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MOLECULAR CHARACTERIZATION OF CELLULOSE DEGRADING BACTERIA ON THE BASIS OF 16S rRNA

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ABSTRACT

Culturable mesophilic (37°C) and thermophilic (60°C) cellulose degrading bacterial flora from weathered wood-like sample collected from the moist place of institute campus,were isolated. Cellulase activities were checked on plate by Congo red staining method. Molecular characterization were determined by phylogenetic analysis of the 16S rRNA sequence by various bioinformatics tools like BLAST, ClustalW, Drawtree etc. The results showed 93% resemblance with *Enterobacter luduwigi*, *Leclercia adecorboxylata*, *Enterobacter cloacae* and Endophytic bacterium. This cellulose is not consumed directly by the organism, but cellulose degrading bacteria has ability to degrade this cellulose molecule into its monomer called glucose.

KEY WORDS: Molecular characterization . bioinformatics tool, cellulose degrading bacteria

INTRODUCTION

Microbial enzymes have received increasing attention, The cellulose which is produced due to photosynthesis every year is estimated to be approximately 40 billion tons (Schwarz *et al.,* 2001). The carbon cycle is closed primarily as a result of the action of cellulose-utilizing microorganism present in soil and the guts of animals. Cellulose is renewable polymer on earth and is the major structural component of all the higher plants and animals (Sharma *et al*., 1990).Cellulose is a linear unbranched biopolymer having anhydroglucose held together by β-1, 4-linkage. Cellulose chain has strength due to interchain and intrachain hydrogen bonding. After hydrolysis with cellulase this polymer is converted into its monomer i.e. glucose. Cellulase refers to a class of enzymes produced by bacteria, fungi and protozoan's that catalyze the cellulolysis (hydrolysis) of cellulose. Cellulases are the enzymes which degrade the insoluble, abundant polymer cellulose. There are large numbers of microorganism present in the environment that degrade cellulose very efficiently.

The 16S rRNA is the most conserved gene in all the cells. Portions of 16S rRNA sequence from distantly related organism are remarkably similar. This means that sequence from distantly related organism can be precisely aligned, making the true difference easy to measure. For this reason, genes that encode the rRNA have been used extensively to determine taxonomy, phylogeny and estimate the rate of species divergence among the bacteria. Thus comparison of 16S rRNA sequence can show evolutionary relatedness among microorganism.

For this project purpose, cellulose degrading bacteria was isolated from the degraded wood sample. The morphological analysis of the isolated microbes was conducted using Gram staining test. The Congo red test is done to check for the activity of cellulose degrading bacteria. Total genomic DNA was extracted by modified CTAB method (Doyle and Doyle., 1990). This DNA was amplified by universal primer specific to 16S rRNA genes. The amplified PCR products were sequenced and BLAST analysis was done against the nucleotide sequence databases for finding out the new strain or related strain of bacteria.

Oligonucleotide Catalogin is Partial sequence characterization, in term of a comparative cataloging approach, has been used for many years since it was not feasible to determine

complete rRNA sequences (Sobieski *et al.,* 1984; Stackebrandt *et al.,* 1987; Woese, 1987). Each purified 16S rRNA is digested into short oligonucleotides, of lengths up to 20 or more bases, with ribonuclease T1 (which cleaves specially at G residues) and the resulting oligonucleotides (or sequence fragments) are collected and given as an "oligonucleotide catalog". The oligonucleotides are then separated and sequenced by two dimensional paper electrophoresis technique. The resulting oligonucleotide fingerprint comprises of particular pattern is further sequenced by digestion with various secondary and tertiary enzymes. Consequently, data from all oligonucleotide catalogs are analyzed and expressed in terms of "percentage sequence similarity" and "binary association coefficients" (SAB value) by comparisons of any pair of catalogs. Finally, dendrograms (phylogenetic tree that reveals the genetic relatedness among bacteria) are constructed using any of several clustering algorithms. There are at present three main procedures used for this purpose, distance matrix methods, maximum parsimony methods, and cluster analysis. In essence, basic to all of these methods is some mathematical criterion that allows one particular tree to be selected from the many possible alternatives for a given set of orthologous macromolecular sequences. Choosing the phylogenetic tree that minimizes the difference between the tree distances (phyletic distances) and the observed sequence differences is one such example of these methods. In addition, the higher order structures of the 16S rRNA sequence comparisons (by multiple alignment the signature positions of 16S rRNAs) also help this classification. Direct 16S rRNA Sequencing Technique To rapidly obtain sequence data, direct 16S rRNA sequencing technique has been used well, though it requires relatively large amounts of cellular RNAs and is prone to errors because only one strand is sequenced and problem of secondary structure of RNAs is often encountered. However, because of abundant ribosomal RNAs (up to 50% of total cellular RNA) in the cell, the idea of rRNA as a template for direct sequencing was possible to perform and was interesting (Lane *et al.,* 1985; Ash *et al.,* 1991; Reysenbach *et al.,* 1992). This method is carried out by using enzyme avian myeloblastosis virus reverse transcriptase and based on the knowledge of conserved sequence elements among widely divergent species which are distributed along the length of 16S rRNA gene as universal primers, and dideoxynucleotide chain termination technique to sequence nearly entire gene directly from extracted RNA (cellular RNA). The sets of sequence data from individual bacteria generated by this means serve as a oligonucleotide catalog, and analyses of these data are subsequently evaluated by the same algorithm(s) as mentioned earlier.

MATERIALS AND METHODS

Isolation of cellulose degrading bacteria

Cellulose degrading bacterial strain was isolated from degraded wood sample. Samples were collected from moist place . Take 1g of degraded wood sample and dissolve in water and make total volume 10 ml. Different dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} made by serial dilution. Take 1ml of solution from each dilution and spread on LB CMC agar plate and kept it for overnight at 37□C for growth. Colony was observed on next day. Take Single colony from LB-CMC agar plate with the help of inoculating loop in laminar air flow and subculture it on fresh LB CMC plate and kept it for overnight at 37□C for growth. This plate used as master plate. Bacterial colonies were observed on next day. Subculture this colony for screening.

Screening of bacterial strain

Cellulase activities of cells were checked on plate by Congo red staining method. Here, Congo red intercalates between the cellulose fibers, if there is any cellulase activity then the cellulose will be degraded. As a result the stains were removed during washing with NaCl and show a clear white zone. The following procedure was followed. Take bacterial colony from master plate and spot it on fresh LB CMC agar plate and kept it for overnight at 37□C for growth, Thick bacterial colonies were observed on next day. This plates were flooded with 0.01% Congo red dye, and kept it for overnight at 37□C with mild shaking. On next day distains this plate with 1M NaCl, each after every 2 hour

Morphological test

Christian Gram (1884) discovered Gram staining. It is a differential staining technique by which we can distinguish the gram positive and gram negative bacteria. Bacteria are divided into two groups, based on whether they retain or lose crystal violet after treatment with iodine and alcohol and counter staining with safranin

It is a four steps procedure, which uses certain dyes to make bacterial cell stand out against its background. Bacteria that retain the purple blue color are termed as gram positive. This is carried out by following way.

Isolation of bacterial genomic DNA.

Genomic DNA isolated by CTAB Method The bacterial cells were grown in LB CMC broth. The broth was centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded The cells were resuspended in TE buffer. Adjust the OD=1, At 600nm,the OD was adjusted by TE buffer. Transfer 740 micro liter of cell suspension was transfered to a clean centrifuge tube. 20 micro liter of lisozyme was added. It was incubated for 5 minute at room temperature. 40 micro liter of 10% SDS and 10μl of proteinase k were added It was incubated for 1 hours at room temperature. 100μl of 5M NaCl was added and it was mixed well. 100μl of CTAB/NaCl was added (heated at 65˚C). 0.5 ml of chloroform: isoamyl alcohol (24:1),were added and mixed well. The mixture was spin at 10000 rpm for 10 minute at room temperature. Upper aqueous phase was transfered to clean eppendrof tube. 0.5ml Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to aqueous phase. This mixture was centrifuge at 10000 rpm for 10 minute at room temperature. Upper aqueous phase was transfered to clean eppendrof tube and add 0.6 volume of isopropanol (-20˚C) It was incubate at room temperature for 30 minute. The spin was given at 12000 rpm for 15 minute. The pellet was washed with 70% ethanol and spin at 12000 rpm for 10 minute. The supernatant was discarded and let the pellet dry at room temperature for 5-10 minute. The pellet was resuspend in TE plus RNase (99μl +1μl).

Electrophoresis of DNA sample

After isolation of genomic DNA, this DNA was run on agrose gel, in which ethidium bromide was added. Ethidium bromide is a fluorescent dye, which intercalates between the stacked bases. The fluorescent yield of the dye: DNA complex is much greater than the unbound dye. UV irradiation at 260nm is absorbed by the DNA and transmitted to the dye and the bound dye itself absorbs radiation at 302nm and 366nm. This energy was retransmitted at 590nm, the reddish-orange region of the visible spectrum. The following protocol was followed

Quantification of DNA sample

Analysis of UV absorption by the nucleotides provides a simple and accurate estimation of the concentration of nucleic acids in a sample. Purines and pyrmidines in nucleic acid show absorption maxima around 260nm (e.g., dATP: 259nm; dCTP: 272nm; dTTP: 247nm) if the DNA sample is pure without significant contamination from proteins or organic solvents. The ratio of OD_{260}/OD_{280} should be determined to assess the purity of the sample. The following protocol was followed. 1 ml TE buffer was taken in a cuvette and calibrate the spectrophotometer at 260nm as well as 280nm. 3 μl of each DNA sample was added to 997μl TE (Tris-EDTA) buffer. TE buffer was used as a blank in the other cuvette of the spectrophotometer) and mix well. Note the OD_{260} and OD_{280} values on spectrophotometer. Calculate the OD_{260}/OD_{280} ratio. The amount of DNA was quantified using the formula.

DNA concentration (ng/μl) = OD260 x (dilution factor) x 50 μg/m

Polymarase Chain reaction

Prepare the mastermix of 50μl , in which 1μl of DNA, 38.5 μl of double distilled water, $MgCl_2$ 1μl, 1μl of dNTP, 1μl of forward primer , 1μl of reverse primer and 0.5μl of taq polymerase and kept it into the thermocycler and set the following programme. Denaturation at 94□C for 2 minute. Denaturation at 94□C for 1 minute. Annealing of primer at 50°C for 1.5 minute. Extenson of primer at 72°C for one minute for 35 cycle. Extension for 5 minute at 72°C. Storage at 4°C .

Formation of competent cell The culture was activated by keeping them at 37°C for 1 hour. The culture were incubated in kanamycin containing medium and a medium without kanamycin, to check is it kanamycin working or not. A single colony picked from the plate and was transfer to 50 ml LB broth in 500 ml flask. The culture was incubated for 3 hours at 37°C with shaking (180 cycle/minute).For efficient transformation, number of viable cell should be not more than 10^8 cell/ml (absorbance-0.4-0.6 at 600nm). The cells were aseptically transferred to sterile disposable ice cold 50ml polypropylene tube. The cultures were cooled to 0°C by storing the tube on ice for 10 minute. The cells were recovered by centrifuged at 4000 rpm for 10 minute at 4°C. The cell was resuspended in 10 ml of 0.1M CaCl and stores it on ice for 40 minute. The cells were recovered by centrifugation at 4000 rpm for 10 minute at 4°C.

Primer name	Seq.Text (5'-3')	Length	Tm (^{0}C)	Stock conc.(μM)	Working conc.(μM)
SUN27F (forward)	AGAGTTTGATCMTGGCTCAG	2O	50	100	20
SUN1492 (reverse)	TACGGYTACCTTGTTACGA	19	47	100	20

Each pellet was resuspended in 2 ml of ice cold 0.1M CaCl for each 50 ml of original culture. These are competent cell.

Transformation of PCR product:

100μl of competent cells were taken and thaw it on ice. 5μl of ligation mixture was taken and mix with competent cell, then stored on ice for 30 minute. Tubes were subjected to heat shock by keeping them in hot water bath at 42°C for 90 second. After that tubes were immediately transfer to ice. The cells were kept on ice for 1or 2 minute and then 900μl of LB broth was added. The cultures were kept on incubators shaker at 37°C for 1 hour. 100μl of culture from tube was taken and plated on agar plate containing kamamycin and a plate without kanamycin (control). The plate was then kept in incubators at 37C for overnight.

Phylogenetic analysis of 16SrRNA gene:

The sequence of PCR product was used for phylogenetic analysis by different type of bioinformatics tools, these tools includes BLAST, ClustalW and Drawtree.

RESULTS

Isolation of bacteria

Cellulolytic bacterial colonies were isolated on the basis of their ability to grow on cellulose containing media.

Screening of bacterial colonies

The bacterial colonies were spotted on fresh LB-CMC agar media from the master plate. The cellulases producing bacterial strain were identified by zone of clearance.This was revealed by Congo red staining (as shown in fig.1).

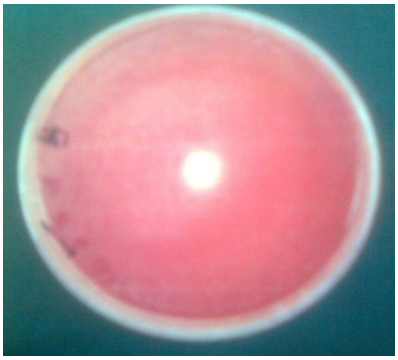

Fig 1: Screening of bacterial colonies after Congo Red treatment showing clear zone around bacterial colonies.

Identification and Morphology of bacterial strain

The isolated bacterial strain was Gram negative, because it takes pink color with safranin in Gram staining method**.**The isolated bacterial strain was rod shaped.

Isolation of bacterial genomic DNA

For the PCR amplification of 16S gene, isolation of genomic DNA was carried out by CTAB method (as shown in fig.2)

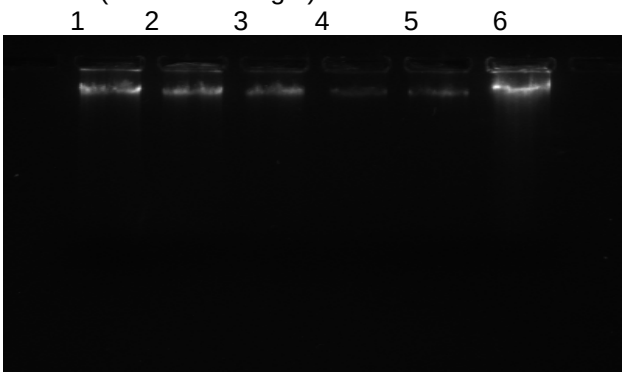

Fig 2: Lane 1, 2, 3 and 6 showing genomic DNA after running on agrose gel.

Amplification of 16S rRNA gene by PCR

The PCR of 16S rRNA gene was carried out in PTC 100 thermocycler by using universal primer (27F and 1492R) specific to 16S rRNA genes.

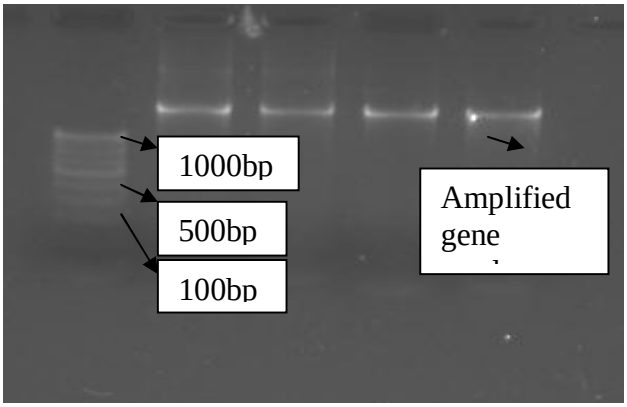

Figure 3:Shows lane1 as marker (100bp), lane 2,3,4 show the PCR amplified product .

Quantification of DNA:

Quantification of genomic DNA was carried out by spectrophotometer. The genomic DNA was diluted 333 times with TE buffer. The value at 260nm, 280nm, the ratio of 260/280 and the concentration of genomic DNA as given below:

Sample. No	Reading at 260nm	Reading at 280nm	260/280	Concentration (ng/μl)
1	.003	.002	1.5	49.95
2	.010	.008	1.25	166.5

Isolation of plasmid

Isolation of plasmid (DH5α E.coli strain) was carried out by miniprep method.

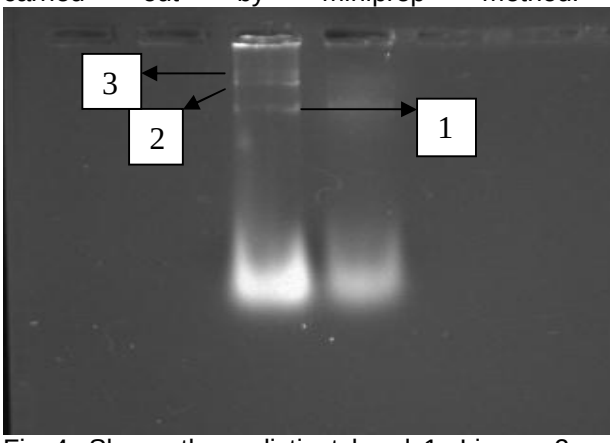

Fig 4: Shows three distinct band 1- Linear, 2-Circular and 3-Supercoiled plasmid

Ligation and Transformation:

After amplification of 16S gene, this gene was cloned by TA cloning method. But the ligase was used in this experiment was degraded due to some storage problem. So after transformation of this ligated vector to competent cell of DH5 strain of E.Coli, there was no appearance of any colonies on selected medium (ampicilin).

Phylogenetic analysis of 16SrRNA gene sequence

Forward primer sequence

CGTACGCGGTATACGCTTAGCAGTCGAACG
GTAGCACAGAGAGCTTGCTCTCGGGTGACG
AGTGGCGGACGGGTGAGTAA
TGTCTGGGAAACTGCCTGATGGAGGGGGAT
AACTACTGGAAACGGTAGCTAATACCGCATA
ACGTCGCAAGACCAAAGAG
GGGGACCTTCGGGCCTCTTGCCATCAGATG
TGCCCAGATGGGATTAGCTAGTAGGTGGGG
TAATGGCTCACCTAGGCGAC
GATCCCTAGCTGGTCTGAGAGGATGACCAG
CCAGGCTGGAACTACTTGACGGCCCCCACT
CCTTCCGGAGGTTTATCACT
GGAATATTCCTTTGAGTGCCCGGCCCTGCC
GCTGGCATCCCACGAGAATGGTTGAGGTCT
TTGCGGGACTTAACCCTTTA
TTTCAGAACAAAAGCTGACGACAGCCATGCA
GCACCTGTCTCAAAGTTCCCGAAGGCACCA
AAGCATCTCTGCTAAGTTC
TCTGGATGTCAAGAGTAGGTAAGGTTCTTCG
CGTTGCATCGAAATAAAACACATGCTGCACC
GCTTGTGCGGGCGCCCGT
CAATACATTATAGTTTTAACCATGCTGCGGT
ACTCCCCACGAGGTCGACTGATCGCGTTAT
CTCCAGAGGCCACTACTCC
AGGGGAGCAACCTCCAAATGGACATCGTTT
AGACGGAGTACTACTACGGGATCGAATACT
GTTGGCTGCTCACGATGTCT
CACCTGAGAATCAGTGGTTTTCCAACGAGGA
CGACCATCGTCAGGAGGAGTCCTCCTGATA
ATTTCTAAGTTTTCAGCTG
CATCCGGTAGAGATCCGCCCTCTACAACAAA
GATTTAAGCAGCTTGTAAGAAGGTACTTCGT
TGAGTGCGCCGATGTTAA
TTACTTACTGGTGGACCGACGGCGGGGAGT
CTCTGCCGTCAATTCGATCAAACAGGAACGA
CCTCCTCATTATCTAAGCT
GCTGGAAGGAAAATGAAACTATGGCGTTGTT
CTCTCTGGTGAGAGAACACATGCATGCGGG
TGATGACCTGAAAAACAAT
ACCCCTCGGCCAAGAAAATTGAGTATCCAAC
CCGCGAGTGTCACAATAACACATGGTG

Reverse primer sequence

CGGCGGAACCACTATGTGCTAGCGCCCTCC
CGAAGGTTAAGCTACCTACTTCTTTTGCAAC
CCACTCCCATGGTGTGACG
GGCGGTGTGTACAAGGCCCGGGAACGTATT
CACCGTAGCATTCTGATCTACGATTACTAGC
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CGAGTTGCAGACTCCAATCCGGACTACGAC
GCACTTTATGAGGTCCGCTTGCTCTCGCGA
GGTCGCTTCTCTTTGTATGC
GCCATTGTAGCACGTGTGTAGCCCTACTCGT
AAGGGCCATGATGACTTGACGTCATCCCCA
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CAAAGCATCTCTGCTAAGTT
CTCTGGATGTCAAGAGTAGGTAAGGTTCTTC
GCGTTGCATCGAATTAAACCACATGCTCCAC
CGCTTGTGCGGGCCCCCG
TCAATTCATTTGAGTTTTAACCTTGCGGCCG
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GCTCCGGAAGCCACTCCTC
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GTTTGCTCCCCACGCTTTC
GCACCTGAGCGTCAGTCTTTGTCCAGGGGG
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TCTACGCATTTCACCGCTAC
ATCTGGAAGTCTATCCCCCCTCTACAAGACT
CTAGCCTGCCAGTTTCGAATGCAGTTTCCCC
ACGTTGAGCGCGGGGAGT
TCACATCCTACTTGACAGACCGCCTGCGTG
CGCTTTACGCCCAGTAAATCCGATCAACGCT
TGGCACCCTCCGCTATTAC
TGAGGCTGCTGGCACGGAAGTTAAGACTGG
TGCGGTTCCTGCCAGCGAAGATCAATTGACT
GCGGTGATGACAACAACAC

Multiple Alignment Sequence

CLUSTAL 2.0.12 multiple sequence alignment

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G-GCGGTCTGTCAAGTCGGA 553
gi|220967120|gb|FJ603034.1|
ATCGGAATTACTGGGCGTAAAGCGCACGCA
G-GCGGTCTGTCAAGTCGGA 550
gi|226897562|gb|FJ859683.1|
ATCGGAATTACTGGGCGTAAAGCGCACGCA
G-GCGGTCTGTCAAGTCGGA 592
gi|162286803|emb|AM778415.1|
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gi|157073875|dbj|AB273740.1|
TTGAGGAGTGGCTTCCGGAGCTAACGCGTT
AAGTCGACCGCCTGGGGAGT 884
gi|282182211|gb|GU253335.1|
TTGAGGAGTGGCTTCCGGAGCTAACGCGTT
AAGTCGACCGCCTGGGGAGT 883
gi|281322989|gb|GU265700.1|

Sf10
TAGAGATCCGCCCTCTACAACAAA-
GATTTAAGCAGCTTGTAAGAAG-GT 855
gi|239505188|gb|FJ605381.1|
ACGGCCGCAAG-
GTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGC 898
gi|131054037|gb|EF446899.1|
ACGGCCGCAAG-
GTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGC 891
gi|220967120|gb|FJ603034.1|
ACGGCCGCAAG-
GTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGC 888
gi|226897562|gb|FJ859683.1|
ACGGCCGCAAG-
GTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGC 928
gi|162286803|emb|AM778415.1|
ACGGCCGCAAG-
GTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGC 934
gi|157073875|dbj|AB273740.1|
ACGGCCGCAAG-
GTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGC 933
gi|282182211|gb|GU253335.1|
ACGGCCGCAAG-
GTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGC 932
gi|281322989|gb|GU265700.1|

Sf10
ACTTCGTTGAGTGCGCCGATGTTAATTACTT
ACTGGTGGACCG-ACG-GC 903
gi|239505188|gb|FJ605381.1|
GGTGGAGCATGTGGT-
TTAATTCGATGCAACGCGAAGAACCTTACCT
ACT 947
gi|131054037|gb|EF446899.1|
GGTGGAGCATGTGGT-
TTAATTCGATGCAACGCGAAGAACCTTACCT
ACT 940
gi|220967120|gb|FJ603034.1|
GGTGGAGCATGTGGT-
TTAATTCGATGCAACGCGAAGAACCTTACCT
ACT 937
gi|226897562|gb|FJ859683.1|
GGTGGAGCATGTGGT-
TTAATTCGATGCAACGCGAAGAACCTTACCT
ACT 977
gi|162286803|emb|AM778415.1|
GGTGGAGCATGTGGT-
TTAATTCGATGCAACGCGAAGAACCTTACCT
ACT 983
gi|157073875|dbj|AB273740.1|
GGTGGAGCATGTGGT-
TTAATTCGATGCAACGCGAAGAACCTTACCT
ACT 982
gi|282182211|gb|GU253335.1|
GGTGGAGCATGTGGT-
TTAATTCGATGCAACGCGAAGAACCTTACCT
ACT 981
gi|281322989|gb|GU265700.1|

Sf10 GG-
GGAGTCTCTGCCGTCAATTCGATCAAACAG
GAACGACCTCCTCATTA 952
gi|239505188|gb|FJ605381.1|
CTTGACATCCAGAGAACTTTCCAGAGATGGA
TTGGTGCCTTCGGGAACTC 997
gi|131054037|gb|EF446899.1|
CTTGACATCCAGAGAACTTTCCAGAGATGGA
TTGGTGCCTTCGGGAACTC 990
gi|220967120|gb|FJ603034.1|
CTTGACATCCAGAGAACTTAGCAGAGATGCT
TTGGTGCCTTCGGGAACTC 987
gi|226897562|gb|FJ859683.1|
CTTGACATCCAGAGAACTTAGCAGAGATGCT
TTGGTGCCTTCGGGAACTC 1027
gi|162286803|emb|AM778415.1|
CTTGACATCCAGAGAACTTAGCAGAGATGCT
TTGGTGCCTTCGGGAACTC 1033
gi|157073875|dbj|AB273740.1|
CTTGACATCCAGAGAACTTAGCAGAGATGCT
TTGGTGCCTTCGGGAACTC 1032
gi|282182211|gb|GU253335.1|
CTTGACATCCACGGAACTTAGCAGAGATGCT
TTGGTGCCTTCGGGAACCG 1031
gi|281322989|gb|GU265700.1|

Sf10
TCTAAGCTGCTGGAAG------
GAAAATGAAACTATGGCGTTGTTCTCTC 996
gi|239505188|gb|FJ605381.1|
TGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGG 1047

gi|131054037|gb|EF446899.1|
TGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGG 1040
gi|220967120|gb|FJ603034.1|
TGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGG 1037
gi|226897562|gb|FJ859683.1|
TGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGG 1077
gi|162286803|emb|AM778415.1|
TGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGG 1083
gi|157073875|dbj|AB273740.1|
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TCGTGTTGTGAAATGTTGGG 1082
gi|282182211|gb|GU253335.1|
TGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGG 1081
gi|281322989|gb|GU265700.1|

Sf10 TG-
GTGAGAGAACACATGCATG--
CGGGTGATGACCTGAAAAA 1036
gi|239505188|gb|FJ605381.1|
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TTTGTTGCCAGCGGTNCGG 1097
gi|131054037|gb|EF446899.1|
TTAAGTCCCGCAACGAGCGCAACCCTTATCC
TTTGTTGCCAGCGGTCCGG 1090
gi|220967120|gb|FJ603034.1|
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TTTGTTGCCAGCGGTTAGG 1087
gi|226897562|gb|FJ859683.1|
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TTTGTTGCCAGCGGTTAGG 1127
gi|162286803|emb|AM778415.1|
TTAAGTCCCGCAACGAGCGCAACCCTTATCC
TTTGTTGCCAGCGGTYMGG 1133
gi|157073875|dbj|AB273740.1|
TTAAGTCCCGCAACGAGCGCAACCCTTATCC
TTTGTTGCCAGCGGTTAGG 1132
gi|282182211|gb|GU253335.1|
TTAAGTCCCGCAACGAGCGCAACCCTTATCC
TTTGTTGCCAGCGGTTCGG 1131
gi|281322989|gb|GU265700.1|

Sf10
CAATACCCCTCGGCCAAGAAAAT--
TGAGTATCCA--ACCCGCGAGTGTC 1082
gi|239505188|gb|FJ605381.1|
CCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGAT 1147
gi|131054037|gb|EF446899.1|
CCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGAT 1140
gi|220967120|gb|FJ603034.1|
CCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGAT 1137
gi|226897562|gb|FJ859683.1|
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AACTGGAGGAAGGTGGGGAT 1177
gi|162286803|emb|AM778415.1|
CCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGAT 1183
gi|157073875|dbj|AB273740.1|
CCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGAT 1182
gi|282182211|gb|GU253335.1|
CCGGGAACTCAAAGGAGACTGCCAGTGATA
AACTGGAGGAAGGTGGGGAT 1181
gi|281322989|gb|GU265700.1|

Sf10
ACAATAACACATGGTG--------- 1098
gi|239505188|gb|FJ605381.1|
GACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCTACAA 1197
gi|131054037|gb|EF446899.1|
GACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCTACAA 1190
gi|220967120|gb|FJ603034.1|
GACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCTACAA 1187
gi|226897562|gb|FJ859683.1|
GACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCTACAA 1227
gi|162286803|emb|AM778415.1|
GACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCTACAA 1233
gi|157073875|dbj|AB273740.1|
GACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCTACAA 1232
gi|282182211|gb|GU253335.1|
GACGTCAAGTCATCATGGCCCTTACGAGTA
GGGCTACACACGTGCTACAA 1231
gi|281322989|gb|GU265700.1|---------Sf10
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GAGCAAGCGGACCTCATAAA 1247
gi|131054037|gb|EF446899.1|
TGGCGCATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGACCTCATAAA 1240
gi|220967120|gb|FJ603034.1|
TGGCGCATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGACCTCATAAA 1237
gi|226897562|gb|FJ859683.1|
TGGCGCATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGACCTCATAAA 1277
gi|162286803|emb|AM778415.1|
TGGCGCATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGACCTCATAAA 1283

gi|157073875|dbj|AB273740.1|
TGGCGCATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGACCTCATAAA 1282
gi|282182211|gb|GU253335.1|
TGGCATATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGACCTCATAAA 1281
gi|281322989|gb|GU265700.1|---------Sf10
gi|239505188|gb|FJ605381.1|
GTGCGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGA 1297
gi|131054037|gb|EF446899.1|
GTGCGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGA 1290
gi|220967120|gb|FJ603034.1|
GTGCGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGA 1287
gi|226897562|gb|FJ859683.1|
GTGCGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGA 1327
gi|162286803|emb|AM778415.1|
GTGCGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGA 1333
gi|157073875|dbj|AB273740.1|
GTGCGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGA 1332
gi|282182211|gb|GU253335.1|
GTATGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGA 1331
gi|281322989|gb|GU265700.1--------Sf10

gi|239505188|gb|FJ605381.1|
ATCGCTAGTAATCGTAGATCAGAATG----------
---------- 1323
gi|131054037|gb|EF446899.1|
ATCGCTAGTAATCGTAGATCAGAATGCTACG
GTGAATACGTTCCCGGGCC 1340
gi|220967120|gb|FJ603034.1|
ATCGCTAGTAATCGTAGATCAGAATGCTACG
GTGAATACGTTCCCGGGCC 1337
gi|226897562|gb|FJ859683.1|
ATCGCTAGTAATCGTAGATCAGAATGCTACG
GTGAATACGTTCCCGGGCC 1377
gi|162286803|emb|AM778415.1|
ATCGCTAGTAATCGTAGATCAGAATGCTACG
GTGAATACGTTCCCGGGCC 1383
gi|157073875|dbj|AB273740.1|
ATCGCTAGTAATCGTAGATCAGAATGCTACG
GTGAATACGTTCCCGGGCC 1382
gi|282182211|gb|GU253335.1|
ATCGCTAGTAATCGTGGATCAGAATGCCACG
GTGAATACGTTCCCGGGCC 1381
gi|281322989|gb|GU265700.1----------Sf10

gi|239505188|gb|FJ605381.1| ----------
----

gi|131054037|gb|EF446899.1|
TTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGG 1390
gi|220967120|gb|FJ603034.1|
TTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGG 1387
gi|226897562|gb|FJ859683.1|
TTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGG 1427
gi|162286803|emb|AM778415.1|
TTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGG 1433
gi|157073875|dbj|AB273740.1|
TTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGG 1432
gi|282182211|gb|GU253335.1|
TTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGG 1431
gi|281322989|gb|GU265700.1| ----------

Sf10 ----------

gi|239505188|gb|FJ605381.1| ----------
----
gi|131054037|gb|EF446899.1|
TAGCTAACTTT----------
1401
gi|220967120|gb|FJ603034.1|
TAGCTTAACCTTCGGGAGGGCGCTTACC-----
---------- 1415
gi|226897562|gb|FJ859683.1|
TAGCTTAACCTTCGGGAGGGCGCTTACCAC
TTTGTGATTCATGACTGGGG 1477
gi|162286803|emb|AM778415.1|
TAGCTTAACCTTCGGGAGGGCGCTTACCAC
TTTGTGATTCATGACTGGGG 1483
gi|157073875|dbj|AB273740.1|
TAGCTTAACCTTCGGGAGGGCGCTTACCAC
TTTGTGATTCATGACTGGGG 1482
gi|282182211|gb|GU253335.1|
TAGCTTAACCTTCGGGAGGGCGCTTACCAC
TTTGTGATTCATGACTGGGG 1481
gi|281322989|gb|GU265700.1| ----------

Sf10 ----------

gi|239505188|gb|FJ605381.1| ----------
----
gi|131054037|gb|EF446899.1| ----------
----
gi|220967120|gb|FJ603034.1| ----------
----
gi|226897562|gb|FJ859683.1|
TGAAGTCGTAACAAGGTACCGT----------
---------- 1499

gi|162286803|emb|AM778415.1|
TGAAGTCGTAACAAGGTAACCGTAGGGGAA
CCTGCGGYTGGATCACCTCC 1533
gi|157073875|dbj|AB273740.1| TGAAGT------
---------- 1488
gi|282182211|gb|GU253335.1|
TGAAGTCGTAACAAGGTAACCGTAA----------
---------- 1506
gi|281322989|gb|GU265700.1|----------

Sf10
gi|239505188|gb|FJ605381.1|:0.00841,
gi|131054037|gb|EF446899.1|:0.00747)
:0.00619,
gi|281322989|gb|GU265700.1|:-0.16834,
Sf10:0.50648)
:0.18717,
gi|282182211|gb|GU253335.1|:0.00113)
:0.00587,
gi|157073875|dbj|AB273740.1|:-0.00141)
:0.00466)
:0.00184,

gi|220967120|gb|FJ603034.1|:0.00237,
gi|226897562|gb|FJ859683.1|:0.00187)
:0.00105,
gi|162286803|emb|AM778415.1|:0.00607);

The multiple sequence alignment of 16SrRNA gene sequence of our bacterial strain with other related bacterial strain of databases.
Red = different nucleotides Green= insertion
1. >gi|239505188|: Enterobacter cloacae strain
2.>gi|281322989|: Leclercia adecarboxylata strain
3. >gi|131054037|: Pantoea agglomerans strain
4. >gi|282182211|: Klebsiella oxytoca strain
5. >gi|157073875|: Leclercia adecarboxylata
6. >gi|220967120|: Endophytic bacterium
7. >gi|226897562|: Enterobacter ludwigii strain
8.>gi|162286803|emb|AM778415.1|
Enterobacter cloacae

DISCUSSION

The present work was undertaken to isolate cellulose degrading bacteria from degraded wood sample and characterize on the basis of 16S rRNA gene. The bacteria were grown on LB-CMC agar. For the screening of bacteria, Congo red test was performed. Congo red is the sodium salt of benzidinediazo-bis-1-naphthylamine-4-sulfonic acid (formula: $C_{32}H_{22}N_6Na_2O_6S_2$) and it has a strong, though apparently non-covalent affinity to cellulose fibers but not have affinity for glucose. Cellulase enzyme was produced by this bacteria hydrolyzed cellulose to its monomer .i.e glucose. Glucose does not bind to Congo red dye, so after treatment with NaCl form clear zone around the bacterial colony because NaCl destains the region where Congo red dye was not bind.

The bacteria was tested negative for Gram staining, because the outer layer of Gram negative bacteria cell-wall is made up of Lypopolysacchride and Protein and it covers a very few thin layers of Peptidoglycan as compaired to Gram positive bacteria. So in Gram' stain test, they do not retain the crystal violet color in their cell wall.The bacteria cell-wall hold the pink dye once a counterstain chemical is used.

This bacteria characterize on the basis of 16S rRNA gene, The use of 16S rRNA sequence to study the bacterial phylogeny and taxonomy has been by far the most common housekeeping gene genetic marker used for number of reason. These reasons includes: 1.its presence in almost all bacteria, often existing as multigenic family or operons; 2. the function of the 16Sr RNA gene over the time has not changed, suggesting that random sequence changes are a more accurate measure of evolution and 3.The 16S rRNA gene (1500) is large enough for informatics purpose. The explosion in the number of recognized taxa is directly attributable to the ease in performance of 16S rRNA gene sequencing studies as opposed to the more cumbersome manipulations involving DNA-DNA hybridization (Janda and Abbott, 2007).

Genomic DNA was isolated from the bacteria by CTAB method. Quantification of this was carried out by spectrophotometer and showed that concentration of this DNA was very low due to the small growth bacteria in LB-CMC broth.

PCR amplification of this DNA was carried out using universal primer specific to 16S rRNA gene. The amplified product was run on 1% agarose gel with 100bp DNA ladder. After running the gel for 1hour, the size of amplified PCR product was around 1300bp with respect to DNA ladder. This result gave us the conformation that the region amplified was 16Sr RNA region because size of 16SrRNA lies in this range.
For sequence analysis of this PCR product, cloning of PCR product was carried out by TA cloning method. For this purpose competent cell

(DH5α strain of *E.Coli*) were prepared. To check, whether cells were competent or not, transformation with isolated plasmid containing selected marker was carried out. This result proved, the competency of the cells .

For cloning of PCR product, ligation of PCR product with TA vector was carried out by ligases. After ligation, this vector was transformed in competent cell. But transformation failed due to degradation of ligase due to some storage problem.

For sequence analysis, direct sequencing of PCR product was carried out. The sequence of this 16Sr RNA was analysed by various bioinformatics tools like BLAST, ClustalW, Drawtree etc. The results showed 93% resemblance with *Enterobacter luduwigi*, *Leclercia adecorboxylata*, *Enterobacter cloacae* and Endophytic bacterium.All these results showed that the isolated organism was different from already known organism.This study is beneficial as by having a detailed knowledge about the cellulose degrading bacteria. Because cellulose is the most abundant molecule on the earth. This cellulose is not consumed directly by the organism, but cellulose degrading bacteria has ability to degrade this cellulose molecule into its monomer called glucose. The glucose is one of the major energy source for all kind of organism from plants to animals. So characterizing this cellulose degrading bacterial species is one of the very good strategies to obtain energy from this abundant cellulose molecule.

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