# *IN VITRO* ANTIBACTERIAL EFFICACY AND MOLECULAR IDENTIFICATION OF ENDOPHYTIC BACTERIA ISOLATED FROM *DENDROCALAMUS ASPER* AND *DENDROCALAMUS STRICTUS*

**Karthik M.B[1*], Jayashankar. M[2]**

**Abstract**

Endophytes are a group of microorganisms which colonize in the tissue of live plant which benefits plants for uptake of nutrition, nitrogen fixation, defence against pathogens, and symbiosis associated without harming the host plant. Bamboos belong to angiosperms, order monocotyledons grass family Poaceae. Green gold is a nickname for bamboo. The shoots which are a very healthy vegetable. The present research study is focused to isolate endophytic bacteria from edible Bamboo shoot species *Dendrocalamus asper* and *Dendrocalamus strictus*, collected from Mekeri, Kodagu region.

Eight endophytic bacteria isolates were obtained from these shoots. which were screened for antibacterial activity by well diffusion assay. Three prominent species (B1, B3, B8.) revealed significant zone of inhibition against clinical pathogens *Escherichia coli*, *Proteus mirabilis. Klebsiella pneumonia* and *Shigella sonnei*. SEM images are obtained for the three isolates. Genome were quantified and identified using partial genome 16S rRNA sequencing. ITS region obtained are compared with closely related species from NCBI data base. The bacteria are from the genus Bacillus. Phylogenetic tree is constructed the obtained bacteria are identified as, *Bacillus licheniformis* - ON254199, *Bacillus velezensis* - ON259692 and *Bacillus subtilis* - ON340723.

**Keywords-** Endophytic bacteria, 16S rRNA sequencing, Bamboo shoot, SEM

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## Introduction

Entophytic bacteria colonize enormous number of plants in the healthy tissue which are not causing injury to the host and symbiosis associate with the plant. The word "endophytes" was first used by scientist De Bary in 1866 to describe microorganisms found in plant tissues [1]. Bacteria colonize in all part of plants leaf, stem, flower, roots, fruits [2].

The young, edible bamboo plants that have just sprung from the earth during the rainy season are known as bamboo shoots [3]. The freshly harvested shoot is cream yellow in colour, has a strong smell and tastes sweet. Bamboo shoots are a delicacy, a nutritional dish of many countries However, all species of bamboo shoots available worldwide are not edible. Bamboo shoots have excellent anti-microbial qualities [4].

Use of antagonistic endophyte as Biocontrol compounds are a viable option for managing some plant diseases with no negative environmental impact[5]. The bacteria, fungi and actinomycetes that make up the plant microbiome include bacterial endophytes, which can invade living plant tissues or intracellular cells without harming the host plant[6]. Endophytic bacteria have many benefits, including increasing mineral absorption, increasing plant growth by producing phytohormones, survival in extreme climate conditions, nitrogen fixation and increasing plant defence against bacterial and fungal diseases [7].

Bamboo shoots that have been fermented contain a wealth of microorganisms, many of which are probiotics. They provide a variety of health advantages when taken, including antioxidant and anti-cancer properties, blood pressure reduction, heart disease prevention, and weight loss, to mention a few. In addition to these, they can be used in several industries, including the food, pharmaceutical industries, and ayurvedic treatments [8].

In this study, endophytic bacteria from the edible bamboo shoots of *Dendrocalamus strictus* and *Dendrocalamus asper* are isolated for the first time. The best isolate with antimicrobial characteristics is screened and identified by the molecular 16s rRNA Gene Sequence Analysis method.

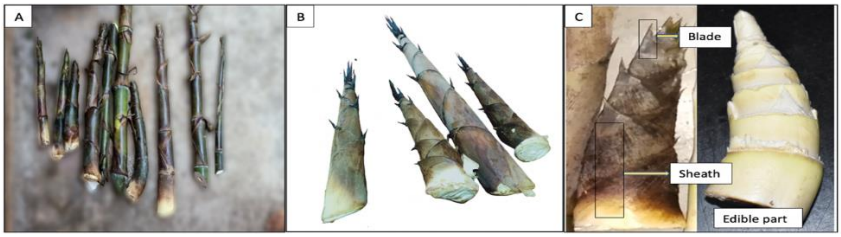


**Figure 1.** A- Photograph of *Dendrocalamus strictus* shoots, B- *Dendrocalamus asper* shoots, C- Bamboo shoot its part.

## Materials and Methods

***Study area:***

The shoot sample is collected from bamboo species *Dendrocalamus asper* and *Dendrocalamus strictus* from the Kodagu district of Karnataka state.

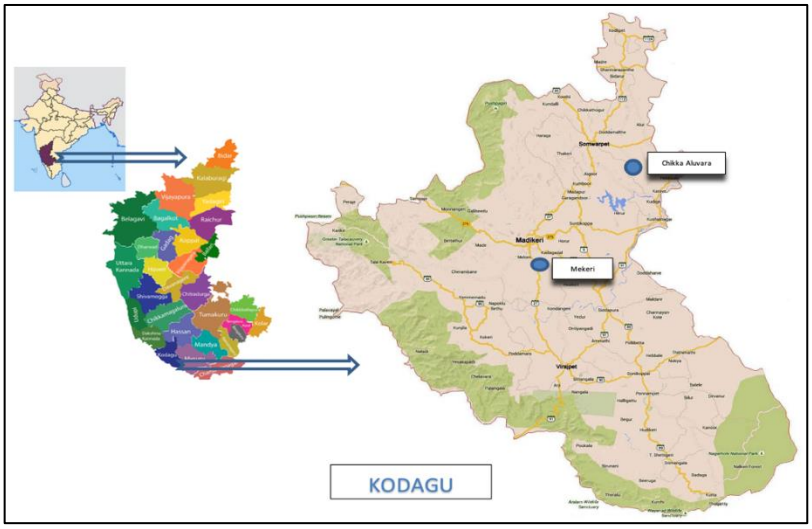


**Figure 2.** Map showing the location of the sampling area

***Collection of samples and isolation of endophytic bacteria:***

*Preparation of shoot segments-*

To prevent interference from plant infections, healthy bamboo shoots were chosen. It was removed from the bottom using a clean knife and brought to the lab in a cleaned, sterile polythene bag within 24 hours.

*Pre-treatment of plant shoots-*

The shoots' upper culm sheath was removed. To eliminate surface soil particles, germs, and adherent detritus, the edible parts are first washed in tap water for five minutes. They are then cleansed once more with distilled water.

Surface Sterilization was done by using the protocol described by Jayashankar [9] with few modifications. Shoots are cut into 1cm long segments/pieces from different parts of a shoot with a sterile surgical SS blade inside the laminar air flow cabinet to avoid contamination. The segments which are occurred from different parts of the shoot are mentioned below (Figure- 3)

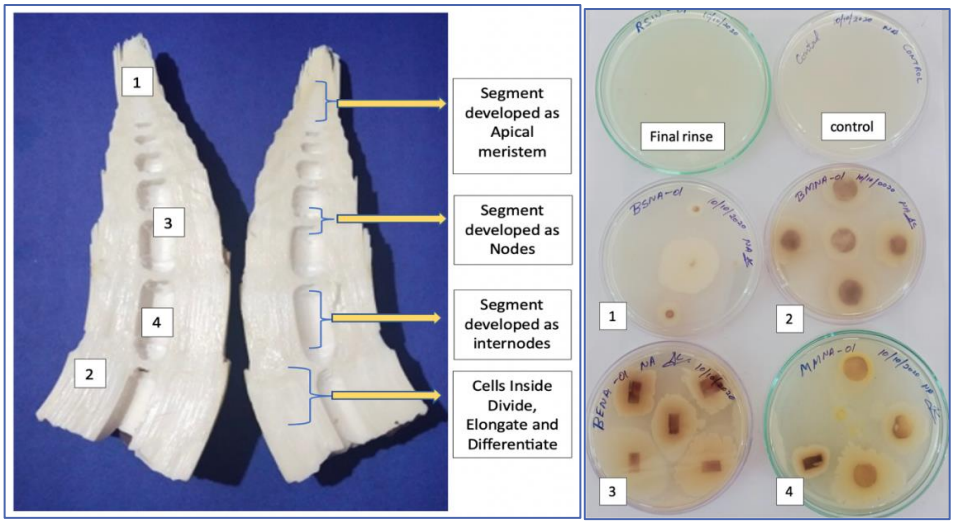


**Figure 3.** Photograph of *Dendrocalamus asper* Bamboo shoot sectioned lengthwise and the segments plated on NA media showing growth of Endophytic bacteria

Shoot segments were submerged in 70% ethanol for three minutes, twice in 2% sodium hypochlorite aqueous solution for five minutes, and then once more for one minute. The shoot samples should then be washed twice in sterile distilled water for 5 minutes to remove the surface sterilising agents, and then rinsed 6-8 times with sterile water in a laminar air flow hood further dried in the sterilized paper in a laminar air flow hood.

*Isolation of endophytic bacteria-*

Sterility Check- To confirm that the shoot surface was effectively decontaminated sterile distilled water that was used in the final rinse of surface sterilization procedure were plated on the Nutrient agar medium and incubated at 37ºC for 24h. Bacterial growth was observed colony was selected based on morphology characters obtained pure cultures.

***Microscopic observation and Biochemical tests:***

Morphological and biochemical studies were used to identify the endophytic bacterial isolates. For each isolate, the following tests are Gram staining, catalase test, oxidase activity, gelatinase, Indole test, and Methyl Red test.

***Pathogenic cultures and growth conditions:***

The pathogenic cultures were cultivated for 24 hours at 37°C with continual shaking in Brain Heart Infusion (BHI) medium (150 rpm)

***Test Culture and Growth Condition:***

The test bacterial cultures were grown fresh in Luria Bertani Media (LB) at 37°C for 24 h. later centrifuged for 10 min at 10,000 rpm. cell-free supernatant (CFS) are collected and filter sterilized which was then used for antibacterial assay

***Antibacterial activity by agar well diffusion in-vitro method:***

BHI agar plates were prepared by inoculating four freshly grown pathogenic culture *E.coli, Proteus mirabilis, Klebsiella pneumonia* and *Shigella sonnei*. Wells of 6 mm in diameter was made in the plate by using sterile cork borer Then 70µl of the given CFS of test culture was added in each well Later, plates are incubated at 37°C for 24 to 48 hour. After incubation zone of inhibition was

measured in mm and recorded Antibiotic Chloramphenicol was added as positive control the results are expressed as mean ± standard error of mean of three replicate. Mean differences in inhibition zones were evaluated with ANOVA.

***Molecular identification of bacteria using 16s rRNA Gene Sequence Analysis method:***

Pure bacterial culture is grown in LB broth for 24 hrs. Cultures are centrifuged at 15000 g for 1 min and bacterial pellet is collected DNA is isolated by CTAB method. The DNA thus obtained has to be quantified.

*Preparation of PCR reaction mixture-*

Each PCR reaction for testing the amplification efficiency and development of multiplex PCR assays for DNA barcode primers contained 1µl DNA template (25 ng), 2µl 10X reaction buffer, 0.5µl of MgCl2 (50pM), 1µl of dNTPs mix (10mM), 1µl forward primer (10pM), 1µl reverse primer (10pM), 0.5µl Taq polymerase (5 U/pi) and the final volume 25µl is adjusted with molecular grade water. Marker gene for 16S ribosomal DNA was used. The PCR used universal primers 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3' to amplify approximately 1500 bp. The electrophoresis of the PCR data was followed by purification..

***Data analysis:***

The acquired sequences are then altered and analysed using bio edit software. Sequences obtained are compared with existed sequence by using Basic local alignment search tool (BLAST) program from National Centre for Biotechnology Information site (NCBI). Then the results are analysed by using MEGHA 11 software to determine the level of kinship. Phylogenetic trees are created using maximum likelihood and character-based parameter models.

**Results and Discussion**

*Dendrocalamus asper* and *Dendrocalamus strictus* edible bamboo shoots are collected from Kodagu region Karnataka. Characteristics of the bamboo species collected are noted and tabulated in (Table 1). *Dendrocalamus asper*, also referred to as sweet bamboo, is the species most commonly used for culinary shoots worldwide[10]. *Dendrocalamus strictus* shoots are in high demand, and this species is the most significant one economically in India, providing 66.6% of the raw materials needed to make paper [11].

The effort was made to explore the endophytes from the shoots to know there association and contribution to the development of a bamboo plant. Hence Different parts of the tender shoot which are selected for visualizing the endophytes are shown in above (Figure 3).

**Table 1-** Characteristics Features of the Bamboo Species we collected

| Sl.no | Location | Bamboo species | Habitat | Soil type | Appearance | Height | Inter node length | Culm Diameter | Life span and Flowering | Uses |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | KODAGU (Mekeri) | *Dendrocalamus asper* | Native To Indonesia | They favour soil mixtures with sand and stones that are properly drained. | They are Non-thorny, closely packed clumps with protruding nodes | 20-30 meter | 20-45 cm | 08 to 20 cm having a thickness of 1.2 to 2 cm | The life span of 70 to 100 years with gregarious or sporadic flowering | Shoots are Edible used for ladders and constructio n-related crafts |
| 2 | KODAGU (Chikka Aluvara) | *Dendrocalamus strictus* | Native to India | They favour open deciduous forests with river streams nearby and dry Alluvial soil. | They are Non-thorny, very densely packed clumps with prominent nodes | 13-16 meter | 13-16 cm | 08 to 10 cm having a thickness of 1.5 to 3 cm | life span of 25to30 years with gregarious flowering | Building materials, furniture, artwork, baskets, mats, and decorative things can be manufactur ed and there shoots are edible. |

***Isolation of endophytic bacteria:***

The different shoots segments which are obtained is surface sterilised by slight modification of protocol by Jayashankar [12] . They are placed on nutrient agar media control was maintained no growth in the final rinse was confirmed which shows the effectiveness of surface sterilization shown in above (Figure 3).

Total fifteen endophytic isolates are obtained from *Dendrocalamus asper* and *Dendrocalamus strictus* species they are emerged from different segments

of apical meristem, nodes and internodes. Total four morphologically different endophytic bacteria are obtained from *Dendrocalamus asper* and four from *Dendrocalamus strictus.*

Colony morphology was observed [13] and recorded. The B1 isolate featured a raised elevation, a filamentous edge, a motile rhizoid shape, and a white colony. The B2 colony had a convex elevation, was motile, circular in shape, and had filamentous margins. The B3 colony has filamentous margins, a motile, circular shape, and a convex elevation. The B4 isolate colony have a undulate edge, a motile, circular form, and a convex elevation. The B5 isolate has a white colony morphology, a circular form, an undulate edge, and convex elevation. It is also nonmotile. The physical traits of B6 bacteria include a bright yellow colour, nonmotility, circular form, whole edge, and elevated elevation. B7 endophytic bacteria with a convex elevation, white colony morphology, nonmotile, circular shape, and all-around margin. B8 bacteria isolates that had a white colony, were mobile, had a rhizoid shape, had filamentous margins, and had a flat elevation.

***Microscopic and Biochemical test:***

Isolates obtained are subjected to grams staining they are observed under microscope results are tabulated. Total 7 isolates are gram positive one is gram negative. All eight isolates were rod shape, which reveals all belongs to *Bacillus* species. Isolates are characterised by biochemical test,[14 15] . Obtained result are tabulated below (Table 2). and (Figure 4).

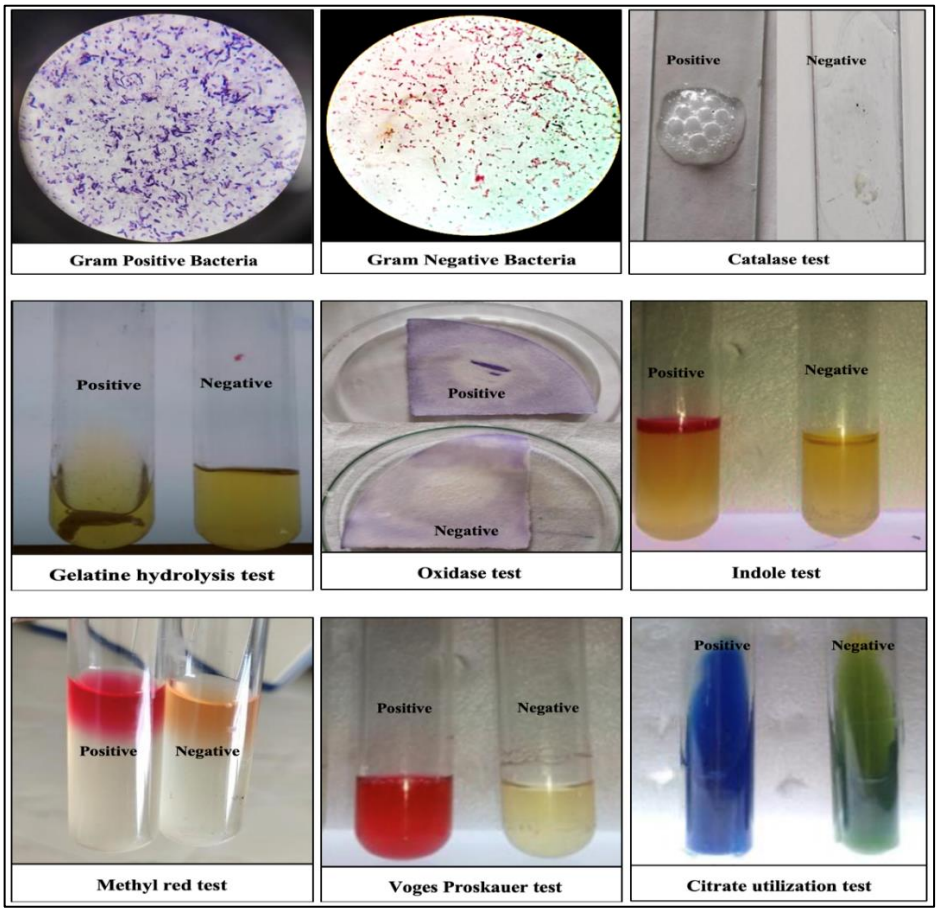


**Figure 4.** Microscopical and Biochemical characterization of endophytic bacteria cultures response to tests performed

**Table 2-** Microscopical and Biochemical characterization of endophytic bacteria

| Bamboo species | Culture Code | Grams reaction | Microscopic observations | Biochemical characterization | | | IMVIC Test | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Catalase | Gelatine | Oxidase | Indole | MR | VP | Citrate |
| *Dendrocalamus asper* | B1-1A | Gram positive | *Rod shape* | + | + | - | - | - | + | + |
| *Dendrocalamus asper* | B2-2A | Gram positive | *Rod shape* | + | + | - | - | - | | |
| *Dendrocalamus strictus* | B3-1S | Gram positive | *Rod shape* | + | + | - | - | - | + | + |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Dendrocalamus strictus* | B4-2S | Gram positive | *Rod shape* | + | - | + | - | + | - | - |
| *Dendrocalamus asper* | B5-3A | Gram negative | *Rod shape* | + | - | - | + | + | - | - |
| *Dendrocalamus asper* | B6-4A | Gram positive | *Rod shape* | + | + | + | - | - | + | + |
| *Dendrocalamus strictus* | B7-3S | Gram positive | *Rod shape* | + | + | + | - | - | - | + |
| *Dendrocalamus strictus* | B8-4S | Gram positive | *Rod shape* | + | + | + | - | - | + | + |

***Anti-bacterial activity:***

Antibacterial activity of the crude extract of endophytes were performed by agar well diffusion method[16] against clinical pathogens *E.coli, Proteus mirabilis, Klebsiella pneumonia and Shigella sonnei* which are collected from Kodagu Institute of Medical sciences, Madikeri. These extraintestinal pathogenic clinical microorganisms that affect human health Gram-negative Escherichia coli bacteria in food is a serious threat to both human health and food safety. [17] UTIs linked with catheter use are frequently caused by *Protease mirabilis*, a gram-negative, rod-shaped bacteria known for its swarming motility and urease activity. It is a catalyst for catheter biofilm development.[18]. Many illnesses, including pneumonia, blood stream infections, and urinary tract infections, are brought on by *Klebsiella species*, which are thought to be opportunistic pathogens. Recent excellent reviews have covered the epidemiology of *Klebsiella* triggered infections, the mechanisms of resistance to antibiotics and the description of some of the virulence factors of this pathogen[19]

*Shigella* species belong to the *Enterobacteriaceae* family and are Gram-negative, rod-shaped, facultatively anaerobic, non-spore-forming, and non-motile bacteria. Given that it is the second most prevalent infectious species of shigellosis bloody diarrhoea, *Shigella sonnei* is an emerging disease on a global scale[20]. These clinical pathogens were selected for antibacterial assay where result revealed high positive control of the microbial inhibition by the crude extracts of B1, B3 and B8 isolates which is shown in (Figure 5) which confirm these three isolates produce high antibacterial extracellular components which supress the growth of clinical pathogens by different mechanisms the outcomes are tabulated in below (Table 3).

**Table 3-** Antibacterial activity of Endophytic bacteria isolated from shoots

| Culture Code | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | *E.Coli* | *Proteus mirabilis* | *Klebsiella pneumonia* | *Shigella sonnei* |
| B1 | 22±0.51 | 21±0.57 | 19±0.44 | 25±0.48 |
| B2 | 09±0.21 | 08±0.30 | 00 | 00 |
| B3 | 16±0.18 | 17±0.44 | 15±0.45 | 16±0.15 |
| B4 | 11±0.42 | 07±0.21 | 07±0.51 | 07±0.21 |
| B5 | 00 | 00 | 00 | 00 |
| B6 | 12±0.31 | 10±0.22 | 08±0.20 | 07±0.32 |
| B7 | 00 | 00 | 00 | 00 |
| B8 | 14±0.20 | 17±0.28 | 15±0.32 | 17±0.22 |
| Control | 00 | 00 | 00 | 00 |
| Standard | 23±0.21 | 25±0.26 | 16±0.22 | 20±0.46 |

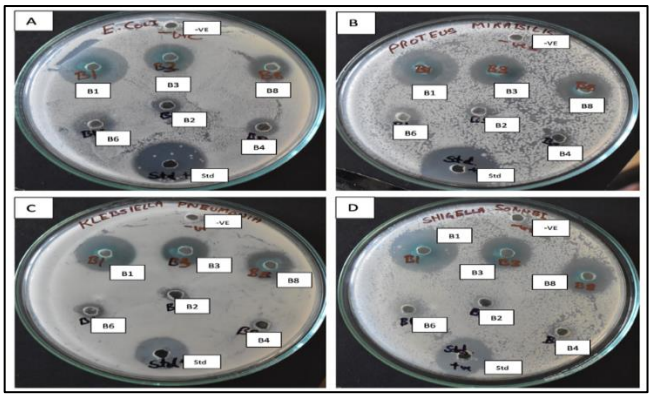


**Figure 5.** Antibacterial activity of Endophytic bacteria isolated from shoots against clinical pathogens A- *E.Coli*, B - *Proteus mirabilis*, C - *Klebsiella pneumonia* and D- *Shigella sonnei*

*B1* isolate shows high antibacterial activity against *Shigella sonnei* 25±0.48 mm followed by *Escherichia coli* 22±0.51mm, *Proteus mirabilis* 21±0.57mm. and *Klebsiella pneumonia* 19±0.44 mm. B3 and B8 isolates also show a fine antagonistic activity against these pathogens followed by B6, B4 and B2. Isolate B5 and B7 did not show any zone of inhibition.

***Field Emission Scanning Electron Microscope imaging:***

FESEM images are obtained for the B1, B3 and B8 strains which are visualized at 1-2 μm confirmed they are bacillus rod shape bacteria.

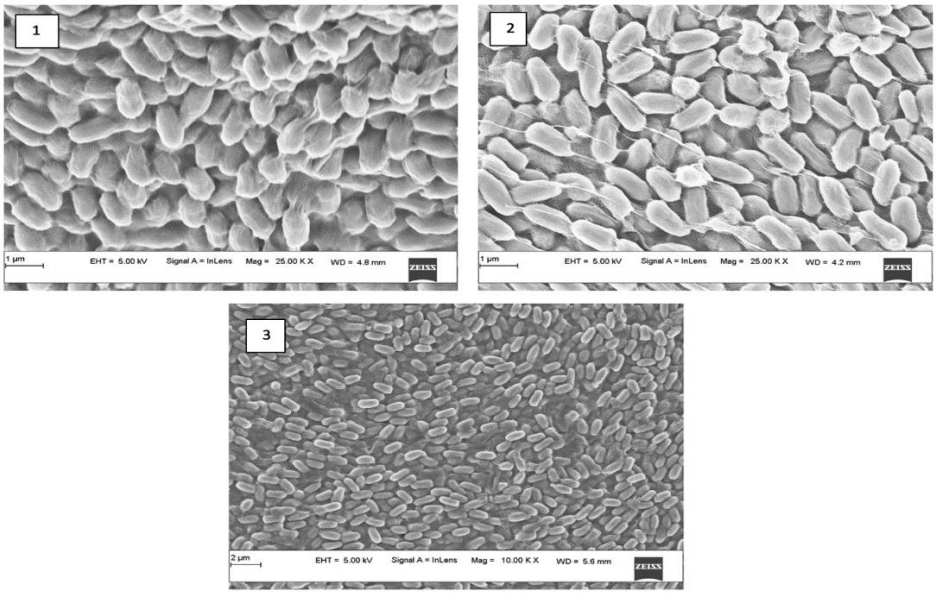


**Figure 6.** Scanning Electron Microscope Images of B1- *Bacillus licheniformis*, B3- *Bacillus velezensis*, B8- *Bacillus subtilis*

***Molecular identification* :**

Efficient three isolates B1, B3 and B8 pure culture are grown in LB broth. Genomic DNA was extracted by CTAB method and quantified by the electrophoresis method (Figure 7)

DNA was successfully amplified by PCR using universal primers 27F 5'-AGAGTTTGATCC TGG CTCAG-3' and 1492R 5'-GGTTACCTTGTTAC GACTT-3' used to amplify approximately 1500 bp of 16S rDNA gene. The PCR findings were then purified and electrophoresed (figure 8). The 16S rDNA gene, a barcoding gene used to identify bacteria, was used for identification. In the (NCBI) National Centre for Biotechnology Information site, retrieved sequences are compared with an existing sequence using the Basic local alignment search tool (BLAST) programme. The bacterial isolates' 16S rDNA sequences were submitted to NCBI with the accession numbers listed in (Table 4). Maximum likelihood character-based parameter models are used to build phylogenetic trees. To investigate the phylogenetic tree, the bootstrap method with 1500 replications was applied.

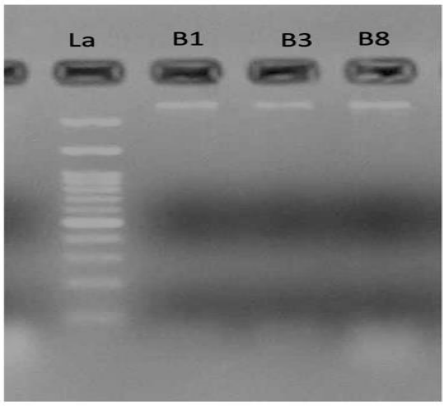


**Figure 7.** 1% Agarose gel data showing Genomic DNA: Lane 1-La: DNA Ladder, 2-B1, 3-B3, 4- B8

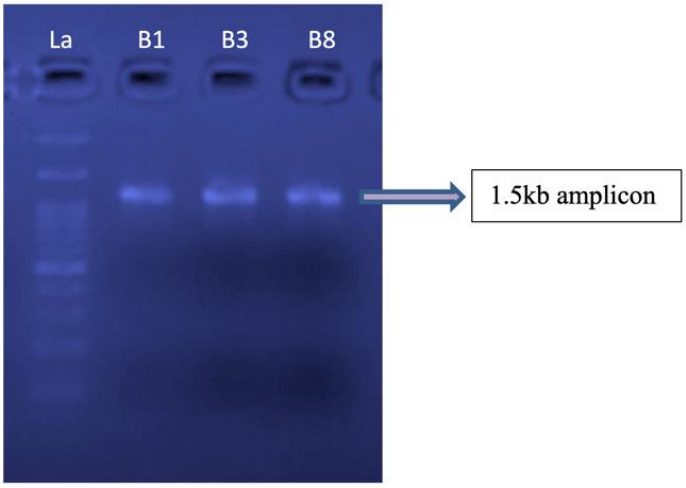


**Figure 8.** 16SrDNA amplification showing 1.5kb amplicon lane 1-La: DNA Ladder, 2-B1, 3-B3, 4- B8

**Table 4-** Following are the endophytic bacteria isolated from Bamboo shoots

| SL NO | CULTURE CODE | SOURCE | ENDOPHYTIC BACTERIA | ACCESSION NUMBER |
|---|---|---|---|---|
| 1 | B1 | *Dendrocalamus asper* | *Bacillus licheniformis* | ON254199 |
| 2 | B3 | *Dendrocalamus strictus* | *Bacillus velezensis* | ON259692 |
| 3 | B8 | *Dendrocalamus strictus* | *Bacillus subtilis* | ON340723 |

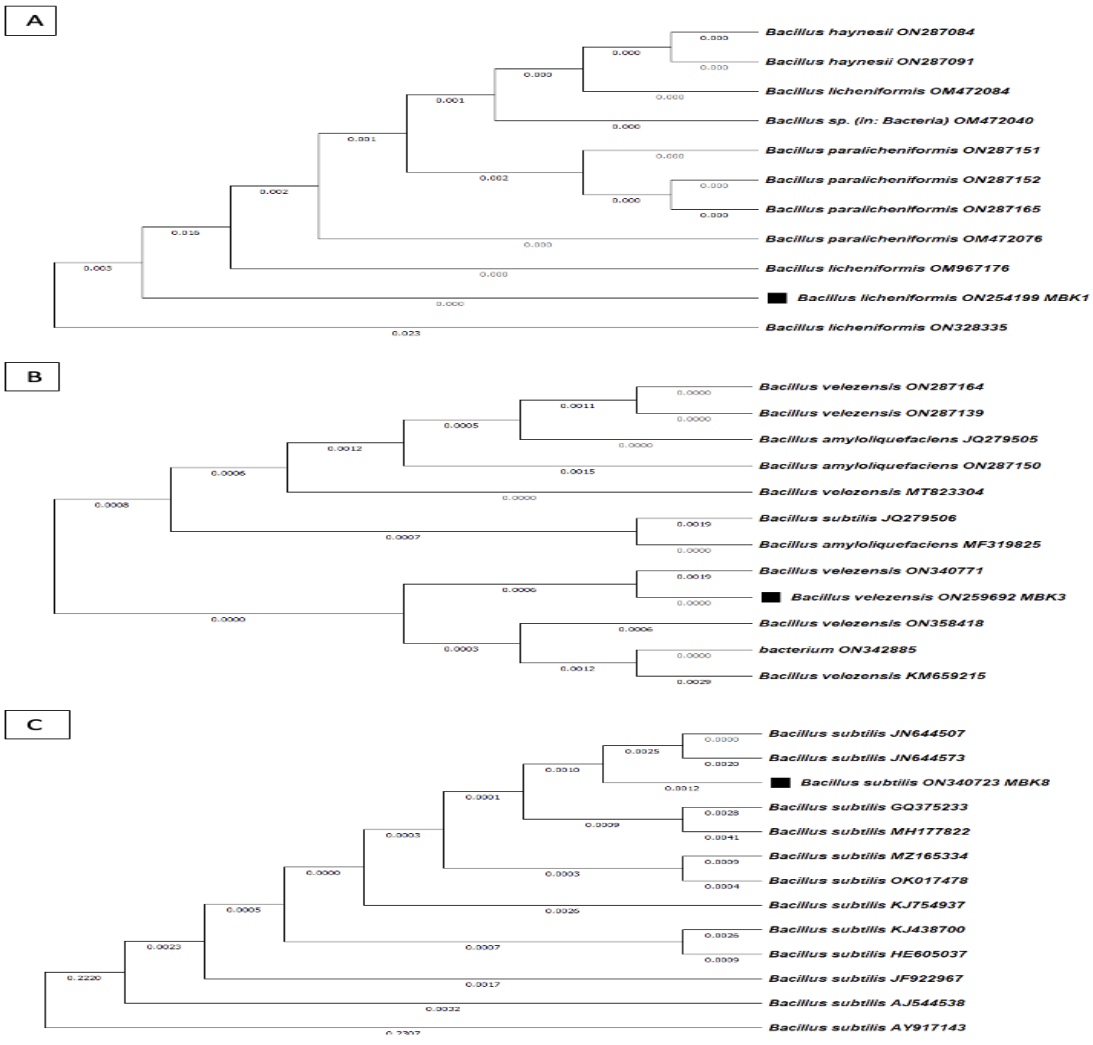


**Figure 9.** Phylogenetic tree constructed by boot strap method for obtained isolates A-B1 *Bacillus licheniformis***,** B-B3 *Bacillus velezensis*, C-B8 *Bacillus subtilis*

A strong tool for the quick identification of bacterial species, the 16S rDNA gene sequence data allow appropriate classification of organisms at the subspecies level. Which revealed B1 isolate are closely related to *Bacillus licheniformis*, B3 are closely related to *Bacillus velezensis* and B8 are closely related to *Bacillus subtilis.*

*Bacillus licheniformis* were previously reported as endophytes from *Vitis vinifera- Bacillus licheniformis* The biocontrolling characteristics of GL174 According to the findings of antagonism tests, the strain could both in vitro and in vivo diminish and impede the mycelium growth of a variety of plant pathogens. [21]. Even after sterilisation, *Bacillus licheniformis* CHM1 reduced the *in vitro* mycelial growth of *Rhizoctonia solani, Fusarium oxysporum, Colletotrichum gossypii, Dothiorella gregaria, Gibberella zeae and Botrytis cinereapers*[22].

*Bacillus velezensis* ZSY-1 strain have antifungal activity against *Alternaria solani* and *Botrytis cinerea*[23]. Bacillus velezensis DMW1, an isolate from potato tubers, showed robust biocontrol activity[24]. The most frequent host of the endophytic bacteria *Bacillus* is a plant. *Bacillus* functions in plants as a biocontrol agent and promotes plant growth[9].

*Bacillus velezensis* SQR9 strain was reported as a plant growth promoting rhizobacteria (PGPR) which was isolated from cucumber rhizosphere soil[25]. By generating auxin and gibberellin, *Bacillus* was also able to stimulate plant growth and adapt to dryness. They functions in plants as a biocontrol agent and promotes plant growth [26]. *Bacillus subtilis* NPROOT3 from *salicornia brachiata* as antimycobacterial agents Bacillibactin class siderophores[27]. The passion fruit leaf-isolated *Bacillus* subtilis Strain GUCC4 exhibits Plant Growth Promotion and Biocontrol of Leaf Blight Induced by *Nigrospora sphaerica* [28]. *Bacillus subtilis* is a endophytic bacteria which have a biocontrol potential against *F. oxysporum, F. proliferatum, F. culmorum,* and *F. verticillioides*[29]. Mentha spicata microplants contain Bacillus subtilis to encourage growth and pathogen tolerance[30]. To combat tomato Verticillium wilt, *Prunus cerasifera's- Bacillus* subtilis P10 strain is a biocontrol agent [31]. which had proven a great sources of bio control agents. Considering these literatures on the obtained bacterial species which have other benefits of these organism for the growth of host plant, for bio control in plant, defence mechanism on pathogens.

**Conclusion**

Our result revealed *Bacillus licheniformis, Bacillus velezensis* and *Bacillus subtilis* are efficient Endophytic bacteria isolated from two different bamboo shoots species *Dendrocalamus asper* and *Dendrocalamus strictus.* Which have high antibacterial activity against the clinical pathogens. Isolates are capable of producing bioactive components which is useful for production of active pharma ingredients. According to a research analysis, these species also significantly influence plant growth. So, it can be said that this isolate has a growth-promoting impact on bamboo shoots, and this work can be further continued for the benefit of the farmers and society.

**Conflict of interest:**

There is not any conflict of interest.

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