



ISOLATION OF ENDOPHYTIC BACTERIA FROM THE LEAF OF *Cymbopogon winterianus* AND ITS CHARACTERIZATION

Microbiology

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ABSTRACT

Cymbopogon are aromatic grasses containing essential oil as secondary metabolite. The essential oil possess high amount of anti-microbial activity. *Cymbopogon winterianus* is the citronella oil-yielding species cultivated in India. Its essential oil can kill up to 99.9% of the bacteria so it was assumed that no endophytic bacteria can survive in the leaf of *Cymbopogon winterianus*. We isolated the endophytic bacteria from the leaf of *Cymbopogon winterianus* collected from the foothill of Karbi Angalong in Assam India. The endophyte bacteria were isolated by modified leaf impression method. The bacteria were grown from the edge of the cut site of the leaf and in presence of essential oil of the leaf the bacteria shows growth which confirms it as endophytic bacteria. Two specific types of bacteria were seen after multiple cultures. The culture were identified by 16S ribosomal RNA using 27F (AGAGTTTGATCATGGCTCAG) as forward primer and 1492R (TACGGCTACCTGTTCAGACTT) as reverse primer found that two species of bacillus were found which were identified by NCBI BLAST. The strain were submitted in nucleotide database of NCBI

KEYWORDS

INTRODUCTION:-

The genus *Cymbopogon* (Poaceae) is known to include about 140 species. Among these, more than 52 have been reported to occur in Africa, 45 in India, six each in Australia and South America, four in Europe, two in North America and the remaining are distributed in South Asia (Jagadish Chandra, 1975b). Most of these species produce characteristic aromatic essential oils that have commercial importance in perfumery, cosmetics and pharmaceutical applications (2). About 20 species of *Cymbopogon* are reported to occur in India (Christopher & Sam Raj, 1986) (3). The citronella oil-yielding species cultivated in India are *C. winterianus* Jowitt and *C. nardus* Rendle, commercially known in trade as 'Java citronella' and 'Ceylon citronella', respectively (Husain, 1994) (4). The *Cymbopogon* essential oils are characterised by monoterpene constituents like citral, citronellol, citronellal, linalool, elemol, 1,8-cineole, limonene, geraniol, β -carophyllene, methyl heptenone, geranyl acetate and geranyl formate. Citral is one of the important components of the oil present in several species of *Cymbopogon* with wide industrial uses such as raw material for perfumery, confectionery and vitamin A₂. After Sprengel named this genus in 1815, a number of taxonomists have attempted to classify the species of *Cymbopogon*. Hackel (1887) and Hooker (1897) have treated this genus as a subgenus of *Andropogon* (3, 4). However, Stapf (1906) raised *Cymbopogon* to its original rank of a genus and this has been accepted by all the later taxonomists. Taxonomically, the species of *Cymbopogon* have been divided into three series viz. 'Shoenantithi', 'Rusae' and 'Citratii' (Stapf, 1906) (5). The leaves of the species in 'Schoenanthi' series are thin, in 'Rusae' subcordate and in 'citratii' lanceolate. The identification and classification of *Cymbopogon* species have become difficult because of the occurrence of numerous transitional forms which are supposed to have arisen by hybridisation (Bor, 1960) (6) and the existence of many varieties and races (7) (Jagadish Chandra, 1975a). Some species, such as *Cymbopogon citratus*, are not known to flower, others like *Cymbopogon nardus* and *Cymbopogon winterianus* flower only rarely and the polyploid forms of *Cymbopogon flexuosus* and *Cymbopogon coloratus* flower, but do not set seed (7). The morphological variation and oil characteristics of various species and varieties of *Cymbopogon* have been reported (Husain, 1994) (8), but such information is not sufficient to define precisely the relatedness among the morphotypes and chemotypes. For

instance, *C. martinii* var. *sofia* and *C. martinii* var. *motia*, are morphologically almost indistinguishable, but show distinct chemotypic characteristics in terms of oil constituents (Guenther, 1950) (9). Conversely, phenotypically and taxonomically well distinguishable species produce oils of almost identical chemical compositions such as lemongrass oils from *C. citratus* and *C. flexuosus* (Anonymous, 1988) (10). Such phenotypic traits, whether morphological or chemotypic are basically the phenotypic expression of the genotype, while DNA markers are independent of environment, age and tissue and expected to reveal the genetic variation more conclusively in assessing such variations. Introgression of various traits, intermittent mutations and selection through human intervention may lead to variation in chemotypic characters across geographical distributions (Kuriakose, 1995) (11). While natural hybridisation may lead to the formation of morphological or chemotypic intermediates, defining taxa purely on this basis may not be appropriate. The earlier works on phytochemical (Patra et al., 1990; Dhar et al., 1993) (12, 13), biochemical and physiological parameters (Nandi and Chatterjee, 1987) (14), development of agrotechnology (Nair et al., 1979; Rao et al., 1985) (15, 16), genetic improvement (Ganguly et al., 1979; Maheshwari and Sethi, 1987) (17, 18) and breeding approaches (Kulkarni and Rajgopal, 1986; Rao and Sobti, 1987a; Shyalaraj and Thomas, 1993) (19, 20, 21) of different *Cymbopogon* species and varieties appear scattered, and do not address the question of relationships among different taxa. *Cymbopogon winterianus* (Jowitt), Java citronella indigenous to Sri Lanka, was introduced into India from Indonesia during 1959 (Virmani et al., 1979) (24) and subsequently acquired by CIMAP to improve and develop agronomic practices for its wide cultivation. During an acclimatization trial at CIMAP, two distinct forms (designated Java I and Java II) were identified from the introduced material. Java II was found to be superior to Java I in growth and yield attributes, and was subsequently adopted as a cultivated variety in India, covering a sizeable area in Assam, West Bengal, Uttar Pradesh, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu (Gupta, 1973) (25). But the production of essential oil differs from place to place. *Cymbopogon* generally grow in bushes and give aroma around that region. This aroma is due to the diffusion of essential oil present in the vacuole of the leaf. The essential oil is present in almost all part of the *Cymbopogon* plant like leaf, root, stem.

But most prominently found in the leaf. The essential oil of different species of *Cymbopogon* species constitute of different aromatic compound as well as different compound. The major compounds are specific to specific species with specific quantity which can be found with the help of GC MS. Different *Cymbopogon* plant has different optimum temperature for the release of its essential oil present on it because of its texture. The essential oil of *Cymbopogon* is known for its anti-microbial activity and its potential to use as an alternative to drug resistant microbes. The volatility nature of the essential oil seems to be a region of it success against the microbes as the compound can easily act on the cell wall similar to that of ethanol. The microbes die due to the polarity which can easily diffuse through the peptidoglycan of the bacteria.

Endophytes are the bacteria living inside the plant system exposing to all the plant metabolite and play major role in plant metabolic activity. Endophytes are plant-associated prokaryotes that form association with their host plants by colonizing the internal tissues, which has made them valuable for agriculture as a tool in improving crop performance²⁶ (Sagarika Mohanta & et.al 2012). Although the interaction between endophytes and host plants has not been fully understood, many bacterial species are reported to promote plant growth and the mechanism attributed includes nitrogen fixation, production of growth-promoting substances and increased resistance to pathogens and parasites²⁶. There are various definitions of endophytes: Microorganisms living within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence are defined as endophytes (Wilson, 1993)²⁷. Bacon and White (2000) defined endophytes as 'microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects'²⁸. Many reports found in literature strongly suggest that these endophytes have an excellent potential to be used as plant growth promoters with legumes and non-legumes (e.g. Bai et al., 2002)²⁹. Plants benefit extensively by harbouring these endophytic microbes and confer enhanced resistance to various pathogens (Clay and Schardl, 2002; Hofflich, 2000; Arnold et al., 2007)^{30, 31, 32} by producing antibiotics (Ezra et al., 2004)³³. Eight hundred root segments from 25 plants of *Cymbopogon* sp. were processed for the isolation of endophytic diazotrophs. A total of five different diazotrophic bacteria belonging to six species were isolated. In Cachar district, highest population was found of *Pseudomonas fluorescens* (40%) where as *Klebsiella pneumoniae* (52.75%) and *Azospillum* sp.(39%) were higher in Karimganj district and Hailakandi district respectively³³. Plant endophyte may be bacteria, fungi, protozoa or any other small multicellular organism which generally stay in a symbiotic association with the plant without causing any damage in the plant, leaving as an important part of plant²⁶(Sagarika Mohanta & et.al 2012) It has been seen that Correlations were found between biological activity and biotope, e.g. a higher proportion of the fungal endophytes, in contrast to the soil isolates, inhibited at least one of the test organisms for anti-algal and herbicidal activities.²⁶ The potential role of the endophyte and its biologically active metabolites in its association with its host has been investigated. The fungal endophytes possess the coenzymes necessary to colonize their hosts and they grow well in the apo-plastic washing fluid of the host. Hydroxy citronella has a sweet flowery odour and is being extensively used in manufacture of high value perfumes. The citronella is also used as - anti-infectious, antiseptic, antibacterial, antidepressant, antispasmodic, anti-inflammatory, deodorant, diaphoretic fungicidal, insect repellent (mosquito), stomach ache, excessive perspiration, rheumatism and arthritic pains etc. The essential oil obtained from *Cymbopogon citratus* are geraniol (40.9 %), neral (29.7 %), myrcene (11.3 %), linalool (1.7 %) and geranylacetate (1.6 %)³³. When the roots of larch are colonized, the association with the host may be mutualistic, improving growth of the host and supplying the mycobiont with enough nourishment to extensively colonize the host's roots. Endophytes with this capacity might profit from association with the plant, because colonization is enhanced³⁶. In turn, host plants benefit by stress reduction and increased root growth³⁶. This mechanism leads to the concept of 'competent' endophytes, defined as endophytes that are equipped with genes important for maintenance of plant-endophyte associations³⁶. The endophytic bacteria equipped with the appropriate degradation pathway improve the in planta degradation of toluene, so there are several function of endophyte still unknown.

Materials and Methods

1. Collection of plant sample

The plant sample was collected from the foothills of Singhason hill, Karbi Anglong, Assam. The sample is collected from the wild and recognized as *Cymbopogon winterianus* (Reinwardtia, 1977).

2. Isolation of endophytes:- Isolation of endophyte from the *Cymbopogon* plant is quite difficult due to the leaf structure and presence of hard silica coat and the presence of essential oil: Different methods are used in the present study for isolation of endophytes from plant leaf which are as follows:

a) Type 1

Take the leaf of a healthy *Cymbopogon* plant Wash it thoroughly with sterile distilled water for 8-10 times, to remove the grease is present on the surface layers.

Cut the leaf into small pieces and dip it in 1% -5% concentration of HgCl₂ individually for different periods (5 min, 10 min and 15 min).

Wash with 95 % ethanol to remove HgCl₂

Wash with sterile distilled water till the removal of all the above entities and place it on Neutral Agar media (NA media), incubate it in room temperature by sealing with paraffin tap.

Note: all the step must be done under laminar airflow and take precautionary steps before starting the work like fumigation, 30 minutes of UV ray, wiping the bench with 70% ethanol and cleaning our hand properly as per norms.

b) Type 2

After performing the sterilization process as mentioned above and washing with distilled water, grind the leaf in motor and pestle with sterile distilled water and homogenized. Add 5 ml of sterile distilled water to it.

Cut the whatman filter paper into disc shape, then autoclave the disk properly to keep it aseptic.

Dip the disc in the homogenized solution under aseptic condition and placed it on NA media and seal it properly, incubate in room temperature for the growth of microbes.

c) Type 3

This is another method used for the growth of endophyte and slightly modified method from Type 1.

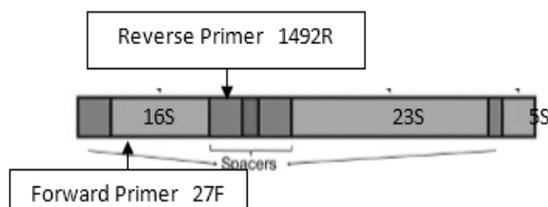
Here the leaves are crushed gently after sterilization process. The leaf is pressed in the appropriate media under aseptic condition and sealed with paraffin tape and kept for incubation.

Media for growth of the endophytes

Two types of media were used namely Nutrient Agar (NA) and Mueller Hinton (MH) agar media, out of which NA media was modified to check the characters of endophyte isolated by addition of the specific essential oil to the NA media at the ratio of 100 microliter in 20 ml before solidification. The addition of essential oil in hot condition will denature its character.

3. 16 s ribosomal sequencing

Genomic DNA extraction: Genomic DNA was isolated by using the InstaGene™ Matrix Genomic DNA isolation kit (Catalog # 732-6030)



Ribosomal Gene organization and Target region amplified

Primer Details: Ribosomal RNA 16S Region Universal primers

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCATGGCT CAG	20
1492R	TACGGCTACCTTGTTAC GACTT	21

Polymerase Chain Reaction: Target gene fragment was amplified using TeledynePrime Thermal Cycler

PCR Protocol:

DNA fragments are amplified using 1 μ l of template DNA in 20 μ l of total PCR reaction mixture using 27F/1492R primers (50 pmol) and 35 amplification cycles with following program:

1. Denaturation: 94°C for 45 sec,
2. Annealing: 52°C for 60 sec,
3. Chain Elongation: 72°C for 60 sec.

Purification of PCR products

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

Sequencing

The PCR product was sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using 16S rRNA gene universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Bio systems).

RESULT AND DISCUSSION:

Endophytes are microorganism which is present in the living tissue of various plants establishing mutual relationship without apparently showing any symptoms of disease³³. *Cymbopogon* has high amount of essential oil which is known for its antimicrobial activity to almost all microbes and also thought to be an alternative of broad spectrum antibiotic in coming future. It was also reported that the leaf part of the *Cymbopogon* species such as *C. flexuosus* and *C. citratus* possess endophytic fungi but not endophytic bacteria. Endophytic bacteria was found in the root of *C. winterianus* by Sagarika Mohanta et.al.³³ and has found 5 different types of bacteria which are listed as *Pseudomonas fluorescens*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Bacillus pumilus*, *Burkholderia cenocepacia*, *Klebsiella pneumoniae* 342 isolated from the roots. These endophytes are directly in contact with the soil and have more proximity to the soil bacteria. The essential oil concentration is higher in leaf than in root so endophytic bacteria have a higher challenge to survive in leaf. Several studies reveal that essential oil found in leaf of *C. winterianus* has higher antimicrobial activity and can kill almost 99.9% of the known bacteria³⁴. We tried to isolate the endophytes taking it a concept that every plant must have endophytes as it help for the synthesis of many plant metabolite and microbes has the capability to live in extreme condition. By taking this in account I tried to isolate endophyte first by normal leaf impression method and the result was very poor then went for modified version by crushing the leaf and incubated it in incubator and in room temperature. The growth was seen in the plates kept at room temperature but not in incubator, which give two concepts that the endophyte are light sensitive and grow well in presence of light and require room temperature. The confirmation of the bacteria as an endophyte can be known as it growth starts form the edge of the crush leaf and in absence of the leaf the growth of the bacteria is very slow and limited. The bacteria does not grow over the leaf and does not cause any disease to the leaf which in one way tells us that this are endophytes, as well as it grows better in plant extract compared to the nutrient broth. One other fact is noted that the leaf loses its chlorophyll after 3 month and also till then the growth of the bacteria is seen. As the chlorophyll disappears the growth of bacteria reduced drastically. Which give us an idea that the endophyte is directly related to the

metabolic activity of the plant. The photo of the bacteria will give a clear idea how the bacteria grows. The

Sample 3



Fig 1 (essential oil containing media)



figure 2 (essential oil free media)

This figure is showing the endophytic bacteria isolated from the leaf of *Cymbopogon winterianus* in NA media, the figure 1 shows the growth of the bacteria in presence of essential oil isolated from the same species and the same form of colony in the figure 2 without essential. The figure 1 say it as an endophytic as it grows with any effect on it. This was done by the 3 method of leaf impression where the leaf was crushed before impressing it to media.

Sample 7



Figure 3(essential oil containing media)

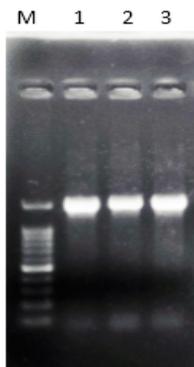


Figure:-5(Essential oil free media)

The figure 1 & 2 shows the colony of bacillus safensis grown in essential oil and essential oil free media. The figure 3,4 shows the bacterial colony of sample 7. The study in 120 bacterial isolate confirm the occurrence of this two bacteria in a ratio of 65% of Sample 3 to 30% occurrence of sample 7.

The characterization of the two bacteria sample 3 and sample 7 has been done by basic biochemical test and genome sequencing. The gram staining shows that both the bacteria belong to gram positive and possess spores, which give us an idea how it tolerate the essential oil as this essential oil has high amount of antimicrobial affect as said in reference34. The isolated sample were subjected 16s RNA study which give us a conformation that two different species of endophyte was found in the leaf of *C. winterianus* with distinctly having higher percentage of deviation to its nearby species. The two bacterial species were subjected to 16 ribosomal sequencing.

Results: sample 3



PCR amplicon of 16s rRNA gene

M-Molecular size ladder, 1- sample 3, 2- Sample 6, 3- Sample 7

Forward Sequence 27F

>Bacterial isolate 3_27F sequence_771bp
 GGGTGTAAAGGTTAGCTGCAGCACTAAGGGGCGGAAACCC
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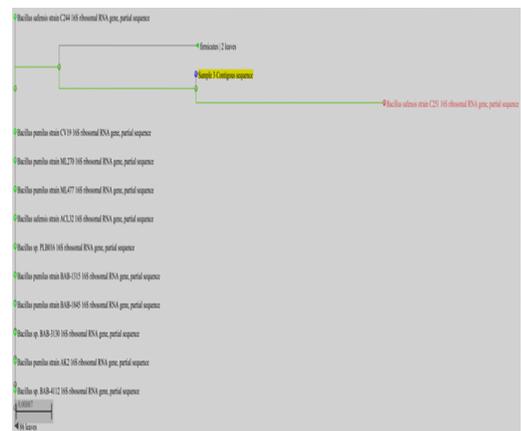
Reverse Sequence 1492R

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 TCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCT
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 TCGTGTCTGAGATGTTGGGTTAAGTCCCGCAACGAGCGC
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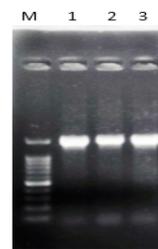
>Sample 3 Contiguous sequence_1400bp

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 GATGGGGTGAATCACAATAATGCGGCCCTGGGCTTGG



Phylogenetic Tree of Bacterial isolate 3

Conclusion: Based on sequence alignment and Phylogenetic analysis the bacterial isolate 3 has been matched to *Bacillus safensis*



PCR amplicon of 16s rRNA gene

M-Molecular size ladder, 1- sample 3, 2- Sample 6, 3 – Sample 7

Forward Sequence 27F

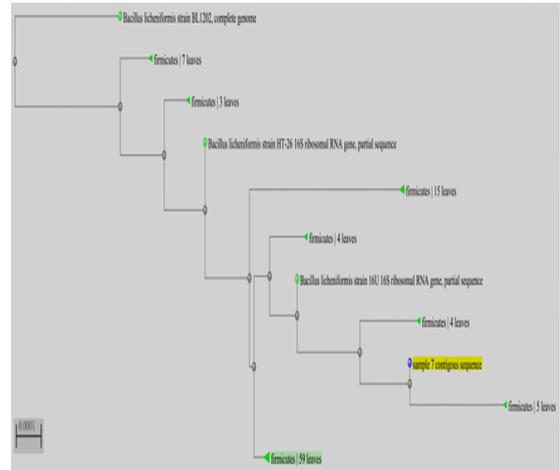
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Reverse Sequence 1492R

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>Bacteria isolate_7_Assembled Sequence_1434bp
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 TTGATCTTAGTTGCCAGATTAGTTGGGCACTTAAGGTG
 ACTGCCGGTGACAAACCGGAGAAGGTGGGGATGACGTCA
 AATCATCATGCCCTTATGACCTGGGCTACACACGTGTAC

AATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAG
 CCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGC
 AACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGA
 TCACATGCCGCGGTGAATACTTCCCGGGCTTGTACACACC
 GCCCCTACACCACGAGAGTGTGTAACACCCGAAGTCGTG
 AGGTACTTTTGGAGCCAGCCGATGGGACAGAGATGGTG
 AATCTAAGCCAAGCCGGCGCTAACCCCTTGC



Phylogenetic Tree of Bacterial isolate 7

Conclusion: Based on sequence alignment and Phylogenetic analysis the bacterial isolate 7 has been matched to Bacillus licheniformis

The two bacterial species are submitted to NCBI nucleotide sequence with accession number

1. (ky552928) Bacillus licheniformis strain TMA1 16S ribosomal RNA gene, partial sequence
2. (ky552929) Bacillus safensis strain TMA2 16S ribosomal RNA gene, partial sequence

Conclusion: Cymbopogon winterianus is known for its antimicrobial activity and anti-insecticide activity. The repeated isolation of the endophyte shows only two types of bacteria grown from the edge of the leaf. These bacteria grow well in crude plant extract compared to its growth in nutrient broth shows that the bacteria is closely related to the plant extract. The bacteria were identified by 16s ribosomal partial sequencing shows that two unique strain of Bacillus licheniformis and Bacillus safensis.

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