ISOLATION, CHARACTERIZATION AND MOLECULAR IDENTIFICATION OF BURKHOLDERIA CEPACIA FROM *CURCUMA LONGA* USING 16S RRNA SEQUENCING

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ABSTRACT

Curcuma longa is well known for its use as spice and medicine. The remarkable feature of the plant is the presence of rhizome, which provides an interesting habitat for association by various groups of bacteria. The present study deals with isolation, identification and analysis of bacteria from *Curcuma longa* through 16S rRNA based molecular technique and samples were obtained from *Curcuma longa.* The Bacterial strain was isolated and characterized using various biochemical tests and confirmed through molecular approach and 16S rRNA gene was amplified using suitable primers. The amplified 16S rRNA gene sequence was compared with the sequence in NCBI sequence database the bacterial strain was identified. Phylogenetic and molecular evolutionary analyses were conducted using 16S rRNA sequencing. The sequences when submitted to NCBI gene bank database using BLAST showed 99 – 100% maximum identity and have shown good similarity with Burkholderi sps. 2xiao7 strain Burkholderi cepacia strain RRE5, Burkholderi cepacia strain RRE3, Burkholderi cepacia strain CG4, Burkholderi cepacia T-34gene. In the present study we investigated extracts of *Curcuma longa* rhizomes. Among the isolates, Burkholderi cepacia isolate was the most active.

KEYWORDS: *Curcuma longa,* rhizomes, Burkholderia cepacia*, NCBI.

INTRODUCTION

Plants have the major advantage of being the most effective and cheaper alternative source of drugs. Historically, pharmacological screening of compounds of natural origin has been the

source of innumerable therapeutic agents. Burkholderia cepacia was first described by Walter Burkholder of Cornell University in 1949 when he determined it to be the cause of bacterial rot of onion bulbs. They are rod-shaped, free-living, motile Gram- negative bacteria Since then, few species have been described: Burkholderi sps. 2xiao7 strain Burkholderi cepacia strain RRE5, Burkholderi cepacia strain RRE3, Burkholderi cepacia strain CG4, Burkholderi cepacia T-34gene .Keeping these above facts in view Identification of bacteria is traditionally performed by isolation of the organisms and study of their phenotypic characteristics, including Gram staining, morphology, culture requirements and biochemical reactions. To study bacterial phylogeny and taxonomy, the 16S rRNA gene sequences are very useful due to its presence in almost all bacteria, often existing as a multigene family, or operons, the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and the 16S rRNA gene (1,500 bp) is large enough for informatics purposes. Its gene sequence informatics is to provide genus and species identification, where the isolates that do not fit any recognized biochemical profiles or for taxa that are rarely associated with human infectious diseases. The usefulness of 16S rRNA gene sequencing as a tool in microbial identification is dependent upon two key elements, deposition of complete unambiguous nucleotide sequences into public or private databases and applying the correct label‖ to each sequence. The primary structure of the 16s rRNA is highly conserved and species having 70% or greater DNA similarity usually have more than 99% sequence identity. These 3% or 45-nucleotide differences are not evenly scattered along the primary structure of the molecule but are concentrated mainly in certain hyper variable regions. Phenotypic biochemical analysis is not sufficient to identify genus of bacteria, but sequencing of 16S rRNA gene sequencing is essential for exact identification. Universal primers are used for experimental purpose. These primers used to polymerize entire 16S rRNA gene or part of it. In order to know new species, there is a requirement of entire length of sequence (1500bp). Unknown bacterial 16S rRNA sequences can be compared with database sequences, in order to know taxonomic position of unknown bacteria. From this, at least genus of particular unknown strain can be defined. 16S rRNA sequencing starts with DNA isolation from particular strain. This DNA used as template for PCR amplification. Targeting of particular part of 16S rRNA can be done by using universal primers. EzTaxon is database of 16S rRNA gene and a tool for analysis of sequences. It contain BLAST program to identify closest relative to query sequence by pair wise alignment.

MATERIALS AND METHODS

### Preparation of extracts from rhizomes of *Curcuma longa*.

The rhizomes of *Curcuma longa* were collected from Allavaram, Amalapuram, Andhra Pradesh, India during the month of March 2012. Shade dried rhizomes of *Curcuma longa* were powdered and separately extracted in a Soxhlet apparatus for 6 hrs successively with hexane, chloroform and methanol, concentrated to dryness under vacuum at temperature of 45°C by using rotary evaporator (Buchi, Switzerland), dried completely and stored in desiccator. The samples were collected in a sterile plastic container and transported to laboratory for bacteriological analysis. Bacterial isolates were screened on nutrient Agar (NA) plates by the standard pour plate method. Plates were incubated at 37°C/24 h and a total of one hundred and forty four isolates were obtained, from that one isolate was selected and used for further studies. The isolated bacteria were identified based on colony characteristics, gram staining methods shown in Figure 1 and Figure 2. The shape and colour of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of Oxidase, Catalase, MR-VP test, Urease test, Motility, Indole production and Citrate utilization shown in Table 1. The tests were used to identify the isolates according to Bergey's Manual of determinative bacteriology. Bacterial genomic DNA was isolated as per the standard protocol (Hoffman and Winston, 1987). [28] The extracted genomic DNA was used as template DNA for amplification of the 16S rRNA gene.

Test organisms

The microorganisms used in the experiment were procured from MTCC, IMTECH-Chandigarh.

PCR Amplification of 16S rRNA Gene

Add 1 µl of template DNA in 20 µl of PCR reaction solution. The universal primers (Forward primer 5'-CCAGCAGCCGCGGTAATACG -3' and reverse primer 5' TACCAGGGTATCTAATCC-3') were used for the amplification of the 16S rRNA gene fragment. Using 16S rRNA Universal primers gene fragment was amplified using MJ Research PTC-225 Peltier Thermal Cycler Use 27F/1492R primers for bacteria. Bacterial Genomic DNA was isolated by using the Insta Gene TM Matrix Genomic DNA isolation kit Catalog # 732-6030. Use 27F/1492R primers for bacteria DNA fragments are amplified about 1,400 bp in the case of bacteria.

RESULT
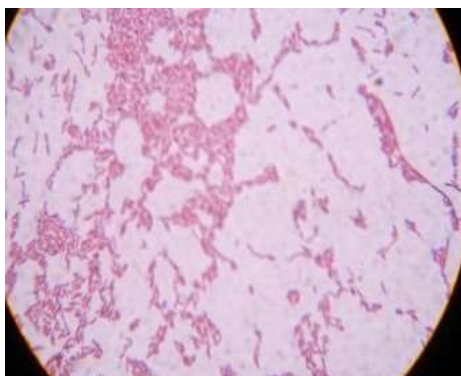
Figure 1:- Gram stain of Burkholderia cepacia
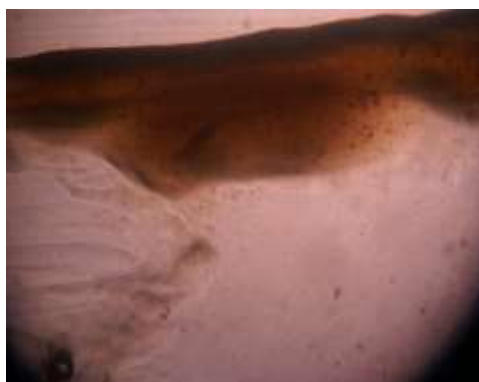
Figure 2:- Colony morphology of Burkholderia cepacia

Table 1:- Biochemical Characteristics of Isolated Bacteria

Colour Morphology

Size	Medium
Colour	Opaque
Form	Irregular
Texture	Mucoid
Elevation	Raised
Margin	Entire

Microscopic observation

Gram's reaction	Negative

Oxygen requirement O/F

Facultative	Positive

Oxygen relation/reaction

Catalase test	Positive
Oxidase test	Positive

Nitrate test   Negative

Urease test   Positive

IMViC tests

Indole   Negative

Voges proskauer   Positive

Citrate   Positive

Hydrolysis

Gelatine   Negative

Pathogenic properties

Hemolysin   Negative

Amino acid &derv

Arginine   Negative

Lysine   Positive

Ornithine   Negative

carbohydrate utilization   polysaccharides

Starch   Negative

Raffinose   Negative

Disaccharides

Sucrose   Positive

Lactose   Negative

Maltose   Positive

Cellobiose   Positive

Pentoses

Ribose   Positive

Arabinose   Negative

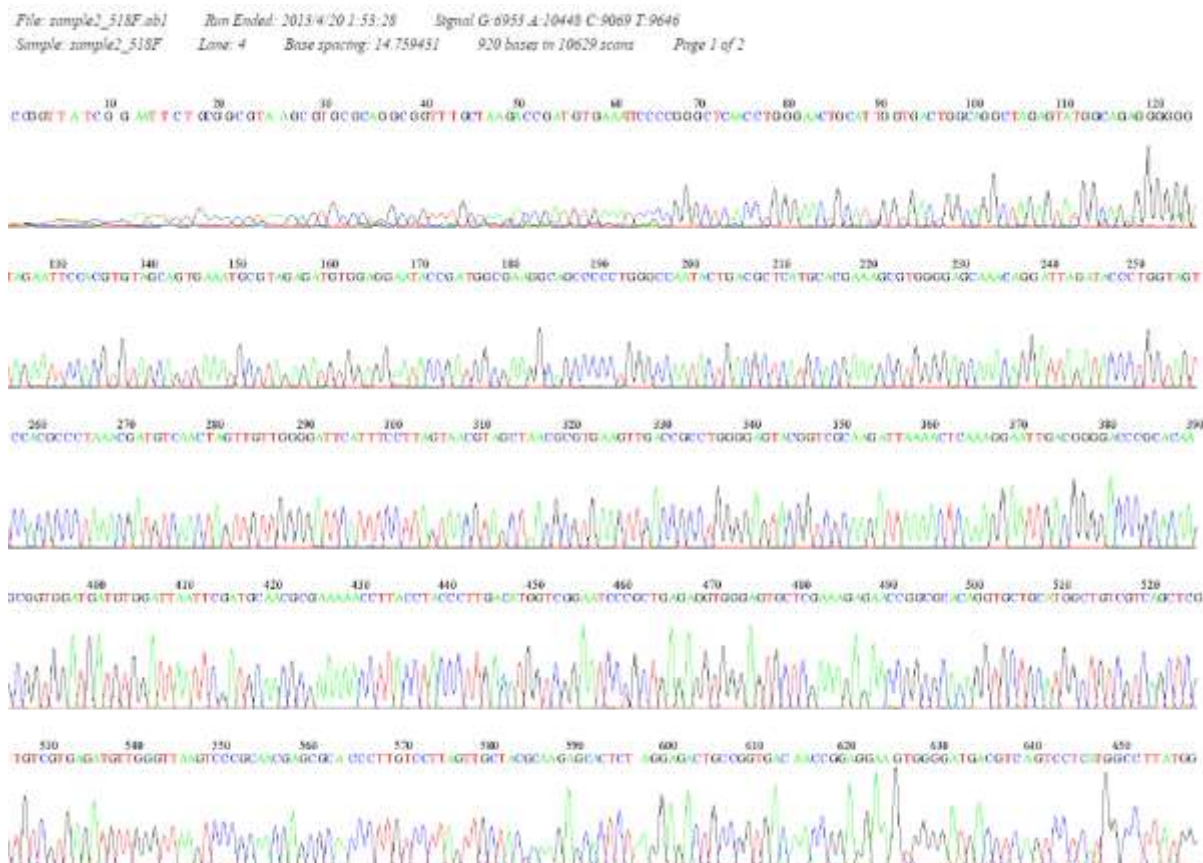Xylose   Negative

Rhamnose   Negative

Hexoses

Dextrose   Positive

Manose   Negative

Galactose   Negative

Fructose   Positive

Various physiological tests confirm characterizations of the selected isolates and biochemical test. The bacterial culture of the *Curcuma longa* expressed morphologically different colonies. The colony from the culture on genomic analysis by 16S rRNA polymerase chain reaction was found to be as **Burkholderia cepacia.**

::: sample2_contig_1 Report :::::::::::::::::::::::::::::::

1. Analysis Report

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
sample2_contig_1	1435	1259	1246	54.91289198606272
sample2_F	889	793	774	54.105736782902135
sample2_R	723	713	710	56.293222683264176

File: sample2_518F.ab1   Run Ended: 2013/4/20 1:53:28   Signal G:6953 A:10448 C:9069 T:9646
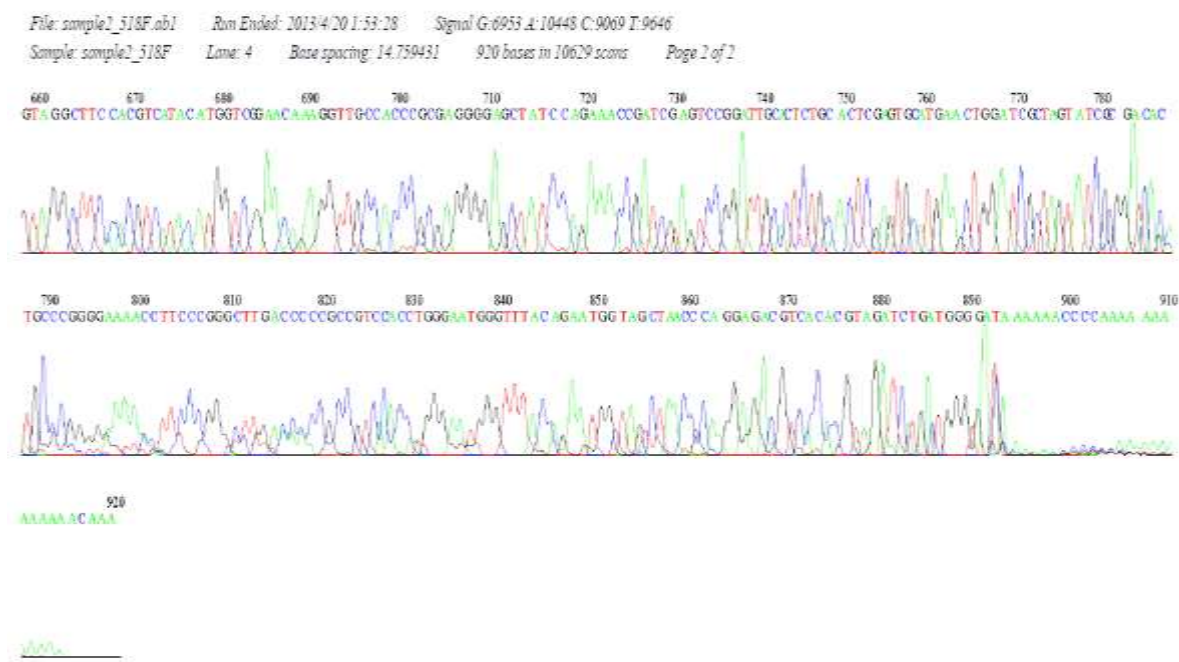Sample: sample2_518F   Lane: 4   Base spacing: 14.759451   920 bases in 10629 scans   Page 1 of 2

Figure 3: *16s rRNA gene sequence of the Burkholderi cepacia sp. obtained by forward and reverse primers*

Include a positive control (E.coli genomic DNA) and a negative control in the PCR. The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISMR BigDyeTM Terminator Cycle Sequencing Kits with Ampli TaqR DNA polymerase (FS enzyme) (Applied Biosystems) PCR reaction was performed in a gradient thermal cycler and then perform 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec and 72oC for 60 sec. Single pass sequencing was performed on each template using below 16s rRNA universal primers. The Fluorescent labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The sequence obtained was subjected to BLAST search and the bacterial species were determined shown i n figure 4. The percentages of sequence matching were also analyzed and the sequences was submitted to NCBI-Gen Bank and obtained accession number.

ASSEMBLED SEQUENCE FOR SAMPLE

Shown in Figure 3.

2. BlastN Report

- Query name : sample2_contig_1

- Query length : 1435

Query		Subject					Score			Identities			
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Total	Pct.(%)	Strand
1	1407	Burkholderia sp. 2xiao7 16S ribosomal RNA gene, partial sequence	FJ606689.1	1520	13	1466	2348	1271	0.0	1401	1454	96	Plus/Plus
1	1407	Burkholderia cepacia strain RRE5 16S ribosomal RNA gene, partial sequence	AY946011.1	1520	13	1466	2348	1271	0.0	1401	1454	96	Plus/Plus
1	1407	Burkholderia cepacia strain RRE3 16S ribosomal RNA gene, partial sequence	AY946010.1	1517	10	1463	2348	1271	0.0	1401	1454	96	Plus/Plus
1	1407	Burkholderia sp. GG4 16S ribosomal RNA gene, partial sequence	HQ728437.1	1498	9	1462	2342	1268	0.0	1400	1454	96	Plus/Plus
1	1407	Burkholderia sp. T-34 gene for 16S rRNA, partial sequence	AB480713.1	1493	19	1472	2342	1268	0.0	1400	1454	96	Plus/Plus
1	1407	Burkholderia cenocepacia J2315 chromosome 2, complete genome	AM747721.1	3217062	2827676	2826223	2342	1268	0.0	1400	1454	96	Plus/Minus
1	1407	Burkholderia cenocepacia J2315 chromosome 1, complete genome	AM747720.1	3870082	246320	247773	2342	1268	0.0	1400	1454	96	Plus/Plus
1	1407	Uncultured Pseudomonas sp. clone TCCC 11168 16S ribosomal RNA gene, partial sequence	EU567048.1	1525	20	1473	2342	1268	0.0	1400	1454	96	Plus/Plus
1	1407	Uncultured Burkholderia sp. clone TCCC 11164 16S ribosomal RNA gene, partial sequence	EU567046.1	1550	19	1472	2342	1268	0.0	1400	1454	96	Plus/Plus
1	1407	Uncultured bacterium clone JPL2-36 16S ribosomal RNA gene, partial sequence	DQ532153.1	1490	20	1473	2342	1268	0.0	1400	1454	96	Plus/Plus

Figure 4: *Burkholderi cepacia sp.***BLAST results**

CONCLUSION

We showed the use of 16S rRNA gene sequence to characterize the bacterial isolate from the *Curcuma longa* and were found to be **Burkholderia cepacia** strain. Thus, the genotyping method using 16S rRNA gene sequence is both simple and effective in strain identification.

The most current research on Burkholderia cepacia includes its use in the agricultural industry to protect plants from pathogens. Burkholderia cepacia can colonize the roots of many plants where it produces compounds that protect against soil-borne pathogens. In absence of these pathogens, plant growth has been noted to improve. The eradication of Burkholderia cepacia as a human pathogen will become increasingly important in the years to come

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