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RESEARCH ARTICLE

16S rDNA IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *XANTHOMONADACEAE NML 03-0222* BACTERIAL ISOLATES FROM AGRICULTURAL SOIL OF JHARKHAND.

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ABSTRACT

The traditional method for identification of bacterial species depends upon different biochemical testing and colonial morphology study. Biochemical tests are simple to execute, require minimal equipment, and generally accurately differentiate between the more common species. However, they are time-consuming and results delay in final identification due to long incubation time period. Several genes common to all bacteria have been studied in sequence-based identification. The objective o present study is to isolate, identify and characterize the bacteria from agricultural farm field of Jharkhand. The use of 16S rDNA gene sequence analysis method, is considered as recent standard approach for identification for all bacteria and is widely recognized as a rapid and accurate method for identification of novel bacterial culture. Fragment of 16S rDNA gene of the DNA was isolated from the bacterial species and was amplified by PCR. DNA sequencing reaction of purified PCR amplicon was carried out with (16sF) and (16sR) forward and reverse primers. Basic Local Alignment Search Tool (BLAST) was performed and the evolutionary distances were computed using the Kimura 2-parameter method and expressed in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in (MEGA 4.0) software for 1447 nt contig region of sample G. It was observed to be homologous with sequence of Genbank having accession no. EU313791.1. and was identified as *Xanthomonadaceae NML 03-0222.*

INTRODUCTION

The bacterial microorganisms always played an important role in increasing soil fertility of agricultural fields. The traditional methods of bacterial identification have resulted in major drawbacks as they are extremely slow and difficult for the identification of slow growing organisms, non cultivable organisms and with the organisms exhibiting biochemical characteristics that do not fit into patterns of any known genus and species. (Woo *et al.,* 2003). Several methods and approaches are nowadays available to generate information on microorganisms that resides in soil. The 16S rDNA molecular gene sequencing method is one of the advanced and recently used methods for the identification of microorganisms. Furthermore, 16S rDNA gene sequencing approach also permits the identification of the surviving and culturable bacterial species, based on employing highly conserved 16S rDNA oligonucleotide primers for the eubacteria with an intervening hypervariable gene sequence, which could be used as signature sequence to aid in species identification (Amann *et al*., 1995; Ward *et al*., 1990). Bacteria classified as members of the genus *Xanthomonadaceae*, the sole family in the *Xanthomonadales* order, in the γ-Proteobacteria has fluctuated since the first description of the genus (Dowson, 1939). The genus *Xanthomonadaceae* is now defined as including in gram-negative protobacteria, that are strictly aerobic, motile, catalase and oxidase positive (Schaad, *et. al*., 2001). Xanthomonas species found in soil can increase plant growth in several ways. They may produce a compound that inhibits the growth of pathogens or reduces invasion of the plant by a pathogen. They may also produce compounds (growth factors) that directly increase plant growth (Ann Kennedy). Initially, biochemical and physiological tests were used for rapid differentiation among *Xanthomonas* isolates. However, these tests proved insufficient for inter and intra-species differentiation (Dye, 1962). The present study aims to characterize the identification bacterial flora isolated from agricultural soil using morphology, biochemical and 16S rDNA gene sequence analysis approach.

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MATERIALS AND METHODS

Soil samples were collected from the agricultural field of ICAR, Research Complex for Eastern Region, Plandu, Ranchi, Jharkhand on february, 2012 as per the method of (Dutta *et al*., 2010). The isolation of bacterial culture was obtained by spread

Table 1. Morphological characteristics of (Sample G) *Xanthomonadaceae NML 03-0222*

Sample Name	Isolate	Cell morphology	Colonial shape	Elevation	Color	Gram stain
G	Xanthomonadaceae NML 03-0222	Rods or coccobacilli	rod	Convex	Yellow	Negative

Table 2. Biochemical characteristics of (Sample G) *Xanthomonadaceae NML 03-0222*

Isolated Sample	Nitrate reduction	Glucose fermantation	Catalase	Oxidase	Motility	urease
Xanthomonadaceae NML 03-0222	-	-	+	+	+	+

Table 3. (BLAST) Sequence alignment analysis of (Sample G) *Xanthomonadaceae NML 03-0222* having Significant E-value and maximum identitity

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
EU313791.1	Xanthomonadaceae bacterium NML 03-0222 16S ribosomal RNA gene	2612	2612	100%	0.0	100%
JF240812.1	Uncultured bacterium clone ncd2808b04c1 16S ribosomal RNA gene	2518	2518	96%	0.0	99%
JF240656.1	Uncultured bacterium clone ncd2805b12c1 16S ribosomal RNA gene	2512	2512	96%	0.0	99%
JF045126.1	Uncultured bacterium clone ncd558d08c1 16S ribosomal RNA gene	2512	2512	96%	0.0	99%
HM274483.1	Uncultured bacterium clone ncd558d08c1 16S ribosomal RNA gene	2512	2512	96%	0.0	99%
JF125670.1	Uncultured bacterium clone ncd1456f10c1 16S ribosomal RNA gene	2479	2479	96%	0.0	99%
JF092066.1	Uncultured bacterium clone ncd1073e05c1 16S ribosomal RNA gene	2479	2479	96%	0.0	99%
JF021770.1	Uncultured bacterium clone ncd491h07c1 16S ribosomal RNA gene	2479	2479	96%	0.0	99%
HM336262.1	Uncultured bacterium clone ncd1073e05c1 16S ribosomal RNA gene	2479	2479	96%	0.0	99%
HM316065.1	Uncultured bacterium clone ncd491h07c1 16S ribosomal RNA gene	2479	2479	96%	0.0	99%

Table 4. Distance Matrix (Sample G) *Xanthomonadaceae NML 03-0222*

Sample-G	1		0.000	0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002
EU313791.1	2	0.000		0.001	0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002
JF240812.1	3	0.001	0.001		0.001	0.001	0.001	0.002	0.002	0.002	0.002	0.002
JF240656.1	4	0.001	0.001	0.001		0.001	0.001	0.002	0.002	0.002	0.002	0.002
JF045126.1	5	0.001	0.001	0.001	0.001		0.000	0.002	0.002	0.002	0.002	0.002
HM274483.1	6	0.001	0.001	0.001	0.001	0.000		0.002	0.002	0.002	0.002	0.002
JF125670.1	7	0.006	0.006	0.005	0.006	0.006	0.006		0.002	0.002	0.002	0.002
JF092066.1	8	0.006	0.006	0.005	0.006	0.006	0.006	0.004		0.002	0.002	0.002
JF021770.1	9	0.006	0.006	0.005	0.006	0.006	0.006	0.004	0.004		0.002	0.002
HM336262.1	10	0.006	0.006	0.005	0.006	0.006	0.006	0.004	0.000	0.004		0.002
HM316065.1	11	0.006	0.006	0.005	0.006	0.006	0.006	0.004	0.004	0.000	0.004	

plate technique (Ahmed and Ahmed, 2006). The pure colony streaked out, and was sub cultured on Nutrient Agar and labeled as sample "G". Morphological characteristics and different biochemical tests were performed by methodologies as described (Green and Bousfield, 1983) for further identification. The isolation of DNA from the pure culture (marked as sample "G") was obtained as per the protocol of (Van *et al.,* 1997), which included mechanical lysis of cells, phenol and chloroform extractions, a potassium acetate precipitation. Its quality was evaluated on 1.2% Agarose gel (Peixoto *et al*., 2002) .The 16S rDNA gene was amplified as per the method of (Li *et al*, 2004) using universal primer and PCR product for the isolate was sequenced in both directions.

DNA sequencing in forward and reverse directions for PCR amplicon was carried out with forward primer (16sF) and reverse primer (16sR) using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer and chromatogram was plot using Chromas Lite 2.0 version Applied Biosystems and Amersham MegaBace automated sequencers. The 16S rDNA gene sequence was further analyzed using Basic Local Search Alignment Search Tool (BLAST) against nrdatabase of NCBI GenBank database to find closely related bacterial 16S rDNA sequences. Based on maximum percent (%) identity score the first ten sequences were selected and aligned using (MSA) multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed according to Neighbour-joining method (Saitou and Nei, 1987) using MEGA 4.0 Molecular Evolutionary Genetics Analysis software version 4 (Tamura *et al.*, 2007) for studying the evolutionary relationship. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in (< 50%) of bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset using complete deletion option.

RESULTS AND DISCUSSION

The colony marked as (sample G) was rod shaped or coccobacilli, convex, yellow in colour and slightly opaque (Table 1). It showed negative reaction for gram stain, and was found to be related as *Xanthomonas sp*. as confirmed by the

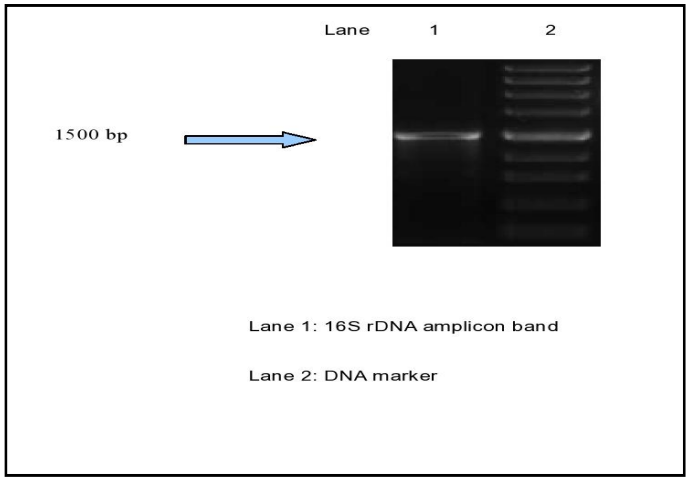
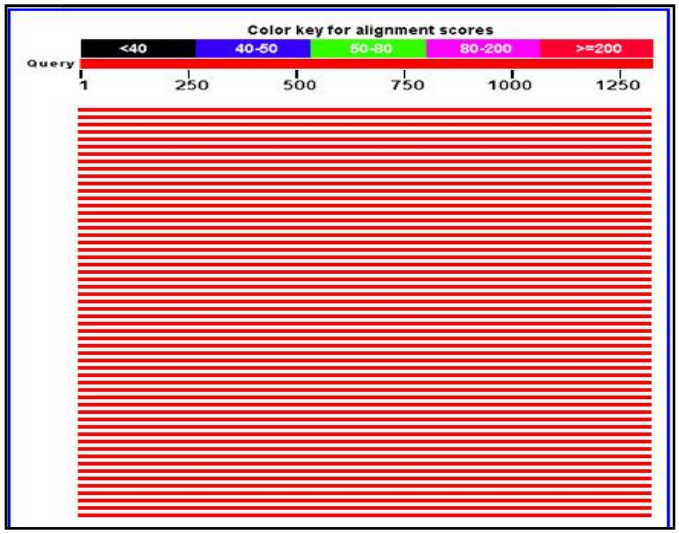


Fig. 1. Gel Image of 16S rDNA amplicon of (Sample G) *Xanthomonadaceae NML 03-0222*

findings of (Schaad and White., 1974) and (Soudi, *et. al*., 2006). Similar results were obtained by (Gomes, *et. al*., 2003) for morphological characterization of *Xanthomonas* colonies. Further biochemical characteristics were studied and it was observed that bacterial isolate was identified as *Xanthomonas* as shown in (Table 1). In biochemical characterization, *Xanthomonas* showed negative result for urease, Glucose fermentation, Nitrate reduction and positive results with oxidase test, Catalase, motility (Table 2) which is favoured by similar observations of (Saudi, *et al*., 2011) who stated that *Xanthomonas sp*. showed positive results with oxidase, catalase, urease and thus was catalase, urease, oxidase positive as in our case also. On quality evaluation of all isolated DNA, a single band of high molecular weight DNA was observed on 1.2% Agarose Gel . A single discrete PCR amplicon band of 1500 bp of 16S rDNA was observed when resolved on agarose Gel (Kimura, 1980) as in (Fig. 1). Similar results were obtained by (Orengo *et. al*., 2010) on amplification of 16S rDNA using

Fig. 2. Chromatogram plot of (Sample G) *Xanthomonadaceae NML 03-0222*

universal primer an amplicon band of 1.5kb was obtained on electrophoresis. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 1414 positions in the final dataset of sample "G". Table 3 and Table 4 describes the significant alignments produced and distance matrix respectively. BLAST search tool was carried out with the nrdatabase of NCBI GenBank database as shown in (Fig. 3).



Distribution of 100 Blast Hits on the Query Sequence

Fig. 3. Basic Local Alignment Search Tool (BLAST) Alignment view using combination of NCBI GenBank data for (Sample G) *Xanthomonadaceae NML 03-0222*

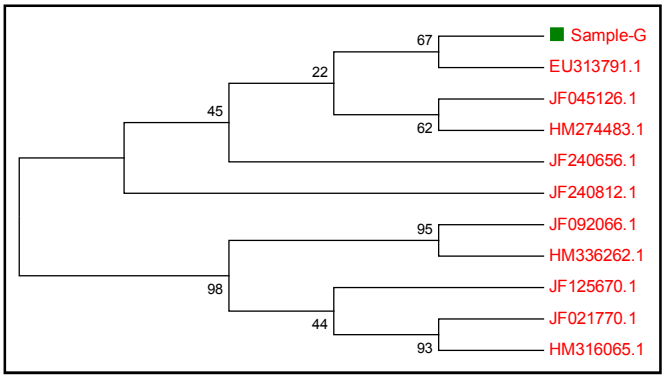


Fig. 4. Phylogenetic Tree for the evaluation of evolutionary relationships of 11 taxa including (Sample G) *Xanthomonadaceae NML 03-0222*

The sequence generated were homologous to sequences with GenBank Accession No:- EU313791.1. Chromatogram (Fig 2.) gives the detailed gene sequence of the sample obtained. Based on nucleotide homology and phylogenetic analysis, (Gurtner *et al*., 2001) sample marked as "G" was found to be *Xanthomonadaceae NML 03-0222*. A phylogenetic tree or evolutionary tree is a branching diagram or tree showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. Fig.4. shows the phylogenetic tree of sample "G" with the ten most similar sequences. Similar results were obtained by (Hauben, *et al*., 1997) while carrying out identification of *Acinetobacter* by 16 S rDNA technique. The taxa joined together in the tree are implied to have descended from a common ancestor. Unrooted trees illustrate the relatedness of the leaf nodes without making assumptions about ancestry at all. In the case of unrooted trees, branching relationships between taxa are specified by the way they are connected to each other, but the position of the common ancestor is not (Maher, 2002).

Conclusion

Xanthomonadaceae NML 03-0222 was considered as beneficial soil bacterial species and was identified successfully by 16S rDNA method.

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