Identification, Characterization, And Analysis of Bioactive Compounds of Endophytic Bacteria from *Eclipta Prostrata*

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***Abstract*—Plants are considered as the best source of medicinal compounds to combat diseases and are used for treatment worldwide - secondary metabolites extracted from non-pathogenic microbes residing in tissues of medicinal plants, popularly known as endophytes. The present work was undertaken to isolate and identify endophytic bacteria from the leaves of *Eclipta prostrata.* Based on morphological, biochemical Characterization, and 16S rRNA sequencing technique, the isolate was identified as *Bacillus stratosphericus* 41KF2a. The production of secondary metabolites from selected bacterial isolates was further characterized by Fourier Transform infrared (FTIR) spectroscopy and Gas Chromatography – Mass spectroscopy (GC-MS) analysis. The optimization of the growth of the isolate was analyzed with different growth parameters such as pH, temperature, incubation time, and NaCl concentration. The antibiotic susceptibility test was performed for the bacterial isolate using seven different antibiotics using the disc diffusion method. Thus, endophytes are beneficial in producing a wide range of natural products that can be used in agriculture, pharmaceutical, and food industries. There is an increasing interest in developing the potential biotechnological applications of endophytes for improving phytoremediation and the sustainable production of non-food crops for biomass and biofuel production.**

***Index Terms*—Endophytic bacteria, *Eclipta prostrata*, 16S rRNA sequencing, Secondary metabolites, FTIR, GC-MS.**

I. INTRODUCTION

Medicinal plants are the vital source of a wide variety of therapeutic drugs, playing an essential role in treating and preventing diseases all over the Globe. Medicinal plants are the source of many bioactive compounds used in producing therapeutic drugs due to their economical, efficient, and accessible form (Anjum & Chandra,2015). De Barry in 1866 coined the term Endophyte, Endophytes are the mutualists microorganisms such as bacteria, fungi, archaea and protists which reside inside the plant tissues such as leaves, stems, roots, flowers, seeds, fruits, ovules, legume nodules, intercellular and intracellular spaces (Sturz *et al*.,1997, Uma and Maheshwari., 2018, Rhoden *et al*., 2015). The study of endophytic bacteria has provoked great interest due to their less explored nature, medicinal, pharmaceutical, industrial, and agricultural applications (Smita *et al*., 2015). Endophytic microorganisms are the source of diverse novel bioactive metabolites having remarkable therapeutic properties such as antimicrobial, antitumor, antidiabetic, antioxidant, and immunomodulatory (Strobel *et al*., 2004). Endophytic secondary metabolites, namely alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, and lignans, revealed potential applications in plant growth enhancements, phytoremediation, abiotic and biotic stress tolerance, nitrogen fixation, and medicinal applications (Jain *et al*., 2017; Arunachalam & Gayathri, 2010). Endophytic bacteria can produce secondary metabolites based on plant environmental conditions like season, latitude, altitude, climate, and soil conditions.

Eclipta prostrata, also known as *Eclipta alba*, belongs to the Asteraceae family and is commonly regarded as false daisy Fig.1. *E. prostrata* shows a variety of medicinal values, such as treating gastrointestinal disorders, respiratory tract disorders, hair loss, hair graying, liver disorders, skin disease, spleen enlargement, cuts and wounds, snakebites, syphilis,

filariasis, and leprosy (Li *et al*., 2019). Phytoconstituents of *E. prostrata* show significant antimicrobial, antidiabetic, antioxidant, analgesic, and anti-inflammatory properties (Duan *et al*.,2006).

Fig 1: *Eclipta prostrata*

The aim of exploring the biodiversity of endophytic strains for novel bioactive metabolites that would lead to the identification of new drugs for effective treatment of diseases, the present study was undertaken to investigate and identify endophytic bacteria present in the leaves of the medicinal plant, *Eclipta prostrata*, also extraction of secondary metabolites by conventional methods, and its analysis through FTIR and GC-MS.

II. MATERIALS AND METHODS

2.1 Collection of Plant Sample

Healthy leaf specimens of *the Eclipta prostrata* plant were collected from their natural habitat at the greenhouse of Sri Krishna Arts and Science College, Coimbatore, Tamil Nadu, India. These plants were maintained under controlled environmental conditions in a laboratory for further experimental analysis.

2.2 Isolation and purification of endophytic bacteria (Abhini & Zuhara, 2018)

Surface sterilization is considered as the most desirable step in isolating endophytes without harming them. The collected leaves were washed under running tap water for 20 minutes to remove the adhered soil particles and rinsed 4-5 times with sterile distilled water. The sample was immersed in 70% ethanol for 1 minute, followed by surface sterilization in 2% sodium hypochlorite solution for 2 minutes, washed with sterile distilled water two to three times, and dried under sterile conditions. The leaves were dried, cut into small pieces, placed on nutrient agar plates under aseptic conditions, and incubated at 37°C for 24 hours. Isolates with distinct colonies were selected and streaked in nutrient agar plates, and the available purified endophytes were stored at 4°C for further analysis.

2.3 Characterization of endophytic bacteria

The selected strains of endophytic bacteria were analyzed by preliminary Characterization, such as colony morphology, Gram staining, and biochemical tests, such as carbohydrate fermentation test, indole test, citrate test, catalase test, oxidase, MR-VP, hydrogen sulphide production test, gelatine test, starch and lipid activity, and motility test.

2.4 16S rRNA sequencing

The identification of an endophytic bacterial strain by 16S rRNA partial sequencing was performed at Acme ProGen Biotech Pvt. Ltd., Salem, India, by using primers, and the sequence was analyzed. The 16S rRNA gene of the selective isolate was sequenced and presented in FASTA format, and the sequence data were identified by BLAST analysis.

2.5 Production of Ammonia (Geetha et al., 2014)

Endophytic bacteria were tested for ammonia production in peptone water by inoculating the bacterial culture and incubating for 48 hours at 30 °C. Then, 0.5 ml of Nessler's reagent was added to each tube, and the change in color from brown to yellow was observed as a positive indicator of ammonia production.

2.6 Production and extraction of metabolites (Swarnalatha *et al*., 2015)

The inoculum culture of bacteria was prepared by inoculating 100 ml of nutrient broth and incubating for 24 hours at 37°C. The 24-hour-old bacterial culture was inoculated into nutrient broth and incubated at 37°C in a shaking incubator at 180 rpm for 5 days. Bacterial culture was centrifuged at 8000 rpm for 5 minutes to obtain supernatant.

An equal volume of solvents such as butanol and acetone were added and centrifuged at 1000 rpm for 60 to 120 minutes to separate the endophytic extract.

2.7 FTIR analysis of acetone and butanol extract

The Fourier Transform Infrared Spectroscopy (FTIR) analysis was done on a Shimadzu 8400S spectrophotometer (Shimadzu Corporation, Japan) in the mid-IR region of 500 to 4000 cm-1. The analysis was carried out by using solvent extract and performed to identify the structural composition of the given sample, to know the different functional groups present in the extracts.

2.8 GC-MS analysis

The solvent extracts were separated, and the bioactive compounds were identified through Gas chromatography-mass spectroscopy (GC-MS). This analysis was performed with an Agilent 7820A gas chromatograph equipped with a –DB-5 column (30 m × 0.25 mm; 0.25 μm film thickness), interfaced with an Agilent mass selective detector 5977E inter MSD. Oven temperature program was from 100 to 270°C at 10°C/min; helium was used as carrier gas, and the flow rate was 1.2 ml/min.

2.9 Antibiotic Sensitivity Test

Antibiotic sensitivity test of the endophytic bacterial isolate was determined by the disc diffusion method. All the cultures were inoculated into nutrient agar plates using a sterile swab. Antibiotics such as Ampicillin (10 mg), Amoxicillin (10 mg), Tetracycline (10 mg), Vancomycin (10 mg), Penicillin (10 mg), Ciprofloxacin (10 mg), and Gentamycin (10 mg) were placed on nutrient agar plates and incubated at 30°C for 24 hours. After incubation, the antibiotic susceptibility pattern was determined by measuring the zone of inhibition.

2.10 Optimization (Gupta et al., 2015, Soni Muhsinin et al., 2016)

The bacterial isolates were optimized under various growth parameters such as pH, temperature, incubation time, and NaCl concentration. Optimization and standardization of growth of the isolates on a range of pH was studied by inoculating the isolates on nutrient broth media having a pH range from 5 to 9 and incubating at 37°C for 48 hours. The growth of bacteria was measured at 610 nm in the ELICO CL 223 Colorimeter, and values were recorded. Effect of temperature on growth of isolates was determined by inoculating the isolates in nutrient broth and incubating at different temperatures, that is, at 4°C, 27°C, 37°C, and 50°C for 48 hours. The biomass concentration of bacteria was measured at 610 nm in an ELICO CL 223 Colorimeter, and the values were recorded. Optimization and standardization of growth of the isolates on various incubation times were studied by inoculating the isolates on nutrient broth media and incubating at 37°C for 48 hours. The data were taken at every interval of 24, 48, and 72 hours, and the absorbance was measured using a colorimeter at a wavelength of 610 nm. To study the effect of sodium chloride on the growth of isolates, the nutrient broth medium was supplemented with different concentrations of NaCl, and the endophytic bacterial isolates were inoculated and incubated at 27°C for 3-4 days. Biomass was observed by measuring the optical density at 610 nm.

III. RESULTS AND DISCUSSION

3.1 Isolation and purification of endophytic bacteria

The medicinal plant *Eclipta prostrata* was collected and maintained under controlled conditions for the endophytic bacterial extraction. The leaf samples were surface sterilized to remove the dirt and epiphytic microorganisms without harming endophytic bacteria. Among the four samples, only one sample, KK, showed the growth of endophytic bacteria around the margins of the leaf sample on the nutrient agar plate. The bacterial isolates were subcultured to get the pure culture, stored at 4°C for further analysis, and presented in Fig. 2.

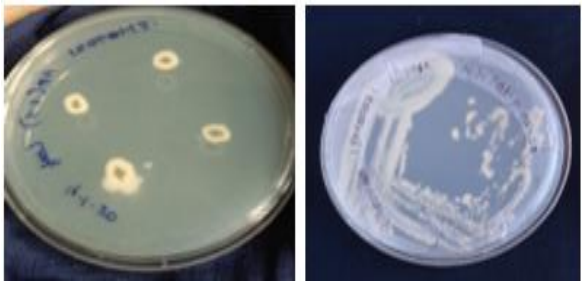

Fig 2a Growth of endophytic bacteria in Agar Plate
2b Purified bacterial culture

3.2 Characterization of endophytic bacteria

3.2 a) Morphological Characterization

The morphological characters of the selected endophyte were studied, and Gram staining was also performed for the isolate named KK. The isolate showed positive in Gram stain, rod-shaped, motile, and morphological observations as pale white in color,

creamy consistency with flat elevation, opaque, and irregular.

3.2.b) Biochemical Characterization

The biochemical analysis of the endophytic bacterial isolate showed positive to methyl red, Oxidase, Starch and lipid hydrolysis, and other tests like Indole, Voges–Proskauer, Citrate utilization, Catalase, Urease, H_2S production, and amylase test. The isolate showed acid production in carbohydrate fermentation broth supplemented with sucrose, which indicates sucrose as a carbon source. The isolate utilized glucose, fructose, and maltose, and the results are similar to those of (Panigrahi *et al*, 2018). The isolate KK showed negative results for Casein hydrolysis and lactose carbohydrate fermentation (Vikas Kumar, *et al*, 2014).

3.2.c) 16S rRNA sequencing

The endophytic bacterial isolates from the medicinal plant were identified based on molecular basis using 16S rRNA gene sequencing, and the results are indicated in Table 1

Table 1: 16S rRNA sequence analysis of endophytic isolates from *Eclipta prostrata*

Plant	Endophytic isolate	Accession number	Closest NCBI database	Percentage identity
Eclipta prostrata	S1	(NR_042336.1)	*Bacillus stratosphericus* strain 41KF2a	99%

The bacterial isolate showed the highest similarity (99%) with *Bacillus stratosphericus* 41KF2a strain, and the sequence ID (NR_042336.1) with a length of 1531 base pairs. *B. stratosphericus* is a motile, rod-shaped, gram-positive bacterium that is endospore-forming. It is a facultative anaerobe capable of multiple modes of cellular respiration. *Bacillus stratosphericus* bacteria are important in the medical, agricultural, and medicinal fields. Durairaj *et al.*, 2017 reported that *B. stratosphericus* produces a variety of antimicrobial substances and is applied in bioremediation processes.

B. stratosphericus strains show promising biopesticide activity against bacterial phytopathogens. They can produce protease, amylase, L-asparaginase activity, and also show phosphate solubilization activity to promote plant growth (Pola *et al*., 2018). Hentati *et al*., 2019 utilized *Bacillus stratosphericus* strain FLU5 for the production of biological surfactants utilizing various carbon sources.

3.3 Production of ammonia

The endophytic bacteria tested positive for ammonia production in peptone water after 48 hours of incubation. The production of ammonia is an important source of nitrogen for plants, and it plays a significant role in plant metabolism and improves yield. The production of ammonia as the secondary metabolite indicates its indirect influence on plant growth (Vikas Kumar *et al.*, 2014).

3.4 Production and extraction of secondary metabolites

Endophytic secondary metabolites have a variety of applications in agriculture and the pharmaceutical field due to their unique niche. The secondary metabolites of endophytic bacterial culture were extracted by Swarnalatha *et al*. (2015) using acetone and butanol as solvents to identify the best among them.

The endophytic bacterial extract was screened to detect the presence of diverse secondary metabolites by FTIR and GC–MS analysis to further use in the medicinal and agricultural fields. The bacterial endophytes are reported to produce novel, highly active, low-toxic secondary metabolites (Bahig *et al*., 2012).

Bacterial endophytes of plants can produce several unique secondary metabolites with pharmaceutical applications.

Mehanni and Safwat., 2010 reported that bacterial endophytes isolated from medicinal plants can produce similar or the same metabolites as their hosts. Secondary metabolites produced by endophytes improve disease resistance, enhance growth, and act as biocontrol agents.

FTIR analysis of *Bacillus stratosphericus* 41KF2a

The presence of a functional group is determined using FTIR spectroscopy analysis spectrum in the range 300 – 4000 cm^{-1}. Figure 3 and Table 2 show the FTIR spectrum of the acetone extract's bacterial isolate.

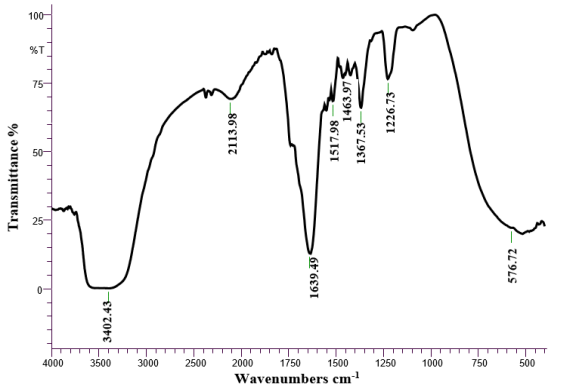

Fig. 3 FTIR analysis of bacterial isolate – acetone extract

Table 2 FTIR analysis of acetone extract of *Bacillus stratosphericus* 41KF2a

S. No	Class of compounds	Absorption, cm^{-1}	Intensity	Assignment
1.	Halo compound	576.72	Strong	C-Br stretch
2.	Amine	1226.73	Medium	C-N stretch
3.	Alcohol	1367.53	Medium	O-H bend
4.	Alkane	1463.97	Medium	C-H bend
5.	Nitro Compound	1517.98	Strong	N-O stretch
6.	Alkenes	1639.49	Strong	C=C stretch
7.	Alkyne	2113.98	Weak	C≡C stretch
8.	Alcohols	3402.43	Strong, broad	O-H stretch

The IR result of the acetone extracts of the bacterial isolate showed a broad-spectrum range at 3402 cm^{-1}, indicating a carboxylic group (O-H). The band at 2113 cm^{-1} indicates alkyne (C≡C), 1639.49 at the isolates showed alkene group (C=C), 1517 cm^{-1} indicates nitro compound, and 1463 cm^{-1} refers to alkane group (C-H). The band at 1367.53 cm^{-1} denotes the presence of alcohol (O – H) bend, 1226 cm^{-1} shows amine group, and the band at 576 cm^{-1} indicates halo compounds (C-Br).

(Nu Yin aye., 2013) The similar results band at 1367 cm^{-1} were 1381 cm^{-1}, and indicate the presence of alcohol (O-H), and the absorption peak at 3402 cm^{-1} indicates the presence of (O-H) stretch. Stretching 1332.86 cm-1 denotes the possibility of alcohol (O-H) bending as reported in (Lal M., 2016).

The FTIR analysis of the bacterial isolate butanol extract is shown in Fig. 4 and Table 3.

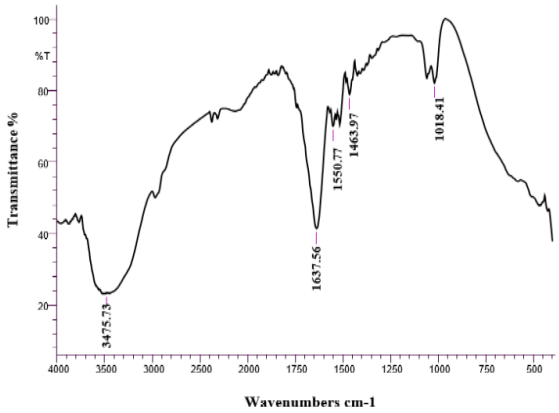

Fig. 4 FTIR analysis of bacterial isolate – Butanol extract

Table 3: FTIR analysis of butanol extract of *Bacillus stratosphericus* 41KF2a

S. No	Class of compounds	Absorption, cm^{-1}	Intensity	Type of bond
1.	Fluro compounds	1018.41	Strong	C-F stretch
2.	Alkane	1463.97	Medium	C-H bend
3.	Nitro compound	1550.77	Strong	N-O stretch
4.	Alkene	1637.56	Medium	C=C stretch
5.	Alcohol	3475.73	Strong, broad	O-H stretch

The IR result of the butanol extracts of the bacterial isolate S1 showed that a broad-spectrum range stretching at 3475.73 cm^{-1} indicates a carboxylic group (O-H stretch). The band at 1637 cm^{-1} indicates an alkene group, and 1550 cm^{-1} refers to an alkane nitrogen compound. The band at 1463.97 cm^{-1} denotes an alkane group, and the band at 1018.41 cm^{-1}indicates the presence of fluoro compounds.

The strong band at 3404 and 3475 cm-1 was assigned to the hydroxyl stretching vibration. The bands in the 1500 and 1463 cm-1 region were assigned to C-H deformation vibration, and the bands 1075 and 1018 cm-1 corresponded to fluoro compounds. A characteristic absorption at 1550 cm-1 could be due to

the stretching vibration of a nitrogen compound. Lal M (2016) also observed a similar spectrum. Thus, FTIR analysis of the acetone extract of the bacterial isolate contains many functional compounds compared to the butanol extract, which is considered a suitable solvent for secondary metabolite extraction of *Bacillus stratosphericus.*

3.5 GC – MS analysis of *Bacillus stratosphericus* 41KF2a acetone extract

The acetone extract of *Bacillus stratosphericus 41KF2a* was analyzed by gas chromatography-mass spectrometry analysis, and the results are given in Fig. 5 and Table 4. The compound was identified based on peak area and mass by charged ions. The primary secondary metabolites present in the acetone extract of S – 1 were tert-Butyldimethylsilyl 14-acetoxy-3,6,9,12-tetraoxatetradecan-1-oate, 1,4-Bis(trimethylsilyl)benzene, Trimethyl [4-(2-methyl-4-oxo-2-pentyl) phenoxy] silane, 1,2-Benzisothiazol-3-aminetbdms, Methyltris(trimethylsiloxy)silane, 1,2 Bis(trimethylsilyl)benzene, Silane, Trimethyl [5-methyl-2-(1-methylethyl) phenoxy]-, 4-Methyl-2-trimethylsilyloxy-acetophenone.

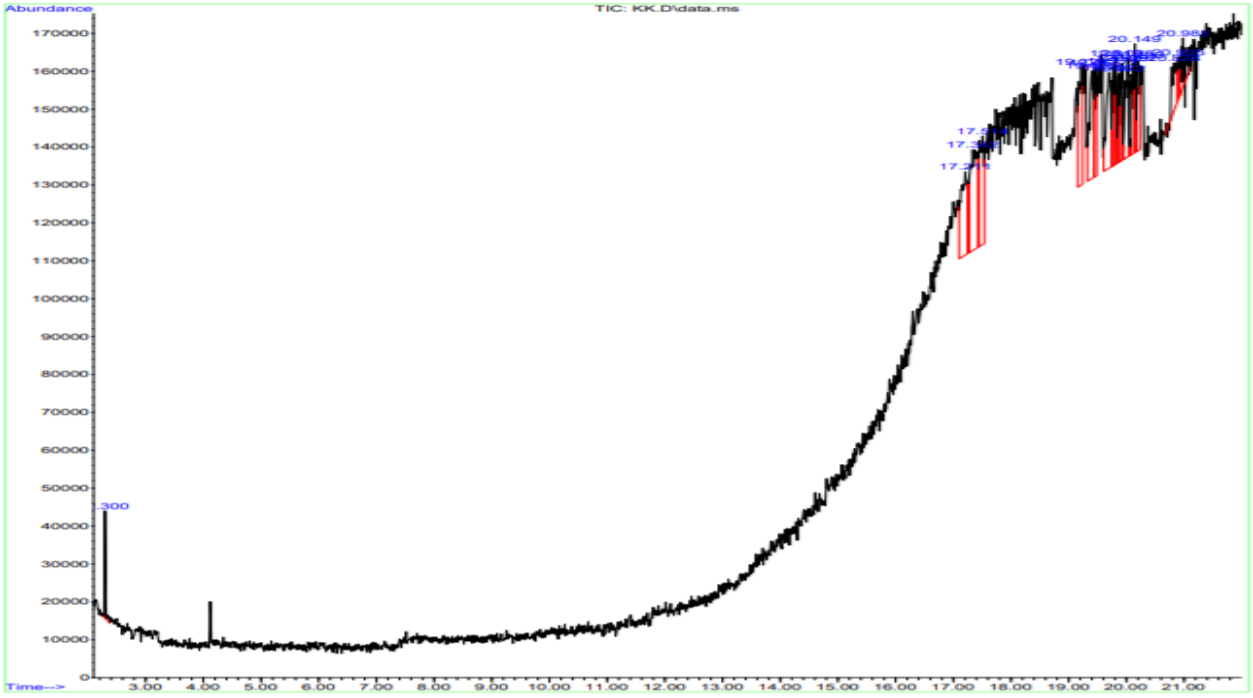

Fig. 5 GC – MS analysis of bacterial isolate – acetone extract

Table 4 GC-MS spectral analysis of acetone extract of *B. stratosphericus* 41KF2a

S. No	Retention time	Compound name	Area (%)
1	2.304	tert-Butyldimethylsilyl 14-acetoxy-3,6,9,12-tetraoxatetradecan-1-oate	2.06
2	17.215	1,4-Bis(trimethylsilyl)benzene	10.05
3	17.338	Trimethyl[4-(2-methyl-4-oxo-2-pentyl) phenoxy] silane	13.84
4	17.518	Trimethyl[4-(2-methyl-4-oxo-2-pentyl) phenoxy] silane	9.25
5	19.229	1,4-Bis(trimethylsilyl)benzene	8.10
6	19.409	1,4-Bis(trimethylsilyl)benzene	7.55
7	19.456	1,4-Bis(trimethylsilyl)benzene	3.92
8	19.702	1,4-Bis(trimethylsilyl)benzene	7.95
9	19.778	1,4-Bis(trimethylsilyl)benzene	3.01
10	19.815	1,2-Benzisothiazol-3-amine tbdms	3.50
11	19.872	1,2-Benzisothiazol-3-amine tbdms	2.25
12	19.929	Methyltris(trimethylsiloxy)silane	3.56

13	20.033	1,2-Bis(trimethylsilyl)benzene	5.81
14	20.099	1,4-Bis(trimethylsilyl)benzene	4.17
15	20.146	Silane, trimethyl[5-methyl-2-(1-methylethyl) phenoxy]-	2.45
16	20.203	Trimethyl[4-(2-methyl-4-oxo-2-pentyl) phenoxy] silane	4.30
17	20.827	1,2-Bis(trimethylsilyl)benzene	3.62
18	20.903	1,2-Benzisothiazol-3-amine tbdms	1.22
19	20.988	4-Methyl-2-trimethylsilyloxy-acetophenone	3.37

3.6 Antibiotic sensitivity test

Endophytic bacterial isolate was observed to be sensitive to Ampicillin, Tetracycline, Vancomycin, Penicillin, Ciprofloxacin and gentamycin at higher concentration and the isolate were observed to be resistant to amoxicillin and the results are presented in Table 5. Similar results were reported in (Arunachalam and Gayathri.,2010) the gram-positive isolates were sensitive to Ampicillin, Gentamycin and Ciprofloxacin.

Table 5 Antibiotic sensitivity test of bacterial isolate

Antibiotics	Bacterial isolate	
	Zone of inhibition (in mm)	Susceptibility Pattern
Ampicillin	32	S
Amoxicillin	5	R
Tetracycline	30	S
Vancomycin	15	S
Penicillin	32	S
Ciprofloxacin	25	S
Gentamycin	15	S

R – Resistant, S – Sensitive

3.7 Optimization of the growth of endophytic bacteria

The endophytic bacterial isolates were optimized to check their growth parameters at different pH, temperature, incubation time, and NaCl concentration. Even a slight change in climatic and soil composition affects the endophytic bacterial growth and secondary metabolite production.

To identify the effect of cultural variation in bacterial growth, a bacterial isolate was inoculated in nutrient media at specific cultural conditions, incubated, and the growth of bacteria was measured after 48 hours in an ELICO CL 223 Colorimeter at 610 nm, as mentioned in Fig.6.

The optimum growth of Bacteria was observed at pH 7 (Bind & Nema,2019) obtained similar results during the study of isolation of endophytic bacteria from *Pigeon pea* plant.

The isolate showed optimum temperature at 37°C, similar to the results of (Gupta et al, 2015; Bind & Nema,2019) research work. The endophytic bacterial isolates showed maximum growth at 5% NaCl.

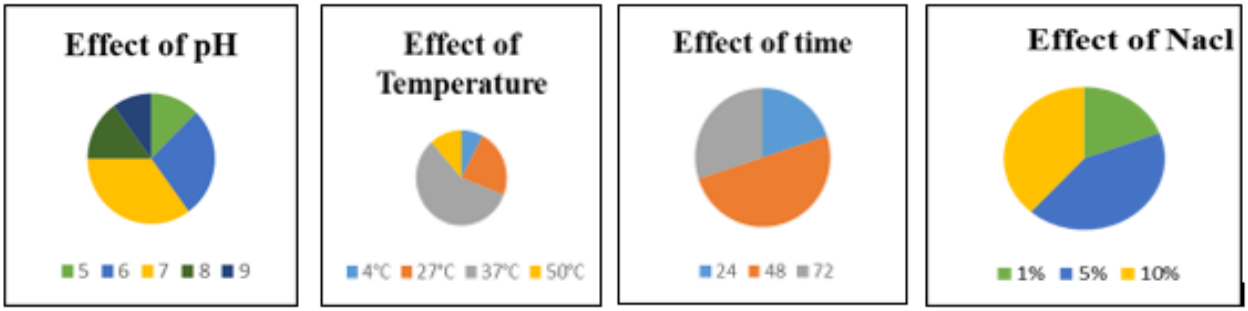

Fig. 6 Effect of pH, Temperature, Incubation time, and NaCl concentration on Bacterial growth

IV. CONCLUSION

Medicinal Plants have been reported to identify and screen the bioactive natural organic compounds, which are beneficial in developing new pharmaceuticals to meet the therapeutic needs. The present study focused on isolating endophytic bacteria from the leaves of *Eclipta prostrata.* The molecular

Characterization was done by 16S rRNA sequencing, and the bacteria were identified as *Bacillus stratosphericus 41KF2a*. The secondary metabolites were produced by using acetone and butanol extracts. The bacterial secondary metabolites were analyzed by FTIR analysis, which indicated the presence of carboxylic acid, nitrites, ketones, alkenes, aliphatic amines, alkanes, and alkyl functional groups, and GC-MS analysis. The optimum growth of the isolate was observed at pH seven and temperature 37°C, 5% NaCl, and 48 hours of incubation time. The antibiotic susceptibility test pattern was observed, and the bacterial isolate was resistant to amoxicillin, but other antibiotics were sensitive.

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