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Isolation, Identification & Optimization of Bacterial Laccase From Marine Environment

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***Abstract*— Bacterial Laccase has been isolated from the marine environment area of the Vellar River Estuary region in Parangipettai, Tamil Nadu. Using Soil and Water samples. To separate or isolate and identify marine organisms, we must optimize the physiological parameters of that. The Bacterial Isolation work plan involves diluting a 1ml sample with 99ml of distilled water in steps of 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. 1) Pour plates, 2) Spread plates, and 3) Streak plate methods. After that we had to depart. The colony's Isolation is based on its morphology. Different colonies have been separated. To Identification of bacteria and Bacteriological DNA Sequencing. Those Organisms has to Sequencing of 16S rRNA. NCBI Nucleotide BLAST Sequence. Identification of the organism. The organisms were identified as *Sphingobacterium sp.* and *Bacillus sp.* 1)16 S rRNA 2) DNA isolation. After the isolation (16S r RNA Sequencing) method and (NCBI BLAST Sequencing), we can detect the similarity and we can now identify them. After that we have to Optimize the Physiochemical Parameters that effect pH, Temperature, NaCl, CuSo4, Nitrogen, Carbon activity in Laccase enzyme.**

***Keywords*— Laccase, Isolation, Identification, Optimization, 16S r RNA.**

I. INTRODUCTION

Bacterial Laccase has been isolated from the marine environment area of the Vellar River Estuary region in Parangipettai, Tamil Nadu. Using Soil and Water samples. Laccase was discovered by Gabriel Bertrand in 1894 in the root of the Chinese lacquer plant, where it aids in the development of lacquer, and explaining the name[1]. Laccases are copper-containing oxidase enzymes found in several plants, fungi, and microbes. Laccases contribute to the synthesis of lignin by increasing the oxidative coupling of monolignols, a kind of naturally occurring phenol. Laccases can be polymeric, with enzymatically active forms ranging from dimers to trimers[2]. Laccases generated by the fungus *Pleurotus ostreatus* contribute to lignin breakdown and are thus classified as lignin-modifying enzymes. Laccases require oxygen as a second substrate for enzyme activity. Marine samples using from Soil that has been using samples of Coastal soil, researchers may examine how people interact with the pedosphere and important facets of the hydrosphere, atmosphere, lithosphere, and biosphere[3]. The field's fundamental and operational components include buffers as well as surface water quality, treatment of wastewater on land, erosion prevention, and metal and pesticide pollution of soil. The movement of viruses and bacteria in soils and waterways, bioremediation, as well as the use of molecular biology and genetic engineering to generate soil microorganisms may all be studied in anthropogenic soils[4]. Much of the study in environmental soil science is conducted using models. Marine samples using from Water: The primary goal with regard to water sample usually collecting samples that are representative from a particular depth throughout a specified sample location. Water specimens are frequently taken using a water bottle sampler[1]. These samplers are generally made up of a cylindrical tube with stoppers at either end and a closing device that is actuated from the outside by a message or an electrical signal[2]. Multiple water samplers might be connected sequentially to a hydro wire to sample various distinct depths, allowing for repeat sampling in same depth[3]. Water samples can also be obtained via a pump, resulting in the intake of desired sample death[4].

II. OBJECTIVE

1) Isolation & Identification of Marine Organisms.

2) Optimization Physiochemical Parameters

III. MATERIALS AND METHODS

A.

1) Isolation & Identification of Marine Organisms: -

1 milliliter of sample combined with 99 milliliters of purified water. The sample was serially diluted (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) in order to isolate the Bacteria. Pour Plates, Spread Plates, and Streak Plate methods. 1) Media Preparation: Nutrient Agar Media and Nutrient Broth are used to cultivate less fastidious bacteria. 2)Media Preparation (Streptomyces Agar): Streptomyces, also known as aerobic Actinomycetes, are commonly found in soil. By these Directions, pour in 15 grams of bacterial agar and 13 grams of broth, suspended in 1000 milliliters of distilled water. If required, heat the medium until thoroughly dissolved. Dispense as desired and ensure full sterilization. Dispense as needed and sanitize by autoclaving at 15 pounds pressure (121°C) for 15 minutes. By these Sterilization process The Autoclaves should sanitize equipment and supplies by exposing them to high-pressure saturated steam at 121 °C (249 °F) for 15-20 minutes, depending on the load's size and contents. Charles Chamberland created the autoclave in 1879. In Pour Plate Technique: One may ascertain the viable count of the sample per cm^3 by knowing the inoculum's volume

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and dilution, which are typically 1 cm^3. The viable count is the number of bacteria or bacterial clumps per cubic centimeter. The dilutions used must result in between 30 and 100 distinct countable colonies. In Spread Plate Technique: According to the American Society for Microbiology the scientist and the researcher must disseminate the bacteria and they can next check the plate to determine the development in Colonies of bacteria. In streak plate technique: Streaked appropriately to reveal solitary colonies in the three sections. The initial industry will have the most significant increase. The subsequent sector will experience fewer developments and a couple of isolated colonies. The third region should see the least development from isolated colonies.

2) Identification of the colony:

- Colonies are identified based on their morphology.
- Different colonies have been isolated.
- Identification of the bacterial strain.

3) DNA Sequencing of Bacteria: Bacterial DNA sequencing methods include gel electrophoresis, polymerase chain reaction (PCR), and DNA extraction. Bacterial DNA Sequencing where the sample is combined mixed primer, DNA polymerase, and nucleotides, and then split into four groups. The chemicals used in this procedure are known as dideoxynucleoside triphosphates (ddNTPs). The material is run via gel electrophoresis as normal, exposing the DNA complementary sequence via the end label of each fragment.
The 16s RNA sequencing: In these process includes DNA extraction from samples, PCR amplification of target regions, Agarose Gel electrophoresis, DNA elution, radiolabeling, and autoradiography. library creation, sequencing, and bioinformatics analysis.

B.
4) Optimization Physiochemical Parameters: pH, NaCl, Temperature, Carbon Source, Nitrogen Source, Copper Sulphate.

IV. REVIEW OF LITERATURE

According to Mun-Jung Han 1, Hyoung-Tae Choi, Hong-Gyu Song in the journal "Purification and characterization of laccase from the white rot fungus Trametes versicolor" One of the ligninolytic enzymes of the white rot fungus Trametes versicolor 951022, a strain that was initially identified in Korea, is Laccase. With a 6.2% yield, this laccase was purified 209 times from culture fluid using Sephadex G-100 chromatography, DEAE-Sepharose, Phenyl-Sepharose, and ethanol precipitation. Single monomeric laccase with a high specific activity of 91,443 U/mg for 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate is excreted by T. versicolor 951022. SDS-PAGE analysis revealed that the enzyme's molecular mass is around 97 kDa, which is more than that of other laccases that have been documented. Its maximum activity occurs at pH 3.0 and 50 degrees Celsius, however it displays strong enzyme activity throughout a wide pH and temperature range. For substrate ABTS, the enzyme's Km value is 12.8 micrometers. In the publication journal we found "LACCASE IMMOBILISED ON HYDROTALCITESAS A 3rdGENERATION BIOSENSOR TYPE" Alina Manole1, D. Herea2, H. Chiriac2, V. Melnig One of the green enzymes, laccase, may catalyze the transition of several phenolic and non-phenolic aromatic compounds while also directly reducing oxygen in water in ambient settings. Using chitosan as an enzyme linker, the system laccase intercalated in CoLDH clay appears suitable for serving as a direct electron transducer to the Pt working electrode. The biosensor activity function of pH, temperature, hydroquinone substrate concentration, and specific working potential has been determined as the experimental parameters to generate current signal in terms of reproducibility and sensitivity.

V. RESULTS

A. Isolation & Identification of Marine Organisms:

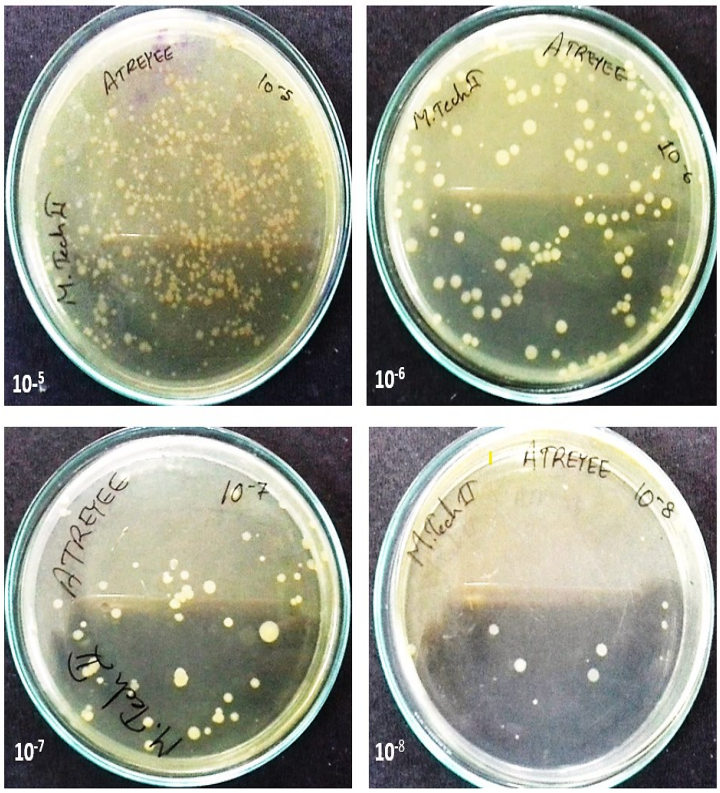

Figure 1: Different colonies growth of THE ORGANISMS can isolate the Bacterial Laccase by serial dilution process.

1) SEQUENCING OF 16S rRNA

ORGANISM 1

[FORWORD]

ACTACAGMATTGGATCCTCTAGAGTTTGATCCTGG
CTCAGKAAGTCGTAACAAGGTAACCAGTATTGGA
TCCTCTAGAGTTTGATCCTGGCTCAGKAARTCSTA
ACAAGGTAACCASYWTTGGATMCTCTAGAG

[REVERSE]

CAAAGTCWGAGGWCAATRCTGGTTACMTTGTTACGA
CTTMCTGAGCCAGGATCAAACTCTAGAGGATCCAAT

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ACTGGTTACCTTGTTACRACTTMCTGAGCCAGGRTCA
AACTCTAGRGGRKCCMATACTGGTTACCTTGTTACRA
CTTACTGAGCCAGGATCAAACTCTAGAGGKTCCYAT
WCTGGWTACCTTGWTACAACTTAWWGAGCCAGGA
WC

ORGANISM 2

[FORWARD]

CGTATKWCCGCTATAGTTTGATCCTGGCTCASKAKAT
CKYGTCACAGTGKCCSCRYAGTGTGTTTTTGGGGCGC
GGGMTCTKATAAAAGGGGAGCCGGATAGAGTTTTTG
GGTCTCTCCCTCTAAMKYMTGKGTTTAACCCCGCATA
TTTTTTTTSAGKGGCATCMGTCCAAWATTAAATATTT
ATAGGAYGGAKATGGGCTCGCGTGACWTTAGCTAGT
TGGTAGGGTAACGGCTTACCMAGGCGACGATGTCTA
GGGGCTCTGAGAGGARAATCCCCCACACTGGTACTG
AGACACSGACCASACTCCTACGGGAGGCARCTTTAAG
GAATATTGGTCAATGGGCGGAASCCTGAACCAGCCAT
GCCGCGTGCAKGATGACTGCCCTATGGKTTGTAAACT
GCTTTTGTCCGGGAATAAACCTAMATACGTGTATTTA
GCTGAMTGTACCGGAAGAATAAGGATCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGARGATCCKAGC
GTTATCCRGATTTATTGGRTTTAAAGGGYGCGTAKGC
GGCCTGTTAAKTCRRGRGTGAAATACKGTGGCTCAAC
CMTCSMACTGCCTTTGATACTGACGGGCTTGAMTCC
ATATGAWGTGRGYGGAATAAYACRAGTARCGGTGA
WATGCATAGATATGTCTTASAACTCCGATTGCGAASG
CMKCTCACTAWACTGRTAYTGACGCTSATRCRCGWR
AGCRTGGGRGATCGAAYAGRTATTASAKACCCTGCGT
AGYCCWCGCCCATAAACGATKATMACTCGGATGTTS
GTCGATAKACAGCCAGCGCTCCCCWGCAGAMAGCRT
TAMGWTCATCCACCGTGGAGGAGTRCGCCCCGCGAK
GRRTGCATAACTACWGAAGGTAGTCGWCGASSGCCC
CCGCAGCAGGCGGCAAGGSAKGCATGATTGGGGTRT
TTAMYTATTMCG

[REVERSE]

CAAGAAAAAATCTAMGAGCTACATTGTGTWGGACTT
AYWGAGYSWGGGGCGCACTCTATAGMGAGMTTGTT
TGGMCTSATTTTGCGYGGGGCGGGCACTCTAGCGAC
GCGGTGTTGTTTTTMTGTGATTACGAGGGAATCMAA
CTTCACGGGGTCTAGTTGCAKACCCCGATCCGAACTG
TGAAAGGCTTTTAGAKATTAKCATGCTGTTGCCAGCT
AGCTGCCCGCTGWACCKTCCATTGTASCACGKGTGTA
GCCCCGGACGTAAGGGCCATGATGACTTGACGTCGTC
CCCACCTTCCTCACTGTTTGCACAGGSAGTCTGTTTAK
AGTCCCCACCATAACATGCTGGCAACTAAACATARG
GGTTGCGCTCGTTGCSGGACTTARCCCAACACCTCAC
GGYMCSAGCTGACGACWGCCATGCASCACCTAGTTT
CSTGTCCCRAAGGACSGAWGCGTCYCTGCMTCCTTCA
STAACTTTCAMGSCCGGGYAAGGTTCSTCRCGKATCW
TCGAATTAAACCACATGMWCCTCCGCTTGYGCGGGC
CCGCGTCRATTCCTTTGAGTTTCATCCTTGCKGGCGTA
CTCCCCAGGWGGATCACTTARCGCTTTCKMTGGGAC
KCTGGCTGYCTATCGCCAACATCGAGTTATCATCGTT
TAKGGMGTGGACTACCRGGGTRTMTAATCCTGTTCK
ATCCCCACGCTTKCKYGCATCASYGTCAATACYAGCT
TAGTGAGCTGCCTTCGGWRATCSGSAGTTCTAMSACR

TAKCTAYGYATTWYAYCGCTACTTGTCYTAGTTCMC
GCCSACWTWATAYGSSAYTCAWGCSCGATCASTATC
AWACGGCACTGYKAKGRGTTGAKTCCCACCGKATRT
CACCCSSSTGAACTTTACAGSCARTCCGTACGCAACCC
CATCTAAGCGSCGACTYATATAWTACCSSGCGCAGTA

2) [NCBI NUCLEOTIDE BLAST SEQUENCE]

Fig 2: BLAST view of the *Bacillus sp.* alignment score

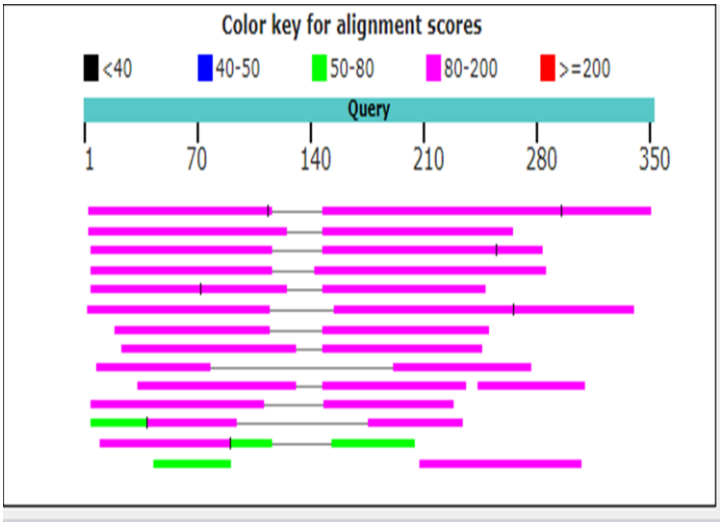
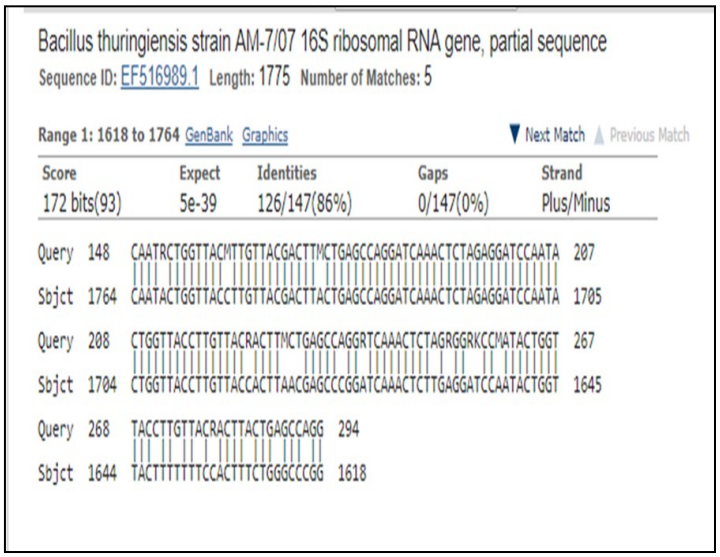


Fig 3: Bacillus thuringiensis strain AM-7/07's 16S ribosomal RNA gene has been found to be 86% identical, with a partial



sequence length of 1775 and a sequence id of "EF516989.1." There have been 5 matches to this query, which has the molecule type nucleic acid and a query length of 352.

[NCBI NUCLEOTIDE BLAST SEQUENCE]

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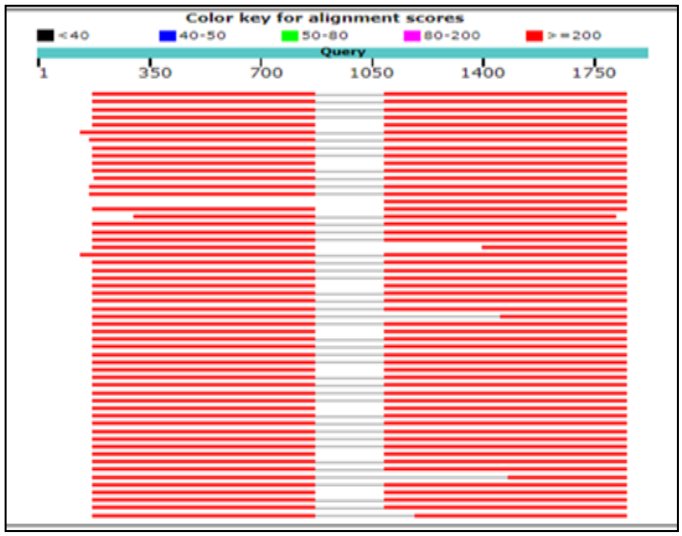

Fig 4: BLAST alignment score of Sphingobacterium sp.

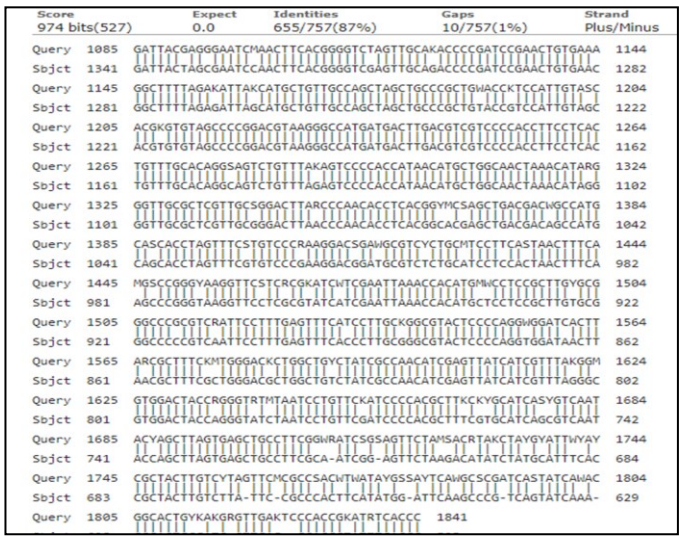

Fig 5: Partial sequencing of the 16S ribosomal RNA gene for the Sphingobacterium sp. strain of the LH-X. ID for sequence: MF062570.1, Query ID: lcl|Query_214771, Query Length: 1911, Program: BLASTN 2.7.0+, Length: 1497, Identical=87% The 16S ribosomal RNA gene of Sphingobacterium sp. strain LH-X has been identified as the first organism. There are two matches.

Isolation of the organism: Organisms were isolated as Sphingobacterium sp. and Bacillus sp.

Figure 6: Water Sample of Bacterial Laccase by streak plate method. The 16SrRNA can be good identification method.

Water 16S r RNA

[FORWORD]

ACTACAGMATTGGATCCTCTAGAGTTTGATCCTGGCT
CAGKAAGTCGTAACAAGGTAACCAGTATTGGATCCT
CTAGAGTTTGATCCTGGCTCAGKAARTCSTAACAAGG
TAACCASYWTTGGATMCTCTAGAG

[REVERSE]

CAAAGTCWGAGGWCAATRCTGGTTACMTTGTTACGA
CTTMCTGAGCCAGGATCAAACTCTAGAGGATCCAAT
ACTGGTTACCTTGTTACRACTTMCTGAGCCAGGRTCA
AACTCTAGRGGRKCCMATACTGGTTACCTTGTTACRA
CTTACTGAGCCAGGATCAAACTCTAGAGGKTCCYAT
WCTGGWTACCTTGWTACAACTTAWWGAGCCAGGA
WC

Figure 7: Soil Samples of Bacterial Laccase by streak plate method. The 16SrRNA can be good identification method.

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Soil 16S r RNA

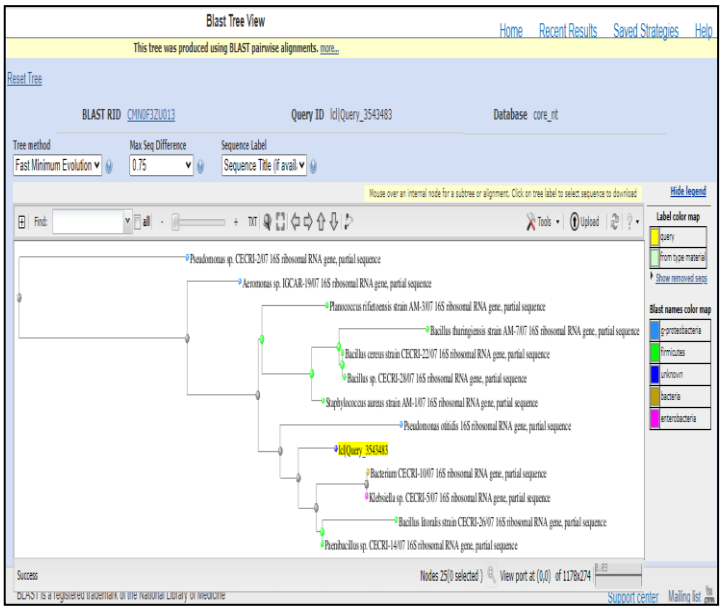
[FORWARD]

CGTATKWCCGCTATAGTTTGATCCTGGCTCASKAKAT
CKYGTCACAGTGKCCSCRYAGTGTGTTTTTGGGGCGC
GGGMTCTKATAAAAGGGGAGCCGGATAGAGTTTTTG
GGTCTCTCCCTCTAAMKYMTGKGTTTAACCCCGCATA
TTTTTTTTSAGKGGCATCMGTCCAAWATTAAATATTT
ATAGGAYGGAKATGGGCTCGCGTGACWTTAGCTAGT
TGGTAGGGTAACGGCTTACCMAGGCGACGATGTCTA
GGGGCTCTGAGAGGARAATCCCCCACACTGGTACTG
AGACACSGACCASACTCCTACGGGAGGCARCTTTAAG
GAATATTGGTCAATGGGCGGAASCCTGAACCAGCCAT
GCCGCGTGCAKGATGACTGCCCTATGGKTTGTAAACT
GCTTTTGTCCGGGAATAAACCTAMATACGTGTATTTA
GCTGAMTGTACCGGAAGAATAAGGATCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGARGATCCKAGC
GTTATCCRGATTTATTGGRTTTAAAGGGYGCGTAKGC
GGCCTGTTAAKTCRRGRGTGAAATACKGTGGCTCAAC
CMTCSMACTGCCTTTGATACTGACGGGCTTGAMTCC
ATATGAWGTGRGYGGAATAAYACRAGTARCGGTGA
WATGCATAGATATGTCTTASAACTCCGATTGCGAASG
CMKCTCACTAWACTGRTAYTGACGCTSATRCRCGWR
AGCRTGGGRGATCGAAYAGRTATTASAKACCCTGCGT
AGYCCWCGCCCATAAACGATKATMACTCGGATGTTS
GTCGATAKACAGCCAGCGCTCCCCWGCAGAMAGCRT
TAMGWTCATCCACCGTGGAGGAGTRCGCCCCGCGAK
GRRTGCATAACTACWGAAGGTAGTCGWCGASSGCCC
CCGCAGCAGGCGGCAAGGSAKGCATGATTGGGGTRT
TTAMYTATTMCG

[REVERSE]

CAAGAAAAAATCTAMGAGCTACATTGTGTWGGACTT
AYWGAGYSWGGGGCGCACTCTATAGMGAGMTTGTT
TGGMCTSATTTTGCGYGGGGCGGGCACTCTAGCGAC
GCGGTGTTGTTTTTMTGTGATTACGAGGGAATCMAA
CTTCACGGGGTCTAGTTGCAKACCCCGATCCGAACTG
TGAAAGGCTTTTAGAKATTAKCATGCTGTTGCCAGCT
AGCTGCCCGCTGWACCKTCCATTGTASCACGKGTGTA
GCCCCGGACGTAAGGGCCATGATGACTTGACGTCGTC
CCCACCTTCCTCACTGTTTGCACAGGSAGTCTGTTTAK
AGTCCCCACCATAACATGCTGGCAACTAAACATARG
GGTTGCGCTCGTTGCSGGACTTARCCCAACACCTCAC
GGYMCSAGCTGACGACWGCCATGCASCACCTAGTTT
CSTGTCCCRAAGGACSGAWGCGTCYCTGCMTCCTTCA
STAACTTTCAMGSCCGGGYAAGGTTCSTCRCGKATCW
TCGAATTAAACCACATGMWCCTCCGCTTGYGCGGGC
CCGCGTCRATTCCTTTGAGTTTCATCCTTGCKGGCGTA
CTCCCCAGGWGGATCACTTARCGCTTTCKMTGGGAC
KCTGGCTGYCTATCGCCAACATCGAGTTATCATCGTT
TAKGGMGTGGACTACCRGGGTRTMTAATCCTGTTCK
ATCCCCACGCTTKCKYGCATCASYGTCAATACYAGCT
TAGTGAGCTGCCTTCGGWRATCSGSAGTTCTAMSACR
TAKCTAYGYATTWYAYCGCTACTTGTCYTAGTTCMC
GCCSACWTWATAYGSSAYTCAWGCSCGATCASTATC
AWACGGCACTGYKAKGRGTTGAKTCCCACCGKATRT
CACCCSSSTGAACTTTACAGSCARTCCGTACGCAACCC
CATCTAAGCGSCGACTYATATAWTACCSSGCGCAGTA

Figure 8: Water Sample of NCBI nucleotide BLAST sequence the phylogeny tree can identify that *Bacillus sp.* and *Sphingobacterium sp.* are the bacteria samples.



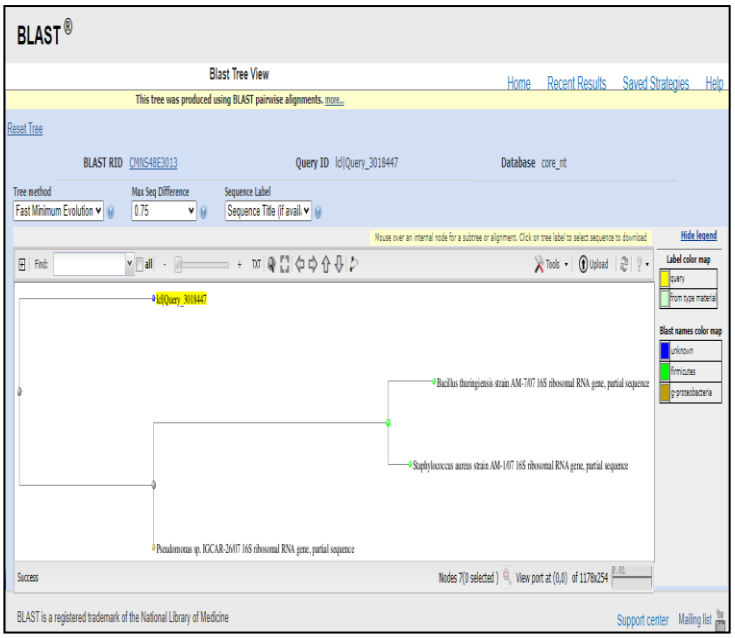


Figure 9: Soil Sample of NCBI nucleotide BLAST sequence the phylogeny tree can identify that Bacillus sp. and Sphingobacterium sp. are the bacteria samples.

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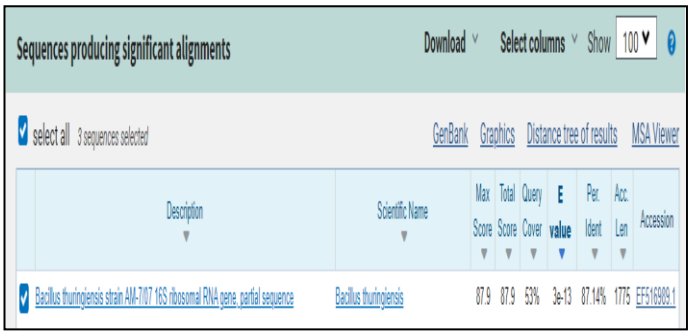

Fig: 10 The organisms were identified using their 16S ribosomal RNA genes from Sphingobacterium sp. strain LH-X and Bacillus thuringiensis strain AM-7/07, both of which had partial sequences (Sequence IDs: MF062570.1 and EF516989.1), lengths of 1497 and 1775, and numbers of matches (2 and 5). Comparable=87% and 86%. Total and maximum score 87.9 that has E value 3e-13.

Identification of the organism: Organisms were identified as Sphingobacterium sp. and Bacillus sp.

B. OPTIMIZATION Physiochemical Parameters are: pH, NaCl, Temperature, Carbon Source, Nitrogen Source, Copper Sulphate.

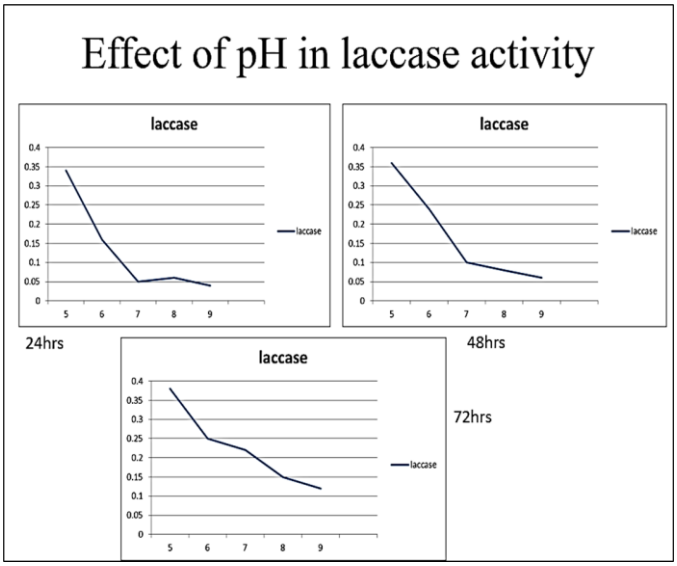

Fig 11: The laccase activity has been increased in different hours speculation in different sources formation. In 50 mM citrate/phosphate buffer (4.0–8.0) and 50 mM Tris/HCl buffer (8.0–9.5), the impact of pH on laccase activity was measured at 60 °C.

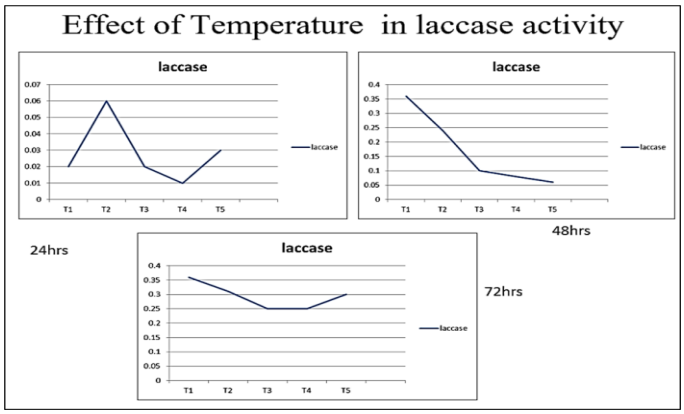

Fig 12: Temperature is very important in laccase activity because in every hour (T1-T5) it has been changed. By culturing each substrate of Laccase and in the temperature range of 4–80 °C, the influence of temperature was ascertained.

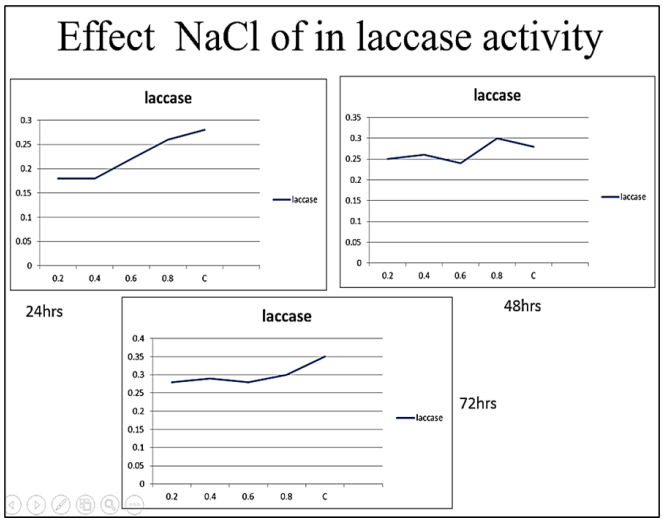

Fig 13: Different concentration of NaCl the laccase activities has been changes in different time.

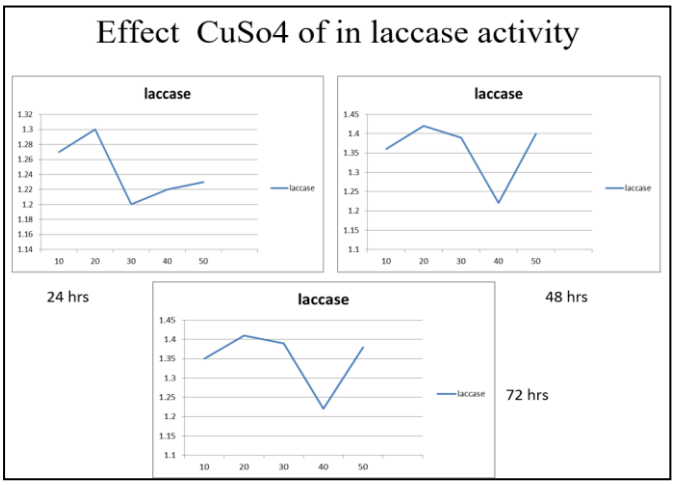

Fig 14: CuSo4 activity of laccase in different time speculate in 24,48,72 hrs in different concentration.

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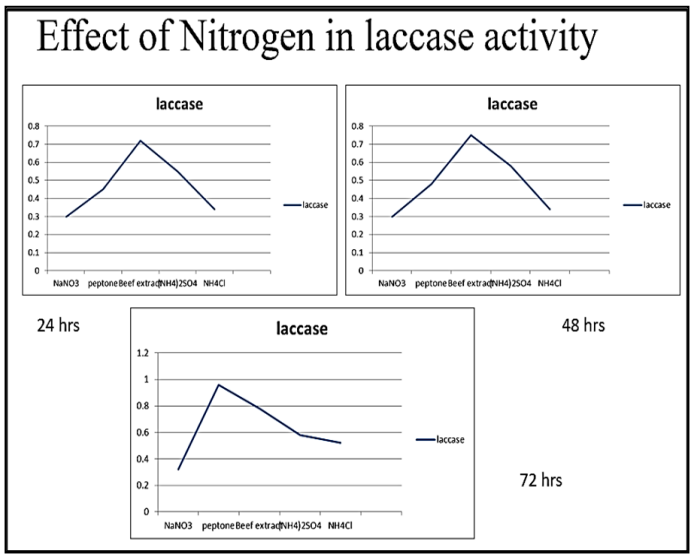

Fig 15: $NaNo_3$, Peptone, Beef extract, $(NH_4)_2SO_4$, NH_4Cl in all the Nitrogen source there are the different Laccase activity has been seen. Synthesis of laccase employing various organisms that operate in nitrogen-sufficient and nitrogen-deficient environments. The results showed a greater level of laccase synthesis (1.51 × 105 U/g of dry substrate).

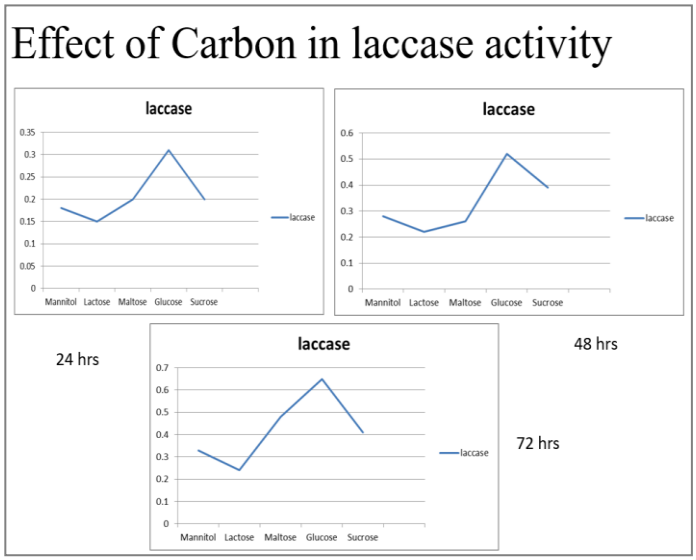

Fig16: Mannitol, Lactose, Maltose, Glucose, Sucrose in all the carbon source there are the different Laccase activity we have seen. The co-culture process's carbon source succession essentially enables the laccase enzyme to excessively produce.

FURTHER STUDY

In further study we have to do marine environment Bacterial Laccase Purification and the Characterization process and for that in the Purification process to achieve 90% saturation, ammonium sulphate was gradually added to the crude enzyme while being stirred gently. After being stored at 4°C for 30 hours, the mixture was centrifuged for 10 minutes at 4°C at 10,000 rpm. The resulting pellet was dissolved in a small amount of pH 7.5 sodium phosphate buffer (0.1 M), and the laccase activity was measured. Dialyzing the protein solution against sodium phosphate buffer (0.01 M, pH 7.5) took 12 hours, with a 6-hour buffer change in between and for Characterization we have to do the purified enzyme's UV–vis absorption spectrum in sodium acetate buffer was evaluated using a UV–vis spectrophotometer in a UV quartz glass cell with a 1 cm path length at ambient temperature (27 °C).

CONCLUSION

In my work done at the Genetic Engineering Lab of SRM Institute of Science and Technology, I have found that after Isolation (16S r RNA Sequencing) process and (NCBI BLAST Sequencing) we see the similarity. Now we can Identify that there are two types of organism: - 1) Sphingobacterium sp. 2) Bacillus sp. and also Optimization the Physiochemical Parameters are pH, Temperature, NaCl, $CuSo_4$, Nitrogen, Carbon of Bacterial Laccase from Marine Environment important for Laccase Purification and characterization.

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