JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Isolation and Identification of Bioremediating Enzyme Laccase producing bacteria from soil samples

Rayudu Venkata Ramadevi[1], IfraErum Mohammad[1], R. VenkataBhargavi[1], Krishna Satya Alapati[1]*

[1]DepartmentofBiotechnology, Acharya Nagarjuna University ,Guntur522510, Andhra Pradesh, India

[2]Rajiv Gandhi University of Knowledge Technologies (RGU IIIT), Nuzvid 521202, AndhraPradesh,India

ABSTRACT:

There is currently an urgent need to reverse the pollution of global water bodies caused by humans. Despite the diversity of pollutants discharged into water bodies, such as plastics, herbicides, fertilizers, synthetic dyes and pharmaceuticals, laccases appear to be an efficient biocatalytic tool with the ability to oxidize these molecules, yielding less toxic and harmful inactive compounds. They act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants which help researchers to put them in various biotechnological applications. They can be effectively used in paper and pulp industries, textile industries, xenobiotic degradation as bioremediation agents and can also act as biosensor. Due to the increase in the usage of this enzyme in various fields of industries ,it is gaining more attention by many industries. To meet the demand of the industry's requirement it is necessary to increase the yield as well as to discover new strains which can produce higher titers of this enzyme. In the present study high yielding bacterial strain was isolated from soil samples. The isolated bacteria were identified as *Pseudomonas alcaliphila* by both biochemical and molecular methods.

KeyWords: Laccase, Bioremediation, lignin related compounds, *Pseudomonas alcaliphila*

1.Introduction

Globally increasing urbanization and industrial activities have distorted ecosystems and had an adverse effect on both human and animal health by producing and incorporating foreign pollutant compounds (1). Strategies have

been used to remove these toxins from water bodies under anthropogenic stress using physical, chemical, and biological methods (2). In particular, laccases, which are broad spectrum biocatalysts, have been utilized to degrade a number of chemicals, including those that can be found in the effluents from businesses and hospitals (3).

Unfortunately, urbanization and industrialization have seriously contaminated water sources, harming ecosystems in the process (4-5). To counteract the effects of global pollution, biotechnologists around the world are investigating and creating cutting-edge technologies and non-polluting procedures (6-7).This is difficult, though, because of the volume and variety of pollutants that are dumped into bodies of water , including plastics, herbicides, fertilizers, synthetic dyes, polycyclic aromatic hydrocarbons (PAHs), chlorinated paraffin phthalates, and others, such as the so-called emerging pollutants, which could include pharmaceuticals (such as painkillers, antibiotics, hormones, and endocrine disruptors), plasticizers, and substances found in self-care products (8-9). To retain or change these molecules into less damaging ones, a variety of therapeutic modalities have been investigated, ranging from physical and chemical techniques to biotechnological tactics (such as the use of enzymes) (10) .Water body contamination is a technical, social, and environmental concern caused by ongoing population growth, insufficient waste elimination methods, and subpar public management of water contaminants (11-13). In reality, a wide range of compounds from domestic and industrial sources are released into water bodies without being regulated, including new contaminants that may have an impact on the environment and human health (14).

Biotechnological procedures incorporating microorganisms and enzymes have been used for this purpose (15). When employing free enzymes or crude enzyme extracts to biotransform a variety of contaminants, laccases have demonstrated significant promise (16).The discovery, investigation, and use of laccase-mediated processes constitute an intensive research topic with the aim of producing ecofriendly and efficient instruments for treating and enhancing water quality. This is necessary to remove pollutants from contaminated water (17). The enzyme family of multi-copper oxidases (MCOs) includes laccases, which are also referred to as urushiol oxidases and p-diphenol oxidases (18). Laccases are classed as benzenediol oxygen reductases (EC 1.10.3.2). Due to their low substrate specificity, use of oxygen as an electron acceptor, and ability to produce water as a byproduct, they are regarded as versatile enzymes capable of oxidizing a wide range of phenolic and non-phenolic compounds (19).

Laccases are widely expressed in nature and may be found in a variety of fungi, plants, bacteria, lichen, and insects. The laccases from each species have unique catalytic properties and sequences, and they are found in abundance in nature (20). About 7300 cellular-organism sources were found in the UniProtKB search results for "laccase" with sequence length between 220 and 800 amino acids, including 1026 bacteria, 6258 eukaryotes, and 16 halo bacteria (archaea)(21).

Based on current demand, there is a greater necessity to find a new microorganism with the capability to produce a higher titer of Laccase that has bioremediating properties. Based on this background, in this current study, various bacteria were isolated from the soil samples that have Laccase production capacity and primary screening studies were conducted.

2.0. Materials and Methods

2.1. Sample Collection:

Soil samples were collected from a depth of 10-15 cm below the earth's surface, from the root nodule area of *Clitoria ternatea* present at the herbal garden of Acharya Nagarjuna University Campus, Guntur, Andhra Pradesh, India. The collected samples were stored in an air-tight polyethylene bag and maintained at 4ºC until used (22).

2.2. Isolation of Laccase-Producing Bacteria and Preparation of Culture media:

Isolation was based on serial dilution technique (23). Initially 1.0 gm of collected soil sample was suspended in 100 ml of sterile distilled water and mixed well for an hour at room temperature. From this solution, 1.0 ml of the sample was added to a tube containing 9 ml of sterile water (10^{-1}) and mixed vigorously for 30 s. Dilution was repeated till 10^{-7} and 0.1 ml of suspension was spread from each dilution tube on nutrient agar with 0.1% guaiacol and 0.005% cycloheximide and incubated at 37^0C for three days. Cycloheximide was added as antifungal agent to prevent its growth (24). The colonies showing reddish brown color were selected. The positive colonies from all the samples were sub-cultured for three times to get pure colony. The isolated bacterium was streaked on the same medium namely nutrient agar with 0.1% guaiacol to confirm the positive activity. The morphological, physiological, and biochemical characteristics of laccase producing strains were examined. Isolated bacteria cultures were maintained on nutrient agar and stored at 4°C for future studies.

2.3. Biochemical Characterization of Laccase Producing Bacterial Isolates:

Biochemical tests were performed (Catalase, Oxidase, Indole tests) and carbohydrate utilization tests (Glucose,

adonitol, lactose, arabinose, and sorbitol). The fermentation tests were performed to study acid and gas production in synthetic broth containing arabinose, dextrose, lactose, by inoculating bacterial isolate with their respective control in triplicates.

2.4. Colony morphology:

The bacteria have the characteristic feature of growing as colonies on solid media, and different bacterial types will show different colony morphology. The Color, elevation, shape, surface, type of margin, and the pigmentation of bacteria were noticed by culturing the bacterial strains on nutrient agar media.

2.5. Gram-staining:

Gram-staining is one type of differential staining method which is named after Dr. Christian Gram and requires minimum of three chemical reagents to be applied to heat-fixed bacterial smear.

Principle: The bacterial smear was stained with the primary stain crystal violet. After adding the primary strain, a mordant is added, which is an iodine solution that acts as a mordant, which causes an increase in the interaction between bacterial strain and the dye. The smear is then washed with 95% ethanol which acts as a decolorizer. Gram- positive bacteria retain the crystal violet-iodine complex, whereas a Gram-negative bacterium loses its crystal violet-iodine complex and becomes colorless. Finally, the smear will be counterstained with safranin which stains the Gram-negative bacteria, appearing in pink color. Still, it does not alter the purple color of the Gram-positive bacteria.

Procedure: The glass slides were washed thoroughly and rinsed with 95% alcohol. After air-drying the slide, a drop of the culture was transferred onto the slide using an inoculating loop. A smear was prepared and fixed by rapidly passing the slide upon the flame (film-side up). On separate slides, smears of five different strains were prepared using the procedures described earlier.

The bacterial smears were then stained with primary basic dye crystal violet and allowed to air dry for one minute. The slides were then rinsed gently with double distilled water. To this slide, the Gram's iodine solution was added and incubated for 1 minute, followed by washing the solution with water. Drop by drop, 95% alcohol was then added until all excess color was rinsed away.

The slides having bacterial smears were rinsed with double distilled water, air-dried, flooded with the counterstain safranin, and left aside for 20-30 seconds. The rinsing with distilled water step was repeated, slides were air-dried. The slides were examined at 100X using the microscope's oil immersion lens (Beveridge TJ 2001).

2.6.Shape:

The bacteria usually exhibit and adapt to three basic shapes: rod, round, or spiral- shaped. The rod-shaped bacteria are termed bacilli, and the round-shaped bacteria are termed cocci. The shape of the bacterial cells is of prime importance in identifying and classifying bacteria. The shape of sub-cultured bacteria was examined microscopically.

2.7. Motility test:

Principle: The motility of bacteria can be evaluated and is directly related to the presence of bacterial flagella. The 'hanging drop' technique is widely used to identify the motility of live bacteria in hanging drops.

Procedure: In the present study, the motility test agar technique is used to test the motility of the bacterial strains. This process of semisolid growth medium allows bacteria to move through the medium. After inoculation into the media, the test tubes were incubated at the temperature 37°C for 24-48 hrs. Motile bacteria spread out from the inoculation zone and established a broad growth zone in various patterns. Non-motile on the other hand, showed no such mobility patterns.

2.8.Endospore test:

Usually, Bacillus and Clostridium genera bacteria develop a resistant structure that allows them to survive for lengthy periods of time in an adverse environment. Because it is developed within the bacterial cell, this arrangement is called an endospore. Endospores are spheroidal to elliptical and can be either shorter or more significant than the parent bacterial cell.

It's challenging to stain endospores, but they strongly withstand decolorization once stained. The bacteria cells were stained with malachite green after the induction of an unfavorable survival environment to test the capabilities of endospore formation. Stain penetration can be achieved by using heat. The rest of the cell is then decolorized and counterstained using safranin.

Procedure: On a glass slide, a bacterial culture smear was made and then covered with blotting paper to fit into the slide. Using malachite green stain solution, the blotting paper was saturated and then exposed to steam for five minutes; more dye was added as and when required to keep the blotting paper moist. Under tap water the slide was then washed gently. Counterstain-safranin was added and incubated for 30 seconds, and then washed with tap water and blotting paper was removed. The slide was examined for the detection of the presence of endospores

using the oil immersion lens. Endospores appear bright green, while vegetative cells exhibit brownish red to pink color.

2.9. Indole test:

Principle: Indole, pyruvic acid, and ammonia are the metabolic end products of the bacterial metabolism of tryptophan. This metabolic process is due to the presence of the enzyme tryptophanase. The bacteria can use ammonia and pyruvic acid for their metabolism. Indole is not used or processed further, and it accumulates as an extracellular metabolite in the culture medium. The production of indole is identified using Ehrlich's or Kovac's reagents. Then the gentle agitation causes the dark red color formation in the reagent layer which indicates a positive test for the presence of indole (*Darkoh C et al., 2015*).

Procedure: The bacterial strains were sub-cultured in peptone water containing amino acid tryptophan and incubated at the temperature of 37°C for 24 hours. After incubation a few drops of Kovac's reagent were added to the bacterial strain. Kovac's reagent contains para-dimethyl amino benzaldehyde, isoamyl alcohol, and concentrated HCl. The color change was observed after 24 hours of incubation.

2.10. Methyl red test:

Principle: Methyl red test detects the bacteria's ability for stable acid end products production from glucose metabolism. The pH indicator Methyl Red exhibits red color at a pH of 4.4 or less.

Procedure:

The bacterial strains were inoculated into glucose phosphate broth and incubated at the temperature of 37°C for 48 hours. After incubation, the mixed-acid-producing bacteria produce enough acid to withstand the phosphate buffer and continue to remain acidic. The medium's pH was assessed by adding five drops of M.R. reagent. The appearance of the red color was an indication of positive results. The yellow color was observed for MR-negative organisms.

2.11. Citrate utilization test:

Principle: This test is employed to test the bacteria's ability to utilize citrate as the only source of carbon for metabolism and energy. Citrate utilization is due to the enzyme citrate lyase, which catalyzes the citrate to Oxaloacetate and acetate, and Oxaloacetate is processed further into pyruvate and carbon-di-oxide. The ammonium and the sodium citrate salt in the culture medium result in ammonia and Sodium carbonate production by bacterial strains. It influences a change in the color of the medium from green to blue due to the development

of alkaline pH.

Procedure: Simmons's citrate agar slants were prepared and inoculated with the bacterial test culture. The bacterial culture inoculated slants were incubated for 24 hours at 37°C. If citrate is utilized by the bacteria, the medium color is changed from green to blue (Jeffrey C. Pommerville 2010).

2.12. Catalase test:

Principle: The bacteria and other microorganisms developed a defense mechanism to escape oxidative damage due to hydrogen peroxide (H_2O_2). Few bacteria can produce catalase enzymes, which neutralizes the H_2O_2 and promotes cellular detoxification. Catalase exerts its function by catalyzing the H_2O_2 into water and oxygen. The rapid formation of bubbles confirms this. The reaction process can be represented as follows:

$2H_2O_2$ + Catalase → $2H_2O_2$ + O_2

Procedure: The bacterial strains were sub-cultured, and after 24 hours, a small fraction of bacterial cells was transferred to the slides. Using Pasteur's pipette, one drop of 3% H_2O_2 was added to the slide containing bacterial culture, and the Petri plate was covered immediately with a lid and then observed for the formation of bubbles.

2.13. Oxidase Test:

To find out whether our isolate can perform the electron transport chain was investigated with oxidase test. In general, the last electron receiver for the system was oxygen. In this test, in the place of oxygen, N, N', N'-tetramethylphenylenediaminedihydrochloride an artificial final electron receiver or acceptor was used. Electron transport chain, when the last element of cytochrome oxidase transfers electron to this electron receiver or acceptor, changes its color to dark blue or purple. Overnight culture from agar Petri plate was taken with the help of sterile swab. The oxide test reagent of 1 drop should be placed on to the culture on the swab. If the bacteria change its color from violet to purple immediately or within 10 to 30sec, is a sign of positive reaction. Time lapse reactions should be ignored.

2.14. Carbohydrate fermentation:

All the sub-cultured isolates were inoculated in the nutrient broth of p^H7 with 1% carbohydrate, 0.1%Andrade's indicator in Durham's tubes. 1% of carbohydrate used in present wok include, arabinose, cellobiose, dextrose, galactose, lactose, mannitol, maltose, melibiose, mannose, ribose, Salicin, sorbitol, trehalose, xylose. Tubes were incubated at 37^0C at the anaerobic condition for 24hours. After incubation time bubbles appear in the Durham's

tube indicates the production of gas and colorless to pink/red indicates fermentation.

Carbohydrate fermentation protocol: (Glucose utilization test)

To determine the acid production of our isolates, 18-24hrs or overnight culture of isolate is about 2 or 3 loopful was added to the broth containing glucose or fructose or sucrose or any other carbohydrate used in the study and at 30ºC for 24-48 hrs. Tubes were incubated. After incubation period in Durham's tube if the gas was collected indicates positive result for gas formation, and color change to yellow color records as a positive means acid production. No gas formation and no color change record as no acid formation i.e., negative.

2.15. Screening of suitable media for Laccase production:

Composition:

S No	Chemicals	Quantity
01	Peptone	10gms
02	Beef extract	10gms
03	Sodium chloride	5gms
04	pH after sterilization	7.3±0.1

Table 01 Composition of media

2.16. Isolation of DNA and Molecular characterization:

The DNA from the isolates was isolated using Marmur's technique (1961). 100 mL of bacterial cultures were grown in suitable media and collected after 10 minutes at 6000 rpm. The cell pellet was centrifuged and rinsed with 0.9 percent NaCl before being suspended in 50 mL STE buffer. Cells were lysed with bacterial lysozyme (10 g/mL) and incubated for 1 hour at 37 °C before being treated with 10% SDS and incubated for 10 minutes at 55 °C. After lysozyme treatment, 15 mL of 5M NaCl and 75 mL of chloroform: iso-amyl alcohol (24:1) was added, and the flasks were gently mixed before centrifuging at 6000 rpm for 15 minutes at 4 °C. The top aqueous phase was recovered, and 7.5 mL of 20% sodium acetate and 60 mL isopropanol were added, followed by 2 minutes of centrifugation at 6000 rpm at 4 °C. The resulting DNA pellet was washed with 70% Ethanol; air dried and resuspended in a small volume of TE buffer for PCR amplification investigations. The DNA's purity was tested using 0.8 percent agarose gel electrophoresis.

STE buffer

Tris-HCl	0.1M
Sodium chloride	0.1M
Ethylene diamine tetra acetic acid	1.0M
pH	8.5±0.2

TE buffer

Tris-HCl	10.0mM
Ethylene diamine tetra acetic acid	1.0mM
pH	8.0±0.2

Amplification of DNA:

To amplify the extracted DNA, the polymerase chain reaction was used. Denaturation at 94 °C, annealing at 54 °C, and extension at 72 °C were all repeated for 30-40 cycles. The reagents listed below were added to a PCR tube set up for a 50 L reaction. 10X buffer (5 L), dNTPs (1 L), each primer (forward and reverse) (2 L), template (4 L), Taq polymerase (1 L), and 35 L doubly distilled water. Universal primers 8F-5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'GGTTACCTTG TTACGACTT-3' were used. By following the procedure, the amplified products were run on a 1% agarose gel.

16s rRNA sequencing:

The PCR products were purified using a PCR purification spin kit as directed by the manufacturer (HiMedia, Mumbai, India). The purified PCR products were then immediately sequenced using an automated sequencer (ABI 3730xl Genetic Analyzer).

Phylogenetic analysis:

Clustal W software (24) was used to organize the acquired sequences. To put the isolated strain phylogenetically, its sequences were compared with the available sequence databases using the NCBI web portal's BLAST search. Phylip 3.6 is used to build trees.

2.17. Laccase enzyme production under Submerged Fermentation:

Laccase production was performed in a 500ml Erlenmeyer flask under shaking at 100rpm. The inoculum was prepared by picking the colonies and inoculating it in 250mL Luria-Bertani broth supplemented with 1M Copper Sulfate and incubated at 37°C under shaking conditions. Under optimal conditions, the batch culture was carried

out to produce Laccases in 500ml flasks containing 250mL of culture medium inoculated with seed culture at 37°C on shaker incubator for 24 hrs. After the incubation period, the cells were lysed and subjected to centrifugation at 8000 rpm at 4°C for 20min. The supernatant obtained was labeled as crude enzyme extract and used for further enzyme purification.

3.0 RESULTS AND DISCUSSION

3.1. Isolation of Laccase-Producing Bacteria:

Fresh soil samples were collected from various areas of root nodule regions of Clitoria *ternatea* present at the herbal garden of Acharya Nagarjuna University Campus, Guntur, Andhra Pradesh, India. The samples were serially diluted and plated on Nutrient Agar plates enriched with Cycloheximide (25) and Guaiacol for bacterial cultivation. Morphologically different colonies were identified initially. The reddish-brown colonies were identified. The positive colonies from all the samples were sub-cultured for three times to get pure colony. The isolated bacterium was streaked on the same medium namely nutrient agar with 0.1% guaiacol to confirm the positive activity and stored for further use.

Fig 01 – Bacterial isolate on Nutrient Agar media

3.2. Growth, Motility & Gram staining

The growth of the isolate was seen in Nutrient Agar medium. At first the culture was isolated from Nutrient Agar (NA) medium plates. Motility of the bacteria is present hence they are positive to motility test. The isolate took

the pink stain in the gram staining technique and hence tested Gram –ve.

Isolate	Motility	Gram Stain
Result	+ve	-ve

Table 02 Motility and Staining Technique

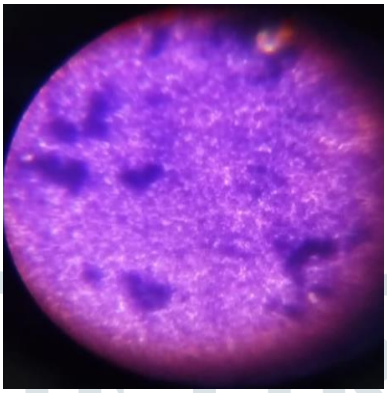

Fig 02- Gram's Staining

3.3. Indole Test

The bacterial isolate was grown on tryptone broth and Kovac's reagent was added after incubation at 37^0C for 24 hrs. The Cherry-red color was not observed as a layer in the broth due to lack of indole production. Hence it is considered as negative to Indole test.

Fig – 03 No color change due to lack of indole production

3.4. Catalase test

The isolated strain was grown on trypticase soy agar slants and incubated for 24hrs at 37^0C. When the drops of

hydrogen peroxide were added the bubbles were formed which indicated positive to the catalase test.

3.5. Oxidase Test

When a droplet of oxidase test reagent was added to the culture swab the color changed to purple immediately which indicated positive to oxidase test.

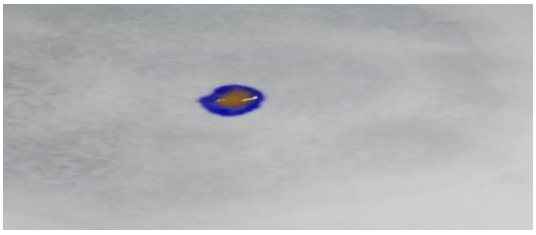

Fig-04 Change of color indicates the positive result.

S No	Name of the Test	Result
01	Oxidase Test	+ve
02	Catalase Test	+ve

Table 03 Respiration Tests

3.6. Carbohydrate fermentation test

The isolated bacterial strain was cultured on different media that differ in the carbohydrate source. 3 different sources were used – Glucose, Sucrose and Mannitol in which the Durham's tubes with phenol red indicator were inverted. After the inoculation with the selected strain the tubes were incubated for 24 hrs and the absence of change in the color indicated negative for carbohydrate fermentation test.

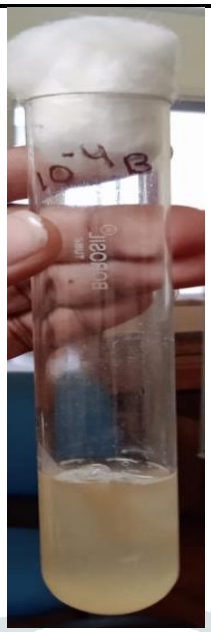

Fig – 05 Absence of color change indicates negative result.

3.7. Molecular Identification

The sample was found to be ***Pseudomonas acaliphila,*** and showed high similarity based on nucleotide homology and phylogenetic analysis.

gDNA 16SrRNA amplicon

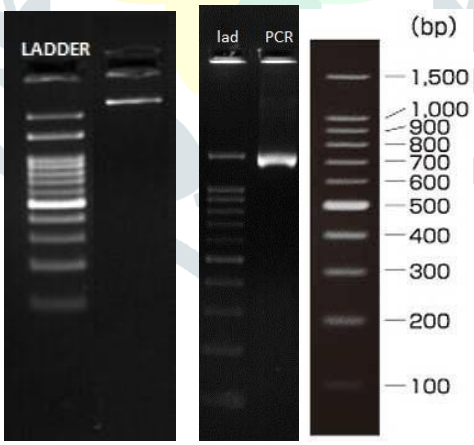

Fig 06gDNA and 16SrRNA Amplicon QC data

Microbial Identification using 16S rRNA based method.

Sanger Seq Chromatogram data file Data:

>Forward Seq data

CCGTTGGGTTCCTTGAGAACTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAG

GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA

AGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAG

GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCC

TTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGC
CGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCG

GAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA

CCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCTTCGGGGGGACGGTTACCACGGAGTGATTCATG
ACTGGGGTGAAGTCGT

>Reverse Seq Data

GTTCCCAGGCGGTCACTTAATGCGTTAGCTGCGCCACTAAGTTCTCAAGGAACCCAACGGCTAGTTGACATCGT

TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCC

AGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCC

TCTACCGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCACATCCAACTTAAC

GAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGG

CACGAAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACACTAACGTATTAGGTTAATGCCCTTCCTCCCA

ACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTC

CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAG

ACCAGTTACGGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAG
CGCAAGGCCCGAAGGTCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTTCCTTTCGGAACGTTATCC
CCCACTACCAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTAAATCAAGGAGCAAGCTCCTCTCATCCG
CTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCAGA

>Reverse complement

TCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAGAGGAGCTTGCTCCTT

GATTTAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCCGAAAGGAAC

GCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTC

GGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTC

ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAA

GCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGG

CATTAACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG

GTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGG

ATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTG

GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACT
GATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC

GATGTCAACTAGCCGTTGGGTTCCTTGAGAACTTAGTGGCGCAGCTAACGCATTAAGTGACCGCCTGGGAAC

> Consensus data

CTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAGAGGAGCTTGCTCCTTGATTTA

GCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCCGAAAGGAACGCTAAT

ACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATT

AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACT

GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG

ATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTA

ACCTAATACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT

ACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTG

AAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATT

TCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATAC
TGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT
CAACTAGCCGTTGGGTTCCTTGAGAACTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGG

CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC

AACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCA

GACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACC

CTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTG

GGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTT

GCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGT

GAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCC

CGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCTTCGGGGGGACGGTTACCACGGAGTG
ATTCATGACTGGGGTGAAGTCGT

Table 04 Sequences producing significant alignments:

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Pseudomonas alcaliphila strain K5	2717	2717	100%	0.0	100.00%	KY928092.1
Pseudomonas sp. strain JXJ CY 57	2712	2712	100%	0.0	99.93%	MZ708739.1
Pseudomonas mendocina strain S178S	2712	2712	100%	0.0	99.93%	JF513150.1
Pseudomonas alcaliphila strain D15	2710	2710	99%	0.0	99.93%	ON088982.1
Pseudomonas chengduensis strain PMB32	2708	2708	100%	0.0	99.86%	KU378105.1
Pseudomonas sp. strain BAB-6007	2708	2708	99%	0.0	100.00%	KX609725.1
Bacterium strain PAH11	2706	2706	100%	0.0	99.86%	MF278994.1
Pseudomonas alcaliphila strain SpW03	2706	2706	100%	0.0	99.86%	OP810792.1
Uncultured bacterium clone FMSB2	2706	2706	100%	0.0	99.86%	KF975545.1
Pseudomonas chengduensis strain MBR	2706	2706	100%	0.0	99.86%	NR_125523.1

Evolutionary analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (26). The tree with the highest log likelihood (-2098.29) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms (27) to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1473 positions in the final dataset. Evolutionary analyses were conducted in MEGA11(28).

Phylogenetic Tree:

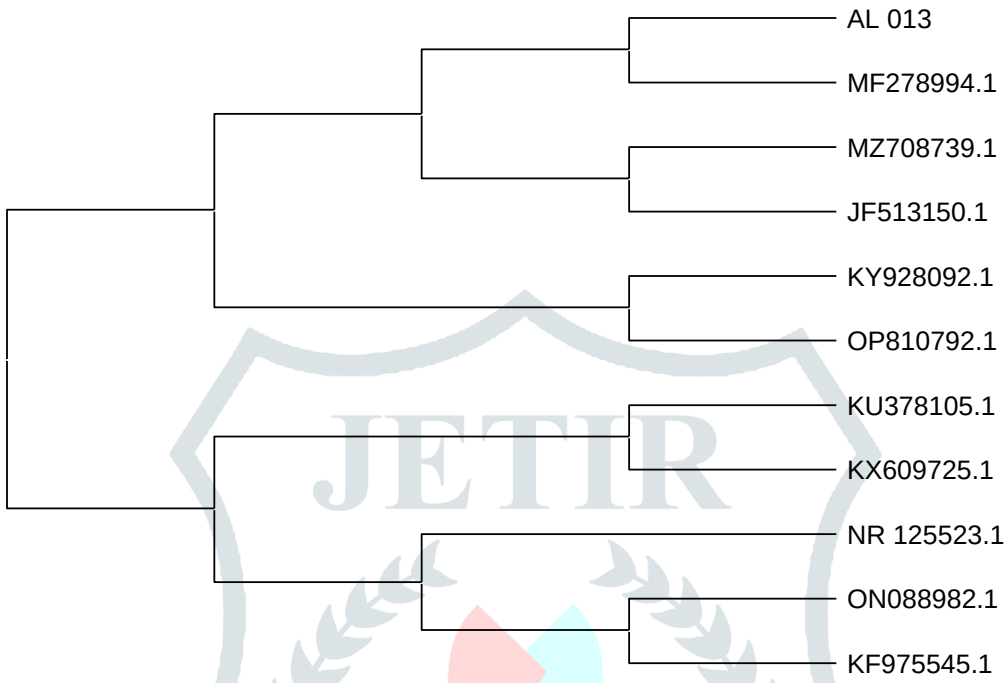

Fig 07 Evolutionary analysis by Maximum Likelihood method

Table 05 Distance Matrix:

AL_013		0.000	0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.001
KY928092.1	0.000		0.001	0.001	0.001	0.000	0.000	0.001	0.001	0.001	0.001
MZ708739.1	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
JF513150.1	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001	0.001
ON088982.1	0.001	0.001	0.001	0.001		0.001	0.001	0.001	0.001	0.001	0.001
KU378105.1	0.000	0.000	0.001	0.001	0.001		0.000	0.001	0.001	0.001	0.001
KX609725.1	0.000	0.000	0.001	0.001	0.001	0.000		0.001	0.001	0.001	0.001
MF278994.1	0.001	0.001	0.002	0.002	0.002	0.001	0.001		0.001	0.001	0.001
OP810792.1	0.001	0.001	0.002	0.002	0.002	0.001	0.001	0.003		0.001	0.001

KF975545.1	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.003	0.003		0.001
NR_125523.1	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.003	0.003	0.001	

4.0 CONCLUSION

The present study depicts the efficient method for the isolation of Bioremediating enzyme Laccase-producing microorganisms from soil samples. By following the plate method and broth studies one bacteria was isolated and it was characterized by morphological, biochemical, and molecular methods. Based on identification studies the isolate was recognized as ***Pseudomonas alcaliphila***. The enzyme production studies were carried out in submerged fermentation. This study signifies the importance of proper isolation methods for the selective screening of microbes for enzyme production without altering the genetic material.

ACKNOWLEDGMENTS

The author Rayudu Venkata Ramadevi is thankful to the Head, Department of Biotechnology, Acharya Nagarjuna University, Guntur, A.P, India for allowing to use the departmental facilities which were acquired previously out of the grants from various projects.

REFERENCES:

1. United Nations (2017). World Urbanization Prospects. https://www.unfpa.org/urbanization
.
2. N. Abdullah *et al.*Recent trends of heavy metal removal from water/wastewater by membrane technologiesJ. Ind. Eng. Chem.(2019)

3. Daughton CG. Non-regulated water contaminants: emerging research. Environ Impact Assess Rev. 2004;24:711–32. https://doi.org/10.1016/j.eiar.2004.06.003.

4. Afroz, R., Rahman, A., and Rahman, A. (2017). Health Impact of River Water Pollution in Malaysia. *Int. J. Adv. Appl. Sci.* 4 (5), 78–85. doi:10.21833/ijaas.2017.05.014

5. Ahmed, S., and Ismail, S. (2018). Water Pollution and its Sources, Effects and Management: a Case Study of Delhi. *Int. J. Curr. Adv. Res.* 7 (2), 10436–10442. doi:10.24327/ijcar.2018.10442.1768

6. R. Avtar, S. Tripathi, A.K. Aggarwal, P. Kumar Population–urbanization–energy nexus: a reviewResources, 8 (2019), p. 136

7. IISD International institute for sustainable development: world population to reach 9.9 billion by 2050 https://sdg.iisd.org/news/world-population-to-reach-9-9-billion-by-2050/ (2020)

8. Alcalde M. Laccases: biological functions, molecular structure and industrial applications. In: Polaina J, MacCabe A, editors. Industrial enzymes: structure, function and applications. Dordrecht: Springer; 2007. p. 461–76. ISBN 978-1-4020-5377-1.

9. Baldrian P. Fungal laccases–occurrence and properties. FEMS Microbiol Rev. 2006;30:215–42. https://doi.org/10.1111/j.1574-4976.2005.00010.x.

10. Tjhung K.F., Shokhirev M.N., Horning D.P., Joyce G.F. An RNA polymerase ribozyme that synthesizes its own ancestor. Proc. Natl. Acad. Sci. USA. 2020;117:2906–2913. doi: 10.1073/pnas.1914282117.

11. Jensen, P. K., Jayasinghe, G., Hoek, W., Cairncross, S., and Dalsgaard, A. (2004). Is There an Association between Bacteriological Drinking Water Quality and Childhood Diarrhoea in Developing Countries? *Trop. Med. Int. Health* 9 (11), 1210–1215. doi:10.1111/j.1365-3156.2004.01329.x

12. Kochhar, N., Gill, G. S., Tuli, N., Dadwal, V., and Balaram, V. (2007). Chemical Quality of Ground Water in Relation to Incidence of Cancer in Parts of SW Punjab, India. *Asian J. Water, Environ. Pollut.* 4 (2), 107 doi:10.1086/114154

13. Lu, Y., Song, S., Wang, R., Liu, Z., Meng, J., Sweetman, A. J., et al. (2015). Impacts of Soil and Water Pollution on Food Safety and Health Risks in China. *Environ. Int.* 77, 5–15. doi:10.1016/j.envint.2014.12.010

14. A.M. Botero-Coy, D. Martínez-Pachón, C. Boix, R.J. Rincón, N. Castillo, L.P. Arias-Marín, L. Manrique-Losada, R. Torres Palmae, A. Moncayo-Lasso, F. Hernández
An investigation into the occurrence and removal of pharmaceuticals in Colombian wastewater

15. Adrio J.L., Demain A.L. Microbial Cells and Enzymes—A Century of Progress. In: Barredo J.L., editor. Methods in Biotechnology. Microbial Enzymes and Biotransformations. Volume 17. Humana Press; Totowa, NJ, USA: 2005. pp. 1–27.

16. Debaste F., Songulashvili G., Penninckx M. J. (2014). The potential of *Cerrena unicolor* laccase immobilized on mesoporous silica beads for removal of organic micropollutants in wastewaters. Desalin. Water Treat.52, 2344–2347. 10.1080/19443994.2013.877851

17. Chatha SAS, Asgher M, Iqbal HMN. Enzyme-based solutions for textile processing and dye contaminant biodegradation—a review. Environ SciPollut Res Int. 2017;24:14005–18. https://doi.org/10.1007/s11356-017-8998-1.

18. Agrawal K., Chaturvedi V., Verma P. Fungal laccase discovered but yet undiscovered. Bioresour. Bioprocess. 2018;5:4.

19. Bourbonnais R., Paice M.G. Oxidation of non-phenolic substrates, an expanded role for laccase in lignin biodegradation. FEBS Lett. 1990;267:99–102.

20. Dittmer, N. T., and Kanost, M. R. (2010). Insect multicopper oxidases: diversity, properties, and physiological roles. *Insect Biochem. Mol. Biol.* 40, 179–188. doi: 10.1016/j.ibmb.2010.02.006

21. Rodgers CJ, Blanford CF, Giddens SR, Skamnioti P, Armstrong FA, Gurr SJ. Designer laccases: a vogue for high-potential fungal enzymes? Trends Biotechnol. 2010;28:63–72. https://doi.org/10.1016/j.tibtech.2009.11.001.

22. Carrigg C RiceO Kavanagh S Collins G O'Flaherty V (2007) DNA extraction method affects microbial community profiles from soils and sediment ApplMicrobiolBiotechnol 77: 955–964.

23. Sanders., E.R. (2012) Aseptic Laboratory Technique: Plating Methods. JoVE 63:e3063

24. Higgins DG, Bleasby AJ, Fuchs R. CLUSTAL V: improved software for multiple sequence alignment. ComputApplBiosci. 1992 Apr;8(2):189–191.,

25. *"Cycloheximide – The Antimicrobial Index Knowledgebase – TOKU-E"*. antibiotics.toku-e.com.,

26. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 2004;5:113.

27. Saitou, N.; Nei, M. (1 July 1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees". Molecular Biology and Evolution. 4 (4): 406–425. doi:10.1093/oxfordjournals.molbev.a040454

28. Galai S, Limam F, Marzouki N. A New Stenotrophomonasmaltophilia strain producing laccase. use in decolorization of synthetics dyes. ApplBiochemBiotechnol. 2009; 158:416–431. doi: 10.1007/s12010-008-8369-y.

29. Ghasemi Y, Yarahmadi E, Ghoshoon MB, Dabbagh F, Hajighahramani N, Ebrahimi N, Mobasher MA, Najafabdy NM. Cloning, expression and purification of laccase gene from Bacillus subtilis in Escherichia coli. Minerva Biotech. 2014; 26:295–300.

30. Gray HB, Malmstrom BG, Williams RJP. Copper coordination in blue proteins. J BiolInorg Chem. 2000; 5:551–559. doi: 10.1007/s007750000146.

31. Gunne M, Höppner A, Hagedoorn PL, Urlacher VB. Structural and redox properties of the small laccase Ssl1 from Streptomyces sviceus. FEBS J. 2014;281(18):4307–4318. doi: 10.1111/febs.12755.

32. Gupta V, Garg S, Capalash N, Gupta N, Sharma P. Production of thermo-alkali-stable laccase and xylanase by co-culturing of Bacillus sp. and B. halodurans for biobleaching of kraft pulp and deinking of waste paper. Bioprocess Biosyst Eng. 2015; 38:947–956. doi: 10.1007/s00449-014-1340-0.

33. Giese, E.C., R.F.H. Dekker and A.M. Barbosa, 2008. Orange bagasse as a substrate for the production of pectinase and laccase by Botryosphaeriarodhina MAMB-05 in submerged and solid-state fermentation. Bioresorces, 3: 335-345

34. Glazer, A.N. and H. Nikaido, 2007. Microbial Biotechnology: Fundamentals of Applied Microbiology. 2nd Edn., Cambridge University Press, Cambridge, UK., ISBN-13: 9780521842105, Pages 554.

35. Harris, Z.L., S.R. Davis-Kaplan, J.D. Gitlin and J. Kaplan, 2004. A fungal multicopper oxidase restores iron homeostasis in aceruloplasminemia. Blood, 103: 4672-4673.

36. Huttermann, A., C. Mai and A. Kharazipour, 2001. Modification of lignin for the production of new compounded materials. Applied Microbiol. Biotechnol., 55: 387-394

37. Hakulinen N, Rouvinen J. Three-dimensional structures of laccases. Cell Mol Life Sci. 2015;72:857–868. doi: 10.1007/s00018-014-1827-5.

38. Ikeda, R., H. Tanaka, H. Oyabu, H. Uyama and S. Kobayashi, 2001. Preparation of artificial urushi via an environmentally benign process. Bull. Chem. Soc. Jpn., 74: 1067-1073.

39. Ihssen J, Jankowska D, Ramsauer T, Reiss R, Luchsinger R, Wiesli L, Schubert M, Thöny-MeyerLFaccio G. Engineered Bacillus pumilus laccase-like multi-copper oxidase for enhanced oxidation of the lignin model compound guaiacol. Protein Eng Des Sel. 2017

40. Jhadav, A., K.K. Vamsi, Y. Khairnar, A. Boraste and N. Gupta et al., 2009. Optimization of production and partial purification of laccase by Phanerochaetechrysosporium using submerged fermentation. Int. J. Microbiol. Res., 1: 9-12.

41. Kalogeris, E., F. Iniotaki, E. Topakas, P. Christakopoulos, D. Kekos and B.J. Macris, 2003. Performance of an intermittent agitation rotating drum type bioreactor for solid-state fermentation of wheat straw. Bioresour. Technol., 86: 207-213.

42. Kiiskinen, L.L., K. Kruus, M. Bailey, E. Ylosmaki, M. Siika-Aho and M. Saloheimo, 2004. Expression of Melanosporaalbomyces laccase in Trichoderma reesei and characterization of enzyme. Microbiology, 150: 3065-3074.

43. Kirk, O., T.V. Borchert and C.C. Fuglsang, 2002. Industrial enzyme applications. Curr. Opin. Biotechnol., 13: 345-351.

44. Kumar, S.V., P.S. Phale, S. Durani and P.P. Wangikar, 2003. Combined sequence and structure analysis of the fungal laccase family. Biotechnol. Bioeng., 83: 386-394.

45. Kalyani DC, Munk L, Mikkelsen JD, Meyer AS. Molecular and biochemical characterization of a new thermostable bacterial laccase from Meiothermusruber DSM 1279. RSC Adv. 2016;6:3910–3918. doi: 10.1039/C5RA24374B.

46. Kameshwar AKS, Qin W. Qualitative and quantitative methods for isolation and characterization of lignin-modifying enzymes secreted by microorganisms. Bioenerg Res. 2016.