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RESEARCH ARTICLE

ISOLATION, CHARACTERIZATION, AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM PICHAVARAM MANGROVE FOREST WITH HAEMOLYTIC, ANTIBACTERIAL, AND ANTIOXIDANT ACTIVITY

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Abstract

Background: Endophytes are known to be important providers of probiotics and antibiotics and live in plant tissues without appearing to cause any harm. The hemolytic, antibacterial, and antioxidant properties of endophytic bacteria are investigated in this work.

Materials and Methods: Rhizophora mucronata and Ceriopsdecandra are common in the coastal area of the Pichavaram mangrove forest in Tamil Nadu. From their leaves, endophytic bacterial strains were recovered. The isolates were characterized biochemically and molecularly. Assays for hemolytic activity, DPPH free radical scavenging, and antibacterial activity using the agar well diffusion method were carried out.

Results: The isolates were identified as Bacillus siamensis, Bacillus paramycoides, and Cytobacillus firmus based on molecular characteriza tion.

Conclusion: The isolates demonstrated significant haemolytic, antimicrobial, and antioxidant activity. These findings indicate that endophytic bacteria from Rhizophora mucronata and Ceriopsdecandra have the potential to serve as sources for novel antibacterial compounds.

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Introduction:-

Mangroves are woody trees and shrubs widely seen in marshy areas [1]. These plants produce phytochemicals, and secondary metabolites have high medicinal potential. The mangrove leaves, roots, and bark are used for the treatment of haemorrhages, angina, and haematuria [2]. This group factors due to their specially developed adaptive features such as their upright roots with buttresses, pneumatophores, and the vast distribution of salt (4). Endophytes are microorganisms, particularly bacteria and fungi, that live within mangroves and are beneficial to the host plant. They play various roles in plant health, including promoting growth, enhancing resistance to pathogens, and improving tolerance to environmental stresses. This symbiotic relationship can enhance plant health and resistance

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to diseases or pathogens, which leads to studies of the antimicrobial activities of bacterial endophytes, focusing on their role in suppressing soil-borne plant pathogens (5). Endophytes are a source of novel antibiotics that can be used to treat human diseases. For example, the endophytic fungus Pestalotiopsis microspora produces pestalotiopsin, which has antibacterial properties. The endophytic fungus Penicillium chrysogenum produces penicillin, one of the earliest discovered and widely used antibiotics. The endophytic strain produced from Rhizophora mucronatahad the benefits of antibacterial, cytotoxic, analgesic, and antiviral activities. These compounds can inhibit viral replication and have potential applications in treating viral infections. Rhizophora mucronata is a small to medium-sized evergreen tree growing to a height of about 20 to 25 meters on the banks of rivers. On the fringes of the sea, 10 or 15 meters is a more typical height.

Fig. 1:- The leaves and root of Rhizophora mucronata.

Fig. 2:- The leaves and root of Ceriopsdecandra.

Ceriopsdecandrais a shrub or small tree reaching 2 to 5 m in height. The leaves are oval to obovate, 4-9 cm long and 2.5-6 cm wide. Ceriopsdecandra is a shrubby mangrove tree species belonging to the Rhizophoraceae family. It is commonly known as the Indian Mangrove. The plant leaves were used for the treatment of gastrointestinal disorders, infection, inflammation, and cancer. [3]. Endophyte strains of Ceriopsdecandrahave unique metabolic pathways that enable the synthesis of secondary metabolites. These pathways are often different from those found in other microorganisms, leading to the production of novel compounds. Endophytic bacteria, especially from the genus Bacillus, produce lipopeptides like surfactin, fengycin, and iturin. (10). These compounds have strong surfactant properties and can disrupt cell membranes, leading to hemolysis. Haemolytic activity refers to the ability of a substance to lyse red blood cells (erythrocytes) (12), leading to the release of haemoglobin into the surrounding fluid. This activity can be indicated by the production of certain enzymes of microorganisms, highlighting the potential of bacterial endophytes in producing novel antibiotics (6, 7). The strain of Bacillus has shown antimicrobial activity against Staphylococcus aureus. The antioxidant activity of Bacillus has significant implications for plant health, human medicine, and various industrial applications. Endophytic Bacillus can enhance plant resistance against environmental aspects, such as drought, salinity, and pathogens, by producing antimicrobial compounds (8, 9). The present study is screening the antimicrobial, antioxidant, and haemolytic activity of Bacillus siamensis BBWCVES01, Bacillus paramycoides BWCVES05, Bacillus paramycoidesBWCVES07, and Cytobacillus firmus BWCVES08 isolated from Rhizophora mucronataand Ceriopsdecandra. The cell-free isolates were processed for antimicrobial, antioxidant, and haemolytic activity by the agar well diffusion method.

Methods and Materials:-

Sample Collection and Isolation

Leaves of Rhizophora mucronata and Ceriopsdecandra were collected from the Pichavaram mangrove forest located on the northeastern coast of Tamil Nadu, near Chidambaram. The samples were transported in sealed containers and stored at 4°C for 24 hours before processing. Endophytic bacteria were isolated under aseptic conditions following the method described by Santos et al. (2003). Surface sterilization involved washing the plant material with 70% ethanol for 2–5 minutes, followed by drying for 4–5 hours. The sterilized leaves were then placed on agar plates and incubated at 37°C for 7–10 days. Emerging colonies were subcultured and purified.

Preliminary Identification of Endophytic Bacteria

Four endophytic bacterial isolates were identified based on biochemical and molecular characteristics.

Biochemical Characterization

Biochemical tests were done using the HiAssorted Biochemical Test Kit (HiMedia # KB002), which comprises 12 tests: 1) Citrate utilization, 2) Lysine utilization, 3) Ornithine utilization, 4) Urease detection, 5) Phenylalanine deamination, 6) Nitrate reduction, 7) H_2S production, and five different carbohydrate utilization tests 8) Glucose, 9) Adonitol, 10) Lactose, 11) Arabinose, and 12) Sorbitol. The culture has been processed for the Indole Test, Methyl Red Test, and Voge's Proskauer test for further biochemical characterization analysis.

Indole Test:

Cultures were inoculated into peptone water and incubated overnight at 37°C. Kovac's reagent was added post-incubation. A red ring indicated a positive result; yellow indicated negative.

Methyl Red Test:

Cultures were inoculated into glucose peptone broth and incubated for 48 hours at 37°C. Methyl red indicator was added. A red colour signified a positive result; yellow signified negative.

Voges-Proskauer Test:

Cultures were grown in glucose peptone broth, followed by the addition of 5 drops of Barritt's A reagent and 3 drops of Barritt's B reagent. A pink colour indicated a positive result.

Growth Curve Analysis

Bacterial cultures were inoculated into sterile broth and incubated under optimal growth conditions. Cell growth over time was measured by optical density (OD) at 660 nm to produce a sigmoid (standard) growth curve.

Haemolytic Activity
Haemolytic activity was assessed using 24-hour nutrient broth cultures. Supernatants were stored at −80°C. Human blood agar plates were prepared by mixing 5 mL of human blood with 100 mL of nutrient agar. Wells were punched in the plates, and 10 µL of each sample was added. Plates were incubated at 37°C for 24 hours, and haemolysis was observed.

Antimicrobial Activity
The antimicrobial assay was performed by the agar well diffusion method in Muller Hinton Agar (MHA) plates. The culture was inoculated in nutrient broth and incubated overnight at 37°C to adjust the turbidity to 0.5 McFarland standards, giving a final inoculum of 1.5 x 108 CFU/ml. MHA plates were cultured with standardized microbial culture broth. The concentration of the sample varied from 150 to 200 µg/ml, with positive control as streptomycin 25 mcg and 100% negative solvent control as DMSO, respectively. The plate was incubated for 18-24 hours at 37°C. The zone of inhibition was measured in mm.

Antioxidant Activity—DPPH Free Radical Scavenging Assay
The antioxidant potential was measured using the DPPH assay. A 4.3 mg sample of DPPH was dissolved in 3.3 mL of methanol and protected from light. Various concentrations (20–200 µg/mL) of the test samples and quercetin (standard) were mixed with DPPH and incubated for 15 minutes. Absorbance was measured at 517 nm. The percentage of inhibition was calculated using
% Inhibition = [(A0 − A1)/A0] × 100
where A0 was the absorbance of the control and A1 was the absorbance in the presence of the sample or positive control.

Molecular Identification of Isolates
Genomic DNA was extracted as described by Arun Dell and Jas Preet (2005). Cultures were incubated in nutrient broth at 30°C overnight. Cells were harvested, lysed with SDS and lysozyme, and DNA was purified using PCI (phenol: chloroform: isoamyl alcohol). DNA was precipitated using sodium acetate and isopropanol, washed with 70% ethanol, and treated with RNase. The final DNA product was stored at −20°C and analysed by 0.8% agarose gel electrophoresis.

16S rRNA Gene Amplification
The 16S rRNA genes of the genomic DNA of all four isolates were amplified using the following bacterial universal primers:

Primers
27 F: 5’ AGAGTTTGATCC TGGCTCAG 3’
1492 R: 5’ GGTTACCTTGTT ACGACTT 3’

Each amplification reaction included 12.5 µL of premix (2x master mix red) containing 2.5 U Taq DNA polymerase, PCR buffer, 1.5 mM $MgCl_2$, and 200 µM dNTPs (Ampliqon, Denmark), 1 1µL of template DNA, 1 µL (20 pmol) of each primer, and 9.5 µL of sterile double distilled water in a final volume of 25 µL.PCR was performed in an automated My Gene TM Peltier Thermal Cycler (MG96G) with the following conditions:

PCR conditions
Initial denaturation: 94°C for 4 min
Denaturation: 94°C for 1 min - 35 cycles
Annealing: 55°C for 1 min
Extension: 72°C for 2 min

Each PCR product was analysed on a 1.2% agarose gel with ethidium bromide (0.5 μgmL^{-1}) and 1×TAE buffer. Electrophoresis was carried out at 100 V until the tracking dye migrated to the end of the gel. Ethidium bromide-stained DNA bands were viewed under a UV transilluminator and photographed for documentation. PCR products were sequenced after purification with the support of a service provider, Eurofins Genomics India Pvt. Ltd., Bangalore, India. DNA bands were viewed under a UV transilluminator and photographed for documentation. PCR products were sequenced after purification with the support of a service provider, Eurofins Genomics India Pvt. Ltd., Bangalore, India. The identification of the isolates was performed using the BLAST

(http://blast.ncbi.nlm.nih.gov/blast/Blast.in) in NCBI. Using the sequence match application and BLAST to verify the similarity of experimental sequences with the reference sequence in the database (14) and classifying them at the geniuslevel.

Results:-

Biochemical Characterization

S. No.	Test	Biochemical Characterization			
		Bacillus SiamensisBWCVES01	Bacillus Paramycoides BWCVES05	Bacillus Paramycoides BWCVES07	Cytobacillus FirmusBWCVES08
1.	Citrate utilization	-	+	+	+
2.	Lysine utilization	+	-	-	-
3.	Ornithine utilization	-	+	+	+
4.	Urease detection	+	+	+	+
5.	Phenylalanine deamination	-	+	+	+
6.	Nitrate reduction	-	+	+	+
7.	H_2S production	+	+	+	+
8.	Glucose	+	-	+	+
9.	Adonitol	+	-	+	+
10.	Lactose	-	+	-	-
11.	Arabinose	-	+	-	-
12.	Sorbitol	+	-	+	+

Table 3.1.1 presents the biochemical test results for four endophytic bacterial isolates: Bacillus siamensis BWCVES01, Bacillus paramycoides BWCVES05 and BWCVES07, and Cytobacillus firmus BWCVES08. The tests included utilization of citrate, lysine, and ornithine; urease activity; phenylalanine deamination; nitrate reduction; H_2S production; and carbohydrate utilization. Variation in metabolic characteristics was observed among the isolates, indicating biochemical diversity.

Growth Curve Analysis

Growth kinetics of the four isolates were monitored by measuring optical density (OD) at 660 nm over an 84-hour period. **Table 3.1.2(a)** shows that B. paramycoides BWCVES05 demonstrated the highest OD, indicating robust growth, followed by C. firmus BWCVES08.

Endophyte isolates	Bacillus SiamensisBWCVES01	Bacillus Paramycoides BWCVES05	Bacillus Paramycoides BWCVES07	Cytobacillus FirmusBWCVES08
Time	O.D at 660 nm			
0	0.06	0.06	0.06	0.06
12	0.1	0.31	0.1	0.13
24	0.16	0.36	0.15	0.18
36	0.2	0.41	0.21	0.22
48	0.24	0.44	0.23	0.26
60	0.26	0.45	0.24	0.28
72	0.28	0.35	0.23	0.27
84	0.25	0.32	0.2	0.25

Doubling times, presented in **Table 3.1.2(b)**, ranged from 5.84 minutes (C. firmus) to 233.15 minutes (B. paramycoides BWCVES05), suggesting significant variation in growth dynamics.

Endophyte isolates	Bacillus SiamensisBWCVES01	Bacillus Paramycoides BWCVES05	Bacillus Paramycoides BWCVES07	Cytobacillus FirmusBWCVES08
Time	O.D at 660 nm			
0	0.06	0.06	0.06	0.06
12	0.1	0.31	0.1	0.13
24	0.16	0.36	0.15	0.18
36	0.2	0.41	0.21	0.22
48	0.24	0.44	0.23	0.26
60	0.26	0.45	0.24	0.28
72	0.28	0.35	0.23	0.27
84	0.25	0.32	0.2	0.25

Antimicrobial Activity

The antimicrobial efficacy of the isolates was evaluated against E. coli, Pseudomonas aeruginosa, and Streptococcus aureus using the agar well diffusion method. **Table 3.1.3** displays the zone of inhibition (in mm). B. siamensis BWCVES01 showed the highest activity across all tested pathogens. C. firmus BWCVES08 exhibited moderate activity against E. coli and S. aureus, but none against P. aeruginosa. Streptomycin was used as the positive control, and DMSO served as the negative control.

Pathogens	Bacillus SiamensisBWCVES01(mm)	Bacillus ParamycoidesBWCVES05(mm)	Bacillus Paramycoides stainBWCVES07(mm)	Cytobacillus FirmusBWCVES08(mm)
E.Coli	8.67±3.06	7.67±1.53	7.±3	7±3
Pseudomonas aeruginosa	8.33±2.08	3.00±3.00	7.00±2.64	0
Streptococcus aureus	8.66±1.52	0	0	7.00±2.00
CONTROL	11±11.00			
DSMO	0			

Antioxidant Activity

The DPPH free radical scavenging activity was assessed at various concentrations (20–200 µg/mL). As shown in **Table 3.1.4(a)**, antioxidant activity increased in a dose-dependent manner. C. firmus BWCVES08 exhibited the highest scavenging effect at 200 µg/mL, followed by B. paramycoides BWCVES07.

Conc. µg/mL	Bacillus Siamensis BWCVES01	Bacillus ParamycoidesBWCVES05	Bacillus Paramycoides BWCVES07	Cytobacillus Firmus BWCVES08	Standard (Quercetin)
20	0.85	12.76	21.7	30.21	23.53
40	3.82	33.19	23.82	33.19	41.96
60	18.72	38.29	31.06	39.57	48.82
80	21.7	40	33.19	42.55	72.94
100	28.08	44.68	43.82	46.8	92.78
120	31.48	48.93	48.51	53.61	95.37
140	35.74	51.48	51.06	55.31	96.87
160	49.36	54.46	61.7	56.59	97.21
180	60	57.8	68.51	61.27	97.93
200	71.48	58.29	74.46	69.36	98.67

IC_{50} values, presented in **Table 3.1.4(b)**, indicated that C. firmus BWCVES08 had the lowest IC_{50} (127.22 µg/mL), suggesting strong antioxidant potential. Quercetin was used as the standard.

Strain	**Scavenging activity**	IC50 mg/ml
Bacillus SiamensisBWCVES01	32.12±22.9	162.07
Bacillus Paramycoides BWCVES05	43.98±13.84	138.96
Bacillus Paramycoides strain BWCVES07	45.78±18.46	127.58
Cytobacillus FirmusBWCVES08	48.84±12.56	127.22
Standard (Quercetin)	76.60±28.27	78.48

Molecular Identification

The isolates were identified based on 16S rRNA gene sequencing. The sequences were compared using NCBI BLAST, and the identities were confirmed:

Bacterial Isolate	BWC 01	BWC05	BWC07	BWC08
Accession Number	MW644759	MZ540882	MW714680	MW431011
National Center for Biotechnology Information (NCBI)	Bacillus siamensis strain BWCVES01	Bacillus paramycoides strain BWCVES05	Bacillus paramycoides strain BWCVES07	Cytobacillus firmus strain BWCVES08
Percentage Similarity	99.72	98	99.86	99.79
Microscopic Identification	Gram-positive rods	Gram-positive rodsGram positive, rods	Gram-positive rodsGram positive, rods	Gram-positive rods

All isolates were Gram-positive rods. The partial 16S rRNA sequences were submitted to NCBI, and the alignment showed ≥99% similarity, confirming their identities.

Fig. 3:- Hemolytic activity of samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus ParamycoidesstainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08.

Fig. 4(a):- Biochemical characterization of Bacillus SiamensisBWCVES01, Bacillus ParamycoidesBWCVES05, Bacillus Paramycoides stainBWCVES07, Cytobacillus FirmusBWCVES08.

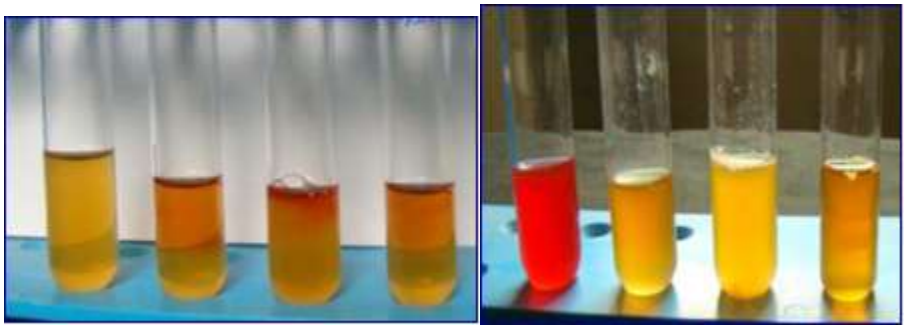


Fig. 4(b):- Indole and Methyl Red test: (1) Bacillus Siamensis BWCVES01, (2) Bacillus Paramycoides BWCVES05, (3) Bacillus Paramycoides strain BWCVES07, and (4) CytobacillusBWCVES08

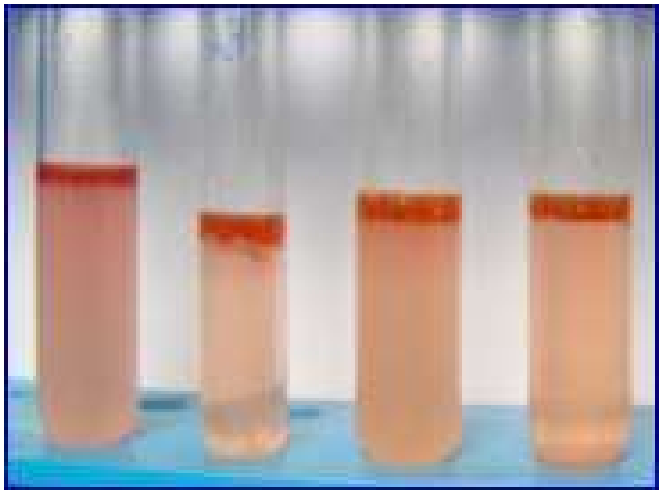
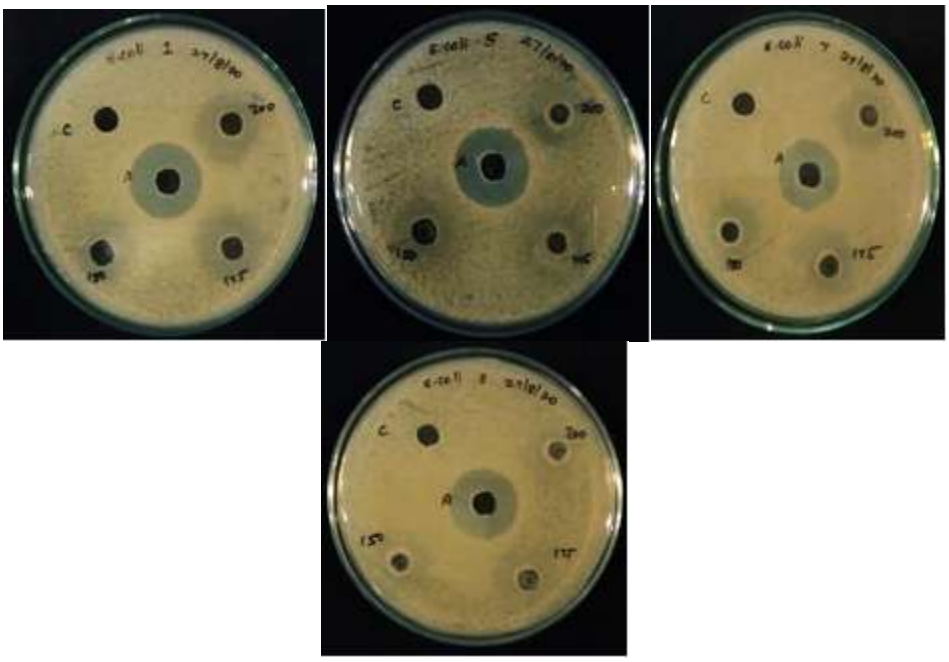


Fig. 4(c):- Voge's Proskauer test.



Tube 1:- Bacillus SiamensisBWCVES01; **Tube 2:-** Bacillus ParamycoidesBWCVES05; **Tube 3:-** Bacillus Paramycoides stainBWCVES07; **Tube 4:-** Cytobacillus FirmusBWCVES08.

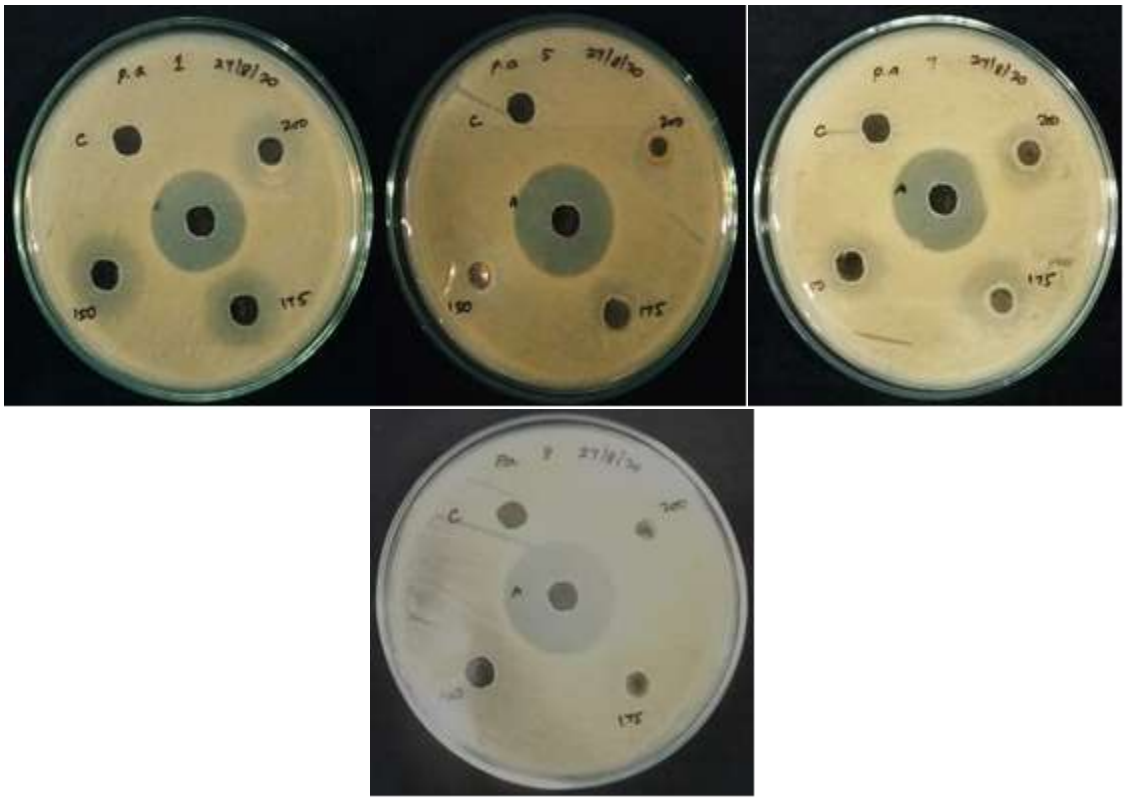


Fig. 5(a):- Antimicrobial activity Against E.Coliof samples 1- Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08.

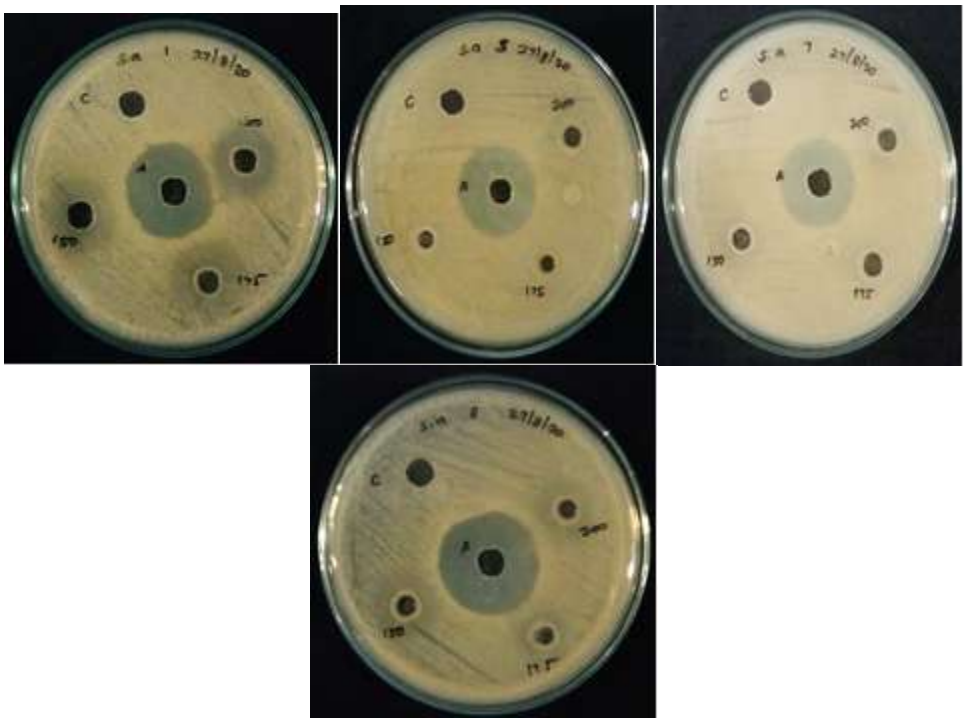
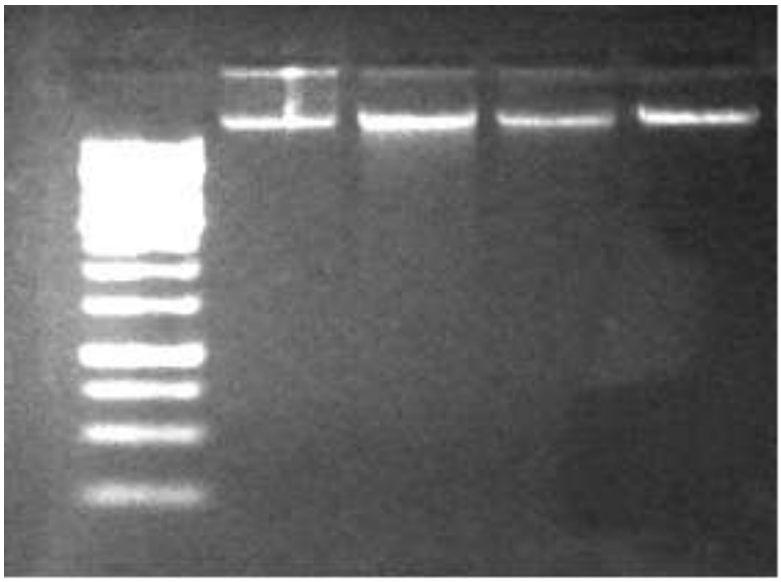


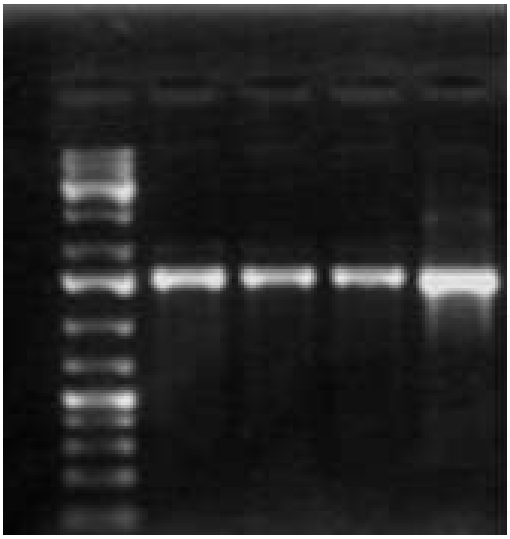
Fig. 5(b):- Antimicrobial activity Against Pseudomonas aeruginosa of samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8-Cytobacillus FirmusBWCVES08.



Lane 1 Lane 2 Lane 3 Lane 4 Lane 5

Fig. 5(c):- Antimicrobial activity Against Streptococcus aureus of samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8-Cytobacillus FirmusBWCVES08.

Lane1: Marker; Lane 2: Bacillus SiamensisBWCVES01 Lane 3Bacillus ParamycoidesBWCVES05; Lane 4: Bacillus ParamycoidesstainBWCVES07; Lane 5: Cytobacillus FirmusBWCVES08



Lane 1 2 3 4 5

Fig 6:- Genomic DNA isolation of the isolates.

Lane1: Marker; Lane 2: Bacillus SiamensisBWCVES01; Lane 3:Bacillus ParamycoidesBWCVES05; Lane 4: Bacillus Paramycoides stainBWCVES07; Lane 5:Cytobacillus FirmusBWCVES08

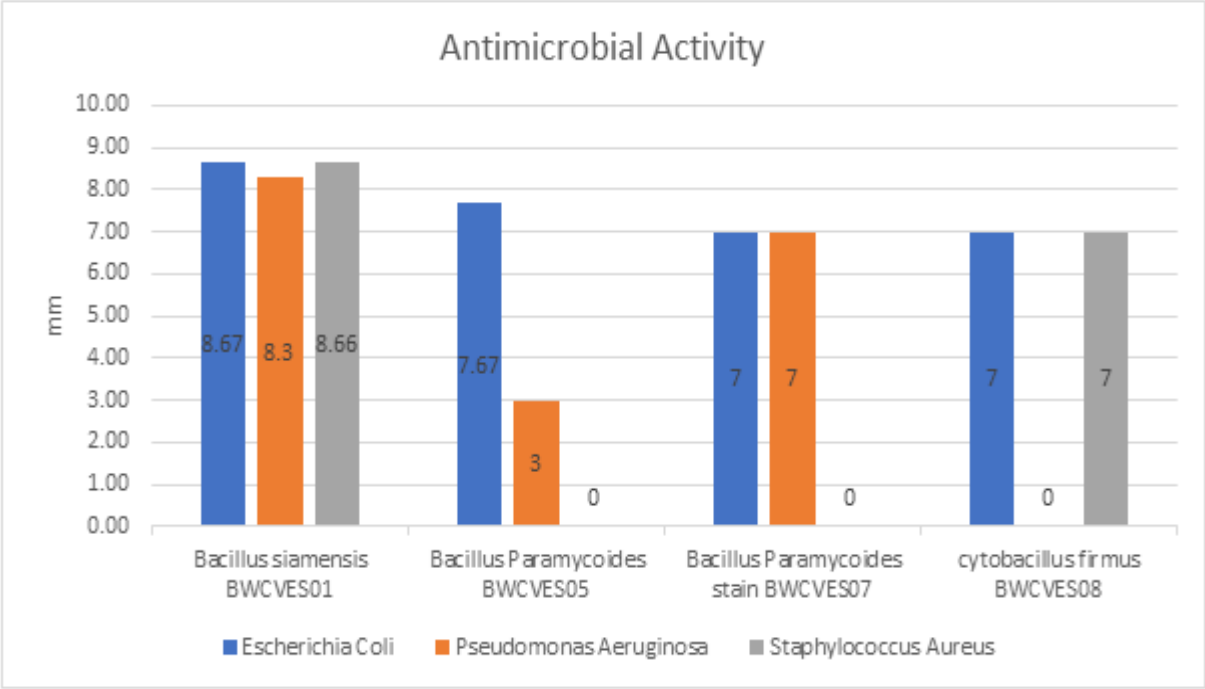
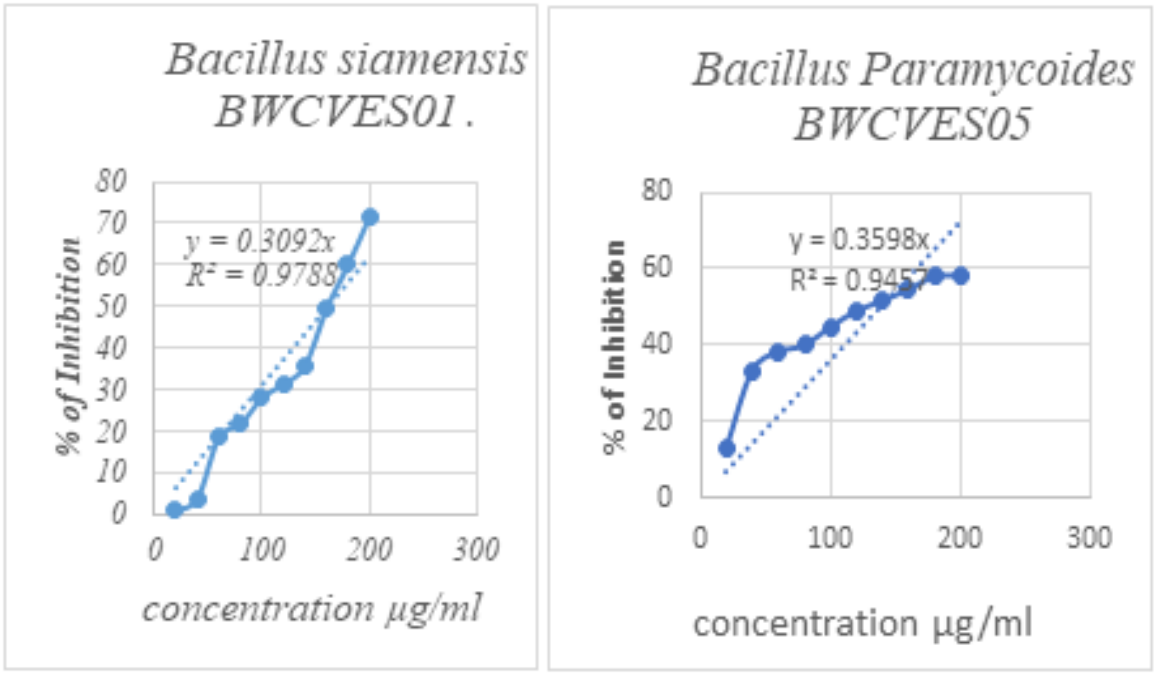


Fig. 7:- 16S rRNA amplified products of the isolates.



Sample1 Sample 2

Figure 8:- Antimicrobial Activity of various endophytic bacteria against pathogens.

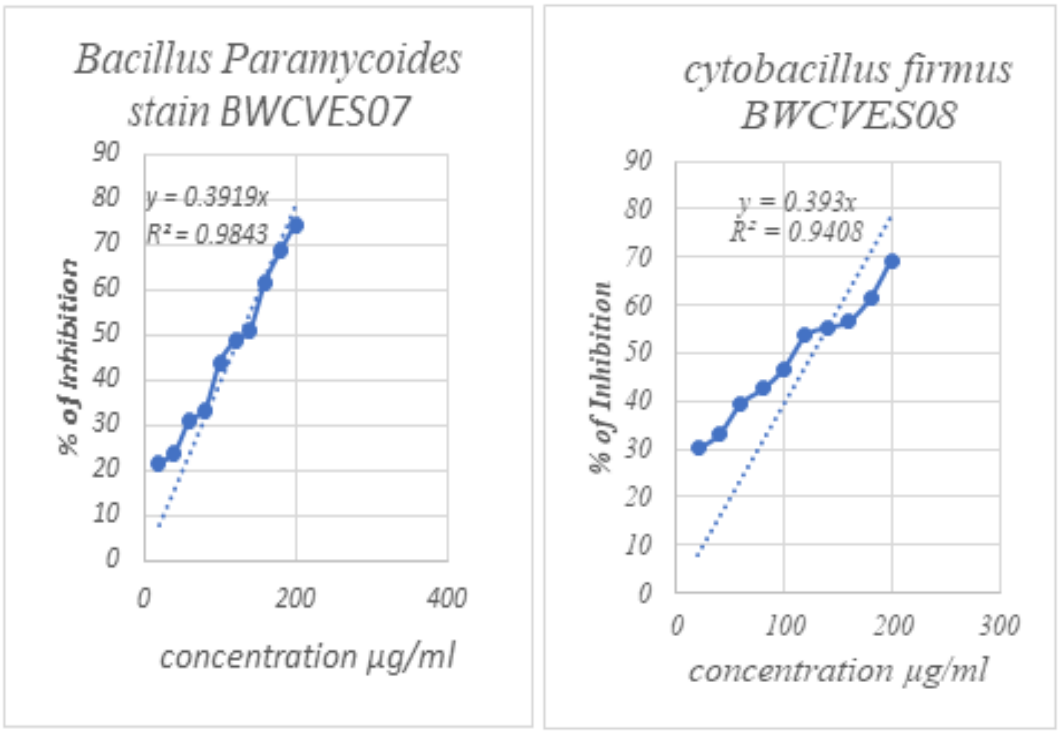


Fig. 9(a):- Antioxidant activity of samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05

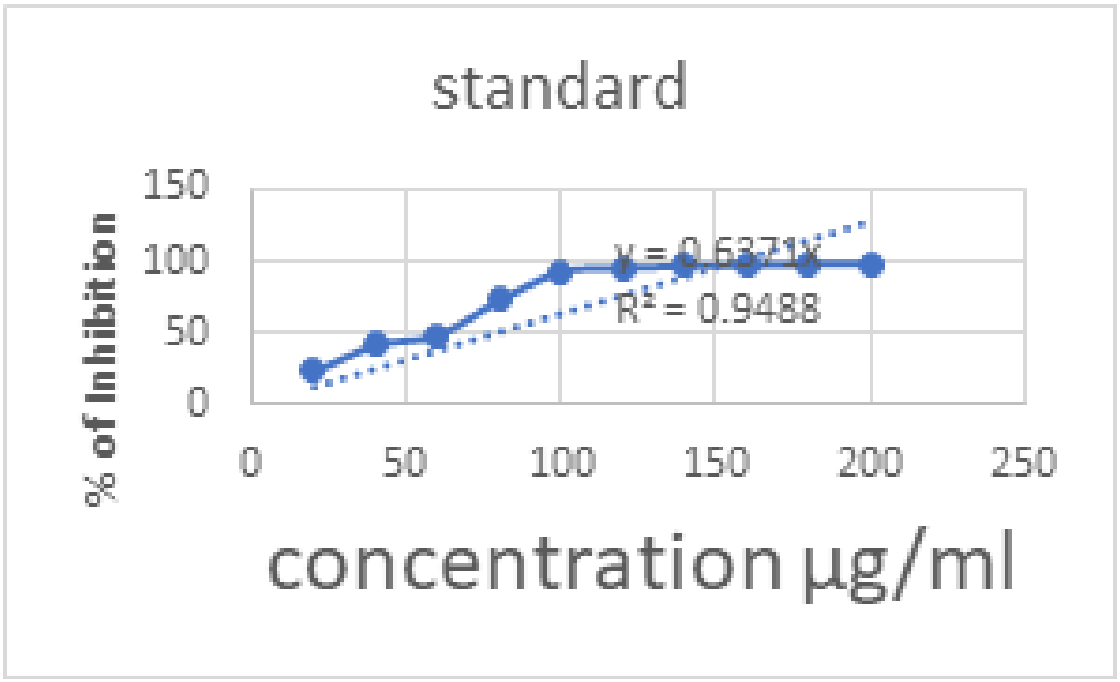


Fig. 9(b):- Antioxidant activity of sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08 with standard.

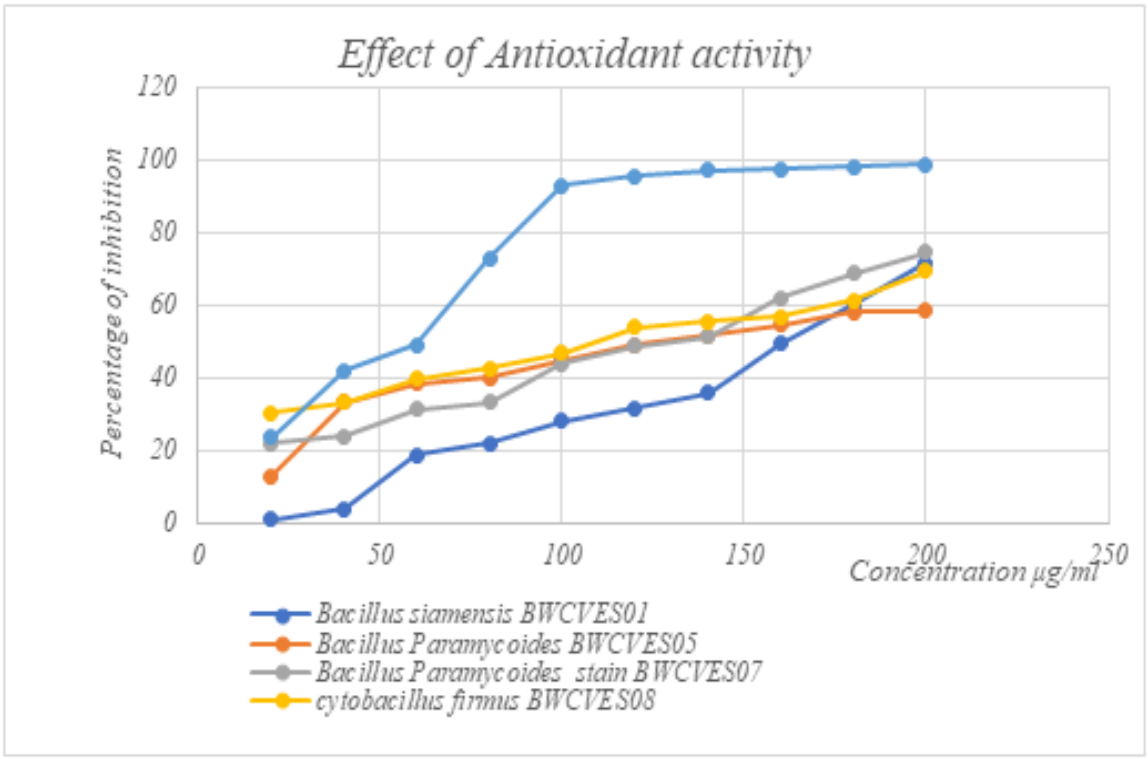


Fig. 10:- Antioxidant activity of samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08.

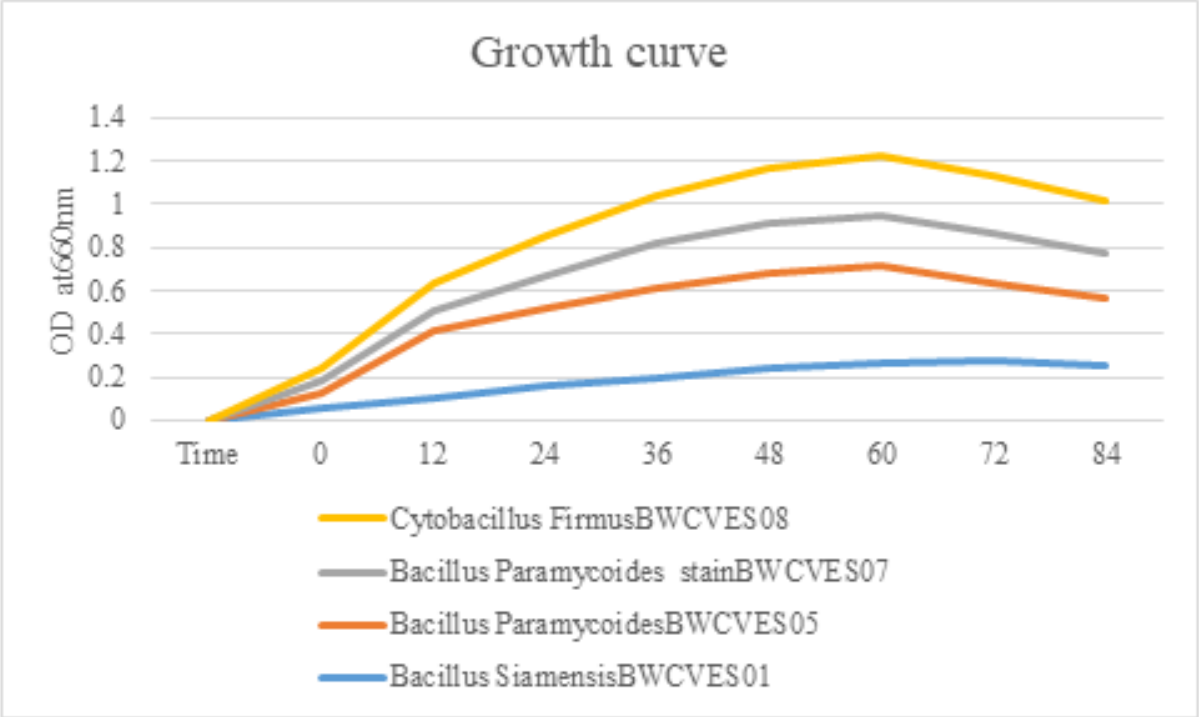


Fig. 11:- Growth Curve analysis samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08

Bacillus SiamensisBWCVES01

TGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTCTGAACCG
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TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCG
CGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAG
CTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTATC

Bacillus ParamycoidesBWCVES05

GAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAAC
CTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGG

TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG
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AGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTA
AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGA
AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAA
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GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAA
CGCATTAAGCACTCCGCCTGGGGAGTACCGCCGCAAGGCTAAACTCAAAGGAATTGACGGGGGCCCG
CACCAGCGGTGGAGCATGTGGTTTAATTTGGAGCCACGCGGAGAACCTTACCCGGTCTTGACATCCTT
TGACAACCCCAGAGATAGGGGTTTTCCTTTGGGAGCAGAATGACCGGTGGTGCCTGGTTGTTGTCAGC
TTGTGTTCGGAGATGTTGGGTTAAGTCCCGCAACGAGGGCAACCCCTGATTTTAGTTGCCCTCAATTAG
TTGGGCCATTTAAGGTGACCGCCGGTGACAAACCGGAGGAAGGTGGGGAAGAAGTCAAATCATCCTG
CCCCTTATGACCTGGGGTACCCACCTGGTACAATGGACGGTACAAAGAGGTGCAAGACCCCGAGGTG
GAGGTAATTTTATAAAACCCTTTTCCGTTTGGATTGTTGGGTGCAAATTGCCTACCTGAAGGCGGAATC
GGTTGTAATCGCGGATCAGCCTGCCGCGGGGAATACGTTCCCGGGCCTTGTACACCCCCCCCGTCACC
CCCCGAGAGGTTGTAACCCCCGAAGTCGGGGGGGTAAT

Bacillus Paramycoides stainBWCVES07

AGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTG
GGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAG
GCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTG
AGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTG
GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAA
AGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGA
ATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGT
CTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCAC
TCCGCCTGGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGAGGGGGCCCGCACCAGCGGTG
GAGCATGTGGTTTAATTTGGAGCCACGCGAAGAACCTTACCAGGTCTTGACCTCCTTTGACAACCCTA
GAGATAGGGGTTTTCCTTTGGGAGCAGAATGACAGGTGGTGCATGGTTGTTGTCAGCTTGTGTTGTGA
GAAGTTGGGTTAAGTTCCGCAACGAGCGCAACCCCTGATTTTAGTTGCCCTCAATTAGTTGGGCCCTTT
AAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAAGACGTCAAATCATCATGCCCCTTATGAC
CTGGGGTACACACCTGGTACAATGGACGGTACAAAGAGGTGCAAGACCGCGAGGTGGAGGTAATTTT
ATAAAACCGTTTTCAGTTTGGATTGTAGGGTGCAAATTGCCTACCTGAAGCCGGAATCGGTTGTAATTG
CGGATCAGCCAGCCGCGGTGAATACGTTCCCGGGCCTTGTACACCCCGCCCGTCACCCCCCGAGAGTT
TGTAACCCCCGAAGTCGGTGGGGTAACC

Cytobacillus FirmusBWCVES08

TGCAAGTCGAGCGGACGGATGGGAGCTTGCTCCCACGACCGTCAGCGGCGGACGGGTGAGTAACACG
TGGGCAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATTCTTTCCC
TCACATGAGGAAAAGCTGAAAGATGGCATCTCGCTATCACTTACAGATGGGCCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
GTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTCAGGGAAGA
ACAAGTACCGGAGTAACTGCCGGTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGC
GGTTCCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTG
AGTGCAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA
GTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTG

CTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTG
AGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTC
TTGACATCTCCTGACAACCCTAGAGATAGGGCGTTCCCCTTCGGGGGACAGGATGACAGGTGGTGCAT
GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGT
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GACCGCGAGGTTAAGCGAATCCCATAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCA
TGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACAC
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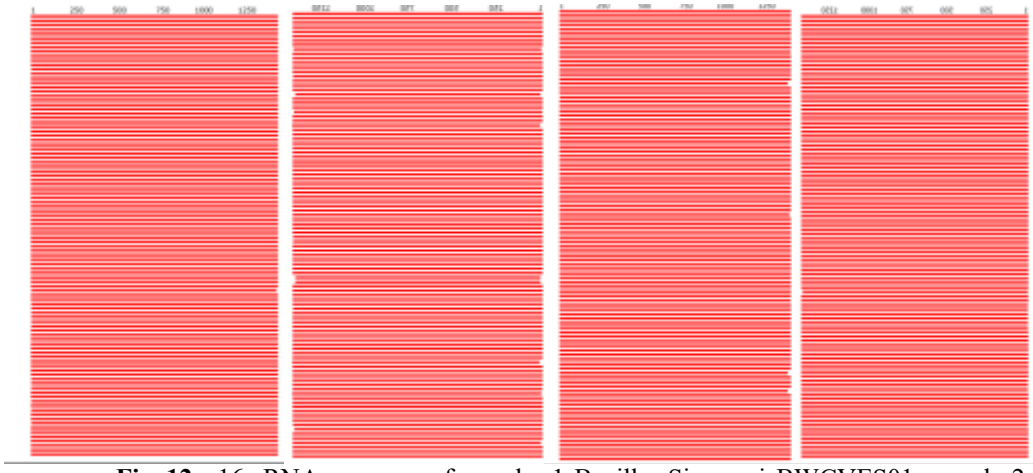


Fig. 12:- 16srRNA sequence of samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08.

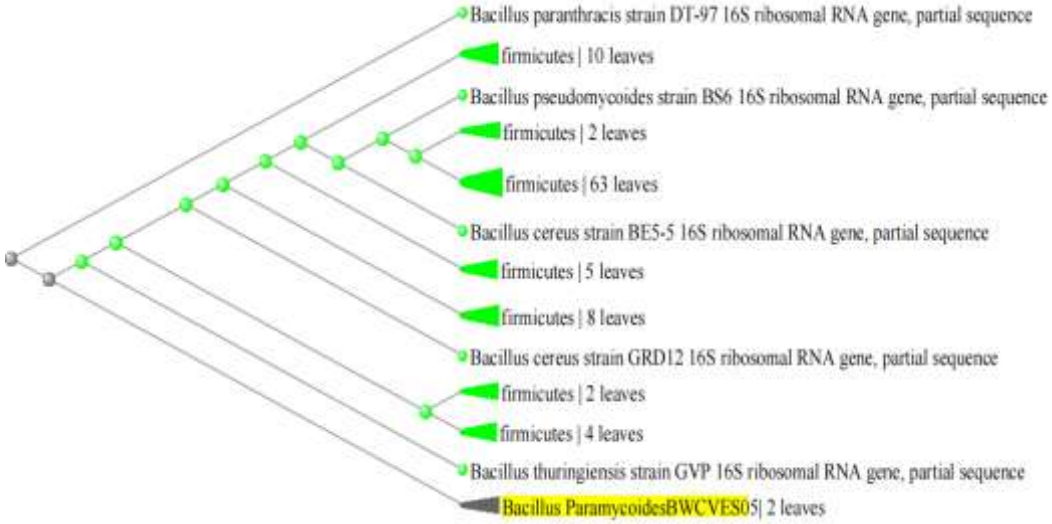
Blast Analysis

Bacillus SiamensisBWCVES01

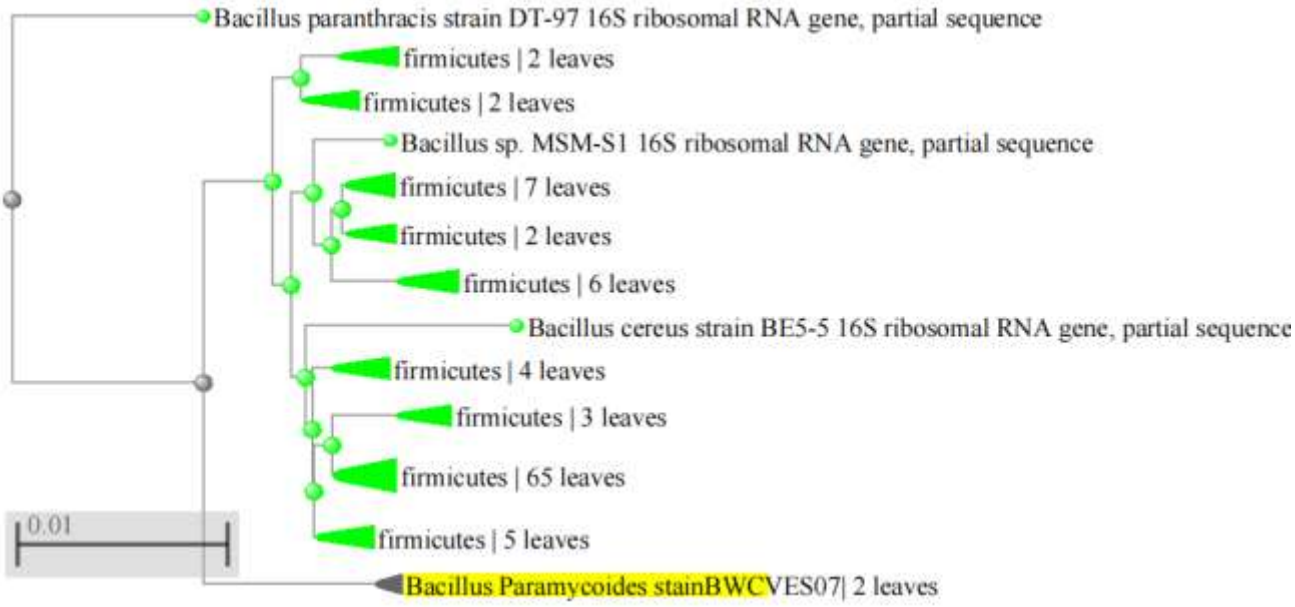
Fig. 13:- BLAST analysis samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus

ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08.

Bacillus ParamycoidesBWCVES05



Bacillus Paramycoides stainBWCVES07



Cytobacillus FirmusBWCVES08

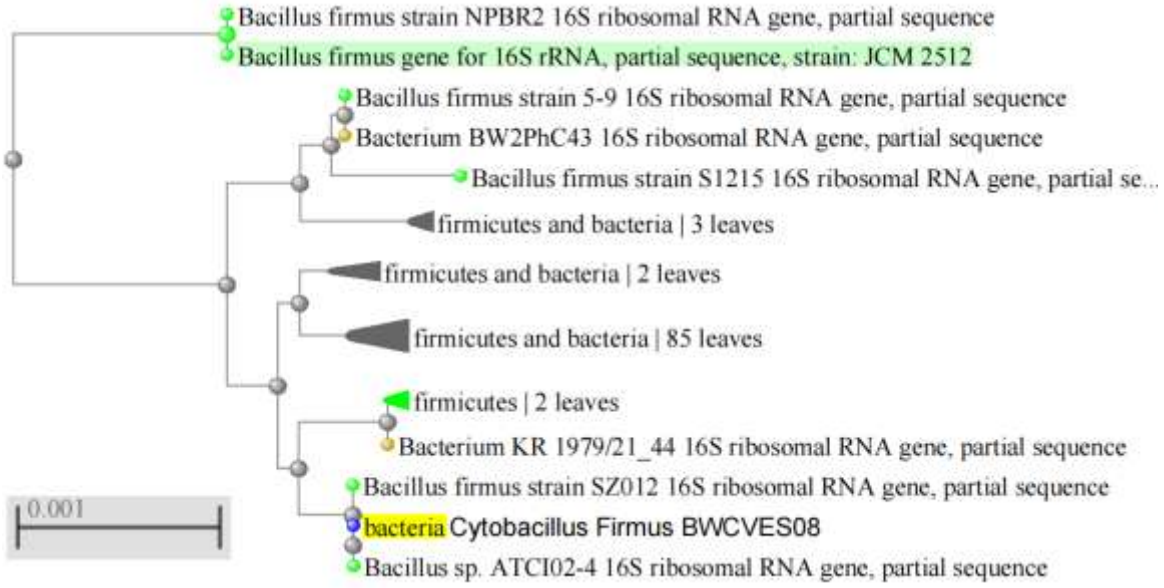


Fig. 14:- Phylogenic tree analysis samples 1-Bacillus SiamensisBWCVES01, sample-2- Bacillus ParamycoidesBWCVES05, sample-7- Bacillus Paramycoides stainBWCVES07, and sample-8- Cytobacillus FirmusBWCVES08.Phylogenetic tree constructed using MEGA7 and the neighbor-joining method.

Discussion:-

Endophytic bacteria, often residing in the rhizosphere, play a vital role in promoting plant growth, enhancing resistance, and protecting plants from pathogens. These microorganisms can stimulate seedling emergence and contribute to nitrogen fixation, phosphate solubilization, and iron chelation in both leguminous and non-leguminous plants (20,21).

In the present study, endophytic bacterial strains were isolated from the leaves of Rhizophora mucronata and Ceriopsdecandra and were identified through biochemical and molecular analyses as Bacillus siamensis BWCVES01, Bacillus paramycoides BWCVES05 and BWCVES07, and Cytobacillus firmus BWCVES08. These isolates demonstrated antimicrobial, haemolytic, and antioxidant activities, highlighting their biotechnological potential.

The biochemical properties shown in Table 3.1.1 confirm distinct metabolic capabilities among the isolates, supporting their taxonomic diversity. The growth curve analysis (Table 3.1.2a) and doubling time (Table 3.1.2b) indicated that C. firmus BWCVES08 had the fastest growth rate, while B. paramycoides BWCVES05 had the slowest.

The isolates exhibited broad-spectrum antibacterial activity, as seen in Table 3.1.3. Notably, B. siamensis BWCVES01 displayed significant inhibition zones against E. coli, Pseudomonas aeruginosa, and Streptococcus aureus. These findings align with previous studies reporting Bacillus species as prolific producers of antimicrobial compounds such as lipopeptides (e.g., surfactin, fengycin, iturin)(7,9). The relatively lower activity of C. firmus against P. aeruginosa may suggest pathogen-specific limitations or differences in compound efficacy.

Haemolytic activity is an indicator of membrane-disrupting potential, often linked to bioactive compounds like lipopeptides. As shown in Figure 3, all isolates exhibited some degree of haemolysis, suggesting the production of potent bioactive metabolites. However, balancing haemolytic activity with antimicrobial potency is critical to ensure safety for therapeutic applications (11,12).

Oxidative stress plays a significant role in cellular damage and disease progression. The isolates demonstrated considerable DPPH radical scavenging activity (Table 3.1.4a), with C. firmus BWCVES08 showing the highest antioxidant potential, closely followed by B. paramycoides BWCVES07. The IC_{50} values (Table 3.1.4b) further confirm the strong antioxidant capacities of these isolates compared to the standard, quercetin. These results suggest possible applications in pharmaceuticals and nutraceuticals (10,24).

Molecular analysis using 16S rRNA gene sequencing confirmed the identities of the isolates, with ≥99% similarity to reference strains in NCBI (see Table 3.1.5). The phylogenetic tree (Figure 14) placed the isolates within the expected clades of Bacillus and Cytobacillus, validating their classification. The use of MEGA7 and neighbour-joining methods enhanced the resolution of evolutionary relationships (18,23).
These results are consistent with previous studies that have highlighted endophytic Bacillus strains as valuable sources of antimicrobial and antioxidant compounds (5,6,13). Moreover, the isolates' strong growth and metabolic versatility support their potential for industrial-scale fermentation and bioactive production.

Conclusion:-

Endophytic bacteria represent a promising area of research with vast potential in plant science, medicine, and biotechnology. In this study, endophytic bacterial strains were successfully isolated from the leaves of Rhizophora mucronata and Ceriopsdecandra, and identified as Bacillus siamensis BWCVES01, Bacillus paramycoides BWCVES05 and BWCVES07, and Cytobacillus firmus BWCVES08 through 16S rRNA sequencing and BLAST analysis.

These isolates demonstrated significant antimicrobial activity against both Gram-positive and Gram-negative pathogens, notable haemolytic effects, and strong antioxidant potential based on DPPH radical scavenging activity. The presence of these bioactivities suggests their applicability in the development of novel antibiotics, natural antioxidants, and bio-based therapeutics.
The results also emphasize the importance of mangrove ecosystems as a reservoir for beneficial endophytes with industrial and pharmaceutical value. Further research, including compound purification and in vivo studies, is recommended to explore their clinical and agricultural applications.

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Conflicts of Interest
There is no conflict of Interest

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