INVITRO CULTIVATION OF CURCUMA AMADA AND ISOLATION, MOLECULAR CHARACTERIZATION OF *BURKHOLDERIA CEPACIA* FROM CURCUMA AMADA USING 16S RRNA SEQUENCING

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Abstract:
Curcuma amada, a prominent aromatic zingiberaceous plant in the Indian subcontinent, was studied using rhizome explants, which provide an attractive environment for bacterial interaction. This work reveals effective Curcuma amada *in vitro* propagation as well as the isolation, identification, and analysis of bacteria isolated from Curcuma amada using a 16S rRNA-based molecular approach using samples collected from Curcuma amada. The bacterial strain was isolated and characterised using different biochemical assays, and the molecular technique was used to amplify the 16S rRNA gene using appropriate primers. The bacterial strain was identified by comparing the amplified 16S rRNA gene sequence to the sequence in the NCBI sequence database. 16S rRNA sequencing was used for phylogenetic and molecular evolutionary investigations. When the sequences were submitted to the NCBI gene bank database using BLAST, they revealed 99 - 100% maximum identity and excellent similarity to *Burkholderi sps*. *Burkholderi cepacia* strain RRE5, *Burkholderi cepacia* strain RRE3, *Burkholderi cepacia* strain CG4, and *Burkholderi cepacia* T-34 gene. We studied extracts of Curcuma amada rhizomes in this work. *Burkholderi cepacia* isolate was the most active of the isolates tested.

Keywords: Curcuma amada, Rhizomes, *Burkholderia cepacia*, NCBI.

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Introduction:

Curcuma amada is a prominent aromatic herb in the Indian subcontinent, with morphological similarities to ginger but a raw mango taste. It is a perennial, rhizomatous, aromatic plant of the Zingiberaceae family that is said to have originated in the Indian subcontinent and is found throughout all regions except arid ones. However, it is cultivated in southern India (Prain, 1981). It grows wild across Bangladesh, particularly in marshy and gloomy areas of Gaibanda, Rangpur, Bogra, Rajshahi, Jessore, and Natore, although it is not economically farmed elsewhere (Ghani, 1998). Curcuma amada is used medicinally as a cooling, astringent, and digestive aid.

Curcuma amada is also utilised as an essential ingredient in pickles, preserves, sweets, sauces, curries, salads, and other dishes because of its unusual taste (Verghese, 1990; Shankaracharya, 1982). Its rhizome possesses carminative characteristics as well as stomachic (Hussain et al., 1992), and rhizome pest has historically been employed for wound, cut, and itching healing (Srivastava et al., 2001). In the natural, this plant is replicated by rhizome buds, while in culture, it is spread by parts of subterranean rhizomes. Several typical difficulties, including poor growth, soil-borne illnesses, rhizome loss caused by bacteria and fungus, and insect attacks, have previously been described for this plant (Balachandran 1990; Prakash et al. 2004).

Historically, pharmacological screening of natural-source chemicals has yielded a plethora of therapeutic medicines. Walter Burkholder identified *B. cepacia* in 1949 as the cause of onion peel rot, and it was initially reported as a human disease in the 1950s (Burkholder WH 1950). It was originally isolated in cystic fibrosis (CF) patients in 1977 as *Pseudomonas cepacia* (Laraya-Cuasay, L., et al 1977). Outbreaks of *B. cepacia* in people with CF were related with a 35% fatality rate in the 1980s. *B. cepacia* has a large genome, with twice as much genetic material as *E. coli*. Antinematodal and antibacterial chemicals are produced by *Burkholderia cepacia*. These chemicals protect plants from soil-borne diseases and are being considered as a replacement for ecologically hazardous pesticides and herbicides. Some of these chemicals include pyrrolnitrin, which inhibits the electron transport chain (N El-Banna 1998), pyoluteolin, and the siderophore cepabactin. Volatile ammonia generation has also been shown to reduce soil infections (Mansour Baligh et al. 1999).

Materials and methods:

Preparation of extracts from rhizomes of Curcuma amada:

Curcuma amada rhizomes were collected in March 2012 from Allavaram mandal, Amalapuram district of Andhra Pradesh, India. Curcuma amada rhizomes had been empowdered and separately extracted in a Soxhlet apparatus for 6 hours with hexane, chloroform and methanol, then concentrated to dryness under vacuum at 45°C using a rotary evaporator (Buchi, Switzerland), drained properly and kept in a desiccator.

Identification and Characterization of isolated Bacteria:

Samples were collected in a sterile sealed container before being transferred to a laboratory for bacteriological investigation. The standard pour plate technique was used to screen bacterial isolates on nutritional Agar (NA) plates. Plates were incubated at 37°C for 24 hours, yielding a total of 144 isolates, one of which was chosen and used for future research. The bacteria were identified by colony characteristics and gram staining techniques as illustrated in Figures 5 and 6. Following Gram staining, the colonies' shape and color were examined under a microscope. Table 1 shows the biochemical activity of Oxidase, Catalase, MR-VP test, Urease test, Motility, Indole synthesis, and Citrate utilization in isolates. According to Bergey's Manual of Determinative Bacteriology, the tests were utilized to identify the isolates. Bacterial genomic DNA was extracted in accordance with conventional procedures (Hoffman and Winston, 1987). The isolated genomic DNA was employed as a template for 16S rRNA gene amplification.

Test organisms:

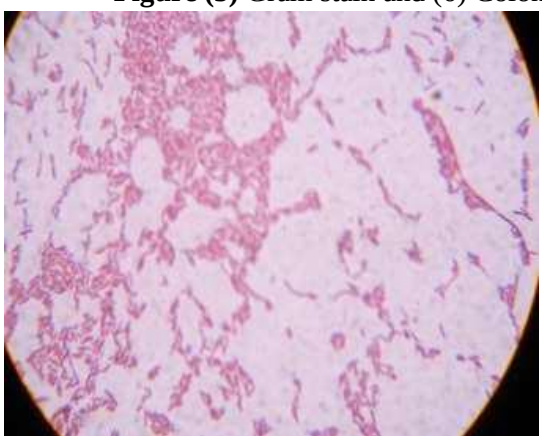
The microorganisms utilized in the study were obtained from IMTECH Chandigarh's MTCC. 16S rRNA gene amplification through PCR. In 20 µl of the PCR reaction solution, add 1 µl of template DNA. The 16S rRNA gene fragment was amplified using the universal primers, forward primer 5'-CCAGCAGCCGCGGTAATACG-3' and reverse primer 5' TACCAGGGTATCTAATCC-3'. Using rRNA (16S) The MJ Research PTC-225 Peltier Thermal Cycler was used for amplification of the gene segment-like universal primers. Add primers 27F/1492R to bacteria. Using the Insta Gene TM Matrix Genomic DNA separation kit (Catalogue # 732-6030), bacterial genomic DNA was extracted. Utilise primers 27F/1492R for microorganisms. In bacteria, DNA fragments are amplified by around 1,400 bp.

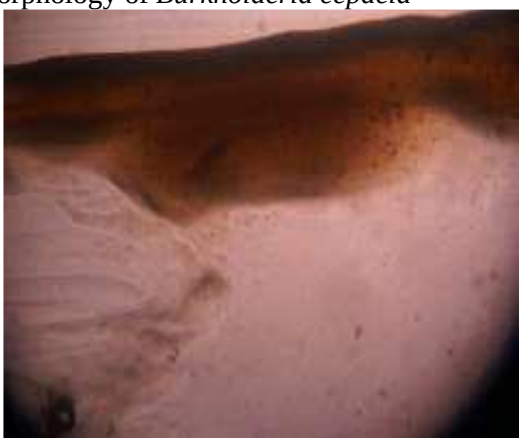
Results:

Figure (1), (2), (3), (4) - In vitro propagation of *Curcuma amada* using rhizome explant:

Colony morphology:

Figure (5) Gram stain and (6) Colony morphology of *Burkholderia cepacia*





Colony 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

Identification of bacteria

Scientific classification	
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Beta Proteobacteria
Order	Burkholderiales
Family	Burkholderiaceae
Genus	Burkholderi
Species	Cepacia

Biochemical Characteristics of Isolated Bacteria *(Burkholderia cepacia)*

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 amada* 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*.**

16s rna sequencing report:

::: 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	54.9128919860627 2
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: 14.759431 920 bases in 10629 scans Page 1 of 2

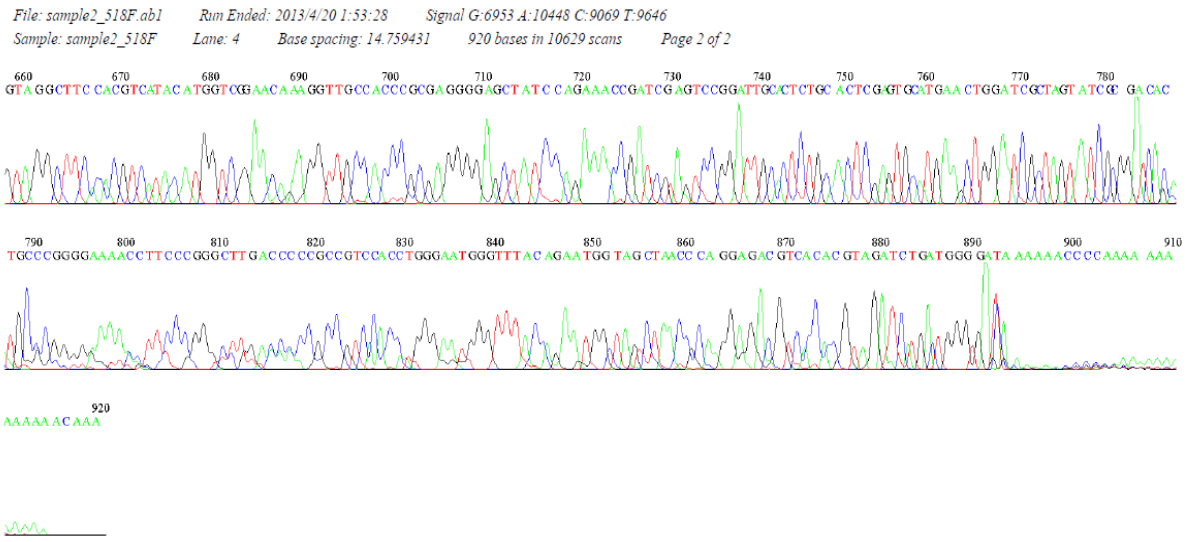


Figure 3: 16s rRNA gene sequence of the Burkholderi cepacia sp. obtained by forward and reverse primers

In the PCR, include a positive control (E.coli genomic DNA) and a negative control. The 518F/800R primers were used to sequence the PCR result. The ABI PRISMR BigDyeTM Terminator Cycle Sequencing Kits with Ampli TaqR DNA polymerase (FS enzyme) (Applied Biosystems) were used for the sequencing processes. The PCR reaction was carried out in a gradient thermal cycler, followed by 35 amplification cycles at 94°C for 45 seconds, 55°C for 60 seconds, and 72oC for 60 seconds.

Each template was sequenced once using the universal 16S rRNA primers listed below. An ethanol precipitation process was used to separate the fluorescently labelled fragments from the unincorporated terminators. The samples were resuspended in distilled water before being electrophoretically separated on an ABI 3730xl sequencer (Applied Biosystems). The resulting sequence was submitted to a BLAST search, and the bacterial species were identified, as shown in figure 4. The percentages of sequence matching were also calculated, and the sequences were submitted to the NCBI-Gen Bank, where they were assigned an accession number.

Assembled sequence for sample was hown 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 used the 16S rRNA gene sequence to characterise a bacterial isolate from Curcuma amada, which turned out to be a Burkholderia cepacia strain. As a result, the genotyping approach based on the 16S rRNA gene sequence is both easy and efficient for strain identification. The most recent Burkholderia cepacia study is its usage in agriculture to protect plants against infections. Burkholderia cepacia may colonise the roots of various plants and create chemicals that protect against diseases found in the soil. Plant growth has been seen to improve in the absence of certain diseases. Burkholderia cepacia elimination as a human disease will become increasingly significant in the coming years.

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