Isolation, Characterization, and Identification of Endophytic Bacteria from Pichavaram Mangrove Forest with Haemolytic, Antibacterial, and Antioxidant Activity

Abstract

Background Endophytes are colonized in the plant tissue, and there is no external sign of effect on their hot environment. These endophytic microorganisms were a revolutionary source for the enlargement of various probiotic and antibiotic progression.This paper gives an overview of the haemolytic activity, antimicrobial and antioxidant activity of endophytic bacteria.

Materials The strain was isolated from the leaves of *Rhizophora mucronate* and *Ceriops decandra* widely distributed in the coastal region of Pichavaram mangrove forest in Tamil Nadu. The Biochemical characterization of Endophytes and molecular identification of Endophytes was analyzed. Haemolytic activity, antimicrobial activity by agar well diffusion method, and DPPH free radical scavenging activity of the isolates was done.

Result The isolated strain was identified as *Bacillus siamensis, Bacillus paramycoide*s, and *Cytobacillus firmus* respectively by molecular identification.

Conclusion: The isolated strain shows appreciable haemolytic and antioxidant activity. The agar well diffusion method of the endophytic isolates shows antimicrobial activity. It can be concluded that endophytic bacteria isolated from *Rhizophora mucronate* and *Ceriops decandra* leaves have a high potential for a new antibacterial source to be developed in the future.

Keywords: Endophytic bacteria, *Bacillus* species, Haemolytic activity, antimicrobial activity, antioxidant activity

1. 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, pneumatophore, as well as 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 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 mucronata* had 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 *Ceriops decandra*

Ceriops decandra is a shrub to small tree reaching 2 to 5m in height. The leaves are oval to obovate,4-9cm long and 2.5-6cm wide. *Ceriops decandra* is a shrubby, mangrove tree species belonging to 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]. Endophytes strain of *Ceriops decandra* have 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 haemolysis. 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 paramycoide*s BWCVES05, *Bacillus paramycoide*s BWCVES07 and *Cytobacillus firmus* BWCVES08 isolated from *Rhizophora mucronata* and *Ceriops decandra.* The cell-free isolates were processed for antimicrobial, antioxidant, and haemolytic activity by agar well diffusion method.

2. Methods and Materials

2.1 Sample Collection and Isolation

The leaves of *Rhizophora mucronate* and *Ceriops decandra* were collected from the Mangrove Forest present in the northeast coastal area of Pichavaram near Chidambaram in Tamil Nadu. The leaves were collected and transported in a sealed holder and kept at a cold room temperature of about 4ºC for 24 hours. Endophytic bacteria were isolated and cultured by following a standard procedure. (5)

The surface-sterilized *Rhizophora mucronate* and *Ceriops decandra* leaves were used for isolation in aseptic conditions. The isolation method of Santos et.al. (2003) is followed. Plant material was washed with 70%ethanol for 2-5 min, and the material was dried for 4-5 hrs. The material was placed on agar medium plates at37ºC temperature for 7-10 days. Different isolates were sub-cultured and purified.

2.2 Preliminary Identification of Endophytic Bacteria

The four isolates of endophytic bacteria were found by biochemical characteristics and molecular investigations**.**

2.2.1 Biochemical characteristic of Endophytes

Biochemical test was done using 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

Each culture was inoculated in peptone water and incubated overnight at 37°C. After incubation, a few drops of Kovac's reagent were added. A red colour ring at the top indicated the positive reaction for indole and yellow colour indicated the negative reaction.

Methyl red

Each Culture was inoculated in glucose peptone broth and incubated at 37°C for 48 h. After incubation, a few drops of methyl red indicator were added. A bright red colour development indicated a positive reaction while the yellow colour indicated a negative reaction.

Voge's Proskauer

Each culture was inoculated in glucose peptone broth and incubated at 37°C for 48 h. After incubation, 5 drops of Barrit's reagent A were added and mixed. Then, 3 drops of Barrit's reagent B were added and mixed. A pink colour development indicated a positive reaction, while a yellow colour indicated a negative reaction.

2.2.2 Growth curve analysis

The organism shows an increase in the cell size at the growth phase. The organisms were inoculated on the sterile broth and incubated under best growth conditions. The dynamics of the bacterial growth were studied by plotting the cell growth and the incubation time. This curve obtained is sigmoid and is known as a standard growth curve.

2.2.3 Haemolytic Activity

Four isolates were taken for the analysis of haemolytic activity. The Haemolytic activity of the isolates was carried out by 24-hour Nutrient Broth culture. The Supernatant was stored at 80º C. Human blood medium was prepared by adding 5ml of human blood in 100ml of Nutrient agar, then poured into a Petrich plate, punctured the well, and 10µl of the sample was added. The result was seen after 24 hours.

2.2.4 Antimicrobial Activity

The antimicrobial assay was performed by agar well diffusion method in Muller Hinton Agar (MHA) plates. The culture was inoculated in Nutrient Broth and incubated overnight at 37º C to adjust the turbidity of 0.5 McFarland standards, giving a final inoculum of 1.5 x108CFU/ml. MHA plates were cultured with standardized microbial culture broth. The concentration of the sample varied from 150-200µg/ml with positive control as streptomycin 25mcg and 100% negative solvent control as DMSO, respectively. The plate was incubated for 18-24 hours at 37ºC. The zone of inhibition was measured in mm.

2.2.5. Antioxidant Property

DPPH free radical scavenging activity

The DPPH assay method was based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (Mohammed et al.,2009). 4.3 mg of DPPH (1,1-Diphenyl-2-picrylhydrazyl) was dissolved in 3.3mL methanol, it was protected from light by covering the test tubes with aluminium foil. 50µL of various concentrations (20-200µg/mL) of the sample, and standard compound (quercetin), were taken, and the volume was made uniformly to 300µL using methanol, followed by the addition of 300µL of DPPH. Absorbance was taken after 15 min at 517 nm using methanol as blank. The IC50 values of the samples and standard were calculated using the formula

$$\% \text{ Inhibition} = [(A0 - A1)/A0] \times 100$$

where A0 was the absorbance of the control and A1 was the absorbance in the presence of the sample or positive control.

2.2.6 Molecular Identification of Isolates

The genomic DNA of the endophytic bacteria was extracted by Arun Dell Land Jas Preet, 2005, and the genomic DNA of all four cultures was isolated and 16s rRNA sequencing was done to confirm their identity. The cultures were separately taken in Nutrient Broth at 30°C overnight. 4ml of each culture was taken in 2ml Eppendorf tube and centrifuged at 5000xg for 5 minutes. The cell pellet was taken and suspended in 200 µL of TE buffer, and 400 µL of Solution I (1% w/v lysosomes, 0.5 M NaCl, 1% w/v SDS) was added and mixed well. The tube was kept for 10 min at 37°C with

intermittent shaking after every 5 min. Immediately, an equal volume of PCI (phenol: chloroform: isoamyl alcohol; 25:24:1) was added and mixed by inversion. Centrifuged at 10000xg for 5 min at 37°C and carefully transferred the supernatant into a new Eppendorf tube,100 µL of 3 M sodium acetate (pH 5.2) and 600 µL of isopropanol were added and mixed gently by inverting the tube four to six times. Centrifuged at 10000xg for 5 min at 37°C. The DNA was precipitated in the pellet. The pellet was washed with 1 mL of 70% ethanol and centrifuged at 10000xg for 5 min. at 37°C.The supernatant was removed, and 50 mg of RNase was added to digest the RNA contamination. The mixture was centrifuged at 10000xg for 5 min at 37°C to remove the supernatant. The pellet was air dried and then suspended in 100 µL of sterile glass distilled water and stored at -20°C for further use. The DNA was analyzed on a 0.8% agarose gel with ethidium bromide.

2.2.7 16s rRNA genes 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 12.5µL of premix (2x master mix red) containing 2.5 U *Taq* DNA polymerase, PCR buffer, 1.5 mM $MgCl_2$, and 200µM dNTPs (Ampliqon, Denmark), 1µL of template DNA, 1µL (20 pmol) of each primer and 9.5µL of sterile double distilled water in a final volume of 25µL. 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 analyzed on a 1.2 % agarose gel with ethidium bromide (0.5µg mL^{-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 classified them at genius level.

Result:

Table 3.1.1 Biochemical Characteristics of Endophytic Bacteria

S. No	Test	Biochemical Characterization			
		*Bacillus Siamensis*BWCVES01	*Bacillus Paramycoides*BWCVES05	*Bacillus Paramycoides* BWCVES07	*Cytobacillus Firmus*BWCVES08
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	+	-	+	+

211 + means presence and -means absence

212 **Table 3.1.2(a)**

213 **Growth curve**

Endophytes isolates	*Bacillus Siamensis*BWCVES01	*Bacillus Paramycoides*BWCVES05	*Bacillus Paramycoides* BWCVES07	*Cytobacillus Firmus*BWCVES08
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

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215

216 **Table 3.1.2(b)**

Strain	Td (Doubling time) in minutes
*Bacillus Siamensis*BWCVES01	99.92279
*Bacillus Paramycoides*BWCVES05	233.1532
Bacillus Paramycoides stainBWCVES07	162.3745
*Cytobacillus Firmus*BWCVES08	5.843814

217 **Table 3.1.3 Antimicrobial Activity of Endophytes**

Pathogens	*Bacillus Siamensis*BWCVES01(mm)	*Bacillus Paramycoides*BWCVES05(mm)	*Bacillus Paramycoides stain*BWCVES07(mm)	*Cytobacillus Firmus*BWCVES08(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			

Table 3.1.4(a) Antioxidant Activity of Endophytes

DPPH free radical scavenging activity

Conc. µg/mL	*Bacillus Siamensis* BWCVES01	*Bacillus Paramycoides* BWCVES05	*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

Table 3.1.4(b)Antioxidant activity expressed in IC50 value of Endophytes

Strain	**Scavenging activity**	IC50mg/ml
*Bacillus Siamensis*BWCVES01	32.12±22.9	162.07
*Bacillus Paramycoides*BWCVES05	43.98±13.84	138.96
*Bacillus Paramycoides stain*BWCVES07	45.78±18.46	127.58
*Cytobacillus Firmus*BWCVES08	48.84±12.56	127.22
Standard (Quercetin)	76.60±28.27	78.48

3.1.5 Identification of Endophytes

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	99.72	98	99.86	99.79

Similarity				
Microscopic Identification	Gram-positive rods	Gram-positive rodsGram positive, rods	Gram-positive rodsGram positive, rods	Gram-positive, rods

The 16 SrRNA sequence was compared and homology with other sequence in NCBI. All the isolates show 99% similarity by BLAST analysis and submitted in NCBI.

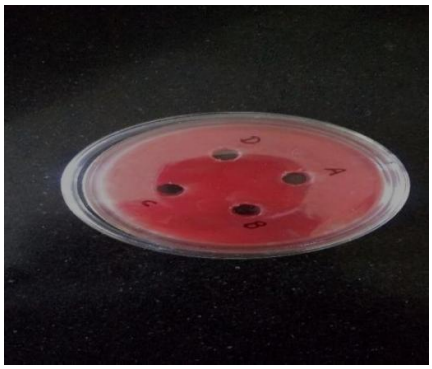
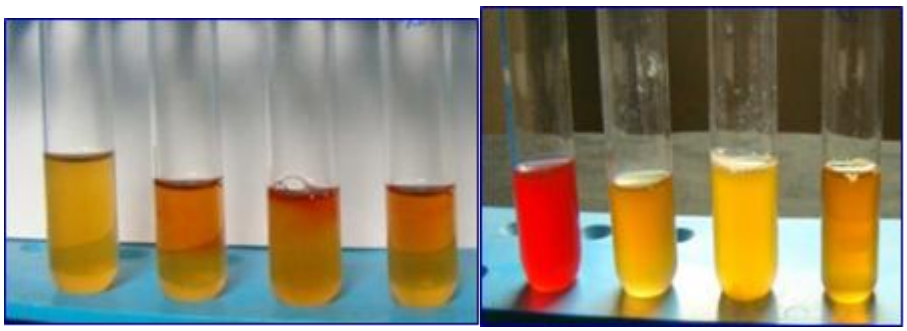


Fig:3 Haemolytic activity of samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08

Fig:4(a) Biochemical characterization of *Bacillus Siamensis*BWCVES01, *Bacillus*

*Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, *Cytobacillus Firmus*BWCVES08



(1) (2) (3) (4) (1) (2) (3) (4)

Fig4(b) Indole and Methyl Red test (1)Bacillus SiamensisBWCVES01 (2) Bacillus ParamycoidesBWCVES05, (3) Bacillus Paramycoides stainBWCVES07, and (4) Cytobacillus FirmusBWCVES08

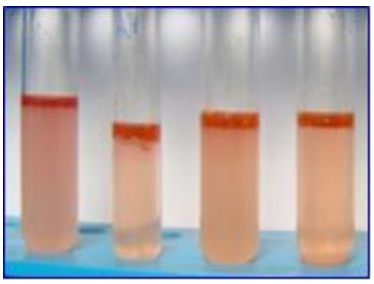
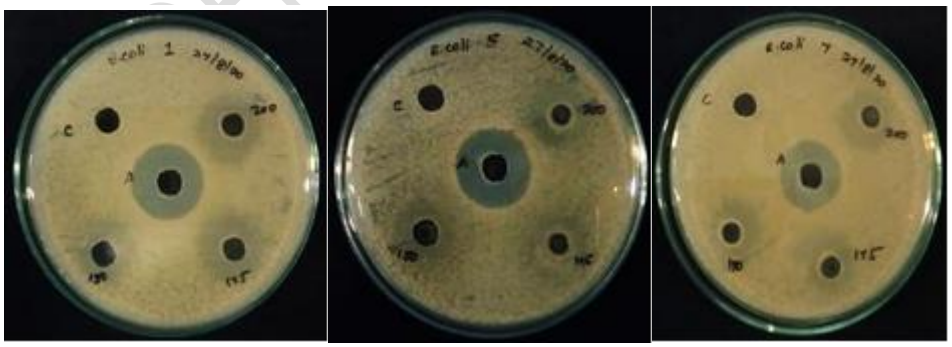


Fig:4(c) Voge's Proskauer test Tube 1: Bacillus SiamensisBWCVES01; Tube 2: *Bacillus Paramycoides*BWCVES05; Tube 3: *Bacillus Paramycoides stain*BWCVES07; Tube 4: *Cytobacillus Firmus*BWCVES08



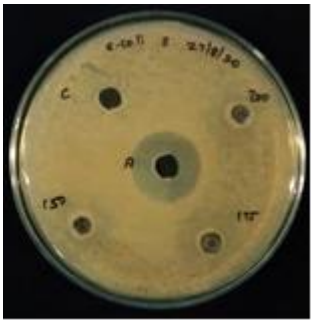


Fig:5(a) Antimicrobial activity Against *E.Coli* of samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08

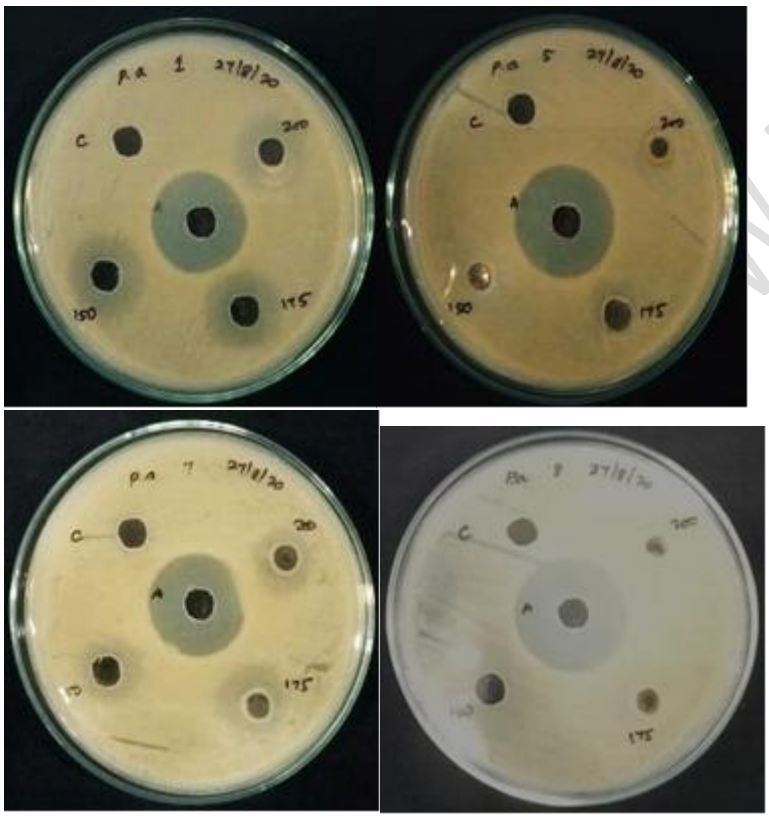


Fig:5(b) Antimicrobial activity Against *Pseudomonas aeruginosa* of samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08.

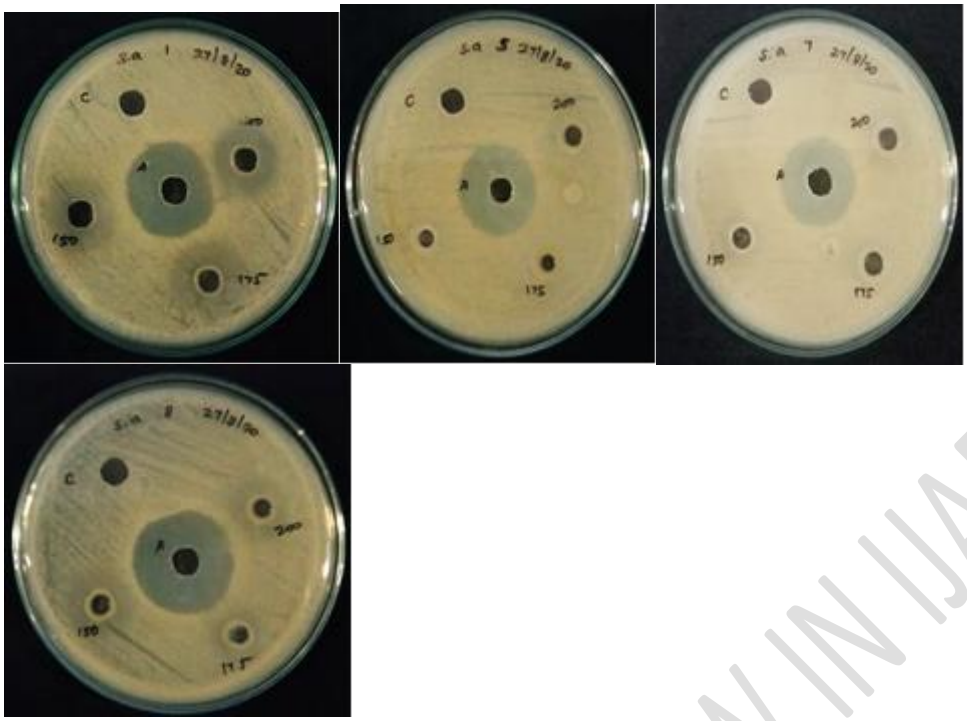
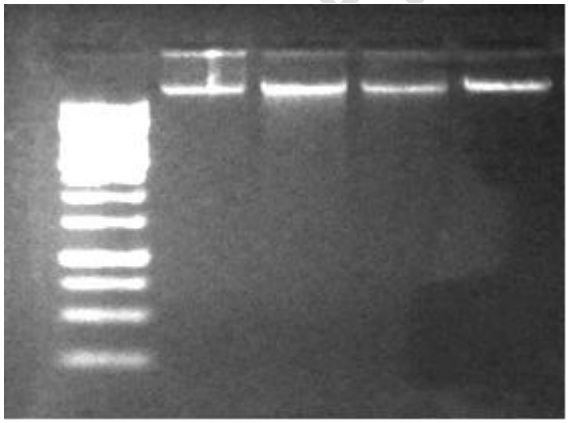


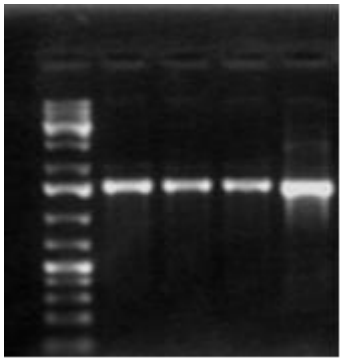
Fig:5(c)Antimicrobial activity Against *Streptococcus aureus* of samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08



Lane 1 Lane 2 Lane 3 Lane 4 Lane 5

Fig 6 Genomic DNA isolation of the isolates

Lane1: Marker; Lane 2: *Bacillus Siamensis*BWCVES01 Lane 3*Bacillus Paramycoides*BWCVES05; Lane 4: *Bacillus Paramycoides* stainBWCVES07; Lane 5: *Cytobacillus Firmus*BWCVES08



Lane 1 2 3 4 5

Fig:7 16S rRNA amplified products of the isolates

Lane1: Marker; Lane 2: *Bacillus Siamensis*BWCVES01 ; Lane 3:*Bacillus Paramycoides*BWCVES05; Lane 4: *Bacillus Paramycoides* stainBWCVES07; Lane 5: *Cytobacillus Firmus*BWCVES08

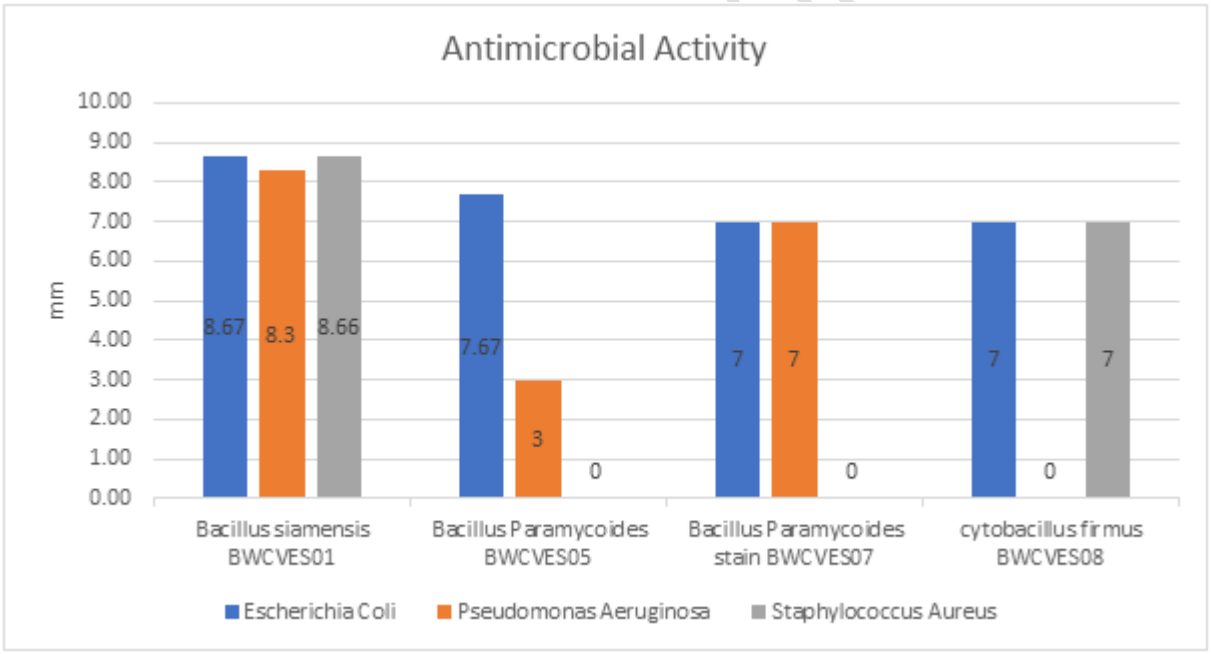
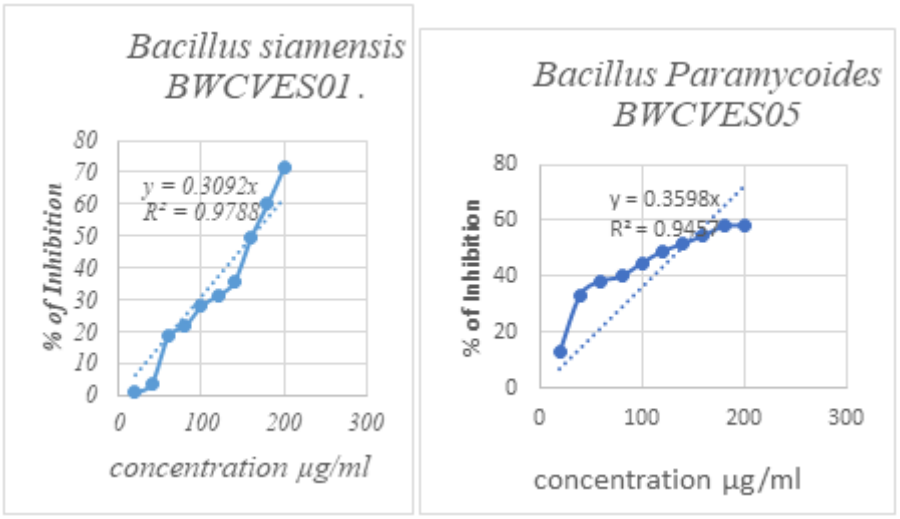
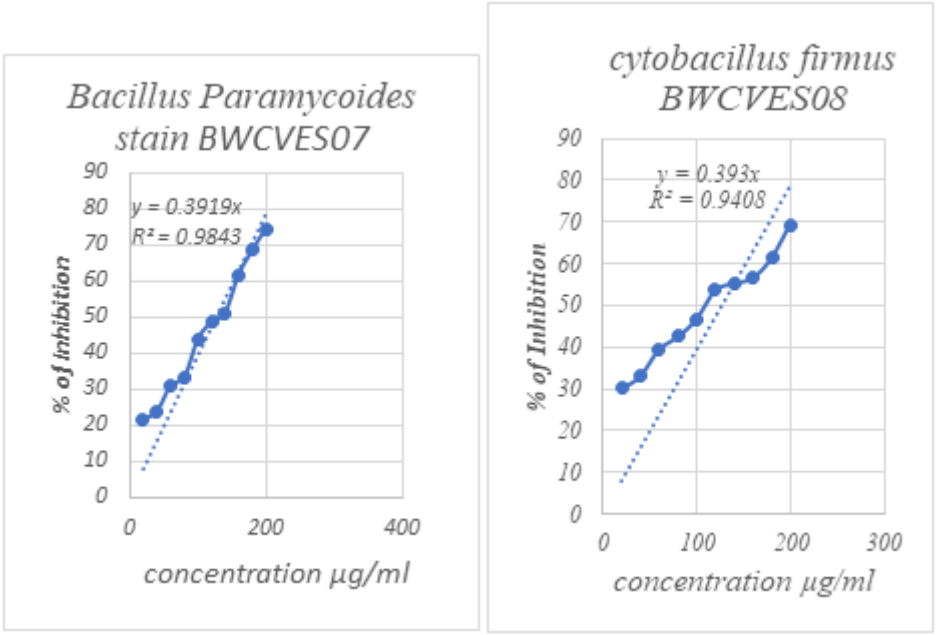


Figure 8 Antimicrobial Activity of various endophytic bacteria against pathogens



Sample1 Sample 2

Fig:9(a) Antioxidant activity of samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05



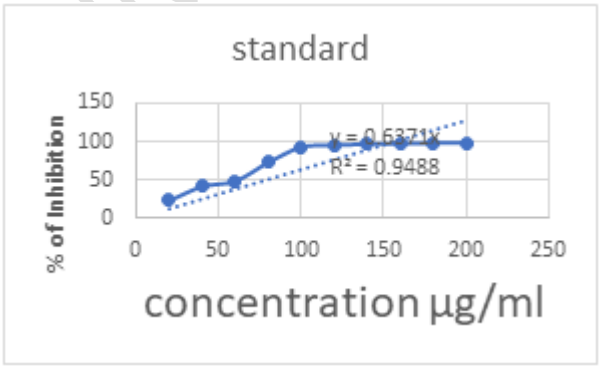


Fig:9(b) Antioxidant activity of sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08 with standard

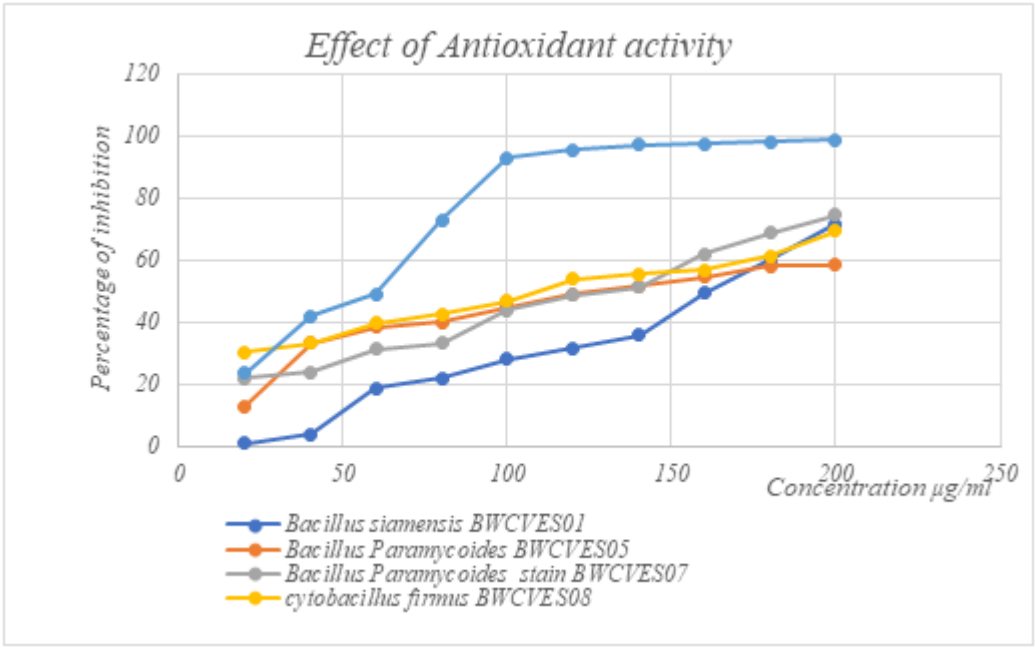


Fig:10 Antioxidant activity of samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08

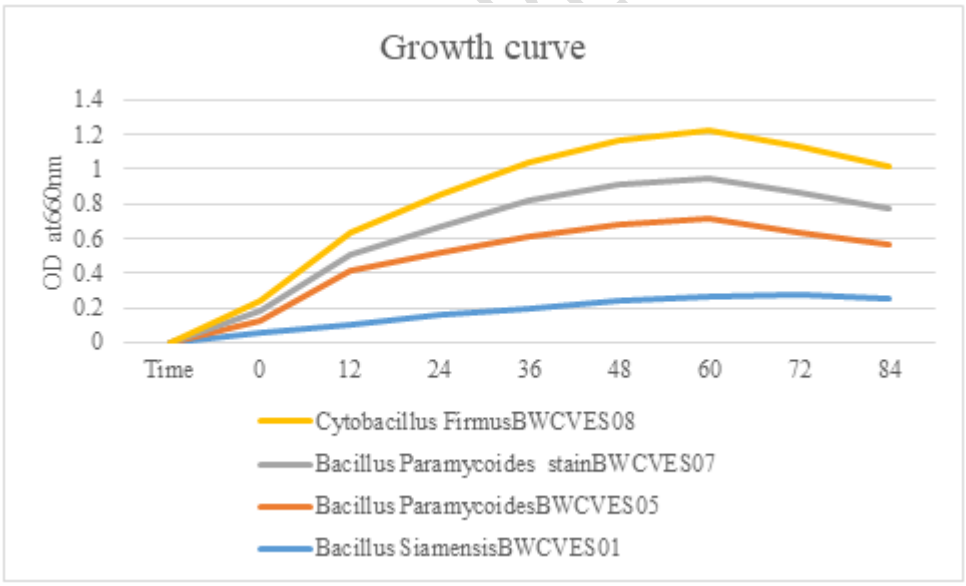


Fig:11 Growth Curve analysis samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08

*Bacillus Siamensis*BWCVES01

TGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAA
CACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGA
TGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATG
GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGC
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GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAG
TGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAAT
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AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAC
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CGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCC
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ACCCGAAGTCGGTGAGGTAACCTTTATC

Bacillus Paramycoides BWCVES05

GAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACA
TTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCG
CGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGC
AGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA
AGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTG
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TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTT
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TAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTAC
CGCCGCAAGGCTAAACTCAAAGGAATTGACGGGGGCCCGCACCAGCGGTGGAGCATGTG
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GATAGGGGTTTTCCTTTGGGAGCAGAATGACCGGTGGTGCCTGGTTGTTGTCAGCTTGTGT
TCGGAGATGTTGGGTTAAGTCCCGCAACGAGGGCAACCCCTGATTTTAGTTGCCCTCAATT
AGTTGGGCCATTTAAGGTGACCGCCGGTGACAAACCGGAGGAAGGTGGGGAAGAAGTCA
AATCATCCTGCCCCTTATGACCTGGGGTACCCACCTGGTACAATGGACGGTACAAAGAGG
TGCAAGACCCCGAGGTGGAGGTAATTTTATAAAACCCTTTTCCGTTTGGATTGTTGGGTGC
AAATTGCCTACCTGAAGGCGGAATCGGTTGTAATCGCGGATCAGCCTGCCGCGGGGAATA
CGTTCCCGGGCCTTGTACACCCCCCCCGTCACCCCCCGAGAGGTTGTAACCCCCGAAGTCG
GGGGGGTAAT

*Bacillus Paramycoides stain*BWCVES07

AGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC
CCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAAC
CGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTC
GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTG
AGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA

GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAA
TAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGC
GCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATT
GGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTG
AAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAC
TGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTA
ACGCATTAAGCACTCCGCCTGGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAAT
TGAGGGGGCCCGCACCAGCGGTGGAGCATGTGGTTTAATTTGGAGCCACGCGAAGAA
CCTTACCAGGTCTTGACCTCCTTTGACAACCCTAGAGATAGGGGTTTTCCTTTGGGAG
CAGAATGACAGGTGGTGCATGGTTGTTGTCAGCTTGTGTTGTGAGAAGTTGGGTTAAG
TTCCGCAACGAGCGCAACCCCTGATTTTAGTTGCCCTCAATTAGTTGGGCCCTTTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAAGACGTCAAATCATCATGCCCC
TTATGACCTGGGGTACACACCTGGTACAATGGACGGTACAAAGAGGTGCAAGACCGC
GAGGTGGAGGTAATTTTATAAAACCGTTTTCAGTTTGGATTGTAGGGTGCAAATTGCC
TACCTGAAGCCGGAATCGGTTGTAATTGCGGATCAGCCAGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACCCCGCCCGTCACCCCCCGAGAGTTTGTAACCCCCGAAGTCGGT
GGGGTAACC

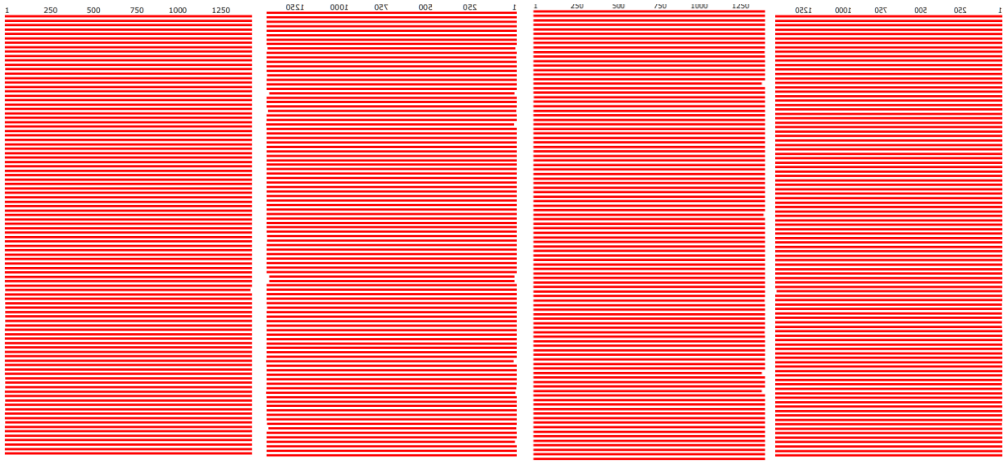
*Cytobacillus Firmus*BWCVES08

TGCAAGTCGAGCGGACGGATGGGAGCTTGCTCCCACGACCGTCAGCGGCGGACGGGTGA
GTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATAC
CGGATAATTCTTTCCCTCACATGAGGAAAAGCTGAAAGATGGCATCTCGCTATCACTTACA
GATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTCAGGGAAGAACAAGTACCGGAGT
AACTGCCGGTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGG
CGGTTCCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG
GGGAACTTGAGTGCAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGA
GATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCC
TGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGAGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCTCCTG
ACAACCCTAGAGATAGGGCGTTCCCCTTCGGGGGACAGGATGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTT
AGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGA
TGGTACAAAGGGCTGCAAGACCGCGAGGTTAAGCGAATCCCATAAAACCATTCTCAGTTC
GGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCA
TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGT
AACACCCGAAGTCGGTGGGGTAACCTTTTGAGCCAGCC

Fig:12 16srRNA sequence of samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08

BLAST ANALYSIS

*Bacillus Siamensis*BWCVES01



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415 Fig:13 BLAST analysis samples 1- *Bacillus Siamensis*BWCVES01, sample-2-
416 *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides*
417 stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08

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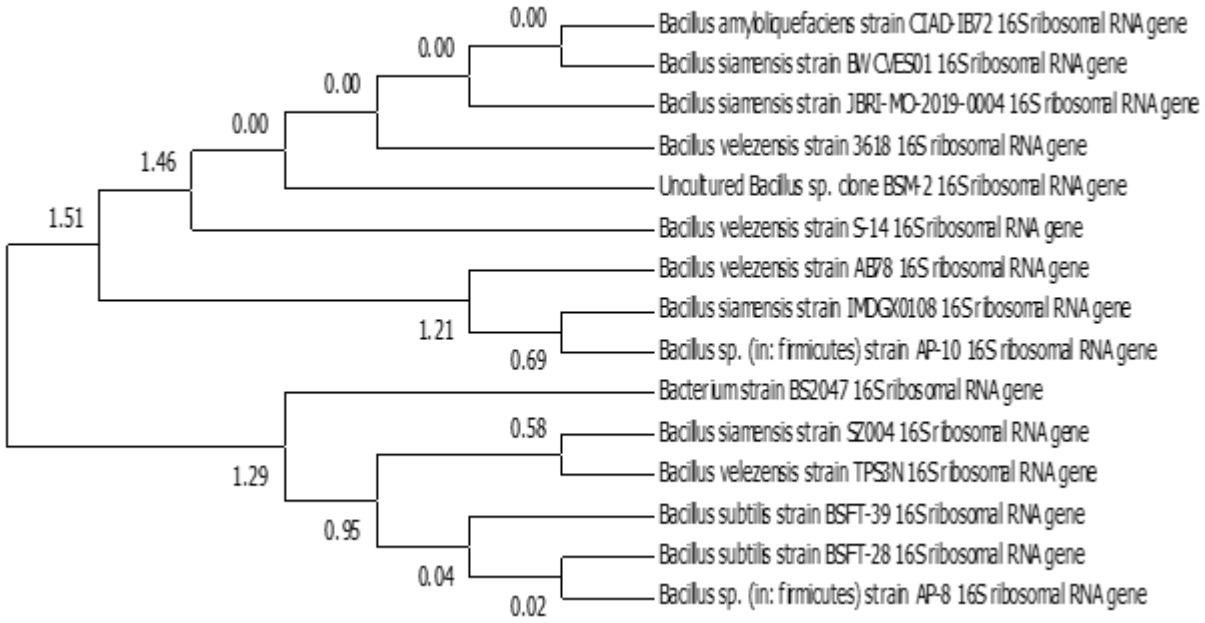

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423 *Bacillus Paramycoides*BWCVES05

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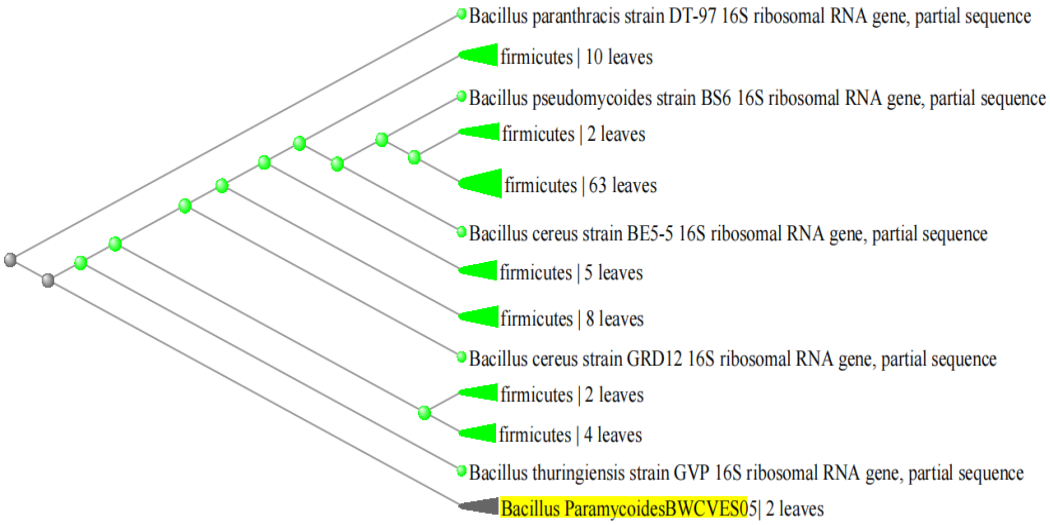
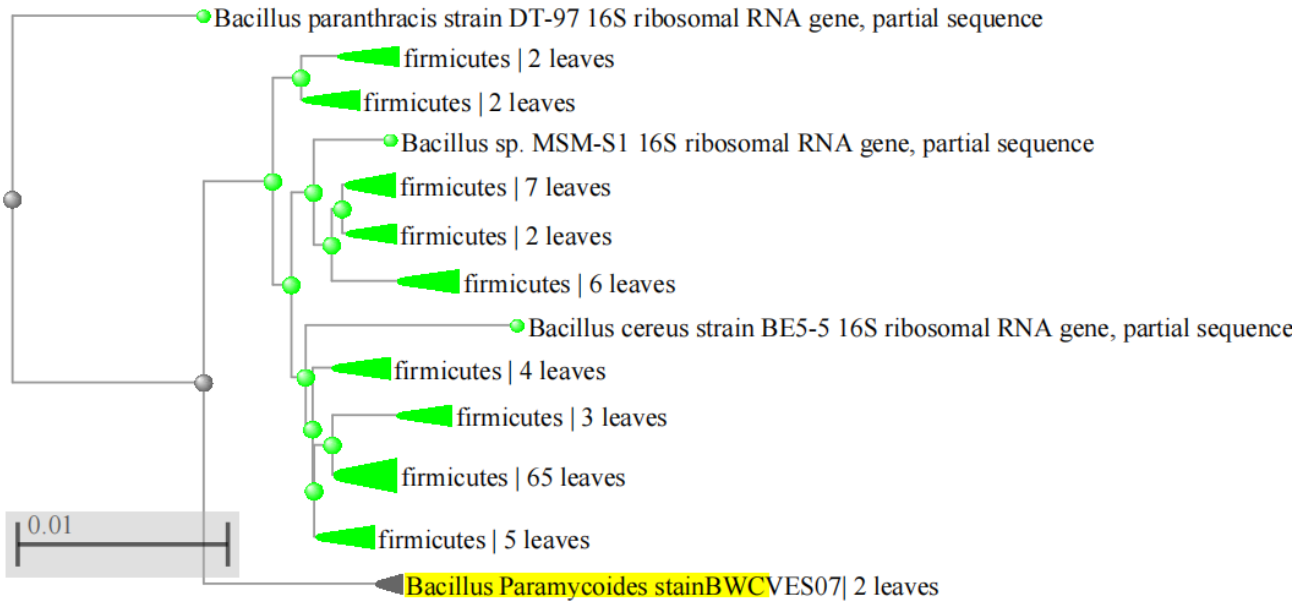


Bacillus Paramycoides stainBWCVES07



*Cytobacillus Firmus*BWCVES08

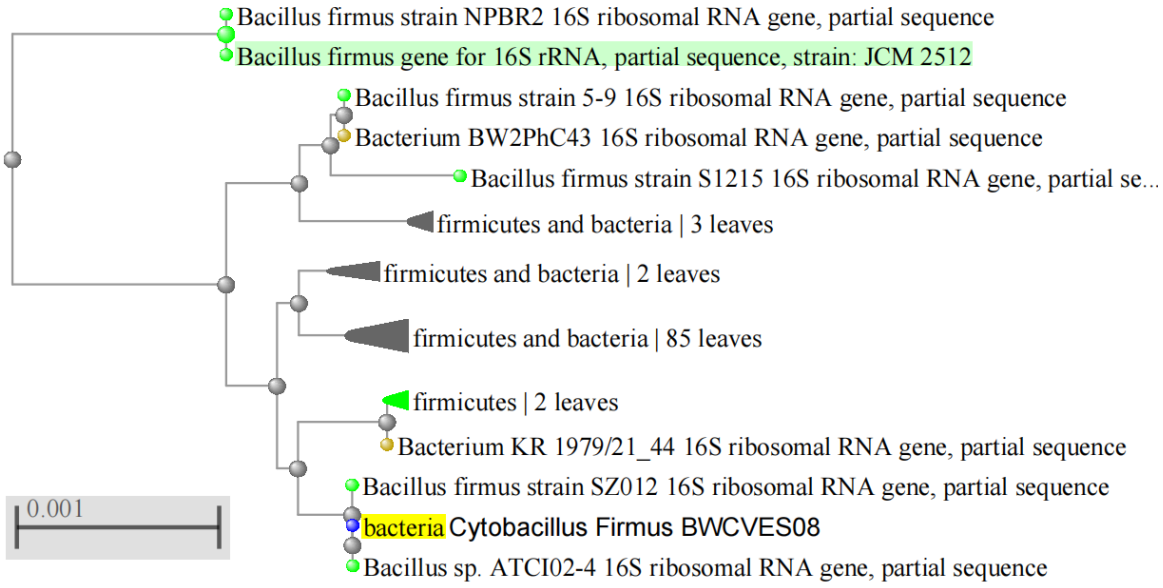


Fig:14 Phylogenic tree analysis samples 1- *Bacillus Siamensis*BWCVES01, sample-2- *Bacillus Paramycoides*BWCVES05, sample-7- *Bacillus Paramycoides* stainBWCVES07, and sample-8- *Cytobacillus Firmus*BWCVES08

Statistical Analysis

The experimental results of biological activity tests were expressed as mean ± standard deviation (SD) of three replicates. The results were processed using Microsoft Excel. The obtained data from PCR amplication were statistically analyzed through MEGA 7, BLAST software packages

Discussion

Endophytes otherwise called rhizosphere bacteria, play a significant role in plant yield and growth promotion for plants and protection of plants. (20; 21). The characteristic of endophytic bacteria can accelerate seedling emergence and help the legume and non-legume plants with Nitrogen Fixation and phosphate solubilization (17) or iron chelation (16). In drug development, it was essential to balance antimicrobial efficacy with safety. Compounds with high antimicrobial activity but low hemolytic activity are ideal candidates (11,12). Research is ongoing to develop compounds that show selective toxicity towards pathogenic cells and cancer cells (13). The table 3.1.1.

shows the biochemical properties of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Table 3.1.2(a) shows the growth curve analysis of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Table 3.1.2(b) shows the doubling time of the isolates in minutes. The endophytic bacterial isolates have antibacterial activity in both gram-positive and gram-negative bacteria. Table 3.1.3 shows the antimicrobial activities of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Table 3.1.4(a) shows the antioxidant activity of the *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08 by DPPH free radical scavenger activity method. Table 3.1.4(b) shows the IC50 value of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Table 3.1.5 shows the molecular identification of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Fig.3 shows the haemolytic activity of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Fig 4(a) shows the biochemical characterization of the isolates and Fig(b) and (c) show the colour formation of the Indole test, Methyl Red test, and Voge's Proskauer test. Fig 5(a) shows the antibacterial activity of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stain BWCVES07, and *Cytobacillus Firmus*BWCVES08 against *E.Coli.* Fig5(b) shows the antibacterial activity of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stain BWCVES07, and *Cytobacillus Firmus*BWCVES08 against *Pseudomonas aeruginosa.* Fig5(c) shows the antimicrobial activity of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stain BWCVES07, and *Cytobacillus Firmus*BWCVES08 against *Streptococcus aureus.* Fig6 shows the genomic DNA isolation of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Fig 7 shows the 16srRNA amplified of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides*

stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Fig 8 shows the graphical presence of antimicrobial activity Fig 9 (a) shows the statical analysis of the antioxidant activity of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. The R-value 0.9 shows the antioxidant activity of the isolates. Fig 10 shows the antioxidant activity of the four isolates. Fig 11 shows the growth curve of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08. Figs 12 and 13 show the 16SrRNA sequences sent in NCBI and BLAST analysis. Fig 14 shows the phylogenic tree of *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08.Partial 16SrRNA sequences were used for the construction of a phylogenetic tree using the Neighbour-joining method. The homologus 16SrRNA sequence were aligned using the multiple-sequence alignment tools 'CLUSTER-W' in MEGA7 software (22). The phylogenetic tree was constructed using the neighborjoining and maximum likelihood method (23). BLAST searches of the 16SrRNA sequences obtained from the isolated bacterial strains were performed in the NCBI databases.

Conclusion

Endophytes are a promising area of research and application in plant science. Their ability to promote growth, enhance stress resistance, and provide biocontrol offers significant potential for sustainable agriculture, environmental remediation, and biotechnology. Further exploration and understanding of these symbiotic organisms can lead to innovative solutions for global agricultural and environmental challenges.

The knowledge of the diversity of endophytic bacteria in plants and medicinal plants is important to explore their capabilities in various fields of biotechnology (24). And hence cultivable endophytic bacterial isolation was confined to the aerial parts of the plant. The isolates of *Bacillus Siamensis*BWCVES01, *Bacillus*

*Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08 were found by 16sRNA and compared the similarity with other sequences in NCBI by Blast analysis. All four isolates show 99% similarity in blast analysis and were named and then sent to NCBI. The strains *Bacillus Siamensis*BWCVES01, *Bacillus Paramycoides*BWCVES05, *Bacillus Paramycoides* stainBWCVES07, and *Cytobacillus Firmus*BWCVES08 show antimicrobial activity, haemolytic activity, and antioxidant activity. The future study expectation is drug development from these endophytes. In conclusion, this study revealed that the endophytic isolates produced bioactive compounds with good antimicrobial and antioxidant activities

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Conflicts of Interest

There is no conflict of Interest

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