Isolation and Identification of Bacillus Subtilis from Paper Industry Effluent for Plant Growth

C. Anuvaishnavi[1], Dr. J. Rengaramanujam[2], M. E. Pavithra[3]
[1]*Second Year, M.Sc. (Microbiology), Department of Microbiology, Dr. N.G.P. Arts and Science College, Coimbatore - 641048, Tamilnadu, India*
[2]*Professor and Head, Department of Microbiology, Dr. N.G.P. Arts and Science College, Coimbatore - 641048, Tamilnadu, India*
[3]*Assistant Professor, Department of Microbiology, KMCH College of Allied Health Science, Coimbatore - 641048, Tamilnadu, India*

***Abstract*— Paper industry is often termed as water inclined one as fresh water is the dire necessity various stages of manufacturing process, as result such paper manufacturing hubs release a large quantity of wastewater and paper effluent elements becomes a challenge .It is described that water is largely used in order to separate the raw material and to give them space to swell and to transport fibers and for laying strong ground for paper formation. In order to enhance the fertility of soil using Bacillus subtilis isolated from paper industry effluent water. The following objective were considered, such as isolation of B.subtilis from paper industrial waste water, Characterization of B.subtilis ,Plant growth promotion using B.subtilis, and isolate and identify the antimicrobial activity of Bacillus against plant pathogen. This study demonstrated that the occurrence of Bacillus subtilis in mungbeans leaves. The morphology and biochemical properties of the isolates had been studied and identified as Bacillus subtilis.**

Index Terms — Paper industry, Bacillus subtilis, Antimicrobial, Protein genes, Cellulolytic enzymes.

I. INTRODUCTION

Bacillus subtilis morphology describes rod-shaped, gram-positive bacteria, and spore producing species that can survive in extremely harsh environment for long period. As Bacillus subtilis biofilm in worm intestine seems to lengthen the worm's lifespan, many human users hope for the same effect. Another use of B.subtilis is in waste treatment. Wastewater must have pH normalized are lower chemical oxygen demand and total suspended solid concentration and be free of excess chloride. These results have contributed to new biodegradation techniques for sewage and wastewater treatment, helping to develop process known as bio augmentation. (Vikas Ghumare et al., 2014) Growing water scarcity and heightened awareness associated with wastewater conservation are prompting more industrial manufactures to explore more water recycling within facilities – a strategy which also reduces wastewater effluent volume. Water recycling is attractive proportion for industries such as P&P that Withdrawal large volume of water or have high polluted waste streams and are subjected to increase charges of fertilizer various materials are generated in paper mills such as ash, dregs, grits, lime mud,pulp mill sludge .mill effluent has high chemical and biochemical oxygen demand some wood derived organic compounds, metals, fatty and resin acids relatively high C:N ratios. (Martin et al., 2001),Various microorganisms or their enzymes such as cellulose ,proteases and lignin degrading enzymes have used as a cost effective and ecofriendly technologists to decrease the consumption of chemicals. Enzymatic pretreatment of effluent reduces the mechanical energy consumption in the process of refining. Some biotic agents however interact symbiotically or synergistically with their host plants .some microbes can be beneficial to plants and perform the same role as chemicals fertilizers and pesticides acting as a bio fertilizer or bio pesticides (Kleinwechter et al., 2016). Plant growth promoting rhizobacteria can significantly enhance plant growth and represent mutually helpful plant microbe interaction. Bacillus sp., major type of rhizobacteria that can form spores that can survive in the soil for long period of time under harsh environmental condition. Plant growth is enhanced by plant growth promoting rhizobium through the induction of

systemic resistance, antibiosis and competitive omission. (Jain, 1998).Thus, the application of the microbes can be used to induce systemic resistance in plants against biotic agents and enhance environmental tolerance. B.subtilis exhibits both a direct and indirect bio control mechanism suppresses disease caused by pathogens. B.subtilis can also solubilize soil enhance, nitrogen fixation, and produce siderophores that promote its growth and suppresses the growth of pathogen. B.subtilis enhances stress tolerance in their plants host by inducing the expressing of stress response genes, phytohormones and stress related metabolites. (Martin et al., 2001).The present review discusses the activity of B.subtilis in the rhizosphere. Bacillus subtilis is one of the most potential biological control agents, because of the broad-spectrum activity of their antibiotics. The current study was carried out to explore in vivo bio control efficacy of Bacillus subtilis alone and combination with plant nutrients against early blight disease of tomato caused by neurotropic fungus. B.subtilis alone in the combination with plant nutrients managed EB disease significantly by 67-83%, while improved plant growth attributes by 20-77%.B.subtilis and plant to fight off the plant hacker by up regulating the production of total phenolic contents and defense enzymes (Baker and Cook 1974).

B.subtilis has a syngernic impact to manage the disease, improve plant growth, enhance physiological and optimistically enhance physiological and biochemical attributes to contest EB stress in plants. B.subtilis promote growth of the host plant by producing inole acetic acid .They synthesis a variety of auxin and cytokines that utilized by the plant for growth and development .Many researchers report the role of PPFMs in enhancing plant growth ,seed germination ,seed vigour index plant yield and systemic resistance of the plant (Goswami et al., 2016). B.subtilis bacteria on plant surface act in a symbiotic relationship with the host plant as they get benefited by methanol ethnol from plants as byproduct of pectin degradation. Bio inoculants used by farmers are sometimes incompatible with the plant and hence do not positively impact the plant growth and yield. (Lugtenberg and Kamilova 2009).To overcome this bio-inoculants should be developed using multi-functional PGPB. These bacteria utilize the plant waste product by producing growth hormones such as IAA ans cytokines, fix the atmosphere nitrogen and enhance stress tolerance by reducing the stress related ethylene production. Phyllosphere bacteria with several plant growth promoting traits may also have the ability to enhance plant growth and tolerance to stress. Impact of Phyllosphere bacteria strains on the plant growth and tolerance to stress .impact of Phyllosphere bacteria strains on plant growth and yield was explained before. (Tripathi et al., 2012).This study was undertaken to evaluate the impact of groundnut genotype of Phyllosphere cultivable bacillus bacterial diversity, and to develop crop-specific bio-inoculants. Based on the plant microbe interaction bacillus sp., are classified as bio fertilizer and bio controller as they directly influence the plant growth by producing phytohormoned especially nutrients supply to the plants and induce systemic resistance in the plants against phytopathogens. In recent years bacillus had received considerable attention for industrial and agricultural application, bio transforms chemical substrates into valuable products with enhanced economic value, Bacillus sp inhabit soil, water and plants and drive the carbon cycle through interactions with neighboring organisms such as plants and bacteria .plants provide Bacillus sp with habitats as well as compound including growth substrates.

In the Phyllosphere, it utilize methanol emitted by plants and affect plant growth (Grover et al., 2011, Vejan et al., 2016). Bacillus strains exhibit their bio control capacity predominantly through inhibitory activity on the growth of plant pathogens, as well as inducing systemic resistance in plants and competing for ecological niches with plant pathogens. Our previous studies showed the presence of multiple biosynthetic operons for synthesis of non-ribosomal lip peptides in the collection of natural isolates of Bacillus, with many strains having more than one of them. Several strains of Bacillus sp. that we have recently characterized showed very strong antibacterial and antifungal activity against phytopathogens. Plant growth-promoting bacteria (PGPB) are bacteria that form specific symbiotic relationships with plants and enhance plant growth through a variety. Many of the PGPB exist in the rhizosphere, the soil within a few millimeters of the plant root surface. PGPB perform various activities to aid plant growth, including nitrogen fixation, siderophores production to reduce iron toxicity to plants and inhibit pathogen growth, production of indolic compounds, and reduction of ethylene gas

production by stressed plants through ACC deaminase and phosphate utilization (Mahdi SS, et al. 2010). Bacillusspp. is PGPB that have been reported to colonize plant roots and exist in a symbiotic relationship with the plant, promoting growth in maize via siderophores production and nitrogen fixation, and rice via ACC deaminase, among other mechanisms . Growth promotion by Bacillus species requires colonization of plants, either externally on roots or as endophytes. Colonization of plant roots with Bacillus subtilisrequires swarming motility and biofilm formation, as well as chemo taxis. (Mazid M, Khan TA 2014).Plant diseases caused by pests, including pathogenic bacteria and fungi, can lead to a reduction in plant growth potential or death. Without proper control measures, as well as the excessive and uncontrolled use of chemical pesticides, plant diseases would lead to decreased crop yields due to the development of pathogen resistance, simultaneously creating a space for atypical pest infestations and pathogen outbreaks. Biological control, being an eco-friendly approach in using a population of beneficial organisms against pathogenic ones, is part of Integrated Crop Management. This implies a combined control of the use of bio control agents (BCAs) together with modified agricultural methods, soil and water conservation practices, to reduce the use of chemical pesticides. (Choudhary, 2011). Hence, developing new methods to combat plant pests and diseases is imperative today, and the application of new bio pesticides could generate a critical change for the ecological and economic sustainability of current food production, and significantly contribute to global food security. Bacteria from Bacillus and Pseudomonas genera are well known producers of secondary metabolites, enzymes and other bioactive compounds with the capacity of biological control of pathogens and beneficial effects on plants via different mechanisms (Beauregard et al., 2013). Healthy nutrition and the urgent need for sustainable agriculture due to emerging pest Colonization of roots by Bacillus subtilis is beneficial to both the bacterium and the host plant. Approximately 30% of the fixed carbon produced by plants is secreted through root exudates. (Garcia-Fraile et al., 2015).

II. MATERIALS AND METHODS

A. Collection of samples

The industrial effluent water was collected from Kavery Riverbed, nearer to local paper industry from Tiruchengode, Namakkal district, Tamilnadu, India.

Figure:1 Collection of sample

B. Isolation and identification Bacillus

The samples were serially diluted, and diluted samples were spreaded by spread plate technique on the surface of nutrient agar and incubated at 37°C for 12-24 hours.

C. Morphology and biochemical characterization of bacteria

Morphological characterization was carried out by gram staining, spore staining and motility test were done .Biochemical procedures such as peptone broth MR-VP broth, and citrare utilization media were prepared and dispensed aseptically into tubes. The proteolytic isolates are incubated into respective medium and incubated at 37° C for 24 hours .After incubation Kovac's reagent, methyl red indicator and barrit's reagent A and B is added to appropriate tubes and observed for the presence or absence of cherry red ring in case of indole, red colour in the case of methyl red, deep pink colour in the case of voges prouskauer and deep Prussian blue in case of citrate utilization teseare done for biochemical characterization.

D. Starch hydrolysis

Sterile starch agar plates are prepared, and the proteolytic isolates are inoculated by single line streak method and incubate at 37° C for 24 hours. After incubation the plates are flooded with iodine reagent for 30 seconds and examined for the presence or absence of blue-black colour around the growth of each isolate.

E. Protein hydrolysis

Skimmed milk agar plate was prepared, and the organisms were streaked on the plate and incubate it for 12-24 hours at 37°C.

F. Urea production test

Medium: Urea broth procedure. Streak the surface of urea agar slant with a portion of well isolated colony or inoculate the slant with 1 to 2 drops from an overnight brain heart infusion broth culture. Leave the cap on loosely and incubate the tube at 35℃ to 37℃ in ambient air for 48 hours to 7 days. Examine for the development of pink colour for as long as 7 days.

G. Motility test

The SIM media was prepared, and the culture was stabbed in the medium and incubates it for 37ºC for 12-24 hours.

H. Gene sequencing

This method not only faster and more attractive than conventional methods, but also allows identification of strains that are difficult to grow in laboratory condition. Furthermore, differentiation of strains that are molecular level enables discrimination between phenotypically identical bacteria.16s rRNA joins with a complex of 19 protein to form 30S subunit of the bacterial ribosome .it is encoded by the 16S rRNA gene, which is present and highly conserved in all bacteria due to its essential function in ribosome assembly, however it also contains variable region which may serve as fingerprints for particular species. These features have made the 16S rRNA gene in ideal genetic fragment classification of bacteria .16S rRNA gene sequencing is based on the polymerase chain reaction followed by DNA sequencing, PCR is a molecular biology method used to amplify specific fragments of DNA through a series of cycles that include 1.Denaturation of a double stranded DNA template 2.Annealing of primers template 3.Extension of primers by the DNA polymerase enzyme, which synthesize a new DNA strand.

I. Inoculation preparation

Nutrient broth culture was prepared and Bacillus subtilis was inoculated into the broth and incubated at 37◦C for 12-24 hours

J. Plant growth promotion

Plant growth promotion of the bacterial isolate has exploited for the agricultural application In this study, the effect of bacterial isolate on the growth of plant were evolved in gardening trays were filled with soil and seeds were transferred to the respective area.

K. Antimicrobial activity of plant pathogen

Muller hinton agar plates were prepared and plant pathogenic organism [i.e.] Pseudomonas sp was swabbed on the surface. The well was cutted and *Bacillus subtilis* was poured and incubated it for 12-24 hours at 37 ºC.

III. RESULTS AND DISCUSSIONS

A. Isolation of Bacillus

In this study, *Bacillus subtilis* were isolated from kavery riverbed, near to local paper industry effluent at Tiruchengode, Namakkal District, Tamil nadu.

B. Morphology and biochemical characterization of isolates

Figure.2 Colony identification by serial dilution

The organism was isolated and identified by serial dilution.

Gram staining – Purple colour rod shaped organism was identified

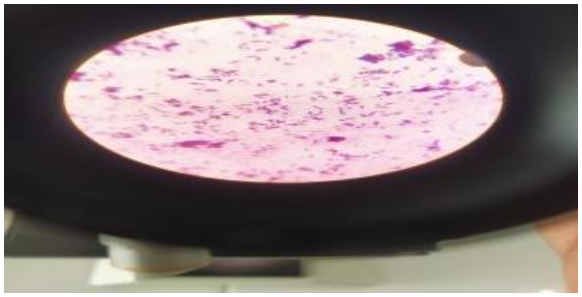


Figure.3 Gram staining

Spore staining –Pink and green colour ellipsoidal organisms were identified. Pink vegetative cells. Green generative cells [Spore forming cells].

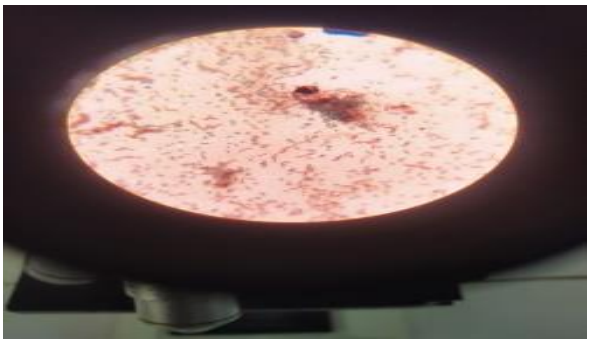


Figure.4 Spore staining

C. Biochemical characterization of bacteria

The isolates were further characterized by a series of biochemical tests. The bacteria were positive for oxidase, urease, catalyze, methyl red, casein hydrolyze, triple sugar iron test, starch. And indole, voges proskauer, citrate were resulted negative.

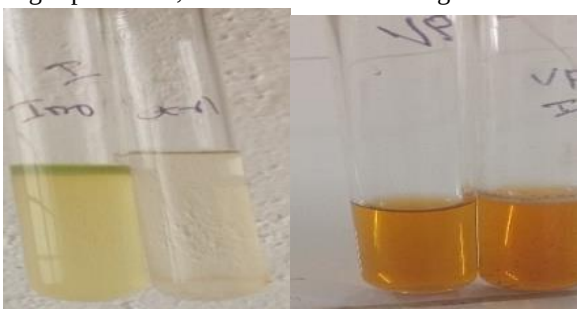


Figure.5 Indole test and Vogus-proskauer

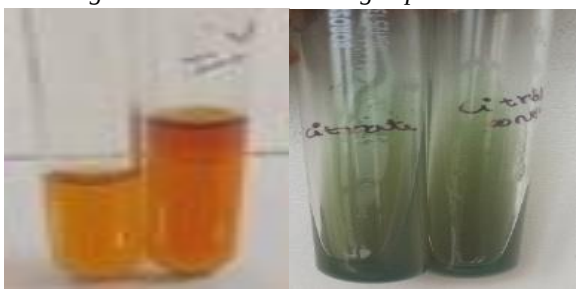


Figure.6 Methyl red and Simmon citrate

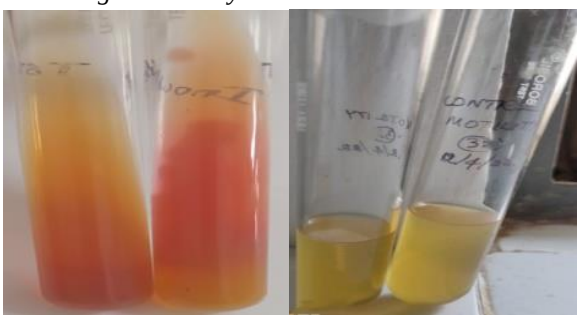


Figure.7 Triple sugar iron and Motility test

Figure.8 Casein hydrolysis

Figure.9 Oxidase test and Catalase test

Figure.10 Starch hydrolysis and Urease test

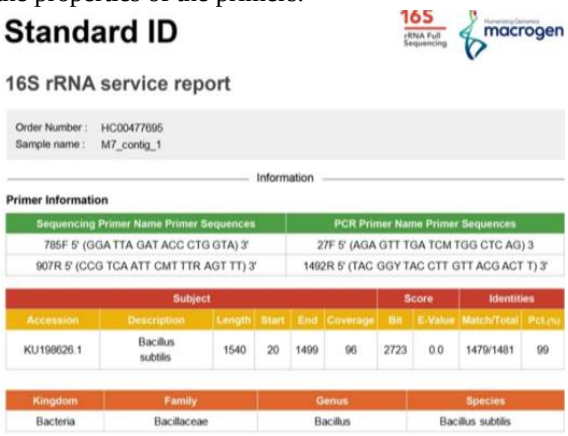
Table 1 Biochemical results

Sl.No.	Characteristics	Results
1	Gram staining	Gram positive rods
2	Spore staining	Green coloured ellipsoidal [spore forming] cell
3	Motility	Positive
4	Indole	Negative
5	Methyl red	Positive
6	Voges proskauer	Negative
7	Citrate	Negative
8	Triple sugar iron	Positive
9	Casein hydrolysis	Positive
10	Urea hydrolysis	Positive
11	Starch hydrolysis	Positive
12	Oxidase test	Positive
13	Catalase test	Positive

D. Gene sequencing

There are several factors that are important for a successful PCR reaction, one of which is quality of the DNA template. Isolation of chromosal DNA from bacteria can be performed using stranded protocols or commercial kits, Special care should be taken to obtain DNA that is free of contaminants that can inhibit the PCR reaction Conserved region of 16SrRNA gene permit the design of universal primer pairs that can

bind to and amplify the target region in any bacterial species. The target region can vary in size. While some primer pairs can amplify most of the 16s rRNA gene, others amplify most of 16s rRNA gene, others amplify only parts of it Cycling condition of PCR are dependent on the type of polymerase that is use and the properties of the primers.



Standard ID

16S rRNA service report

Order Number : HC00477695
Sample name : M7_contig_1

Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
KU198626.1	Bacillus subtilis	1540	20	1499	96	2723	0.0	1479/1481	99

Kingdom	Family	Genus	Species
Bacteria	Bacillaceae	Bacillus	Bacillus subtilis

Figure.11 Gene sequencing report

It is recommended to follow the manufacturer's guidelines for a particular polymerase. The DNA sequence is automatically generated from a DNA chromatogram by a computer and must be carefully checked for quality, as manual editing is sometimes needed, the gene sequence is compared with sequence deposited in the 16S rRNA database. The region of similarity is identified and the most similar sequence is delivered. The 16s rRNA sequencing confirmed the isolate as *Bacillus subtilis.*

E. RNA sequencing

Further isolates were confirmed by using the emerging technique 16S RNA sequencing which confirmed bacterial genus and species as Bacillus subtilis. The below test was carried out at genolite laboratory, Coimbatore.

Table 2 Primer information of the bacteria

Sequencing primer name primer sequence	PCR primer name primer sequence
785F 5′[GGA TTA GAT CTG GTA]3′	27F 5′ [AGA GTT TGATCM TGG CTG AG]3′
907R 5′ [CCG TCA ATT CMT TTR AGT TT]3′	1492R 5′ [TAC GGY TAC CTT GTT ACG ACT]3′

Further isolates were confirmed by using the emerging technique 16S RNA sequencing which confirmed bacterial genus and species as *Bacillus subtilis.* The below test was carried out at genolite laboratory, Coimbatore.

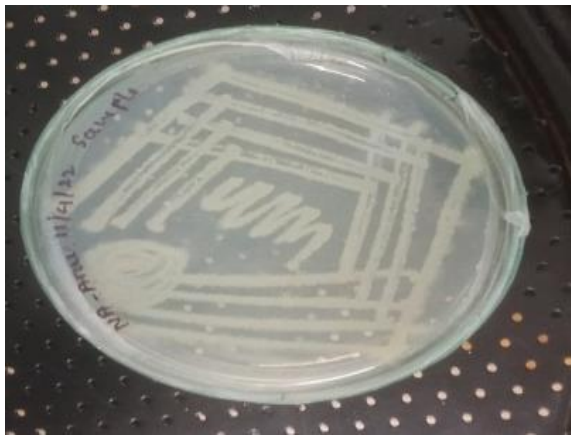


Figure.12 Bacillus subtilis

F. Plant growth promotion experiment

Seed bacterization of *Bacillus subtilis* significantly enhanced root and shoot length and number of leaves per plant. Treatment with *Bacillus subtilis* resulted in enhancement in root length, stem height and leaf height [cm] when compared to the root length, stem height and leaf height [cm] of control. In terms of shoot length and number of leaves, similar significant increase was recorded.

G. Seed germination

The seeds are inoculated with *Bacillus subtilis* for the germination in the concentration of 2 ml, 1.5 ml, 1 ml, 0.5 and control.

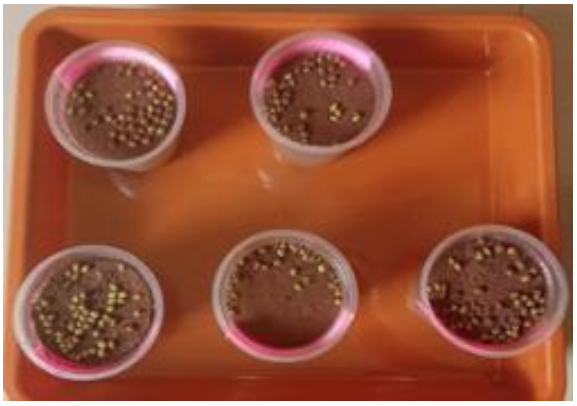


Figure.13 Seed germination

H. Plant growth first observation

The mung bean seeds were germinated in cups. A specific difference was observed in the seeds treated with Bacillus subtilis than uninoculated broth in 2ml dilution the sprout formation was identified on the next day when compared to first observation.

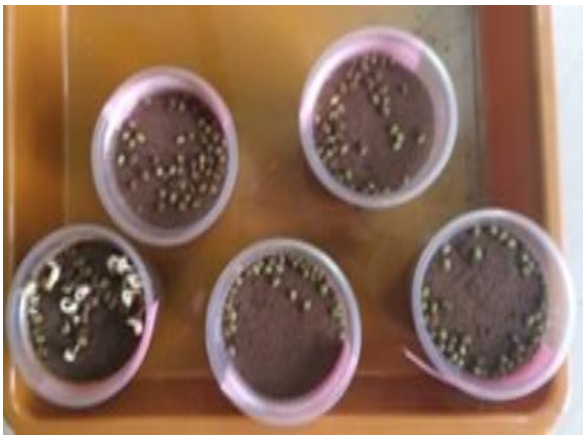


Figure.14 Plant growth first observation

I. Plant growth second observation

The plant growth was observed during the 3rd day of seed inocution.The length of the plant was increase in when compared to the control.The development of the shoot and the leaflets has been increased.

Figure.15 Plant growth second observation

J. Plant growth third observation

The growth was observed during the 7th day of inoculation, the plants grown aseptically at different ratio.

Figure.16 Plant growth third observation

Figure.17 Plant Root formation

Figure.18 Variation of plants based on dilutions

Table 3 Variation of plants based on dilutions

Dilution	2 ml	1.5 ml	1 ml	0.5 ml	Control
Total Height of the Plant	21.4 cm	20.9 cm	20.5 cm	16.5 cm	12.5 cm
Stem	11.7 cm	11.4 cm	11.4 cm	10.5 cm	8.5 cm
Root	8.8 cm	9 cm	7.9 cm	4.5 cm	4.5 cm
Leaf	4 cm	3.5 cm	3.5 cm	3 cm	3 cm

K. Antimicrobial activity

Bacillus subtilis resist the growth of *Pseudomonas sp* with of zone 2.2cm were observed on MHA plates .,It confirms that the *Bacillus subtilis* resist plant pathogen.

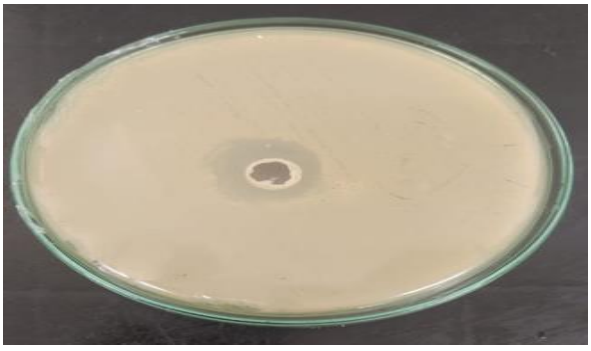


Figure.19 Antimicrobial activity

IV. CONCLUSIONS

This study demonstrated that the occurrence of *Bacillus subtilis* in mungbeans leaves. The morphology and biochemical properties of the isolates

had been studied and identified as *Bacillus subtilis.* Further confirmation was done by gene sequencing. Crop productivity is decreasing due to climatic changes, and human populations are increasing daily, which results in starvation problems in under-developed countries. Research is on-going to enhance crop yields despite various unfavourable environmental conditions. Physical, chemical and biological methods are being used to address the biotic and abiotic stress-induced damage in plants. The mutualistic relationship between plants and microbes is well known, especially the interactions between plants and bacteria either from the soil or inside the plants that help to improve the plant health under adverse stress conditions. The plant-beneficial *Bacillus* spp. produces plant growth-promoting substances (hormones and solubilizing enzymes) to increase plant growth. Some of the physiological alterations in plants during *Bacillus* spp. inoculation in stress environments slow plant aging. For example, the ethylene-suppressing enzyme (ACC deaminase) synthesized by *Bacillus* spp. mitigates the detrimental effects of abiotic and biotic stress in plants by delaying senescence. Exopolysaccharide production by *Bacillus* spp. has been frequently reported to reduce sodium ion transport and regulate plant nutrient uptake during salinity stress. Additionally, the lip peptides and toxic substances secreted from *Bacillus* spp. prevent pathogen growth and reduce disease occurrence in crops. The plant growth-promoting activities of *Bacillus* spp. have been well-documented as evidenced by increased growth of roots, shoots, and leaves as well as enhanced yields. However, very few studies have been conducted regarding the physiological and molecular aspects of these processes. Some of these studies have revealed that *Bacillus* spp. regulate nutrient uptake, water transport, and antioxidant, pigment, hormone and stress-responsive genes and proteins in plants leading to tolerance under adverse environmental conditions.

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