extraction and manufacturing are two steps in the process of paper making. Cellulosic fibres are available for paper production after fibre extraction and lignin removal from pulp (Singh et al., 2019). The actual papermaking process conversion of pulp to wet paper sheets and then, wet sheets are dried by different surface treatments.

2.4. Paper and Pulp Industry a Source of Pollution

The P & P industries are the main consumer of energy (electricity and fossil fuels) and natural resources (water and wood) and also an important contributor of contaminants released to the environment. After cement, oil, steel, textile and leather industries; P & P industry is the 6th major polluting industry and can released different types of solid, gaseous and liquid wastes to the

environment. The effluents generated from these mills is based on certain factors like production methods and the use of materials, these effluents mainly have low biodegradability rate and high COD and also contain more than 700 inorganic and organic compounds (Karrasch et al., 2006). In developing countries these pollutants are released to the enviroenment with out treatment, which have serious threats to the aquatic and terrestrial life. The additives, the feeding water, and the fibrous raw material used in the production of paper are the major source of contamination in the water that produces pollutants such as wax, lignin and its derivatives, dioxins, resinic acids, organic chlorides, and many more. It is widely known that the paper manufacturing process pollutes the environment to a high degree, both in terms of discharge volumes and composition.

Lignin is a naturally occurring irregular and highly recalcitrant biopolymer that consists of phenyl propane units, numerous linkages, and substitutions (--COOH, -- OCH3, --OH). It is a major pollutant responsible for the toxicity of the water. Residual lignin in wood fiber is a key coloring material that also combines with chlorine molecules in the effluent to generate organochlorine compounds. These effluents are hazardous to aquatic species and have a severe mutagenesis effect (Ramos et al., 2009).

Large amounts of solid waste are also generated during the pulping process. These wastes include green liquor silts, scrubber slush, lime grits, boiler ash, pulp screening rejects and dregs, wood processing residues, and lime mud. These pollutants have a serious impact on the environment if not properly disposed off (Simao et al., 2018).

2.5. Effects of Paper and Pulp Industrial Effluent on the Benthic Environment

2.5.1. Bottom-water and sediment deoxygenation

Pulp mill effluents after their discharge into the marine environment may have both short- and long-term hydrographic effects on areas that are receiving it. The dissolved organic content causes an immediate BOD, whereas the suspended solids result in long-term BOD. Both components have major consequences in regions when the value of effluent BOD is high or when the volume of receiving water is small. The complete eradication of benthic fauna because of the formation of an anaerobic blanket deposit of wood chips and fibers at the sediment surface is one of the most significant consequences (Pearson, 1980). Even if the conditions improve i.e., increase in oxygen content or reduction in pollution, the presence of this anaerobic blanket will keep the anoxic

conditions at the bottom. This same case happened on the Canadian west coast inlets (Hamilton, 1976).

2.5.2. Direct toxicity

The organic materials such as lignin and its derivatives like chlorinated phenols, lignosulphonic acid, chlorinated resins and biocides are present in abundance in paper and pulp wastewater (Mazhar et al., 2019). These effluents when discharged into water bodies they pose serious toxic effects on aquatic life. Pentachlorophenol (PCP) is a well-known harmful contaminant. These compounds have adverse or toxic effect on invertebrates and fishes. USEPA (the United States Environmental protection agency) declared it major pollution and its concentration of about 1.0 mg/L is not considered safe for the environment (Kumar at al., 2019). Previously, it has been reported that the physiology of mollusk and fish is at risk because of kraft mill waste. It has also been studied that the pulp mill effluent had negative impact on the oyster's metabolic activity.

2.5.3. Discoloration of Water

Many pulp and paper mills effluent contains high amount of lignin derivatives and dyes, which reduces the clarity of water greatly. This causes far more serious environmental issues in freshwater than it does in the sea but when these effluents are released into the fjords, there is severe reduction of phytoplankton population because of decrease in the transparency of water (Rajput et al., 2013). Similarly, aquatic plants and algae growth are both affected by highly colored water because it reduces the process of photosynthesis as well as the oxygen level in the water.

2.5.4. Effects on Human Health

The communities existing in encompassing zones close to industry are affected by various hazardous compounds, which enter into the food chain and induce bio-magnifications (Rahman et al., 2016). These hazardous compounds can enter the fishes through water, which are devoured by people and cause illnesses like modification of metabolic activities such as an increase in lymphocyte levels, g-glutamyl transpeptidase activity, and increased excretion of 17-hydroxycorticosteroid. These toxic compounds also causes skin diseases such as hepatomegaly, chlorane, dermatitis, and folliculitis. Polychlorinated biphenyl present in wastewater causes lungs and digestive system cancer, which increases mortality rate (Singh et al., 2016). Lignin generates many chlorinated organic compounds like, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin the most

hazardous chemical molecule, is not destroyed by biological systems and is responsible for acne in males (acneiform dermatitis). These compounds also enter into the cytoplasm and bind with the aryl hydrocarbon receptor (AhR). Dioxins cause damages at both molecular and cellular level (Yadav, 2006).

2.6. Remediation of Pulp and Paper Mill Effluent

The P & P industries are facing serious threats from environmental regulation authorties, effluent must be treated before, discharged to the environment. There are various processes for treatment of effluent, which mainly include physiochemical treatment processes and biological treatment processes.

2.6.1 Physiochemical Treatment

Various physiochemical treatment approaches are used for the treatment of P & P mill effluent such as, adsorption, filtration, coagulation and precipitation, ozonolysis, advance oxidation process and reverse osmosis have been used which are as follows:

2.6.1.1. Adsorption

Adsorption is a method used for the removal of pollutants from wastewater. In this process, the contact between the adsorbable solute-containing solution and solid (highly porous surface) results in the deposition of solute molecules present in the solution at the solid surface because of a liquid-solid intermolecular force of attraction. The solute that is deposit on the solid is termed as adsorbate whereas that solid is termed as adsorbent (Rashed, 2013). The various adsorbent are available which includes; activated carbon, activated coke, coal ash, activated charcoal fuller's earth, silica, etc. More than 90% of the color is removed by adsorption by using activated carbon, fuller earth, charcoal, and coal ash. Similarly, it is reported that almost 90% of color and COD is removed from bleached water by using absorbent i.e., activated coke (Shawwa et al., 2001).

2.6.1.2. Precipitation and Coagulation

The principle of this technology is the use of metal salts to create larger flocs from small particles in order to remove contaminants easily. Several chemicals such as polyethyleneimine (PEI), horseradish peroxidase (chitosan), epichlorohydrin polycondensate (HE), and hexamethylene diamine have been reported as effective chemicals to remove colour, adsorbable organic halides,

and total organic carbon (Kalamdhad et al., 2020). About 60-80 % of color reduction from P & P mills effluent via coagulation and precipitation by using aluminum chloride, ferric sulfate, or alum is reported but there are some issues of sludge management and disposal (Gellman, 1974) According to the studies, alum salts as a coagulant removed 96%, 50%, and 20% of COD from paper machine, pulping, and from bleaching effluents (Gökçay et al., 1994). Similarly, it is reported that corn stover's pretreatment using metal catalysts such as MgO, Fe_2O_3, ZnO, and CuO reduced sugar depletion and boost lignin removal (Ge, 2018).

2.6.1.3. Membrane Technologies

The ability of membranes to recover lignin from wastewater has been investigated in a number of experiments. The membrane filtration technique involves the removal of small solid particles, which includes inorganic and organic matter, microorganisms, color, salts, and heavy metals by passing wastewater over a synthetic membrane (Neves et al., 2017). In the first step, lignin is precipitated by the addition of chemicals such as sulfuric acid, chlorine dioxide, and carbon dioxide. After precipitation lignin is isolated via membrane filtration (Humpert et al., 2016). The most common membrane filtration techniques are nanofiltration, microfiltration, ultrafiltration, electro-dialysis, and reverse osmosis. These techniques differ in the type of pollutants, their capacity, and the strength of the driving forces utilized to promote the separation. The removal of TDS, COD, BOD, and color from paper and pulp effluent is reported to be 80% to 90%, after the treatment in a membrane reactor (Chanworrawoot et al., 2012).

2.6.1.4. Chemical Oxidation

In pulp and paper mills, several advanced oxidation techniques such as ozonation, fenton type reactions, photo-catalysis, wet oxidation and photo-oxidation are employed to destroy both chromophoric and non-chromophoric contaminants. These advanced oxidation processes aimed to degrade recalcitrant organic compounds which are even difficult for microbes to break. About 30%-70% of biodegradability of P & P mill effluent is reported through oxidation (Verenich et al., 2000). Similarly, the effectiveness of ozone towards color, COD, and hazardous chemicals is investigated by many researchers. About 95-97% of color reduction was reported when the effluent was exposed to ozone for 15 min under high ozone concentration (Sarikaya et al., 2002).

Designed plants, which comprises physico-chemical treatments, in term of operational cost, can be generally found costly for all type of P & P mill wastes (Buyukkamaci and Koken, 2010). Physico-chemical methods can also contribute to greenhouse gas emission either openly through the processes itself, or ramblingly due to their energy necessity. These processes involve the use of electricity (power consumption), which in turn increases the operational cost (Ashrafi et al., 2015). Biological methods could be alternative to physico-chemical methods, biological methods, which are eco-friendly and cost effective.

2.6.2. Biological Treatment

Biological treatment is well known process in reduction of toxic organic load and intensive research has been carried out to decontaminate the effluent from pulp and paper industries using fungi, bacteria and their enzymes (Mathews et al., 2016; Kumar et al., 2020). These methods are environment friendly, cost-effective, very efficient, and do not require any chemicals as compared to physiochemical processes. Lignin produced by P & P industries is the main sources of water pollution. Lignin is decolorized and degraded through two mechanisms: biosorption or microbial enzymes (Kornillowicz-Kowalska, 2016). In biosorption, the discoloration of lignin from the effluent is aided by the residues of microbial cells. Different fungal and bacterial species have been reported for treatment of paper mills effluent.

2.6.2.1. Fungal Treatment

Fungi have been widely researched for its ability to degrade and decolorize lignin and its associated monomers. Fungal treatment has been proved to be very efficient in removing organic compounds from wastewater, because of the presence of extracellular enzymatic system, white-rot fungi gained attention in the research area (Karim et al., 2016; Schmidt et al., 2016). The cellulose portion present in the wood is used as a carbon source by white-rot wood fungi, so to reach that part, it completely degrades the lignin. The timber degrading white rot fungi are effective to breakdown lignin. The fungal species such as *Tinctoria borbonica, Schizophyllum commune*, *Trametes versicolor* and *Phanerochaete chrysosporium, Trichoderma spp.,* and *Aspergillus Niger* has been utilized for degradation of lignin (Kamali and Khodaparast, 2015; Dashtban et al., 2010).

Schizophyllum commune, a white rot fungus can decolorize the effluent from bagasse-based pulp and paper mills. But, this fungi cannot break down lignin without the readily available carbon

source, this specie first relay on available carbon source and then act on lignin (Dashtban et al., 2010). The readily available nutrients like nitrogen and carbon not only enhance the decolorization by fungus but also results in the better elimination of COD and BOD from the pulp and paper mill waste and the finest co-substrate for the lignin breakdown was sucrose (Kamali and Khodaparast, 2015). The treatment efficiency of sewage with *Schizophyllum commune* was maximum at pH 4-5 and was additionally enriched by continuous aeration. *Schizophyllum commune* can reduce the COD and BOD up to 72% and 70%, respectively and also remove the 90% lignin from the effluent in 2 days incubation under optimum conditions (Lucas et al., 2012, Saritha et al., 2009).

The fungus *Tinctoria borbonica* reduced the color of black liquor approximately upto 90-99% after 4 days of incubation (El-Rahim and Zaki, 2016). A naturally present soil saprophyte *Gliocladium virens* has been used for the treatment of pulp and paper mill effluent. It was perceived that this fungus was capable to grow in the waste and can decolorize it up to 42% and reduce the level of lignin, cellulose and BOD 52%, 75% and 65%, respectively (Kamali and Khodaparast, 2015). Model white rot fungus *Phanerochaete chrysosporium* can effectively degrade lignin and decolorize the effluent, because of specialized capability to degrade the abundant aromatic polymer (Senthilkumar et al., 2014). Another white rot fungus*, Coriolus versicolor*, can also have good potential for the treatment of effluent*. Coriolus versicolor* can produce an extracellular enzyme called laccase. Extracellular laccase plays potential role in lignin biodegradation (Kamali and Khodaparast, 2015, Aftab et al., 2011). Growth substrates like glucose or cellulose are required for the degradation of lignin. Decolorization rate of effluents were friendly and effective, when the inoculum and sucrose concentration were high (Tiku et al., 2010). *Cyrus stercoreus* a gasteromycetes is linked with decomposition of litter, can effectively degrade lignin like other white rot fungus (Saritha et al., 2009). Number of studies was conducted on *Aspergillus foelidus* ability for color removal, lignin depolymerization and COD reduction from raw and alkali pulp and black liquors (Sumathi and Phatak, 1999).

Fungal treatment system for effluent detoxification has limitations of growth conditions. Fungus requires acidic pH for production of ligninolytic enzymes, usually the pH of P & P mills effluent tends to be alkaline in nature so the practice of fungal system for effluent treatment requires pH adjustment which adds extra cost to the process. While bacteria are gaining interest because of nvironmental adaptability and biochemical versatility as compared to fungi (Mathews et al., 2016).

2.6.2.2. Bacterial Treatment

Different bacterial species are used for detoxification of effluent, because of their potential for the color removal, lignin degradation and detoxification of effluent and some of them are also used commercially. Bacteria have enormous environmental flexibility, biochemical adaptability, improved biodegradation ability and wide range of pH tolerance as compared to fungi (Chandra et al., 2011). The indigenous microbes such as *Micrococcus luteus* and *Bacillus subtilis* can effectively reduce COD and BOD up to 94% and 87%, respectively, and were also found proficient for reducing the content of lignin up to 97% within nine days under shaking condition and were also capable of bring up the effluent pH to neutral and increase the concentration of dissolved oxygen from 0.8-6.8 mg/L present in the effluent (Tyagi et al., 2014).

Several bacteria depolymerize lignin monomer but some rare bacterial strains can attack lignin derived from various pulping practices (Chandra and Singh, 2012; Chandra and Bharagava, 2013). Another bacterial species, *Pseudomonas aeruginosa* can decrease Kraft mill waste color by 25-53% or more in aerobic condition (Ramsay and Nguyen, 2002). Xylanase an extracellular enzyme produced by *Bacillus stearothermophilus* T6 is thermo-stable and alkali lenient enzyme, this enzyme was effectively used for bioleaching and decolorizes mash optimally at 65°C and pH 9.0. *Streptomycetes viridosporous* and *S. badius* have been reported to use synthetic kraft lignin as the sole carbon source (Chandra et al., 2011). *Acienetobacter calcoaceticus* and *Pseudomonas putida* were investigated for their ability to degrade the black liquor from P&P mill in a continuous reactor. They were capable of removing 70% COD, 80% lignin and 80% color in 8 days of incubation (El-Rahim and Zaki, 2016). The isolated bacteria such as *Enterobacter* spp., *Pseudolllonas putida* and *Citrobaterer* sp. can effectively remove the color from the effluent up to 97% and also can reduced sulfide, phenolics and COD up to 97%, 94%, 96% and 94%, respectively (Tiku et al., 2010, Keharia and Madamwar, 2003).

Table 2. 2 Summary of microbial degradation of lignin

Microbial strain	Lignin load	Degradation%	Reference
Bacteria			
Bacillus ligniniphilus L1	1 g/L	38.9%	(Zhu et al., 2017)
Pandoraea spp. B-6	3g/L	46.7 %	(Shi et al., 2013)
Novosphingobium sp. B-7	3g/L	30%	(Chen et al., 2012)
Bacillus sp. CS-1	0.05g/L	61%	(Chang et al., 2014)
Pandoraea sp.	5g/L	39.9%	(Zhang et al., 2013)
Citrobacter freundii (FJ581026)	600ppm (w/v)	49%	(Chandra and Bharagava, 2013)
Streptomyces spp. strains F-6	5g/L	37.6%	(Yang et al., 2012)
Fungi			
Aspergillus sp. strain F-3	2 g/L	65%	(Yang et al., 2011)
Fusarium proliferatum	5g/L	19.6%	(Regalado et al., 1997)
CladosporiumBio-1	2g/L	35%	(Jin et al., 2012)
P. chrysogenum	1g/L	83.5%	(Rodríguez et al., 1994)
P. sulphureus Phellinus sp	0.5%	36%	(Arun and Eyini, 2011)

2.6.2.2.1. Bacterial Ligninolytic Enzymes

Many ligninolytic bacteria have been characterized for efficient degradation of lignin containing waste during wood decay processes (Janusz et al., 2017). Lignin is the second most abundant natural organic polymer, it persist in the soil for longer period of the time because of its low biodegradability, recalcitrant nature of lignin is due to the presence of complex chemical bonds between its monomers (Ragauskas et al. 2014). The lignin is composed of three monomers which includes, guaiacyl (G), syringyl (S) and p-hydroxyphenyl monomer (H) and these monomers are linked together through ether bonding, which makes it complex and stable structure (Shin et al., 2019). The complex structure of lignin represents major challenge during utilization for sustainable development.

The oxidation of lignin and other aromatic compounds are carried out mainly by ligninolytic enzymes. Many ligninolytic enzymes produced by fungi and bacteria are previously been identified as lignin degraders. Ligninolytic enzymes directly attacks on β-O-4 ether bond to generate free cation radicals. These cation radicals cleaves C–C bond resulting in hydrophilic products. The structure of lignin contains various β-O-4 aryl-ether linkages and biphenyl linkages which are targeted by ligninolytic enzymes during degradation. Lignin degrading enzymes or ligninolytic enzymes are grouped into copper- containing laccases and iron-containing peroxidases.

2.6.2.2.2. Laccases

Laccase is a glycoprotein consists of monomeric, dimeric, and trimeric glycoproteins. It is also known as p-diphenol oxidases or dioxygen oxidoreductase with molecular weight of 50–300 kDa (Chandra and Chowdhary, 2015). Laccase has been characterized and reported with three domains, 500 amino acid residues, and a Greek key barrel structure. Three different types of copper i.e., Blue Cu (T1), Normal Cu (T2), and coupled binuclear Cu (T3) are found in laccase that maintains the amino acids at active site (Bento et al., 2010). Laccase is the only enzyme that liberates only H_2O as a byproduct, and it is referred to as "Green Catalysts" because it can oxidize a variety of compounds (Piscitelli et al., 2010). Recently some bacterial strains such as *Marinomonas mediterranea, Bacillus* sp.*, S. lavendulae,* and *Pseudomonas* sp. have been reported for laccase production (Bharagava et al., 2019). Microbial laccases have higher efficiency as compared to plant laccases because they exhibit higher redox potential (Guan et al., 2018)

Laccases catalyze two types of reactions, direct substrate oxidation and indirect substrate oxidation. In the first type, there is direct contact of the substrate with the copper present in the laccase that results in the substrate oxidation (Chernykh et al., 2008). However, in many cases, because of the low redox potential of T1 copper ion, it is not possible to have direct oxidation (Yaropolov et al., 2007). In the 2^{nd} type i.e., indirect substrate oxidation, first a mediator is catalyzed by enzyme and then the substrate is oxidized by that mediator.

Laccases are extensively spread in fungi, plants and also in bacteria, like *Sinorhizobium meliloti, A. lipoferum* and *B. subtilis* (Pawlik et al., 2016). Laccase localization is linked with its various physical functions and also depends on the enzyme producing organism. Due to arbitrary polymer lignin nature, laccases mostly interact with free phenolic segments of lignin and also help in

lowering redox potential. Although, laccase can act on non-phenolic aromatic compounds in the low molecular mass mediator with high redox potential (Kunamneni et al., 2007). Oxidation of substrate is single electron generating free radical by laccase. An unstable phenoxy radical is an initial product, which can be converted into quinone in a 2^{nd} reaction catalyzed by enzyme (Shekher et al., 2011).

2.6.2.2.3. Peroxidases

Peroxidase is an enzyme that can catalyze various biosynthesis and biodegradation reactions. Previously, heme-containing peroxidases found to be involved in lignin degradation. Heme peroxidases require oxygen as an electron acceptor which is usually taken from hydrogen peroxide, to catalyze oxidative reactions. Heme peroxidases are grouped into lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), dye-decolorizing peroxidase (EC 1.11.1.19) (Janusz et al., 2017; Ozer et al., 2020).

2.6.2.2.4. Lignin Peroxidase

Lignin peroxidase (LiP) is a glycosylated monomeric protein, containing 300-600 amino acid residues, 2 calcium ions, and 4 carbohydrates having a molecular weight of 40–70 kDa (Bharagava et al., 2019). It also consists of 2 anti-parallel beta-sheets, 8 helices, and 2 domains present on both sides of heminic group. Furthermore, the His amino acid is linked with the heme group and this His amino acid elevates the redox potential of the enzyme by creating the electronic deficiency in the iron's porphyrin ring. (Dan Cullen et al., 2008). Lignin peroxidase is one of the most important lignin degrading enzyme and its activity is detected in various bacterial species such as *Streptomyces* and *Pseudomonas* and *Bacillus* (Schraft et al., 2010). The catalytic mechanism of Lip is based on three steps: the first step involves the two-electron oxidation of the native ferric enzyme [Fe (III)] by H_2O_2 to form a compound I oxo-ferryl intermediate (two-electron oxidized form). In the second step, compound-I is reduced by a reducing substrate such as non-phenolic aromatic substrate and receives one electron to form compound-II (one-electron oxidized form). In the third step, compound II receives a second electron from the reduced substrate returning the enzyme to the native ferric oxidation state to complete the oxidation cycle (Abdel-Hamid et al., 2013). The overall catalytic reaction of Lip is shown in Fig 2.2.

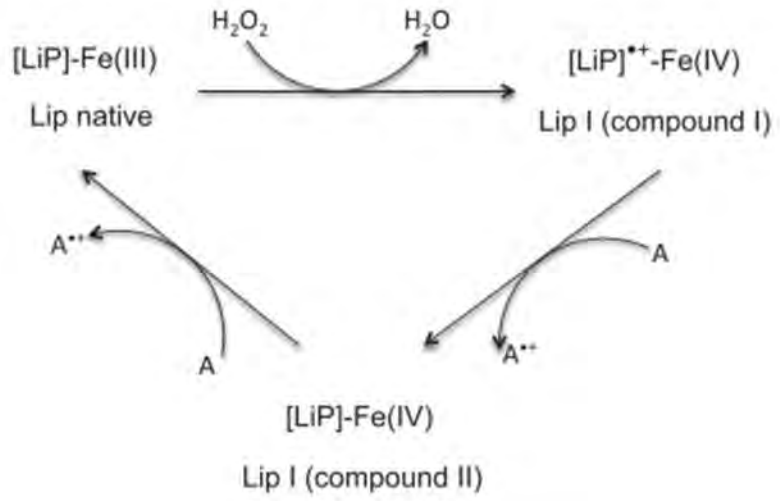


Figure 2. 2. The catalytic reaction of lignin peroxidase

2.6.2.2.5. Manganese Peroxidase

Manganese peroxidase (MnP) is another heme-containing oxidoreductase enzyme having a molecular mass of about 40–50kD. MnP enzyme consists of 350 amino acids, 1 minor and 10 major helices, and a heme sandwiched between two domains. Its active site is maintained by 5 disulfide-bridging elements and 2 calcium ions (Wong, 2009). Different amino acids such as aspartic acid residue bonded with hydrogen, proximal histidine ligand and arginine residues, and catalytic His in a distal side peroxidase-binding pocket are present in its active site. MnP is H_2O_2 dependent enzyme and oxidation by MnP achieved in multiple steps, catalytic cycle starts with the oxidation of heme group by H_2O_2 that results in the production of MnP compound I (oxyferryl porphyrin cation radical and H_2O). MnP compound II (oxyferryl chemical species) and a free radical ion is produced by the oxidation of substrate molecule via MnP compound I. Next is the oxidation of Mn^{2+} into Mn^{3+} via compound III. The final step is the dissociation of Mn^{3+} from the enzyme and its stabilization via various organic acids (chelators) which are oxalate, glyoxylate, fumarate, and malate. Mn^{3+} is responsible for the oxidation of a lignin and variety of aromatic compounds via one electron or proton abstraction (Zainith et al., 2020).

2.6.2.2.6. Dye Decolorizing-Type Peroxidase

Dye decolorizing peroxidase or (DyPs) belongs to a novel superfamily of heme-containing peroxidase, and showing stability in acidic conditions. DyP-type peroxidases have been reported

for their potential applications in the degradation of various dyes, as well as in polymeric lignin degradation (Li et al., 2017). According to the PeroxiBase database, DyPs are classified into four classes A, B, C, and D (Fawal et al., 2012). Class A-type contains TAT-signal sequence serves the role of transporting folded proteins across cell membrane. Crystal structures of class A-type DyPs from *Streptomyces lividans* TK24, *Streptomyces coelicolor*, *Escherichia coli* O157, *Cellulomonas bogoriensis*, *Sphingomonas* sp. A1 and *Thermobifida fusca* were deposited in Protein Data Bank (PDB) (Habib et al., 2019; Rahmanpour et al., 2016; Chaplin et al., 2020). Class B and C types have major role in intracellular metabolism (Yoshida et al., 2016). D-type were reported from fungi, including *Bjerkandera adusta*, *Auricularia* j*udae*, and *Pleurotus ostreatus* and tcrystal structures of class D enzymes were deposited PDB (Linde et al., 2015; Fern´andez-Fueyo et al., 2018). Bacterial DyPs proved to be promising biocatalyst as compared to fungal DyPs and other peroxidases, because the catalytic efficiency of bacterial peroxidases is relatively easier to enhance through recombinant DNA technology (Fern´andez-Fueyo et al., 2018). Thus, bacterial DyPs opens up the opportunity of biocatalytic degradation of lignocellulosic waste.

2.7. Improvement Strategies for Ligninolytic Enzyme Production

For the successful application of ligninolytic enzymes like laccases and DyP-type peroxidases on industrial scale, high yields and excellent catalytic activity and specific properties such as stability at elevated temperature and pH, have significant importance. Additionally, production and application of enzymes should be eco-friendly and cost-effective (Shin et al., 2019). Most of the reported ligninolytic do not have specific properties for industrial application. Several traditional and modern technologies are available for increase production and enhance lignin degradation efficiency of ligninolytic enzymes. The traditional technologies based on screening of efficient ligninolytic enzyme producing organisms from lignin-contaminated sites and optimization of culture conditions for enhance production of enzymes, purification and functional characterization of enzymes. However, the native enzymes are not enough to fulfil the industrial demand, due to low production and stability in harsh industrial conditions (Ahmed et al., 2009). So the modern technologies such as molecular methodologies must be applied for enhance production of ligninolytic enzymes. Various ligninolytic enzymes encoding genes have been cloned and expressed in different expression hosts and achieved high level of expression by use of

recombinant DNA technology (Dhankhar et al., 2020). These recombinant ligniolytic enzymes have shown better properties and more stability than the native enzymes.

2.8. Expression in Bacterial Cells

Escherichia coli expression system is the choice of organisms for expression of recombinant proteins due to well studies genome and easy alteration. For expression of recombinant laccases and peroxidases, *E. coli* proved to be best expression system as compared to yeast and other fungal systems, because the expression host like yeast has the undesirable tendency of hyper-glycosylating the heterologously expressed glycoproteins, which restrains the subsequent downstream processing and resulting in the hindrance for enzymatic applications. Furthermore, 20-fold higher production can be achieved through expression in *E. coli* than yeast (Eggenreich et al., 2016). Previously, laccases and lignin degrading peroxidases from different organisms have been successfully expressed in *E. coli* expression cells. Lignin degrading peroxidases from *Bacillus subtilis*, *Pseudomonas fluorescens*. *Rhodococcus jostii* RHA1, and *Thermobifida fusca* has been successfully cloned and expressed in *E .coli* (Ahmad et al., 2011, Singh et al., 2012). Higher recombinant peroxidase yield of 100mg/l is another benchmark triumph reported using *E. coli* cell factory.

2.9. Industrial Applications of Ligninolytic Enzymes

Ligninolytic enzymes have many application in different industries such as detoxification of effluent from of textile and paper industries, food processing, cosmetic, and production of value added chemicals, and biofuels (Maciel et al., 2010). Laccase has been reported for different application in P & P industries, such as delignification and bleaching of wood pulp (Widsten and Kandelbauer, 2008). Laccase can also be used to enhance the food color and remove the undesirable phenolic compounds. While, peroxidases can be used in food industry to produce aromatic flavors in the food (Capareda et al., 2015), and treatment of dyes containing effluent generated from industries (Kunamneni et al., 2008a; Gomes et al., 2012). Various studies have reported the degradation of different polycyclic aromatic hydrocarbons (PAHs) by using laccases and peroxidases (Anastasi et al., 2009). Laccases have also been used in pharmaceuticals industries, production of polymers, coupling of phenols and steroids, complex natural products synthesis, personal hygienic products, and biosensors (Maciel et al., 2010; Kunamneni et al., 2008a; Barbosa et al., 2008). Ligninolytic have also been utilized to convert lignocellulose waste

material to value-added products such as biofuel, animal feeds, fine chemicals like vanillin, adipic acid, Teraphthalic acid, Pyruvate, Triacylglycerol Lipids, and Polyhydroxybutyrate (Malherbe and Cloete, 2002). Teraphthalic acid and Polyhydroxybutyrate act as precursors for production of biodegradable plastics.

3. MATERIALS AND METHODS

The current study was conducted in Applied, Environmental and Geomicrobiology Laboratory, at Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan, and Department of Biology, Karadeniz Technical University, Trabzon, Turkey. It includes isolation and characterization of most efficient lignin degrading bacteria from pulp and paper mill effluent. Efficient lignin degrading bacterial strains were investigated for production of ligninolytic enzymes including, laccases and peroxidases. The genes of ligninolytic enzymes were cloned and over expressed in mesophilic host. Recombinant proteins were biochemically characterized and used for lignin degradation.

3.1. Collection of Sample

Sludge and effluent samples were collected aseptically from effluent treatment plant (ETP) of Century Paper and Board Mills Limited, District Kasur, Punjab Pakistan. Sludge samples were collected from aeration tank, while effluent samples were collected from six different points of ETP, these points includes: Inlet point, Aeration Tank 1, Aeration Tank 2, Secondary Sedimentation Tank, Final Sedimentation Tank and Black liquor from pulping units. Effluent samples were collected carefully to make them as representative as possible of the whole water. Three grab samples (1L) were collected, at constant time intervals over the sampling period and mixed in 5L sterile plastic bottles to give a 3 h composite sample. All the samples were collected in sterile plastic bottles, and carefully transported to laboratory and analyzed within 48 h of collection.

3.2. Physiochemical Analysis of Samples

In order to assess the pollutant load, All the effluent samples were subjected to physicochemical analysis and analyzed for pH, total suspended solids (TSS), total dissolved solid (TDS), nitrates (NO_3) and sulphates (SO_4) as per the standard methods provided by APHA (2012). Chemical Oxygen Demand (COD) was determined by COD Cell Test kit according to the instructions provided by manufacturer. Lignin concentration and color were measured by following Pearl and Benson (1940) and Canadian Pulp and Paper Mills Association (CPPA, 1974) methods, respectively.

Procedure:

3.2.1. pH Measurement

pH determination is important to find out whether the sample is acidic or basic in nature, and the type of microorganisms thrive in the sample. For pH determination 100 mL of the effluent sample was taken and filtered in-order to remove impurities, and pH was measured by using pH meter.

3.2.2. Sulphates

Sulphates concentration in the effluent sample was measured according to the EPA method 0375 Barium Chrometery (Rice et al. 2017).

Procedure:

Effluent sample (50 mL) was taken in a sterile beaker and buffer A and B (5 mL) were added to the effluent sample. Then, $BaCl_2$ crystals (0.15g) were added and gently mixed for 30 s. Absorbance was then taken spectrophotometrically at 420 nm.

3.2.3. Total Dissolved Solids

In order to remove the suspended solids, the effluent samples were filtered with the help of filter paper. The filtered sample was then transferred to sterilized beaker which was initially weighed. The sample was kept in oven for 5 h at 105°C for drying.

Total dissolved solids was measured according to the formula mentioned below:

Total dissolved solids (mg/l) = (A-B) ×1000/ Volume of sample (ml)

Where;

A= Weight of dried residue and beaker in mg

B= Weight of the beaker in mg

3.2.4. Total Suspended Solids

Dry weight of the filter paper was measured and 100ml of mixed effluent samples were filtered through filter paper, then filter paper having filtrate was dried in oven at 105°C for 1 h. Total suspended solids were measured by using the formula mentioned below:

Total suspended solids (mg/l) = (A-B) ×1000/ volume of sample (ml)

Where;

A= Weight of filter and dried residue in mg

B= Weight of the filter paper in mg

3.2.5. Nitrates

Concentration of nitrates in the effluent was determined by EPA 4500 NO_3-N method. Following the method 25 mL filtered sample was collected in flask and 0.5 mL of 1 molar HCl was supplemented to the sample. After mixing of the sample, the level of the nitrates were measured with the help of spectroquant.

3.2.6. Chemical Oxygen Demand (COD)

Commercially available COD vials were used to measure the concentration of COD in effluent. 3mL of effluent sample was poured into the COD vials using pipette. The sample was carefully allowed to run from pipette down inside of the reaction vial. The screw cap was tightly fixed and vigorously mixed the contents of the vial. The reaction vial was heated (148°C) in the preheated thermo reactor for 120 m. COD vials contains strong oxidizing agent i-e potassium dichromate ($Cr_2O_7^{-2}$), on heating dichromate ion reduces to green chromic ion and the released chromic ion was measured spectrophotometerically.

3.2.7. Color

The color was measured according to the standard method of Canadian Pulp and Paper Association (CPPA, 1974). The suspended solids from effluent samples were separated by centrifugation (10,000 rpm) for 30 m, pellet was discarded, while supernatant was adjusted to pH 7.6. The absorbance was taken spectrophotometerically at 465nm using distilled water as blank. Following formula was used to transform absorbance into color units (PtCO):

$$CU\ (PtCO) = 500\ A_2/A_1$$

Where,

A_1= Absorbance of 500 CU of standard platinum-cobalt solution (0.1214)

A_2 = Absorbance of the sample

3.2.8. Lignin Contents

Lignin contents in effluent samples were analyzed by the method, as previously reported by Pearl and Benson (Pearl and Benson, 1940). 10% $NaNO_3$ and CH_3COOH were prepared and 1 mL of each was added to 50 mL of effluent samples and mixed it throughly, and mixed samples were allowed to incubate at room temperature for 15 min. After incubation time, 2mL of NH_4OH was added to the samples and allowed to incubate for 5 min, and absorbance was taken through spectrophotometer at 430 nm. Blank was prepared by adding 2ml NH_4OH and 1ml CH_3COOH to 50 ml distilled water and allowed to incubate for 15 min, after incubation time 1ml $NaNO_3$ was added, and absorbance was taken after 5 min.

3.3. Isolation of Bacteria from Effluent

The isolation of the bacteria from sludge and effluent samples was carried out on the nutrient agar medium by spread plate method. The samples were serially diluted to nine folds ranging from 10^{-1} to 10^{-9}. 100 µl sample from dilution 10^{-1}, 10^{-3}, $10^{-5}$and 10^{-7} were spread on the nutrient agar plate by means of sterile glass spreader. The inoculated plates were kept in incubator at 37ºC for 24-48 h. Individual bacterial colonies with different morphology purified by repeated streaking on freshly prepared nutrient agar plates. Pure bacterial cultures were stored at 4°C for further study.

3.4. Screening for Lignin Degrading Bacteria

The isolated bacterial strains were screened for their maximum tolerance concentration for lignin on lignin amended minimal salt medium (L-MSM) plates. The composition of L-MSM is as follows (g/L): lignin, 0.5; Na_2HPO_4, 2.4; NH_4NO_3, 0.1; K_2HPO_4, 2.0; $MgSO_4$, 0.01; $CaCl_2$, and agar 20, and lignin was supplemented as the sole source of carbon (Raj et al., 2007). All the salts were mixed, and the pH of the media was adjusted to 7.6, and then autoclaved at 15psi and 121ºC for 20 minutes. Autoclaved media was poured into plates under sterilized condition and the bacterial isolates were streaked on plates using sterile wire loop, and then kept in an incubator at 37ºC for 10 days. The isolated bacterial strains were screened with different concentrations of lignin, ranging from 0.5-3.0 g/L.

3.5. Assays for Lignin Degradation Enzymes from Bacterial Strains

3.5.1. Qualitative Assays for Lignin Degrading Enzymes

The isolated bacterial strains were screened for their potential to produce ligninolytic enzymes i.e. laccase and lignin degrading peroxidase. Initially these strains were screened using plate assays.

3.5.1. Guaiacol Plate Assay

Laccase production was observed on guaiacol agar plates, composition of the medium for plate assay was (g/L): nutrient broth 8.0g, agar 20g and supplemented with 0.02% guaiacol (Coll et al., 1993).The isolated bacterial strains were spot inoculated on substrate amended nutrient agar plates and inoculated plates were incubated for 7 days at 37ºC, and observed for appearance of brown colored zone around bacterial growth.

3.5.2. Azure-B Plate Assay

Lignin peroxidase production was observed on azure-B agar plates. The ingredients of the medium was (g/L): nutrient broth 8.0g, agar 20g and supplemented with and azure-B 0.01% (Archibald, 1992). Bacterial strains were spot inoculated on plates and incubated at 37ºC for 7 days. Peroxidase production was determined by the appearance of decolorization zone around bacterial colonies.

3.6. Lignin Degradation Assay

Ligninolytic bacterial strains were further used to check for lignin degradation efficiency. Degradation experiment was carried out in 1000 ml Erlenmeyer flask, containing 500 ml synthetic waste water. The composition of synthetic waste water was (g/L): Lignin, 3; Na_2HPO4, 2.4; $CaCl_2$, 0.01; NH_4NO_3, 0.1; $MgSO_4$, 0.01; K_2HPO4, 2.0; peptone (0.25 %, w/v) and glucose (0.5 %, w/v), and adjusted to pH 7.6. The flasks were inoculated with 1ml of overnight grown (18h) culture of bacterial strains with an absorbance of 0.6 OD. Control flask (uninoculated) and inoculated flasks were incubated at 37 ºC for 7 days with the shaking speed of 120 rpm in shaker incubator. The samples were withdrawn after every 24 hrs for the analysis of enzyme activity. Degradation was confirmed by decolourization, reduction in lignin contents and SEM.

3.6.1. Determination of Ligninolytic Enzymes

Enzyme assays for laccase and lignin degrading peroxidase were carried out from degradation medium and specific activity was calculated by determining the concentration of proteins.

3.6.1.1. Laccase Assay

Extracellular laccase activity was assasyed by the method described by Niku-Paavola et al. (1988) using ABTS [2, 2-azino-bis-(3-ethylbenzothiazoline)-6-sulphonate], as a substrate and sodium acetate buffer (Appendix 01). Culture supernatant was collected as a source of extracellular enzyme. The laccase activity was measured spectrophotometrically at 450nm.

The reaction mixture for enzyme assay was prepared as follows:

- ABTS (2mM): 1 mL
- Sodium acetate buffer (10mM): 3 mL
- Enzyme source (bacterial supernatant): 1mL

The reaction mixture was incubated at 30ºC for 15 min, and then analyzed at 420 nm. The unit of enzyme activity was measured in international units (IU), which is the amount of enzyme used to oxidize 1 µmol of ABTS/ min. Following formula was used to measure the laccase activity (U/ml):

$$E.A = A \times V/t \times e \times v$$

Where, the absorbance of samples is expressed as A, the total volume of the reaction (ml) is expressed as V, the volume of the enzyme used for reaction (ml) is expressed as v, time for incubation (min) is expressed as t and the extinction coefficient of ABTS (36,000 M−1 cm−1) is expressed as e.

3.6.1.2. Assay for Lignin Degrading Peroxidase

The lignin degrading peroxidase activity was measured against ABTS by the protocoal as previously reported by Santos et al. (2014). The reaction mixture for enzyme assay was contained as:

- 125mM sodium tartrate buffer pH 3: 1mL
- 2 mM ABTS: 0.5mL

- Culture supernatant; 0.5mL
- 2mM hydrogen peroxide: 0.5mL

The reaction was allowed to incubate at 30ºC for 15 minutes and enzyme activity was measured at 420 nm. Lignin peroxidase activity was represented in international units (IU), where 1U is the concentration of enzyme used to oxidize 1 µmol of ABTS/minute under standard conditions. Following formula was used to measure the lignin degrading peroxidase activity in U/ml;

$$E.A = A \times V/t \times e \times v$$

Where, the absorbance of samples is expressed as A, the total volume of the reaction (ml) is expressed as V, the volume of the enzyme used for reaction (ml) is expressed as v, the time for incubation (min) is as t and the extinction coefficient of ABTS (36,000 M−1 cm−1) is expressed as e.

3.6.1.3. Determination of Protein Concentrations

The amount of total protein was calculated by Lowery's method. According to protocol, three different reagents were prepared. While Bovine serum albumin (BSA) was used as standard.

3.6.1.4. Analytical Reagents

Reagent A:

Lowery's reagent A was prepared by dissolving sodium carbonate (10g) and sodium hydroxide (2g) dissolved in 500mlof dH_2O.

Reagent B:

Reagent B was synthesized by mixing copper sulfate (0.05g) and potassium sodium tartrate (15g) in 500ml of dH_2O.

Reagent C:

Reagent C was synthesized by dissolving the reagent A and reagent B in 100:2.

Reagent D or Folin Reagent:

For every test sample, 0.1 ml fresh Folin reagent was prepared by dissolving 1N Folin reagent distilled water

Procedure:

Separate test tubes were taken for test and blank sample. About 1ml of supernatant was added to test tube and 1ml distilled water was added to control. Approximately 1ml of freshly prepared solution C was added to both the tubes and incubated at room temperature for 10 min. After incubation, 100µL of freshly prepared reagent D was added to each tube. Then tubes were gently shaken for immediate and proper mixing and allowed to incubate for 30 min at room temperature in the dark place. Blue color appearance in the tube containing supernatant was taken as positive result (sample containing protein content) while control with no such color after incubation, indicated negative result. Then absorbance was measured at 650nm.

3.6.2. Measurement of Color and Residual Lignin

Color and lignin contents were measured following the methods as previously described in section 3.2.7 and 3.2.8.

3.6.3. SEM

The lignin was examined for changes in surface morphology after treatment by bacterial strains through SEM. Both the treated and untreated samples were vacuum dried into fine powder. Dried prepared samples were mounted on carbon tape and gold plating was done to enhance the conductivity of the samples for viewing and imaging. The SEM analysis was carried out at ×500 and ×1.0 K resolution power.

3.7. Identification and Characterization of Selected Bacterial Strains

Strain SL7 and BL5 were selected for identification on the basis of lignin degradation efficiency.

3.7.1. Morphological Identification

The bacterial colony morphology was observed by streaking each bacterial isolate in quadric streak pattern on nutrient agar plates and then incubated for 48 h at 37ºC. The bacterial colonies were observed for their shape, size, color, elevation, margins and pigmentation.

3.7.2. Microscopic Identification

Gram staining

Strains SL7 and BL5 were stained by Gram's staining technique. A thin bacterial smear was made on glass slide from freshly grown bacterial isolates on nutrient agar plates. After heat fixation, crystal violet was poured on slide for 1 min, then was washed with distilled water. Gram's iodine was poured on slide as mordant and washed after 1min. The slide was then washed with ethanol-acetone for 10-15 s. The slide was washed again with distilled water to avoid excessive decolorization as well as cells washout. At the end safranin was added as a counter stain for 1 min and again washed with water. The slides were air dried and observed under microscope (Nikon YS100, Japan) at 100X lens.

3.7.3. Biochemical Identification

The bacterial strains were subject to different tests like triple sugar iron (TSI), citrate test, oxidase, motility, catalase test and indole test, then Bergey's Manual of Determinative Bacteriology (9th Edition) was concerned for interpretation of the results.

i. Triple Sugar Iron Test

In this test, TSI agar medium was poured in different test tubes and kept in slope state at 45ºC angle for solidification in sterile conditions. After this, a well-isolated colony of each strain was taken from pure culture by using inoculating needle, and then TSI agar slant was inoculated by stabbing in center of the medium up to bottom and then streaking the surface of the agar slant. Inoculated tubes were allowed to incubate for 24 h at 37°C.

ii. Citrate Test

In citrate utilization test, slants of Simmons citrate agar medium was prepared. After this, a well-isolated colony of each strain was taken from pure culture by using inoculating needle, and then agar slant was inoculated by stabbing and incubated for 24 h at 37ºC. The citrate utilization turned the green colored medium to blue.

iii. Oxidase Test

Oxidase test was performed by filter paper test method to determine the activity of oxidase enzyme. Kovac's oxidase reagent was freshly prepared by adding 1% tetra-methyl-*p*-phenylenediamine dihydrochloride into distilled water. Then 3 drops of 1% solution of oxidase reagent were added

on strip of Whatman No. 1 filter paper placed in petri plate. Colony of test strain was picked and rubbed on filter paper using platinum loop and observed from pink to purple to dark purple.

iv. Motility Test

Hanging drop method was used to perform motility test. Bacterial strain was grown to early exponential phase (~0.2 OD_{600}) and one drop of young bacterial culture was added to a dry and clean glass slide. The slide was covered with a clean cover slip and examined under 40x for motility.

v. Catalase Test

In order to perform catalase test, 3% hydrogen peroxide was prepared in a dark brown bottle. Using dropper 2-3 drops of 3% of H_2O_2 were transferred on dry glass slide and then colony of test strain was mixed in hydrogen peroxide by sterile wooden stick. After 5 – 10 s results were noted down.

vi. Indole Test

Tryptophan broth was prepared and poured into test tube. The prepared broth was inoculated with overnight prepared inoculum of bacteria and allowed to incubate at 35^{o}C for 24 h. After incubation of 24 h, few drops of Kovac's reagent was pipette to the culture medium, and observed for the appearance of cherry red colored layer.

3.7.4. Molecular Characterization

Isolates were further identified on the basis of 16S rRNA sequence analysis. The DNA were extracted and sent to Macrogen, Netherlands, for sequencing.

3.7.4.1. DNA Extraction

For 16S rRNA gene, overnight broth culture of strain SL7 and BL5 were prepared in Luria-Bertani (LB) medium and genomic DNA was purified by using Wizard® Genomic DNA Purification Kit (Promega).

Procedure

Strain SL7 and BL5 were grown over night and 3 mL of culture was centrifuged for 2mins at 14,000 ×*g* and supernatant was discarded, and pellet was suspended thoroughly in 480µl of 50mM EDTA. 120µl of lytic enzyme was gently added, and samples were incubated at 37°C for 30–60

min. After incubation, samples were centrifuged for 2 min at 14, 000 × *g* and supernatant was removed. Nuclei Lysis Solution (600µl) was added to resuspend the pellet and allowed to incubate at 80°C for 5 min. 3µl RNase solution was added to cell lysate, and mixed properly by inverting tube 2–5 times and then incubated at 37°C for 60 mins and allowed to cool at room temperature. Then, protien precipitation solution (200 uL) was mixed to cell lysate at ice for 5 minutes. Following incubation, the mixture was centrifuged at 16,000 x g for 3 min, and supernatant was collected in separate tube containing isopropanol alcohol (600 µL). Then, the samples were centrifuged at 14,000 × *g* for 2 minutes and the supernatant was carefully discarded and 70% ethanol (600µl) was added. The samples were centrifuged for 2 min at 14,000 × *g* and supernatant was discarded. Ethanol was carefully aspirated and pellet was allowed to dry for 10–15 min. In the next step, DNA rehydration solution (100 uL) was added and allowed to incubate for 1 h at 65°C and purified DNA was stored at 2–8°C. DNA purification was visualized through gel electrophoresis.

3.7.4.2. Gel Electrophoresis

The purified sample of DNA was run on 1% agarose gel for DNA confirmation. In order to prepare 1 % agarose gel, 0.45g of agarose powder was weighed and mixed with 45 ml TBE (Tris-Borate EDTA, (Appendix 02) buffer. The mixture was heated in microwave for 1 minute after which 5 µL of ethidium bromide was added. The mixture was then poured in the electrophoresis tray along with comb until it gets solidified. After the gel was solidified the comb was removed and plates were placed in the chamber with well in direction of negative end of chamber. The 1µL of 6X loading dye was mixed with 5µL DNA sample and loaded in wells with the help of pipette. The electrophoresis was run for 40 mins at 120 volts. The DNA in gel was then viewed under the U.V transilluminator.

3.7.4.3. 16S rRNA Gene Amplification by PCR

For identification of strain SL7 and BL5, 16S rRNA gene was amplified by universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). The PCR reaction mixture contained 12.5 µL of DreamTaq master mix (ThermoFisher, USA), 10 µL of deionized water, 0.5 µL of each primer of 10 nM, and 1 µL template (25 ng/µL). The PCR conditions were set as, initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 58°C for 30 s, 1 min extension at 72°C and final extension at 72°C for 5 min.

Amplification of 16S rRNA was confirmed gel electrophoresis using 1 % agarose gel containing ethidium bromide, and analysed using the Bio-Rad Gel Doc imaging system.

3.7.4.4. PCR Product Purification and Sequencing

The amplified PCR products were refined by DNA purification kit (Promega). Equal volume of DNA binding buffer was added to the PCR product and the mixture was transferred to spin column in collection tube and centrifuged at ≥ 12000 g for 1 min., then 400 µL DNA pre-wash buffer was added to the column and centrifuged again. After that 700 µL DNA wash buffer was added to the column and centrifuged for 1 min. This step was repeated with 200 µl DNA wash buffer. Finally, the column was transferred to a clean collection tube, 40 µL elusion buffer was added and centrifuged after 5 min incubation for 1 min at ≥ 12000 x g at room temperature. DNA concentration and purity were checked by NanoDrop 2000 (Thermo Fisher, USA). The amplified 16S rRNA gene product were sequenced by Macrogen Company (Netherlands).

3.7.4.5. Phylogenetic Analysis

The obtained sequences were trimed to remove unwanted sequences using Bioedit-7.2. The trimed sequences were BLAST (Basic local algnment search tool) searched in NCBI (National Centre for Biotechnology Informaion) data bases (http://www. ncbi.nlm.nih.gov/BLAST/) to find the most homologous species and then closely related sequences were downloaded. Mega-X software was used to construct phylogenetic trees by neighbor joining method (Kumar et al. 2018). The 16S rRNA nucleotide sequence of SL7 (Appendix 03) and BL5 (Appendix 04) were submitted to the NCBI Gene Bank nucleotide sequence data base under the accession numbers MZ400969 and MZ413436, respectively.

3.8. Laccase and Lignin Degrading Peroxidase Production

Optimization of different culture conditions for production of laccase (Lac) and lignin degrading peroxidase (LDP) from strain SL7 and BL5, respectively were performed.

3.8.1. Optimization of Physico-chemical Parameters for Production of Lac and LDP

Optimization experiment was analysed in Erlenmeyer flask (500mL), consisting of 100 ml production media. The composition of production media was as follows (g/100mL): starch, 3; yeast extract, 3; K_2HPO_4, 0.32; KH_2PO_4, 0.23; NH_4SO_4, 0.41; $MgSO_4$, 0.3; $FeSO_4$, 0.001 and lignin,

0.5. These flasks were inoculated with 2% inoculum and incubated for 96 hours at 150 rpm. Samples were observed after 24 hours and centrifuged at 12,000 rpm for 15 min. Supernatant (crude enzyme) was saved and used for enzyme assay and protein estimation.

3.8.1.1. Preparation of Inoculum

For enzyme production experiment, inoculum of strain SL7 and BL5 was prepared by inoculating the pure bacterial strains in nutrient broth. The composition of nutrient broth was as follows g/L: NaCl, 5; peptone, 5; yeast extract, 1.5 and beef extract 1.5. Fresh culture of strain SL7 and BL5 was inoculated into the flask containing nutrient broth and incubated at 37°C for 24 h in shaking incubator. In the optimization experiment, uniform inoculum load was used in all experiments.

3.8.1.2. Effect of Incubation Time

The effect of incubation time on production of Lac and LDP was determined by incubating the fermentation media for different incubation times (0, 24, 48, 72 and 96 hours) at 35ºC and pH 7.5.

3.8.1.3. Effect of Temperature

The temperature effect on production of Lac and LDP from selected bacterial strains was checked by incubating them at different temperature, ranges from 25 – 40°C, and optimized temperature was determined on the basis of maximum enzymes activity.

3.8.1.4. Effect of pH

The effect of pH on production of Lac and LDP was analyzed by carrying out fermentation at different pH (5, 6, 7, 8, 9, 10, 11 and 12). pH of the media was adjusted by 1N HCl and 1N NaOH solution. Media containing lignin concentration of 0.5% was inoculated with 2% inoculum and incubated for 72 h at optimized temperature.

3.8.1.5. Effect of Carbon Sources

For selection of best carbon sources for production of Lac and LDP, different carbon sources like, glucose, sucrose, starch, lactose and maltose were provided in production media. Mineral salt medium supplemented with specific carbon source was inoculated with 2% inoculum and incubated for 72 hours at optimized temperature.

3.8.1.6. Effect of Nitrogen Sources

For selection of best nitrogen sources for production of Lac and LDP, various nitrogen sources like ammonium nitrate peptone, ammonium nitrate, beef extract, yeast extract, urea and ammonium sulphate were provided in production medium, containing optimized carbon source.

3.8.2. Optimization of Nutritional Factors by Placket-Burman Design

The Placket- Burman design was used for optimization of various ingredients of production medium through selection of the interface of various ingredients and outcome of vital factors in media. Experiment was designed by using Design Expert 7 software (Stat-Ease Inc.). Eleven components of medium were optimized in fifteen run. Maximum laccase production was achieved under following components of the medium: inoculum size, starch, yeast extract, $CaCl_2$, $(NH_4)_2SO_4$, $MgSO_4$, $FeSO_4.7H_2O$, $CuSO_4$, K_2HPO_4, KH_2PO_4 and lignin, while maximum lignin degrading peroxidase production was achieved under following components of the medium: inoculum size, glucose, peptone, $CaCl_2$, $(NH_4)_2SO4$, $MgSO_4$, $FeSO_4.7H_2O$, $CuSO_4$, K_2HPO_4, KH_2PO_4 and lignin. Experiments were carried out under optimized temperature and pH. Crude laccase and lignin degrading peroxidase activity and specific activity was assayed after every 24 h for 72 h. Response to the factors was calculated in terms of specific activity of enzymes (U/mg). Significant factors were found by evaluating the responses.

3.8.3. Central Composite Design (CCD)

CDC was analysed to optimize the mutual interactive actions of the significant factors obtained from Plackett-Burman design, on production of laccase and lignin degrading peroxidase. For laccase production, 4 significant factors obtained from PB were included in CCD model and their influence on enzyme production was measured in 20 different runs. The factors observed in CCD were ammonium sulphate (D), MgSO4 (E), K_2HPO_4 (F) and Fe_2SO_4. $7H_2O$ (J). For lignin degrading peroxidase production, 5 factors were showing significant effect on production of enzyme in PB, their effect on enzyme production was analyzed in CCD in 50 different runs. The factors observed in the CCD design for lignin degrading peroxidase were inoculum size (A), glucose (B), peptone (C), ammonium nitrate (D) and lignin (E). Response was calculated in terms of specific activity of enzymes (U/mg).

3.9. Purification of Lac and LDP

3.9.1. Acetone Precipitation

Acetone is the most widely used solvent for protein precipitation, which dissolve non-polar compounds such as lipids and inhibits protein dispersion in water-based solvents, and results in protein aggregation and precipitation. Lac and LDP were partially purified through acetone precipitation. Supernatant was collected from production media as crude enzymes and were purified in ice cold environment by using ice chilled acetone. In order to optimize the concentration of acetone at which maximum proteins were precipitated out, different concentration of acetone, like 20%, 30%, 40%, 50%, 60% and 70% were used. Experiment was carried out on wet ice and being stirred by magnetic stirrer, to achieve desired saturation. Sample with different saturation levels were then centrifuged, pellets were collected and dried by evaporating the remaining acetone in fume hood. Then pellets were dissolved in sodium phosphate buffer pH 7 (Appendix 05), and enzyme assay and protein estimation were carried out for each level of saturation. The level with highest enzyme activity was considered to be optimized saturation.

3.9.2. Gel Filtration Chromatography

Since proteins varies in their characteristics features as in shape, size and net charges. They can be separated using these differentiations. After the removal of acetone, the sample (proteins) was allowed to pass through column chromatography, which separates proteins on the basis of their size. Chromatography column was packed with gel filtration medium (Sephadex G-100). The column was properly washed with antibacterial (amoxicillin), antifungal (fluconazol) and in the end with 50mM citrate buffer (Appendix 06). The sample was allowed to pass through the column gently with a low speed. After every twelve minutes 3mL of sample was being taken, in this way different fractions were obtained. Protein estimation and enzyme assay of every fraction was conducted (Coskun *et al*., 2016). Fractions with enzyme activities were collected and stored at -20˚C

3.9.3. Protein Electrophoresis SDS – PAGE Analysis

SDS-PAGE was utilized to identify the purity and molecular weight of purified enzyme. This technique relies on the electrophoresis, which may be defined as the moving of a molecule (with a net charge) under electric field's influence. This technique assess homogeneity and subunit

structure of purified protein through determination of molecular weight (Fling and Gregerson, 1986).

Procedure

Mini-Protean® Tetra Electrophoresis System (Bio-Rad) was used to perform protein gel electrophoresis. Acrylamide gel was prepared by using 30% Acryl-Bisacrylamide Mix (Bio-Rad) and TEMED (Roth), and poured in an appropriate apparatus included in the electrophoresis system. For better definition of molecular weight ranges from greater to lower, concentration of acrylamide was varied from 8% to 15%, respectively. Stacking gel was prepared with 5% acrylamide concentration. Prior to loading protein samples into acrylamide gel, proteins were disaggregated by mixing with 3X loading buffer (Appendix 07) and heating at 95°C for 5 min. Separation of proteins were performed in running buffer (Appendix 08). Proteins were separated through stacking gel (Appendix 09) at 200 volts and resolving gel (Appendix 10) at 180 volts. After acrylamide gel electrophoresis, proteins were stained in a Coomassie solution (Appendix 11) and destained with desatining solution I (Appendix 12) for 1 hour at shaking speed of 100 rpm, solution was discarded and gel was washed with distilled water and the solution II (Appendix 13) was added.

3.9.4. Protein Bands Analysis

Molecular weight (MW) of the proteins were determined by comparing the migration distance of proteins bands in gels electrophoresis with that of the molecular weight standards. The relative migration distance (*Rf*) was used to calculate the molecular weight of protein.

Following formula was used to determine the migration distance:

$$Rf = \frac{\text{Migration distance of the protein}}{\text{Migration distance of the dye front}}$$

3.10. Cloning of Laccase and Lignin Degrading Peroxidase

3.10.1. Media, Antibiotics, Microbial Strains and Plasmids

3.10.1.1. Culture Media

All the strains used for cloning were grown and stored in Luria-Bertani (LB) broth, because LB is a nutrient rich medium and favors the growth of most chemoheterotrophic microorganisms. The LB medium was prepared and sterilized by autoclaving at 121°C for 20 min. The composition of LB medium is stated in Appendix 14.

3.10.1.2. Antibiotics

For the growth of recombinant bacterial strains, ampicillin and/or kanamycin were added in culture media. Stock solutions of all the antibiotics were prepared and filtered through 22µm pore filters prior to storage at -20°C. Preparation of all the antibiotics and their storage temperatures are mentioned in Appendix 15.

3.10.1.3. Isopropyl-β-D-thiogalactoside

Expression of recombinant protein was induced by different concentrations of isopropyl-β-D-thiogalactoside (IPTG). 1 M stock solution was prepared by dissolving 238.3 mg IPTG in 1mL distilled water. Prepared solution was filtered by 22µm filter paper, and stored at -20°C. For optimal protein expression, the final concentration of IPTG was varied from 0.1-1mM depending on the protein and strain.

3.10.1.4. Microbial Strains

For cloning and expression of enzymes, *E. coli* JM101 (Stratagene Technical Services) and *E. coli* BL21 (DE3) (Invitrogen) were used, respectively.

3.10.1.5. Plasmids Vectors

E coli plasmid used in the present work includes, pET-28a (+) (Novagen) having kanamycin resistant genes, and pGEM-T Easy vector (Promega) having ampicillin resistant genes.

3.10.2. Amplification of Laccase and Lignin Degrading Peroxidase by PCR

3.10.2.1. Primers Designing

For amplification of laccase gene, primers were designed on sequence of *B. altitudinis* and efeb gene encoding lignin degrading DyP-type peroxidase primers were designed on sequence of *Bacillus subtilis* obtained from GenBank and conserved regions were identified by alignments of the sequence by Clustal W program, implemented in MEGA- 4.0. (Molecular Evolutionary Genetics Analysis Program).

Laccase primers

- 5'- Cgg ATC CgG ATg AAC CTA gAA AAg TTT gTT g -3' 31 nt (*BamHl*)
- 5'- AAg CTT CTA AAT gAT ATC CAT Cgg C -3' 25 nt (*HindIII*)

Lignin degrading DyP- type peroxidase primers

- 5'- CCA TGG ggC gAT gAA CAg AAA AAg C – 3' 25 nt *NcoI*
- 5' – AAg CTT CTA TgA TTC CAg CAA ACg – 3' 24 nt *HindIII*

3.10.2.2. Isolation of Genomic DNA of strain SL7 and BL5

Genomic DNA of strain SL7 and BL5 was extracted as previously described in section 3.7.4.1. Isolated DNA was visualized in gel electrophoresis.

3.10.2.3. Gel Electrophoresis

Gel electrophoresis was run as previously described under section 3.7.4.2.

3.10.2.4. Quantification of Nucleic acid

The spectrophotometer ND-100 NanoDrop® was used to measure the concentration and degree of purity of nucleic acid. 1µL of sample was pipette onto the end of optic fiber cable, and flash lamp which was connected to the instrument provided the light to measure the absorbance of the sample using a 0.2 mm path length. The concentration of sample was measured at OD_{260}, and purity of samples was assessed by absorbance ratio at 260 and 280 nm.

3.10.2.5. Amplification of Lac and LDP Genes by PCR

Laccase and DyP- type peroxidase genes were amplified from the genomic DNA of strain SL7 and BL5, respectively. The reaction mixture contained: 50ng DNA template, 1.5 mM $MgCl_2$, 50mM *Taq* buffer, 1% $MgCl_2$, Nonidet P40, 10 mM dNTPs, 1.5 units recombinant *Taq* DNA polymerase (Fermentas UAB Lithuania) and each primer (25 pM). Reaction was carried out with initial heating at 94°C for 2 min, denaturation of DNA strands at 95°C for 1 min followed by annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. PCR amplification was confirmed by gel electrophoresis using 1% agarose gel, and analyzed by BioRad Gel Doc imaging system.

3.10.3. Ligation of PCR Products into pGEMT-Easy Vector

The amplified PCR products were ligated into pGEMT-Easy vector. T4 DNA ligase was used to carry out ligation between vectors and insert. Reaction was performed according to the manufacturer's specification (BioLabs). The appropriate amounts of the reagents of reaction was calculated by using online tool found at http://www.insilico.uni-duesseldorf.de/Lig_Input.html

Reagents:

Reaction volume: 10 µL

- Double distilled water: 5 µL
- Buffer: 2 µL
- T4 Ligase: 1 µL
- pGEMT vector: 1 µL
- Insert: 1uL

In order to get the maximum transformants ligation reactions was incubated at 16ºC for 16h (overnight).

3.10.4 Transformation

Competent *E. coli* JM (101) cells were prepared before transformation, and transformation was carried out by the method described by Cohen et al (1972).

3.10.4.1 *E. coli* Competent Cells Preparation

E. coli cells were grown in LB for 16 hour at 37°C and OD_{600} was measured in order to calculate the amount of culture required to inoculate in fresh LB. 0.86 mL culture was transferred into 30 mL fresh LB and maintained under agitation at 37°C until the OD_{600} was approximately 0.45 -0.55 (mid-exponential phase). The bacterial culture was centrifuged at 4500 rpm for 5 mins, supernatant was discarded and 10 mL of 0.1 M ice cold $CaCl_2$ was added to the pellet and then homogenized. The homogenous pellet was incubated at 4°C for 30 mins. After incubation, mixture was centrifuged at 4500 rpm for 5 mins, supernatant was discarded while pellet was again homogenized in 2 mL $CaCl_2$ and incubated at 4°C for 3 h. After incubation, competent cells were transformed with ligated pGEMT vector.

3.10.4.2. Transformation of Ligated Plasmids to *E. coli*

200 µL of prepared competent cells were transferred to a sterile tube and about 10 µL of the ligation product was mixed in it. Transformation was carried out by heat shock method by incubating reaction mixture in ice for 30 min, then incubated at 42°C for 120 s, then quickly placed in ice again for 10 min, freshly prepared 200 µL LB broth was added to transformation mixture and allowed to incubate for 120 min at 37°C. 100 µL of transformed culture was spread on LB agar plates containing with 50µg/mL ampicillin and 50 µg/mL of IPTG/X-Gal for screening of blue white colonies. Inoculated plates were allowed to incubate at 37°C for the growth of transformed colonies.

3.10.5. Screening of Transformed/recombinant Colonies

Successful cloning of inserts into the pGEMT-Easy Vector interrupts the coding sequence of β-galactosidase. Recombinant clones can be identified by color screening on indicator plates which is known as blue white screening. Recombinant colonies appeared as white and other appeared as blue. Further confirmation was done by digesting extracted plasmids with *E.coRI* restriction endonucleases.

3.10.5.1. Plasmid Isolation of Transformed Cells

Plasmid isolation of transformed cells was done by using "Promega Pure Yield™ Plasmid Miniprep kit". 600 µl of overnight grown culture of bacteria was taken in 1.5 mL microcentrifuge

and pellet was obtained by centrifugation at 1400 rpm for 30 s. Pellets were re-suspended in 100 µL of Cell Lysis Buffer, and mixed by gently inverting the tubes. Then, 350 µL of Neutralization Solution added, and mixed thoroughly by inverting the tubes so the solution become clear and viscos. Then, cell suspension was centrifuged at 14,000 rpm for 3 min and supernatant was carefully pipette into Mini column. Mini column was centrifuged at maximum speed for 15 s and flow through was discarded, and column was placed on new collection tube. Spin column was washed by using 200 µL of Endotoxin Removal Wash, and column was centrifuged at maximum speed in a microcentrifuge for 15 s. The washing step was repeated by using 400 µl of Column Wash Solution (CWC), again the column was centrifuged and flow through was discarded. After washing column was transferred to a clean 1.5 mL microcentrifuge tube, and plasmid DNA was eluted from column by adding 30 µL of elution buffer. Eluted plasmid DNA was stored at –20°C.

3.10.5.2. Confirmation of Cloning

The extracted plasmid was digested with specific restriction endonucleases for confirmation of cloning. The restriction mixture contained: 250 ng of plasmid, restriction buffer (10X) diluted to 1X by nanopure water and 5.0 U of *Eco*RI (Fermentas UAB Lithuania). All the reagents were mixed and allowed to incubate at 37ºC for 1 h and visualized on gel electrophoresis to check cloned plasmid. After the confirmation of successful cloning, recombinant plasmids were designated as pGEMT-Lac-SL7 and pGEMT-DyP-BL5, and sent for sequencing.

3.10.6. Cloning of pGEMT-Lac-SL7 and pGEMT-DyP-BL5 into pET28a (+) Vector

3.10.6.1. Digestion of Plasmids with Specific Enzymes

Sequential digestion of pET28a (+) vector, pGEMT-Lac-SL7 and pGEMT-DyP-BL5 was carried out with specific restriction endonucleases, using the most suitable buffer for restriction endonucleases. The maximum quantity of DNA in the digestion reactions was 500 ng for plasmid vectors. Reaction mixture was digested at 37ºC for 2 h, and then digestion was verified by gel electrophoresis.

3.10.6.2. Digestion of Plasmids with *Nco1*

In order to create adhesive ends both expression plasmid pET28a (+) and recombinant pGEMT plasmids were digested with *NcoI.* Reaction mixture contained as: *NcoI* (5 U/µL), 1 µL: Buffer E,

5 µL: Plasmid, 25 µL. After incubation the plasmids digestion were confirmed by gel electrophoresis.

3.10.6.3. Inactivation of *NcoI*

After first digestion *NcoI* was inactivated by heat, and inactivation of enzyme was achieved at 80 ºC with incubation time of 20 min.

3.10.6.4. Digestion of Plasmids with *HindIII*

The *NcoI* digested pET28a (+) vector, pGEMT-Lac-SL7 and pGEMT-DyP-BL5 were digested with *HindIII* restriction enzyme. Reaction mixture was as follows: *HindIII* (5 U/µL), 1 µL; Buffer B, 5 µL; Plasmid, 25 µL, and incubated at 37ºC for 2 h.

3.10.6.5. QIA quick Gel Extraction Kit Protocol (QIAGEN)

Digested pET28a (+) vector, pGEMT-Lac-SL7 and pGEMT-DyP-BL5 were purified using commercially available Gel Extraction Kit (QIAGEN), and visualized through gel electrophoresis.

3.10.6.6. Ligation of Digested Plasmids

The digested laccase and DyP-type peroxidase gene inserts were ligated directly into pET28a plasmid as given in instructions. Two separate reactions were performed for both laccase and DyP-type peroxidase. The ligation mixture composition for laccase enzyme was:

- Ligase Buffer 5 µL
- T4 DNA Ligase 1 µL
- Laccase gene insert 3 µL
- pET28a (+) plasmid 1 µL

The ligation mixture composition for DyP-type peroxidase enzyme was:

- Ligase Buffer 5 µL
- T4 DNA Ligase 1 µL
- DyP-type peroxidase gene insert 3 µL
- pET28a (+) plasmid 1 µL

The ligation reaction was incubated at 16°C for 16 h and digestion was confirmed by gel electrophoresis.

3.10.6.7. Transformation of Ligated Plasmid in *E. coli* BL21 Cells

E. coli BL21 competent cell ($CaCl_2$-treated) suspensions of 200 µL was transferred to 1.5 mL Eppendorf® tube and mixed with 10 µL ligated plasmid. The reaction mixture was allowed to incubate for 30 min on ice, then transferred to 42°C for 2 min without mixing, and then again transferred to ice. Then, incubated at 37°C for 2 hrs for the expression of antibiotic gene marker. 100 µL of transformed culture was spread on LB agar supplemented with kanamycin (50 µg/mL). Cloning was confirmed by digesting the transformed plasmids by respective restriction endonucleases.

3.10.6.8. Verification of Cloning

Plasmids were isolated from transformed colonies using Thermo Scientific GeneJET Plasmid Miniprep Kit. Isolated plasmids were double digested with *NcoI* and *HindIII* enzymes. The digestion reaction was containing respective restriction buffer (10X), 8 uL; *NcoI* and *HindIII* (5 U/µL), 2 µL; plasmid 50 µL and nanopure water, 9 µL. All the reagents were mixed and allowed to incubate for 2 h at 37°C. Restriction digestion was confirmed by 1% agarose gel electrophoresis. Recombinant plasmids were named as pET28-Lac-SL7 and pET28-DyP-BL5.

3.10.6.9. Sequencing of Cloned Gene

Recombinant plasmids pET28-Lac-SL7 and pET28-DyP-BL5 were sent for sequencing to Applied Biosystems (Macrogen, Netherlands).

3.10.6.10. Bioinformatics Analysis

NCBI programs BLASTp and BLASTn were used for the analysis of deduced amino acids and their nucleotides (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. CLUSTALW program was used for multiple sequence alignment of laccase and lignin degrading DyP-type peroxidase by using online tool (http://www.ebi.ac.uk/clustalW) and MEGA 6.0 software was used for construction of phylogenetic trees (with minimum evolution).

3.11. Expression of Recombinant Laccase and DyP- type Peroxidase

After confirmation of DNA sequence of recombinant plasmids, laccase which was amplified from the genomic DNA of SL7 was named as LacSL7 and lignin degrading DyP-type peroxidase was amplified from genomic DNA of strain BL5, named as DyPBL5. Both enzymes were expressed in *E.coli* BL21 cells under T7 RNA polymerase. Inoculum of recombinant strains was prepared by inoculating the strains in LB broth supplemented with kanamycin antibiotic (50 µg/mL) at 37ºC for 16 hours (overnight). 100 µL freshly prepared sterile LB medium containing same antibiotic was taken in Erlenmeyer flask and inoculated with appropriate amount of inoculum, and incubated at 37ºC. When the culture reached an OD_{600} nm of 0.5, roughly 3 h later, protein expression was induced using IPTG at 1 mM concentrations and 0.1 mg/mL of hemin for DyPBL5 and 1mM $CUSO_4$ for LacSL7 and incubated at 20 °C for 16 h.

3.12. Purification of LacSL7 and DyPBL5

3.12.1. Ion- exchange Chromatography

The ion exchange chromatography works on the principle of components separation on the basic of their charges and for interaction with ion exchange resin under specific conditions. LacSL7 and DyPBL5 were purified through Q-Sepharose Fast Flow column.

3.12.2. Q-Sepharose Chromatography

LacSL7 and DyPBL5 were purified through anionic resin Q-Sepharose Fast Flow column (50 × 1.5 cm). Resion was pre swollen in ethanol (20%). Column (1.5 × 50 cm) was properly packed with prepared gel slurry. For DyPBL5, Q-Sepharose column was pre-equilibrated with 30mM glycine hydroxide buffer pH 9 and for LacSL7, column was pre-equilibrated with phosphate buffer pH 8, and washed at flow rate of 0.5 mL/min using same buffer and then enzyme solution was flow through the column, and elution was achieved by linear gradient of (0.5 M) NaCl in glycine hydroxide buffer for DyPBL5 and phosphate buffer for LacSL7. 50 fractions (3 mL) were collected. Each fraction was analyzed for enzymes activity and protein estimation. The active fractions were polled and stored at –20°C. Purity of proteins was confirmed by SDS-PAGE analysis, as described in previous section.

3.13. Enzyme Activity Assays and Characterization

3.13.1. Determination of LacSL7 Activity

The LacSL7 activity was measured according to the methods reported by Niku-Paavola et al. (1988), using ABTS, [2, 2-azino-bis-(3-ethylbenzothiazoline)-6-sulphonate], as a substrate. The reaction mixture (1mL) contained; 1mM ABTS, 50 mM citrate buffer (pH 5.0), 0.5 µg enzyme, and allowed to incubate at 55°C for 10 min. A control was setup in separate with no enzyme in the reaction mixture.

3.13.2. Determination of DyPBL5 Activity

The DyPBL5 activity was measured against ABTS as described earlier by Santos et al. (2014). The activity mixture (1mL) contained; 1 mM ABTS, 50 mM citrate buffer (pH 4.0), 0.1 mM H_2O_2, 0.75 µg enzyme, and incubated at 30°C for 20 min. A control was setup in separate vial with no enzyme in the reaction mixture. The ABTS cations released, were measured through spectrophotometer at 420 nm. The amount of DyPBL5 required to catalyze 1 µmol of ABTS per min under standard assay conditions was referred to as one unit of enzyme activity.

3.13.3. Protein Estimation

The protein contents present in enzymes fractions was measured by Bradford (1976) method using Bovine serum albumin as a standard. The Bradford reagents used for estimation of protein are stated in Appendix 16. Concentration of proteins were estimated on the basis of color change from brown to blue, color change is caused due to binding of protein molecules Coomassie dye at low pH.

Procedure

90 µL of 0.15 M NaCl solution was taken in test tube and 10 µL of protein sample was added in solution, while was run with 100 µL of 0.15 M NaCl without protein sample. Then, 3 mL Bradford reagent dye was pipette in to reaction tubes and incubated at 25°C for 15 min, and absorbance was taken using spectrophotometer at 595nm.

13.3.4. Protein Standard Curve

Protein standard curve was plotted using bovine serum albumin (BSA). Stock solution of bovin serum albumin was prepared in dH_2O by dissolving the BSA (1 mg/1mL) and then was serially diluted to 10 dilutions. Protein contents in each dilution was determined by Bradford (1976) method and absorbance was taken spectrophotometrically at 595 nm. Then, Microsoft office™ Excel program was used to draw a graph in which optical density of each dilution was plotted against their relative concentrations. Following formula was obtained from standard curve and used to estimate the protein content in unknown samples:

$x = (y + 0.061)/0.092$

Here, the concentration of proteins (mg/mL) in unknown samples is represented as X, and the optical density of unknown sample at 595nm is represented as Y.

3.14. Characterization of Recombinant LacSL7 and DyPBL5

The recombinant purified enzymes were characterized for different parameters:

3.14.1. Effect of Temperature on Activity and Stability of LacSL7 and DyPBL5

The effect of temperature on purified recombinant LacSL7 and DyPBL5 were measured by calculating the relative activity at different temperatures (25-90°C). The maximum enzyme activity was represented as 100% and used as a control to measure the relative activity at different temperatures. Temperature stability of the LacSL7 and DyPBL5 were measured by incubating the enzymes at 25-60°C till the 50% decrease in activity was observed. All samples were processed in triplicates.

3.14.2. Effect of pH on Activity and Stability of LacSL7 and DyPBL5

The relative activity of recombinant LacSL7 and DyPBL5 were determined over a wide range of pH 3.0- 10.0. 0.1 M Sodium citrate buffer with pH 3.0-6.0, 0.1 M Sodium Phosphate buffer with pH 7.0-8.0 and 0.1 M Glycine NaOH buffer with 9.0-10.0, were used in present study.

Recombinant LacSL7 and DyPBL5 were incubated at pH 3.0-10.0 for determination of stability. Residual activity was measured till the 50% decrease in activity was observed.

3.14.3. Effect of Metal Ions on Activity of LacSL7 and DyPBL5

Effect of metal ions such as $CoCl_2$, $FeSO_4$, $MnSO_4$, $CaCl_2$, $MgSO_4$, $NiCl_2$, CuSO4, $ZnSO_4$, KCl, NaCl and $HgCl_2$, on the activity of recombinant LacSL7 and DyPBL5 was determined. Metal effect was determined under varying concentration like 0.5 mM, 1 mM, 2 mM and 5 mM at optimized temperature and pH.

3.14.4. Effect of Inhibitors on Activity of LacSL7 and DyPBL5

Effect of inhibitors such as phenyl methyl sulphonyl fluoride (PMSF), dithiothreitol (DTT), diethyl pyrocarbonate (DEPC), ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol on activity of purified recombinant LacSL7 and DyPBL5 was assayed. Enzymes were incubated at different concentrations of inhibitors (1 mM, 5 mM and 10 mM). The enzymes activity was analyzed and residual activity was calculated till the 50% reduction in activity was observed. All samples were processed in triplicates.

3.14.5. Effect of Organic Solvents on Activity of LacSL7 and DyPBL5

Organic solvents such as isopropanol, methanol, ethanol, butanol, DMSO and dioxane were used to check the stability of recombinant purified LacSL7 and DyPBL5, under the standard conditions.

3.14.6. Effect of Detergents on Activity of LacSL7 and DyPBL5

Effect of detergent such as SDS, Triton-X100 and Tween 20 on activity of purified recombinant LacSL7 and DyPBL5 were determined. The enzymes were incubated with 1% and 5% concentration of detergents at optimized pH and temperature. Residual activity was measured till the 50% decrease in activity was observed.

3.14.7. Determination of Kinetic Parameters of LacSL7 and DyPBL5

K_m shows the substrate concentration at which enzyme 50% of its maximum velocity (*Vmax*). The Michaelis-Menten plot was constructed to estimate the Michaelis–Menten constant (*Km*), maximum velocity (*Vmax*) and catalytic constant (*Kcat*).

Protocol

The kinetic parameters of LacSL7 and DyPBL5 were determined by measuring the oxidation of ABTS at different concentrations and with fixed concentration of enzyme. For calculation of *Km*

and *Vmax,* Michaelis-Menten graph was plotted by using the reciprocal of V against the reciprocal of substrate concentration (S).

3.15. Alkali Lignin Degradation through Recombinant LacSL7 and DyPBL5

3.15.1. Enzymatic Hydrolysis of Alkali Lignin

The enzymes, LacSL7 and DyPBL5 were used separately and in combination to degrade alkali lignin. An alkali lignin solution (2 g/L) was prepared and adjusted to pH 5. All the experiments were carried out in Erlenmeyer flasks (1000 mL) containing 500 mL of degradation medium.

The degradation medium for LacSL7 contained:

Alkali lignin:	2g
50 mM Citrate buffer pH 5.0:	500µL
LacSL7 :	5 µg
0.2 mM ABTS:	50µL

The degradation medium for DyPBL5 contained:

Alkali lignin:	2g
50 mM Citrate buffer pH 5.0:	500µL
LacSL7 :	5µg
0.2 mM ABTS:	50µL
0.1mM H_2O_2:	50µL

The degradation medium for combined reaction contained:

Alkali lignin:	2g
50 mM Citrate buffer pH 5.0:	500µL
LacSL7 :	5µg
DyPBL5:	5µg
0.2 mM ABTS:	50µL
0.1 mM H_2O_2:	50µL

For LacSL7 and DyPBL5 degradation medium was incubated at 55°C and 35°C, respectively, for 20 h in shaking incubator at 100 rpm and analysed for lignin degradation at regular intervals. The degradation potential was measured by reduction in lignin contents, structural and compositional changes by FTIR and SEM, and detection of degradation products by GC-MS. All the experiments were carried in triplicates and negative control was setup in seprarate flasks without any enzyme**.**

3.15.2. Determination of Alkali Lignin Contents

The residual lignin content was measured by the method as previously reported by Chandra et al. 2007. The pH of the supernatant was adjusted to 7.6 by phosphate buffer and its absorbance was measured at 280 nm by UV-visible spectrophotometer. Lignin standard curve was constructed using various concentrations of lignin (100 to 3000 mg/L) in order to calculate the lignin concentration. The reduction in lignin contents was calculated by the following equation:

Lignin contents (%) = (C1 − C2)/C1 × 100

where C1 is the lignin concentration in control and C2 is the lignin concentration in treated sample.

3.15.3. Determination of Structural Modification by SEM and FTIR

3.15.3.1. SEM

Scanning electron microscopy (ZEISS EVO LS 10) was performed to analyze changes in the surface of lignin during degradation. Both the treated and untreated samples were vacuum dried

into fine powder. The samples were prepared by mounting on carbon tape, and gold plating was done to enhance its conductivity for viewing and analysis was carried out at x650 resolution power.

3.15.3.2. FT-IR

FT-IR was performed to analyze changes in the functional group of heteropolymeric lignin during enzymatic hydrolysis. 2 mg of vacuum dried sample was mixed with 200 mg of potassium bromide (KBr), mixture was homogenized and compressed under continuous pressure of 40 MPa to form thin pellets. The samples were analyzed by FT-IR spectra (Perkin Elmer Spectrum One FT-IR, Waltham, USA) within the range of 4000 to 400 cm^{-1} and time for analysis of each sample was set at 60 s.

3.15.2.4. Determination of Lignin Degradation Products by GC-MS

Supernatant was taken from 50 mL of sample by centrifugation (12,000 rpm) for 15 min. supernatant was acidified to pH 1.0-2.0 by using 6 M HCL and acidified supernatant was thoroughly extracted with three-volume ethyl acetate. The organic layer was formed on the top of the aquous medium and collected by seprarting funnel, and organic layer was dehydrated by using anhydrous Na_2SO_4, then rotary vacuum evaporator was used to dry the residues. The dried residues were derivatized by adding dioxane (100 µL), pyridine (10 µL), trimethylchlorosilane (10 µL), and followed by 50 µL of N-methyl-N-trimethylsilyl fluoroacetamide. The derivatized sample was allowed to heat at 60ºC for 15 min and residues were dissolved by continuous shaking. 3µL of derivatized sample was injected to the GC-MS injector port, which was equipped with DB-FFAP capillary column (30 m × 0.25 µm × 0.25 mm) (Agilent Technologies, Wilmington, DE, USA). The column temperature was set to 120-280ºC (10ºC per min rise) with flow rate of 1.5 mL per min. A solvent removal time was set to 3.0 min and mass spectra in the range of 50–750 (m/z). The detection of lignin degradation products was accomplished by comparing the RT and mass spectra of products in test sample with available mass spectra in the National Institute of Standards and Technology (NIST) library.

4. RESULTS

4.1. Physiochemical Analysis of Effluent

Sludge and effluent samples were collected from effluent treatment plant (ETP) of Century Paper and Board Mills Limited, District Kasur, Punjab Pakistan, and labelled as; Sludge (SL), Black liquor (BL), Inlet point (IP), Aeration tank 1 (AT_1), Aeration tank 2 (AT_2), Secondary sedimentation tank outlet (ST), and Final sedimentation tank outlet (FS). Collected samples were processed for physico-chemical analysis and isolation of lignin degrading bacteria. The physico-chemical analysis showed that effluent generated from pulp and paper mill was slightly alkaline in nature with high concentration of pollutants as indicatd by high values of chemical oxygen demand (COD), sulphates, total suspended solids (TSS), nitrates, total dissolved solids (TDS), color and lignin. The effluent was treated through aerated lagoon at industrial site that reduced the pollutant concentration but still it remained beyond the permissible limits as recommended by Environmental Protection Agency (EPA) (USEPA, 2002), results are stated in Table 4. 1.

Table 4. 1 Physico-chemical analysis of effluent from ETP

Parameters	Inlet point	Aeration tank 1	Aeration tank 2	Secondary sedimentation tank	Final sedimentation tank	Permissible limit
pH	8.0	7.3	7.3	7.4	7.6	5-9
COD	994	997	962	650	407	120
Sulphate	440	540	670	620	740	252
TDS	440	439	440	426	408	_
TSS	600	700	700	400	200	_
Color	1954	2636	2272	2136	1909	_
Nitrates	144	200	188	224	64	10
Lignin	7416	7958	5916	7790	279	0.05

*(–): not specified. All the values are presented in mg/mL except color which is in CU.

4.2. Isolation of Bacteria from Effluent

The total of 55 bacterial strains with distinct culture characteristics were isolated on nutrient agar plates, from effluent samples (Table. 4.2).

Table 4. 2 Bacterial isolates from pulp and paper mill effluent.

No.	Name of Sample	Sample code	Number of Isolates
1	Black Liquor	BL	5
2	Inlet Point	IP	5
3	Aeration tank 1	AT1	13
4	Aeration tank 2	AT2	10
5	Secondary Sedimentation Tank	SST	7
6	Final Sedimentation Tank	FST	7
7	Sludge (Aeration tank)	SL	8
	Total isolates		55

4.3. Screening of Bacteria for Lignin Tolerance Limit

All bacterial isolates were subjected to minimal salt medium supplied with lignin (L-MSM) to evaluate their potential to use lignin as a sole carbon source. These strains were grown in different concentrations of lignin, ranging from 500-3000 mg/L. Eight isolates showed good growth in the presence of highest tested concentration of lignin (3000 mg/L) in comparison to other bacteria. The growth pattern of all bacteria in the presence of various lignin concentration is presented in Table 4.3.

4.4. Screening for Lignin Degrading Enzymes

All these isolates were screened for laccase and lignin peroxidase activity on nutrient agar plates supplemented with specific substrate in each case. Laccase activity was determined on guaiacol agar plates, and indicated by appearance of brown color around bacterial growth. Three bacterial isolates, designated as SL7, BL5 and BL2 were found to produce laccase**.** While lignin peroxidase activity was determined on azure-B agar plates. Azure-B is one of the most commonly used substrates for screening of lignin peroxidase as the action of enzyme on azure B leads to a visible change in color of the medium around colonies. Four bacterial isolates, designated as SL4, BL5, BL2 and AT4 showed the production of lignin peroxidase enzyme.

Table 4. 3 Bacterial growth in the presence of various concentrations of lignin

Sample	Concentration of Lignin (mg/L)					
	500	1000	1500	2000	2500	3000
BL	4	4	4	4	3	3
IP	3	3	3	3	1	1
AT_1	5	5	4	4	4	1
AT_2	6	5	5	5	4	1
SST	1	1	1	1	0	_
FST	4	4	4	4	3	_
SL	5	3	3	3	4	2
Total No. of isolates	28	25	24	24	18	8

*(–): No growth

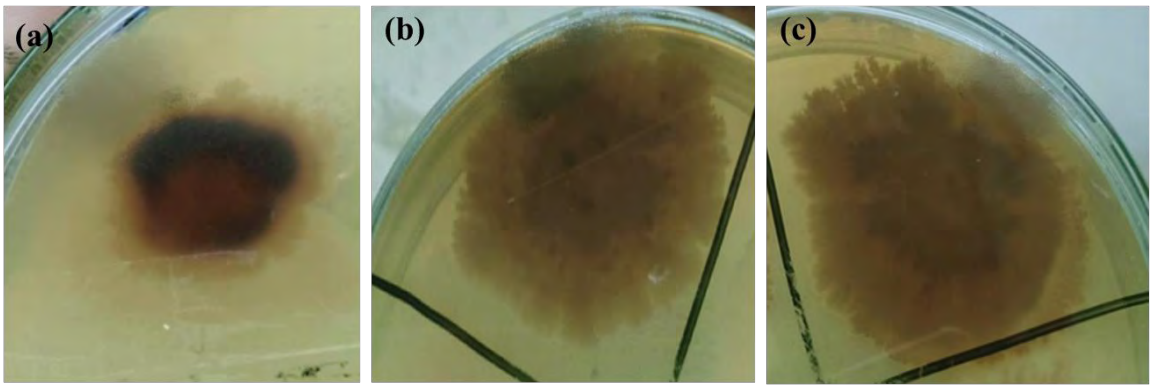


Figure 4. 1 Appearance of brown color around bacteria growth on guaiacol agar plates after 7 days of incubation. (a) SL7 (b) BL5 (c) BL2

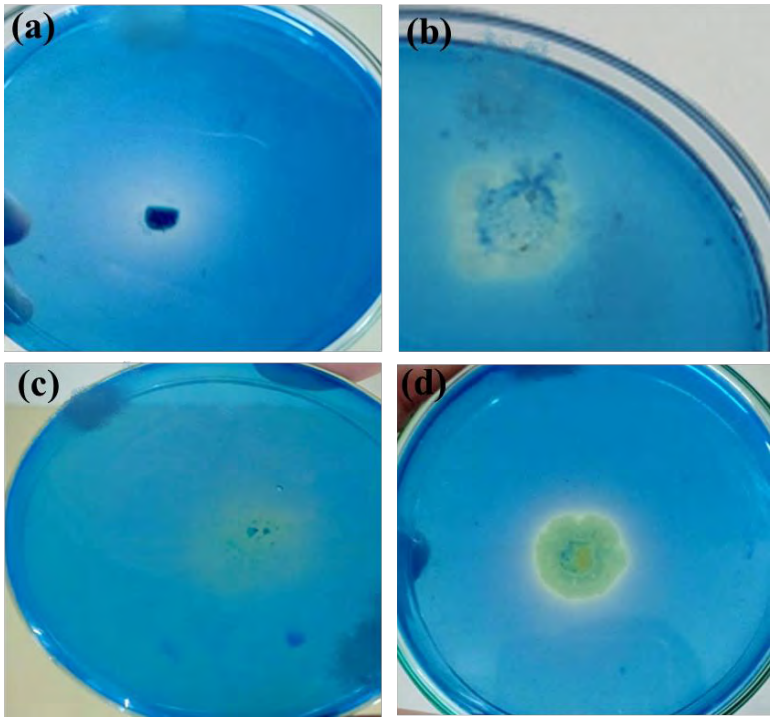


Figure 4. 2 Appearance of clear zone around bacterial growth on azure-B agar plates after 7 days of incubation. **(a)** SL4 **(b)** BL5 **(c)** IP4 **(d)** AT4 **(e)** BL3

4.5. Biodegradation of Alkali Lignin

Eight bacterial strains, designated as AT4, AT_24, IP4, SL7, SL4, BL2, BL3 and BL5, were selected based upon their growth on L-MSM plates and their ability to produce ligninolytic enzymes, and used to check their efficiency towards lignin degradation. The degradation efficiency was checked by using MSM with 3000 mg/L of lignin. The experiment was run for 7 days along with a control in separate flask and samples were withdrawn at regular intervals in order to evaluate reduction in lignin, color and enzymes activity. The degradation was further confirmed by Scanning Electron Microscopy. Among eight bacterial strains, only strain BL5 and SL7 efficiently degraded lignin with 35-44% reduction in lignin and 22-26% reduction in color within 7 days of incubation as shown in Figure 4.3 and 4.4, respectively. During the course of degradation, ligninolytic activity was measured from degradation medium. Both the strains SL7 and BL5 showed maximum laccase and lignin peroxidase production with activity around (1.3 IU/mL) and (1.19 IU/ml) after 5 days of incubation, respectively (Fig. 4.5 & 4.6). Based on lignin degradation efficiency and ligninolytic activity, strains BL5 and SL7 were selected for further study.

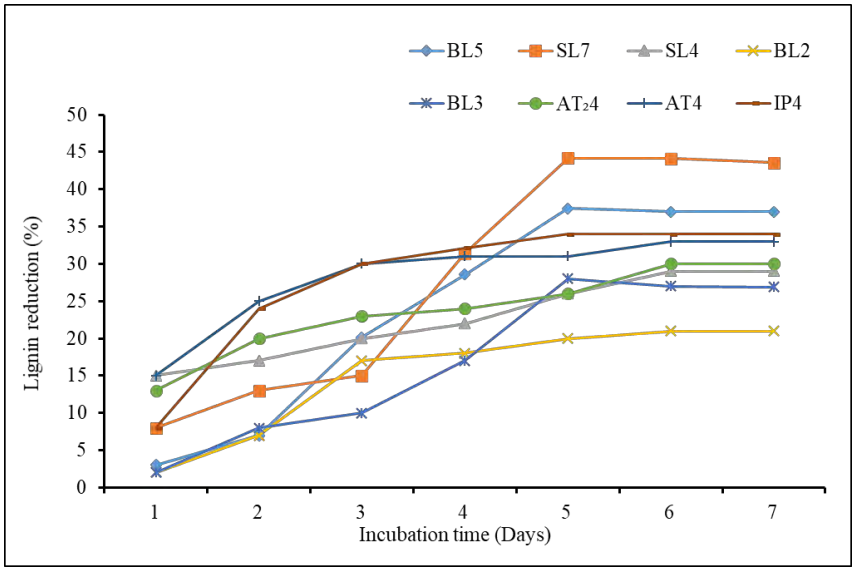


Figure 4. 3 Lignin reduction by strain BL5, SL7, SL4, BL2, BL3, AT_24, AT4 and IP4, in AL-MSM with 3 g/L lignin concentration over 7 days of incubation at 37^0C.

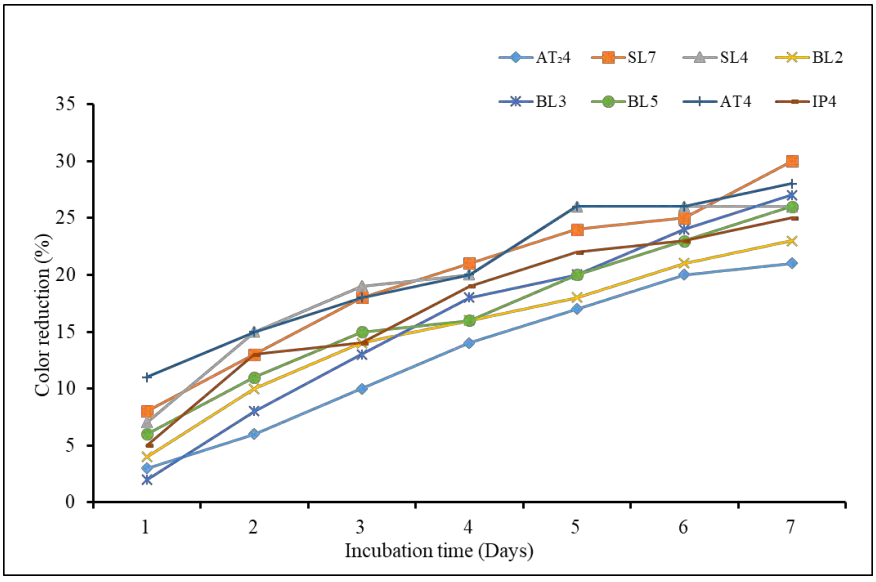


Figure 4. 4 Color reduction by strain AT_24, SL7, SL4, BL2, BL3, BL5, AT4 and IP4, in AL-MSM with 3 g/L lignin concentration over 7 days of incubation at 37^0C.

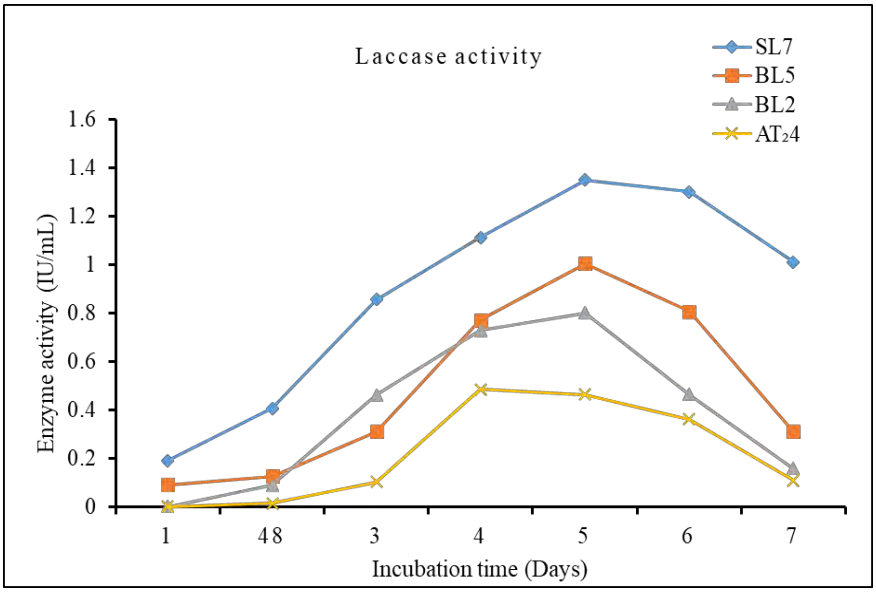


Figure 4. 5 Laccase activity by strain SL7, BL5, BL2, and AT$_2$4 in lignin degradation medium over 7 days of incubation

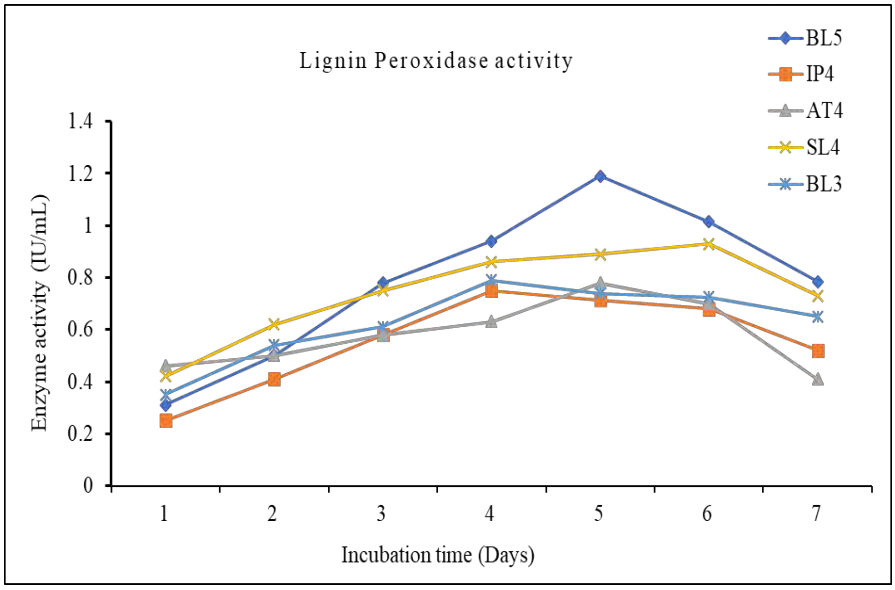


Figure 4. 6 Lignin peroxidase activity by strain BL5, IP4, AT4, SL4 and BL3 in lignin degradation medium over 7 days of incubation.

4.5.1. Confirmatory Analysis of Lignin Degradation by SEM

The changes in surface morphology of alkali lignin as a result of treatment using strain SL7 and BL5 were investigated by Scanning Electron Microscopy. Surface of treated lignin samples were

completely eroded in comparison to un-treated lignin samples, as shown in Figure 4.7. It clearly indicates the degradation of lignin by strain SL7 and BL5.

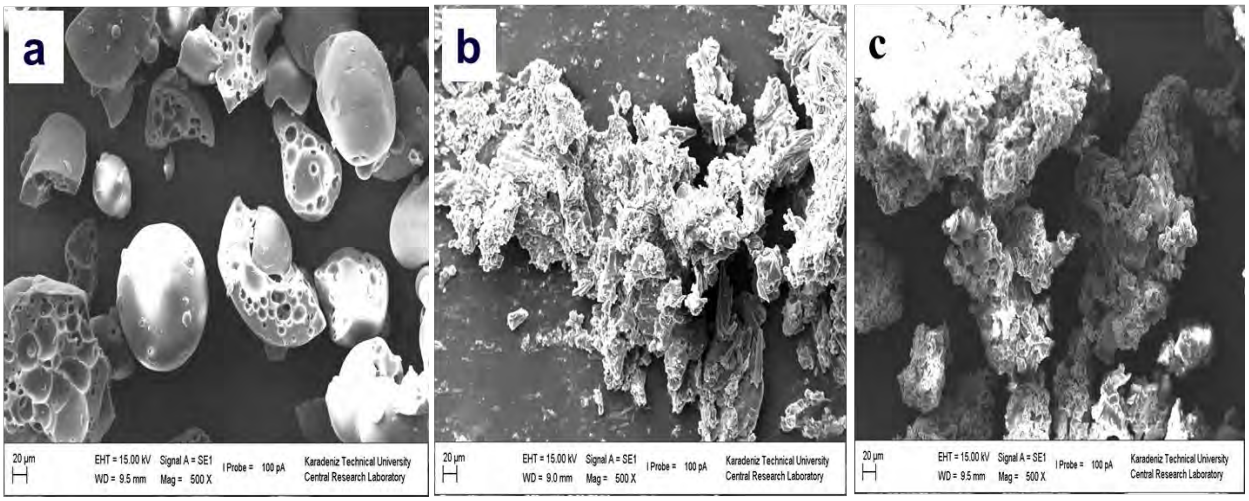


Figure 4. 7 SEM analysis carried out at resolution 500X for lignin treated with strain SL7 and BL5 (a) Un-treated lignin (b) SL7 (c) BL5

4.6. Identification of Selected Strains

4.6.1. Morphology

Strains SL7 and BL5 were grown on nutrient agar and its culture characteristics were observed after 24 h. Both strains were observed to have medium size, off-white, slightly raised centered and mucoid colonies (Fig. 4.8).

4.6.2. Microscopy

The cellular morphology of bacterial strains SL7 and BL5 was observed under microscope through Gram's staining. The microscopic analysis revealed that both strains were Gram positive short rods in shape (Fig. 4.9).

4.6.3. Biochemical Tests

Both the strains SL7 and BL5 were positive for catalase, oxidase, motility, citrate and VP while negative for indole, methyl red and oxidase, indole and H_2S production, as shown in Table 4.4.

4.6.4. Molecular Identification

The phylogenetic analysis of strain SL7 indicated its relationship to genus *Bacillus* showing 100 % similarity with *Bacillus altitudinis* strain SGAir0031 (CP022319); thus, strain SL7 was identified as *Bacillus altitudinis*. The 16S rRNA sequence of strain SL7 reported here can be obtained from the NCBI nucleotide sequence database under accession number MZ400969. While strain BL5 showed 99% similarity with *Bacillus* sp. and 16S rRNA sequence has submitted to NCBI database under accession number MZ413436. For an exact identification of strain BL5, further analyses and hybridization studies are required. Phylogenetic trees of the obtained sequence of strains SL7 and BL5 were constructed with help of NCBI BLAST-N and Mega-X software, shown in Figure 4.10 & 4.11.

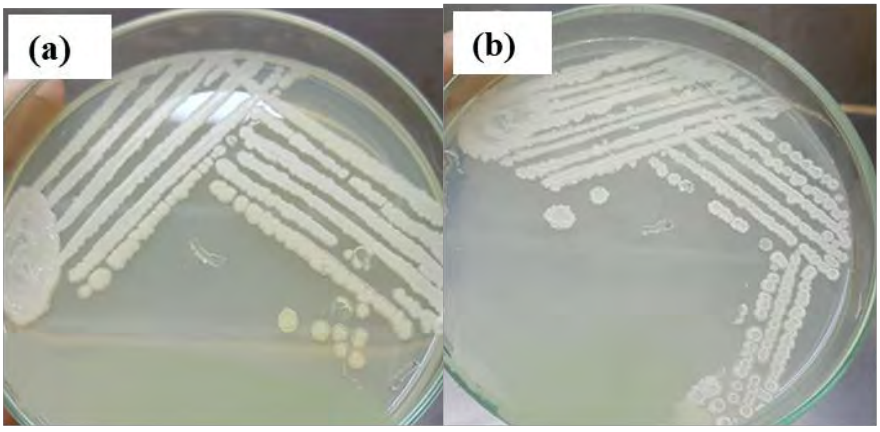


Figure 4. 8 Bacterial isolates SL7 and BL5 on nutrient agar plates (a) SL7, (b) BL5

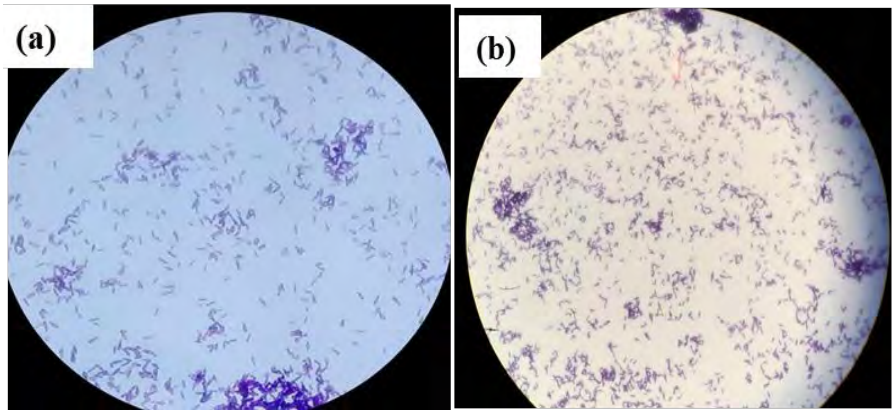


Figure 4. 9 Microscopic examination of strains SL7 and BL5 with Gram staining under 100X (a) SL7, (b) BL5

Table 4. 4 Biochemical test profile of strains SL7 and BL5

Biochemical Tests	**Results**	
	BL5	**SL7**
Catalase test	+	+
Oxidase test	+	+
Citrate utilization	+	+
Motility	+	+
V/P	+	+
Indole	-	-
Methyl red	-	-
H_2S production	-	-

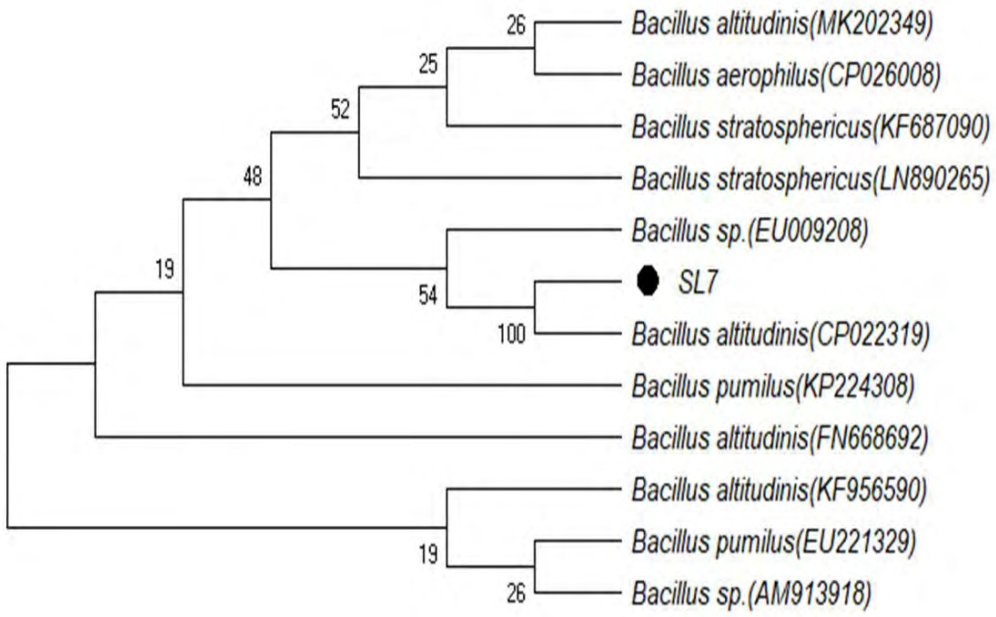


Figure 4. 10 Phylogenetic tree of strain SL7 by Neighbor-Joining method constructed using MEGA-X software.

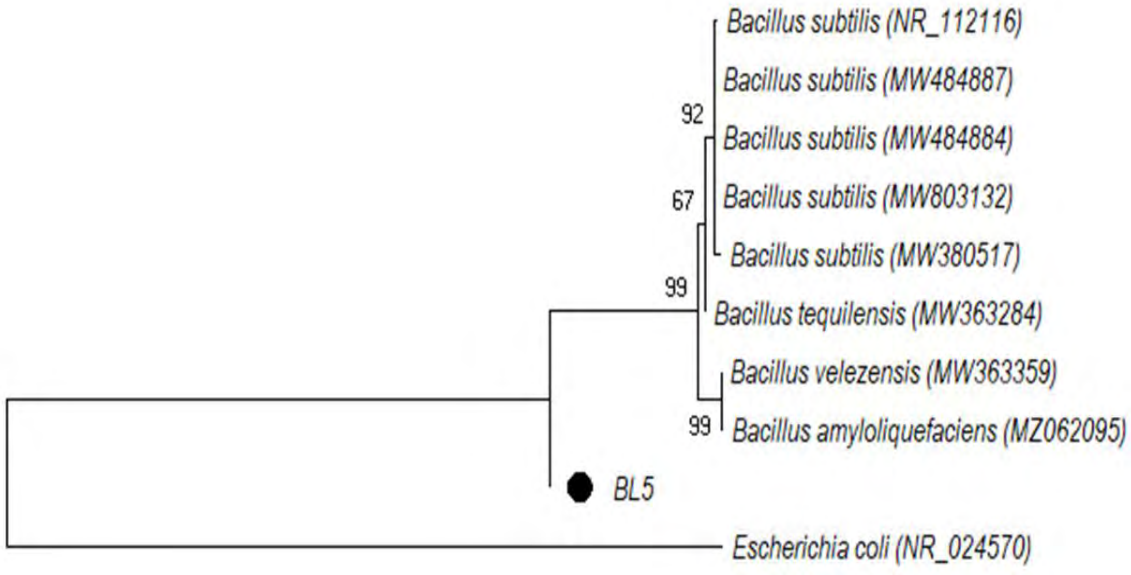


Figure 4. 11 Phylogenetic tree of strain BL5 by Neighbor-Joining method constructed using MEGA-X software.

4.7. Laccase and Lignin Degrading Peroxidase Production

Culture conditions were optimization for the production of laccase (Lac) from strain SL7 and lignin degrading peroxidase (LDP) from strain BL5 in lignin amended minimal salt medium.

4.8. Optimization of Physico-chemical Parameters for Production of Lac and LDP

4.8.1. Effect of Temperature

Optimum temperature for the production of Lac and LDP from *Bacillus altitudinis* SL7 and *Bacillus* sp. BL5, respectively was determined by incubating the production media at temperature ranges from 30–45°C for 96 h. The samples were withdrawn every 24 h for enzyme assay and protein estimation. The maximum specific activity of Lac and LDP was found at 30°C after 72 h of incubation. While the activity was low at temperature below 30˚C and above 35˚C (Fig 4.12 & 4.13).

4.8.2. Effect of pH

Optimum pH for the production of Lac and LDP from *Bacillus altitudinis* SL7 and *Bacillus* sp. BL5, respectively, was assessed growing at various pH from 5.0–12.0 at 30°C. Maximum specific activity of Lac and LDP was observed at pH 8.0 and 7.0, respectively, after 72 h of incubation. Both acidic and alkaline pH was not favourable for production of enzyme (Fig 4.14 & 4.15).

4.8.3. Effect of Carbon Sources

Various carbon sources were screened for production of Lac and LDP from *Bacillus altitudinis* SL7 and *Bacillus* sp. BL5, respectively. These carbon sources includes: glucose, sucrose, starch, lactose and maltose. Among all carbon sources, starch and glucose were found to be the most suitable source for maximum production of Lac and LDP, respectively (Fig 4.16 & 4.17).

4.8.4. Effect of Nitrogen Sources

For maximum Lac and LDP production, nitrogen sources including both organic and inorganic sources such as: peptone, yeast extract, beef extract, ammonium nitrate, ammonium sulphate and urea were screened. Maximum specific activity of Lac and LDP were observed in the presence of yeast extract and peptone, respectively (Fig 4.18 & 4.19)

4.9. Optimization of Nutritional Factors by Placket-Burman and Central Composite Design

Placket-Burman (PB) design was used for optimization of numerous nutritional factors for production of Lac and LDP enzymes. Total eleven (11) factors were analyzed in fifteen runs, and the factors that affect the process of fermentation have also been specified in coded value by the PB design, as shown in Table (4.5 & 4.6). Response to factors were measured in specific activity. The highest laccase activity (8.26 U/mg) was found in run number 15 and lowest activity (1.32 U/mg) was found in run number 04 (Fig. 4.20). While highest lignin degrading peroxidase activity (6.73 U/mg) was found in run number 03 and lowest activity (0.29 U/mg) was found in run number 15 (Fig 4.21). Moreover, the significant factors towards Lac and LDP activity were determined with the help of Pareto chart, four factors showed significant impact towards laccase activity, these include; $(NH_4)_2SO_4$, $MgSO_4$.7H2O, $FeSO_4.7H_2O$ and KH_2PO_4, and five factors showed significant impact towards LDP activity, these include; inoculum size, lignin, peptone, glucose and $(NH_4)_2$NO3, results are shown in Fig 4.22 & 4.23. For further optimization studies two-level central composite design (CCD) was used on the significant factors that achieved from PB Design, that have positive effect on Lac and LDP production. Total four factors for Lac and five factors for LDP production were analyzed in CCD, details are present in Table 4.7 & 4.8. Response to factors was measured in specific activity; results are shown in Fig. 4.24 & 4.25.

4.10. Purification of Lac and LDP

4.10.1. Precipitation with Acetone

Lac and LDP from *Bacillus altitudinis* SL7 and *Bacillus* sp. BL5, respectively, were partially purified by using acetone in various concentrations (40-80%). Maximum precipitation of Lac and LDP was found at 60% concentration of acetone, while 10, 20, 30, 40, and 50% concentration was not enough to get maximum precipitation of enzymes.

4.10.2. Size Exclusion Chromatography

Partially purified enzymes were further purified by size exclusion column chromatography using Sephadex (G-100) gel resin eluted by 50 mM citrate buffer. 30 fractions were collected about 3 ml of each, collected fractions were assayed for Lac and LDP. The highest activity was determined in fractions 10 to 17 for both enzymes. All these fractions were pooled up to concentrate the Lac and LDP for maximum activity.

4.10.3. Total yield of Purified Lac and LDP

The total yield and purification folds for precipitated and gel filtered Lac and LDP from strain SL7 and BL5, respectively were calculated. Total yield of Lac was 6.18% and purification fold was 57.6, and total yield for LDP was 5.94% and purification fold was 38.04. The purification folds and yield are given in Table 4.9.

4.10.4. Molecular Weight Determination (SDS-PAGE)

The purity and size of Lac and LDP were analyzed by SDS-PAGE with 12% acrylamide gel. The Lac and LDP were purified to homogenicity level and only single band for both enzymes was visualized in SDS-PAGE. Standard protein marker was used to determine the molecular weight of Lac and LDP, and it was found to be approximately 55 and 45 kDa, respectively (Fig. 4.26).

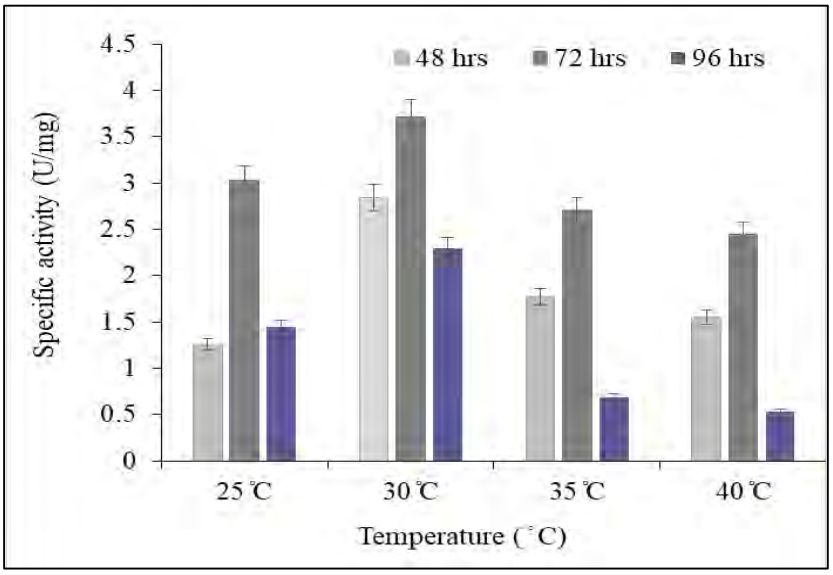


Figure 4. 12 Effect of different temperatures on production of laccase from *Bacillus altitudinis* strain SL7 within 48 hours to 96 hours of incubation. Highest specific activity (3.7 U/mg) was found at 30℃ after 72 hours of incubation. The line on each bar represents the standard deviation (n=3).

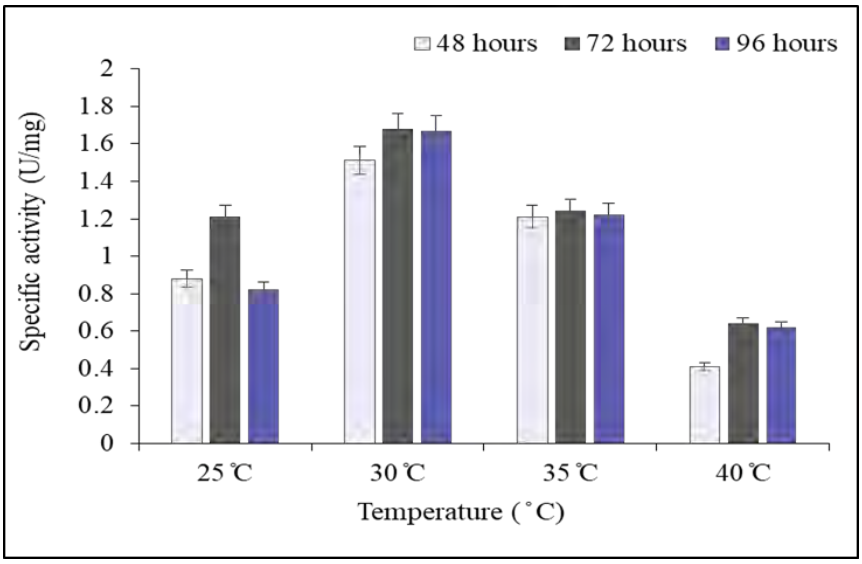


Figure 4. 13 Effect of different temperatures on production of LDP from *Bacillus* sp. strain BL5 within 48-96 hours of incubation. Highest specific activity (1.68 U/mg) was found at 30℃ after 72 hours of incubation. The line on each bar is the standard deviation (n=3).

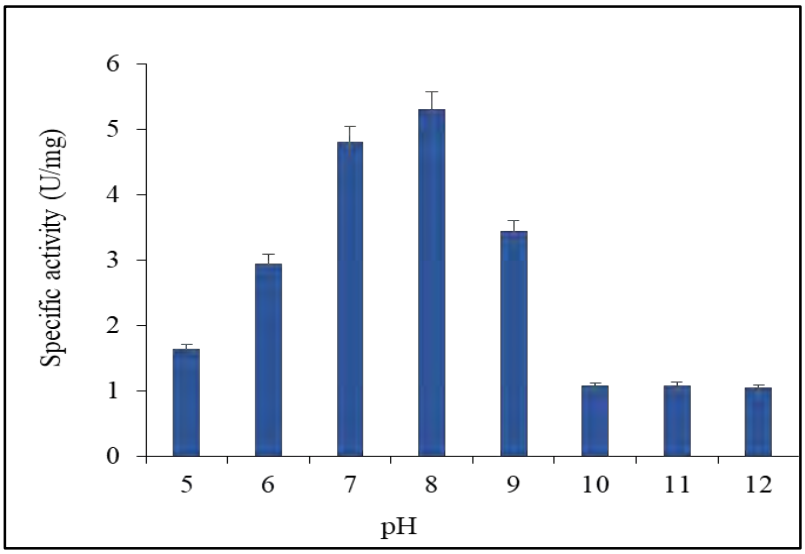


Figure 4. 14 Effect of pH on production of laccase from *Bacillus altitudinis* strain SL7 after 72 hours of incubation. Highest enzyme activity (5.2 U/mg) was found at pH 8. The line on each bar is the standard deviation (n=3).

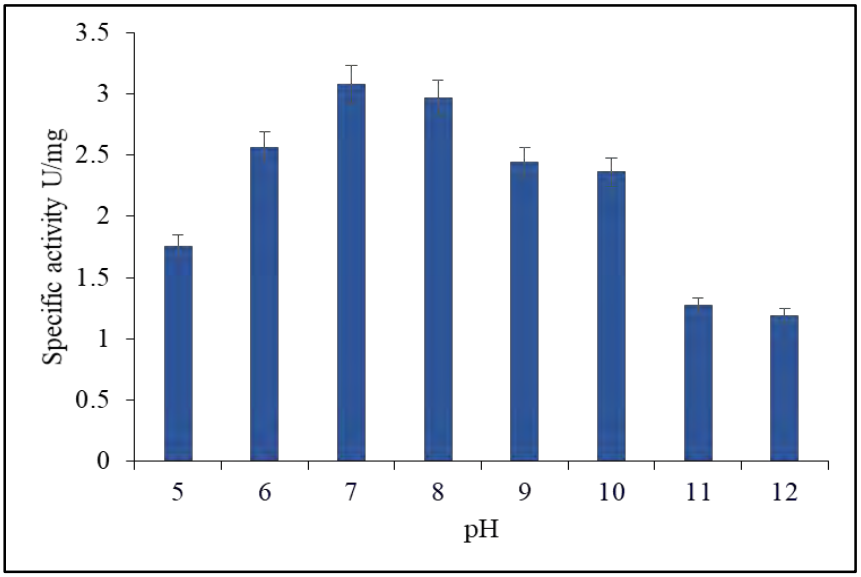


Figure 4. 15 Effect of pH on production of LDP from *Bacillus* sp. strain BL5 after 72 hours of incubation. Highest enzyme activity (3.07 U/mg) was found at pH 7. The line on each bar is the standard deviation (n=3).

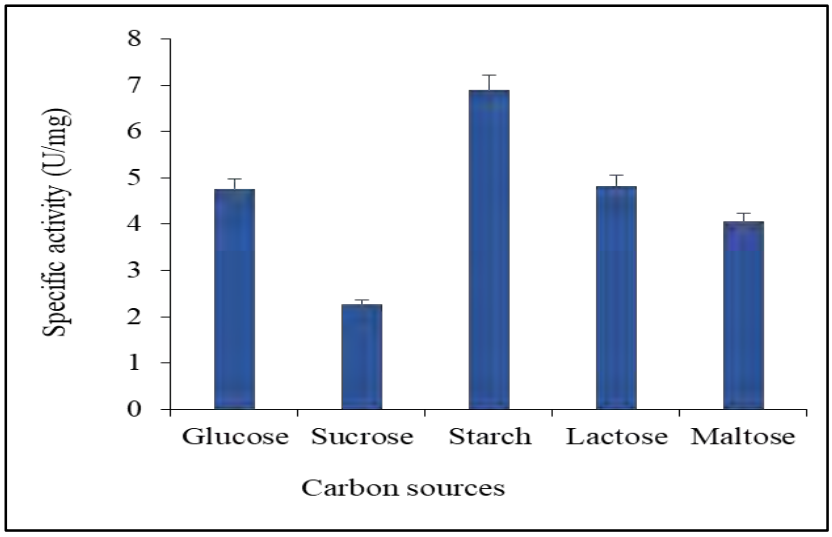


Figure 4. 16 Effect of different carbon sources on production of laccase from *Bacillus altitudinis* strain SL7 within 72 hours of incubation. Highest enzyme activity (6.88 U/mg) was found in the presence of starch. The line on each bar is the standard deviation (n=3).

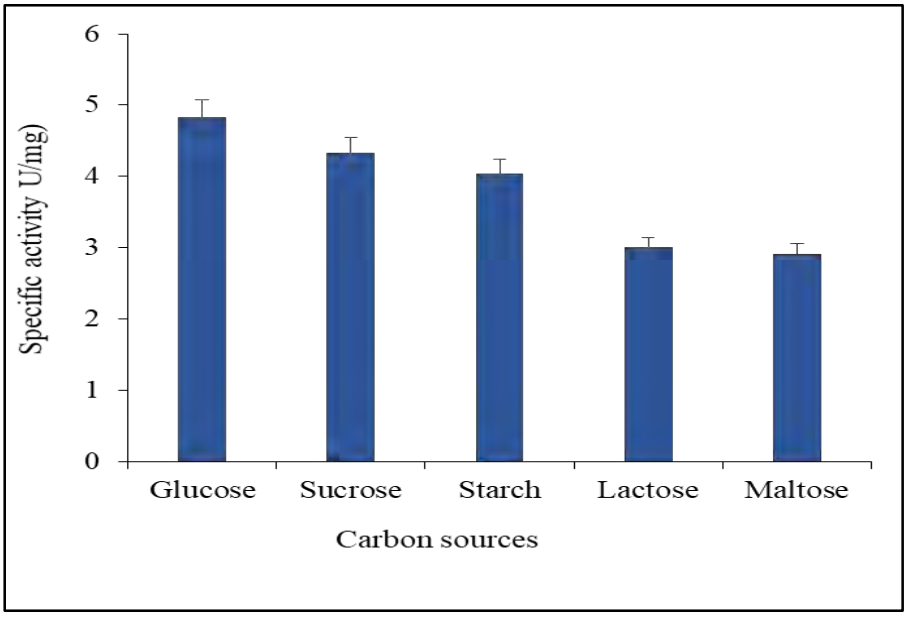


Figure 4. 17 Effect of different carbon sources on production of LDP from *Bacillus* sp. strain BL5 within 72 hours of incubation. Highest enzyme activity (4.82 U/mg) was found in the presence of glucose. The line on each bar is the standard deviation (n=3).

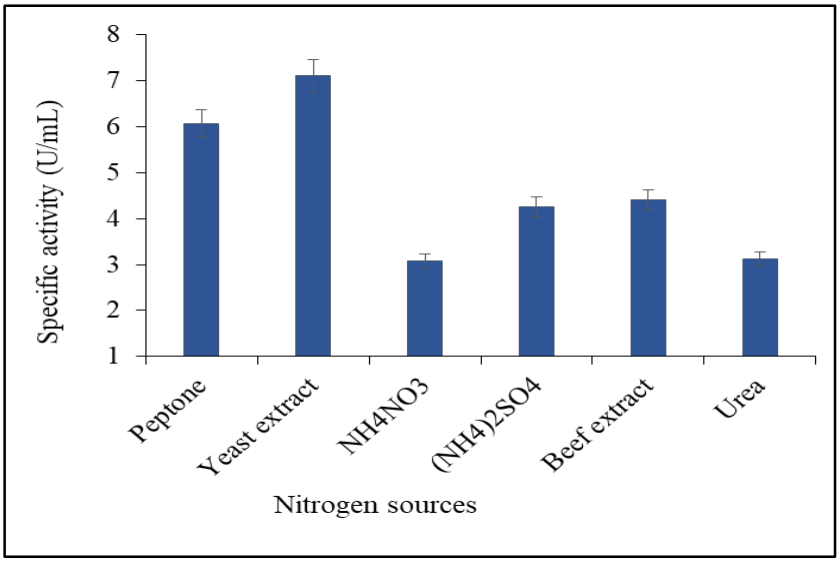


Figure 4. 18 Effect of different nitrogen sources on production of laccase from *Bacillus altitudinis* strain SL7 within 72 hours of incubation. Highest enzyme activity (7.1 U/mg) was found in the presence of yeast extract. The line on each bar is the standard deviation (n=3).

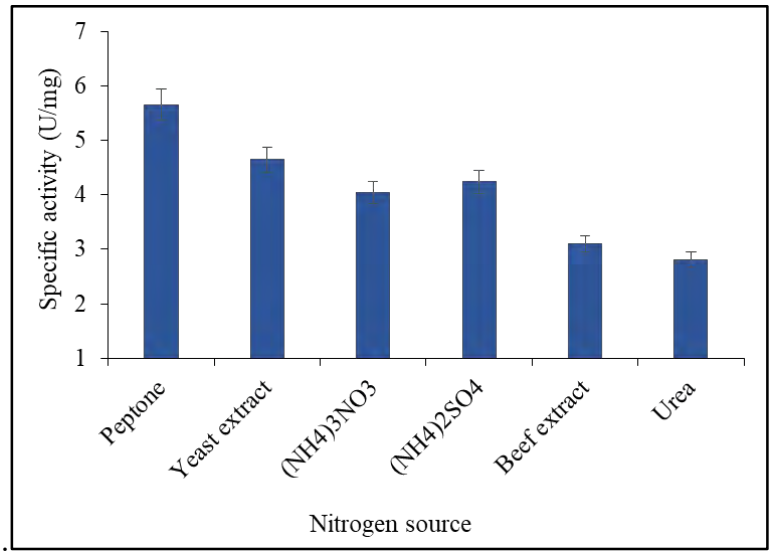


Figure 4. 19 Effect of different nitrogen sources on production of LDP from *Bacillus* sp. strain BL5 within 72 hours of incubation. Highest enzyme activity (5.65 U/mg) was found in the presence of peptone. The line on each bar is the standard deviation (n=3).

Table 4. 5 Optimization of nutritional factors for laccase production by Placket Burman design

Run no.	Factor A: Inoculum size	Factor B: Starch	Factor C: Yeast extract	Factor D: $(NH_4)_2SO_4$	Factor E: $MgSO_4$	Factor F: K_2HPO_4	Factor G: KH_2PO_4	Factor H: $CaCl_2$	Factor I: $FeSO_4.7H_2O$	Factor J: Lignin	Factor K: $CUSO_4$	Response I: Specific activity (U/mg)
1	6	2	1.25	0.16	0.01	0.23	0.32	0.01	0.01	0.2	0.55	2.87
2	10	3	2	0.01	0.01	0.16	0.4	0	0.01	0.3	0.1	2.57
3	2	1	0.5	0.01	0.01	0.16	0.23	0	0	0.1	0.1	1.81
4	6	2	1.25	0.16	0.01	0.23	0.32	0.01	0.01	0.2	0.55	1.32
5	10	1	2	0.3	0.01	0.3	0.4	0.01	0	0.1	0.1	6.14
6	2	3	2	0.3	0.01	0.16	0.23	0.01	0	0.3	1	2.58
7	2	3	2	0.01	0.02	0.3	0.4	0	0	0.1	1	7.08
8	6	2	1.25	0.16	0.01	0.23	0.32	0.01	0.01	0.2	0.55	1.37
9	2	3	0.5	0.3	0.02	0.16	0.4	0.01	0.01	0.1	0.1	4.59
10	2	1	0.5	0.3	0.01	0.3	0.4	0	0.01	0.3	1	4.59
11	10	1	0.5	0.01	0.02	0.16	0.4	0.01	0	0.3	1	3.44
12	10	1	2	0.3	0.02	0.16	0.23	0	0.01	0.1	1	2.47
13	2	1	2	0.01	0.02	0.3	0.23	0.01	0.01	0.3	0.1	3.09
14	10	3	0.5	0.01	0.01	0.3	0.23	0.01	0.01	0.1	1	3.30
15	10	3	0.5	0.3	0.02	0.3	0.23	0	0	0.3	0.1	8.26

Table 4. 6 Optimization of nutritional factors for lignin degrading peroxidase production by Placket Burman design

Run no.	Factor A: Inoculum size	Factor B: Glucose	Factor C: Peptone	Factor D: $(NH_4)_2SO_4$	Factor E: $MgSO_4$	Factor F: K_2HPO_4	Factor G: KH_2PO_4	Factor H: $CaCl_2$	Factor I: $FeSO_4.7H_2O$	Factor J: Lignin	Response I: Specific activity (U/mg)
1	0.6	0.9	1.8	0.009	0.018	0.27	0.207	0.009	0.009	0.27	1.062
2	3	2.7	1.8	0.009	0.009	0.144	0.36	0	0.009	0.27	0.704
3	3	0.9	1.8	0.27	0.018	0.144	0.207	0	0.009	0.09	6.73
4	3	0.9	1.8	0.27	0.009	0.27	0.36	0.009	0	0.09	5.69
5	0.6	2.7	1.8	0.009	0.018	0.27	0.36	0	0	0.09	3.07
6	0.6	0.9	0.45	0.27	0.009	0.27	0.36	0	0.009	0.27	0.81
7	3	0.9	0.45	0.009	0.018	0.144	0.36	0.009	0	0.27	1.63
8	1.8	1.8	1.125	0.144	0.009	0.207	0.288	0.009	0.009	0.18	0.31
9	0.6	0.9	0.45	0.009	0.009	0.144	0.207	0	0	0.09	1.36
10	3	2.7	0.45	0.27	0.018	0.27	0.207	0	0	0.27	0.73
11	3	2.7	0.45	0.009	0.009	0.27	0.207	0.009	0.009	0.09	0.98
12	0.6	2.7	1.8	0.27	0.009	0.144	0.207	0.009	0	0.27	0.96
13	1.8	1.8	1.125	0.144	0.009	0.207	0.288	0.009	0.009	0.18	1.13
14	0.6	2.7	0.45	0.27	0.018	0.144	0.36	0.009	0.009	0.09	0.37
15	1.8	1.8	1.125	0.144	0.009	0.207	0.288	0.009	0.009	0.18	0.29

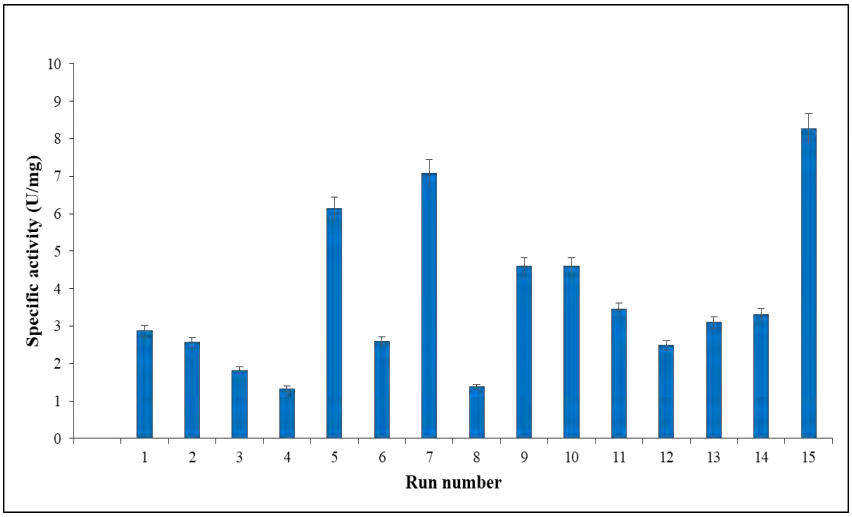


Figure 4. 20 Placket-Burman design was used for optimization of numerous nutritional factors for production of lacasse. Total eleven factors were analyzed in fifteen runs and highest enzyme activity was found at run number 15 (8.26 U/mg) and lowest activity (1.32 U/mg) was found at run number 08. Error bars represent standard deviation of the mean (n = 3).

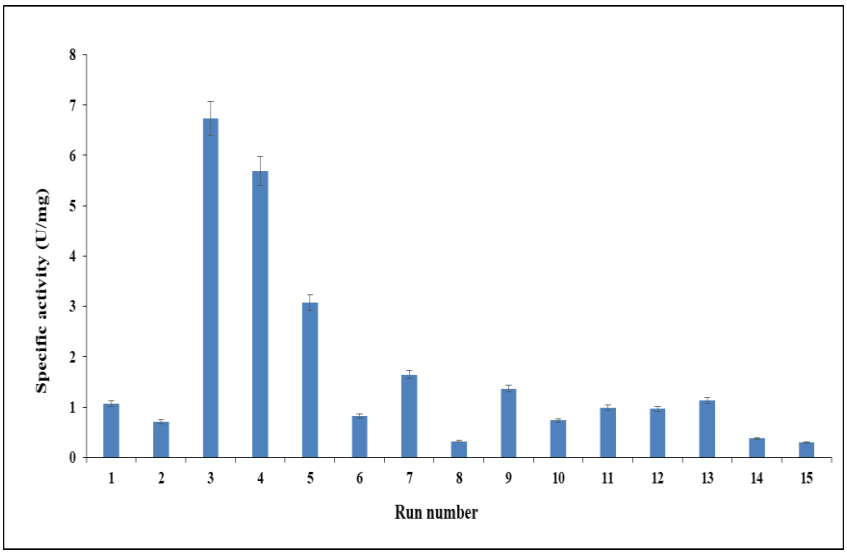


Figure 4. 21 Placket-Burman design was used for optimization of numerous nutritional factors for production of LDP. Total eleven (11) factors were analyzed in fifteen runs and highest enzyme activity was found at run number 03 (6.73 U/mg) and lowest activity (0.29 U/mg) was found at run number 15. Error bars represent standard deviation of the mean (n = 3).

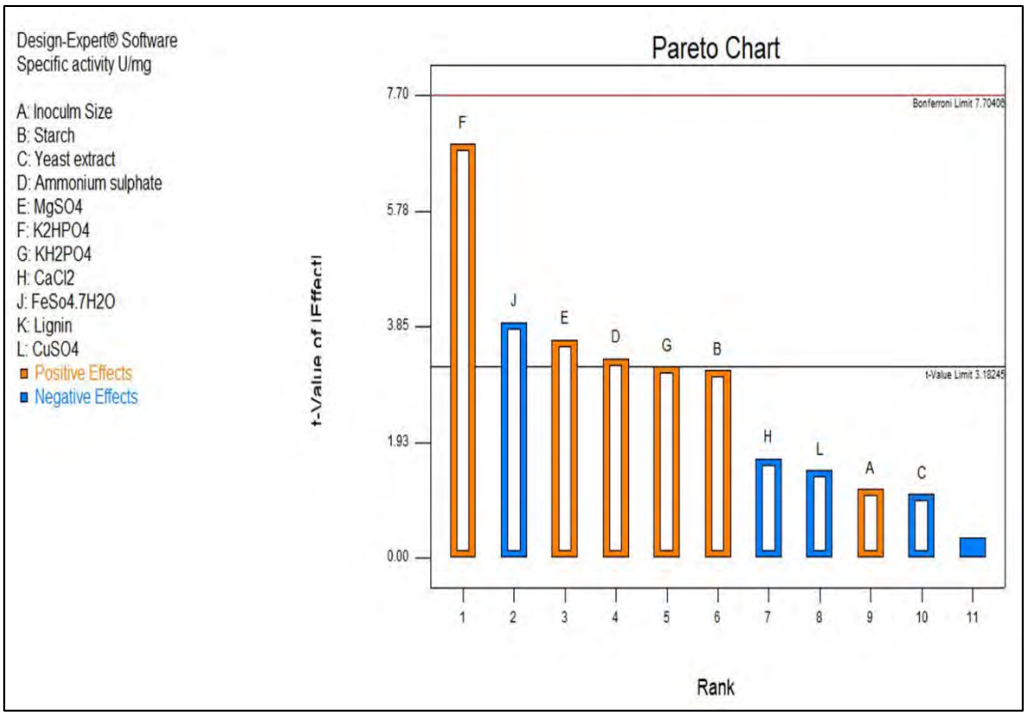


Figure 4. 22 Pareto chart showing the effect of t value by important factors (D: Ammonium sulphate, E: MgSO4, F: K2HPO4 and J: FeSO4.7H2O,) generated by PB design with respect to the effect on the production of laccase.

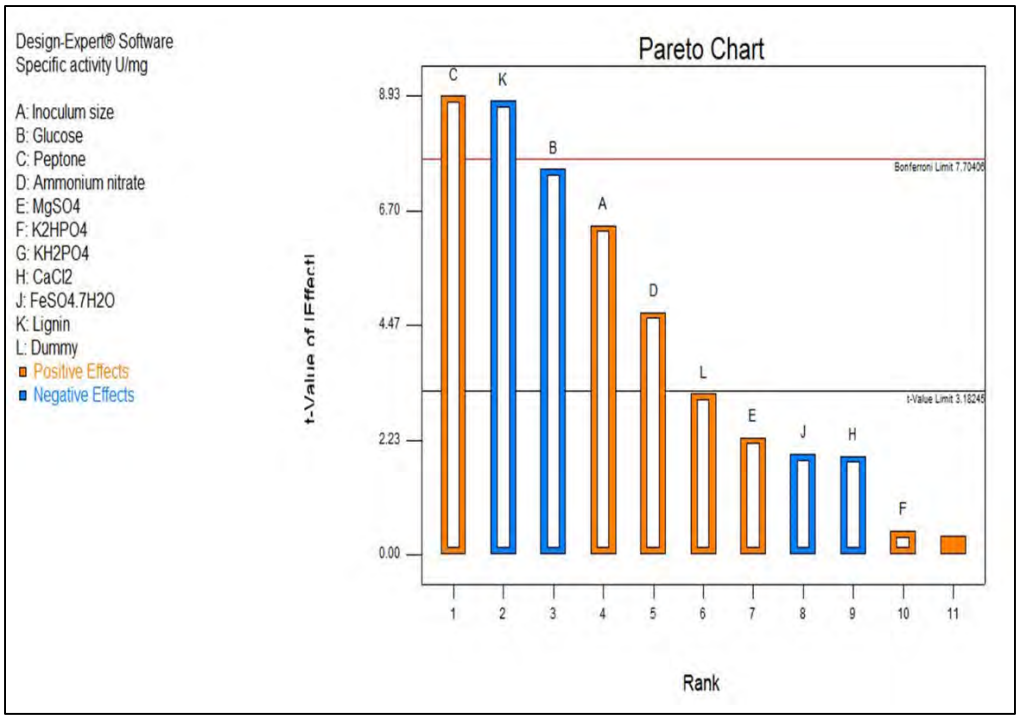


Figure 4. 23 Pareto chart viewing the influence of t value by important factors (C: Peptone, K: Lignin, A; Inoculum size, B; Glucose, D; Ammonium nitrate generated by PB design with respect to the effect on the LDP production.

Table 4. 7 Central Composite Design of factors for laccase production from *Bacillus altitudinis* strain SL7

Run no.	K_2HPO_4	MgSO4	$(NH_4)_2SO_4$	Inoculum	Starch	Yeast extract	KH_2PO_4	Lignin	CU_2SO_4	Enzyme activity (U/mg)
1	0.324	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	5.8
2	0.324	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	5.8
3	0.288	2.079	0.279	3	2.7	0.45	0.207	0.27	0.09	4.3
4	0.324	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	5.3
5	0.36	2.079	0.369	3	2.7	0.45	0.207	0.27	0.09	17.3
6	0.324	1.782	0.324	3	2.7	0.45	0.207	0.27	0.09	9.8
7	0.288	2.97	0.279	3	2.7	0.45	0.207	0.27	0.09	12.75
8	0.288	2.079	0.369	3	2.7	0.45	0.207	0.27	0.09	6
9	0.324	3.267	0.324	3	2.7	0.45	0.207	0.27	0.09	6.9
10	0.324	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	8.26
11	0.324	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	5.7
12	0.36	2.97	0.279	3	2.7	0.45	0.207	0.27	0.09	4.4
13	0.36	2.97	0.369	3	2.7	0.45	0.207	0.27	0.09	21.48
14	0.324	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	6.3
15	0.2691	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	5.54
16	0.324	2.574	0.396	3	2.7	0.45	0.207	0.27	0.09	27.1
17	0.36	2.079	0.279	3	2.7	0.45	0.207	0.27	0.09	17.9
18	0.288	2.97	0.369	3	2.7	0.45	0.207	0.27	0.09	29.5
19	0.387	2.574	0.324	3	2.7	0.45	0.207	0.27	0.09	5.7
20	0.324	2.574	0.252	3	2.7	0.45	0.207	0.27	0.09	5.4

Table 4. 8 Central Composite Design of factors for lignin degrading peroxidase production from *Bacillus* sp. BL5

Run No.	Peptone	Inoculum	$(NH_4)_2 SO_4$	Lignin	Glucose	$MgSO_4$	K_2HPO_4	KH_2PO_4	$FeSO_4.7H_2O$	Specific activity (U/mg)
1.	1.89	3.3	0.27	0.045	0.45	0.018	0.144	0.207	0.009	13.9
2	2.7	3.3	0.27	0.081	0.45	0.018	0.144	0.207	0.009	15.5
3	2.7	3.3	0.72	0.045	0.45	0.018	0.144	0.207	0.009	28.57
4	1.89	4.5	0.27	0.081	0.81	0.018	0.144	0.207	0.009	8.5
5	2.295	3.9	0.495	0.108	0.63	0.018	0.144	0.207	0.009	10.2
6	3.258	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	19.7
7	2.7	3.3	0.72	0.081	0.45	0.018	0.144	0.207	0.009	11.6
8	1.89	3.3	0.27	0.045	0.81	0.018	0.144	0.207	0.009	5.5
9	1.89	4.5	0.72	0.045	0.45	0.018	0.144	0.207	0.009	12.4
10	1.89	4.5	0.72	0.081	0.45	0.018	0.144	0.207	0.009	18.7
11	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	37.1
12	2.295	5.31	0.495	0.063	0.63	0.018	0.144	0.207	0.009	27.5
13	1.89	3.3	0.72	0.045	0.45	0.018	0.144	0.207	0.009	17.5
14	2.7	4.5	0.72	0.045	0.45	0.018	0.144	0.207	0.009	29.2
15	2.295	3.9	0.495	0.018	0.63	0.018	0.144	0.207	0.009	20.4
16	2.7	4.5	0.72	0.045	0.81	0.018	0.144	0.207	0.009	3.9
17	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	2.8
18	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	34.3
19	2.7	4.5	0.72	0.081	0.81	0.018	0.144	0.207	0.009	56.9
20	1.89	4.5	0.27	0.045	0.81	0.018	0.144	0.207	0.009	11.8
21	1.89	3.3	0.27	0.081	0.45	0.018	0.144	0.207	0.009	10
22	2.295	3.9	-0.036	0.063	0.63	0.018	0.144	0.207	0.009	12.4
23	1.89	4.5	0.72	0.045	0.81	0.018	0.144	0.207	0.009	11.7
24	1.89	3.3	0.72	0.081	0.45	0.018	0.144	0.207	0.009	26.5
25	2.7	3.3	0.27	0.081	0.81	0.018	0.144	0.207	0.009	19.6
26	2.7	3.3	0.27	0.045	0.81	0.018	0.144	0.207	0.009	8.8

27	2.295	3.9	0.495	0.063	1.062	0.018	0.144	0.207	0.009	37
28	2.7	4.5	0.27	0.045	0.81	0.018	0.144	0.207	0.009	33.6
29	2.7	4.5	0.27	0.081	0.81	0.018	0.144	0.207	0.009	30.6
30	2.7	3.3	0.72	0.081	0.81	0.018	0.144	0.207	0.009	16.4
31	2.295	2.472	0.495	0.063	0.63	0.018	0.144	0.207	0.009	20.1
32	1.89	3.3	0.72	0.081	0.81	0.018	0.144	0.207	0.009	10.5
33	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	39.1
34	1.89	4.5	0.27	0.081	0.45	0.018	0.144	0.207	0.009	23.5
35	2.7	3.3	0.27	0.045	0.45	0.018	0.144	0.207	0.009	8.8
36	1.89	3.3	0.27	0.081	0.81	0.018	0.144	0.207	0.009	13.9
37	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	27.8
38	1.89	4.5	0.72	0.081	0.81	0.018	0.144	0.207	0.009	7.8
39	2.295	3.9	0.495	0.063	0.198	0.018	0.144	0.207	0.009	12.8
40	2.295	3.9	1.026	0.063	0.63	0.018	0.144	0.207	0.009	14.7
41	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	13.4
42	1.89	3.3	0.72	0.045	0.81	0.018	0.144	0.207	0.009	25.5
43	2.7	4.5	0.27	0.081	0.45	0.018	0.144	0.207	0.009	3.9
44	1.89	4.5	0.27	0.045	0.45	0.018	0.144	0.207	0.009	29
45	1.332	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	7.1
46	2.7	4.5	0.72	0.081	0.45	0.018	0.144	0.207	0.009	5.5
47	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	9.1
48	2.7	4.5	0.27	0.045	0.45	0.018	0.144	0.207	0.009	2.5
49	2.295	3.9	0.495	0.063	0.63	0.018	0.144	0.207	0.009	15.5
50	2.7	3.3	0.72	0.045	0.81	0.018	0.144	0.207	0.009	40.4

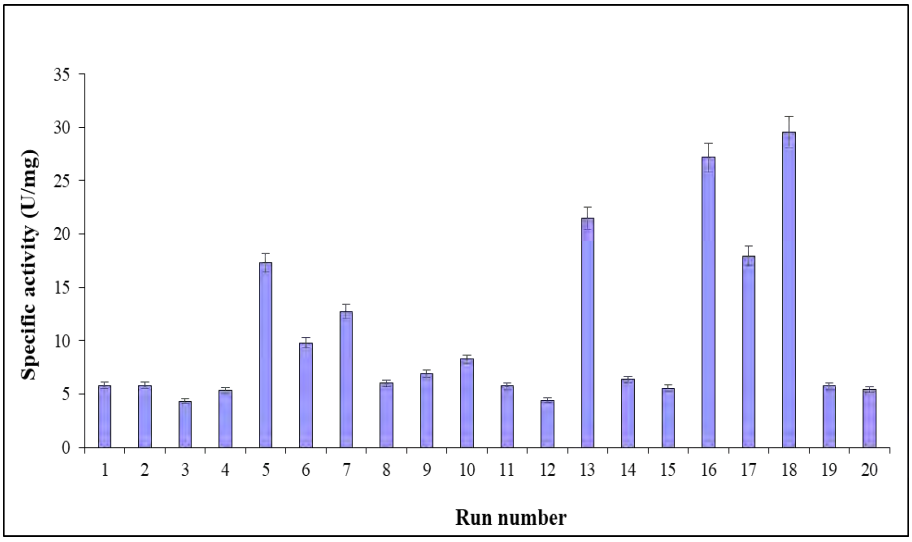


Figure 4. 24 Central composite design was used for optimization of different concentration of signification factors for laccase production. Different concentration of four factors were analyzed in twenty runs and highest enzyme activity was found at run number 18 (29.5 U/mg) and lowest activity (4.33 U/mg) was found at run number 03.

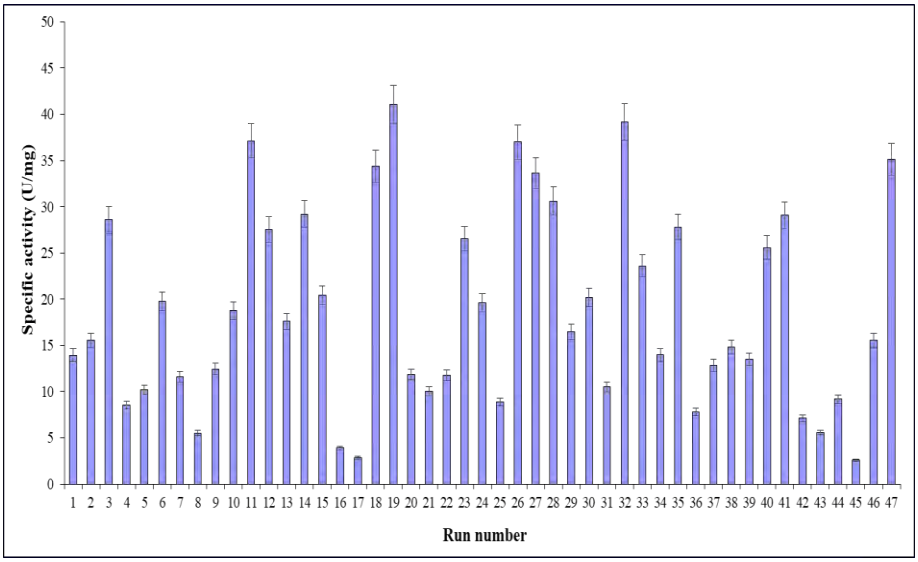


Figure 4. 25 Central composite design was used for optimization of different concentration of signification factors for LDP production. Different concentration of five factors were analyzed in fifty runs and highest enzyme activity was found at run number 19 (56.9 U/mg) and lowest activity (2.8 U/mg) was found at run number 48.

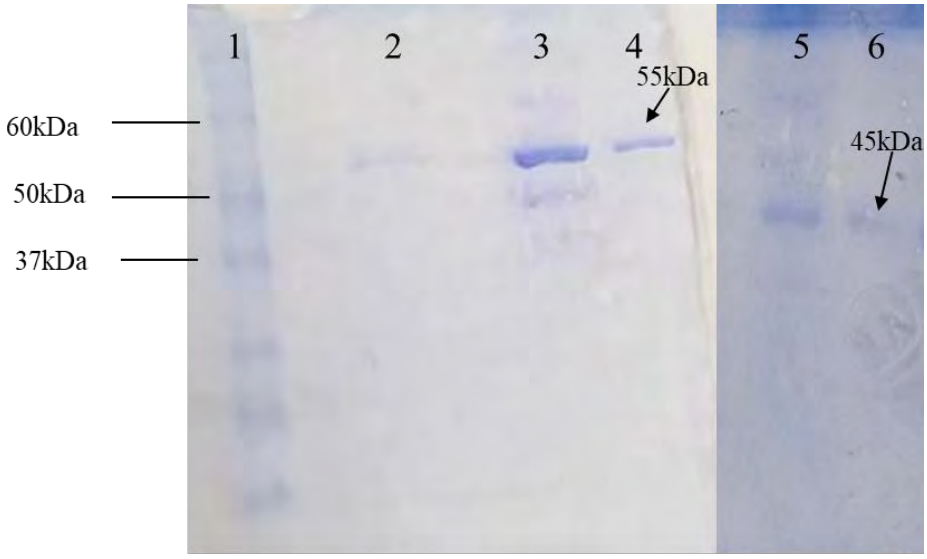


Figure 4. 26 SDS-PAGE for laccase and lignin degrading peroxidase **(1)** Protein marker (Thermo Fisher# 10748010), **(2)** Crude laccase, **(3)** Proteins obtained after acetone precipitation for laccase, **(4)** Fully purified laccase by gel chromatography, **(5)** Proteins obtained after acetone precipitation for LDP, **(6)** Fully purified LDP by gel chromatography.

Table 4. 9 Purification steps of laccase and lignin degrading peroxidase

Enzyme	**Purification steps**	**Total Protein (mg)**	**Total Activity (U/ml)**	**Total Specific Activity (U/mg)**	**Yield (%)**	**Purification Fold**
Laccase	Crude extract	400	24000	60	1	100
	Precipitate	7.5	260	34.6	0.57	1.08
	Gel permeation	0.7	1500	214.2	6.18	57.6
Lignin degrading peroxidase	Crude extract	40	12000	300	1	100
	Precipitate	12.5	205	16.4	0.054	1.70
	Gel permeation	0.8	78	97.5	5.94	38.04

4.11. Cloning and Expression of Laccase and Lignin Degrading Peroxidase

4.11.1 Isolation of DNA

Genomic DNA of *Bacillus altitudinis* SL7 and *Bacillus* sp. BL5 was extracted using commercially available Wizard ® Genomic DNA Purification kit (Fig. 4.27). The Lac and LDP were amplified from extracted DNA.

4.11.2. Amplification of Lac and LDP genes by PCR

Laccase encoding gene and efeb gene encoding lignin degrading DyP-type peroxidase were amplified from the genomic DNA of *Bacillus altitiudinis* SL7 and *Bacillus* sp. BL5, respectively, with translational initiation codon ATG. Amplification of approximately 1.2 kb size of lignin degrading peroxidase and 1.5 kb of laccase encoding genes were achieved, results are shown in Fig. 4.28 & 4.29.

4.11.3. Cloning of Lac and LDP Gene in Cloning Vector

Laccase gene from strain SL7 (LacSL7) and lignin degrading DyP-type peroxidase gene from strain BL5 (DyPBL5) were ligated with pGEM®-T cloning vector and transformed to *E.coli* JM101 competent cells by heat shock method. Cloning was confirmed by blue-white screening, cells having plasmids with LacSL7 and DyPBL5 gene products were shown to be white (figure 4.30). Cloning was further confirmed by sequencing and digestion of plasmids with restriction endonucleases *EcoRI.* Digestion of pGEM®-T cloned plasmids with *EcoRI* produced the desired band size of 1.5 kb and 1.2 kb for LacSL7 and DyPBL5, respectively with 3 kb of its own size (Fig 4.31 & 4.32).

4.11.4. Preparation of pGEMT-LacSL7, pGEMT-DyPBL5 and pET-28a Vector for Cloning

In-situ cloning of LacSL7 and DyPBL5 genes into pET-28a (+) vector was performed by constructing gene-vector map (Fig. 4.33 & 4.34). pET-28a (+) vector and pGEMT cloned LacSL7 and DyPBL5 were digested with *NcoI* and *HindIII* restriction endonucleases and visualized using 1% agarose gel through gel electrophoresis under gel documentation system. The digested pET-28a (+) vector showed approximately 5300bp product size of single band while digested LacSL7 and DyPBL5 showed approximately 1500bp and 1251bp, respectively. LacSL7 and DyPBL5 digested product also released the pGEM-T vector of about 3000bp (Fig 4.35 & 4.36). The digested

pET-28a (+) vector and gene products were excised from gel and purified by using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer instructions. The excised gene products and pET-28a (+) vector were visualized by gel electrophoresis and used as insert for ligation.

4.11.5. Cloning of LacSL7 and DyPBL5 into Expression Vector

The digested pET-28a (+) vector and LacSL7 and DyPBL5 were ligated using T4 ligase enzyme. The ligated expression vector and gene insert were transformed into competent cells of *E.coli* JM101 by heat shock method (Fig. 4.37). Confirmation of cloning was done by digesting plasmids with specific restriction endonucleases. White colonies were grown in LB medium containing kanamycin antibiotic, as pET-28a (+) vector have kanamycin resistant gene, and plasmids were isolated. Isolated plasmids were digested with restriction enzymes and further confirmed by sequencing.

4.11.6. Protein Sequence Analysis

The Lac and DyP genes encoded open reading frame of 502 and 418 amino acids, respectively. Nucleotide sequence of LacSL7 and DyPBL5 are presented in Appendix 17 & 18. The molecular mass for LacSL7 and DyPBL5 was calculated using Expasy server ProtParam tool (https://web.expasy.org/protparam/) and calculated molecular mass was around 58 and 46 kDa, respectively. The sequence analysis of the deduced amino acids was confirmed to represent the Lac and DyP-type peroxidase encoding protein, and showing more than 99% similarity to other laccase and DyP proteins present in genome of neighboring species. The phylogenetic analysis of LacSL7 and DyPBL5 are shown in Fig. 4.38 & 4.39.

4.12. Expression and Purification of Recombinant LacSL7 and DyPBL5

pET-28a vector cloned with LacSL7 and DyPBL5 from *E.coli* JM101 were transformed into *E. coli* BL21 (DE3) expression cells (Fig. 4.40). Recombinant enzymes were expressed under T7 promoter, induction of recombinant LacSL7 and DypBL5 was achieved through 1 mM IPTG, along with 1 mM $CuSO_4$ in case of LacSL7. Clearly expressed proteins of LacSL7 and DyPBL5 were visualized by SDS-PAGE with molecular mass of 55 kDa and 46 kDa, respectively (Fig. 4.41 & 4.42). Recombinant LacSL7 and DyPBL5 were purified through anionic resin Q-Sepharose Fast Flow, and elution was achieved by linear gradient of (0.5 M) NaCl in glycine hydroxide buffer for DyPBL5 and phosphate buffer for LacSL7. 50 fractions of about 3 mL each were collected and

assayed for enzymes activity and protein estimation. Purity of proteins was confirmed by SDS-PAGE analysis (Fig. 4.43 & 4.44). The total yield and purification folds for crude and purified LacSL7 and DyPBL5 were calculated. Total yield of LacSL7 was 39% and purification fold was 33, and total yield for DyPBL5 was 11% and purification fold was 37. The purification folds and yield are given in Table. 4.10 & 4.11.

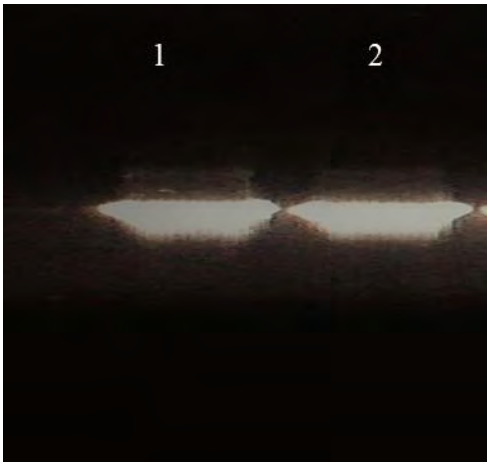


Figure 4. 27 Genomic DNA of strain SL7 anf BL5, 1: SL7 DNA, 2: BL5 DNA

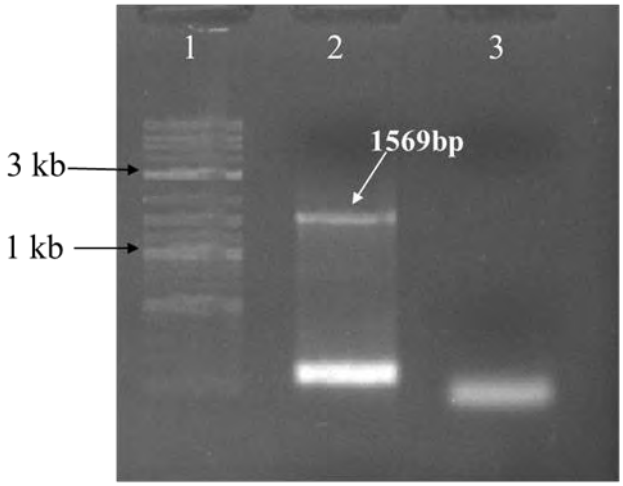


Figure 4. 28 PCR amplification of laccase gene from *Bacillus altitudinis* strain SL7, 1: DNA ladder (NEB# B7025), 2: Laccase gene PCR product, 3: Negative control

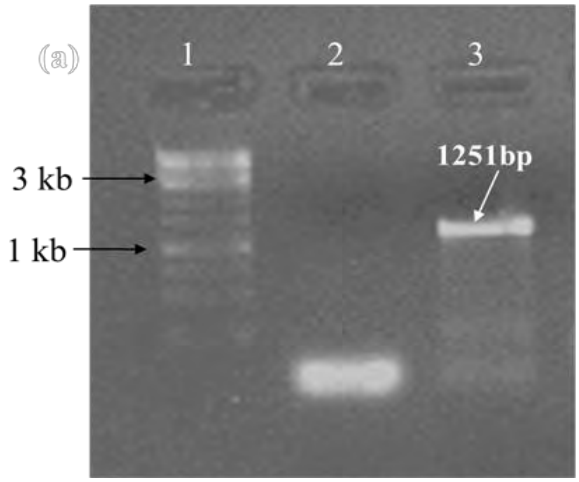


Figure 4. 29 PCR amplification of lignin degrading peroxidase gene from *Bacillus* sp. strain BL5, 1: DNA ladder (NEB# B7025), 2: Negative control, 3: Lignin degrading peroxidase gene PCR

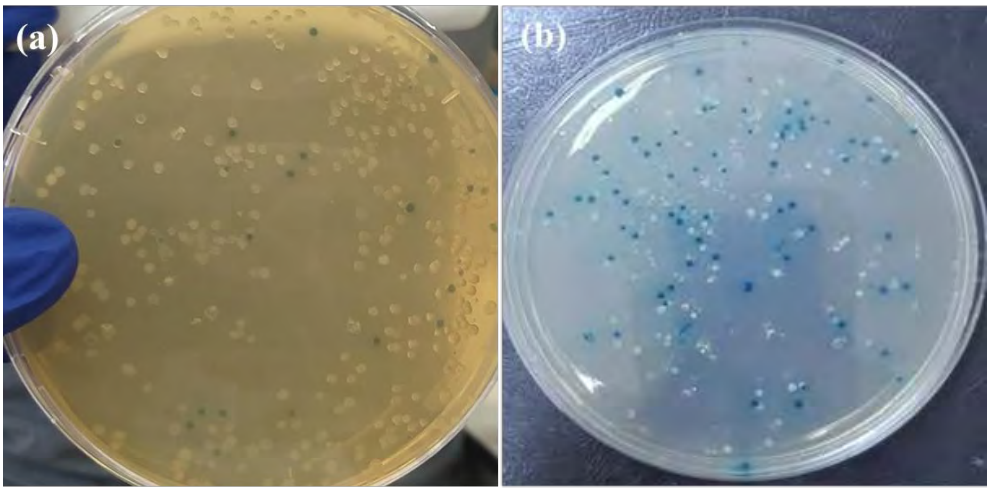


Figure 4. 30 Blue/white Screenig of pGEM-T easy vector transformed *E.coli* JM101 cells with laccase and LDP genes, a: Transformed vector carrying Lac gene, b: Tranformed vector carrying LDP gene

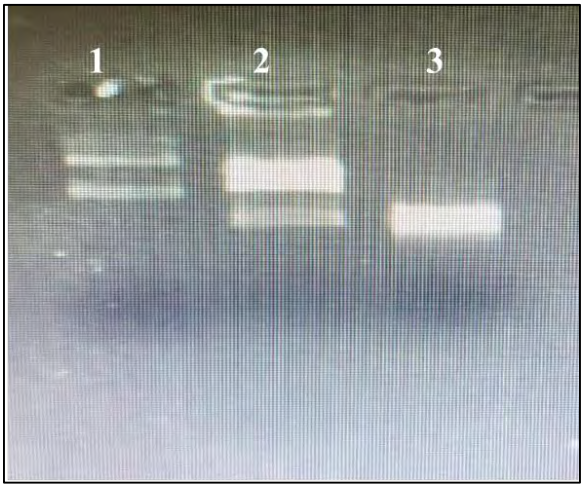


Figure 4. 31 Digestion of strain SL7 laccase cloned into pGEM-T easy vector with *EcoRI* restriction enzyme, 1: Undigested Lac clone, 2: Digested Lac clone with *EcoRI*, 3: Lac PCR product

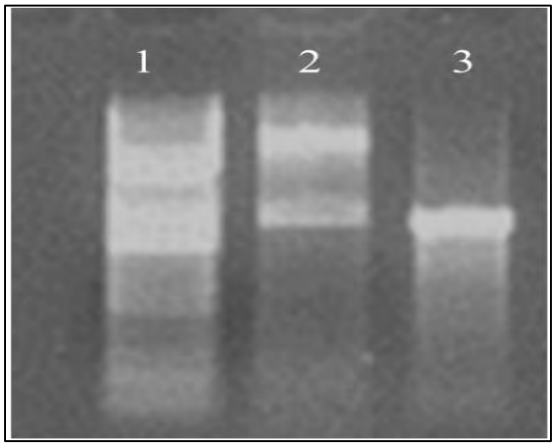


Figure 4. 32 Digestion of strain BL5 LDP cloned into pGEM-T easy vector with *E.coRI* restriction enzyme, 1: Undigested LDP clone, 2: Digested LDP clone with EcoRI, 3: LDP PCR product

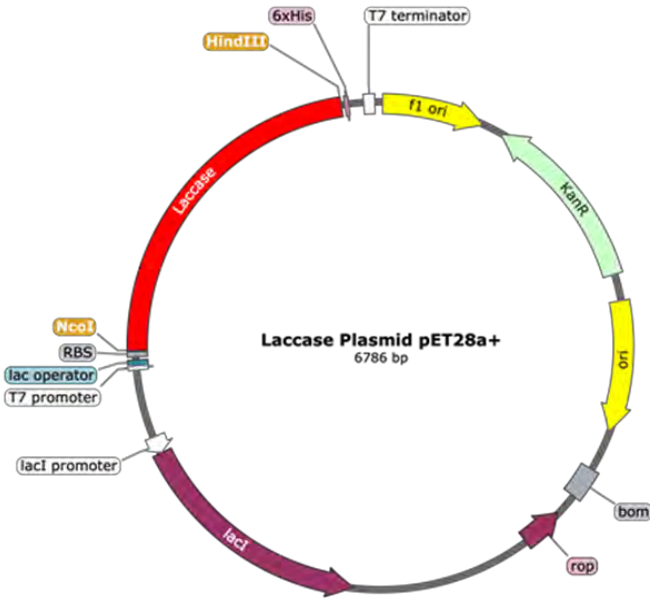


Figure 4. 33 In situ cloning of laccase gene from *Bacillus altitudinis* strain SL7 designed in pET28a (+) expression vector

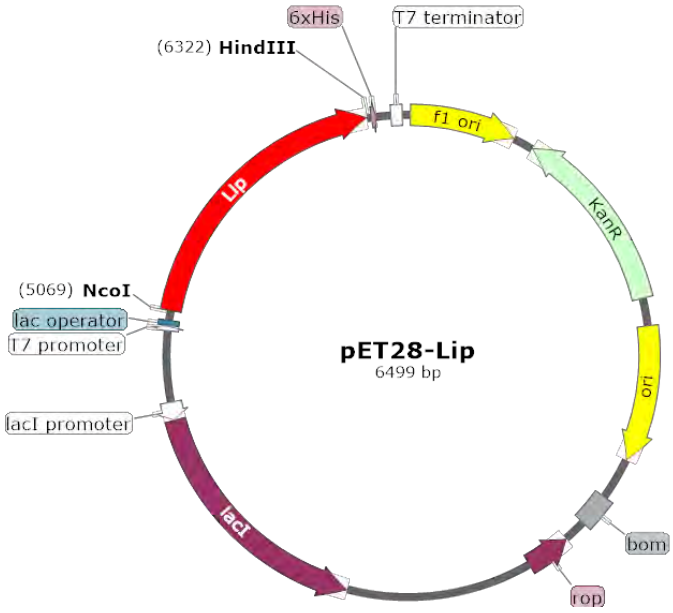


Figure 4. 34 In situ cloning of lignin degrading peroxidase gene from *Bacillus* sp. strain BL5 designed in pET28a (+) expression vector

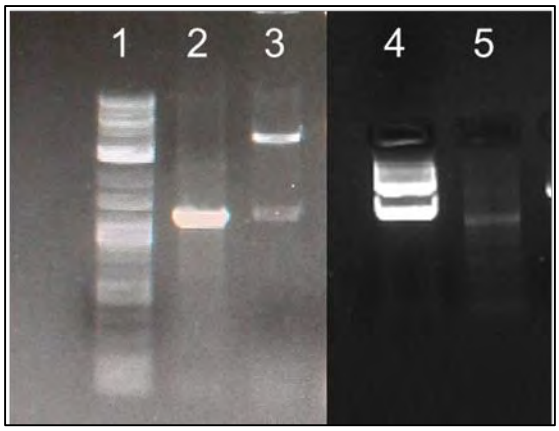


Figure 4. 35 Digestion of pGEM-T laccase clone and pET-28a (+) plasmid with *NcoI* and *HindIII* restriction enzymes, 1: DNA ladder, 2: Lac PCR product, 3: *NcoI* and *HindIII* digested pGEM-T Lac clone, 4: Undigested pET-28a (+) plasmid, 5: *NcoI* and *HindIII* digested pET-28a (+) plasmid

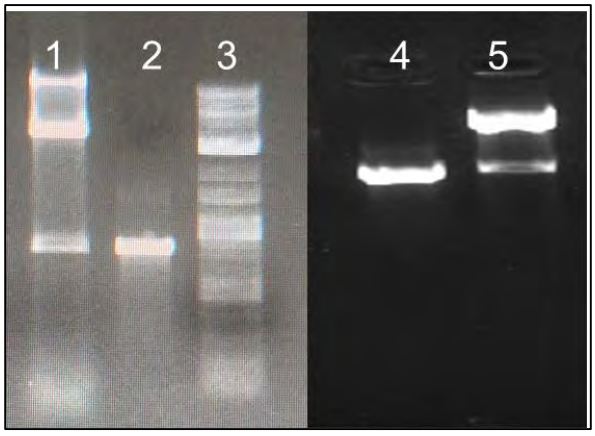


Figure 4. 36 Digestion of pGEM-T LDP clone and pET-28a plasmid with *NcoI* and *HindIII* restriction enzymes, 1: *NcoI* and *HindIII* digested pGEM-T LDP clone, 2: LDP PCR product, 3: DNA ladder, 4: *NcoI* and *HindIII* digested pET-28a plasmid, 5: Undigested pET-28a (+) plasmid

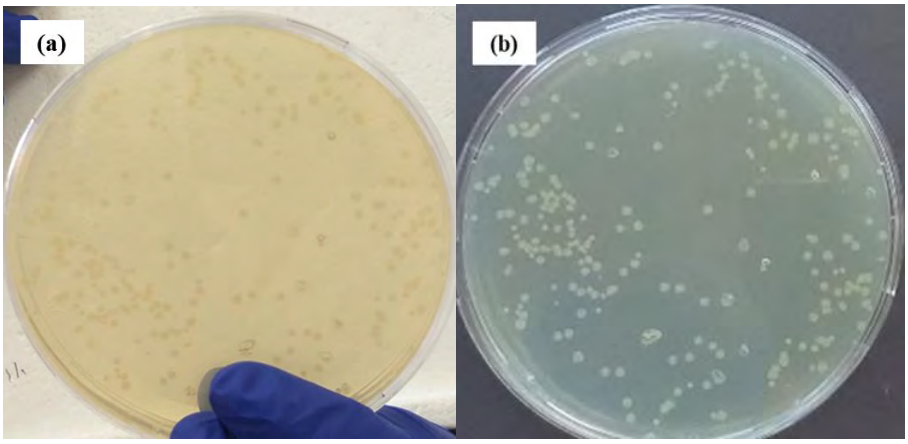


Figure 4. 37 Transformation of pET-28a (+) ligated with Lac and LDP genes into *E.coli* JM101, a: Lac gene, b: LDP gene

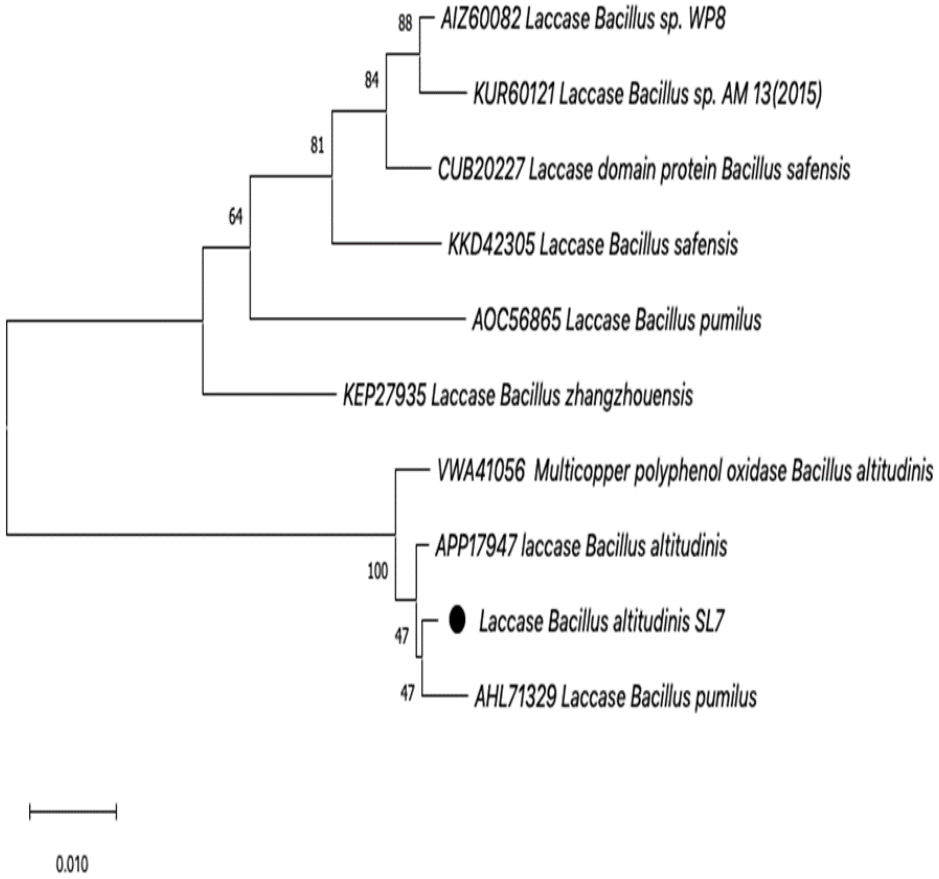


Figure 4. 38 Phylogenetic relationship of laccase from *Bacillus altitudinis* strain SL7 with other laccases available in NCBI database

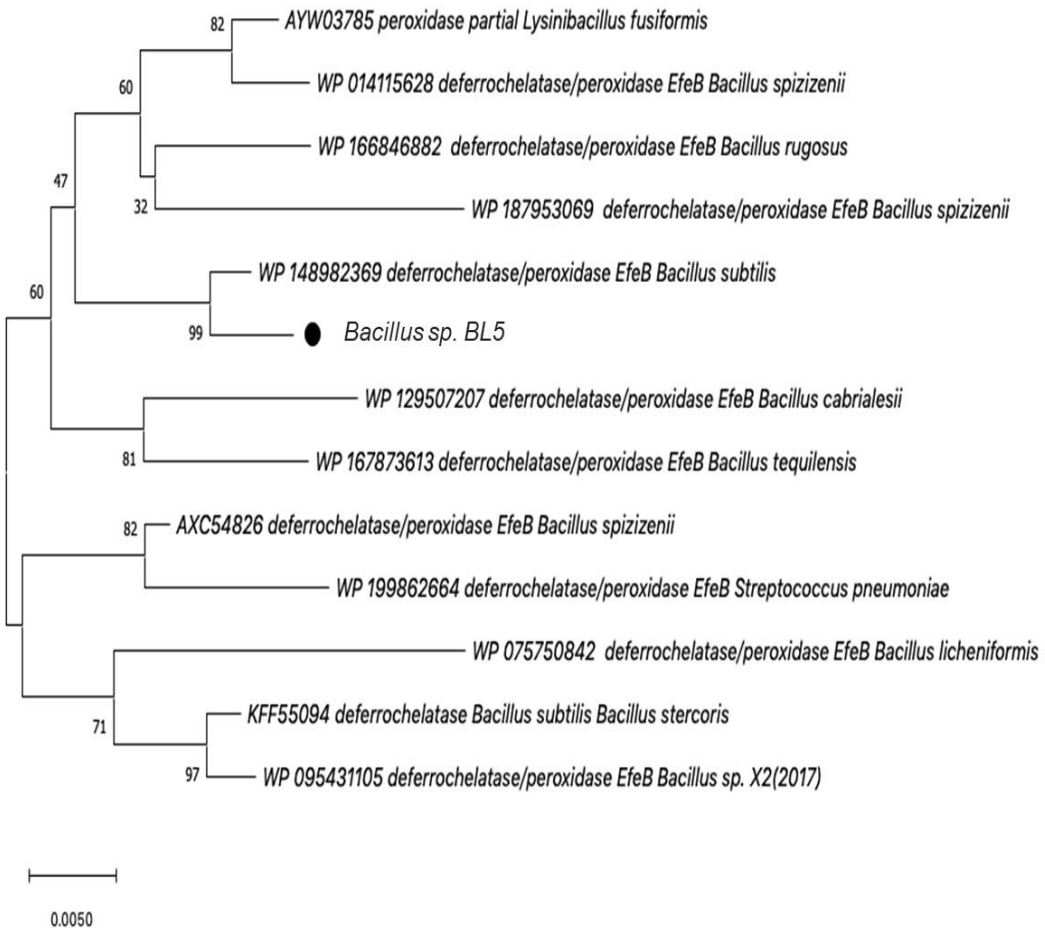


Figure 4. 39 Phylogenetic relationship of DyP-peroxidase from *Bacillus* sp. strain BL5 with other peroxidases available in NCBI database

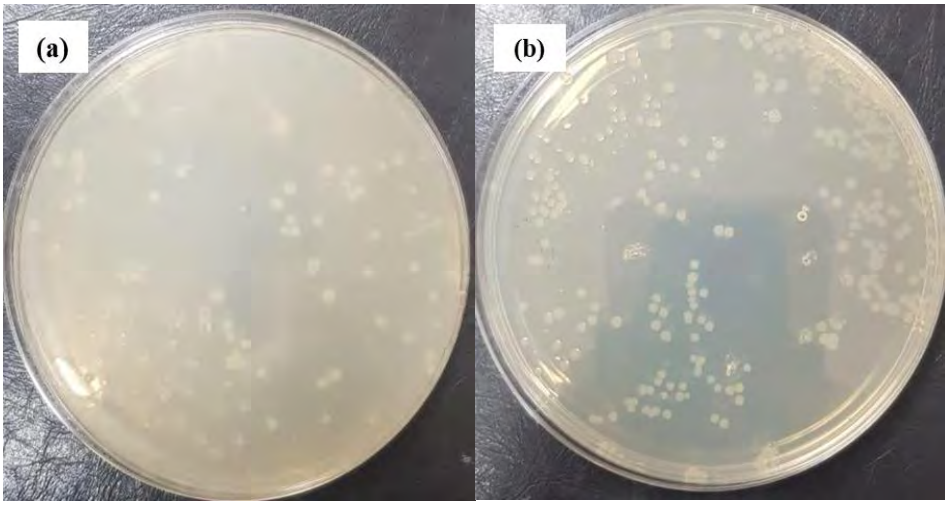


Figure 4. 40 Transformation of pET-28a plasmid with Lac and LDP genes into E.coli BL21 (DE3) expression cells, a: Lac gene, b: LDP gene

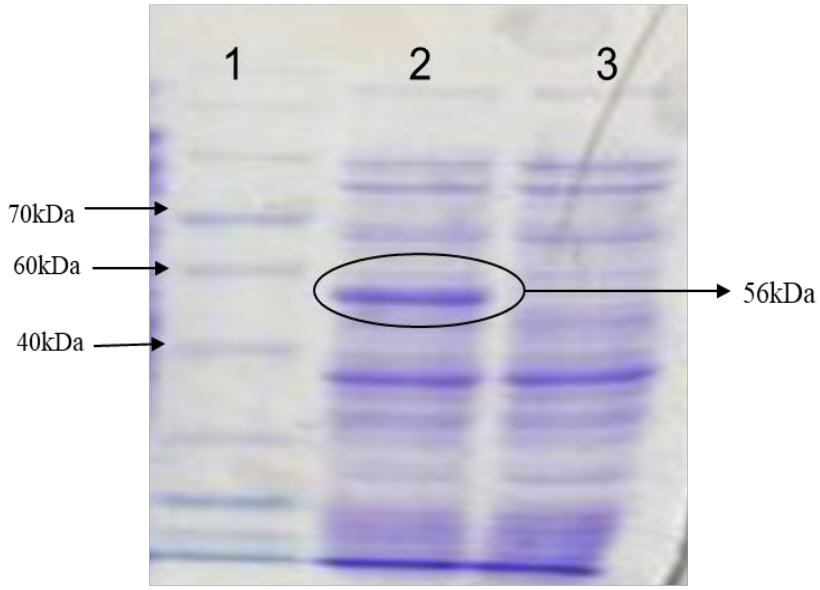


Figure 4. 41 SDS PAGE visualization for recombinant laccase, 1: Protein Marker (Thermo Fisher #26616), 2: Induced proteins of *E. coli* BL21 (DE3) with pET28a-LacSL7 plasmid, 3: Induced proteins of *E. coli* BL21 (DE3) with pET-28a (+) plasmid

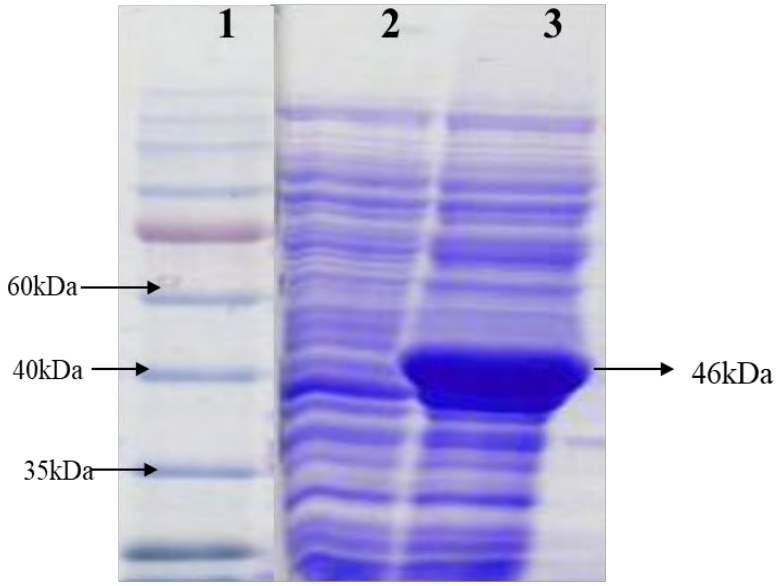


Figure 4. 42 SDS PAGE visualization for recombinant DyPBL5, 1: Protein Marker (Thermo Fisher #26616), 2: Induced proteins of *E. coli* BL21 (DE3) with pET-28a (+) plasmid, 3: Induced proteins of *E. coli* BL21 (DE3) with pET28-DyPBL5 plasmid

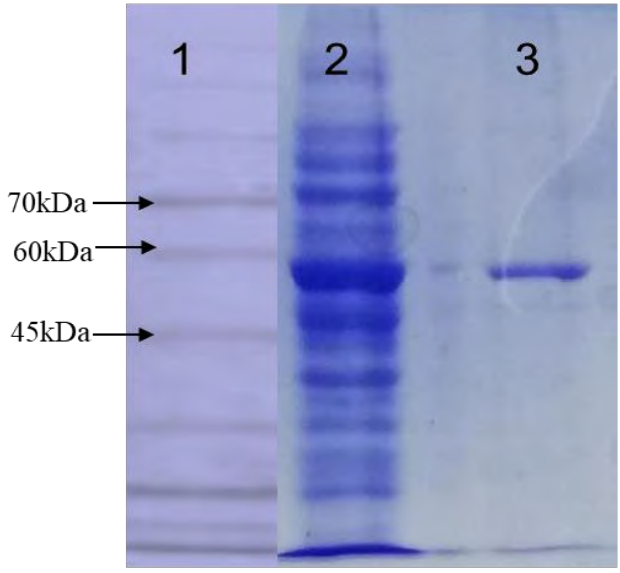


Figure 4. 43 SDS–PAGE visualization of purified LacSL7, 1: Protein marker, 2: Crude LacSL7, 3: Purified LacSL7

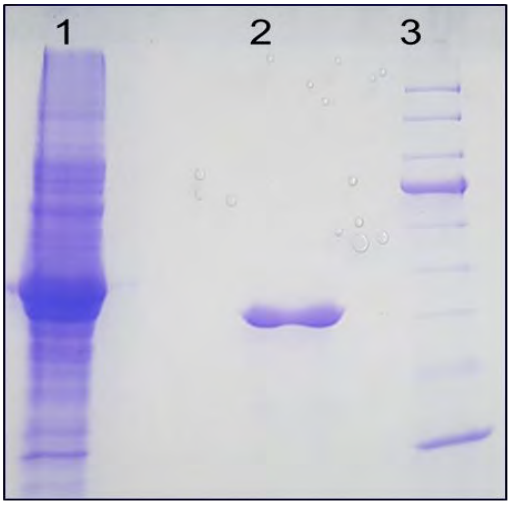


Figure 4. 44 SDS–PAGE visualization of purified DyPBL5, 1: Crude DyPBL5, 2: Purified DyPBL5, 3: Protein marker

Table 4. 10 Summary of LacSL7 purification steps

Purification Steps	**Total Protein (mg)**	**Total Activity (AU)**	**Specific Activity (U/mg)**	**Purification (fold)**	**Recovery (%)**
Crude enzymes	400	30650	76.5	1	100
Purified enzyme	4.8	12144	2530	33	39

Table 4. 11 Summary of DyPBL5 purification steps

Purification Steps	**Total Protein (mg)**	**Total Activity (AU)**	**Specific Activity (U/mg)**	**Purification (fold)**	**Recovery (%)**
Crude enzyme	2000	69444	34.7	1	100
Purified enzyme	6	7916.55	1319	37.9	11.3

4.13. Functional Characterization of Purified LacSL7 and DyPBL5

4.13.1. Temperature Effect on Activity of Purified LacSL7 and DyPBL5

Temperature effect was analyzed by incubating LacSL7 and DyPBL5 with substrate at different temperatures ranging from 25-90°C and activity was calculated in term of residual activity. Both LacSL7 and DyPBL5 showed activity over the wide range of temperature with optimum activity at 55°C and 35°C, respectively. LacSL7 retained more than 70% activity at 35-70°C, while DyPBL5 retained more 70% activity at 25-40°C (Fig. 4.45).

4.13.2. pH Effect on Activity of Purified LacSL7 and DyPBL5

pH effect on activity of purified LacSL7 and DyPBL5 was analyzed by deremning the enzyme activity in buffers with pH ranges from 3.0-10.0, and activity was calcualted in terms of residual activity. Optimum pH for LacSL7 and DyPBL5 was revealed to be 5 and 4, respectively. More than 50% of activity was retained from pH 3 to 7 for both enzymes (Fig. 4.46).

4.13.3. Temperature Effect on Stability of Purified Enzymes

Stability of purified enzymes was analyzed by incubating the enzymes at different temperatures, LacSL7 was incubated at 30-80°C and DyPBL5 was incubated at 25-55°C, and then activities were performed on optimized conditions. Incubation was carried out until residual activities were lost less than 50%. LacSL7 was stable over the wide range of temperatures and maintained more than 50% of activity at 30-70°C for 24 h and more than 80% at 30-60°C for 16 h (Fig. 4.47). DyPBL5 was active at 30–40 °C and maintained its stability between 25-35 °C, and retained more than 70% activity for 16 h. The activity was declined to half, at 40 °C after 8 h of incubation and enzyme lost its half of activity after 30 mins of incubation at 50 °C and 55 °C (Fig. 4.48)

4.13.4. pH Effect on Stability of Purified Enzymes

Stability of purified LacSL7 and DyPBL5 was analyzed by incubating in buffers with different pH ranges from 3.0-10.0 and the residual activities were calculated at optimum pH of assays. Purified LacSL7 retained more than 80% of its activity at pH 7-8 for 16 h, and more than 50% at pH 5-6 up to 8 h and lost 50% of activity at pH 9 after 2 h (Fig. 4.49). Purified DyPBL5 retained more than 50% activity after exposure to pH 4-6 for 16 h and lost half of its activity after 8 h at pH 7-8 (Fig. 4.50).

4.13.5. Effect of Metals Ions

The effect of different metal ions in various concentration ranging from 0.5-5 mM on activity of purified recombinant LacSL7 and DyPBL5 was investigated. In general, with increased concentration of metal ions, the activity of both the enzymes was decreased and at lower concentration (1-2 mM) activity of enzymes was enhanced. Hg^{++} caused strong inhibition of activity of LacSL7 and DyPBL5 at 5mM concentration. LacSL7 activity was increased in the presence of 1 mM concentration of $CaCl_2$, $NiCl_2$, CuSO4, ZnSO4, KCl, and NaCl (Fig. 4.51). While FeSO4, $CaCl_2$, MgSO4, KCl and NaCl has increased the activity of DyPBL5 at 1 mM concentration (Fig. 4.52). 5 mM concentration of all the used metals partially or strongly effected the activity of both the enzymes.

4.13.6. Effect of Detergents

The LacSL7 was showing stability in the presence of all the tested detergents (Fig. 4.52.1). DyPBL5 was strongly inhibited in the presence of even at 1% SDS concentration. Triton-X 100, Tween 80 and Tween 20 increased the activity of enzyme at 1% while 5% concentration of Tween 80 and Tween 20 strongly inhibited activity of DyPBL5 (Fig. 4.53).

4.13.7. Effect of Inhibitors

The effect of inhibitors at concentration of 1 to 10mM on activity of both the enzymes was investigated. The LacSL7 and DyPBL5 were quite stable in the presence of all the tested inhibitors, except DTT and β-mercaptoethanol which strongly inhibited the activity of enzymes even at 1mM concentration. PMSF, DEPC, EGTA, EDTA only inhibited 20% to 30% of enzymes activity at 1mM and 5mM concentration (Fig.4.54 & 4.55).

4.13.8. Effect of Organic Solvent

The effect of organic solvent at 10% and 25% (v/v) final concentration was evaluated. The activity of purified LacSL7 and DyPBL5 was increased in the presence of ethanol and methanol. The LacSL7 was strongly inhibited by DMSO and Butanol at 25% concentration, and DyPBL5 was strongly inhibited by DMSO, Butanol and Dioxane even at 10% concentration (Fig. 4.56 & 4.57). DyPBL5 was substantially influenced by isopropanol at all the tested concentration.

4.13.9. Kinetic Parameters

Kinetic parameters for LacSL7 and DyPBL5 were determined spectrophotmetrically using ABTS with different concentrations (0.5-5 mM). The catalytic efficiency was calculated by Michaelis-Menten plot. LacSL7 exhibited *Km*, *Vmax*, and *Kcat* values 0.28 mM, 2073 µmol/min/mg and 3878 S^{-1}, respectively (Fig. 4.58). The DyPBL5 exhibited *Km*, *Vmax*, and *Kcat* values 1.06 mM, 519.75 µmol/min/mg and 365 s^{-1}, respectively (Fig. 4.59).

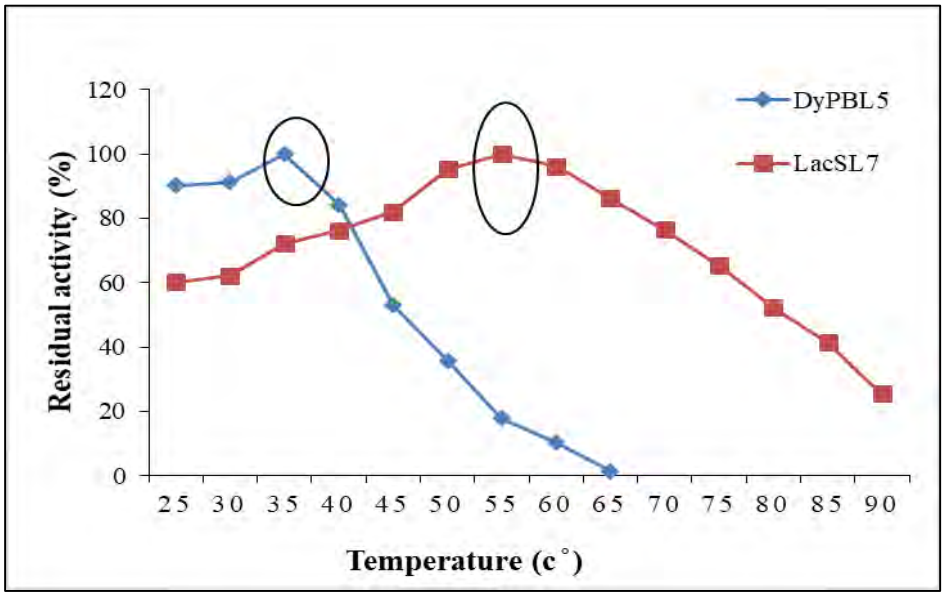


Figure 4. 45 Temperature effect on activity of purified recombinant LacSL7 and DyPBL5

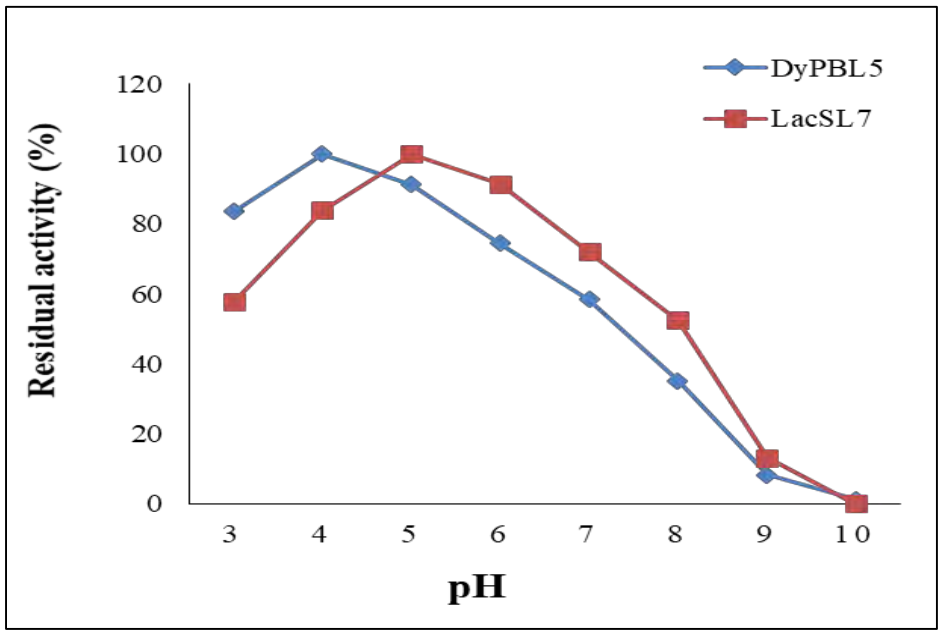


Figure 4. 46 pH effect on activity of purified recombinant LacSL7 and DyPBL5

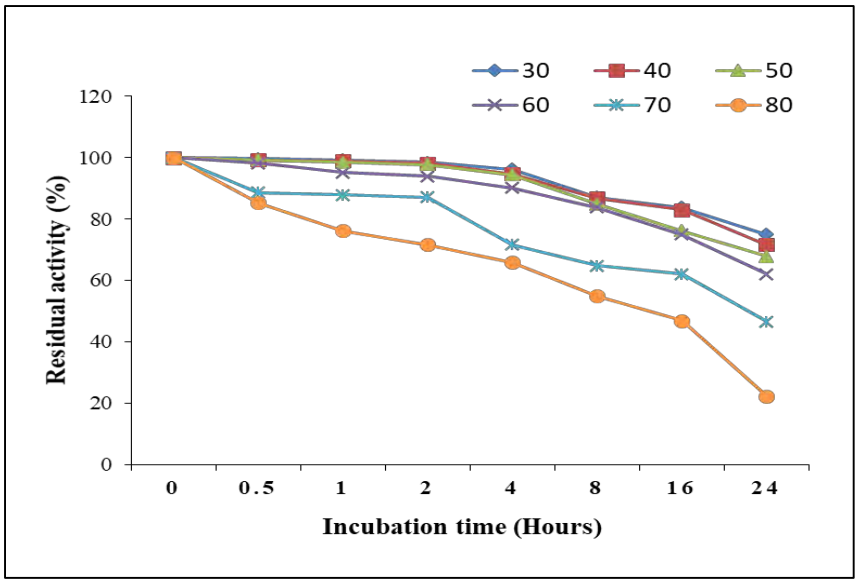


Figure 4. 47 Temperature effect on stability of purified recombinant LacSL7

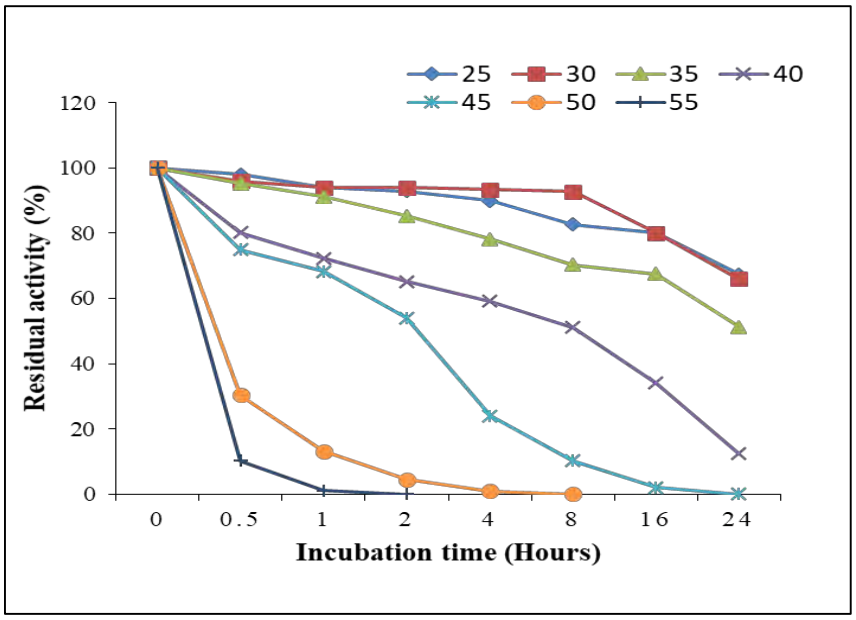


Figure 4. 48 Temperature effect on stability of purified recombinant DyPBL5

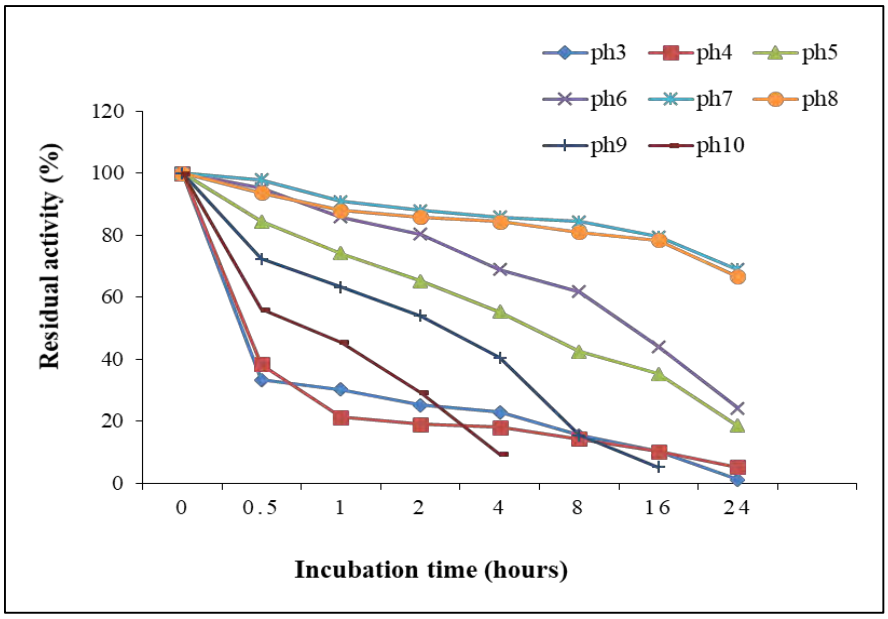


Figure 4. 49 pH effect on stability of purified recombinant LacSL7

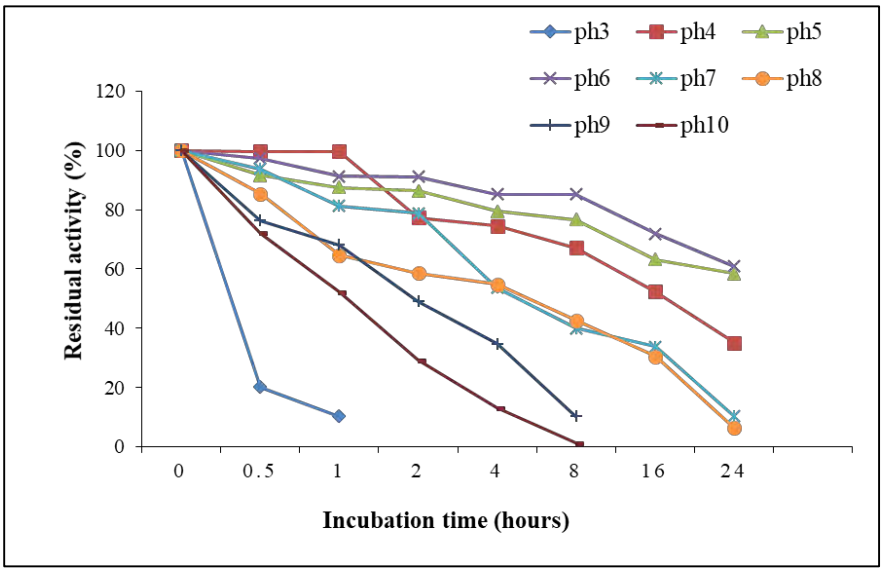


Figure 4. 50 pH effect on stability of purified recombinant DyPBL5

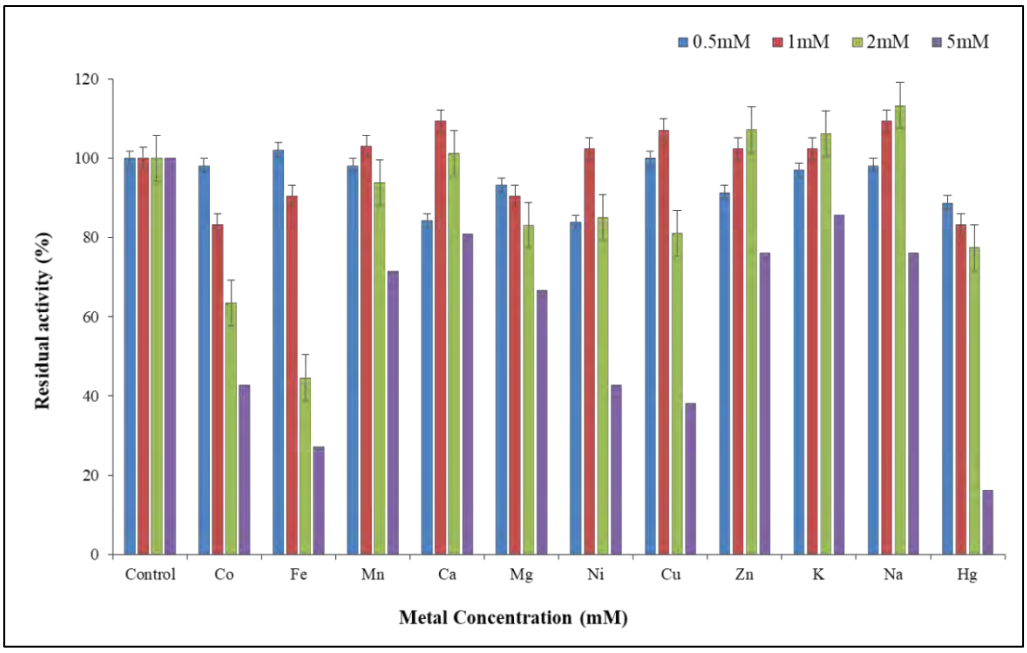


Figure 4. 51 Metal ions effect on activity of purified LacSL7. Error bars represent standard deviation of the mean (n = 3).

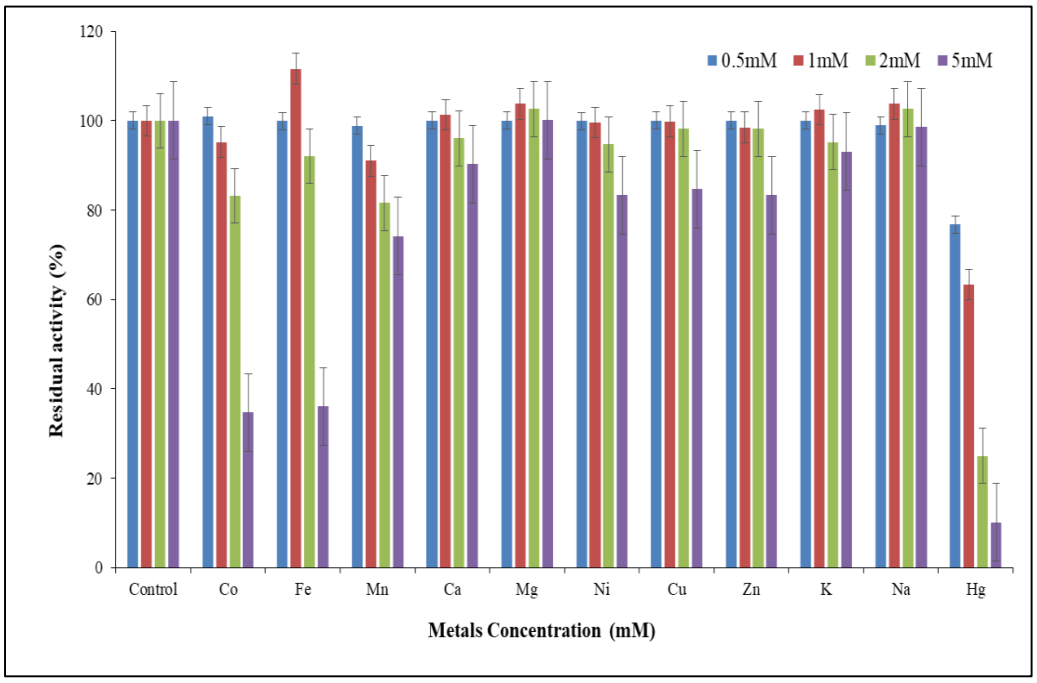


Figure 4. 52 Metal ions effect on activity of purified DyPBL5. Error bars represent standard deviation of the mean (n = 3).

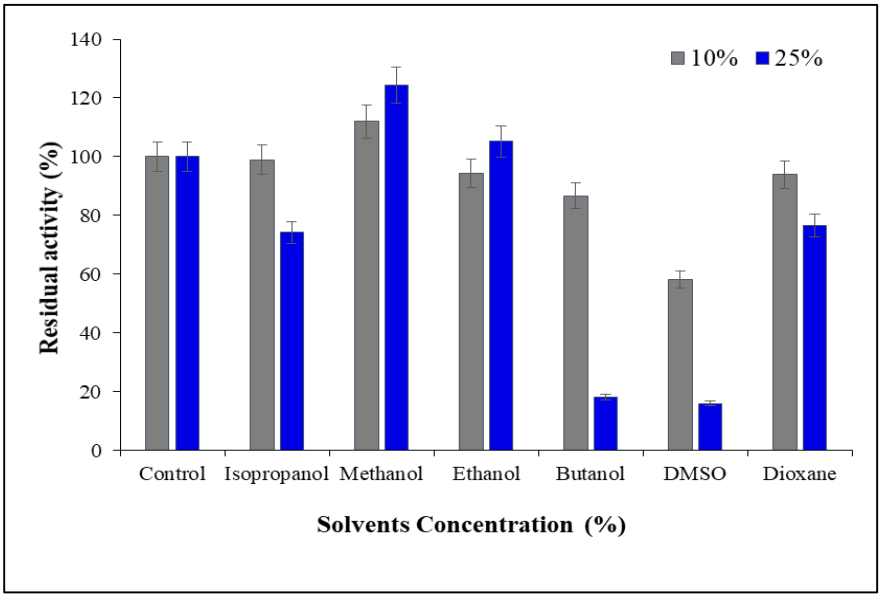


Figure 4. 53 Organic solvents effect on activity of purified LacSL7. Error bars represent standard deviation of the mean (n = 3).

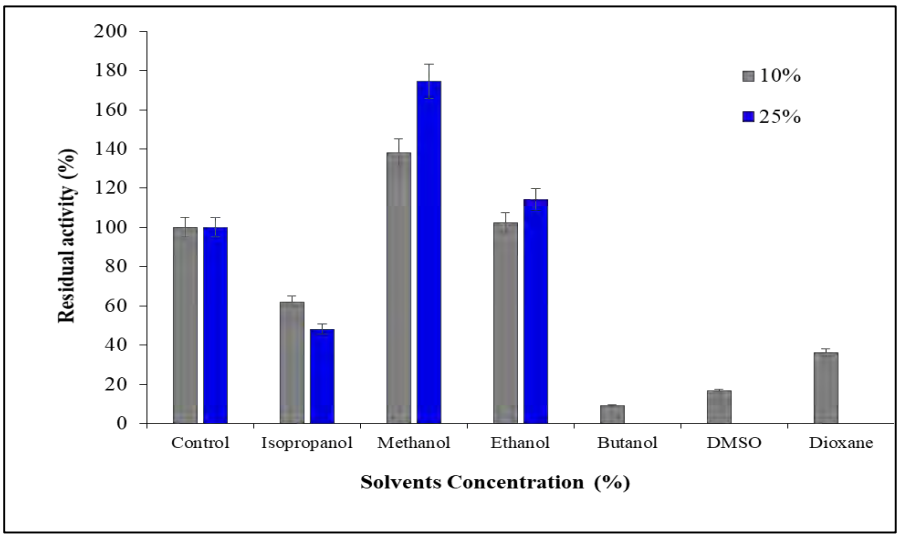


Figure 4. 54 Organic solvents effect on activity of purified DyPBL5. Error bars represent standard deviation of the mean (n = 3).

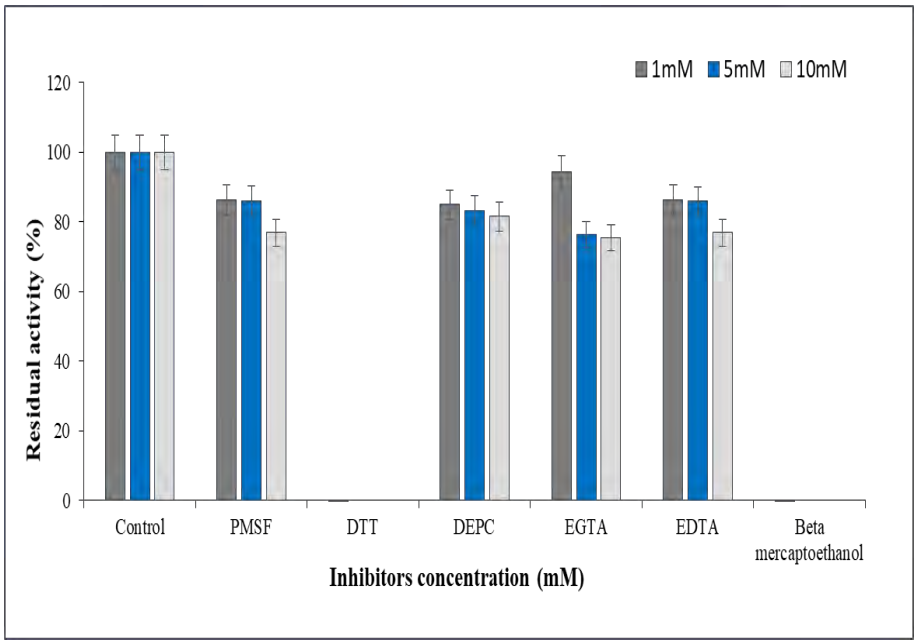


Figure 4. 55 Inhibitors effect on activity of purified LacSL7. The line on each bar is the standard deviation (n=3).

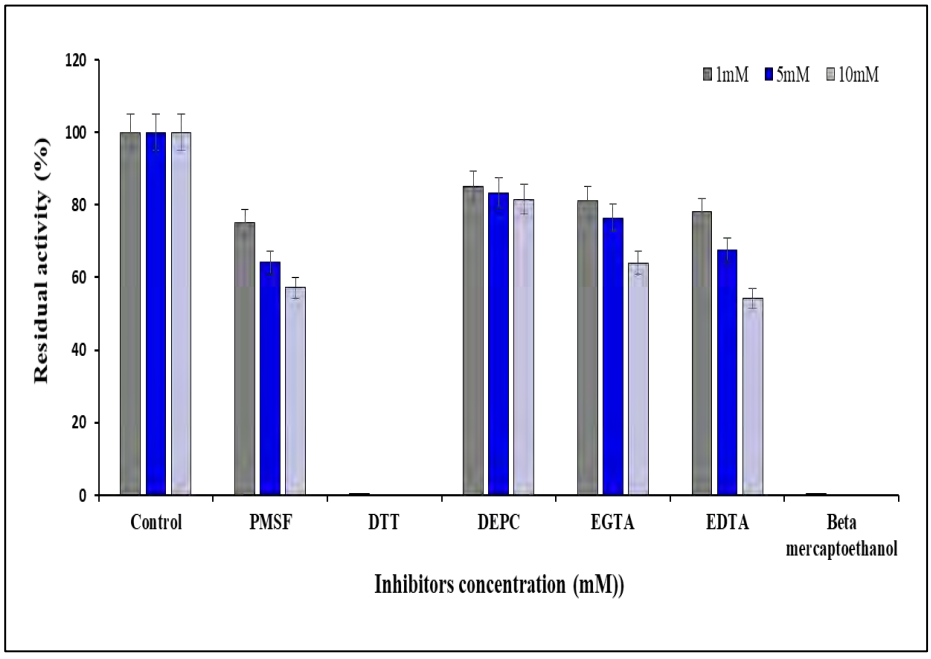


Figure 4. 56 Inhibitors effect on activity of purified DyPBL5. The line on each bar is the standard deviation (n=3).

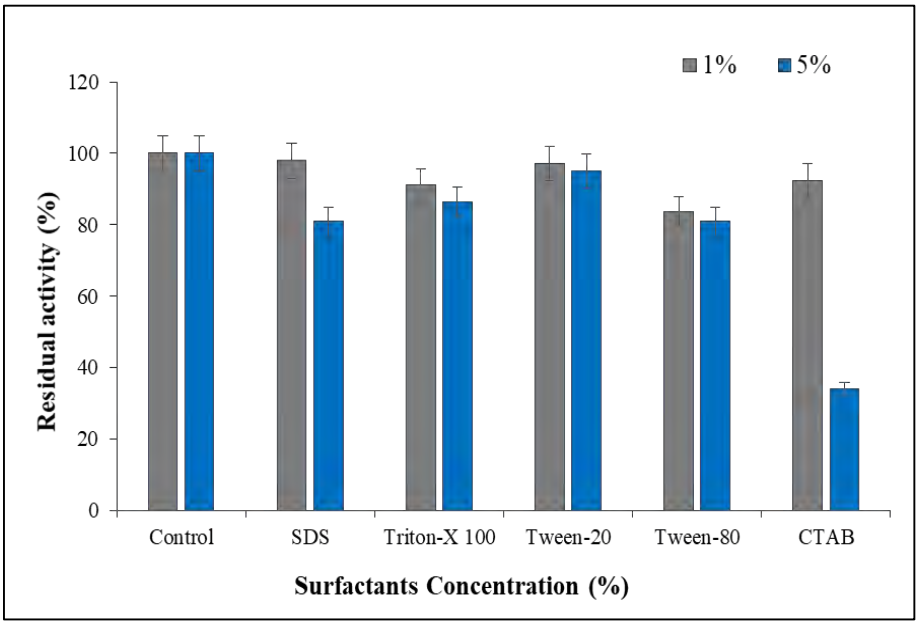


Figure 4. 57 Surfactants effect on activity of purified LacSL7. The line on each bar is the standard deviation (n=3).

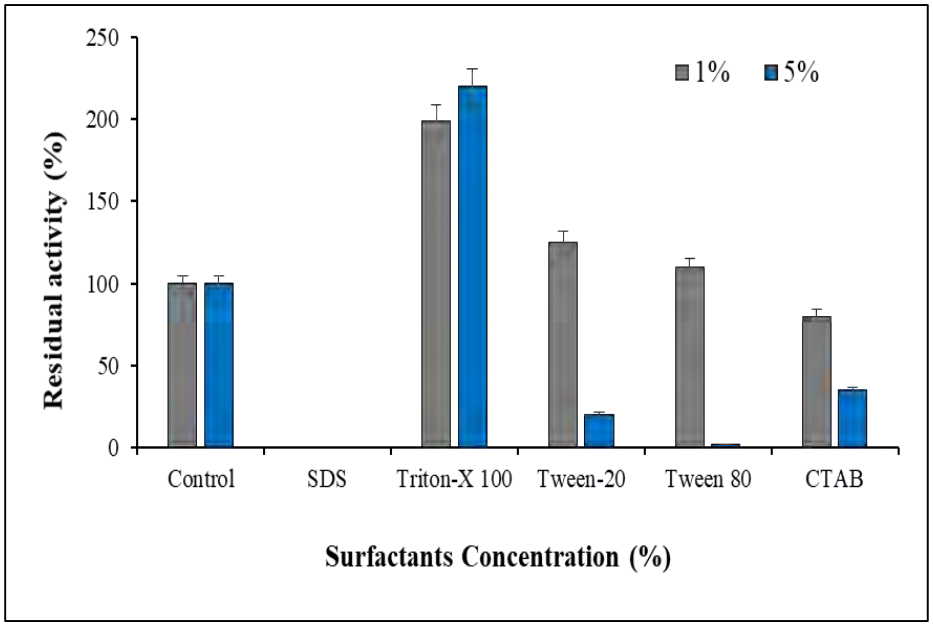


Figure 4. 58 Surfactants effect on activity of purified DyPBL5. Error bars represent standard deviation of the mean (n = 3).

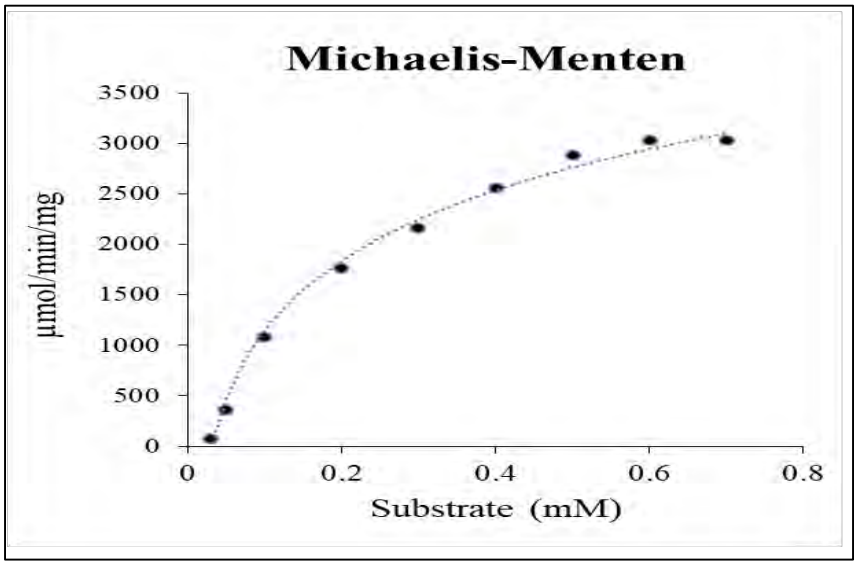


Figure 4. 59 Michaelis-Menten plot of LacSL7: *K*m, *V*max and *K*cat values were determined according to Michaelis-Menten plot.

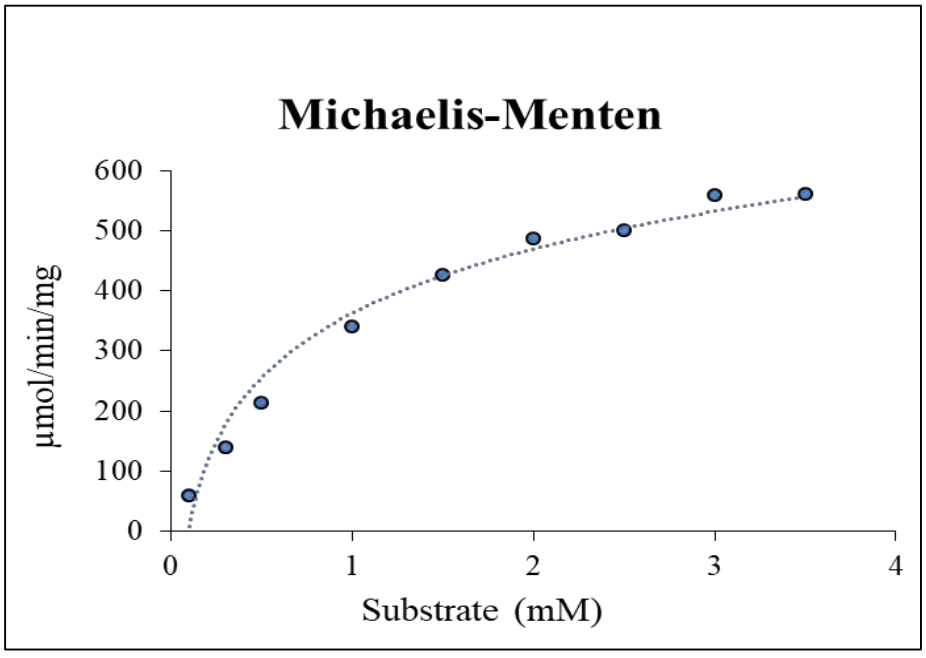


Figure 4. 60 Michaelis-Menten plot of DyPBL5: *K*m, *V*max and *K*cat values were determined according to Michaelis-Menten plot.

4.14. Enzymatic Hydrolysis of the Alkali Lignin

LacSL7 and DyPBL5 were used separately and in combination to degrade alkali lignin. Lignin degradation was carried out using medium containing 2.0 g of lignin and 5 μg of each purified enzyme at pH 5.0 in shaker incubator for 20 h. The reduction in lignin content was analyzed at regular intervals. During the course of degradation, the amount of alkali lignin degraded by LacSL7 reached to 31.2% which was higher than that of DyPBL5 (26.04%). When 2 enzymes were used in combination, the amount of alkali lignin degradation was reached to 62.4%. Results are presented in Fig. 4.61.

4.14.1. SEM

SEM analysis was performed to investigate changes in the surface of lignin particles after incubation with purified LacSL7 and DypBL5. Figure 4.57 indicates the breakdown of lignin particles after treatment with purified enzymes in comparison to the abiotic control that remained unchanged.

4.14.3. FT-IR

The FT-IR spectra of the enzymatically hydrolyzed lignin were assigned according to the literature. IR spectrum of samples are shown in Fig. 4.62. During the course of hydrolysis, the absorbance was decreased in the region of 3500-3000 cm^{-1} attributed to stretching frequency of -OH bonds of alcohol and phenol in lignin, which indicates lignin degradation. The peak in the region 1575 cm^{-1} corresponds to carbonyl groups in lignin, an increase in peak intensity was observed that indicates stretching of C═C bonds in the aromatic ring. The absorption peaks at 1240 cm^{-1} corresponds to production of degradation products, such as ethers, phenol, and alcohols. Increase in intensity of peaks in this region represents the high amount of lignin degradation products. The presence of guaiacyl (G) and syringyl (S) group in the untreated lignin sample assigned to the peaks of 1134 and 1038 cm^{-1}, absence of both peaks from treated samples showed enzymatic hydrolysis of both the (G) and (S) groups.

4.14.4. GC–MS

GC–MS analysis was performed to detect the degradation products of lignin after incubation with purified LacSL7 and DyPBL5. Fig. 4.63a & b indicates Total Ion Chromatograph (TIC) of low

molecular weight compounds extracted with ethyl acetate from acidic supernatant of enzymatically treated lignin and untreated control. The chromatographic peaks were assigned to compounds by using NIST standard mass spectral library on the basis of their RT and mass to charge ratio. Various low molecular weight compounds were detected in enzymatically treated lignin sample and were identified as Butyl alcohol, Acetic acid, Vanillin, Valeric acid, 2-methyoxyphenol, 3,4-dimethoxybenzyl alcohol, 3,4-dimethoxybenzyl alcohol, 4-Methoxy-3,5-dihydroxybenzoic acid, and p-Hydroxybenzoic acid. Details are presented in Table. 4.12.

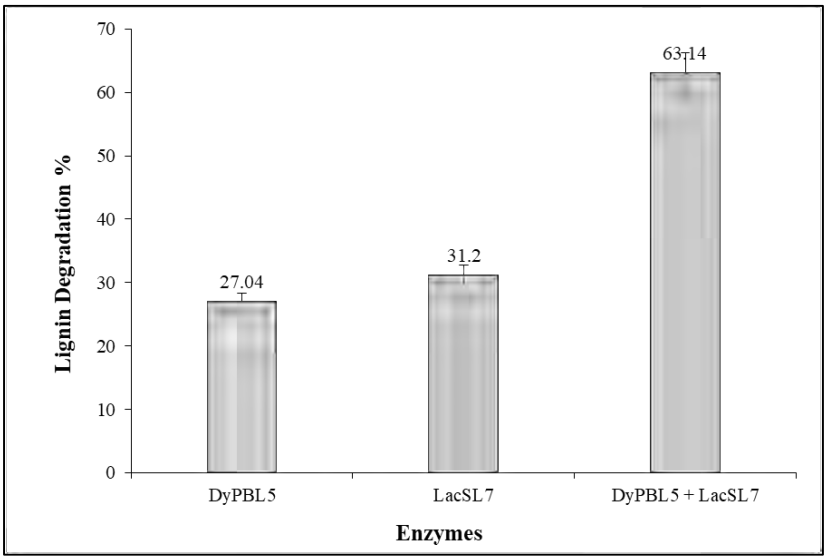


Figure 4. 61 Degradation of lignin by recombinant purified LacSL7, DyPBL5 and in combination of both enzymes. Error bars represent standard deviation of the mean (n = 3).

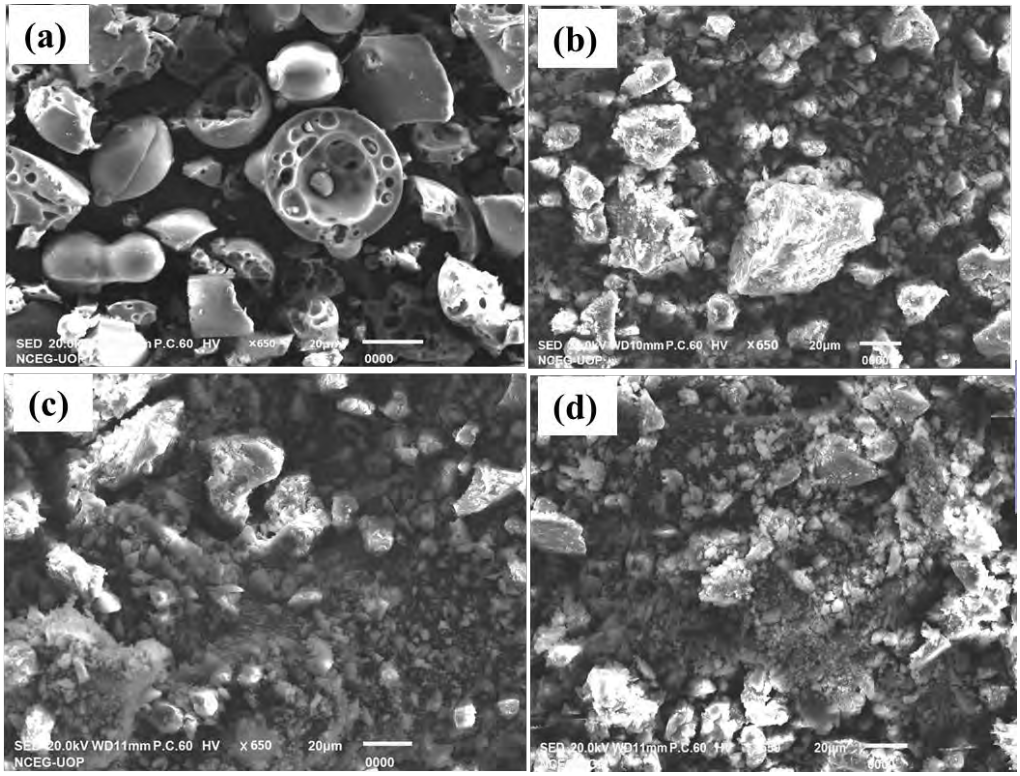


Figure 4. 62 SEM analysis carried out at resolution 650X for enzymatically treated lignin, a: Control (Uninoculated), b: DyPBL5 treated lignin, c: LacSL7 treated lignin, d: DyPBL5+LacSL7 treated lignin.

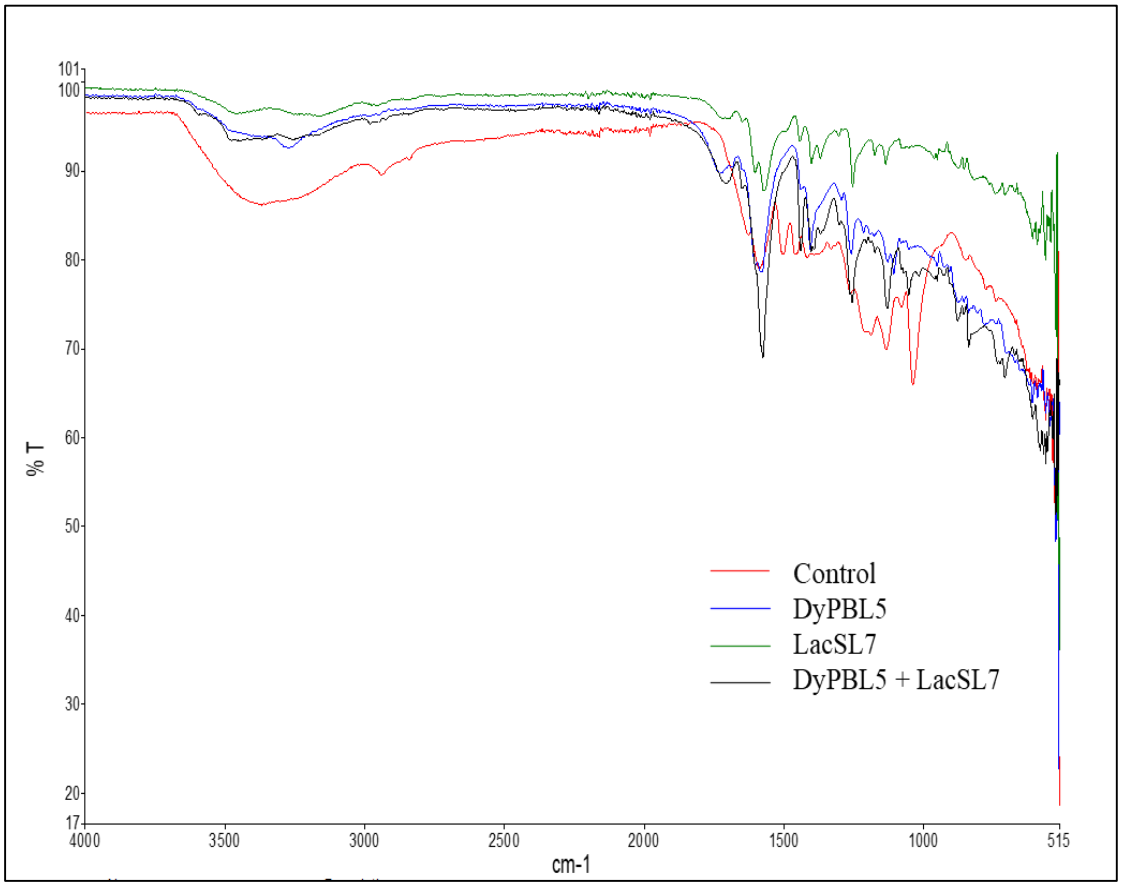


Figure 4. 63 FTIR spectra of enzymatically treated lignin samples compared with untreated lignin

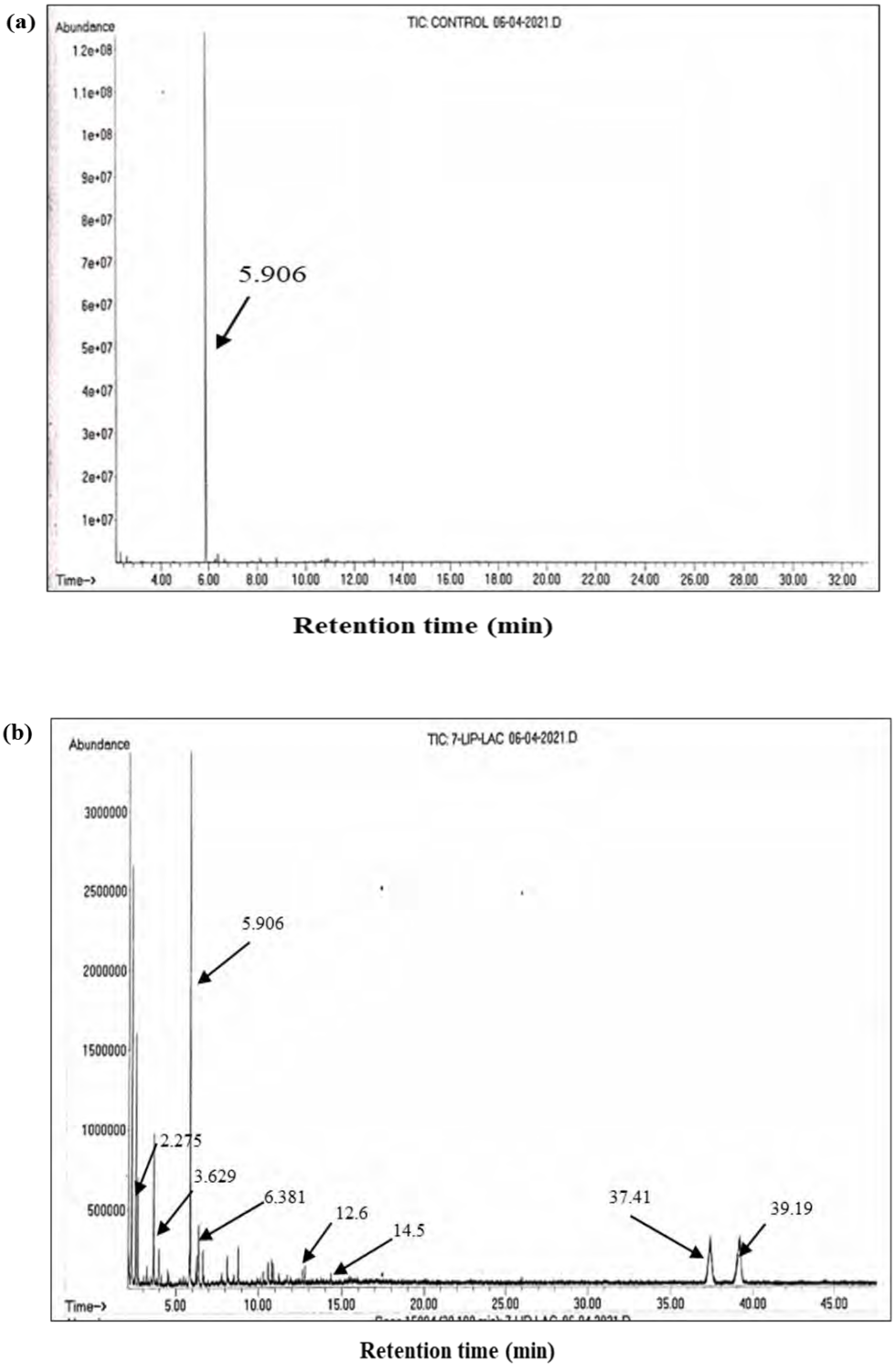
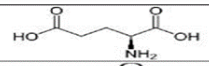
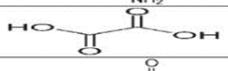
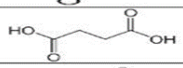
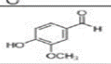
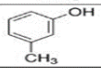
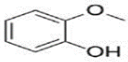
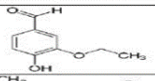
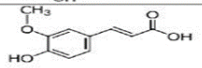
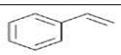
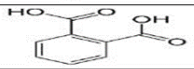
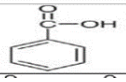


Figure 4. 64 Total ion chromatograph of TMS derivatized detected products, a: Control (untreated lignin), b: DyPLip+LacSL7 treated lignin

Table 4. 12 Lignin degradation products identified by GC-MS

Retention time (Min)	Product name	Product structure	m/z
2.275	Glutamic acid		147
3.629	Oxalic acid		91
	Succinic acid		119
	Vanillin		148
6.381	3-methylphenol		104
	2-methyoxyphenol		129
	Ethyl vanillin		167
	Ferulic acid		190
12.6	Styrene		169
14.5	Phthalic acid		166
37.41	3,5-dihydroxy-benzoic acid		159
	Benzoic acid		316

5. DISCUSSION

Several phenylpropane units such as syringyl alcohol, p-coumaryl alcohol and guaiacyl alcohol linked together to form heterogenous structure of lignin, linked through one of the most prevalent intermolecular bonds i.e β-O-4-ether bond. Rapidly growing cellulosic ethanol (Zhang et al., 2018) and pulp & paper industries are producing large amount of lignin residues as by a product annually (Naron et al., 2018). Majority of the lignin residues either burned as low-cost fuel to provide heat and power to industries or released in the aquatic resources without any treatment. The release of lignin accounts for undesirable coloration of water resources along with deterioration of aquatic flora and fauna (Singh et al., 2019) and only 2% of the available lignin residues is utilized for production of value-added chemicals (Gordobil et al., 2016). Decomposition and sustainable use of lignin represents the major challenges due to heterogeneous structure and recalcitrant nature of lignin (Bugg et al., 2015).

Several strategies have been implemented to depolymerize the industrial lignin, such as membrane filtration, sedimentation, chemical oxidation, ozonation and biological treatment (Abdelaziz et al., 2016). Among these strategies, biological treatment involving the use of ligninolytic enzymes is considered as one of the promising routes, due to targeted oxidation of substrate, mild reaction conditions, low energy requirements, and less production of toxic waste (Zhang et al., 2020). Several researchers have reported bacteria from soil that are able to metabolize lignin and lignin model compounds through the production of extracellular peroxidases but the enzymology of bacterial lignin depolymerization is not well characterized. The recombinant DNA technology has improved the production of enzymes from bacteria, animals and plants, and applications of enzymes on industrial level by improving the stability and specificity of enzymes.

In the present study, it was aimed to isolate the efficient ligninolytic bacteria from pulp and paper mill effluent, by assuming the presence of potential lignin-degraders in the area that contains lignin and other toxic chemicals. Several researchers have reported the isolation of lignin-degrading bacteria from lignin contaminated sites that were successfully applied for remediation of pulp and paper mills effluent (Barapatre et al. 2017; Menon & Hartz-Karp 2019). The characteristic features of wastewater are good indicators of effluent toxicity. The collected effluent sample was dark brown and alkaline in nature with high concentration of lignin, sulphates, nitrates and chemical oxygen demand. The effluent was treated through an aerated lagoon system at industrial site that

reduced the pollutant concentration but still, it remained beyond the permissible limits as recommended by EPA (USEPA 2002). Lignin is recalcitrant in nature and major constituent of lignocellulosic biomass, presence of lignin and its derivatives possibly contributed to the dark brown color of effluent and high concentration of COD (Rice et al. 2017). The source of sulfate in the effluent might be sodium sulfite, which is used during pulping process (Singhal and Thakur, 2009).

Initially, qualitative and quantitative tests were performed for screening of ligninolytic bacteria. 55 bacterial strains were isolated from effluent, suggesting that the study site is rich in bacterial microbiota. Further investigation of the site with modern technique such as culture independent technique will explore more potent strains with potential application in bioremediation technology. Lignin tolerance experiments were carried out on minimal salt medium plates containing lignin as a sole carbon source. 08 bacterial strains showed remarkable ability to grow well in the presence of high concentration of lignin (3 g/L), utilizing lignin as a sole carbon source. Only potent bacteria can survive in the presence of a high concentration of lignin because lignin-derived aromatics have harmful effects and causes cell death by membrane disruption, DNA damage, and enzyme inhibition (Zeng et al. 2014), therefore, isolated strains were selected and screened for laccase and peroxidase production on substrates amended nutrient agar plates. Only SL7, BL2 and BL5 strains were found efficient laccase producers, indicated by the appearance of brown color around bacterial growth on guaiacol agar plates. The guaiacol has been previously used for laccase assay and proved to be the most suitable substrate because laccase causes oxidative depolymerization of guaiacol and appears in the form of brown color, which is easy to detect (Kumar et al. 2020a). While strain SL4, BL5, IP4 and AT4 were found positive for peroxidase activity, indicated by the appearance of decolorization zone around bacterial colonies. The azure B was turned to colorless compound by the reduction of C=N to –NH due to the action of peroxidases, and then the –NH further combines with –OH and forms colorless compound by dehydration reaction (Li et al., 2014).

Alkali lignin degradation study was performed with newly isolated bacterial strains AT4, $AT_2$4, IP4, SL7, SL4, BL2, BL3 and BL5, and degradation was investigated in synthetic wastewater prepared by using 3000 g/L lignin. Additionally, 0.25 % peptone and 0.5 % glucose were added to the degradation medium as a source of nitrogen and carbon. Among 08 bacterial strains, only

BL5 and SL7 efficiently degraded lignin with 35-44% reduction in lignin and 22-26% reduction in color within 7 days of incubation. A significant reduction in lignin was observed after 3 days of incubation with maximum reduction on day 05. Despite of fast bacterial growth during the initial days of incubation, a significant reduction in color and lignin contents was achieved on day 5, which indicates the phenomena of co-metabolism adopted by bacteria. It could be possible that bacteria utilized glucose and peptone as a carbon and nitrogen source to initiate their growth followed by the utilization of lignin as a co-substrate. Similarly, various authors have also reported lignin degradation by co-metabolism by fungi and bacteria (Singhal & Thakur 2009; Singh & Chandra 2019). Chandra et al. 2009 has reported *Bacillus cereus* strain ITRC-S6 and *Serratia marcescens* strain ITRC-S7 with reduction in lignin by 30-40% and color by 45-52 % of 0.5 g/L of lignin. In another study when lignin degradation was investigated by using *Bacillus ligniniphilus* L1, 38.9 % lignin reduction was achieved (Zhu et al., 2017). Strain BL5 and SL7 could be of interest for the degradation of high load of lignin from pulp and paper mills effluent. Lignin degradation was further confirmed by evaluating the surface changes and structural changes of lignin through SEM. SEM analysis clearly indicated significant transformation of lignin by strain SL7 and BL5 during treatment. Kumar et al. (2015) also observed similar surface destruction of lignin after bacterial treatment.

Bacterial peroxidases and laccases are involved in breakdown of lignin by oxidizing aromatic unit of lignin in the presence H_2O_2 and molecular oxygen, respectively. One electron is extracted from lignin, leading to the generation of free radicals from the substrate molecules, and oxygen molecules are reduced to water. The generated free radicals are unstable and undergo polymerization or depolymerization reactions, causing lignin degradation (Zeng et al., 2014). During the course of lignin degradation extracellular laccase (Lac) and lignin degrading peroxidase (LDP) activities were measured. Strain SL7 attained maximum Lac activity with 1.3 IU/ml and strain BL5 attained maximum LDP activity with 1.19 IU/mL on day 05 of the incubation which is in accordance with maximum lignin degradation. Lignin degrading peroxidase and laccase activities obtained in this study were higher as compared to previous reports on *Bacillus* strains (Chen et al., 2012a; Shi et al., 2013). On the basis of lignin degradation efficiency and ligninolytic enzymes production, strain BL5 and SL7 were selected for production of recombinant enzymes. Strain SL7 was identified as *Bacillus altitudinis*, and BL5 was identified as *Bacillus* sp.

Production of enzymes can be enhanced through optimization of different culture conditions using conventional and statistical methods. Temperature and pH are significant factors that can affect the growth of microorganism, enzymes production and stability of their enzymes by modifying 3D structure of proteins (Lei et al., 2000). Maximum Lac and LDP production was observed at 30°C. Yadav and Chandra, 2015 reported maximum yield of ligninolytic enzymes at 32 °C, similar to other metabolic enzymes.

Several carbon sources such as maltose, fructose, sucrose, starch, and glucose were used to investigate their influence on the production of lignin degrading peroxidase. The highest enzyme activity was found in the presence of glucose as carbon source and these results were similar to the findings of Batool et al. (2018), who reported glucose as best carbon source for maximum production of enzyme. In another study, Kanwal and Ready (2011) used different carbon sources for ligninolytic enzyme and found maximum activity with glucose as a carbon source. While, maximum laccase production was observed in the presence of starch. Previously, different carbon sources for laccase production has been reported. Ding et al. (2012) has reported laccasse production from *Ganoderma lucidum* with glucose as an effective co-substrate. However, fructose was observed by Johnsy and Kaviyarasan (2011) as best co-substrate for laccase production by *Lentinus kauffmanii*.

Nitrogen source is essential for growth of microorganisms as it is prime building block of nucleic acids, proteins and other cellular components. Different sources of nitrogen have been used in production media. It can be utilized in inorganic and organic form. In current study, organic nitrogen sources used for LDP and Lac were peptone, yeast extract, urea and beef extract while inorganic source was ammonium nitrate. Maximum lignin degrading peroxidase production and laccase production was found in the media containing peptone and yeast extract, respectively. Chandra et al. (2007) reported medium having glucose and peptone as additional carbon and nitrogen sources were found to enhance the growth of lignin degrading bacteria and production of ligninolytic enzymes. To optimize the rest of the components for optimal production of LDP and Lac, the Placket-Burman and central composite designs were used. Various studies reported the usage of these software for optimization of medium compositions for enzyme productions (Mazzucotelli et al., 2015). Using central composite design the highest LDP activity was observed as 56.9 U/mg and lowest activity was 0.29 U/mg, and inoculum size, glucose, peptone, lignin and

ammonium nitrate were found to be significant factors in production of LDP. While, highest laccase activity was observed to be 29.5 U/mg and lowest was 4.33 U/mg, and $MgSO_4$, $(NH_4)_2SO_4$, $FeSO_4.7H_2O$ and K_2HPO_4 were found to be significant factors. According to the literature review the best parameters for lignin breakdown and enhance production of ligninolytic enzymes in effluent differ substantially from one bacterial strain to other and environmental variables. These variables include pH, temperature, nitrogen and carbon sources. Response surface methodology was proved to be a valuable and dependable tool for the optimization of laccasse and lignin degrading peroxidase production from *Bacillus* species.

After attaining the best media composition by conventional optimization LDP from *Bacillus* sp. and Lac from *Bacillus altitudinis* were purified through acetone precipitation and gel permeation, purity of enzymes was analyzed through SDS-PAGE. The molecular weight of LDP was 45 kDA, which is lower than the 65 kDa of *Bacillus megaterium* (Patil et al., 2014). Lignin peroxidase of *Serratia liquefaciens* was fully purified by DEAE and was reported to be only 28 kDA (Haq et al., 2016). Lac from *Bacillus altitudinis* was observed with molecular weight of 55 kDa, and laccase from *Bacillus licheniformis* was reported to be 65 kDa (Lu et al., 2013).

The lignin degrading DyP-type peroxidase gene of 1251 bp from *Bacillus* sp. strain BL5 (DyPBL5) and laccase gene of 1500 bp from *Bacillus altitudinis* strain SL7 (LacSL7) was cloned and expressed in *E. coli* BL21 (DE3) cells. Recombinant enzymes were functionally active and enzyme activity was also higher as compared to the native enzymes, which can be attributed to higher expression levels of protein. Various studies reported the peroxidase and laccase gene from Gram positive and Gram negative bacteria including *Bacillus* have also been cloned and expressed in *E. coli* (Verma et al., 2013). Therefore, it was determined that *E. coli* is suitable host for cloning of ligninolytic enzymes.

The DyP gene encoded open reading frame of 418 amino acids, and calculated molecular mass was around 46 kDa. The sequence analysis of the deduced amino acids was confirmed to represent the DyP-type peroxidase encoding protein, and showing 32-99% similarity to other DyP proteins present in genome of neighboring species (Sahinkaya et al., 2019; Yang et al., 2019; Min et al., 2015). Laccase gene encoded 500 amino acids and calculated molecular weight was around 56kDa. The sequence analysis of recombinant laccase was confirmed to represent the laccase encoding protein, and showing more than 99% similarity with laccases from other *Bacillus* species (Liu et

al., 2017; Muthukumarasamy et al., 2015; Yuan et al., 2015). DyPBL5 and LacSL7 protein was purified by ion exchange column chromatography, active fractions were polled and analyzed by SDS-PAGE. The online molecular weight calculator ExPASy was used to estimates the molecular mass of the proteins based on molecular weight of each amino acid residues included in protein. The molecular weight of recombinant DyPBL5 and LacSL7 was observed to be 46 kDa and 56 kDa on SDS-PAGE, respectively which is in accordance with calculated protein mass. 50 kDa of *Bacillus amyloliquefaciens* (Yang et al., 2019) and 50 kDa of *Streptomyces avermitilis* DyP-type peroxidases (Sugawara et al., 2017) were the most similar in size with the *Bacillus* sp. strain BL5, and 30 kDa of *Rhodococcus* sp. T1 DyP-type peroxidase has been reported, which is lower than the peroxidase of strain BL5 (Sahinkaya et al., 2019). Kumar et al (2017) reported recombinant laccase from *Pandoraea* sp. ISTKB with molecular weight of 49 kDa.

The temperature dependent activity and stability of recombinant purified DyPBL5 and LacSL7 were tested in the range of 25-95ºC. The DyPBL5 exhibited optimum activity at 35ºC, most of the previously studied DyP-type peroxidases from *Bacillus* and other bacteria have been reported to operate at an optimum temperature of 30-35ºC (Yang et al., 2019; Sahinkaya et al., 2018; Min et al., 2014; Santos et al., 2014). The DyPBL5 retained more than 60% residual activity at 25-35ºC for 24 h, followed by a gradual decrease in stability with increase in temperature. The temperature of effluent containing lignin released from pulp and paper industries is usually in the range of 35-40ºC, since DyPBL5 has optimum temperature of around 35ºC, it might be therefore useful to use this enzyme directly to the effluent. Several researchers have reported DyP peroxidases from *Streptomyces avermitilis*, *Rhodococcus* sp. T1 and *Pseudomonas putida* that were found stable at mesophilic temperature (Sahinkaya et al., 2019; Rahmanpour et al., 2015). A DyP-type peroxidase was reported from *Bacillus adusta*, retained 50% of its activity at 50ºC for 2h (Ahmed et al., 2011). LacSL7 exhibited optimum activity at 55°C, and was stable over the wide range of temperatures. It maintained more than 50% of activity at 30-70°C for 24 h, and more than 80% at 30-60°C for 16 h. Previously, different studies have reported thermo-stable bacterial laccases. *Bacillus tequilensis* SN4 laccase was also active at 80°C and stable at high temperature (Sondhi et al., 2014). Extracellular laccase from *Bacillus subtilis* OH67 and *Pandoraea* sp. ISTKB is optimally active at 50°C (Hajipour et al., 2020; Kumar et al., 2018). Almost similar temperature profile was also observed in *Klebsiella pneumonia* recombinant laccase and *Bacillus coagulans* laccase

(Ihssen et al., 2015; Liu et al., 2017). The stability of laccase from *Bacillus altitudinis* at high temperature makes LacSL7 valuable for other applications such as bio-bleaching and bio-pulping.

The pH of activity and stability for DyPBL5 and LacSL7 were tested in the range from 3-10. The optimum DyPBL5 activity was found at pH 4, and on the other hand, DyPBL5 retained more than 50% of its activity at pH 5-6 after 24 h of incubation, followed by low stability at pH other than extreme acidic or alkaline range. The peroxidases reported from *Rhodococcus* sp. T1, *Bacillus subtilis*, *Pseudomonas putida* and *Cellulomonas bogoriensis* were found to have optimum activity between pH 4.0-5.0 (Sahinkaya et al., 2019; Santos et al., 2014; Habib et al., 2019). The presence of aspartic acid D240 at the catalytic site of DyPBL5 accounts for activities at low pH. Sugano et al. (2007) highlighted the role of aspartic acid in the active site of DyP from *Bacillus adusta* by point mutation at D171N position. The optimum pH of laccase from *Bacillus altitudinis* SL7 was found to be 5, which differ from *Alcaligenes faecalis* laccase, reported to work under neutral to alkaline pH range (Mehandia et al., 2020). Other laccases such as a non-blue laccase from *Bacillus amyloliquefaciens*, *Pantoea ananatis* Sd-1 and *Paenibacillus glucanolyticus* work optimum under acidic conditions (Sondhi et al., 2021). The pH profile of white laccase from fungus *Myrothecium verrucaria* NF-05 was almost similar with present study (Zhou et al., 2014).

Both, lignin degrading peroxidase and laccase are metal containing enzymes, therefore addition of metals can modify and stabilize enzyme activity. The activity of DyPBL5 and LacSL7 was stable at low concentration of tested metals, while the activity was decreased upon increase in metal concentration. As per the previous reports, the activity of dye degrading peroxidases from *Anabaena* sp. (Sun at al., 1999), *Pseudomonas aeruginosa* (Li et al., 2012) and *Streptomyces* sp. (Fodil et al., 2012) and laccase from *Alcaligenes faecalis*, *Cerrena* sp. HYB07 and *Pandoraea* sp. ISTKB (Kumar et al., 2018; Mehandia et al., 2020; Yang et al., 2014) decreased with increase in metals concentration. DyP-type peroxidases are metal containing H_2O_2 dependent enzymes, their activity is negatively affected with increase in metal concentration as a result of decomposition of H_2O_2 (Ogola et al., 2009). Fe^{2+}, Co^{2+} and Hg^{2+} strongly inhibited the DyPBL5 and LacSL7 activity at 5 mM concentration. Similar results were also observed for DyP peroxidases from *Pseudomonas aeruginosa* and *Irpex lacteus* (Li et al., 2012; Salvachúa et al., 2013), and laccases from *Bacillus tequilensis* and *Bacillus* sp. MSK-01 (Sondhi et al., 2020). Fe^{2+} and Co^{2+} have high potential for binding to enzymes and disrupt the electron transport system of enzyme. Hg^{2+} ions causes

inactivation of enzymes by binding with sulfhydryl groups on an enzyme, which are parts of the enzyme that contain a sulfur atom that is attached to a hydrogen atom (-SH). Binding of mercury can change the shape of the enzyme and block its activity (Goulding et al., 2021).

Surfactants have significant role in industries so these are widely used in different industries, therefore it is desirable property of an enzyme to work in the presence of surfactants. Thus, the influence of different surfactants on the stability of DyPBL5 and LacSL7 was also investigated. Among these surfactants, non-ionic surfactants such as Triton X, Tween 20 and Tween 80 enhanced the enzyme activity at low concentration (1% v/v). The non-ionic surfactants increases the stability of enzymes due to their hydrophobic interactions to the enzymes which increases protein folding (Gulcin et al., 2003). SDS strongly inhibited the DyPBL5 activity, inhibition of enzyme by SDS can be due to the fact that it is a harsh ionic detergent and reacts with hydrophobic regions of enzymes, which causes modification in its 3-D structure. While LacSL7 activity was not much effected by SDS. Laccase from *Bacillus* sp. MSK-01 has also reported to be stable in the presence of SDS (Sondhi et al., 2021).

Chelating and reducing agents are known to inhibit the activity of ligninolytic enzymes because of their obvious nature. The behavior of enzyme in the presence of different inhibitors such as PMSF, DTT, DEPC, EGTA, EDTA and β- mercaptoethanol was also studied. DDT and β-mercaptoethanol completely inhibited the DyPBL5 and LacSL7 activity. DTT and β-Mercaptoethanol are reducing agents and decrease enzyme activity by preventing the formation of disulfide bridges in cysteine-containing proteins (Sahinkaya et al., 2019). These results are in confirmation with the results obtained by *Bacillus* sp. MSK-01 (Sondhi et al., 2021), *Bacillus* sp. ADR (Telke et al., 2011) and *Bacillus tequilensis* SN4 (Sondhi et al., 2014) which showed significant inhibition in laccase activity in the presence of DTT.

The activity of DyPBL5 and LacSL7 in the presence of organic solvents was also investigated. Among the tested organic solvents, DMSO and dioxane strongly inhibited the DyPBL5 activity at lowest tested concentration (10% v/v). The effect of organic solvents on enzyme activity depends on the polarity of active site, which could lead to activity inhibition by reorientation of the bound substrate. The activity of DyPBL5 and LacSL7 was increased in the presence of methanol and ethanol, while decreased in the presence of butanol. Since ethanol have two carbon atoms in main

carbon chain while butanol have four carbons, so the extra carbon atoms might be involved in imparting competitive binding with substrate in active site.

Kinetic parameters were determined spectrophotometrically using different concentrations of ABTS ranging from 0.5-5 mM. The catalytic efficiency was calculated by Michaelis-Menten plot for both enzymes. The DyPBL5 exhibited *Km*, *Vmax*, and *Kcat* values 1.06 mM, 519.75 µmol/min/mg and 365 s^{-1}, respectively. Kinetic parameters reported in this study are higher than DyP-type peroxidases of *Thermobifida fusca* (*Km* 0.86 mM and *Kcat* 28.1 s^{-1}) (Rahmanpour et al., 2016), *Streptomyces avermitilis* (*Km* 0.97 mM and *Vmax* 1.46 µmol/min/mg) (Sugawara et al., 2017), *Pseudomonas fluorescens* (*Km* 1.13 mM and *Kcat* 13.5 s^{-1}) (Rahmanpour et al., 2015) *Bacillus subtilis* (*Vmax* 66.6 µmol/min/mg) (Min et al., 2014) and lower than *Rhodococcus jostii* RHA1 peroxidase (*Km* 0.94 mM and *Vmax* 1417.53 µmol/min/mg) (Sahinkaya et al., 2018). LacSL7 exhibited *Km*, *Vmax*, and *Kcat* values 0.28 mM, 2073 µmol/min/mg and 3878 s^{-1}, respectively. Laccases of *Bacillus tequilensis* SN4 (*Km* 0.08 mM and *Kcat* 261 s^{-1}) (Sondhi et al., 2014), *Bacillus* sp. WT (*Km* 0.132 mM and *Kcat* 309 s^{-1}) (Siroosi *et al.,* 2016), *Klebsiella pneumoniae* (*Km* 0.47 mM and *Vmax* 398 µmol/min/mg) (Niladevi et al., 2008), *Bacillus subtilis* OH67 (*Km* 1.107 mM and *Vmax* 19 µmol/min/mg) (Hajipour et al., 2020), *S. cyaneous* CECT 3335 (*Km* 0.38 mM and *Vmax* 55.55 µmol/min/mg) (Selin Ece et al., 2017) and *Trichoderma harzianum* WL1 (*Km* 0.18 mM and *Vmax* 302 µmol/min/mg) (Sadhasivam, et al., 2008) was reported with lower catalytic efficiency than LacSL7.

The recombinant purified DyPBL5 and LacSL7 were used separately and in combination for degradation of alkali lignin. Lignin degradation was studied in the degradation medium containing 2.0 g of lignin. The amount of alkali lignin degraded by LacSL7 was found to be 31.2 % which was higher than that of DyPBL5 (26.04%). When both enzymes were used in combination, the amount of alkali lignin degradation was reached to 62.4 %. The lignin reduction was higher than reported by Zhang et al. (2020), with reduction of 16 %, 22.15 %, and 26 % by lignin peroxidase, laccase and combination of both enzymes, respectively. Several researchers have reported the lignin degradation by DyP class of peroxidases from different organisms such DyP from *Pseudomonas fluorescens* pf-5 (Rahmanpour et al., 2015), TfuDyP from *Thermobifida fusca* (Van Bloois et al., 2014), SviDyP from *Saccharomonospora viridis* DSM43017 (Yu et al., 2014) and DyP from *Rhodococcus jostii* (Singh et al., 2013). It has been suggested in studies that,

microorganism producing multiple ligninolytic enzymes can degrade lignin more efficiently than the ones producing only single ligninolytic enzyme. White rot fungi have been reported to degrade lignin effectively due to the coexistence of multiple ligninolytic enzymes (Knezevic et al., 2013). So, it is concluded that microbial degradation of lignin is achieved by synergy between enzymes.

SEM analysis also confirmed the breakdown of lignin particles after treatment with purified DyPBL5 and LacSL7 in comparison to the abiotic control that remained unchanged. Zhang et.al (2020) reported similar surface changes in lignin after treatment with ligninolytic enzymes. FT-IR analysis showed that the absorbance was decreased in the region of 3500-3000 cm^{-1} attributed to stretching frequency of -OH bonds of alcohol and phenol in lignin, which indicates lignin degradation (Rahmanpour et al., 2015). The absorbencies and shapes of the peaks in the region of 1600-1400 cm^{-1} has been changed, which corresponds to the stretching of C=C bonds in the aromatic skeleton of lignin, meaning that aromatic ring was destroyed during degradation (Wang et al. 2021). The ligninolytic enzymes specifically attack C=C bonds in aromatic skeleton of lignin that leads to enzymatic depolymerization of lignin structure (Zeng et al. 2014). The absorption peaks at 1240 cm^{-1} corresponds to production of lignin degradation products, such as ethers, phenol, and alcohols. The presence of guaiacyl (G) and syringyl (S) group in the untreated lignin sample assigned to the peaks of 1134 and 1038 cm^{-1}, absence of both peaks from treated samples showed enzymatic hydrolysis of both the (G) and (S) groups (Pandey et al., 2003). Increase in intensity of peaks represents the high amount of lignin degradation products by recombinant DyPBL5 and LacSL7. Previously, different studies have reported the similar spectrum for enzymatic degradation of lignin (Sonkar et al. 2019). Based on previous studies and combined with the results of current study, it has been shown that the representative chemical bonds in alkali lignin were disrupted and indicated that the combination of recombinant DyPBL5 and LacSL7 facilitated alkali lignin degradation.

Various low molecular weight compounds were also detected in enzymatically treated lignin sample and were identified as Butyl alcohol, Acetic acid, Vanillin, Valeric acid, 2-methyoxyphenol, 3, 4-dimethoxybenzyl alcohol, 3, 4-dimethoxybenzyl alcohol, 4-Methoxy-3, 5-dihydroxybenzoic acid and p-Hydroxybenzoic acid. The detection of phenolic compounds in degradation medium was clear indication of lignin depolymerization by DyPBL5 and LacSL7. The production of acids during lignin degradation was possibly due to the enzymatic degradation of

phenolic side chain into ketones, which were further degraded through Cα–Cβ cleavage to form acids (Zhang et al., 2020). Formation of vanillin and methoxyphenol confirmed the oxidation of coniferyl and sinapyl groups of lignin polymer (Raj et al., 2007), and similar branch destruction was also indicated by FT-IR analysis of treated samples. Lignin degrading peroxidases catalyze the α, β and β-ether bonds by using H_2O_2 as an electron acceptor, and leads to efficient depolymerization of lignin into derivatives such as 2- methoxyphenol and 3,4-dimethoxybenzyl alcohol. This finding was consistent with previous reports (Feng et al., 2019; Munk et al., 2018; Rich et al., 2016). We demonstrated here that high catalytic efficiencies and lignin degradation rate makes DyPBL5 and LacSL7 ideal bio-catalysts for remediation of lignin-contaminated sites.

6. CONCLUSIONS

i. *Bacillus* sp. BL5 isolated from black liquor and *Bacillus altitudinis* SL7 isolated from sludge were found to be most efficient lignin degrading bacteria.

ii. Lignin degrading peroxidase gene from strain BL5 and laccase gene from strain SL7 were successfully cloned into intracellular expression vector pET28a (+) and expressed into *E.coli* BL21 (DE3), and purified through ion exchange chromatography.

iii. Purified *Bacillus* sp. BL5 lignin degrading peroxidase provides optimum activity at pH 4 and temperature 35˚C, while *Bacillus altitudinis* SL7 laccase provides optimum activity at pH 5 and temperature 55˚C. Both enzymes worked efficiently in the presence of majority of metals ions at low concentration but slightly inhibited at high concentration.

iv. Recombinant enzymes were highly active and kinetic parameters for lignin degrading peroxidase were 1.06 mM, 519 µmol/ min/mg and 395 S^{-1} for Km, Vmax and Kcat, respectively and kinetic parameters for laccase were 0.28 mM, 2073 µmol/min/mg and 3878 s^{-1} for Km, Vmax and Kcat, respectively.

v. 62% lignin was removed from lignin degradation medium when treated with recombinant enzymes. Enzymatic treatment caused significant structural changes in lignin which was confirmed by SEM and FT-IR analysis and 12 major lignin degradation products were identified by GC-MS.

7. FUTURE PROSPECTS

i. Application of ligninolytic enzymes can be extended to other industries like chemicals, textile, food, biofuel and can also be used for other applications in pulp and paper industries like biopulping and biobleaching

ii. Thermal stability of lignin peroxidase can be enhanced through molecular biology techniques such as adding proline tag to the protein sequence to enhance its thermal stability.

iii. Lignin degrading peroxidase and lacasse can be more efficiently tailored made through protein engineering for utilization of lignin degradation products.

iv. The experiments can be extended to pilot scale and industrial effluent degradation

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9. APPENDICES

Appendix 01: Sodium acetate Buffer

Componants	**Volume**
Sodium acetate	7.721 g
Acetic acid	0.353 g
Water	800 mL

Appendix 02: 10X TBE Buffer

Tris Base	108g
Boric acid	55g
0.5 M EDTA	40mL
Total volume	1000 mL

Appendix 03: *Bacillus altitudinis* strain SL7 16S ribosomal RNA gene, partial sequence (MZ400969)

ATGGTACCGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTA
GCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTAAACC
TTGCGGTCTCGCAGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGA
CGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCA
AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACT
CTGTCCCCGAAGGGAAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTT
CGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCC
CCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTAC
GGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGA
GTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCTTCTTCTGC
ACTCAAGTTTCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTG
CGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGC
CGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCAAGCAGTTACTCTTGCACTTGTTCTTCCCTAACAACAGAGCTTTA
CGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTG
CCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGC
CTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGACAGCCGAAACCGTCTTTC
ATCCTTGAACCATGCGGTTCAAGGAACTATCCGGTATTAGCTCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCA
GGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCCGGGAGCAAGCTCCCTTCTGTCCGCTCGACTTGCATGT
ATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCATGATCAAACTCTAGAAT

Appendix 04: *Bacillus* sp. strain BL5 16S ribosomal RNA gene, partial sequence (MZ413436)

ATGGTACCGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTA
GCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCT
CGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGAC
GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAA
GGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTC
TGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTTCTTGCGACCGTACTCC
CCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTA
CGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCGCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAG
AGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTG
CACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCT
GCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAG
CCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTT
TACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGC
TGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTT
GCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTT
TTATATTTGAACCATGCGGTTCAAATAAGCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGC
AGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCAT
GTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCATGATCAAACTCTAGAAT

Appendix 05: 1M Sodium Phosphate Buffer

Components	**Amount**
Na_2HPO_4. $7H_2O$	10.9 g
NaH_2PO_4. $2H_2O$	3.1 g
Water	1000mL

Appendix 06: Sodium Citrate Buffer

Components	**Amount**
Sodium Citrate	17.099 g
Citric acid	8.041 g
Water	1000 mL

Appendix 07: SDS-PAGE Loading Dye

Reagents	**Volume**
10% SDS	400 µL
80% glycerol	250µL
Tris (pH 6.8)	150µL
1% bromophenol blue	140µL
β-mercaptoethanol	60µL
Total volume	1000µL

Appendix 08: SDS-PAGE Running Buffer

Reagents	**Volume**
Glycin	21.6 g
Tris base	4.5 g
10 % SDS	15 mL
Water	1.5L

Appendix 09: SDS Resolving Running Gel (12%)

Reagents	**Volume**
30% and 8% acrylamide/bis	2
Tris (1.5 M, pH 8.8)	1.3
SDS 10%	0.05 mL
Water	1.6
TEMED	0.002 mL
10% APS	0.05

Appendix 10: SDS-PAGE Stacking Gel

Reagents	**Volume**
30% and 8% acrylamide/bis	0.5
Tris (1.5 M, pH 8.8)	0.38
SDS 10%	0.03
Water	2.1
TEMED	0.02
10% APS	0.03

Appendix 11: Protein Dye Solution

Reagents	Volume
Water	40 mL
Methanol	50 mL
Acetic acid	10 mL
Coomassie brilliant blue R-250	0.125 g
Total volume	100mL

Appendix 12: Destaining Solution I

Reagents	**Volume**
Water	40 mL
Methanol	50 mL
Acetic acid	10 mL
Total Volume	100 mL

Appendix 13: Destaining Solution II

Reagents	**Volume**
Water	132 mL
Methanol	7.5 mL
Acetic acid	10.5 mL
Total volume	150 mL

Appendix 14: Luria-Bertani Broth

Bacterial tryptone	10 g
Yeast extract	5 g
Nacl	5 g
Total volume	1000 mL

Appendix 15: Antibiotics

Antibiotic	**Working concentration**	**Stock solution**
Ampicillin (Amp)	50 µg·mL-1	50 mg·mL-1 in H_2O Stored at -20°C
Kanamycin (Kan)	50 µg·mL-1	50 mg·mL-1 in H_2O Stored at -20°C

Appendix 16: Bradford Reagents

Reagents	**Volume**
Coomassie brilliant blue R-250	50mg
Methanol	50 mL
85 % Phosphoric acid (w/v)	100ml
Water	500 mL

Appendix 17: Laccase gene sequence from *Bacillus altitudinis* strain SL7

ATGCCTCCTTCAACCCGACAAAAGACATCATACGTCCTGTTTTCCCTTGATCACGCCGATGTGAGAAGAA
ATGCTGCTCATTGCAACTTGTGCACATCGAACTGACATGTATGTTTTCTTCTTTTAATCCTGCATGAAGTA
ATACGTTTTTGTTTAATGTTTTTAAGTCAATTTTGTATTGTCCTTGTGACATCTCGGAATACACATCCTCTG
TTGAAAAAGGAAGCTGTTTCACTTGATCTATGACAACATCATCTACTATGTAGCAGCAGCTGCCAATGGA
TGGTCCAATGACGACTTGTATATGATCGGCATCAGCGCCTTCTTGGCGAACCCATGCATCAACCATGTTG
GCGCCAATTTGTTTGACGGTTCCTTTCCAGCCGGCATGAGCCGTTCCAATCAGCCCAGATTGCGGCGCTA
GAAAATAAAGCGGAACACAGTCAGCGAAACAAAGAGCGAGCATGATGTTTGGTTCAGAGGTGTAAAAC
CATCTGTTCTAGAGAGTGCTTCATCATACTGAAGACTTCCTCTTCCCTTATGTTCCTTTGTGACTTTCATGA
TGCGATCCTCGTGTGTTTGGTCAGCAAACACCCAATTAGAGAGCGGCACAGCAGTAGCATCTGCATGAC
TTGACGATTCATTTGGACACTAGAAGCATCATCTCCTACATGGAGTCCAGTGTTCATTGATTGATATGGA
GAAAGACTAAAGCCGCCATTTTTTGTCGTAAAACCGGCCAATATCTCTTTTCCAGAACATGTCATGTTCGT
CCAATCATGAATCAGCATTGCATCAGGTGATTTCTGCTGAAAGGGGTCATATTTGTCAAGCGGAATCCCA
AAGGGAAACGTCAAATCCGGACCCTCAAGGGCCAAACCTTTCCGGTTGGCTTGAACGCCCCAAAAAAAA
ATATTTCCGGGGGGGGATGGGACACCAAAATTTTATAATTCTCAAAAAATCGCGGTTTTTTTACAAATTTTT
CCGGGGTGAACAACCGCCCCCTTTTCTTCAAAAAACGGGAAAAAGAAGAGGCGCGGGGGGGGATGATT
TATTCCCCCGCCGCGTCAACACCGGGCGGAACAATTTGTTTTGTGTTTCCACCACCTCCGCCCCCCCCAAT
TTGCGGAATTCCCAAGAG

Appendix 18: Lignin degrading peroxidase gene sequence from *Bacillus* sp. strain BL5

ATGGGCGATGAACAGAAAAAGCCAGAACAAATTCATAGACGGGACATCCTAAAATGGGGAGCGATGGCAGGCG
CGGCCGTTGCGATCGGTGCCAGCGGACTCGGTGGTCTCGCTCCGCTTGTTCAGACCGCGGCTAAGTCGTCGAAAA
AGGATGAAAAAGAGGATGAGCAGGTCGTTCCGTTTTACGGAAAGCATCAAGCCGGAATCACAACTGCCCATCAGA
CGTATGCCTATTTTGCGGCGCTGGATGTTACTGCAAAAGAGAAGAGCGACATCATTACATTATTCAGAAACTGGAC
AAGTCTGACACAGATGCTGACGTCTGGAAAGAAAATGTCGGCTGAGCAGAAAAATCAATATCTGCCGCCGCAGGA
TACAGGTGAATCGGTTGATTTATCCCCTTCCAATTTAACGGTCACAGTCGGATTCGGGCCTGGCTTTTTTGAAAAAG
ACGGAAAGGACCGCTTTGGGCTGAAAAACAAAAAACCGAAGCATCTTGCCGCTCTTCCAGCGATGCCGAATGACA
ACCTGGATGAGAAGCAGGGAGGCGGAGACATCTGCATTCAAGTATGCGCAGACGATGAACAAGTGGCATTTCAC
GCACTGCGGAACCTGCTGAATCAAGCGGTCGGAACCTGTGAGGTCCGCTTTGTGAACAAAGGCTTTTTAAGCGGA
GGAAAAAATGGCGAAACGCCGCGCAACCTCTTCGGGTTTAAAGATGGAACAGGCAACCAGAGCACGAAGGATGA
CACCTTGATGAACTCGATCGTGTGGATTCAGTCCGGTGAACCCGACTGGATGACGGGCGGTACCTATATGGCCTTT
CGGAAAATCAAAATGTTCCTTGAGATATGGGACCGCTCTTCCCTCAAGGATCAAGAGGATACCTTCGGCCGCAGA
AAAAGCTCGGGAGCGCCATTTGGCCAGAAAAAAGAAACAGACCCCGTGAAGCTGAATCAAATCCCGGCAAATTCA
CACGTTAACCTTGCGAAATCTACGGGAAAACAAATTTTGCGAAGAGCTTTCTCTTACACAGAAGGACTTGATCCGA
AAACCGGCTATATGGATGCGGGTCTCCTGTTTATCAGCTTTCAAAAAAATCCCGACAATCAGTTCATCCCCATGCTG
AAGGCTCTTTCAGCAAAGGATGCGTTAAACGAATACACGCAAACAATCGGTTCTGCTTTATATGCATGCCCAGGCG
GCTGCAAAAAAGGAGAATATATTGCCCAGCGTTTGCTGGAATCATAG

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Degradation of lignin by *Bacillus altitudinis* SL7 isolated from pulp and paper mill effluent

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ABSTRACT

Lignin is a major by-product of pulp and paper industries, and is resistant to depolymerization due to its heterogeneous structure. Degradation of lignin can be achieved by the use of potential lignin-degrading bacteria. The current study was designed to evaluate the degradation efficiency of newly isolated *Bacillus altitudinis* SL7 from pulp and paper mill effluent. The degradation efficiency of *B. altitudinis* SL7 was determined by color reduction, lignin content, and ligninolytic activity from degradation medium supplemented with alkali lignin (3 g/L). *B. altitudinis* SL7 reduced color and lignin content by 26 and 44%, respectively, on the 5th day of incubation, as evident from the maximum laccase activity. Optimum degradation was observed at 40 °C and pH 8.0. FT-IR spectroscopy and GC-MS analysis confirmed lignin degradation by emergence of the new peaks and identification of low-molecular-weight compounds in treated samples. The identified compounds such as vanillin, 2-methyoxyhenol, 3-methyl phenol, oxalic acid and ferulic acid suggested the degradation of coniferyl and sinapyl groups of lignin. Degradation efficiency of *B. altitudinis* SL7 towards high lignin concentration under alkaline pH indicated the potential application of this isolate in biological treatment of the lignin-containing effluents.

Key words: alkaline pH, *Bacillus* sp., degradation products, laccase, lignin depolymerization

HIGHLIGHTS

- *Bacillus altitudinis* SL7 isolated from effluent could efficiently degrade lignin under alkaline conditions.
- Degradation efficiency was determined by analyzing lignin content, color reduction, and ligninolytic enzyme activity.
- Extracellular laccase from *B. altitudinis* SL7 can play a significant role in the depolymerization of lignin.
- Various low-molecular-weight lignin degradation products were determined through GC-MS.

INTRODUCTION

The pulp and paper (P & P) industry is among the largest fast-growing industries in the world that utilize lignocellulosic biomass for paper production. It consumes an enormous amount of fresh water and inorganic compounds during pulping, bleaching and washing processes and generates highly contaminated wastewater (Kumar *et al.* 2015). The lignocellulosic biomass utilizing industries are a major threat to environmental health and the magnitude of problem is indicated by lignocellulosic waste generation from these industries: agricultural waste (200 billion tons/year), food industry (1.3 billion ton/year), P & P industry (effluent 150–200 m^3/ton) and sugarcane molasses-based distilleries (effluent 15 lit/1) (Chandra *et al.* 2011; Kharayat 2012; Kadam *et al.* 2013; Ravindran & Jaiswal 2016; Taha *et al.* 2016). The P & P industry generates effluent that is characterized as dark brown with fluctuating pH (generally alkaline), and high chemical oxygen demand (COD) (1,110–1,272 mg/L), suspended solids (1,160–1,380 mg/L), dissolved solids (1,043–1,293 mg/L) and lignin contents (Singh 2015). The concentration of lignin in effluent depends on the type of lignocellulosic biomass used for production of pulp. Disposing off untreated effluent accounts for undesirable coloration of aquatic resources along with deterioration of aquatic flora and fauna by obstructing the passage of sunlight. Lignin derivatives such as chlorolignin affect the reproductive system of fish by causing delayed maturity, lower sex hormone, and reduction in gonad size (Singh & Chandra 2019). In

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Cloning, expression and biochemical characterization of lignin-degrading DyP-type peroxidase from *Bacillus* sp. Strain BL5

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ABSTRACT

Lignin is a major byproduct of pulp and paper industries, which is resistant to depolymerization due to its heterogeneous structure. The enzymes peroxidases can be utilized as potent bio-catalysts to degrade lignin. In the current study, an Efeb gene of 1251bp encoding DyP-type peroxidase from *Bacillus* sp. strain BL5 (DyPBL5) was amplified, cloned into a pET-28a (+) vector and expressed in *Escherichia coli* BL21 (DE3) cells. A 46 kDa protein of DyPBL5 was purified through ion-exchange chromatography. Purified DyPBL5 was active at wide temperature (25−50 °C) and pH (3.0–8.0) range with optimum activity at 35 °C and pH 5.0. Effects of different chemicals on DyPBL5 were determined. The enzyme activity was strongly inhibited by SDS, DDT and β-mercaptoethanol, whereas stimulated in the presence of organic solvents such as methanol and ethanol. The kinetic parameters were determined and K_m, V_{max} and K_{cat} values were 1.06 mM, 519.75 μmol/min/mg and 395 s^{-1}, respectively. Docking of DyPBL5 with ABTS revealed that, Asn 244, Arg 339, Asp 383 and Thr 389 are putative amino acids, taking part in the oxidation of ABTS. The recombinant DyPBL5 resulted in the reduction of lignin contents up to 26.04 %. The SEM and FT-IR analysis of test samples gave some indications about degradation of lignin by DyPBL5. Various low molecular weight lignin degradation products were detected by analyzing the samples through gas chromatography mass spectrometry. High catalytic efficiency and lignin degradation rate make DyPBL5 an ideal bio-catalyst for remediation of lignin-contaminated sites.

1. Introduction

Lignin is a heterogenous polymer comprising of several linked phenylpropane units i.e. p-coumaryl alcohol, guaiacyl alcohol and syringyl alcohol. The β-O-4-ether linkage is the most prevalent intermolecular bond in lignin [1,2]. Rapidly growing cellulosic ethanol [3] and pulp & paper industries are producing large amounts of lignin residues as by product annually [4]. Majority of the lignin residues either burned as low-cost fuel to provide heat and power to industries [5] or released in the aquatic resources without any treatment. The release of compounds accounts for undesirable coloration of water resources along with deterioration of aquatic flora and fauna [6] and only 2% of the available lignin residues is utilized for sustainable technologies for production of biofuels, chemicals and bio-based materials such as bio-plastics [7]. Most sustainable technologies focus on cellulose and hemicellulose valorization, whereas lignin is usually considered to be a low value product. Decomposition and sustainable use of lignin represents the major challenge due to heterogeneous structure and recalcitrant nature of the lignin, considerable effort has been devoted to understanding the major natural pathways involved in lignin degradation [8].

Several strategies have been implemented to degrade lignin, such as membrane filtration, sedimentation, chemical oxidation, ozonation and biological treatment [9,10]. Among these strategies, biological treatment involving the use of ligninolytic enzymes is considered as one of the promising routes, due to targeted oxidation of substrate, mild reaction conditions, low energy requirements, and less production of toxic waste [8,11]. Lignin modifying enzymes are known as ligninases or ligninolytic enzymes, produced by various fungus and bacterial species.

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