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OPTIMIZATION OF PROCESS PARAMETERS FOR IMPROVED PRODUCTION OF BIO-ACTIVE METABOLITES BY ENDOPHYTIC FUNGUS *CLADOSPORIUM CLADOSPORIOIDES* ISOLATED FROM MANGROVE PLANT *LUMNITZERA RACEMOSA* LINN.

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Keywords:

Cladosporium cladosporiodes, Bioactive metabolite production, Optimization, Endophytic fungi

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ABSTRACT: A study has been undertaken with an aim to isolate potent endophytic fungi from mangrove plant *Lumnitzera racemosa* Linn. Among the 10 different fungal strains isolated, one potent strain with broad-spectrum antagonistic activity was found. Basing on the morphological, cultural and molecular characteristics, the potent fungal strain was identified as *Cladosporium cladosporioides.* Production of bioactive metabolites by the strain was high in Malt Extract Broth compared to other media tested. The culture utilized Mannitol and Beef extract as good carbon and Nitrogen sources for the elaboration of bioactive metabolites. The optimum pH and Temperature for bioactive metabolite production of the strain were recorded at 4.0 and 30 °C. The secondary metabolites produced by the strain grown under optimal conditions exhibited high antagonistic activity against gram-positive as well as Gram-negative bacteria and fungi. This is the first report of *Cladosporium cladosporioides* from mangrove plant *Lumnitzera racemosa* Linn.

INTRODUCTION: The word "mangrove" is attributed to the collection of plants that colonized and dominate the intertidal areas of tropical regions [1]. There has been much work done regarding the diversity of mangrove tree species [2, 3], but a lot of research remains to be done in assessing the mycobiota of mangroves [4, 5]. The fungi which colonize mangrove trees, and all higher plants for that matter, can be generally classified into two main groups – the exophytes which grow on the outside of the plant, and endophytes which grow from within the host [6]. Endophytic fungi are a group of organisms unique in the sense that they live virtually their entire life cycles within the tissues of a host.

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They have been known to confer beneficial properties to the plants they inhabit in terms of protection from predation and tolerance against abiotic stress. For all their unique properties, much work lies ahead in understanding these innocuous organisms. Mangrove endophytic fungi are the more interesting group among endophytes, as they have adapted not only to the host plant but also to the extreme environment those mangrove plants are constantly subjected to. There has been limited work in Gilakaladindi, Krishna District, Andhra Pradesh regarding mangrove endophytic fungi, with more focus being given to fungi that are associated with mangrove plants. The most common endophytes are anamorphic members of the Ascomycota, and some are closely related to fungi known to cause disease in plants and animals.

The presence of endophytic fungi in plant tissues was discovered more than 75 years ago from *Lolium* grass [7]. Marine-derived microbes especially fungi have long been recognized as a potential source of novel and biologically potent metabolites [8].

Many of the microbes live in extreme environments such as high temperatures, high salt concentrations, low pH, and high radiation. Some of the physical factors also influence the fungal growth and metabolite productions, the natural environment is still the most important contributor of novel drugs in the face of the development of combinatorial chemistry, which quickly generated thousands of new chemicals. An attempt has been made in the present study to isolate and identify potential endophytic fungus possessing antimicrobial activity from *Lumnitzera racemosa* collected from mangrove areas [9]. The pure culture of *Cladosporium cladosporioides* isolated from mangrove plant *Lumnitzera racemosa* maintained in Sabourauds dextrose agar medium at 4 °C was used. Further studies were carried out to optimize the culture conditions of the potential isolate to enhance the growth and production of biologically active compounds.

MATERIALS AND METHODS:

Collection of Plant Material: Healthy leaves of Mangrove plant *Lumnitzera racemosa* (Authentication no. ANUBH01191), were collected from Gilakaladindi, Krishna District A.P. The plant material was brought to the laboratory in sterile bags and processed within a few hours after sampling. Fresh plant materials were used for isolation work to reduce the chance of contamination.

Isolation of Endophytic Fungi: All the leaf samples were washed twice in distilled water and then surfaced sterilized by immersion for 1min in 70% (v/v) ethanol, 4 min in Sodium Hypochlorite [3%(v/v) available chlorine], 30s in 70%(v/v) ethanol, and washed 3 times in sterilized distilled water for 1min each time. After surface sterilization, the samples were cut into 5.0-7.0 mm long segments and aseptically transferred to Petri plates containing Sabouraud's dextrose agar (SDA) medium and incubated at 27 ± 2 °C for a week. The isolates were characterized morpho typically using lactophenol cotton blue using scotch tape technique [10]. The colonies were picked up and maintained as pure cultures on SDA slants and stored at 4°C for further study.

Extraction of the Bioactive Metabolite: Endophytic fungal isolates were inoculated into 250 ml Erlenmeyer flasks containing 100 ml Sabourauds dextrose broth (SDB) and incubated at room temperature for 21 days under stationary conditions and filtered to separate the mycelium and the filtrate [14, 15].

Screening Bioactive Properties of Fungal Metabolites: Antibacterial activity of secondary metabolites extracted from endophytic fungi was tested against microorganisms such as *Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* using agar well diffusion method. Wells were made with the help of a borer extract were inoculated in separate wells. The zone of inhibition was detected after 24-48 h of incubation at 37 °C. The presence of a zone of inhibition on plates was used as an indicator of bioactive nature of the strain. Basing on the zone of inhibition, the potent metabolite producing fungal culture is selected for further studies.

Identification of Potent Fungal Strain: The potent fungal strain is identified based on colony characteristics (colony size, color, shape, appearance, pigment production) and micro morphological (mycelium, conidiophores, and conidia) characteristics [11, 12, 13].

Cultural and Morphological Characteristics of VJLB 37: The strain was grown on several culture media such as Sabourauds dextrose Agar (SDA), Czapek Dox Agar (CDA), Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Yeast Extract Malt Extract Dextrose Agar (YMD) for one week to study the colony characteristics (10).

Molecular Identification of Strain VJLB 37: Molecular identification was done using 18s rRNA sequence analysis. These sequences were deposited in the gene bank (NCBI). Phylogenetic and molecular evolutionary analysis was conducted using Molecular Evolutionary Genetics Analysis (*MEGA*) version 5.0 [16].

Growth Pattern of the Strain VJLB 37: The strain was inoculated into SD broth and incubated at 30 ± 2 °C on a rotary shaker at 180 rpm. At every 72 h interval up to 25 days, the flasks were harvested and the growth of the strain was measured in terms of the dry weight of biomass. The antimicrobial metabolite production was

determined in terms of its antimicrobial activity. The culture filtrate extracted with ethyl acetate was tested for antimicrobial activity by agar well diffusion method [17].

Selection of the Culture Media: To select the suitable growth medium, the isolate was grown on different culture media such as Czapek's Dox broth, Sabourod's broth, Potato dextrose broth, Malt extract broth, and Nutrient broth. The medium in which the isolate exhibited maximum antibiotic production expressed in terms of zone of inhibition was used as the optimized medium for further study. All the media were procured from HiMedia Laboratories, Mumbai, India.

Effect of Temperature on Biomass and Bioactive Metabolite Production: The fungus was subjected to different temperature ranges (15 to 45°C) to study the optimum temperature required for growth and bioactive metabolite yield. Under aseptic conditions, the medium was inoculated with the culture and incubated for 18 days. After incubation, the dry mycelial weight and the production of antimicrobial metabolites were recorded.

Effect of pH on Biomass and Bioactive Metabolite production: The effect of pH on the growth and bioactive metabolite production of the isolate was tested at different pH levels (pH 4-9). The medium was adjusted to the desired pH by adding 0.1N NaOH or 0.1N HCl. Each flask was inoculated with mycelial discs (5mm) in sterile conditions. Inoculated flasks were incubated at 30 ± 2 °C for 18 days, and the dry mycelial weight and bioactive metabolite productions were recorded.

Effect of NaCl Concentration on Biomass and Bioactive Metabolite Production: The effect of salinity on mycelial growth and bioactive metabolite produced by the isolate was carried out by growing the culture in medium with different NaCl concentrations, ranging from 1-6%. The biomass, as well as the bioactive metabolite production at different sodium chloride concentrations, was estimated and recorded.

Effect of Carbon Sources on Biomass and Bioactive Metabolite Production: To study the effect of different carbon sources, glucose, starch, sucrose, fructose, lactose, mannitol, carboxy methylcellulose, and maltose were used. Carbon source@ 1% was added to the SDB medium individually. The flasks were inoculated with 5 mm mycelial discs of seven-day-old fungal culture and incubated for 18 days. After the incubation period, biomass (mycelial dry weight) and the production of bioactive metabolites were recorded.

Effect of Nitrogen Source on Biomass and Bioactive Metabolite Production: To study the effect of different nitrogen sources, beef extract, yeast extract, peptone, ammonium sulphate, urea, malt extract, and sodium nitrate were used. Each nitrogen source @ 1% was added to the SDB medium and dextrose was used as the source of carbon in all the treatments. Flasks were inoculated with 5 mm mycelial discs of seven-day-old fungal culture under aseptic condition and incubated for 18 days. The mycelial weight and antimicrobial compound production were recorded at the end of the incubation period.

Fermentation, Extraction and Antimicrobial Assay of Bioactive Compounds Produced by VJLB 37: The pure culture of the strain was transferred aseptically into the seed medium (SD broth). After 7 days of incubation, the seed culture at a rate of 10% was inoculated into the production medium of the same composition. Fermentation was carried out at 30 ± 2 °C for 18 days under agitation at 180 rpm. After incubation, the dry weight of biomass was recorded and expressed as mg/100ml. The secondary metabolites produced by the strain were extracted twice with ethyl acetate and the pooled solvent extracts were concentrated under vacuum to yield a crude residue. The residue dissolved in ethyl acetate was used for testing antimicrobial activity [18].

Antimicrobial Spectrum of *Cladosporium cladosporioides* Grown on Optimized Medium: The culture inoculated into the optimized medium was incubated at 30 °C with shaking at 180 rpm for 18 days. The broth was then harvested and the growth of the strain was measured in terms of the dry weight of biomass. Antimicrobial metabolite production was determined in terms of its antimicrobial activity.

Statistical Analysis: Results obtained are statistically analyzed by using AGRISTAT and MINITAB16 software.

RESULTS AND DISCUSSION:

Sample Collection, Isolation, and Identification of Endophytic Fungi: A systematic study about the endophytic fungal isolates of Mangrove plant, *Lumnitzera racemosa,* was carried out to evaluate their capacity to produce bioactive compounds.

TABLE 1: ANTIMICROBIAL METABOLITE PRODUCTION OF THE STRAINS VJLB 31 TO VJLB 40 AGAINST TEST MICROORGANISMS

Fungal strains	Antimicrobial activity represented as Diameter of inhibition zone (mm)					
	Bacteria					Fungus
	I	II	III	IV	V	VI
VJLB31	08	08	08	08	06	07
VJLB32	07	07	07	08	07	07
VJLB33	06	06	06	06	07	07
VJLB34	12	11	12	10	12	09
VJLB35	06	08	06	09	06	07
VJLB36	07	07	07	07	07	08
VJLB37	28	29	28	26	28	28
VJLB38	11	11	11	12	10	12
VJLB39	20	22	20	22	22	20
VJLB40	10	11	10	09	13	08

*I = *Staphylococcus aureus*, II = *Bacillus subtilis*, III = *Bacillus megaterium*, IV = *Escherichia coli*, V = *Pseudomonas aeruginosa*, VI = *Candida albicans*. The results are analysed statistically and found to be significant at 5% level.

A total of 10 fungal strains designated as VJLB 31 to VJLB 40 were isolated from the leaf samples of *Lumnitzera racemosa.* All the fungal strains were screened for bioactive metabolites. All the 10 isolates showed antimicrobial activity in **Table 1**. Among the 10 isolates, VJLB 37 was found potent against test bacteria and fungi. Hence an attempt was made to identify the VJLB 37 strain.

Cultural and Morphological Characteristics of VJLB37: Cultural characteristics of **VJLB37** were studied on 6 different media *viz*. SD, NA, CDA, PDA YMA, and MEA**.** VJLB37 grew luxuriously on MEA followed by YMA and PDA.

Morphological characteristics like morphology of mycelium, conidiophore, and conidia were assessed by using a slide culture technique. Colonies are slow-growing, olivaceous-brown to blackish brown due to aging of the culture, often becoming powdery due to the production of abundant conidia. The reverse side is olivaceous black. Vegetative hyphae, conidiophores, and conidia are equally pigmented. Conidiophores are distinct from the vegetative hyphae, erect, straight, unbranched, with geniculate sympodial elongation. Conidia are 1 to 2 celled, smooth, with a distinct dark hilum and are produced in branched acropetal chains **Plate 1**.

The identification of the strain based on the molecular approach was also carried out based on 18s rRNA analysis. The partial sequence of the isolate was submitted to the GenBank database with accession number MG769026. The phylogenetic tree was constructed based on the Maximum Parsimony method **Fig. 1** and the strain was identified as *Cladosporium cladosporioides.*

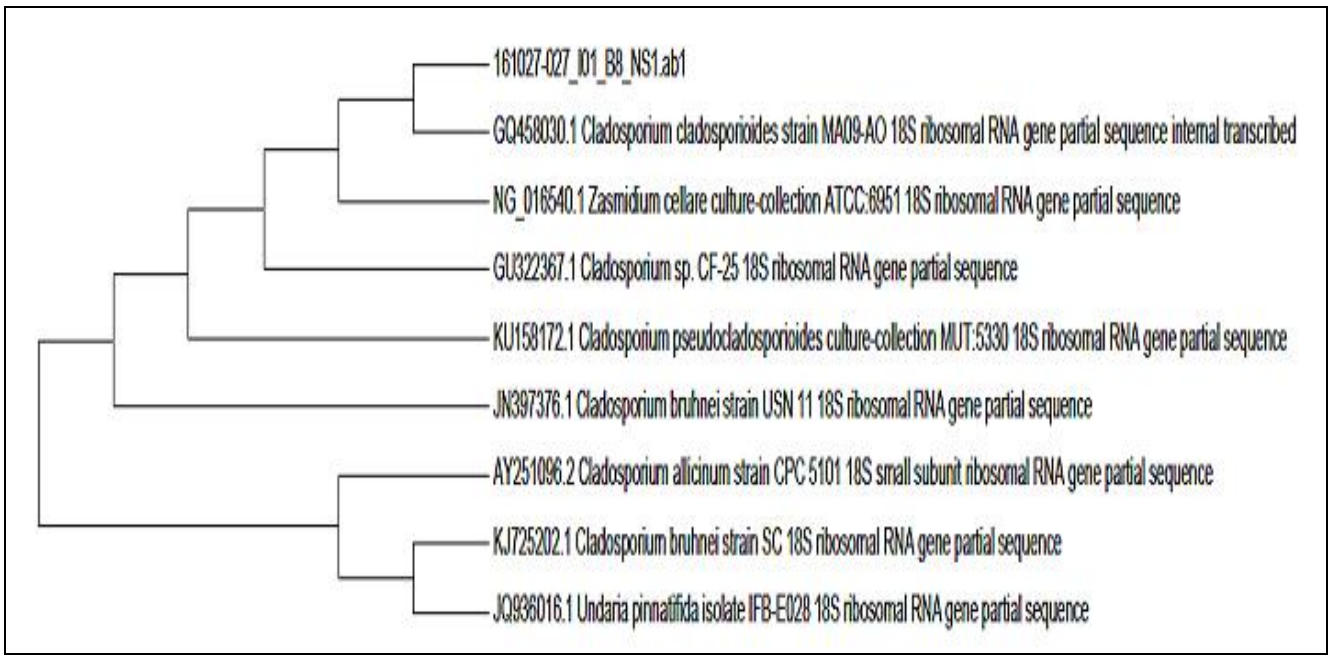
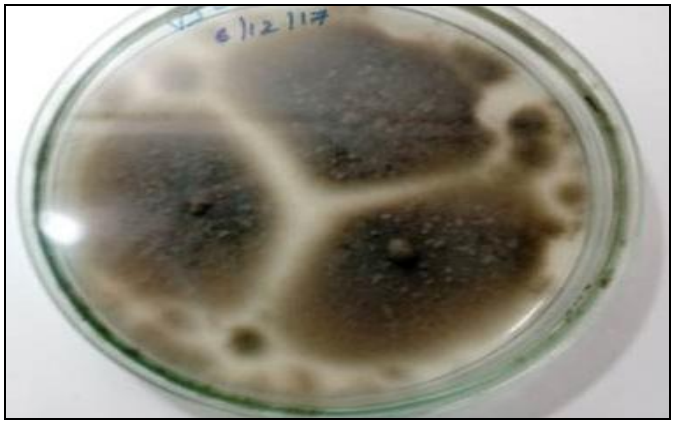
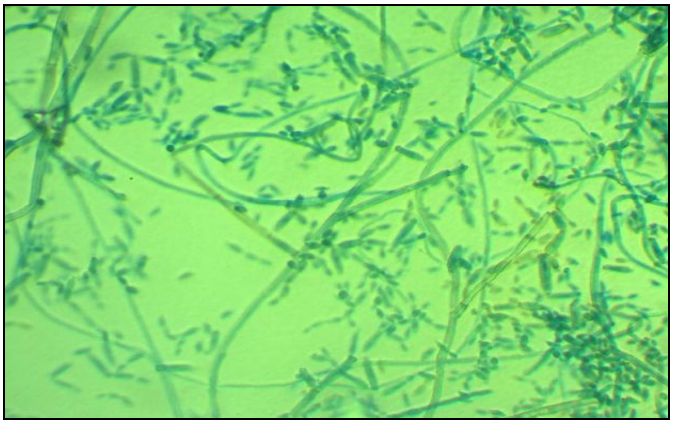


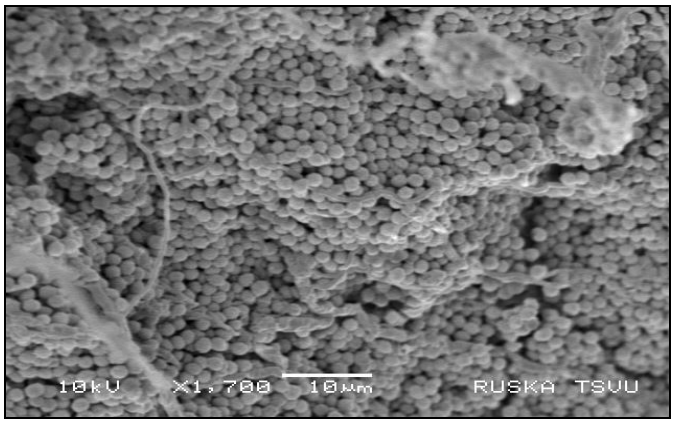
FIG. 1: MAXIMUM PARSIMONY TREE BASED ON 18S rRNA GENE SEQUENCE SHOWING RELATIONSHIP BETWEEN ISOLATE VJLB37 AND RELATED MEMBERS OF THE GENUS *CLADOSPORIUM*



FUNGUS GROWN ON MALT EXTRACT



AGAR MICROMORPHOLOGY OF THE FUNGUS (400x)



SCANNING ELECTRON MICROSCOPIC IMAGE OF *CLADOSPORIUM CLADOSPORIOIDES*

PLATE 1: CULTURAL CHARACTERISTIC OF *CLADOSPORIUM CLADOSPORIOIDES*

Growth Pattern of VJLB37: Analysis of growth pattern revealed that the culture entered into the log phase on 6th day of incubation which extended up to 14th day followed by stationary phase from 15-18 days and then entered into decline phase **Fig. 2**.

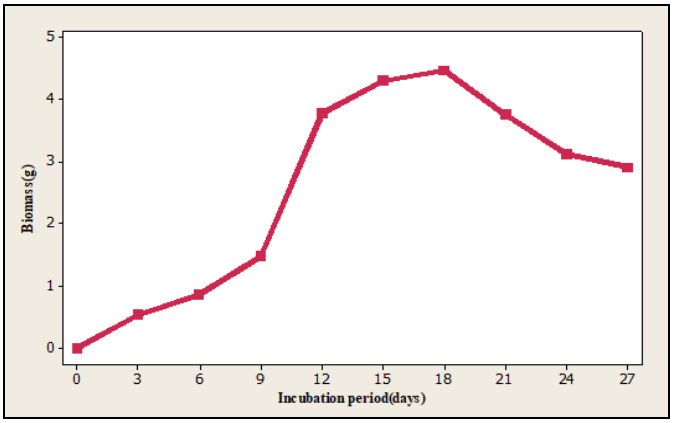


FIG. 2: GROWTH PATTERN OF *CLADOSPORIUM CLADOSPORIOIDES*

Effect of Culture Media on Antimicrobial Metabolite Production by *C. Cladosporioides*: Among the 6 culture media tested, ME broth was found to support antimicrobial metabolite production followed by YM broth, PD broth **Table 2**.

TABLE 2: INFLUENCE OF CULTURE MEDIA ON ANTIMICROBIAL METABOLITE PRODUCTION BY *CLADOSPORIUM CLADOSPORIOIDES* AGAINST TEST MICROORGANISMS

Media	Diameter of inhibition zone (mm) against microorganisms					
	*I	II	III	IV	V	VI
CDB	11	10	10	09	10	09
SDB	12	14	12	12	14	12
YMB	13	14	14	14	15	14
MEB	28	30	30	30	30	28
NB	04	03	03	04	05	04
PDB	12	13	14	14	14	15

*I = *Staphylococcus aureus*, II = *Bacillus subtilis*, III = *Bacillus megaterium*, IV = *Escherichia coli*, V = *Pseudomonas aeruginosa*, VI = *Candida albicans*. The results are analysed statistically and found to be significant at 5% level.

Effect of pH on Biomass and Antimicrobial Metabolite Production of *C. cladosporioides*: The influence of pH on growth and bioactive metabolite production was determined by adjusting the pH of MEB from 4.0 to 9.0.

Maximum growth was observed at pH 6.0 followed by pH 7.0 and 5.0 **Table 3**.

TABLE 3: EFFECT OF pH ON BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION BY *CLADOSPORIUM CLADOSPORIOIDES*

pH	Biomass (g/100ml)	Antimicrobial activity in terms of zone of inhibition (mm)					
		I	II	III	IV	V	VI
4	1.065	50	60	52	53	50	55
5	2.275	14	25	10	14	14	14
6	6.625	12	15	12	12	12	14
7	3.79	14	18	11	10	13	14
8	2.435	12	16	11	12	12	14
9	0.435	08	25	11	08	09	12

I = *Staphylococcus aureus*, II = *Bacillus subtilis*, III = *Bacillus megaterium*, IV = *Escherichia coli*, V = *Pseudomonas aeruginosa*, VI = *Candida albicans.* The results are analysed statistically and found to be significant at 5% level.

Effect of Temperature on Biomass and Antimicrobial Metabolite Production of *C. cladosporioides:* Temperature has a profound effect on the growth of the strain as well as bioactive metabolite production. The yield of bioactive metabolites and biomass were recorded when grown at a temperature ranging from 20 to 40 °C, optimum being 30 °C indicating mesophilic nature of the strain **Table 4**.

TABLE 4: EFFECT OF TEMPERATURE ON BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION BY *CLADOSPORIUM CLADOSPORIOIDES*

Temp (°C)	Biomass (g/100ml)	Antimicrobial activity in terms of zone of inhibition (mm)					
		I	II	III	IV	V	VI
20	1.5	07	14	14	18	14	13
25	3.1	13	17	17	25	17	15
30	3.4	30	23	23	29	33	21
35	1.3	05	08	10	14	08	08
40	0.2	00	02	02	00	02	02

I = *Staphylococcus aureus*, II = *Bacillus subtilis*, III = *Bacillus megaterium*, IV = *Escherichia coli*, V = *Pseudomonas aeruginosa*, VI = *Candida albicans.* The results are analyzed statistically and found to be significant at 5% level.

Effect of NaCl on Biomass and Antimicrobial Metabolite Production of *C. cladosporioides*: The influence of NaCl on growth and bioactive metabolite production was determined by adjusting the NaCl of MEB from 1% to 6%. Maximum growth was observed at 4% NaCl followed by 5% NaCl. Antimicrobial metabolite production was high at 5% NaCl **Table 5.**

Effect of Carbon Sources on Biomass and Antimicrobial Metabolite Production of *C. cladosporioides*: The strain exhibited good growth in terms of biomass as well as antimicrobial activity in mannitol followed by starch and Carboxy Methyl Cellulose (CMC) as carbon source while it was moderate with fructose, maltose, lactose compared to dextrose and sucrose **Table 6**.

TABLE 5: EFFECT OF NACL ON BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION BY *CLADOSPORIUM CLADOSPORIOIDES*

Nacl conc. (%)	Biomass (g/100ml)	Antimicrobial activity in terms of zone of inhibition (mm)					
		I	II	III	IV	V	VI
1	0.32	11	16	12	16	14	12
2	1.005	13	20	14	10	12	08
3	1.85	22	51	28	30	30	32
4	2.1	34	30	30	34	32	35
5	1.46	42	40	35	40	36	38
6	0.245	24	26	28	24	26	28

I = *Staphylococcus aureus*, II = *Bacillus subtilis*, III = *Bacillus megaterium*, IV = *Escherichia coli*, V = *Pseudomonas aeruginosa*, VI = *Candida albicans.* The results are analysed statistically and found to be significant at 5% level.

TABLE 6: EFFECT OF CARBON SOURCES ON BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION BY *CLADOSPORIUM CLADOSPORIOIDES*

Carbon sources (1%)	Biomass (g/100ml)	Antimicrobial activity in terms of zone of inhibition (mm)					
		I	II	III	IV	V	VI
Fructose	0.28	20	20	14	10	20	11
Dexrose	0.18	12	25	12	12	25	12
Sucrose	0.135	12	18	12	12	18	12
Maltose	0.24	13	12	12	10	20	12
Lactose	0.21	18	15	15	12	24	12
Mannitol	3.285	24	26	25	25	24	24
CMC	0.335	10	12	10	10	20	10
Starch	1.485	14	13	12	12	16	11

I = *Staphylococcus aureus*, II = *Bacillus subtilis*, III = *Bacillus megaterium*, IV = *Escherichia coli*, V = *Pseudomonas aeruginosa*, VI = *Candida albicans.* The results are analyzed statistically and found to be significant at 5% level.

Effect of Nitrogen Sources on Biomass and Antimicrobial Metabolite Production of *C. cladosporioides*: The strain exhibited good growth with beef extract and tryptone followed by ammonium sulphate. Growth is moderate with malt extract, yeast extract, and peptone while growth is poor in sodium nitrate and urea. Ammonium sulphate as nitrogen source supported good metabolite production in **Table 7**.

Antimicrobial Spectrum of *C. cladosporioides* Grown on Optimized Culture Medium: *C.cladosporioides* was cultured on optimized MEB (1% mannitol, 1% ammonium sulphate, 5% NaCl, Temp. – 30 °C, pH – 4.0) at optimal conditions for 18 days and the metabolite was harvested and tested for antimicrobial activity against test bacteria and fungi. High antimicrobial activity **Plate 2** was recorded when cultured under optimized conditions **Table 8**. Attempts are in progress for the identification of bioactive metabolites produced by *C. cladosporioides.*

TABLE 7: EFFECT OF NITROGEN SOURCES ON BIOMASS AND ANTIMICROBIAL METABOLITE PRODUCTION BY *CLADOSPORIUM CLADOSPORIOIDES*

Nitrogen Sources (1%)	Biomass (g/100ml)	Antimicrobial activity in terms of zone of inhibition (mm)					
		I	II	III	IV	V	VI
Tryptone	2.545	34	26	34	30	28	26
Ammonium sulphate	1.205	42	60	42	52	56	60
Malt Extract	0.74	14	20	14	18	20	20
Urea	0.1	12	12	12	12	12	12
Peptone	0.295	18	25	18	24	25	25
Sodium Nitrate	0.105	16	24	16	24	24	24
Yeast Extract	0.47	10	19	10	12	15	19
Beaf Extract	3.050	25	28	25	22	26	28

I = *Staphylococcus aureus*, II = *Bacillus subtilis*, III = *Bacillus megaterium*, IV = *Escherichia coli*, V = *Pseudomonas aeruginosa*, VI = *Candida albicans*. The results are analyzed statistically and found to be significant at 5% level.

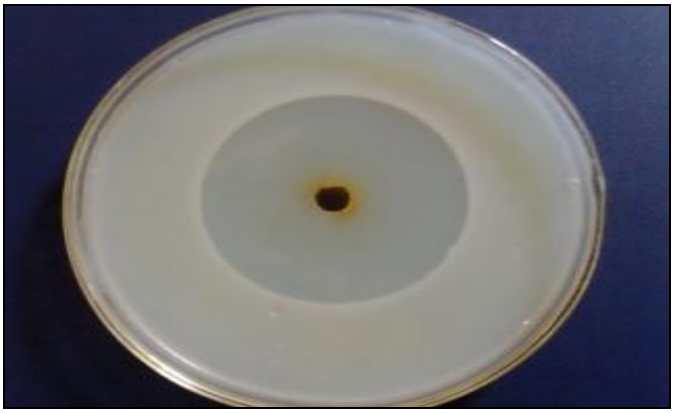


PLATE 2: ANTIMICROBIAL METABOLITE PRODUCTION AGAINST *BACILLUS MEGATERIUM*

TABLE 8: ANTIMICROBIAL ACTIVITY OF METABOLITES PRODUCED BY *CLADOSPORIUM CLADOSPORIOIDES* ON OPTIMIZED MALT EXTRACT BROTH

Test organism	Diameter of Inhibition zone (mm)
Bacillus megaterium	66
Staphylococcus aureus	62
Bacillus subtilis	58
Escherichia coli	64
Pseudomonas aeruginosa	63
Candida albicans	59

The results are analyzed statistically and found to be significant at 5% level

CONCLUSION: This is the first report of *C. cladosporioides* isolated from mangrove plant *Lumnitzera racemosa* of Gilakaladindi. In this study, *C. cladosporioides* was cultivated on CDA, PDA, NAM, SDA, MEA and YMA culture media. MEA promoted good growth as well as antimicrobial metabolite production. Optimized MEA promoted good growth and high metabolite yield reflected by high antimicrobial activity. Hence, *C. cladosporioides* is considered to be a potent strain as it exhibited good antimicrobial activity. Attempts are in progress for the identification of bioactive metabolites produced by *Cladosporium cladosporioides*.

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CONFLICTS OF INTEREST: None declared

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