BIOPROSPECTING OF THERMOPHILIC BACTERIA FROM HOT WATER SPRINGS OF HIMACHAL PRADESH FOR LACCASE ENZYME PRODUCTION

Thesis

by

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Submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MOLECULAR BIOLOGY AND BIOTECHNOLOGY



COLLEGE OF HORTICULTURE
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This is to certify that the thesis entitled, **"Bioprospecting of thermophilic bacteria from hot water springs of Himachal Pradesh for laccase enzyme production"** submitted in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY** in **MOLECULAR BIOLOGY AND BIOTECHNOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (HP) is a record of bonafide research work carried out by **Ms. Ruchika Sharma (H-2011-07-D)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigations has been fully acknowledged.

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ॐ भूर्भुवः स्वः, तत्सवितुर्वरेण्यं।
भर्गोदेवस्य धीमहि, धियो यो नः प्रचोदयात्।

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LIST OF ABBREVIATIONS

ABTS	-	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
µl	-	microlitre
BLAST	-	Basic Local Alignment Search Tool
Bp	-	Base pair
$CaCl_2$	-	Calcium chloride
Cm	-	Centimeter
$CoSO_4$	-	Cobalt sulphate
$CuSO_4$	-	Copper sulphate
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxy nucleotide 5' triphosphate
EBI	-	European Bioinformatics Institute
EDTA	-	Ethylenediamine tetraacetate
EMBL	-	European Molecular Biology Laboratory
et al.	-	Co-workers
EtBr	-	Ethidium bromide
etc.	-	et cetera
$FeSO_4$	-	Ferrous sulphate
g/l	-	Gram per liter
h	-	hours
HCl	-	Hydrogen chloride
i.e.	-	id est (that is)
KCl	-	Potassium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
l	-	litres
L	-	Litre
m	-	molar
mg	-	milligram
min	-	minutes
ml	-	milliliter
mM	-	millimolar
$MnSO_4$	-	Manganese sulphate
N	-	Normal

Na_2CO_3	-	Sodium carbonate
Na_2HPO_4	-	Disodium hydrogen phosphate
Na_2SO_4	-	Disodium sulphate
NaCl	-	Sodium chloride
$NaNO_3$	-	Sodium nitrate
NaOH	-	Sodium hydroxide
NCBI	-	National Centre for Biotechnology Information
ng	-	nanogram
NH_4Cl	-	Ammonium chloride
NJ	-	Neighbor Joining
No.	-	number
nt	-	Nucleotide
^oC	-	degree centigrade
OD	-	Optical density
PCR	-	Polymerase chain reaction
PHYLIP	-	Phylogeny Inference Package
pmoles	-	picomoles
RAPD	-	Random amplified polymorphic DNA
RBBR	-	Remazol Brilliant Blue R
rpm	-	revolutions per minute
SDS-PAGE	-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sp.	-	Species
Taq	-	*Thermus aquaticus*
U	-	units
UPGMA	-	Unweighted Pair Group Method of Arithmetic Averages
URL	-	Uniform Resource Locator
µg	-	microgram
µM	-	Micromolar

Chapter-1

INTRODUCTION

The microbial world is the largest unexplored reservoir of biodiversity on the earth, and is an important front in biology under investigation. Microbial diversity involves investigation of microorganisms in relation to their biotic and abiotic environments. The vast array of microbial activities and their importance to the biosphere and to human economics provide strong rationale for understanding their diversity, conservation and exploitation. This is done with a variety of approaches and tools, including microscopy, culturing, biochemistry and molecular biology. The microorganisms have been adapted to a variety of habitats all round the biosphere. The opportunities of new industrial applications from microorganisms has been as large as the variety of environments, they confront. Microbial diversity also describes the complexity and genetic variability of the communities. The total genomic complexity denotes the confines of diversity in terms of genetic information present and provides information about the overall taxonomic and functional variability at the community level.

The microorganisms are the living entities which have ability to survive under extreme environmental conditions in which other life forms are unable to survive.Microorganisms, specifically bacteria, continue to be discovered living under extreme environmental conditions such as high temperature, low pH, high salinity and low temperature etc.Thermophiles were the first extreme microbes to be discovered andare a group of extremophiles whose optimal growth temperature lies between 45-80°C, while extremophiles whose optimum temperature is above 80°C are called hyperthermophiles. Natural environments for thermophilic microorganisms are widespread on Earth's surface (Bale *et al.,* 1997; Barns *et al.,* 1994), such as hot water springs, volcanic eruption areas etc. Microbial diversity has been a great source for biotechnological exploration of novel microorganisms, products and processes. The discovery of life at high temperatures and the isolation of *Thermusaquaticus*from the Yellowstone

National park were major initial steps in the field of microbial biotechnology. The success of *Thermusaquaticus*achieved commercially has interested and directed scientists towards isolation and identification of thermophilic microorganisms in geothermal areas, which have not been identifiedas yet. After elucidating thermostable properties of thermophilic microorganisms,attention has been drawn towardsrevealing potential industrial uses of various thermostable enzymes, such as laccase, lipase, protease, cellulase, amylase etc.Thermostable enzymes offer major biotechnological advantages over commonly usedmesophilic enzymes, as these possess higher resistance to chemical denaturants, high alkalinity, extreme acidity and can also withstand increased substrate concentrations without losing their catalytic efficiency. Due to their stability at elevated temperatures, reactions are faster and lesssusceptible to microbial contaminations (Li *et al.,*2005).

Industrially important laccases are a part of broad group of enzymes called polyphenol oxidases, containing copper atoms in the catalytic centre and are also called multicopper oxidases. Laccases contain three types of copper atoms, one of which is responsible for their characteristic blue colour. Laccases (EC: 1.10.3.2) are considered to be ideal green catalysts, since these catalyse the oxidation of phenols, polyphenols, aromatic amines and different non-phenolic substrates by using molecular oxygen from air as an electron acceptor (Kiiskinen*et al.,* 2002; Viswanath*et al*., 2008). Phylogenetically, these enzymes have evolved from small sized prokaryotic azurins to eukaryotic plasma protein ceruloplasmin. The bacterial azurins e.g. crystallized rusticyanin from *Thiobacillusferrooxidans*, which contains only one copper, can be regarded as a precursor protein of laccase. These enzymes have been found to be ubiquitious in nature and the first laccase was reported in 1883 from *Rhusvernicifera*, the Japanese lacquer tree (Thurston, 1994). Thereafterlaccases have been found to be widely distributed in higher plants, bacteria, fungi, insects and lichens (Arakane*et al.,* 2005), where these are involved in melanin formation, cell pigmentation and in a variety of different physiological functions like fungal morphogenesis, plant pathogenesis, bacterial endospore coat component, bacterial copper resistance and fungal virulence.

Whereas, the foremost study on prokaryotic laccaseshas beenreported from *Azospirillumlipoferum*, a rhizospheric bacterium(Alexander and Zhulin, 2000) which was followed bymore reports, suggesting that laccases are widespread in bacteria including*Bordetellacompestris, Caulobactercrescentus, Mycobacterium tuberculosum, Pseudomonas syringae, Pseudomonas aeruginosa,Stenotrophomonasmaltophilia*and *Yersinia pestis*(Enguita*et al.,* 2003; Galai*et al*., 2009; Arora and Sharma, 2010).Bacterial laccases differ significantly from fungal laccases and have an edge over their fungal counterparts in terms,that genetic tools and biotechnological manipulations for bacterial system are well established in industry, are easy to handle and have much faster manipulation rate than fungi. In addition bacteria are more versatile and can prefer anything from acidophilic to alkalophilic, whereas fungi prefer an acidic environment.Bacterial laccase induction system has not been studied although this system offers many advantages over fungal system as described above. Laccase from *Bacillus halodurans*is stimulated rather than inhibited by chloride, which has been a novel trait of great importance for industrial processes. Most importantly, several pH-tolerant bacterial laccases with pH ranges from 4.0-9.5 have been discovered from *γ-Proteobacterium* (Singh *et al.,* 2007), *Streptomyces* (Dube*et al*., 2008; Ye *et al.,* 2010), *Bacillus halodurans* and metagenomic sources. For industrialand biotechnological purposes, laccases were among the first oxidoreductases providing larger-scale applications such as removal of polyphenols, bleaching and removal of lignin etc. Due to harsh industrial process conditions that may include high temperature and pressure, high salt concentrations, acidic or alkaline pH, oxidative conditions, high shear forces or short delays, resistant enzymes are required. In addition there is a current need for enzymes with new catalytic properties for potential innovations and breakthroughs in industrial biotechnology. One of the general prerequisites for an enzyme to be applicable in industrial processes is thermotolerance(Vieille*et al*.,1996; Vieille and Zeikus, 2001). In order to facilitate novel and more efficient bio-catalytic process applications, there is a need for laccases with improved biochemical properties, such as thermotolerance.

The thermostablelaccases have been isolated from bacteria whereas the fungal laccases usually rapidly drop their activity at temperatures above 60^0C (Baldrian, 2004).First report onprokaryotic thermostable laccase was also from *Azospirillumlipoferum*, (Alexander and Zhulin, 2000), whereas,the best studied thermostable bacterial laccase to date is the CotA, the endospore coat component of *Bacillus subtils* which is encoded by *cot*A gene(Martins *et al.,* 2002).
Recent studies have been conducted with thermophilic organisms including laccase derived from *Streptomyces lavendula*, *Pyrobaculumaerophilum*, *Aquifexaeolicus,Thermusthermophilus*, *Thermusthermophilus* 14, *Bacillus pumilus*and*Thermusthermophilus* HB27.

Most of the laccases are monomeric, dimeric or tetrameric glycoproteins in nature (Thurston, 1994) having fewer saccharide compounds (10-25%) in fungi and bacteria as compared to plant enzymes. The carbohydrate compounds containmonosaccharides such as hexoamines, glucose, mannose, galactose and arbinose (Rogalski and Lenowicz, 2004). Laccase glycoprotein usually contains four copper atoms per monomer, bound to three redox sites (type 1, type 2, and type 3 Cu pair). Glycosylation plays an important role in copper retention, thermal stability, susceptibility to proteolytic degradation, and secretion. The mononuclear copper centre contains one type-1(T1) copper atom that is trigonally coordinated to two histidines and a cysteine. The coordination bond is highly covalent showing absorption at around 600 nm and gives characteristic blue colour to the enzyme. The trinuclear clusters contain one type-2 (T2) copper atom and a pair of type-3 (T3) copper atom. The T2 copper is coordinated by two and the T3 copper atoms by six conserved histidines. The T1 and T2 copper atoms are paramagnetic and can be identified in electron paramagnetic resonance (EPR) spectrum. The T3 copper is antiferromagnetically coupled by bridging a hydroxide, which makes T3 copper EPR-silent.

Laccase catalysis comprises of three major steps *i.e*. type 1(T1) Cu reduction by the reducing substrate, internal electron transfer from type1 Cu to type2 (T2) and type3 (T3) Cu trinuclear cluster at a distance of 12.5 A^o. And O_2 reduction (to water) at T2 and T3 Cu centre. Laccases are essentially

biocatalystsandin the presence of air to produce water as the only by-product and thus they are also known as ecofriendlyenzymes. The ability of laccase to oxidise phenolic compounds as well as their ability to reduce molecular oxygen to water has lead to intensive studies of these enzymes.

Laccase producers can be discovered from environmental samples by plate test screening methods. Guaiacol is a sensitive substrate for screening laccase producing micro-organisms, other substrates such as ABTS, dimethoxy phenol and tannic acid have also been found useful for screening. In case of bacteria, most of laccases are located intracellularly and areperiplasmic protein in nature. The bacterial cells must have some strategy, like rearrangement of the electron transport system, to cope with the intracellular presence of laccases and its toxic by-products. In bacteria, the perceived role of laccases has mostly been limited to oxidation of metals and pigment formation. The later function is based onwell studiedCotAlaccase located in the spore coat of *Bacillus subtilis*, which produces a melanin-like pigment for the protection of the spore against UV-light (Martins *et al*., 2002).

The catalytic ability, non-specific reaction catalysis and broad potential substrate range of laccase has raised interest in use of these enzymes in several industrial applications and biotechnological applications. They have been extensively used for the transformation of aromatic compounds such as dyes, aromatic pollutants or for waste water treatments. Laccasemediater system (LMS) has already found applications such as lignozyme process for biobleaching of pulp(Bourbonnais and Paice, 1990). Laccases activate the fibre-bound lignin during manufacturing of the composites, thus resulting in boards with good mechanical properties without toxic synthetic adhesives. Laccase has the ability to decrease odour arising from the garbage disposal sites, livestock farms and pulp mills. Since laccasescatalyze the electron transfer reactions without additional cofactors, they can also be used as biosensors to detect various phenolic compounds, oxygen, and azide. As biosensor, laccase enzyme biomolecules can detect morphine, codeine, catecholamine, estimate phenol or other enzymes in fruit juice in addition to plant flavonoides. Polycyclic aromatic

hydrocarbons (PAHs) which arose from natural oil deposits and utilisations of fossil fuels havebeen also found to be degraded by laccases. Laccases also degrade herbicides, insecticides and reduce 2,4,6-trinitrotoluene (TNT) metabolites to an organic soil matrix, which resulted in detoxification of residue (Nunez *et al*., 2001). One of the major problems, faced by the world today, is the contamination of soil and water by toxic compounds, which include pentachlorophenols (PCP), polychlorinated biphenyls (PCB), 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), benzene, toluene, ethylbenzene, xylene (BTEX) and trinitrotoluene (TNT), laccase catalysis could result in direct degradation or polymerisation, of these hazardous compounds, thus, laccaseshave a huge potential in bioremediation. Cosmetic and dermatological preparations containing proteins for skin lightening have also been developed using laccases. Laccasesare also used in dye transfer blocking functions in detergents and washing powders industry.

The aim of this research has been to study the presence and diversity of laccase producing thermophilicbacteria in hot water springs of Himachal Pradesh; as industrial and environmental interests in these unique ecosystems. Useful bacteria are nature's gift to man and Himachal Pradesh has been bestowed with hot water springs too. It's worth exploring microbial diversity of these hot water springs of the state to assess the status of the microbial wealth in Himachal Pradesh.

Himachal Pradesh is located in northern India, between 30"22' and 33"12' north latitude and between 75"47' and 79"04' east longitude. The mountainous state has altitudes ranging from 350 to 7000 meters above the sea level. Nature has blessed Himachal Pradesh with a number of hot water springs located at Manikaran, Vashist, Khirganga, and Tattapani in the districts of Kullu and Mandi. Keeping this in view, it has been proposed to isolate laccase producing thermophilic bacterialstrains from these hot water springs and analyze them for their phylogeny followed by production of laccase enzyme and its partial purification followed by examination of its industrial status.

The present study, therefore, has been proposed to accomplish the following objectives:

OBJECTIVES

i) Isolation, enumeration and characterization of laccase producing thermophilic bacteria from hot water springs of Himachal Pradesh.

ii) To determine suitable conditions for the laccase production by the bacterial isolate(s).

iii) Partial purification and biochemical characterization of enzyme laccase.

Chapter-2

REVIEW OF LITERATURE

Laccase enzyme (*p*-diphenol: dioxygen oxidoreductases; benzenediol dioxygen oxidoreductases; EC: 1.10.3.2) is a member of large blue copper proteins. Proteins that contain copper atoms as cofactors are crucial for numerous reactions in cellular metabolism. They are involved in photosynthesis, oxidative phosphorylation, metal ion homeostasis and catabolism of various nutrients and toxic chemical compounds. The incorporation of copper atoms in protein structures allow the proteins to perform electron transfer reactions involved in the above-mentioned processes, because copper atoms are able to switch their oxidation states between Cu-I and Cu-II. The protein structure functions as a complex polymeric ligand for the catalytically active coppers, providing them with a coordination environment where switches between the reduction states are thermodynamically feasible. The structurally simplest copper-containing proteins, such as plant plastocyanins and bacterial azurins, are typically electron-carriers involved in electron-transfer reaction chains. More complex copper proteins are generally oxidoreductases, *i.e.* enzymes that catalyze oxidation/reduction reactions and various examples of these are microbial galactose oxidase, laccase and nitrite reductase, mammalian ceruloplasmin and plant ascorbate oxidase. Several copper-containing enzymes have more than one copper atom in the active center. The combination of various copper sites in one protein molecule allow the enzyme to catalyze reactions that involve transfer of several electrons at a time. This is especially important when molecular oxygen is used as an electron acceptor in the catalytic cycle, since oxygen derivatives generated by single electron transfers are highly detrimental to the cell. Multicopper oxidases typically contain two or four copper atoms per protein molecule and they catalyze oxidation reactions in which electrons are removed from the reducing substrate molecules and transferred to oxygen to form water or hydrogen peroxide. Examples of multicopper oxidases are ceruloplasmin, ascorbate oxidase, ferredoxin, phenoxazinone synthase, bilirubin oxidase and

laccase. Ceruloplasmin (EC: 1.16.3.1) is a ferredoxin which is essential for iron homeostasis in plasma. Its corresponding representative in yeast has been shown to be yeast ferredoxin Fet3p (Hasset *et al.,* 1998). Plant ascorbate oxidase (EC: 1.10.3.3) is apparently involved in balancing the reduction potential in growing cells, but its actual function is not yet known (Pignocchi *et al.,* 2003). Laccases (p-benzenediol: oxygen oxidoreductase; EC: 1.10.3.2) are multicopper oxidases present mainly in plants, fungi and bacteria. They are structurally homologous to ceruloplasmin and ascorbate oxidase and are interesting as model enzymes for multicopper oxidases. Laccases are also of particular interest with regard to potential industrial applications, because of their capability to oxidize a wide range of industrially relevant substrates.

Laccases and their homologues form the protein superfamily of multicopper oxidases (MCO). These enzymes have shown a significant potential to replace chemical based technology for treatment of industrial wastes, textile industry, pulp and paper industry wastes and from food applications to bioremediation processes. Because of their broad substrate specificity laccases harbour great biotechnological potential and current applications for these enzymes include pulp delignification, textile dye bleaching, effulent detoxification, washing powder components, removal of phenolics from wine in addition to transformation of antibiotics and steroids. The catalytic properties of laccases also have a great impact on the development of biosensors for both environmentally important pollutants and clinically relevant metabolites. A variety of xenobiotics that can be degraded by laccases start from simple phenols, anilines, benzene thiols to polycyclic aromatic hydrocarbons, organophosphorus insecticides and even the nerve agents. The biotechnological importance of laccases stimulated fundamental studies on biochemistry and structure of these enzymes. Laccases are glycoproteins which are ubiquitious in nature, they have been reported in higher plants and every fungus that have examined for them (Thurston, 1994). Until recently only fungal laccases have been the subject of biotechnological applications. In contrast little is known about bacterial laccases, although recent progress in the whole genome analysis suggests that the enzymes are widespread in bacteria. Identification of bacterial laccases for which genetic

tools and biotechnological applications and processes have been recognised will be of significant importance. An important prerequisites for an enzyme to be applicable in industrial processes is efficient bio-catalytic process applications and a need for laccases with improved biochemical properties such as thermotolerance. Since fungal laccases drop their activity rapidely at temperatures above 60^0C, whereas the thermostable laccases isolated from bacteria can withstand at high temperature (Baldrian, 2004). First report on prokaryotic thermostable laccase has been communicated from *Azospirillum lipoferum*, (Alexander and Zhulin, 2000). In nature thermophilic microorganisms are widespread on earth's surface (Bale *et al.,* 1997; Barns *et al.,* 1994), in form of hot water springs, volcanic eruption areas, peat bogs, compost and deep sea hydrothermal vents etc. The discovery of life at high temperatures and isolation of *Thermus aquaticus* from the thermal springs of Yellowstone National Park were major initial steps in the field of extremophilic microbial biotechnology.

2.1 THERMAL SPRINGS

A thermal spring is a hot water body produced by the emergence of geothermally heated groundwater from the earth's crust. There are geothermal hot springs in many locations all over the crust of the earth. Thermal springs are high temperature aquatic ecosystems and usually maintain a constant temperature and flow of water, thus can be considered as 'laboratories in the natural environment. The thermal springs are widely distributed in the world but are most numerous in volcanically active areas. The chemical properties of the springs have been related with medicinal properties of certain springs (Waring, 1965). Many hot water springs have significant amount of hydrogen sulphide. The concentration of elements such as fluoride, arsenic, rare earths and gold varies very much from spring to spring. Many springs are highly radioactive and some springs precipitate silica, others deposit travertine ($CaCO_3$) and still others form elemental sulphur.

In India, thermal springs are scattered throughout the country and occur either as solitary or in groups from sea levels up to nearly three thousand meters

above the sea level. Early studies were mainly concerned with the medicinal properties of the springs (Newbold, 1848; Buist, 1851; Macpherson, 1855; Schlagintweit, 1862). Oldham & Oldham (1882) prepared an exhaustive catalogue of Indian hot water springs and reported more than three hundred thermal springs in the country. Some springs in Bombay have been recorded as being permanently radioactive (Sierp and Steichen, 1911) containing radium salts in solutions. Nag (1933) and Chatterjee (1936) studied the radioactive properties of Rajgir thermal spring. Ghosh (1948) reported hot water springs at Jarom and studied 112 springs and recognized 4 main broad belts in which majority of the thermal springs occur in India (Fig.1) viz. Bihar, along the west coast of India, the Himalayan belt, East India. Further on the basis of chemical nature, the thermal springs are classified into four main groups viz. simple or indifferent waters, having low mineral contents; alkaline waters; sulphur waters; chloride or saline waters. In recent years a number of studies concerning primary productivity, nutrient cycle, trophodynamics and succession of communities have been undertaken in hot springs which has been helpful in understanding the hot spring ecosystem (Brock, 1967; 1970 and 1973; Brock and Brock, 1966; 1968; Castenholz, 1967; 1970 and 1976). Stockner (1968) and Naiman (1976) extensively studied the different aspects of thermal spring ecosystem and reviewed extensively the environmental requirements and biology of thermophilic microbes.

2.2 THERMOPHILES

Hyperthermophiles grow optimally at temperatures between 80 and 110°C. Only represented by bacterial and archaeal species, these organisms have been isolated from all types of terrestrial and marine hot environments, including natural and man-made environments. Enzymes from these organisms (hyperthermophilic enzymes) developed unique structure-function properties of high thermostability and optimal activity at temperatures above 70°C. Some of these enzymes are active at temperatures as high as 110°C and above.

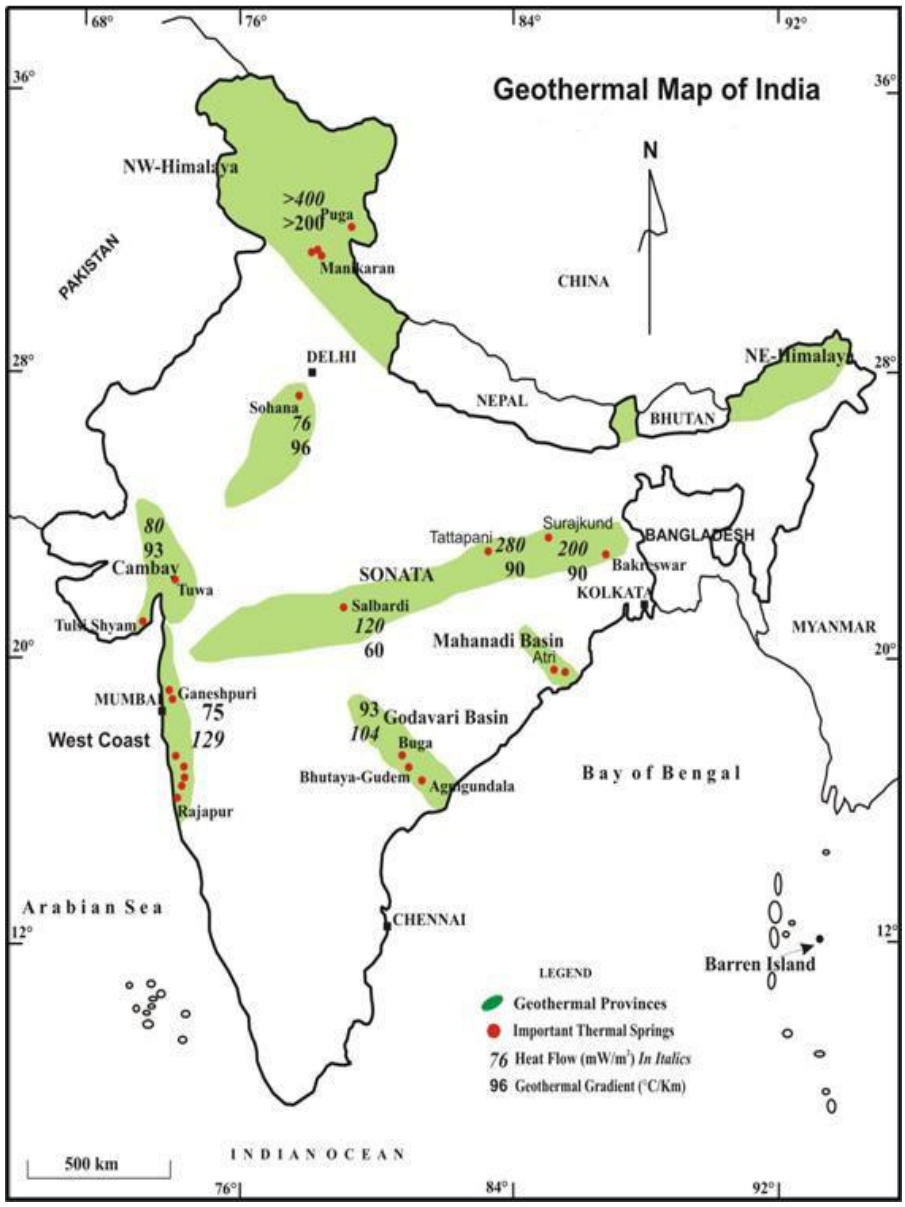


Fig. 1: The distribution of hot water springs in India

Thermophilic organisms grow optimally between 50 and 80°C. Their enzymes (thermophilic enzymes) show thermostability properties which fall between those of hyperthermophilic and mesophilic enzymes. These thermophilic enzymes are usually optimally active between 60 and 80°C. Active at high temperatures, thermophilic and hyperthermophilic enzymes typically do not function well below 40°C.

Thermophiles are the organisms which are adapted to live at high temperatures and these high temperature environments are widely distributed on the earth. The availability of thermophiles to grow at temperatures above 60^{o}C is associated with extremely thermostable macromolecules present in them. Thermophilic bacteria have been reported in most boiling water environments, where they reproduce normally (Brock, 1967). Thermophilic microorganisms have been found to be potential and good alternative sources of thermostable enzymes. The investigation and application of thermostable enzymes from thermophilic microorganisms have received a lot of attention in industries ranging from food to waste management industries.

A great bacterial diversity has been reported in various solar heated environments ranging from rod shaped spore forming genera viz. *Bacillus, Clostridium, Thermoactinomyces* to rod shaped non-spore forming genera viz. *Thermus anaerobium* (Zeikus, 1979). The major nutritional categories include phototrophic species viz. *Chloroflexus auratiacus*, chemoautotrophic species viz. *Methanobacterium, Thermoautotrophicum,* chemoheterotrophic species viz. *Thermoanareobium brockii,* chemosynthetic mixotrophic species that grow autotrophically or heterotrophically viz. *Sulfolobus acidocaldarius* (Transey and Brock, 1978).

Thermophilic bacteria may have tremendous potential in future microbial and enzyme technology because of their unique property to work at high temperature and enables development of improved or new biotechnology industry (Zeikus, 1979).

2.3 THERMOSTABLE ENZYMES

Thermostable enzymes were the result of discovery of thermophilic microorganisms from hot water springs in 1960s. It was discovered that many bacteria in hot springs not only survived but also thrive at higher temperatures. The first thermostable enzyme Taq polymerase was obtained from *Thermus aquaticus* in 1974 and it was followed by more thermostable enzymes listed in Table-1.

Table 1: Summary depicting reported thermophilic microorganisms

Enzymes	Organisms	Optimal temperature (^{o}C)	References
Laccase	*Bacillus subtilis*	70-80	Alexandre and Zhulin, (2000)
	Streptomyces lavendulae	70	Suzuki *et al.,* (2003)
	Bacillus pumilus	55-60	Reiss *et al.,* (2011)
	Trametes hirsute	65-85	Zang *et al.* , (2009)
α-Amylase	*Bacillus stearothermophilus*	70-80	Vihinen and Mantsala (1990)
	Bacillus stearothermophilus	70	Jeayoung *et al*., (1989)
	Myceliophthora thermophila	100	Ramkrishna *et al*., (1993)
	Pyrococcus furiosus	100	Laderman *et al,*. (1993a,b)
	Thermococcus aggreganes	100	Canganella *et al*., (1994)
β-Amylase	*Clostridium thermosulphurogenes*	75	Shen *et al.,* (1988)
Pullulan	*Pyrococcus furiosus*	98	Brown and Kelly, (1993)
	Thermococcus aggregans	100	Canganella *et al.,* (1994)
	Thermococcus hydrothermalis	95	Gantlet and Duchiron, (1998)
Xylanase	*Pyrococcus furiosus*	100	Bauer *et al.,* (1999)
	Scytalidium thermophilum	65	Nazan and Zumrut (2000)
	Sulfolobus solfataricus	105	Nucci *et al*., (1993)
	Thermotoga thermarum	80	Sunna *et al.,* (1996a)
Cellulase	*Pyrococcus furiosus*	102-105	Kengen *et al*., (1993)
	Rhodothermus marinus	95	Hreggvidsson *et al*., (1996)
	Thermotoga neapoltana	95	Bok *et al.,* (1998)
Protease	*Bacillus stearothermophilus*	85	Rahman *et al.,* (1994)
	Bacillus thermoruber	45	Manchini *et al*., (1988)
	Thermococcus celer	95	Klingberg *et al.,* (1991)
Lipase	*Bacillus stearothermophilusa*	68	Gupta *et al*., (1999)
	Pseudomonas sp.	90	Rathi *et al*., (2000)
	Pyrococcus horikoshii	95	Yan *et al.,* (2000)
	Bacillus sp. RSJ-1	50	Sharma *et al*., (2002)
	Bacillus thermoleovorans	75	Lee *et al.,* (1999)
	Acinetobacter sp.	94	Bisht and Panda (2011)
	Geobacillus sp.	94	Zuridah *et al*. (2011)

Although the importance of enzymes as catalysts in industrial processes has been widely recognized, very few have high market value for industrial and household applications (Jarnagin and Ferrari, 1992). However, thermophilic bacteria, having growth at high temperature and specific macromolecular properties, possess high metabolic rates, physically and chemically stable enzymes. Thermophilic processes appear to be more stable, rapid and less expensive and facilitate reactant activity and product recovery and replaced the traditional methods to some extent (Zeikus, 1979). In addition the ability to clone and express virtually any desired gene has removed this obstacle and increased the number of enzymes conceivably available for industrial experimentation from a few hundred to several thousand (MacQuitty, 1988). Expression of newly available neutral enzymes or novel ones created by protein engineering require the availability of reliable production hosts, that are easy to transform. In present era, thermophiles have become the subject of special interest for structural investigations and biotechnological applications (Herbert, 1992)

2.4 DISTRIBUTION OF LACCASES

Laccases have been only found in eukaryotes (fungi, higher plants, insects) until recently, but now there is strong evidence for their widespread distribution in prokaryotes and the first crystal structure of a bacterial laccase is already available.

2.4.1 Higher plant laccases

In plants, their potential function is the biosynthesis of lignin. Laccases are common enzymes in nature and the first laccase was reported in 1883 from *Rhus vernicifera*, the Japanese lacquer tree (Reinhammar, 1984), from which designation laccase was derived. Subsequently laccases have been discovered in numerous other plant species, e.g., peach (Lehman *et al*., 1974), Cell cultures of *Acer pseudoplatanus* have been shown to produce and secrete laccase, sycamore (Bligny and Douce, 1983), *Pinus taeda* tissue contain eight laccases, all expressed predominantly in xylem tissue (Sato *et al.,* 2001), tobacco and poplar (Ranocha *et al*., 1999). Laccases have also been characterized from a few monocots such as ryegrass and maize (Gavnholt *et al*., 2002; Caparros- Ruiz

et al., 2006) The plant laccases have not been characterized or used extensively despite their wide occurrence, because their detection and purification is often difficult, as the crude plant extracts contain a large number of oxidative enzymes with broad substrate specificities (Ranocha *et al*., 1999). Other reports on the presence of laccase in leaves of *Aesculus parviflora* (Wosilait *et al*., 1954) and in green shoots of tea (Gregory and Bendall, 1966). Five distinct laccases have been shown to be present in the xylem tissue of Populus euramericana (Ranocha *et al.,* 1999). Plant laccases participate in the radical-based mechanisms of lignin polymer formation. They are found in the xylem, where they presumably oxidize monolignols in the early stages of lignification (Gavnholt *et al.,* 2002). In addition, laccases have been shown to be involved in the first steps of healing in wounded leaves. Detection and purification of plant laccases is often difficult because crude plant extracts contain a large number of oxidative enzymes with broad substrate specificities (Ranocha *et al*., 1999), which could probably explain the lack of detailed information on the biochemical properties of plant laccases.

2.4.2 Insect laccases

Laccases have also been detected and reported in several dipterans like *Drosophila virilise* (Yamazaki, 1969) and *Lucila cuprina* (Barrett, 1987), as well as in the lepidopteran *Bombyx mori* (Yamazaki, 1972). In insects, several forms of laccases have been identified. 'Laccase-1' and Laccase-2 have been reported in *Manducca sexta* and *Tribolium castaneum* (Arakane *et al*. 2005, Dittmer *et al*., 2004), in *Drosophila melanogaster, Anopheles gambiae* (Arakane *et al*., 2005) and *Nephotettix cincticeps* (Hattori *et al*., 2005).

2.4.3 Fungal laccases

In fungi, they are supposed to be involved in lignin degradation, pigment production and plant pathogenesis.Fungal laccases are significant ligninases, and have been found to be widespread in many fungal species belonging to Ascomycetes, Deuteromycetes and Basidiomycetes (Assavanig *et al.,* 1992). Laccase from *Monocillium indicum* was the first fungal laccase to be characterized from Ascomycetes. Laccases are mostly produced by white-rot

fungi such as *Trametes versicolor, Trametes hirsute, Trametes ochraceaa, Coriolposis polyzoan* and litter-decomposing fungi. Basidiomycetous fungi such as *Phanerochaete chrysosporium*, *Theiophora terrestris*, *Lenzites*, *betulina* and wood degrading Ascomycetes like *Trichoderma and Botryosphaeria* have also been shown to possess laccase activity. Laccase activity has never been reported in the lower fungi belonging to the Zygomycetes and Chytridiomycete. Owing to the higher redox potential of +800 mV of fungal laccases compared to plants or bacterial laccases they are implicated in several biotechnological applications, especially degradation of lignin.

The majority of laccases characterized so far have been derived from fungi especially from white-rot basidiomycetes that are efficient lignin degraders. Well-known laccase producer fungi belong to the ascomycetes, deuteromycetes, basidiomycetes, and cellulolytic class. The enhanced formation of laccase by the white rot fungi *Trametes pubenscens* in the presence of copper was reported by Galhaup *et al.,* (2001). The influence of copper on laccase production has been reported earlier in *Neurospora crassa* (Huber and Lerch, 1987). A blue laccase of litter decaying basidiomycete *Stropharia rugosoannulata* capable of oxidizing Mn^{2+} in the presence of Mn^{2+} chelators has also been suggested (Schlosser and Hofer, 2002). The important laccase producing white rot fungi are *Neurospora crassa* (Froehner and Eriksson, 1974), *Agaricus bisporus* (Wood, 1980), *Pleurotus ostreatus* (Sannia *et al.,* 1986), *Phlebia radiata* (Niku-Paavola *et al*., 1988), *Ganoderma lucidum* (Perumal, 1997), *Trametes versicolor* (Swamy and Ramsay, 1999), *Cyathus stercoreus* (Sethuraman *et al*., 1999), *Pycnoporus cinnabarinus* (Otterbein *et al*., 2000), *Pleurotus ostreatus* (Palmieri *et al*., 2000), *Pycnoporus sanguineus* (Pointing *et al*., 2000), *Coriolus hirsutus* and *Coriolus sonatus* (Koroleva *et al*., 2001), *Trametes modesta* (Nyanhongo *et al*., 2002), *Bjerkandera adusta*, *Pleurotus sajor caju* (Reddy *et al*., 2003) and *Panus tigrinus* (Zavarzina *et al*., 2004). The ascomycete fungi like *Podospora anserine* (Frese and Stahl, l992), *Trichophyron rubrum* (Jung *et al*.,2002), *Botryophaeria sp* (Da-Cunha *et al*., 2003) and *Physisporinus rivulosus* (Hakala *et al*., 2005) also exhibits laccase activity. Laccase activity has also been reported from *Agaricus bisporus* (Smith *et al*., 1998), the hyphomycete, *Chalara paradoxa* (Robles *et al*.,

2000) and ascomycetes such as *Mauginiella* sp (Palonen *et al*., 2003), *Trichoderma atroviride*, *Trichoderma harzianum* (Holker *et al*., 2002) and *Melanocarpus albomyces* (Kiiskinen and Saloheimo, 2003).

A specific type of laccase termed phenol oxidase has been reported in actinomycetes, which is involved in the lignin degradation and has been reported only in a few *Streptomyces* species such as *Streptomyces badius* (Borgmeyer and Crawford, I985), *Streptomyces cyaneus* (Berrocal *et al*., 1997), *Streptomyces griseus* (Endo *et al*., 2003), *Streptomyces lavendulae* (Suzuki *et al.,* 2003) and *Streptomyces coelicolor* (Machczynski *et al.*, 2004).

2.4.4 Bacterial laccases

In bacteria, they are suggested to play a role in melanin production, spore coat resistance, morphogenesis and detoxification of copper. Laccase in bacteria is present intracellularly and as periplasmic protoplast (Claus, 2003; Arora and Sharma, 2010). Bacterial laccases have certain advantages over fungal laccases- these have been reported to be less sensitive towards alkaline conditions, halides as well as fast growth rate (Verma and Madamwar, 2003). Spore coat laccase can withstand high temperature and extreme pH and thus have a potential to be used for bioremediation or application in reactor (Hullo *et al.,* 2001). So, keeping in view the importance of bacterial laccases in pesticide pollution degradation, new bacterial strains displaying laccase activity are the need of the time. The lack of commercially available robust and inexpensive laccase is a major barrier to the widespread applications of laccases in various industrial sectors. Isolating laccase from bacteria is of commercial importance since bacterial genetics and their use in industry is well established. The first bacterial laccase was found in the plant root associated bacterium, *Azospirillum lipoferum* (Givaudan *et al.,* 1993; Sharma *et al.,* 2007; Sharma and Kuhad, 2008), where it was shown to be involved in melanin formation (Faure *et al.,* 1994; Sharma and Kuhad, 2008).Thereafter laccase has been discovered in a number of bacteria including *Bacillus subtilis, Bordetella compestris, Caulobacter crescentus, Escherichia coli, Mycobacterium tuberculosum, Pseudomonas syringae, Pseudomonas aeruginosa,* and *Yersinia pestis* (Alexandre and Zhulin, 2000; Enguita *et al.,* 2003; Arora and Sharma,

2010). Recently, *Stenotrophomonas maltophilia* strain has been found to be laccase producing, which was used to degrade synthetic dyes (Galai *et al.,* 2009; Arora and Sharma, 2010).

Givaudan *et al*., 1993 were the first to report prokaryotic laccase activity in non-motile *Azospirillum lipoferum* isolated from the rhizosphere of rice. The polyphenol oxidase activity was observed which was correlated with production of dark-brown pigment. Both laccase activity and melanisation could potentially contribute to adaptive mechanism of *Azospirillum lipoferum* in rhizosphere of rice or toxic phenol scavenging. This laccase occurs as a multimeric enzyme composed of a catalytic subunit with one or two large chains. This was followed by isolation of *Azospirillum lipoferum* 4T TN5 mutant, with affected bacterial laccase activity and laccase dependent melanisation (Faure *et al*., 1994, Faure *et al.,* 1995) and activity of bacterium *Azospirillum lipoferum* laccase on phenol derivatives. Studies carried out by spectrophotometry, HPLC and GC/MS analysis and it was reported that this laccase transformed phenolic compounds of syringic type into 2, 6- dimethoxy-1, 4-bezoquinone (Faure *et al*., 1995).

A laccase-like spore protein of a marine *Bacillus* strain SG1 has been shown to oxidize Mn (II). Mutation in genes coding for multi-copper oxidases have lost their metaloxidizing activities. On polyacrylamide gels, laccase activity was present as molecular mass complexes or multimers in enzymes from this bacterium (Van Waasbergen *et al.* 1996). A laccase-like enzyme activity was also found in spores of *Bacillus sphaericus* (Claus and Filip, 1997). Laccase has been reported from a melanogenic marine bacterium *Marinomonas mediterranea* producing two different polyphenol oxidases (PPO), an unusual multi-potent PPO able to oxidize substrates characteristic of both tyrosinase and laccase (Solano *et al*., 1997).

Alexander and Bally (1999) reported another non-motile strain of *Azospirillum lipoferum* isolated from rice rhizosphere which revealed the most unusual polyphenol oxidase activity. It was further reported that the enzyme laccase activity was present intracellularly in *Azospirillum lipoferum* 4T which resulted in melanin production which may contribute to scavenging of toxic

phenol components. Laccase containing six putative copper binding sites were discovered in marine bacterium *Marinomonas mediterranea*, but no functional role was assigned to this enzyme (Amat *et al.,* 2001; Sharma and Kuhad, 2008)

A putative multi-copper oxidase encoded by the *yacK* gene of *E. coli* had been cloned and expressed in *E. coli* strain TG-1 containing plasmid pWLFO-5. The expressed enzyme contained the predicted copper centers that harbored both phenol oxidase and ferroxidase activities. The enzyme caused oxidation of a phenolate siderophore that released chelated iron in such a way that the level of free iron was increased in periplasm and as a consequence Fe II and Cu II uptake via *feo* system became appropriately balanced. Such a mechanism *Pseudomonas* conferred a level of protection from elevated copper on the bacterium and left the more soluble form of Fe II available for uptake (Kim *et al.,* 2001).

A bacterial laccase named CotA has been reported in *Bacillus subtilis*. The *cotA* gene codes for a 65-kDa protein belonging to the outer spore coat. CotA participates in the biosynthesis of the brown spore pigment, which is also thought to be a melanin-like product (Driks, 2004) and seems to be responsible for most of the protection afforded by the spore coat against UV light and hydrogen peroxide. CotA protein displays similarities with multi-copper oxidases. The protein exhibits a higher thermal stability with a half-life of about 2 h at 80°C and optimum temperature 75°C (Martins *et al.,* 2002). Ligands involved in T1 copper binding are two histidines, a cysteine, and a fourth ligand that varies between the members of the multi-copper oxidase family. In the equivalent sequence position CotA has a methionine residue (Met-502) and, thus, presents a T1 copper site similar to that of ZAO but also to other fungal laccases. The single amino acid substitution of histidine 497 by an alanine (H497A) or replacement of methionine 592 with a leucine (M502L) impair the copper coordination by the surface-exposed T1 center, thus altering drastically the enzymological properties of the protein.

Another laccase has been reported from non-melanogenic alkalotolerant γ-proteobacterium JB from industrial waste water drained soil. This bacteria found intense reddish brown colonies on M162 media and bacterium was

identified from its partial 16S rRNA gene sequencing (Gene bank accession No. AF574088) analysis as belonging to the order α-proteobacterium (Bains *et al*., 2003). Recently, a protein encoded by ORF bh2082 of *Bacillus halodurans* C-125 (Ruijssenaars and Hartmans, 2004) has been identified as a potential bacterial laccase by genome mining. The enzyme showed an alkaline pH optimum with syringaldazine as the substrate and was stimulated rather than inhibited by chloride. This finding underlines the potential of bacteria as a source of unusual laccases that may not have some of the disadvantages of the classical laccases.

Some of the reported laccases have the ability to perform the activity at very crucial conditions like in the presence of high concentration of Cl^- and Cu^{2+} and even at neutral pH values. The enzyme produced by *Sinorhizobium meliloti* is a one of the examples of such enzymes and is a protein having two subunits with pI 6.2 and the molecular weight of the subunits is 45 kDa each (Morozova *et al.,* 2007), whereas laccase produced by *Pseudomonas putida* is also an example of such enzyme and is a single subunit 59 kDa protein which works well at pH 7.0 (Morozova *et al.,* 2007). Both enzymes can oxidize syringaldazine.

A new laccase gene (*cotA*) was cloned from *Bacillus licheniformis* and expressed in *Escherichia coli*. The recombinant protein CotA was purified and showed spectroscopic properties typical for blue multi-copper oxidases. The enzyme has a molecular weight of ~65kDa and demonstrates activity towards canonical laccase substrates 2, 2'- azino-bis (3-ethylnenzothiazoline-6sulphonic acid) (ABTS), syringaldazine (SGZ) and 2, 6- dimethoxyphenol (2, 6-DMP). Kinetic constants Km and kcat for ABTS were of 6.5±0.2 μM and 83s-1, for SGZ of 4.3+0.2 μM and 100s-1, and for 2, 6- DMP of 56.7+1.0 μM and 28 s-1. Highest oxidizing activities towards ABTS were obtained at 85^{o}C (Koschorreck *et al.,* 2009). This was followed by characterization of an alkali resistant and organic solvents tolerant laccase from *Bacillus licheniformis* LS04 isolated from forest soil. The laccase of *Bacillus licheniformis* LS04 demonstrated a broad pH range for catalysing substrates (Lu *et al*., 2011). Reiss *et al*., (2011) reported a heat stable enzyme with wide substrate spectrum. A novel CotA-type laccase

from *Bacillus pumilus* was cloned, expressed and purified and characterized. The molecular weight of the purified laccase was estimated to be 58 kDa and enzyme was found to be associated with four copper atoms.

Table 2: Summary depicting reported laccase or laccase like activity in other prokaryotes

Species	Molecular mass (kDa)	Referances
Aquifex aeolicus	59.3	Deckert *et al.*,(1998)
Azospirillum lipoferum	48.9,97.8 and 179.3	Givaudan *et al.,* (1993)
Bacillus Sp.	multimeric	Van Waasbergen *et al.*,(1996)
Bacillus sphaericus	-	Claus and Filip, (1997)
Bacillus subtilis	65	Hullo *et al*., (2001)
Baccilus halodurans C-125	56	Ruijssenaars and Hartmans, (2004)
Escherichia coli	56.5	Kim *et al.*, (2001); Roberts *et al.,* (2002)
Marinomonas mediterranea	54	Sanchez-Amat and Solano, (1997)
α-proteobacterium SD 21	150 and 250	Francis and Tebo, (2001)
γ-proteobacterium JB	120	Bains *et al.,* (2003)
Pseudomonas putida GB1	50	Brouwers *et al.,* (1999)
Pseudomonas sp	50	Francis and Tebo, (2001)
Pseudomonas aerophilum	52.9	Fitz-Gibbon *et al.,* (2002)
Streptomyces griseus	114	Endo *et al*., (2003)
Thermos themophilus HB 27	53	Miyazaki, (2005)
Xanthomonas campestris	69.5	Lee *et al.,*(1994)

Laccase-homologous sequences identified in thermophiles are very rare. Some of the reports include: laccase (NCB Accession No. JC8030) from *Streptomyces lavendulae* REN-7 (Suzuki *et al.,* 2003); a laccase-like protein (NCB Accession No. NC_003364) from the hyperthermophilic archaeon, *Pyrobaculum aerophilum* IM2 (Fitz-Gibbon *et al.* 2002); *Pseudomonas putida* strain LUA15.1and LHB7.1, *Pseudomonas umsongenesis* strain LHB91., *Pseudomonas mohnii* strain LHN12.2, *Pseudomonas chlororaphis* strain LUD7.1, *Pseudomonas jessenii* strain LHN9.1, *Pseudomonas lurida* strain LB6.2, *Pseudomonas graminis* strain LHN8.1, *Pseudomonas veronii* strain

LUA14.1, *Pseudomonas fulva* strain LR5.1, *Lysnibacillus* fusiformis strain LKM7.1, *Lysnibacillus sphaericus* strain LH3.4 and *Bacillus subtilis* strain LB6.1 (Karuna, 2013), a laccase-like protein (NCB Accession No. NC_000918) from the hyperthermophilic bacterium *Aquifex aeolicus* VF5 (Deckert *et al.,* 1998) and laccase (NCB Accession No. AAS81712) from *Thermus thermophilus* HB27 (Miyazaki, 2005). There are few reports of laccase or laccase like activity in other prokaryotes listed in Table-2.

2.5 CELLULAR LOCALIZATION OF LACCASE

Ligninolytic enzymes have mostly been reported to be extracellular but there are evidences in literature of the occurrence of intracellular. Intracellularly as well as extracellularly laccases are identified while, most laccases purified so far are extracellular. In plants and fungi, extracellular localization of the enzymes helps them circumvent the problem of the reactive species, such as semiquinones and quinones that are generated by laccases while oxidizing aromatic substrates. These reactive species are powerful inhibitors of the electron transport system in both bacteria and mitochondria. The localization of laccase is probably connected with its physiological function and determines the range of substrates available to the enzyme. The cell wall and spore associated laccases were linked to the possible formation of melanin and other protective cell wall compounds. In the case of bacteria, most of the laccases so far studied are located intracellularly as in *Azospirillum lipoferum* (Diamantidis *et al.,* 1999), *Marinomonas mediterranea* (Solano *et al*., 1997), *Bacillus subtilis* (Martins *et al.,* 2002), *Sinorhizobium meliloti* (Sowinski *et al*., 2002) and *Bacillus licheniformis* LS04 (Lu *et al*., 2011). Bains *et al*. (2003) found both intracellular and extracellular laccase activity in γ-proteobacterium. The bacterial cells must have some strategy to cope with the intracellular presence of laccase and its toxic by-products. Rearrangement of the electron transport system has been hypothesized to be one of the ways in which the laccase-positive cells adapt to endogenous substituted quinones generated as products of laccase catalyzed reaction.

2.6 STRUCTURAL ORGANIZATION

2.6.1 Genetic orgnisation

Marinomonas mediterranea ppoA is the first prokaryotic laccase sequenced containing laccase and tyrosine activites and first PPO showing such multi-potent catalytic activity. The amino acid sequence shows the existence of a signal peptide and four copper-binding sites characteristics of the blue multi-copper proteins and additional putative copper-binding sites near its N-terminus (Sanchez-Amat *et al.,* 1997). The genetic and molecular basis of copper resistance of *Xanthomonas campestris* pv. *juglandis* have been well studied, and hybridization analysis indicates that the copper resistance genes are located on the chromosomes, sharing nucleotide sequence similarity with other copper resistance genes from *Pseudomonas syringae* pv. *tomato*, *Pseudomonas syringae* and *Xanthomonas campestris* pv. *vesicatoria.* DNA sequence analysis of the fragment revealed four open reading frames (ORF1–ORF4) in the same direction. Four histidine-rich polypeptide regions in the ORF1 of *Xanthomonas campestris* pv. *juglandis* and copA of *Pseudomonas syringae* pv. *tomato* strongly resembles the copper-binding motifs of small blue copper proteins and multi-copper oxidases, such as fungal laccases, plant ascorbate oxidase (Asox), and human ceruloplasmin (Lee *et al.,* 1994). *Pseudomonas putida* GB-1 catalyzes the oxidation of Mn2+ and nucleotide sequence analysis of the transposon insertion site revealed a gene *cumA* encoding a protein homologous to multi-copper oxidase (Brouwers *et al.,* 1999).

Secondary structure

The structural comparison of bacterial laccases with fungal laccases is done using CotA as a model structure and this is a monomeric protein (Hullo *et al.,* 2001). The primary sequence of CotA was aligned with that of monomeric multi-copper oxidases of known three-dimensional structures: CueO, the laccase CcLa and Asox using Multalin as shown in Fig. 2.

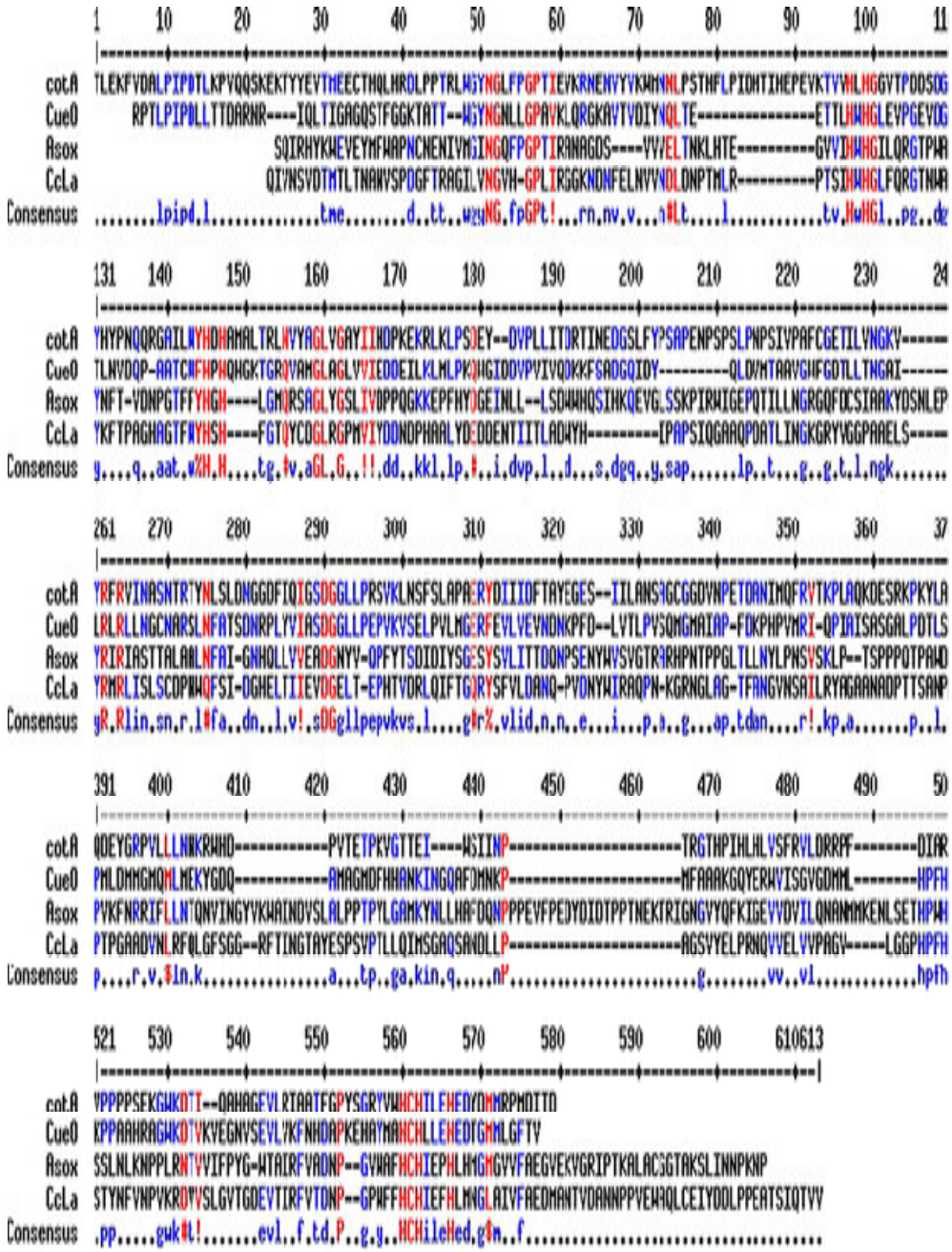


Fig. 2: Sequence alignment by Multalin: CotA (*Bacillus subtilis*), CueO (*Escherichia coli*), Asox (Ascorbate oxidase), CcLa (*Coprinus cinereus*) (Hullo *et al.*, 2001)



Fig. 3: Secondary structure of *Bacillus subtilis* CotA (PDB code 1GSK) (Hullo *et al.,* 2001)

The overall CotA fold comprizes three cupredoxin-like domains, as in case of fungal laccases but all the three domains are structurally different from fungal and similar to the laccase-like protein of *E. coli* (CueO) (Roberts *et al.,* 2002). The cupredoxin fold is mainly formed by an eight-stranded Greek key β-barrel, comprising two β-sheets composed by four strands, arranged in a sandwich conformation. The first (N-terminal, domain 1 in Fig. 3) cupredoxin like domain of CotA, residues 2–176, has a somewhat distorted conformation in comparison with the equivalent domain in other multi-copper oxidases. It comprizes of eight strands organized in a β-barrel form, starting with a coiled section (residues 2–25; Fig. 3) that connects domains 1 and 2, and is stabilized by hydrogen bonds, contributing to the packing between these domains. This coiled section is absent in plant and fungal multi-copper oxidases such as the laccase

from *C. cinereus* (CcLa) and Asox. However, a similar coiled section present in the *E. coli* CueO protein (Fig. 2).

The overall fold of the second cupredoxin-like domain of CotA (domain 2, in Fig. 3) comprizes a β-barrel composed of 12 strands residues (183–340) very similar to the fold of domain 2 in Asox. Domain 2 of CotA acts as a bridge between domains 1 and 3, but a short α-helical fragment, encompassing residues 177–182, makes the connection between domains 1 and 2, whereas a large loop segment including residues 341–368 links domains 2 and 3. In both the structures of CotA and CueO, this region represents an external connection between domains 2 and 3, whereas in plant and fungal multi-copper oxidases the corresponding link is made through an internal connection. Therefore, this feature may be a characteristic of the prokaryotic variants of these enzymes. Together with the coiled section that links domains 1 and 2 of CotA (which also has an equivalent in CueO; residue 169–174 in Fig.3), this external loop motif contributes decisively to the closer resemblance between the overall folds of the prokaryotic proteins CotA and CueO, relative to other structurally characterized multicopper oxidases. Finally, domain 3 of CotA (Fig. 4; residues 369–501) not only contains the mononuclear copper center, but also contributes to the formation of the binding site of the trinuclear copper center, which is located in the interface between domains 1 and 3 (Fig. 4). Moreover, domain 3 includes the putative substrate-binding site, located at the surface of the protein, close to the type I mononuclear copper center. A protruding section formed by a loop and short α-helix (Fig.4), comprising amino acids from 434 to 454, forms a lid-like structure over the substrate-binding site. No similar element has been found in the previously analyzed multicopper oxidases with the known three-dimensional structure. Therefore, this structural element represents a distinctive feature of bacterial laccases. The laccase from *B. subtilis* (Martins *et al.,* 2002) and *S. lavendulae* (Suzuki *et al.,* 2003) have 47% similarity to each other but are different from other laccases.

2.6.2 Protein characterization

Putative laccase–like multi-copper oxidases have been detected in the genomes of many bacterial species, suggesting that laccases are widespread in

bacteria (Alexandre and Zhulin, 2000). However, of three laccases that have been structurally characterized, those from *C. cinereus* (Ducros *et al.,* 1998), from *Trametes versicolor* (Bertrand *et al.,* 2002) and from *Melanocarpus albomyces* (Hakulinen *et al.,* 2002) none is of bacterial origin. The first three dimensional structure on bacterial laccase was reported for *B. subtilis* CotA laccase as determined by X-ray crystallography. The 65-kDa CotA protein is a highly thermostable laccase which is associated with high-proline content. CotA contains 46 proline residues, a number that does not differ greatly from that observed for other laccases. Atomic packing has been recognized as an important measurement for characterizing protein structure. Several methods have been defined for determining atomic packing, and OSP values for CotA protein showed three intervals in which this value corresponded to a more packed structure. These highly packed residues are localized in the interface regions between domains 1 and 2 and close to the mononuclear copper center (Fig-4). Together, they constitute a pocket around the copper atoms and contribute to the overall structure packing of CotA protein (Enguita *et al.,* 2003).



Fig. 4: Ribbon representation of the CotA structure, with the localization of the residues with higher OSP values (represented in yellow). Copper atoms are represented as magenta balls (Enguita *et al.,* 2003).

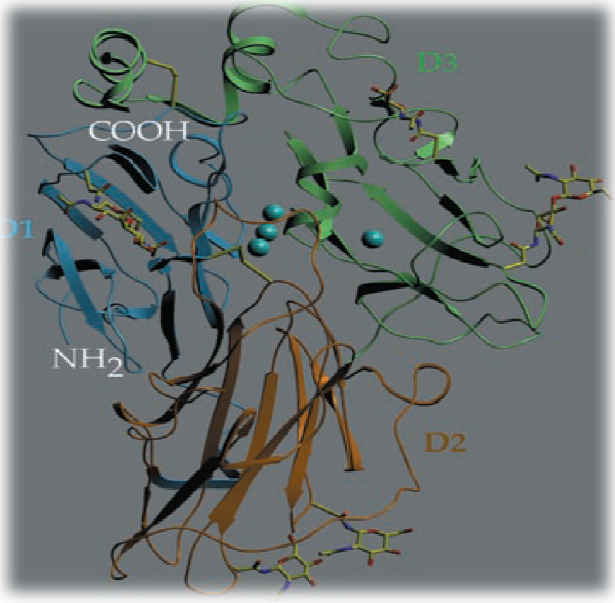


Fig. 5: Ribbon diagram of *Trametes versicolor* laccase, The arrangement of the domain structure is depicted in different colour coding (D1–D3). Copper ions are drawn as *blue spheres*. Carbohydrates and disulfide bonds are included as stick models (Enguita *et al.,* 2003).

2.7 CATALYTIC PROPERTIES OF LACCASE

Laccase nomenclature is defined in the Enzyme Commission (EC 1.10.3.2), where EC 1 indicates oxidoreductases, EC 1.10 denotes action on diphenols and related substances as donors. Laccases, (benzenediol: oxygen oxidoreductase) are either mono or multimeric copper-containing oxidase which catalyze the reduction of oxygen to water accompanied by the oxidation of a phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water (I).

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O \qquad \text{(I)}$$

As one electron oxidation of a substrate is coupled to a four-electron reduction of oxygen the reaction mechanism cannot be straight forward (Thurston, 1994). When oxidized by a laccase, the reducing substrate loses a single electron and usually forms a free radical (II). The unstable radical may undergo further laccase catalyzed oxidation or non-enzymatic reactions including hydration, disproportionation, and polymerization (Thurston, 1994). Laccase-catalyzed oxidation of phenolic hydroxyl groups to phenoxy.

$$PhOH + O_2 \xrightleftharpoons{\text{laccase}} 4PhO^{+} + 2H_2O \qquad \text{(II)}$$

Laccase catalysis involves:

(i) Reduction of the type 1 copper by reducing substrate

(ii) Internal electron transfer from the type 1 to the type 2 and type 3 copper

(iii) Reduction of oxygen to water at the type 2 and type 3 copper site

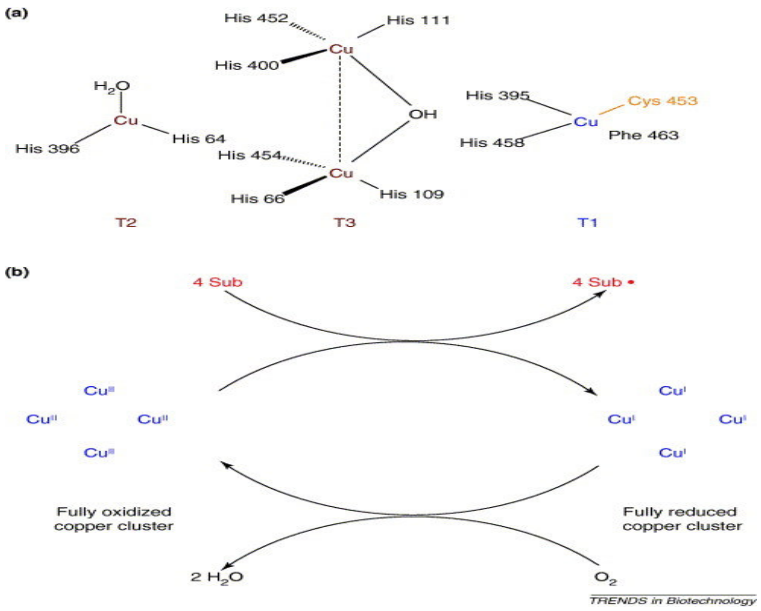


Fig. 6: Laccases: active-site structure and catalytic cycle. (a) Model of the catalytic cluster of the laccase from *Trametes versicolor* made of four copper atoms. Type I (T1) copper confers the typical blue colour to the protein and is the site where substrate oxidation takes place. Type 2 (T2) and Type 3 (T3) copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place . (b) Schematic representation of a laccase catalytic cycle producing two molecules of water from the reduction of one molecule of molecular oxygen and the concomitant oxidation (at the T1 copper site) of four substrate molecules to the corresponding radicals. Sub: substrate molecule; Sub-oxidized substrate radicals (Thurston, 1994).

A characteristic blue appearance is exhibited with purified laccases from electronic absorption around 600nm. UV-visible spectra of laccase (at resting state) typically show two maxima around 280 nm to that at 600 nm are generally 14 to 30, and the ratio of the absorbance at 330nm to that at 600 nm is 0.5 to 2 (Xu, 1999). Most laccases have four copper atoms per monomer in holoenzyme

form (Coll *et al*., 1993), the prosthetic group laccase from phlebia was reported to have two copper and one pyrroloquinoline quinone. White laccase was described in one of the laccases from *Pleurotus ostreatus* (Palmieri *et al*., 2000). Using UV/visible and electron paramagnetic resonance (EPR) spectroscopy the copper atoms are classified in three groups (Leontievsky *et al*., 1997). Blue colour of the enzyme is due to Type I copper (T1), and is the site for oxidation with standard redox potential. It has a strong electronic absorption around 600nm, EPR is detectable. The type II copper (T2) is colourless, but EPR is detectable and the type 3 copper (3) gives a weak absorbance using the UV spectrum at 330 nm and consists of a pair of copper atoms but no EPR signal. Copper sites of T2 and T3 are close together and form a trinuclear centre (Leontievsky *et al*.,1997), place where binding of dioxygen and four - electron reduction to water occur (Piontek *et al.,* 2002). For some laccases that T1 is the primary center at which electrons are accepted from reducing substrates [Xu, 1996]. High redoxpotential T1 site are of special interest in biotechnology e.g., bioremediation processes (Mayer *et al.,* 2002), for different bleaching (Balakshin *et al.,* 2001).

Polyphenol oxidases are associated with three types of activities

- Catechol oxidase or o- diphenol: oxygen oxidoreductase (EC: 1.10.3.1)
- Laccase or p- diphenol: oxygen oxidoreductase (EC: 1.10.3.2)
- Cresolase or monophenol monooxygenase (EC: 1.18.14.1)

It is difficult to define laccase by its reducing substrate due to its very broad substrate range, which varies from one laccase to another and overlaps with the substrate range of another enzyme the monophenol mono-oxygenase tyrosinase (EC: 1.14.18.1). Although laccase was also called diphenol oxidase, monophenols like 2,6-dimethoxyphenol or guaiacol are often better substrates than diphenols, e.g. catechol or hydroquinone. Laccases can oxidize a wide range of molecules as their reducing substrates. Thurston (1994) stated in a review that simple diphenols like hydroquinone and catechol are substrates (for most laccases, but not for all), but that guaiacol and 2,6 - dimethoxyphenol (DMP) are often better. Para -phenylenediamine is widely used substrate, and syringaldazine (N,N'-bis 3,5 dimethoxy -4 hydroxy benzylidene hydrazine) is a unique substrate

for laccase only (Harkin *et al*., 1973) as long as hydrogen peroxide is avoided in the reaction, as this compound is also oxidized by peroxidases. Many of the compounds occur naturally and/or can be important industrial precursors, products, or byproducts. Their transformability by laccases might allow various viable applications of these enzymes.

Mediators of Laccase

A mediator is a small molecule that acts as an 'electron shuttle'. Once oxidised by the enzyme generating a strongly oxidizing intermediate, the co-mediator (Medox), it diffuses away from the enzyme pocket and in turn oxidises any substrate that, due to its size, could not directly entre the enzymatic pocket (Banci *et al.*, 1999). An ideal redox mediator must be a good laccase substrate. Its oxidised and reduced forms must be stable but should not inhibit the enzymatic reaction. Redox mediators have sufficiently high redox potentials and can perform multiple catalytic reactions without chemical degradation (Morozova *et al.*, 2007).

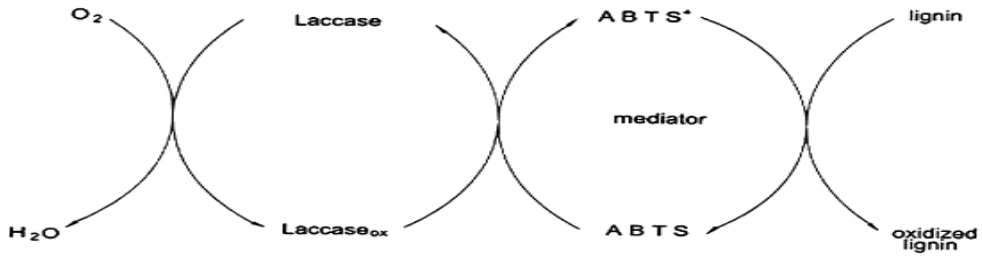


Fig 7: Oxidative pathway for catalytic action of laccase on lignin during biobleaching of pulps (Paice and Bourbonnais , 1990).

For instance, in the presence of the suitable redox mediator, 2, 2′-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), laccases from *T. versicolor* oxidise non-phenolic model compounds, which are not substrates for laccase alone (Bourbonnais and Paice, 1990, Archibald *et al.*, 1997). The laccase mediator system (LMS) was originally developed to solve problems in bio-bleaching of wood pulps and was firstly described by Bourbonnais and Paice (1990) with the use of ABTS as the first mediator. Laccases are thought to play a role in the biodegradation of lignin but this is restricted to phenolic compounds

because of the low oxidation potentials of these enzymes (Reid and Paice, 1994). Application of these enzymes in the presence of the so-called mediator compounds resulted in high redox potential (>900 mV) leading to the oxidation of nonphenolic lignin model compounds. It is suggested that delignification of Kraft pulp with laccase/ABTS system is likely to proceed by a combination of two actions: first, Cα-Cβ cleavage of nonphenolic sites in lignin, and second, solubilisation of the lignin fragments by formation of hydrophilic lignin ABTS. Recently, laccase-catalysed oxidation reaction of ABTS was studied by means of mass spectrometry and the mechanism was revealed. Under laccase catalysis ABTS formed ions, which could be directly detected using either positive (protonated ABTS, ABTS radical cation, ABTS dication) or negative (ABTS anion, ABTS dianion) ionization mode MS (Marjasvaara *et al.*, 2008). The application of the LMS on hardwood Kraft pulp resulted in reduction of kappa number (lignin number), demethylation and depolymerisation of Kraft lignin (Reid and Paice, 1994, Paice *et al.*, 1995, Archibald *et al.*, 1997).

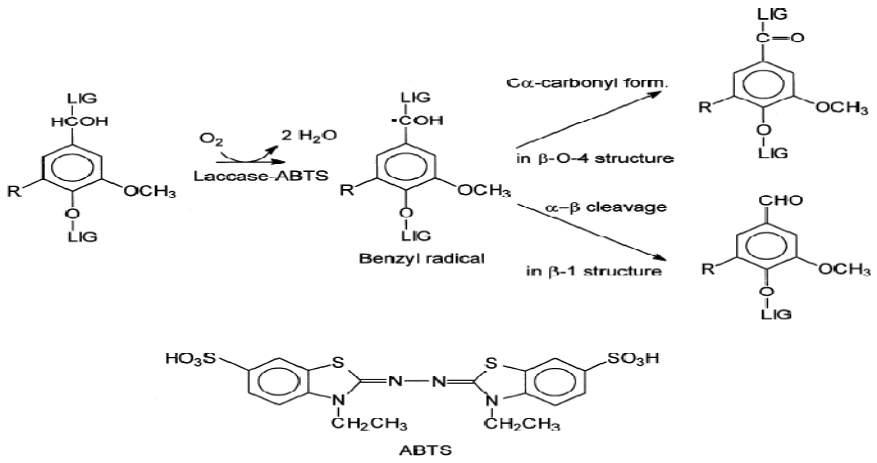


Fig. 8: Oxidation of non-phenolic lignin subunits by laccases and a mediator ABTS (Archibald *et al.*, 1997)

The LMS was successfully applied to the oxidation of aromatic methyl groups, benzyl alcohols (Johannes *et al.*, 1998), polycyclic aromatic hydrocarbons (Johannes *et al.*, 1998, Majcherczyk *et al.*, 1998) and degradation of textile dyes (Hardin *et al.*, 2000). The oxidised mediator can follow an electron transfer, a radical hydrogen atom transfer or an ionic route for the oxidation of substrates. The activity of a LMS towards lignin is dependant on two main

factors. Firstly, the redox potential of the enzyme and, secondly, the stability and reactivity of the radical resulting from the oxidation of the mediator. It has been shown that laccases from different organisms react variably with different mediators and different substrates (Bourbonnais *et al.*, 1997). Approximately 100 different potential mediator compounds have been described for the LMS, but ABTS and HBT remain the most commonly used (Bourbonnais *et al.*, 1997, Majcherczyk and Johannes, 2000).

Recently, further studies have revealed the delignifying action of natural mediators in the LMS. Natural mediators include phenol red, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol (Majcherczyk and Johannes, 2000, D'acunzo and Galli, 2003). A natural mediator, 3-hydroxyanthranilic acid (HAA), has been detected in the white rot fungus *P. cinnabarinus* culture broth (Eggert *et al.*, 1996a, Geng and Li, 2002). The use of natural mediators has proved to be as efficient as the commonly used ABTS and HBT (Majcherczyk and Johannes, 2000). Phenol red and its derivatives particularly dichlorophenol red are mediators of *Poliporus pinsitus* laccase. It has been shown that their efficiency in oxidation of the nonphenolic substrate 4-methoxybenzyl alcohol is at least 10 times higher than with HAA (D'Acunzo and Galli, 2003).

2.8 PHYSICO-CHEMICAL PROPERTIES

Laccase enzyme occurs as isozymes recurrently or monomers that oligomerize to form multimeric complexes. Each laccase isozyme has four copper atoms and is able to individually carry on its catalytic mechanism. The molecular mass of the laccase monomers ranges from 40-130 kDa with a covalently linked carbohydrate content of 10–25% in fungi and 20-45% in plants. The carbohydrate moiety typically consists of mannose, N-acetylglucosamine and galactose, which may contribute to the high stability of the enzymes (Claus, 2003).

The acidic isoelectric point of laccases is around pH-4.0. The pH optima of laccases are highly dependable on the substrate. Usually, fungal laccases exhibit pH optima in the range of 3.0-5.0 and bacterial laccases in the range of

5.0-6.0, when the substrate is a hydrogen atom donor compound (i.e., ABTS) (Heinzkill *et al.,* 1998). When the substrate is a phenolic compound (e.g., syringaldazine), the optimal pH is shifted to 6.0-7.0. This shift in pH is a result of the balance of redox potentials between the substrate and the inhibition of the T2/T3 copper site by binding of an OH^- ion.

The optimal temperature differs with the source of laccase, being 40°C for *Fusarium* sp. BOL35 laccase, 50°C for *Trametes versicolor*, 60°C for *Galerina* sp HC1, and 70 °C for *Myceliophthora thermophila*. In general, laccases are stable at 30-50°C and rapidly lose activity at temperatures above 60°C (Xu *et al.,* 1996). The most thermostable laccases have been isolated from bacteria; the half-life of *Streptomyces lavendulae* laccase was 100 minutes at 70°C and that of *Bacillus subtilis* CotA was 112 minutes at 80°C (Martins *et al.*, 2002). The typical half-lives of fungal laccases are clearly below one hour at 70°C and below 10 minutes at 80°C (Xu *et al.,* 1996).

The redox potential ranges from 0.4-0.5 V in plants and bacteria to 0.4-0.9 V in fungal laccases. The redox potential of the Type 1 site has been determined for many laccases using different mediators and varies from 430 mV for the laccase from *R. vernicifera* tree up to 780 mV for fungal laccase from *Polyporus versicolor*. It was previously found that the catalytic efficiency (*kcat*/*Km*) of laccases for some reducing substrates depended linearly on the redox potential of the Type 1 copper, in the sense that the higher the potential of the Type 1 site the higher the catalytic efficiency. That is why laccases with a high redox potential of the Type 1 site are of special interest in biotechnology, e.g., for efficient bleaching and bioremediation processes. The catalytic action of an enzyme is quantitatively described by the Michaelis constant *Km* and the catalytic efficiency constant *kcat*. The turnover is heterogeneous over a broad range depending on the source of enzyme and substrate/type of reaction. The kinetic constants differ in their dependence on pH. *Km* is pH-independent for both substrate and co-substrate, while *Kcat* is pH-dependent.

Three-dimensional structural analysis of several fungal, bacterial and plant laccases reveals that all are composed of three sequentially arranged

cuprodoxin-like domains; each of them with a greek key β-barrel topology, highly related to small copper proteins such as azurin and plastocyanin. The multiple alignment of primary sequences of laccases shows that the copper binding motifs are highly conserved in all sequences, which reflects a common mechanism for copper oxidation and oxygen reduction. However, putative binding pocket analysis reveals that bacterial laccases have larger binding cavities when compared to those from plants and fungi.

Until recently, the three-dimensional structure of five fungal laccases has been reported: *Coprinus cinereus* (in a copper Type 2-depleted form) (V.Ducros, 1998), *Trametes versicolor* Piontek *et al.,* (2002), *Pycnoporus cinnabarinus*. *Melanocarpus albomyces and Rigidoporus lignosus*, the latter four enzymes with a full complement of Cu ions. Moreover, thethree-dimensional structure of the CoA laccase from *Bacillus subtilis* endospore hasalso recently been published (Enguita, 2003).

2.9 ISOLATION OF LACCASE PRODUCING MICROBES

In nature microbes including bacteria which are ubiquitous exist as mixed communities and their identification depends upon isolation of individual colonies. The isolation of axinic culture from various environmental sources is basic and important in all research work related to microbes. Since bacteria are ubiquitous and live in microbial communities of mixed populations. Population in microbial communities or ecosystem may interact and co-operate in their efforts to obtain nutrients from the environment and with the waste products from one group of microbes serving as nutrients for another. The wastes/metabolic waste from various industries and fermentation processes also provide a favourable ecological niche for bacteria. Laccase producing microbes can be isolated from various sources like soil, hot water textile effluent, municipal waste, pulp and paper industry waste water, tree barks etc. Some of the laccase producing fungi belonging to class *Ascomycete* and *Basidiomycete* have been isolated from the marine samples (Verma *et al*., 2010).

Kiiskinen *et al.,* (2004) isolated and screened novel laccase-producing microbes from various environmental sample such as decomposing tree stump, oak leaf compost, birch log, spruce chip pile, test compost of municipal biowaste at different composting stages, crude cork material, process waters of a Portuguese cork factory, soiled pulp sample from a pulp mill. Fungi were cultivated on solid media containing indicator compounds that enabled the detection of laccases as specific colour reactions. The indicators used were Remazol Brilliant Blue R (RBBR), Poly R-478, guaiacol and tannic acid. The screening work resulted in isolation of 26 positive fungal strains. However, Viswanath *et al.* (2006) isolated laccase producing fungi from different environmental samples using the indicator compound guaiacol. Out of 12 cultures tested, six cultures were found to be laccase-positive with *Stereum ostrea* and *Phanerochaete chrysosporium* being the best potential cultures.

Gochev and Krastanov (2007) isolated laccase producing *Trichoderma* sp from four soil samples of farming, forest, city surroundings and mountain from Ploudiv Province of Bulgaria on crystal violet media. Based on its dye decolorization activities four *Trichoderma* strains were screened as laccase producers. The strains were identified as *Trichoderma atroviride*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, and *Trichoderma viride*. Liang, Han and Chu (2007) isolated a new thermotolerant laccase producing species, *Paecilomyces biformis* from a soil sample collected of Tengchong Geothermal National Geopark, Yunnan Province, PR China on potato dextrose agar plates containing different concentrations of O-methoxyphenol, the fungus exhibited laccase activities.

Ticlo D'Souza *et al.,* (2009) isolated a thermostable metal-tolerant laccase producing marine-derived fungus from decaying mangrove wood of Charao Island in Goa. The fungus was isolated on Boyd and Kohlmeyer agar medium prepared with half strength sea water and identified as *Cerrena unicolor* based on the D1/D2 region of 28S and the 18S rDNA sequence. However, Liu *et al*. (2009) isolated *Paecilomyces* sp. WSH-L07 for laccase production. They identified *Paecilomyces* sp. WSH-L07 according to the morphological characteristics and

the comparison of internal transcribed spacer (ITS) ribosomal DNA (rDNA) gene sequences. Production of laccase by *Paecilomyces* sp. WSH-L07 was greatly enhanced by the nontoxic inducers of copper sulphate and methylene blue. Under the induction of 50 μM copper sulphate and 20 μM methylene blue, the maximum laccase production was obtained. When these inducers were added into cultivation medium at 24 h and 12 h, respectively, an increment of about 100 times of laccase activity compared with that of in inducer-free medium and about two times of that of in single copper-supplemented medium was observed.

Desai and Dhala (2009) isolated laccase producing fungi from soil and tree bark scrapings of farms and forests of different places in and around Hubli were screened for isolation of laccase producing fungi. Fungi were cultivated on potato dextrose agar (PDA) plates containing indicator compounds namely guaiacol and tannic acid. It resulted in isolation of 9 fungal strains, among which one of the strains was presumed to be potent depending on its growth characteristics and was used for further experiments. It was identified as belonging to *Trichoderma* genus.

Mabrouk *et al*. (2010) screened some marine-derived fungal isolates for lignin degrading enzymes (LDEs) production. A study was carried out to establish the lignin-degrading enzymes (LDEs) activity of a large number of diverse marine ascomycetes. Eighty eight fungal isolates obtained from different algae, sea grasses and decaying wood samples collected from Abou-keer, Alexanderia and Egypt, were screened for the presence of laccase (Lac), lignin-peroxidase (LiP) and manganese-dependent peroxidase (MnP) activities. Results obtained from both qualitative and quantitative assay showed that the marine fungal isolate *Trematosphaeria mangrovei* measured the highest zone diameter and colony diameter in agar plate screening test with guaiacol. It was recommended that the marine fungal isolate *Trematosphaeria mangrovei* was the most promising one for laccase enzyme production.

More *et al.* (2011) isolated non-spore forming Basidiomycetes *Pleurotus* sp. fungal laccase from samples of decomposed stumps, leaf litter, biowaste of municipal compost and fruiting bodies of mushrooms. Isolation was carried out

on culture medium (B and K agar) and pure culture was tested for the presence of laccase using α-naphthol. Similarly, Christie and Shanmugam (2012) isolated fungal cultures from soil samples of Anamalai hills on Rose Bengal Agar plates for laccase enzyme production. Fungus was then screenend for laccase activity on B and K agar medium containing ABTS and Guaiacol as indicator compounds. Fungi which showed positive reaction were identified using intergenic spacer region sequence (ITSF) and Internal transcribed spacer region (ITSR) primer and found to be belonging to *Ascomycetes* species i.e. *Aspergillus niger, fusarium oxysporium, Alternaria arborescence* and *Pencillium marnefii*.

Abraham and Singh (2013) isolated eleven laccase producing fungal isolates from compost soil by serial dilution. They were cultivated on potato dextrose agar (PDA) plate with indicator compound 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to screen for the laccase production ability. Whereas, Alfarra *et al.,* (2013) isolated laccase producing fungi from samples trees barks of International Islamic University Malaysia, Kuantan campus using different chromogenic substrates on the potato dextrose agar plates such as ABTS, Tannic Acid, Azure B and Guaiacol. They found isolates of fungi has excellent ability to produce laccase. The microscopic visualization showed the characteristics of white-rot fungi.

Kalra and Chauhan (2013) isolated laccase producing *Trichoderma* sp. from water samples. They inoculated cultures on potato dextrose agar plates supplemented with 0.04% guaiacol for the isolation of fungal species. The cultures were purified by repeated transfer to potato dextrose agar plates and grown at 25°C for different periods of time.

2.9.1 Isolation of laccase producing bacteria

The first laccase producing bacteria was *Azospirillum lipoferum* from the rice rhizosphere. This advantage makes bacterial laccases as biocatalyst more important because of their high capacity of oxidising phenolics, aromatic compounds and suitable for some a biotechnological application such as biodegradation of xenobiotic compounds including methoxyphenol, aniline and benzene thiols (Solano *et al.,* 1997; Xu *et al.,* 1996). Bacterial laccases have been

found to possess certain advantages over their fungal counter parts, such as withstanding high temperature, extreme pH, less sensitivity towards halides and fast growth. It has been recognised that they possess better detoxification efficancy for pollutants and increase their industrial potential in pollution degradation. Laccase producing bacteria can be isolated from various sources like composted municipal waste, decomposing tree stump, oak leaf compost, birch log, crude cork material, soiled pulp from pulp mill, paper industry waste water and rhizosphere of rice. Media most commonly used for isolation and screening of laccase producing bacteria reported so far were M162, M9, LB, NA, TY and TYM containing indicator compounds such as guaiacol, ABTS, dimethoxyphenol, pyrogallol and napthol.

Givaudan *et al*. (1993) isolated first laccase producing non-motile forms of *Azospirillum lipoferum* from the rice rhizosphere of rice. Using a combination of substrate/inhibitor specificity tests, intracellular enzyme extracts of non-motile strains were demonstrated to have laccase activity by oxidising various *o*-diphenols and *p*-diphenols. This work was followed by isolation of *Azospirillum lipoferum* 4T TnS mutants. *Azospirillum lipoferum* 4T isolate was found to possess original properties of nonmotility, melanin synthesis, and laccase activity. Following random Tn5 mutagenesis in *A. lipoferum* 4T, 10 mutants were obtained which were affected in melanization and laccase activity. The class 1 mutants, with intermediate levels of laccase activity, showed some coloration whereas the class 2 mutants were found completely negative for laccase activity and these were also colorless (Faure *et al*., 1994). Alexander and Bally (1999) reported another non-motile strain of *Azospirillum lipoferum* isolated from rice rhizosphere on TY medium which revealed the most unusual polyphenol oxidase activity. It was further reported that enzyme laccase activity was present intracellularly in *Azospirillum lipoferum* 4T, which resulted in melanin production contributing to scavenging of toxic phenol components.

Rhizobial bacterial strains have also been reported to produce laccase and it was first reported in *Sinorhizobium meliloti* melanin producing strain**.** Sowinski *et al.* (2002) reported Tryptone Yeast medium supplemented with tyrosine and

$CuSO_4$ for isolation and maintenance of *Sinorhizobium meliloti*. Twenty four *Sinorhizobium meliloti* strains were isolated from Uruguayan soil. Laccase like activity was determined using syringaldazine as a substrate in cell free extract of CE52G, CE46 and U143 starins.

Bains, Capalash and Sharma (2003) reported laccase from a non-melanogenic, alkalotolerant γ -*Proteobacterium* JB isolated from industrial wastewater drained soil on M162 medium supplemented with guaiacol. Two hundered isolates were obtained initially from various soil samples and only one bacterium found to produce laccase, was identified as *γ-Proteobacterium* from its partial 50 bp 16S rRNA sequence analysis (Genebank accession No. AF574088).

A thermostable laccase was reported in an extremely thermophilic bacteria *Thermus thermophilus* HB27. This bacterium grows at 68-85^{0}C. Laccase activity was detected and the enzyme was named as Tth-laccase. The medium used for isolation of bacteria reported was TYM medium supplemented with $CuSO_4$ (Miyazaki, 2005). However, Raj *et al*. (2007) isolated a new kraft lignin degrading bacterial strain, *Aneurinibacillus aneurinilyticus* (ITRC S7) from sludge of pulp and paper mill using mineral salt medium and characterized as *Aneurinibacillus aneurinilyticus* by biochemical tests and 16S rRNA gene sequencing.

Another laccase positive bacteria *Stenotrophomonas maltophilia AAP56* was isolated and identified by biochemical and molecular methods. It was cultured in Luria Bertani (LB) medium containing 0.4 mM copper sulfate. The addition of $CuSO_4$ in culture medium improved production of laccase activity. Decolorization activity of some synthetic dyes such as methylene blue, methyl green, toluidine blue, congo red, methyl orange, and pink and the industrial effluent SITEX Black was successfully achieved (Galai *et al*., 2009).

Nikitina *et al*. (2010) investigated intracellular and extracellular phenol oxidase activity in a number of *Azospirillum* strains of various species. Three species of *Azospirillum* were used, four strains of *Azospirillum brasilense*, two strains of *Azospirillum lipoferum*, two strains of *Azospirillum irakense* and single

strain of *Azospirillum amazonense*. Synthetic medium contains KH_2PO_4, 0.4; $FeSO_4$. $7H_2O$, 0.01; $CaCl_2$, 0.026; $MgSO_4$, 0.2; KNO_3, 0.05 and glucose, 1.44 at pH 6.8 was used for culturing, followed by qualitative detection of oxidase activity using ABTS, 2,6-dimethoxyphenol and syringaladazine and laccase activity was determined by green appearance of bacteria in first case, brown coloration in second and pink in third. *Azospirillum brasilense* strains Sp245 and Sp7 and *Azospirillum irakense* strain KA-3 and KBC-1 can oxidise 2,6-dimethoxyphenol whereas syringaldazine was oxidised by only *Azospirillum brasilense* strains Sp245 and Sp7 and substrate ABTS by *Azospirillum lipoferum* 43 and *Azospirillum irakense* strains KA-3 and KBC-1.

Wang *et al.* (2010) isolated and characterized a novel *Bacillus subtilis* WD23 exhibiting laccase activity from forest soil. The M9 medium containing Cu^{2+} was used for enriching and isolating bacterial strains capable of oxidizing syringaldazine. One isolated strain was identified as *Bacillus subtilis* WD23 based on the results of physiological and biochemical tests and 16S rDNA sequence analysis. The strain WD23 could grow at temperatures ranging from 20 to 55°C and showed optimum growth temperature and pH at 25°C and 7.0, respectively. The temperature half-life of the spore laccase was 2.5 h at 80°C and the pH half life time was 15 d at pH 9.0. Its spore laccase could decolorize 50 - 90% of Remazol brilliant blue R (RBBR), alizarin red, congo red, methyl orange and methyl violet, which suggests the potential application of spore laccase in dyestuff treatment.

Another thermostable laccase was found to be present in *Bacillus sp.* XJT-7 isolated from marine environment using toluene enriched media. The vegetative cells possessed thermostable laccase which remain active at 100^0C shock. These heated spores also termed as spore ghost have been found useful in biodegradation due to high laccase activity (Abari *et al*., 2012).

Sheikhi *et al.* (2012) screened laccase producing bacteria from pulp and paper industry weastwater. Bacteria isolated from collected samples on Nutrient agar medium containing 0.5mM Guaiacol. Bacterial DNA was extracted for phylogenic identification and the 16S rDNA amplified using Universal 16S

rDNA PCR primers. Product of PCR found to have 1500 bp band on 1% agarose gel. Phylogenic relationship determined by Blast and comparative analysis of 16S rDNA sequence revealed it as a member of *Bacillus subtilis* WPI (Gene Bank accession No JN082773).

Huang *et al.* (2013) isolated 140 bacterial strains from soils of a biodiversity-rich rainforest in Peru and isolates screened based on their oxidative activity on ABTS, a laccase substrate. Strain C6 (*Bacillus pumilus*) and strain B7 (*Bacillus atrophaeus*) were selected for their high laccase activity and identified by 16S rDNA analysis. Whereas, Dhiman (2014) isolated sixty seven bacterial isolates from peddy fields and paper milis of Himachal Pradesh, India. Bacteria were isolated on TY agar plates supplemented with 5mM guiacol.

2.10 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

Determining colony morphology is important to characterize a bacterial colony in cultures. The term "colony morphology" refers to the visible characteristics of a colony. Colonies that differ in appearance are typically different bacterial strains, species, or genera. However, colony morphology is not a reliable way to identify bacteria, as many different types of bacteria have similar colony morphology. Still, recognizing differences in colony morphology is useful when trying to isolate bacteria. Although bacterial colonies can differ in the details of their appearance, a colony basically looks like a dot growing on the medium. This dot is composed of millions of bacteria that arose through binary fission from one initial bacterium, the parent. Description of a colony's morphology includes its shape, the margins or edges of the colony, the colony's color, as well as surface features. Some colonies are round and smooth; others can have wavy edges and a wrinkled appearance. Classification based on morphological and biochemical features highlights the evolutionary relationships of bacteria remains the most practical way to identify these organisms. A definitive identification scheme for bacteria was first presented in 1984 in **Bergey's Manual of Systematic Bacteriology.** In this scheme, bacteria are classified on the basis of many characteristics. Cell shape, nature of multicell

aggregates, motility, formation of spores, and reaction to the Gram stain are important. Morphological features are important in microbial taxonomy for many reasons. Morphology is easy to study and analyze, particularly in eukaryotic microorganisms and the more complex prokaryotes. In addition, morphological comparisons are valuable because structural features depend on the expression of many genes, are usually genetically stable, and normally do not vary greatly with environmental changes. Thus, morphological similarity often is a good indication of phylogenetic relatedness. The different morphological features employed in the classification and identification of microorganisms are: cell shape, cell size, colonial morphology, ultrastructural characteristics, staining behavior, mechanism of motility, endospore shape and location, spore morphology and location, cellular inclusions and color.

Biochemical/metabolic characteristics are also very useful because they are directly related to the nature and activity of microbial enzymes and transport proteins. Microbial metabolism is the means by which a microbe obtains the energy and nutrients (e.g. carbon) it needs to live and reproduce. Microbes use many different types of metabolic strategies and species can often be differentiated from each other based on metabolic characteristics. The specific metabolic properties of a microbe are the major factors in determining that microbe'secological niche, and often allow for that microbe to be useful in industrial processes or responsible for biogeochemical cycles. Since proteins are gene products, analysis of these characteristics provides an indirect comparison of microbial genomes. Biochemical tests help in the identification of a genus and species of bacteria, including the determination of the kinds of nutrients a cell can use, the products of its metabolism, the response to specific chemicals, and the presence of particular characteristic enzymes. Other criteria used for the identification of some types of bacteria might be their antigenic composition, habitat, disease production, and requirement for specific nutrients. Some tests are based on the ultrastructure of the bacteria revealed under the electron microscope by negative staining and preparation of thin sections.

Both morphological as well as biochemical characterization technologies have been immensely useful and have been applied in carrying out characterization of most of the microorganisms.

Wang *et al.* (2010) isolated and characterized a novel *Bacillus subtilis* WD23 exhibiting laccase activity from forest soil on the results of physiological and biochemical tests and 16S rDNA sequence analysis. Whereas, Zhao *et al*. (2011) characterized a laccase producing strain on the basis of morphological characterization and biochemical. They found out a strain LSO4 was a gram positive, spore forming and motile long rods. The colonies were rough, white and opaque, with irregular edge. Good growth could occur at temperature of 50^0C and pH 9.0. It was also able to grow in medium containing up to 11% NaCl. Strain LS04 gave positive tests for catalase and oxidase activity, starch and gelatin hydrolysis as well as for V–P test, indole test and nitrate reduction. The strain showed negative reactions for methyl red test. It was capable of using sodium citrate, sodium malonate and sodium propionate as the sole carbon source. Positive tests were also observed for acid production from glucose, maltose, rhamnose, mannose, arabinose, fructose, mannitol, inositol and sorbitol.

Three Gram-positive rods and one Gram-positive coccus laccase producing bacteria was identified by Abari *et al.* (2012) on the basis of morphological and biochemical characterization. The Gram positive, spore-forming, oxidase- negative and catalase-positive rods which produce convex orange colonies were identi-fied as *Bacillus amyloliquefaciens*. The Gram-positive coccus, which had aerobic growth and which was capable of nitrate reduction, oxidase and catalase positive and capable of endospore forming, was identified as *Sporosarcina halophila*. The Gram-positive spore-forming, oxidase negative catalase-positive rods which produce slime white colonies were identified as *Bacillus firmus*.

Sheikhi *et al.* (2012) also identified a laccase producing bacteria as *Bacillus subtilis* WPI on the basis of biochemical tests and 16S rDNA sequence. This strain presented colonies of 1–3 mm in diameter, white colour and was Gram positive. It was motile and capable to hydrolyze gelatine. The strain was

urease, catalase, casein protease, cytochorme C oxidase and amylase positive. It was also able to reduce NO^{3-} to $NO^{2-}$and Voges-Proskauer (VP) tests reaction was positive. Citrate test and Indole production were negative. The strain was facultative anaerobic and was able to produce acid compounds from utilized glucose and sucrose. The strain was not able to use manitol, lactose and xylose. The morphological and biochemical characteristics of this strain according to Bergey's Manual of Systematic Bacteriology showed that it was close to the members of *Bacillus subtilis*.

Karthikeyan and Anbusaravanan (2013) isolated, identified and characterized bacteria from textile effluents mixed with sewage from river Amaravathy, Karur, Tamilnadu, India. Based on the results of these various biochemical tests, the isolates were identified, which were confirmed by 16S rRNA sequencing as *Bacillus cereus* AK1968 (accession numbers JN689235) and *Pseudomonas* sp. AKDYE14 (accession numbers JN674167).

2.11 MOLECULAR CHARACTERIZATION AND PHYLOGENETIC STUDIES

The traditional biochemical and morphological methods for identification of bacterial species depends upon different biochemical testing and colonial morphology study though are simple to execute, require minimal equipment and accurately differentiate between more common species but these are time-consuming due to long incubation time period and delay final identification. Several genes common to all bacteria have been studied in sequence-based identification. The use of molecular characterization technology is considered as recent standard approach for identification of all bacteria and is widely recognized as a rapid and accurate method for identification of novel bacterial culture. The analysis of DNA has been used in a large number of studies on bacterial taxonomy, bacterial typing, and also added to our understanding of the basic mechanisms of evolution. The availability of DNA sequences for the complete genome of a number of bacteria has paved the way for the identification of orthologous families of genes within genomes based on DNA sequence and gene function (Eisen, 1998a,b). The characterization of orthologous gene families

has been termed phylogenomics (Eisen, 1998b). Related to phylogenomics is the concept of exaptation defined as the recruitment of DNA or RNA sequences into variant or novel function: RNAs from all categories have been identified that may be exapted as novel genes or regulatory elements (Brosius, 1999; Brosius & Gould, 1992). Similarly, bacterial morphology provides significant evolutionary insights when mapped onto rRNA phylogeny since bacterial morphology has been shown to be remarkably consistent with rRNA phylogeny (Siefert & Fox, 1998). A phylogenomic approach that includes information about the function of genes, exaptation and bacterial genetics of morphology and or biochemistry in the phylogenetic analysis may in the future be of value in inter-genomic as well as intra-genomic analyses of isolates.

The evolution of bacteria has long been viewed primarily through the perspective of rRNA sequences (Woese, 1987). The gene content of the genome (Snel *et al.,* 1999), the evolutionary rates of different classes of genes (Jain *et al*., 1999), genome signatures (defined as the ratios between the observed dinucleotide frequencies and the frequencies expected if neighbours were chosen at random). G+C content (Sueoka, 1999) and the occurrence and location of gene duplications, rearrangements, insertions, gene capture (Szpirer *et al.,* 1999) and horizontal gene transfer (Lawrence & Hartl, 1992) are all indices of genome organization that vary between species. A much more complete analysis of the taxonomy and evolution of bacteria may be possible with the current rapid DNA analysis techniques if these parameters are used as a framework.

The analysis of DNA sequences has significantly furthered knowledge about the relatedness of different isolates in at least three categories including random whole-genome analysis, specific gene variation and mobile genetic elements. The numerous studies in these areas has provided considerably more information than traditional phenotypic methods and with this more questions have been raised that were previously not evident.

The analysis of whole genomes by DNA band pattern analysis has been facilitated by the discovery of repetitive elements and their analysis by a number of PCR based techniques (including RAPD, ERIC, BOX and REP) and the

analysis of restriction enzyme sites on bacterial chromosomes by PFGE and AFLP. These two advances led to a plethora of studies using these techniques in a wide range of bacterial species. All of these techniques recognize random sites on the genome that cannot be predicted without the whole genome sequence. These random whole-genome analysis techniques have been applied mainly in strain differentiation (RAPD, PFGE) and to a smaller extent in taxonomy (AFLP). But technically, RAPD suffers from non-reproducibility and non-portability.

The recent techniques for molecular typing, taxonomy and evolution of bacterial isolates include both single-locus and multi-locus approaches. The single-locus approach uses highly variable genes. In contrast, multi-locus approaches include multi-locus sequence typing (MLST) and the analysis of multigene families such as *rrn* operons and trna genes. The latter includes 16S rRNA variations: intergenic (16S-23S rRNA) spacer regions (ISR) typing; ISR concerted evolution; ISR trna genes and trna genes not associated with *rrn* operons.

2.11.1 16S rRNA gene technology

16S rRNA has generated as a powerful method for the identification of microorganisms and defining or restructuring prokaryotic taxa according to their natural relationships. Rapid progress in sequencing and *in vitro* nucleic acid amplification technology has developed rapid and easy to apply routine techniques such as 16S rRNA. The analysis of small rrna gene sequences is another important landmark in the study of evolution and classification of living organisms. Traditionally, living organisms were classified, according to

Table 3: Genomic approaches to typing, taxonomy and evolution of bacterial isolates

Genetic Element	Species	References
Genome organization/function		
Horizontal transfer	All	Lawrence & Hartl, 1992
Operational informational genes	All	Fitz-Gibbon & House, 1999
tRNA	All	Sueoka, 1999
G+C content	All	Franco *et al*., 1999
Gene content	All	Snel *et al*., 1999
Genome signatures	All	Campbell *et al*., 1999

Random whole genome analysis		
Randomly amplified polymorphic DNA (RAPD)	All	Caetono-Anolle, 1993
Enterobacterial repetitive intergenic consensusd sequences (ERIC)	*Enterobacteria*	Hulton *et al*., 1991
Invertedly repeated elements (BOX)	*Streptococcus*	Martin *et al*., 1992
Repetitive extragenic palindromic sequence (REP)	*E. coli*	Gilson *et al*., 1984
Pulse-field gel electrophoresis (PFGE)	All	Tenover *et al*., 1995
Amplified fragment length polymorphism (AFLP)	All	Savelkoul *et al*., 1999
Spoligo typing	*Mycobacterium*	Aranaz *et al*., 1996
Binary typing	*Staphylococcus*	Van Leeuwen *et al*., 1999
Specific gene variation-single locus		
Pathogenicity islands	*E. coli*	Franco *et al*., 1999
mecA	*Staphylococcus*	Salisbury *et al*., 1997
Van A, B, C	*Enterococcus*	Miele *et al*., 1995
Neurotoxins	*Clostridium*	Collins & East, 1996
Specific gene variation-multi locus		
Multilocus sequence typing (MLST)	*Neisseria*	Maiden *et al*., 1998
16S rRNA	*Thermus* sp., *Thermotoga* sp., *Deinococcus* sp., *Meiothermus* sp., *Bacillus* sp., *E. coli*, *Clostridium* sp.	Rainey *et al*., 1997
Intergenic spacer region-polymerase chain reaction (ISR-PCR)	All	Fisher & Triplett, 1999
tRNA-PCR	*Streptococci*	Maes *et al*., 1997
Mobile genetic elements		
30 ISs	*Mycobacterium*	Gordon *et al*., 1999
Tn916	*Enterococcus*	Thai *et al*., 1997
IS1548	Group B *Streptococci*	Granlund *et al*., 1998
Tn1546	*Enterococcus*	Jensen *et al*., 1998

similarities and differences in their phenotypic characteristics, into prokaryotes and eukaryotes, and these were in turn further classified into various kingdoms, phyla, classes, orders, families, genera and species. However, objective taxonomic classification by these methods can be difficult because of variations in phenotypic characteristics. Three decades ago, Carl Woese and others started to analyse and sequence the 16S rRNA genes of various bacteria, using DNA/RNA sequencing, and used the sequences for phylogenetic studies. The

invention of PCR and automated DNA sequencing two decades ago, and subsequent work on 16S rRNA gene sequencing of bacteria, as well as 18S rrna gene sequencing of eukaryotes, has led to the accumulation of a vast amount of sequence data on the rRNA genes/rDNA of the smaller subunit of the ribosomes in a large number of living organisms. Comparison of these sequences has shown that the rrna gene sequences are highly conserved within living organisms of the same genus and species, but that they differ between organisms of other genera and species.

In addition to the calculation of similarity coefficients among organisms, a detailed analysis of 16S rRNA oligonucleotide catalogues or complete sequences reveals that there are both quite variable and highly conserved regions of the molecule, presumably reflecting different degrees of functional constraint on different parts of the molecule. Separate comparison of the variable and conserved regions allows determination of both close and distant relationships. In addition, careful inspection of the conserved regions has revealed that there are sequences that are characteristic of major groups of bacteria, termed signature sequences. At great phylogenetic distances these signature sequences are important in allowing the determination of relationships.

Using these rrna gene sequences for phylogenetic studies, three domains of life, Archaea, Bacteria and Eukarya, as opposed to the traditional classification of living organisms into prokaryotes and eukaryotes only, were described. Using 16S rRNA gene sequences, numerous bacterial genera and species have been reclassified and renamed, classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial species has been facilitated. In the last decade, sequencing of various bacterial genomes and comparison between genome and 16S rRNA gene phylogeny has confirmed the representativeness of the 16S rRNA gene in bacterial phylogeny.

Studies based on 16S rDNA sequencing analyses revealed enough evidence supporting reclassifi cation of thermophilic members of the genus *Bacillus* as *Amphibacillus, Alicyclobacillus, Paenibacillus, Aneurinibacillus* and

Brevibacillus, Halobacillus, Virgibacillus, Gracilibacillus, Sulfobacillus and Salibacillus, Anoxybacillus, Coprobacillus, Thermobacillus, Filobacillus, Geobacillus, Ureibacillus, Jeotgalibacillus, Sulfobacillus, and *Marinibacillus* (Adiguzel *et al.,* 2009).

To get in depth information about the sequence of various microorganisms and to look for its relationship with other related species, sequence analysis and phylogenetic studies conducted by different scientists were reviewed critically. Some of the available literature has been presented as follows:

Weisburg *et al*. (1991) described 16S ribosomal RNA amplification for phylogenetic study. A set of oligonucleotide primers capable of initiating enzymatic amplification (polymerase chain reaction) on a phylogenetically and taxonomically wide range of bacteria is described along with methods for their use and examples. One pair of primers is capable of amplifying nearly full-length 16S ribosomal RNA from many bacterial genera; the additional primers are useful for various exceptional sequences. Methods for purification of amplified material, direct sequencing, cloning, sequencing, and transcription are outlined. An obligate intracellular parasite of bovine erythrocytes, Anaplasma marginale, is used as an example; its 16S rDNA was amplified, cloned, sequenced, and phylogenetically placed. Anaplasmas are related to the genera *Rickettsia* and *Ehrlichia*. In addition, 16S rDNAs from several species were readily amplified from material found in lyophilized ampules from the American Type Culture Collection. By use of this method, the phylogenetic study of extremely fastidious or highly pathogenic bacterial species can be carried out without the need to culture them. In theory, any gene segment for which polymerase chain reaction primer design is possible can be derived from a readily obtainable lyophilized bacterial culture.

Rainey *et al.* (1997) reported phylogenetic diversity of the *Deinococci* as determined by 16S ribosomal DNA Sequence comparison. 16S ribosomal DNA (rDNA) sequences were determined for the five species of the genus *Deinococcus (Deinococcus erythromym, Deinococcus proteolyticus, Deinococcus rudioduruns, Deinococcus radiophilus,* and *Deinococcus radiopugnuns)* and the single species

of the genus *Deinobucter (Deinobucter grandis).* With the exception of *Deinococcus erythromyxa,* the deinococci formed a coherent phylogenetic cluster which was related to the *Themus-Meiothemus* lineage. An analysis of the 16s rDNA sequence of *Deinococcus erythromyxa* revealed that this organism was an actinomycete and a member of the genus *Kocuriu. Deinobucter grandis* falls within the radiation of the genus *Deinococcus* and phylogenetically can be considered a member of this genus. The results of the phylogenetic analyses were consistent with chemotaxonomic data. On the basis of our data, *Deinobacter grandis* was transferred to the genus *Deinococcus* as *Deinococcus grandis* comb. nov., the description of the genus *Deinococcus* was amended accordingly, and *Deinococcus erythromyxa* was transferred to the genus *Kocuriu* as *Kocuriu erythromym* comb. nov. The description of the family *Deinococcuceae* was amended to include organisms with rod-shaped cells, and a set of 16S rDNA signature nucleotides was designated for this group. On the basis of the distinct phylogenetic position of the *Deinococcus* lineage and a set of 16S rDNA signature nucleotides, the order *Deinococcules* ord. nov. was described.

Shi and Xia (2005) reported characterization of viable bacteria from Siberian Permafrost by 16S rDNA sequencing. Viable bacteria were found in permafrost core samples from the Kolyma-Indigirka lowland of Northeast Siberia. The samples were obtained at different depths; the deepest was about 3 million years old. The average temperature of the permafrost was reported to be −10°C. Twenty-nine bacterial isolates were characterized by 16S rDNA sequencing and phylogenetic analysis, cell morphology, Gram staining, endospore formation, and growth at 30°C. The majority of the bacterial isolates were rod shaped and grew well at 30°C; but two of them did not grow at or above 28°C, and had optimum growth temperatures around 20°C. Thirty percent of the isolates could form endospores. Phylogenetic analysis revealed that the isolates fell into four categories: high G+C Gram-positive bacteria, b-proteobacteria, g-proteobacteria, and low G+C Gram-positive bacteria. Most high G+C Gram-positive bacteria and b-proteobacteria, and all g-proteobacteria, came from samples with an estimated age of 1.8–3.0 million years (Olyor suite). Most low G+C Gram-positive bacteria came from samples with an estimated age of 5,000–8,000 years (Alas suite).

Philip *et al*. (2000) proposed phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. An unusually thick (1cm) slime developed on a slump of finely disseminated pyrite ore within an extreme acid mine drainage site at Iron Mountain, near Redding, California. The subaerial form of the slime distinguished it from more typical submerged streamers. Phylogenetic analysis of 16S rRNA genes revealed a diversity of sequences that were mostly novel. Nearest relatives to the majority of sequences came from iron-oxidizing acidophiles, and it appeared that iron oxidation was the predominant metabolic characteristic of the organisms in the slime. The most abundant of the 16S rRNA genes detected were from organisms related to *Leptospirillum* species. The dominant sequence (71% of clones) represented a new genus. Sequences within the *Archaea* of the *Thermoplasmales* lineage were detected. Most of these were only distantly related to known microorganisms. Also, sequences affiliating with *Acidimicrobium* were detected. Some of these were closely related to "*Ferromicrobium acidophilus*," and others were affiliated with a lineage only represented by environmental clones. Unexpectedly, sequences that affiliated within the delta subdivision of the *Proteobacteria* were detected. The predominant metabolic feature of bacteria of this subdivision is anaerobic sulfate or metal reduction. Thus, microenvironments of low redox potential possibly existed in the predominantly oxidizing environments of the slime.

16S rRNA technology has been exploited for identification of some laccase producing bacteria and literature available has been cited as follows:

Alexander and Bally (1999) used partial 16S DNA sequences to identify a variant of *Azospirillum lipoferum*. Total DNA was amplified using primers FGPS6-63 (5'-GGAGAGTTAGATCTTGGCTC-3') and FGPS659-64 (5'-CACCGCTACACCAGGAATTC-3'). Amplified DNA fragments were sequenced in both directions using the method of Sanger *et al.,* $1953_{a,b}$ and it was revealed that *Azospirillum lipoferum* strain 4B and the *Azospirillum lipoferum* variant $4V_1$ found to be 100% identical.

Bains *et al*. (2003) used 16S rRNA technology for identification of *γ-Proteobacterium* JB isolated from industrial waste water drained soil. DNA was isolated, purified and PCR product comprised of a partial 16S rRNA sequence was generated. The sequence of both strands was determined by automated fluorescence sequencing. The GenBank database was used to align pairwise BLAST and match sequence of unknown samples. The bacterium was identified from its partial (first 510 bp) 16S rRNA sequence (GenBank accession no: AF574088) analysis.

Zhao *et al*. (2011) also exploited 16S rRNA technology for decolourization and identification of a novel alkali resistant, salt/solvent tolerant spore laccase producing *Bacillus licheniformis* LS04. Genomic DNA was isolated with bacterial DNA kit and 16S rRNA gene amplified using the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP1 (5'-ACGGTTACCTTGTTACGACTT-3') and amplification was carried out followed by 16S rDNA sequencing leading to identification of *Bacillus licheniformis*

Another novel bacteria, *Bacillus subtilis* WD23 which produces an extremely important heat stable and alkaline pH tolerant spore laccase having potential for detoxification and bioremediation was also identified using 16S r DNA technology. After DNA extraction and purification PCR amplification was carried out using primers specific for eubacteria 16S rDNA 27 F: 5'-GAGTTTGATCMTGGCTCAG-3' and 1492 R: 5'-TACGGYTACCTTGTACGACTT-3' leading to identification of the cot A producing *Bacillus subtilis* WD23 (Wang *et al*., 2010)

Sheikhi *et al*. (2012) also identified a laccase producing *Bacillus subtilis* WPI strain using 16S rDNA sequencing. Bacterial DNA was extracted and purified using DNA extraction kit and 16S rDNA was amplified using universal primers. The comparative analysis of 16S rDNA sequence showed that it was close to the members of *Bacillus subtilis.*

2.11.2 RAPD- PCR Technology

Randomly amplified polymorphic DNA (RAPD) has been found to be a powerful technology for determining genetic diversity and discrimination among bacterial strains. Several Polymerase Chain Reactions (PCR) based DNA fingerprinting methods exist for molecular typing of bacterial species. RAPD-PCR technology involves decamer RAPD markers which are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. RAPD-PCR technology has been used to analyse genetic diversity of an individual using random primers and is an inexpensive yet powerful typing method for many bacterial species. This approach has been used for bacterial identification as it is relatively fast and easy (Williams *et al*., 1990). The polymorphism within the set of DNA fragments generated has been used in discriminating microorganisms both at the interspecies and intraspecies level (Welsh and McClelland, 1990). The PCR along with RAPD analysis has been used successfully for typing different bacteria.

Development of RAPD technology or arbitrarily primed PCR fingerprinting gave an advantage in which molecular preliminary information of the species studied is not required necessarily (Welsh and McClelland, 1990) and polymorphic patterns obtained usually vary among the species.. Although variability of RAPD fingerprints has been observed (Muralidharan and Wakeland, 1993), it is reported that reproducibility can be achieved under carefully controlled conditions (Farber and Addison, 1994).

RAPD-PCR has been successfully used for genetic fingerprinting and molecular typing for many microorganism such as *Lactobacillus*, *Salmonella*, *E. coli*, yeast, *Bacillus* (Miyata *et al*., 1995; Giraffa *et al*., 2004; Elegado *et al.,* 2004; Rengua-Mangia *et al*., 2004; Foschino *et al*., 2004; Svensson *et al*., 2004) whereas Shi and Xia (2005) studied the genetic variation in clones of *Pseudomonas pseudoalcaligenes* using RAPD technology. Quednau *et al.* (1997) used the same technique for rapid, reliable and easy identification of

species of *Enterococcus* within a day due to simplicity and speed for assessing genetic diversity even when no information about the genome is available (Hadrys *et al*., 1992). RAPD analysis also has been used to evaluate genetic diversity and to discriminate different strains like *Pseudomonas aeruginosa* (Ortiz-Herrera *et al*., 2004), *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (Sudheesh *et al*., 2002), *Acanthamoeba divineness* (Ortega-Rivas *et al*., 2003), *Pseudomonas pseudoalcaligenes* (Shi and Xia, 2005) and *Rhizobial* strains (Fiki, 2006). RAPD-PCR technology has also been used to study genetic relatedness among *Arthrobacter* strains by phylogenetic analyses of DNA fingerprint patterns (Saxena and Singh, 2013)

2.12 BIOINFORMATICS APPLICATIONS

Advances in computational biology and bioinformatics has been remarkable in the last few decades, that established large scale sequencing, structure and function determination, gene prediction, and specific landmarks on the genome as well as proteome analysis on strong foundations. Several services emerged as the need for access to bioinformatics increased considerably and the most two significant were the European Molecular Biology Laboratory (EMBL) and the United States National Center for Biotechnology Information (NCBI). There are various tools in bioinformatics and they are needed whenever sets of homologous sequences are compared and are essential precursors to numerous further analyses. Use of these tools in sequence alignment, phylogenetic studies and proteomics is being reviewed in the following sections:

2.12.1 Multiple Sequence Alignment

One of the cornerstones of modern bioinformatics is the comparison or alignment of nucleic acid/protein sequences. With the aid of multiple sequence alignments, biologists are able to study sequence patterns conserved through evolution and the ancestral relationships between different organisms. The most widely used programs for global multiple sequence alignment are from the Clustal series of programs. The first Clustal program was written by Des Higgins and Sharp. This program constructed guide tree using the UPGMA (Unweighted Pair Group Method of Arithmetic Averages) method. In 1992, Higgins *et al*.,

released Clustal V which generated trees from the multiple alignments using Neighbour-joining (NJ) method. ClustalW was released in 1994 by Thompson *et al*. and it included sequence weighting, position-specific gap penalties and the automatic choice of a suitable residue comparison matrix at each stage in the multiple alignments. In this version the dendrogram construction by UPGMA was replaced by NJ.

2.12.2 Phylogenetic Tree Construction

Phylogenetic tree is a construction which describes the ancestor-descendants relationships of a set of entities. Usually the leaves of the trees represent the present-day entities and the internal nodes represent common ancestors which existed in the past. Thus, a phylogenetic tree is a model of evolutionary divergence events. These trees contain two types of information, the tree topology, which illustrates the order of divergence events and the branch lengths, which indicate the extent of sequence divergence. It is now well known that in genomics, the phylogeny of the sequences is of more interest than the phylogeny of the organisms.

2.12.2.1 Computational Phylogenetics

Computational phylogenetics is the application of computational algorithms, methods and programs for phylogenetic analyses. The goal is to assemble a phylogenetic tree representing a hypothesis about the evolutionary ancestry of a set of genes, species or other taxa. Traditional phylogenetics relies on morphological data obtained by measuring and quantifying the phenotypic properties of representative organism, while, the more recent field of molecular phylogenetics uses nucleotide sequence encoding genes or amino acid sequence encoding proteins as the basis for classification. Many forms of molecular phylogenetics are closely related to and make extensive use of sequence alignment in constructing and refining phylogenetic trees, which are used to classify the evolutionary relationships between homologous genes represented in the genomes of divergent species.

2.12.2.2 Types of phylogenetic trees

Phylogenetic trees generated by computational phylogenetics can be either rooted or unrooted depending on the input data and the algorithm used. A rooted tree is a directed graph that explicitly identifies a most recent common ancestor (MRCA), usually an imputed sequence that is not represented in the input. Genetic distance measures can be used to plot a tree with the input sequences as leaf nodes and their distance from the root, proportional to their genetic distance from the hypothesized MRCA (Most Recent Common Ancestor).

By contrast, uprooted trees plot the distances and relationship between input sequences without making assumptions regarding their decent. An unrooted tree can always be produced from a rooted tree, but a root cannot usually be placed on an unrooted tree without additional data on divergence rates, such as assumption of the molecular clock hypothesis (Mount, 2004).

2.12.2.3 Methods to construct phylogenetic trees

There are different methods for phylogenetic tree analysis, but the most widely used methods can be broadly classified into two types:

- Distance-based methods or Algorithmic methods
- Character-based methods

1. Distance/Algorithmic methods

Distance-matrix methods of phylogenetic analysis explicity rely on a measure of "genetic distance" between the sequences being classified, and therefore they require a multiple sequence alignment (MSA) as an input. Distance is often defined as the fraction of mismatches at aligned positions, with gaps either ignored or counted as mismatches (Mount, 2004). Distance methods attempt to construct an all-to-all matrix from the sequence query sets, describing the distance between each sequence pair. From this, is constructed a phylogenetic tree that places closely related sequences under the same interior node and whose branch lengths closely reproduce the observed distances between sequences. Distance matrix methods may produce either rooted or unrooted trees depending

on the algorithm used to calculate them. The two most popular distance methods are Unweighted Pair Group Method of Arithmetic Averages (UPGMA) and Neighbor-joining (NJ) and both of these are algorithmic methods.

Unweighted Pair Group Method of Arithmetic Averages (UPGMA)

The simplest distance-based method, Unweighted Pair Group Method of Arithmetic Averages (UPGMA), dates back to 1958 in its earliest version (Sokal and Michener, 1958; Sneath and Sokal, 1973). This method simply joins the two nodes with the shortest distance at each stage of clustering. The newly formed cluster is given a distance to the remaining nodes calculated as arithmetic averages. In this approach all distances from the leaves to the root become the same. If the assumption of a molecular clock holds, this approach delivers acceptable topological results. However, UPGMA yields poor results if substitution rates very.

Neighbor-joining (NJ)

Saitou and Nei in 1987 gave the Neighbor-joining algorithm which is a heuristic method for obtaining a tree from a matrix of distance values according to the criterion of minimum evolution (in which the goal is the tree with minimum sum of branch lengths). Neighbor-joining methods thus apply general data clustering techniques to sequence analysis using unrooted trees, but it does not assume a constant rate of evolution (i.e., a molecular clock) across lineages. Its relative, UPGMA produces rooted trees and requires a constant-rate assumption. It assumes an ultrametric tree in which the distances from the root to every branch tip are equal.

2. Character-based methods

These methods include Parsimony, Maximum Likehood and Bayesian methods (a recent variant of Maximum Likehood- seeks those trees with the greatest likehood given the data). All of these use multiple alignment directly by comparing characters within each column (each site) in the alignment.

Parsimony

Parsimony is a method of identifying the potential phylogenetic trees that requires the smallest total number of evolutionary events to explain the observed sequence data. This is a useful approach in case where, not every possible type of event is equally likely, for example, in cases when particular nucleotides or amino acids are known to be more mutable than others. An algorithm referred to as *branch-and-bound* is guaranteed to find the optimal tree according to the datasets with larger than 20-30 sequences. Thus, one typically searches for the MP tree using different new strategies that can evaluate some subsets of tree space. MP is generally applied in an unweighted fashion (i.e. all substitutions are assumed to occur with equal frequency).

Rogers and Swofford in 1998 developed parsimony method for reconstructing ancestral nucleotides and stated that it allows calculation of initial branch lengths that are good approximations to optimal maximum-likelihood estimates under several commonly used substitution models. Use of these approximate branch lengths (rather than fixed arbitary values) as starting points has significantly reduced the time required for iteration to a solution that maximizes the likelihood of a tree. These branch lengths were close enough to the optimal values that they can be used without further iteration to calculate approximate maximum-likelihood scores that were very close to the "exact" scores found by iteration. Several strategies were, hence described for using these approximate scores to substantially reduce times needed for maximum-likelihood tree searches.

Maximum Likehood (ML)

Maximum Likehood (ML) is an explicitly statistical criterion for tree construction (Felsentein, 1985) and it is often considered the best approach. The likelihood of a phylogenetic tree is the probability of observing the sequence data under a specific model of substitution and for a given tree topology, assuming both to be correct. The principle of Maximum likehood (ML) is to find the topology with the highest likelihood. In order to do this, the algorithm must investigate the probability of all possible residues (amino acids or nucleotides)

for each position in the alignment at each of the internal nodes of the tree. Some residues are more likely than others, to give rise to the nucleotides at the tips of the tree (i.e., the residues in the extant sequences) and therefore will contribute more to the likelihood of that topology. Since Maximum likehood is only a criterion to choose among trees and does not provide a quick way to search for the best trees, it can be very computationally intensive, requiring examination of many different trees as with Maximum likehood, but it is considerably slower than either Parsimony or Neighbour-joining.

PHYLIP Package

PHYLIP is a free comprehensive phylogenetic analysis package created by Joseph Felsenstein at the University of Washington (Felsenstein, 1985). The name is an acronym for PHYLogeny/Inference package. It provides numerous software modules for wide variety of phylogenetic methods, and consists of 35 portable programs. The source code is written in C and precompiled executables are available for Windows (95/98/NT/2000/me/XP), MacOS 8 and 9, MacOS X and Linux systems. Methods that are available in the package include parsimony, distance matrix and likelihood methods, including bootstrapping and consensus trees. Data types that can be handled include molecular sequences, gene frequencies, restriction sites, distance matrices and 0/1 (binary) discrete characters.

2.13 ENZYME PRODUCTION

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. The practical application and industrial use of enzymes to accomplish certain reactions dates back many centuries and was practiced long before the nature or function of enzymes was understood. Use of barley malt for starch conversion in brewing, and of dung for bating of hides in leather industry exemplify the ancient use of enzymes and with the turn of century causative agents for bringing about such biochemical reactions were recognised as enzymes. The first crude preparations were made from animal tissues such as pancreas, stomach mucosa, from plant tissues such as malt and papaya fruit,

which found technical applications in the textile, leather, brewing, and other industries. Once the favourable results of such enzyme preparations were established, it led to a search for better, less expensive, and more readily available sources of such enzymes. Then it was found that certain microorganisms also produce enzymes similar in action to the amylases of malt and pancreas and proteases of the pancreas and papaya fruit and this led to the development of processes for producing these microbial enzymes on a commercial scale. Technical cultivation of fungal enzymes and their introduction to industry was first reported by Takamine (1894) whereas Boidin and Effront (1917) pioneered in the production of bacterial enzymes about 20 years later. Technological progress in this field during the last half century has been so great that, for many uses, microbial cultivated enzymes have replaced the animal or plant enzymes for a number of industrial applications. At present, significant numbers of microbial enzymes have found commercial applications and the number is increasing with a significant potential of expansion in the future. Another important factor in microbial enzyme production is that the absolute and relevant amounts of various individual enzyme produced differ between species and even between strains of the same species. Thus selection of best strains for commercial production of specific enzymes which have the capacity for producing highest amounts of the particular enzymes is highly desired. Use of microbial enzymes in food, pharmaceutical, textile, paper, leather and other industries are numerous and are increasing rapidly, paving way for search of more and more enzymes newer.

2.13.1 Production of laccase enzyme

For an industrial enzyme production process, production medium and cultivation conditions play critical role because these affect the formation, concentration and yield of a particular end product thus affecting the overall process economics. Therefore has been important to consider the optimization of production medium and process conditions in order to maximize the profits from fermentation process (Schmidt *et al.,* 2005). Each organism or strain has its own special conditions for the maximum enzyme production. The general rules for the optimization of microbial enzyme production have been affected by various

physical factors which include pH, cultivation temperature, shaking condition and aeration. These factors have been found to be important to promote, stimulate, enhance and optimize the production of enzyme. However, optimization of cultivation conditions has been essential in successful production of an enzyme, while optimization parameters, such as pH and temperature have been important in developing the cultivation process. Since laccase is a very unique enzyme capable of degrading multiple substrates leading to a number of applications, gives this enzyme considerable market value. Therefore for effective laccase production, it has been found essential to optimize simultaneously the culture conditions and composition of media. Enhanced production of laccase using optimised factors may help in various biotechnological applications at industrial level. Owing to its vivid biotechnological applications, studies on laccase producing organisms have been intensified in the recent years and the optimization of laccase production from different microorganisms has been carried out. The ever increasing demand for this enzyme requires the production process to be economical. Identifying inexpensive raw materials for enzyme production could be viewed as a solution to make the entire process cost effective and further enhancement using inducers may add to the benefit. The production of laccase from different microorganisms by submerged fermentation has been reported (Tong *et al*., 2007; Janusz *et al*., 2007). Submerged fermentation is the cultivation of organisms in liquid culture media. The mode of fermentation has many advantages with respect to the purity of the product and easiness in controlling different process parameters and scale-up processes. The optimization of physicochemical conditions is inevitable in a fermentation process and it is usually performed by varying the levels of one independent variable while fixing other variables at a certain level. The optimization of various parameters by this method for laccase production has been reported by Songulashvili *et al.,* (2007).

2.13.2 Factors influencing laccase production

Several factors influence laccase production such as type of cultivation (submerged or solid state), carbon limitation, and nitrogen source along with various cultural conditions. The optimization of various physical, cultural and

nutritional parameters for production of laccase enzyme by many fungi, some actinomycetes and bacteria have been studied and reported in literature.

Influence of different substrates

Microbes produce enzymes to acquire energy and nutrients through breakdown of complex organic substrates and indeed the activity of extracellular enzymes has been linked to rates of decomposition of these substartes (Carneiro *et al*., 2000) and nutrient mineralization (Alison and Vitousck, 2005). Along with the abiotic factors the activity of extracellular enzymes is regulated by inputs of resources in the form of substrates which provide energy and elements necessary for enzyme synthesis. Variation in terms of substrate inputs has potential to cause variation in extracellular enzyme activity. Laccase has a broader range of substrates which can induce enzyme production in microorganisms. As laccases have potential to oxidize various types of substrates, several different compounds could be used as substrates for production, such as guaiacol, syringaldazine, tannic acid, pyrogallol etc. Guaiacol has been reported to induce laccase in *Rhodococcus* sp., *Enterobacter* sp., *Staphylococcus saprophyticus* and *Delftia tsuruhatensis* (Mongkolthanaruk *et al*., 2012). Many agricultural wastes such as grape seeds, grape stalks, barley bran, cotton stalk, molasses waste water and wheat bran have also been reportedly used as substrates for laccase production (Shraddha *et al*., 2012). Utilization of industrial and agricultural wastes for laccase production is an effective way to reduce production costs (Risna and Suhirman, 2002). Increased production of extracellular laccases in many species of white rot fungi when grown on natural substrates, such as cotton stalk (Ardon *et al*., 1996), molasses waste water (Kahraman and Gurdal, 2002), wheat bran (Souza *et al.,* 2002) and barley bran (Couto and Toca*,* 2006) has been reported. The use of wheat bran for the production of laccases has been reported widely from fungal as well as actinomycetes strains (Rodriguez and Sanroman, 2005). Arias *et al.* (2003) used soya flour for laccase production by *Streptomyces cyaneus*. Jing and Wang (2012) reported use of wheat bran and rice straw as promising substrates for laccase production by *Streptomyces psammoticus* and *Streptomyces cinnamomensis* respectively, while the production of laccase using

sugarcane bagasse was found to be very low in *Streptomyces psammoticus* and *Streptomyces cinnamomensis.*

Influence of temperature on laccase production

Temperature is a critical parameter that must be controlled for maximum laccase production and it varies from one microorganism to another and it has been reported that effect of temperature has been limited in case of laccase production. The optimal temperature for laccase production differs greatly from one strain to another and the optimum temperature range has been reported to be 25-30^{0}C in various fungi (Pointing *et al*., 2000, Shraddha *et al*., 2012). However, preincubation of enzymes at 40^{0}C-50^{0}C has been reported to increase the laccase activity (Farnet *et al.,* 2000) and laccase from *Pleurtus ostreatus* was found to be fully active in the temperature range of 40^{0}C– 60^{0}C, with maximum activity at 50^{0}C and activity remained unaltered after prolonged incubation at 40^{0}C after more than 4 hours (Palmieri *et al.,* 1993). Similar study was reported with *Trametes modesta* (Nyanhongo *et al.* 2002). In case of bacteria and streptomyces, optimum temperature range has been reported to be 28^{0}C- 40^{0}C and laccase from *Rhodococcus sp.* and *Delftia tsuruhatensis* was found to be active at 37^{0}C whereas laccase from *Enterobacter* sp showed maximum activity at 45^{0}C. In case of *Streptomyces psammoticus* MTCC 7334 optimization studies revealed that the laccase yield has been found to be maximum at 32^{0}C (Niladevi and Prema, 2008) and at 28^{0}C in *Streptomyces lydicus* (Mahmoud *et al*., 2013)

Influence of pH

The pH of the culture medium is another very critical factor for growth of the microorganisms and enzyme production. The culture pH strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Paruleka, 1991). The effect of pH has been reported to be limited in case of laccase production (Thurston, 1994). At high pH value the potential difference between the phenolic substrate and the T1 copper could increase the substrate oxidation while the hydroxide anion (OH^-) bound to the T2/T3 copper centre and these effects determined the optimum value of pH for laccase enzyme (Kunamneni *et al.,* 2007). The optimum pH range for laccase

production has been reported to be 4.5-7.0 in a number of fungi such as *Agaricus bisporus* (Wood, 1980), *Gaeumannomyces graminis* var. tritici (Edens *et al*., 1999), *Botryosphaeria sp* (Vasconcelos *et al*., 2000),*Melanocarpus albomyces* (Kiiskien *et al.,* 2002), *Magnaporthe grisea* (Iyer and Chatto, 2003), *Schizophyllum commune*, *Ganoderma lucidum* (Ding *et al*., 2012) etc. It has been reported by Cordi *et al., (*2007) that the optimum pH for L1 (isozyme of laccase) was 4.0, whereas the optimum pH for L2 was 5.0 using syringaldazine as a substrate. Han *et al.,* (2005) extracted laccase from *Trametes versicolour* which showed high enzyme activity at broad range of pH and temperature range but the optimum activity was reported at pH 3.0 and 50^0C temperature. In case of bacteria optimum pH range for *γ-Proteobacterium* has been reported to be 7.0-10.0 and it was able to grow and produce laccase enzyme in this pH range (Bains *et al*., 2003) whereas in *Streptomyces* the optimum pH range reported has been 7.0-7.5 and laccase yield was found to be maximum at a pH 7.5 for *Streptomyces psammoticus* MTCC 7334 (Niladevi and Prema, 2008) and at a pH 7.0 for *Streptomyces lydicus* (Mahmoud *et al*., 2013).

Influence of agitation

Agitation is another important factor which affects laccase production and it has been found to considerably decrease the laccase production in case of fungi such as *Trametes multicolour* (Hess *et al.,* 2002), *Trametes versicolour* (Tavares *et al,* 2006.). Whereas in case of some actinomycetes and bacteria laccase production has been reported to be enhanced by agitation. Agitation rate of 175 rpm was found to effectively increase the laccase production in *Streptomyces psammoticus* MTCC 7334 (Niladevi and Prema, 2008). The agitation rate influenced the enzyme production in such a way that the yield increased 0.064 U/ml from static culture to 0.30 U/ml at 100 rpm The enhancement in enzyme production at higher agitation rate was due to the better aeration in the well-agitated flasks that was essential for the growth and enzyme production by *Streptomyces lydicus* which is an aerobic organism (Mahmoud *et al*., 2013). High production of laccase was noticed in *Streptomyces cinnamomensis* at 150 rpm agitation rate (Jing and Wang, 2012) and by *Streptomyces sp*. 200 rpm (Arias *et al.,* 2003)

Influence of inducer and metal ions

Laccases are generally produced in low concentrations by laccase-producing microbes, but a higher concentration of laccase has been reported with the addition of various supplements such as xenobiotic compounds to media. The addition of aromatic compounds such as 2,5- xylidine, lignin, and veratryl alcohol were found to increase and induce laccase activity . Veratryl alcohol, an aromatic compound lead to an increase in laccase production. Addition of 2,5-xylidine after 24 hours of cultivation gave the highest induction of laccase activity and increased laccase activity by ninefold, whereas at higher concentrations the 2,5-xylidine had a reducing effect due to toxicity . The promoter region encoding for laccase contains various recognition sites that are specific for xenobiotics and heavy metals; they bind to the recognition sites and induce laccase production. The addition of inducer increases the concentration of a specific laccase enzyme. Lee *et al.,*1999 found that alcohol enhanced laccase activity more in comparison to xylidine and has been found to be a vary economical way to enhance laccase production. D'Souza-Ticlo *et al.,* 2009 determined the effect of inhibitor on the activity of Lac-II in the presence of sodium azide, SDS, and mercaptoethanol. It has been found out that 32–37% activity of Lac-II was inhibited in the presence of Sn, Ag, and Hg while 56% and 48% Lac-II activity has been inhibited in the presence of Cr and W, respectively.

Filazzola *et al.* (1999) reported influence of xenobiotics on catalytic activity of constitutive forms of laccases. Metal ions and several organic molecules have also been assayed for their ability to enhance activity of inducible form of laccases (Sheel *et al*., 2000; Singh *et al*., 2007). Gallic and ferulic acids were used, mainly because of their structural analogy with lignin model compounds (Gianfreda *et al.,* 1999). Dyes that affect laccase synthesis include Ethidium Bromide, Malachite Green, Phenol Red and Thymol Blue (Dhawan *et al*., 2003;). Malhotra *et al.,* (2004) found out that copper and dyes enhance laccase production in *γ-Proteobacterium*. It has been reported that genotoxic textile dyes, xenobiotics and substrates could increase laccase activity. Aromatic compounds such as guaiacol, phenol red and black liquor (from pulp and paper industry has been repored to enhance laccase production in bacterial species

(Mongkolthanaruk *et al*., 2012) whereas $CuSO_4$, L-tryptophan and L-leucine were found to be the best inducers for laccase production for *Streptomyces lydicus* (Mahmoud *et al*., 2013). Addition of metal ions in culture medium also improved laccase activity, whereas in case of *Streptomyces lydicus* (Mahmoud *et al*., 2013) Mn^{2+}, Zn^{2+}, Fe^{+2}, EDTA and arbutin decreased the production, while Ag^{+}, sodium azide and L-cystein completely inhibited the enzyme production

Significant literature is available on optimization of various physical, cultural and nutritional parameters in case of fungi. However only few reports have been found for optimization of various physical, cultural and nutritional parameters in case of bacteria and the available literature has been cited as below:

Petroski *et al.* (1980) carried out production of an extracellular laccase from *Polyporus anceps*. A simple quantitative colorimetric assay based on the oxidation of syringaldazine to syringaldazine quinine was described and consistently high titers of laccase were obtained using a defined supplemented with cysteine and histidine amino acids. The enzyme was isolated directly from fermentation medium using ion exchange method and could be stored for 6 months at -70^0C. Similarly, Wood (1980) also compared production of extracellular laccase from *Agaricus bisporus* in malt extract medium and in basal medium containing range of sole carbon source such as glucose, mannitol, fructose, trehalose, xylose, cellobiose, maltose, sucrose and ribose to determine if extracellular enzyme production was inducible or constitutive. The fungus produced extracellular laccase on all the carbon sources which were examined with approximately a two fold variation in the yield of enzyme per unit weight of mycelium. In malt extract medium the enzyme yields were found to be three- to four fold greater than the best yields from the single carbon source media and for this reason malt extract medium was used for bulk enzyme production. The maximum yield of enzyme was found at the end of the exponential growth phase of the fungus when grown in shaken or static cultures and enzyme production paralleled fungal growth in both types of cultures but enzyme yield and fungal biomass were slightly greater in shaken cultures. Whereas treatment with cycloheximide or with other potential inducers such as toluidine or xylidine lead

to no enzyme induction. Sclomczynski *et al.* (1995) reported production of laccase from *Botrytis cinerea* and found that an isolate of *Botrytis cinerea* constitutively expressing substantial amounts of extracellular laccase on a modified Danshina medium for 8 days at room temperature. A comparison of enzyme production with time for aerated fermentation versus shake flasks demonstrated doubling of the laccase yields under former conditions.

Bains *et al.* (2003) reported production of intracellular as well as extracellular laccase enzyme from *γ-Proteobacterium* JB and it was found that growth was maximum at 8 hours using M162 medium range at initial pH range from 7.0 to 9.0 but enzyme production was maximum at pH 10.0 and at an optimum temperature of 37^0C. Malhotra *et al.* (2004) reported copper and dyes enhanced laccase production in γ-*Proteobacterium* JB. 0.1 mM $CuSO_4$ enhanced production by 13-fold and 2.5 µm ethidium bromide, 10 µm malachite green, 10 µm phenol red and 10 µm thymol blue enhanced production by 17, 19, 4 and 2-fold, respectively. Among the fourteen aromatic/organic compounds which were tried, *p*-aminobenzoic acid and an industrial effluent showed 1.2- and 1.26-fold increase in production.

Another report of two novel thermotolerant laccase isoforms were produced by a thermotolerant strain of *Pycnoporus sanguineus*. It was reported that the strain was able to grow at 47°C and in GMY medium containing 500 mM NaCl. The strain was also tolerant to presence of 30,000 ppm of crude Maya oil. A 68-kDa protein purified from submerged cultures exhibited laccase activity towards 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), guaiacol, syringaldazine, and *o*-dianisidine, for which it presented the highest affinity (*K*m = 43 µM). The purified enzyme showed high thermostability, retaining 40% of its original activity after 3 hours at 60°C (Gonazlez *et al*., 2008).

Jhadav *et al*. (2009) optimized production of laccase from *Phanerochaete chrysosporium* using submerged fermentation. Laccase production and activity was measured by culturing the fungi in a composite medium containing different primary carbon sources such as rice bran, wheat straw and maize bran. Standard incubation time for production of laccase was found to be ten days and slight

increase in the laccase activity was observed when culture is grown in rice and maize bran containing medium as compared to glucose medium but glucose containing medium in presence of guaiacol gave higher laccase activity.

Laxmi and Khan (2010) studied effect of natural phenolic and lignin rich inducers on the production of laccases by *Streptomyces griseus* MTCC 4734. The production of laccase by this organism grown in submerged cultures in the presence of different natural phenolic and lignin rich sources was studied in comparison to aromatic inducers which have been traditionally used. Among different inducers studied, *Spirulina* was shown to enhance laccase production to a greater extent more than the aromatic inducers (190 Ug-1 in the presence of *Spirulina* and (132 Ug-1) in the presence of Tween 80). Bajra and grapes also showed a significant rise in laccase activities. The optimum pH and temperature for the partially purified enzyme from this strain were found to be 4.5 and 30^0C. The enzyme obtained was stable at high pH and temperature.

Another thermostable laccase was produced by the mushroom, *Ganoderma lucidum*, using submerged fermentation. The effects of variations in the medium nutrient composition or culture conditions was studied and it was reported out that low concentrations of glucose and yeast extract stimulated *Ganoderma lucidum* laccase production. Addition of lignocellulosic substrates to the media stimulated laccase production. Pomelo peel induced the highest laccase activity of 11842.13 U/L and banana peel reduced laccase production, compared to wheat bran substrate (Ding *et al.,* 2012).

Christie and Shanmugam (2012) analysed fungal cultures isolated from Anamalia hills for laccase enzyme production. Laccase positive fungal cultures i.e. *Aspergillus niger*, *Fusarium oxysporium*, *Alternaria arborescence* and *Penicillium marnefei* was inoculated into basal medium at different temperature, pH, agitation and incubation time and it was reported that all fungal cultures showed maximum production at 150 rpm in acidic pH with a temperature range between 35-45^0C after 12 days of incubation.

Mongkolthanaruk *et al*. (2012) studied independent behavior of bacterial laccases to inducers and metal ions during production and activity. Bacterial strains i.e. *Rhodococcus* sp., *Enterobacter* sp., *Staphylococcus saprophyticus* and *Delftia tsuruhatensis* were investigated for laccase production and for its activity. The medium for production was a high nitrogen containing aromatic compounds as inducers such as guaiacol, phenol red and black liquor from pulp and paper industry and it was reported that guaiacol gave higher laccase production in all bacterial strains.

2.14 PURIFICATION AND CHARACTERIZATION OF LACCASE

Purification is the first step in the study of physio-chemical, kinetic and biological properties of the enzyme. Purification generally involves a series of fractionations by which the enzyme protein was separated from the other proteins. The basic aim in enzyme purification is to isolate enzyme from other contaminating proteins so that its structure and other properties can be studied. Once a suitable cellular source of the protein has been identified, it is liberated into solution and then separated from contaminating materials by sequential use of a series of different fractionation techniques or separations. These separations exploit one or more of the following basic properties of the protein such as its solubility, size, charge or specific binding affinity. These separations are generally carried out using chromatographic techniques such as ion exchange, gel filtration or affinity chromatography, hydrophobic interaction chromatography, in which a protein binds to a hydrophobic material, or electrophoretic techniques such as isoelectric focusing. Other electrophoretic procedures, mainly SDS-PAGE and native PAGE are used to monitor the extent of purification and to determine the molecular mass and subunit composition of the purified protein.

2.14.1 Ammonium sulphate precipitation and dialysis

Ammonium sulphate precipitation is a technique used to precipitate proteins from solution by increasing the ionic strength of the solution using ammonium sulphate in the range of 0-80%. The technique is reliant on the hydrophobic nature of proteins, since they contain hydrophilic and hydrophobic groups. When the proteins are dissolved, water is forced into contact with the

protein's hydrophobic groups and in the process becomes ordered around the proteins. Increasing the concentration of salt ions by the addition of ammonium sulphate causes water to be removed from around the protein exposing the hydrophobic portions of the proteins. Precipitation of the proteins will then occur due to the aggregation of proteins via the exposed hydrophobic portions. This technique is used to fractionate the proteins from solution since proteins with larger or more hydrophobic portions will aggregate and precipitate before those with smaller or fewer proteins of hydrophobic groups.

Many types of salts have been employed to effect protein separation and purification through salting-out. Of these salts, ammonium sulfate has been the most widely used chemical because it has high solubility and is relatively inexpensive. Because enzymes are proteins, enzyme purification can be carried out by following the same set of procedures as those for proteins, except that some attention must be paid to the consideration of permanent loss of activity due to denaturation under adverse conditions. In ammonium sulphate precipitation, chart of Gomori (1955) as mentioned by Dixon and Webb (1964) has been applied to calculate the solid ammonium sulphate to be added to achieve any given concentration of the cell free filtrate under investigation. Dhiman, (2014) used ammonium sulphate technique for laccase enzyme purification.

Dialysis is the process of passage of solvent molecules from a region of higher concentration to a region of lower concentration through a semi permeable membrane. This technique is used for purification of crude enzymes. It is used for enzyme concentration as well as enzyme purification. Other sophisticated methods such as electro dialysis, electro filtration, isoelectric focusing, forced flow electrophoresis, electro decantation and isotachophoresis are also developed and used. Membrane based processes such as reverse osmosis and pervaporation are also gaining popularity.

2.14.2 Chromatographic techniques

Chromatography was first employed by Russian scientist Mikhail Tswett in 1903. New types of chromatography developed during the 1930s and

1940s made the technique useful for many separation processes. Chromatography technique developed substantially as a result of the work of Archer John Porter Martin and Richard Laurence Millington Synge during the 1940s and 1950s. They established the principles and basic techniques of partition chromatography, and their work encouraged the rapid development of several chromatographic methods: paper chromatography, gas chromatography, and high performance liquid chromatography. Since then, the technology has advanced rapidly. Researchers found that the main principles of Tswett's chromatography could be applied in many different ways, resulting in the different varieties of chromatography described below. Advances are continually improving the technical performance of chromatography, allowing the separation of increasingly similar molecules.

2.14.3 Gel filtration column chromatography

This technique was invented by Grant Henry Lathe and Colin R Ruthven, working at Queen Charlotte's Hospital, London. They later received the John Scott Award for this invention. While Lathe and Ruthven used starch gels as the matrix, Jerker Porath and Per Flodin later introduced dextran gels, other gels with size fractionation properties include agarose and polyacrylamide. There were also attempts to fractionate synthetic high polymers; however, it was not until 1964, when J. C. Moore of the Dow Chemical Company published his work on the preparation of Gel Permeation Chromatography (GPC) columns based on cross-linked polystyrene with controlled pore size, that a rapid increase of research activity in this field began. It was recognized almost immediately that with proper calibration, GPC was capable to provide molar mass and molar mass distribution information for synthetic polymers. Because the latter information was difficult to obtain by other methods, GPC came rapidly into extensive use.

The chromatographic media used in this technique are porous, polymeric organic compounds with molecular sieving properties. These are cross- linked polymers which swell considerably in water forming a gel of three dimensional network of pores. The size of pore is determined by degree of cross linking of polymeric chains. Different solutes in a mixture get separated on the basis of their

molecular size and shape during their passage through a column packed with the swollen gel particles. The terms 'exclusion chromatography', 'gel filtration' and 'molecular sieve' chromatography are used for this separation process. The large molecules in sample are unable to penetrate through the pores into the gel and thus remain excluded while the small molecules enter into the gel beads. The volume of solvent accessible to large molecules is very much less, where as small molecules, which can freely penetrate into the gel have access to solvent inside as well as outside the spherical beads.

As component of the sample travel down the column, the compounds whose moleculer size exceeds the fractionation range of the gel, are unable to enter into the gel particles and hence remain completely excluded from beads and travel through the interstitial spaces. Molecules of the smaller compound, however diffuse into the gel matrix through the pores and get distributed between the mobile phase inside as well as outside the gel particles.These thus follow a longer path then the larger molecules and hence their movement down the column is retarded. Consequently different components in the sample get separated from each other with larger molecules getting eluted first followed by smaller molecules.

2.14.4 Ion exchange chromatography

English researchers H Thompson and J T Way first used ion methods to treat clays with ammonium sulfate or carbonate in solution in order to extract the ammonia and release calcium. Nearly 80 years later, the first zeolite mineral column was used to remove calcium and magnesium ions from a solution so that the sulfate content of water could be accurately determined. And in World War II, when the US, UK and Canada were working on the Manhattan Project to develop the first atomic bomb, the modern version of ion chromatography was developed. Scientists needed a way to separate and concentrate the radioactive elements and chose adsorbents that latch onto charged uranium elements, which could then be differentially eluted. This technique was developed into the systems used today to purify biologicals and inorganics.

Ion exchange chromatography is a type of adsorption chromatography in which retention of a solute occurs due to its reversible electrostatic interaction with the oppositely charged groups on an ion exchanger. Hence this technique is useful for separation of compounds which bear a net electric charge such as proteins, nucleic acids and amino acids etc. Ion exchangers are prepared from either certain synthetic resins which were insoluble porous organic molecules or naturally occurring biopolymers such as cellulose to which various groups known as fixed ions are covalently attached. These fixed ions are balanced by equal and oppositely charged ions from the solution referred to as counter ions. Depending on nature of the counter ions, these ions exchanger are of two types: cation exchanger in which the counter ions are cationic or positively charged ions and anion exchangers which have negatively charged counter ions. Counter ions are mobile and can be easily exchanged by other similarly charged molecules in the sample. Nature of resin matrix remains unchanged during this exchange process.Generally resin based ion exchangers are used for separation of low moleculer weight by molecules and cellulosic ion exchanger are more suitable for isolation of macro molecules such as proteins and nucleic acids.

Ion exchangers have to be precycled to get an appropriate charge and their complete swelling. In case of anion exchanger, it is treated first with alkali and then with acid and finally washed with water till it was neutral. On the other hand, cation exchanger is first given the acid treatment and then alkali treatment and finally washed with water till neutral. After precycling, the ion exchanger is packed into a column and it is equilibrated with the counter ion by passing 2-3 bed volume of the starting buffer of required pH. In case of anion exchanger, Tris-HCl buffer and for cation exchanger K^+ or Na^+ buffer is used.The process of ion exchange takes place in different steps. The ion exchanger has positive fixed ion which are balanced by negatively charged counter ions. When sample containing mixture of compounds is applied on the top of the exchanger bed, the solute molecules having charge similar to that of counter ions get exchanged with the counter ions and bind reversibly but strongly to the ion exchanger. Strength of binding depends upon degree of the charge and charge density of the solute.The other solute particles have no affinity for the stationary phase and are washed

down along with starting buffer. The bound solute molecules are then released in succession by altering pH or ionic strength of the elution buffer.

In general, plant laccases have been purified from sap or tissue extract, whereas extracellular and intracellular fungal as well as bacterial laccases are purified from culture medium (fermentation broth) and by cell disruption of the selected organism. Various protein purification techniques are frequently used in purifying laccase. Typical purification protocol involves ultrafiltration, ion exchange, gel filtration, hydrophobic interaction, or other electrophoretic and chromatographic techniques. Affinity chromatography using a phenolic group as ligand can increased purification efficiency. The purity of laccase preparation is often measured by SDS-PAGE and by the ratio of the absorbance at 280 nm to that at 600 nm.

2.14.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

In this technique, protein are dissociated into their constituent subunits using anionic detergent such as sodium dodecyl sulphate (SDS) or sodium lauryl sulphate. The protein mixture are denatured by heating the sample at 100^{o}C in the presence of excess SDS. Under these conditions most polypeptides bind SDS in a constant weight ratio (1.4gm SDS per gm of polypeptide).The intrinsic charges of the polypeptides are insignificant compared to negative charges provided by the bound detergent. Consequently SDS polypeptide complexes have essentially identical charge densities and these migrate in polyacrylamid gels of an appropriate porosity strictly according to polypeptide size or their moleculer mass. Thus in addition to analysis of polypeptide composition of the sample,the moleculer weight of the polypeptides of the sample protein can also be determined by using standard moleculer weight markers.

Persual of the literature has shown some studies on purification and characterization of laccase enzyme from fungus as well as bacteria which are discussed as under:

Froehner and Eriksson (1974) reported purification and properties of laccase produced from *Neurospora laccase.* The extracellular laccase enzyme has

been purified to apparent homogeneity using classical purification techniques such as ammonium sulphate, gel filteration and ion exchange chromatography. It was reported that enzyme purified consisted of mainly one form with a molecular weight of 64,800 and 11% carbohydrate content. Using ultraviolet, visible, and electro-paramagnetic resonance spectra it was found out that both type I and type II copper have been present in enzyme and phloroglucinol, para- and ortho-diphenols reported to be effective substrates for the enzyme.

Coll *et al.* (1993) purified and characterized a phenoloxidase (laccase) from the lignin-degrading Basidiomycete PM1 (CECT 2971). Laccase was purified to homogeneity by gel filtration chromatography, anion-exchange chromatography, and hydrophobicity chromatography and the enzyme was reported to be a monomeric glycoprotein containing 6.5% carbohydrate with a molecular weight of 64,000. Enzyme possessed an isoelectric point of 3.6, and was found stable in a pH range from 3.0 to 9.0, with an optimum pH of 4.5. Whereas optimal reaction temperature for laccase was found to be 80°C and it was found stable for 1 hour at 60°C.

Perez *et al*. (1996) carried out purification and partial characterization of laccase from the white rot Fungus *Phanerochaete flavido-alba.* This protein has been purified to homogeneity using ultrafilteration, anion-exchange chromatography, fast protein liquid chromatography (FPLC) and Superdex 75 HR gel filtration chromatography and it was reported that enzyme have a molecular weight of 94,000 and an isoelectric point lower than 3.55. UV-visible spectrum of this enzyme has been typical of copper-containing proteins.

Chefetz *et al.* (1998) studied purification and characterization of laccase from *Chaetomium thermophilium* and it has been purified using techniques such as ultrafiltration, anion-exchange chromatography, and affinity chromatography. The purified enzyme has been identified as a glycoprotein with a molecular mass of 77 kDa and an isoelectric point of 5.1. This laccase was found to be stable for 1 hour at 70°C and had half-lives of 24 and 12 hour at 40 and 50°C, respectively and the enzyme was reported to be stable at pH 5.0 to 10.0, and the optimum pH

for enzyme activity was 6.0. The purified laccase efficiently catalyzed a wide range of phenolic substrates but not tyrosine.

Diamantidis *et al.* (1999) purified and characterized the first bacterial laccase from the rhizospheric bacterium *Azospirillum lipoferum*. Purification was carried out after cell disruption by sonication, protein solubilisation using Triton X-100, acetone and protamine precipitation, hydroxyapatite column chromatography in the presence of urea (5 M) and NaCl (2M), and finally by conventional hydroxyapatite column chromatography. The purified enzyme was found to be thermostable up to 70^{o}C for 10 minutes, had an optimal pH of 6.0, and was inhibited by tropolone which is found to be a known inhibitor of metal-containing enzymes.

A new member of the laccase family was purified and characterized from the white-rot basidiomycete *Coriolus hirsutus* by Shin and Lee in 2000. Laccase produced by *Coriolus hirsutus* has been purified to electrophoretic homogeneity by acetone precipitation, DEAE Sepharose CL-6B, Sephacryl S-200 HR, Hitrap SP, and Mono S chromatography. The purification purity was found to be 14.5-fold with an overall yield of 32.3%. The enzyme purified was a monomeric glycoprotein with 11% carbohydrate content having an isoelectric point of 7.4 and 73 kDa molecular mass. It was found out that N-terminal amino acid sequence of this enzyme showed low homology with the laccases of other white-rot basidiomycetes but spectroscopic analyses revealed a typical laccase active site in the *Coriolus hirsutus* enzyme and all three Cu centers have been identified. The highest rate of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) oxidation for the enzyme was found at 45°C, and in the pH range of 2.5-4.0. The enzyme was reported to oxidize a variety of the laccase substrates, including lignin-related phenols and had highest affinity toward guaiacol. Under standard assay conditions, the apparent *Km* value of the enzyme toward guaiacol was 10.9 mM and the enzyme catalyzed single electron transfer via the phenoxy radical as an intermediate and was completely inhibited by L-cysteine and sodium azide but not by EDTA.

Kiiskinen *et al*. (2002) reported purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces* using anion exchange chromatography, hydrophobic interaction chromatography and gel filtration and it was reported that it showed activity towards typical substrates of laccases including 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate), dimethoxyphenol, guaiacol, and syringaldazine. The laccase found to possess unusual properties of good thermostability (60–70°C) and neutral pH optimum (6.0-7.0). The molecular weight of the laccase was found to be about 80 kDa with 4.0 isoelectric point. The ultraviolet-visible absorption and electron paramagnetic resonance spectra of the purified laccase indicated that the typical three types of copper were present in the enzyme.

Martins *et al.* (2002) described molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. By using comparative techniques, a model was derived for CotA based on the known X-ray structures of Zucchini ascorbate oxidase and *Cuprinus cereneus* laccase. The CotA protein was purified to homogeneity from an overproducing *Escherichia coli* strain. The purified CotA showed an absorbance and a EPR spectra typical of blue multicopper oxidases. Optimal enzymatic activity was found at <pH 3.0 and at pH 7.0 for ABTS or SGZ oxidation, respectively. Maximal enzyme activity was observed at 75°C with ABTS as substrate. Remarkably, the coat-associated or the purified enzyme showed a half-life of inactivation at 80°C of about 4 and 2 h, respectively, indicating that CotA was intrinsically highly thermostable.

Baldrian (2004) purified and characterized laccase from the white-rot fungus *Daedalea quercina*. Laccase was purified using anion exchange and size-exclusion chromatographies and the purified laccase enzyme was found to be a monomeric protein of 69 kDa using SDS-PAGE with an isoelectric point near 3.0. The optimum pH for activity was reported below 2.0 for 2,2′- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Km=38 μM), 4.0 for 2,6-dimethoxyphenol (Km=48 μM), 4.5 for guaiacol (Km=93 μM) and 7.0 for syringaldazine (Km=131 μM) substrate respectively. The optimum temperature

was between 60 and 70°C depending on the pH and buffer used and the enzyme was stable up to 45°C at alkaline pH. It was found that enzyme activity was increased by the addition of Cu^{2+} and inhibited by Mn^{2+}, sodium azide, dithiothreitol, and cysteine.

Suzuki *et al.* (2003) reported purification, characterization, nucleotide sequencing and expression of a thermostable laccase from *Streptomyces lavendulae* REN-7. About 8 mg of purified POP was obtained from 200g of mycelium. Enzyme was capable of oxidation of syringaldazine, substrate of laccase. REN-7-PPO was active after 20 min of incubation at 70°C. Another thermostable laccase was purified and partially characterized from a unidentified basidiomycete *UD4* (Jordaan *et al*., 2004)**.** Enzyme was found to show an unusually high affinity for ABTS as a substrate (low K_m) and inhibited by sodium azide. The high specificity constant, substrate affinity and broader substrate range of this thermostable laccase indicates that it is a highly favourable candidate enzyme for industrial application.

Lu *et al.* (2007) also purified and characterized laccase from *Pycnoporus sanguineus*. Laccase was purified using ultrafiltration, anion-exchange chromatography, and gel filtration. The molecular weight of the purified laccase was estimated as 61.4 kDa by SDS polyacrylamide gel electrophoresis and enzyme oxidized typical substrates of laccases including 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate), 2,6-dimethoxyphenol, and syringaldazine. The optimum pH and temperature for the purified laccase were 3.0 and 65°C, respectively. The enzyme was stable up to 40°C, and high laccase activity was maintained at pH 2.0–5.0, whereas sodium azide, L-cysteine, and dithiothreitol strongly inhibited laccase activity.

McMahon *et al.* (2007) reported purification of laccase and tyrosinase from soil bacterium *Pseudomonas putida* F6. Both enzymes were purified to homogeneity separately using a combination of anion exchange chromatography, gel filtration and gel slicing technique. Molecular mass of tyrosinase was found to be 39,000 and that of laccase was 59,000. It was reported that tyrosinase

showed maximum activity using tyrosine and diphenolase as subtrates and laccase with syringaldazine at 30°C and7.0 pH.

Singh *et al.* (2007) reported purification and characterization of a pH-stable laccase from alkali-tolerant *γ-Proteobacterium* JB. The enzyme was purified to homogeneity by ammonium sulphate precipitation, DEAE-sepharose anion exchange chromatography and preparatory polyacrylamide gel electrophoresis. The purified enzyme was found to be a monomeric polypeptide with 120 kDa molecular weight and when absorbed at 590 nm it indicated the presence of Type I Cu^{2+}-centre. It was found to work optimally at 55^0C and showed different pH optima for different substrates whereas it was highly stable in the pH range 4–10 even after 60 days at 4^0C.

Abou-mansour *et al.* (2009) carried out purification and partial characterization of a 60 KDa laccase from *Fomitiporia mediterranea.* A 60-kDa extra-cellular laccase enzyme was purified to homogeneity from a liquid culture of *Fomitiporia mediterranea* and it was found out that the enzyme exhibited thermal stability. From substrate specificity and inhibitor studies it was found out that the enzyme was a typical fungal laccase and could oxidise several natural phenolic and polyphenolic compounds.

Shaikly (2009) reported purification and characterization of *Trametes hirsute* DSMZ No.5072 laccase. Laccase purification was done by precipitating the enzymes with ammonium sulphate at 90% saturation, followed by gel filtration chromatography using Sephadex-G25 and ion exchange (DEAE-Sephadex). Purified enzyme was concentrated by ultra filtration membrane and analyzed by SDS-PAGE. The optimum pH for the laccase activity of the fungal laccase was observed at acidic pH values of 3.5-4.6 while the optimum temperature was 70^0C.

Another thermostable laccase with unique oxidative characteristics from *Trametes hirsute* was purified and characterized by Zang *et al.* in 2009 . A laccase was purified from *Trametes hirsute* using ultrafilteration, ion exchange and gel filteration chromatography. Laccase showed that a pH 2.4 was optimal

for the oxidation of ABTS and pH 2.5 for DMP at optimal temperature of 85^{o}C. It was reported that the half-life of this laccase was 70 minutes at 75^{o}C, and 5 hour at 65^{o}C and enzyme was not inhibited by Cu^{2+}, Mn^{2+}, or EDTA.

Reiss *et al.* (2011) characterized *Bacillus pumilus* laccase as a heat stable enzyme with a wide substrate spectrum. The molecular weight of the purified laccase was found to be 58 kDa and associated with four copper atoms. Its catalytic activity towards 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (2,6-DMP) and syringaldazine (SGZ) was investigated and it was reporterd that KM and kcat for ABTS were 80 ± 4 μM and 291 ± 2.7 s-1, for 2,6-DMP 680 ± 27 μM and 11 ± 0.1 s-1 and for SGZ only kcat could be estimated to be 66 ± 1.5 s-1. The pH optimum for ABTS was 4, for 2,6-DMP 7 and for SGZ 6.5 and temperature optima for ABTS and 2,6-DMP were found to be around 70°C. The screening of 37 natural and non-natural compounds as substrates for *Bacillus pumilus* laccase revealed 18 suitable compounds and three of them served as redox mediators in the laccase-catalyzed decolorization of the dye indigo carmine (IC), thus assessing the new enzyme's biotechnological potential.

2.15 ISOLATION AND CHARACTERIZATION OF LACCASE GENE

Modern biotechnology has a steadily increasing demand for novel genes for application in various industrial processes. Identification and isolation for novel genes at a reasonable pace is the main driving force behind the development of unprecedented experimental approaches. As there is an increasing demand for novel biocatalysts which has prompted the development of novel experimental approaches to find and identify novel biocatalyst encoding gene. One of the methods of isolating and identifying a novel gene is PCR amplification using specific/designed primers which allows the specific amplification of rare sequences within complex genomic DNA. Thus PCR amplification using designed primers have been used to amplify a number of specific and novel genes e.g. lipase gene, cellulose gene, cry I gene, chitinase gene and many others from various bacteria and fungi. As laccase is a very important enzyme both for biotechnological and industrial applications so

research was emphasized on the isolation and characterization of its gene from microbes producing it so that this gene could be further cloned and expressed.

The first gene and/or cDNA sequences were recorded for laccase from the fungus, *Neurospora crassa* (Germann and Lerch, 1986). Since then, number of fungal laccase genes sequencing has increased considerably. However, a significant number of these are only partial stretches of putative laccase genes that have been found in genome-wide sequencing projects and have been annotated on the basis of sequence homology with known laccases. The number of laccase genes of which the corresponding protein products have been experimentally characterized is significantly lower. The sequences mostly encode polypeptides of approximately 500 to 600 amino acids (including the N-terminal secretion peptide). All the laccases are secreted proteins, and typical eukaryotic signal peptide sequences of about 21 amino acids have been found at the N-termini of the protein sequences. The one cysteine and ten histidine residues involved in the binding of copper atoms were conserved for laccases which were used to design primers using various bioinformatic tools to amplify laccase specific gene. Laccase gene family could be used as a potential tool to define its moonlighting functions It was found out that a number of laccase gene vary among fungi such as *Trametes villosa* which posseses five genes coding for five laccase isoforms, three laccase genes were reported in *Rhizoctonia solani,* two-phenol oxidase genes (*pox1* and *pox2*) were isolated from *Pleurotus ostreatus* encoding for two isoforms of laccase (Giardiana *et al*., 1998) and three genes from *Coprinopsis cinereus*. Phylogenetic data of the moonlighting protein i.e., Fet3, CotA and polyphenol oxidase from various sources proves that diverse paralogus laccase genes may have descended from progenitor gene or master gene, which had duplicated and diverged prior to speciation. These paralogus genes could give information on the early ancestors of families of proteins now residing in many contemporary organisms and showing moonlighting function.

In contrast to number of fungal laccase genes sequenced there has been very few reports on bacterial laccase gene isolation/sequencing. Ausec *et al.* (2012) revealed high diversity of bacterial genes for laccase-like enzymes using

bioinformatic analysis. Laccase-like enzymes were searched for in over 2,200 complete and draft bacterial genomes and four metagenomic datasets, using the custom profile Hidden Markov Models for two and three domain laccases. More than 1,200 putative genes for laccase-like enzymes were retrieved from chromosomes and plasmids of diverse bacteria. In 76% of the genes, signal peptides were predicted, indicating that these bacterial laccases may be exported from the cytoplasm. Moreover, several examples of putatively horizontally transferred bacterial laccase genes were described and many metagenomic sequences encoding fragments of laccase-like enzymes could not be phylogenetically assigned, indicating considerable novelty. Laccase-like genes were also found in anaerobic bacteria, autotrophs and alkaliphiles, thus opening new hypotheses regarding their ecological functions. Bacteria identified as carrying laccase genes represent potential sources for future biotechnological applications. However few reports on isolation and characterization of bacterial laccase gene have been found in literature, such as, cotA gene which codes for highly stable and thermotolerant laccase. It was reported that the *cotA* gene codes for a 65-kDa protein belonging to the outer spore coat of *Bacillus subtilis* which displays similarity with multicopper oxidases like laccase (Hullo, 2001).

Various biotechnological and bioinformatics tools have been exploited for characterization and comparision of laccase gene and literature available has been cited as follows: Sualoheimo, carried out isolation and structural analysis of the laccase gene from the lignin degrading fungus *Phlebia radiate* and it was found out that the gene has nine introns as well as recognizable fungal promoter elements. The derived laccase amino acid sequence shows homology to plant ascorbate oxidases, suggesting that the basic structure of the laccase is similar to the three-fold repeated p-barrel of the ascorbate oxidases. In another report, a laccase gene isolated and characterized from *Podospora anserine* and it was reported that the genome of the filamentous ascomycete contained four non-adjacent regions which are homologous to the laccase gene of *Neurospora crassa*. One of the regions out of four non-adjacent regions contained a gene (*lac2*) encoding protein that displayed 62% identity with the *Neurospora crassa* laccase. (Larrea and Stahl, 1996).

Berka *et al.* (1997) reported characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila.* Genomic DNA segment encoding an extracellular laccase was isolated from the thermophilic fungus and the nucleotide sequence was determined, which showed that amino acid sequence of *Myceliophthoro thermophila* laccase (MtL) showed homology to laccases from diverse fungal genera; Rajendran *et al*. (2008) carried out cloning and characterization of a laccase gene from *Ganoderma* spp. causing basal stem rot disease in coconut. Genomic DNA of the laccase-positive isolates were amplified by polymerase chain reaction (PCR) using degenerate primers for the partial sequence, which showed the fragment size of 200 bp and nucleotide sequence of the DNA of this isolate showed high homology with those of the laccase genes of other basidiomycetes.

Fang *et al.* (2011) reported a new bacterial laccase gene of 1.32 kb obtained from a marine microbial metagenome of the South China Sea by using a sequence screening strategy. The protein named as Lac15 of 439 amino acids encoded by the gene found to contain three conserved Cu^{2+} binding domains, but shared less than 40% of sequence identities with all of the bacterial multicopper oxidases characterized.

Li *et al*. (2012) carried out isolation of a multicopper oxidase gene from *Ochrobactrum sp*. 531. Based on two conserved sequences at copper-binding sites of the CueO-type multicopper oxidases of several bacteria , two degenerate primers, 531F1 and 531R1 were designed using the program CODEHOP, which were used to amplify genomic DNA of the strain. 1029 PCR product of the gene was eluted and sequenced which showed that amino acid sequence derived from translationg the nucleotide sequence, gave 98% similarity to *Ochrobactrum anthropi* (gi/153011149) and more than 75% to *Brucella suis* (gi/23500444).

Yang *et al.* (2012) reported isolation of another laccase gene from *Bacillus subtilis* and it was found to be composed of 1542 bp and encoded a 514-amino acid protein showing 86% homology with a published laccase with GeneID 936023. The lac2 gene was deposited in GenBank as a new nucleotide sequence and this new sequence was cloned into the multiple cloning site of

pPIC9K to generate pPIC9K-lac2, which was then transformed into *Pichia pastoris* GS115 via electroporation

2.16 PROTEIN SEQUENCING AND STRUCTURE DETERMINATION OF LACCASES

Protein sequencing is another powerful technology that has played a vital role in proteomics, as the information obtained could be used to deduce structure, function and location of the particular amino acid sequence, which in turn aids in identifying novel proteins. The advent of protein sequencing can be traced to two parallel discoveries by Frederick Sanger's mass spectrometry technique and Pehr Edman's Edman degradation technique. Discovering the structures and functions of proteins in living organisms has been an important tool to understand the cellular processes and determining the amino acid composition and involved hydrolysis, separation of amino acids followed by quantitative analysis.

Many proteins/peptide sequences have been stored in various protein databases such as CluSTr, CSA, HPI, IntEnz, InterPro, IPI, UniProt etc. The names of amino acids are either recorded as three letters or one letter abbreviation. Techniques in sequence analysis can be applied to learn more about the proteins. These techniques generally consist of comparing the query sequence of protein to other sequences of proteins from sequence databases. Findings about these sequences may be applicable to the sequence under investigation.

Now a days many companies offer protein sequencing services involving N-terminal sequencing, complete protein sequencing, protein deblocking sequencing and internal sequence determination. Protein sequence can also be generated from DNA/mRNA sequence that codes for the protein. There are several applications of protein sequencing such as identification of the protein family to which a particular protein belongs and finding the evolutionary history of that protein, prediction of the cellular localization of the protein based on its target sequence, prediction of sequence of the gene encoding the particular protein, discovering the structure and function of a protein through various computational methods and experimental methods.

Laccase proteins have been the subject of increasing attention because of their established or potential novel uses in biotechnology and it was found out that the overall structure of laccases comprised of three cupredoxin-like domains; A, B and C, that are of equal size (Ducros *et al*., 1998; Piontek *et al*., 2002). All three domains are important for the catalytic activity of laccases: the substrate binding site is located in a cleft between domains B and C, a mononuclear copper centre is located in domain C, and a trinuclear copper centre is located at the interface between domains A and C. The crystal structures of laccases from different fungi have also been elucidated. Three of the fungal laccases that have been structurally characterized were from *Coprinus cinereus* (Ducros *et al*., 1998), *Trametes versicolor* (Piontek *et al.,* 2002), and *Melanocarpus albomyces* (Hakulinen, 2006). Whereas in case of bacteria, crystal structure of coat component bacterial laccase (CotA) from *Bacillus subtilis* (Enguita *et al*., 2003) and crystal structure of *E. coli* laccase CueO have been determined (Li *et al.*, 2007). Some of reports available on the crystal structure determination of laccase have been described as follows:

Ducros *et al.* (1998) reported the crystal structure of a laccase from the fungus *Coprinus cinereus*. This was found to be a copper type-2-depleted form in which the putative T2 copper was completely absent and therefore was in a catalytically incompetent state. The difficulties in successfully crystallizing the active form of laccase have been unanimously attributed to the occurrence of extensive microheterogeneity, presumably caused by variable glycosylation of the enzyme. The crystal structure of a *Trametes versicolor* laccase (TvL) was described by Piontek *et al.* (2002) and it was found out that the geometry of the trinuclear copper cluster in TvL was similar to that found in the ascorbate oxidase and that of mammalian ceruloplasmin structures. The TvL structure was reported to be a monomer organized in three sequentially arranged domains and had dimensions of about 65 x 550 x 45 A3. The trinuclear copper cluster (T2/T 3) was found to be embedded between domains l and 3 with both domains providing residues for the coordination of the coppers. The third domain had the highest helical content with one 3, 0-helix and two α-helices located in the connecting regions between the strands of the different B-sheets. Finally, at the C terminal

end of domain 3, three sequentially arranged cc-helices completed the fold. A l3-aminoacid- long oc-helix at the C-terminal portion was stabilized by a disulfide bridge to domain l (Cys-85—Cys-488), and a second disulfide bridge (Cys-1 l7—Cys-205) connected domains 1 and 2.

Enguita *et al.* (2003) described the crystal structure of a bacterial laccase which existed as a component of the spore coat of *Bacillus subtilis*. The overall CotA fold comprised of three cupredoxin-like domains and cupredoxin fold has been mainly formed by an eight-stranded 3-barrel, comprising two B-sheets composed by four strands, arranged in a sandwich conformation. The first domain comprises eight strands organized in a B-barrel form, starting with a coiled section which has been found absent in plant and fungal multicopper oxidases. Domain 2 of CotA acts as a bridge between domains l and 3 but a short fragment, encompassing residues 177-182, made the connection between domains l and 2, whereas a large loop segment including residues 341-368 linked domains 2 and 3. Therefore, this feature may be a characteristic of the prokaryotic variants of these enzymes. Domain 3 of CotA not only contains the mononuclear copper center, but also contributed to the formation of the binding site of the trinuclear copper center, which has been located in the interface between domains l and 3. Moreover, domain 3 included the putative substrate binding site, located at the surface of the protein, close to the type I mononuclear copper center. A protruding section formed by a loop and a short on-helix, comprising amino acids from 434 to 454, formed a lid-like structure over the substrate binding site. No similar element has been found in the previously analyzed multicopper oxidases with known three-dimensional structure.

Li *et al.* (2007) determined crystal structures of *E. coli* laccase CueO at different copper concentrations. Low Structural comparison between CueO and other three fungal laccase proteins indicated that Glu106 in CueO constitutes the primary counter-work for reconstitution of the trinuclear copper site. Mutation of Glu106 to a Phe enhanced CueO oxidation activity and thus supported this hypothesis. In addition, an extra α-helix from Leu351 to Gly378 covers substrate

biding pocket of CueO and might compromises the electron transfer from substrate to type I copper.

2.17 APPLICATIONS OF LACCASES

Laccases have received much attention from researchers in last decades due to their ability to oxidise both toxic and nontoxic substrates including highly recalcitrant environmental pollutants. This property of laccases has been found to be very useful for application to several biotechnological processes (Couto and Herrera, 2006). The important applications of laccases include detoxification of dyes containing industrial effluents from textile and leather industries (Wong and Yu, 1999), pulp biobleaching (Arias *et al.,* 2003), use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases have also been used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics. Laccases have also found applications in food industry that includes wine, fruit juice and beer processing (Minussi *et al*., 2002). The major sectors where laccases are being have been used are as follows:

2.17.1 Dye decolourization

Synthetic dyes are released into the environment in wastewater streams from textile and dye manufacturing industries. These dyes have been considerably recalcitrant to biodegradation in waste water treatment facilities, and pose a severe environment risk since these might be mutagenic and carcinogenic (Zille *et al*., 2003; Cordi *et al*., 2007). Current methods for dye-decolourization have been found to be chemically derived and include absorption, chemical transformation, and incineration. It has been suggested that enhanced microbial decolourization might provide a less expensive and more environmentally acceptable alternative to chemical treatment (Selvam *et al*., 2003; Ferry and Leech, 2005). Laccases have been used for the decolourization of dyes belonging to different categories such as azo, anthraquinone, heterocyclic, triphenylmethane dyes etc. and have been extensively studied for their degradation of azo dyes (Zille *et al*., 2005). It was found out that some

chemicals serve as redox mediators, facilitate the dye degrading activity of laccase and enhance its specificity to wide range of dyes. Chivukula and Renganathan (1995) have reported the oxidation of phenolic azo dyes by the laccase from *Pyricularia oryzae* and similarly laccase from *Thelephora species* has been reported to decolourize azo dyes at different rates. The enzyme decolourized 19% of orange G, 12.0% of congo red and 15.0 % of amido black 10B (Selvam *et al*., 2003). Purified laccase from *Trametes hirsute* has been reported as capable of degrading triarylmethane, indigoid, azo, and anthraquinonic dyes. Anthraquinonic dyes and indigo carmine were degraded more than twofold faster than the azo dyes by the purified laccase (Abadulla *et al.,* 2000). Nyanhongo *et al*. (2002) have screened the ability of laccase from different *Trametes species* to decolorize synthetic dyes viz., anthraquinone, azo, indigo and triarylmethane. Specificity of *Trametes hirsuta* laccase towards different dye structures has also been investigated by Couto *et al*., 2006. The enzyme decolourized dyes at different rates and 83 % decolourization of the azo dye methyl orange was attained with this enzyme. In another study, Cameselle *et al*., 2003 have observed that the azo dye methyl orange was easily degraded than the triphenyl methane dye, bromophenol blue. Laccases immobilized on different supports have also been implemented in dye decolourization. Peralata-Zamora *et al*. (2003) have reported decolourization of different reactive dyes by immobilized laccase. Laccase from *Sclerotium rolfsii* immobilized on alumina pellets has been employed for the decolourization of wool azo dye Diamond Black PV 200 (Ryan *et al*., 2003). The decolourization of the commonly used anthroquinone dye RBBR by laccases has also been cited (Hou *et al.,* 2004). Held *et al.* (2005) has reported for the first time that spores from *Bacillus* SF with laccase activity could be used for continuous decolorization of dyes when immobilized on alumina carriers. Whereas decolorization of reactive dyes by laccase immobilized in alginate/gelatine blend with polyethylene glycol was also reported.

2.17.2 Phenol removal

Removal of hazardous phenolic components from industrial aqueous effluents has been a major problem currently experienced by many chemical

industries including paper, textile, coal conversion and petroleum refining (Atlow *et al.,* 1984). Many of these phenolics have been considered as phytotoxic, antimicrobial, mutagenic and carcinogenic in nature (Zille *et al*., 2003). Current methods for the removal of these phenols from wastewater include solvent extraction, microbial degradation, adsorption and chemical oxidation .The use of free laccase, tyrosinase and peroxidase, which catalyse the oxidative coupling of phenol compounds resulting in the formation of water insoluble oligomeric and polymeric products, which are then removed by sedimentation or filtration, has been proposed (Shuttleworth and Bollag, I986). The use of enzymes has been suggested as an altemative method for other physico-chemical methods that suffer from serious drawbacks such as high cost and formation of hazardous by-products (Atlow *et al*., 1984). The efficiency of laccases in the degradation of individual phenolic compounds as well as phenolic mixtures has been proved by Lante *et al.* (2000) and Krastanov (2000) whereas decolourization of phenolic effluents from different industrial sources has been reported by Davis and Burns in 1990. The effect of reactive co-substrates such as guaiacol and 2, 6-dimethoxyphenol on the removal of chlorinated phenols by horseradish peroxidase (HRP) and a laccase from the fungus *Trametes versicolor* has been investigated by Roper *et al.* (1995) and it was reported that the addition of co-substrates would enhance the precipitation of phenols by laccase. D'Annibale *et al.* (2000) reported the efficiency of an immobilized laccase from *Lentinula edodes* in removing the phenolics from olive mill waste water and it was reported that the laccase immobilized on Eupergit C was able to remove the phenols efficiently. Recently in 2007, Niladevi and Prema used immobilized laccase of *Streptomyces psammoticus* for phenol removal.

2.17.3 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs), also known as poly-aromatic hydrocarbons or polynuclear aromatic hydrocarbons, are potent atmospheric pollutants that consist of fused aromatic rings and do not contain heteroatoms or carry substituents and these include naphthalene, anthracene, benzopyrene, pyrene, phenanthrene and many others. These are one of the most widespread organic pollutants. These are present in fossil fuels and are formed by incomplete

combustion of carbon-containing fuels such as wood, coal, diesel, fat, tobacco, and incense. Some of these have been known or suspected carcinogens, and have been linked to other health problems. The degradation of PAHs are of great significance in maintaining a proper ecosystem for human life. The laccase-mediator system has been applied for the degradation of environmental xenobiotics such as polycyclic aromatic hydrocarbons (PAHs). Oxidation of anthracene and benzo aminopyrene by laccase from *Trametes versicolor* has been reported by Collins *et al.* (1996). Oxidation of five polycyclic aromatic hydrocarbons; anthracene, benzopyrene, fluoranthene, phenanthrene and pyrene by have been reported by laccase from *Coriolus hirsutus* in the presence of the redox mediators (Cho *et al*., 2002). The ability of laccase from *Pleurotus ostreatus* to degrade different PAHs have been investigated by Pozdnyakova *et al*. (2006) and was reported that PAH degradation by the enzyme differed with the type of the aromatic compound as well as with the mediator used.

2.17.4 Denim washing

Denim washing includes one of the new areas of application for laccases. Cellulase enzyme has been usually used in denim garment processing to get stone wash look on to the denim garments. Laccases are new generation enzymes for the finishing of denim jeans. Laccase can be used for bleaching denim fabrics as well as for making fashionable effects without compromising the strength of the fabric. Campos *et al.* (2001) reported the degradation of indigo both in effluents and on fabrics using purified laccases from *Trametes hirsuta* and *Sclerotium rolfsii* in combination with redox-mediators and reported that bleaching of fabrics by the laccases correlated with the release of indigo degradation products. Pazarlogliu *et al.* (2005) showed that a phenol-induced laccase from *Trametes versicolor* was an effective agent for stone washing effects of denim fabric without using a mediator.

2.17.5 Pulp and paper industry

The major problem encountered in the pulping process is the characteristic brown colour of the pulp due to the presence of residual lignin. Pulp bleaching has been currently achieved by treating pulps with chlorine-based chemicals. Although the chemical treatments are effective method to achieve this

task, the high cost and related pollution harms make them unattractive. Chemical method results in the formation of chlorinated aliphatic and aromatic compounds that could be acutely toxic, mutagenic and carcinogenic (Bermek *et al*., 2002; Bajpai *et al*., 1999). Hence, there arises the necessity for alternate methods of pulp bleaching. Biobleaching is ecofriendly treatment of pulp that involves either microorganisms or their enzymes and this process helps in the selective removal of lignin and hemicellulose components without degrading cellulose. Intensive studies have been performed to develop enzymatic, environmentally benign bleaching technologies (Camarero *et al*., 2004). Xylanases are the most widely used enzyme for biobleaching however, the role of Iaccases in the process has also been elucidated in the recent years. Sealey and Ragauskas (l998) have demonstrated that the laccase/n-hydroxybenzotriazole bleaching system can very effectively remove lignin from kraft pulps via a series of oxidative degradation reactions and Balakshin *et al*. (2001) have studied the biobleaching of pulp with dioxygen in laccase-mediator system. Laccases from three different white rot fungi; *Pycnoporus cinnabarinus*, *Trametes versicolor* and *Pleurotus eryngii* have been reported to be efficient bio-bleaching (Camarero *et al*., 1996). The laccase from thermostable laccase from the fungus *Pycnoporus cinnabarinus* along with the chemical mediator hydroxybenzotriazole has been employed for the biobleaching of eucalyptus kraft pulp (Ibarra *et al.*, 2006). Prokaryotic laccase from *Streptomyces cyaneus* (Arias *et al*., 2003) has been used for biobleaching of eucalyptus kraft pulp, whereas, Singh *et al.* (2007) used alkalophillic laccase from *γ-Proteobacterium* JB for biobleaching of wheat straw-rich soda pulp.

2.17.6 Food industry

In the food industry laccase finds application in different processes like fruit juice processing, wine stabilization, sugar beet pectin gelation, baking and in improving food sensory parameters (Minussi *et al*., 2002). The color and taste of the wines depend particularly on the phenolic compounds present in different types of wines (Brenna & Bianchi, 1994). The polyphenols present in musts and wines should be selectively removed to prevent any alterations in taste and colour imparted mainly by the oxidation of polyphenols. Different methods have been used in order to prevent the decolorization and flavor alteration in wines, such as

the removal of phenolic groups with polyvinylpolypyrrolidone, and the use of sulfur dioxide to block oxidizers, among others (Minussi *et al*., 2002). An alternative for the physical chemical adsorbents could be the use of enzymes that selectively target specific polyphenols during the madeirization process. These polyphenolic substances would be oxidized by the enzyme, polymerized and then removed by clarification (Peralata-Zamorani, 2003). Laccases has been effectively used for this purpose (Cantarelli, 1986). There are several studies in the literature which state that laccase treatment promotes wine stabilization (Cantarelli & Giovanelli, 1990; Plank and Zent, 1993; Servili *et al*., 2000). Maier *et al*. (1990) have shown that wines made by laccase treatment were the best, suggesting that a stable and high quality wine can be made with little or no added $S0_2$ and feasibility of using laccase for phenol removal from white must have also been suggested (Minussi *et al.,* 2007).

Table 4: Commercial preparations based on laccases for industrial processes

Industry	**Main application**	**Brand name**	**Manufacturer**
Food industry	Brewing	Flavourstar	Advanced Enzyme Technologies Ltd. (India)
	Colour enhancement in tea, etc.	LACCASE Y120	Amano Enzyme USA Co. Ltd.
	Cork modification	Suberase	Novozymes (Denmark)
Paper industry	Pulp bleaching	Lignozym-process	Lignozym GmbH (Germany)
	Paper pulp delignification	Novozym 51003	Novozymes (Denmark)
Textile Industry	Denim bleaching	Bleach Cut 3-S	Season Chemicals (China)
		DeniLite	Novozymes (Denmark)
		ZyLite	Zytex Pvt. Ltd. (India)
	Denim finishing	Cololacc BB	Colotex Biotechnology Co. Ltd. (Hong Kong)
		Ecostone LC10	AB Enzymes GmbH (Germany)
		IndiStar	Genencor Inc. (Rochester, USA)
		Novoprime Base 268	Novozymes (Denmark)
	Denim bleaching and shading	Primagreen Ecofade LT100	Genencor Inc. (Rochester, USA)

It is well known that browning, both enzymatic and chemical, is one of the major faults in beverages (Giovanelli and Ravasini, 1993). Various pre- and post treatments are available to avoid post-turbidity and discoloration of fruit juices. Various enzymatic treatments have been proposed for fruit juice stabilization, including the use of laccase (Piacquadio *et al*., 1998). Stutz (1993) proved that is possible to produce clear and stable juices concentrates with a light colour by means of ultrafiltration and laccase, without any large additional investment. Ritter and Dietrich (1996) and Piacquadio *et al.* (1998) have reported that the use of laccase improves stability in apple juice. In the baking industry, the use of laccase results in an increased volume, an improved crumb structure and softness of the baked product, as well as increased strength, stability and reduced stickiness and thereby improved machinability of the dough (Minussi *et al*., 2002). Some commercial preparations based on laccases for industrial processes are listed below (Table-4).

2.17.7 Cosmetics industry

Laccases have also found application even in production of cosmetics. Laccase-based hair dyes were found to be less irritant and easier to handle than currently available hair dyes, since laccases replace H_2O_2 as an oxidizing agent in the dye formulation (Roure *et al*., 1992; Aaslyng *et al*., 1996; Lang and Cotteret, 1999). More recently, cosmetic and dermatological preparations containing proteins for skin lightening have also been developed using laccase enzyme (Golz-Bemer *ét al*., 2004).

2.17.8 Nanobiotechnology

Since laccases are able to catalyse electron transfer reactions without additional cofactors, their use has also been exploiting in biosensor technology to detect various phenolic compounds and azides. Moreover, biosensors for detection of morphine and codeine, catecholamines, plant flavonoids, for determination of glucose, aromatic amines, phenolic compounds and electro immuno assay have been developed. Nanotechnology contributes to the development of smaller and more efficient biosensors through controlled deposition and specific adsorption of biomolecules on different types of surfaces,

achieving micro and nanometer order. Typical molecules used in this process are Polyl ectrolytes such as PSS: poly (styrene sulfonate); PAA: Poly(acrylic acid), PAH: poly (allylamine hydrochlorid), PDADMAC: poly (diallyldimethylammonium chloride).

Chen *et al.* (1998) showed a biotechnological application of micropatterned surfaces: the production of islands of micrometer size of extracellular matrix, where the pattern of these islands could determine the position and distribution of bovine and endothelial cells. The control of the nature and the density of the groups (e.g. alkys, amides, alcohols) of a surface built with assembled monolayers has been used succesfully to investigate the non-specific adsorption of proteins. Regarding laccases, the immobilisation has an important influence on the biosensor sensitivity.

Cabrita *et al.* (2005) immobilised laccase from *Coriolus versicolor* on N-Hydroxysuccinimide-terminated self-assembled monolayers on gold. This procedure could be useful for the further development of biosensors. In addition, an enzyme electrode based on the co-immobilisation of an osmium redox polymer and a laccase from *T. versicolor* on glassy carbon electrodes has been applied to ultrasensitive amperometric detection of the catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, attaining nanomolar detection limits. Laccase can also be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems. Biofuel cells are extremely attractive from an environmental point of view because electrical energy is generated without combusting fuel, thus, providing a cleaner source of energy.

Parshetti *et al.,* (2006) studied phytotoxic effect of malachite green dye and its degraded product upon *Triticum aestivum* under *in vitro* conditions. They found no germination inhibition, when seeds were treated with degradation products of malachite green, revealed that degradation products were non-toxic nature as compaired to dye it self. Similarly, Christie and Shanmugam (2012) studied textile effluents on vegetation of *Capsicum annum*. They study toxicity by watering (5 mL) the seeds with textile mill effluents and extracted enzyme

sample. The control was run by watering the seeds with distilled water. The watering was done twice a day.Germination and length of shoot and root were recorded after 13 days. They found that germination percentage combines measurements of relative root and shoot elongation as both are sensitiveto the presence of phytotoxic compounds.

2.18 IMMOBLIZATION OF LACCASE ENZYME

Enzymes exhibit a number of features that make their use advantageous as compared to conventional chemical catalysts. However, a number of practical problems exist that reduce their operational life-time, such as their high cost of isolation and purification, their non-reusability, the instability of their structures and their sensitivity to harsh process conditions. The current demands of the world's biotechnological industries are enhancement in enzyme productivity and development of novel techniques for increasing their shelf life. These requirements have been inevitable to facilitate large-scale and economic formulation. Enzyme immobilization provides an excellent base for increasing availability of enzyme to the substrate with greater turnover over a considerable period of time. Several natural and synthetic supports have been assessed for their efficiency for enzyme immobilization. Nowadays, immobilized enzymes are preferred over their free counterpart due to their prolonged availability that curtails redundant downstream and purification processes. Immobilization is "the imprisonment of an enzyme in a distinct phase that allows exchange with, but is separated from the bulk phase in which the substrate, effector or inhibitor molecules have been dispersed and monitored" (Trevan, 1980). An immobilized enzyme method is attached to an inert, insoluble material such as calcium alginate which has been produced by reaction between a mixture of sodium alginate solution and enzyme solution with calcium chloride. This provides increased resistance to changes in conditions such as pH or temperature in addition that it also allows enzyme to be held in place throughout the reaction, following which they are easily separated from the products and may be used again and so is widely used in industry for enzyme catalysed reactions.

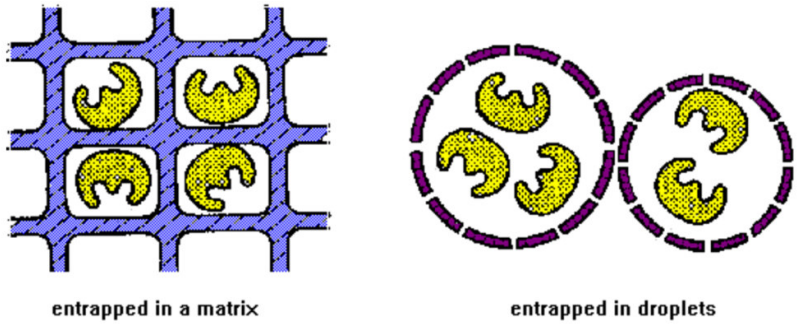


Fig. 9: Immoblization of enzyme (Duran *et al.,* 2002)

The broad substrate specificity of immobilized laccases holds promise to use them for biotechnological purposes such as biomechanical pulping, bleaching of pulp, degradation of dye and transformation and detoxification of xenobiotic and other aromatic compounds (Nyanhongo *et al.,* 2002)Moreover, immobilized laccase showed a broadening of the temperature range of enzyme activity (Al-Adhami *et al.,* 2002; Dodor *et al.,* 2004). Consequently, many supports have been proposed for immobilization of laccase (Duran *et al.,* 2002). Laccase has been immobilized on gels like Sepharose (agarose), Sephadex (dextran), cellulose-based materials (Duran *et al.*, 2002; Reku *et al.*, 2009) and allowed good activity retention since these materials are highly hydrophilic. However these gels tend to may compress or expand and cannot be used in packed bed reactors. Laccases can also be immobilized on inorganic supports like alumina (Kandelbauer *et al.*, 2004; Zille *et al.*, 2003), and silica (Peralta-Zamora *et al.*, 2003; Zhu *et al.*, 2007). Satisfactory laccase activities were achieved by first activating the material surface with 3-aminopropyltriethoxysilane (APTES) to introduce amines and cross-link the enzyme to the surface with glutaraldehyde (Duran *et al.*, 2002). However, these materials tend to be brittle and therefore mechanical agitation must be limited. More recently, laccase was immobilized on acrylic supports like poly (butyl acrylate-co-ethyleneglycol dimethacrylate), poly (glycidyl methacrylate-co-ethyleneglycol dimethacrylate) (Arica *et al.*, 2009), commercial acrylic carriers like Dilbeads and Sepabeads (Kunamneni *et al.*, 2008) and very high laccase activities were reported. Acrylic carriers have good mechanical and chemical stability and can be made highly hydrophilic (Mosbach *et al.*, 1976).

Frohener *et al.* reported the immobilization of Laccase from *Neurospora crassa* on CNBr-activated sepharose 4B by covalent attachment in 1974. Thereafter many studies have been done on immobilization of laccase enzyme such as *Rhizoctonia praticola* Laccase was found to covalently coupled to activated Celite®, *Polyporus versicolor* Laccase was reported to be immobilized by entrapment and adsorption on several carriers (gelatin, polyurethane, sepharose 4B-Epi-IDA-Cu2, i.e. a metal-chelate affinity matrix) and used for different purposes. *Coriolus versicolor* Laccase co-polymers entrapped in calcium alginate gel or Lac covalently immobilized on activated carbon were used in the treatment of effluents from the pulp and paper industry whereas immobilization of laccases isolated from fungus *Polyporus versicolor, Trametes versicolor, Coriolus versicolor, Rhus vernicifera* and *Pyricularia oryzae* was carried out by a number of immobilization method using different supports.In contrast to fungal laacase immobilization there are very few reports of bacterial laccase immobilisation, Held *et al.* (2005) immobilized laccase containing spores from *Bacillus* SF on alumina pellets whereas Singh *et al.* (2010) immobilized from γ-*Proteobacterium* on different supports viz., agarose–guar gum, nylon and nitrocellulose membranes, egg shell membrane and polyvinyl alcohol (PVA). Immobilized laccase enzyme rendered feasible their use in a variety of applications including synthetic and analytical purposes, bioremediation of contaminated soils, wastewater treatment and must and wine stabilization. Among them wastewater treatment, and wine and beverages stabilization appear to be very promising and, with regard to this aspect, it is worthwhile to make some considerations.

Chapter-3

MATERIALS AND METHODS

The present investigation on "**Bioprospecting of thermophilic bacteria from hot water springs of Himachal Pradesh for laccase enzyme production"** was carried out in the department of Biotechnology, Dr. Y S Parmar, UHF, Nauni, Solan (HP). The experimental details with respect to materials and methodology adopted for the study have been described here in.

3.1 Survey and selection of the sites

3.2 Sample collection from selected sites

3.3 Physico-chemical analysis of water samples

3.4 Standardization of different parameters for isolation of laccase producing thermophilic bacteria

3.5 Eumeration of laccase producing thermophilic bacteria

3.6 Isolation of laccase producing thermophilic bacteria

3.7 Secondary screening of thermophilic bacterial isolates for thermostable laccase production

3.8 Biochemical characterization of laccase producing thermophilic bacterial isolates

3.9 Qualitative and quantitative screening for laccase activity

3.10 Molecular characterization of selected laccase producing thermophilic bacterial isolates

3.11 Optimization of culture conditions for maximum laccase production

3.12 Enzyme production and partial purification of thermostable laccase enzyme

3.13 Characterization of partially purified thermostable laccase enzyme

3.14. Applications of partially purified thermostable laccase enzyme

3.14.1 Textile dye degradation

3.14.2 Immobilization of enzyme

3.14.3 Phytotoxicity studies

3.15 Isolation of thermostable laccase encoding gene

3.1 SURVEY AND SELECTION OF THE SITES

A survey was conducted for selection of various sites of hot water springs from Himachal Pradesh, districts of Mandi and Kullu of Himachal Pradesh were surveyed for hot water springs. A total of four sites viz., Manikaran, Vashisht, Khirganga of Kullu district and Tattapani of Mandi ditsrict were selected finally. The geographical locations of these sites along with their altitudes (m) and code numbers of samples are shown in Table-5.

Table 5: Geographical location of selected sites

S. No.	Sites	Districts	Sample	Sub site code	Sample code	Altitude (m)
1.	Manikaran	Kullu	Water	A	MAW1	1735
					MAW 2	
					MAW 3	
					MAW 4	
				B	MBW 1	
					MBW 2	
					MBW 3	
					MBW 4	
				C	MCW 1	
					MCW 2	
					MCW 3	
					MCW 4	
				D	MDW 1	
					MDW 2	
					MDW 3	
					MDW 4	
				E	MEW 1	
					MEW 2	
					MEW 3	
					MEW 4	
			Soil	A	MAS1	
					MSA2	
				B	MBS1	
					MBS2	
				C	MCS1	
					MCS2	
				D	MDS1	
					MDS2	
				E	MES1	
					MES2	

			Rock matting	A	MAR1	
					MAR2	
				B	MBR1	
					MBR2	
				C	MCR1	
					MCR2	
				D	MDR1	
					MDR2	
				E	MER1	
					MER2	
			Pebbels	A	MAP1	
					MAP2	
				B	MBP1	
					MBP2	
				C	MCP1	
					MCP2	
				D	MDP1	
					MDP2	
				E	MEP1	
					MEP2	
2.	Vashisht	Kullu	Water	A	VAW1	2050
					VAW 2	
					VAW 3	
					VAW 4	
				B	VBW 1	
					VBW 2	
					VBW 3	
					VBW 4	
				C	VCW 1	
					VCW 2	
					VCW 3	
					VCW 4	
				D	VDW 1	
					VDW 2	
					VDW 3	
					VDW 4	
				E	VEW 1	
					VEW 2	
					VEW 3	
					VEW 4	
			Soil	A	VAS1	
					VSA2	
				B	VBS1	
					VBS2	
				C	VCS1	
					VCS2	

				D	VDS1	
					VDS2	
				E	VES1	
					VES2	
			Rock matting	A	VAR1	
					VAR2	
				B	VBR1	
					VBR2	
				C	VCR1	
					VCR2	
				D	VDR1	
					VDR2	
				E	VER1	
					VER2	
			Pebbels	A	VAP1	
					VAP2	
				B	VBP1	
					VBP2	
				C	VCP1	
					VCP2	
				D	VDP1	
					VDP2	
				E	VEP1	
					VEP2	
3.	Khirganga	Kullu	Water	A	KAW1	2960
					KAW 2	
					KAW 3	
					KAW 4	
				B	KBW 1	
					KBW 2	
					KBW 3	
					KBW 4	
				C	KCW 1	
					KCW 2	
					KCW 3	
					KCW 4	
				D	KDW 1	
					KDW 2	
					KDW 3	
					KDW 4	
				E	KEW 1	
					KEW 2	
					KEW 3	
					KEW 4	
				A	KAS1	
					KSA2	

			Soil	B	KBS1	
					KBS2	
				C	KCS1	
					KCS2	
				D	KDS1	
					KDS2	
				E	KES1	
					KES2	
			Rock matting	A	KAR1	
					KAR2	
				B	KBR1	
					KBR2	
				C	KCR1	
					KCR2	
				D	KDR1	
					KDR2	
				E	KER1	
					KER2	
			Pebbels	A	KAP1	
					KAP2	
				B	KBP1	
					KBP2	
				C	KCP1	
					KCP2	
				D	KDP1	
					KDP2	
				E	KEP1	
					KEP2	
4.	Tattapani	Mandi	Water	A	TAW1	680
					TAW 2	
					TAW 3	
					TAW 4	
				B	TBW 1	
					TBW 2	
					TBW 3	
					TBW 4	
				C	TCW 1	
					TCW 2	
					TCW 3	
					TCW 4	
				D	TDW 1	
					TDW 2	
					TDW 3	
					TDW 4	
				E	TEW 1	
					TEW 2	

					TEW 3	
					TEW 4	
			Soil	A	TAS1	
					TSA2	
				B	TBS1	
					TBS2	
				C	TCS1	
					TCS2	
				D	TDS1	
					TDS2	
				E	TES1	
					TES2	
			Rock matting	A	TAR1	
					TAR2	
				B	TBR1	
					TBR2	
				C	TCR1	
					TCR2	
				D	TDR1	
					TDR2	
				E	TER1	
					TER2	
			Pebbels	A	TAP1	
					TAP2	
				B	TBP1	
					TBP2	
				C	TCP1	
					TCP2	
				D	TDP1	
					TDP2	
				E	TEP1	
					TEP2	

3.2 SAMPLE COLLECTION FROM SELECTED SITES

Twenty samples of water and ten samples of soil, rockmatting and pebbles from each site were collected for studies. Different samples were collected in sterilized screw capped vials and these samples were brought to the laboratory and kept at $4^{o}C$ in refrigerator till further processing. Sample codes were given to each sample and shown in Table-5.

3.3 PHYSICO-CHEMICAL ANALYSIS OF WATER SAMPLES

Water samples from twenty subsites were studied for various physico-chemical characters. The following parameters were evaluated during the survey:

3.3.1 pH

The pH of the water samples was recorded both at the sites as well as in the laboratory.

3.3.2 Temperature

The temperature of all the samples was recorded at the site using temperature probe.

3.3.3 Chloride (American Public Health Association (APHA), 1976)

- Silver nitrate (0.141N)
- Potassium nitrate indicator (100.0 g/l)

Procedure

Water samples were assayed titrametrically. 10 ml of the sample was taken in the flask and 5 ml of chromate reagent was added to it. The yellow coloured solution was then titrated against standard solution of silver nitrate (0.141N), till the appearance of brick red colour. A blank titration was also done.

Calculations

$$\text{Chloride mg/l} = \frac{(V1\text{-}V2)\times \text{Normality}\times 35.46 \times 1000}{\text{Vol. of sample taken}}$$

Where, 35.46 is the equivalent weight of chloride.
V1-V2 = ml of titrant used

3.3.4 Sulphate (APHA, 1976)

- Barium chloride (20-30 crystals)
- NaCl-HCl (100.0 g NaCl added to 800 ml distilled water and then the volume was made up to 1 litre).
- Conditioning reagent (Glycerol-ethanol solution mixed 50 ml glycerol in 100 ml ethanol).
- 0.147 g anhydrous Na_2SO_4 in 1 litre of distilled water: 1ml of solid contains 100.0 µg SO_4.

Procedure

Diluted 10 ml of the sample to 100 ml. Five ml of the conditioning

added to the flask with constant stirring using a magnetic stirrer. A few crystals of $BaCl_2$ were added and mixed for 1 minute. Immediately absorbance was read at 420 nm against a blank. Estimated the sulphate concentration of the sample by comparing the turbidity reading with a calibration curve prepared by carrying the sulphate standard through entire procedure. Sulphate was expressed then in mg/l.

Calculations

$$SO_4 \text{ mg /l} = \frac{MgSO_4 \times 1000}{\text{Vol. of sample taken} \times 1000}$$

3.3.5 Total hardness (APHA, 1976)

- Standard EDTA titrant (0.01M)
- Ammonium buffer solution, 114 ml concentrated NaOH was added to 13.5 g NH_4Cl and made the final volume 200 ml.
- Erichrome Black-T indicator: Dip 5.0 g in 100 ml of 80% ethyl alcohol.

Procedure

To 50 ml of water sample, 1ml of ammonium buffer and 5 drops of indicator were added. The wine red coloured solution was then titrated against the standard EDTA solution (0.01M) till the blue colour appeared.

Calculations

$$\text{Total hardness as mg/l as } CaCO_3 = \frac{(V1\text{-}V2) \times B \times 1000}{\text{Vol. of sample taken}}$$

Where, V1-V2 = ml of titrant used

B= mg $CaCO_3$ equivalent to 1.0 ml EDTA titrant at calcium indicator end point B=1.05.

3.3.6 Calcium hardness (APHA, 1976)

- Standard EDTA titrant (0.01M)
- Sodium hydroxide solution (8.0%)
- Murexide indicator mixture.

Procedure

To 50 ml of the sample, added 1 ml NaOH solution and a pinch of murexide indicator, added till the pink coloured solution changed to purple.

Checked the end point by adding 1 to 2 drops of titrant in excess till no further change in colour occurred.

Calculations

$$\text{Calcium hardness as mg/l } CaCO_3 = \frac{(V1\text{-}V2) \times B \times 1000}{\text{Vol. of sample taken}}$$

$$\text{Calcium carbonate hardness as mg/l } Ca^{++} = \frac{(V1\text{-}V2) \times B \times 400.8}{\text{Vol. of sample taken}}$$

Where, V1-V2 = ml of titrant used

B= mg $CaCO_3$ equivalent to 1.0 ml EDTA titrant at calcium indicator end point B=1.05.

400.8= correct EDTA use for any blank value obtained in calcium calculation, calculate calcium concentration for sample by multiplying by 400.8 and reported as mg/l.

3.3.7 Magnesium content (APHA, 1976)

Procedure

If the calcium hardness as calcium carbonate is known, the magnesium content can be calculated as follows:

Magnesium in mg/l = (Total hardness – Calcium hardness) × 0.243.

Where, 0.243= correct EDTA use for any blank value obtained in calcium calculation, calculate calcium concentration for sample by multiplying by 0.243 and reported as mg/l.

3.3.8 Statistical data analysis of physicochemical characters

3.3.8.1 Simple correlation studies

Simple correlation coefficient (Karl Pearson's coefficient) between characters was worked out by using the following formula.

$$r(xy) = \frac{Cov(xy)}{\sqrt{V(x), V(y)}}$$

Where:

r (xy) = Simple correlation between x and y

V (x) = Variance of x character

V (y) = Variance of y character

For testing of simple correlation the't' value was calculated by the following formula given by Gosset (1908), also known as student-t-test.

$$T = \frac{r \times \sqrt{n-2}}{\sqrt{1-r}}$$

Where:

r = Simple correlation

n = Number of coefficients in the sample

This value was compared with the tabulated't' value at (n-8) degrees of freedom.

3.3.8.2 Variability analysis

In order to compare the consistency, the coefficient of variation has been worked out for each of the observed characteristics under each site as given below:

$$CV = (\sigma / \bar{x}) \times 100$$

Where,

$\overline{X}$ = (Arithmetic mean) = $n^{-1} \sum_{i=1} xi$

σ (standard deviation) = $\left(n^{-1} \sum_{i=1}^{N} (x_i - \overline{x})^2 \right)^{½}$

Analysis of variance technique (ANOVA)

Analysis of variance technique (one way classification of data) was applied to know the average performance of various characters viz. temperature, pH, sulphate, total hardness, calcium hardness and magnesium content w.r.t. sites.

3.4. STANDARDIZATION OF DIFFERENT PARAMETERS FOR ISOLATION OF LACCASE PRODUCING THERMOPHILIC BACTERIA

3.4.1 Medium for isolation

Five different culture media were investigated for the enrichment and isolation of laccase producing thermophilic bacterial isolates (Table-6) viz., Nutrient agar (NA) containing 5 mM guaiacol, M162 containing 5 mM guaiacol

(Bains *et al.*, 2003), Tryptone Yeast Medium (TYM) containing 40 mg/l $CuSO_4$ and 5 mM guaiacol (Givaudan *et al.*, 1993), Luria Bertani (LB) medium containing 0.4 mM $CuSO_4$ and 5 mM guaiacol (Galai *et al.*, 2009) and M9 medium containing 0.2 mM $CuSO_4$ and 5 mM guaiacol (Wang *et al.*, 2010). Medium producing maximum growth OD at 540 nm along with best proliferation of reddish/brown/yellowish colonies of laccase producing thermophilic bacteria was selected further for enrichment and isolation of laccase producing thermophilic bacterial isolates.

Table 6: Composition of various media (A1-A5) used for the isolation of laccase producing thermophilic bacteria

Media Code	Composition of media	Reference
A_1	Tryptone 5.0 g, Yeast Extract 3.0 g, 1M $CaCl_2$ Solution 10 ml, $CuSO_4$ 40 mg, 5 mM Guaiacol and distilled water to make one litre having pH 7.0±2	Givaduan *et al.,* 1993, Sowinski *et al*. 2002
A_2	Tryptone 10 g, Yeast Extract 5.0 g, NaCl 5.0 g, 0.4 mM $CuSO_4$, 5 mM guaiacol and distilled water to make one litre having pH 7.0±2	Galai *et al*., 2009
A_3	Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 10 g 1M $MgSO_4$ solution 2ml, 1 M $CaCl_2$ solution 0.1 ml, 20% (w/v) glucose solution 1ml, 0.2 mM $CuSO_4$, 5 mM guaiacol and distilled water to make one litre having pH 7.4±2	Wang *et al*., 2010
A_4	$CaSO_4.2H_2O$ 0.04 g, $MgCl_2.6H_2O$ 0.2 g, Nitrilotriacetic acid 0.1 g, Micronutrient solution (H_2SO_4 0.05 ml, $MnSO_4.2H_2O$ 0.228 g, $ZnSO_4.7H_2O$ 0.05 g, H_3BO_3 0.05 g, $CuSO_4.5H_2O$ 0.0025 g, $Na_2Mo_4.2H2O$ 0.0025 g and $CaCl_2.6H_2O$ 0.0045 g) 0.1 ml, 0.01M Ferric Citrate solution 0.5 ml, Tryptone 2 g, Yeast Extract 2 g, 5 mM guaiacol and distilled water to make one litre having pH 7.2±2	Bains *et al*., 2003
A_5	Peptone 3 g, Beef Extract 5 g , NaCl 5 g, Guaiacol 5 mM and distilled water to make one litre having pH 7.2	-

A_1: TYM
A_2: LB medium
A_3: M9 Medium
A_4: M162
A_5: NA medium

3.4.2 Incubation temperature

Effect of incubation temperature on enumeration and isolation of laccase producing thermophilic bacteria was investigated at a temperature range of 55^{o}C-70^{o}C. The optimum condition of temperature producing maximum growth OD at 540 nm was selected for both enumeration and isolation of laccase producing thermophilic bacteria.

3.4.3 Incubation time

Effect of incubation time on enumeration and isolation of laccase producing thermophilic bacteria was investigated in the time range of 12-120 hrs. The optimum condition of incubation time producing maximum growth OD at 540 nm was selected for enumeration and isolation of laccase producing thermophilic bacteria.

3.4.4 Substrate concentration

Effect of different concentrations of substrate *i.e.* guaiacol was also studied on the enumeration and isolation of laccase producing thermophilic bacteria and medium containing different guaiacol concentrations ranging from 1–9 mM was investigated. Optimum substrate concentration depicting maximum growth OD at 540 nm of laccase producing bacteria was selected for further for enumeration and isolation of laccase producing thermophilic bacteria.

3.4.5 pH

Effect of pH on enumeration and isolation of laccase producing thermophilic bacteria was also investigated in a pH range of 5.0-8.0. The optimum condition of pH producing maximum growth OD at 540 nm was selected for enumeration as well as for isolation of laccase producing thermophilic bacteria.

Best medium along with a specific substrate (guaiacol) concentration, pH, incubation time and optimum incubation temperature, standardized above used for enumeration and isolation of laccase producing thermophilic bacteria from 200 samples collected from hot water springs.

3.5 ENUMERATION OF LACCASE PRODUCING THERMOPHILIC BACTERIA

Enumeration of laccase producing thermophilic bacterial population from 80 water samples of four hot water springs was carried out using serial dilution technique, which involves dilution of samples upto $10^{-9}/10^{-10}$. One ml of each water sample was transferred into 9.0 ml sterile dilution blank, which was vigorously shaken using vortex mixer. From this tube 0.1 ml is transfered to next 9.9 ml tube diluting the sample 10 times. And in this way dilutions were carried out upto 10^{-9}. Simultaneously five petridishes containing 15-20 ml TYM agar were taken and marked as 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}. To each of these petridis 1.0 ml of sample from the corresponding tube was transfered and was spread all over agar surface using a sterile L-shaped glass rod. All the plates were incubated at an optimum temperature of 60^0C for 24-48 hrs.

3.6 ISOLATION OF LACCASE PRODUCING THERMOPHILIC BACTERIA

Isolation of laccase producing thermophilic bacteria was carried out using selected TYM along with a specific guaiacol concentration, pH, incubation time and optimum incubation temperature. One gm of each soil/rock matting/pebbles samples and 1.0 ml each of water samples were inoculated in 10 ml of TYM broth containing 5 mM guaiacol and incubated in an orbital incubator shaker for 24 hrs at 60^oC of optimum temperature, at 150 rpm, at an optimum temperature of 60^0C. Turbid cultures were streaked on plates of solidified TYM agar followed by incubation for 24 hrs at an optimum temperature of 60^0C. Since mixed populations of different bacteria were found in form of different colony morphotypes, each of the colony morphotype was transferred to 10 ml of TYM broth containing 5 mM guaiacol to obtain axenic culture of each bacteria followed by incubation for 24 hrs at an optimum temperature of 60^0C followed by streaking of each axenic culture on TYM agar plates.

All the thermophilic bacterial isolates were studied for morphological characterization:

3.6.1 Morphological characterization (Kristjansson *et al*., 1986)

The laccase producing thermophilic bacterial isolates were further studied for various morphological characters which included the following colony and microscopic characters.

3.6.1.1 Colony characters of thermophilic bacterial isolates

a) Colour

1) White
2) Cream
3) Yellow
4) Creamish yellow
5) Brown
6) Light brown
7) Pink
8) Red

b) Size

1. Pinpointed (Punctiform)
2. Small
3. Large
4. Very large

c) Shape

1. Circular
2. Irregular

d) Texture

1. Smooth
2. Rough

e) Elevation

1. Flat
2. Raised

f) Pigment formation

1. Present (+)
2. Absent (-)

3.6.1.2 Microscopic characters of thermophilic bacterial isolates (Aneja, 2003)

a) Gram's reaction

- Crystal violet
- Gram's iodine solution
- 95% ethyl alcohol
- Safranin

Procedure

Thin smears of each bacterial culture were prepared on glass slides and then heat fixed. Few drops of crystal violet were added on the smear for 60 secs. The slides were washed with distilled water and each smear was then covered with Gram's iodine solution for 60 secs. Then the slides were washed with alcohol followed by safranin applied to the smears for 60 secs. Washed the slides with distilled water and observed under low power magnification and oil immersion objective of microscope to observe the colour, shape and arrangement of the bacterial cells.

b) Shape of the cells

Shape of the cells was observed under microscope using oil immersion objective (1000X magnification).

c) Spore formation

- Malachite green (5% aqueous)
- Safranin (0.5% aqueous)

Procedure

Bacterial smears were heat fixed and malachite green was applied to the smears. The slides were then heated to steaming for 5 mins and more stain was

kept on adding from time to time. After that the slides were washed under running tap water and counterstained with safranin for 60 secs. Then washed the slides with distilled water and observed under microscope.

d) Motility

Motility of the cells was observed in motility medium.

3.6.1.3 Pellicle in liquid culture

Pellicle formation was observed directly by seeing the suspension cultures.

All morphological characters were studied visually and by using microscope.

3.7 SECONDARY SCREENING OF THERMOPHILIC BACTERIAL ISOLATES FOR LACCASE PRODUCTION

All the bacterial isolates obtained were further screened for laccase activity using tannic acid agar plate test (Harkin and Obst, 1973), dimethoxyphenol (Kiiskinen *et al*., 2004) and syringaldazine (Rayner and Boddy, 1988). Tannic acid agar plate test involved addition of 0.5 % tannic acid into TYM agar. Bacterial isolates were inoculated on this medium and plates were incubated for 3-10 days at optimum incubation temperature of 60^0C. Bacterial colonies which were able to produce black zone/brown colonies were selected to be positive for laccase activity. In case of dimethoxyphenol, 0.2% dimethoxyphenol in 100 mM of phosphate buffer (pH 6.5), was dropped on bacterial colonies cultured on TYM agar. Bacterial isolates were then incubated for 4-5 hrs at optimum incubation temperature of 60^0C. Bacterial colonies which were able to produce orange coloured pigment were selected to be positive for laccase activity. Similarly in case of syringaldazine, 3 mM of syringaldazine in 200 ml of DMSO was dropped on bacterial colonies cultured on TYM agar. Bacterial isolates were then incubated for 4-5 hrs at optimum incubation temperature of 60^0C. Bacterial colonies which were able to produce purple coloured pigment were selected to be positive for laccase activity.

3.8 BIOCHEMICAL CHARACTERIZATION OF LACCASE PRODUCING THERMOPHILIC BACTERIAL ISOLATES

All the selected thermophilic bacterial isolates were studied for biochemical characterization.

a) Methyl red & Voges-Proskauer (MR-VP) test (Case and Johnson, 1984)

I. MR-VP broth (1% glucose supplemented in nutrient broth).

II. Methyl red (0.1 g methyl red dissolved in 300 ml of 95% ethanol).

III. VP reagent I (6 g α-napthol dissolved in 100 ml of 95% ethanol)

IV VP reagent II (40% KOH)

Procedure

The tubes containing MR-VP broth were inoculated with bacterial isolate and incubated at optimum temperature for 12 hrs - 24 hrs. Five drops of methyl red were added to one of the tubes and change in colour (brick red) if any was recorded. To the other tube, 0.6 ml of VP reagent I and 0.2 ml of VP reagent II were added. After shaking the tubes gently with their caps off to expose them to air, these were allowed to stand for 30 mins. and the change in the colour from pink to red was taken as positive and no change in colour or black colour formation was taken as negative.

b) Catalase test (Harrigan, 1998)

I. Hydrogen peroxide (3%)

Procedure

Add few drops of hydrogen peroxide over the surface of an agar culture and observed the effervescence if any, caused by the liberation of free oxygen as gas bubbles.

c) Indole production test (Aneja, 2003)

I. Kovac's reagent

II. 1% trypton broth

Procedure

Tubes containing 1% trypton broth were inoculated with bacterial isolates and incubated at optimum temperature for 48 hrs. 1 ml of Kovac's reagent was added to the tubes. After shaking the tubes gently, these were allowed to stand to permit the reagent to come to the top and development of a cherry (deep) red colour in the top layer of the tube. This taken as positive test for indole production.

d) Citrate utilization test (Aneja, 2003)

Procedure

Simmon's citrate agar slants were inoculated with bacterial isolates and incubated at optimum temperature for 48 hrs, change in colour of the medium from green to blue was taken as positive test.

e) Urease test (Aneja, 2003)

Procedure

Urea agar slants were inoculated with bacterial isolates and incubated at optimum temperature for 48 hrs. Change in colour from yellow to red or deep pink was taken as positive reaction for the degradation of urea by means of the production of an enzyme urease.

f) Oxidase test (Aneja, 2003)

I. Oxidase reagent

Procedure

Autoclaved filter paper strips were dipped in wurster reagent and bacterial cultures were inoculated on it with the help of loop. Production of blue or purple colouration was taken as positive test.

g. Fermentation of sugars (Aneja, 2003)

I. Glucose broth

II. Sucrose broth

III. Lactose broth

IV. Arabinose broth

Procedure

Tubes containing glucose, sucrose, lactose and arabinose broth were inoculated with bacterial isolates and incubated at optimum temperature for 48 to 72 hrs, change in colour from pink to the colour of broth in all the type of fermentation broths indicates that fermentation occurred and the test was taken as positive for fermentation of sugars.

h) Triple sugar iron agar test (TSI)

Procedure

TSI agar slants were inoculated with bacterial isolates and incubated at optimum temperature for 48 hrs. Change in colour of slants from red to pink and butt from red to yellow was taken as positive to ferment glucose and not to ferment sucrose or lactose whereas if both slant and butt were pink in colour means none of the sugar was fermented and H_2S gas formation was showed by black colouration in the butt.

3.9 QUALITATIVE AND QUANTITATIVE SCREENING FOR LACCASE ACTIVITY

Selected thermophilic bacterial isolates were further screened for laccase activity by both qualitative and quantitative methods. The culture supernatant was obtained from selected bacterial isolate by centrifugation of overnight grown cultures at 10,000 X g, 4^0C for 10 mins and used as crude extracellular enzyme. Cells thus obtained were washed twice and suspended in 2.0 ml of 100 mM phosphate buffer (pH 6.5) and disrupted by ultrasonication using sonicator (MP Biomedical) giving 5-6 bursts of 1 min each at 100 % power, with 5 mins intervals. The cell debris was removed by centrifugation and cell free supernatant was obtained which was used as crude intracellular enzyme. Crude intracellular and extracellular enzyme preparations were used for both qualitative as well as quantitative assays.

Qualitative determination of laccase activity

Plate assay is a rapid determination of presence of laccase in both extracellular and intracellular enzyme extracts. Fifteen ml of sterile agar containing 1.0 % dimethoxy phenol was poured into petriplates and after solidification wells of appropriate size were made. Hundered µl of crude enzyme extract was added to first well and commercial laccase enzyme was added to the second well followed by incubation at 60^0C for 5-10 hrs. Orange coloured zone would be formed due to the oxidation of substrate which interpreted laccase activity.

Quantitative determination of laccase activity

The intracellular as well as extracellular activity was measured by monitoring the oxidation of ABTS. Laccase activity was determined spectrophotometrically at 420 nm with ABTS as a substrate The reaction mixture contained 5.0 µl aliquots of crude intracellular and extracellular enzyme respectively and 3 ml of 0.2 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) in a total volume. The reaction was held at 60^0C for 10 min and then 0.5 ml of 80% trichloroacetic acid (TCA) was added to stop the reaction. One unit of enzyme was defined as the amount of enzyme required to oxidize 1µmol of ABTS per min. The molar extinction coefficient of ABTS was found to be 3.6 X 10^4 M^{-1} cm^{-1} (Sowinski *et al*., 2002).

The bacterial isolate which produced maximum orange zone during qualitative assay and showed highest thermolaccase enzyme activity during quantitative assay was selected for further studies.

3.10 MOLECULAR CHARACTERIZATION OF SELECTED LACCASE PRODUCING THERMOPHILIC BACTERIAL ISOLATES

3.10.1 Extraction of genomic DNA

Total genomic DNA of selected isolates was extracted using Genomic DNA extraction kit-Mini (Real Genomics) using the following protocol:

Preparation of bacterial suspension

Twenty five ml of TYM broth (150 ml conical flask) was inoculated with two loop fulls of culture of each of the selected laccase producing bacterial isolates and incubated at 60^{o}C, in an orbital incubator shaker for 24 hrs.

Cell harvesting for Gram –ve bacteria

GT buffer : Genomic DNA extraction kit, Real Genomics

Overnight grown bacterial culture (approx 10^9 cells/ml) was transferred to 1.5 ml microfuge tubes and centrifuged at 13,000 rpm for 1 min in a microcentrifuge (REMI). Supernatant obtained was discarded and to each tube 200 µl of GT buffer (Genomic DNA extraction kit, Real Genomics) was added. There after the cell pellet was resuspended by vortexing which was followed by incubation at room temperature for 5 mins.

Cell harvesting for Gram +ve bacteria

Table 7: Lysozyme buffer

S. No.	Components	Quantity
1	Lysozyme	20 mg/ml
2	Tris HCl	20mM
3	EDTA (pH 8.0)	2mM
4	Triton X-100	1%.

Overnight grown bacterial culture (approx 10^9 cells/ml) was transferred to 1.5 ml microfuge tubes and centrifuged at 13,000 rpm for 1 min in a microcentrifuge (REMI). Supernatant obtained was discarded and to each tube 200 µl of lysozyme buffer (freshly prepared) was added. There after the cell pellet was resuspended by vortexing which was followed by incubation at room temperature for 10 mins.

Cell lysis

Two hundred µl of GB buffer (lysis buffer) was added to sample and mixed by vortexing for 5 secs. Then the microfuge tubes containing samples were incubated at 70^{o}C for 10 mins until the sample lysate was clear. During

incubation the tubes were inverted every 3 mins. Elution buffer (200 µl per sample) was preheated at 70^0C which was used for DNA elution later.

DNA binding

Two hundred µl of 96-100% ethanol was added to the sample lysate and mixed immediately for 10 secs by vortexing. During mixing precipitation was avoided by pipetting. Then a GB column (Genomic DNA extraction kit, Real Genomics) was placed in a 2.0 ml collection tube and the mixture from previous step was applied to the GB column. After closing the caps, centrifugation was done at 13,000 rpm for 2 mins.

Washing

Four hundred µl of W1 (Wash buffer) buffer was added in the GB column (Genomic DNA extraction kit, Real Genomics) and centrifuged at 13,000 rpm for 30 secs. The flow-through was discarded and the GB columns were placed back in a 2.0 ml collection tube. Six hundred µl of wash buffer was added in the GB column followed by centrifugation at 13,000 rpm for 30 secs. Flow through was discarded and GB column matrix was dried by centrifugation at 13,000 rpm for 3 mins.

DNA elution

Dried GB column was transferred into a clean 1.5 ml microfuge tube and 100 µl of preheated elution buffer was added into the center of the column matrix. The tube was allowed to stand for 3-5 mins until elution buffer got absorbed by the matrix followed by centrifugation at 13,000 rpm for 1 min to elute the purified DNA. The eluted purified DNA was stored at -20^{o}C for further use.

3.10.2 RAPD-DNA finger printing

Genomic DNA of selected laccase producing thermophilic bacterial isolates was analyzed by RAPD-PCR technology for determining genetic relatedness among these thermophilic bacterial isolates.

3.10.2.1 Standardization of RAPD-PCR technology

Genomic profile of selected eighteen maximum laccase producing thermophilic bacteria was screened by RAPD-PCR technology and 35 random primers were used for RAPD analysis. The details of primers used in the study have been depicted in table-8 mentioned below.

Table 8: List of RAPD primers investigated in the study

S. No.	Primers used	Sequence 5'- 3'
1.	OPA-01	CAGGCCCTTC
2.	OPA-02	TGCCGAGCTG
3.	OPA-03	AGTCAGCCAC
4.	OPA-04	AATCGGGCTG
5.	OPA-05	AGGGGTCTTG
6.	OPA-06	GGTCCCTGAC
7.	OPA-07	GAAACGGGTG
8.	OPA-08	GTGACGTAGG
9.	OPA-09	GGGTAACGCC
10.	OPA-10	GTGATCGCAG
11.	OPA-11	CAATCGCCGT
12.	OPA-12	TCGGCGATAG
13.	OPA-13	CAGCACCCAC
14.	OPA-14	TCTGTGCTGG
15.	OPA-15	TTCCGAACCC
16.	OPA-16	AGCCAGCGAA
17.	OPA-17	GACCGCTTGT
18.	OPA-18	AGGTGACCGT
19.	OPA-19	CAAACGTCGG
20.	OPA-20	GTTGCGATCC
21.	OPB-01	GTTTCGCTCC
22.	OPB-02	TGATCCCTGG
23.	OPB-03	CATCCCCCTG
24.	OPB-04	GGACTGGAGT
25.	OPB-05	TGCGCCCTTC
26.	OPB-06	TGCTCTGCCC
27.	OPB-07	GGTGACGCAG
28.	OPB-08	GTCCACACGG
29.	OPB-09	TGGGGGACTC
30.	OPB-10	CTGCTGGGAC
31.	OPC-06	GAACGGACTC
32.	OPC-07	GTCCCGACGA
33.	OPC-08	TGGACCGGTG
34.	OPC-09	TGGGGGACTC
35.	OPC-10	TGTCTGGGTG

The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume with composition as depicted in Table-9. Amplifications were performed using thermal cycler (Biorad) and with a temperature profile standardized for RAPD amplification (Table-10).

Table 9: Composition of PCR reaction mixture

S. No.	Reaction mixture	Quantity (µl)
1	PCR buffer (10X) with $MgCl_2$ (1.5 mM)	2.0
2	dNTPs mixture (0.5 mM each)	2.0
3	Taq DNA polymerase (5U/µl)	0.5
4	Random Primer (10nM)	1.0
5	Template DNA	1.0
6	Distilled water	13.5
Total volume		**20.0**

Table 10: PCR reactions followed in RAPD amplification studies

Stage of PCR		Time (mins)	Temp (^oC)	No. of cycle
Pre-PCR	Hot start	5 mins	94	1
PCR	Denaturation	1 mins	94	40
	Annealing	1 mins	36	
	Extension	2 mins	72	
Post-PCR	Final extension	7 mins	72	1
Hold at 4^0C				

3.10.2.2 Gel electrophoresis

Amplified PCR products were separated by agarose gel electrophoresis using 1.0 % agarose gels, in TAE buffer (242 g Tris base, Tris, 57.1 ml glacial acetic acid, 100 ml 0.5M EDTA; pH 8.0) containing ethidium bromide (0.5 µg/ml) and 100 bp -5 kb DNA ladder as a size marker for 2 hrs at 6 V cm^{-1}. The gels were viewed and image were captured using gel documentation system.

The amplified products of selected laccase producing thermophilic bacterial isolates were scored for the presence and absence of bands. Presence of an amplified product was designated as '1' and absence was marked as '0'. A pair wise similarity matrix was constructed by using dice similarity index. This was further subjected to UPGMA clustering analysis and a dendrogram was constructed (Rohlf, 1993). NTSYS-pc, version 2.02 (Numerical Taxonomy

System, Exeter Software) was used to perform cluster analysis of the complete RAPD data.

3.10.3 Characterization of isolates using 16S rRNA gene technology

The extracted genomic DNA of selected thermophilic bacterial isolates was amplified by using universal primers for 16S rRNA gene of selected bacterial isolates (Dhiman, 2014). The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume with composition as depicted in Table-11. Amplifications were performed using thermal cycler (Biorad) and with a temperature profile standardized for 16S rRNA gene amplification (Table-12).

Universal Primers for 16S rRNA gene:

B27F 5`-AGAGTTTGATCCTGGCTCAG-3'

U1492R 5`-GGTTACCTTGTTACGACTT-3'

Table 11: Composition of PCR reaction mixture

S. No.	Reaction mixture	Quantity (µl)
1	PCR buffer (10X) with $MgCl_2$ (1.5 mM)	2.0
2	dNTPs mixture (0.5 mM each)	2.0
3	Taq DNA polymerase (5U/µl)	0.5
4	Primer F (10nM)	1.0
5	Primer R (10nM)	1.0
6	Template DNA	1.0
7	Distilled water	12.5
Total volume		**20.0**

Table 12: PCR reactions followed in amplification studies

Stage of PCR		Time (mins)	Temp (^{0}C)	No. of cycle
Pre-PCR	Hot start	5 mins	94	1
PCR	Denaturation	1 mins	94	30
	Annealing	1 mins	48	
	Extension	1.5 mins	72	
Post-PCR	Final extension	8 mins	72	1
Hold at 4^0C				

3.10.4 Agarose gel electrophoresis of PCR products

The amplified PCR products of each test isolate was resolved by electrophoresis using 1.0% agarose gel in 1 X Tris-acetate EDTA buffer (2 M

Tris base, 57.10 ml acetic acid and 0.5 M EDTA (pH 8.0, 50X) containing ethidium bromide (0.5 µg/ml). DNA ladder of 100 bp -5 kb was used as a marker. The gel was run at 80 V for 2-3 hrs using Bangalore Genei power system. The gels were viewed and images were captured using gel documentation system (AlphaImager 2200, Alpha Infotech Corporation, USA). To counter possible stochastic effects of PCR (Wagner *et al*. 1994), five amplifications were carried out on each sample and pooled prior to purification. Amplified PCR products were eluted from the gel using gel extraction kit (Hi Yield Gel/ PCR DNA Extraction Kit from Real Genomics).

3.10.5 Gel extraction protocol

Gel dissociation

Agarose gel slices containing relevant DNA fragments were excised and extra agarose was removed to minimize the size of the gel slices. 300 mg of the gel slices were transferred into a microcentrifuge tube. 500 µl of DF buffer was added to each sample and mixed by vortexing followed by incubation at 55ºC for 10-15 mins until the gel slices were completely dissolved. During incubation, the tubes were inverted at every 2-3 mins and dissolved sample mixtures were cooled down to room temperature.

DNA binding

A DF column was placed in 2.0 ml collection tube. 800 µl of sample mixture from previous step was applied into the DF column followed by centrifugation at 13,000 rpm for 30 secs. Flow through was discarded and DF column was placed back in 2 ml collection tube.

Wash

Four hundred µl of W1 (Wash buffer) buffer was added in the DF column (Genomic DNA extraction kit, Real Genomics) and centrifuged at 13,000 rpm for 30 secs. The flow-through was discarded and the DF columns were placed back in the 2.0 ml collection tubes. Six hundred µl of wash buffer was added in the DF column followed by centrifugation at 13,000 rpm for 30 secs. Flow through were

discarded and all GB column matrixes were dried by centrifugation at 13,000 rpm for 3 mins.

DNA elution

The dried columns were transferred in a new microfuge tubes and 20 µl of elution buffer was added into the centre of each column matrix. The microfuge tubes were allowed to stand for 2 mins until elution buffer got absorbed by the matrix followed by centrifugation for 2 mins at 13,000 rpm to elute the purified DNA from all the samples.

3.10.6 16S rRNA gene sequencing

Sequencing is the process of determining the nucleotide (A, T, G and C) order of a given DNA fragment. DNA sequencing has been performed using the chain termination method developed by Sanger and his coworkers in 1977. The PCR products obtained through amplification with universal primers targeting 16S rRNA gene using same upstream and downstream primers.

3.10.7 *In silico* analysis of sequences

In silico is an expression used to mean "performed on computer or via computer simulation". Raw sequence data were obtained as a set of two sequences for each sample one with forward and other with reverse universal primers of sequencing reaction. Analysis of raw sequences data was done using Chromas Lite (version 2.1) of Technelysium Pvt. Ltd. Sequences of the selected bacterial isolates were analyzed using different approaches which have been discussed below briefly.

3.10.7.1 BLASTn

Basic Local Alignment Search Tool (BLAST) uses an algorithm of Altshul *et al*. (1990) for searching similarities above certain threshold between a query sequence and all other sequences present in a database. 16S rRNA gene sequences of selected bacterial isolates were analyzed using BLASTn to align them with corresponding sequences of 16S rRNA from the database.

Reference URL

http://www.ncbi.nlm.nih.gov/blast

3.10.7.2 Phylogenetic analysis

Phylogenetic analysis has been used in comparative genomics and proteomics to depict evolutionary relationships. The analysis began with aligning of sequences using tools like ClustalW and after alignment, phylogenetic trees are constructed.

During present investigations entire set of nucleotide sequences (nucleotide sequences of selected isolates and rest all sequences from NCBI) were placed in a single notepad in their Fasta format, and these were aligned through EBI tool, CLUSTALW program, integerated with MEGA (Molecular Evolutionary Genetics Analysis; Tamura *et al*., 2007) version 4 and the tree was generated using MEGA version 4 and analyzed. The evolutionary history/phylogenetic analysis were inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 100 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) has been shown above the branch (Felsenstein, 1985).

3.11 OPTIMIZATION OF CULTURE CONDITIONS FOR MAXIMUM LACCASE PRODUCTION

The culture conditions for industrially useful microorganisms are generally optimized to obtain higher yields of their industrially important products including enzymes. The bacterial isolate selected in step 3.7 by quantitative and qualitative assays depicting maximum laccase activity has further investigated to study effect of different factors such as incubation temperature, pH, incubation time, nitrogen source, carbon source, different metal ions, different $CuSO_4$ concentrations, different concentrations of guaiacol and

inoculum sizes on laccase production as well as growth of the thermophilic bacterial isolate.

3.11.1 Effect of incubation time

Effect of different incubation times for maximum laccase activity were investigated ranging from 24, 48, 72, 96, 120 to 144 hrs in addition to growth of bacterial isolate using TYM containing 40 mg/l $CuSO_4$ and 5 mM guaiacol. The optimum incubation time leading to maximum production of laccase enzyme as well as best growth of the thermophilic bacterial isolate was selected for further studies.

3.11.2 Effect of incubation temperature

Effect of incubation temperature on the growth of thermophilic bacterial isolate and laccase production was studied. The bacteria was grown at a temperature range of 30°C-80°C using TYM containing 40 mg/l $CuSO_4$ and 5 mM guaiacol and optimum temperature was selected.

3.11.3 Effect of pH

To find optimum pH for the optimum thermophilic bacterial growth and maximum laccase production by bacterial isolate, a pH range of 4.0-11.0 was examined using TYM containing 40 mg/l $CuSO_4$ and 5 mM guaiacol at an optimum temperature and optimum time, the best conditions were selected and used in all further experiments.

3.11.4 Effect of different concentration of guaiacol

TYM basal containing 40 mg/l $CuSO_4$ was prepared with guaiacol concentration ranging from 1.0-10.0 mM for bacterial growth and laccase production. Concentration of guaiacol which produced maximum laccase and led to best growth of organism was selected and used further in all experiments.

3.11.5 Effect of different concentration of $CuSO_4$

TYM containing standardized concentration of guaiacol was prepared with different concentration of $CuSO_4$ ranging from 10 mg/l to 70 mg/l and used

for growth and laccase production. Concentration of $CuSO_4$ which produced maximum laccase and led to best growth of organism was selected and used further in all experiments.

3.11.6 Effect of inoculum size

Effect of different inoculum sizes ranging from 1.0-5.0% was investigated on the laccase production and growth of thermophilic bacterial isolate using TYM containing 40 mg/l $CuSO_4$ and 5 mM guaiacol at an optimum time, temperature and pH. The inoculums size that produced maximum laccase enzyme and showed best growth of the thermophilic bacterial isolate was selected.

3.11.7 Effect of carbon source

Effect of 1 % concentration of glucose, sucrose, maltose and mannitol on growth and laccase production was studied using TYM containing 40 mg/l $CuSO_4$ as basal medium. Stock solution of each sugar was prepared and autoclaved separately and sugars were added to the medium prior to inoculation with starter cultures and monitored for its effect on laccase production and growth of bacterial isolate.

3.11.8 Effect of nitrogen

TYM was prepared with 0.1, 0.2, 0.3, 0.4 and 0.5 % of yeast extract and 0.3, 0.4, 0.5, 0.6 and 0.7 % of tryptone in combination and studied for maximum laccase production and best growth of bacterial isolate. Combination of tryptone and yeast extract which gave maximum laccase production and best growth was selected.

3.11.9 Effect of different metal ions

The effect of metals ions was investigated on laccase production and growth of microorganism by addition of each metal ion i.e. $MgSO_4$, $LiCl_2$, $FeSO_4$, $NaNO_3$, $CaCl_2$ and $CuSO_4$ at final concentration of 1 mM in TYM

containing 5 mM guaiacol. Effect of each metal ion was investigated on laccase production and bacterial growth.

3.12 ENZYME PRODUCTION AND PARTIAL PURIFICATION OF THERMOSTABLE LACCASE ENZYME

3.12.1 Enzyme production

3.12.1.1 Preparation of inoculum

An axenic culture of selected bacterial isolate, which produced maximum orange zone during qualitative assay and showed highest thermolaccase enzyme activity during quantitative assay as described in section 3.9 was inoculated into 10-20 ml of standardized TYM for laccase production followed by incubation at optimum temperature of 60^{o}C for optimum period of time of 24 hrs with the shaking speed of 150 rpm.

3.12.1.2 Preparation of bacterial culture for laccase production

Standardized inoculum of 1.0 ml (overnight culture) was inoculated into the standardized TYM for laccase production followed by incubation at optimum temperature of 60^{o}C for an optimum period of time of 24 hrs at 150 rpm**.** The cells were collected by centrifugation at 10,000 X g, 4^{o}C for 10 mins determine intracellular laccase activity whereas extracellular laccase activity was tested for the supernatant itself.

3.12.1.3 Preparation of extracellular / intracellular crude enzyme extract

The culture supernatant obtained by centrifugation in the previous step was used as crude extracellular enzyme. Cells thus obtained were washed twice and suspended in 2.0 ml of 100 mM phosphate buffer (pH 9.0) followed by disruption using ultrasonication carried out using ultra-sonicator (MP Biomedical) giving 5-6 bursts of 1 min each at 100% power, with 5 mins intervals. The cell debris was removed by centrifugation and obtained cell free supernatant, which was used as crude intracellular enzyme.

3.12.1.4 Protein estimation of crude enzyme extract by Lowry's method (Lowry *et al*., 1951)

Protein concentration estimated in crude enzyme extract by standard Lowry's method.

Reagents

A. 1 percent copper sulphate

B. 2 percent sodium carbonate in 0.1 N sodium hydroxide

C. 2 percent sodium potassium tartarate

D. To 50 ml of reagent b, 0.5 ml of reagent a and 0.5 ml of reagent c were added. This solution was prepared fresh.

E. 1 N Folin's Ciocalteau reagent (prepared fresh)

Procedure

Five ml of Reagent D was added to 1 ml of suitably diluted protein solution and mixed immediately. 1N Folin's reagent (0.5 ml) was added after 10 mins and the test tube was shaken vigorously to ensure proper mixing. After 30 mins, the intensity of colour developed was measured at 660 nm against the reagent blank. The amount of protein was calculated from standard curve using Bovine Serum Albumin (0-100 µg per ml).

3.12.1.5 Enzyme Assay

The intracellular as well as extracellular activity was measured by monitoring the oxidation of ABTS. Laccase activity was determined spectrophotometrically at 420 nm with ABTS as a substrate The reaction mixture contained 5.0 µl aliquots of crude intracellular and extracellular enzyme respectively and 3 ml of 0.2 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) in a total volume. The reaction was held at 60^0C for 10 min and then 0.5 ml of 80% trichloroacetic acid (TCA) was added to stop the reaction. One unit of enzyme was defined as the amount of enzyme required to oxidize 1µmol of ABTS per min. The molar extinction coefficient of ABTS was found to be 3.6 X 10^4 M^{-1} cm^{-1}.

Determination of laccase activity (U/L)

Enzyme activity = (A xV)/(t x e x v)

A = Absorbance

V = Total volume of reaction mixture (ml)

v = Enzyme volume (ml)

t = Incubation time (min)

e = Extinction coefficient

3.12.2 Partial purification of laccase enzyme

All steps of purification were performed at a temperature of 4^{o}C using 100 mM sodium phosphate buffer, pH 9.0. Techniques used for the purification of laccase enzyme were ammonium sulfate precipitation, dialysis, gel filtration chromatography and ion exchange chromatography. The enzyme preparations at various stages of laccase enzyme purification were analyzed for protein concentration and enzyme activity.

3.12.2.1 Ammonium sulphate precipitation

The cell free extract was subjected to various saturation/concentration of ammonium sulfate (0-90%). The ammonium sulfate precipitation table (Scopes, 1982) was followed to calculate the required amount of ammonium sulfate to be added in cell free extract. Ammonium sulfate was added with continuous stirring and stored at 4^{o}C for overnight. The precipitated proteins were recovered by centrifugation at 20,000 X g for 20 mins and the pellet was dissolved in minimum quantity of 100 mM sodium phosphate buffer (pH 9.0).

3.12.2.2 Preparation of dialysis bag

The dialysis was carried out for 24 hrs against three successive changes of dialysis buffer 100 mM sodium phosphate buffer (pH 9.0) in dialysis bag. The dialysis bag was rinsed in distilled water and then boiled in excess of distilled water containing 50 µM EDTA to remove the impurities and eliminate the release of ultraviolet light absorbing material during dialysis. The dialysis bag was washed thrice with distilled water. One end of the dialysis bag was tied with

thread and the crude extract was poured into the bag. The other end of the dialysis bag was also tied and was kept in dialysis buffer at 4^{o}C for concentration till the volume was reduced considerably. Protein concentration and laccase activity were estimated in protein sample using Lowry's method and enzyme assay respectively. The fraction or percentage cut exhibiting maximum activity of laccase enzyme was termed as ASF (Ammonium Sulphate Fraction) and was taken for further purification of laccase enzyme.

3.12.2.3 Gel filtration column chromatography

Four grams of Sephadex G-100 was suspended in 200 ml of sodium phosphate buffer (pH 9.0) for 24 hrs. It was packed into glass column having dimensions of (50 x 1.5cm) avoiding entrapment of any air bubble in the gel bed. It was then washed with sufficient amount of Phosphate buffer (pH 9.0). Then the 4 mg/ml blue dextran solution was loaded on the column and 3 ml fractions were collected. The absorbance was recorded at 600 nm. This was done to check the uniformity of packed column and for calculating the void volume. 2 ml of concentrated ASF fractions from dialysis was loaded on the Sephadex G-100 column. This column was eluted with phosphate buffer and 3 ml fractions were collected. The collected fractions were analyzed for protein content at 280 nm and for enzyme assay. The most active fractions were pooled and stored at 4^{o}C till further purification step.

3.12.2.5 Ion exchange chromatography

Two grams of DEAE-Sephadex was suspended in 200 ml of phosphate buffer (pH 9.0) for 48-72 hrs. It was packed in a glass column (8 x 2.5cm). The column was washed with sufficient amount of phosphate buffer. Then the active pooled fractions obtained from G-100 gel filtration were loaded on ion exchange column. The column was first eluted with phosphate buffer (pH 9.0) to wash out unbound proteins. The bound proteins were eluted with linear salt gradient using four bed volumes of 0.1 M NaCl, 0.2 M NaCl, 0.3 M NaCl, 0.4 NaCl and 0.5 M NaCl in phosphate buffer (pH 9.0). Fractions of 2 ml each were collected and monitored for protein content at 280 nm and for enzyme assay. The active fractions were pooled together and concentrated by dialysis against solid sucrose.

The concentrated sample was used for carrying out native-PAGE as well as for further studies.

3.12.2.6 Native PAGE (Polyacrylamide gel electrophoresis)

The purity of the laccase enzyme obtained after the course of partial purification was checked by native polyacrylamide gel electrophoresis on vertical slab gel using 7.5 per cent acrylamide gels and the method adopted was anionic system of Davis, (1964).

3.12.2.6.1 Reagents

i) Acrylamide-bisacrylamide stock solution

Acrylamide	-	30.0 g
Bisacrylamide	-	0.8 g

Dissolved in water and the final volume was made to 100 ml with distilled water. Filtered the solution through Whatman No. 1 filter paper and stored in brown bottle at 0-4^{o}C.

ii) Stacking gel buffer stock (Tris-HCl, pH-6.8)

Tris	-	6.0 g
1M HCl	-	48.0 ml

Adjusted its pH to 6.8 and the final volume was made to 100 ml with distilled water. Filtered it through Whatman No. 1 filter paper and stored at 0-4^{o}C.

iii) Resolving gel buffer stock (Tris-HCl, pH 8.8)

Tris	-	36.3 g
1 M HCl	-	48.0 ml

Adjusted its pH to 8.8 and the final volume was made to 100 ml. It was filtered through Whatman No. 1 filter paper and stored at 0-4^{o}C.

iv) Reservoir buffer (Tris-glycine, pH 8.3)

Tris	-	3.0 g
Glycine	-	14.4 g

Adjusted its pH to 8.3 and the final volume was made to 1 litre with distilled water.

v) 1.5 per cent (w/v) ammonium persulfate (APS):

This solution was prepared by dissolving 0.15 g of APS in 10 ml of distilled water. This reagent was prepared fresh just before use.

vi) Staining solution

Coomassie brilliant blue R-250	-	1.25 g
Methanol	-	200 ml
Glacial acetic acid	-	35 ml

After mixing the above components, the final volume was made to 500 ml with distilled water. It was filtered to remove undissolved materials and stored at room temperature.

vii) Destaining solution

Glacial acetic acid	-	75 ml
Methanol	-	50 ml

The above components were mixed and the final volume was made to one litre with distilled water.

viii) Sample buffer

Sample loading buffer was prepared by mixing 25 % glycerol, 1 % bromophenol blue and 62.5 mM Tris-HCL (pH 6.8).

3.12.2.6.2 Sample preparation

Mix equal proportion of partially purified sample and sample buffer prior to loading on the gel.

3.12.2.6.3 Gel preparation and electrophoresis of sample

Glass plates were washed with distilled water and oven dried. The plates were placed on gel casting assembly. The plates were clamped properly avoiding any leakage and air bubble entrapment. Various components of resolving gel were mixed as specified in the Table-13 and were poured into the glass plate assembly after addition of TEMED. The gel was allowed to polymerize for 30 mins. After the polymerization of gel, the whole assembly was placed in reservoir buffer tank. Buffer tank was filled with prechilled reservoir buffer and 30 µl of protein sample was loaded in each well with a few drops of marker dye (bromophenol blue) in one of the wells as tracking dye. Electrophoresis was carried out at a constant current of 3 mA/well. The current was applied till the tracking dye reached the other end of the gel.

Table 13: Composition of stacking and resolving gel for native-PAGE

Reagents	Stacking gel (2.5 %)	Resolving gel (7.5 %)
Acrylamide- bisacrylamide stock solution	2.50	7.50
Stacking gel buffer stock solution (Tris HCl, pH 6.8)	5.00	-
Resolving gel buffer stock solution (Tris HCl, pH 8.8)	-	3.75
1.5 per cent APS	1.50	1.50
Water (ml)	11.00	17.25
TEMED(ml)	0.015	0.15
Total volume (ml)	20	30

3.12.2.7 Staining and destaining of polyacrylamide gel

After electrophoresis, gel was removed from the electrophoresis apparatus and transferred to a small gel staining box containing Coomassie blue gel stain (approximately 50 ml), and incubated at room temperature for 2-3 hours with agitation. It was then destained by changing the destaining solution frequently.

3.12.2.8 Zymography/Activity staining

After completion of the run, the gel was removed from the glass plates and soaked with 0.1 M sodium acetate buffer (pH 4.5) containing ABTS or dimethoxyphenol for laccase activity detection. The gel was incubated at 60°C until a color band appeared.

3.13 CHARACTERIZATION OF PARTIALLY PURIFIED THERMOSTABLE LACCASE ENZYME

Characterization of the purified thermolaccase enzyme involved determination of molecular weight of thermolaccase enzyme using SDS-PAGE in addition to effect of different factors such as temperature, pH and salt on its activity and stability was studied. Effect of substrate concentrations and inhibitors was also determined on laccase activity.

3.13.1 SDS- Polyacrylamide gel electrophoresis

SDS-PAGE was carried out to determine the purity and molecular weight of thermolaccase enzyme. Molecular weight of the purified laccase was determined by SDS-PAGE as per the method of Laemmli, (1970).

3.13.1.1 Reagents for SDS-PAGE

The analytical grade reagents were used for SDS-PAGE. Acrylamide, bis-acrylamide (N,N'-methylenebisacrylamide), sodium dodecyl sulfate (SDS), bromophenol blue and dithiothreitol (DTT), Tris Base, TEMED (N, N, N', N'-tetramethylene-ethylenediamine), ammonium persulfate, 2-mercaptoethanol, glycerol, glycine and hydrochloric acid were from procured from SRL, Pvt Ltd, India.

3.13.1.2 Stock solutions for SDS-PAGE

(i) 2 M Tris-HCl (pH 8.8)
(ii) 1 M Tris-HCl (pH 6.8)
(iii) 10 % (w/v) SDS
(iv) 50 % (v/v) glycerol
(v) 1 % (w/v) bromophenol blue
(vi) 2-mercaptoethanol

3.13.1.3 Working solutions for SDS-PAGE

All working solutions to perform SDS-PAGE were stored at 4-10^{o}C.

(i) Solution A (Acrylamide stock solution)

The acrylamide stock solution for SDS-PAGE was prepared by dissolving 29.2 g acrylamide and 0.8 g bis-acrylamide in distilled water to make volume 100 ml and stir until completely dissolved.

(ii) Solution B (4X Separating gel buffer)

It was prepared by mixing 75 ml of 2 M Tris-HCl (pH 8.8), 4 ml of 10 % SDS and 21 ml of H_2O for SDS-PAGE.

(iii) Solution C (4X Stacking gel buffer)

To make 100 ml of staking gel buffer for SDS-PAGE, 50 ml of 1 M Tris-HCl (pH 6.8) was mixed with 4 ml of 10% SDS and 46 ml of H_2O.

(iv) 10% Ammonium persulphate

One gm ammonium persulphate was dissolved in double distilled water to final volume of 10 ml.

(v) Electrophoresis buffer

One litre of electrophoresis buffer was prepared for SDS-PAGE by dissolving 3.0 g Tris, 14.4 g glycine and 1.0 g SDS in distilled water and its pH was adjusted to 8.3 before making final volume one litre.

(vi) 5X Sample buffer

Ten ml of sample loading buffer was prepared by mixing 0.6 ml of 1M Tris-HCl (pH 6.8), 5 ml of glycerol (50 %, v/v), 2 ml of SDS (10 %, w/v), 0.5 ml of 2-mercaptoethanol, 1 ml of bromophenol blue and 0.9 ml of H_2O.

3.13.1.4 Preparation of gel for SDS - PAGE

The plates of gel electrophoresis system were cleaned with distilled water and dried in air. To prepare one gel of 1.0 mm thickness for SDS-PAGE, 6 ml of separating (10 %) gel mixture was transferred to the glass plate sandwich and over layered with distilled water and allowed to polymerize for 30 mins (Table-

14), followed by removed of water layer using filter paper and 1 ml of stacking (5 %) gel mixture was poured over it. The comb was inserted gently to avoid air bubbles and kept for 2 hrs for polymerization.

Table 14: The composition of stacking gel and resolving gel for SDS-PAGE

Stock solution	Stacking gel (2.5 %)	Resolving gel (12.5 %)
Acrylamide – bis acrylamide stock solution	2.50	12.50
Stacking gel buffer (Tris HCl, pH 6.8)	5.00	-
Resolving gel buffer (Tris HCl, pH 8.8)	-	3.75
10 per cent SDS	0.20	0.30
1.5 per cent APS	1.00	1.50
Water (ml)	11.30	11.95
TEMED(ml)	0.015	0.015
Total volume (ml)	20.00	30.00

The distance travelled by the dye and laccase enzyme was recorded and the Rm values were calculated by the formula.

$$\text{Rm value} = \frac{\text{Distance travelled by sample protein}}{\text{Distance travelled by tracking dye}}$$

3.13.1.5 Protein molecular weight marker

Molecular weight of the purified thermolaccase enzyme was determined by SDS-polyacrylamide gel electrophoresis using calibration curve prepared by plotting Rm value versus log molecular weight of marker proteins. High range protein molecular weight markers (Genei, Banglore) were used to determine the molecular mass of purified laccase of the selected bacterial isolates. The standard protein markers consisted of five precisely sized recombinant proteins of molecular weight 205,000; 97,400; 66,000; 43,000 and 29,000 daltons.

3.13.1.6 Sample loading and electrophoresis

The protein sample and 5 X sample loading buffer were mixed in the ratio of 4:1 (v/v) in a microfuge tube and heated to 100℃ for 2-5 mins for SDS-PAGE. The samples were loaded in wells. Electrophoresis was carried with constant current (100 mA) at 4^{o}C. Power was turned off when the dye reached to

the bottom of the gel. Gel was stained with Coomassie Blue R 250 to visualise the band of protein.

3.13.1.7 Staining and destaining of polyacrylamide gel

After electrophoresis, gel was removed from the electrophoresis apparatus and transferred to a small gel staining box containing Coomassie blue gel stain (approximately 50 ml), and incubated at room temperature for 2-3 hrs with agitation. It was then destained by changing the destaining solution frequently.

3.13.2 Effect of pH on thermolaccase activity

To find out the optimum pH for laccase enzyme activity, a pH range of 4.0-10 was investigated. ABTS was used as a substrate with the following four buffers: citrate buffer (pH: 4.0-6.0); Tris-buffer (pH: 6.0-8.0) and phosphate buffer (pH: 8.0-10.0) at 60°C. The activity was measured using standard ABTS assay as described in Section 3.9.

3.13.3 Effect of temperature on thermolaccase activity

The effect of temperature on enzyme activity was investigated within the range of 20-90°C at pH 9.0. The laccase activity was measured using standard ABTS assay as described in Section 3.9.

3.13.4 Effect of substrate concentration

Effect of varying substrate concentrations 0.2-2.0 mM of ABTS was investigated for laccase activity. K_m value was determined by plotting Lineweaver Burk (1934) plot.

3.13.5 Effect of inhibitors

Two potential laccase inhibitors sodium azide and dithiothreitol were evaluated to study their effect on the laccase activity from the selected bacterial isolate. The enzyme was incubated with various concentrations of inhibitors ranging from 0.1-0.9 mM for l0 mins at optimum temperature and laccase activity was measured using ABTS as a substrate.

3.14 APPLICATIONS OF PARTIALLY PURIFIED THERMOSTABLE LACCASE ENZYME

3.14.1 Textile dye degradation

To study the ability of the thermophilic bacteria and enzyme preparation on degradation of textile dyes, which are significant contributors to water pollution, six different dyes were investigated viz., Remazol brilliant blue R (RBBR), Indigo Carmine, Congo Red, Aniline Blue, Brilliant Blue and Bromophenol Blue.

3.14.1.1 Dye decolourization by bacteria

The laccase producing bacterial isolate was tested for its ability to decolorize the selected six synthetic dyes: RBBR, Indigo carmine, Congo red, Aniline blue, Brilliant blue and Bromophenol blue. In each case 0.5 g/l of the dye was incorporated in both TYM agar and TYM broth. The plates were inoculated with selected bacterial isolate and observed for decolorization up to 10 days. Whereas in case of TYM broth supplemented with 50 mg/l of each dye, 48 hrs grown liquid culture of selected bacterial isolate was used as an inoculum and 10 % of inoculum was used further. The inoculated flasks were put on a rotary shaker at a speed of 150 rpm under the optimum conditions of temperature of 60°C for 10 days. Decolourization of each dye was determined visually as well as by monitoring the decrease in absorbance at the peak of maximum visible absorbance and expressed as percentage of decolourization.

3.14.1.2 Dye decolourization by crude and partially purified laccase

Crude enzyme and purified enzyme extracts were also investigated for dye decolourization of selected six different dyes namely Remazol brilliant blue R (RBBR), Indigo Carmine, Congo Red, Aniline Blue, Brilliant Blue and Bromophenol Blue. All the dyes were solubilised in distilled water to the final concentration of 50 mg/l. The reaction mixture (6.0 ml) contained 2.0 ml acetate buffer (pH: 4.6), 2.0 ml of individual dye solution and 2.0 ml of crude enzyme extract/ laccase enzyme followed by incubation at 60^0C for 3-10 hrs. Control samples were run in parallel without addition of crude extract/laccase enzyme

and one sample containing commercial laccase from *Trametes* (SIGMA chemicals) was also run to compare the decolourization efficiency. The decolurization percentage was determined spectrophotometrically as the relative decrease of absorbance at each maximal absorbance wavelength of the dyes. All reactions were performed in triplicates. Dye decolourisation was expressed in terms of percentage calculated according to the equation.

$$\text{Decolourisation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where, A_0 is an absorbance at an appropriate wavelength of the dye immediately measured after adding the enzyme solution and A_t is an absorbance after each time intervals.

3.14.2 Immoblization of enzyme

Immobilization of enzymes offers several improvements for enzyme applications because the storage and operational stabilities are frequently enhanced. Moreover, the reusability of immobilized enzymes represents a great advantage as compared with free enzymes. Different methodologies of enzyme immobilization that have been reported for laccases are adsorption, entrapment, encapsulation, covalent binding and self-immobilization. In this study, immobilisation of laccase enzyme was done using calcium alginate for entrapment of enzyme and immobilised enzyme was investigated for decolourization of dyes.

3.14.2.1 Materials and enzymes

Alginic acid sodium salt from brown algae (Sodium alginate) was obtained from Himedia. All other chemicals were of analytical grade and used without further purification. Laccase enzyme preparation from selected bacterial isolate was prepared as described in Section 3.9.

3.14.2.2 Enzyme immobilization on alginate using entrapment method

Sodium alginate solution was prepared by dissolving 0.1 gm of sodium alginate in 10 ml of distilled water under continuous stirring at room temperature followed by addition of 10 ml of enzyme solution into the sodium alginate

solution under continuous stirring at room temperature to ensure complete mixing for 20 mins. This solution was added drop by drop to 50 ml of 100 mM $CaCl_2$ solution at the flow rate 3 ml min^{-1} leading to formation of calcium alginate beads followed by hardening, washing and filteration. Each of the filtered hardening solution and two washings were collected for immobilization percentage determination according to following equation:

$$\text{Immobilization (\%)} = \frac{A_{load} - A_{wash}}{A_{load}} \times 100$$

where, A_{load} is total loaded activity into the mixture of Na-alginate solution assayed using ABTS as substrate and A_{wash} is laccase activity detected in hardening solution and two washing solutions assayed using the same substrate. All the immobilized laccase alginate enzymes in the form of beads were kept in distilled water at 4°C.

3.14.2.3 Enzyme activity assay

The laccase activity of immobilized beads was assayed based on the oxidation of ABTS according to the method described in Section 3.12.1.5. Laccase activity of immobilized enzymes was assayed similarly as free enzyme in aqueous solution.

3.14.2.4 Synthetic dyes decolorization by immobilized laccase

Six synthetic dyes such as RBBR, Congo red, Aniline blue, Indigo carmine, Brilliant blue and Bromophenol blue were investigated for decolorization using immobilized Ca-alginate laccase. The reaction mixture (6.0 ml) contained 2.0 ml acetate buffer (pH: 4.6), 2.0 ml of individual dye solution and 8-10 beads of immobilized Ca-alginate laccase. Then the reaction mixture was incubated at 60^0C for 3-10 hrs. Control samples were run in parallel without addition of immobilised laccase enzyme and one sample containing beads of immobilised commercial laccase enzyme from *Trametes* sp. (SIGMA chemicals) was also run to compare the decolourization efficiency. The decolourization percentage was determined spectrophotometrically as the relative decrease of absorbance at each maximal absorbance wavelength of the dyes. All reactions

were performed in triplicates. Dye decolourisation was expressed in terms of percentage calculated according to the equation.

$$\text{Decolourisation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where, A_0 is an absorbance at appropriate wavelength of the dye immediately measured after adding the enzyme solution and A_t is an absorbance after each time intervals.

3.14.3 Phytotoxicity studies

Phytotoxicity assay was carried out in order to evaluate the toxicity of treated and untreated textile dyes by partially purified thermolaccase enzyme preparation in three replications. Three textile dyes viz., Congo red, Indigo carmine and RBBR were selected for this experiment on the basis of their efficiency in the previous experiment, producing maximum degradation by the partially purified thermolaccase enzyme. Further each dye was dissolved in sterile distilled water to a final concentration of 500 ppm, to investigate its phytotoxicity effect, at room temperature on three plant species namely *Phaseolus mungo, Calendula officinalis* and *Tagetes patula,* important in the Indian agriculture and floriculture. Twenty seeds of each of the three plant species were kept in a separate petridish containing a moist filter paper. These were regularly supplied with distilled water as a control, Congo red, Indigo carmine and RBBR dyes and their degradation products for a period of 7 days. Volume of distilled water, each of the three dyes and degraded dye metabolites were kept same, i.e. 10 ml per day for 20 seeds of each plant species. Toxicity effect was measured in terms of percent germination, lengths of plumule and radicle of the plants after 7 days and the germination percentage has been considered as an estimate of viability of seeds (Parashetti *et al.,* 2006) and was calculated using following formula:

$$\text{Seed germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seed}} \times 100$$

3.15 ISOLATION OF THERMOSTABLE LACCASE ENCODING GENE

Genomic DNA of selected thermophilic bacterial isolate which produced maximum laccase activity was analysed for laccase encoding gene using the degenerate primer pair which were designed based on the conserved regions I and IV of copper-binding sites in bacterial laccases.

3.15.1 Isolation of genomic DNA

Genomic DNA of selected thermophilic bacterial isolate was isolated using Genomic DNA extraction kit-Mini (Real Genomics) using the same protocol as described in section 3.10.1.

3.15.2 PCR amplification of laccase encoding gene

The isolated DNAs of selected isolate was used further for PCR amplification of gene encoding laccase using degenerate primers which were designed based on the conserved regions I and IV of copper-binding sites in bacterial laccases (Dhiman, 2014). The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume with composition as depicted in Table-15. Amplifications were performed using thermalcycler with a standardized temperature profile used for DNA amplification (Table-16).

CuIF : 5`-ACMWCKGTTCAYTGGCACGG-3`

CuIVR: 5`-TGNTCNAGNAWGTGRCARTG-3`

Table 15: Composition of PCR reaction mixture for laccase gene amplification

S. No.	Reaction mixture	Quantity (µl)
1	PCR buffer (10x) with $MgCl_2$ (1.5 mM)	2.5
2	dNTPs mixture (0.5 mM each)	2.0
3	Taq DNA polymerase (5U/µl)	0.3
4	Primer F (10nM)	0.6
5	Primer R (10nM)	0.6
6	Template DNA	4.0
7	Distilled water	10.0
Total volume		**20.0**

Table 16: PCR reactions followed in laccase gene amplification study

Stage of PCR		Time (mins)	Temp (^{o}C)	No. of cycle
Pre-PCR	Hot start	3.0 mins	95	1
PCR	Denaturation	0.5 mins	95	35
	Annealing	0.5 mins	50	
	Extension	2.0 mins	72	
Post-PCR	Final extension	10.0 mins	72	1
Hold at 4^{o}C				

The PCR products were analyzed on 1.2% agarose gel in 1X Tris-acetate EDTA buffer (2 M Tris base, 57.10 ml acetic acid and 0.5 M EDTA, pH 8.0, 50X) containing ethidium bromide (0.5 µg/ml). DNA ladder of 100 bp- 5 kb was used as a marker. The gel was run at 80 V for 2-3 hrs using Bangalore Genei power system. The gels were viewed and images were captured using gel documentation system (AlphaImager 2200, Alpha Infotech Corporation, USA). Amplified PCR product of selected bacterial isolate which produced maximum laccase activity out of the fifty bacterial isolates was eluted from the gel using gel extraction kit (Real genomics Hi Yield^{TM} Gel/PCR DNA Mini Kit). To counter possible stochastic effects of PCR five amplifications were carried out on each sample and pooled prior to purification and eluted fragment.

3.15.3 Sequencing

The PCR product obtained through amplification with degenerate primers targeting laccase gene using same upstream and downstream primers.

3.15.4 *In silico* analysis of sequence

Raw sequence data was obtained as a set of two sequences for each sample one with forward and other with reverse degenerate primers of sequencing reaction. Analysis of raw sequence data was done using Chromas Lite (version 2.1) of Technelysium Pvt. Ltd. Gene sequence of the selected bacterial isolate was analyzed using different approaches which have been discussed below briefly.

3.15.4.1 Translation of sequence

Laccase encoding gene sequence of selected bacterial isolate was translated into the corresponding protein sequence using protein translator tool-ExPASy (Expert Protein Analysis System) server, a bioinformatics resource portal operated by the Swiss Institute of Bioinformatics (SIB).

Reference URL

http://www.expasy.ch/tools/dna.html

3.15.4.2 BLASTp

Searches were run for protein sequences (BLASTp). Laccase encoding gene sequence of selected bacterial isolate was analyzed using BLASTp to know their homology among other sequences of laccases, already submitted to NCBI.

3.15.4.3 Multiple Sequence Alignment

Multiple sequence alignment of amino acid sequence was generated using MULTALIN program between the laccase gene translated amino acid sequences obtained from selected bacterial isolate with other laccase gene amino acid sequences of worldover, retrieved from NCBI database. From the multiple sequence alignment inference of sequence homology, phylogenetic analysis and motif detection was carried out.

3.15.4.4 Phylogenetic analysis

MEGA version 6 was used for computational phylogenetic studies as described in section 3.10.7.3.

3.15.4.5 Conserved Domain Search

Conserved domain (CD) can be defined as recurring units in molecular evolution which appears in protein sequences as conserved blocks of amino acid residues that have distinct functions. Conserved domains are displayed in MMDB (Molecular Modeling Database) structure summaries. Conserved domain search

(Marchler and Bryant, 2004) was performed in the test laccase encoding gene sequence obtained from selected bacterial isolate.

Reference URL

http:// www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

3.15.4.6 Structure prediction

Phyre 1 and 2, (Protein Homology/Analogy Y Recognition Engine) a web based services for protein structure prediction were used to predict secondary and tertiary structure of protein.

Chapter-4

EXPERIMENTAL RESULTS

The major objectives of the present study were to isolate laccase producing thermophilic bacteria from various hot water spring sites of Himachal Pradesh and production of the enzyme from these indigenous strains. To achieve the objectives a number of experiments were conducted and the results obtained during the course of this study have been presented in this chapter under the following sections:

4.1 Survey and selection of the sites

4.2 Sample collection from selected sites

4.3 Physico-chemical analysis of water samples

4.4 Standardization of different parameters for isolation of laccase producing thermophilic bacteria

4.5 Enumeration of laccase producing thermophilic bacteria

4.6 Isolation of laccase producing thermophilic bacteria

4.7 Secondary screening of thermophilic bacterial isolates for thermostable laccase production

4.8 Biochemical characterization of laccase producing thermophilic bacterial isolates

4.9 Qualitative and quantitative screening for laccase activity

4.10 Molecular characterization of selected laccase producing thermophilic bacterial isolates

4.11 Optimization of culture conditions for maximum laccase production

4.12 Enzyme production and partial purification of thermostable laccase enzyme

4.13 Characterization of partially purified thermostable laccase enzyme

4.14. Applications of partially purified thermostable laccase enzyme

4.14.1 Textile dye degradation

4.14.2 Immobilization of enzyme

4.14.3 Phytotoxicity studies

4.15 Isolation of thermostable laccase encoding gene

4.1 SURVEY AND SELECTION OF THE SITES

Four hot water springs of Manikaran, Vashisht, Khirganga and Tattapani located in two districts of Kullu and Mandi in the state of Himachal Pradesh, were surveyed and selected for sample collection (Fig. 10, Plate-1). Manikaran thermal spring has been found to be one of the hottest water spring of H.P. with a temperature of water around 106^{o}C. It is located at a distance of 46 km from Kullu in a village Manikaran at an altitude of 1735 m along the bank of the river Parvati. Vashisht hot spring is located in the village Vashisht located at a distance of 5 km from Manali and 44 km from Kullu, at an altitude of 2050 m from sea level. Khirganga is also located in Kullu district of H.P. and situated at a distance of 46 km from Kullu and 12 km from village Bershani at an altitude of 2960 m, whereas Tattapani is located in Mandi district at an altitude of 680 m and it is 51 km from Shimla and 29 km from Naldhera (Figure-10, Plate-1). From each selected site five subsites were selected for the collection of the samples and appropriate codes have been given (Table-5), in materials and methods.

4.2 SAMPLE COLLECTION FROM SELECTED SITES

Twenty samples of water were collected by using a 50 ml syringe with a 1.0 cm wide opening and ten samples each of soil, rock matting and pebbles from each site were collected (Plate-2). Different samples were collected in sterilized screw capped vials (Plate-2). The temperature at the collection site was measured by sticking the temperature probe into the openings of the hot water springs. The samples were given codes and these were listed in Table-3 and their altitudes (m).

4.3 PHYSICO-CHEMICAL ANALYSIS OF WATER SAMPLES

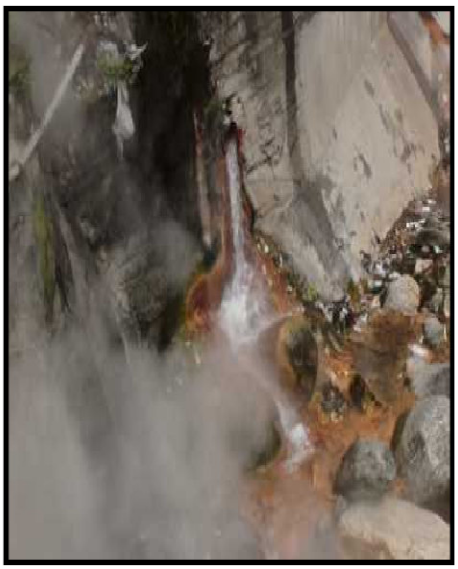
4.3.1 pH

Variability in pH was studied for 80 water samples and it was found to range from a minimum of 4.8 to a maximum of 9.5. (Table-17, Fig.11).

The maximum pH of 9.5 was exhibited by MEW2 sample of Manikarn site and the minimum pH of 4.8 was observed in TBW2 and TDW2 sample of site Tattapani.



Fig. 10: Map showing four selected sites of hot water springs in Himachal Pradesh



Manikaran



Vashisht



Khirganga



Tattapani

Plate 1: Selected hot water springs of Himachal Pradesh, India.



Plate 2: Water, Soil, and rock matting samples collected from the selected sites

Table 17: Variability in pH amongst eighty samples of four hot water springs in H.P.

Sites	pH																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	5.8	5.6	6.8	6.0	7.8	6.2	6.8	8.2	6.9	6.7	6.0	9.2	7.5	7.7	5.7	6.4	6.7	**9.5**	6.9	6.0
S_2	6.9	6.2	6.1	6.5	6.1	6.0	5.9	6.0	5.9	5.7	6.2	5.2	5.7	5.4	5.9	6.0	6.3	5.9	4.9	5.0
S_3	5.8	5.6	6.8	6.0	6.8	6.2	5.8	5.2	5.9	5.7	6.0	5.2	5.5	5.7	5.7	6.4	6.7	6.8	5.9	6.0
S_4	6.1	5.6	5.8	5.0	5.8	5.2	**4.8**	6.2	6.9	4.9	6.0	5.2	5.0	4.8	5.7	6.0	6.1	6.3	5.2	6.5

Table 18: Variability in temperature amongst eighty samples of four hot water springs in H.P.

Sites	Temperature (^oC)																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	95	96	94	95	99	93	94	97	98	99	**106**	97	96	99	96	98	99	95	97	100
S_2	46	45	42	49	52	57	59	44	45	47	44	42	48	55	53	57	54	50	46	44
S_3	51	50	42	45	**41**	44	48	44	50	45	47	44	42	48	51	47	44	42	49	46
S_4	50	55	56	54	58	57	59	52	50	47	51	58	53	57	59	49	51	55	59	44

S1- Manikaran **S2**- Vashisht **S3**- Khirganga **S4**- Tattapani **I to XX-** Water samples

4.3.2 Temperature

Temperature was also studied for all the 80 samples and it was found to range from a minimum of 41.0^{o}C to a maximum of 106.0^{o}C. Variability in temperature among 80 samples of four sites of HP has been depicted in Table-18 (Fig.12). It was found that MCW3 sample of Manikaran site of district Kullu showed a maximum temperature of 106.0^{o}C and sample KBW1 of Khirganga site of Kullu district showed the minimum temperature of 41^{o}C.

4.3.3 Chloride content

Variability in chloride content was studied in 80 samples only, rest of the samples were in the form of pebbles, soil & rock matting. It was observed that chloride content ranged from 495- 2673 mg/l.

It is clear from Table-19 (Fig.13) that maximum chloride content of 2673 mg/l was recorded in TAW3 sample of Tattapani site of Mandi district. The minimum chloride content of 495 was observed in MBW1, MEW2 and MCW3 sample of the Manikaran site.

4.3.4 Sulphate content

Sulphate content was also tested in 80 samples, whereas rest of the samples were in the form of pebbles, soil & rock matting. It was observed that the values ranged from a maximum 31.8 to a minimum of 10.0 mg/l of sulphate content.

Variability in sulphate amongst 80 samples of hot water springs from four sites in HP has been shown in Table-20 (Fig.14). It was found that maximum value of sulphate content (31.8 mg/l) was recorded in KCW1 and KDW1 sample of site Khirganga of Kullu district whereas the minimum value of sulphates (10.0 mg/l) was found in MDW4 sample of Manikaran site of Kullu district.

4.3.5 Total hardness

The 80 samples from four hot water springs were analyzed for total hardness whereas rests of the samples were in the form of pebbles, soil & rock matting. The total hardness values ranged from 105.0-315.0 mg/l.

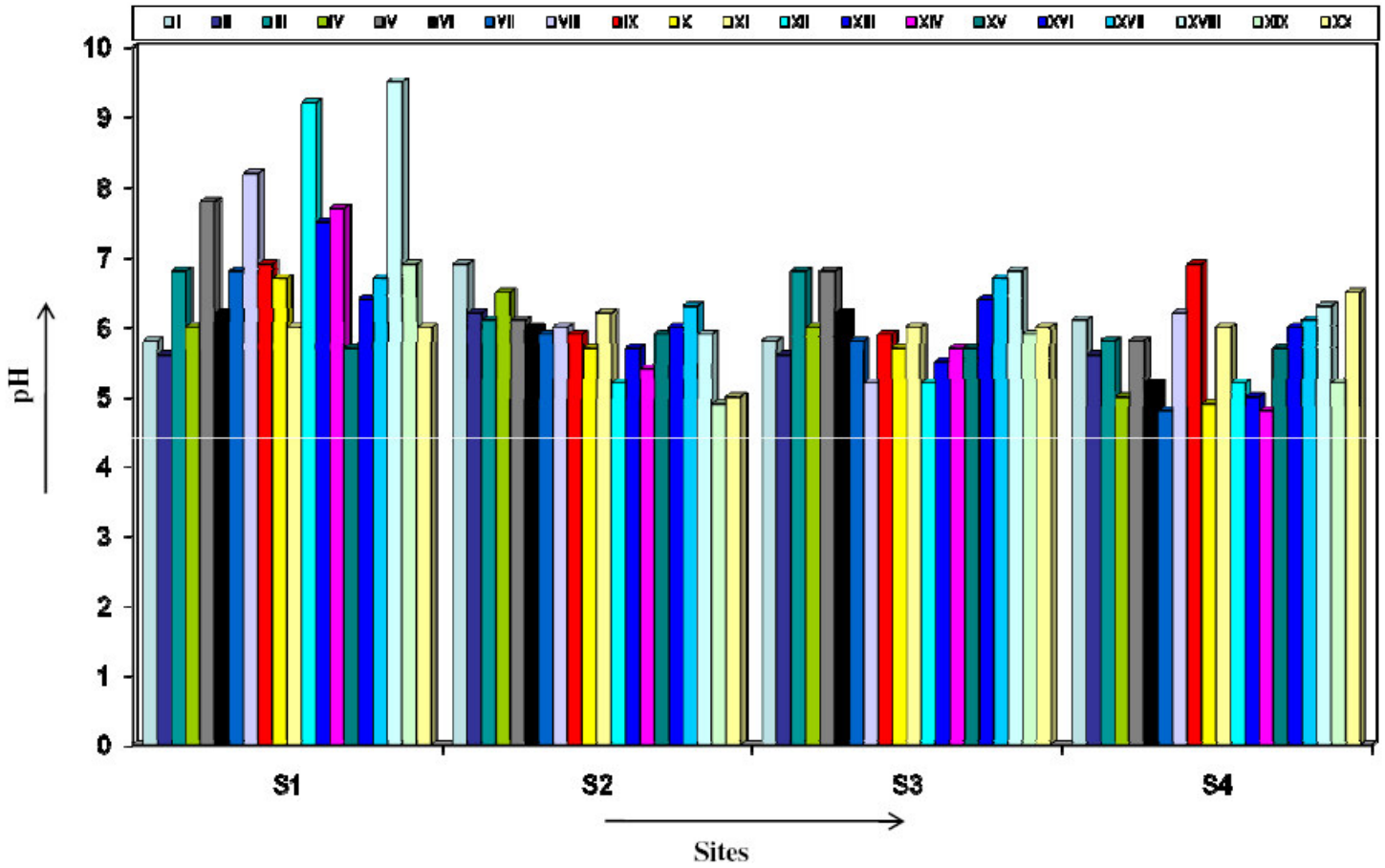


Fig. 11: Variability in pH amongst eighty samples of four hot water springs in H.P.

S1- Manikaran S2- Vashisht S3- Khirganga S4- Tattapani **I to XX-** Water samples

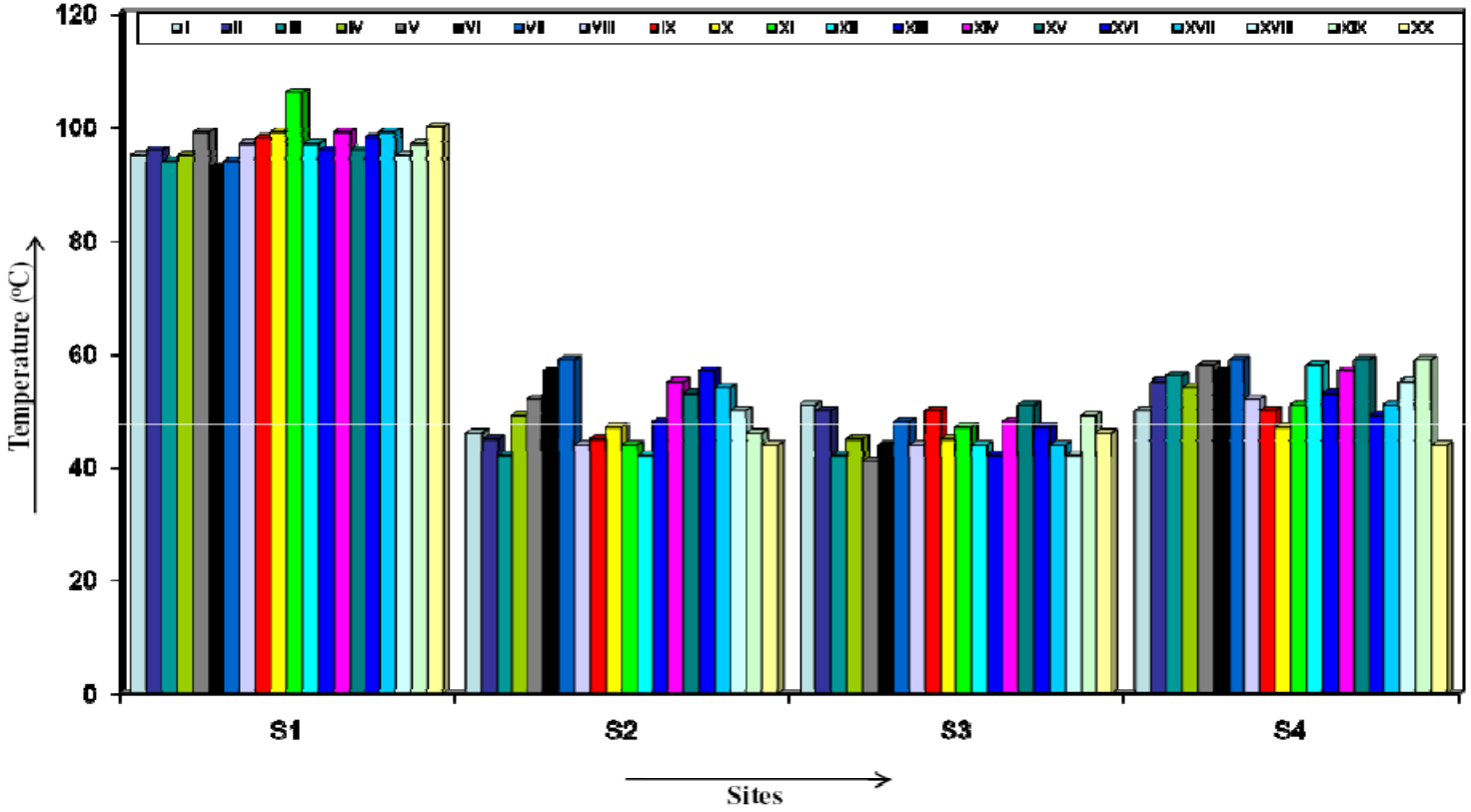


Fig. 12: Variability in temperature (°C) amongst eighty samples of four hot water springs in H.P.

S1- Manikaran **S2**- Vashisht **S3**- Khirganga **S4**- Tattapani **I to XX**- Water samples

Table 19: Variability in chloride content amongst eighty samples of four hot water springs in H.P.

Sites	Chloride (mg/l)																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	1180	792	792	999	**495**	891	792	792	000	891	**495**	0	792	891	1287	1683	594	**495**	792	999
S_2	1089	891	1188	0	0	1386	1089	1485	1287	1089	792	0	1188	693	594	0	1188	789	1089	999
S_3	891	792	643	999	0	891	909	1089	871	792	801	992	978	999	0	891	899	1189	699	659
S_4	2178	0	**2673**	1181	1485	2475	0	1734	1485	1081	1268	1675	0	1181	1485	1141	1398	1181	1485	2475

Table 20: Variability in sulphate content amongst eighty samples of four hot water springs in H.P.

Sites	Sulphate (mg/l)																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	0.0	14.0	19.0	20.0	18.0	20.6	16.0	0.0	20.0	20.0	16.0	20.0	14.0	18.0	20.4	**10.0**	14.0	20.0	18.0	16.0
S_2	20.8	0.0	20.8	21.8	0.0	21.0	20.6	21.0	21.6	0.0	20.8	14.0	40.0	21.0	20.0	20.6	0.0	40.0	20.8	20.6
S_3	11.0	29.0	20.0	20.8	15.0	20.8	21.8	14.0	**31.8**	20.8	22.0	20.0	**31.8**	0.0	20.0	23.6	21.0	20.8	0.0	20.8
S_4	19.0	20.0	18.0	20.6	16.0	0.0	20.0	20.0	16.0	20.0	19.0	20.0	20.8	0.0	20.8	21.8	0.0	21.0	20.6	21.0

S1- Manikaran **S2**- Vashisht **S3**- Khirganga **S4**- Tattapani **I to XX-** Water samples

Table-21 (Fig.15) depicts the variability in total hardness among 80 samples of four sites in Himachal Pradesh and maximum value of 315.0 mg/l was exhibited by TBW2 sample of Tattapani site of Mandi district and the minimum value of 105.0 mg/l was observed in VDW2 sample of Vashisht site of Kullu district.

4.3.6 Calcium hardness

Variability in calcium hardness in the form of was studied in 80 water samples whereas rest of the samples were in the form of pebbles, soil & rock mattings. The values ranged from 33.66-74.28 mg/l.

Variability in calcium hardness among 80 samples of four sites in H.P has been shown in Table-22 (Fig.16) and it was found to be maximum 74.28 mg/l for TAW2 and TCW4 sample of Tattapani site of Mandi district whereas the minimum value of 33.66 was found in KBW4 and KDW2 sample of Khirganga site of Kullu district.

4.3.7 Calcium carbonate hardness ($CaCO_3$)

The 80 water samples were also analyzed for calcium hardness ($CaCO_3$) whereas rests of the samples were in the form of pebbles, soil and rock matting. The values have been found to range from 37.7 -268.0 mg/l.

It is clear from Table-23 (Fig-17) that maximum value of 268.0 was observed in KDW3 sample of Khirganga site of Kullu district whereas the minimum (37.7) was recorded in VDW4 sample of Vashisht site of Kullu district.

4.3.8 Magnesium content

Magnesium content was also tested in 80 hot water samples because rests of the samples were in the form of pebbles, soil & rock matting. The values ranged from a maximum of to a minimum of 1.46 – 55.16 mg/l.

Variability in magnesium content among 80 samples from hot water spring of four sites in Himachal Pradesh was shown in Table-24 (Fig.18). The maximum value of 55.16 was observed in VCW1 sample of Vashisht site of Kullu district whereas the minimum 1.46 was recorded in TEW1 sample of Tattapani site of Mandi district.

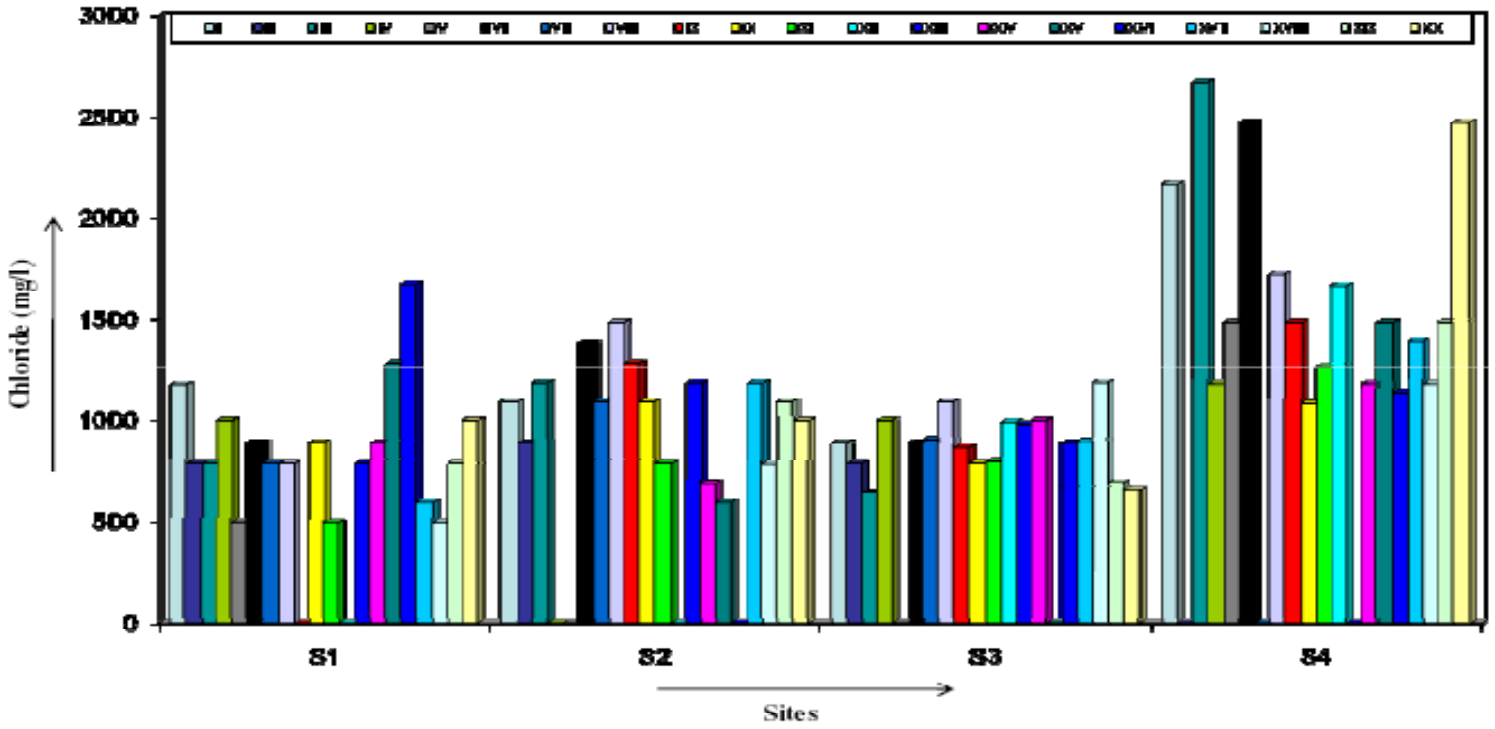


Fig. 13: Variability in chloride content amongst eighty samples of four hot water springs in H.P.

S1- Manikaran S2- Vashisht S3- Khirganga S4- Tattapani I to XX- Water samples

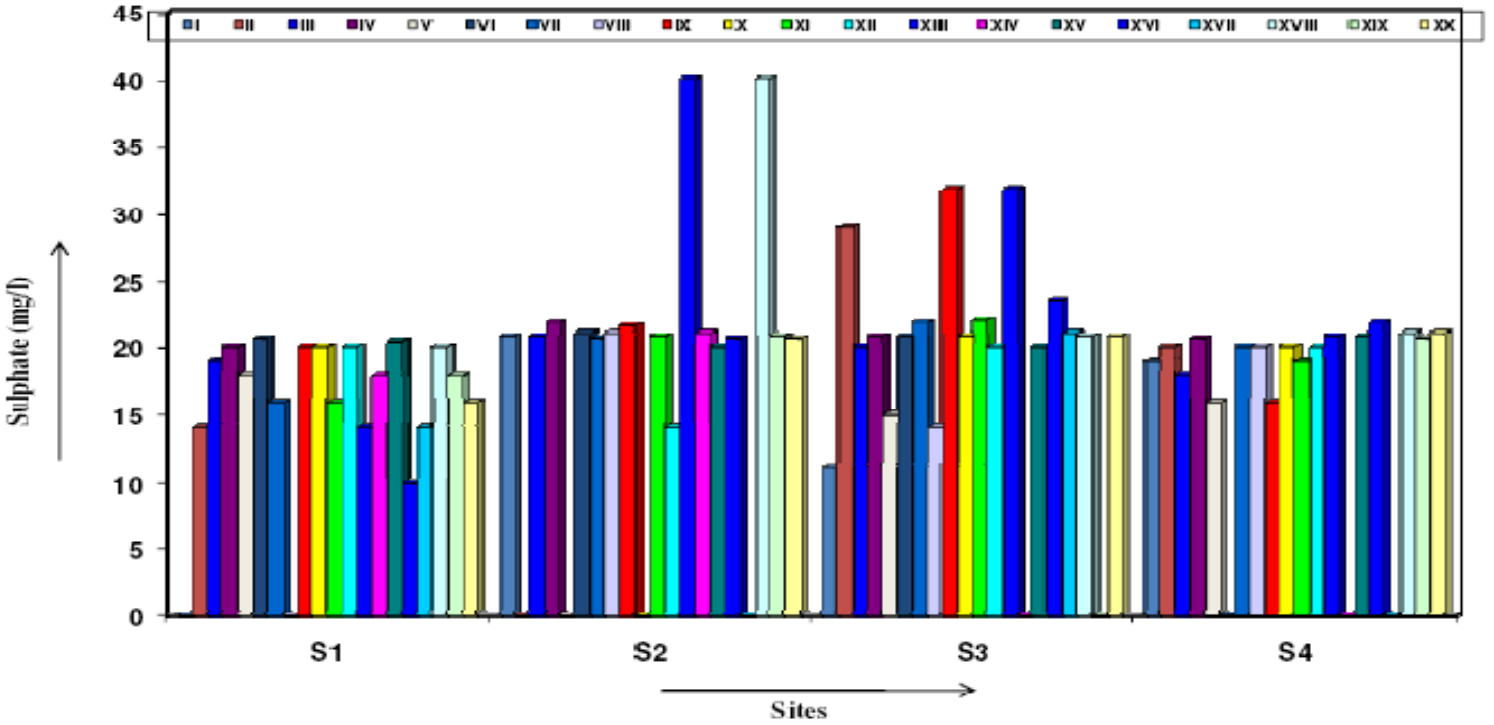


Fig. 14: Variability in sulphate content amongst eighty samples of four hot water springs in H.P.

S1- Manikaran S2- Vashisht S3- Khirganga S4- Tattapani I to XX- Water samples

Table 21: Variability in total hardness amongst eighty samples of four hot water springs in H.P.

Sites	Total hardness (mg/l)																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	231.0	231.0	252.0	210.0	126.0	189.0	231.0	310.0	189.0	189.0	231.0	210.0	193.0	147.0	189.0	189.0	252.0	210.0	252.0	249.0
S_2	168.0	138.0	200.0	216.0	189.0	168.0	270.0	168.0	168.0	210.0	210.0	147.0	189.0	189.0	210.0	**105.0**	252.0	210.0	147.0	189.0
S_3	250.0	252.0	213.0	158.0	138.0	200.0	216.0	189.0	238.0	168.0	238.0	210.0	116.0	189.0	268.0	170.0	138.0	168.0	138.0	230.0
S_4	252.0	210.0	126.0	189.0	231.0	**315.0**	189.0	189.0	231.0	210.0	252.0	210.0	168.0	138.0	200.0	216.0	189.0	168.0	270.0	168.0

Table 22: Variability in calcium hardness (Ca^{++}) amongst eighty samples of four hot water springs in H.P.

Sites	Calcium hardness (mg/l)																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	61.72	43.28	57.71	74.28	52.10	80.16	0.00	61.72	57.72	64.12	95.39	57.71	64.12	95.39	48.09	72.95	57.71	70.54	61.72	43.28
S_2	40.08	48.09	0.00	61.72	34.66	0.00	61.72	0.00	67.33	61.0	67.33	40.08	48.09	62.52	0.00	67.33	40.08	0.00	48.09	43.28
S_3	47.71	57.71	64.28	40.08	48.09	0.00	61.72	**33.66**	41.72	40.08	48.09	0.00	61.72	**33.66**	0.00	41.72	0.00	40.08	48.09	0.00
S_4	57.71	**74.28**	52.10	80.16	0.00	61.72	57.72	64.12	95.39	57.71	57.71	**74.28**	40.08	48.09	0.00	61.72	34.66	0.00	61.72	0.00

S1- Manikaran **S2**- Vashisht **S3**- Khirganga **S4**- Tattapani **I to XX-** Water samples

Table 23: Variability in calcium carbonate hardness ($CaCO_3$) amongst eighty samples of four hot water springs in H.P.

Sites	Calcium carbonate hardness (mg/l)																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	169.3	187.7	194.3	135.7	73.9	108.8	231.0	253.3	131.3	124.9	135.6	152.3	128.9	51.6	140.9	116.1	194.3	139.5	190.1	205.7
S_2	127.9	89.9	200.0	154.3	155.3	168.0	208.3	168.0	100.7	149.0	142.7	106.9	140.9	126.5	210.0	**37.7**	211.9	210.0	98.9	145.7
S_3	202.3	194.3	148.7	117.9	89.9	200.0	154.3	155.3	196.3	127.9	189.9	210.0	54.3	155.3	**268.0**	128.3	138.0	127.9	89.9	230.0
S_4	194.3	135.7	73.9	108.8	231.0	253.3	131.3	124.9	135.6	152.3	194.2	135.72	127.9	89.9	200.0	154.3	155.3	194.3	135.7	73.9

Table 24: Variability in magnesium hardness amongst eighty samples of four hot water springs in H.P.

Sites	Magnesium hardness (mg/l)																			
	A				B				C				D				E			
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX
S_1	18.23	0.00	12.64	26.24	2.92	17.98	7.05	11.66	13.85	9.72	4.86	0.00	2.00	0.00	11.66	10.45	14.58	6.56	10.45	17.25
S_2	17.25	8.50	0.00	8.26	10.94	11.91	12.39	0.00	**55.16**	0.00	29.16	22.36	12.15	24.30	0.00	0.00	10.21	10.49	0.00	26.73
S_3	26.73	11.42	16.76	10.45	2.67	8.02	25.51	10.45	0.00	3.65	0.00	0.00	0.00	0.00	10.45	14.82	0.00	16.03	14.82	21.87
S_4	21.87	3.15	26.00	0.00	32.07	0.00	0.00	10.45	28.00	29.60	0.00	25.30	10.45	10.45	10.45	3.88	**1.46**	11.42	3.88	11.66

S1- Manikaran **S2**- Vashisht **S3**- Khirganga **S4**- Tattapani **I to XX-** Water samples

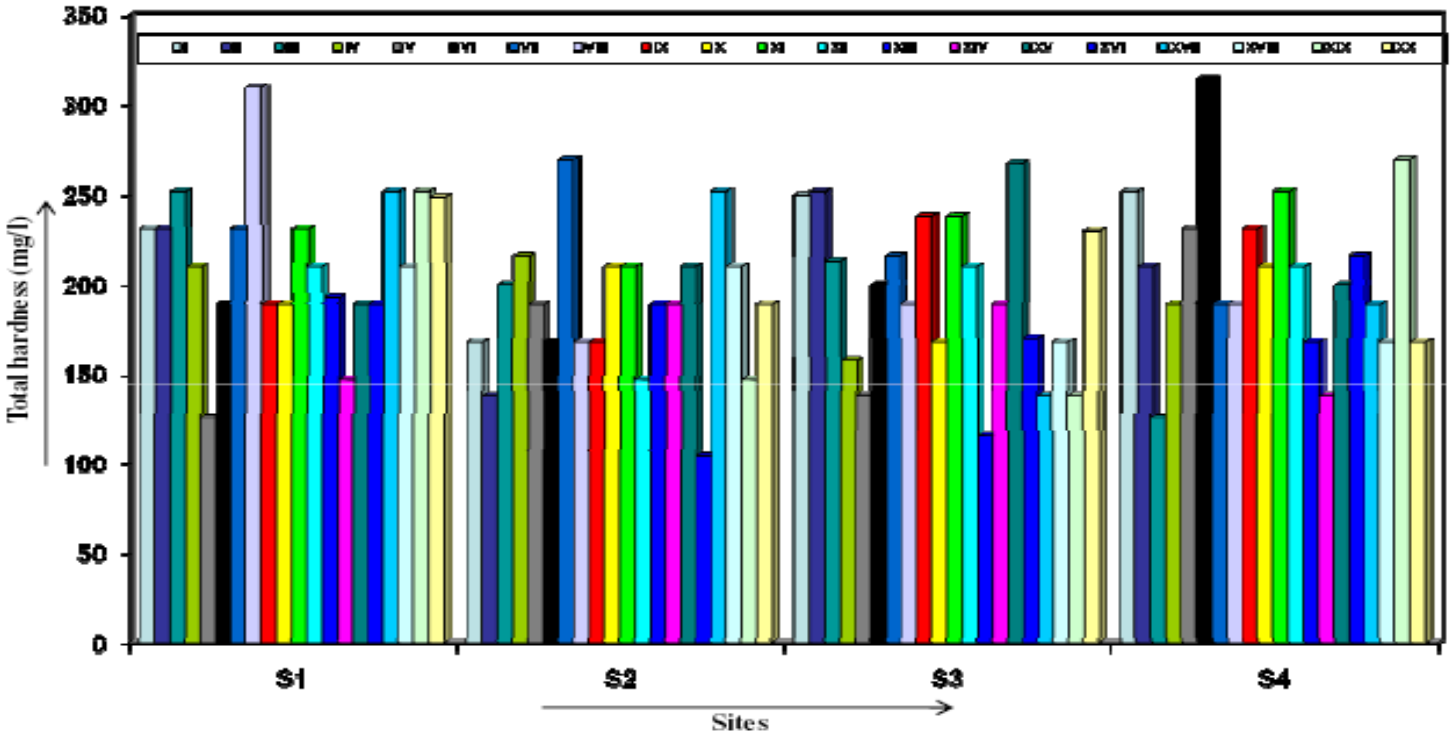


Fig.15: Variability in total hardness amongst eighty samples of four hot water springs in H.P.

S1- Manikaran S2- Vashisht S3- Khirganga S4- Tattapani I to XX- Water samples

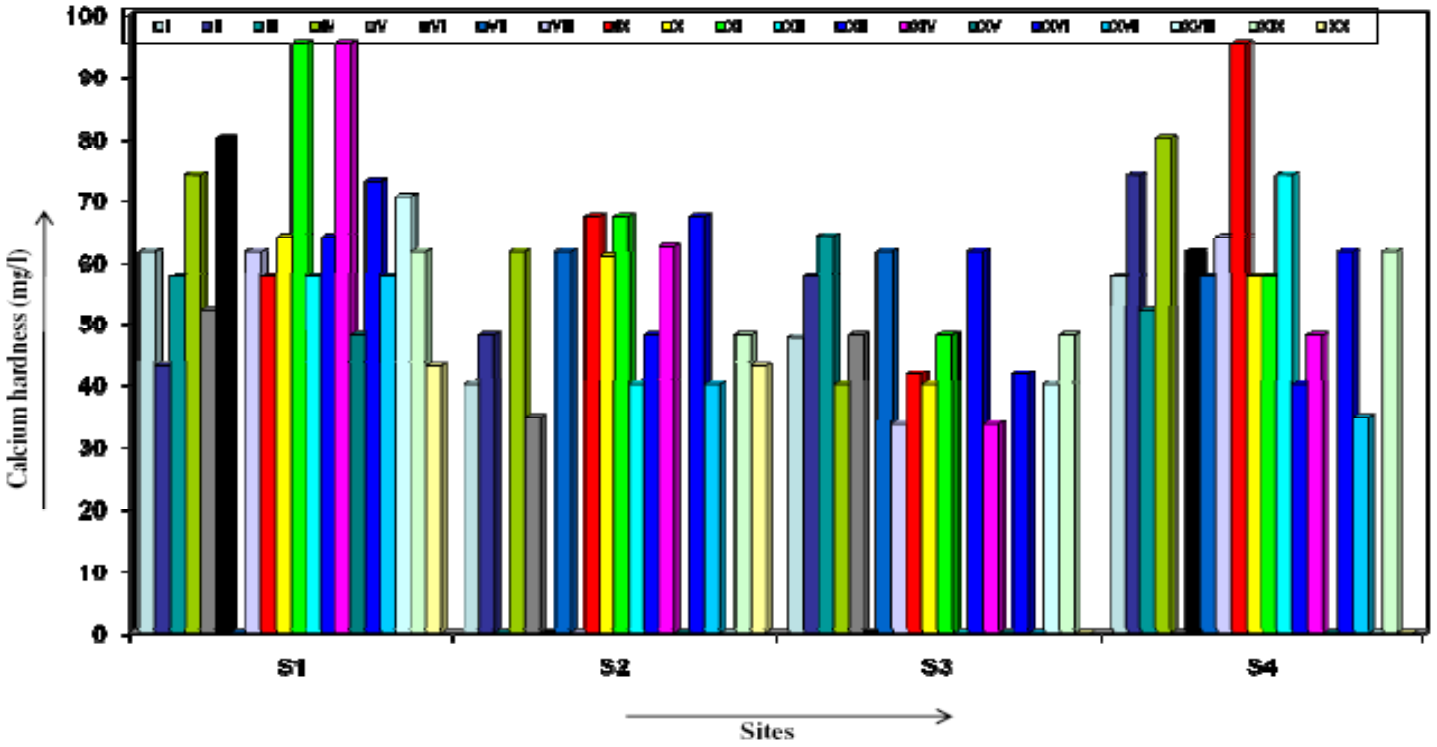


Fig. 16: Variability in calcium hardness (Ca++) amongst eighty samples of four hot water springs in H.P.

S1- Manikaran S2- Vashisht S3- Khirganga S4- Tattapani I to XX- Water samples

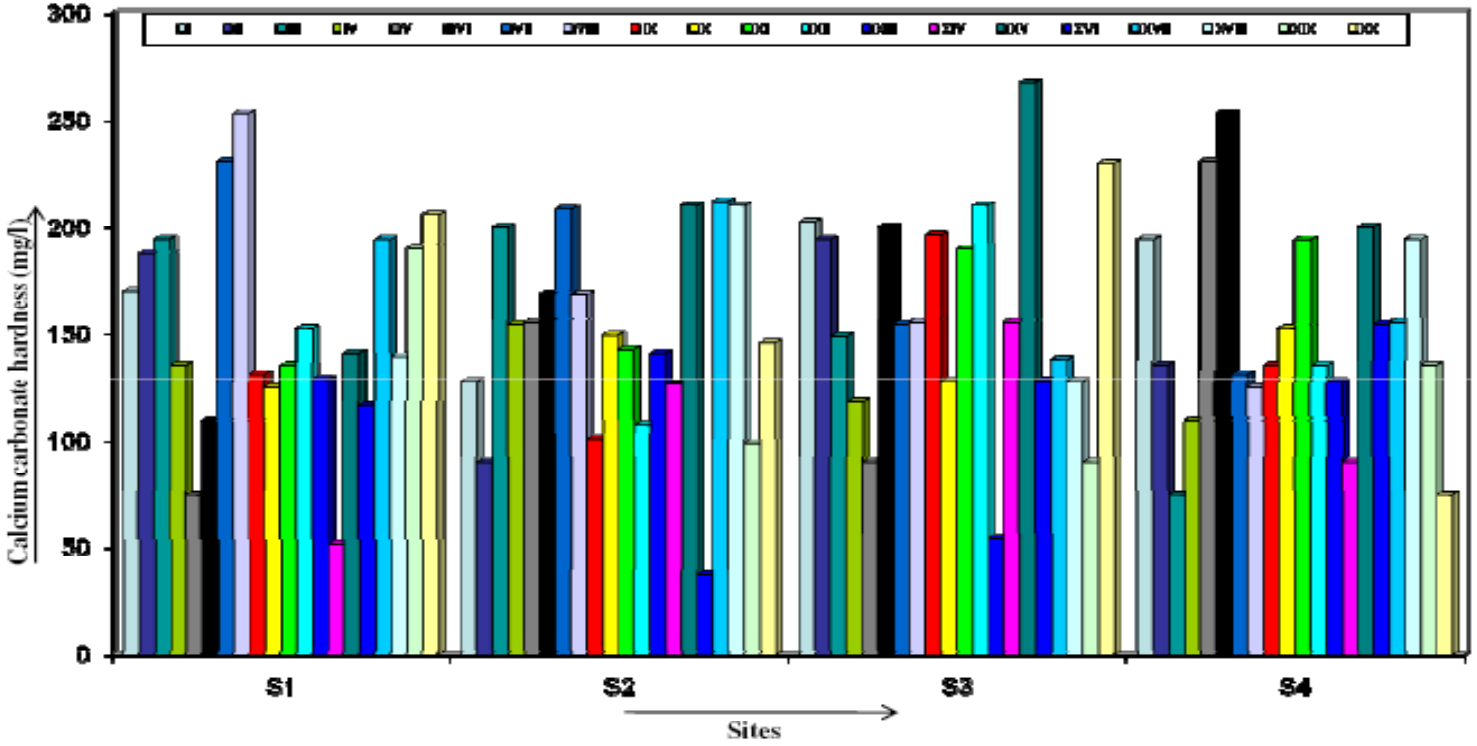


Fig. 17: Variability in calcium carbonate hardness ($CaCO_3$) amongst eighty samples of four hot water springs in H.P.

S1- Manikaran S2- Vashisht S3- Khirganga S4- Tattapani I to XX- Water samples

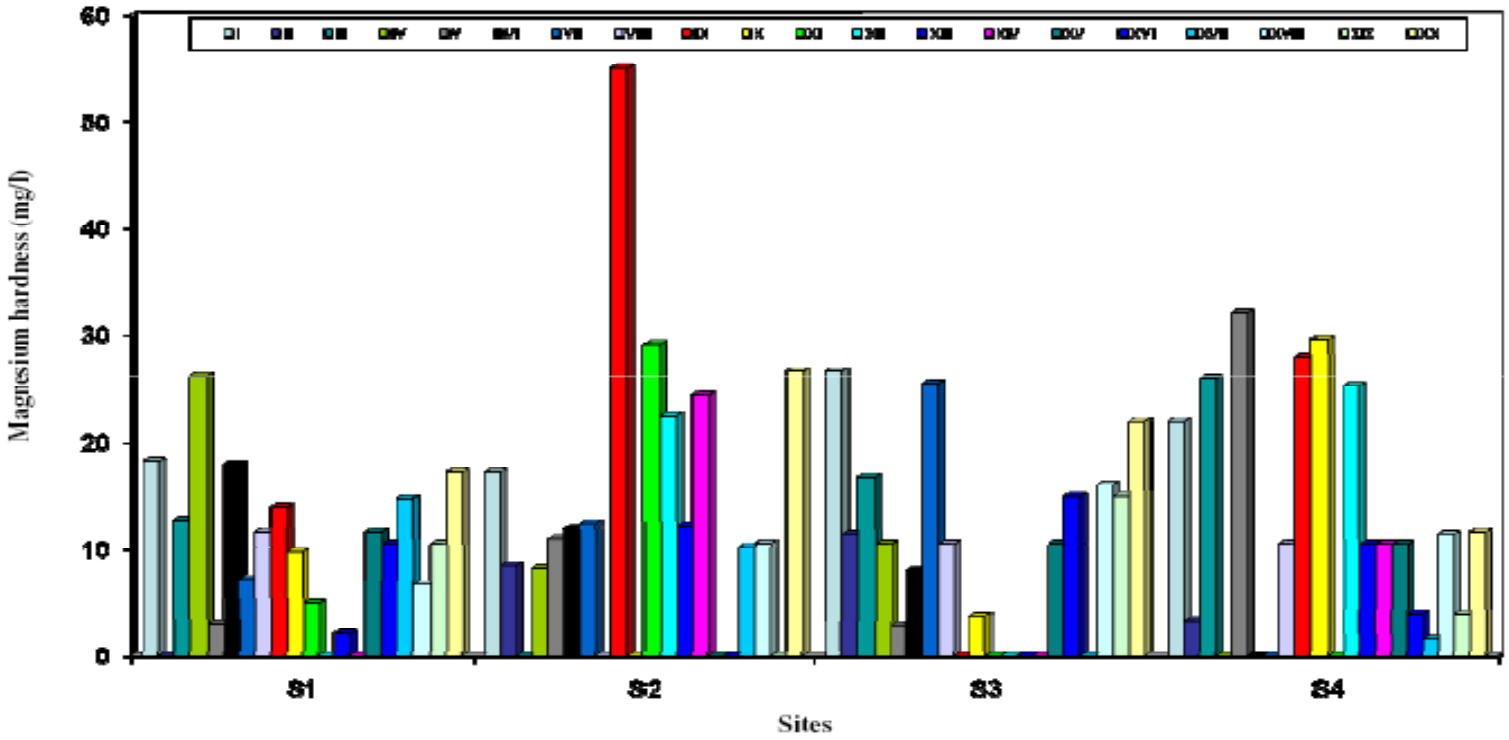


Fig. 18: Variability in magnesium hardness amongst eighty samples of four hot water springs in H.P.

S1- Manikaran S2- Vashisht S3- Khirganga S4- Tattapani I to XX- Water samples

4.3.9 Statistical data analysis of physico-chemical characters

4.3.9.1 Correlation studies

To study the correlation amongst eight variables obtained from physico-chemical characters were subjected to standard statistical analysis (Panse and Sukhatme, 1978) and a correlation matrix was obtained.

Table 25: Correlation Matrix

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8
X_1	1.0							
X_2	-0.322	1.0						
X_3	-0.154	0.983*	1.0					
X_4	-0.154	0.983*	1.00*	1.0				
X_5	-0.154	0.983*	1.00*	1.00*	1.0			
X_6	-0.228	0.994*	0.997*	0.997*	0.997*	1.0		
X_7	-0.175	0.987*	1.00*	1.00*	1.00*	0.999*	1.0	
X_8	-0.180	0.988*	1.00*	1.00*	1.00*	0.990*	1.00*	1.0

***Significant values at 5% level of significance**

X_1: pH
X_2: Temperature
X_3: Chloride
X_4: Sulphate
X_5: Total hardness
X_6: Ca^{++}
X_7: $CaCO_3$
X_8: Magnesium content

Correlation matrix showing pairwise correlation coefficients has been presented in Table-25. The significance was tested at 5% level of significance and it has been observed that all variables are significantly correlated.

4.3.9.2 Variability analysis

Coefficient of variability and other related parameters with respect to various physico-chemical characters under study for different sites were further analyzed by standard statistical methods followed by Bartlett's test for homogeneity of variance. The maximum value of 32.65% for coefficient of variation was observed for sulphate content and a minimum of 1.50% for pH at S3 site (Table-26).

Comparison of all the sites amongst themselves depicted that for pH maximum value of coefficient of variation of 9.00% was found for S1 site and minimum value of 1.50% was found for S2 site. Similarly, for temperature, coefficient of variation was maximum 13.42% for site S1 and minimum 2.71% for site S3. Sulphate, coefficient of variation was maximum 129.33% for site S6. In case of chloride maximum value of coefficient of variation 7.10% was for S2 site and minimum 5.78% for S1 site and for total hardness maximum value of coefficient of variation 22.45% was for S3 site and minimum 15.46% for S2 site. Also for calcium hardness when noticed as Ca++ maximum value of coefficient of variation 32.65% was for S3 site and minimum 23.65% for S4 site and as $CaCO_3$ maximum value of coefficient of variation 9.45% was for S4 site and minimum 5.46% for S2 site. It is interesting to add that for magnesium content maximum value of coefficient of variation 13.45% was for S4 site and minimum 10.47% for S1 site. To test whether differences in variation with respect to the sites varied significantly, Bartlett's test was applied. The calculated values of Chi-square under Bartlett's statistics was found to be significant for all the parameters, at 5% level of significance indicating that the sites differed significantly w.r.t these parameters/characteristics. The significant values of Chi-square for Bartlett's test under different parameters viz. pH, temperature, chloride content, sulphate content, total hardness, calcium hardness and magnesium content have largely occurred due to the differences in population density and a small contribution may be because of climatic variations under different places.

4.3.9.3 Analysis of variance technique (ANOVA)

Average performance of various characters was studied using analysis of variance technique (ANOVA) and it has been observed that in case of pH, maximum mean pH of 6.92 was observed in site S1, followed by site S3, S2 and S4 (Table-27). In case of temperature, the maximum mean temperature of 97.15 was observed for S1 whereas minimum of 46.00 for S3 and all the parameters are statistically different from each other. For chloride content, maximum mean of 1379.05 was observed for S3 where as minima of 782.60 was observed in S1. In case of sulphate content, maximum mean value of 19.25 was observed in S3

Table 26: Site-wise coefficient of variability and other related parameters

Sites /Variables	pH		Temperature (^oC)		Chloride content		Sulphate content		Total hardness		Ca^{++} content		$CaCO_3$ content		Magnesium content	
	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV	σ	CV
S1	0.73	9.00	12.66	13.42	105.29	5.78	11.45	30.45	11.89	20.44	11.45	30.45	11.45	8.45	0.45	10.47
S2	0.12	1.50	3.10	6.28	43.29	7.10	3.56	25.46	13.43	15.46	3.56	25.76	9.56	5.46	0.53	15.00
S3	0.16	2.60	1.54	2.71	62.23	5.90	3.45	32.65	16.45	22.45	13.45	32.65	6.45	7.65	0.44	12.6o
S4	0.31	5.50	2.53	4.04	69.35	6.76	9.67	23.45	19.67	23.45	5.78	23.65	9.67	9.45	0.67	13.45

σ : Standard deviation **CV:** Coefficient of variation

Table 27: Mean Value Table

Sites /Variables	pH	Temperature (^oC)	Chloride content	Sulphate content	Total hardness	Ca^{++} content	$CaCO_3$ content	Magnesium content
S1	6.92	97.15	782.60	15.70	214.00	60.98	153.26	9.90
S2	5.89	48.95	841.80	18.27	187.15	39.57	147.63	12.99
S3	5.98	46.00	799.20	19.25	194.35	35.42	158.92	9.68
S4	5.65	53.70	1379.05	16.73	206.05	48.95	150.11	12.00
lsd	0.34	0.94	0.35	0.50	0.68	1.55	0.41	0.59

S1- Manikaran **S2**- Vashisht **S3**- Khirganga **S4**- Tattapani

followed by S2 (18.27) and it has been interesting to note that S1 and S4 are statistically at par with each other. But for total hardness maximum value of 214.00 was observed in S1 followed by S4 (206.05) and it is interesting to add that S2 and S3 are statistically at par with each other. For Ca++ maximum mean value of 60.98 was observed in S1 whereas minimum of 35.42 was observed in S3. Also, for $CaCO_3$ maximum mean performance of 158.92 was seen in S3 followed by S3 (158.93), S1 (153.26), S4 (150.11) and S2 (147.63). For magnesium content, S2 and S4 was giving maximum mean performance of 12.99 and 12.00 respectively, followed by S1 and S3 statistically at par with each other mean value of 9.90 and 9.68 respectively (Table-27).

4.4 STANDARDIZATION OF DIFFERENT PARAMETERS FOR ISOLATION OF LACCASE PRODUCING THERMOPHILIC BACTERIA

4.4.1 Medium for isolation

For selection of the best medium, five different media were investigated, for isolation of thermostable laccase producing bacteria (Table-6) and TYM was found to be the best isolation medium with growth OD of 0.939 at 540 nm wavelength along with maximum number of colonies, as compared to other four media. Growth OD of 0.218, 0.204, 0.158 and 0.157 was observed for NA, M162, LB and M9 media respectively (Table-28). Both M9 and Luria-Bertani medium were found unable to support laccase producing bacterial colonies, whereas nutrient agar was able to produce only a single colony and M162 supported only three colonies (Plate-3). Thus TYM was selected for isolation of laccase producing bacteria from samples collected from hot water springs and further experiments (Fig.19).

Table 28: Effect of medium for isolation of laccase producing thermophilic bacteria

Sr. No.	Medium	OD (540 nm)	Colonies
1	TYM	0.939	12
2	M9 medium	0.157	0
3	LB medium	0.158	0
4	NA medium	0.218	1
5	M162	0.204	3

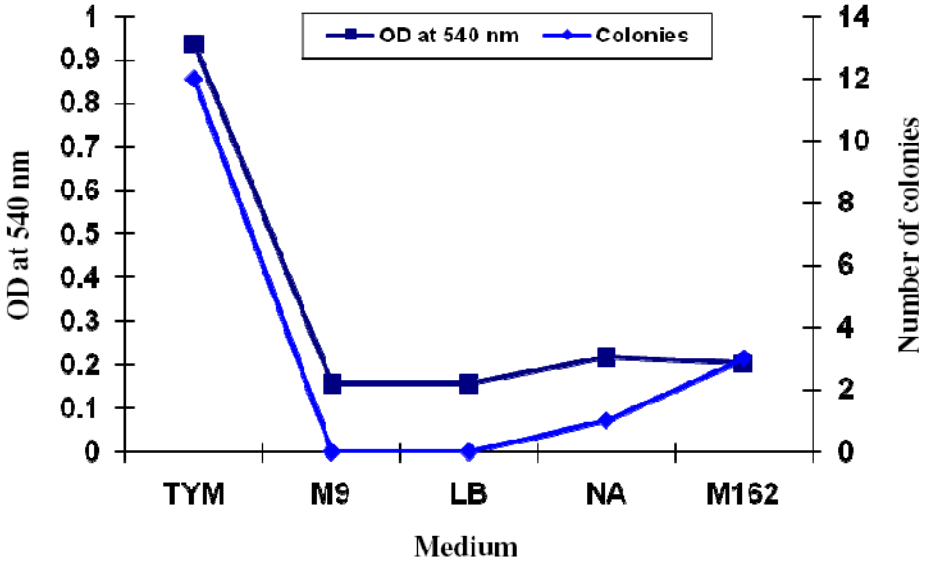


Fig. 19: Effect of medium for isolation of laccase producing thermophilic bacteria

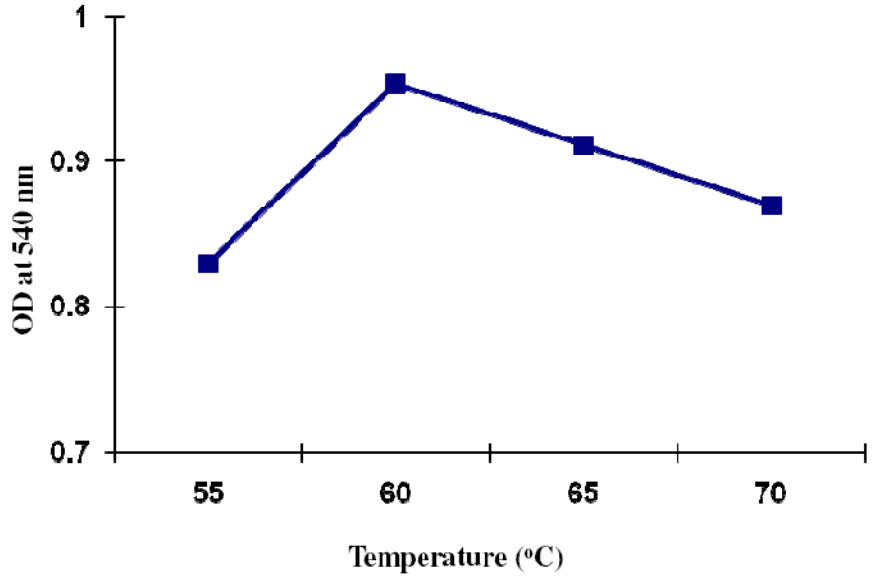


Fig. 20: Effect of temperature for isolation of laccase producing thermophilic bacteria

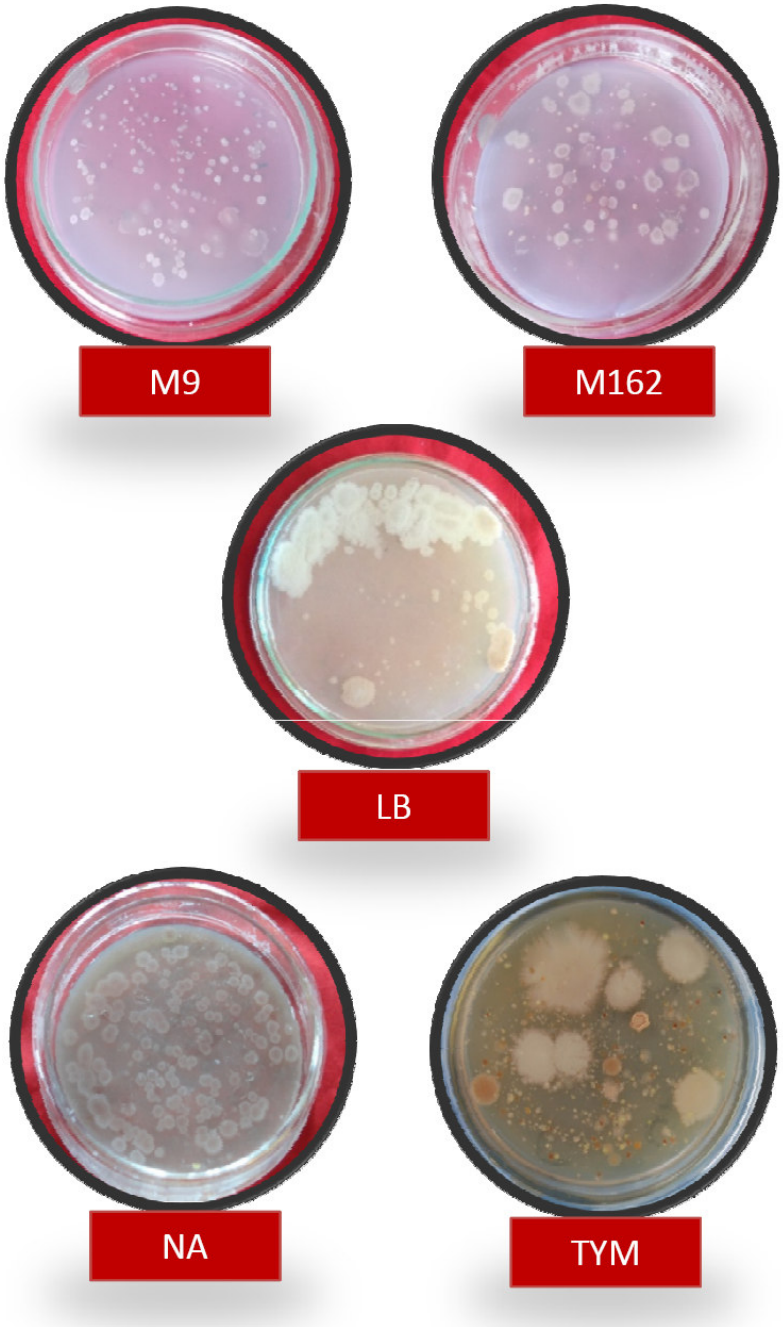


Plate 3: **Effect of medium for isolation of laccase producing thermophilic bacteria**

4.4.2 Incubation temperature

To select optimum temperature, a temperature range of 55-70^{o}C was investigated using the TYM broth and bacterial growth was measured spectrophotometrically at 540 nm. The maximum OD of 0.954 was observed at 60^{o}C, followed by 0.912, 0.870 and 0.830 at 65^{o}C, 70^{o}C and 55^{o}C, respectively (Table-29). Thus optimum incubation temperature selected was 60^{0}C for isolation of thermostable laccase producing bacteria from samples collected from hot water springs and further experiments (Fig.20).

Table 29: Effect of temperature for isolation of laccase producing thermophilic bacteria

Sr. No.	Temperature (^{o}C)	OD (540 nm)
1	55^{o}C	0.830
2	60 ^{o}C	0.954
3	65 ^{o}C	0.912
4	70 ^{o}C	0.870

4.4.3 Incubation time

To select optimum incubation time, a time range of 12-120 hrs was investigated using the TYM broth at an optimum temperature of 60^{o}C and bacterial growth OD was measured at 540 nm wavelength. The maximum growth OD of 0.940 was observed after 24 hrs, followed by 0.838, 0.824, 0.720 and 0.527 after 72, 48, 96 and 120 hrs, respectively (Table-30). Thus optimum incubation time selected was 24 hrs for isolation of thermostable laccase producing bacteria from samples collected from hot water springs and further experiments (Fig.21).

Table 30: Effect of incubation time period for isolation of laccase producing thermophilic bacteria

Sr. No	Time (Hrs)	OD (540 nm)
1	12	0.452
2	24	0.940
3	48	0.824
4	72	0.838
5	96	0.720
6	120	0.527

4.4.4 Substrate concentration

Guaiacol has been used as a substrate for isolation of laccase producing bacteria and to select the best substrate concentration, guaiacol concentration range of 1–9 mM was investigated using the TYM broth at 60^{o}C for 24 hrs and bacterial growth OD was observed at 540 nm wavelength. The maximum growth OD of 1.290 was observed with 5 mM guaiacol concentration, followed by 0.870, 0.810, 0.720 and 0.390 with 7 mM, 9 mM, 3 mM and 1 mM respectively (Table-31). Thus the best guaiacol concentration was selected 5 mM in TYM, for isolation of laccase producing thermophilic bacteria from samples collected from hot water springs and further experiments (Fig.22).

Table 31: Effect of substrate concentration for isolation of laccase producing thermophilic bacteria

Sr. No	Substrate concentration (mM)	OD (540 nm)
1	1	0.390
2	3	0.720
3	5	1.290
4	7	0.870
5	9	0.810

4.4.5 pH

To select optimum pH, a pH range of 5.0-10.0 was investigated using the TYM broth with 5 mM guaiacol concentration at 60^{o}C for 24 hrs and bacterial growth OD was measured at 540 nm wavelength. The maximum growth OD of 1.230 was observed at pH 8.0, followed by 0.920, 0.882, 0.860, 0.856 and 0.480 OD at pH 6.0, 7.0, 9.0, 10.0 and 5.0, respectively (Table-32). Thus optimum pH was selected 8.0 for isolation of thermostable laccase producing bacteria from samples collected from hot water springs and further experiments (Fig.23).

Table 32: Effect of pH for isolation of laccase producing thermophilic bacteria

Sr. No	pH	OD (540 nm)
1	5.0	0.480
2	6.0	0.920
3	7.0	0. 882
4	8.0	1.230
5	9.0	0.860
6	10.0	0.856

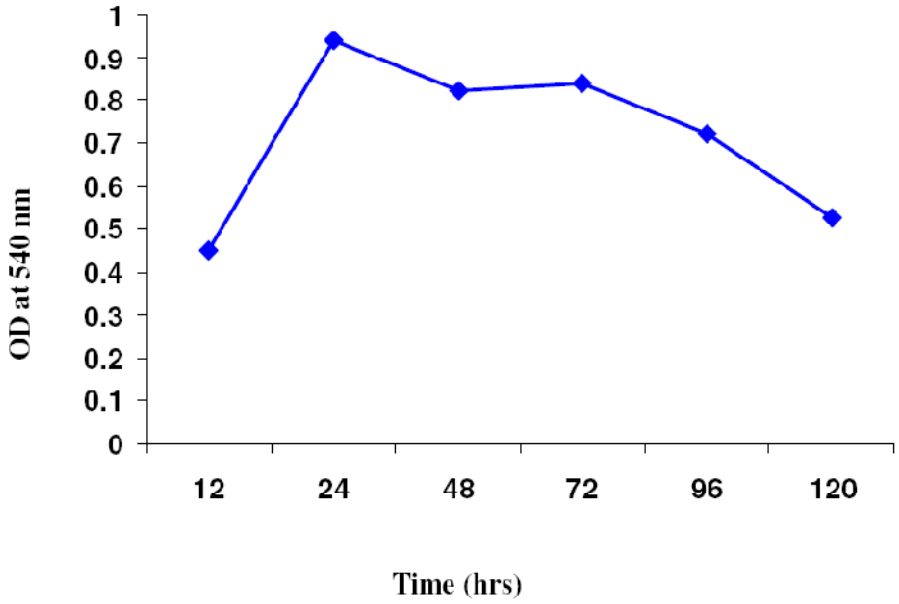


Fig. 21: Effect of incubation time period for isolation of laccase producing thermophilic bacteria

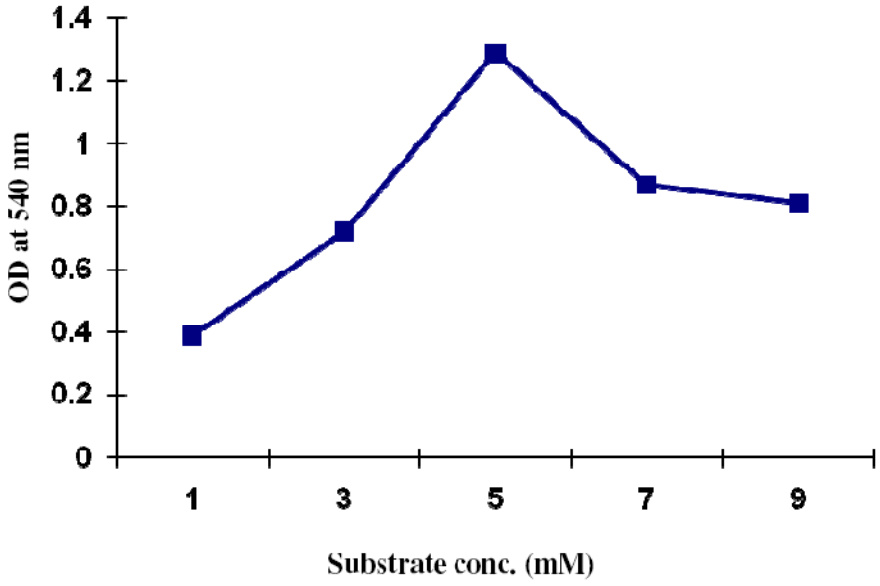


Fig. 22: Effect of substrate concentration for isolation of laccase producing thermophilic bacteria

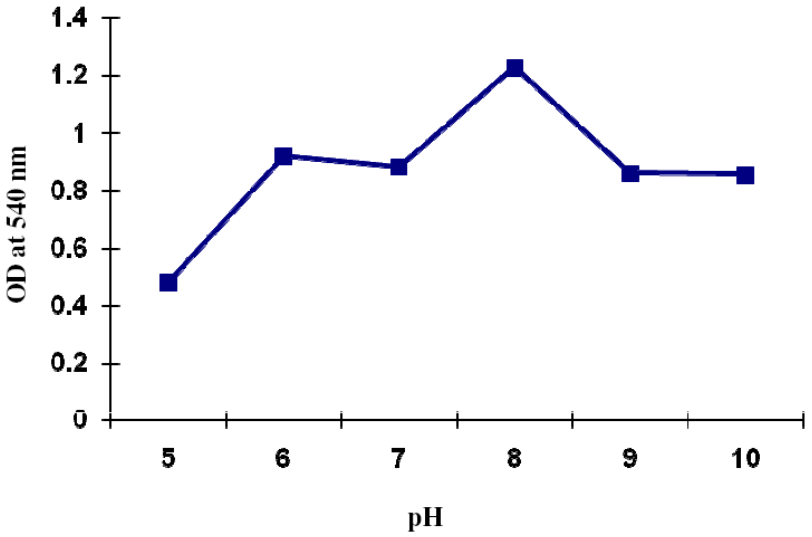


Fig. 23: Effect of pH for isolation of laccase producing thermophilic bacteria

4.5 ENUMERATION OF LACCASE PRODUCING THERMOPHILIC BACTERIA

Thermostable laccase producing was analyzed using water samples collected from hot water springs using serial dilution technique. TYM with optimum substrate concentration, pH, optimum temperature and optimum incubation time period selected in previous experiment were applied. Bacterial population in water was maximum (2.98 cfu/ml) at B sub site of Tattapani and minimum (0.72 cfu/ml) at E sub site of Manikaran as presented in Table-33.

Table 33: Enumeration of laccase producing thermophilic bacteria from water samples

Sites	Water sample (10^2 cfu/ml)				
	A	**B**	**C**	**D**	**E**
Manikaran	1.87	1.56	1.32	2.15	**0.72**
Vashisth	1.56	2.05	2.32	1.56	1.73
Khirganga	1.22	1.92	1.49	1.27	2.76
Tattapani	2.23	**2.98**	1.34	1.62	1.20

A, B, C, D and **E** are the subsites of four hot water springs

4.6 ISOLATION OF LACCASE PRODUCING THERMOPHILIC BACTERIA

A total of 118 laccase producing thermophilic bacterial isolates were obtained from four hot water spring sites of Himachal Pradesh using TYM (pH 8.0) supplemented with 5 mM guaiacol, at an incubation temperature of 60^{o}C and incubation time period of 24 hrs. Maximum numbers of laccase producing thermophilic bacterial isolates obtained from Tattapani site were 39, followed by 34, 25 and 20 bacterial isolates from Khirganga, Vashisht and Manikaran sites, respectively (Table-34).

Table 34: Number of putative laccase producing thermophilic bacterial isolates from different sites of Himachal Pradesh

Sites / Samples	Manikaran	Vashisht	Tattapani	Khirganga
Water	9	12	8	14
Soil	11	10	16	9
Rock matting	0	3	15	11
Pebble	0	0	0	0
Total	**20**	**25**	**39**	**34**

4.6.1 Isolation and morphological characterization of laccase producing thermophilic bacterial isolates

4.6.1.1 Manikaran site

Twenty laccase producing thermophilic bacterial isolates were obtained from fifty samples in form of water, soil, rock matting and pebbles collected from five locations of Manikaran hot water springs (Table-35). It has been further observed that from 10 soil samples 11 laccase producing thermophilic bacterial isolates were obtained and from 20 water samples 9 laccase producing

Table 35: Colony characterization of laccase producing thermophilic bacterial isolates from Manikaran

Sr. No	Isolate	COLONY CHARACTERISTICS						
		Colour With guaiacol	Colour Without guaiacol	Size (mm)	Shape	Texture	Elevation	Pigment formation
1.	MS6	Y	Y	4.0	I	R	R	-
2.	MS1	Y	C	3.0	C	S	F	+
3.	MS2.1	C	C	2.7	C	R	F	-
4.	MS2.2	Y	Y	3.0	I	S	F	-
5.	MW2	Y	Y	2.1	C	R	F	-
6.	MW2.1	Y	W	2.0	C	R	F	+
7.	MS1.1	V	V	5.4	I	R	F	-
8.	MW17.1	W	W	3.7	C	R	F	-
9.	MW9	Y	Y	2.0	C	S	F	-
10.	MS2	Y	Y	4.0	C	R	F	-
11.	MS3	C	C	2.0	C	R	R	-
12.	MS9	R	R	2.5	C	S	F	-
13.	MW6	B	B	2.5	C	R	F	-
14.	MS5	B	C	2.7	C	R	F	+
15.	MS6	R	C	1.7	C	R	F	+
16.	MS8	Y	Y	3.1	C	R	R	-
17.	MW15	Y	Y	2.0	I	R	F	-
18.	MW11	Y	C	4.0	C	R	R	+
19.	MW20	B	C	2.0	I	R	F	+
20.	MW17	B	C	2.0	C	R	F	+

Isolates
M-Manikaran
S-Soil
W-Water
R-Rock matting

Colour
Y-Yellow
C-Cream
W-White
R-Red
B-Brown
O-Orange

Shape
C-Circular
R- Rough

Elevation
F-Flat
R-Raised

Texture
S- Smooth
I-Irregular



Plate 4: Different colony morphotypes obtained from Manikaran hot water springs

thermophilic bacterial isolates were obtained, whereas from rock matting and pebble samples no laccase producing thermophilic bacterial isolates were obtained. All the laccase producing thermophilic bacterial isolates obtained were found to be different in their colony morphotypes on basis of colour, size, shape, texture and elevation (Plate-4). The laccase producing thermophilic bacterial isolates when grown in TYM without substrate i.e. guaiacol showed colony colour variation ranging from white, cream, light brown, yellow to brown, whereas colony morphotypes obtained using TYM containing 5 mM guaiacol resulted in colour variation ranging from reddish brown, yellow orange, red, pink and brown in addition to all the above colour. As described in the previous section oxidation of guaiacol leads to reddish brown/brown colour of the colonies, thus reddish brown/brown coloured colonies were supposed to be putative laccase producing thermophilic bacterial isolates.

Variation in size of colonies of laccase producing thermophilic bacterial isolates ranged from 1.7- 5.4 mm depicting small to medium type of colonies. On the basis of texture of the colonies only 20% were found to be smooth, whereas 80% colonies were rough. On the basis of elevation 15% colonies were found to be flat, whereas 85% were raised. On the basis of shape 75% were found to be circular and only 25% colonies were found to be irregular in shape (Table-35).

Laccase producing thermophilic bacterial isolates obtained from five locations of Manikaran site were further investigated for various microscopic characters. Persual of Table-36 has shown that 35% laccase producing thermophilic bacterial isolates were found to be pink in colour and thus gram negative in nature, whereas 65% were found to be purple in colour and thus gram positive in nature (Plate-5). Variation in shape of the cells showed cocci and rods. Only 45% of laccase producing thermophilic bacterial isolates possessed coccus shape whereas rest of the 55% laccase producing thermophilic bacterial isolates were found to be rod shaped. Arrangement of cells produced variation from singles to chains and majority 75% were found to be in singles. Out of total 20 laccase producing thermophilic bacterial isolates only 20% isolates were found to be spore forming in nature (Table-36). All 20 laccase producing thermophilic bacterial isolates were tested for their ability to form pellicle in the suspension

culture and it was found that all bacterial isolates were able to form pellicle and were found to be positive for the morphological descriptor of pellicle formation (Table-36).

Table 36: Microscopic characterization of laccase producing thermophilic bacterial isolates from Manikaran site

Sr. No	Isolate		MICROSCOPIC CHARACTERISTICS				
		Gram's reaction	Shape	Arrangement of cells	Spore formation	Pellicle formation	Motility
1	MS6	+	Coccus	Single	+	+	+
2	MS1	+	Coccus	Single	-	+	+
3	MS2.1	-	Rods	Chains	-	+	+
4	MS2.2	-	Rods	Single	-	+	+
5	MW2	+	Rods	Single	-	+	+
6	MW2.1	+	Rods	Single	-	+	+
7	MS1.1	-	Rods	Chains	-	+	+
8	MW17.1	+	Rods	Chains	-	+	+
9	MW9	-	Rods	Single	-	+	+
10	MS2	+	Coccus	Single	-	+	+
11	MS3	-	Rods	Single	-	+	+
12	MS9	-	Rods	Single	-	+	+
13	MW6	+	Coccus	Single	-	+	+
14	MS5	+	Coccus	Single	-	+	+
15	MS6	-	Coccus	Chains	-	+	+
16	MS8	+	Coccus	Single	+	+	+
17	MW15	+	Coccus	Single	+	+	+
18	MW11	+	Rods	Chains	-	+	+
19	MW20	+	Coccus	Single	-	+	+
20	MW17	+	Rods	Single	+	+	+

4.6.1.2 Vashisht site

Twenty five laccase producing thermophilic bacterial isolates were obtained from fifty samples in form of water, soil, rock matting and pebbles collected from five locations of Vashisht hot water springs. It has been further observed that from 20 water samples 12 laccase producing thermophilic bacterial isolates were obtained followed by 10, 3 laccase producing thermophilic bacterial isolates from 10 samples each of soil and rock matting respectively, whereas from pebble samples no laccase producing thermophilic bacterial isolate was obtained. All the laccase producing thermophilic bacterial isolates obtained were found to be different in their colony morphotypes on basis of colour, size, shape, texture and elevation (Plate-6). The laccase producing thermophilic bacterial isolates when grown in TYM without substrate i.e.

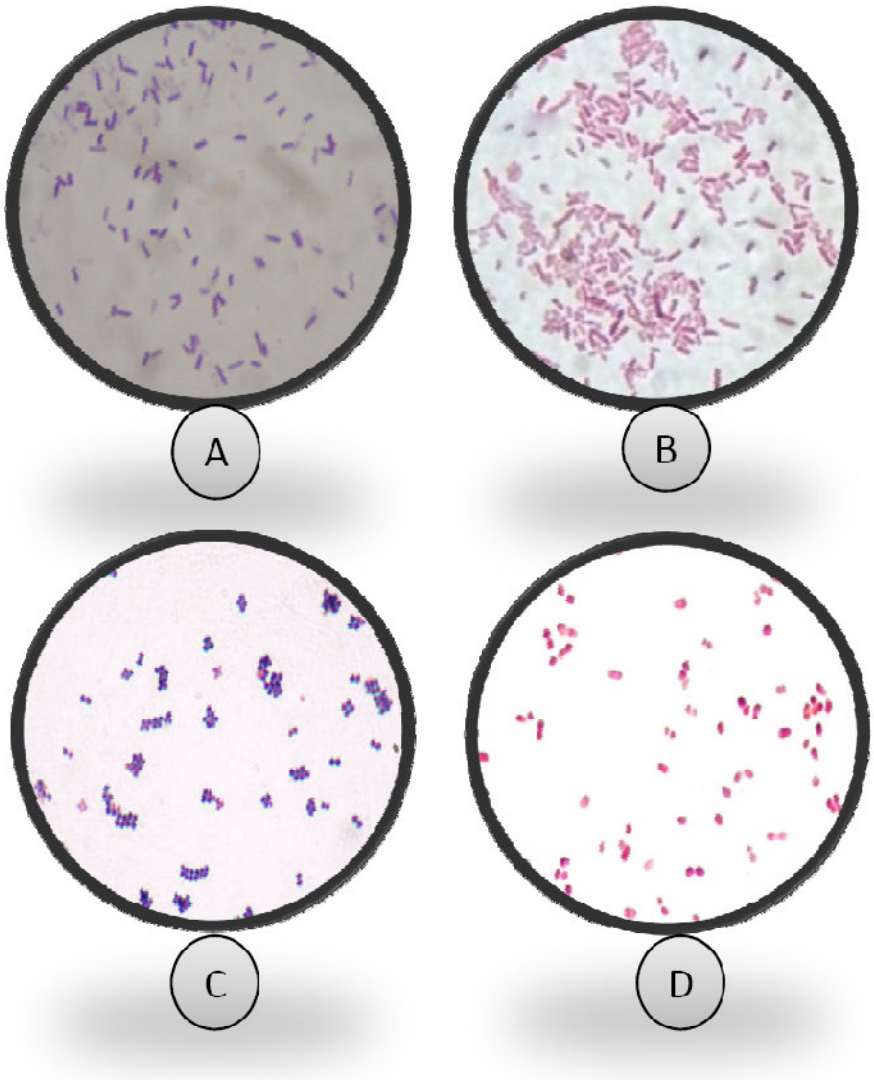


Plate 5: Gram stained bacteria at 1000 X magnification.

- **A) Gram positive rod**
- **B) Gram negative rod**
- **C) Gram positive cocci**
- **D) Gram negative cocci**

guaiacol showed colony colour variation ranging from white, cream, light brown, yellow to brown, whereas colony morphotypes obtained using TYM containing 5 mM guaiacol resulted in colour variation ranging from reddish brown, yellow orange, red, pink and brown in addition to all the above colour. As described in the previous section oxidation of guaiacol leads to reddish brown/brown colour of the colonies, thus reddish brown/brown coloured colonies were supposed to be putative laccase producing thermophilic bacterial isolates.

Table 37: Colony characterization of laccase producing thermophilic bacterial isolates from Vashisht site

Sr. No	Isolate	COLONY CHARACTERISTICS						
		Colour With guaiacol	Colour Without guaiacol	Size (mm)	Shape	Texture	Elevation	Pigment formation
1.	VW20	Y	Y	5.0	I	R	R	-
2.	VW13	Y	Y	2.5	I	S	F	-
3.	VW5	B	C	3.0	C	S	F	+
4.	VS5	C	C	3.5	C	R	R	-
5.	VW2	B	W	3.0	C	S	F	-
6.	VW9	B	B	6.0	I	R	F	-
7.	VS7	Y	C	2.2	C	S	R	+
8.	VS10	C	C	2.9	I	R	F	-
9.	VS8	B	W	7.0	I	S	R	-
10.	VS5.1	Y	Y	3.5	C	S	F	-
11.	VS7.1	Y	Y	2.5	I	R	R	-
12.	VS1	C	C	2.9	C	R	F	-
13.	VS3	R	W	1.2	C	R	F	+
14.	VR5	Y	C	4. 5	I	R	F	+
15.	VR2	Y	C	9.0	C	S	R	-
16.	VR9	V	V	4.5	I	R	R	-
17.	VW10	W	W	4.0	C	S	F	-
18.	VW17	Y	Y	2.1	I	S	R	-
19.	VW16	Y	Y	3.0	I	R	R	-
20.	VW2.1	C	C	3.0	I	R	F	-
21.	VS9	B	C	7.0	C	R	F	-
22.	VW1	Y	C	1.0	C	S	F	-
23.	VW1.1	C	C	6.0	I	R	F	-
24.	VS5.2	R	R	3.5	C	R	R	-
25.	VW7	Y	Y	1.7	I	S	F	-

Isolates
M-Manikaran
S-Soil
W-Water
R-Rock matting

Colour
Y-Yellow
C-Cream
W-White
R-Red
B-Brown
O-Orange

Shape
C-Circular
R- Rough

Elevation
F-Flat
R-Raised

Texture
S- Smooth
I-Irregular

Variation in size of colonies of laccase producing thermophilic bacterial isolates ranged from 1.2- 9.0 mm depicting small to large type of colonies. On the basis of texture of the colonies only 44% were found to be smooth, whereas rests of 56% colonies were rough. On the basis of elevation 60% colonies were found to be flat, whereas 40% were raised. On the basis of shape 52% colonies were found to be irregular and only 48% were found to be circular in shape (Table-37).

All 25 laccase producing thermophilic bacterial isolates from five locations of Vashisht site were also investigated for various microscopic characters (Table 38).

Table 38: Microscopic characterization of laccase producing thermophilic bacterial isolates from Vashisht site

Sr. No	Isolate		MICROSCOPIC CHARACTERISTICS				
		Gram's reaction	Shape	Arrangement of cells	Spore formation	Pellicle Formation	Motility
1	VW20	+	Coccus	Single	+	+	+
2	VW13	+	Coccus	Single	-	+	+
3	VW5	+	Rods	single	+	+	+
4	VS5	+	Rods	Single	-	+	+
5	VW2	+	Rods	Single	-	+	+
6	VW9	+	Rods	Single	-	+	+
7	VS7	-	Rods	Chains	-	+	+
8	VS10	+	Rods	Single	-	+	+
9	VS8	+	Rods	Single	+	+	+
10	VS5.1	+	Coccus	Single	-	+	+
11	VS7.1	-	Rods	Single	-	+	+
12	VS1	-	Rods	Single	-	+	+
13	VS3	+	Coccus	Single	-	+	+
14	VR5	+	Coccus	Single	-	+	+
15	VR2	+	Rods	Single	+	+	+
16	VR9	+	Coccus	Single	+	+	+
17	VW10	+	Coccus	Single	+	+	+
18	VW17	-	Rods	Chains	-	+	+
19	VW16	+	Rods	Single	-	+	+
20	VW2.1	+	Rods	Single	-	+	+
21	VS9	+	Rods	Single	+	+	+
22	VW1	+	Rods	Single	-	+	+
23	VW1.1	+	Coccus	Single	-	+	+
24	VS5.2	-	Rods	Chains	-	+	+
25	VW7	+	Rods	Single	-	+	+

Gram's reaction showed that 20% laccase producing thermophilic bacterial isolates were pink in colour and thus gram negative in nature whereas



Plate 6: Different colony morphotypes obtained from Vashisht hot water springs

80% were found to be purple in colour and thus gram positive in nature. Variation in shape of the cells showed cocci and rods (Plate-5). Only 32% of laccase producing thermophilic bacterial isolates possessed coccus shape whereas rests of the 68% isolates were found to be rod shaped. Arrangement of cells produced variation from singles to chains and majority 88% were found to be in singles. Out of total 25 laccase producing thermophilic bacterial isolates only 28% were found to be spore forming in nature (Table-38). All 25 laccase producing thermophilic bacterial isolates were tested for their ability to form pellicle in the suspension culture and it was found that all bacterial isolates were able to form pellicle and were found to be positive for the morphological descriptor of pellicle formation.

4.6.1.3 Tattapani site

In case of Tattapani maximum 39 laccase producing thermophilic bacterial isolates were obtained from fifty samples in the form of water, soil, rock matting and pebbles collected from five locations of hot water spring. It has been further observed that 18 laccase producing thermophilic bacterial isolates were obtained from 20 water samples followed by 16, 15 laccase producing thermophilic bacterial isolates from 10 samples each of soil and rock matting respectively, whereas from pebble samples no laccase producing thermophilic bacterial isolate was obtained. All the laccase producing thermophilic bacterial isolates obtained were found to be different in their colony morphotypes on basis of colour, size, shape, texture and elevation (Plate-7). The laccase producing thermophilic bacterial isolates when grown in TYM without substrate i.e. guaiacol showed colony colour variation ranging from white, cream, light brown, yellow to brown, whereas colony morphotypes obtained using TYM containing 5 mM guaiacol resulted in colour variation ranging from reddish brown, yellow orange, red, pink and brown in addition to all the above colour. As described in the previous section oxidation of guaiacol leads to reddish brown/brown colour of the colonies, thus reddish brown/brown coloured colonies were supposed to be putative laccase producing thermophilic bacterial isolates (Table-39).

Table 39: Colony characterization of laccase producing thermophilic bacterial isolates from Tattapani site

Sr. No	Isolate	COLONY CHARACTERISTICS						
		Colour With guaiacol	Colour Without guaiacol	Size (mm)	Shape	Texture	Elevation	Pigment formation
1.	TW2.1	P	C	2.0	I	R	R	+
2.	TW2.2	Y	C	3.5	C	S	F	-
3.	TW3	Y	Y	5.0	C	R	R	-
4.	TW4	B	B	2.5	C	R	F	-
5.	TW5	B	C	2.0	C	R	R	+
6.	TW14	B	B	1.5	C	R	R	-
7.	TW17	B	B	2.5	I	R	F	+
8.	TS1	B	B	9.0	I	R	F	-
9.	TS2.1	B	C	3.5	C	R	F	-
10.	TS2.2	Y	Y	6.5	C	S	F	+
11.	TS2.4.	Y	Y	5.5	C	R	F	-
12.	TS2.3	Y	Y	1.7	C	R	R	+
13.	TS4.1	R	C	5.0	I	S	F	-
14.	TS4.2	R	C	3.0	I	S	F	-
15.	TS5.1	R	R	1.5	I	R	R	-
16.	TS5.2	B	B	5.0	I	S	F	+
17.	TS8	O	C	5.0	I	R	F	+
18.	TR4	Y	Y	3.5	C	S	F	-
19.	TR9	B	Y	2.5	C	R	F	-
20.	TR4.1	B	C	6.0	I	R	F	+
21.	TR4.2	C	C	3.0	I	R	F	-
22.	TR1	B	C	4.0	I	R	F	-
23.	TR6.1	Y	C	6.0	I	R	F	+
24.	TR6	Y	Y	2.2	C	R	F	-
25.	TR9.1	Y	Y	3.0	C	R	F	-
26.	TW2	Y	W	7.0	C	R	F	-
27.	TS4	B	B	3.0	I	R	F	-
28.	TS5	B	B	2.5	I	R	F	-
29.	TR6.2	P	C	2.0	I	R	F	+
30.	TR8	R	R	1.2	I	R	F	-
31.	TR10	Y	C	4.2	I	R	F	+
32.	TR10.2	B	B	2.0	I	R	F	-
33.	TR8	B	B	4.5	I	R	F	-
34.	TR2	B	C	3.0	I	R	F	+
35.	TS7	B	B	2.1	C	R	F	-
36.	TS3	W	W	3.0	C	R	F	-
37.	TS3.1	P	P	3.0	C	R	F	-
38.	TR2.1	C	C	3.0	I	R	F	-
39	TS3.2	Y	C	2.0	I	R	F	+

Isolates
T-Tattapani
S-Soil
W-Water
R-Rock matting

Colour
Y-Yellow
C-Cream
W-White
R-Red
B-Brown
O-Orange

Shape
C-Circular
I-Irregular

Elevation
F-Flat
R-Raised

Texture
S- Smooth
R- Rough

Variation in size of colonies of laccase producing thermophilic bacterial isolates ranged from 1.2- 9.0 mm depicting small to large type of colonies. On the basis of texture of the colonies only 18% were found to be smooth, whereas 82% colonies were rough. On the basis of elevation majority 82% colonies were found



Plate 7: Different colony morphotypes obtained from Tattapani hot water springs

to be flat, whereas only 18% were raised. On the basis of shape 66% colonies were found to be irregular and 44% were found to be circular in shape (Table-39).

A total of 39 laccase producing thermophilic bacterial isolates from five locations of Tattapani hot water springs were also investigated for various microscopic characters (Table-40).

Table 40: Microscopic characterization of laccase producing thermophilic bacterial isolates from Tattapani site

Sr. No	Isolate	MICROSCOPIC CHARACTERISTICS					
		Gram's reaction	Shape	Arrangement of cells	Spore formation	Pellicle Formation	Motility
1	TW2.1	+	Coccus	Single	+	+	+
2	TW2.2	+	Coccus	Single	-	+	+
3	TW3	-	Rods	Chains	-	+	+
4	TW4	+	Rods	Single	-	+	+
5	TW5	+	Rods	Single	+	+	+
6	TW14	+	Rods	Single	-	+	+
7	TW17	-	Rods	Chains	-	+	+
8	TS1	+	Rods	Chains	-	+	+
9	TS2.1	-	Rods	Single	-	+	+
10	TS2.2	+	Coccus	Single	-	+	+
11	TS2.4.	-	Rods	Single	-	+	+
12	TS2.3	-	Rods	Single	-	+	+
13	TS4.1	+	Coccus	Single	-	+	+
14	TS4.2	+	Coccus	Single	-	+	+
15	TS5.1	-	Coccus	Chains	-	+	+
16	TS5.2	+	Coccus	Single	+	+	+
17	TS8	+	Rods	Single	+	+	+
18	TR4	-	Rods	Chains	-	+	+
19	TR9	+	Rods	Single	-	+	+
20	TR4.1	+	Rods	Single	-	+	+
21	TR4.2	+	Rods	Single	+	+	+
22	TR1	-	Rods	Chains	-	+	+
23	TR6.1	+	Rods	Chains	-	+	+
24	TR6	-	Rods	Single	-	+	+
25	TR9.1	+	Coccus	Single	-	+	+
26	TW2	-	Rods	Single	-	+	+
27	TS4	-	Rods	Chains	-	+	+
28	TS5	+	Rods	Chains	-	+	+
29	TR6.2	-	Rods	Single	-	+	+
30	TR8	+	Coccus	Single	-	+	+
31	TR10	-	Rods	Single	-	+	+
32	TR10.2	-	Rods	Single	-	+	+
33	TR8	+	Coccus	Single	-	+	+
34	TR2	+	Rods	Single	+	+	+
35	TS7	-	Rods	Chains	-	+	+
36	TS3	+	Coccus	Single	+	+	+
37	TS3.1	+	Coccus	Single	+	+	+
38	TR2.1	+	Rods	Chains	-	+	+
39	TS3.2	+	Coccus	Single	-	+	+

Persual of table -39 has shown that 29% laccase producing thermophilic bacterial isolates were found to be pink in colour and thus gram negative in nature and 71% were found to be purple in colour and thus gram positive in

nature (Plate-5). Variation in shape of the cells showed cocci and rods. Majority 77% of laccase producing thermophilic bacterial isolates were found to be rod shaped whereas only 23% possessed coccus shape. Arrangement of cells produced variation from singles to chains and majority 82% were found to be in singles. Out of total 39 laccase producing thermophilic bacterial isolates only 24% bacterial isolates were found to be spore forming in nature. Laccase producing thermophilic bacterial isolates were tested for their ability to form pellicle in the suspension culture and it was found that all bacterial isolates were able to form pellicle and were found to be positive for the morphological descriptor of pellicle formation (Table-40).

4.6.1.4 Khirganga site

Thirty four laccase producing thermophilic bacterial isolates were obtained from fifty samples in the form of water, soil, rock matting and pebbles collected from five locations of Khirganga hot water springs. It has been further observed that 14 laccase producing thermophilic bacterial isolates were obtained from 20 water samples followed by 11, 9 laccase producing thermophilic bacterial isolates from 10 samples each of soil and rock matting respectively, whereas from pebble samples no laccase producing thermophilic bacterial isolate was obtained. All the laccase producing thermophilic bacterial isolates obtained were found to be different in their colony morphotypes on basis of colour, size, shape, texture and elevation (Plate-8). The laccase producing thermophilic bacterial isolates when grown in TYM without substrate i.e. guaiacol showed colony colour variation ranging from white, cream, light brown, yellow to brown, whereas colony morphotypes obtained using TYM containing 5 mM guaiacol resulted in colour variation ranging from reddish brown, yellow orange, red, pink and brown in addition to all the above colour. As described in the previous section oxidation of guaiacol leads to reddish brown/brown colour of the colonies, thus reddish brown/brown coloured colonies were supposed to be putative laccase producing thermophilic bacterial isolates.

Variation in size of colonies of laccase producing thermophilic bacterial isolates ranged from 1.0-9.0 mm depicting small to large type of colonies. On the basis of texture of the colonies only 15% were found to be smooth, whereas 85%



Plate 8: Different colony morphotypes obtained from Khirganga hot water springs

colonies were rough. On the basis of elevation majority 82% colonies were found to be flat, whereas only 18% were raised. On the basis of shape only 36% were found to be circular and 64% colonies were found to be irregular in shape (Table-41).

Table 41: Colony characterization of laccase producing thermophilic bacterial isolates from Khirganga site

Sr. No	Isolate	COLONY CHARACTERISTICS						
		Colour With guaiacol	Colour Without guaiacol	Size (mm)	Shape	Texture	Elevation	Pigment formation
1.	KS2.1	R	R	6.0	I	R	F	-
2.	KS3	C	C	3.5	C	R	F	-
3.	KW7	C	C	1.7	I	R	F	-
4.	KR1	O	C	4.0	I	R	F	+
5.	KR3	B	Y	3.7	I	R	F	+
6.	KW5	O	O	2.7	C	R	F	-
7.	KW8	Y	Y	6.0	I	R	F	-
8.	KW9	O	O	2.5	C	R	F	-
9.	KR7	Y	C	2.0	I	R	F	+
10.	KS2	R	R	5.5	I	R	F	-
11.	KR8	W	W	3.7	C	R	F	-
12.	KW9.1	Y	C	7.0	C	S	F	-
13.	KS5	Y	C	4.0	I	R	F	+
14.	KW3	Y	Y	2.0	I	R	F	-
15.	KW14	B	C	2.5	I	R	F	+
16.	KW17	R	R	2.5	I	R	F	-
17.	KR7.1	C	C	2.7	I	R	F	-
18.	KW3.1	C	C	2.0	I	R	F	-
19.	KR1.1	W	W	3.5	I	R	F	-
20.	KW9.2	Y	Y	5.0	I	R	F	-
21.	KS4	Y	Y	2.5	I	S	R	-
22.	KR7.2	Y	W	2.0	I	R	F	-
23.	KR10	R	C	1.5	C	R	F	+
24.	KS9	B	C	2.5	C	R	F	+
25.	KS10	C	C	7.0	I	S	R	-
26.	KS8	R	R	3.0	C	R	F	-
27.	KW3.2	B	C	4.0	C	S	F	-
28.	KW12	Y	C	5.0	C	R	R	+
29.	KW17.1	Y	Y	1.0	I	R	F	-
30.	KS2.1	C	C	9.0	I	S	F	-
31.	KR2	Y	W	3.0	I	R	R	+
32.	KR 1.1	Y	Y	1.5	I	R	R	-
33.	KR10.1	W	W	5.1	C	R	F	-
34.	KW4	Y	C	3.5	C	R	R	-

Isolates
K-Khirganga
S-Soil
W-Water
R-Rock matting

Colour
Y-Yellow
C-Cream
W-White
R-Red
B-Brown
O-Orange

Shape
C-Circular
I-Irregular

Elevation
F-Flat
R-Raised

Texture
S- Smooth
R- Rough

Axenic cultures were obtained by transfer of individual colonies using TYM agar plate containing 5 mM guaiacol. The stock cultures were prepared by

inoculating agar slants from the second plate and for storage purposes; all isolates have been preserved at 4^0C.

Thirty four laccase producing thermophilic bacterial isolates from Khirganga hot water springs were investigated further for various microscopic characters (Table-42).

Table 42: Microscopic characterization of laccase producing thermophilic bacterial isolates from Khirganga site

Sr. No	Isolate	MICROSCOPIC CHARACTERISTICS					
		Gram's reaction	Shape	Arrangement of cells	Spore formation	Pellicle formation	Motility
1	KS2.1	+	Coccus	Single	+	+	+
2	KS3	+	Coccus	Single	-	+	+
3	KW7	-	Rods	Chains	-	+	+
4	KR1	+	Rods	Single	-	+	+
5	KR3	+	Rods	Single	-	+	+
6	KW5	+	Rods	Single	-	+	+
7	KW8	+	Rods	Single	-	+	+
8	KW9	+	Coccus	Single	-	+	+
9	KR7	+	Coccus	Single	-	+	+
10	KS2	-	Rods	Chains	-	+	+
11	KR8	+	Rods	Single	-	+	+
12	KW9.1	+	Rods	Single	+	+	+
13	KS5	+	Rods	Single	-	+	+
14	KW3	-	Rods	Chains	-	+	+
15	KW14	+	Rods	Chains	-	+	+
16	KW17	-	Rods	Single	+	+	+
17	KR7.1	+	Coccus	Single	+	+	+
18	KW3.1	-	Rods	Single	-	+	+
19	KR1.1	-	Rods	Single	-	+	+
20	KW9.2	-	Rods	Single	-	+	+
21	KS4	+	Coccus	Single	+	+	+
22	KR7.2	+	Rods	Single	-	+	+
23	KR10	-	Rods	Chains	-	+	+
24	KS9	+	Rods	Single	-	+	+
25	KS10	+	Rods	Single	-	+	+
26	KS8	+	Rods	Single	-	+	+
27	KW3.2	+	Rods	Chains	+	+	+
28	KW12	+	Rods	Chains	-	+	+
29	KW17.1	-	Rods	Single	-	+	+
30	KS2.1	+	Coccus	Single	-	+	+
31	KR2	-	Rods	Single	-	+	+
32	KR 1.1	-	Rods	Single	-	+	+
33	KR10.1	+	Coccus	Single	-	+	+
34	KW4	+	Rods	Single	+	+	+

Gram's reaction showed that only 32% laccase producing thermophilic bacterial isolates were pink in colour and thus gram negative in nature and majority 68% were found to be purple in colour and thus gram positive in nature (Plate-5). Variation in shape of the cells showed cocci and rods. Majority of 77% laccase producing thermophilic bacterial isolates were rod shaped whereas 23% of isolates possessed coccus shape. Arrangement of cells produced variation from singles to chains like and only 16% were found to be in chains. Only 21% isolates were found to be spore forming in nature. Laccase producing thermophilic bacterial isolates when tested for their ability to form pellicle in the suspension culture and it was found that all bacterial isolates were able to form pellicle and were found to be positive for the morphological descriptor of pellicle formation (Table-42).

4.7 SECONDARY SCREENING OF THERMOPHILIC BACTERIAL ISOLATES FOR THERMOSTABLE LACCASE PRODUCTION

A total of 118 laccase producing thermophilic bacterial isolates have been further screened on TYM supplemented with tannic acid and by dropping dimethoxyphenol and syringaldazine on bacterial colonies for laccase activity. Laccase producing thermophilic bacterial isolates which produced black zones/brown colonies on tannic acid agar plates (9a) and bacterial isolates which produced orange coloured colonies (9b) and purple coloured colonies (9c,d) on adding dimethoxyphenol and syringaldazine, respectively were confirmed and selected to be positive for laccase activity. On the basis of result obtained only 50 laccase producing thermophilic bacterial isolates have been found to test positive (Table- 43). In both cases of dimethoxyphenol test and syringaldazine test laccase producing thermophilic bacterial isolates showed orange colour and purple colour respectively, within 4-5 hours of incubation at an optimum temperature of 60^{o}C.

Table 43: Secondary screening of laccase producing thermophilic bacterial isolates

Sr. No.	Bacterial Isolates	Tannic acid Test	Dimethoxyphenol Test	Syringaldazine Test
1.	TW2.1	-	-	-
2.	TW2.2	-	-	-
3.	TW3	-	-	-
4.	TW4	-	-	-

Sr. No.	Bacterial Isolates	Tannic acid Test	Dimethoxyphenol Test	Syringaldazine Test
5.	TW5	+	+	+
6.	TW14	+	+	+
7.	TW17	-	-	-
8.	TS1	-	-	-
9.	TS2.1	-	-	-
10.	TS2.2	-	-	-
11.	TS2.4.	-	-	-
12.	TS2.3	-	-	-
13.	TS4.1	-	-	-
14.	TS4.2	-	-	-
15.	TS5.1	-	-	-
16.	TS5.2	-	-	-
17.	TS8	+	+	+
18.	TR4	-	-	-
19.	TR9	+	+	+
20.	TR4.1	-	-	-
21.	TR4.2	+	+	+
22.	TR1	-	-	-
23.	TR6.1	-	-	-
24.	TR6	-	-	-
25.	TR9.1	-	-	-
26.	TW2	-	-	-
27.	TS4	-	-	-
28.	TS5	-	-	-
29.	TR6.2	-	-	-
30.	TR8	-	-	-
31.	TR10	-	-	-
32.	TR10.2	-	-	-
33.	TR8	-	-	-
34.	TR2	+	+	+
35.	TS7	-	-	-
36.	TS3	-	-	-
37.	TS3.1	-	-	-
38.	TR2.1	-	-	-
39.	TS3.2	-	-	-
40.	KS2.1	+	+	+
41.	KS3	-	-	-
42.	KW7	-	-	-
43.	KR1	+	+	+
44.	KR3	-	-	-
45.	KW5	+	+	+
46.	KW8	+	+	+
47.	KW9	-	-	-
48.	KR7	+	+	+
49.	KS2	+	+	+
50.	KR8	-	-	-
51.	KW9.1	+	+	+
52.	KS5	+	+	+
53.	KW3	+	+	+
54.	KW14	+	+	+
55.	KW17	-	-	-
56.	KR7.1	+	+	+
57.	KW3.1	+	+	+
58.	KR1.1	+	+	+
59.	KW9.2	+	+	+
60.	KS4	-	-	-
61.	KR7.2	+	+	+
62.	KR10	+	+	+
63.	KS9	+	+	+

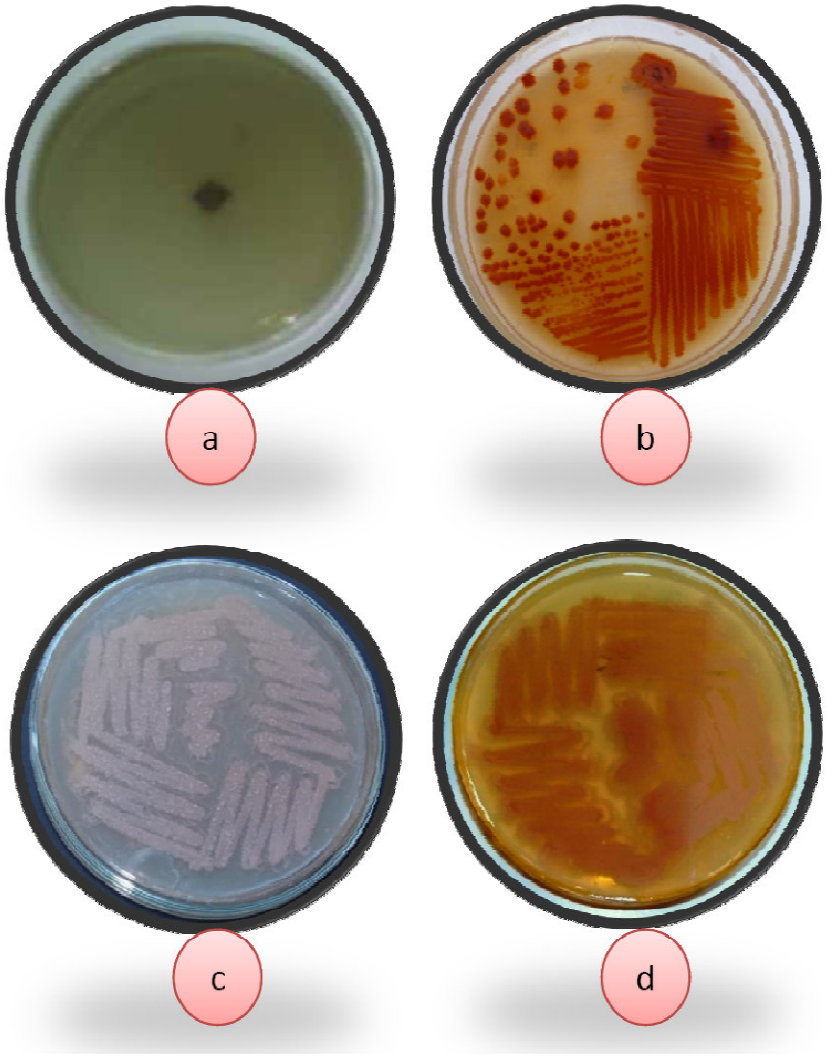


Plate 9: Secondary screening of laccase producing thermophilic bacterial isolates

a) Black zone / colony 0.5 % tannic acid agar plate.
b) Orange colour formation after dropping dimethoxyphenol on bacterial colonies
c-d) Purple colour formation after dropping syringaldazine on bacterial colonies

Sr. No.	Bacterial Isolates	Tannic acid Test	Dimethoxyphenol Test	Syringaldazine Test
64.	KS10	-	-	-
65.	KS8	-	-	-
66.	KW3.2	+	+	+
67.	KW12	-	-	-
68.	KW17.1	-	-	-
69.	KS2.1	-	-	-
70.	KR2	-	-	-
71.	KR 1.1	-	-	-
72.	KR10.1	-	-	-
73.	KW4	+	+	+
74.	VW20	+	+	+
75.	VW13	-	-	-
76.	VW5	+	+	+
77.	VS5	+	+	+
78.	VW2	+	+	+
79.	VW9	-	-	-
80.	VS7	-	-	-
81.	VS10	+	+	+
82.	VS8	+	+	+
83.	VS5.1	+	+	+
84.	VS7.1	-	-	-
85.	VS1	+	+	+
86.	VS3	-	-	-
87.	VR5	-	-	-
88.	VR2	+	+	+
89.	VR9	+	+	+
90.	VW10	-	-	-
91.	VW17	-	-	-
92.	VW16	-	-	-
93.	VW2.1	-	-	-
94.	VS9	+	+	+
95.	VW1	+	+	+
96.	VW1.1	-	-	-
97.	VS5.2	+	+	+
98.	VW7	+	+	+
99.	MS6	-	-	-
100.	MS1	+	+	+
101.	MS2.1	+	+	+
102.	MS2.2	+	+	+
103.	MW2	-	-	-
104.	MW2.1	+	+	+
105.	MS1.1	-	-	-
106.	MW17.1	-	-	-
107.	MW9	-	-	-
108.	MS2	-	-	-
109.	MS3	-	-	-
110.	MS9	+	+	+
111.	MW6	+	+	+
112.	MS5	+	+	+
113.	MS6	-	-	-
114.	MS8	-	-	-
115.	MW15	+	+	+
116.	MW11	+	+	+
117.	MW20	+	+	+
118.	MW17	+	+	+

4.8 BIOCHEMICAL CHARACTERIZATION OF LACCASE PRODUCING THERMOPHILIC BACTERIAL ISOLATES

4.8.1 Biochemical characterization of thermophilic bacterial isolates

A total of 50 laccase producing thermophilic bacterial isolates selected in the previous experiment were further characterized for various biochemical tests viz. Catalase, oxidase, urease, indole test methyl red & Voges-Proskauer test citrate test (IMViC), fermentation of sugars [glucose, sucrose, lactose, and arabinose] and triple sugar iron test (Table-44).

a) Catalase test

All laccase producing 50 thermophilic bacterial isolates were found to be test positive for catalase test (Table-44). Thus catalase test did not depict any variation in the 50 laccase producing thermophilic bacterial isolates.

b) Oxidase test

This test also depicted variation for 50 laccase producing thermophilic bacterial isolates. Only four laccase producing thermophilic bacterial isolates viz., TR9, KR1, VS5.1 and VW7 tested negative whereas rest of the laccase producing thermophilic bacterial isolates tested positive for oxidase enzyme activity (Table-44).

c) Urea production

Thirty five laccase producing thermophilic bacterial isolates were positive for urea production whereas only fifteen laccase producing thermophilic bacterial isolates were found to be negative for urease test (Table-44). So this test showed variation for 50 laccase producing thermophilic bacterial isolates.

d) Indole test

All 50 laccase producing thermophilic bacterial isolates were found to be tested negative for indole production test (Table-44). Thus no variation was observed for the 50 laccase producing thermophilic bacterial isolates.

e) Methyl red and Voges-Proskauer test

Methyl red test was found to be positive for 45 laccase producing thermophilic bacterial isolates whereas only 5 laccase producing thermophilic bacterial isolates found to be test negative and for VP test 33 laccase producing thermophilic bacterial isolates were found to be negative whereas only 17 were found to be positive (Table-44). Thus variation was observed for the 50 laccase producing thermophilic bacterial isolates.

f) Citrate test

Thirty laccase producing thermophilic bacterial isolates were found to be negative for citrate test whereas 20 laccase producing thermophilic bacterial isolates were found to be positive (Table-44). Thus variation was observed for the 50 laccase producing thermophilic bacterial isolates.

g) Fermentation of sugars (glucose, sucrose, lactose, arabinose)

Laccase producing thermophilic bacterial isolates showed significant variation to ferment different sugars viz., glucose, sucrose, lactose and arabinose. Glucose was fermented by 96% laccase producing thermophilic bacterial isolates and sucrose was fermented by all laccase producing thermophilic bacterial isolates (Table-44). It was depicted that lactose sugar was fermented by only 48% laccase producing thermophilic bacterial isolates whereas 52% were not able to ferment lactose. Only 46% laccase producing thermophilic bacterial isolates were not found to ferment arabinose and 54% isolates were able to ferment arabinose.

h) Triple Sugar Iron test

Laccase producing thermophilic bacterial isolates also showed variation to ferment triple sugar and produce H_2S gas. It was observed that in case of 60% laccase producing thermophilic bacterial isolates, slant was pink in colour and butt was yellow in colour which means laccase producing thermophilic bacterial isolates were not able to ferment sucrose or lactose but able to ferment glucose, whereas in other 8% laccase producing thermophilic bacterial isolates both butt and slant was found pink means none of the sugar was fermented. H_2S gas formation was not showed by any laccase producing thermophilic bacterial

isolates, whereas 32% laccase producing thermophilic bacterial isolates were yellow in colour which means bacterial isolates ferment all three sugars (Table-44).

Table 44: Biochemical characterization of fifty laccase producing thermophilic bacterial isolates

Sr. No.	Isolate	Catalase	Oxidase	Urea	IMViC test				Fermentation of sugars				TSI
					Indole	MR	VP	Citrate	Glucose	Sucrose	Lactose	Arabinose	
1.	TW5	+	+	-	-	+	+	+	+	+	+	-	+
2.	TW14	+	+	+	-	+	-	-	+	+	-	+	+
3.	TS8	+	+	-	-	+	-	+	+	+	+	-	+
4.	TR9	+	-	+	-	+	-	-	+	+	-	+	+
5.	TR4.2	+	+	+	-	+	-	+	+	+	+	-	+
6.	TR2	+	+	-	-	+	+	+	+	+	+	-	+
7.	KS2.1	+	+	+	-	+	-	-	+	+	-	+	+
8.	KR1	+	-	+	-	+	-	-	+	+	+	+	+
9.	KW5	+	+	+	-	-	-	-	+	+	-	+	+
10.	KW8	+	+	-	-	+	+	+	+	+	+	-	+
11.	KR7	+	+	+	-	+	-	-	+	+	-	+	+
12.	KS2	+	+	+	-	+	-	-	+	+	-	+	+
13.	KW9.1	+	+	-	-	+	+	+	+	+	+	+	+
14.	KS5	+	+	+	-	+	-	-	+	+	+	-	-
15.	KW3	+	+	+	-	+	-	+	+	+	-	+	+
16.	KW4	+	+	+	-	+	-	-	+	+	-	+	+
17.	KR7.1	+	+	+	-	+	-	-	+	+	-	+	+
18.	KW3.1	+	+	-	-	+	+	+	+	+	+	-	+
19.	KR1.1	+	+	+	-	-	-	-	-	+	-	+	+
20.	KW9.2	+	+	+	-	+	-	-	+	+	-	-	+
21.	KR7.2	+	+	-	-	+	+	+	+	+	+	-	+
22.	KR10	+	+	+	-	+	-	-	+	+	-	+	+
23.	KS9	+	+	+	-	+	-	-	+	+	-	+	+
24.	KW3.2	+	+	+	-	+	-	-	+	+	-	+	+
25.	KW4	+	+	-	-	+	+	+	+	+	+	-	+
26.	VW20	+	+	+	-	+	-	-	+	+	-	+	+
27.	VW5	+	+	-	-	+	+	+	+	+	+	-	+
28.	VS5	+	+	+	-	+	-	+	+	+	+	+	+
29.	VW2	+	+	-	-	+	+	+	+	+	+	-	+
30.	VS10	+	+	-	-	+	+	+	+	+	+	-	+
31.	VS8	+	+	+	-	-	-	-	+	+	-	-	+
32.	VS5.1	+	-	+	-	+	-	+	-	+	+	+	+
33.	VS1	+	+	+	-	+	-	-	+	+	-	+	-
34.	VR2	+	+	-	-	+	-	+	+	+	+	-	+
35.	VR9	+	+	+	-	+	-	-	+	+	-	-	+
36.	VS9	+	+	-	-	+	-	+	+	+	+	-	+
37.	VW1	+	+	-	-	+	-	+	+	+	+	-	+
38.	VS5.2	+	+	+	-	+	-	-	+	+	+	+	+
39.	VW7	+	-	+	-	+	-	-	+	+	-	+	+
40.	MS1	+	+	+	-	+	-	-	+	+	-	+	+
41.	MS2.1	+	+	+	-	+	-	-	+	+	+	+	+
42.	MS2.2	+	+	+	-	+	-	+	+	+	-	-	+
43.	MS1.2	+	+	+	-	+	-	-	+	+	-	+	+
44.	MS9	+	+	+	-	+	-	+	+	+	+	-	+
45.	MW6	+	+	+	-	+	-	-	+	+	+	+	+
46.	MS5	+	+	+	-	+	-	+	+	+	-	-	+
47.	MW15	+	+	+	-	+	-	-	+	+	-	-	+
48.	MW11	+	+	+	-	+	-	-	+	+	+	-	-
49.	MW20	+	+	+	-	+	-	-	+	+	-	+	+
50.	MW17	+	+	-	-	+	-	+	+	+	-	-	+

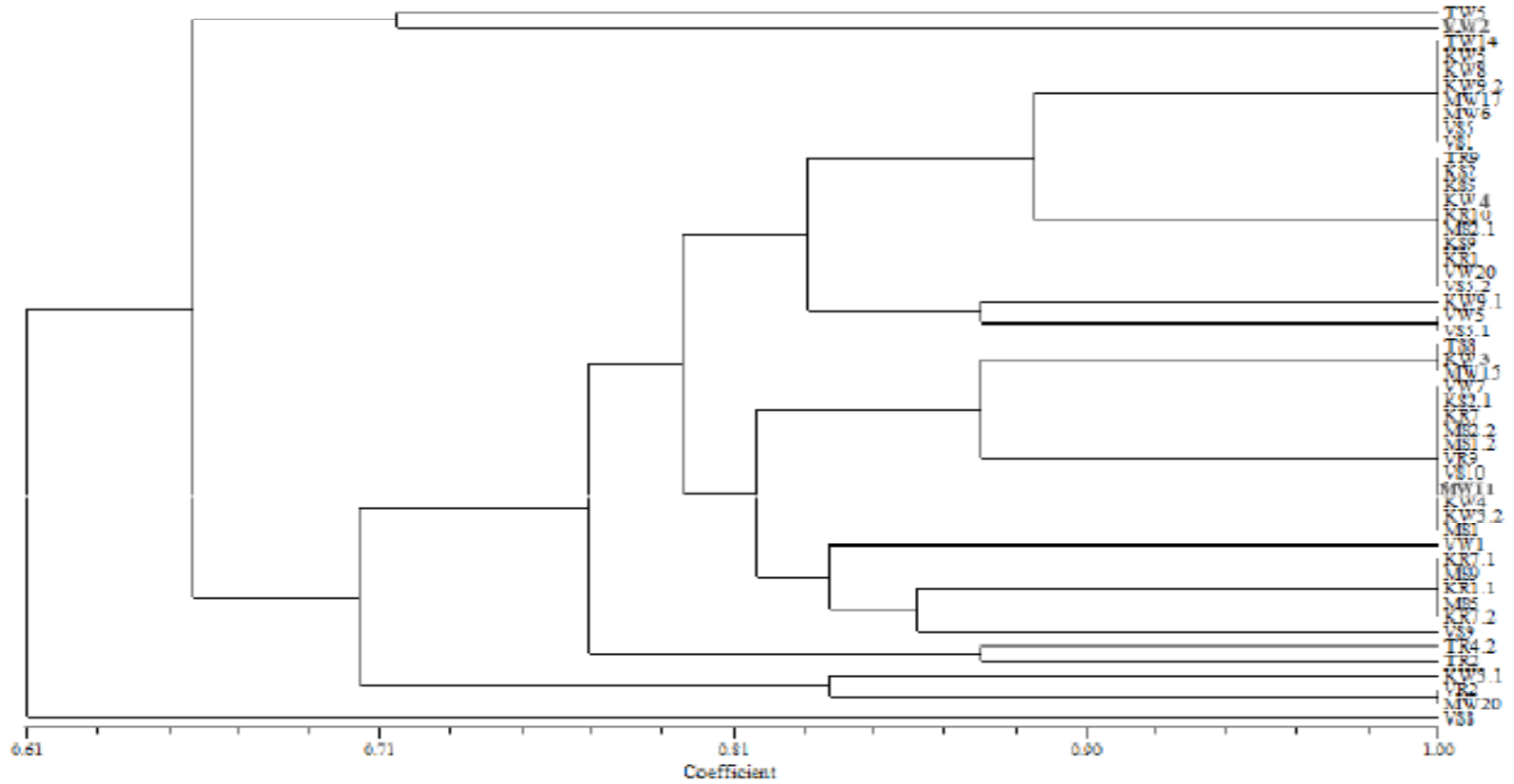


Fig. 24: Dendrogram showing relatedness among 50 laccase producing thermophilic bacterial isolates based on biochemical characters

Biochemical characters of selected fifty laccase producing thermophilic bacterial isolates were studied and the data matrices were analysed by the SAHN module of NTSYS-pc version 2.20 and similarities between isolates were estimated using the Jaccard's coefficient, calculated as J= A/(N-D), where A is the number of positive matches, D is the number of negative matches and N is the total sample size including both the number of matches and unmatches (Rohlf & Milligan, 1994). Dendrogram was produced from the resultant similarity matrices using the UPGMA method. According to dendrogram (Fig-24), the dendrogram bifurcates into two clusters A and B, B cluster clearly separate SV8 isolate from rests of 50 laccase producing thermophilic bacterial isolates. Cluster A further divided into two subclusters, whereas D subcluster was further divided into sub-subclusters and each sub-subclusters was further divided into different groups, whereas subcluster C was divided only into 2 groups. Thus dendogram of biochemical characters divided 50 laccase producing thermophilic bacterial isolates into 16 groups. Each laccase producing thermophilic bacterial isolates of the group was further examined for its laccase activity and the laccase producing thermophilic bacterial isolates which produce maximum laccase enzyme was selected for sequencing and *in silico* analysis.

4.9 QUALITATIVE AND QUANTITATIVE SCREENING FOR LACCASE ACTIVITY

Qualitative screening of both extracellular and intracellular crude laccase enzyme preparations of fifty selected laccase producing thermophilic bacteria was assessed by orange-brown coloured zone formation potential using dimethoxyphenol indicator and it has been observed only extracellular crude laccase enzyme preparations produced coloured zones, whereas intracellular crude laccase enzyme preparations were unable to do so (Table-45, Plate-10). All 50 laccase producing thermophilic bacterial isolates were observed for laccase enzyme activities after 24, 48, 72 to 96 hrs of incubation. Persual of table-46 shows extracellularly and intracellularly laccase enzyme activity. It was found out that thermophilic bacterial isolate TR2 showed maximum extracellular laccase activity of 0.00381 U/l after 24 hrs of incubation, whereas TR9 laccase

Table 45: Intracellular and extracellular qualitative screening of fifty laccase producing thermophilic bacterial isolates

Sr. No.	Bacterial Isolates	Laccase enzyme zone (mm)	
		IC	EC
1.	TW5	0.0	1.8
2.	TW14	0.0	0.2
3.	TS8	0.0	1.5
4.	TR9	0.0	1.0
5.	TR4.2	0.0	1.4
6.	TR2	0.0	1.7
7.	KS2.1	0.0	1.3
8.	KR1	0.0	1.0
9.	KW5	0.0	0.2
10.	KW8	0.0	1.5
11.	KR7	0.0	1.0
12.	KS2	0.0	0.2
13.	KW9.1	0.0	2.0
14.	KS5	0.0	0.2
15.	KW3	0.0	0.5
16.	KW3.1	0.0	2.0
17.	KR7.1	0.0	1.0
18.	KW14	0.0	0.2
19.	KR1.1	0.0	0.5
20.	KW9.2	0.0	0.8
21.	KR7.2	0.0	1.9
22.	KR10	0.0	0.2
23.	KS9	0.0	0.7
24.	KW4	0.0	2.1
25.	KW3.2	0.0	1.0
26.	VW20	0.0	0.4
27.	VW5	0.0	1.7
28.	VS5	0.0	1.3
29.	VW2	0.0	2.0
30.	VS10	0.0	1.6
31.	VS8	0.0	1.7
32.	VS5.1	0.0	0.8
33.	VS1	0.0	1.0
34.	VR2	0.0	2.2
35.	VR9	0.0	1.2
36.	VS9	0.0	1.9
37.	VW1	0.0	1.6
38.	VS5.2	0.0	1.0
39.	VW7	0.0	0.2
40.	MS1	0.0	0.3

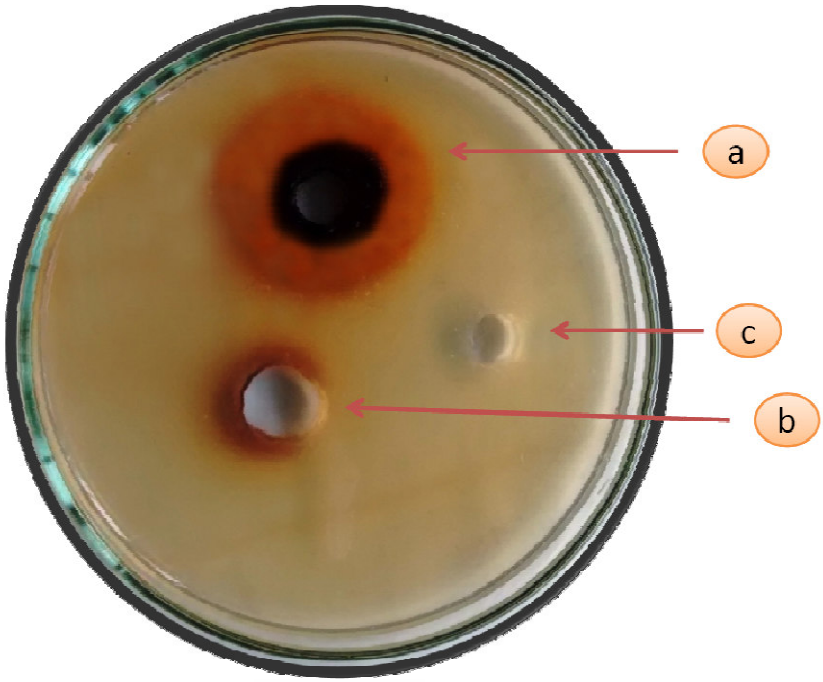


Plate 10: Qualitative screening for laccase activity

a) Commercial laccase enzyme zone formation

b) Extracellular laccase enzyme zone formation

c) Intracellular laccase enzyme zone formation

41.	**MS2.1**	0.0	0.6
42.	**MS2.2**	0.0	2.3
43.	**MS1.2**	0.0	1.0
44.	**MS9**	0.0	0.2
45.	**MW6**	0.0	0.8
46.	**MS5**	0.0	0.6
47.	**MW15**	0.0	0.3
48.	**MW11**	0.0	1.0
49.	**MW20**	0.0	1.0
50.	**MW17**	0.0	2.0

EC -Extracellular laccase enzyme activity
IC - Intracellular laccase enzyme activity

producing thermophilic bacterial isolate showed minimum extracellular activity of 0.00070 U/l after 24 hrs of incubation. In case of intracellular activity VS1 bacterial isolate showed maximum activity of 0.00166 U/l after 24 hrs of incubation, whereas bacterial isolate VW20 showed minimum activity 0.0002 U/l. Out of 50 laccase producing thermophilic bacterial isolates 80% showed extracellular activity after 24 hrs whereas only 20% isolates were not able to show any activity. In case of intracellular laccase enzyme activity only 34% laccase producing thermophilic bacterial isolates showed no activity whereas 66% laccase producing thermophilic bacterial isolates showed laccase enzyme activity.

After 48 hrs of incubation 72% laccase producing thermophilic bacterial isolates showed extracellular activity whereas 28% isolates were not able to show any activity. In case of intracellular laccase enzyme activity only 24% laccase producing thermophilic bacterial isolates showed no activity whereas 76% laccase producing thermophilic bacterial isolates showed laccase enzyme activity. Out of 50 laccase producing thermophilic bacterial isolates 46% showed extracellular activity after 72 hrs of incubation whereas 54% isolates were not able to show any activity. In case of intracellular laccase enzyme activity only 40% laccase producing thermophilic bacterial isolates showed no activity whereas 60% laccase producing thermophilic bacterial isolates showed laccase enzyme activity. After 96 hrs of incubation only 22% laccase producing thermophilic bacterial isolates showed extracellular activity whereas 88% isolates

were not able to show any activity. In case of intracellular laccase enzyme activity 70% laccase producing thermophilic bacterial isolates showed no activity whereas 30% laccase producing thermophilic bacterial isolates showed laccase enzyme activity.

Table 46: Intracellular and extracellular laccase activity of fifty laccase producing thermophilic bacterial isolates

Sr. No.	Bacterial Isolates	Laccase activity after 24 hrs (U/l)		Laccase activity after 48 hrs (U/l)		Laccase activity after 72 hrs (U/l)		Laccase activity after 96 hrs (U/l)	
		IC	EC	IC	EC	IC	EC	IC	EC
1.	**TW5**	0.00045	0.00234	0.00073	0.00045	0.0000	0.0000	0.0000	0.0
2.	**TW14**	0.00089	0.00075	0.00402	0.00089	0.0000	0.0000	0.0000	0.0000
3.	**TS8**	0.00000	0.000218	0.00090	0.00180	0.0000	0.0000	0.0000	0.0000
4.	**TR9**	0.00016	0.00070	0.0014	0.00045	0.0000	0.0000	0.0000	0.0000
5.	**TR4.2**	0.0000	0.00323	0.00124	0.00211	0.0000	0.00117	0.0000	0.00011
6.	**TR2**	0.00020	0.00381	0.00234	0.00322	0.0000	0.00232	0.00098	0.00201
7.	**KS2.1**	0.00098	0.00121	0.00234	0.00098	0.0000	0.00321	0.00056	0.00234
8.	**KR1**	0.00033	0.00133	0.00032	0.00033	0.00043	0.0000	0.0000	0.00389
9.	**KW5**	0.00012	0.00022	0.00189	0.0000	0.0000	0.0000	0.0000	0.0000
10.	**KW8**	0.00132	0.00277	0.00055	0.00204	0.0000	0.0000	0.0000	0.0000
11.	**KR7**	0.00111	0.00098	0.00321	0.00111	0.0000	0.0000	0.0000	0.0000
12.	**KS2**	0.00033	0.00132	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
13.	**KW9.1**	0.00034	0.00255	0.00056	0.0210	0.00047	0.0020	0.0000	0.0000
14.	**KS5**	0.00123	0.00087	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
15.	**KW3**	0.00134	0.00076	0.00102	0.00104	0.0000	0.0000	0.0000	0.0000
16.	**KW3.1**	0.00056	0.0156	0.00121	0.00256	0.00234	0.00033	0.00189	0.0000
17.	**KR7.1**	0.00012	0.00022	0.0000	0.0000	0.00111	0.00012	0.00022	0.0000
18.	**KW14**	0.00014	0.00034	0.00012	0.00022	0.00011	0.00022	0.00089	0.00098
19.	**KR1.1**	0.0000	0.00007	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
20.	**KW9.2**	0.00142	0.00098	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
21.	**KR7.2**	0.0000	0.00323	0.0000	0.00285	0.0000	0.0000	0.0000	0.0000
22.	**KR10**	0.0000	0.0009	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
23.	**KS9**	0.00012	0.00022	0.00098	0.0000	0.00121	0.00058	0.0012	0.0000
24.	**KW4**	0.0000	0.0028	0.0013	0.0022	0.0021	0.00088	0.0010	0.0000
25.	**KW3.2**	0.00085	0.00056	0.00099	0.00085	0.00321	0.00022	0.00211	0.0000
26.	**VW20**	0.00012	0.00022	0.00122	0.00187	0.00111	0.00089	0.0000	0.0000
27.	**VW5**	0.00089	0.00327	0.00066	0.00312	0.0000	0.0000	0.0000	0.0000
28.	**VS5**	0.0000	0.0027	0.00112	0.00187	0.0000	0.0000	0.0000	0.0000
29.	**VW2**	0.00056	0.0026	0.0012	0.0025	0.0023	0.00033	0.0019	0.0000
30.	**VS10**	0.00120	0.00276	0.00032	0.0021	0.0000	0.0198	0.0000	0.0000
31.	**VS8**	0.0016	0.0030	0.0011	0.0028	0.0000	0.0000	0.0000	0.0000

32.	**VS5.1**	0.00056	0.00154	0.00065	0.00106	0.0000	0.0000	0.0000	0.0000
33.	**VS1**	0.00166	0.00092	0.00156	0.00166	0.0000	0.0000	0.0000	0.0000
34.	**VR2**	0.00304	0.00287	0.00167	0.00304	0.0000	0.0000	0.0000	0.0000
35.	**VR9**	0.00012	0.00022	0.0089	0.0000	0.0000	0.0000	0.0000	0.0000
36.	**VS9**	0.0010	0.00253	0.00133	0.00219	0.0000	0.0000	0.0000	0.0006
37.	**VW1**	0.00009	0.00262	0.00246	0.00202	0.0000	0.00178	0.0000	0.00123
38.	**VS5.2**	0.00111	0.7800	1.567	0.00111	0.0000	0.0000	0.00432	0.00098
39.	**VW7**	0.00012	0.00022	0.0000	0.0000	0.0000	0.0000	0.00321	0.00056
40.	**MS1**	0.00011	0.00156	0.00156	0.00211	0.00189	0.00043	0.0000	0.0000
41.	**MS2.1**	0.00086	0.00121	0.00130	0.00086	0.00086	0.00012	0.0000	0.0000
42.	**MS2.2**	0.00123	0.00345	0.00356	0.00123	0.00211	0.00088	0.00102	0.0000
43.	**MS1.2**	0.00134	0.00178	0.00098	0.00134	0.00021	0.00022	0.00211	0.0000
44.	**MS9**	0.00012	0.00022	0.0000	0.0000	0.00104	0.00033	0.00189	0.0000
45.	**MW6**	0.00089	0.00134	0.00189	0.00089	0.00086	0.00012	0.0000	0.0000
46.	**MS5**	0.0000	00.0012	0.009	0.0016	0.0000	0.0000	0.0000	0.0000
47.	**MW15**	0.00012	0.00022	0.00011	0.00012	0.00011	0.0011	0.0000	0.0000
48.	**MW11**	0.00121	0.00234	0.00234	0.00121	0.00321	0.0000	0.00432	0.00098
49.	**MW20**	0.0002	0.00017	0.0000	0.0000	0.00234	0.0004	0.0000	0.0000
50.	**MW17**	0.00045	0.00265	0.00073	0.00145	0.00089	0.00143	0.0000	0.0000

EC -Extracellular laccase enzyme activity
IC - Intracellular laccase enzyme activity

It was found from the dendrogram (Fig-24) constructed on the basis of biochemical characters the fifty laccase producing isolates were divided into 16 groups. One laccase producing thermophilic bacterial isolate which showed maximum laccase activity from each group was selected further for sequencing and for *in silico* analysis. However from 3rd and 8th group two laccase producing thermophilic bacterial isolates showing maximum laccase activity were selected. Thus a total of eighteen laccase producing thermophilic bacterial isolates were selected on the basis of biochemical characters and maximum laccase activity (Table-46) (Plate-11-15).

4.10 MOLECULAR CHARACTERIZATION OF SELECTED LACCASE PRODUCING THERMOPHILIC BACTERIAL ISOLATES

Eighteen laccase producing thermophilic bacterial isolates selected from previous experiments were further subjected to molecular characterization using RAPD-PCR technology and 16S ribosomal DNA-PCR technology.

4.10.1 Extraction of genomic DNA

Total genomic DNA of each of the selected 18 laccase producing thermophilic bacterial isolates was extracted successfully using Genomic DNA extraction Mini kit (Real Genomics). Presence of DNA and its quality was checked using 1.0% agarose gel. Sharp DNA bands in lanes wherein the samples were loaded were clearly revealed without any contamination of proteins and RNA (Plate-16).

4.10.2 Random amplified polymorphic DNA (RAPD)-PCR technology

Genetic relatedness amongst these 18 laccase producing thermophilic bacterial isolates, which were selected on the basis of maximum laccase activity as investigated using RAPD-PCR technology. The PCR conditions for RAPD analysis were optimized by investigating the quality and concentration of genomic DNA, primer annealing and extension temperature as well as denaturation time and temperature as described in material and method section (3.10.2.1). The quality of genomic DNA extracted was found well, to be used as a template for PCR amplification. The annealing temperature of 36ºC was found to be optimum for good resolution of RAPD banding pattern. However, decreasing or increasing the optimum annealing temperature led to insufficient amplification products.

Thirty five random decamer oligonucleotide primers (Table-8) were screened initially and it was found that only 19 random decamer oligonucleotide primers viz: OPA-01, OPA-03, OPA-06, OPA-09, OPA-11, OPA-12, OPA-16, OPA-18, OPA-20; OPB-01, OPB-02, OPB-03, OPB-04, OPB-06, OPB-07, OPB-08, OPB-10; OPC-06 and OPC-07 were able to amplify the genomic DNA of all the 18 laccase producing thermophilic bacterial isolates. Whereas 16 random primers were unable to amplify the genomic DNA of laccase producing thermophilic bacterial isolates. Out of 19 random primers 16 were found to polymorphic in nature, whereas 3 were found to monomorphic. The distribution of polymorphic bands among these 18 laccase producing thermophilic bacterial isolates have been presented in Plates 17-24 and summarized in Table-47. Total number of RAPD bands varied from a minimum of 18 with primer OPB-02 and OPB-08 to a maximum of 74 with primer OPC-07.



Plate 11: Selected laccase producing thermophilic bacterial isolates

A) TW5 on TYM+ 5mM Guaiacol
B) TW5 on TYM
C) TR4.2 on TYM+ 5mM Guaiacol
D) TR4.2 on TYM
E) TR2 on TYM+ 5mM Guaiacol
F) TR2 on TYM
G) KW8 on TYM+ 5mM Guaiacol
H) KW8 on TYM

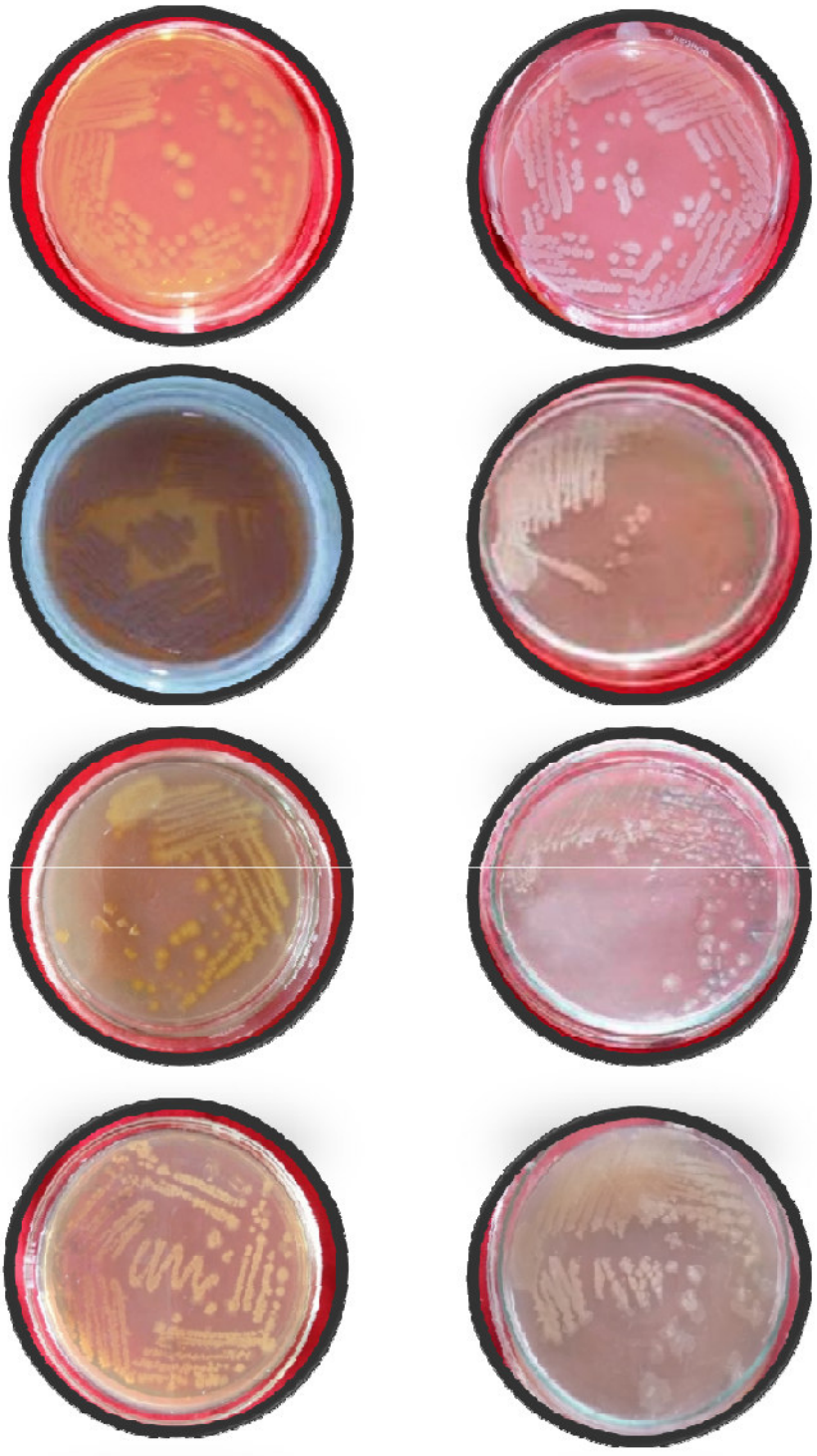


Plate 12: Selected laccase producing thermophilic bacterial isolates

A)	KW9.1 on TYM+ 5mM Guaiacol	B)	KW9.1 on TYM
C)	KW3.1 on TYM+ 5mM Guaiacol	D)	KW3.1 on TYM
E)	KR7.2 on TYM+ 5mM Guaiacol	F)	KR7.2 on TYM
G)	KW4 on TYM+ 5mM Guaiacol	H)	KW4 on TYM

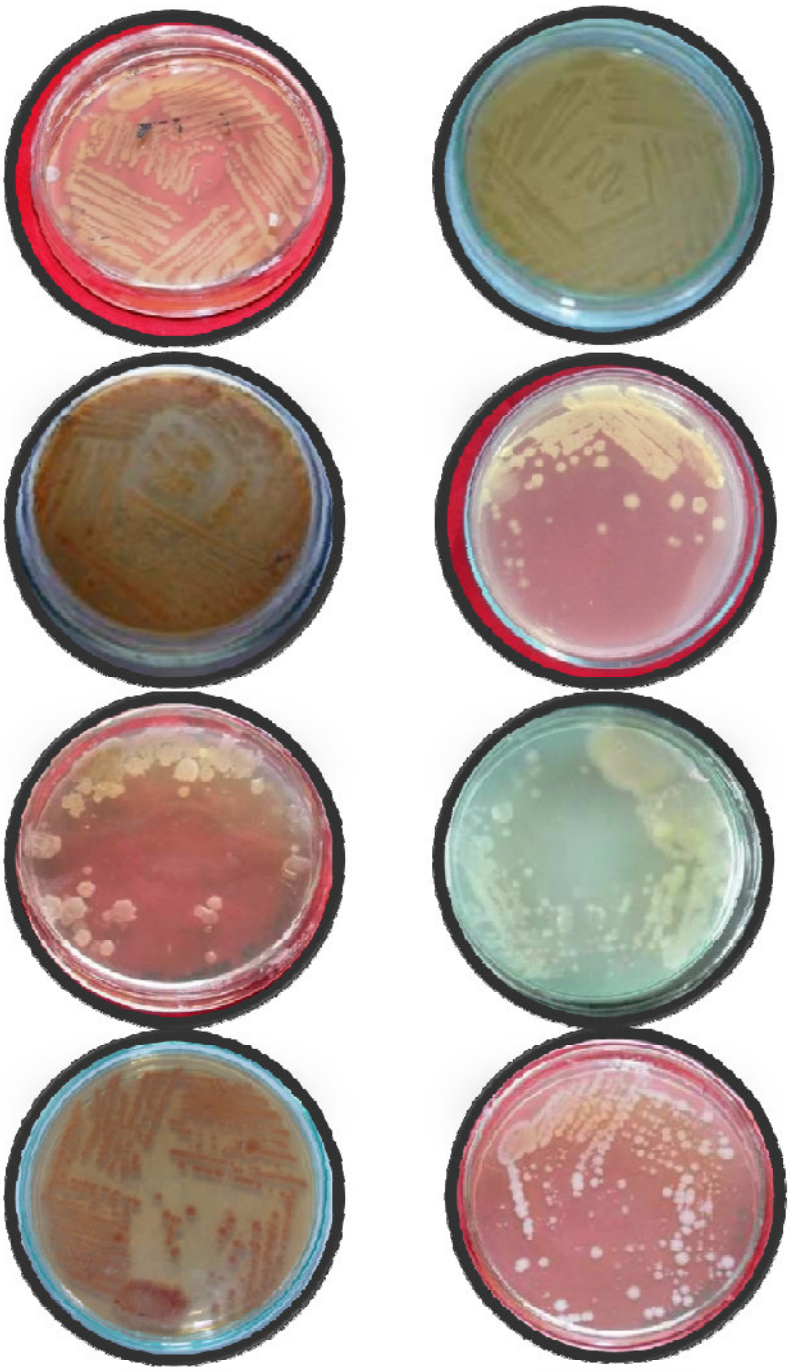


Plate 13: Selected laccase producing thermophilic bacterial isolates

A)	VS10 on TYM+ 5mM Guaiacol	B)	VS10 on TYM
C)	VS9 on TYM+ 5mM Guaiacol	D)	VS9 on TYM
E)	VR2 on TYM+ 5mM Guaiacol	F)	VR2 on TYM
G)	VW2 on TYM+ 5mM Guaiacol	H)	VW2 on TYM



Plate 14: **Selected laccase producing thermophilic bacterial isolates**

A) **VS8 on TYM+ 5mM Guaiacol** **B)** **VS8 on TYM**

C) **MS2.2 on TYM+ 5mM Guaiacol** **D)** **MS2.2 on TYM**

E) **MW17on TYM+ 5mM Guaiacol** **F)** **MW17 on TYM**

G) **TS8 on TYM+ 5mM Guaiacol** **H)** **TS8 on TYM**

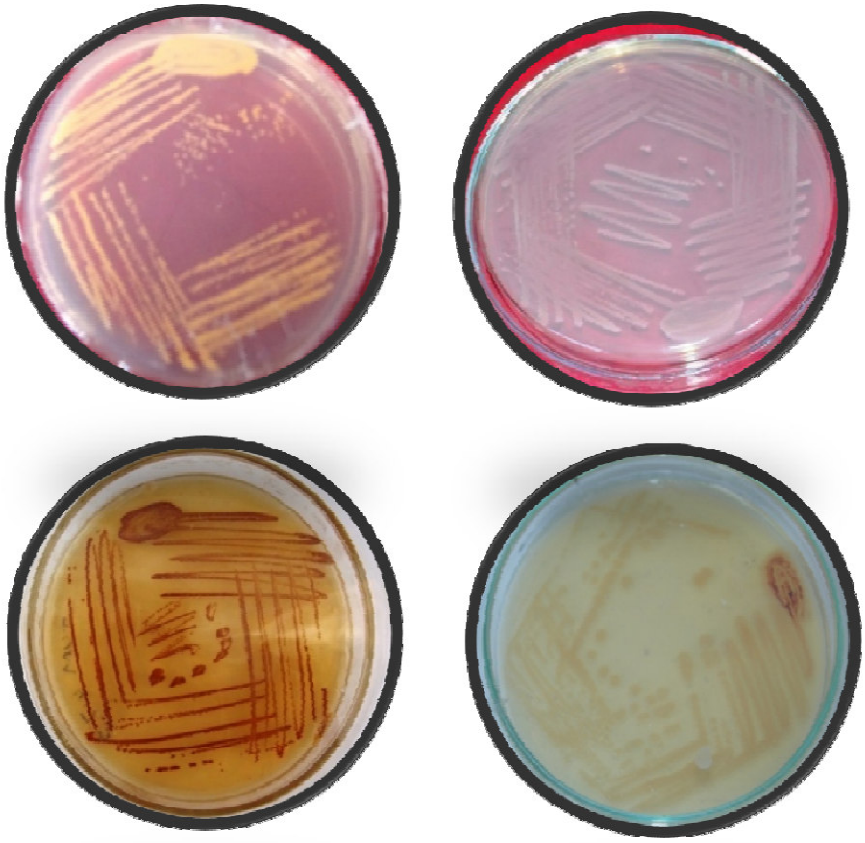


Plate 15: Selected laccase producing thermophilic bacterial isolates

A) VW1 on TYM+ 5 mM Guaiacol B) VW1 on TYM

C) VW5 on TYM+ 5 mM Guaiacol D) VW5 on TYM

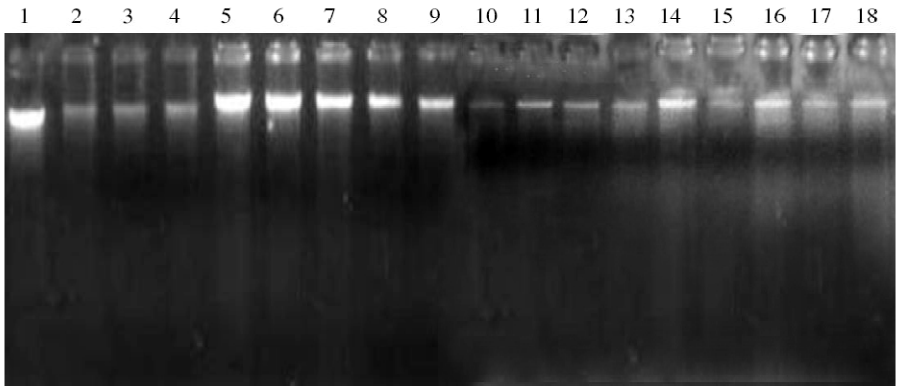


Plate 16: **Gel image showing genomic DNA extracted from selected eighteen laccase producing bacterial isolates.**

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Table 47: Summary of the RAPD amplified products obtained from selected 18 laccase producing thermophilic bacterial isolates

Total number of primers screened	35
Total number of primers produced significant amplification	19
Total number of bands amplified	679
Total number of polymorphic bands identified	463
Total number of monomorphic bands identified	216
Size range of amplified products	110-5000 bp
Percentage of total polymorphic bands	68.25 %

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4.10.2.1 RAPD pattern with OPA primer series

All the 20 random primers of OPA series were evaluated for PCR-amplification with DNA of 18 laccase producing thermophilic bacterial isolates. Only nine OPA series random primers were able to amplify the genomic DNA of the 18 laccase producing thermophilic bacterial isolates. Eight random primers viz; OPA-01, OPA-03, OPA-09, OPA-11, OPA-12, OPA-16, OPA-18 and OPA-20 were found to be polymorphic and only one primer OPA-06 was found to be monomorphic in nature.

With OPA-01 primer, total numbers of scorable bands enumerated were 28 and all were found to be polymorphic in nature. Whereas one laccase producing thermophilic bacterial isolate VS8 showed no amplification with OPA-01 primer. Three unique markers OPA-01_{200}, OPA-01_{240} and OPA-01_{260} were found in VW2, TR2 and VS10 laccase producing thermophilic bacterial isolates respectively. Primer OPA-03 was found to produce 28 amplification products. OPA-03_{3600} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates. Three unique RAPD markers were found with this primer. Laccase producing thermophilic bacterial isolate KW9.1 showed two unique markers OPA-03_{1300} and OPA-03_{1700}, whereas Laccase producing thermophilic bacterial isolate VR2 showed one unique marker OPA-03_{1500}. The OPA-06 primer showed amplification in all the 18 laccase producing thermophilic bacterial isolates and thus no polymorphism was detected by this primer. OPA-06_{2500} OPA-06_{3000} and OPA-06_{4000} bands were found to

monomorphic in nature, as they were present in all 18 laccase producing thermophilic bacterial isolates.

Primer OPA-09 showed amplification with all 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 37 and all were found to be polymorphic in nature. RAPD analysis with OPA-11 primer showed amplification in all 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 26 and out of them 18 were monomorphic and 8 were polymorphic. OPA-11_{4000} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates. OPA-11_{2700} and OPA-11_{3000} bands were found to be present only in isolates VW5, MW17, TR4.2 and VR2, whereas rest of the laccase producing thermophilic bacterial isolates showed no amplified product of this size. Primer OPA-12 was found to be highly polymorphic. The total numbers of scorable bands were 65, and all bands were polymorphic. Size of polymorphic bands ranged from OPA-12_{200} - OPA-12_{5000}.

RAPD analysis with OPA-16 primer showed amplification in all 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 34 and out of them 18 were monomorphic in nature and 16 were polymorphic. OPA-16_{3100} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates. OPA-16_{500} band was found only in VW2, TW5 and MS2, whereas rest of other laccase producing thermophilic bacterial isolates of this size. OPA-16_{2000} band was found in VW5, VW2, VS8, MS2, VS9, MW17 and VW1. OPA-16_{1000} band was found in VW2, VS8, and MW17 laccase producing thermophilic bacterial isolates, however rest of other laccase producing thermophilic bacterial isolates of this size. OPA-16 primer showed a unique band OPA-16_{3500} present in laccase producing thermophilic bacterial isolate VW5. Primer OPA-18 showed amplification in 16 laccase producing thermophilic bacterial isolates and two isolates KR7.2 and KW4 showed no amplification with this primer. Total numbers of scorable bands were found to be 51 and all were polymorphic in nature. Polymorphic bands obatned using OPA-18_{1000}, band was found to be

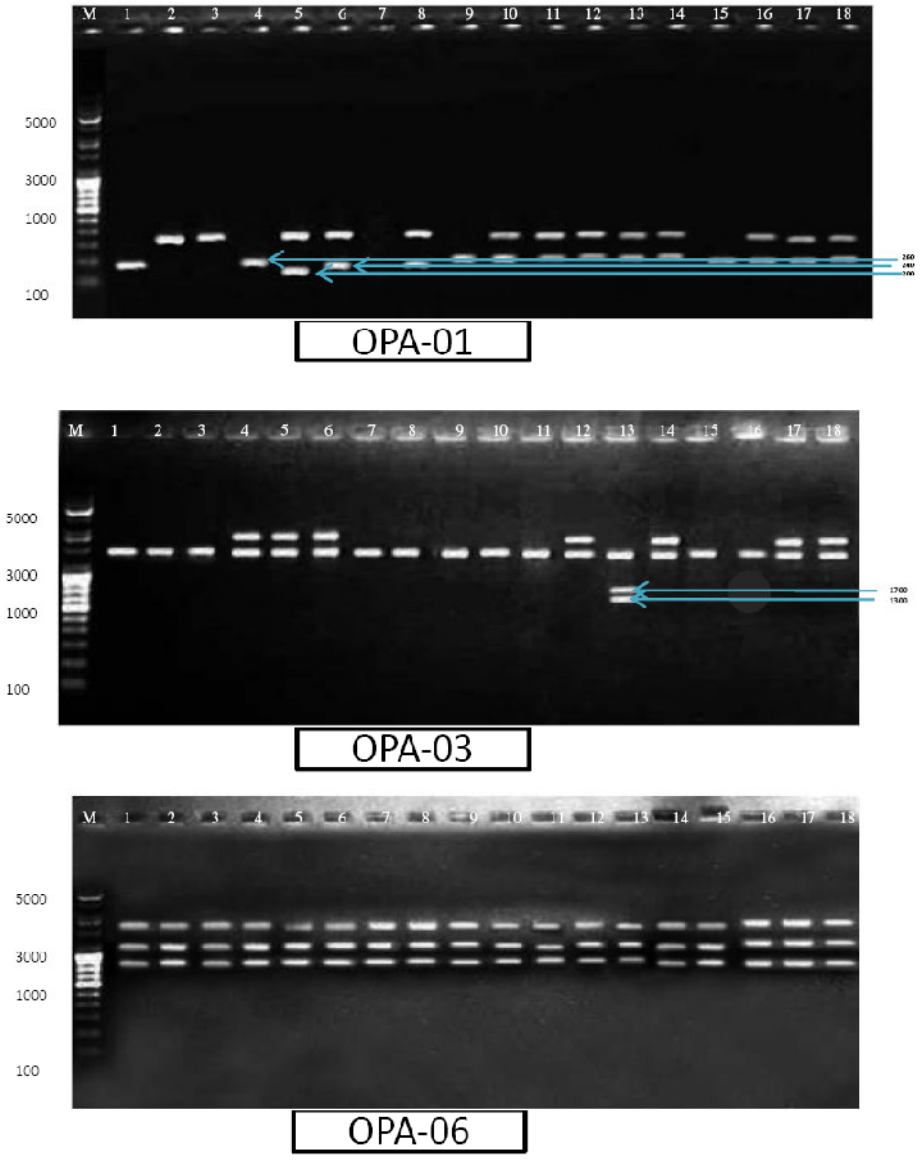


Plate 17: RAPD amplified products of laccase producing bacterial isolates (18) using OPA-01, OPA-03 and OPA-06 primers.

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M :Molecular marker (100 bp -5 kb DNA ladder)

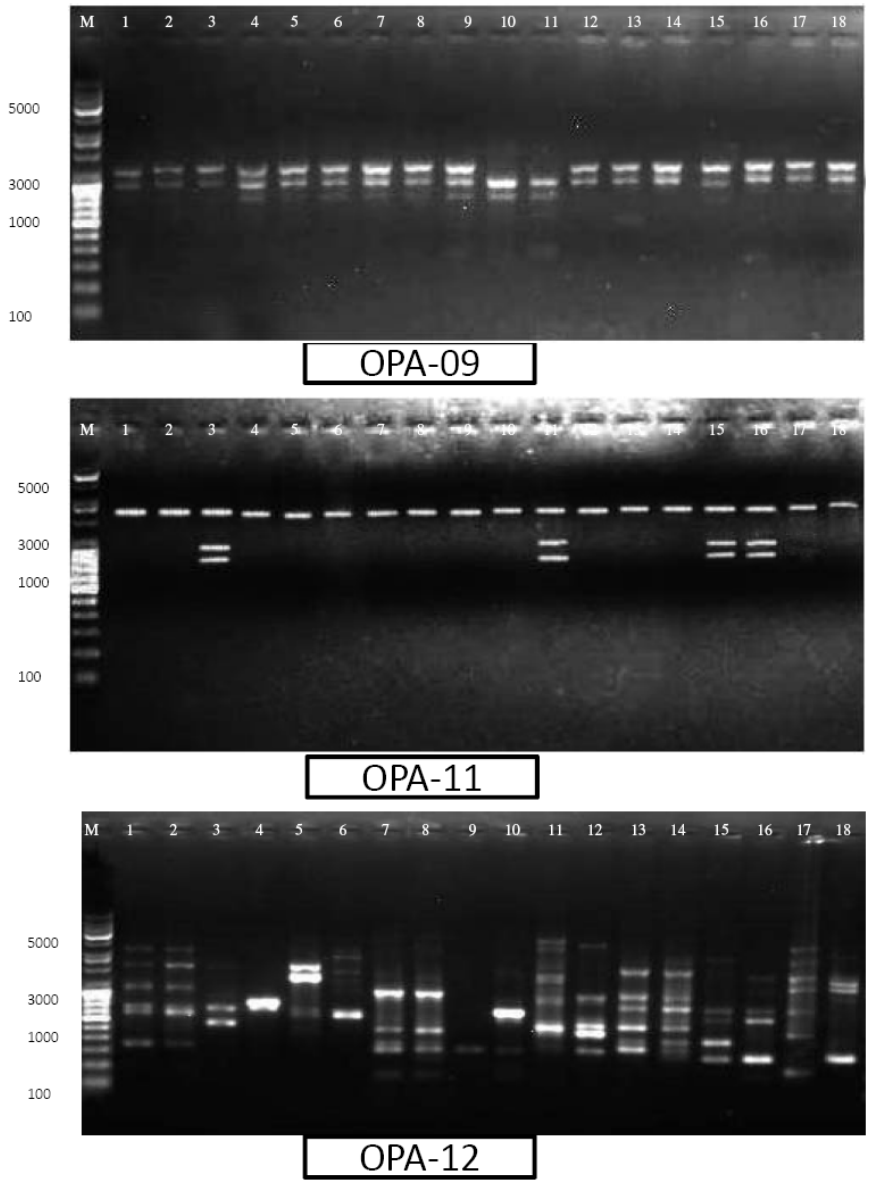


Plate 18: RAPD amplified products of laccase producing bacterial isolates (18) using OPA-09, OPA-11 and OPA-12 primers.

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M : Molecular marker (100 bp -5 kb DNA ladder)

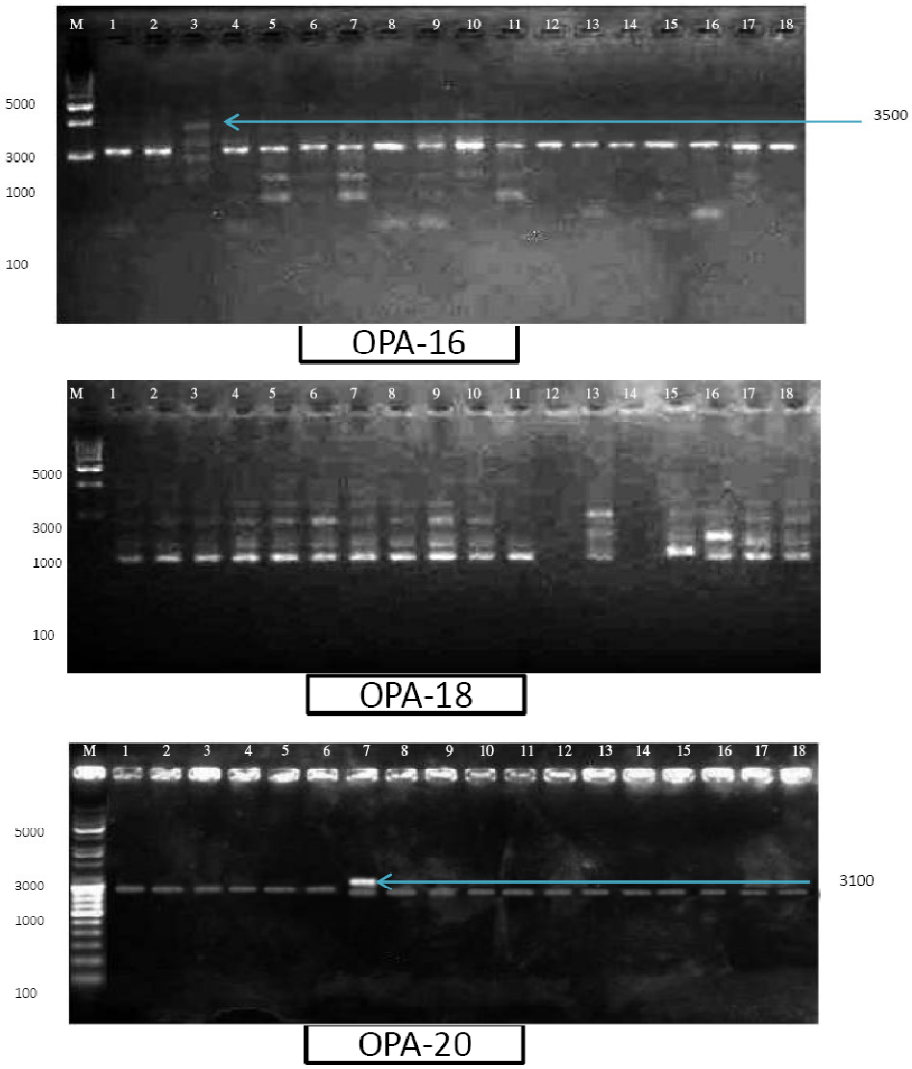


Plate 19: **RAPD amplified products of laccase producing bacterial isolates (18) using OPA-16, OPA-18 and OPA-20 primers.**

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M : Molecular marker (100 bp -5 kb DNA ladder)

present in 16 laccase producing thermophilic bacterial isolates and absent in two isolates KR7.2 and KW4. OPA-18_{2000}, OPA-18_{3000}, and OPA-18_{3100}, bands were found to be absent in TS8, KW3.1, KW9.1, KR7.2, MW17 and KW4 laccase producing thermophilic bacterial isolates. With OPA-20 primer, total numbers of scorable bands were 19 and all were found to be monomorphic. RAPD analysis with OPA-20 primer showed a unique marker OPA-20_{3100} present in SV8 laccase producing thermophilic bacterial isolate. OPA-20_{3000} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates.

4.10.2.2 RAPD pattern with OPB primer series

Ten OPB series primers were evaluated for PCR-amplification with DNA of 18 laccase producing thermophilic bacterial isolates. Only 8 OPB series random primers were able to amplify the genomic DNA of the 18 laccase producing thermophilic bacterial isolates. Eight random primers viz; OPB-01, OPB-02, OPB-03, OPB-04, OPB-06, OPB-07, OPB-08 and OPB-10 were found to be polymorphic bands in all the 18 laccase producing thermophilic bacterial isolates.

Primer OPB-01 showed amplification in 17 laccase producing thermophilic bacterial isolates, whereas VS8 laccase producing thermophilic bacterial isolate showed no amplification with OPB-01 primer. Total numbers of scorable bands were found to be 36 and two bands OPB-01_{600} and OPB-01_{3200} were found to be present in all 17 laccase producing thermophilic bacterial isolates. Two laccase producing thermophilic bacterial isolates KW9.1 and VR2 showed OPB-01_{1000} band, whereas rest of laccase producing thermophilic bacterial isolates band was absent. The OPB-02 primer showed amplification in all the 18 laccase producing thermophilic bacterial isolates and thus no polymorphism was detected by this primer. OPB-02_{600} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates. RAPD analysis with OPB-03 primer showed amplification in all 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 25 and out of them 18 were monomorphic and 7 were

polymorphic. OPB-03_{3000} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates. OPB-03_{3300} band were found to be present only in 7 laccase producing thermophilic bacterial isolates viz; TR2, VW2, VS10, KW4, KR7.2, VW1 and KW8.

RAPD analysis with OPB-04 primer showed amplification in all 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 38 polymorphic in nature. OPB-04_{500} band was amplified by nine laccase producing thermophilic bacterial isolates whereas rest showed no amplified product of this size. All 17 laccase producing thermophilic bacterial isolates showed amplification with OPB-04 primer whereas laccase producing thermophilic bacterial isolate VS8 showed no amplified product. OPB-04_{3600} band was amplified by ten laccase producing thermophilic bacterial isolates whereas rest showed no amplified product of this size. OPB-04 primer showed two unique markers OPB-04_{200} and OPB-04_{800} present in KW3.1 laccase producing thermophilic bacterial isolate. Primer OPB-06 showed amplification in all the 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 21 and out of them 18 were monomorphic and 3 were polymorphic. OPB-06_{3000} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates. OPB-06 primer showed three unique markers of OPB-06_{1500}, OPB-06_{3500} and OPB-06_{4000} present in SV8 laccase producing thermophilic bacterial isolate. With OPB-07 primer, total numbers of scorable bands were 47 and all were found to be polymorphic.

The OPB-08 primer showed amplification in all the 18 laccase producing thermophilic bacterial isolates. OPB-08_{3500} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates and thus no polymorphism was detected by this primer. RAPD analysis with OPB-10 primer showed amplification in all 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 32 and out of them 18 were monomorphic and 14 were polymorphic. Bands OPB-10_{3700} and OPB-10_{4000} were found to be present only in 7 laccase producing

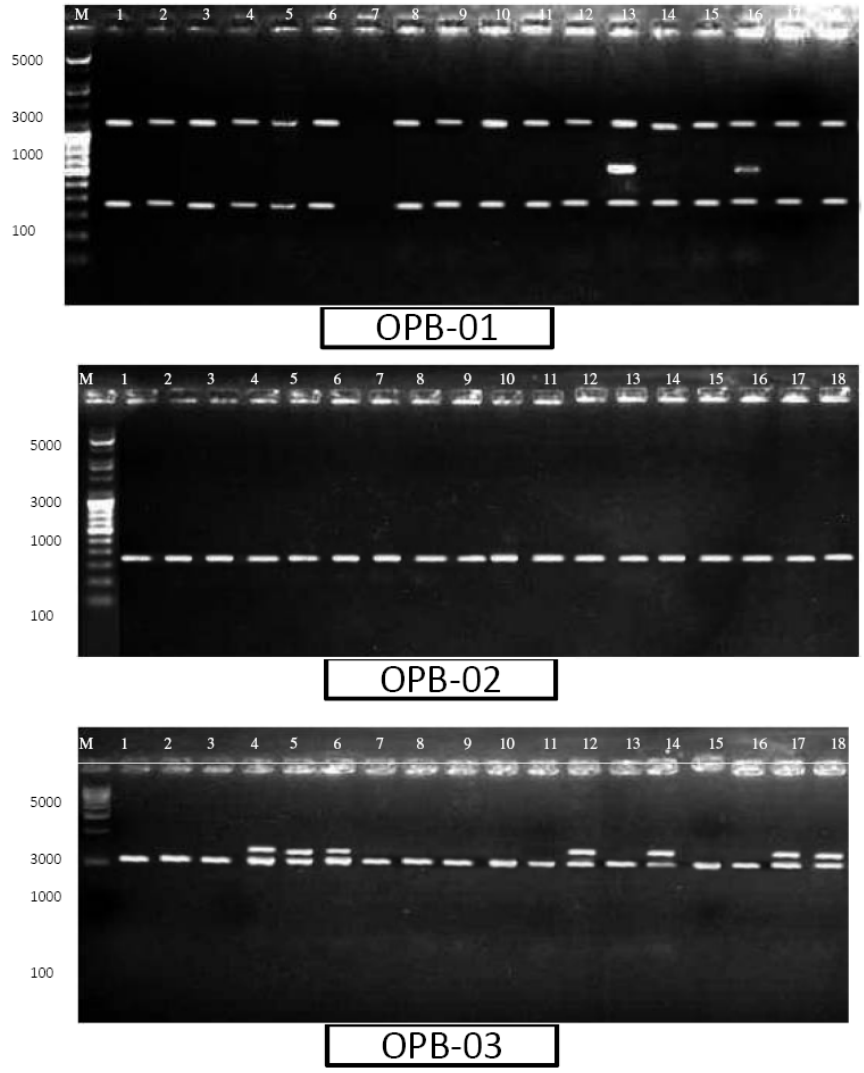


Plate 20: RAPD amplified products of laccase producing bacterial isolates (18) using OPB-01, OPB-02 and OPB-03 primers.

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M : Molecular marker (100 bp -5 kb DNA ladder)

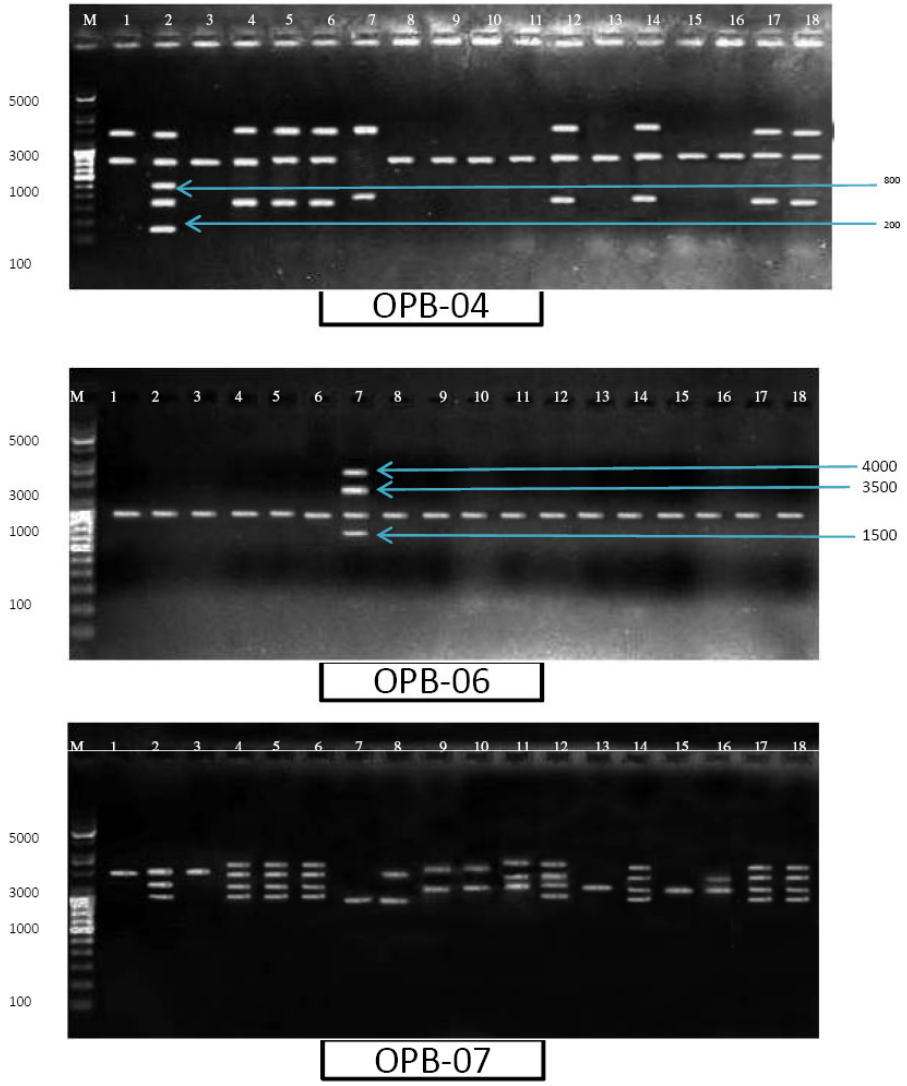


Plate 21: RAPD amplified products of laccase producing bacterial isolates (18) using OPB-04, OPB-06 and OPB-07 primers.

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M : Molecular marker (100 bp -5 kb DNA ladder)

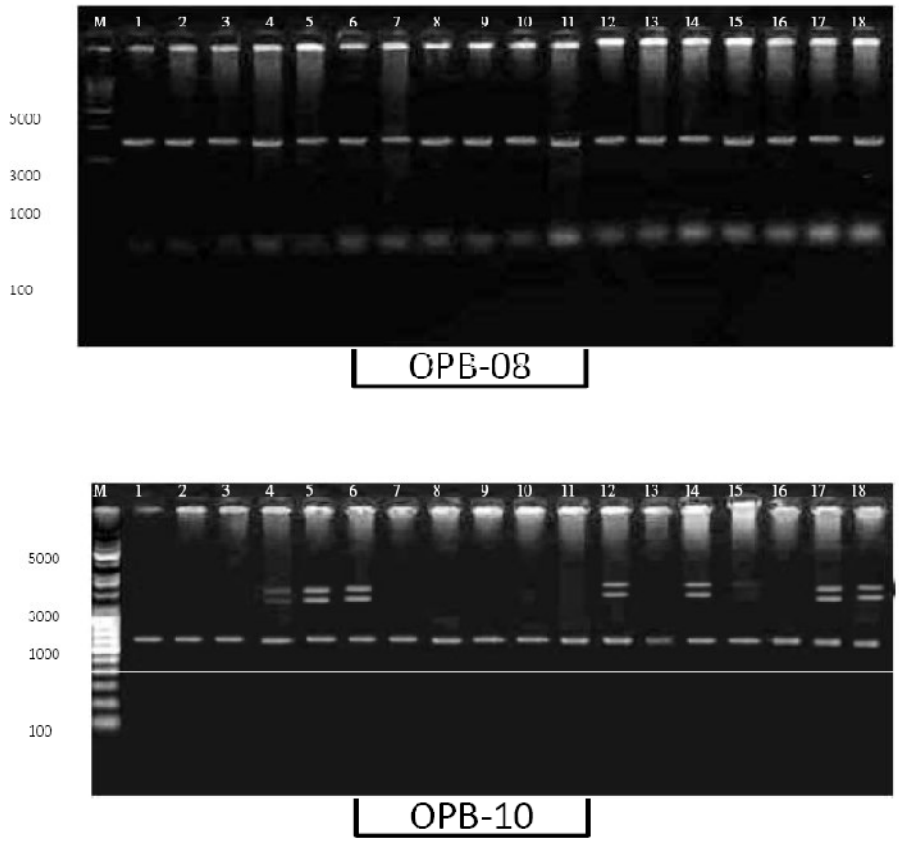


Plate 22: RAPD amplified products of laccase producing bacterial isolates (18) using OPB-08 and OPB-10 primers.

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M : Molecular marker (100 bp -5 kb DNA ladder)

thermophilic bacterial isolates viz; TR2, VW2, VS10, KW4, KR7.2, VW5 and KW8. OPB-10_{2500} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates.

4.10.2.3 RAPD pattern with OPC primer series

In this OPC series, 5 primers used and only 2 primers viz; OPC-06 and OPC-07 showed amplification in all the 18 laccase producing thermophilic bacterial isolates. Total numbers of scorable bands with OPC series were 32 and out of them 18 were monomorphic and 14 were polymorphic.

Primer OPC-06 showed amplification in all the 18 laccase producing thermophilic bacterial isolates and total numbers of scorable bands were 30 and out of them 18 were monomorphic and 12 were polymorphic. OPC-06_{3700} band was found to monomorphic in nature, as it was present in all 18 laccase producing thermophilic bacterial isolates. Bands OPC-06_{500} and OPC-06_{3500} were found to be amplified by 4 laccase producing thermophilic bacterial isolates viz: TW8, VS9, KW9.1 and KR7.2, whereas OPC-06_{1000} and OPC-06_{3000} bands were amplified by only VS10 laccase producing thermophilic bacterial isolate. OPC-06 primer showed unique markers OPC-06_{4000} present in laccase producing thermophilic bacterial isolate MS2.2. OPA-12 primer was found to be highly polymorphic. The total numbers of scorable bands were 74 and all bands were polymorphic. OPC-07_{110} band was found to be present only in two isolates viz; KW9.1 and VR2, whereas absent in rest of 16 laccase producing thermophilic bacterial isolates. TR2 laccase producing thermophilic bacterial isolate showed only one amplified band of 800 bp. Laccase producing thermophilic bacterial isolate KW8 showed unique marker OPC-07_{5000}.

RAPD analysis of 18 laccase producing thermophilic bacterial isolates produced a total of 679 amplified bands, in the size range of ~110-5000 bp. Sixteen preliminary informative DNA-based markers (unique bands), suggesting that these DNA-based markers can be used for identification of these laccase producing bacterial isolates (Table-48).

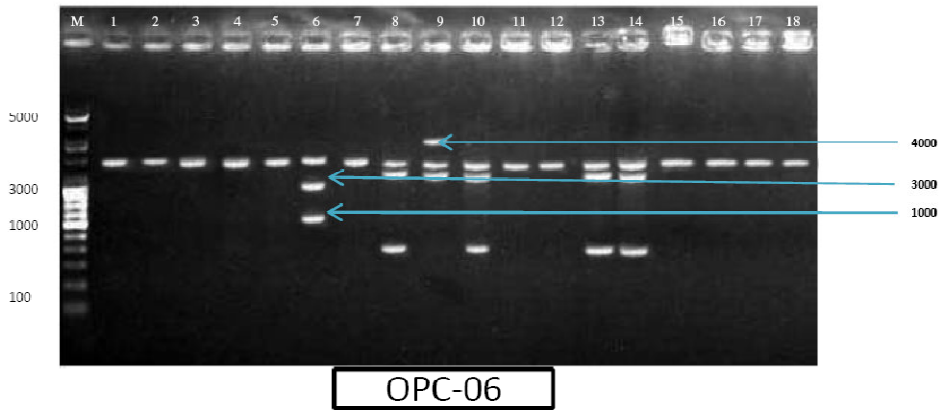
Table 48: RAPD markers specific for selected laccase producing thermophilic bacterial isolates

Sr No.	Unique Bands	Specific isolate
1.	OPA-01_{200}	VW2
2.	OPA-01_{240}	TR2
3.	OPA-01_{260}	VS10
4.	OPA-03_{1300}	KW9.1
5.	OPA-03_{1700}	KW9.1
6.	OPA-16_{3500}	VW5
7.	OPA-20_{3100}	VS8
8.	OPB-04_{200}	KW3.1
9.	OPB-04_{800}	KW3.1
10.	OPB-06_{1500}	VS8
11.	OPB-06_{3500}	VS8
12.	OPB-06_{4000}	VS8
13.	OPC-06_{4000}	MS2.2
14.	OPC-06_{1000}	VS10
15.	OPC-06_{3000}	VS10
16.	OPC-07_{5000}	KW8

RAPD-PCR profile was analyzed by the SIMQUAL program of NTSYS-pc (version 2.20) and similarities were estimated using the Jaccard's coefficient to develop a dendrogram using the UPGMA program. The similarity coefficient revealed that the similarity among 18 laccase producing bacterial isolates ranged between 64 to 91 percent. The results (Fig-25) revealed that the first major bifurcation of the dendrogram segregated the 18 laccase producing thermophilic bacterial isolates into two main clusters A and B. Cluster B was clearly separate out SV8 from other isolates and A cluster further devided into sub-clusters C and D, where C further devided into Sub sub clusters, similarly s D devided into two sub clusters and further devided into Sub sub clusters. Strain TS8 and KW 3.1 Showed 91 percent similarity.

4.10.2.4 Characterization of thermophilic bacterial isolates using 16S rDNA technology

The eighteen thermolaccase producing thermophilic bacterial isolates were further characterized using 16S rRNA gene technology and all the 18 genomic DNA samples extracted from these isolates were selectively amplified



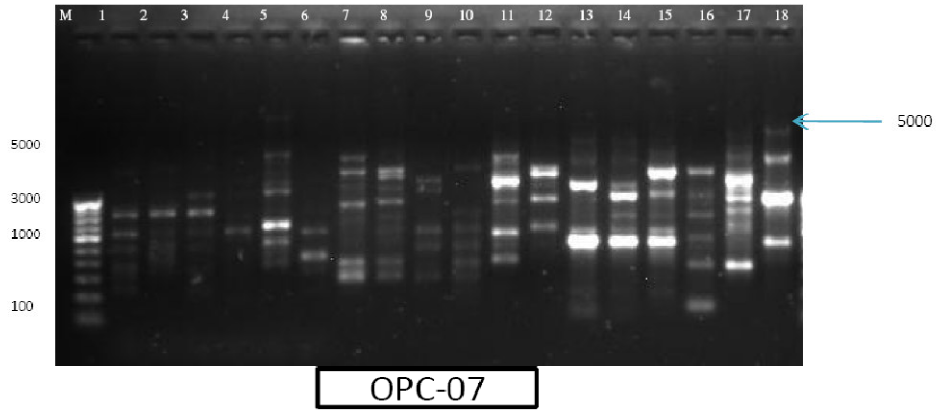


Plate 23: RAPD amplified products of laccase producing bacterial isolates (18) using OPC-06, and OPC-07 primers.

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M : Molecular marker (100 bp -5 kb DNA ladder)

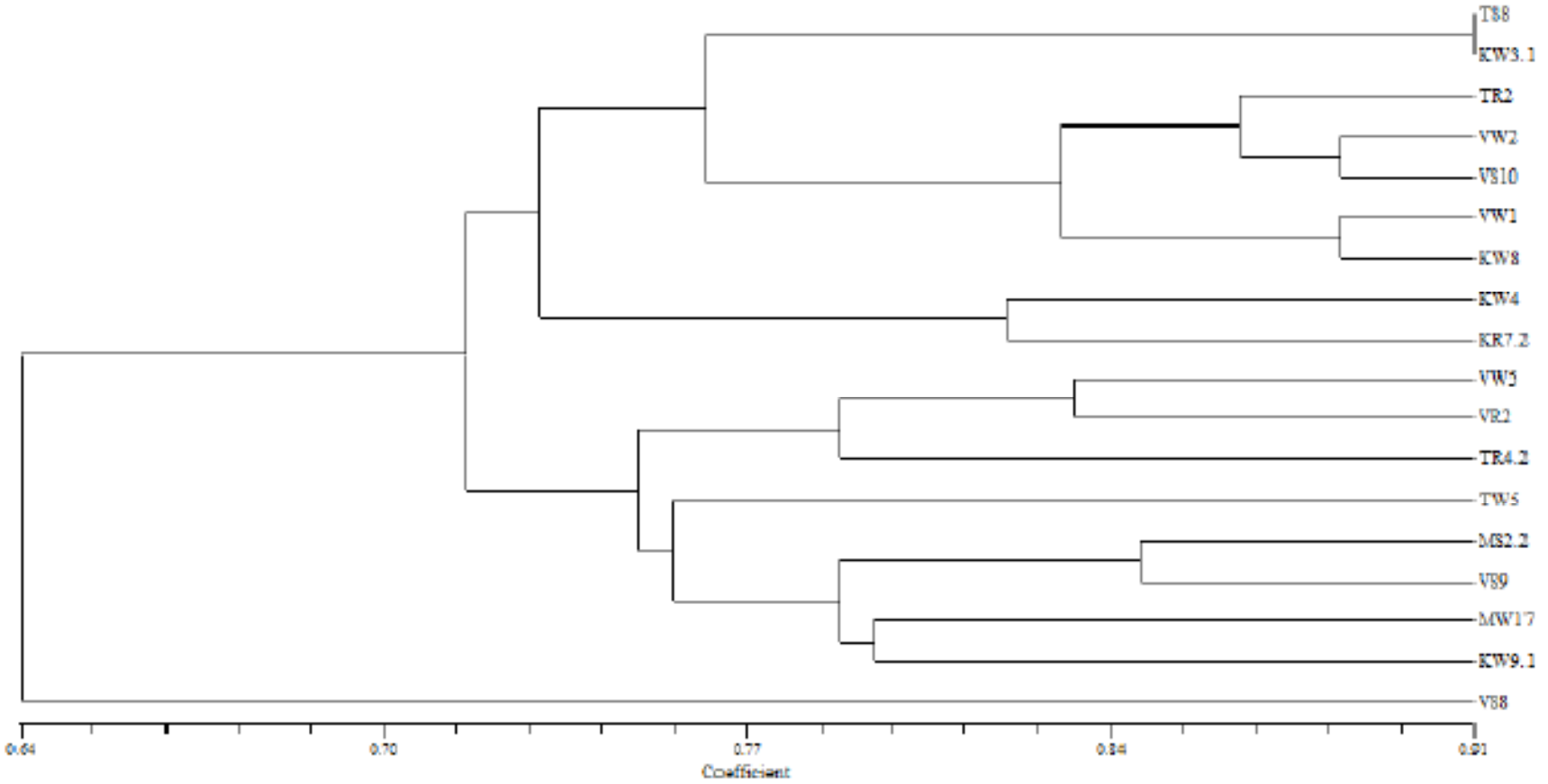


Fig. 25: Dendrogram showing relatedness among 18 laccase producing thermophilic bacterial isolates based on RAPD-PCR markers

using PCR technology. Universal primer B27F and U1492R for 16S rRNA gene was used for this experimentation. After 30 cycles of PCR amplification as described under section 3.10.3, universal primer for 16S rRNA gene were able to successfully amplify 16S rRNA gene of all the 18 thermophilic bacterial isolates and produced an amplicon of expected size i.e. 1500 bp (Plate-24).

4.10.3 Sequencing

The selected 18 bacterial isolates were sequenced followed by *in silico* analysis. To determine the nucleotide sequence of the PCR products of the 16S rRNA gene of all the 18 isolates, sequencing was carried out using same upstream and downstream primers which were used for amplification and as a result, the size of the 16S rRNA gene sequences of all the 18 bacterial isolates was found to range between 304 bp - 1135 bp. On the basis of results obtained from 16S rRNA gene analysis and in addition to G+C content analysis, the selected 18 laccase producing bacterial isolates were found to belong to two genera *Bacillus* and *Pseudomonas* (Table-49-66).

Seventeen isolates showed similarity to *Bacillus* species on the basis 16S rRNA gene analysis and G+C content. Whereas rest of one bacterial isolate was found to be similar to genus *Pseudomonas*. The nucleotide sequences of these fourteen selected laccase producing bacterial isolates, obtained after sequencing of 16S rRNA gene have been depicted here as under:

Table 49: Nucleotide base composition in the query sequence (TR2 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	136	20.2
Thymine (T)	171	25.4
Cytosine (C)	192	28.6
Guanine (G)	173	25.7
G+C	365	54.3
A+T	307	45.7

Table 50: Nucleotide base composition in the query sequence (VS8 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	234	20.6
Thymine (T)	283	24.9
Cytosine (C)	342	30.1
Guanine (G)	276	24.3
G+C	618	54.4
A+T	517	45.4

Table 51: Nucleotide base composition in the query sequence (VS10 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	220	20.2
Thymine (T)	273	25.0
Cytosine (C)	335	30.7
Guanine (G)	262	24.0
G+C	597	54.8
A+T	493	45.2

Table 52: Nucleotide base composition in the query sequence (MS2.2 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	221	19.9
Thymine (T)	276	24.9
Cytosine (C)	346	31.3
Guanine (G)	264	23.8
G+C	610	55.0
A+T	497	45.0

Table 53: Nucleotide base composition in the query sequence (VW20 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	166	20.3
Thymine (T)	209	25.6
Cytosine (C)	241	29.5
Guanine (G)	200	24.5
G+C	441	54.0
A+T	375	46.0

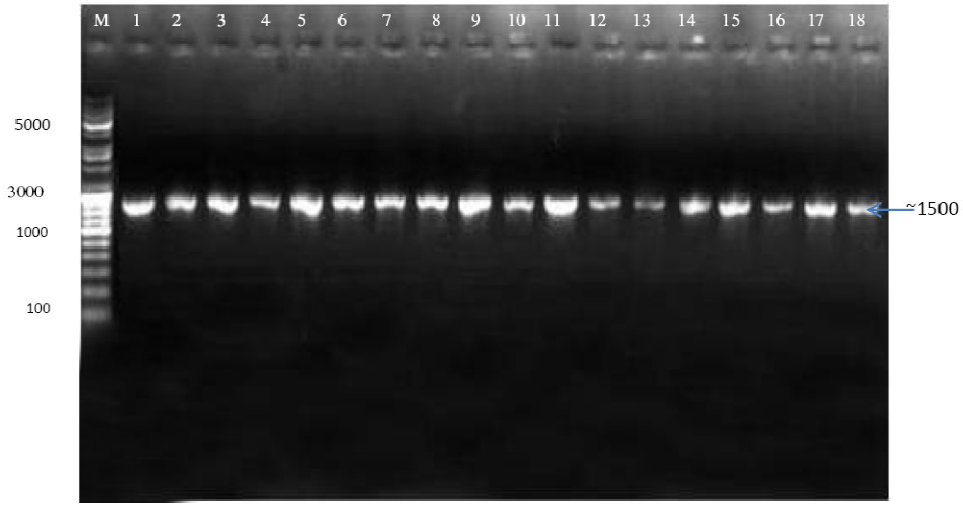


Plate 24: Amplicons of 16S rRNA gene of selected eighteen laccase producing bacterial isolates.

Lane1: TS8	**Lane 7: VS8**	**Lane13: KW9.1**
Lane 2 : KW3.1	**Lane 8: TW5**	**Lane14: KR7.2**
Lane 3: VW5	**Lane 9: MS2.2**	**Lane15: TR4.2**
Lane 4: TR2	**Lane 10: VS9**	**Lane16: VR2**
Lane 5 : VW2	**Lane 11: MW17**	**Lane17: VW1**
Lane 6: VS10	**Lane12: KW4**	**Lane18: KW8**

Lane M : Molecular marker (100 bp -5 kb DNA ladder)

Table 54: Nucleotide base composition in the query sequence (TR4.2 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	167	23.9
Thymine (T)	117	16.8
Cytosine (C)	171	24.5
Guanine (G)	242	34.7
G+C	413	59.3
A+T	284	40.7

Table 55: Nucleotide base composition in the query sequence (KR7.2 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	149	26.84
Thymine (T)	108	19.45
Cytosine (C)	133	23.9
Guanine (G)	165	29.7
G+C	298	53.7
A+T	257	46.3

Table 56: Nucleotide base composition in the query sequence (KW4 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	132	26.6
Thymine (T)	89	17.9
Cytosine (C)	139	27.9
Guanine (G)	137	27.6
G+C	276	55.5
A+T	221	44.5

Table 57: Nucleotide base composition in the query sequence (KW8 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	75	24.7
Thymine (T)	60	19.7
Cytosine (C)	73	24.0
Guanine (G)	96	31.6
G+C	169	55.6
A+T	135	44.4

Table 58: Nucleotide base composition in the query sequence (VR2 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	96	24.4
Thymine (T)	74	18.8
Cytosine (C)	101	25.6
Guanine (G)	123	31.2
G+C	224	56.9
A+T	170	40.1

Table 59: Nucleotide base composition in the query sequence (TW5 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	124	20.7
Thymine (T)	146	24.3
Cytosine (C)	174	29.0
Guanine (G)	156	26.0
G+C	330	55.0
A+T	270	45.0

Table 60: Nucleotide base composition in the query sequence (KW9.1 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	150	25.2
Thymine (T)	129	21.7
Cytosine (C)	126	21.2
Guanine (G)	190	31.9
G+C	316	53.0
A+T	279	47.0

Table 61: Nucleotide base composition in the query sequence (VW1 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	95	20.7
Thymine (T)	111	24.1
Cytosine (C)	129	28.0
Guanine (G)	125	27.2
G+C	254	55.2
A+T	206	44.8

Table 62: Nucleotide base composition in the query sequence (MW17 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	122	27.3
Thymine (T)	97	21.7
Cytosine (C)	107	23.9
Guanine (G)	121	27.1
G+C	228	51.0
A+T	219	49.0

Table 63: Nucleotide base composition in the query sequence (VS9 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	154	25.5
Thymine (T)	123	20.4
Cytosine (C)	145	24.0
Guanine (G)	181	30.0
G+C	326	54.1
A+T	227	45.9

Table 64: Nucleotide base composition in the query sequence (KW3.1 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	231	25.4
Thymine (T)	181	19.9
Cytosine (C)	211	23.2
Guanine (G)	286	31.5
G+C	497	54.7
A+T	412	45.3

Table 65: Nucleotide base composition in the query sequence (ST8 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	145	21.3
Thymine (T)	165	24.2
Cytosine (C)	218	32.0
Guanine (G)	153	22.5
G+C	371	54.5
A+T	310	45.5

Table 66: Nucleotide base composition in the query sequence VW5 isolate)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	144	20.3
Thymine (T)	174	24.5
Cytosine (C)	230	32.4
Guanine (G)	161	22.7
G+C	391	55.1
A+T	318	44.9

4.10.4 *In silico* analysis of the sequences

Further *in silico* analysis pertaining to the sequences, so obtained, was carried out using various bioinformatic tools available online such as, BLASTn, ClustalW and MEGA 6. Analysis of 16S rRNA gene of the selected 18 isolates revealed their homology with various other 16S rRNA gene sequences. Various online approaches were followed to assess and predict relationship of the selected isolates with other 16S rRNA gene sequences.

4.10.5 BLASTn

BLASTn search of these 18 sequences with the most similar 16S rRNA gene sequences of the GenBank database (http://www.ncbi.nlm.nih.gov/blast) revealed the closest sequence identities from the sequence database. All the similarities have been summarized (Table-67). Characterization of the laccase producing bacterial isolates on basis of the 16S rRNA gene coding genes revealed that the all the 18 laccase producing bacterial isolates showed maximum similarity with *Pseudomonas* species and *Bacillus* species. Sequences similarity between 18 laccase producing bacterial isolates was compared with those from database using FASTA programme.

4.10.6 Phylogenetic analysis

To gain insight of the evolutionary pattern, phylogenetic tree was constructed using MEGA 6. Neighbour-Joining (NJ) technique of mathematical averages (UPGMA) was used. The results have been presented in the form of phylogenetic tree.

Table 67: Similarity values of 16S rRNA gene sequences of selected eighteen laccase producing bacterial isolates

Bacterial Isolate	Closest Match	Accession Number	Per cent Similarity
VS9	*Bacillus aerius* strain CCMMB963 16S ribosomal RNA gene	KF879291.1	96
MS2.2	*Bacillus sonorensis* strain SXYC19 16S ribosomal RNA gene, partial sequence	JN999894.1	96
WM17	*Bacillus subtilis* strain 1051 16S ribosomal RNA gene, partial sequence	JF322958.1	91
TS8	*Bacillus sonorensis* strain NR1 16S ribosomal RNA gene	KF895393.1	96
VW1	*Bacillus licheniformis* strain P5 16S ribosomal RNA gene, partial sequence	KP052704.1	100
VW5	*Bacillus pumilus* strain ATHA27 16S ribosomal RNA gene, partial sequence	KF680995.1	98
KR7.2	*Bacillus licheniformis* strain CFR2 16S ribosomal RNA gene, partial sequence	EF427891.1	98
KW4	*Bacillus licheniformis* strain MU4 16S ribosomal RNA gene, partial sequence	KF631227.1	99
VW2	*Bacillus licheniformis* strain hswX145 16S ribosomal RNA gene, partial sequence	JQ236837.1	98
VS10	*Bacillus licheniformis* strain SDS12 16S ribosomal RNA gene, partial sequence	KM437881.1	96
VS8	*Pseudomonas taiwanensis* strain IHB B 14565 16S ribosomal RNA gene, partial Sequence	KM817286.1	99
VR2	*Bacillus aerius* strain CCMMB963 16S ribosomal RNA gene, partial sequence	KF879291.1	97
TW5	*Bacillus sonorensis* strain RSV8 16S ribosomal RNA gene	KM518206.1	100
TR4.2	*Aneurinibacillus thermoaerophilus* strain BillorisVO18 16S ribosomal RNA gene, partial sequence	KJ913705.1	99
TR2	*Bacillus licheniformis strain LZBL-19* 16S ribosomal RNA gene, partial sequence	JX847129.1	98
KW8	*Bacillus licheniformis* partial 16S rRNA gene, isolate DV7	HE586589.1	98
KW9.1	*Bacillus sonorensis* strain NR1 16S ribosomal RNA gene, partial sequence	KF895393.1	97
KW3.1	*Bacillus amyloliquefaciens* strain H55 16S ribosomal RNA gene, partial sequence	KC441795.1	96

4.10.7 Phylogenetic tree analysis

To trace out the evolutionary pattern of the selected 18 test bacterial isolates, and assess their relationship with other selected sequences at NCBI, phylogenetic tree was constructed using Neighbour-Joining (NJ) method of mathematical averages (UPGMA) among 16S rRNA gene sequences and corresponding gene sequences. The bacterial isolates selected were united with quite high statistical support by the bootstrap method estimates for 1,000 replications and values inferred greater than 50 percent, only were presented (Fig.26).

By Neighbor-Joining algorithm, the phylogenetic tree (Fig.27) has been divided into clusters and sub divided into sub clusters, named A, B, C, D with bootstrap values ranging from 27 to 85. The two big clusters i.e. cluster A & B was produced. Cluster A was subdivided further into two subclusters C at bootstrap value of 73 and D at bootstrap value of 40. These clusters were subcluster further and verified that bacterial isolate TR2 as *Bacillus licheniformis* RSM8, as the isolate *Bacillus licheniformis* RSM8 clustered closely with *Bacillus licheniformis* strain LZBL-01 (JX847129.1) with boot strap value of 72 .

4.10.9 IDENTIFICATION AND CHARACTERIZATION OF SELECTED THERMOLACCASE PRODUCING THERMOPHILIC BACTERIA

Among these fourteen thermolaccase producing thermophilic bacterial isolates only one bacterial isolate TR2 which produced maximum laccase activity was selected on the basis of its protein content and laccase production. Therefore, TR2 was selected for identification and for laccase production, purification and characterization and also for the studies on gene encoding laccase (Table-68).

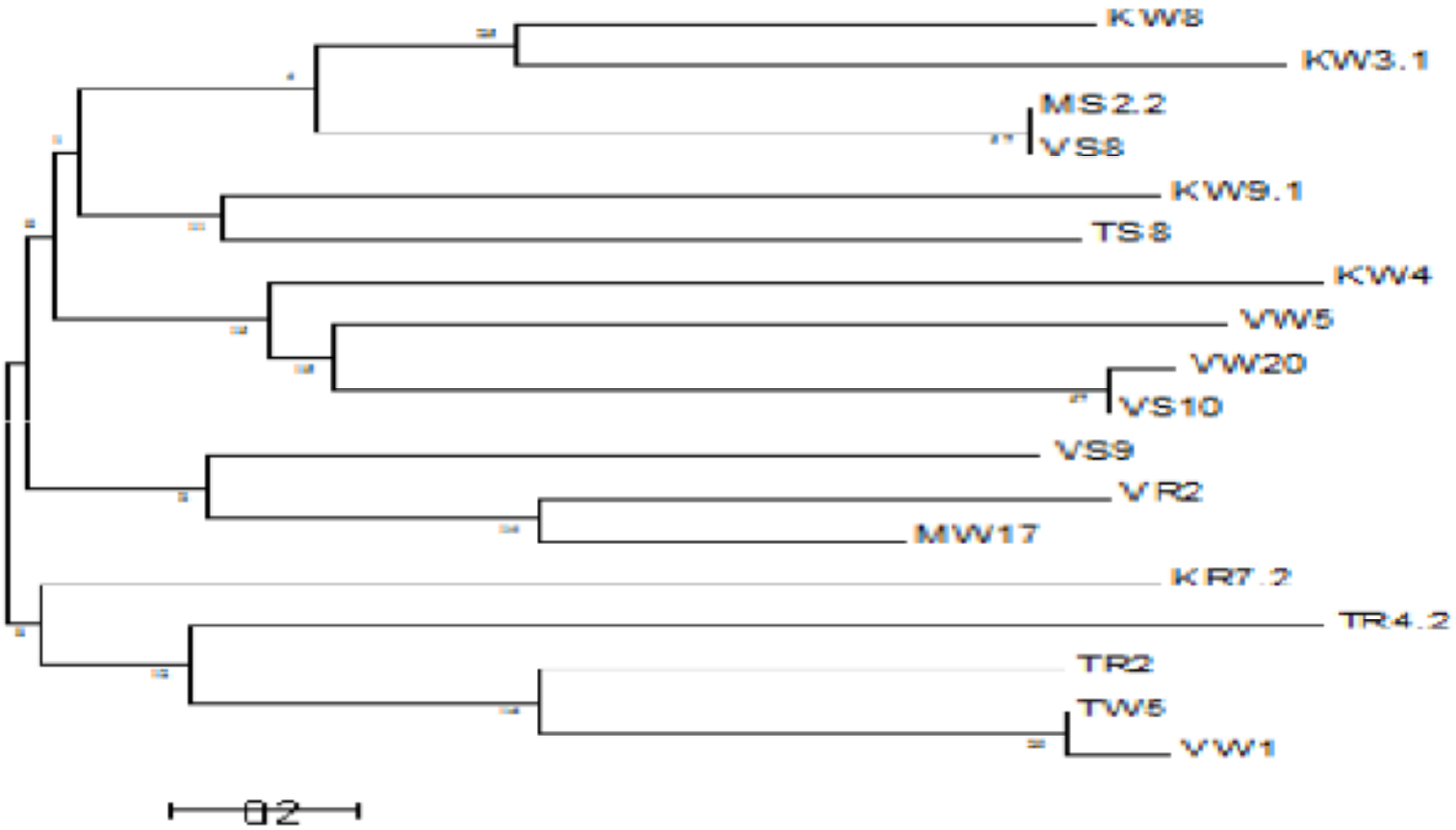


Fig. 26: Phylogenetic tree of thermolaccase producing bacteria on the basis of 16S*rrna* gene analysis

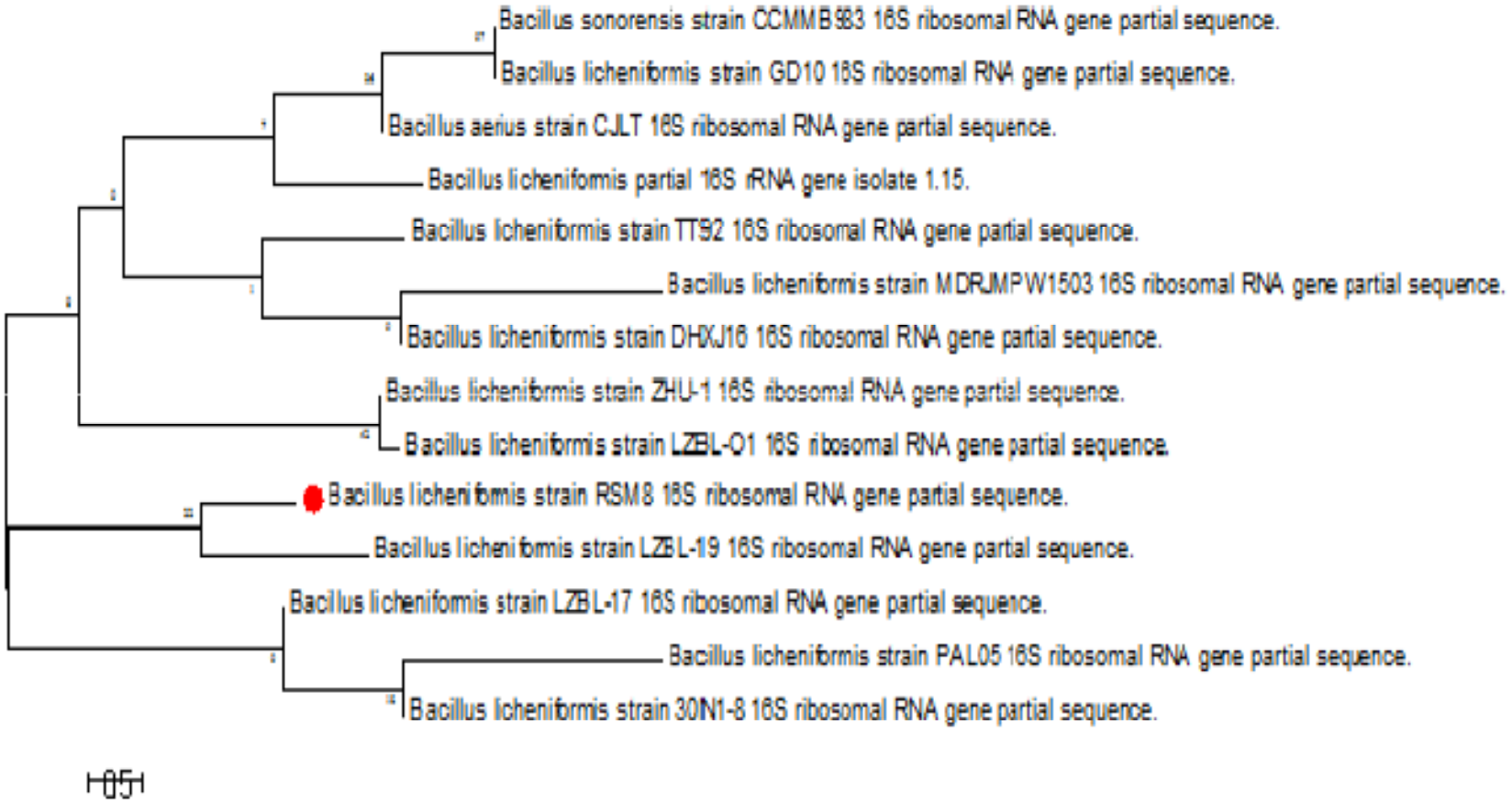


Fig.27: Phylogenetic tree of *Bacillus licheniformis* strain RSM8 on the basis of 16S *rrna* gene analysis

Table 68: Morphological, biochemical and physiological characterization of *Bacillus licheniformis* strain RSM8

Morphological characteristics					
Colony morphology	Cream, flat surface, irregular shape, rough texture				
Pigment Formation	+				
Grams reaction/ cell shape/cell size	+/rods/long/motile				
Physiological characteristics					
Growth at different temperature (55 to 70ºC)	+				
Growth at different pH (5.0 to 10.0)	+				
Biochemical tests/ carbohydrates utilization					
Catalase	+	Sucrose	+	Tyrosinase	+
Oxidase	+	Glucose	+	Cellulase	-
Voges Proskauer's	-	Arabinose	+		
Citrate utilization	-	Lactose	-		
Nitrate Reduction	+				
Hydrogen sulphide production	-				
Gelatin hydrolysis	-				
Starch hydrolysis	+				
Casein decomposition	+				
Indole production	-				
Urea test	+				

The isolate TR2 was preliminary characterized by morphological physiological and biochemical characteristics (Table-66). The isolated colonies on TYM containing 5 mM guaiacol and 40 mg/l $CuSO_4$ after 24 hrs of incubation were brown in colour, having flat surface irregular shape with rough texture. The isolated colonies in TYM containing no substrate and 40 mg/l $CuSO_4$ are cream in colour with flat surface irregular shape with rough texture. Pigment formation was observed even after 5 days whereas pellicle formation and slight turbidity was observed when the isolate was grown in TYM broth under unshaken conditions. The microscopic characteristics of the isolate are as follows: the cells were Gram postive, non-spore forming, rods occurring in single. The biochemical characteristics of the isolate are as follows: The isolate was positive for catalase,

oxidase, urease, methyl red and TSI test and negative for gelatin hydrolysis, starch hydrolysis, Voges Proskauer's, Indole production, citrate utilization test and could utilize glucose as sole carbon source.

Phylogenetic analysis of selected isolate TR2 by 16S rRNA gene analysis

Universal primer B27F and U1492R were successfully used for the amplification of 16S rRNA gene from bacterial isolate TR2. As a result of amplication, an amplicon of expected size *i.e.* ~ 1500 bp was obtained. To determine the nucleotide sequence of the PCR products of the 16S rRNA gene of TR2, sequencing was carried out using same upstream and downstream primers which were used for amplification. 672 bp sequences corresponding to 16S rRNA gene of TR2 was obtained. It was further observed that the sequence consisted of 136 adenines, 192 cytosines, 173 guanines and 171 thymines. The genomic DNA G+C content was 54.30 %.

The sequence of 16S rRNA gene from TR2 was then analysed using BLASTn analysis (http://www.ncbi.nlm.nih.gov/blast) and was found to have 99 percent homology with several *Bacillus* isolates reported from different parts of the world (Table-69). Sequence analysis revealed that TR2 (RSM8) belongs to *Bacillus licheniformis* as it showed maximum homology (98%) with *Bacillus licheniformis* strain PAL05 16S ribosomal RNA gene, partial sequence.

To trace out the evolutionary patterns of the test isolate and to find out relationship of the same with other selected sequences at NCBI, phylogenetic tree was also constructed using Neighour-Joining (J) method of mathematical averages (UPGMA) among 16S rRNA sequence of TR2 and corresponding sequence of different *Bacillus* sp. Isolate RSM8 was united with quite high statistical support by the bootstrap method estimates for 1,000 replications and values inferred greater than 50 per cent are only presented. Phylogenetic tree (Fig.28) also verified TR2 as *Bacillus licheniformis* as the isolate RSM8 clustered closely with *Bacillus licheniformis.*

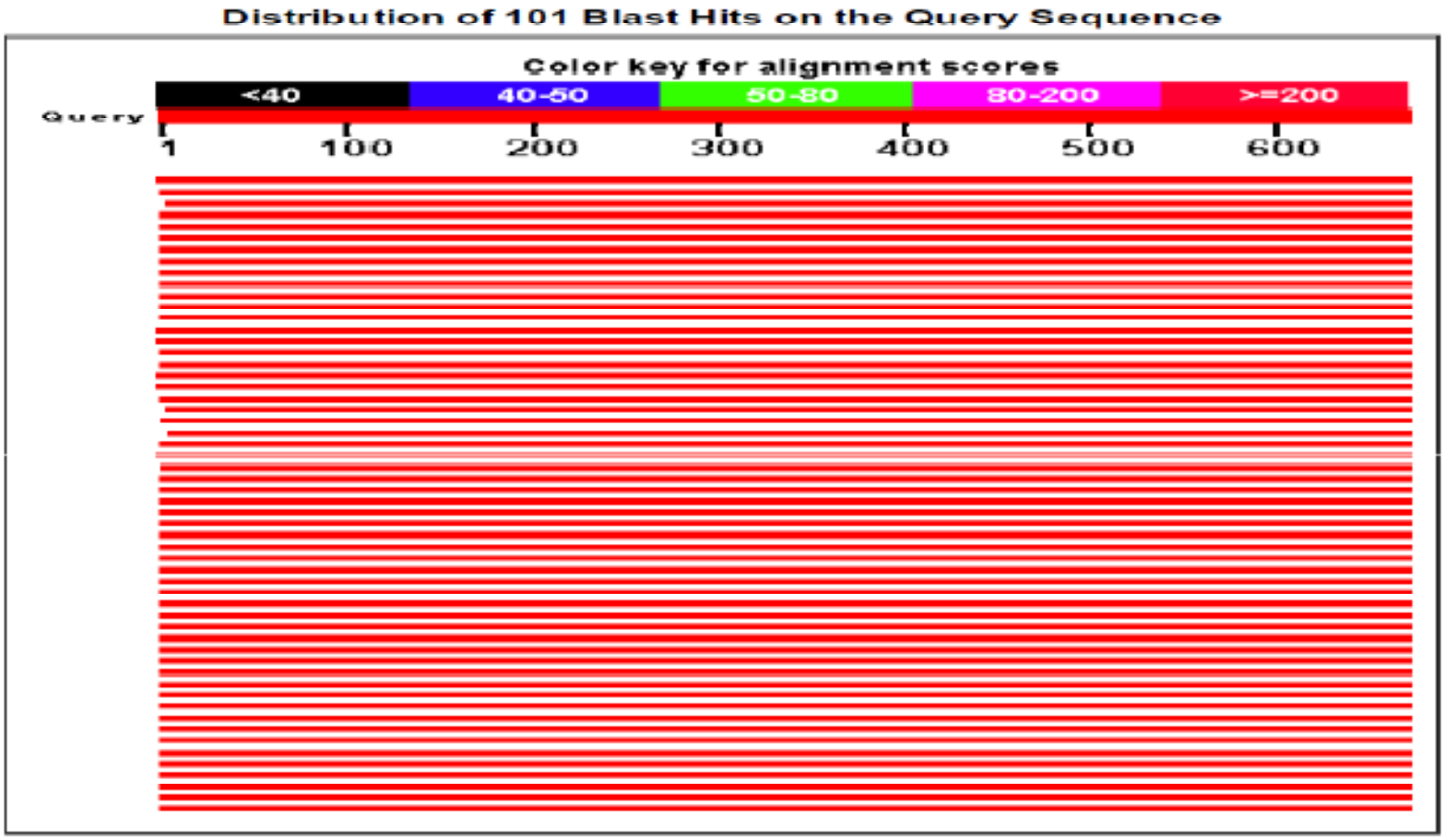


Fig. 28: Colour key for alignment score of nucleotide query sequence of TR2 isolate with other nucleotide sequence present in the database using BLASTn analysis (Red lines show the similarity of the query sequence is more than 200)

Table 69: Percent homology of 16S rRNA gene sequence of TR2 isolate with other nucleotide sequences present in the database using BLASTn analysis.

Accession Number	Closest match	Per cent Similarity
JX847129.1	*Bacillus licheniformis* strain LZBL-19 16S ribosomal RNA gene, partial sequence	98
KC437094.1	*Bacillus licheniformis* strain PAL05 16S ribosomal RNA gene, partial sequence	98
HE804780.1	*Bacillus licheniformis* partial 16S rRNA gene, isolate 1.15	98
KF664027.1	*Bacillus licheniformis* strain DAS-1 16S ribosomal RNA gene, partial sequence	98
KC197213.1	*Bacillus licheniformis* strain ZHU-1 16S ribosomal RNA gene, partial sequence	98
JX068653.1	*Bacillus licheniformis* strain GD10 16S ribosomal RNA gene, partial sequence	98
GQ340514.1	*Bacillus licheniformis* strain TT52 16S ribosomal RNA gene, partial sequence	98

Based on morphological, biochemical and molecular characterization, the isolate TR2 was eventually identified as *Bacillus licheniformis* RSM8 strain.

4.11 OPTIMIZATION OF CULTURE CONDITIONS FOR MAXIMUM LACCASE PRODUCTION

To achieve the final objective of thermostable laccase enzyme production and purification, only one maximum laccase producing thermophilic bacterial isolate identified as *Bacillus licheniformis* strain RSM8 was selected. Optimization of various parameters such as pH, incubation temperature, incubation time, different concentrations of guaiacol, different $CuSO_4$ concentrations, carbon sources, inoculum sizes, nitrogen sources and different metal ions was carried out for maximum laccase production as well as for best growth of the selected *Bacillus licheniformis* strain RSM8.

4.11.1 Effect of pH

A pH range of 4.0-11.0 of TYM was investigated and it was found that growth of the laccase producing thermophilic bacterial isolate increased gradually with increase in pH of the medium up to pH 9.0 and thereafter it was found to decrease (Fig.29). Maximum growth OD was found to be 0.87 at 540 nm wavelength, thus pH 9.0 was selected as optimum for best growth of *Bacillus licheniformis* strain RSM8 (Table-70).

Table 70: Effect of pH of TYM on laccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

pH	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
4.0	0.11	0.312	4329	0.0001
5.0	0.37	1.336	4580	0.0002
6.0	0.49	6.564	5200	0.0013
7.0	0.55	8.689	5746	0.0015
8.0	0.62	8.771	5296	0.0017
9.0	**0.87**	**10.926**	5240	**0.0021**
10.0	0.78	9.812	**6018**	0.0016
11.0	0.74	8.726	5219	0.0017

Enzyme production in terms of laccase enzyme activity was found to be increasing gradually with the increase in initial pH, reached the maximum at pH 9.0, then decreased on further increase in pH, which depicted that pH in the alkaline range was found to be ideal condition for laccase production by this strain RSM8. At pH 9.0 maximum extracellular enzyme activity of 10.926 U/l was observed. Thus pH optimization studies indicated that pH 9.0 was the optimum pH for growth as well as for maximum laccase production by *Bacillus licheniformis* strain RSM8 (Table-70). However, extracellular protein increased with increase in pH up to pH 10.0 and then a decrease was observed at pH 11.0 (Fig.30). Maximum value of extracellular protein of 6018 mg/l was observed at pH 10.0 (Table-70), whereas maximum extracellular specific activity was found to be 0.0021 U/mg at pH 9.0. On the basis of results obtained alkaline pH of 9.0 was selected as optimum to be used in further experiments for growth and extracellular laccase enzyme production of *Bacillus licheniformis* strain RSM8.

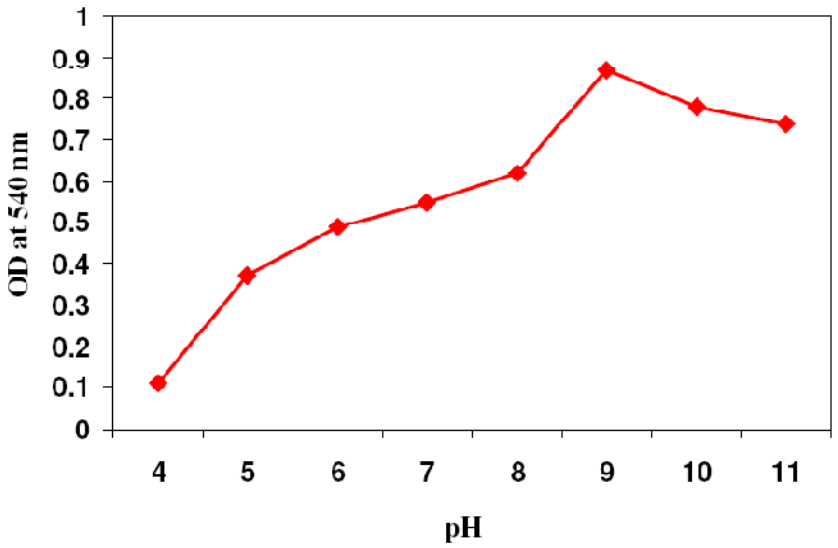


Fig. 29: Effect of pH on growth of *Bacillus licheniformis* strain RSM8

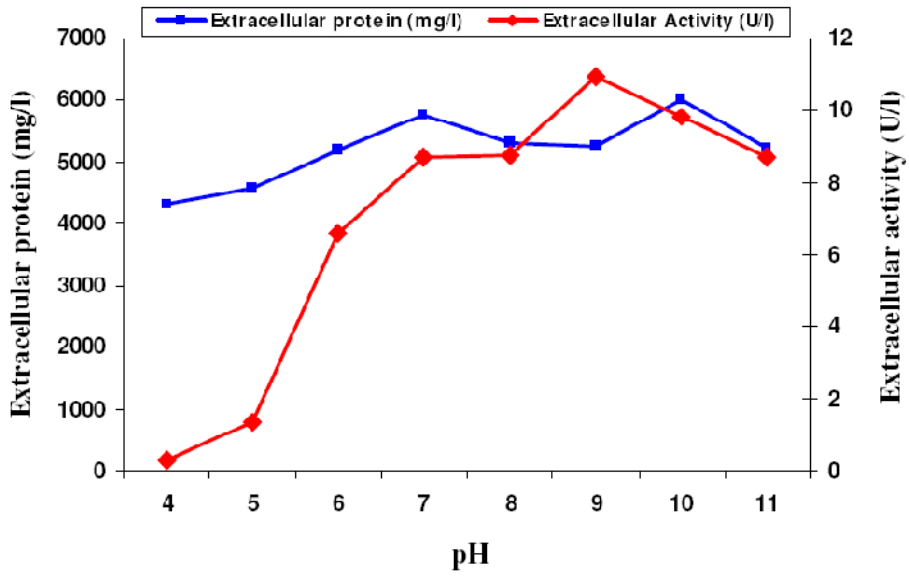


Fig. 30: Effect of pH on laccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

4.11.2 Effect of incubation temperature

Growth and laccase enzyme production was also investigated at different temperatures using TYM broth at pH 9.0. Growth of *Bacillus licheniformis* strain RSM8 was observed at a temperature range of 30-80^{o}C for 24 hrs and growth was found to increase with increase in temperature up to 60^{o}C followed by decrease up to 80^{o}C. The selected bacterial strain RSM8 showed a maximum growth OD of 0.953 at 540 nm at an incubation temperature of 60^{o}C and thus selected as optimum incubation temperature for best growth of RSM8 strain (Fig.31).

Similarly in case of laccase enzyme production, extracellular enzyme activity was found to increase with increase in temperature up to 60^{o}C followed by decrease up to 80^{o}C. Thus optimum incubation temperature selected for best growth and laccase production was also found to be 60°C (Fig.32) with maximum extracellular activity of 12.12 U/l (Table-71). Extracellular protein produced by *Bacillus licheniformis* strain RSM8 also decreased with increased temperature up to 60^{o}C showing maximum extracellular protein of 6245 mg/l at 30^{o}C temperature, whereas specific activity was found to be maximum (0.0031 U/mg) at temperature 60^{o}C. On the basis of results obtained temperature 60^{o}C was selected to be used in further experiments for growth and extracellular laccase enzyme production of *Bacillus licheniformis* strain RSM8.

Table 71: Effect of incubation temperature on laccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Temp. (^{o}C)	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
30	0.234	3.10	**6245**	0.0005
40	0.677	4.87	5986	0.0008
50	0.710	8.72	5411	0.0016
60	**0.953**	**12.12**	4216	**0.0031**
70	0.756	10.56	5329	0.0020
80	0.451	7.81	5412	0.0014

4.11.3 Effect of time of incubation

Bacterial growth of *Bacillus licheniformis* strain RSM8 was monitored up to 144 hrs in TYM broth (pH 9.0) at optimum temperature of 60^{o}C and it has been observed that bacterial growth increased at a very fast rate depicting an OD of 0.983 at 540 nm wavelength during the first 24 hrs and then there was a gradual decrease from 48 to 144 hrs with the growth OD value of 0.918, 0.909, 0.876, 0.789 and 0.774 respectively (Fig.33). The selected isolate exhibited maximum growth OD of 0.983 at wavelength of 540 nm after 24 hrs. Thus 24 hrs was selected as optimum incubation time for best growth of *Bacillus licheniformis* strain RSM8 (Table-72).

Table 72: Effect of time course (hrs) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Time (hrs)	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
0	0.000	00.00	0000	0.0000
24	0.983	**10.80**	6073	**0.0022**
48	0.918	10.20	6880	0.0015
72	0.909	04.10	7700	0.0005
96	0.876	02.50	**7840**	0.0003
120	0.789	00.00	4005	0.0000
144	0.777	00.00	2506	0.0000

Effect of incubation time at different time intervals on extracellular enzyme activity produced by *Bacillus licheniformis* strain RSM8 was also determined. It was found that maximum extracellular enzyme activity of 10.80 U/l was observed after 24 hrs of incubation and was found to be higher than the activity estimated at different time intervals and thus for production of extracellular thermolaccase, 24 hrs incubation time was found optimum (Fig.34). Extracellular protein content was found to be increased with increase in time up to 96 hrs followed by decrease up to 144 hrs. Maximum extracellular protein value of 7840 mg/l was obtained after 96 hrs (Table-72), whereas maximum specific activity of 0.0022 U/mg was obtained after 24 hrs of incubation. On the

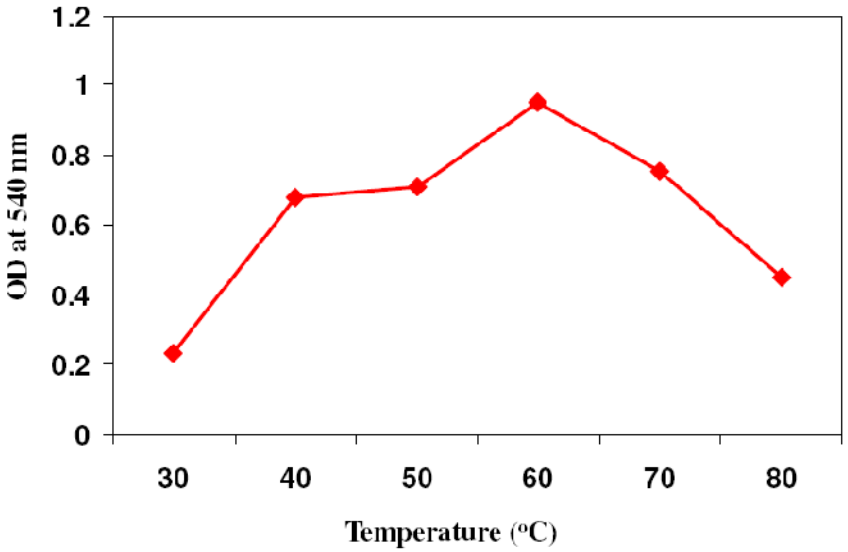


Fig. 31: Effect of incubation temperature (°C) on growth of *Bacillus licheniformis* strain RSM8

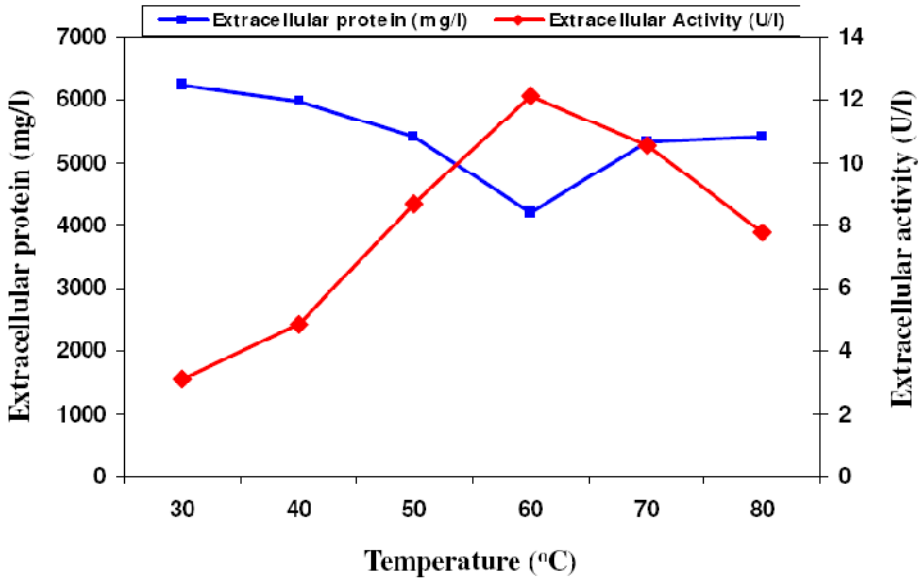


Fig. 32: Effect of incubation temperature (°C) on laccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

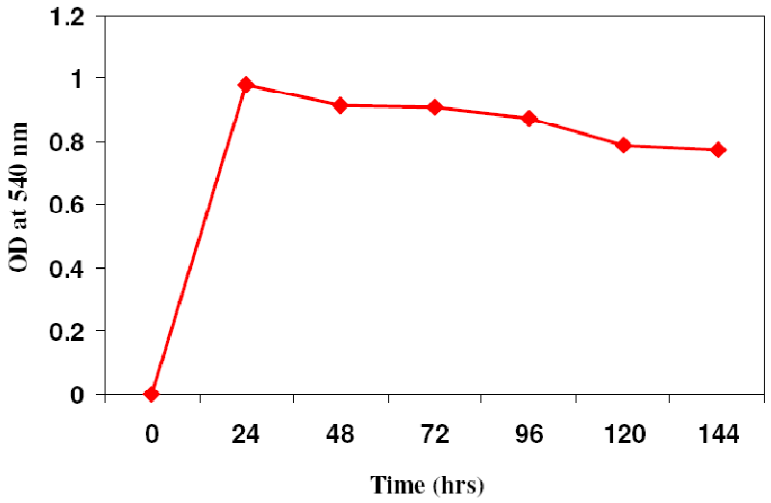


Fig. 33: Effect of time course (hrs) on growth of *Bacillus licheniformis* strain RSM8

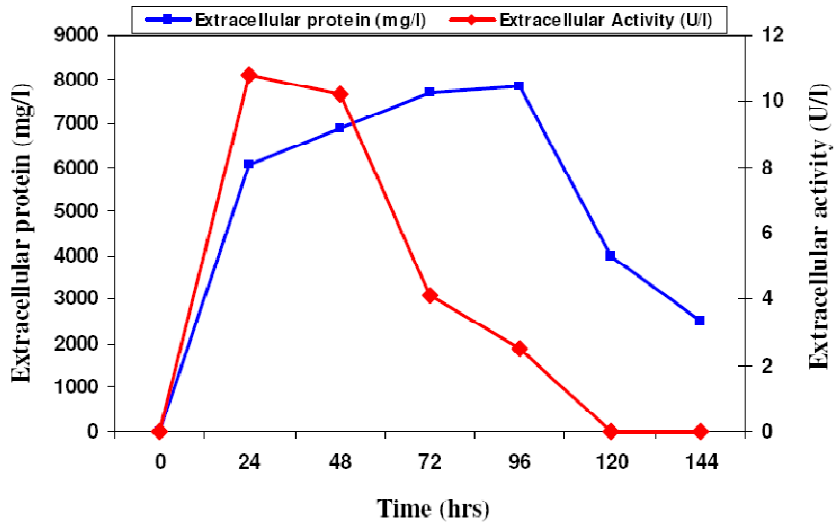


Fig. 34: Effect of time course (hrs) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

basis of these results obtained the optimum incubation time of 24 hrs was selected to be used in further experiments for growth and extracellular thermolaccase enzyme synthesis of *Bacillus licheniformis* strain RSM8.

4.11.4 Effect of different guaiacol concentration

Effect of different guaiacol concentrations was evaluted on growth and thermolaccase production using TYM (pH 9.0) at 60^{o}C for 24 hrs of incubation period. Growth of *Bacillus licheniformis* strain RSM8 was studied at different guaiacol concentrations ranging from 1.0-10.0 mM. The selected bacterial isolate showed a maximum growth OD of 0.985 at a wavelength of 540 nm in TYM broth containing 5.0 mM guaiacol followed by decrease with the increasing concentrations of guaiacol (Fig.35).

Similarly thermolaccase activity was found to increase from 1.0-5.0 mM guaiacol concentration followed by decrease. Maximum extracellular enzyme activity of 18.260 U/l was obtained with 5.0 mM guaiacol concentration. Laccase enzyme activity was found to be negligible, when no guaiacol was added to the medium and thus it was observed that guaiacol acts as an inducer of laccase production (Table-73, Fig.36). Whereas in case of protein production,

Table 73: Effect of different guaiacol concentration (mM) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Guaiacol Concentration (mM)	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
1	0.400	0.008	4216	0.0000
2	0.621	2.174	4212	0.0005
3	0.892	7.870	3419	0.0023
4	0.850	12.327	3627	0.0034
5	**0.985**	**18.260**	2264	**0.0081**
6	0.982	14.201	6219	0.0023
7	0.689	10.565	**7242**	0.0015
8	0.691	7.202	6441	0.0011
9	0.624	4.111	6212	0.0006
10	0.687	3.290	6211	0.0005
No guaicoal	0.267	0.002	2312	0.0000

extracellular protein content first showed a gradual decrease with increase in guaiacol concentration up to 5.0 mM and after that increase was observed in two guaiacol concentrations of 6.0 and 7.0 mM with maximum extracellular protein value of 7242 mg/l followed by gradual decrease. However maximum extracellular specific activity of *Bacillus licheniformis* strain RSM8 was found to be 0.0081 U/mg at 5.0 mM guaiacol concentration. On the basis of results obtained 5.0 mM guaiacol concentration was selected as optimum to be used in further experiments for growth and extracellular thermolaccase enzyme production of *Bacillus licheniformis* strain RSM8.

4.11.5 Effect of $CuSO_4$ concentration

Evaluation of growth and thermolaccase production was also carried out at different concentrations of $CuSO_4$ using TYM broth medium containing 5.0 mM guaiacol (pH 9.0) at 60^{o}C for 24 hrs. Growth of *Bacillus licheniformis* strain RSM8 was observed at different $CuSO_4$ concentration ranging from 10-70 mg/l and it was found that growth of the bacterial isolate depicted a gradual decrease with increase in $CuSO_4$ concentration. The selected bacterial isolate showed a maximum growth OD of 1.16 at 540 nm in TYM broth containing 10 mg/l $CuSO_4$ and further at higher concentration of $CuSO_4$ (Fig.37).

Thermolaccase enzyme production depicted variability in results with increase in concentration of $CuSO_4$. At concentration of 10-20 mg/l thermolaccase enzyme activity increased followed by gradual decrease up to concentration 40 mg/l and maximum thermolaccase enzyme activity was obtained at a concentration of 50 mg/l $CuSO_4$ followed by decrease. *Bacillus licheniformis* strain RSM8 showed maximum extracellular enzyme activity of 15.50 U/l using 50 mg/l $CuSO_4$ concentration in TYM broth containing 5.0 mM guaiacol. No thermolaccase activity was detected in the absence of Cu^{+2} ion. Thus, it has been depicted that $CuSO_4$ acts as an inducer to thermolaccase enzyme production (Table-74, Fig.38).

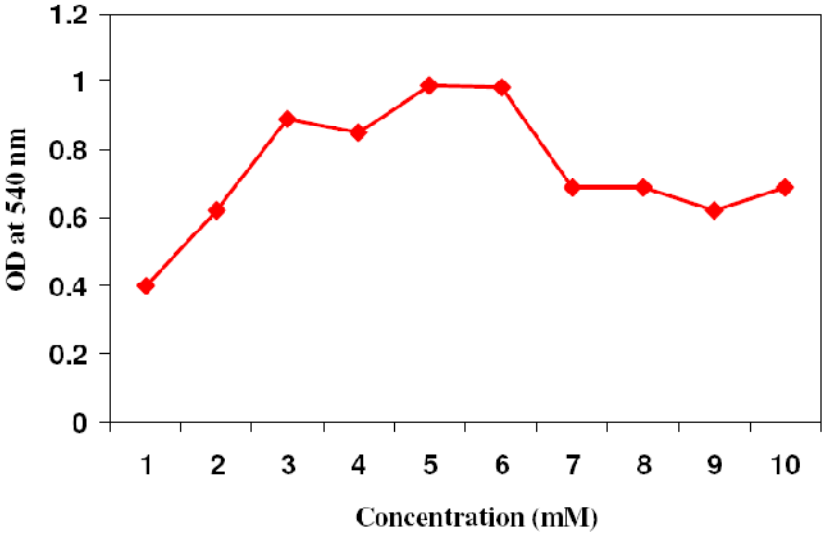


Fig. 35: Effect of different guaiacol concentration (mM) on growth of *Bacillus licheniformis* strain RSM8

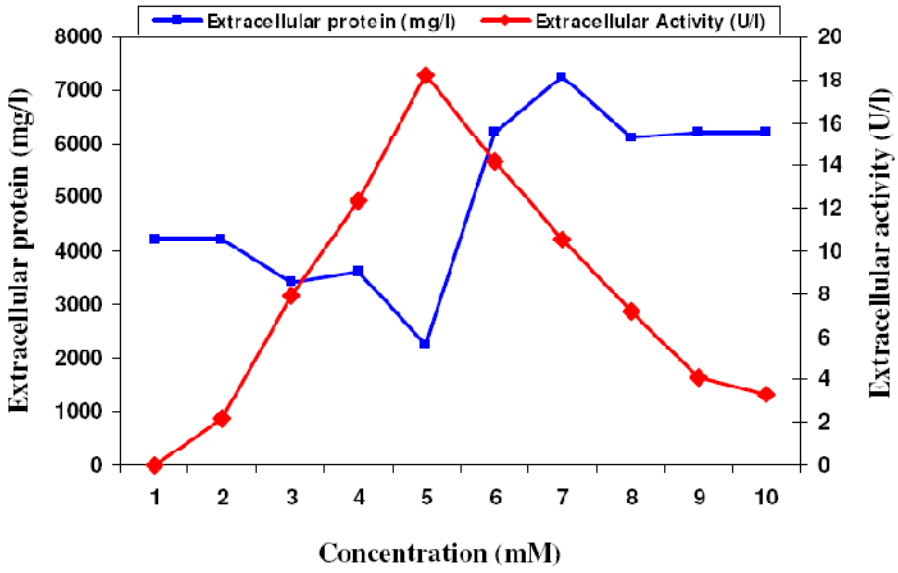


Fig. 36: Effect of different guaiacol concentration (mM) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

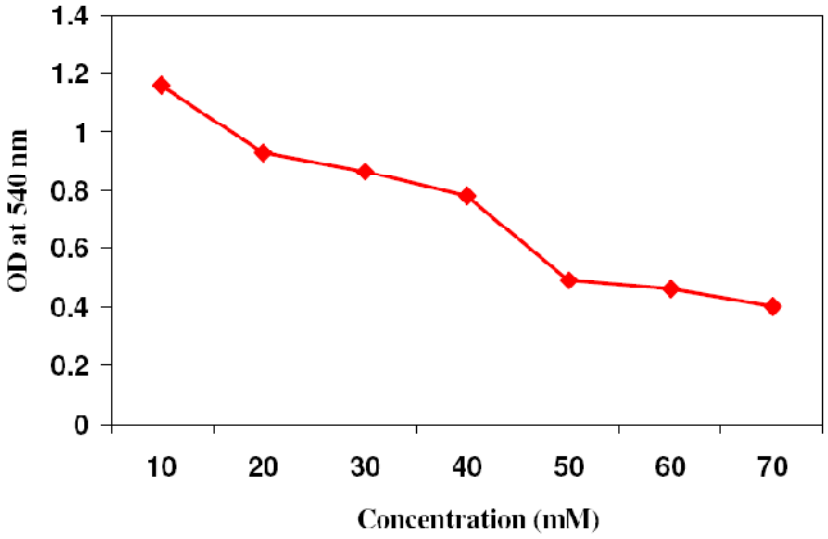


Fig. 37: Effect of $CuSO_4$ concentration (mg/l) on growth of *Bacillus licheniformis* strain RSM8

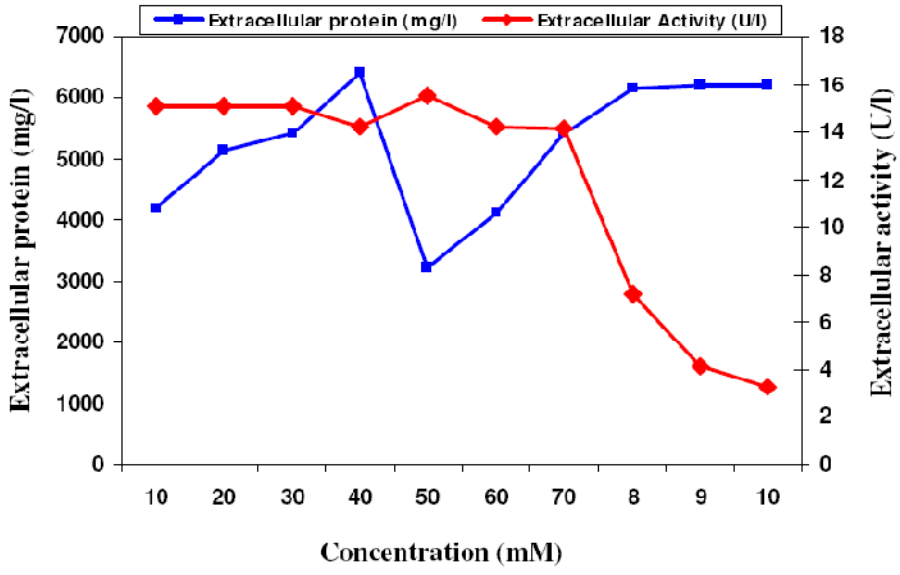


Fig. 38: Effect of CuSO4 concentration (mg/l) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Table 74: Effect of $CuSO_4$ concentration (mg/l) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

$CuSO_4$ Concentration (mg/l)	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
0 (Control)	0.27	0.00	5445	0.0000
10	**1.16**	15.10	4216	0.0036
20	0.93	15.12	5129	0.0030
30	0.86	15.09	5412	0.0028
40	0.78	14.26	**6418**	0.0022
50	0.49	**15.50**	3216	**0.0048**
60	0.46	14.26	4129	0.0035
70	0.40	14.18	5412	0.0026

Contrary to growth and extracellular enzyme activity, extracellular protein produced by *Bacillus licheniformis* strain RSM8 showed increase with increased $CuSO_4$ concentration upto 40 mg/l followed by a decline at 50 mg/l and again showed gradual increase at 60-70 mg/l concentration. Maximum extracellular protein 6418 mg/l was produced, when 40 mg/l $CuSO_4$ was added to TYM broth containing 5.0 mM guaiacol, whereas maximum extracellular specific activity of 0.0048 U/mg was obtained at 50 mg/l $CuSo_4$ concentration. On the basis of results obtained 10 mg/l $CuSo_4$ concentration was selected for growth and 50 mg/l $CuSo_4$ concentration was selected for extracellular thermolaccase enzyme production of *Bacillus licheniformis* strain RSM8 in further experiments.

4.11.6 Effect of different carbon sources

Different carbon sources viz., glucose, sucrose, maltose and mannitol were investigated for their effect on growth as well as thermolaccase enzyme production. Maximum growth OD of 1.27 at a wavelength of 540 nm for *Bacillus licheniformis* strain RSM8 was observed in the presence of glucose as carbon source followed by OD values of 1.25, 1.22 and 0.99 for sucrose, mannitol and maltose, respectively (Fig.39).

In case of extracellular thermolaccase production no activity was observed in the presence of glucose and maltose after an incubation of 24 hrs, whereas very less amount of thermolaccase enzyme production was observed in the presence of sucrose and mannitol (Table-75). Whereas maximum extracellular protein production 4126 mg/ml was observed, with sucrose as carbon source followed by mannitol, maltose and glucose (Fig.40). Maximum extracellular specific activity was found to be 0.0017 U/mg with sucrose as carbon source. On the basis of results obtained no single carbon source was selected to be used in further experiments for growth and extracellular thermolaccase enzyme production of *Bacillus licheniformis* strain RSM8.

Table 75: Effect of different carbon sources on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Carbon Source	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
Glucose	1.27	0.00	2250	0.0000
Sucrose	1.25	1.12	4126	0.0002
Mannitol	1.22	2.12	4001	0.0005
Maltose	0.99	0.00	2947	0.0000

4.11.7 Effect of inoculum size

The effect of inoculum size ranging from 1.0-5.0 % was examined on growth and extracellular thermolaccase enzyme production using TYM broth (pH 9.0) containing 5.0 mM guaiacol and 50 mg/l $CuSo_4$ at 60^oC for 24 hrs.. Growth of the *Bacillus licheniformis* strain RSM8 increased with increase in inoculums size up to 3.0 % with maximum growth OD of 1.25 at 540 nm wavelength followed by decrease up to 5.0 % inoculum size. Thus 3% inoculum size was found best for the optimum growth of *Bacillus licheniformis* strain RSM8 (Fig.41).

For thermolaccase enzyme production, it has been revealed that an inoculum size of 1.0 % was found to be optimum with maximum extracellular laccase activity of 15.72 U/l. An inoculum size below this level was found

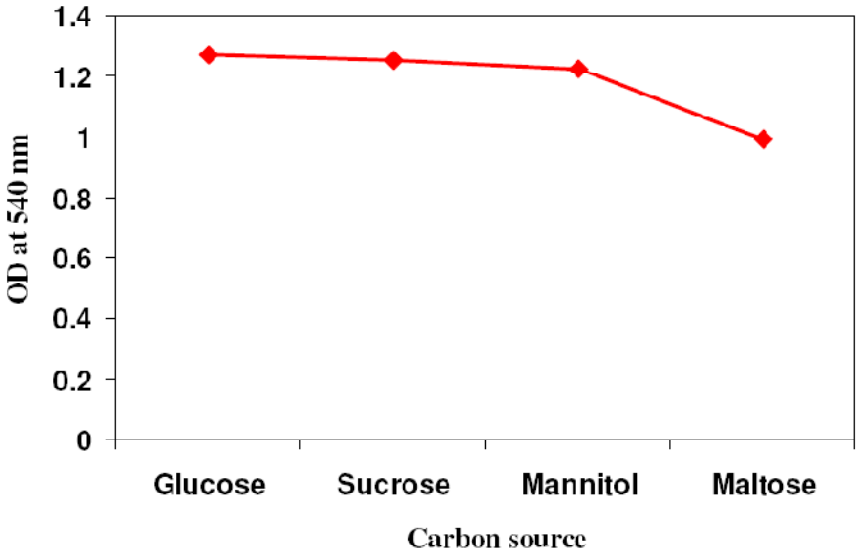


Fig. 39: Effect of different carbon source on growth of *Bacillu licheniformis* strain RSM8

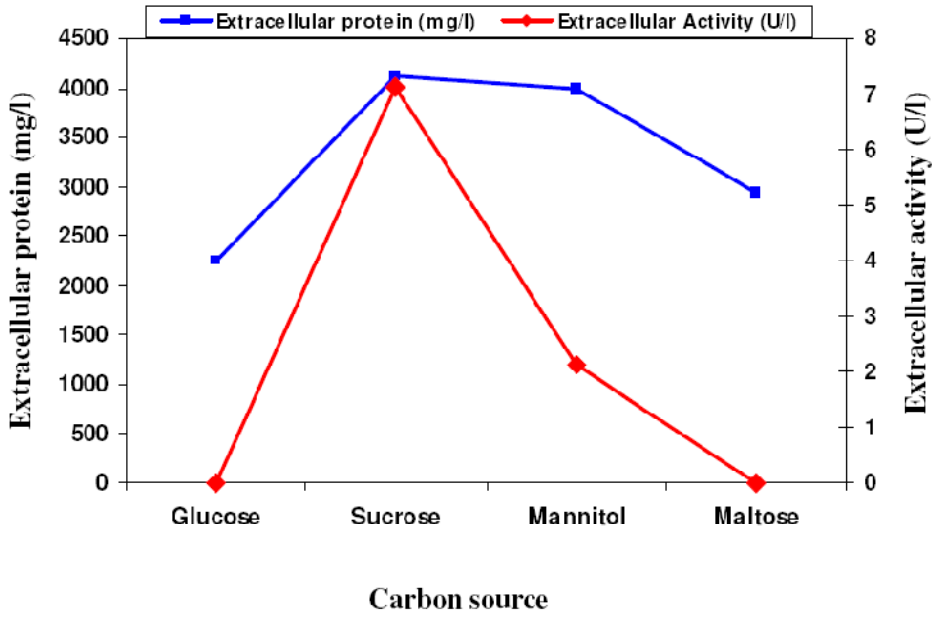


Fig. 40: Effect of different carbon source on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

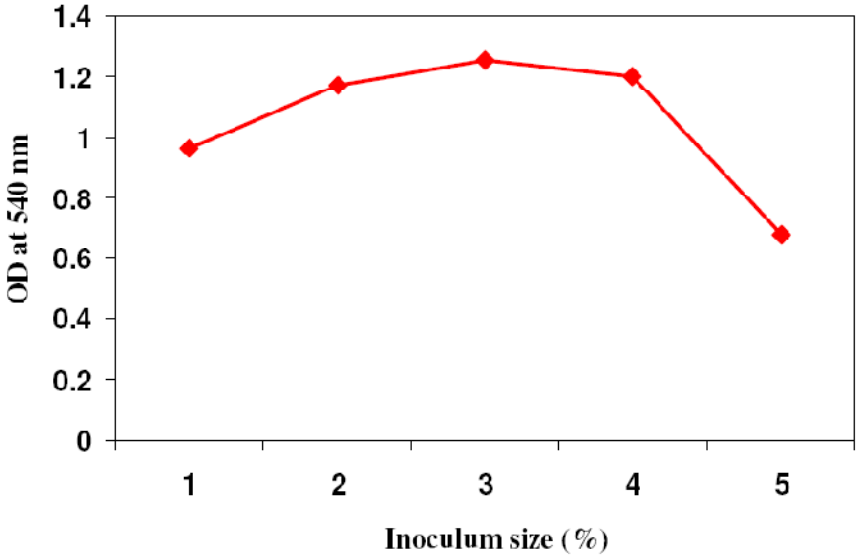


Fig. 41: Effect of inoculum size on growth of *Bacillus licheniformis* strain RSM8

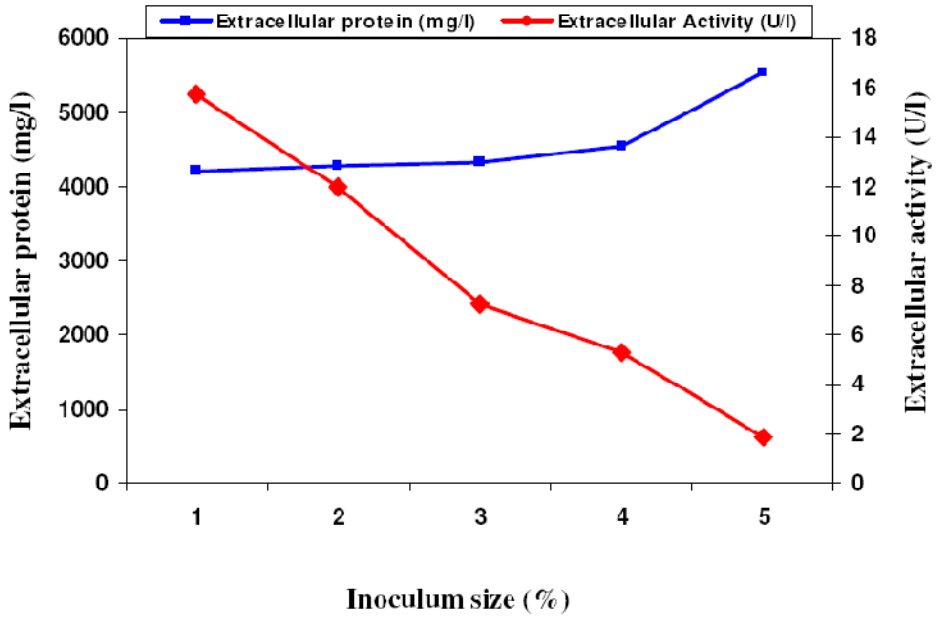


Fig. 42: Effect of inoculum size on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

inadequate for enzyme production while the higher levels resulted in rapid depletion of nutrients in the media and reduced the enzyme yield. Also higher cell density would have led to the production of inhibitory metabolites that may be interfering with the enzyme production. Whereas extracellular protein content was found to be gradually increased with inoculum size, maximum value of 5540 mg/l was found at 5.0 % inoculum size. However maximum specific activity was found to be 0.0037 U/mg at 1.0 % inoculum size. Thus results of optimization studies indicated that 3.0 % inoculum size was selected as optimum for best growth of *Bacillus licheniformis* strain RSM8, whereas 1.0 % inoculum size was found to be optimum for thermolaccase enzyme production of the *Bacillus licheniformis* strain RSM8 (Table-76, Fig.42).

Table 76: Effect of inoculum size on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Inoculum Size (%)	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
1.0	0.96	**15.72**	4216	**0.0037**
2.0	1.17	12.01	4289	0.0028
3.0	1.25	7.26	4326	0.0021
4.0	1.20	5.29	4557	0.0012
5.0	0.68	1.87	**5540**	0.0003

4.11.8 Effect of tryptone and yeast extract concentrations

Different combinations of tryptone and yeast extract were investigated for growth as well as on thermolaccase enzyme production. Growth of the *Bacillus licheniformis* strain RSM8 was found to be maximum at 0.98 OD at 540 nm using 0.2 % of yeast extract and 0.4 % of tryptone in combination (Table-77, Fig.43). 0.5 % Tryptone and 0.3 % of yeast extract in combination gave maximum extracellular thermolaccase enzyme activity of 16.10 U/l (Fig.44). Whereas maximum value of 3219 mg/l extracellular protein was observed using 0.4 % of tryptone and 0.2 % of yeast extract in combination. Maximum extracellular specific activity of 0.0070 was obtained using 0.5 % tryptone and 0.3 % of yeast extract in combination. On the basis of results obtained 0.2 % of yeast extract and 0.4 % of tryptone in combination was selected as optimum for

the growth of *Bacillus licheniformis* strain RSM8 and 0.5 % tryptone and 0.3 % of yeast extract in combination was selected as optimum for extracellular thermolaccase enzyme production to be used in further experiments.

Table 77: Effect of tryptone and yeast extract (Nitrogen source) concentrations on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Nitrogen source		OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
Tryptone (%)	Yeast extract (%)				
0.3	0.1	0.72	10.29	2112	0.0049
0.4	0.2	**0.98**	12.01	**3219**	0.0037
0.5	0.3	0.82	**16.10**	2296	**0.0070**
0.6	0.4	0.77	10.84	2689	0.0040
0.7	0.5	0.74	9.22	2810	0.0033

4.11.9 Effect of metal ions

Effect of metals ions was also investigated for growth and thermolaccase enzyme production by addition of different metal ions at final concentration of 1 mM in TYM. Growth of *Bacillus licheniformis* strain RSM8 was found to be maximum when $CaCl_2$ was added into the medium and with growth OD of 1.95 at a wavelength of 540 nm. However CuSO4, $FeSO_4$, $LiCl_2$, $MgSO_4$ and NaNO3 showed growth OD values of 1.85, 1.50, 1.10, 1.09 and 0.90, respectively (Table-78).

Table 78: Effect of metal ions (mM) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Metal Ions	OD at 540 nm	Extracellular Activity (U/l)	Extracellular protein (mg/l)	Extracellular Specific Activity (U/mg)
Control	0.98	8.29	8221	0.0009
$MgSO_4$	1.09	3.40	4210	0.0008
$LiCl_2$	1.10	10.12	2791	0.0036
$FeSO_4$	1.50	4.86	3501	0.0014
$NaNO_3$	0.90	0.91	7412	0.0001
$CaCl_2$	1.95	14.72	5310	0.0028
$CuSO_4$	1.85	18.96	6126	0.0031

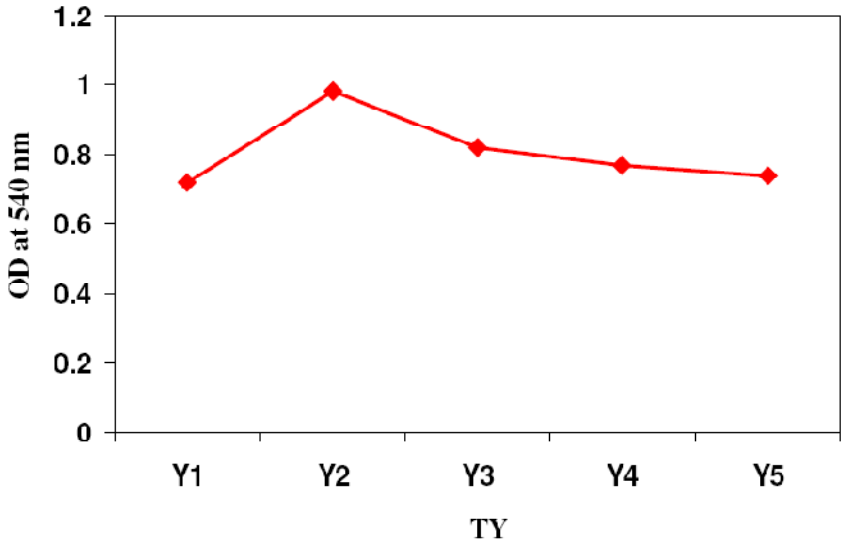


Fig. 43: Effect of tryptone and yeast extract concentration on growth of *Bacillus licheniformis* strain RSM8

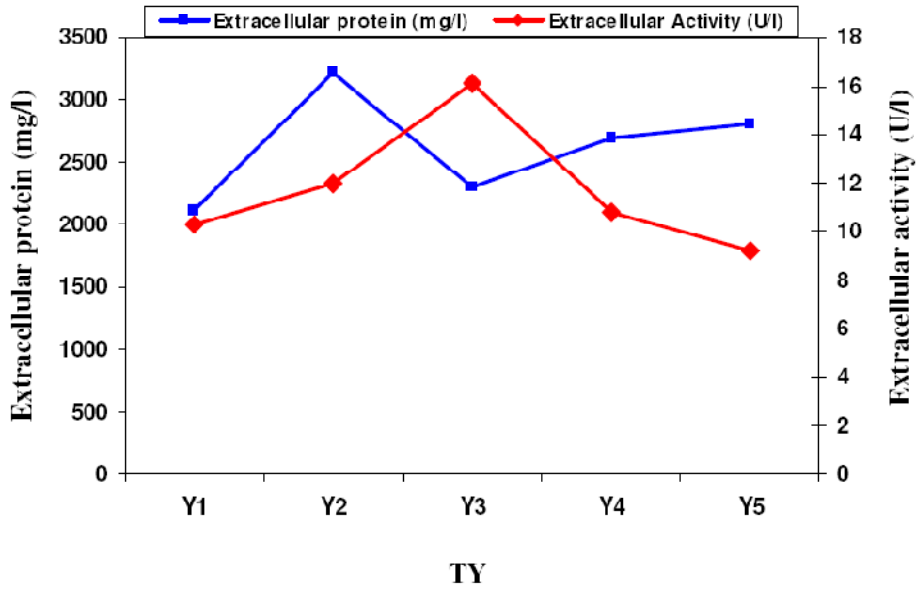


Fig. 44: Effect of tryptone and yeast extract concentration on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

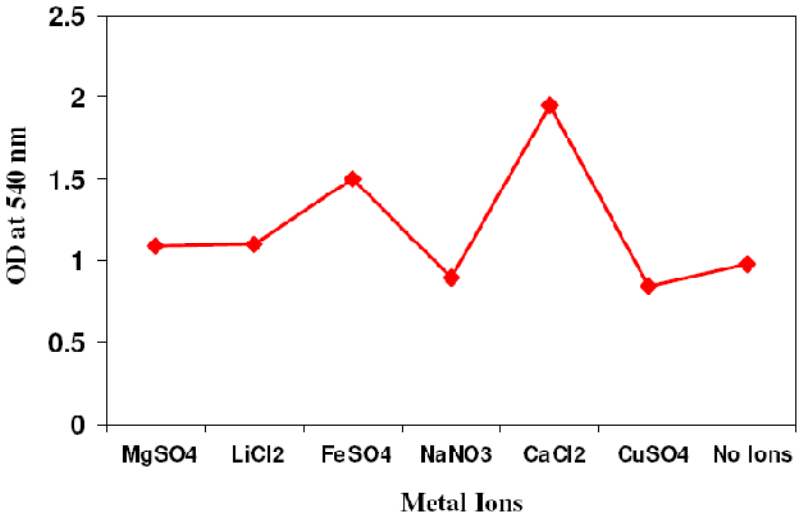


Fig. 45: Effect of metal ions (mM) on growth of *Bacillus licheniformis* strain RSM8

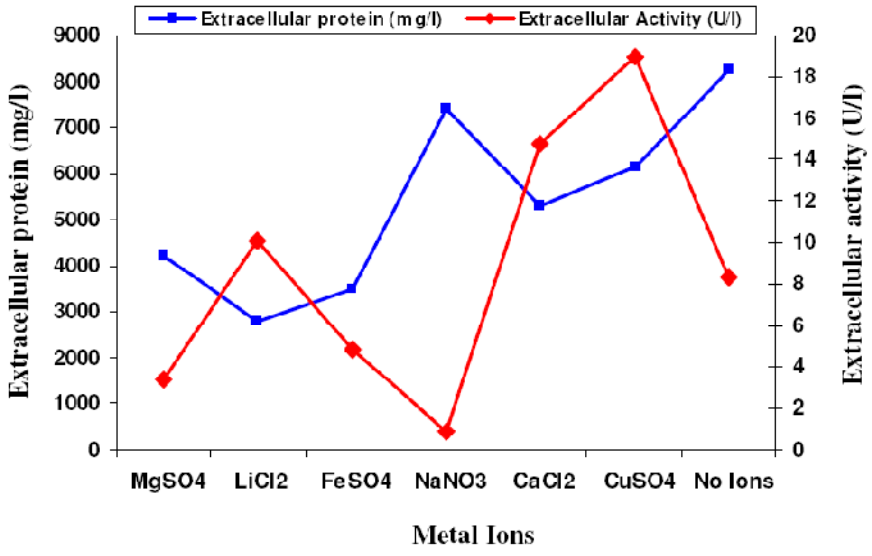


Fig. 46: Effect of metal ions (mM) on thermolaccase extracellular activity and extracellular proteins produced by *Bacillus licheniformis* strain RSM8

Among all the metal ions studied $CuSO_4$ was found to be most effective in stimulating thermolaccase production showing maximum extracellular enzyme activity of 18.96 U/l followed by $CaCl_2$ and $LiCl_2$. However metal ions $FeSO_4$, $NaNO_3$ and MgSO4 showed negligible extracellular enzyme activity. Extracellular protein was found to be maximum when no ions were added into the TYM (Table-79, Fig.46). However maximum extracellular specific activity was obtained with $LiCl_2$. On the basis of section 4.9.5 and results obtained $CuSO_4$ was selected as optimum to be used in further experiments for growth and extracellular laccase enzyme production of *Bacillus licheniformis* strain RSM8.

Table 79: Optimization of extracellular thermostable laccase production using one variable at a time

Parameters	Range Tested	Optimum
Time	24-144 hrs	24 hrs
Temperature	30-80^{0}C	60^{o}C
pH	4.0-11.0	9.0
Guaiacol concentration	1.0-10.0 mM	5.0 mM
$CuSO_4$ concentration	10-70 mg/l	50 mg/l
Inoculum size	1.0-5.0 %	1.0%
Carbon source	Glucose, Sucrose, Manittol, Maltose	None
Nitrogen source combination	Combination of tryptone and yeast extract	0.5 % Tryptone + 0.3 % Yeast extract
Metal ions	$MgSO_4$, $LiCl_2$,$FeSO_4$, $NaNO_3$,$CaCl_2$, $CuSO_4$	Cu^{+2} ions

4.12 ENZYME PRODUCTION AND PARTIAL PURIFICATION OF THERMOSTABLE LACCASE ENZYME

4.12.1 Production of thermolaccase enzyme

Thermolaccase production investigations was carried out for extracellular preparations of the enzyme using all the standardized and selected optimum conditions of time, pH, inoculums size, temperature, guaiacol and $CuSO_4$ concentrations. 1 % primary inoculum size was used to inoculate TYM broth containing 5 mM guaiacol and 50 mg/l $CuSO_4$, pH: 9.0 at 60^{0}C for 24 hrs (Table-79). Extracellular enzyme preparation was prepared as described in 3.12.1.

Crude extract was further used to purify laccase enzyme using different purification techniques (Table-80).

4.12.2 PURIFICATION OF THERMOLACCASE ENZYME

Crude extracellular enzyme extract was further purified using different purification techniques such as ammonium sulphate precipitation, gel filtration and ion exchange chromatography.

4.12.2.1 Ammonium sulphate precipitation

Crude extracellular enzyme extract was subjected to ammonium sulphate (0-90%) saturation and it was found out that maximum laccase enzyme activity was detected in 50-90 percent level of saturation. The enzyme preparation at this stage was treated as ammonium sulphate fraction (ASF) (Table-80).

4.12.2.2 Gel filtration column chromatography

The elution profile of blue dextran on Sephadex G-100 column, shown in Fig.47. The void volume (V_o) of Sephadex G-100 was calculated as 30 ml by loading 4mg/ml blue dextran on Sephadex G-100 column.

The ASF (proteins precipitated after the overnight dialysis of 50-90 % ammonium sulphate cut) was applied on Sephadex G-100 column equilibrated with sodium phosphate buffer (pH 9.0) used as start buffer. Maximum absorbance at 280 nm was detected in the fractions numbering 15-28 and the elution profile obtained on Sephadex G-100 column has been shown in Fig.48. Laccase activity for each 15-28 fractions was calculated using ABTS assay and it was found that maximum laccase activity was obtained in the fraction numbering 19-23 (Fig.48). These most active fractions were pooled.

4.12.2.3 Ion exchange chromatography

The active pooled fractions prepared previously were loaded on ion exchange column equilibrated with sodium phosphate buffer pH 9.0 as a start buffer. The proteins were eluted with linear salt gradient of 0.1 M – 0.5 M NaCl and maximum absorbance at 280 nm was detected in the fractions numbering 38-46. It was found that laccase fractions were eluted with 0.3 M NaCl gradient and the elution profile obtained on DEAE sephadex has been depicted in Fig.49.

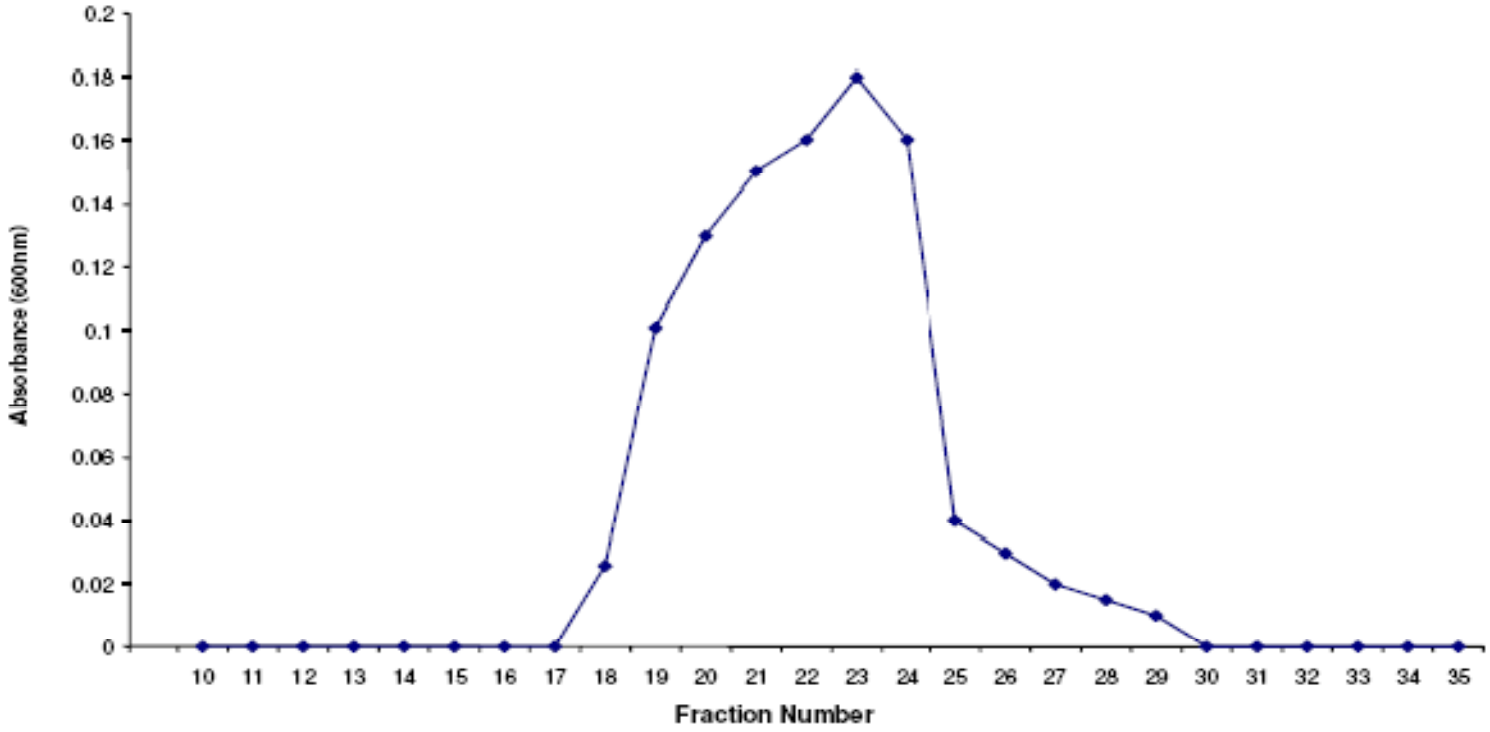


Fig. 47: Elution profile of Blue Dextran on Sephadex G–100 column

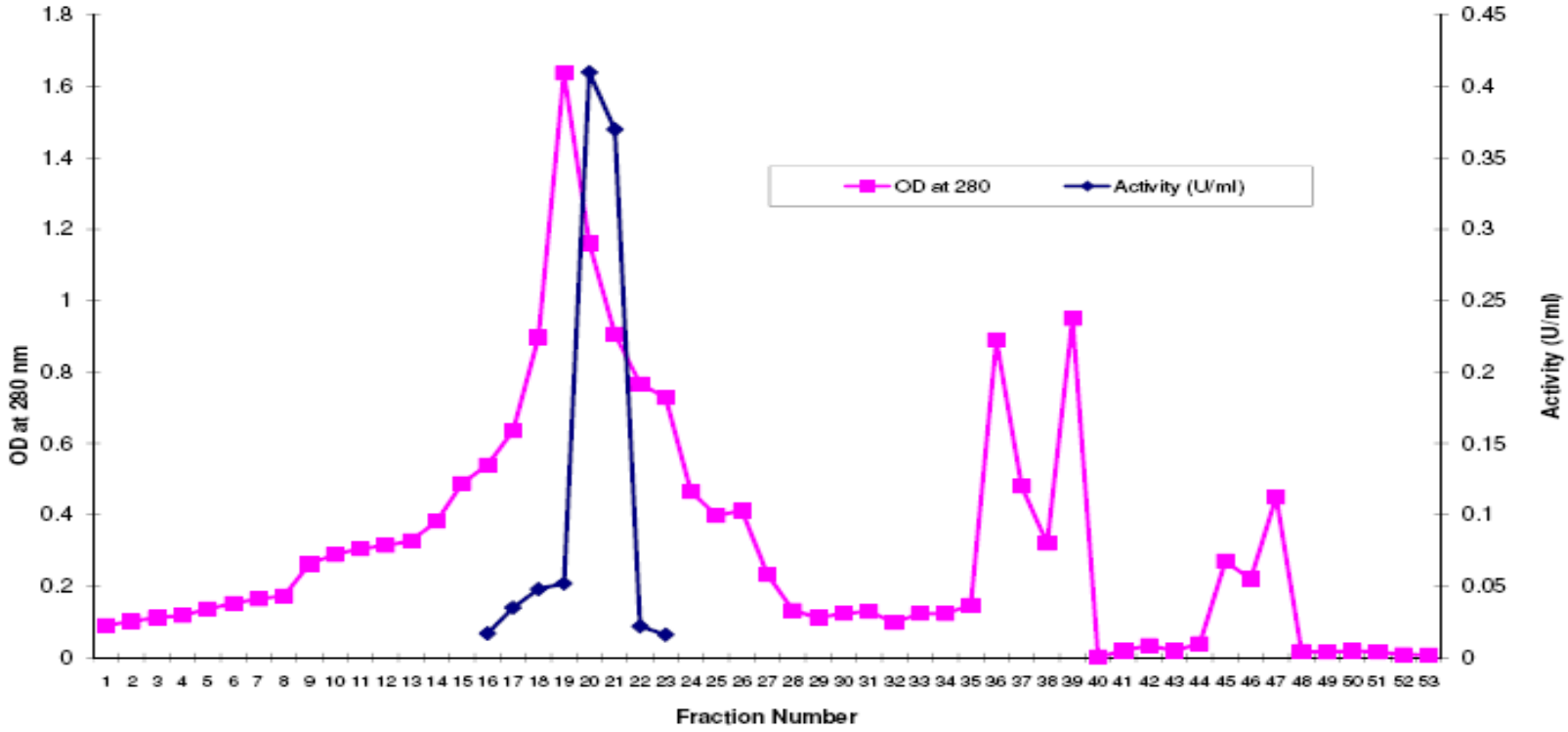


Fig. 48: Elution profile of thermostable laccase enzyme on Sephadex G-100 column

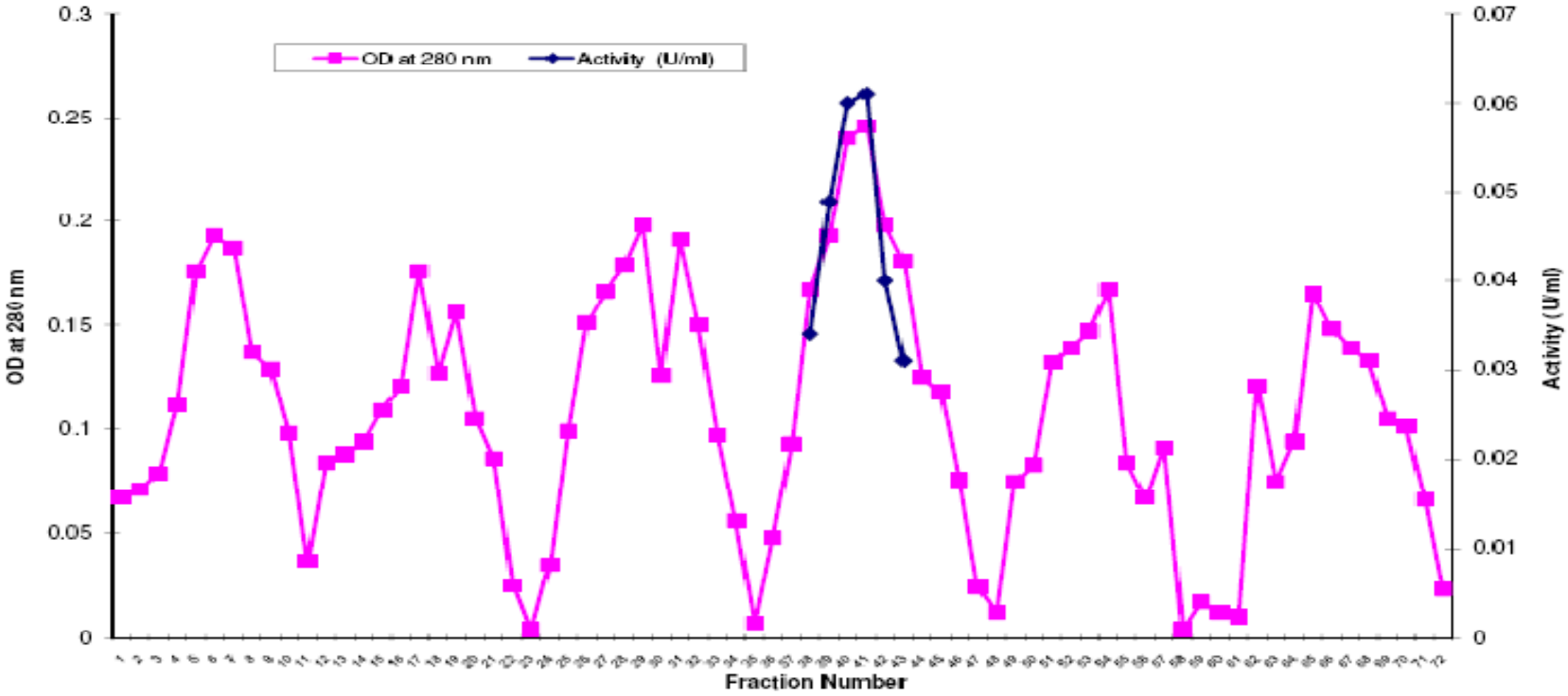


Fig. 49: Elution profile of thermostable laccase enzyme on DEAE-sephadex

Table 80: Partial purification of thermolaccase from *Bacillus licheniformis* strain RSM8

Steps	Total Enzyme Activity (U)	Total soluble protein (mg)	Specific activity (U/mg protein)	Fold purification	Percent yield
Crude extract	1.42	686	0.0020	1.0	100
Ammonium sulfate precipitation	1.13	253	0.0044	2.2	79.85
Gel filtration chromatography	0.302	55	0.0055	3.5	21.26
Ion exchange chromatography	0.21	10.88	0.0198	10.5	15.21

The data of total laccase activity (U), protein content, specific activity, fold purification and yield at different stages of purification has been presented in Table-80. The laccase enzyme was partially purified up to 10.5 fold with 15.21 percent recovery. The specific activity increased significantly from 0.00020 U/mg in crude extract to 0.0198 U/mg in ion exchange chromatography fraction.

4.12.2.4 Native PAGE (Polyacrylamide gel electrophoresis)

The purity as well activity after each step of purification was checked by native-PAGE technology, using 7.5% acrylamide gel and anionic system of Davis (1964) on vertical slab gel electrophoresis. Native PAGE of fraction after gel filtration chromatography followed by ion exchange chromatography revealed a single band of purified protein (Plate-25). Activity staining/zymography of gel with ABTS and Dimethoxyphenol substrates also showed single green and orange coloured bands respectively of purified protein, thus showing laccase activity on the stained gel (Plate-25).

4.13 CHARACTERIZATION OF PARTIALLY PURIFIED THERMOSTABLE LACCASE ENZYME

Purified enzyme was further characterized biochemically to determine its molecular weight, optimum temperature and pH and effect of inducers on enzyme activity. Km value was also determined using ABTS as substrate by plotting Lineweaver-Burk plot.

4.13.1 Determination of molecular weight of laccase of *Bacillus licheniformis* strain RSM8

SDS polyacrylamide gel electrophoresis was carried out to determine the purity as well as molecular weight of laccase enzyme. The purified fractions of ion exchange chromatography showed the presence of a distinct protein band (Plate-26), which confirmed the purity of enzyme. The plot of Rm value versus log of molecular weight is shown in Fig.50. Protein molecular weight markers were used for determining molecular weight of the enzyme and it was found that molecular weight of the partially purified laccase preparation was 72.5 KDa (Plate-26, Fig.50).

4.13.2 Effect of pH on laccase enzyme activity

An optimum pH was found to be 9.0 (Phosphate buffer) for maximum thermolaccase enzyme activity. A maximum value was achieved at pH 9.0 and after that the activity decreased up to the pH 10.0 (Table-81, Fig. 51)

Table 81: Effect of pH on the thermolaccase enzyme activity

Buffer	pH	Enzyme activity (U/l)
Citrate buffer	4.0	0.312
	5.0	0.336
	6.0	0.564
Tris-buffer	6.0	0.689
	7.0	0.771
	8.0	0.786
Phosphate buffer	8.0	0.774
	9.0	**0.926**
	10.0	0.812

4.13.3 Effect of temperature on thermolaccase enzyme activity

Optimum temperature of the purified fraction of ion exchange chromatography of laccase was determined at optimum pH of 9.0 using ABTS as the substrate and maximum thermolaccase enzyme activity of 0.620 U/l was observed at a temperature of 60^0C. It has been observed that the enzyme activity increased with increase of temperature up to 60^0C and after that gradual decrease was observed in the enzyme activity up to 80^0C (Table-82, Fig. 52)

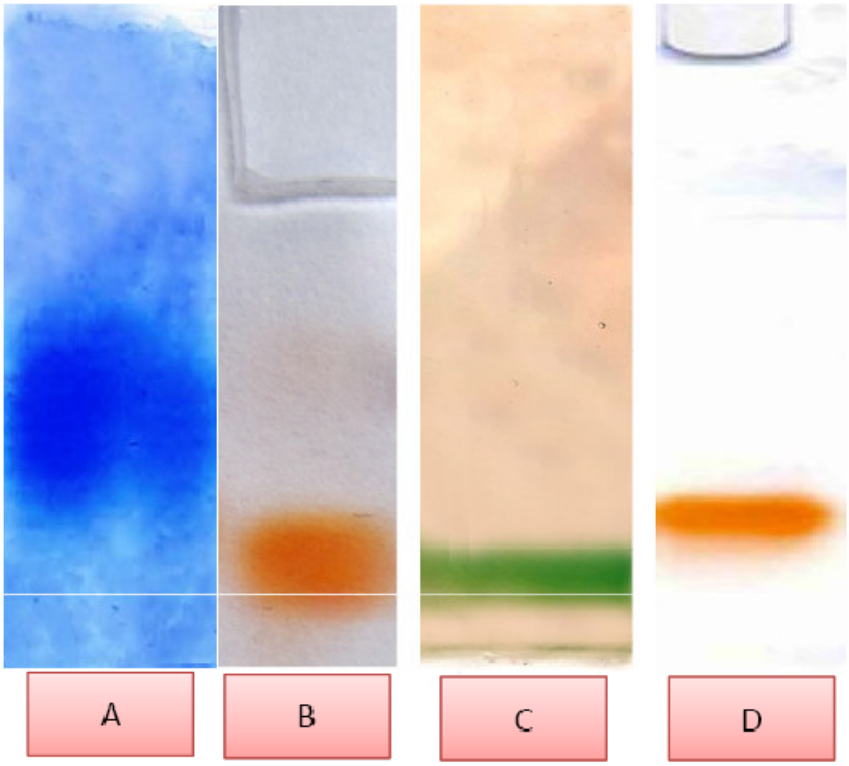


Plate 25: Native polyacrylamide gel electrophoresis of crude and partially purified thermolaccase enzyme of *Bacillus licheniformis* strain RSM8

A) Staining of crude laccase enzyme with brilliant blue

B) Activity staining of crude laccase enzyme with dimethoxyphenol

C) Activity staining of partially purified enzyme with ABTS

D) Activity staining of partially purified protein with dimethoxyphenol

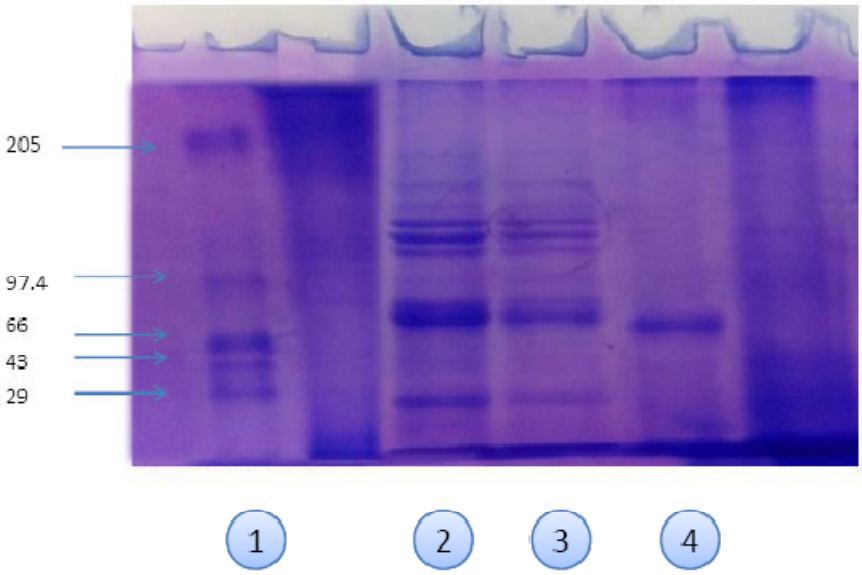


Plate 26: SDS polyacrylamide gel electrophoresis of thermolaccase enzyme *Bacillus licheniformis* strain RSM8

Lane 1: Protein molecular weight marker (KDa) GeNei

Lane 2: Extracellular crude extract

Lane3: Protein fraction after ammonium sulphate fraction,

Lane 4: Partially purified protein eluted from ion exchange chromatography

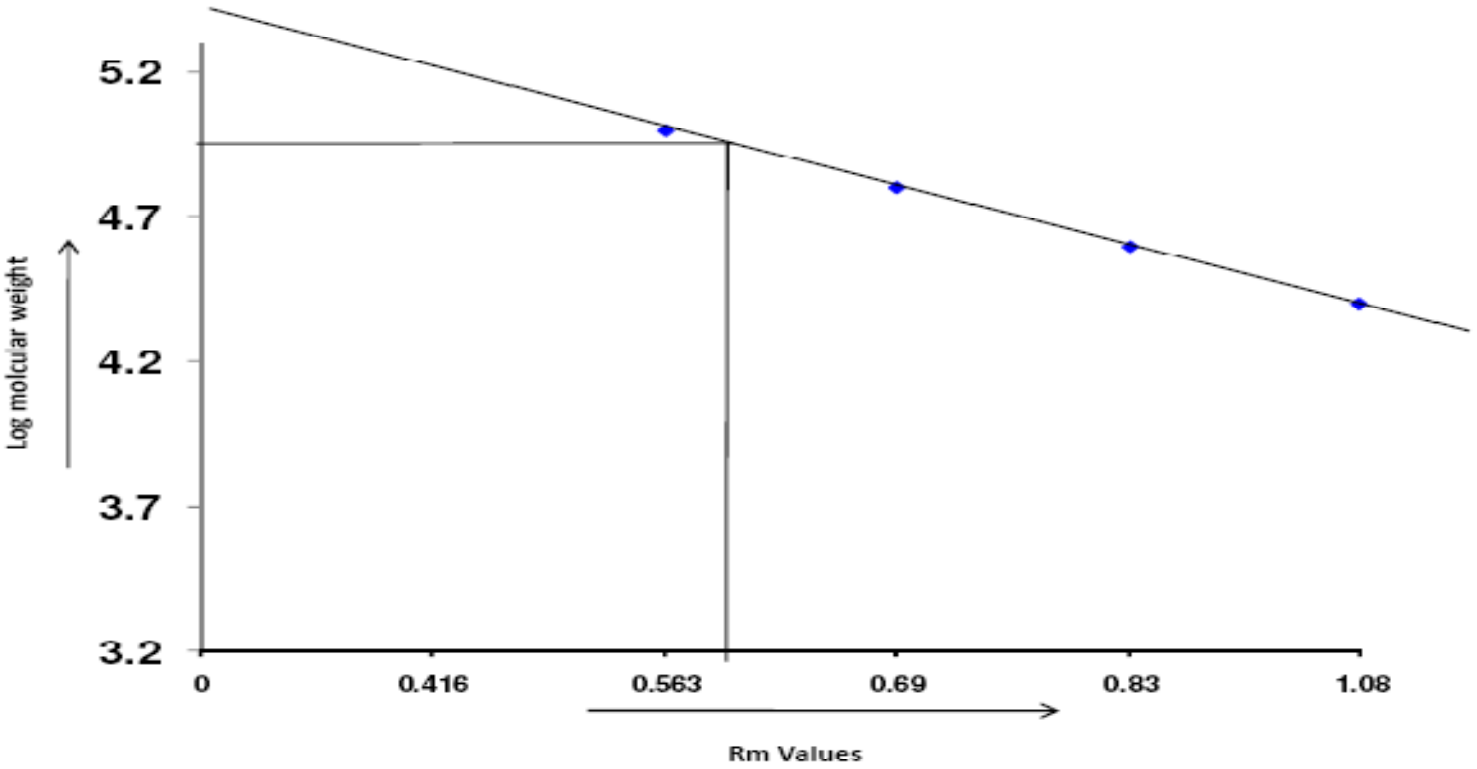


Fig. 50: Molecular weight determination

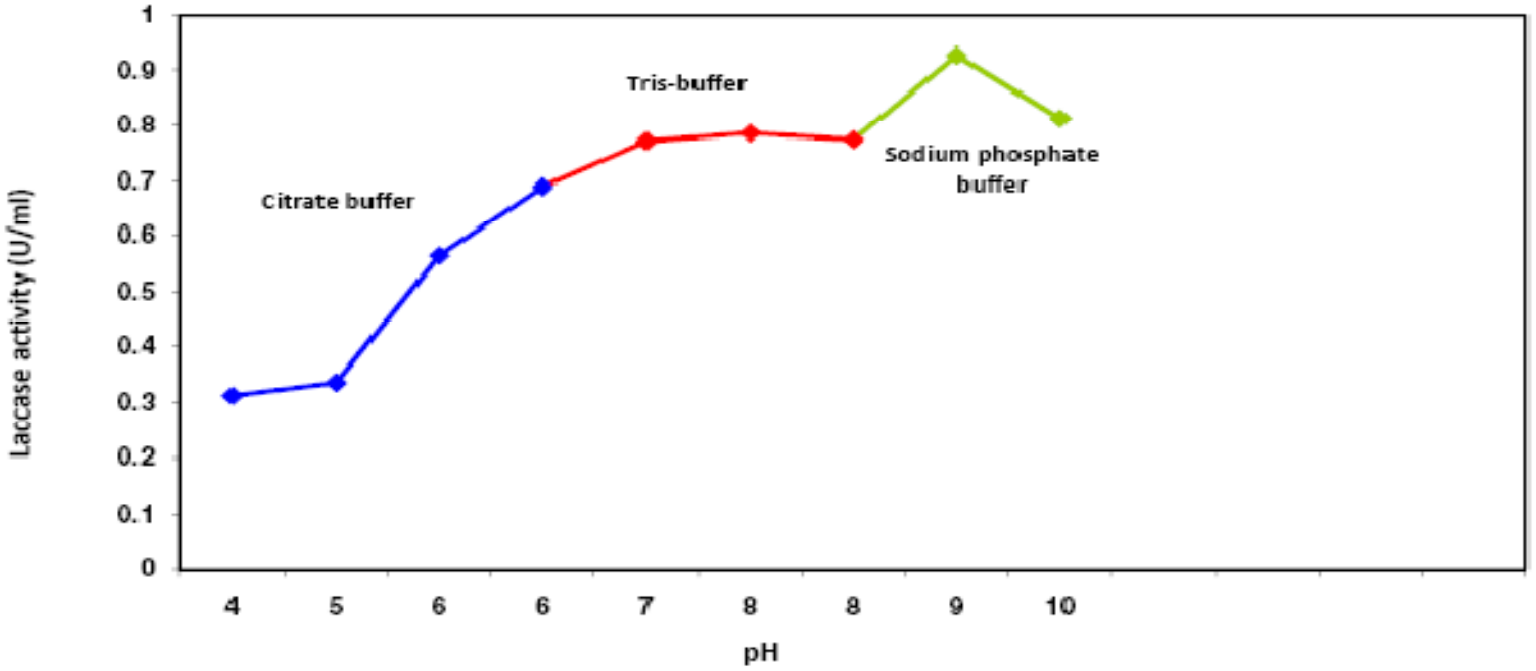


Fig. 51: Effect of pH on thermolaccase enzyme activity

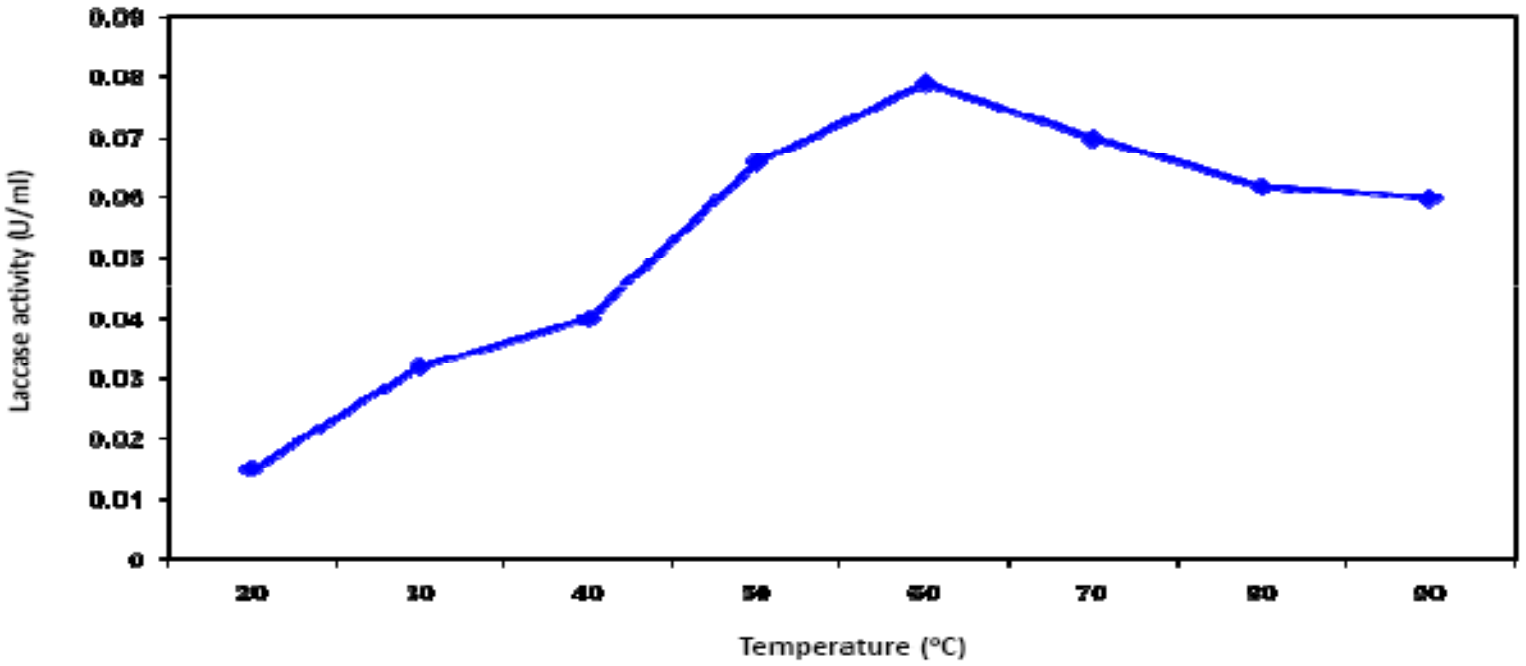


Fig. 52: Effect of temperature on thermolaccase activity

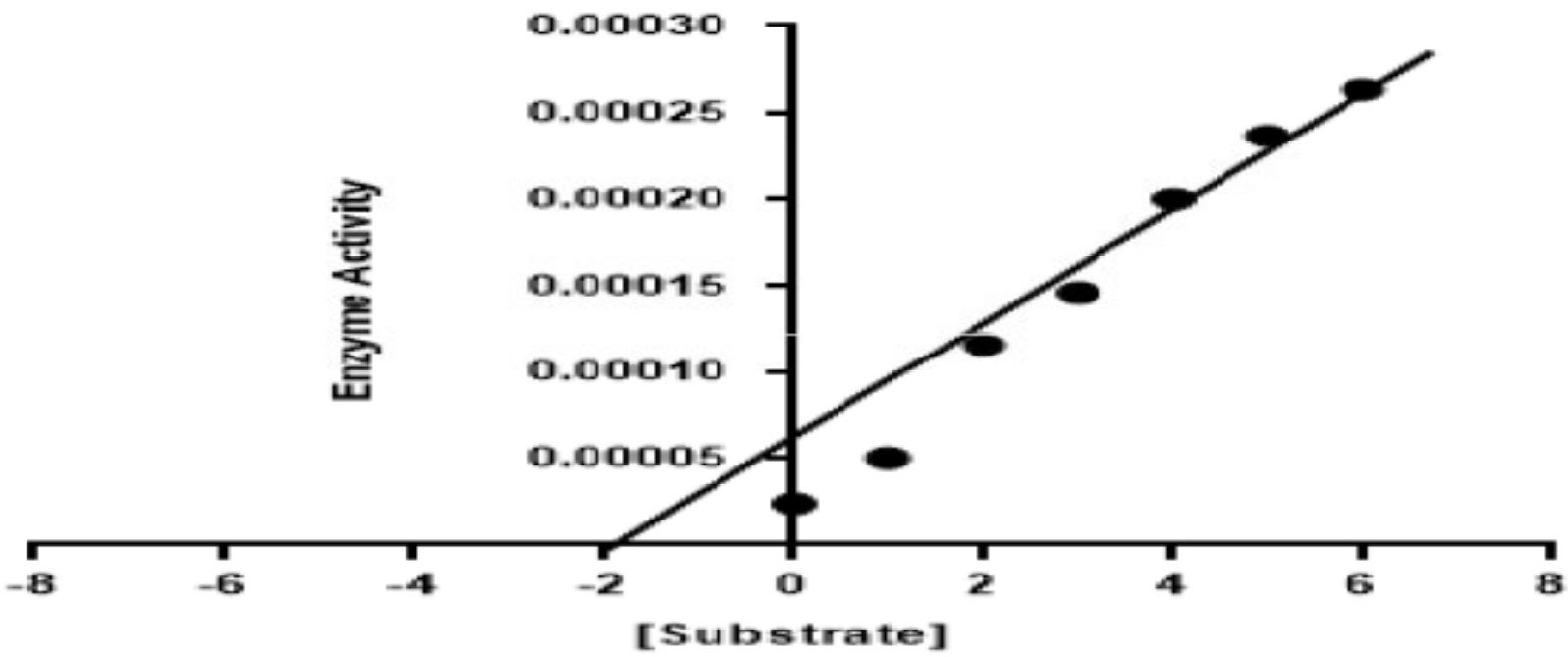


Fig. 53: Lineweaver burk plot of thermolaccase enzyme activity

Table 82: Effect of temperature on thermolaccase enzyme activity

Temperature	Enzyme activity (U/l)
20°C	0.106
30°C	0.178
40°C	0.382
50°C	0.498
60°C	**0.620**
70°C	0.570
80°C	0.487
90°C	0.472

4.13.4 Effect of substrate concentration

Michaelis-Menten constant (K_M) was calculated using Lineweaver- Burk plot (Fig.53) and was found to be 16.22 µM.

4.13.5 Effect of inhibitors

Two potential laccase inhibitors sodium azide and dithiothreitol at a concentration 0.1 mM- 0.9 mM were evaluated to study their effect on the thermolaccase enzyme activity after 10 mins of incubation and it was found that both of them were the potential inhibitors of laccase enzyme at a specific concentration (Table-83). A decrease in thermolaccase enzyme activity was observed when enzyme was incubated with different concentrations of sodium azide and DTT for 10 mins. It was observred that 94.44% and 92.59% activity was

Table 83: Effect of inhibitors (Sodium azide and Dithiothreitol) on thermolaccase enzyme activity

Inhibitor	Inhibitor concentration (mM)	Control activity (U/l)	Activity (U/l)
Sodium Azide	0.1	4.2	4.0
	0.3	4.2	2.4
	0.5	4.2	1.9
	0.7	4.2	0.2
	0.9	4.2	0.0
DTT	0.1	4.2	3.2
	0.3	4.2	2.2
	0.5	4.2	1.2
	0.7	4.2	0.4
	0.9	4.2	0.1

left after incubation with 0.1 mM sodium azide and dithiothreitol and then activity decreased with increase in concentration of both substrates. Only 1.9% activity was left after incubation with 1 mM inhibitors concentration leading to almost lost of the activity.

4.14 APLLICATIONS OF PARTIALLY PURIFIED THERMOSTABLE LACCASE ENZYME

4.14.1 Textile dye decolourization

Bacillus licheniformis strain RSM8 and its partially purified extracellular thermolaccase enzyme preparation was investigated for its ability to degrade six different textile dyes, viz., RBBR, Indigo Carmine, Congo Red, Aniline Blue, Brilliant Blue and Bromophenol Blue, which are significant contributors to water pollution.

4.14.1.1 Dye decolourization by thermophilic bacterial isolate

The thermolaccase producing thermophilic bacterial isolate *Bacillus licheniformis* strain RSM8 was tested using plate assay for its ability to decolorize the selected six synthetic dyes and it was found that this bacterial isolate was able to degrade all these dyes effectively. Significant decolourization has been observed with indigo carmine, brilliant green, congo red and RBBR dyes (Plate-27-29) whereas comparatively lesser decolurization of bromophenol blue and aniline blue was observed after 10 days of incubation.

In case of TYM broth supplemented with 0.5 g/l of each dye, percent decolourization of each dye was determined after a regular interval of time. Significant decolourization of 95% was observed after 10 days in case of Congo red dye, whereas comparatively lesser decolourization of 20% was observed with brilliant blue. Thus, it has been depicted that bacterial isolate *Bacillus licheniformis* strain RSM8 can degrade textile dyes successfully.

4.14.1.2 Dye decolourization by crude and partially purified thermolaccase

Crude and partially purified enzyme extracts were also investigated for dye decolourization ability of selected six different dyes. In case of congo red dye,

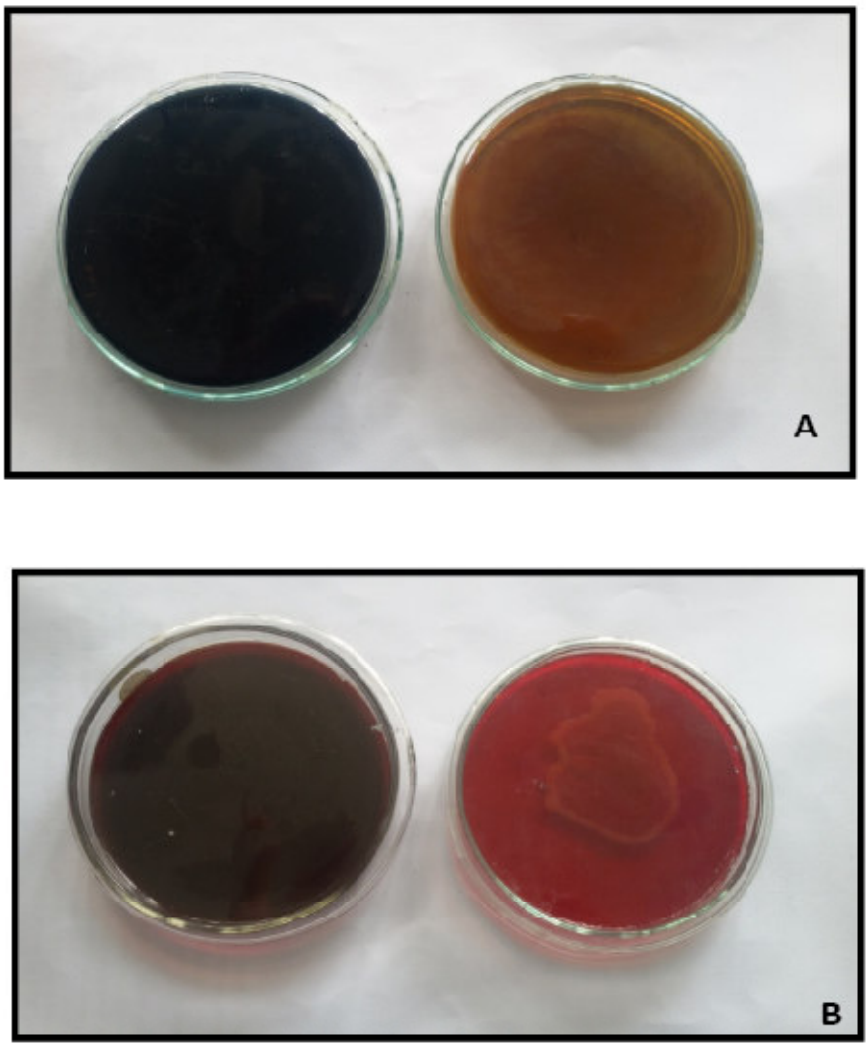
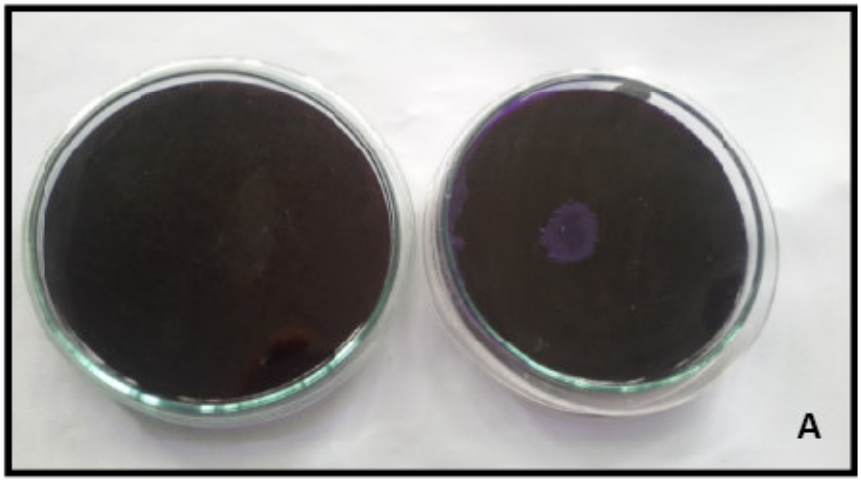


Plate 27: Dye decolourization by bacterial isolate *Bacillus licheniformis* strain RSM8 on TYM agar.
A) Aniline blue dye decolourization
B) Congo red dye decolourization



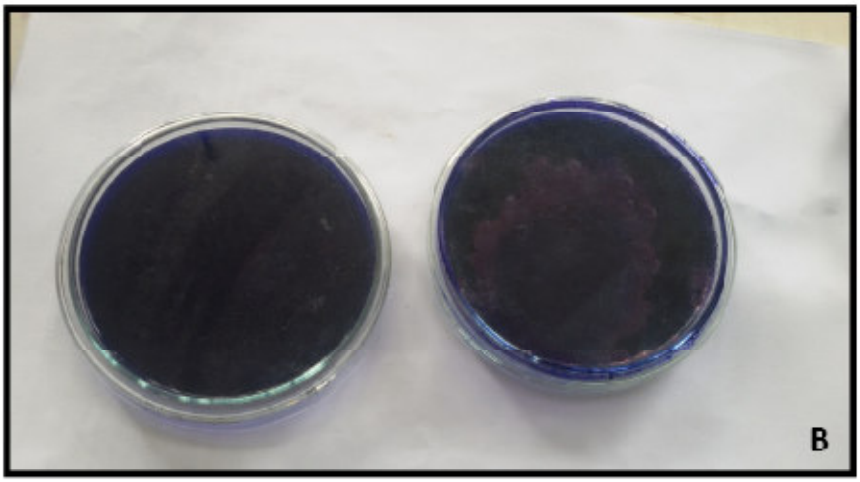


Plate 28: Dye decolourization by bacterial isolate *Bacillus licheniformis* strain RSM8 on TYM agar.

A) Bromophenol blue dye decolourization
B) Brilliant blue dye decolourization

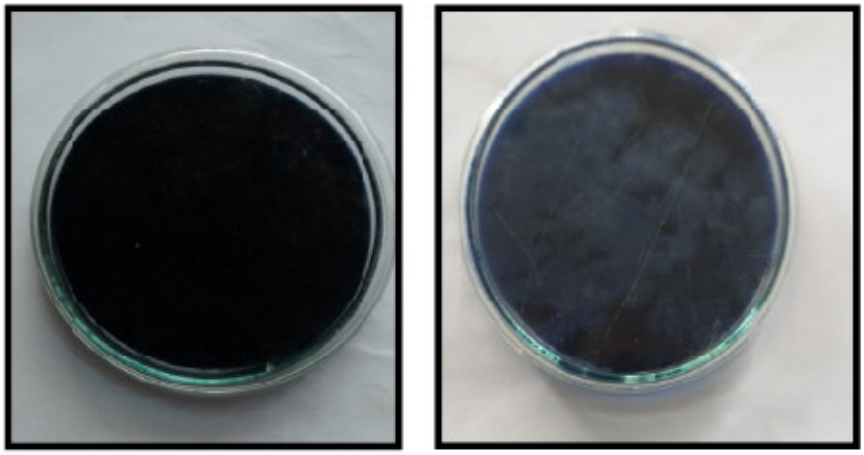


Plate 29: Dye decolourization by bacterial isolate *Bacillus licheniformis* strain RSM8 on TYM agar
A) RBBR dye decolourization

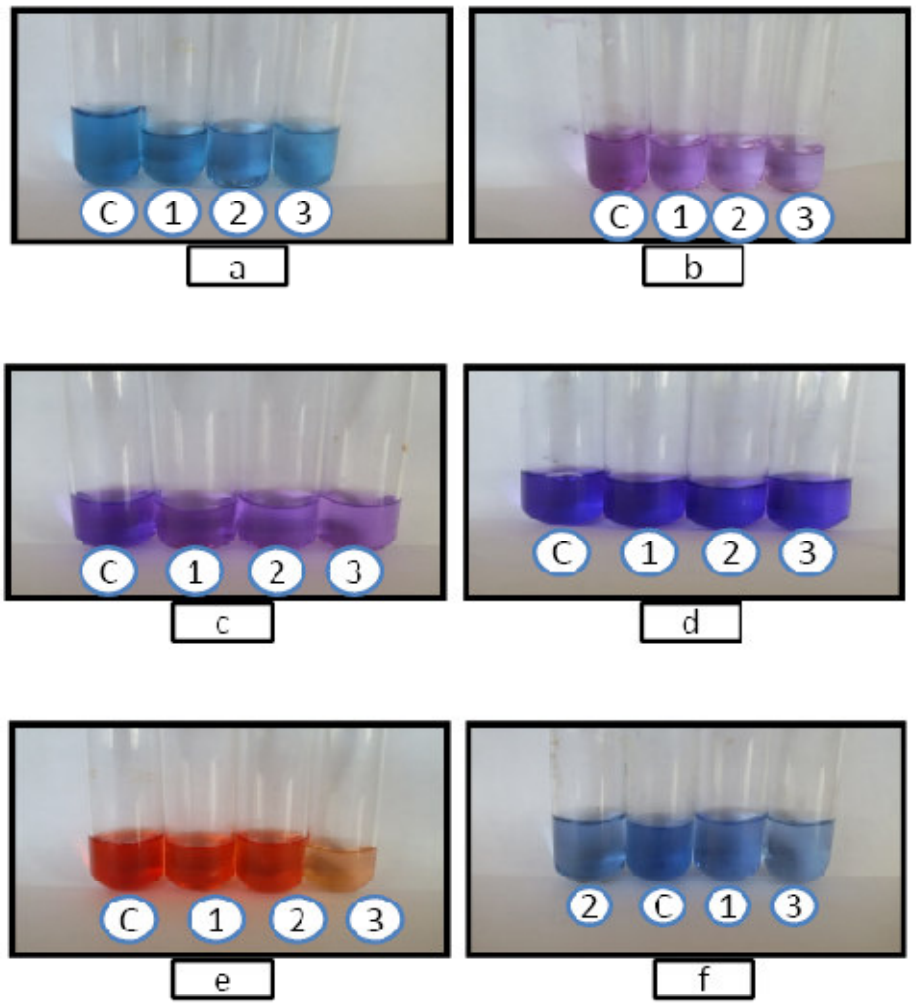


Plate 30: Decolourization of synthetic dyes using thermolaccase enzyme preparations.

a) Aniline blue decolourization
b) Brilliant blue decolourization
c) RBBR decolourization
d) Bromophenol blue decolourization
e) Congo red decolourization
f) Indigo carmine decolourization

C Control
1 Crude enzyme
2 Ammonium sulphate fraction
3 Partially purified enzyme

it was found that 56% of decolourization occurred after 24 hrs of incubation and then after 48 hrs of incubation, 86% of decolourization was observed whereas in case of Inodigo carmine dye, 51% of decolourization was measured after 24 hrs of incubation and it decolourized up to 73% after 48 hrs of incubation. In case of RBBR, 68% of decolourization was determined after 48 hrs of incubation. The rest of three dyes, Brilliant blue, Bromophenol blue and Aniline blue were found to show lesser decolourization comparatively. Percent decolourization of 40%, 51% and 54% was observed in Brilliant blue, Bromophenol blue and Aniline blue dyes, respectively even after 48 hrs of incubation (Plate-30, Table-84). Thus it was observed that crude as well as purified enzyme preparation also have ability to effectively degrade the textile dyes.

Table 84: Decolourization of dyes by using thermolaccase enzyme preparations

Dye	Decolourization percentage					
	Crude enzyme			Partially purified enzyme		
	12hr	24hr	48hr	12hr	24hr	48hr
Congo Red	15.00	56.00	86.00	72.89	86.00	91.66
Brilliant Blue	7.10	28.57	40.32	12.00	46.00	60.43
Indigo Carmine	38.57	51.42	73.00	56.60	72.70	80.00
RBBR	21.00	27.77	68.88	45.00	64.81	74.07
Bromophenol Blue	48.00	48.33	51.66	54.28	60.00	64.12
Aniline Blue	50.00	62.96	54.05	43.00	54.76	67.80

4.14.2 IMMOBLIZATION OF THERMOLACCASE ENZYME

Laccase enzyme with 1.89 U/l activity was entrapped in alginate beads after treating with 0.1% sodium alginate solution and 100 mM calcium chloride and immobilization percentage was found to be 76%. Laccase enzyme activity after immobilization was showed a little decrease and it was 1.70 U/l (Plate-31). It was further observed that stability of immobilized enzyme was more as compared to the free enzyme preparation and immobilized enzyme showed maximum activity at a pH of 10.0 and at an optimum temperature of 70^0C.

4.14.2.1 Synthetic dyes decolorization by immobilized thermolaccase

Six synthetic dyes viz., RBBR, Indigo Carmine, Congo Red, Aniline Blue, Brilliant Blue and Bromophenol Blue, were investigated for decolorization

using immobilized (Ca-alginate) laccase enzyme beads. Immobilized enzyme beads of Ca-alginate were also found to be effective in decolourization of all four synthetic dyes (Plate-32). In case of congo red dye, it was found that 78% of decolourization occurred after 24 hrs of incubation and then after 48 hrs of incubation 92% of decolourization was achieved. Similarly in case of RBBR, Indigo carmine and Aniline blue, 83%, 90% and 63% of decolourization was observed after 24 hrs of incubation, whereas 58% and 66% decolourization was observed in case of Bromophenol blue after 24 and 48 hrs of incubation respectively. Brilliant Blue dye was comparatively less decolourized and percent decolourization of 43% was measured after 48 hrs of incubation (Table-85).

Table 85: Decolourization of dyes using immobilized thermolaccase enzyme beads

Dye	Decolourization percentage		
	Immobilized thermolaccase enzyme		
	12 hr	24 hr	48 hr
Congo Red	42	78	92
Brilliant Blue	12	30	43
Indigo Carmine	69	82	90
RBBR	47	62	83
Bromophenol Blue	36	58	66
Aniline Blue	32	58	63

4.14.3 Phytotoxicity studies

In the previous experiment successful decolourization of six dyes has been achieved by crude and partially purified enzyme preparations in addition to *Bacillus licheniformis* culture. However decolourization does not essentially implies detoxification, thus it was important to evaluate the phytotoxicity of dyes before and after degradation. Therefore it was significant to perform toxicity assay on the textile dyes before and after treatment with partially purified enzyme preparation. Only three dyes viz., Congo red, Indigo carmine and RBBR have been used on the basis of results of previous experiment. Application of dyes and partially purified enzyme treated dyes on agriculturally important *Phaseolus mungo* seeds (dessicated, healthy, round) and two floricultural important plant



Plate 31: Immobilization of partially purified thermolaccase enzyme

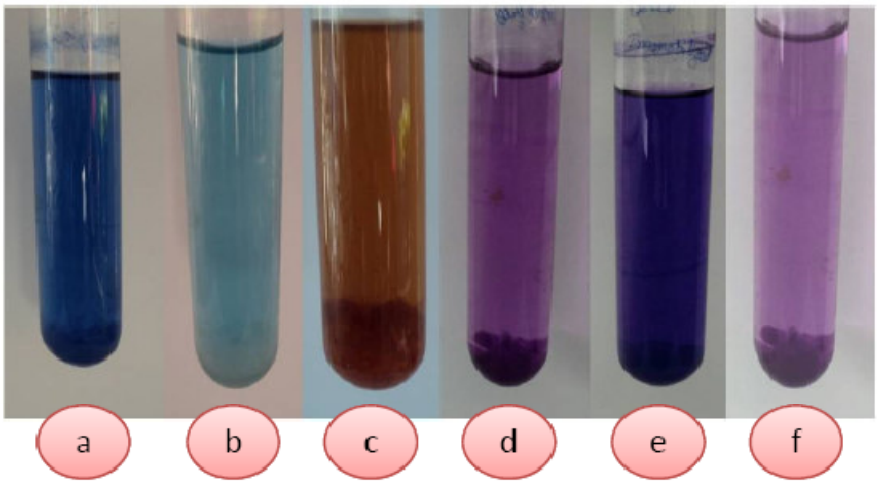


Plate 32: Decolourization of synthetic dyes using immobilized thermolaccase enzyme

a) Indigo carmine decolourization
b) Aniline blue decolourization
c) Congo red decolourization
d) RBBR decolourization
e) Bromophenol blue decolourization
f) Brilliant blue decolourization

species *Calendula officinalis* seeds (dessicated, healthy, crescent shaped) and *Tagetes patula* seeds (dessicated, healthy, strap-shaped) have been investigated. The germination percentage, plumule and radicle seedling emergence has been used as a parameter to assess toxicity.

Relative sensitivity of *Phaseolus mungo* towards three dyes viz., Congo red, RBBR and Indigo carmine and enzyme treated dyes were examined under *in vitro* conditions. Hundred percent seed germination was observed with water as control under *in vitro* conditions after 7 days. Whereas, seed germination of only 30%, 80% and 60% was observed with Congo red, RBBR and Indigo carmine dyes respectively. Thus 70%, 20% and 40% inhibition of seed germination indicates the toxic nature of the respective dyes to plants (Table-86, Plate-33). In case of enzyme treated dyes, seed germination of 85% was observed in presence of all the three enzyme treated dyes. In case of enzyme treated dyes the germination percent increased to 85% with Congo red showing 55% increase, whereas, enzyme treated RBBR showed 5% increase in seed germination as compared to the dye as such. In case of enzyme treated Indigo carmine 35% increase has been observed when compared to untreated dye. The length of plumules and radicles were also found to be affected significantly (Table-86, Plate-33) by Congo red, RBBR and Indigo carmine dyes, indicating toxic nature of these dyes.

Table 86: *In vitro* phytotoxicity evaluation of dyes (500 ppm) and enzyme treated dyes for *Phaseolus mungo*

Sr. No.	Treatment	Germination percentage	Plumule length (cm) (Average value)	Radicle length (cm) (Average value)
1	**Control**	100	8.0 cm	6.0 cm
2	**Congo red**	30	1.5 cm	3.0 cm
3	**Enzyme treated Congo red**	85	3.0 cm	5.0 cm
4	**RBBR**	80	1.0 cm	2.0 cm
5	**Enzyme treated RBBR**	85	5.0 cm	3.0 cm
6	**Indigo Carmine**	60	1.0 cm	4.0 cm
7	**Enzyme treated Indigo Carmine**	85	2.0 cm	5.0 cm

Average length of plumule with water as control was found to be 8.0 cm after 7 days, which was reduced to 1.5 cm, 1.0 and 1.0 cm, when seeds were treated with Congo red, RBBR and Indigo carmine dyes respectively. However enzyme treated dyes showed increase in plumule length to 3.0 cm, 5.0 cm and 2.0 cm respectively with Congo red, RBBR and Indigo carmine dyes. Average length of radicle with water as control was found to be 6.0 cm, which was reduced to 3.0 cm, 2.0 and 4.0 cm when seeds were treated with Congo red, RBBR and Indigo carmine dyes respectively after 7 day. Whereas enzyme treated dyes showed increase in radicle length to 5.0 cm, 3.0 cm and 5.0 cm respectively with Congo red, RBBR and Indigo carmine dyes after 7 days. Thus on the basis of this experiment it has been observed that in *Phaseolus mungo*, there was significant germination inhibition, a slower rate of plumule and radicle lengths in the presence of Congo red, RBBR and Indigo carmine dyes as compared to enzyme treated dyes (Table-87).

In case of *Calendula officinalis* plant species relative sensitivity towards three dyes viz., Congo red, RBBR and Indigo carmine and enzyme treated dyes were examined under *in vitro* conditions. Hundred percent seed germination was observed with water as control under *in vitro* conditions after 7 days. Whereas, seed germination of only 50%, 30% and 20% was observed with Congo red, RBBR and Indigo carmine dyes respectively. Thus 50%, 70% and 80 % inhibition of seed germination indicates the toxic nature of the respective dyes to plants (Table-87, Plate-34). In the case of enzyme treated dyes, seed germination of 75%, 60% and 80% was observed with enzyme treated Congo red, RBBR and Indigo carmine dyes respectively. In case of enzyme treated dyes the germination percent increased to 75% with Congo red showing 25% increase, whereas, enzyme treated RBBR showed 30% increase in seed germination as compared to the dye as such. In case of enzyme treated Indigo carmine 60% increase has been observed when compared to untreated dye. The length of plumules and radicles were also found to be affected significantly (Table-87, Plate-34) by Congo red, RBBR and Indigo carmine dyes, indicating toxic nature of these dyes. Average length of plumule with water as control was found to be 4.0 cm, which was reduced to 2.0 cm, 1.5 and 2.0 cm, when seeds were treated with Congo red,

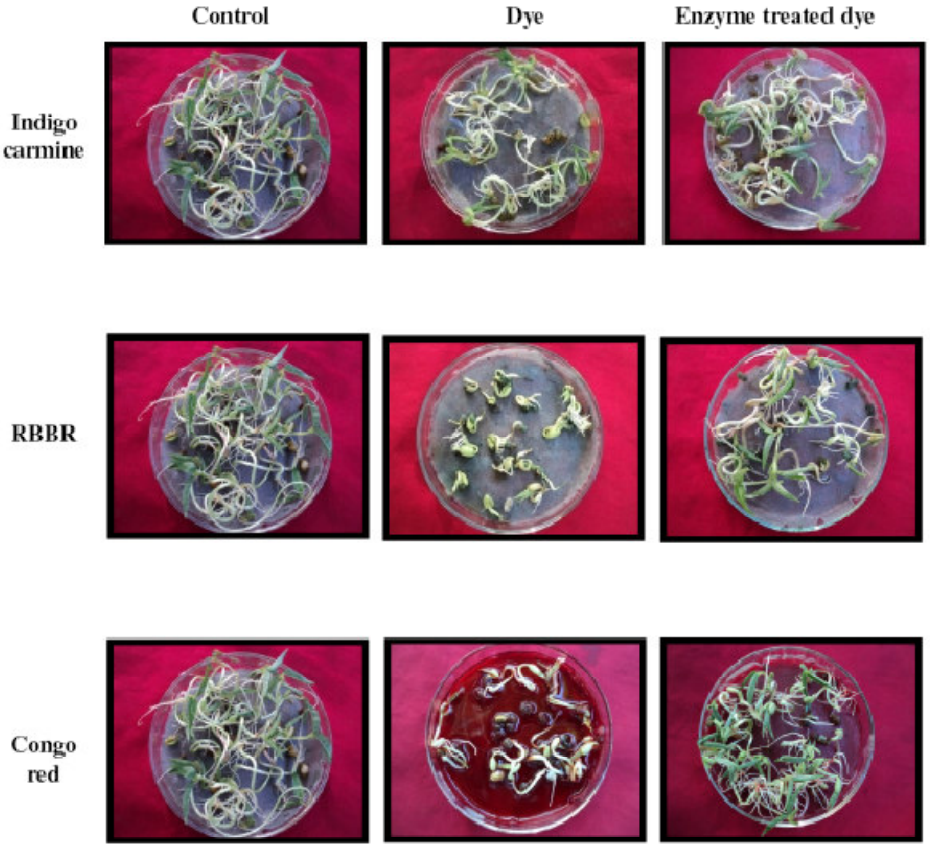


Plate 33: *In vitro* phytotoxicity study of dyes and enzyme treated dyes on *Phaselous mungo*

RBBR and Indigo carmine dyes respectively after 7 days. Whereas enzyme treated dyes showed increase in plumule length to 2.5 cm, 3.5 cm and 3.0 cm with Congo red, RBBR and Indigo carmine dyes respectively. Average length of radicle with water as control was found to be 3.5 cm, which was reduced to 1.5 cm, 3.0 and 2.0 cm when seeds were treated with Congo red, RBBR and Indigo carmine dyes respectively after 7 days. However enzyme treated dyes showed increase in radicle length to 2.5 cm, 3.0 cm and 3.0 cm respectively with Congo red, RBBR and Indigo carmine dyes after 7 days. Thus on the basis of this experiment it has been observed that in *Calendula officinalis*, there was significant germination inhibition, a slower rate of plumule and radicle lengths in the presence of Congo red, RBBR and Indigo carmine dyes as compared to enzyme treated dyes (Table-87).

Table 87: *In vitro* phytotoxicity evaluation of dyes (500 ppm) and enzyme treated dyes for *Calendula officinalis*

Sr. No.	Treatment	Germination percentage	Plumule length (cm) (Average value)	Radicle length (cm) (Average value)
1	**Control**	100	4.0 cm	3.5 cm
2	**Congo red**	50	2.0 cm	1.5 cm
3	**Enzyme treated Congo red**	75	2.5cm	2.5 cm
4	**RBBR**	30	1.5 cm	3.0 cm
5	**Enzyme treated RBBR**	60	3.5 cm	3.0 cm
6	**Indigo Carmine**	20	2.0 cm	2.0 cm
7	**Enzyme treated Indigo Carmine**	80	3.0 cm	3.0 cm

The relative sensitivity of *Tagetes patula*, towards three dyes viz., Congo red, RBBR and Indigo carmine and enzyme treated dyes were examined under *in vitro* conditions. Fifty percent seed germination was observed with water as control under *in vitro* conditions after 7 days. Seed germination of only 10 % was observed with Congo red dye whereas, no seed germination was observed with RBBR and Indigo carmine dyes. Thus 40%, 50% and 50 % inhibition of seed germination indicates the toxic nature of the respective dyes to plants (Table-88, Plate-35). In case of enzyme treated dyes, seed germination of 40%, 20% and

10% was observed with enzyme treated Congo red, RBBR and Indigo carmine dyes respectivaly. In case of enzyme treated dyes the germination percent increased to 40% with Congo red showing 30% increase, whereas, enzyme treated RBBR showed 20% increase in seed germination as compared to the dye as such. In case of enzyme treated Indigo carmine 10% increase has been observed when compared to untreated dye. The length of plumules and radicles were also found to be affected significantly (Table-88, Plate-35) by Congo red, RBBR and Indigo carmine dyes, indicating toxic nature of these dyes after 7 days. Length of plumule with water as control was found to be 2.0 cm, which was reduced to 0.5 cm when seeds were treated with Congo red dye. However enzyme treated dyes showed increase in plumule length to 0.8 cm, 0.2 cm and 0.1 cm respectively with Congo red, RBBR and Indigo carmine dyes. Length of radicles with water as control was found to be 1.0 cm, which was reduced to 0.2 cm when seeds were treated with Congo red dye. However enzyme treated dyes showed increase in radicle length to 0.4 cm, 0.1 cm and 0.1 cm with Congo red, RBBR and Indigo carmine dyes respectively after 7 days. Thus on the basis of this experiment it has been observed that in *Tagetes patula*, there was significant germination inhibition, a slower rate of plumule and radicle lengths in the presence of Congo red, RBBR and Indigo carmine dyes as compared to enzyme treated dyes (Table-88).

Table 88: *In vitro* phytotoxicity evaluation of dyes (500 ppm) and enzyme treated dyes for *Tagetes patula*

Sr. No.	Treatment	Germination percentage	Plumule length (cm) (Average value)	Radicle length (cm) (Average value)
1	**Control**	50	2.0 cm	1.0 cm
2	**Congo red**	10	0.5 cm	0.2 cm
3	**Enzyme treated Congo red**	40	0.8 cm	0.4 cm
4	**RBBR**	00	0.0 cm	0.0 cm
5	**Enzyme treated RBBR**	20	0.2 cm	0.1cm
6	**Indigo Carmine**	00	0.0 cm	0.0 cm
7	**Enzyme treated Indigo Carmine**	10	0.1 cm	0.1 cm

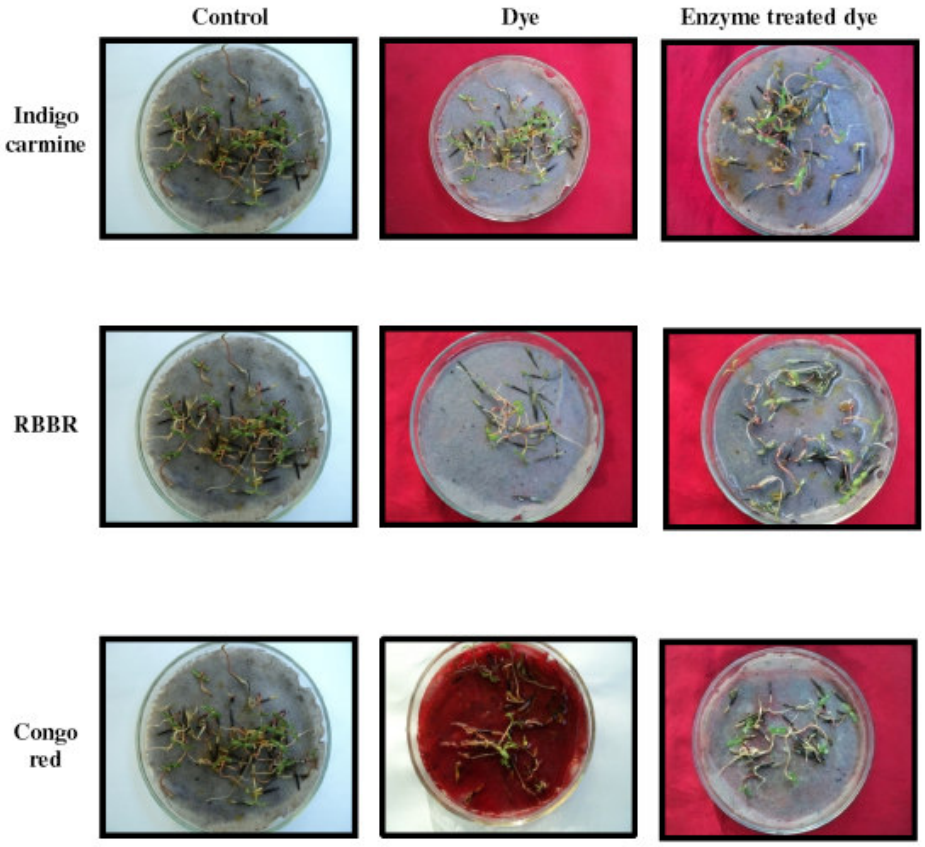


Plate 34: ***In vitro*** **phytotoxicity study of dyes and enzyme treated dyes on** ***Calendula officinalis***

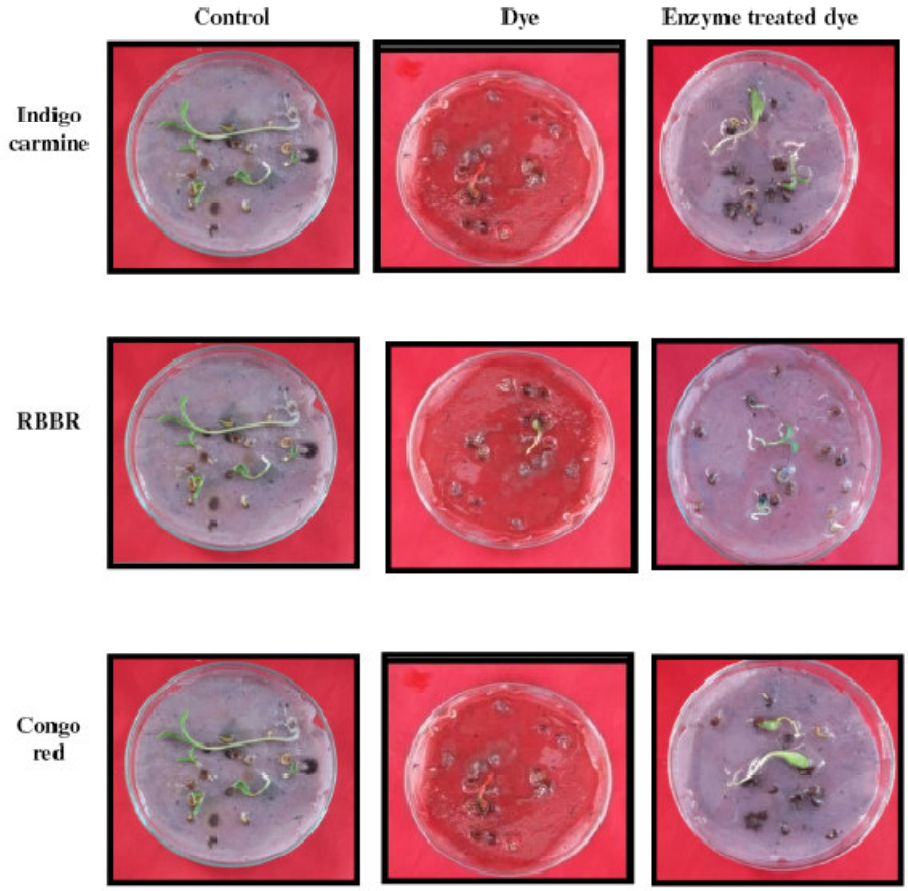


Plate 35: ***In vitro*** **phytotoxicity study of dyes and enzyme treated dyes on** ***Tagetes patula***

Control Dye Enzyme treated dye



15 days



30 days

Plate 36: *In vivo* phytotoxicity study of Congo red dye and enzyme treated Congo red on *Phaselous mungo*

On the basis of these results *Phaseolus mungo* was selected for studying further *in vivo* effect of Congo red dye on this plant species. Relative sensitivity of *Phaseolus mungo* towards Congo red dye as such and partially purified thermolaccase enzyme treated Congo red dye has been investigated under *in vivo* conditions. Sixty percent seed germination was observed with water as control under *in vivo* conditions after 15 and 30 days. Whereas, seed germination of only 40% was observed on treatment with Congo red dye, thus 20% inhibition was observed indicating the toxic nature of the dye to plants (Table-89, Plate-36). In case of enzyme treated dye the germination percent increased to 50% showing 10% increase as compared to the dye as such. The length of plumules was also found to be affected significantly (Table-89, Plate-36) by Congo red dye after 15 and 30 days. Average length of plumules with water as control was found to be 10 cm after 15 days, which was reduced to 4.0 cm when seeds were treated with Congo red dye. Whereas enzyme treated dye showed increase in average plumule length to 6.0 cm. Similarly average length of plumule after 30 days with water as control was found to be 15.0 cm, which was reduced to 5.0 cm when seeds were treated with Congo red dye. However enzyme treated dye showed increase in average plumule length to 8.0 cm. Thus on the basis of this experiment it has been observed that in *Phaseolus mungo* there was significant germination inhibition, a slower rate of plumule length in the presence of Congo red dye as compared to partially purified enzyme treated dye (Table-89), indicating reduction of toxic effect of Congo red dye after treatment with partially purified thermolaccase enzyme preparation on *Phaseolus mungo* germination status.

Table 89: *In vivo* phytotoxicity assessment of Congo red dye and enzyme treated Congo red dye for *Phaseolus mungo*

Sr. No.	Treatment	Germination percentage (15 days & 30 days)	Plumule length (cm) (Average value)	
			15 days	30 days
1	**Control**	60	10.0 cm	15.0 cm
2	**Congo red**	40	4.0 cm	5.0 cm
3	**Enzyme treated Congo red**	50	6.0 cm	8.0 cm

4.15 ISOLATION OF THERMOSTABLE LACCASE ENCODING GENE

4.15.1 Selective amplification of thermolaccase encoding gene

The selected laccase producing bacterial isolate *Bacillus licheniformis* strain RSM8 was investigated for the presence of gene, encoding for laccase enzyme. The genomic DNA sample, extracted from this isolate was selectively amplified using PCR technology and degenerate primers CuLVF and CuLVR based on the conserved copper sites of laccases. After 35 cycles of PCR amplification as described under section 3.15.2 , these primers were able to successfully amplify laccase encoding gene and produced an amplicon of expected size i.e. 1000 bp (Plate-37).

4.15.2 Sequencing

To determine the nucleotide sequence of the PCR product of the laccase encoding gene, sequencing was carried out using same upstream and downstream primers and as a result, a sequence of 150 bp corresponding to laccase encoding gene of RSM8 was obtained. The nucleotide sequence of the query gene of bacterial isolate *Bacillus licheniformis* strain RSM8, obtained after correction has been depicted here as under:

>Query gene sequence

ATTGCGACCTTTGCGCCGTATAGCGGCCGCTATGTGTGGCATTGCCAT
ATTCTGGAACATGAAGATTATGATATGATGCGCCCGCTGGAAGTGAC
CGATGTGCGCGAAGATTATGATATGATGCGCCCGCTGGAAGTGACCG
ATGTGCGC

It was clearly depicted in the table that query sequence contain 22.00% Adenine, 25.33% Thymine, 22.00% Cytosine and 30.67% Guanine out of a total of 150 query sequenence with a G+C content of 52.70% (Table-90).

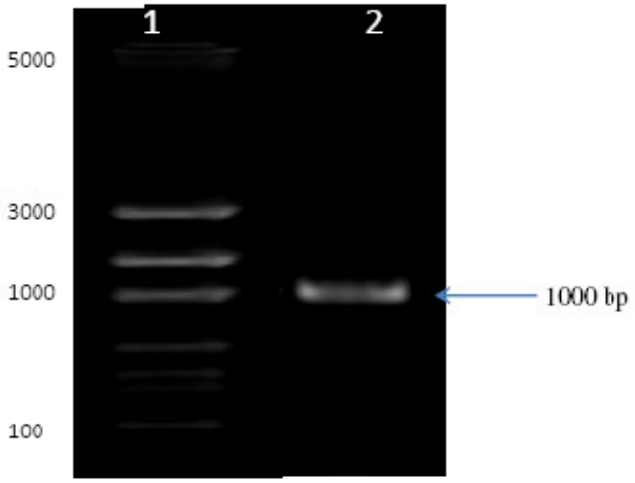


Plate 37 : Gel image showing amplification of thermolaccase encoding gene.

Lane 1 : Molecular marker (100 bp -5 kb DNA ladder)

Lane 2 : Amplified thermolaccase gene

Table 90: Nucleotide base composition in the query thermolaccase gene nucleotide sequence of *Bacillus licheniformis* strain RSM8

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	33	22.00
Thymine (T)	38	25.33
Cytosine (C)	33	22.00
Guanine (G)	46	30.67
G+C	79	52.70
A+T	71	47.30

4.14.3 Translation of the laccase gene nuclotide sequence of *Bacillus licheniformis* strain RSM8

Laccase gene nucleotide sequence of *Bacillus licheniformis* strain RSM8 was translated into corresponding amino acid residual sequences using protein translator tool ExPASy (Expert Protein Analysis Syatem). Analysis of amino acid sequence produced a longest reading frame (5`3`Frame-1) with a total of 50 amino acids having isoleucine amino acid at the start position and amino acid arginine at the end of the sequence.

5'3' Frame 1

>Amino acid sequence decoded from the laccase gene nucleotide sequence of *Bacillus licheniformis* strain RSM8

IATFAPYSGRYVWHCHILEHEDYDMMRPLEVTDVREDYDMMRPL
EVTDVR

4.15.4 *In silico* analysis of the sequences

In silico analysis pertaining to amino acid sequences, so obtained, was carried out using various bioinformatics tools available online. Various online approaches investigated are as follows:

4.15.4.1 BLASTp

Translated amino acid sequence of laccase gene nucleotide sequence of *Bacillus licheniformis* strain RSM8 was analysed with corresponding sequences of different laccase enzymes reported from different parts of the world. BLASTp

search of protein sequence with the most similar sequences of the protein database (http://www.ncbi.nlm.nih.gov/blast) revealed the closest homology from the database. All the similarities have been summarized in the Table-86. Sequence analysis revealed that test laccase amino acid sequence of *Bacillus licheniformis* strain RSM8 showed maximum homology of 97% with copper oxidase *Bacillus licheniformis* (Table-91).

Table 91: Percent homology of query laccase protein sequence of *Bacillus licheniformis* strain RSM8 with other protein sequences present in the database using BLASTp analysis

Accession Number	Closest match	Percent Similarity
WP_035337064.1	Copper oxidase *Bacillus licheniformis*	97.0
AGT45479.1	Spore coat protein (*Bacillus licheniformis*)	97.0
WP_023855578.1	Multispecies : copper oxidase *(Bacillus)*	97.0
WP_026578945.1	Copper oxidase *Bacillus* sp SB47	97.0

4.15.4.2 Multiple Sequence Alignment

Multiple sequence alignment of test amino acid sequences of laccase of *Bacillus licheniformis* strain RSM8 with that of the selected amino acid sequences was performed using MULTALIN programme. Similarity score of amino acid sequences with test laccase amino acid sequence have been depicted in Annexure-3.

4.15.4.3 Computational Phylogenetics

To gain the insight of laccase nucleotide sequence of *Bacillus licheniformis* strain RSM8, phylogenetic trees were constructed using MEGA (Molecular Evolutionary Genetics Analysis; Version 4). The trees were inferred from amino acid sequences of *Bacillus licheniformis* strain RSM8. Relationship between query sequence and other sequences taken from NCBI were investigated using Neighbour Joining (NJ) method and phylogenetic trees were drawn supported with high bootstrap values. Phylogenetic trees also verified, amino acid

sequences as laccase gene and enzyme respectively, as they clustered closely with copper oxidase *Bacillus licheniformis* (WP_035337064.1).

Phylogenetic tree analysis of laccase amino acid sequence

This phylogenetic tree was clustered into two clusters A and B (Fig.54) which were further sub clustered. Cluster A was subclustered into two subclusters C and D and Cluster B was sub clustered into two sub clusters E and F, which further sub, sub-sub divided into sub clusters. These sub-sub clusters further divided into groups. Query sequence of *Bacillus licheniformis* strain RSM8 was separated out by sub cluster D, which showed similarity with other Spore coat protein (*Bacillus licheniformis*) and copper oxidase *Bacillus licheniformis.* Thus, Phylogenetic tree has also verified that test laccase amino acid sequences of the present study showed maximum similarity to other *Bacillus licheniformis* laccase enzyme.

4.15.5 Conserved Domain Search

Proteins often contain several domains or modules, each with distinct evolutionary origin function. National Centre for Biotechnology Information (NCBI) houses Conserved domain database which is a collection of sequence alignments and profiles representing protein domains conserved in evolution. It also includes alignments of the domains to known 3-dimensional protein structure. The conserved domain (CD) search was used to find putative conserved domains present in test amino acid sequence of laccase encoding gene of *Bacillus licheniformis* strain RSM8 . Results of the CD-search have been presented in Fig-55. For putative CD-search, reverse position-specific BLAST (PSI-BLAST) algorithm was used. Suf I (COG2132, Putative multicopper oxidases [Secondary metabolites biosynthesis, transport, and catabolism] Conserved Domain was identified at amino acid position 1-33 with E-value of 5.27e-07 (Fig.55).

4.15.6 Secondary Structure Prediction

Secondary structure of the amino acid sequence of laccase enzyme in the present study was predicted using Phyre 1 and 2, (Protein Homology/Analogy Y

Recognition Engine) a web based services for protein structure prediction. Results of the secondary structure prediction for the protein sequences of laccase of *Bacillus licheniformis* strain RSM8 have been presented in Fig.56 which clearly depicts the absence of alpha helix, beta strand (blue in colour) and coils indicated by the faint lines in the secondary structure. SS confidence line in the secondary structure showed confidence in prediction with red colour showing high confidence in prediction and blue colour showing low confidence in prediction. In the secondary structure of the query sequence high (9) value of confidence key was predicted (Fig.56).

4.15.7 Tertiary Structure Prediction

Tertiary structure was also predicted for the amino acid sequences of laccase enzyme of *Bacillus licheniformis* strain RSM8 using Phyre 1 and 2. Results of the tertiary structure prediction for the protein sequences of laccase of *Bacillus licheniformis* strain RSM8 have been presented in Fig.57. Tertiary structure was formed with 100 % confidence along with 98 % coverage which means 109 residues i.e. 98 % of query sequence have been modelled with 100% confidence by the single highest scoring template. Tempelate identification was done by the server itself and it was found that d1kv7a2, cupredoxin superfamily was selected as template for the tertiary structure prediction detailed information of template was shown in Fig.57. Interactive 3D structure of protein model was also predicted using Jmol software and conserved histidine residues was found out in the structure which was shown in Fig.58.

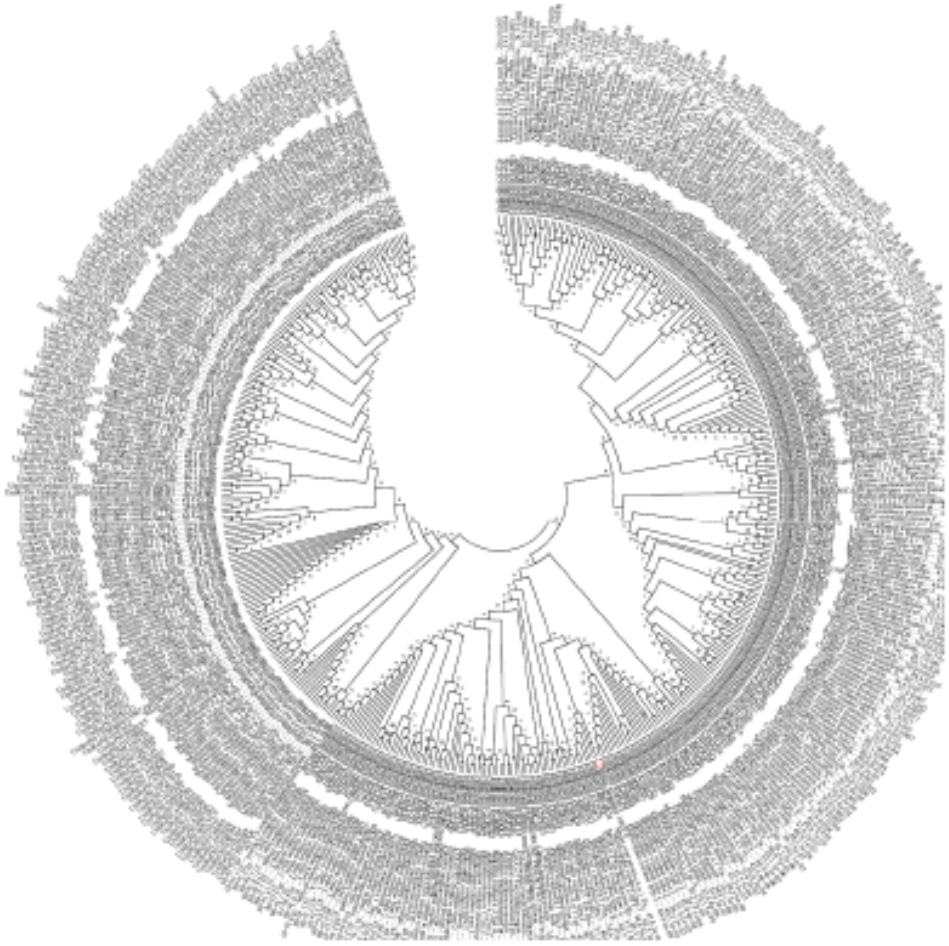


Fig. 54. Phylogenetic tree of laccase/thermolaccase protein sequences of *Bacillus licheniformis* strain RSM8. GenBank accession numbers of the analyzed sequences are shown. A total of 100 bootstrap replicates were performed, and the bootstrap values are indicated at the branching points.

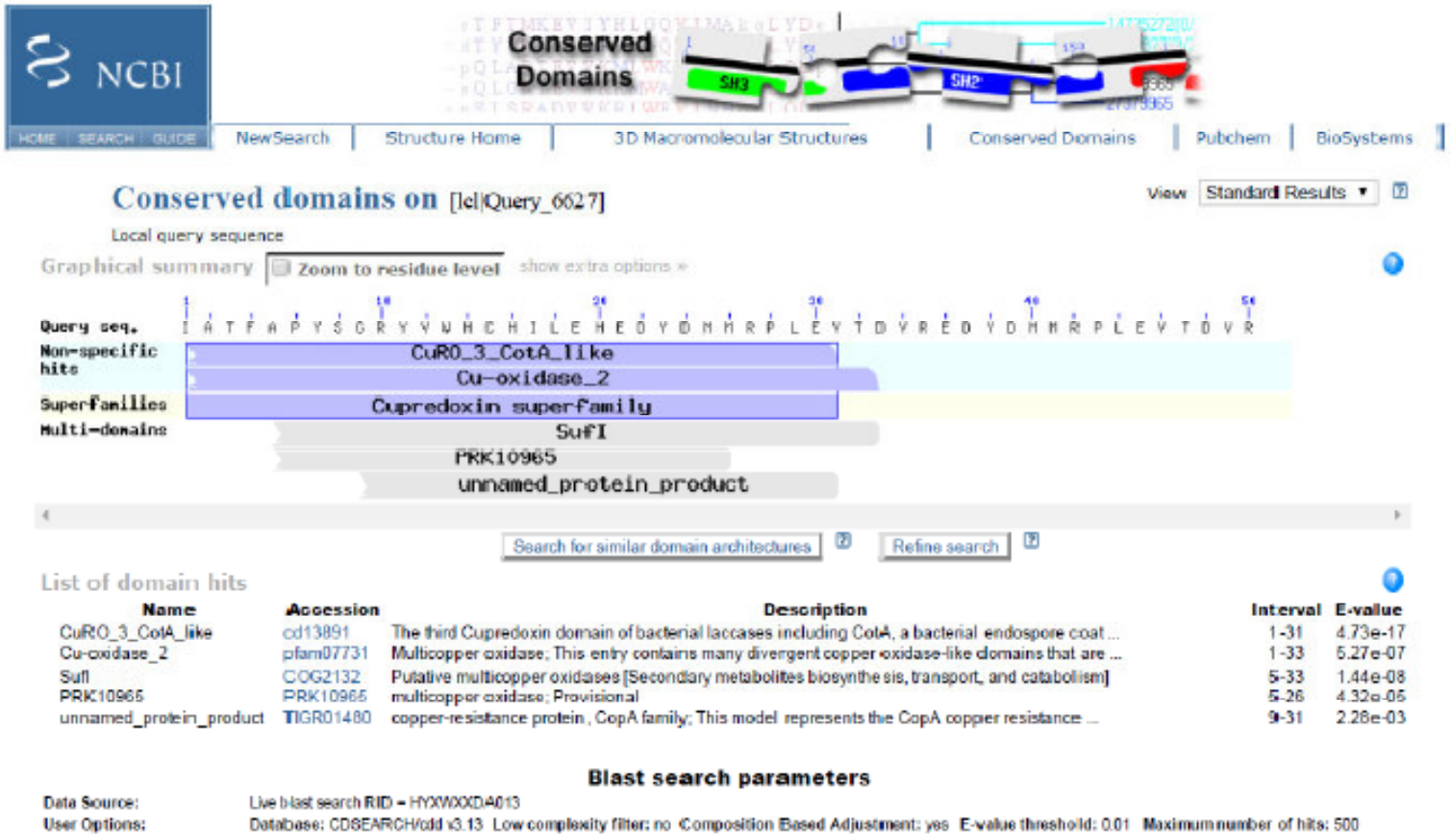


Fig . 55: Conserved domains present in thermolaccase protein sequence of *Bacillus licheniformis* strain RSM8

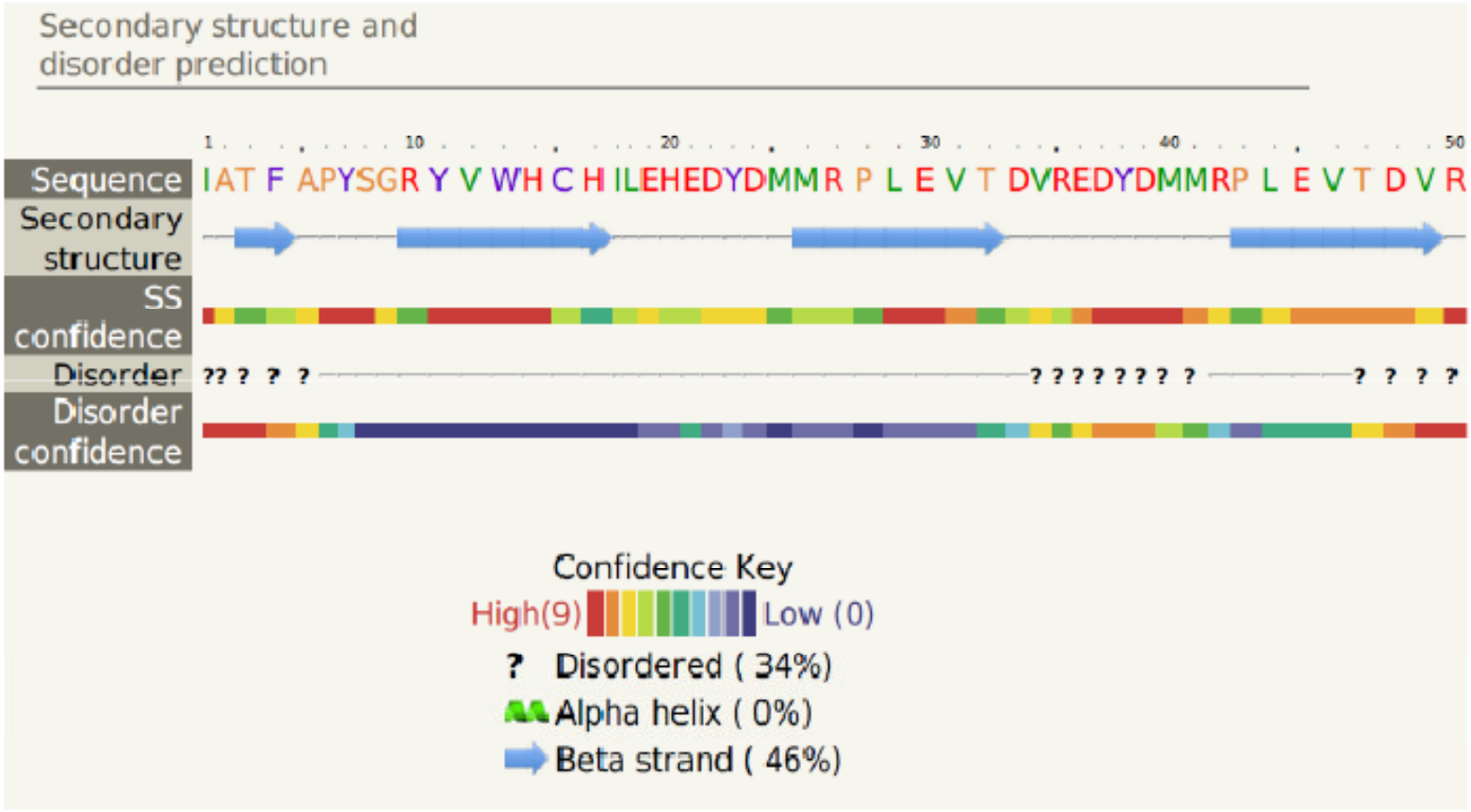


Fig . 56: Secondary structure of thermolaccase enzyme obtained from *Bacillus licheniformis* strain RSM8

Detailed template information

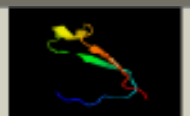
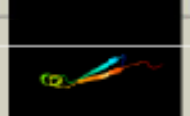
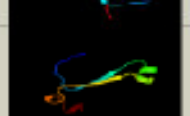
#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c1ofdA_	Alignment		99.7	88	**PDB header:**oxidoreductase **Chain:** A; **PDB Molecule:**spore coat protein a; **PDBTitle:** crysta structure of bacillus subtilis cota after 1h2 soaking with ebs
2	c2xdlC_	Alignment		99.7	42	**PDB header:**oxidoreductase **Chain:** C; **PDB Molecule:**bilirubin oxidase; **PDBTitle:** the crystal structure of bilirubin oxidase from myrothecium2 verrucaria
3	c3abgA_	Alignment		99.7	42	**PDB header:**oxidoreductase **Chain:** A; **PDB Molecule:**bilirubin oxidase; **PDBTitle:** x-ray crystal analysis of bilirubin oxidase from myrothecium2 verrucaria at 2.3 angstrom resolution using a twin crystal
4	c2q23G_	Alignment		99.7	48	**PDB header:**oxidoreductase **Chain:** G; **PDB Molecule:**phenoxazinone synthase; **PDBTitle:** the crystal structure of hexameric phenoxazinone synthase
5	c3zx1A_	Alignment		99.6	45	**PDB header:**oxidoreductase **Chain:** A; **PDB Molecule:**oxidoreductase, putative; **PDBTitle:** multicopper oxidase from campylobacter jejuni: a metallooxidase
6	d1gska3	Alignment		99.6	88	**Fold:**Cupredoxin-like **Superfamily:**Cupredoxins **Family:**Multidomain cupredoxins
7	c3sqrA_	Alignment		99.6	21	**PDB header:**oxidoreductase **Chain:** A; **PDB Molecule:**laccase; **PDBTitle:** crysta structure of laccase from botrytis aclada at 1.67 a resolution

Fig. 57: Detailed template information showing identity of template sequences of thermolaccase enzyme of *Bacillus licheniformis* strain RSM8 with 3-D structures of other laccases.

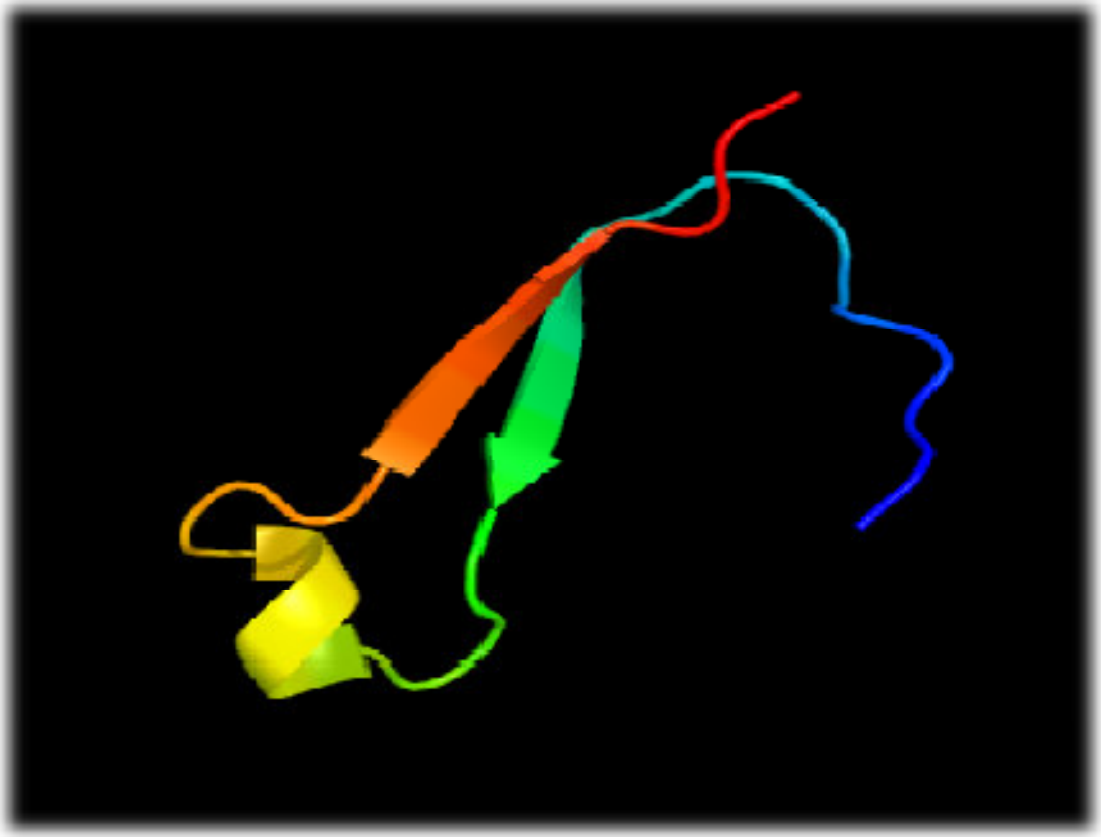


Fig. 58: 3-D structure of thermolaccase enzyme obtained from *Bacillus licheniformis* strain RSM8

Chapter-5

DISCUSSION

The discovery of extremophiles has put vitality into the biotechnology industry and dreams of stock options in the mind of biologists. The discipline of life in extreme environments has exploded during the past decades and various extremophiles have been now recognised as cell factories. Advances in biotechnology and associated areas have enhanced the value of biodiversity and indigenous biological wealth and knowledge. It has been reported that global markets for some products derived from genetic resources and biodiversity lies in the range of US $500-800 billion (Kate *et al.*, 1999b). The Indian biotechnological sector is expected to grow from the current US $5-7 billion to US $100 billion by 2025 by doubling the growth rate of this sector from 15% to 30% according to Association of Biotechnology Led Enterprises (OECD, 2011). Over the past few decades the development of new approaches in the field of biology, genomics, chemistry and information technology has given impetus to the pace of change in the industry, setting new targets for development. This in turn has created greater demands for adequate supply of bioresources, further encouraging bioprospecting-the exploration of biodiversity for commercially valuable biological and genetic resources. This activity involves the applications of advanced technologies to develop new industrial enzymes in addition to many more products from biodiversity. Such advances in laboratory based biotechnology have increased the value of genetic resources and indigenous microbial wealth, providing important leads to commercially exploitable properties of resources. Bioprospecting and commercialization of bioresources by indigenous biological communities in India is moving towards the new paradigm. Though bioprospecting was largely centred on plants previously, however in recent times various other forms of biodiversity specially the microbial diversity has been explored with considerable success. The opportunity of new industrial applications from microorganisms is as large as the variety of environments they confront. Success of *Thermus aquaticus* achieved commercially has interested

and directed scientists towards isolation and identification of thermophilic microorganisms in geothermal areas, not identified designated as yet. Microbes evolve very quickly than we can study them, providing an even increasing diversity of functions for industrial applications. The rapid evolutions are predicted on the great diversity and apparent plasticity of microbial products which have the characteristics of high chemical diversity, biochemical specificity and other molecular properties. Most of the microorganisms that contribute to the microbial diversity describe variability of communities. After elucidating thermostable properties of thermophilic microorganisms, attention has been drawn towards revealing potential industrial uses of various thermostable enzymes, such as laccase, lipase, protease, cellulase, amylase etc. Thermostable enzymes offer major biotechnological advantages over commonly used mesophilic enzymes, as these possess stability at high temperatures, higher resistance to chemical denaturants, high alkalinity, extreme acidity and can also withstand increased substrate concentrations without losing their catalytic efficiency. Due to their stability at elevated temperatures, reactions are faster and less susceptible to microbial contaminations (Li *et al.,* 2005). The present study was carried out to explore the status of indigenous thermolaccase producing thermophilic bacteria from hot water springs of Himachal Pradesh, which harbours plenty of diverse forms of microbial life and has remained largely unexplored with the need to search for novel forms of commercial uses of biodiversity and associated knowledge by indigenous communities.

Laccases are exceptionally versatile enzymes and ubiquitous in nature being produced by wide variety of eukaryotes and prokaryotes. The functions of enzymes differ from organism to organism and it has been observed during the perusal of literature that these enzymes have a wide range of applications because of their potential to curtail different types of the pollutions caused by toxic chemicals, xenobiotics and industrial effluents. In the recent years laccases have been recognised for their use in the diverse fields of industrial applications. So these oxidative enzymes are also known as ‘green catalyst’ which work with air producing water as only by-product and this property makes them more useful for various scientific applications. The broad substrate specificity of laccases

harbours great potential and currently it has been recognised for use in wide variety of applications. These are the model enzymes of multicoppper oxidase family and have received much attention from researchers in last decades due to their ability to oxidize both phenolic and nonphenolic lignin related compounds as well as environmental pollutants, which makes them very useful in biotechnological processes such as detoxification of industrial effluents and in bioremediation. Due to harsh industrial process conditions that may include high temperature and pressure, high salt concentrations, acidic or alkaline pH, oxidative conditions, high shear forces or short delays, resistant enzymes are required. In addition there is a current need for enzymes with new catalytic properties for potential innovations and breakthroughs in industrial biotechnology. One of the general prerequisites for an enzyme to be applicable in industrial processes is transient ability to maintain catalytic activity at elevated temperatures and ability to resist irreversible inactivation at high temperatures and to keep activity at least at 60^0C for a prolonged period of time (Vieille *et al*., 1996; Vieille and Zeikus, 2001). In order to facilitate novel and more efficient bio-catalytic process applications, there is a need for laccases with improved biochemical properties, such as thermotolerance.

It is certainly worthwhile to intensify screening programmes to explore and identify rare genera and species of microorganisms, culture and conserve them *in vitro* and intensively investigate them for enzymes with novel properties. Indeed such screening programmes in combination with advances in molecular biology have already been yielding spectacular results in the developed world. It is essential that similar studies are intensified in other developing countries of the world including India to remain competent and competitive in biotechnology in the future decades.

In India also thermal springs have been found scattered throughout the country and occur either as solitarily or in groups upto 3000 m above sea level. In the state of Himachal Pradesh, which is located in northern India, between 30"22' and 33"12' north latitude and between 75"47' and 79"04' east longitude, thermal springs occur as terrestrial biotopes of thermophiles and have been found located

in mainly the districts of Kullu and Mandi at places known as Manikaran, Vashisht, Khirganga and Tattapani. The mountainous state has altitudes ranging from 350-7000 meters above the sea level. Physico-chemical analysis of water of these thermal springs reveals a pH range of 4.8-9.5 with temperature range of 41-106°C and Manikaran thermal spring has been found to be one of the hottest water spring of the region having a temperature as high as 106°C. The chloride and sulphate contents have been found to range from 495-2673 mg/l and 10.0-31.8 mg/l respectively, whereas magnesium and calcium ion content vary from 1.46-55.16 mg/l and 33.66-74.28 mg/l respectively. In the present study values of total hardness and $CaCO_3$ hardness were found to be varie from 105-315 mg/l and 37.7-268 mg/l respectively. In other study Kumar *et al.,* (2003) reported total hardness of 165.2 mg/l at pH 7.0 from Vashisht hot water spring samples. In another study Pathak and Rekadwad (2013) reported pH of 7.33, temperature of 60.7^{o}C, total hardness 127.7 mg/l and calcium hardness of 28.9 mg/l from Unkeshwar hot water spring located in Maharashtra. Olivier *et al.,* (2008) reported pH range 6.0-9.0, temperature range 30-52^{o}C, magnisisum hardness range 1.80-6.44 mg/l, calcium hardness range 13.00-36.13 mg/l and sulphate content range 2.16-92.82 mg/l from thermal springs in the Waterberg area in Limpopo Province, South Africa. Correlation matrix obtained by statistical analysis depicted pair wise correlation coefficient and all the variables were found to be significantly correlated with few exceptions (Table-25). Coefficient of variability and other related parameters were further analysed by ANOVA and depicted average performance of various characters. Maximum mean values of pH, temperature, chloride, sulphate, total hardness, calcium ions, $CaCO_3$ and magnesium content was found to be 6.92, 97.15, 1379.05, 19.25, 214.0, 60.98, 158.92 and 12.99 respectively (Table-27).

Isolation and screening of laccase producing bacteria

The perusal of literature has revealed the presence of laccase exclusively in plants and fungi with fungal laccases reported to be extensively being used for biotechnological applications. However databank searches provided evidence for their distribution in prokaryotes also and a survey showed that laccase like enzyme occurs in many gram-negative and gram-positive bacteria harbouring

corresponding genes which have been thought to have branched off early during evolution. Alexander and Zhulin (2000) reported presence of several protein sequences in bacterial genomes with significant similarity to fungal laccases. The first report of prokaryotic laccase was from rhizospheric bacterium *Azospirillum lipoferum* isolated from the paddy fields followed by a number of other bacteria including *Bacillus subtilis, Bordetella compestris, Caulobacter crescentus, Escherichia coli, Mycobacterium tuberculosum, Pseudomonas syringae, Pseudomonas aeruginosa, Yersinia pestis* and *Stenotrophomonas maltophilia* (Alexandre and Zhulin, 2000; Enguita *et al.,* 2003; Arora and Sharma, 2010; McMahon *et al*., 2007; Galai *et al.,* 2009). First report on prokaryotic thermostable laccase was also from soil bacterium *Azospirillum lipoferum* with $T_{1/2}$ value of 43 min. at 70^{o}C (Alexander and Zhulin, 2000), whereas, the most studied thermostable endospore-coat bacterial laccase to date has been CotA, the endospore coat component of *Bacillus subtils* which has been encoded by *cot*A gene (Martins *et al.,* 2002). Recent studies have also been reported with thermophilic organisms including laccase derived from *Aquifex aeolicus* (Deckert *et al*., 1998), *Streptomyces griseus* (Endo *et al.,* 2003), *Streptomyces lavendula* (Suzuki *et al.,* 2003), *Thermus thermophilus* HB27 (Miyazaki, 2005) and *Bacillus pumilus* (Reiss *et al.,* 2011).

In the present investigation, 118 thermolaccase producing thermophilic bacterial isolates were obtained from various soil, water and rock matting samples successfully, whereas no isolate was obtained from the pebble samples (Table-5, Plate 1-2). The pH of water samples was found to range from 4.8-9.5 and temperature varied from 41-106^{o}C of hot water springs investigated in the present study. Popovitch, (2012) has also reported isolation of laccase producing thermophilic bacteria from Mickey hot spring found in the Alvord Desert of Oregon with temperature condition of 66.9-80.9°C and an alkaline range of pH 8.52-8.92. However, isolation of bacterial species with laccase activity have also been reported from seawater (Solano *et al*., 1997), river sludge/top-soil containing organic litter (Ruijssenaars and Hartmans, 2004), soil contaminated with dye, textile industry effluent and lignocellulosic wastes (Senan and Abraham, 2004),

forest soil (Wang *et al*., 2010) and soil samples containing sawdust and dairy effluents (Kuddus *et al*., 2013).

In the present study, Tryptone Yeast Medium (TYM) containing 5mM guaiacol has been shown to be the best medium for the isolation of thermostable laccase producing thermophilic bacterial isolates. Guaiacol is a substrate, which is a chromogen in nature leading to successful screening of laccase producers, which were able to produce extracellular guaiacol oxidising enzymes, and additionally it can also act as best source of carbon for laccase production. Similar reports of TYM for successful isolation of thermostable laccase producing bacteria *Thermus thermophilus* HB27 have been communicated (Miyazaki, 2005; Sowinski *et al.,* 2002). However TY, M162, Luria Bertani and M9 medium have also been reportedly used for the isolation of laccase producing bacteria (Alexzender and Belly, 1999; Bains *et al*., 2003; Galai *et al*., 2009 and Wang *et al.*, 2010). Bains *et al*., (2003) isolated *γ-proteobacterium* JB using M162 medium containing 5mM guaiacol and Mongkolthanaruk *et al*., (2012) reported isolation of *Rhodococcus* species*, Enterobacter* species*., Staphylococcus saprophyticus and Delftia tsuruhatensis* using M162 medium containing 0.1% guaiacol. However more laccase producing bacteria have been cultured using other media such as Luria Bertani (LB) medium containing 0.4 mM copper sulphate for isolation of *Stenotrophomonas maltophilia* (Galai *et al*., 2009), M9 medium containing Cu^{2+} for isolation of *Bacillus subtilis* WD23 (Wang *et al*., 2010), toluene enriched media for isolation of *Bacillus* species XJT-7 (Abari *et al*., 2012) and nutrient agar medium containing guaiacol has also been reported for isolation of *Bacillus subtilis* (Sheikhi *et al*., 2012). In the present study, a total of 118 putative laccase producing bacteria have been successfully isolated from 200 samples excluding all pebble samples, using TYM containing 5 mM guaiacol (Plate-4, 6-8).

All the 118 putative laccase producing thermophilic bacterial isolates obtained were found to be different in their colony morphotypes on basis of colour, size, shape, texture and elevation (Table-35, 37, 39 and 41). Bains *et al*., (2003) reported isolation of 200 putative laccase producing bacteria from soil samples possessing different colony morphotypes. Wang *et al*., (2010) also

reported isolation and screening of 400 bacterial isolates differing in their colony morphotypes. Sheikhi *et al.*, (2012) isolated 96 putative laccase producers differing in their colony characters from pulp and paper waste water. Morphological characterization has been immensely useful and has been applied in carrying out characterization of most of the microorganisms. In the present study different morphological descripters viz., colour, size, texture, elevation and margin of the colonies (Table-36, 38, 40 and 42) along with variations microscopic characters such as gram reaction, shape and arrangement of cells, spore formation, motility and pellicle formation were studied and it was established that 71% of laccase producers were gram postive in nature and 66.7% were rod shaped with singles to chains arrangement. Whereas only 29% were found to be gram negative in nature, 33.3 % were coccus in shape and only 23.2% were found to be spore formers. It has also been noted that all 118 putative laccase producers were able to form pellicle and were motile in nature. Similarly other authors also recorded gram positive, spore forming, motile, long rod-shaped bacteria efficient in laccase production (Lu *et al.*, 2011 and Sheikhi *et al.*, 2012), whereas Bains *et al.*, (2003) reported a gram negative, rod shaped, non-spore former and motile laccase producing bacteria. Thus it has been perceived from present study that laccase producing bacteria can either be Gram negative or positive, coccus or rods, spore former or non-spore former and it has been further observed that thermophilic laccase producers are motile and able to form pellicle.

Discovery of novel laccases with different substrate specificities and improved stabilities has been important for industrial applications. Laccases exhibit an extraordinary substrate range which is a reason for their attractiveness for many biotechnological applications. In the present study guaiacol as substrate was used for primary screening as this is a very sensitive substrate for laccase production and allows visual detection of laccase activity, indicated by the formation of reddish-brown colonies and 118 laccase producing thermophilic bacterial isolates were isolated using guaiacol as primary screening reagent. This was followed by their secondary screening with different substrates viz., tannic acid, dimethoxyphenol and syringaldazine. Tannic acid allows visual detection of laccase activity indicated by the formation of a dark-brown colony/black coloured

zone (Harkin and Obst, 1973), whereas dimethoxyphenol indicated laccase positive microbes by formation of orange coloured colony/zone due to the oxidation of substrate (Kiiskinen *et al*., 2004). Syringaldazine is a substrate with no free ortho or para positions and may be oxidized only to its quinone form and has a strong molar absorption coefficient of 65,000 at 526 nm. The high molar extinction coefficient, coupled with the fact that syringaldazine is oxidized to a single product, makes it an excellent substrate for screening of laccase producing microbes (Petroski *et al*., 1980). In the present investigation, secondary screening led to selection of only 42% of total laccase producing thermophilic bacterial isolates. Thus on the basis of their ability to degrade 0.5% tannic acid leading to zone formation (Plate-9a), change of colour to orange after treatment with laccase specific substrate dimethoxyphenol (Plate-9b) and change of colour to purple after treatment with laccase specific substrate syringaldazine, only 50 laccase producing thermophilic bacterial isolates were selected (Plate-9c,d; Table-43). Kiiskinen *et al*., (2004) had reported use of various indicators such as, Remazol Brilliant Blue R (RBBR), Poly R-478, guaiacol and tannic acid for screening of laccase producing fungi. Kumar *et al.,* (2011) had also reported use of guaiacol and syringaldazine for screening of laccase producing fungi. Dhiman, (2014) also reportedly used guaiacol, dimethoxyphenol and tannic acid for screening of laccase producing mesophilic bacteria isolated from paper mills and paddy fields.

Qualitative screening of both extracellular and intracellular crude laccase enzyme preparations of fifty selected laccase producing thermophilic bacteria were assessed by orange-brown coloured zone formation potential using dimethoxyphenol indicator and it has been observed only extracellular crude laccase enzyme preparations produced coloured zones, whereas intracellular crude laccase enzyme preparations were unable to do so (Plate-10). Further quantitative screening of both extracellular and intracellular crude laccase enzyme preparations of fifty selected laccase producing thermophilic bacteria was assessed using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) which is a non-phenolic substrate and most commonly recruited for assaying laccase activity due to its intrinsic advantages of pH independence and the high molar extinction coefficient of its oxidized product (ABTS, ε_{420nm} = 36,000

M^{-1} cm^{-1}) (Table-46). It has been observed that more activity was present in extracellular crude laccase enzyme preparations as compared to intracellular crude laccase enzyme preparations. Extracellular enzyme activity was found to be maximum (0.00381 U/l) in TR2 laccase producing thermophilic bacterial isolate, whereas intracellular enzyme activity was found to be maximum (0.00166 U/l) in VS1 laccase producing thermophilic bacterial isolate. Similarly ABTS has also been used reported for laccase activity assay of *Sinorhizobium meliloti* (Sowinski *et al*., 2002), *γ-proteobacterium* (Bains *et al*., 2003) and *Bacillus subtilis* (Sheikhi *et al*., 2012). However other substrates viz., syringaldazine, dimethoxyphenol and guiacoal have also reported for evaluating laccase enzyme activity of *Azospirillum lipoferum* (Givaduan *et al.,* 1993), *Sinorhizobium meliloti* (Sownski *et al*., 2002), *Bacillus subtilis* WD23 (Wang *et al.,* 2010), *Bacillus licheniformis* (Lu *et al*., 2011) and *Bacillus subtilis* (Sheikhi *et al*., 2012). Thus on the basis of more laccase activity of extracellular crude laccase enzyme preparations, these were used for further experiments.

Biochemical characterization has been immensely useful and has been applied in carrying out characterization of most of the microorganisms. The biochemical characterization of 50 laccase producing thermophilic bacterial isolates revealed positive result for catalase and fermentation of sucrose, whereas 45 were found positive for oxidase test and methyl red test. However variation has been observed in utilization of three carbon sources, citrate test and urea production (Table-44). As per Bergey's manual of Systemetic Bacteriology and on the basis of results obtained from morphological and biochemical characters, the 50 putative laccase producing thermophilic bacterial isolates were tentatively found to belong to the two genera of *Bacillus* and *Pseudomonas*. On the basis of the biochemical characterization, which on further analysis generated a dendogram dividing these 50 laccase producers into 16 groups. On the basis of qualitative, quantitative and biochemical results, two bacteria from third and eights group and one bacterial representative from rest of each group of dendrogram (Fig.24), with maximum laccase activity were selected and a total of 18 laccase producing thermophilic bacterial isolates were chosen for further molecular characterization. Dhiman (2014) has also reported biochemical

characterization of 67 laccase producers isolated from paddy fields, pulp and paper mills of Himachal Pradesh were positive for citrate test, 64 for catalase test, and 63 for oxidase test and reported variation for utilization of five carbon sources and urea production.

Molecular characterization of selected bacterial isolates

Eighteen thermolaccase producing thermophilic bacterial isolates selected from previous experiments were further subjected to molecular characterization using RAPD-PCR technology and 16S ribosomal DNA -PCR technology.

Since the introduction of RAPD-PCR technology in 1990 (William, 1990), their use in bacterial genetic analysis has increase in an exponential manner due to ease of procedure and very low amount of DNA required for analysis. The present study also employs only 30 ng DNA of each bacterial isolate for single amplification. Though this is the first report of RAPD technology having been successfully used to assess the genetic relatedness of thermophilic laccase producing bacterial isolates, however this technology has been reportedly used for assessing genetic relatedness in some other studies of bacteria. It suggested that these markers could be used for laccase producing thermophilic bacteria, as no significant work has been published.

In this study a high degree of polymorphism (68.25%) amongst 18 laccase producing thermophilic bacterial isolates has been detected, which was expected as the 18 laccase producing thermophilic bacterial isolates have been collected from different locations. However, it is necessary to select, establish the purity, amount of DNA and this fact is even more important, when the purpose is to choose primers that provide clear distinction between the bacterial isolates. In the present study DNA was extracted and purified using Real Genomic DNA extraction kit-mini, but it has been observed that only 19 RAPD primers amplified the genomic DNA of all the 18 laccase producing thermophilic bacterial isolates successfully. Whereas 16 primers were unable to amplify the genomic DNA of all the 18 laccase producing thermophilic bacterial isolates. Only 16 primers were found to be polymorphic in nature and rest of three primers were monomorphic in

nature. The conditions of PCR reaction and analysis used in study were also similar to those found most suitable for the present study. The use of intermediate annealing temperature of 36^{o}C was found to give best results as far as number of amplified fragment and reproducibility of results are concerned. From the 16 polymorphic primers, no single primer was found to produce distinct banding pattern for all the bacterial isolates, but OPA-01, OPA-03, OPA-16, OPA-20, OPB-04, OPB-06, OPC-06 and OPC-07 were found to produce unique markers, which could distinguish 9/18 (50%) of the laccase producing thermophilic bacterial isolates. In order to quantify the level of polymorphism detected, Nei's estimate of similarity, based on the probability that on amplified fragment from one bacteria will also be found in another, was used to generate similarity index (Nei & Li, 1979). The set of 18 laccase producing thermophilic bacteria fell in the range of 0.64 to 0.91. A dendogram (Fig-25) generated on the basis of results obtained using 19 random primers divided these 18 isolates into two clusters. The cluster B clearly separated laccase producing thermophilic bacterial isolate VS8, which belonged to genus *Pseudomonas* from rest of 17 laccase producing thermophilic bacterial isolates, in cluster A belonging to genus *Bacillus.* It has been found to be in synonym with the result of biochemical characterization. In the present study, no single primer was able to distinguish between all the bacterial isolates, however amplification by different primers was informative and produced bacterial specific patterns. In the RAPD analyses the following DNA bands were observed, which can be used as molecular markers, OPA-01_{200} for VW2 bacterial strain, OPA-01_{240} for TR2, OPA-01_{260} for VS10 strain, OPA-03_{1300} and OPA-03_{1700} for KW9.1 strain, OPA-16_{3500} for VW5 strain, OPA-20_{3100} for VS8 strain, OPB-04_{200} and OPB-04_{800} for KW3.1 strain, OPB-06_{1500} , OPB-06_{3500} , OPB-06_{4000} for VS8, OPC-06_{4000} for MS 2.2, OPC-06_{1000} and OPC-06_{3000} for VS10 and OPC-07_{5000} for KW8 strain, suggesting that unique RAPD-markers can be used for identification of these laccase producing thermophilic bacterial isolates (Plate-17-23). To the best of knowledge this is the first report to study genetic relatedness amongst laccase producing thermophilic bacterial isolates using RAPD-PCR technology. Similarly Adiguzel *et al.,* (2009) studied molecular diversity of thermophilic bacterial isolates of Pasinler hot water spring

located in Turkey. Whereas, Dhiman, (2014) reported use of RAPD technology to study genetic relatedness among 14 laccase producing mesophilic bacterial isolates of identification of unique RAPD markers to study genetic relatedness.

16S rRNA gene technology and *in silico* analysis

Molecular characterization using 16S rRNA gene technology, which leads to rapid and accurate identification of bacterial isolates till species level. Although conventional phenotypic methods are relatively inexpensive and allow identification of most common bacteria but certain groups of bacteria have been difficult to identify and specified equipment/expertise may be required. Woese (1987) developed this state of art technology to analyse the sequence of 16S rRNA gene technology to analyse the sequence of 16S rRNA gene of various bacteria. The inventions of PCR and automated DNA sequences two decades ago followed by subsequent research on 16S rRNA sequencing of bacteria has led to accumulation of vast amount of sequence data on the ribosomal RNA gene of smaller subunit of ribosomes. Comparison of these sequences has revealed that ribosomal RNA sequences are highly conserved within living organisms of the same genus and species and differ between organisms of other genera and species. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. In the present study, universal primers B27F 5́-AGAGTTTGATCCTGGCTCAG-3́ and U1492R 5`-GGTTACCTTGTTACGACTT-3́ with an annealing temperature of 48^0C produced an amplified product of 1500 bp of 16S rRNA gene of all selected 18 laccase producing thermophilic bacterial isolates (Plate-24). In other studies also universal primers have been reported to amplify 16S rRNA gene. Bains *et al*., (2003) adopted standard 5F and 531R 16S rRNA gene primers with an annealing temperature of 55^0C. Lu *et al*., (2011) reported the usage of fD1 and rP1 universal primers with an annealing temperature of 55^0C. Wang *et al.,* (2010) used 27F and 1492R universal primers with an annealing temperature of 56^0C. Mongkolthanaruk *et al*., (2012) also used 27F and 1492R universal primers with an annealing temperature of 55^0C, whereas Sheikhi *et al*., (2012) used universal 16S forward primer, 5́-CCGAATTCGTCGACAACAGAGTTTGATCCTGCTCA

G-3́ and reverse primer, 5́-CCCGGGATCCAAGCTTACGGTTACCTTGTTACG ACTT-3́ with an annealing temperature of 48^0C. Other conditions of denaturation and extension were 94^0C and 72^0C, respectively. Amplification followed by agarose gel electrophoresis produced 16S rRNA gene amplified products of 1500 bp size. The amplified products were eluted, sequenced and the size of the 16S rRNA gene sequences of all the 18 laccase producing thermophilic bacterial isolates ranged between 304-1135 bp. On the basis of results obtained from 16S rRNA sequence data and G+C content analysis (Table-49-66), 17 isolates viz., TS8, KW3.1, VW5, TR2, VW2, VS10, TW5, MS2.2, VS9, MW17, KW4, KW9.1, KR7.2, TR4.2, VR2, VW1 and KW8 showed similarity to genus *Bacillus* whereas only one thermophilic bacterial isolate VS8 was found similar to genus *Pseudomonas.* Further *in silico* analysis of these 16S rRNA gene sequences using various bioinformatics tools such as BLASTn, Multiple Sequence Alignment and Phylogenetic tree construction using MEGA6, confirmed that the thermophilic bacterial isolate TS8 showed closest similarity with *Bacillus sonorensis* strain NR1 16S ribosomal RNA gene, partial sequence, KW3.1 with *Bacillus amyloliquefaciens* strain H55 16S ribosomal RNA gene, partial sequence, VW5 with ribosomal RNA gene, partial sequence, TR2 with *Bacillus licheniformis* strain LZBL-19 16S ribosomal RNA gene, partial sequence, VW2 with *Bacillus licheniformis* strain hswX145 16S ribosomal RNA gene, partial sequence, VS10 with *Bacillus licheniformis* strain SDS12 16S ribosomal RNA gene, partial sequence, TW5 with *Bacillus sonorensis* strain RSV8 16S ribosomal RNA gene, partial sequence, partial sequence, MS2.2 with *Bacillus sonorensis* strain SXYC19 16S ribosomal RNA gene, partial sequence, VS9 with *Bacillus aerius* strain CCMMB963 16S ribosomal RNA gene, partial sequence, MW17 with *Bacillus subtilis* strain 1051 16S ribosomal RNA gene, partial sequence, KW4 with *Bacillus licheniformis* strain MU4 16S ribosomal RNA gene, partial sequence, KW9.1 with *Bacillus sonorensis* strain NR1 16S ribosomal RNA gene, partial sequence, KR7.2 with *Bacillus licheniformis* strain CFR2 16S ribosomal RNA gene, partial sequence, TR4.2 with *Aneurinibacillus thermoaerophilus* strain BillorisVO18 16S ribosomal RNA gene, partial sequence, VR2 with *Bacillus aerius* strain CCMMB963 16S ribosomal RNA gene, partial sequence, VW1 with *Bacillus licheniformis* strain P5 16S ribosomal RNA gene, partial

sequence, VS8 with *Pseudomonas taiwanensis* strain IHB B 14565 16S ribosomal RNA gene, partial sequence and KW8 with *Bacillus licheniformis* partial 16S rRNA gene, isolate DV7 (Table-65).

Similarly 16S rRNA gene technology along with *in silico* analysis have been successfully adopted in other studies to identify laccase producing bacterial isolates such as *γ-proteobacterium* (Bains *et al.,* 2003), *Bacillus licheniformis* (Lu *et al*., 2011), *Rhodococcus sp., Enterobacter sp., Staphylococcus saprophyticus and Delftia tsuruhatensis* (Mongkolthanaruk *et al*., 2012), *Bacillus subtilis* WD23 (Wang *et al*., 2010) and *Bacillus subtilis* WPI (Sheikh *et al*., 2012). Although gene encoding laccase protein sequence has been reported in the complete genome of all these bacterial isolates using prokaryotic genome annotation tool of NCBI but to the best of our knowledge, this is the first report to describe the isolation of culturable laccase producing thermophilic *Bacillus licheniformis, Bacillus sonerensis, Bacillus amyloliquefaciens and other Bacillus, Pseudomonas* species from hot water springs of Himachal Pradesh. In other studies also *Bacillus subtilis* WP1 was isolated from wastewater of a pulp and paper industry (Sheikhi *et al*., 2012) whereas *Bacillus subtilis* WD23 has been isolated from forest soil (Wang *et al*., 2010), *γ-proteobacterium* was also isolated from industrial waste water drained soil (Bains *et al.*, 2003), *Bacillus licheniformis* LS04 from forest soil samples (Lu *et al*., 2011) and *Sinorhizobium meliloti* from Uruguayan soil sample (Sowinski *et al*., 2002). The role of laccase enzyme in these bacterial isolates might be in protecting these bacteria from toxic phenolic compounds.

In vitro laccase production and characterization

Laccases are the most promising enzymes for future industrial applications since these can catalyse reactions with high degrees of stereo and regioselectivity under mild conditions. The name laccase was designated from its first report from the Japanese lacquer tree and then followed by discovery in numerous plants. Plant and fungal laccases have found wide application in various industrial applications but problem with plant and fungal laccases is their low substrate specificity and a wide range of reactions. Whereas bacterial laccases have been found to be highly active, much more thermostable and can withstand high pH

values. Laccases, particularly from thermophilic bacteria are going to play an important role in future, because procurement of laccase from bacteria is easier in comparison to the plant and fungal sources. Bacterial laccase can easily be induced to large amounts in few hours and harbour great biotechnological potential due to their broad substrate specificities. In the present study, *Bacillus licheniformis* strain RSM8 is one of the potential laccase producing thermophilic bacterial isolate showing a high level of extracellular laccase activitiy and thus it was selected for the optimization of best growth conditions with the objective to produce maximum laccase enzyme. Each micro-organism has its own special conditions for the maximum enzyme production. The general rules for the optimization of microbial enzyme production have been affected by various physical factors which include pH, incubation temperature, cultivation temperature, shaking conditions and aeration. These factors have been found to be important to promote, stimulate, enhance and optimize the production of a particular enzyme. Enhancement of laccase production, by modifying the nutritional and physiological conditions during cultivation, is a prerequisite for their optimum utilization at industrial scale. Besides nutritional supplements, inducers such as organic solvents and metal ions also play important role in production of laccases (Shraddha *et al*., 2012). In the present study, TYM containing 50 mg/l $CuSO_4$ supplemented with 5mM guaiacol has been found to be best medium for growth as well as laccase production. In another report also same TYM medium containing $CuSO_4$ has been reportedly used successfully for optimum growth as well as laccase enzyme production by *Thermus thermophilus* HB27 (Miyazaki, 2005). However, TY medium containing 40 µg/ml $CuSO_4$ but no aromatic compound/substrate has been reportedly used for growth and laccase production in case of *Azospirillum lipoferum* (Diamantidis *et al*., 1999) and *Sinorhizobium meliloti* (Sowinski *et al*., 2002), whereas in case of *γ-Proteobacterium* JB, M162 medium containing both 5 mM guaiacol and 100 µM $CuSO_4$ was found to be optimum for enzyme production (Bains *et al*., 2003).

In present study optimum incubation time of 24 hrs was selected for *Bacillus licheniformis* strain RSM8, as maximum growth OD of 0.983 and maximum extracellular laccase enzyme activity of 10.80 U/l was obtained after 24

hrs (Table-72). Whereas, Bains *et al*., (2003) reported, 48 hrs of optimum incubation period for maximum intracellular as well as extracellular laccase enzyme activity from *γ-Proteobacterium* JB, whereas maximum laccase production of 94.10 U/ml was recorded in *Pseudomonas putida* MTCC 7525 after 108 hrs of incubation (Kuddus *et al*., 2013). Temperature is another important factor which influences the laccase enzyme yield significantly. Optimum incubation temperature both for growth of bacteria *Bacillus licheniformis* strain RSM8 and laccase enzyme production was found to be 60°C in the present investigation (Table-71). Diamantidis *et al.,* (1999) reportedly found *Azospirillum lipoferum* laccase enzyme was thermostable up to 70^0C with an optimal pH of 6.0. But in other studies of laccase production by mesophilic bacteria it has been noted that, maximum enzyme production was achieved at 30^0C incubation temperature in case of *Pseudomonas putida* MTCC 7525 (Kuddus *et al*., 2013) and 37°C in case of *γ-proteobacterium* JB (Bains *et al*., 2003). Thus in the present study a thermophilic bacterial isolate *Bacillus licheniformis* strain RSM8 isolated from hot water spring is a thermophile producing thermophilic laccase enzyme, which was found stable at 60°C (Table-71)

Hydrogen ion concentration of the production medium strongly affects many enzymatic processes and transport of compounds across the cell membrane thus pH of medium also plays an important role in enzyme production. Optimum pH of 9.0 at an optimum temperature of 60^0C recorded to be best for both maximum laccase production and growth of the *Bacillus licheniformis* strain RSM8. It is interesting to note that this thermophilic bacterial strain was also able to produce thermolaccase at higher alkaline pH range of 10.0-11.0 (Table-70). Optimum pH for maximum laccase enzyme production reported in other studies was 8.0 in case of both *Pseudomonas putida* MTCC 7525 (Kuddus *et al*., 2013) and *γ-Proteobacterium* JB (Bains *et al*., 2003). Laccases enzyme generally produced in low concentrations can be induced to produce higher concentration with addition of inducers. Two inducers guaiacol and metal ions at different concentrations with an objective to increase laccase production were investigated and it was observed that 5 mM guaiacol and 50 mg/l $CuSO_4$ induced increase in laccase production in *Bacillus licheniformis* strain RSM8 (Table-73, 78). Similarly other studies, have communicated that 100 µM $CuSO_4$ metal ion

enhanced laccase production in *γ-Proteobacterium* JB (Malhotra *et al*., 2004) and aromatic compound i.e. guaiacol also induced and increased laccase enzyme in *Rhodococcus* species, *Enterobacter* species, *Staphylococcus saprophyticus* and *Delftia tsuruhatensis* (Mongkolthanaruk *et al*., 2012). Effect of carbon sources and inoculum size were also examined for growth and enzyme production in the present study (Table-75, 76) and it was found that 3% inoculum size was optimum for growth, whereas maximum production was induced with 1% inoculum size. Similarly, Bains *et al*., (2003) also showed 1% inoculum size to be optimum for laccase production. Whereas carbon sources were found to inhibit the laccase production to a significant level suggesting that sugars were causing catabolic repression of the laccase production. Similar results were expressed with, *γ-Proteobacterium* JB strain which showed complete suppression of laccase production in presence of sugars such as glucose, sucrose, maltose and xylose (Bains *et al*., 2003). Effect of nitrogen sources on growth and laccase production in *Bacillus licheniformis* strain RSM8 (Table-77) revealed that 0.5% tryptone and 0.3% yeast extract gave maximum production of laccase extracellularly (22.94 U/l) after 24 hrs, whereas growth of bacterial strain was enhanced using combination of 0.2% yeast extract and 0.4% tryptone. In another study, effect of nitrogen source on growth and laccase production in *γ-Proteobacterium* JB, revealed that 0.3% of yeast extract and 0.3% tryptone in combination produced maximum laccase after 24 hrs (Bains *et al*., 2003). However in case of *Pseudomonas putida* MTCC 7525, 10% skim milk and 1 mM sodium nitrate were used as additional nitrogen source for maximum laccase production (Kuddus *et al*., 2013).

Purification of an enzyme is an essential step for the determination of accurate kinetic parameters due to the possible presence of compounds from the host that may act as natural mediators, or the presence of similar enzymes that may exhibit significantly different reaction kinetics (Rittstieg *et al*., 2003). The most commonly used method for laccase purification is salt elution from an anion-exchange resin, probably due to the higher stability of laccase at neutral to slightly alkaline pH, as well as the isoelectric point (pI) of laccases. White rot fungi are also largely laccase producers and the purification of fungal laccase

enzyme using standard ammonium sulphate precipitaion and ion-exchange chromatography for this group has been reported (Palonen *et al*., 2003; Zouari-Mechichi *et al*., 2006). Actinomycetes are believed to be the potent producer of laccases, next to fungi. Laccases have also been purified and characterized from certain non-filamentous bacteria like *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Bacillus subtilis and γ-Proteobacterium* JB (Diamantidis *et al*., 1999; Fernandez *et al*., l999; Martins *et al*., 2002, Bains *et al*., 2003).

A thermostable bacterial laccase enzyme was extracted and partially purified from indigenous *Bacillus licheniformis* strain RSM8, it was found to be stable in standard sodium phosphate buffer, pH 9.0, which was used for further purification. Similar reports have been communicated, where sodium phosphate at pH 6.5 was found stable with laccase from mesophilic bacteria namely *γ-Proteobacterium* JB (Bains *et al*., 2003) and *Bacillus subtilis* (Sheikhi *et al*., 2012), however Givaduan *et al*., (1993) reported stability of *Azospirillum lipoferum* laccase enzyme in Tris HCL buffer, pH 7.2. Potassium phosphate buffer, pH 8.0 was found to be stable with laccase enzyme of *Pseudomonas putida* LBC1 (Kuddus *et al*., 2013). Thermostable laccase enzyme was purified in the present study by different purification techniques i.e. ammonium sulphate precipitation (50-90%), gel-filteration chromatography (Sephadex G-100) and ion exchange chromatography (DEAE-Sephadex). The procedure yielded 10.88 mg protein with 10.5 fold purification and percent yield of 15.21 (Table-80). In another study laccase enzyme from *Bacillus* species was purified using sucrose dialysis followed by DEAE-cellulose, an anion-exchange chromatography technique, which yielded 0.56 mg of purified enzyme from culture supernatant and with 6.6 fold purification. Similarly Kuddus *et al.*, (2013) also reported purification of laccase enzyme from *Pseudomonas putida* MTCC 7525 using salt precipitation followed by ion exchange chromatography, which showed 6.44 fold purification and *γ-Proteobacterium* JB laccase enzyme was purified using ammonium sulphate precipitation (40-60%), DEAE sepharose and PAGE with a purification fold of 21.5 and 9% yield (Bains *et al*., 2003). Some other reports in literature have used other purification techniques Telke *et al*., (2009) purified laccase from *Pseudomonas* species. LBC1 using acetone precipitation followed

by DEAE-cellulose an anion-exchange chromatography which yielding 1.54 mg of purified enzyme with 6.1-fold purification and partial purification of laccase from cytosolic fraction of *Sinorhizobium meloti* performed by ion-exchange chromatography, using DEAE cellulose with 24% yield and 2.5 fold purification. On the contrary, first bacterial laccase from *Azospirillum lipoferum* 4T was purified using a combination of acetone and protamine precipitation, hydroxyapatite chromatography with a 24% yield.

Characterization of the partially purified thermolaccase enzyme of *Bacillus licheniformis* strain RSM8 produced one band on SDS-PAGE gel with a molecular weight of 72.5 kDa (Plate-26) showing that this enzyme is monomeric in nature. In other studies molecular weight of other monomeric laccase enzymes from mesophilic bacteria has been found to range from 39.5-179.3 KDa, such as 65 kDa from *Bacillus subtilis* (Martins *et al*., 2002), 120 kDa from *γ-Proteobacterium* JB (Bains *et al*., 2003), 70 kDa from *Pseudomonas* species LBC1 (Telke *et al.*, 2009) and 39.5 kDa from *Pseudomonas putida* MTCC 7525 (Kuddus *et al.,* 2013). Whereas multimeric laccase enzyme has been reported in *Azospirillum lipoferum* bacteria with molecular weight 48.9, 97.8, 179.3 KDa (Diamantidis *et al*., 1999). Effect of pH, temperature and different inhibitors on thermolaccase activity was also examined in the present study (Table-81-83). The partially purified laccase of *Bacillus licheniformis* strain RSM8 expressed optimum enzyme activity at pH 9.0 and 60^0C incubation temperature. Diamantidis *et al.,* (1999) reportedly found *Azospirillum lipoferum* laccase enzyme was thermostable up to 70^0C with an optimal pH of 6.0. However in other studies, optimum enzyme activity has been reported at an acidic pH 4.0 and 40^0C temperature in case of *Pseudomonas sp*. LBC1 (Telke *et al*., 2009), laccase enzyme from *Pseudomonas putida* MTCC 7525 had optimal activity at pH 8.0 and 40°C (Kuddus *et al*., 2013) and laccase from *γ-Proteobacterium* JB was found to be stable in the pH range of 4.0-9.0 with an optimum temperature of 55^0C (Bains *et al*., 2003). Inhibitors such as sodium azide and dithiothreitol were found to inhibit enzyme activity of *Bacillus licheniformis* strain RSM8 thermolaccase. Negligible enzyme activity of 0.1% was left after incubation with 0.9 mM of both inhibitor concentrations leading to almost loss of activity. In

some other studies other inhibitors have reported to affect laccase enzyme activity negatively in *Pseudomonas putida* MTCC 7525 laccase activity was inhibited by pCMB (93%), in *Pseudomonas* species LBC1 laccase enzyme activity was inhibited by EDTA (Telke *et al.,* 2009) and *Azospirillum lipoferum* laccase activity was inhibited by tropolone, a known inhibitor of metal-containing enzymes. The kinetic constant (Km) of the purified laccase using ABTS substrate was determined under the optimal conditions and Km value was found to be 16.22 µM (Fig.53). In other studies Km, Vmax and Kcat values were recorded to be 0.25mM, 2.09 Umg^{-1} of enzyme min^{-1} and 2.71 min^{-1}, respectively for purified laccase from *Pseudomonas* species LBC1 (Telke *et al.,* 2009) and Km of 26±2 µM and Vmax of 4±1 µmol min^{-1} mg^{-1} was observed for Cot A laccase from *Bacillus subtilis* (Martins *et al.,* 2002).

Decolourization of various dyes

Release of harmful wastewaters from industries is a serious environmental and health concern. Most of industries and textile in particular, produce huge amount of wastewaters with spent dyes which are extremely difficult to be removed and release of dyes into the environment is also deleterious, not only because of their colour, but also because they are not easily degraded and under action of anaerobic reductive bacteria they can form toxic or mutagenic compounds. Therefore, developing low cost and effective methods of decolourization and degradation of these dyes is significantly important. As common physical or chemical methods for dye removal are expensive and sometimes generate secondary pollution, therefore the decolourization and biodegradation of dyes by use of laccase enzyme appears to be an attractive alternative. In literature there were several reports of dye decolourization from mesophilic laccase, but to the best of our knowledge this is the first report of dye decolourization using thermostable laccase enzyme.

Potential of *Bacillus licheniformis* strain RSM8 and its partially purified extracellular thermolaccase enzyme preparation were investigated for commonly used six different textile dyes, viz., RBBR, indigo carmine, congo red, aniline blue, brilliant Blue and bromophenol blue which are significant contributors to

water and soil pollution and it was revealed that all three-bacterial culture, crude enzyme and partially purified enzyme preparations decolorized these textile dyes significanty. In the present study 91.66% degradation was achieved for Congo red dye, 80.00 % for indigo carmine dye and 74.07% for RBBR dye, when treated with a culture of *Bacillus licheniformis* strain RSM8, whereas lesser degradation was observed with Aniline blue (67.80%), Brilliant blue (60.43%) and Bromophenol blue (64.12%), (Table-84). In other studies, Kuddus *et al*., (2013) reported decolorized various synthetic dyes (0.02%) and industrial effluents (10%) in a range of 74–93% and 58–68%, respectively. However, the culture supernatant of *Pseudomonas putida* revealed 36–94% and 16–86% decolorization of synthetic dyes and effluents, respectively within 24 hrs of incubation. Similarly *Pseudomonas* species LBC1 showed 90% decolorization of Direct Brown MR dye and textile industry effluent with significant reduction (Telke *et al*., 2010). The ability of the *Bacillus licheniformis* strain RSM8 to decolourize recalcitrant dyes such as Congo red, under *in vitro* conditions suggests its potential that this strain could be useful in the management of industrial effluents.

Immoblization of laccase enzyme

The stability and the catalytic ability of free enzymes are dramatically decreased by highly polluted wastewaters due to the instability of their structures and their sensitivity to the process, apart from these being non-reusable (Taylor, 1991). Therefore, additional measures to enhance enzyme's operational lifetime and reduce enzyme waste are need of the time. The use of immobilized enzymes can overcome some of these limitations and provide stable catalysts with longer life times (Krajewska, 2004). In particular, immobilization of laccases by covalent coupling usually provide enzymes with high stability and has proved to be effective in removing phenolic compounds and colour over wide ranges of pH and temperature (Davis and Burns, 1990). Since several limitations prevent the use of free enzymes in bioremediation, thermolaccase from *Bacillus licheniformis* strain RSM8 was immobilised by adsorption method in calcium alginate beads and was used to decolorize six different synthetic dyes, RBBR, Aniline blue, Congo red, Indigo carmine, Bromophenol blue and Brilliant Blue. It was revealed that immobilised enzyme was also able to degrade these textile dyes effectively, 92%

degradation was achieved for of Congo red, 83% for RBBR, 90% for Indigo carmine, 66% for Bromophenol blue, 63% for Aniline blue and 66% for Brilliant blue (Table-85). Thus, both immobilized and free thermolaccase enzyme preparations resulted in significant decolourization. Therefore immobolized laccase enzyme has significant potential for decolourization of industrial effluent and enables its reuse in dyeing processes providing water and energy savings in textile wet processing. Singh *et al*., (2010) immobilised laccase on different support systems such as agarose guar gum, nitrocellulose membrane, egg shell membrane and nylon membrane and showed 100% enzyme activity, when immobilized on the nitrocellulose membrane. Whereas, Wang *et al*., (2012) surface immobilised bacterial laccase (WlacD) into the cell surface of the solvent-tolerant bacterium *Pseudomonas putida* to construct a whole-cell biocatalyst, with relative decolourization values of 91.2% (3 hrs) and 97.1% (18 hrs) toward acid green and decolourization values of 80.5% (3 hrs) and 89.0% (18 hrs) to acid red.

Phytotoxic studies

Despite the fact that, untreated dyeing effluents may cause serious environmental and health hazards and their disposition in water bodies which may be used for agricultural purpose in India (Parshetti *et al*., 2006) having a direct impact on the fertility of the soil. However decolourization does not essentially implies detoxification, thus it was important to evaluate the phytotoxicity of dyes before and after degradation. Therefore it was significant to perform toxicity assay on the textile dyes before and after treatment with partially purified enzyme preparation. Application of three dyes viz., Congo red, Indigo carmine and RBBR and their partially purified enzyme treated dyes were evaluated on agriculturally important *Phaseolus mungo* and two floricultural plant species *Calendula officinalis* and *Tagetes patula* to study the phototoxic effect. The germination percentage, plumule and radicle length were used as parameters to assess toxicity and significant germination inhibition and slower rate of plumule and radicle lengths in the presence of Congo red, RBBR and Indigo carmine dyes was achieved under *in vitro* conditions (Table 86-88) and under *in vivo* conditions in *Phaseolus mungo* plant species significant germination inhibition, a slower rate of

plumule length in the presence of Congo red dye as compared to partially purified enzyme treated dye was observed (Table-89).

Isolation of laccase encoding gene

Modern biotechnology has a steadily increasing demand for novel genes for applications in various industrial processes. Identification and isolation of novel genes at a reasonable pace is the main driving force behind the development of unprecedented experimental approaches. The first gene sequence recorded for laccase was from the fungus, *Neurospora crassa* (Germann and Lerch, 1986) and since then, number of fungal laccase genes sequencing has increased considerably. In contrast to a significant number of fungal laccase genes sequenced, there have been very few reports on bacterial laccase gene isolation/sequencing. Though, Ausec *et al.,* (2011) revealed high diversity of bacterial genes for laccase-like enzymes using bioinformatic analysis. As there has been an increasing demand for novel laccase biocatalysts because laccase is a significant enzyme for various biotechnological and industrial applications. So the present study was emphasized on the isolation and characterization of thermolaccase gene from *Bacillus licheniformis* strain RSM8 for further cloning and expression experiments. One of the methods of isolating and identifying a novel gene is PCR amplification using specific/designed primers which allows the specific amplification of rare sequences within complex genomic DNA. In the present study PCR amplification was also carried out using designed degenerate primers which were based on the conserved regions I and II of copper-binding sites in bacterial laccase viz., CuIF : 5`-ACMWCKGTTCAYTGGCACGG-3` and CuIVR: 5`-TGNTCNAGNAWGTGRCARTG-3` with an annealing temperature of 50^0C. In other studies also designed primers, which have been based on the conserved regions of copper-binding sites in bacterial laccase were used, Li *et al*. (2012) carried out isolation of a multicopper oxidase gene from *Ochrobactrum sp*. 531 using two degenerate primers, 531F1 and 531R1 which were designed based on two conserved sequences at copper-binding sites of the CueO-type multicopper oxidases of several bacteria were used to amplify genomic DNA of the strain. In the present study, an amplification product of 1000 bp encoding laccase gene sequences was produced which was further eluted and sequenced using same

upstream and downstream primers and after sequencing 150 bp of laccase gene sequences was obtained. Nucleotide sequence of the laccase gene of *Bacillus licheniformis* strain RSM8 was translated into corresponding amino acid residue sequence using protein translator tool ExPASy and nucleotide sequence analysis depicted an open reading frame of 150 bp encoding a polypeptide which comprised of 50 amino acids. In other studies, it has been reported that the *cotA* gene which codes for a 65 KDa protein belonging to the outer spore coat of *Bacillus subtilis* displayed similarity with multicopper oxidases like laccase (Hullo *et al*., 2001) and nucleotide sequence analysis showed an open reading frame of 1605 bp encoding a polypeptide comprising of 534 amino acids in case *Aeromonas hydrophila* WL-11 (Wu *et al*., 2010), whereas in case of *Bacillus subtilis* laccase gene composed of 1542 bp nucleotide sequence and encoded a 514-amino acids (Yang *et al*., 2012). A new bacterial laccase gene of 1.32 kb obtained from a marine microbial metagenome (Fang *et al.,*2011) and 1029 bp nucleotide sequence product was isolated from *Ochrobactrum sp*. 531 (Li *et al*., 2012). Further by using various bioinformatics tools, it has been confirmed that the predicted amino acid sequence depicted a high homology with other bacterial laccases in the genome and protein databases and the highest degree of similarity of 97 % was observed with the amino acid sequences of bacterial laccases i.e. copper oxidase [*Bacillus licheniformis*] and with spore coat protein [*Bacillus licheniformis*] (Table-90). Multiple sequence alignment of the amino acid sequence of *Bacillus licheniformis* strain RSM8 thermolaccase with other known bacterial laccases revealed conserved histidine residues in test bacterial laccase and other bacterial laccases. Secondary and tertiary structure of thermolaccase enzyme was predicted using PHYRE 1 and 2, web based software and structure of the enzyme predicted the structural feature characteristics of other laccases, including the conserved regions of four histidine-rich copper-binding sites (Fig-56-58). In other studies also the primary structure of the enzyme predicted the structural features characteristic of other laccases, including the conserved regions of four histidine-rich copper-binding sites (Wu *et al*., 2010), whereas Fang *et al*., (2011) studied that the protein named as Lac15 of 439 amino acids encoded by the isolated gene found to contain three conserved Cu^{2+} binding domains, but

shared less than 40% of sequence identities with all of the bacterial multicopper oxidases characterized.

To the best of our knowledge this is the first report of isolation and characterization of thermolaccase producing thermophilic bacteria, which was identified as *Bacillus licheniformis* strain RSM8 from Tattapani hot water spring of Himachal Pradesh. This bacterium produced an extracellular and alkalophilic thermolaccase enzyme which was monomeric with molecular weight of 72.5 KDa. This thermolaccase showed significant potential of decolorizing water and soil pollution causing textile dyes under *in vitro* and *in vivo* conditions. However thermolaccase enzyme production strategies of gene cloning and mutation can be further investigated in addition to evaluation of thermolaccase enzyme for other important applications.

Chapter-6

SUMMARY AND CONCLUSION

The results of the present investigations so obtained are summarized below:

- Four hot water springs viz., Manikaran, Vashisht, Khirganga and Tattapani were purposely selected for the present studies. Twenty samples of water and ten samples each of soil, rock matting and pebbles from each hot water springs were collected.
- The pH and temperature of the four thermal springs were recorded which ranged from 4.8-9.5 and 41^{o}C-106^{o}C respectively. The chloride, sulphate, total hardness, calcium hardness, calcium carbonate hardness and magnesium content ranged from 495.0-2673.0, 10.0-31.8, 105.0-315.0, 33.66-74.28, 37.7-268.0 and 1.46-55.16 mg/l respectively.
- Different parameters for isolation of laccase producing thermophilic bacterial isolates were standardized and TYM containing 5mMguaiacol(pH 9.0) at an incubation temperature of 60^{o}C for 24 hrs was found best for isolation of laccase producing thermophilic bacterial isolates.
- One hundred eighteen laccase producing thermophilic bacterial isolates were isolated from a total of 200 samples which were investigated for various colonyand microscopic characters viz., colour, size,shape, texture, elevation and pigmentformation of the colonies and Gram reaction (-), shape, arrangement of cells, spore formation (+),pellicle formation and motility.
- Secondary screening led to the selection of only 50laccase producing thermophilic bacterial isolates.On the basis of black zone formation on oxidation of tannic acid, orange colour developed on oxidation of dimethoxyphenol and purple colourdeveloped on oxidation ofsyringaldazine.

- These 50laccase producing thermophilic bacterial isolates were further characterized biochemically and it was found out that these laccase producing thermophilic bacterial isolates were tentatively grooved to belong to the two genera *Bacillus* and *Pseudomonas* as per Bergey's Manual of Systemetic Bacteriology.On the basis of the biochemical characterization which on further analysis generated a dendogram,dividing these 50 laccase producers into 16 groups.
- Qualitative and quantitative screening of extracellular laccase activity of these 50 laccase producing thermophilic bacterial isolates using ABTS, ranged from 0.0007-0.0038 U/l.
- One bacterial representative from each of the 16 groups showing maximum laccase activity was selected for further molecular characterization, whereas from 3^{rd} and 8^{th} group 2 showing maximum laccase activity were selected. A total of 18 bacterial isolates were selected.
- Genomic DNA was isolated from all 18laccase producing thermophilic bacterial isolates and genetic relatedness amongst these 18laccase producing thermophilic bacterial isolates was evaluated using RAPD-PCR technology and it was found out that the results of RAPD-PCR study was in synonym with the results of biochemical characterization as it divided 18 isolate into two groups- seventeen isolates were found to belong togenus *Bacillus*and one to genus *Pseudomonas.*
- Sixteen informative DNA-based markers were identified viz., OPA-$01_{200}$for VW2 bacterial strain, OPA-01_{240} for TR2,OPA-$01_{260}$for VS10 strain, OPA-03_{1300} and OPA-$03_{1700}$for KW9.1 strain, OPA-$16_{3500}$for VW5 strain, OPA-20_{3100}forVS8 strain, OPB-$04_{200}$and OPB-$04_{800}$for KW3.1 strain, OPB-06_{1500} , OPB-06_{3500} , OPB-06_{4000} for VS8, OPC-06_{4000} for MS 2.2, OPC-06_{1000} and OPC-06_{3000} for VS10 and OPC-$07_{5000}$for KW8 strain,suggesting that these DNA-based markers can be used for identification of these laccase producing thermophilic bacterial isolates. To the best of our knowledge, this is the first report to study genetic

relatedness amongst laccase producing thermophilic bacterial isolates using RAPD-PCR technology.

- 16S rRNA gene technology was used for rapid and accurate identification of thesebacteria. Genomic DNA was amplified using universal primers and an amplified product of 1500 bpwas produced and sequenced using similar primers.
- **G+C content analysis,** BLASTn analysis, multiple Sequence Alignment and Phylogenetic tree construction using MEGA6confirmed that the thermophilic bacterial isolate TS8 showed closest similarity with *Bacillus sonorensis* strain NR1 16S ribosomal RNA gene, partial sequence, KW3.1 with *Bacillus amyloliquefaciens* strain H55 16S ribosomal RNA gene, partial sequence, VW5 with ribosomal RNA gene, partial sequence, TR2 with *Bacillus licheniformis strain LZBL-19* 16S ribosomal RNA gene, partial sequence, VW2 with *Bacillus licheniformis* strain hswX145 16S ribosomal RNA gene, partial sequence, VS10 with *Bacillus licheniformis* strain SDS12 16S ribosomal RNA gene, partial sequence, TW5 with *Bacillus sonorensis* strain RSV8 16S ribosomal RNA gene, partial sequence , partial sequence, MS2.2 with *Bacillus sonorensis* strain SXYC19 16S ribosomal RNA gene, partial sequence, VS9 with *Bacillus aeriuss*strain CCMMB963 16S ribosomal RNA gene, partial sequence, MW17 with *Bacillus subtilis* strain 1051 16S ribosomal RNA gene, partial sequence, KW4 with *Bacillus licheniformis* strain MU4 16S ribosomal RNA gene, partial sequence, KW9.1 with *Bacillus sonorensis* strain NR1 16S ribosomal RNA gene, partial sequence, KR7.2 with *Bacillus licheniformis* strain CFR2 16S ribosomal RNA gene, partial sequence, TR4.2 with *Aneurinibacillusthermoaerophilus* strain BillorisVO18 16S ribosomal RNA gene, partial sequence, VR2 with *Bacillus aerius* strain CCMMB963 16S ribosomal RNA gene, partial sequence, VW1 with *Bacillus licheniformis* strain P5 16S ribosomal RNA gene, partial sequence, VS8 with *Pseudomonas taiwanensis* strain IHB B 14565 16S ribosomal RNA gene, partial sequence and KW8 with *Bacillus licheniformis* partial 16S rRNA gene, isolate DV7.

- ❖ Optimization of culture conditions for growth and laccase production of isolate*Bacillus licheniformis* strain RSM8 was carried out successfully.
- The optimum pH for the maximum growth and extracellular enzyme production was found to be 9.0.
- The optimum temperature for the maximum growth and extracellular enzyme production was found to be 60^{o}C.
- The best incubation time for maximum growth and maximum extracellular laccase enzyme activity was recorded to be 24 hrs.
- The optimum substrate concentration ofguaiacol for maximum growth and maximum extracellular laccase enzyme activity was found to be 5 mM.
- The optimum concentration of$CuSO_4$ for maximum growth was found to be 10 mg/l and 50 mg/l $CuSO_4$ concentration was found to be optimum for maximum extracellular laccase enzyme activity.
- Effect of different carbon sources was investigated and it was confirmed no single carbon source was selected to be used for growth and enzyme production.
- 3% inoculum size was confirmed to be best for maximum growth whereas 1% inoculum size produced maximum laccaseextracellularly.
- The effect of nitrogen sources on growth and laccase production revealed that 0.5% tryptone and 0.3% yeast extract resulted in maximum production of laccase whereas extracellularenzyme production was enhanced using 0.2% yeast extract and 0.4% tryptone in combination.
- Effect of different metal ions revealed that $CaCl_2$ enhanced growth of *Bacilluslicheniformis*strain RSM8, whereas no ion was found to increase extracellular laccase enzyme production of *Bacillus licheniformis*strain RSM8.

- ❖ The crude extracellularextract was produced using optimized conditions and further purified using different purification techniques viz., ammonium sulphate precipitation, gel permeation chromatography and ion exchange chromatography. The laccase enzyme was partially purified up to 10.5 fold with 15.21 percent recovery. The specific activity increased

significantly from 0.00020 U/mg in crude extract to 0.0198 U/mg in ion exchange chromatography fraction.

- ❖ Purity as well as activity of the partially purified protein was checked using native-PAGE and revealed a single band of purified protein. Activity staining/zymograph of gel with ABTS and dimethoxyphenol substrates also showed single green and orange coloured bands respectively of purified protein, thus showing laccase activity on the stained gel also.
- ❖ The partially purified enzyme obtained after DEAE-cellulose technique was used for characterization.
- Molecular weight of the partially purified enzyme was confirmed to be 72.5 kDa using SDS-PAGE gel.
- Optimum pH for maximum laccase activity was revealed to be 9.0 whereas optimum temperature was found to be 60^{o}C
- Sodium azide and dithiothreitol were potential inhibitors of *Bacillus licheniformis*strain RSM8 laccase enzyme production.
- Km value was determined for this partially purified enzyme using ABTS as substrate was found to be16.22 µM.

- ❖ *Bacillus licheniformis*strain RSM8 and its partially purified extracellular laccase enzyme preparation was investigated for its ability to degrade six different textile dyes, viz., RBBR, Indigo Carmine, Congo Red, Aniline Blue, Brilliant Blue and Bromophenol Blue, which are significant contributors to water pollution. It has been depicted that both bacterial isolates and enzyme preparations can degrade textile dyes successfully, playing a significant role in the bioremediation of synthetic dyes, thus can be used further for bioremediation of industrial effluents.
- ❖ *Bacillus licheniformis* strain RSM8 enzyme preparations was investigated for phytotoxicity evaluationof three dyes viz., Congo red, RBBR and Indigo carmine and each of enzyme treated dyes for *Phaseolusmungo*and *Calendula officinalis* and *Tagetespatula* plant species respectively, under *in vitro* conditions and*Phaseolusmungo*with Congo red dye under *in vivo*

conditions. Significant germination inhibition, a slower rate of plumuleand radicle seedlings growth was observed for Congo red, RBBR and Indigo carmine dyes as compared to enzyme treated dyes.

- Laccase from *Bacillus licheniformis*strain RSM8 was successfully immobilised by adsorption method in calcium alginate beads and was used to decolorize four different synthetic dyes, RBBR, Congo red, Indigo carmine and Brilliantblue. It was observed that immobilised enzyme was also able to degrade these synthetic dyes effectively.
- *Bacillus licheniformis*strain RSM8 was also investigated for the presence of gene, encoding for laccase enzyme. The genomic DNA sample, extracted from this isolate was selectively amplified using PCR technology and degenerate designed primers CuLVF and CuLVR based on the conserved copper binding sites. An amplified product of 1000 bp was obtained.
- Thus a gene DNA segment encoding an extracellular thermolaccase enzyme from *Bacillus licheniformis*strain RSM8 was sequenced and asequence of 150 bp corresponding to laccase encoding gene was obtained with G+C content of 52.70 %.
- The nucleotide sequence was translated into amino acid sequence using EXPASy translator tool. Further *in silico* analysis of this laccase amino acid sequence, using various bioinformatics tools such as BLASTp, Multiple Sequence Alignment and Phylogenetic tree construction using MEGA 6.
- Analysis of amino acid sequence produced a longest reading frame (5`3`Frame 1) with a total of 50 amino acids and it's*in silico* analysis confirmed that it showed maximum homology of 97 %with copper oxidase*Bacillus licheniformis* (WP_035337064.1).
- A conserved domain, Suf I (COG2132, Putative multicopper oxidases [Secondary metabolites biosynthesis, transport, and catabolism] was identified at amino acid position 30-111 with E-value of 1.16e-09 in the amino acid sequence of *Bacillus licheniformis*strain RSM8.

- ❖ Secondary and tertiary structure of laccase enzyme was predicted using web based server, PHYRE 1 and 2 and structure of the enzyme predicted the structural feature characteristics of other laccases, including the conserved regions of four histidine-rich copper-binding sites.
- ❖ Identification and characterization of extracellular laccase encoding gene was performed for the first time in thermophilic*Bacillus licheniformis*strain RSM8.

CONCLUSION

A total of 118 laccase producing thermophilic bacterial isolates were obtained from 200 hot water spring samples using TYM containing 5 mMguaiacol which were morphologically characterized. These were rescreened on the basis of their ability to oxidise tannic acid, dimethoxyphenol and syringaldazine leading to selection of 50 laccase producing thermophilic bacterial isolates, which were characterized biochemically. Eighteen thermophilic bacterial isolates exhibiting maximum laccase activity of 0.0007-0.0038 U/l were selected for further, molecular characterization using RAPD-PCR and 16S rRNA gene technology. *In silico* analysis of 16S rRNA gene sequences led to identification of these bacterial isolates and they were found to belong to genus *Bacillus,Aneurinibacillus* and *Pseudomonas*, as *Bacillus licheniformis* strain RSV20, *Bacillus licheniformis* strain RSM8, *Bacillus licheniformis* strain RSV10, *Bacillus sonerensis* strain RSM17, *Bacillus sonerensis* strain RSV8, *Bacillus licheniformis* strain RSP1, *Bacillus licheniformis* strain RSP2, *Bacillus licheniformis* strain RSP3, *Bacillus licheniformis* strain RSP7, *Bacillus sonerensis* strain RSP5, *Bacillus sonerensis* strain RSP11, *Aneurinibacillusthermophilus* strain RSP13, *Bacillus aerius* strain RSP4, *Bacillus aerius* strain RSP9, *Bacillus subtilis* strain RSP8, *Bacillus amyloliquifacience*strain RSP10, *Bacillus pumilis* strain RSP12 and *Pseudomonas taiwanensis* strain RSP6.

On the basis of maximum laccase enzyme activity *Bacillus licheniformis* strain RSM8 was selected for production and purification of the laccase enzyme. Maximum extracellular enzyme production was achieved at

60°C, pH 9.0 and 24 hrs incubation with 5 mMguaiacol, 5 % tryptone and 3 % yeast extract in combination with nitrogen source. Crude extracellular thermolaccase enzyme preparation was purified by ammonium salt precipitation (50-90%) followed by gel filtration and ion exchange chromatography which showed 15.21 yield and 10.5 fold purification. The purified enzyme had optimal activity at pH 9.0 and 60 °C, and 16.22 µM Km value. The molecular weight of thermolaccase in the present study was found to be 72.5 kDa. However activity was inhibited by sodium azide and DTT. *Bacillus licheniformis* strain RSM8 as well as its enzyme preparations were investigated for their ability to decolourize dyes which are the potential contributors of water pollution. Six different synthetic dyes were decolourized RBBR (68 %), congo red (86 %), indigo carmine (73 %), brilliant blue (40 %), bromophenol blue (51 %) and aniline blue (54 %) when treated with the crude enzyme preparation of *Bacillus licheniformis* strain RSM8. And partially purified enzyme preparation of *Bacillus licheniformis* strain RSM8 showed greater decolourization of dyes comparatively RBBR (74 %),congo red (91 %), indigo carmine (80 %), brilliant blue (60 %), bromophenol blue (64 %) and aniline blue (67 %).The purified enzyme was successfully immobolized using adsorption method in calcium alginate beads with 76% immobolization percentage and immobolized laccase enzyme beads were studied for their ability to degrade dyes. The stability and reusability of the immobilized enzyme system has the potential to make the entire treatment process inexpensive.*Bacillus licheniformis* strain RSM8 enzyme preparations was investigated for phytotoxicity evaluationof three dyes viz., Congo red, RBBR and Indigo carmine and each of enzyme treated dyes for *Phaseolusmungo*and *Calendula officinalis* and *Tagetespatula* plant species respectively, under *in vitro* conditions and *Phaseolusmungo*with Congo red dye under *in vivo* conditions. Significant germination inhibition, a slower rate of plumule and radicle seedlings growth was observed for Congo red, RBBR and Indigo carmine dyes as compared to enzyme treated dyes.

An extracellular laccase producing gene has been isolated using degenerate primer based on the copper I and II conserved site of laccase enzyme, from the hot water spring bacteria, *Bacillus licheniformis* strain RSM8 followed

by determination of the amino acid which were translated from nucleotide sequence and encodes a polypeptide comprised of 50 amino acids showing 97 % identity with the amino acid sequences of bacterial laccases i.e. copper oxidase [*Bacillus licheniformis*]. Further multiple sequence alignment using MULTALIN and structure prediction using Phyre1 & 2 revealed conserved histidine residues.

In this study a thermolaccase producing thermophilic bacterial isolate has been obtained which was characterised as *Bacillus licheniformis* strain RSM8. This bacterium successfully produced an extracellular, alkaline and heat stable laccase enzyme which showed significant potential to reduce the textile dye pollution of water and soil under *in vitro* and *in vivo* conditions.

Chapter-7

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Title of Thesis	**:**	**"Bioprospecting of thermophilic bacteria form hot water springs of Himachal Pradesh for laccase enzyme production"**
Name of the Student	**:**	Ruchika Sharma
Admission Number	**:**	H-2011-07-D
Major Advisor	**:**	Dr (Mrs.) Poonam Shirkot
Major Field	**:**	Molecular Biology and Biotechnology
Minor Field(s)	**:**	Microbiology
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ABSTRACT

Laccase enzyme has acquired the status of 'green catalyst' as it possesses remarkable bioremediation potential along with numerous applications in effluent detoxification, degradation of textile dyes, herbicide and insecticide degradation, wine clarification, enzymatic conversion of chemical intermediates, biosensors and organic synthesis. In the present study, significantly high diversity of laccase producing bacteria from hot water springs of Himachal Pradesh was assessed. A total of 118 laccase producing thermophilic bacterial isolates were obtained from 200 hot water spring samples using TYM containing 5 mM guaiacol which were morphologically characterized. These were rescreened on the basis of their ability to oxidise tannic acid, dimethoxyphenol and syringaldazine leading to selection of 50 laccase producing thermophilic bacterial isolates, which were characterized biochemically. Eighteen thermophilic bacterial isolates exhibiting maximum laccase activity of 0.0007-0.0038 U/l were selected for further, molecular characterization using RAPD-PCR and 16S rrna gene technology. *In silico* analysis of 16S rrna gene sequences led to identification of these bacterial isolates and they were found to belong to genus *Bacillus, Aneurinibacillus* and *Pseudomonas*, as *Bacillus licheniformis* strain RSV20, *Bacillus licheniformis* strain RSM8, *Bacillus licheniformis* strain RSV10, *Bacillus sonerensis* strain RSM17, *Bacillus sonerensis* strain RSV8, *Bacillus licheniformis* strain RSP1, *Bacillus licheniformis* strain RSP2, *Bacillus licheniformis* strain RSP3, *Bacillus licheniformis* strain RSP7, *Bacillus sonerensis* strain RSP5, *Bacillus sonerensis* strain RSP11, *Aneurinibacillus thermophilus* strain RSP13, *Bacillus aerius* strain RSP4, *Bacillus aerius* strain RSP9, *Bacillus subtilis* strain RSP8, *Bacillus amyloliquifacience* strain RSP10, *Bacillus pumilis* strain RSP12 and *Pseudomonas taiwanensis* strain RSP6.

On the basis of maximum laccase enzyme activity Bacillus licheniformis strain RSM8 was selected for production and purification of the laccase enzyme. Maximum extracellular enzyme production was achieved at 60°C, pH 9.0 and 24 hrs incubation with 5 mM guaiacol, 5 % tryptone and 3 % yeast extract in combination with nitrogen source. Crude extracellular thermolaccase enzyme preparation was purified by ammonium salt precipitation (50-90%) followed by gel filtration and ion exchange chromatography which showed 15.21 yield and 10.5 fold purification. The purified enzyme had optimal activity at pH 9.0 and 60 °C, and 16.22 µM Km value. The molecular weight of thermolaccase in the present study was found to be 72.5 kDa. However activity was inhibited by sodium azide and DTT. Bacillus licheniformis strain RSM8 as well as its enzyme preparations were investigated for their ability to decolourize dyes which are the potential contributors of water pollution. Six different synthetic dyes were decolourized RBBR (68 %), congo red (86 %), indigo carmine (73 %), brilliant blue (40 %), bromophenol blue (51 %) and aniline blue (54 %) when treated with the crude enzyme preparation of Bacillus licheniformis strain RSM8. And partially purified enzyme preparation of Bacillus licheniformis strain RSM8 showed greater decolourization of dyes comparatively RBBR (74 %), congo red (91 %), indigo carmine (80 %), brilliant blue (60 %), bromophenol blue (64 %) and aniline blue (67 %). The purified enzyme was successfully immobolized using adsorption method in calcium alginate beads with 76% immobolization percentage and immobolized laccase enzyme beads were studied for their ability to degrade dyes. The stability and reusability of the immobilized enzyme system has the potential to make the entire treatment process inexpensive. Bacillus licheniformis strain RSM8 enzyme preparations was investigated for phytotoxicity evaluation of three dyes viz., Congo red, RBBR and Indigo carmine and each of enzyme treated dyes for Phaseolus mungo and Calendula officinalis and Tagetes patula plant species respectively, under in vitro conditions and Phaseolus mungo with Congo red dye under in vivo conditions. Significant germination inhibition, a slower rate of plumule and radicle seedlings growth was observed for Congo red, RBBR and Indigo carmine dyes as compared to enzyme treated dyes.

An extracellular laccase producing gene has been isolated using degenerate primer based on the copper I and II conserved site of laccase enzyme, from the hot water spring bacteria, *Bacillus licheniformis* strain RSM8 followed by determination of the amino acid which were translated from nucleotide sequence and encodes a polypeptide comprised of 50 amino acids showeng 97 % identity with the amino acid sequences of bacterial laccases i.e. copper oxidase [*Bacillus licheniformis*]. Further multiple sequence alignment using MULTALIN and structure prediction using Phyre1 & 2 revealed conserved histidine residues.

Signature of Major Advisor **Signature of the Student**

Countersigned

Professor and Head
Department of Biotechnology
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ANNEXURE-I

A. Solutions, Chemicals and Reagents used for DNA extraction

1. Real Genomics, Genomic DNA isolation kit (Mini)

B. Solvent for DNA

10X TNE Buffer:

S. No.	Reagent	Quantity added (1000 ml of 10X TNE)	Final concentration (100 ml 10X TNE)
1.	Tris	12.11 g	100 mM
2.	EDTA	3.72 g	10 mM
3.	NaCl	116.89 g	2 M

C. Gel Electrophoresis

1. Agarose gel (1 per cent)

2.0 g agarose was added to 200 ml of 1 X TAE buffer and molten down. The molten gel was cooled down to 50^{o}C and 18 µl of ethidium bromide solution was added and mixed well. The molten gel was cast in a gel tray with a comb containing 51 teeth to produce wells.

2. Ethidium bromide 10 mg/ml

10 mg of ethidium bromide was dissolved in sterile water and volume made upto 1 ml the solution was stored in an amber coloured bottle at 4^{o}C.

3. Loading dye (6 X solution) (MBI fermentas)

Tris-HCl (pH 7.6)	:	10 mM
Bromophenol blue	:	0.03 %
Xylene cyanol FF	:	0.03 %
Glycerol	:	60 %
EDTA	:	60 mM

The volume was made up to 20 ml with sterile water.

D. PCR cocktail

1. *Thermus aquaticus* DNA polymerase

A stock solution 3 units μl^{-1} was provided by the manufacture that was stored at-20^{o}C.

Dexyribonucleotide Tri-phosphate

dATP (10 mM)	:	100 µl
dCTP (10 mM)	:	100 µl
dGTP (10 mM)	:	100 µl
dTTP (10 mM)	:	100 µl

Sterile water

The solution was mixed well and stored at -20^{o}C.

2. Primer

The manufacturer in a lyophilized form provided the primer. Based on the molecular weight of given primer, a solution of 10 pmoles/l was prepared by adding the required amount of TE buffer. Storage was done at - 20^{o}C.

ANNEXURE-II

The sources of instruments

i) Instruments used in DNA extraction

Microfuge	:	Eppendorf
Waterbath	:	Neolab
Deep freezer (-20^{o}C)	:	Sanyo Ultra Low
Refrigerator	:	Godrej
Water purification system	:	Millipore
Vortex	:	Vortex Genie-2
Pipetteman	:	Eppendorf
Eppendorf tubes	:	Axygen

ii) Instruments used in molecular analysis

PCR machine	:	MJ Research Inc., USA, model PTC-100
Laminar flow	:	Stericlean
Weighing balance	:	Sartorius
Gel electrophoresis unit	:	Biorad
UV transilluminator	:	Hoefer
Camera	:	Kodak
Power Pack 300	:	Biorad
Microwave Oven	:	LG
Pipetteman	:	Finipipette
Thinwalled PCR tubes	:	Axygen
Gel dryer	:	Biorad
Gel plate	:	Hoefer

ANNEXURE-III

Expresso alignment result

MSA

The multiple sequence alignment result as produced by T-coffee.

```
T-COFFEE, Version_11.00.8cbe486 (2014-08-12 22:05:29 - Revision 8cbe486 - Build 477)
Cedric Notredame
SCORE=28
*
 BAD AVG GOOD
*
1aboA    :  28
1aboB    :  28
cons     :   2

1aboA    ----------------IATFAPYS--------GRYVWHCHI---------LEHEDYDMMRPLEVTDVREDYDMMR
1aboB    GFQHERMLFLNWHADEFGACLPFSMGHEAARNGTAGRHLTINGQAGSITELEAGQVVLVRLLNLDNT---WTYRL

cons                      :.:  *:*        *    *  *         **  :  ::* *:: :.   :


1aboA    PLEVTDVR------------------------------
1aboB    NLKGNSARIYALDGNPVTPRALDEYWLGPGMRIFLAIRI

cons      *: ...*
```

Citation

Please cite this result referring the papers at this link.

Result files

9 output files - download them all

Input(s)	Input sequences (177 B)	
System	Command line (326 B)	Log file (33KB)
Multiple Alignment	score_html file (4KB)	clustalw_aln file (494 B) fasta_aln file (257 B) score_ascii file (592 B) phylip file (311 B)
Template List	template_list file (26 B)	

ANNEXURE-IV

16S rRNA partial sequence of TR2

Total Bases: 672

TGGTCCCCTTTCGGCGGCTGGCTCCAAAAGGTTACCTCACCGACTTCGGGT
GTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACG
TATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGC
AGTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGG
CTTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGT
AGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACT
AAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAA
GCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCG
CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA
ATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAA
TGTTTTTGCTGCAGCACTAAAAGGGACGGGATACTATTCTACCACTTAGTT
CTCATCGTTTACG

16S rRNA partial sequence of VS8

Total Bases: 1135

CGTTCCCCTTTCGGCGGCTGGCTCCAAAGGTTACCTCACCGACTTCGGGTG
TTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCA
GTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGC
TTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGT
AGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACT
AAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAA
GCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCG
CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA

ATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAA
TGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTTAGCACT
CATCGTTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCC
ACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCA
CTGATGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTAGAAATTAC
ACTCGTCCTTCTTCCTGCTACTGCAAGTTACCCCAAGTTTCCAATGGACCC
GTCCCCCGGCTTGGAGGCCGGAGGGGCGTTTCACATCAGAACCTTAAGAT
GAACATACCTCGCGGCAGCGGCTGTTACGACCCGACTACATTCCGAGTAC
GATGACATTGCTCATCGCTCACCGCATTAACATCTGCCTTGCCAGGTCCTG
GTGAACCTTCAGCTCTGTGAGACCGTGCCTGTGTTATAGGATGACGGTTCA
CCCGTTTACCAAGGGCGCTAAGTACTGCAAACTGGGTAAGGCGTGGTAAT
ACGTTTGTCATCTATCCCACCAT

16S rRNA partial sequence of VS10

Total bases: 1090

TGTCCCCTTTCGGCGGCTGGCTCCAAGGTTACCTCACCGACTTCGGGTGTT
ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT
TCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGT
CGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTT
AGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGTAG
CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGG
TTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAA
GATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAAGC
CCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCG
TTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAAT
TCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATG
CGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCAT
CGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCT
TTCGCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGT
GTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAAATTCCACTCT
CCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGGTTGAG
CCGGGGGGGCTTTCACATCAGACTTAAGAAAACCGCCCTGCGCGCGCTTT

ACGCCCAATTAATTTCCGGACCACGCTTTGCCACCTACGTATTACCGGCGG
CCTGGCTGGCACGGTAGTTTAGCCCGTGACCTTTCTTGGGTTAAGGTACGT
TCACGTTAACGACCTAGTCCGAACGTACCTTGCTTCTTGCTTACCAACAGA
AGTTTAACGATCCCGGAAAATCCATCA

16S rRNA partial sequence of MS2.2

Total bases: 1107

CGGTTCCCTTTCGGCGGCTGGCTCCAAAGGTTACCTCACCGACTTCGGGTG
TTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCA
GTCGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGC
TTAGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGT
AGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACT
AAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACA
CGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAA
GCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCG
CGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCA
ATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAA
TGCGTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTC
ATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACG
CTTTCGCGCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCCTTCGCCACTG
GTGTTCCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTTCCAC
TCTCCTCTTCTGCACTCAAGTTCCCCAGTTTTCCAATGACCCTCCCCGGGTT
GAGCCGGGGGCCTTTCAACATCAGACTTTAAGAAACCAGCCCTGCGCGCG
CCTTACCGCCCAATTAATTTCGGGAACAACCGCTTGCCCACCCTACGTATT
ACCGCGCTGGCTTGACACGGTTAGTAGCCGTGGCCTTTCTGTTAAGGGTAC
CCGGTCCAACGTACCGCCTATTCGAACGGTACCTGGTTCTTTCCTTAACAA
CCAGAGTTTACCGTATCGATAGTCACAGTCACTCAGCTCGGC

16S rRNA partial sequence of VW20

Total bases: 816

AACGTTACTTCGGCGGCTGGCTCAAAGGTTACCTCACCGACTTCGGGTGTT
ACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT

TCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGT
CGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTT
AGCCTCGCGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGTAG
CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGG
TTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAA
CATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAAGC
CCTATCTCTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCG
TTGCTTCTAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAAT
TTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAAT
GCGTTTTGCTGCAGCACTAAAGGGCGGAAACCCCTCTAACACTTAGCACT
CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCAC
GCTTTTCTCGCCTTCAGCGTCAGTTTACAGACCAAGAGAGTCCGCCTTCGC
CACTGGTGGTTACTTCCACATCTCTACGCATTTTCATCGGCTTACAACGTG
GCAATT

16S rRNA partial sequence of TR4.2

Total bases: 697

GGAAAGGCCTTTGAGCCGCGTACAGATGGGCCTGCGGCGCATTAGCTAGT
TGGTGGGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAG
GGTGAACGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGAGGAAGGTCTTCGGATCGTAAAACTCTGTTGTCAGGGAA
GAACCGCCGGGATGACCTCCCGGTCTGACGGTACCTGACGAGAAAGCCCC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTTGT
CCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCAGGTG
TGAAAGCCCACGGCTCAACCGTGGAGGGCCATCTGAAACTGGGGAGCTTG
AGTGCAGGAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGTAACTGA
CGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTATGTGTTGGGGAGTCCACCTCCTCA
GTGCCGCAGCTAACGCAATAAGCACTCCGCCTGGGAGAGTACGGCCGCAA
GG

16S rRNA partial sequence of KR7.2

Total Bases : 555

CGAAGAGAAGGGCAGGTGGCAAGGGCGCTATCATGCAAGTCGAGCGGAC
CGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATA
CCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAAC
TACCACTTACAGATGGACCCGCGGCGCATTAACTAGTTGGTGAGGTAACG
GCTCACCAAGGCGACGATGCGTAACCGACCTGAAAGGGTGATCGGCCACA
CTGGGACTGAAACACGGCCCAGACTCCTACGGGAGGCAGCAGTAAGGAA
TCTTCCGCAATGGACGAAAGTCTGACGGAACAACGCCCCGTGAGTGATGA
AGGTTTTCCGATCGTAAAACTCTGTTGGTAGGGAAGAACAAGTACCGGTC
GAATAGGGTTTTTTTTTTGGCGGAACCCAACCACAAAGGCACCGCAAAGT
AGGGGGGGAGGTGGGCCCCCCACCTCCCCCCTTGTTAAATTTTTATCACAT
AAATA

16S rRNA partial sequence of KW4

Total Bases: 497

CAAGTTCGGGCGGTCTATAATGCAAGTCGAGCGGACCGACGGGAGCTTGC
TCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTG
TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATT
GAACCGCATGGTTCAATCCTAAAAGGTGGCTTTTGACTAACAATAGAGAA
GGAAAAAAAGAGTTTAGCTTGTGGGGCATTAGCCTCCAAGGGGAGGAAG
GGGTAAACCCAGGTCAAAGGCCCTCGCTGATTGGGCGTCACCACCTACCA
TCCACCGGTTTGGCAGCGGCAGTCACCAAAATTGCCCATTGAATGCTGGC
CACAAAAACACCGGCTCCGGTCGTGCGTTCTCTCACCACCCTCCAGGAGA
CAACGAACACAATCATGCCCAACAGCCCTTAGCCCCTAAAGTGAAACCCG
ACCCCACGGTCCCCTAGTGTTGGAAGGGGGAAAAACCCCCCCGCGCGA

16S rRNA partial sequence of KW8

Total bases : 304

CGCGCATATTATTCGCCAGAAGGGGGGGGGGCTATCCTGCAGTCGAGCGG
ACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACAC

GTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATGCTTGATTGAACCGCATGGTTCCATTCTAAAAAGGGGGTTTTA
ACTAACCATTTAAATTGTCCACCCGGGGGGAATAACTTAGTGGGGCAGGA
AACGGCTCACCAGGGCTACGGGGGGTACCCAACCGGAATTTAGACCTCCA
AAC

16S rRNA partial sequence of VR2

Total bases : 394

CAGGTGGCGCAGCCCTAATACGTGCAAGTCGAGCGGACCGACGGGAGCTT
GCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC
TGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGA
TTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACTG
ACTCCACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CGACGATGCGTACCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
ACTAAAGTCTGACGGTACAACGCCGCCGTGAGTAATGAAGGTT

16S rRNA partial sequence of TW5

Total bases: 600

CTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC
GGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTG
CAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCG
CGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGTAGCCCAGGT
CATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCA
CCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGAC
GACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAAGCCCTATCT
CTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTG
AGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGC
TGCAGCACTAAAGGGCGGAAACCCTCTAACACTTTAGCACTCAT

16S rRNA partial sequence of KW9.1

Total Bases: 595

GCTTGCTCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTG
CCTGGTAGTGGGGGACAACGTTTCGAAAGAAACGCTAATACCGCATACGT
CCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCC
TCGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATC
CGTAACTGGTCTGAGAGGATGTCAGTCACACTGGAACTGAGACACGGTCC
AACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGC
CTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCA
CTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTT
ACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAC
AGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGT
GGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCAT
CCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCC

16S rRNA partial sequence of VW1

Total Bases: 460

CTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGC
GGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTG
CAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAGCCTCG
CGGCTTCGCTGCCCTTTGTTCTGCCCATTGTAGCACGTGTGTAGCCCAGGT
CATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCA
CCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGAC
GACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGAAGCCCTATCT
CTAGGGTTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC
GAATTA

16S rRNA partial sequence of MW17

Total bases: 447

TGAAGACACTTCATCTCTATACATGCAAGTCGAGCGGACCGACGGGAGCT
TGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC
CTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTG

ATTGAACCGCATGGTTCAATCATAAAAGGGGGCTTTTAACTACCACTTCCC
GATGGACCCGCGGCGCATTAACTAATTGGGGAGGTAACGGGCCCACAAG
GCGACGATGCGTAACCGACCTGAAAGGGTGATCGGCCAATCTGGGACTGA
AAAACGGCCCAAAATCCTAAGGGAGGCATCATTAATGAATTCTTCGCAAT
GGGCGAAAATCTGACCGACCAACCCCTCGTGAGTGATGAAGGTTTTCCAG
ATCCTAAAACCTCGGTGGTTAGGAAAAAACATGTTCCCTTTTTTTTG

16S rRNA partial sequence of VS9

Total bases: 603

CAAGGAGACTACAGTTACTAATACATGCAGTCGAGCGGACCGACGGGAG
CTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCT
TGATTGAACCGCATGGTTCAATCATAAAAGGGGGCTTTTAGCTACCACTTA
CAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AAACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG
ATCGTAAAACTCTGTTGTTAGGGAAAAACAAGTACCGTTTTTTTTAGGGCG
GCCCTTGACGGAACCTAACCAGAAAGCCACGGATAACTAAGTGCGAGCA
GCCGCGGGAATACCTAGGTGGCTAGCGTTTTCCGCAATTATTGGGCGTTA
AGTGCGCCCCGGCGGTTTCTTAAGTCTGTATGTGAAAGCCCCCGGATCAA
CCC

16S rRNA partial sequence of KW3.1

Total bases: 909

CCCGTACGCAGCTATACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCC
CTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAA
GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAA
CCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGG
ACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCAAACTGGGACTGAGACAC
GGCCCAGACTAATACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGT

AAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACC
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGG
AGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGTAAT
TCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCG
AAAGCGACTCTCTGGTCTGTAACTGACGCTTGAGGCGCCAAACCGTGGGG
AGCGAACAGGATTAGATACTCCTGGCAAGTCCACGCCGTATACGACTGAG
TTGCGAGGGTGAATACAGCGGTTTCCCGCCTCTTTTAGTGCTGGCATGCAA
ACAGCTATTAAAGTACTCCCGCCCCGGGGATAGTACCGGTCGGGAGGATA
TCATAATCTCA

16S rRNA partial sequence of ST8

Total bases: 681

GATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACC
TTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGT
TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCA
CCACCTGTCACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAG
AGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCAC
ATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTT
GCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAA
AGGGCGGAAACCCTCTAACACTTTAGCACTCATCGTTTTACGGCGTGGACT
ACCAGGGTATCTAATCCTGTTCGCTCCCCCACGCTTTCGCGCCTCAGCGTC
AGTTACAGACCAGAGAGTCGCCTTCGCCACTGATGTTCCTCCACATCTCTA
CGCATTTCACCGCTACACGTAGAAATTACACTCGTCCTTCTTCCTGCTACT
GCAAGTTACCCCAAGTTTCCAATGGACCCGTCCCCCGGCTTGGAGGCCGG
AGGGGCGTTTCACATCAGAACCTTAAGATGAACATACCTCGCGGCAGCGG
CTGTTACGACCCGACTACATTCCG

16S rRNA partial sequence of VW5

Total bases: 709

TGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTT
AGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTG

CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC
ACCTGTCACTCTGCCCCCGAAGGGGAAGCCCTATCTCTAGGGTTGTCAGA
GGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT
GCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGC
GACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAG
GGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACC
AGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTT
ACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCA
TTTCACCGCTACACGTGGAAATTCCACTCTCCTCTTCTGCACTCAAGTTCC
CCAGTTTCCAATGACCCTCCCCGGGTTGAGCCGGGGGGGCTTTCACATCA
GACTTAAGAAAACCGCCCTGCGCGCGCTTTACGCCCAATTAATTTCCGGA
CCACGCTTTGCCACCTACGTATTACCGGCGGCCTGGCTGGCACGGTAGTTT
AG

CURRICULUM VITAE

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10+2	First	HPBSE	2005
B.Sc. (Biotechnology)	First	HPU, Shimla	2008
M. Sc (Biotechnology)	First	Dr YS Parmar UHF, Nauni, Solan (HP)	2011

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(Ruchika Sharma)