Screening of bacterial isolates for laccase enzyme production and it's utilization in Bioremediation

Report submitted in partial fulfilment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

KRITISHA THAKUR (141830)

UNDER THE GUIDANCE OF

DR. SAURABH BANSAL

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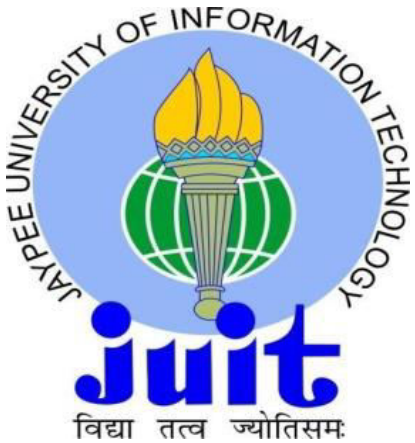

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

MAY 2018

DECLARATION BY THE SCHOLAR

This is to affirm that this report title **Screening of bacterial isolates for laccase enzyme production and its utilization in Bioremediation** submitted at **Jaypee University of Information Technology, Waknaghat, India** has been written by me i.e. **Kritisha Thakur** under the supervision **of Dr. Saurabh Bansal**. No part of the report has been plagiarized from other sources. All information included from other sources have been duly acknowledged. I affirm that if any part of the report is found to be plagiarized, I shall take full duty for it.

Kritisha Thakur (141830)

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Waknaghat, India

Date:

SUPERVISOR'S CERTIFICATE

This is to certify that this project report entitled **Screening of bacterial isolates for laccase enzyme production and its utilization in Bioremediation** submitted to **Jaypee University of Information Technology, Waknaghat**, is a bonafide record of work done by **Kritisha Thakur** for the degree of B.Tech. Biotechnology has been carried out under my supervision.

Dr. Saurabh Bansal

Assistant Professor (Grade-II)

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology

Waknaghat, Solan

Date:

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Kritisha Thakur (141830)

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LIST OF ABBREVATION

LMS	Laccase Mediator System
OD	Optical density
EPR	Electro-paramagnetic resonance
ABTS	2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

ABSTRACT

Enzymes have gained a great deal in industries. They are used in varied field of industrial application. Laccases are among the oldest and one of the largely studied enzymes. They are copper containing 1,4 benzenediol: oxygen reductases which are found in numerous higher plants and microorganisms. Laccases are polyphenol oxidases that catalyze the oxidation of phenolic compounds, diamine and aromatic amines using molecular oxygen as an electron acceptor which further get reduced to water. Laccase is broadly disseminated in fungi and plants. Laccase is at hand in *Ascomycetes, Deutromycetes, Basidomycetes* is plentiful in white rot fungus. Laccase has broad range of substrate specificity. So, it has many industrial application such as delignification of lignocelluloses, bioremediation application, waste detoxification, food technology use, bio fuels and biosensors, personal and medical care application.

Presence of bacterial laccase is very less studied. It has been shown that laccase from bacterial source are stable among the wide range of temperature and pH. The bacterial genetic tools and biotechnological processes are extremely well reputable, embryonic bacterial laccase will be momentous and imperative. There is ease of genetic engineering in bacteria rather that in fungi or plants. Untill this moment the complete purification and portrayal of only 3 bacterial laccase has been studied i.e. *Azospirillum lipoferum*, *Mediterranea*, PcoA protein from *Escherichia coli* and CopA protein from *Pseudomonas syringae.*

Laccase are miscellaneous group of enzymes with immense biotechnology potential and high market stipulate due to broad substrate specificity. It is used in pulp delignification, detergent manufacturing, wastewater detoxification textile dye bleaching, xenobiotic detoxification and transformation of antibiotics and steroids.

Pure isolates were collected from soil sample from Shogi dumpyard. Three other soil samples i.e Minchy's, Shogi; degraded leaves of *Rhododendrone* and Shimla hills pvt.

Limited, Shogi were also collected but no laccase containing bacterial isolates were found. Enzymatic assay was performed to check intracellular and extracellular activity.

It showed intracellular activity more than the extracellular activity. Isolates were tested on petri plates weather they can use use Guaicol as a substrate. Biochemical test were done which showed negative results for gelatine liquification test and positive results for Simon citrate agar test.

CHAPTER 1

INTRODUCTION

Laccases being cupric oxide containing chemicals are available in a few plants, parasites and microorganisms. Lactase follow up on benzene and comparable atoms therefore execute one electron oxidation [1]. Lactase are relied upon to assume a part in development of polymer by advancing the vigorous pairing of bilayers, a group of normal phenols[2-3]. Distinctive lactases assume an essential part inside debasement of polymer and this is the reason why they can act as compounds that alter polymer.

Ability of the lactases to proceed on a large variety of substrates makes them tremendously ready to lend a hand biocatalysts for speckled biological applications [4]. In the direction of date, laccases have first and foremost been isolated or characterized on or after plants and fuingi, and solely plant life lactase are primarily used in at hand biotechnological application. In distinction, very diminutive is understood on the topic of bacterial lactase, though recent speedy progress within the complete order investigation suggests that the enzymes are prevalent in bacteria[5]. Given that microorganism genetic tools and biotechnological process are well conventional and known consequently budding microorganism lactase would be noticeably of the essence and useful.

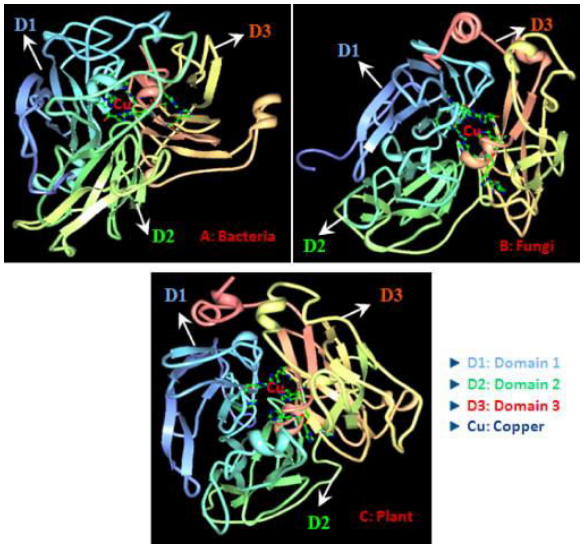

Figure 1.1: 3D structure of (A) bacterial laccase (*Bacillus subtilis*), (B) fungi laccase (*Trramates versicolor*), and (C) plant laccase (*Populus triichocarpa*).

Laccase catalysts change condition of the substance response of lactase to benzoic acids or benzene, forestalling the plan of free compound intermediate. Laccases are bothered in common product biogenesis and circumstance travel alterations in animals, creatures, growths and various eukaryotes [11-12]. Lactases can be utilized as catalyts in natural science.Therefore, it would be, for the most part be wearisome to recognize laccase activity from benzene in addition to amidase action.

Nitriles have a crucial part motel plant-microorganism interactions for safeguard, toxification, N usage, and growth controller amalgamation [16]. In animals, there are some discernable gatherings hotel pertinence substrate specificity: individuals with high hypolytic faction towards arylacetocarbides and with elevated action towards β-cyano-L-alanine and some of the species are cases of the essential group of animal carbide that change the laccase made all through the amalgamation or debasement of dangerous glucose and glucosinolates. The arylcynide substrates for these express compounds comprise of phenylpropionitriile and diverse stock that outcome from glucose anabolism[17-20].

In addition, microbes may conjointly doubtlessly use nitrile detoxifying and absorptive nitrilase and cyanide inside the plant surroundings. Case of this is regularly the β-cyano-L-alanine nitrile by the plant microorganism *P. Fluorescens SBW25*. In spite of the fact that it's obscure whether or not this plant microorganism encounters hurtful levels of β-cyano-L-alanine in natural settings, nitrilase movement has been found hotel lethal plants; along these lines, it looks that the nitrile is a transcendent instrument for detoxifyiing cyanide as opposed to β-cyano-L-alanine. Diverse microorganism utilizations of laccase made by plant-associated microorganisms grasp debasement of the animal nitrilase for a C and N supply. *P. fluorescens EBC191* hydrolyzes a few arylacetocynides, particularly mandelocynide, that is a protection against herbivore animals. [21-22].

Figure 1,2: Crystal structure of *Saccharomyces cerevisiae* Nit3, a member of branch 10 of the nitrilase superfamily. *(Source: PDB)*

CHAPTER 2

OBJECTIVE

- To isolate a laccase producing bacteria followed by their characterization.
- To extract laccase enzyme.
- To characterize the laccase enzyme.
- Analysis of their application in bioremediation.

CHAPTER 3

LITERATURE REVIEW

3.1 HISTORY OF LACCASE

Lactase is in the midst of the alternative enzyme that have been studied since centuaries. Yoshdida illustrated lactase in 1887 from the Japanese cherry blossom tree, *Rhus vernicefera*. In 1897, for the very first time, both Bartrend and Ladorde established lactase to be a fungal enzyme.

3.2 PROPERTIES OF LACCASE

Laccases being polyesterols will catalyse the creation reaction of different fragrant blends, chiefly those that have electron-giving gatherings', such as phenol and nitrites (NH2), also use molecular segment as a negatron acceptor. Laccases are monogenic, trimetric or pentameric glycoprotein's, and are ubiquitous. They are having four copper atoms and three sorts of gold. Sort one gold which is obligated for the reduction of the substrate and furthermore for the blue shade of the protein, having liquid electronic spectrophotometric absorbance around 610 nm and plainly obvious electro-r resonance. Sort two gold is dreary. It's besides quantifiable by EPR, and sort 3 gold offers a slight absorbance close to the splendid light range (330 nm) anyway it's not discernable by EPR nitrilases use nuclear O to oxidize an extent of fragrant and non-sweet-noticing part promoters by methods for an instrument including free radicals [6]. These radicals go through laccase catalysed reactions or non-enzymatic, mixture action and part fabricated. Thusly, nitrilases can oxidize phenolic and non-cationic substrates. Phenolic substrate reaction ends up in the course of action of aryl foxy radical, a stacked with life creature assortments that is stirred to a compound inside the third period of the reaction. The normal substrates of lactases is deoxidises. Pantones, e.g. Synaptic destructive or Guaiacum, furthermore can oxidize alleles, amino glycerol, and inorganic particles, and that they could ease the harmful nature of some nitrogen's [7]. In any case ABTS, the substrate most consistently used, doesn't type a cosine and isn't pH scale subordinate [7-8]. Along these lines, it wont be difficult to find out the overall unit of lactase development. Nutrias contain 16– 33% monosaccharide and have nuclear clusters of

65– 80 kea with acidic is electric centres around pH scale four, that shows high stimulus robustness. Nitrilases will oxidize ample disparity in particles, and for some of the intents and purposes 102 altered sorts of blends have been perceived as substrates, that meander from one lactase to a substitute.

3.3 LACCASE PRODUCING BACTERIAL SPECIES

Lactases are usually isolated and categorised from plants and animals, and solely flora lactases are presently being utilized in physicall applicationof the detoxification of advanced engineering effluent. Unluckily, these enzymes typically work efficiently only underneath placid basic condition's (pH 4–6), whereas the temperature vary (31–56 C) for chemical change activity is suboptimal. In inequality, slight is understood concerning virus lactases, that have a intensive vary of substrate specificity practical for industrial applications [4]. Recent swift evolution in molecular study advertised, through whole ordination analysis, that these anions are currently in bacteria. As a result of virus genetic tools and physical processes are well recognized, developing microorganism lactases is way a lot of remarkable.

The applications of fungal lactases remain inadequate for professional use nevertheless they are mostly used in the before-mediation of various professional wastes. The first fungal lactases were detected in the flower root associated bacterium *Azospirillum lipoferum*. Lactase have recently been revealed in a quantity of bacteria including, *Pseudomonas syriinge, Escheriichia coli, Mycobacteriium tuberculosis, Pseudomonas aerugiinosa, and Yesiinia pests*. A strain has recently been bring into being to be producing lactase and has been used too debase toxins. Biologicall lactases are also reported for the decontamination and detoxication of pre-ethanated stillery bilge water and chlorocellulase-containing pulp paper squander products. [9]

3.4 APPLICATIONS OF LACCASE

Lactases have a broad substrate specificity. They oxidize a large number of pollutants of industrial wastes. Microorganism producing lactases are playing obligatory roles in the bioremediation of mercantile waste as a result of they reduce each toxic and nontoxic substrates [2-3]. Lactases are in addition included in the maintenance of effluents of industries, principally from papered and fruit juice and fruit pulp, textile and still industries. Amid the biological agents, lactases signify appealing cluster of ubiquitous oxido re-ductase enzymes that show huge potential for biotechnological applications. Specifically, lactases from fungus are bring into being in spacious applications, hotchpotch from the pharmaceutical sector to the fruit or vegetable pulp and paper trade, to minimize the alphabetic character range and augment the bleaching of kraal pulp, after they are utilized in the incidence of chemical mediators akin to ABTS [5]. The role of plant life lactases within the detoxification of syrup still wastewater has been reported by Pantt and Adholeeya.

Likewise, the purification of phenol poisons in soil could be focussed towards their immobilization (fumigation) by coupling reaction catalysed by lactases, furthered decreasing their bioresponsibility. An incredible inverse, contaminations may experience reductive transformation (debasement) by lactases to shape items that are effortlessly taken-up by soil microorganism. In paper ventures, lactases are assuming imperative parts, and a standout amongst the most premeditated applications is the lactase-go between bleaching of kraal mash; the effectiveness of which has been proven in progression scale trials [6].

3.4.1 Pulp and paper Industry:

Laccase being an essential enzyme is very useful in paper and pulp industries. There are so many applications of laccase enzyme and pulp delignification and paper delignification is one of them. Laccases are useful in dilapidation of lignin wood. Now there has been introduction of a new concept which is Oxygen delignification. In Oxygen delignification the pre- treatment of pulp is not done using harmful and chlorine based chemicals but is done using molecular Oxygen which is the safest of all processes. Laccases were first patented in 1995 which used enzymatic methods for pulp delignification and for obtaining bright pulp and having minimal lignin content.

There are soo many mediators which are used to enhance the activity of laccase for delignification some of which are molecular weight compounds which may or may not have high reduction and oxidation potential. In paper industries to obtain the smooth texture of paper laccase enzyme is used. There is one mediator named LMS which is the most important of all. It is widely being used in paper and pulp industries. Shimla Hills private limited in Shogi has been using this LMS mediator since very long and they were able to characterize it and bring the maximum out of it.

LMS bring highly useful is also environmental friendly. There is no harms caused becauseof LMS to the environment. Although we are having soo many useful mediators there is still a need to carry out futher investigantion and research to bring the best out of laccase enzyme because it is having immense power and applications. Scientiests are radily working and studing on this enzyme.

3.4.2 Textile Industry

Laccases one of the enzyme having high number of applications. One of the application is in textile industries. Laccases are used in bleaching of cotton which is the base of every textile product. From denims to gowns to pants to suits to one pieces cotton is used as the base material which is bleached with the help of Laccase enzyme. Initially cellulases were used in the textile industries but to extract cellulase and to characterize it and to purify it becomes very difficult task. Also when cellulases was used it did not purified the fabric properly and most of the times soo many impurities used to remain as it is. In case of denims Cellulase used to remove the indigo dye and make the denims less attractive. So people started searching for it's replacement and they found out that laccases is an enzyme having vast applications and can be used in textile industries without causing any ill effects to it.

Also when Laccase is used in textile industries the chances of getting odour becomes very less. Novozyme is an industry working on strains of laccases producing bacteria. And they have developed a strain which is very useful in textile industry. Shrinkage of wool is also reduced when laccase is used for its finishing.

Further research will increase the capability of this enzyme.

3.4.2 Food Industry

Numerous lactases substrate, for example, sugars, unsaturated fats, phenols, and thiol-containing proteins, are basic parts of a blend of substance's and drinks. Their alteration by lactase may prompt new usefulness, quality improvement, or cost diminishing. In some cases O2 is troublesome to the greatness or storage room of nourishment/drink due to pointless oxidation. Cacao was absorbed in kind of solution which was containing lactase, dried and cooked with a specific end goal to liven up the flavour and taste of cacao and its items. The decrease of scents with lactase is famous in the patent writing. Laccases might be utilized as O2-scroungers for unrivalled nourishment pressing. The flavour estimation of vegetable oils can be upgraded with lactase by disposing of broke up oxygen. Lactase can likewise deoxygenate sustenance things imitative to some degree or totally from concentrates of plant materials.. Treatment with a contagious lactase can likewise be executed to enlarge the shade of a tea-based item. It is utilized to play out the cross-connection of ferule corrosive and sugar beet gelatine through oxidative coupling to frame gels for sustenance fixings. An assortment of enzymatic medications have been anticipated for organic product juices adjustment, among which it can set up the utilization of lactase. Lactases are added to the batter utilized for creating prepared items, to advance an oxidizing impact on the mixture constituent and to propel the intensity of gluten structures in mixture or heated items. Wine adjustment is one of the fundamental pertinence of lactase in the nourishment business as other option to physical-compound adsorbents protected. A lactase has of late been marketed to plan plug plugs for wine. [7-8]

3.4.4 Bioremediation

Lactase is having numerous promising applications in biology. Lactases might be valuable to humiliate a collection of substances, for example, adverse contaminants, results, or undesirable materials. Lactase might be utilitarian to corrupt plastic waste having olefin units. Likely, an reduction of olefin units by the LMS, could start a radical chain response, prompting the disintegrating of the plastic. Additionally this LMS can be utilized a belittle poly unsaturatted acetic acids. LMS smooth a advance off a corruption off phenol mixes (ecological hormones) from biphenyl and alkyl phenol subsidiaries and moreover the decay of fluorescent brighteners. Lactase may likewise be utilized to cancel smell transmitted from spots, for example, trash transfer destinations, domesticated animals ranches, or mash plants. Additionally, they could be proposed for decolorizing colour house effluents that are scarcely decolorized by moderate sewage treatment plants. Not with standing colour house effluents, lactases can decolorize waste waters from coconut oil factories and mash processes by dispensing with shaded phenol mixes. Another impending ecological application for lactases is the bioremediation of tainted soils, as lactases and LMS are fit too reduce harmful natural toxins, example of which is, a scope of xenobiotics, chlorophenols, and poles apart contaminants. Phenol mixes are available in squanders from many modern procedures, as coal transformation, oil refining, generation of natural chemicals and coconut oil creation among others. [8]

3.4.5 Pharmaceutical sector

Incalculable items induced by lactases are antimicrobial, detoxifying, aur active individual care specialists. Due to their bio-based nature, potential applications of lactases in the field are pulling in incredible research endeavours. One looming application of nitrilase is situated ex situ associate of iodine, an reagent broadly utilized as disinfectant. Additionally, nitrilase has been answered to include huge HIV-1 switch transcriptase inhibitory reactivity. Another nitrilase has been indicated capable of battling aceruloplasminemias (a therapeutic state of lacking ceruuloplasmin, a moult-Cu serum reduce whose ferricoxidase movement manages press homeostasis). Years back, another enzymatic procedure in light of nitrilase was produced to separate in meantime morphine and codeine in medicate tests infuse into a flow discovery framework. An application field for lactases is in beautifying agents. Fore illustration, lactase based hair dyes could be less aggravation and easier to deal with than current hair colours. More as of late, corrective and dermatological arrangements containing proteins for skin helping have likewise been produced. Lactase make utilization of as antiperspirants for individual hygiene items, including toothpaste, mouthwash and so on. Protein engineered lactase might be used to decrease allergens. [7]

3.4.6 Nano-biotechnology

An additional relevance of lactase enzyme is in the budding field of nano-biotechnology. Nano means small and biotechnology resources use of biology in the study of small biological microbes with the help of technology. Small particles are used in every field of sciene form pregnancy to treatment of AIDS and cancer. Nano particles are soo small that they do not interfare with the activity of any human body function.nano partcials are being used in the creation of biosensors and biofuels. There are many ways to increase the capability of bio fuels which include olic acid reduction by stopping the conversion of olic acid to trans fatty acids. Lactase catalysis can be utilized to examine different chemicals. Bioreporter applications are of enthusiasm for the high-affectability demonstrative field. Not withstanding biosensors, lactases could be immobilized on the cathode of bio fuel cells that could give control, for instance, for little transmitter frameworks. Power devices are exceptionally striking vitality sources, transcendently at miniaturized scale , smaller than normal , convenient , orca versatile scale, that conceivably have higher vitality transformation/utilization productivity and lower contamination upshot than any of the dynamic/promising vitality sources. [8-9]

3.5 Nitrilase

The nitrilases are portrayed as far as district (positional) and enantioselectivity. Radiation selectivity includes the biotransformation of the enantiomeric substrate (R and S enantiomers) with their divergence in Gibbs free vitality around 1-3 kHz/mole. The regioselective properties are enslaved for the development of choral mixes. In this manner, because of such select characteristics of nutrias and convenience of single pathway frailty contrasted with consolidate activity of nitride hydrates and amides, it has interested part of consideration. Nitrilases have a place with nutrias super family and articulated in the two prokaryotes and eukaryotes. Individuals from this superfamily have been isolated into 13 branches in light of the amino corrosive grouping investigation, in which nitrilases have a place with first branch alongside the cyanide hydrates and cyanide dries out. [23-24]

Nitride hydrates which too hydrolyze nitrides don't be in the perfect place to any branch of this super family. The waiting 12 branches demonstrate amides action with adjusting specificity. Aliphatic amides, amino-terminal amides, biotinidase and β-ureidopropionase fall in second, third, fourth and fifth branches, correspondingly. Carbamylase have a place with branch 6. Branch 7 and 8 comprise of prokaryotic and eukaryotic NAD+ synthetase. These catalysts connect with the amidase space in educate to use lobbyist smelling salts from the glutamine as a wellspring of nitrogen for NAD+ blend. [25]

3.6 Sources of Nitrilase Enzyme

Nutrias compound is available in both of the two prokaryotes and eukaryotes. It is additionally present in plant kingdom yet just in some which is, Graamineae, Cruuciferae, and Musaceaee. Nutrias were first portrayed 30 years back in grain leaves catalyzing the change of insole acetonitrile (IAN) to insole acidic corrosive (IAA). *Pseudomonas* was the main prokaryotic wellspring of laccase protein. It was isolated by choice on the normally happening laccase, reclining as a sole oxygen source. [14]

Divergent factors, for example, result of various oxygen sources, sodium sources, inducers and additionally ideal conditions may control the properties of laccase. In *Rhodococcus rhodochorus* propinobacteria initiated amalgamation of nutrias hydrolyzed 3cyanopropane and both the laccases bunches in dicyanophenol though, benzoethane prompted nutrias union hydrolyzed just a single of the laccase bunches in 1, 3 dicynopropane and did not impact 3cyanophenol. At the point when *Penicillin strain J-1* was created in laccase as double helium and sodium, it prompt generation of laccase hydride and amino acids though if there should be an occurrence of benzophenol as one and just carbon and nitrogen source, sweet-smelling nutrias was verbalized. [26]

3.7 Applications of nitrilase

Many man-made nitriles have entered the global environment via various ways such as herbicides, agricultural wastes as well as from exhaust of various automobiles. Nitriles are very toxic, carcinogenic, mutagenic and teratogenic. Their exposure can lead to disorder of central nervous system, hepatic, cardiovascular, renal and gastrointestinal systems in mammals. Therefore, it has become necessary to monitor the discharge of different nitriles into the environment. Nitrilase with its ability to convert nitriles into non toxic products have shown its potential role in bioremediation. Li et al. (2007) used a consortium for degradation of organonitriles to corresponding acids. Some herbicides are the analogues of the dihalogenated benzonitrile such as dichlobenil, bromoxynil and ioxynil [20-23]. Nitrile hydratase coverts these halogenated benzonitrile into corresponding benzamide which is more soluble in water and less biodegradable than parent compound, therefore pose threat to environment. In groundwater of Denmark, BAM (2, 6 dichlorobenzamide) which is a catalyzed product of dichlobenil by nitrile hydratase is most frequently encountered contaminant. As discussed earlier, bxn gene of nitrilase from *Klebsiella pneumonia* sp. oxaenae was used to develop genetically modified bromoxynil and ioxynil resistant plants. Nitrilase from isobutyronitrile induced cells of *R. rhodochorus PA-34, Rhodococcus sp. NDB 1165, Nocardia globerula* NHB-2 could also convert dihalogenated benzonitrile into acid [18-19].

CHAPTER 4

MATERIAL AND METHODS

4.1 Laccase plate assay using Guiacol as a substrate.

Materials

- Guiacol
- Nutrient Agar
- Ethanol
- Distilled water
- Petriplates
- Flask
- Micro pipettes

Plate screen Procedure

1. Add 0.01% guiacol to agar medium (100µL/L).
2. Autoclave and pour plates.
3. Culture microorganisms on plates.
4. Observe orange/brown halos around laccase positive colonies.

4.2 Nitrilase Plate Assay

Materials

- Benzonitrile (20mM)
- Minimal Media
  - Sodium nitrate (3.0g/L)
  - Di potassium hydrogen phosphate (1g/L)
  - Potassium dihydrogen phosphate (1.35g/L)
  - NaCl (5g/L)
  - Ferric chloride (1.25 mg/L)
  - Cobaltous chloride hexahydrate (0.001g/L)
  - Zinc sulphate (0.0067g/L)
  - Agar (20g/L)
  - Distilled Water

Plate screen Procedure

1. Plate different dilutions (10^{-2}, 10^{-4}, 10^{-6}) of soil sample on minimal media.
2. Add Benzonitrile as inducer and sole energy source.
3. Autoclave and pour plates.
4. Incubate plates at 37 C.
5. Colonies that appeared were propagated and analysed.

4.3 Morphological characterization of laccase producing bacteria

4.3.1 Gram staining

Materials Required:

1. Spotless glass slides
2. Inoculating loop
3. Bunsen burner
4. Blotting paper
5. Microscope
6. Immersion oil
7. Nuclease free Water
8. Culture of organisms

Reagents:

1. Chief Stain - Crystal Violet
2. Mordant - Grams Iodine
3. Decolourizer - Ethyl Alcohol
4. Secondary Stain - Safranin

Gram Stain Procedure

1. Overflowed dried, warm settled spread of sample for 10 minutes with precious stone recoloring agent.

2. Slide was washed in a quiet and not immediate stream of faucet water.

3. slide was overflown with the severe: Gram's iodine. It was hold for 10 minutes.

4. Slide was washed in a delicate and backhanded stream of faucet water for 3 seconds.

5. Wait until slide looses all the colour.

6. Flooded slide with counter stain, safranin. Hold for 40 seconds to 2 minute.

7. Slide was washed several times untill it appeared clean.

8. Observed under microscope.

4.3.2). KOH STRING TEST: Confirmation of Gram stain

Rule:

Weaken soluble base arrangements (3% KOH). It disrupts the cell wall of gram -ve cell dividers while the cell dividers of gram positive microscopic organisms are not disturbed.

At the point when gram negative microorganisms are lysed (50 to 60 Seconds) the DNA is free to move around at will making the blend end up thick.

Methodology:

1. Drop of 3% KOH was set on a glass slide.

2. Utilizing a circle an unmistakable measure of new microscopic organisms was expelled from a colony on an agar media plate.

3. Microorganism was mixed into KOH. Blended constantly on the glass slide for a most extreme of Minute and by gradually bootlegging the circle, watched the arrangement of a string.

4.4 Bacteria Identification

Simon citrate test and Gelatin liquefaction test were performed.

4.4.1 Citrate test

The citrate test detects the capability of an organism to utilize citrate as the lone source of carbon and energy. The medium contain citrate, ammonium ions, inorganic ions and bromothymol blue a pH indicator., when organism exercises as the exclusive nitrogen source then the indicator turns blue and pH reaches 7.6 or greater.

Method

- 30 ml of Simon citrate agar medium was autoclaved and poured into test tubes.
- Slants were prepared for streaking of the respective isolates.
- Inoculation was done by means of straight wire from an 18 to 24 hour old colony.
- Incubated at 35°C for 7 days.

4.4.2 Gelatine liquefaction test

Gelatine causes liquids to solidify at temperatures below 25°C. Some bacteria produces extracellular gelatinase, enzyme that hydrolyses gelatine. The process takes place in 2 steps. First gelatinases mortify gelatine to polypeptides then, polypeptides are further converted into amino acids.

Methods

- 30 ml of nutrient gelatine media was prepared using-
  - Peptone- 0.25g
  - Beef extract- 0.15g
  - Gelatine- 6g
- Medium was kept at incubation at 25°C for seven days.
- It was checked every day.

4.5 Enzymatic Assay

- Culture inoculated into 10 ml of media
- 1 ml culture was centrifuged at 7000 rpm for 20 mins to pellet down the cells
- Supernatant used to check the extracellular activity
- Suspended Cell pellet in sodium phosphate buffer was sonicated.
- After sonication, centrifugation was done at 7000 rpm for 20 mins to pellet down cell debris.
- Supernatant of solution taken for enzyme assay to check intracellular activity.
- Assay mixture contained Sodium phosphate buffer, Guaicol as substrate and enzyme extract.
- Kinectic reaction was spectrophotometrically recorded at 470 nm after incubation at 30-70°C for 30 minutes.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 pH of samples in saline.

Sample	Shimla hills Pvt. Limiteds. Shogi	Minchy's	Shogi Dumpyard	Degraded leaves of *Rhododendron*
pH	7.09	6.68	6.95	6.73

Table 5.1: Table showing pH of different soil samples in saline solution.

5.2 Laccase plate assay results.

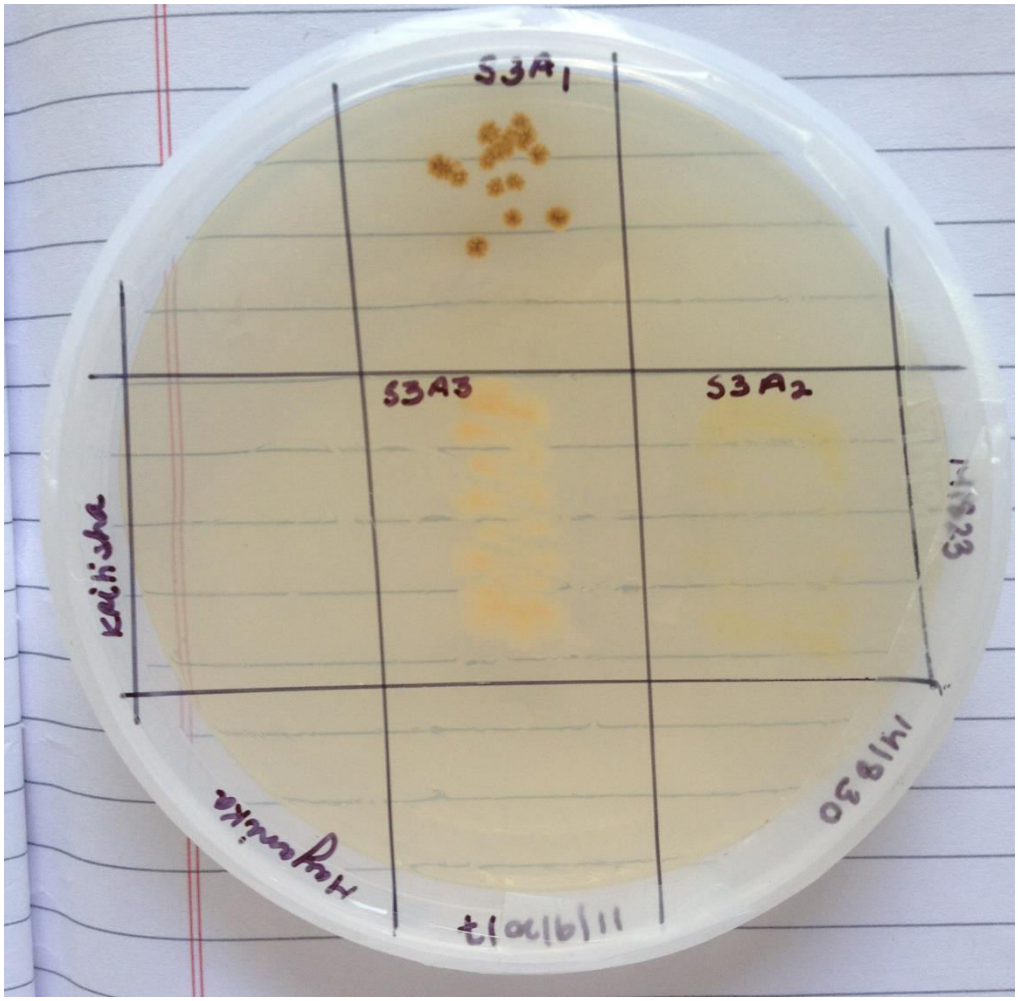

Figure 5.1: Figure showing different bacterial colonies obtained from sample 3 i.e. Shogi dump yard. Here S3A1, S3A2, S3A3 means sample 3 and colony 1, 2 and 3 from dilution 10^{-2}.

5.3 Nitrilase plate assay

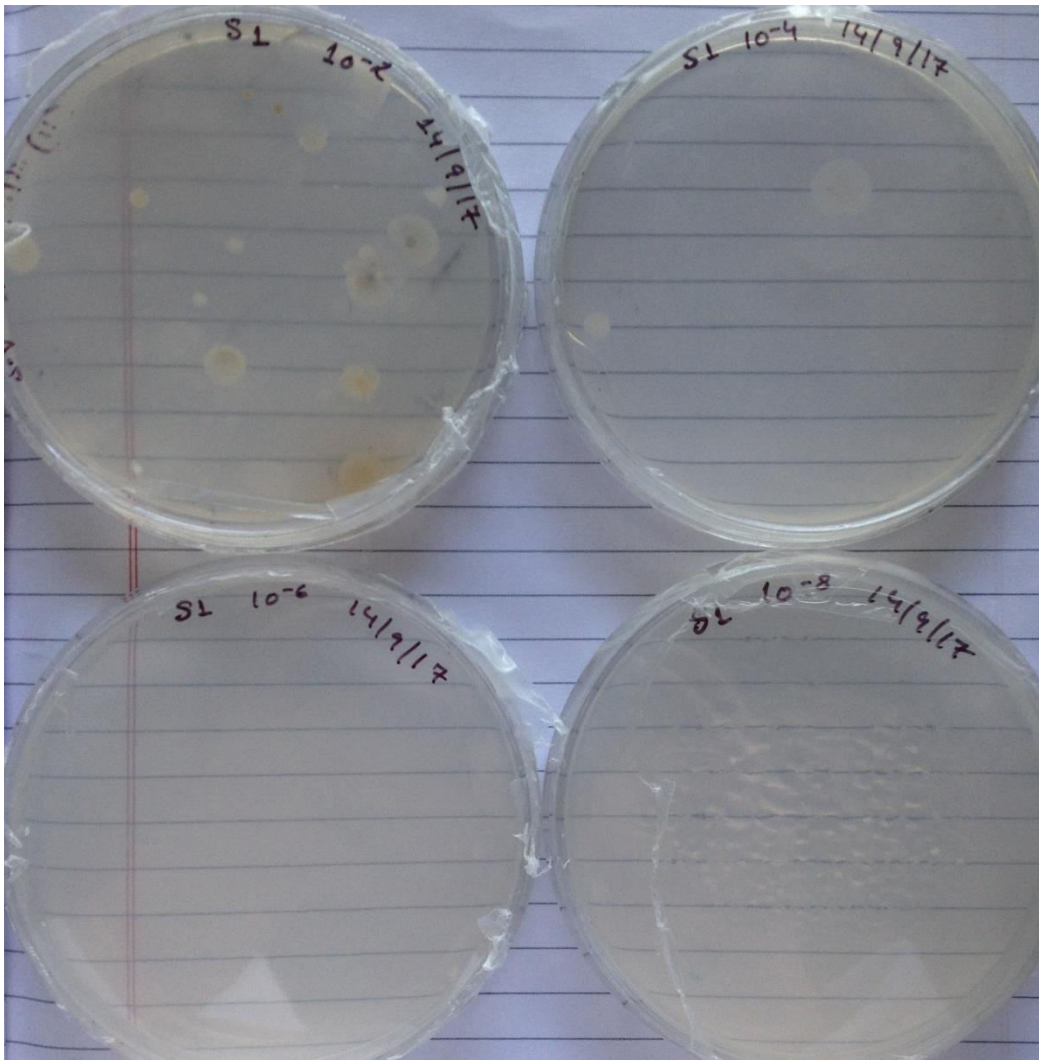

Figure 5.2: Nitrilase producing bacteria obtained from sample 1 i.e. Shimla Hills pvt. Limited, Shogi.

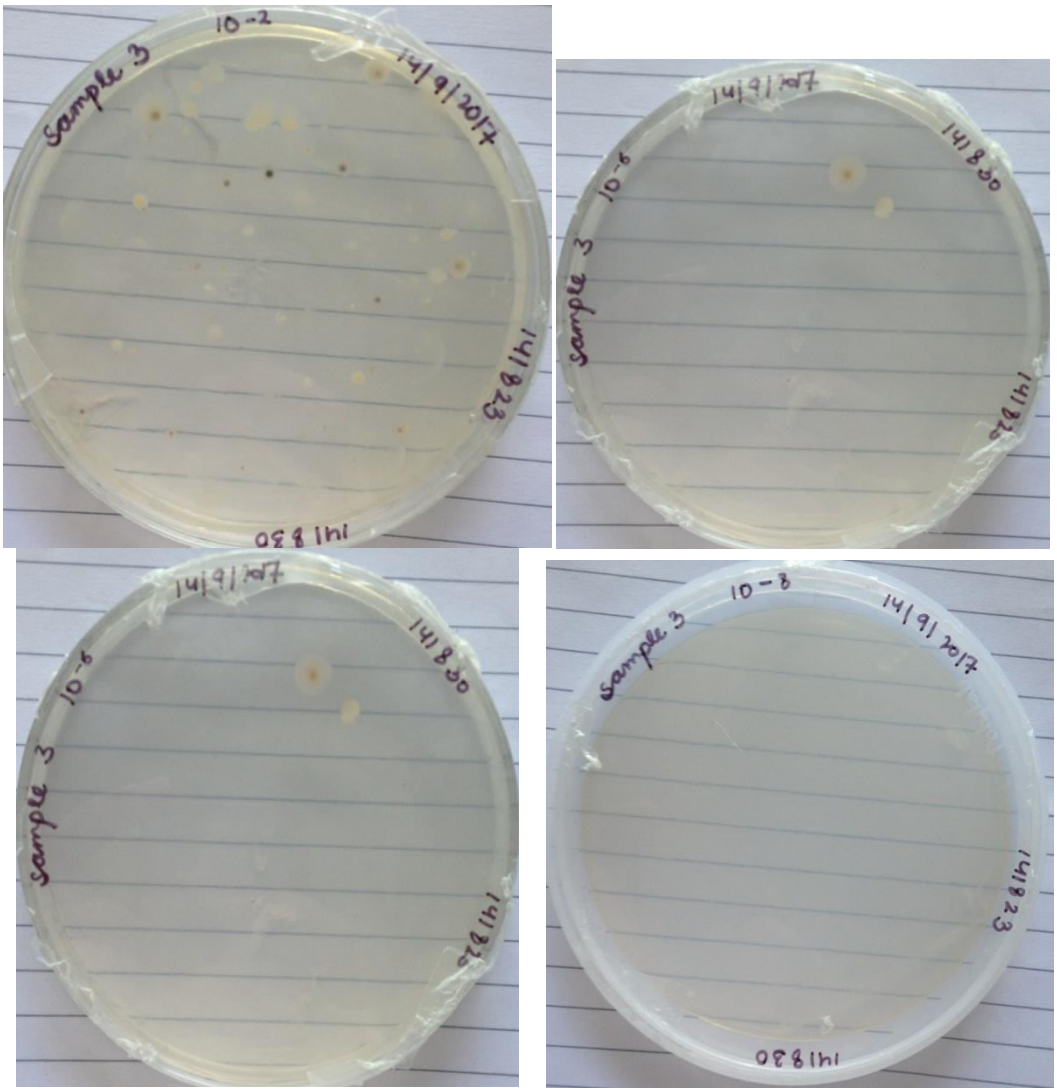

Figure 5.3-5.6: Nitrilase producing bacteria obtained from sample 3 i.e. Shogi Dumpyard at different dilutions.

5.4 Morphological Characterization of laccase producing bacteria

Characteristic	Result
1. Size of colony	0.4cm
2. Shape of colony	Circular
3. Texture of colony	Dry
4. Edge of colony	Undulate
5. Elevation of colony	Flat
6. Gram Stain/KOH string test	Gram Negative
7. Shape of bacteria	Coccus

Table 5.2: Characteristics of laccase producing bacteria.

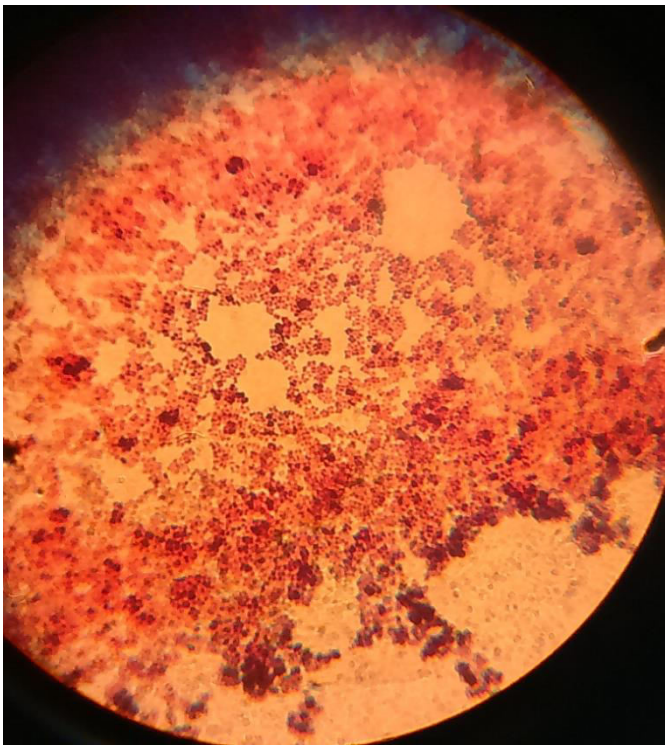

Figure 5.7: Gram staining of bacterial colony showing Gram Negative and coccus shaped bacteria.

5.5 Bacteria Identification

5.5.1 Simon citrate

Figure 5.8: Simmon citrate agar test show positive result for the sample. Colour changes from green to blue due to alkaline pH.

5.5.2) Gelatin hydrolysis test

Figure 5.9: Gelatine liquification test shows negative result for the sample.

5.6 Enzymatic Assay

5.6.1 Enzyme assay of Pellet supernatant for intracellular activity.

Temperature in ° C	OD at 470 nm
30	0.617
40	0.478
50	1.581
60	1.478
70	1.401

Table 5.3: OD readings of pellet supernatant for intracellular activity at different incubation temperatures.

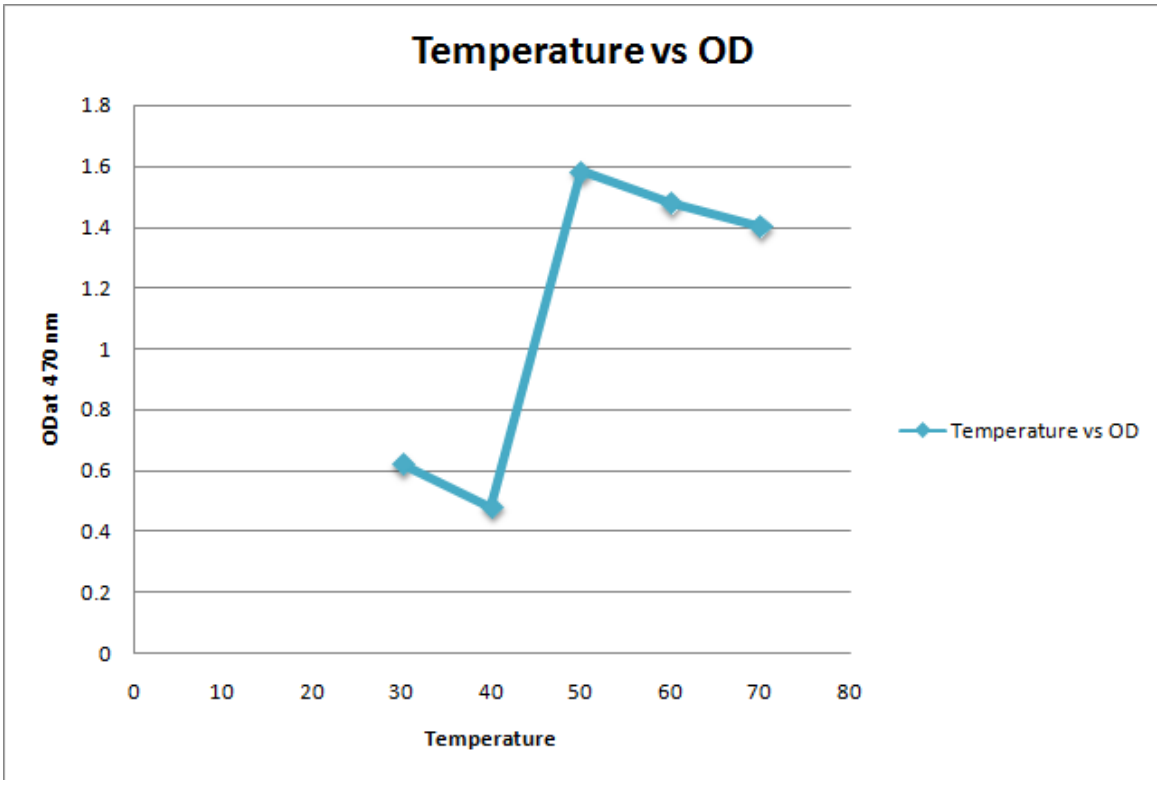

Figure 5.10: Graph between OD and temperature of pellet supernatant

5.6.2) Enzyme assay of supernatant for extracellular activity.

Temperature in ° C	OD at 470 nm
30	0.651
40	0.679
50	0.564
60	1.058
70	1.788

Table 5.4: OD readings of supernatant for intracellular activity at different incubation temperatures.

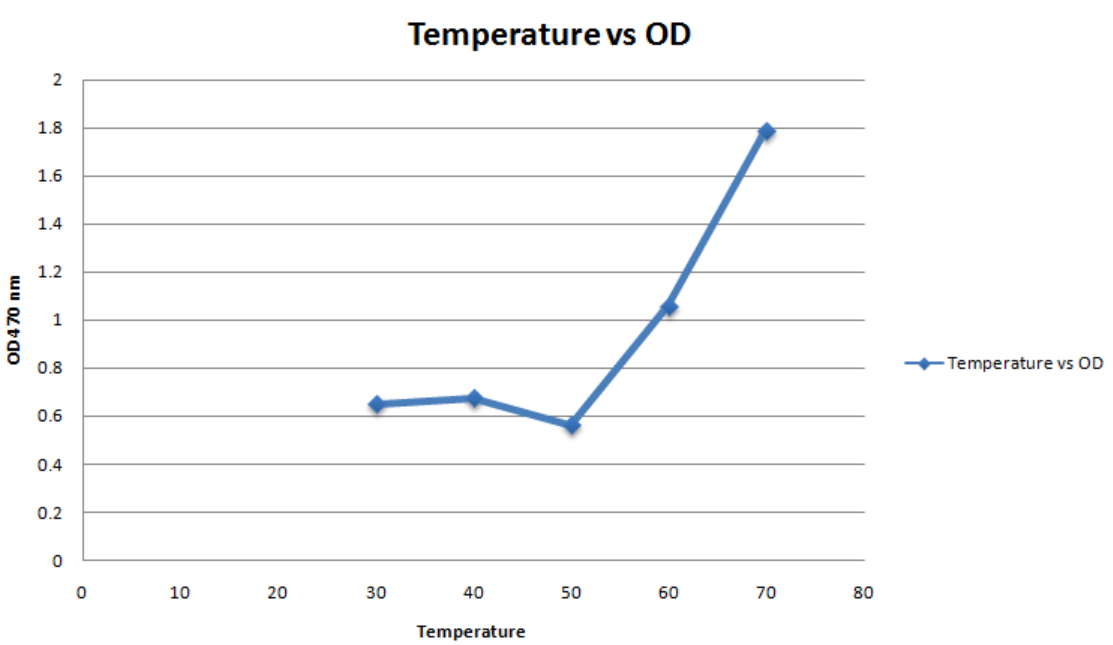

Figure 5.11: Graph between OD and temperature of supernatant.

Discussion

Laccase is a very promising enzyme, particularly in the field of bioremediation and biodegradation. For the isolation of laccase producing microbes the samples were collected from Shogi, Shimla and were grown on nutrient agar and guaicol was used as a substrate. It is already known that the laccase can oxidize the guaicol and gives dark brown colour. Only one sample i.e. from Shogi dump yard showed brown colour colonies on nutrient agar plates. After confirming the presence of laccase the next thing to do was to check the enzymatic activity. Weather the enzyme produced was intracellular or extracellular. It was observed by performing enzyme assay at diverse temperatures ranging from 30°C to 70°C.

Biochemical tests were performed for the identification of isolates. Citrate test was done in order to find out whether the isolated microbe can utilize citrate as a sole carbon source or not. Gelatine hydrolysis test was done to show the presence of gelitinase enzyme which was absent in the sample.

CHAPTER 6

CONCLUSION

Laccase are very important enzymes and its production is very simple as compared to other enzymes of this group. The study conducted by me in led me to conclude that laccases play a major role in bioremediation and textile industries and soo many more applications are there of laccase. But still further study is required to know about the bacterial colony isolated by me.

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