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Molecular Identification of High Laccase Producing *Enterobacter cloacae strain T137* Based on 16s rRNA Sequencing

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ABSTRACT

The present study deals with isolation, identification, and analysis of high laccase producing bacteria from forest soil sample through 16S rRNA based molecular technique. Eight laccase producing bacterial isolates were isolated and screened on LB/Cu^{2+} agar medium containing 10 mM guaiacol for detection of extracellular laccase enzyme. The bacterial isolate (ISL-6) shown high laccase activity in liquid culture (7.12 U/ml) on the 3rd day of incubation at 37°C and pH 7.0 The most potent bacterial isolate-ISL6 was identified as Enterobacter sp. by morphological and biochemical characterization. Further, the Bacterial strain was confirmed through molecular approach. Bacterial 16S rRNA gene was amplified using suitable primers. The amplified 16S rRNA gene sequence was compared with the sequence in the NCBI sequence database. The bacterial strain was identified as Enterobacter cloacae strain T137 (NCBI Gene Bank Accession No: KC764978.1). Phylogenetic and molecular evolutionary analyses were conducted using 16S rRNA sequencing. The sequence when submitted to NCBI gene bank database using BLAST showed 96% maximum identity and E-value equal to 0 for all closely related taxa.

Key words: *Enterobacter sp., Laccase, Forest soil, 16S rRNA sequencing, BLAST.*

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are copper-containing enzymes that belong to the so-called blue copper oxidases. These enzymes are responsible for the oxidation of a variety of aromatic amines as well as phenolic compounds with the reduction of molecular oxygen to water[1]. The classical laccases are associated exclusively with plants and fungi. The rapid increase in industrialization has imposed sustainable utilization of existing renewable resources. The development of novel enzyme-catalyzed industrial processes is gaining importance due to their eco-friendly compatibility in various industries[2]. Until now only fungal laccases were extensively studied while the laccases from potential bacterial species for the same purpose has not been equally assessed so far[3].

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The first bacterial laccase to be extensively studied was from *Azospirillum lipoferum*[4]. The *Bacillus subtilis* laccase crystal structure is now available however there is no report of commercial use[5]. It has been reported that of fungal laccases lack stability at high temperature and pH[6], however, bacterial laccases have the ability to overcome these drawbacks of fungal laccases, making them compatible with almost all industrial processes[7]. ABTS is the most popular substrate in laccase activity assays, however recently, 2, 6-dimethoxyphenol (2, 6-DMP), catechol, guaiacol, and syringaldazine are also commonly used. These laccase-mediator systems (LMS) have been used in a number of processes including pulp delignification, oxidation of organic pollutants and in organic synthesis[8]. This study aims in isolation, screening and molecular identification of high laccase producing *Enterobacter sp*. from Devarayana Durga forest soil sample of Tumkur District, based on guaiacol assay.

MATERIAL AND METHODS

Sample Collection:

The soil samples were collected from Devarayana durga forest, (13.375 N latitude, 77.213 E longitudes), Tumkur District. Soil samples were collected in sterilized plastic bags from a depth of 10–15 cm below the earth's surface. All the samples were kept at 4°C until used.

Isolation and screening of extracellular laccase producing bacterial strains

Eight laccase producing bacterial strains were isolated by enrichment technique by using M9 culture medium supplemented with 0.2 mmol/L Cu^{2+} were inoculated with 10 g of soil sample and incubated at 37°C on a rotary shaker (150 rpm) for 2 days. The 5ml culture was transferred to 100 ml Luria-Bertani (LB) culture medium containing 0.2 mmol/L Cu^{2+} and incubated at 37°C at 150 rpm for 6 days. Stable enrichment cultures were obtained after subculture. To isolate pure cultures, the enriched bacterial cultures were appropriately diluted with the sterile saline solution (0.9% NaCl) before spreading onto LB/Cu^{2+} agar plates supplemented with guaiacol (10 mM) and incubated at 37°C for 3-5 days. The bacterial isolates were reconfirmed for laccase enzyme production on LB/Cu^{2+} agar medium supplemented with guaiacol (10 mM) as substrate and without guaiacol as control[9]. Later the pure, positive cultures of bacterial isolates showing extracellular laccase secretion on guaiacol supplemented plates were developed in LB/Cu^{2+} broth and the cultures were maintained in 15% glycerol as a stock culture for further analysis. The enzyme was produced in 250 ml Erlenmeyer flasks, containing 100 ml of LB/Cu^{2+} broth devoid of guaiacol, and micronutrient solution[10]. Finally, the pH was adjusted to 7.0 prior to sterilization. The production medium was inoculated with 1% inoculum (2.0 A_{610}) and incubated at 37°C under shaking condition (150 rpm) for six days. After incubation, the culture broth was centrifuged at 10,000 RPM for 10 min at 4°C and the cell-free supernatant was used as the crude enzyme to measure laccase activity.

Laccase activity assay:

Laccase activity in the supernatant was determined with guaiacol as substrate. Laccase activity in supernatant of bacterial culture was determined in 30 mM Tris-HCl buffer (pH 8.0), containing 10 mM guaiacol and 10 mM hydroquinone as substrates and incubated at 37°C for 10 min. One unit of laccase was defined as the amount of the enzyme required to transform 1µmol substrate per min under standard assay conditions. All the experiments on laccase activity were performed in triplicates, with an average standard deviation of laccase activity less than 5%. Laccase activity in the supernatant of bacterial culture was determined in Tris-Hcl buffer (pH 8.0), the reaction was started by adding the stock solutions of guaiacol to buffer to make a final concentration of 10 mM, and then absorbance was measured at 470 nm for guaiacol (e=

$6740M^{-1}Cm^{-1}$) in an UV-Visible spectrophotometer.

Genomic DNA isolation

The genomic DNA was isolated from high laccase producing bacterial isolate (ISL-6) as per the standard protocol[11]. The Single colony was inoculated in LB/Cu^{2+} broth and grown for overnight at 37ºC. Cells were harvested from 5 mL of the culture and to this 100µL of lysozyme was added and incubated at RT for 30 min, followed by the addition of 700 µL of cell lysis buffer (Guanidium isothiocyanate, SDS, Tris-EDTA). The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked transparent. 700 µL of isopropanol was added on top of the solution. The two layers were mixed gently until white strands of DNA were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 50µL of 1X TE buffer. The quality of the DNA was checked by running on 0.8% agarose gel stained with ethidium bromide (0.5 µg/µL) A single intense band with slight smearing was noted. The extracted genomic DNA was used as template DNA for amplification of the 16S rRNA gene.

PCR amplification of 16S rRNA gene

PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The universal primers (Forward primer 5'AGAGTTTGATCCTGGCTCAG-3' and reverse primer (5' GGTTACCTTGTTACGACTT 3') were used for the amplification of the 16S rRNA gene fragment. The reaction mixture of 50 µl consisted of 10 ng of genomic DNA, 2.5 Units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris-HCl, 500 mM KCl pH-8.3), 200µM dNTP, 10 picomoles each of the two universal primers and 1.5 mM $MgCl_2$. Amplification was done by initial denaturation at 94°C for 3min, followed by 40 cycles of denaturation at 94°C for 30 seconds, an annealing temperature of primers was set at 55°C for 30 seconds and extension at 72°C for 1 minute. The final extension was conducted at 72°C for 10 minutes.

Agarose gel electrophoresis

From the above mixture, 10 µl of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0 % agarose with ethidium bromide (0.5 µg/µL) as per the standard protocols[12] at 80V/cm and the reaction product was visualized under Gel documentation System (Alpha Innotech).

Purification of PCR Product by Exosap-IT

The PCR product is subjected to purification by using Exosap-IT it is a mixture of Exonuclease I and Shrimp Alkaline Phosphatase that removes leftover primers and free nucleotides from the PCR reaction. 2 µl of Exosap is added to the 5 µl of the above PCR product. Further, the reaction mixture was incubated at 37ºC for 15 minutes to allow the degradation of primers and free nucleotides. The tube was transferred to the 80ºC water bath and incubated for 15 minutes to inactivate the Exosap-IT enzyme. The sample is ready for the sequencing reaction.

DNA sequencing of 16S rRNA gene fragment

The 16S rRNA purified PCR product (100ng concentration) was subjected for the sequencing using ABI DNA 3730 XL sequencer (Applied Biosystem Inc). Sequencing of the 16S rRNA gene of the bacterial isolate (ISL-6) was done from both the directions. The sequence obtained was subjected to BLAST search and the bacterial species were determined. The percentages of sequence matching were also analyzed and the sequences were submitted to NCBI-Gen Bank and obtained accession numbers.

Computational analysis (BLAST) and identification of the bacterial isolate (ISL-6).

BLAST (Basic Local Alignment Search Tool) is a web-based program that is able to align the search sequence to thousands of different sequences in a database and show the list of top matches. BLAST can search through a database of thousands of entries in a minute.

BLAST performs its alignment by matching up each position of search sequence to each position of the sequences in the database. For each position, BLAST gives a positive score if the nucleotides match, it can also insert gaps when performing the alignment[13]. When a single gap inserted it has a negative effect on the alignment score, but if enough nucleotides align as a result of the gap, this negative effect is overcome and the gap is accepted in the alignment. These scores are then used to calculate the alignment score, in the form of bits. This is converted to the statistical E-value. If lower the E-value, the more similar is the sequence found in the database to the query sequence. The most similar sequence is the first result listed.

RESULTS AND DISCUSSION

The conventional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on molecular methods. Assessment of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. The sequence of the 16S rRNA gene has been widely used to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to classify an unknown bacterium to the genus or species level[14]. The use of 16S rRNA gene sequences to study bacterial phylogeny[15] and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its occurrence in almost all bacteria, often accessible as a multigene family, or operons (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes[16]. The rRNA based analysis is a fundamental method used not only to explore microbial diversity but also to identify new strains. The present study has been carried out to identify the bacterial isolate (ISL-6) from forest soil of Tumkur District. From the previous objective work, a total of eight isolates were obtained, from that one isolate was used for further analysis (Table.1) and it was found to be *Enterobacter sp*. based on morphological and Biochemical characterization (Table. 2, 3). Further confirmation was done using a molecular approach. Bacterial genomic DNA was isolated as per the standard protocol[11]. The presence of bacterial genomic DNA isolated was confirmed on 0.8% agarose gel stained with ethidium bromide. An intense single band was seen along with the DNA marker. The extracted DNA was used as a template for amplification of 16S rRNA gene. The universal primers 27F and 1429R were used for the amplification and sequencing of the 16S rRNA gene fragment. The optimum annealing temperature was found to be 55ºC. An intense single band (Fig.1) was visible on 1% agarose gel stained with ethidium bromide. The PCR product was subjected to sequencing using BDT V3.1 cycle sequencing kit on ABI 3730 XL genetic analyzer from both forward and reverse directions. The sequences (Fig. 3, 4) obtained were compared with the NCBI gene bank database using the BLAST search program (http://www.ncbi.nlm.nih.gov)[17,18]. The percentages of sequence matching were also analyzed. The homology search made using BLAST showed 96% maximum identity with that of *Enterobacter cloacae strain T137*, NCBI Gene Bank Accession No: KC764978.1 and E-value equal to 0 for all closely related taxa (Table. 5). It shows unique consensus sequences (Fig.5) Further, the sequences of the bacterial isolates were used for the construction of the phylogenetic dendrogram to know the genetic relatedness between the bacterial isolates. All the closely associated homologs of identified bacteria were used for the construction of the phylogenetic dendrogram to identify their evolutionary origin. The dendrogram showing the relation between *Enterobacter cloacae strain T137* and their close homologs were shown (Fig. 5).

Table 1: Quantitative screening of laccase activity from bacterial isolates (The values are presented as the mean ± SD of triplicate tests)

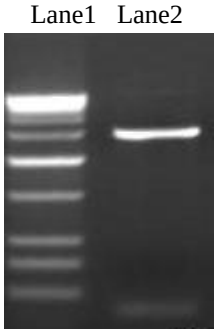
Incubation of Bacterial isolates	Laccase activity of ISL-1 (U/ml)	Laccase activity of ISL-2 (U/ml)	Laccase activity of ISL-3 (U/ml)	Laccase activity of ISL-4 (U/ml)	Laccase activity of ISL-5 (U/ml)	Laccase activity of ISL-6 (U/ml)	Laccase activity of ISL-7 (U/ml)	Laccase activity of ISL-8 (U/ml)
Day 1	1.24±0.11	0.15±0.03	1.51±0.08	1.15±0.08	2.4±0.02	3.56±0.02	2.25±0.02	1.24±0.11
Day 2	1.95±0.02	2.04±0.02	2.4±0.08	1.51±0.08	4.54±0.05	5.6±0.05	4.71±0.05	2.67±0.02
Day 3	2.93±0.02	2.31±0.02	2.67±0.02	2.22±0.02	6.23±0.31	7.12±0.15	6.05±0.31	3.82±0.02
Day 4	3.91±0.15	3.2±0.02	3.56±0.02	3.2±0.02	6.23±0.31	7.12±0.15	6.05±0.31	5.25±0.05
Day 5	4.98±0.15	3.82±0.02	4.45±0.02	3.73±0.02	6.23±0.31	7.12±0.15	6.14±0.54	6.4±0.31
Day 6	4.98±0.15	3.82±0.02	4.45±0.02	3.73±0.02	6.23±0.31	7.12±0.15	6.14±0.54	6.4±0.31

Table 2: Morphological characterization of the bacterial isolate (ISL 6)

Characteristics	*Enterobacter sp.*
Colony diameter	3mm-6mm in diameter
Colony texture	mucoid
Colony colour	Slight pink
Cell morphology	Gram –ve rods
Cell motility	+ve

Table 3: Biochemical characterization of the bacterial isolate (ISL 6)

Sl. No.	Biochemical Test	Test Result*
1	Gelatin hydrolysis	+
2	Urease	-
3	Lipase	-
4	Oxidase	-
5	Catalase	+
6	Casein protease	-
7	Amylase	-
8	Nitrate reduction	+
9	Indole production	+
10	Methyl red	+
11	Voges-Proskauer	+
12	Citrate Utilization	+



Lane1: marker with 100,200, 300, 600, 1000, 1500, 2000, 2500 and 3000bp bands
Lane2: 1.5Kb amplified product

Fig. 1: Gel image of 16S rRNA amplicon, Lane 1: DNA marker; Lane 2: 16S rRNA amplicon band

Forward primer
>1016_187_013_PCR_1C_16S_F_F08.ab1
GGGCATGGCGGCAGCTACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAG
TGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCT
AATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGAT
GGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCG
GGGAGGAAGGCGTTAAGGTTAATAACCTCGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG
CAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAG
GCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCT
TCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTTCT
TGACATCCAGAGACTTACCAGAGATGCATTGGTGCCTTTCGGGACTCTGAGACAGGTGCTGCATGGCTG
TCGTCAGCTCGTGTTTGTGAAATGTTGGTAAGTCCCGCAACGAGCGCAACCCTAATCATTTGTTGCAGCG
GTTCGCGGGACTCAAAGGAAACTGCAAGTGATAACCTGAGACGTTGGATGACGTCAGTCATCATGGCTA
CCGAGTAGCTAACACGTGCTCAATGTCCTACAAGAGCGACCTCGCGAGAGACAGCGACCTCTATAAAAA
GTGGCG

Fig. 3: Forward primer of *Enterobacter cloacae strain T137*

Reverse primer
>1016_187_014_PCR_1C_16S_R_G08.ab1
GAACTGATCCAAAGTGGTAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATG
GTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGC
GATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTT
GCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCA
TGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGA
CCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTG
ACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGCTAAGTTCTCTGGAT
GTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCG
GAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAA
TCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGG
TATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCT
GCCAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTGCGT
GCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGCTGCTGGCACGGAGTT
AGCCAGGTGCTTCTTCTGCGGGTAACGTCAATCGACGAGGTTATTAACTTATCACCTTCCTCCCCGCTGA
ACGTACTTACAACCCGAAGCATCGTCATACACGCGCATGCTGCATCAGCCTGCGCCATGTGCAGATCCC
CACTGCTGCTCCGGTAGAGTCTGAACGTGTCTCAGTTCATGTGCCTGTCATTCGTCCAGAACAGCTAGG
ATCGTCCTAGGTGAGCCGTTACCCACGTACTAGCCTAATTGCCCACTCTCTT

Fig. 4: Reverse primer of *Enterobacter cloacae strain T137*

Consensus sequence:
GGGCATGGCGGCAGCTACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAG
TGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCT
AATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGAT
GGGAAAGAGAGTGGGCAATTAGGCTAGTACGTGGGTAACGGCTCACCTAGGACGATCCTAGCTGTTCTG
GACGAATGACAGGCACATGAACTGAGACACGTTCAGACTCTACCGGAGCAGCAGTGGGGATCTGCACAT
GGCGCAGGCTGATGCAGCATGCGCGTGTATGACGATGCTTCGGGTTGTAAGTACGTTCAGCGGGGAGG
AAGGTGATAAGTTAATAACCTCGTCGATTGACGTTACCCGCAGAAGAAGCACCTGGCTAACTCCGTGCCA
GCAGCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTC
TTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGC
TAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCA
GAGAACTTAGCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCG
GCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATG
GCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCA
AGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCG
CTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
ATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTACCACTTTGGATCAGTTC

Fig. 5: Consensus sequence of *Enterobacter cloacae strain T137*

Table 5: Sequences producing significant alignments from BLAST search

Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterobacter cloacae strain T137 16S ribosomal RNA gene, partial sequence	2327	2327	99%	0.0	96%	KC764978.1
Enterobacter hormaechei strain Ni-1 16S ribosomal RNA gene, partial sequence	2326	2326	99%	0.0	96%	HM446004.1
Enterobacter cloacae strain PCX2 16S ribosomal RNA gene, partial sequence	2322	2322	99%	0.0	96%	KU936831.1
Enterobacter sp. CIFRI D-TSB-9-ZMA 16S ribosomal RNA gene, partial sequence	2320	2320	98%	0.0	96%	JF799886.1
Enterobacter xiangfangensis strain LMG27195, complete genome	2318	18438	99%	0.0	96%	CP017183.1
Enterobacter hormaechei subsp. steigerwaltii strain DSM 16691, complete genome	2318	18493	99%	0.0	96%	CP017179.1
Enterobacter hormaechei strain CAV1176, complete genome	2318	18493	99%	0.0	96%	CP011662.1
Enterobacter hormaechei subsp. steigerwaltii strain 34998, complete genome	2318	18331	99%	0.0	96%	CP012167.1
Enterobacter hormaechei subsp. steigerwaltii strain 34977, complete genome	2318	18493	99%	0.0	96%	CP010376.2
Enterobacter cloacae strain CAV1669, complete genome	2318	18493	99%	0.0	96%	CP011650.1
Enterobacter cloacae strain CAV1668, complete genome	2318	18493	99%	0.0	96%	CP011584.1
Enterobacter cloacae strain CAV1411, complete genome	2318	18493	99%	0.0	96%	CP011581.1
Enterobacter cloacae strain CAV1311, complete genome	2318	18493	99%	0.0	96%	CP011572.1

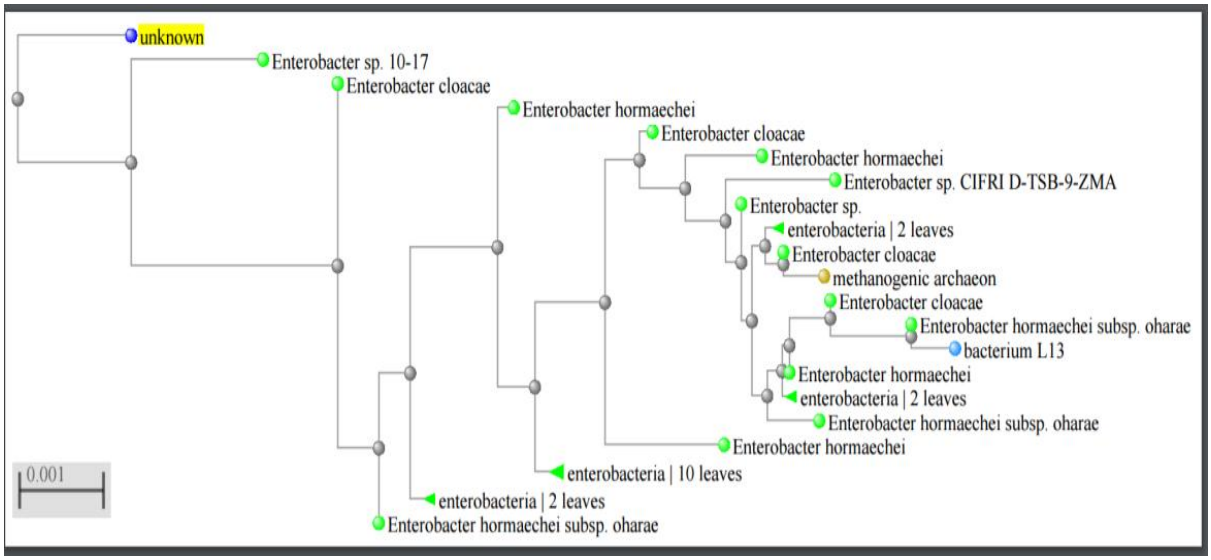


Fig. 5: Phylogenetic tree of 16S rDNA Sequence of *Enterobacter cloacae strain T137 and related taxa.* The scale bar indicates 0.001 substitution per nucleotide position

CONCLUSION

This study showed that bacterial species have at least one copy of the 16S rRNA gene containing highly conserved regions together with hypervariable regions. The use of 16S rRNA gene sequences to identify new strains of bacteria is gaining importance in recent years. This study showed the use of 16S rRNA gene sequence to characterize high laccase producing bacterial isolate (ISL-6) from the forest soil sample and was found to be *Enterobacter cloacae strain T137*. Thus, the 16S rRNA gene sequencing is both simple and is more accurate for the species identification.

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