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Phylogenetic Analysis of the Bacteria Associated with the Fresh Water Micro-Alga *Chlorella* sp.

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ABSTRACT

The importance of the microbial association with fresh water algae is well known and reported by several researchers. However, majority of these studies focus on the interaction between the algae and the microbes and there are very few reports on the phylogenetic studies on these associated microbes. Therefore, in the present investigation, molecular phylogeny of the three different bacterial species associated with the Chlorella sp. was determined by amplifying and sequencing the genomic 16S rRNA region. The DNA sequences were analyzed using online BLASTn tool. The BLAST results were used to find out evolutionary relationship between these bacteria. The phylogenetic tree for these bacteria was constructed by using MEGA 5 software.

Key words: *Polymerase chain reaction, 16S rRNA, Phylogenetic tree, Evolutionary relationship*

INTRODUCTION

Chlorella is a genus of the single celled photosynthetic, green algae which belong to the phylum Chlorophyta. Many species of *Chlorella* have attracted attention of the researchers as a potential source of food and energy. There are many investigations that report the association of several bacteria with these freshwater green algae and these bacteria are reported to play a key role in the flocculation of the host species [1-3]. A perusal of literature indicated that the phylogenetic analysis of these associated bacteria is not carried out to the greater extent. In view of this, the present study was undertaken to isolate three bacterial species associated with *Chlorella* and study them phylogenetically using modern bioinformatics tools.

MATERIALS AND METHODS

Collection of microalga and isolation of the bacteria

The *Chlorella* sp. was obtained from the culture library of Botany Department. The *Chlorella* sp. was grown in freshwater-based medium BG11. The culture was grown in 500 ml media in a 1000 ml flask for 14 days. After 14 days, the culture was sonicated and centrifuged.

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The supernatant was taken further for bacterial isolation. 100 µl supernatant was spread plated on LB agar for the isolation of bacteria. These plates were incubated for 48 h at 30°C. After incubation, the bacterial colonies were isolated, purified and stored on LB agar slants.

DNA extraction and quantification from the bacterial isolates

DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505) following the manufacturer's instructions. DNA was precipitated by adding 200 µl of ethanol to the lysate and vortexing. Lysate was then loaded on HiElute Miniprep Spin column and centrifuged at 10,000 rpm for 3 min. After repeated washings, 200 µl of elution buffer was added to the column, incubated at room temperature for 5 mins and then centrifuged at 10,000 rpm for 3 mins. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at -20°C till further used.

PCR amplification of the isolated DNA

The DNA isolated from the bacteria was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer, 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consisted of a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 50°C, 2 min at 72°C; and an additional cycle of 7 min at 72°C. The reagents used were procured from GeNei. Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approx. 850bp for the partial 16S rRNA region.

DNA sequencing

The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing the PCR product, 519F - 5' CAGCAGCCGCGGTAATAC 3' sequencing primer was used.

Bioinformatics analysis

The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of bacteria. Altogether twenty sequences, including sample were used to generate phylogenetic tree. The tree was constructed by using MEGA 5 software [4, 5]. The evolutionary history was inferred using the Neighbor-Joining method and was conducted in MEGA5.

RESULTS

Three bacteria isolated from the *Chlorella* sp. were given codes viz. CHL3, CHL4 and CHL5. 16S rRNA gene of these bacterial isolates was amplified and partially sequenced (Fig.1, 2 and 3).

Sample code: CHL3

Partial 16S rRNA gene Sequence (Length 840 bp)

TTACTGGGCGTAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTG
GGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGG
TGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG
CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
TGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCT
GGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGA
GATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGA
ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC
CTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGC
AAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGG
AATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCGTAGTAACCAGCG

Fig. 1: Partial 16S rRNA gene sequence of bacterium CHL3

Sample Code: CHL4

Partial 16S rRNA gene Sequence (Length 858 bp)

TATCCGATTTCTGGGCGTAAGCGCGTGTAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTC
AACCTGGGAACTGCATTTTTAACTGGCAAGCTGGAGTCTTGTAAGGGGGGTAGAATTCCAGGTGTA
GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGGATAATAACT
GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAA
CGATGTCGACTTGCTGTTTGGGCCCTTGAGCCTTGGCTTCCGGAGCTAACCCTGAAATTCACCCCC
CGGGGAAGTACGGCCCCAAAGTTAAAACTCAAAGAATTTGACGGGAGCCCGCACAACCGGGGATG
ATGGGGATTTAATTCATGGCACCGCAAAAAACCTTACTACCCCTTGACATCCTGAAAACTTAAAAAA
ATTGCTTTGTTGCCTTCGGGAACTCTGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCACCGATTAGGACTCTAA
CGCGACTGACACTGCCAATGATAAGCTGGGTGAAGATGACGATGACTCCTCGTGATCCTGATCCTT
ACGACTTCGCACACACTACTGCGACCATGACATATGGTCGCAAACCCGACCTCGCGAGAGCATCTC
AAACTCATGATGTATGTCCGGATCCCAATTGGAGACTCCACTGCGACTCCACGAAATCGGAATCAC
TCGTAGATCAGAATCTCAATGCCAATACGATTACGTTCCCTGTACTTGCCGACACCGA

Fig. 2: Partial 16S rRNA gene sequence of bacterium CHL4

Sample code: CHL5

Partial 16S rRNA gene Sequence (Length 850 bp)

ATTGGGCGTAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGG
AGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
AGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTG
GGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAG
ATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCAC
TCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGGATGACGTCAAATCATCATGCCCCT
TATGACCTGGGCTACACACGTGCTACAATGGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTA
AGCCAATCCCACAAATCTGTTCTCAGTTCGGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGG
AATCGCTAGTATCGCGGATCAGCATGCCGCGGTGAATACGTTCCTGGTTTGAGCCCCAGAGAGAG
AGAAGAA

Fig. 3: Partial 16S rRNA gene sequence of bacterium CHL5

The results for sequence analysis carried out using the BLASTn tool for the isolate CHL3 are shown in the Table 1. The bacterial isolate CHL3 was indicated to be most likely from the genus *Enterobacter* as its sequence showed 99% sequence similarity with the existing entries in the nucleotide database. Its other phylogenetic neighbours were found be species from *Cedecea*, *Kluyvera*, *Citrobacter*, *Tatumella*, *Escherichia* and *Klebsiella* genera (Table 1 and Fig. 4).

Table 1: Phylogenetic neighbors of CHL3 culture based on 16S rRNA gene sequence

Description	Max score	Identity	Accession
Enterobacter asburiae strain JCM6051 16S rRNA	1522	99%	NR_024640.1
Enterobacter cancerogenus strain LMG 2693 16S rRNA	1519	99%	NR_044977.1
Enterobacter ludwigii strain EN-119 16S rRNA gene	1517	99%	NR_042349.1
Leclercia adecarboxylata strain CIP 82.92 16S rRNA	1506	99%	NR_104933.1
Enterobacter aerogenes strain KCTC 2190 16S rRNA	1500	99%	NR_102493.1
Enterobacter aerogenes strain JCM1235 16S rRNA	1500	99%	NR_024643.1
Enterobacter hormaechei strain 0992-77 16S rRNA	1496	99%	NR_042154.1
Cedecea davisae strain DSM 4568 16S rRNA	1495	99%	NR_025243.1
Kluyvera cryocrescens strain 12993 16S rRNA gene	1495	99%	NR_028803.1
Serratia ureilytica strain NiVa 51 16S rRNA gene	1489	99%	NR_042356.1
Cedecea neteri strain GTC1717 16S ribosomal RNA	1487	99%	NR_040930.1
Kluyvera intermedia strain 256 16S ribosomal RNA	1483	99%	NR_028802.1
Citrobacter murliniae strain CDC 2970-59 16S rRNA	1483	99%	NR_028688.1
Citrobacter braakii strain 167 16S ribosomal RNA	1483	99%	NR_028687.1
Enterobacter asburiae LF7a strain LF7a 16S rRNA	1478	99%	NR_074722.1
Citrobacter freundii strain ATCC 8090 16S rRNA	1478	99%	NR_028894.1
Tatumella punctata strain SHS 2003 16S rRNA	1472	98%	NR_104937.1
Escherichia vulneris strain ATCC 33821 16S rRNA	1472	98%	NR_041927.1
Klebsiella pneumoniae subsp. ozaenae strain ATCC 11296 16S ribosomal RNA gene, partial sequence	1472	98%	NR_041750.1
Enterobacter cloacae strain 279-56 16S rRNA	1472	98%	NR_028912.1

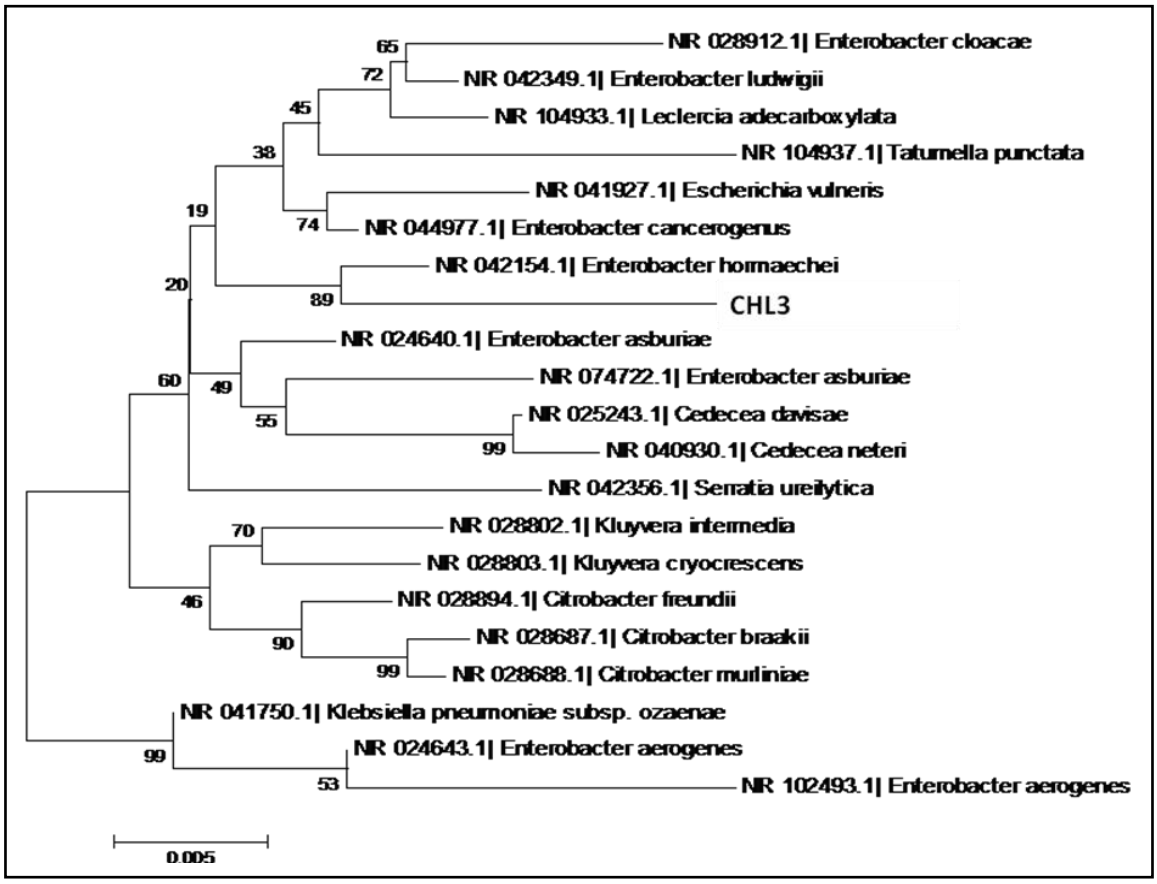

Fig. 4: Phylogenetic tree for CHL3 using partial 16S rRNA gene sequences

The results for sequence analysis carried out using the BLASTn tool for the isolate CHL4 are shown in the Table 2. The bacterial isolate CHL4 was found to be a phylogenetic neighbour of species from the genera *Citrobacter*, *Kluyvera* and *Enterobacter* with a sequence similarity of 83-84%. Its other phylogenetic neighbours were found be species from *Raoultella*, *Serratia* and *Klebsiella* genera (Table 2 and Fig. 5).

Table 2: Phylogenetic neighbors of CHL4 based on partial 16S rRNA gene sequence

Description	Max score	Identity	Accession
Citrobacter freundii strain NBRC 12681 16S rRNA	749	83%	NR_113596.1
Citrobacter werkmanii strain CDC 0876-58 16S rRNA	749	83%	NR_024862.1
Kluyvera cryocrescens strain NBRC 102467 16S rRNA	747	84%	NR_114108.1
Kluyvera cryocrescens strain 12993 16S ribosomal RNA	747	84%	NR_028803.1
*Citrobacter freundi*i strain JCM 1657 16S rRNA gene	745	83%	NR_113340.1
Enterobacter aerogenes strain NCTC10006 16S rRNA	745	84%	NR_114737.1
Citrobacter freundii strain NBRC 12681 16S rRNA gene	743	83%	NR_114345.1
Enterobacter aerogenes strain NBRC 13534 16S rRNA	743	84%	NR_113614.1
Klebsiella oxytoca strain ATCC 13182 16S rRNA gene	743	83%	NR_041749.1
Citrobacter freundii strain ATCC 8090 16S rRNA	743	83%	NR_028894.1
Klebsiella oxytoca strain JCM1665 16S rRNA	743	83%	NR_112010.1
Enterobacter aerogenes strain KCTC 2190 16S rRNA	741	84%	NR_102493.1
Enterobacter aerogenes strain JCM1235 16S rRNA	741	84%	NR_024643.1
Citrobacter murliniae strain CDC 2970-59 16S rRNA	737	83%	NR_028688.1
Citrobacter braakii strain 167 16S rRNA gene sequence	737	83%	NR_028687.1
Klebsiella oxytoca strain NBRC 102593 16S rRNA	736	83%	NR_114152.1
Serratia ureilytica strain NiVa 51 16S rRNA gene	736	84%	NR_042356.1
Klebsiella pneumoniae subsp. rhinoscleromatis strain ATCC 13884 16S rRNA gene, partial sequence	736	84%	NR_114507.1
Kluyvera intermedia strain 256 16S rRNA gene	736	84%	NR_028802.1
Raoultella ornithinolytica B6 strain B6 16S rRNA	730	83%	NR_102983.1

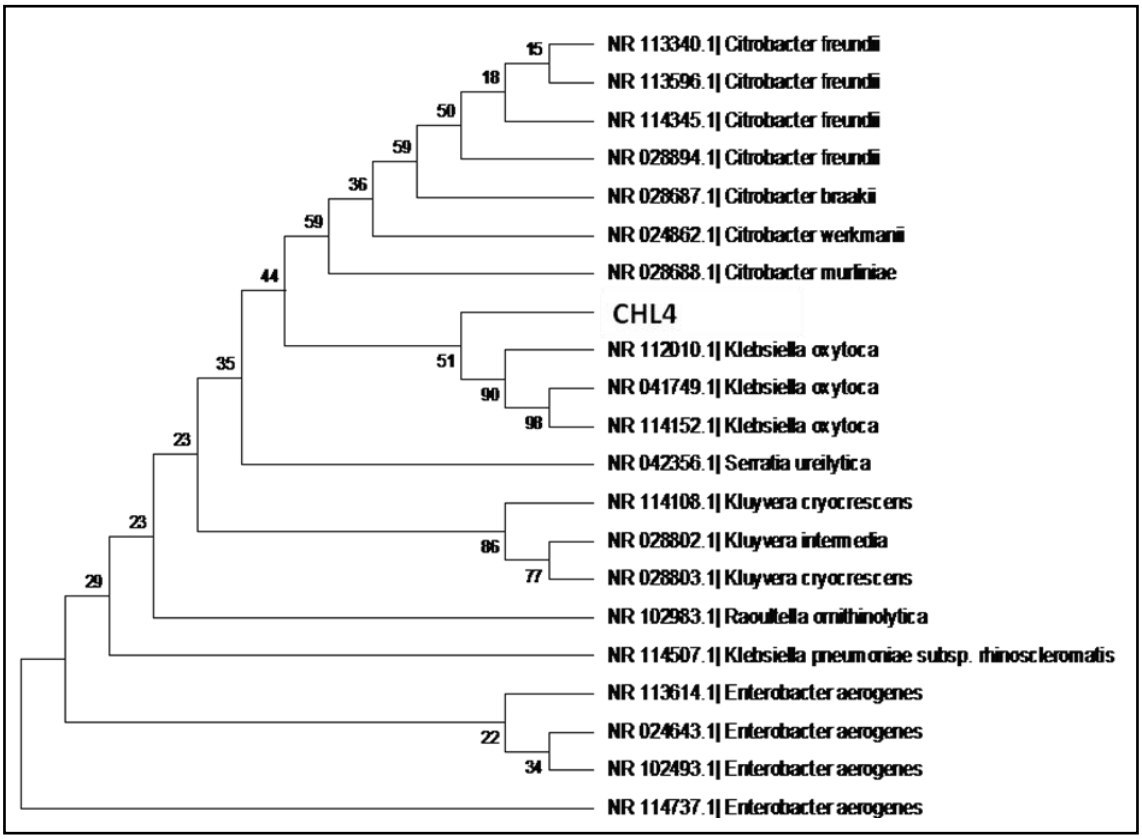

Fig. 5: Phylogenetic tree for CHL4 using partial 16S rRNA gene sequence

The results for sequence analysis carried out using the BLASTn tool for the isolate CHL5 are shown in the Table 3. The bacterial isolate CHL5 was indicated to be most likely from the genus *Bacillus* as its sequence showed 99% sequence similarity with the existing entries in the nucleotide database. Its phylogenetic position with respect to its neighbours was found and a tree was constructed as shown in the Fig. 6.

Table 3: Phylogenetic neighbors of CHL5 based on partial 16S rRNA gene sequence

Description	Max score	Identity	Accession
Bacillus subtilis subsp. spizizenii strain 168 16S rRNA	1491	99%	NR_118591.1
Bacillus subtilis subsp. subtilis strain 168 16S rRNA	1491	99%	NR_102783.1
Bacillus amyloliquefaciens subsp. plantarum strain FZB42 16S ribosomal RNA gene, complete sequence	1491	99%	NR_075005.1
Bacillus subtilis subsp. spizizenii strain ATCC 6633 16S ribosomal RNA gene, partial sequence	1491	99%	NR_118486.1
Bacillus subtilis strain SBMP4 16S rRNA gene	1491	99%	NR_118383.1
Bacillus tequilensis strain 10b 16S rRNA gene	1491	99%	NR_117611.1
Bacillus vallismortis strain NBRC 101236 16S rRNA	1491	99%	NR_113994.1
Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	1491	99%	NR_104873.1
Bacillus amyloliquefaciens strain MPA1034 16S rRNA	1491	99%	NR_117946.1
Bacillus tequilensis strain 10b 16S ribosomal RNA	1491	99%	NR_104919.1
Bacillus subtilis strain JCM1465 16S rRNA gene	1491	99%	NR_113265.1
Bacillus subtilis strain NBRC 13719 16S ribosomal RNA	1491	99%	NR_112629.1
Bacillus subtilis subsp. spizizenii strain NBRC 101239 16S ribosomal RNA gene, partial sequence	1491	99%	NR_112686.1
Bacillus amyloliquefaciens strain NBRC15535 16S rRNA	1491	99%	NR_112685.1
Bacillus amyloliquefaciens strain BCRC11601 16S rRNA	1491	99%	NR_116022.1
Bacillus amyloliquefaciens strain NBRC15535 16S rRNA	1491	99%	NR_041455.1
Bacillus subtilis subsp. subtilis strain OS-109 16S rRNA	1491	99%	NR_115002.1
Bacillus subtilis subsp. subtilis strain OS-44a 16S rRNA	1491	99%	NR_114997.1
Bacillus subtilis subsp. subtilis strain OS-6.2 16S rRNA	1491	99%	NR_114996.1
Bacillus subtilis subsp. subtilis strain DSM10 16S rRNA	1491	99%	NR_027552.1

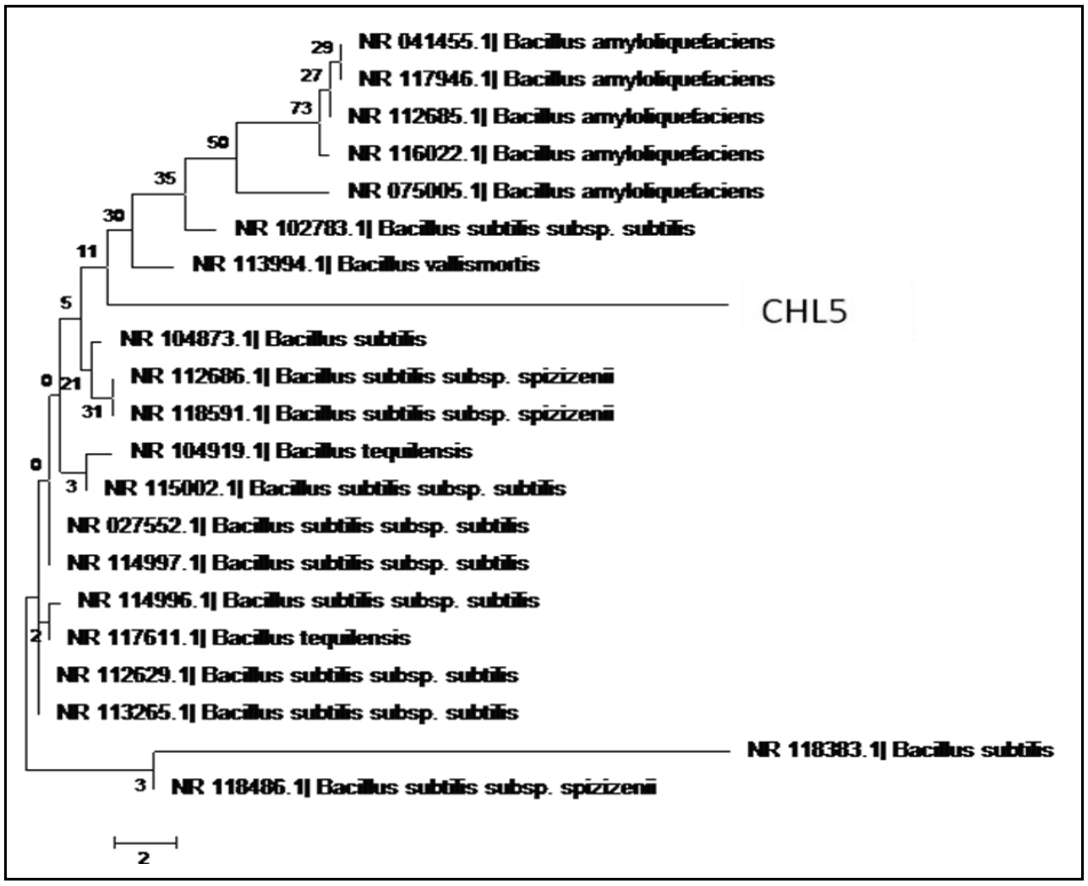

Fig. 6: Phylogenetic tree for CHL5 strain by using partial 16S rRNA gene sequence

DISCUSSION

Microalgae such as *Chlorella* are considered important as a source of dietary and recombinant proteins, novel antimicrobial substances and many other valuable compounds [6]. The microbes associated with them need to studied as understanding of these of algae–bacterial interactions are crucial to exploit their practical applications. These associated bacteria are known to have both stimulative and inhibitory effects on the growth of these microalgae [7]. These associations have been tried out for the effective treatment of wastewaters; therefore highlighting the importance of such studies [8].

In the present investigation, the amplified gene sequences of 16S rRNA gene form the bacterial isolates were efficient in suggesting the genera of these bacteria. The usefulness of bioinformatic softwares is constructing the phylogenetic trees that show the position of the sample isolate in relation with its neighbours was also demonstrated.

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