# Isolation, Characterization And Molecular Identification Of Bacteria From Commercial Source Using 16s Rrna Sequencing For Domestic Waste Water Treatment

**Priyanka Tomar, Kantha D Arunachalam, K.C. Vinuprakash, Harsh Thakur**

***Abstract-*** ***This present study focuses on the isolation, identification and analysis of bacteria from a commercial source using 16S rRNA sequencing based on molecular techniques. Bacterial strain has been isolated, characterized and identified by various biochemical tests and molecular approaches that have been confirmed. The bacterial 16srRNA gene sequences were amplified and compared by using primers which has been compared to NCBI sequence database. The bacterial strains were identified as ribosomal RNA stain clostridium butyricum 16s , Thiobacillus denitrificans ATCC 25259, Thiobacillus thioparus strain Pankhurst T4 and for these stains the national center for biotechnology information gene bank accession no is M59085.1, accession no CP000116.1 accession no HM173633.1 and the closely phylogenetic tree and molecular advancement analyzed by using 16s rRNA sequencing. The intense identity of all three stains closely related data has been calculated 100%, 99-100% and 99-100% and E- value for all the three was 0 by using BLAST after submitting to the national center for biotechnology information gene bank.***

***Keywords: 16s rRNA sequencing, Blast, NCBI sequence database, phylogenic tree, molecular techniques, primers, biochemical test.***

## I. INTRODUCTION

The deterioration of watercourses due to organic pollution was not a serious problem until the last 200 years or so, since a relatively small human population lived in dispersed communities and wastes dumped into rivers could be coped with by natural self - cleaning properties. Water pollution has become a serious problem with nations ' industrialization, coupled with rapid population growth acceleration. Industrialization led to urbanization, with people in the new factories leaving the land to work. In the nearest rivers, domestic waste from rapidly expanding cities and waste from industrial processes has all been poured untreated. Effluent waters are known as sewage, which should be removed from settlements and industrial companies. The origin of the effluent was classified as domestic or public sewage, industrial effluent and atmospheric (rain). The sanitary requirements for the composition and properties of water bodies restrict the discharge of sewage into water bodies significantly. The term "water sludge" or "bio solids" represents the insoluble residue produced during the treatment of wastewater and subsequent stabilization of the sludge, such as aerobic or anaerobic digestion. The term wastewater refers to produce by a community from three different sources: (a) domestic (household) wastewater, (b) industrial wastewater, and (c) rainwater. The performance of isolated designed consortia consisting of Clostridium butyricum, Thiobacillus denitrificans and Thiobacillus thioparus for the treatment of wastewater in terms of the reduction of demand for chemical oxygen, BOD, MLSS (mixed liquor suspended solids), and total suspended solids.Waste water treatment with bacteria includes the stabilization of waste by decaying aerobic and anaerobic process into harmless inorganic solids, which has been included as a good treatment method for domestic waste water and biological treatment of waste water.Activated sludge process (Jawahar et al., 1998; Kadam 1990; Rajamani et al., 1995) is the most ordinary used process for chemical and biological treatment of domestic wastewater in the frequent treatment plants in India. There are many commercially growing microorganisms. The 16S rRNA sequencing generally identifies bacteria rRNA is the least viable in all cells. Significantly the similar portions of rRNA sequence are remotely related to the organisms. It is easy to measure true differences by precisely aligned sequences with remotely related organism. To determine the taxonomy, phylogeny and approximate species state of diverging rates between bacteria, the gene encoding rRNA have been extensively used. The advanced relationship of microorganism can be seen by the comparision of the 16s rRNA sequencing. Due to the hyper variable region, 16s rRNA sequence can differ over the time. These are often bound or lines which shows the strongly preserved regions. To bind and amplify variable regions, the primers has been designed for conserved regions. It has been determined that the DNA sequence of the 16s rRNA gene can be used to find the large number of species. In fact, as in many species, there is no other gene as well characterized. The national center for biotechnology information (www.ncbi.nlm.nih.gov) which provides large

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number of sequences of clinical and environmental isolates on internet. To compare new database sequences, these sites can provide search algorithms. This present study focuses on the molecular identification, biochemical characterization and isolation of different bacteria from the commercial source by using molecular techniques based on the 16s rRNA sequencing.

## II. MATERIAL & METHOD

The experimental work has been carried out in 2018 - 2019 in Kattankulathur, India, in the Department of Civil Engineering and the Department of the Center for Environmental Nuclear Research SRM Institute of Science and Technology.

### A. Isolation of Bacteria

The commercial source of bacterial sample was collected from JM Enviro Technologies Pvt. Ltd. India. The sample was collected in sterilized plastic container and transported to the laboratory for bacteriological analysis. The screening has been done for bacterial isolates by using standard pour plate method on nutrient agar plates. Total no of Two hundred and sixty four isolates were obtained after the incubation period of the plates for 24hrs at 37$^{o}$C and there are three isolates which has been selected for further study and research. On the basis of gram staining colony characterization method and different biochemical tests conducted in the decided bacteriology manual of Bergey (1984).

### B. Biochemical Characterization of Bacteria

The selected strains were grown in nutrient broth culture medium and other solid and liquid medium which contains yeast extract, beef extract, peptone and many more chemicals. Cultures were inoculated with 5% (v/v), broth was added 1.5% agar (Hi-media, India) 50 ml in 250 ml of conical flaskMicroscope has been used to examine the colonies, Color, size and shape after gram staining. Isolates were analyzed for oxidase, catalase, MR-VP test, citrate test, starch hydrolysis, gelatin test, and indole production with the help of biochemical analysis which has been shown in table 1, table 2 and table 3. The tests were used to identify isolates in accordance with the Decided Bacteriology Manual of Bergey.

### C. Bacterial DNA isolation

Single colonies were inoculated for all the three strains in nutrient broth and cultivated at 37$^{o}$C for overnight. By using 5ml of cultivation, cells were harvested and added to 100 µl of lysozyme and incubated at RT for 30 min which has been followed by addition of 700 µl cell lysis buffer (SDS, Tris-EDTA etc.). Gently mix the vial by inverting for 5 min, mix the content until white DNA strands were seen. The DNA was precipitated by ethanol from the aqueous layer. The DNA pellet was dried and dissolved in 50 µl of 1x TE buffer. The DNA quality was checked with 0.8% agarose gel stained with ethidium bromide (0.5 µg/µl). Single intensive DNA was seen which was extracted to use to amplify 16s rRNA gene as a template DNA.

### D. 16S rRNA gene PCR amplification

PCR reaction was carried out. The primers have been for *Clostridium butyricum*, forward primer 27F with sequence details (5' AGAGTTTGATCMTGGCTCAG 3') and reverse primer 1492R with sequence details (5'TACGGYTACCTTGTTACGACTT 3'). Primers for *thiobacillus denitrificans*forward primer Bac967Fd with sequence details (5'-CGACGACCATGCANCACCT 3') and reverse primer Bac967Rd with sequence details (5'-ATACGCGARGAACCTTACC- 3') and primers for *Thiobacillus thioparus* forward primer Bact363F, sequence details (5'-CAATGGRSGVRASYCTGAHS 3') and reverse primer Bact363R with sequence details(5'-CTNYGTMTTACCGCGGCTGC- 3') used for the amplification of the 16s rRNA gene fragments. Add 5 µl of isolated DNA in 25µl of PCR reaction solution (1.5µl of forward & reverse primer, 5µl deionized water, 12µl of Taq master mix). Taq master mix composition follows as:-

DNA polymerase is mixed in 2x taw buffer, 0.4Mm dNTPs, 3.2mM $MgCl_2$ & 0.02% bromophenol blue perform PCR using thermal cycle conditions.

1. Denaturation- DNA template is heated at 94$^{o}$C which breaks the weak hydrogen bonds and holds strands of DNA together in helix which allow to saperate the strands to single stranded DNA.
2. Annealing- Mixture is cooled from 90$^{o}$C to 60$^{o}$C which allows the primers to bind or anneal to complementary sequence in the DNA template.
3. Extension- The reaction is heated at optimal temperature for DNA polymerase to act at 72$^{o}$C. DNA polymerase extends the primers, add nucleotides into primer in a sequence manner by using target DNA as a template. Initial denaturation at 94$^{o}$C for 3 min, denaturation at 94$^{o}$C for 30 sec, 30 cycles and annealing at 60$^{o}$C for 30 sec, extension 72$^{o}$C for 1min and final extension at 72$^{o}$C for 10 min hold at 4$^{o}$C.

### E. Agarose Gel Electrophoresis

The standard protocol according to(Sambrook et al., 2001), the reaction mixture has been taken 10µl and then analyzed by using 1% agarose with ethidium bromide (0.5 µg/µl) with the help of gel electrophoresisand the reaction product has been seen under the gel documentation system (Alpha Innotech).

### F. PCR Product purification

Unincorporated PCR primers and dNTPs removed from PCR products by using montage PCR cleanup kit (Millipore). The universal primer 27F/1492R was used to sequence the product. Sequencing reactions were performed using a ABI PRISM Big Dye TM terminator cycle sequencing kit with AmpliTaq DNA polymerase (FS enzyme) (Applied Bio System).

### G. DNA sequencing of 16S rRNA gene fragment

By using ABI DNA 3730 XL for sequencing sequencer (Applied Bio system), the 16S rRNA

purified PCR product (100ng concentration) was submitted. Sequencing of the bacterial isolate's 16S rRNA gene was carried out in both directions. By using ABI DNA 3730 XL sequencer (Applied Bio system) for sequencing, the 16S rRNA purified PCR product (concentration) was submitted. Sequencing of the bacterial isolate's 16S rRNA gene was carried out in both directions. The bacterial species were determined with the obtained sequence which was searched for BLAST. The sequences were submitted to the NCBI Gen Bank after sequence matching percentages and accession numbers were obtained.

### H. BLAST (computational analysis) and Bacterial species Identification

Basic local alignment search tool is a computer program which can align search sequences in the database of thousands of sequences and can display the list of top most matches. Within a minute, it can search thousands of entries of a database. BLAST can also insert the gaps when performs the alignments for each position if the nucleotides gives suitable match. Each and every inserted gap has a negative effect on the alignment scored, the negative effect is overcome and the gap in alignment is accepted if as a result of the gap has sufficient nucleotides aligned. The scores are used to calculate the score of alignments in "bits" and converted to statistical E- value. It shows the lower E-value when the more similar query sequence is found in the database. The most similar sequence is listed as first result.

## III. RESULTS AND DISCUSSION

The phenotypic characteristics of the traditional identification of bacteria are not as accurate as genotypic identification. The comparision of the bacterial 16s rRNA sequence was done by preferred genetic technique. To estimate the relationship between bacteria (phylogeny), sequence of the 16s rRNA was widely used as a molecular clock but it became more important as a means to identify an unknown genus or species according to (Sacchi et al., 2002). To study the bacterial phylogeny (Amann et al., 1995) and taxonomy the 16s rRNA gene sequencing used and as far as the most common genetic marker used in housekeeping for several reasonsThese reasons include (i) Multi-gene family or operons often exists when the 16s rRNA gene is present in almost all the bacteria. (ii) It has been suggested that the changes in random sequence are more accurate measure of time but the function of 16s rRNA has not changed over time. (iii) The size of gene (1500 bp) which is sufficient for information (Patel, J. B. 2001). It is a key microbiological method which is based on the rRNA analysis not only used to explore microbial diversity but also used to identify new strains. To determine the bacterial strain isolation, this study was conducted from the commercial source of bacterial sample. A total of two hundred and sixty four isolates were obtained for further study. Colonies has been isolated from mixed populations which has been characterized and sub-cultured on nutrient agar plates, to identify isolated bacteria based on biochemically analyzed activities in order to obtain pure culture and identification of *Clostridium butyricum*, *Thiobacillus denitrificans* and *Thiobacillus thioparus* has been confirmed using a molecular approach for the activities of catalase, oxidase, MR-VP test, citrate test, starch hydrolysis, gelatin test , citrate use and indole production which has been shown in (table 1, table 2, table 3) and gram staining shown in ( figure 1, figure 2, figure 3).Bacterial genomic DNA was isolated under the standard protocol (Hoffman and Winston, 1987). The presence of isolated bacterial genome was confirmed by the stained on 0.8% agarose gel with ethidium bromide. An intense band was seen together with the DNA marker. To amplify the 16srRNA gene as a tempelate, the extracted DNA was used. The universal primers 27F/1429R for Clostridium butyricum, Bac967Fd/Bac967Rd for Thiobacillus denitrificans and Bact363F/Bact363R Thiobacillus thioparus have been used for the sequencing and amplification of the 16s rRNA gene fragments. It has been found that the optimum temperature for annealing is 55$^{o}$C. An intense single band are visible on 1% agarose gel stained with ethidium bromide in figure 4.The sequencing has been done from forward and reverse directions for the PCR product by using the BDT V3.1 cycle sequencing kit on the ABI 3730XL genetic analyzer. The figures 5,6 and 7 shows the sequences which has been compared using BLAST search program (www.ncbi.nlm.nih.gov) with the NCBI gene bank database (Marchler- Bauer et al., 2000; Pruitt et al., 2005). It has been analyzed the percentage of sequencing matching for all closely related data and the search conducted for homology using BLAST which shows the identity of *clostridium butyricum* strain CDC 51208 is 100% and NCBI Gene Bank accession no is M59085.1 , E-value is 0. Similarily for *Thiobacillusdenitrificans* strain ATCC 25259 shows 99-100% and NCBI Gene Bank accession no is CP000116.1 and E-value is 0. *Thiobacillus thioparus* strain Pankhurst T4 showed 99-100% identity, NCBI Gene accession no: HM173633.1, E - value equal to 0 for all closely related data.The phylogenic tree has been constructed for the sequences of the bacterial isolates which shows the genetic relationship between the bacterial isolates. All the closely related homologous data is showing the origin of evolutionary identified bacteria with the help if constructed phylogenic tree. *Clostridium butyricum* strain CDC 51208, *Thiobacillus denitrificans* strain ATCC 25259 and *Thiobacillus thioparus*strain Pankhurst T4 is showing the close homologs with the help of phylogenic tree in Figure 8, Figure 9 & Figure 10.

## IV. CONCLUSION

To grow and spread microbial population, the commercial source of bacteria is enriched media and contains many bacteria. Bacterial species have at least one copy of the 16S rRNA gene with highly conserved regions and hyper - variable regions. In has been seen the recent years that the use of 16S rRNA gene sequences to identify new bacteria from strains is gaining momentum. We demonstrated the use of 16S rRNA gene sequence to characterize the commercial source bacterial isolated and found to be Clostridium butyricum strain CDC 51208, Thiobacillus

denitrificans strain ATCC 25259 and Thiobacillus thioparus strain Pankhurst T4. The simple and effective identification of strains method is done of genotyping by using 16s rRNA gene sequencing.

**Table no 1. Physical and biochemical characteristics of the isolate *Clostridium butyricum* strain CDC 51208**

| TEST | OBSERVATION |
|---|---|
| Gram staining | Gram Positive |
| Spore staining | Circular |
| Cell shape | Rod shape cell |
| Colony character | Off white |
| Catalase test | negative |
| Glucose | Positive |
| Sucrose | positive |
| Fructose | positive |
| Lactose | Positive |
| Maltose | Positive |
| Mannitol | negative |
| Arabinose | Positive |
| Sorbitol | Positive |
| Mannose | Positive |
| Starch | Positive |
| Xylose | Positive |
| Glycerol | Negative |
| Citrate test | Positive |
| Indole test | Negative |
| VP test | Negative |
| Test for Methyl red | Positive |
| Starch hydrolysis | Positive |
| Gelatinase | Positive |
| Oxidase | positive |

**Table no 2. Physical and biochemical characteristics of the isolate Thiobacillusdenitrificans strain ATCC 25259**

| TEST | OBSERVATION |
|---|---|
| Gram staining | Gram negative |
| Spore staining | circular |
| Cell shape | Rod shape |
| Colony character | Clear or weakly opalescent colonies |
| Catalase test | Positive |
| Glucose | Positive |
| Sucrose | Positive |
| Fructose | Negative |
| Lactose | Positive |
| Maltose | Positive |
| Mannitol | Positive |
| Arabinose | Negative |
| Sorbitol | Positive |
| Mannose | Positive |
| Starch | Positive |
| Xylose | Positive |

| | |
|---|---|
| Glycerol | Negative |
| Citrate test | Positive |
| Indole test | Negative |
| VP test | Negative |
| Test for Methyl red | Positive |
| Starch hydrolysis | Positive |
| Gelatinase | Positive |
| Oxidase | Negative |

**Table no 3. Physical and biochemical characteristics of the isolate *Thiobacillusthioparus* strain Pankhurst T4**

| TEST | OBSERVATION |
|---|---|
| Gram staining | Gram negative |
| Spore staining | circular |
| Cell shape | Short Rod |
| Colony character | Opaque |
| Catalase test | Positive |
| Glucose | Positive |
| Sucrose | Positive |
| Fructose | Positive |
| Lactose | Positive |
| Maltose | Negative |
| Mannitol | Negative |
| Arabinose | Negative |
| Sorbitol | Positive |
| Mannose | Positive |
| Starch | Negative |
| Xylose | Positive |
| Glycerol | Negative |
| Citrate test | Positive |
| Indole test | Negative |
| VP Test | Negative |
| Test for methyl red | Positive |
| Starch hydrolysis | Negative |
| Gelatinase | Negative |
| Oxidase | Negative |

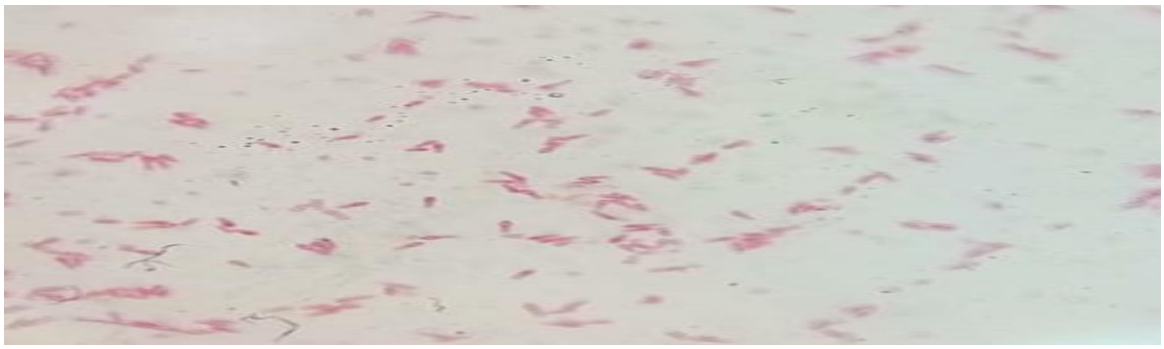

**Fig 1. *Clostridium butyricum* strain**

**Fig 2. *Thiobacillus denitrificans* strain**

**Fig 3. *Thiobacillus thioparus* strain**

**PCR products**

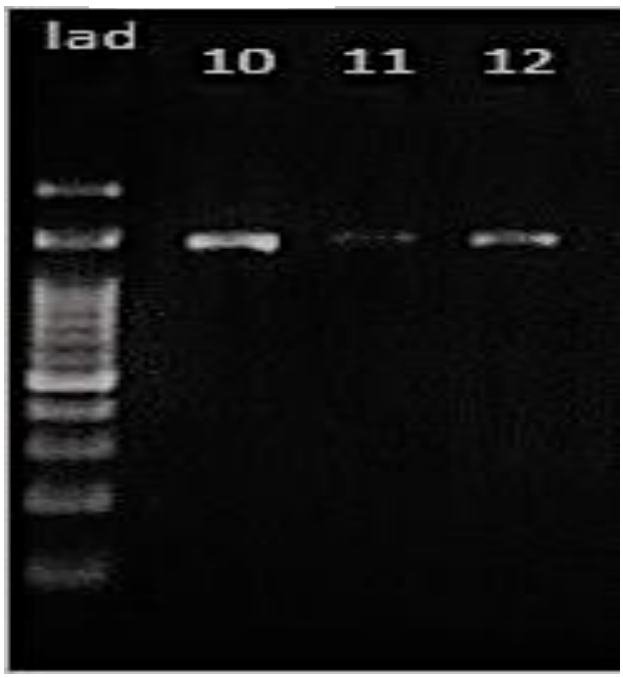


**Fig 4. Gel image of 16s rRNAamplicon for *Clostridium butyricum, Thiobacillus thioparus, Thiobacillus denitrificans***

>TTTTATTGAGAGTTTGATCCTGGCTCAGGACGAAC
GCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGA
TGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTCATAGAGGG
GAATAGCCTTTCGAAAGGAAGATTAATACCGCATA
AGATTGTAGTACCGCATGGTACAGCAATTAAAGGA
GTAATCCGCTATGAGATGGACCCGCGTCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACATT
GGGACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGGGAAACCCT
GATGCAGCAACGCCGCGTGAGTGATGACGGTCTTC
GGATTGTAAAGCTCTGTCTTTAGGGACGATAATGA
CGGTACCTAAGGAGGAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCNAGCGTT
GTCCGGATTTACTGGGCGTAAAGGGCGCGTAGGTG
GATATTTAAGTGGGATGTGAAATACCCGGGCTTAA
CCTGGGNCCTGCATTCCAAACTGGATATCTAGAGT
GCAGGAGAGGAAAGGAGAATTCCTAGTGTAGCGG
TGAAATGCGTAGAGATTAGGAAGAATATCAGTGGC
GAAGGCGCCTTTCTGGACTGTAACTGACACTGAGG
CNNNAAAGCGTGGGGAGCAAACAGGATTAGATAC
CCTGGTAGTCCACGCCGTAAACGATGAATACTAGG
TGTAGGGGTTGTCATGACCTCTGTGCCGCAGCTAA
CG<

**Fig 5: Partial sequencing of isolated *Clostridium butyricum* by using PCR product of 16S rRNA gene.**

>CTCCTGGTGGCGAGTGGCGAACGGGTGAGTAATG
CGTCGGAACGTACCGAGTAATGGGGGATAACGCAS
CGAAAGKTGTGCTAATACCGCATACGCCCTGAGGG
GGAAAGGGGGGACCGCAAGGCCTCACGTTATTCGA
GCGGCCGACGTCTGATTAGCTAGTTGGTGGGGTAA
AGGCCTACCAAGGCGACGATCAGTAGCGGGTCTGA
GAGGATGATCCGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTT
GGACAATGGGSGAASCCTGATCCAGCCATTCCGCG
TGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTT
CAGCTGGAAGAAACGSTCGSGCKAAYATCSYGKGS
TAATGACGGTACCRGCAGAAGAAGCACCGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCG
AGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCG
CAGGCGGATTGTTAAGCAAGATGTGAAATCCCCGG
GCTTAACCTGGGAATGGCATTTTGAACTGGCAGTC
TAGAGTGCGTCAGAGGGGGGTGGAATTCCACGTGT
AGCAGTGAAATGCGTAGAGATGTGGAGGAACACC
GATGGCGAAGGCAGCCCCCTGGGATGACACTGACG
CTCATGTACGAAAGCGTGGGTAGCAAACAGGATTA

GATACCCTGGTAGTCCACGCCCTAAACGATGTCAA
CTGGTTGTTGGGGGAGTGAAATCCCTTAGTAACGA
AGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACG
GTCGCAAGATTAAAACTCAAAGGAATTGACGGGGA
CCCGCACAAGCGGTGGATGATGTGGATTAATTCGA
TGCAACGCGAAAAACCTTACCTACCCTTGACATGT
CCGGAATCCTGCAGAGATGCGGGAGTGCCCGAAAG
GGAATCGGAACACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTATCATTRGTTGCTACGCAAGG
GCACTCTAATGAGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTA
TGGGTAGGGCTTCACACGTCATACAATGGTCGGTA
CAGAGGGTTGCCAAGCCGCGAGGTGGAGCCAATCC
CAGAAAGCCGATCGTAGTCCGGATTGTTCTCTGCA
ACTCGAGAGCATGAAGTCGGAATCGCTAGTAATCG
CGGATCAGCATGTCGCGGTGAATACGTTCCCGGGT
CTTGTACACACCGCCCGTCACACCATGGGAGTGGA
ATCTGGCAGAAGTAGGTAGCCTAACCGCAAGGAGG
GCGCTTACCACGCTGGGTTTCATGACTGGGGTGAA
GTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGCT
GGATCACCT<

**Fig 6: Partial sequencing of isolated *Thiobacillus denitrificans* by using PCR product of 16S rRNA gene.**

GTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAAC
ACCAATGGCGAAGGCAGCCCCCTGGGATGACACTG
ACGCTCATGTACGAAAGCGTGGGTAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCCTAAACGATGT
CAACTGGTTGTTGGGGGAGTGAAATCCCTTAGTAA
CGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGT
ACGGTCGCAAGATTAAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGATGATGTGGATTAATT
CGATGCAACGCGAAAAACCTTACCTACCCTTGACA
TGTCCAGAACCCTGCAGAGATGCGGGGGTGCCCGA
AAGGGAATTGGAACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTATCATTAGTTGCTACGCAA
GGGCACTCTAATGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCT
TATGGGTAGGGCTTCACACGTCATACAATGGTCGG
TACAGAGGGTTGCCAAGCCGCGAGGTGGAGCCAAT
CCCAGAAAGCCGATCGTAGTCCGGATTGTTCTCTG
CAACTCGAGAGCATGAAGTCGGAATCGCTAGTAAT
CGCGGATCAGCATGTCGCGGTGAATACGTTCCCGG
GTCTTGTACACACCGCCCGTCACACCATGGGAGTG
GAATCTGGCAGAAGTA<

**Fig 7: Partial sequencing of isolated *Thiobacillus thioparus* by using PCR product of 16S rRNA gene.**

>TCAGCTGGAACGAAACGGTACAGGCCAATACCCT
GTGCTAATGACGGTACCGGCAGAAGAAGCACCGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
TGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCG
TGCGCAGGCGGATTGTTAAGCAAGATGTGAAATCC
CCGGGCTTAACCTGGGAATGGCATTTTGAACTGGC
AGTCTAGAGTGCGTCAGAGGGGGGTGGAATTCCAC

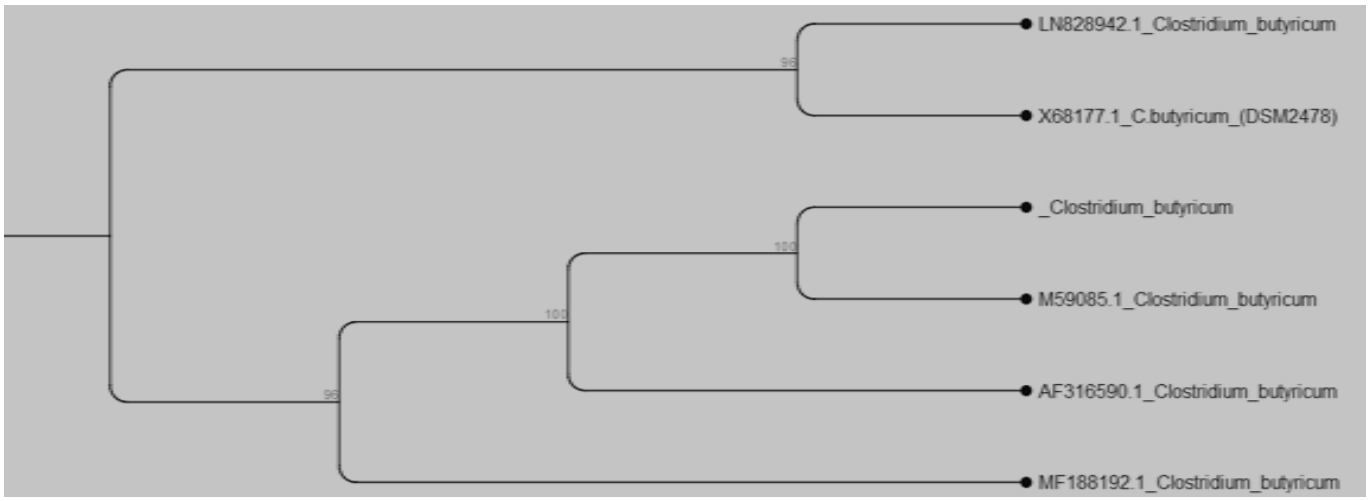


**Fig 8: Close homologs of *Clostridium butyricum* strain CDC 51208 shown in phylogenic tree.**

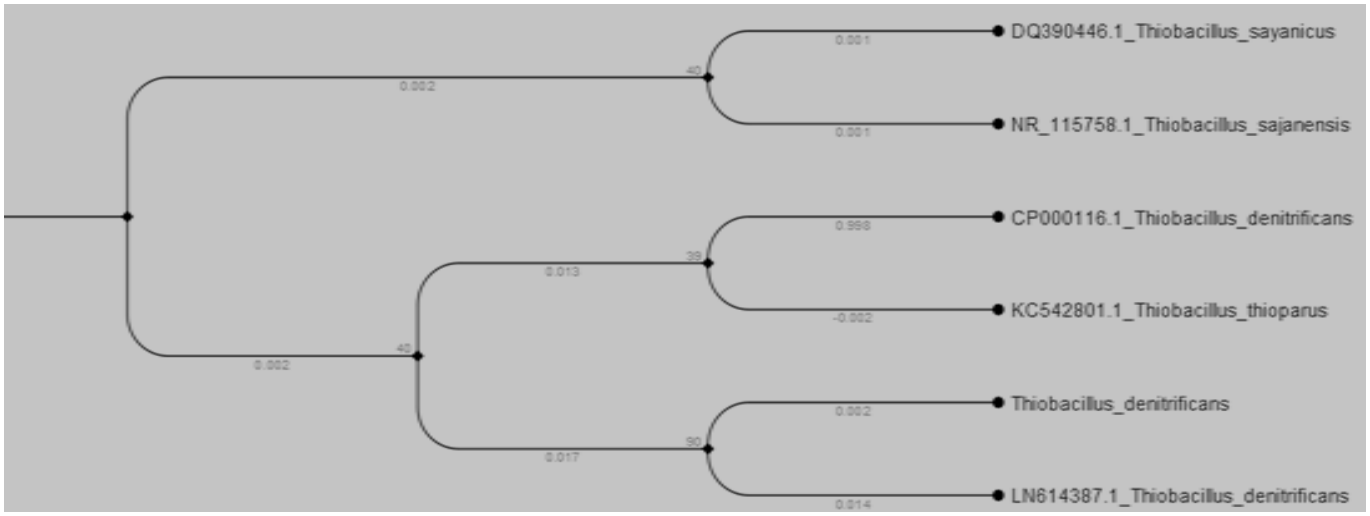


**Fig 9: Close homologs of *Thiobacillusdenitrificans* strain ATCC 25259shown in phylogenic tree.**

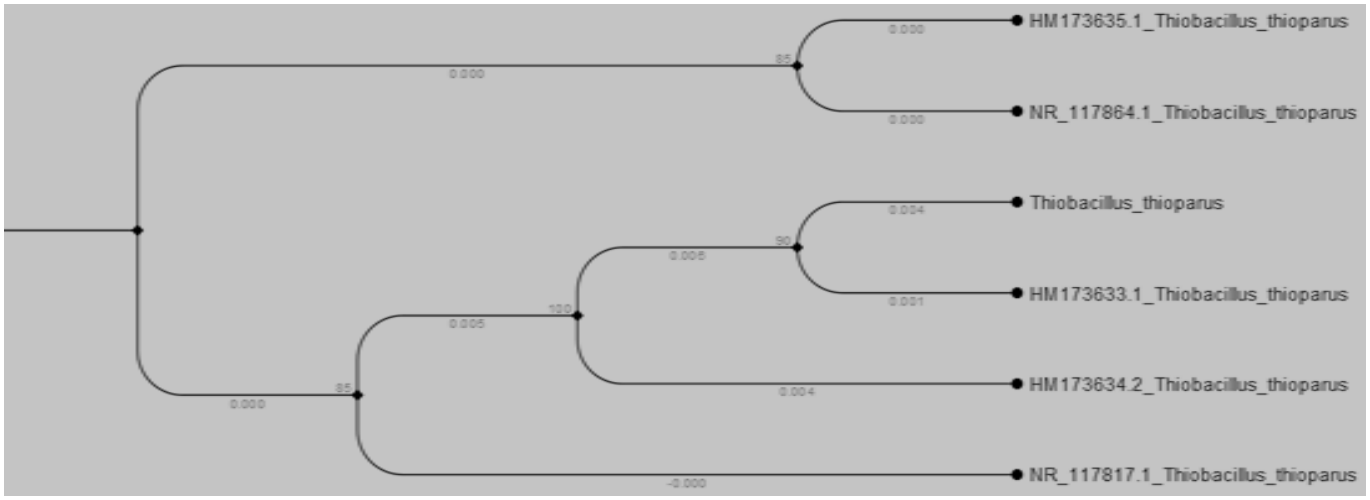


**Fig 10: Close homologs of *Thiobacillus thioparus* strain Pankhurst T4 shown in phylogenic tree.**

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