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Molecular identification and screening the antagonistic and cellulolytic activities of native strain *Bacillus subtilis* isolated from Myanmar marine sediment

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Abstract

In order to combat plant diseases and produce profitable agricultural crops, biocontrol agents derived from bacteria offer sustainable and eco-friendly options. Numerous fungal diseases have been successfully treated by some advantageous strains of *Bacillus subtilis*. The bacterial strain used in this study, designated S1-10, was isolated from a marine sediment sample. The antifungal qualities of the isolate were evaluated against eight out of ten plant-pathogenic fungi. The highest cellulolytic activity of the isolate was observed after four days of incubation. The association of the strain with *Bacillus subtilis* was confirmed by phylogenetic analysis based on the sequences of the *gyrB* and 16S rRNA regions. The NCBI has received strain S1-10, which has been assigned accession codes OR708648 for 16S and PV007904 for *gyrB*.

Keywords: *Bacillus subtilis*, marine sediment, antifungal effect, cellulolytic activity, phylogenetic analysis

Introduction

Marine sediments represent one of the largest habitats on our planet. Their distinctive ecological characteristics, such as elevated salinity, high pressure, and low oxygen levels, can trigger the activation of previously dormant genes in marine microbes. This process leads to the emergence of unique microbes, enzymes, bioactive compounds, and specialized metabolic pathways that have adapted to these specific environmental conditions. The microorganisms sourced from marine sediments and their bioactive metabolites hold significant importance and present promising opportunities for commercial development in sectors such as food, pharmaceuticals, chemicals, agriculture, environmental protection, and human nutrition and health [1]. The unique living conditions found in marine environments compared to terrestrial ones make marine microorganisms highly promising candidates for producing innovative natural compounds [2]. Marine *Bacillus* isolates represent a diverse collection of bacteria, exhibiting both phylogenetic and phenotypic variations within the broader microbial world. These bacteria are widely distributed throughout the marine environment and possess remarkable resilience, thriving in challenging conditions like high temperatures, pressure, salinity, and pH levels.

Typically, *Bacillus* strains necessitate ample nutrients and space to achieve their maximum growth rates, a competitive advantage they utilize against other organisms. The vastness of the ocean, with its constant dilution, compels marine organisms to develop powerful bioactive compounds for survival, either to outcompete rivals or to defend against smaller predators [3]. Numerous bioactive compounds, such as cyclic peptides, glycopeptides, lipopeptides, and bacteriocins, are produced by *Bacillus* species, which are well known for their wide spectrum of antibacterial properties [4]. Chemical pesticides are currently the main tool used by agricultural producers to control or prevent crop diseases. The accumulation of these pesticide residues in food, soil, and aquatic environments poses significant threats to human health, the environment, and ecosystems [5]. As a result, there is a growing demand from consumers and environmental advocates to substitute chemical pesticides with natural, eco-friendly microorganisms that have innovative modes of action for the sustainable cultivation of crops [6]. In nature, biomass rich in lignocellulose and agricultural residues is widely available as a source of carbon. Utilizing microorganisms or their enzymes for the biological

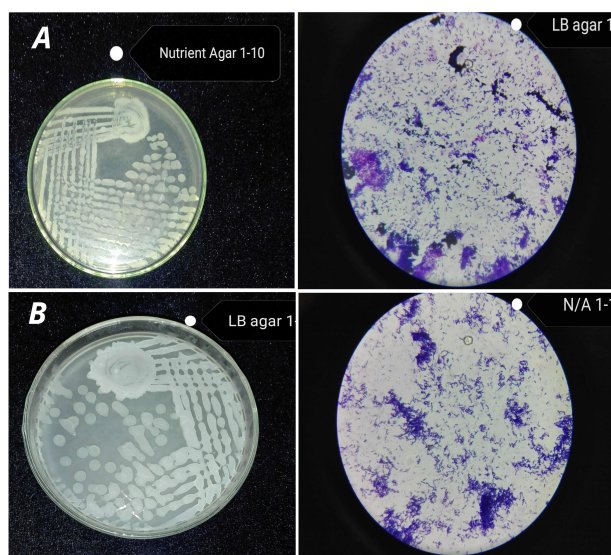


Figure 1. Colony and microscopic morphology (100×) of (S1–10) isolate on LB (Luria-Bertani) and N/A (Nutrient Agar)

pretreatment of lignocellulosic biomass provides an environmentally friendly method for processing biomass. The use of cellulases and hemicellulases to hydrolyze lignocellulosic biomass results in the formation of monomeric sugars that can be easily fermented by microorganisms [7]. Numerous studies have shown that both bacterial and fungal species can produce cellulolytic enzymes. Bacterial species, particularly those from the *Bacillus* genus, have been predominantly utilized in industrial applications due to their ability to secrete a variety of hydrolytic enzymes [8]. The cellulolytic enzymes derived from *B. subtilis* have been extensively researched because of their significant production capacity and usefulness in biomass valorization [9].

Bacillus subtilis is a catalase-positive, rod-shaped bacterium that is Gram-positive. Like other members of the *Bacillus* genus, it can produce an endospore that enables it to endure extreme conditions such as high temperatures and dryness. *B. subtilis* is regarded as the most extensively studied model organism for investigating bacterial chromosome replication and cell differentiation among Gram-positive bacteria [10]. Additionally, it is frequently utilized as an industrial cell factory for synthesizing vitamins, inositol, acetoin, hyaluronan, and various other chemicals. Research in agriculture has shown that incorporating a suitable amount of *B. subtilis* can significantly enhance the humus and carbon levels in compost, thereby improving soil quality and supporting crop growth [11].

Myanmar's economy is largely based on agriculture, making the agricultural sector crucial for business. Thus, enhancing agricultural output, ensuring food safety, and improving soil quality are fundamental strategies. The agricultural inputs currently in use are primarily chemical in nature. Poor management practices on farms and the incorrect application of agrochemicals have led

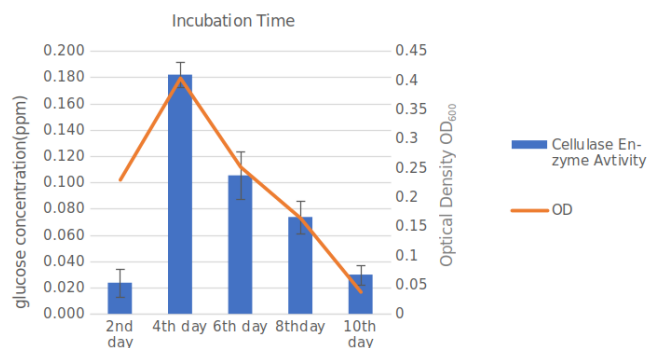


Figure 2. Detection of cellulase and optimum growth condition

to soil degradation and environmental harm. This study aims to isolate and identify *Bacillus subtilis* from marine sediment and to explore the effective antagonistic and cellulolytic properties of this bacterial isolate for use as a biofungicide and in composting.

Materials and Methods

Sample collection and strain isolation

Strain S1-10 was isolated from a sample of wet sediment taken from the sea near Panga Village, located in Thanbyuzayat Township, Mawlamyine District, Mon State, Myanmar (15° 54' 18" N, 97° 43' 31" E). Ten milliliters of a 0.85% saline solution were used to suspend one gram of the marine sediment, which was then shaken for half an hour at 30°C. After settling for two hours, the sample solution was serially diluted (10^{-1} to 10^{-4}). Using these dilutions, 100 μ L was plated onto a 3% NaCl-enriched nutritional agar medium and cultured for three days at 30°C [12]. Repeated streaking on the same medium allowed for the selection and purification of the colonies that emerged. For future research, the pure culture of strain S1-10 was stored at -80°C in a 20% (v/v) glycerol solution after being regularly maintained and incubated at 30°C.

Screening of antagonistic activity and cellulolytic activity

Plant pathogenic fungi were generously supplied by Professor Dr. Hee-Young Jung from the Department of Plant Medicine at Kyungpook National University in Daegu, South Korea. The dual culture technique was employed to assess the efficacy of a *Bacillus* strain against these fungi, utilizing Petri dishes measuring (90 × 15 mm) filled with potato dextrose agar (PDA). In this method, a 6 mm diameter circle containing a suspension of conidia and mycelium, serving as the pathogen inoculum, was placed at the center of the dish. The antagonist was positioned 3 cm away from the center. Each experiment was conducted in triplicate, with control fungal colonies not exposed to the antagonists. The inoculated Petri dishes were incubated at 30°C for 14 days. After this incubation period, the inhibition zone between the phytopathogen and antagonist colonies was observed on the Petri dish [13].

The assessment of cellulolytic activity was conducted using LB medium supplemented with 1% cellulose powder. Colonies of the isolate were inoculated onto this medium and incubated at room temperature for 48 hours. Following incubation, the plate was treated with 0.1% Congo red for a duration of 15 to 20 minutes, then rinsed with 1 M NaCl for an additional 15 to 20 minutes. The presence of a clear zone surrounding the colony was noted, indicating cellulase activity [14]. This transparent area indicates the cellulolytic activity of the isolate. According to Miller GL's 1959 description, the 3,5-dinitrosalicylic acid (DNS) method was used for the quantitative analysis of cellulase. By adding 3 mL of DNS reagent—which is made up of 1 g dinitrosalicylic acid, 16 g NaOH, 300 g potassium sodium tartrate, and distilled water to a final volume of 1 L—to the 1 mL reaction mixture, the enzymatic reaction was stopped. The optical density of this mixture was then measured at 540 nm after it had been boiled for five minutes in capped glass tubes and cooled in cold water. D-glucose-based calibration curves were used to measure the cellulase activity.

Analysis of Morphology and Physiology

A variety of agar media, such as nutrient agar (NA; Difco), marine agar 2216 (MA; Difco), tryptic soy agar (TSA; Difco), and Luria Bertani agar (LB; Difco), were used to assess the growth of the isolated bacterial strain S1-10. On LB agar, the temperature range for growth was determined at 4, 10, 15, 20, 25, 30, 37, 42, and 45 °C. Furthermore, after a 7-day incubation period, the pH range for growth was evaluated on LB agar at pH levels ranging from 4.0 to 10.0, in increments of 1.0 pH unit. To adjust the pH of the medium, 1 M NaOH and HCl were used. Additionally, growth was observed on nutrient agar after 7 days in the presence of NaCl concentrations ranging from 1.0% to 10.0% (w/v), with 1.0% increments.

Phylogenetic Analysis

The HiGene Genomic DNA Prep Kit (Biofact, Daejeon, Korea) was used to extract the genomic DNA of strain S1-10 from 24-hour-old cultures grown on nutrient medium. The methodology developed by Weisburg *et al.* was followed in the polymerase chain reaction (PCR) procedure used in this study to amplify the 16S rRNA region. Primers 9F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1512R (5'-ACG GCT ACC TTG TTA CGA CTT-3') were used in this investigation [15].

For phylogenetic analysis and species identification within the *Bacillus* group, the DNA gyrase B subunit gene, *gyrB*, was selected for examination. DNA sequencing was conducted using degenerate primers for *gyrB*, specifically *gyrB*-F (5-GAA GTC ATC ATG ACC GTT CTG-3) and *gyrB*-R (5-AGC AGG GTA CGG ATG TGC GAG-3) [16]. The PCR product cleaning reagent ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) was used to purify the amplified PCR products.

The full sequence of the 16S rRNA gene was assembled using SeqMan software (DNASTAR, Madison, WI, USA). Manual adjustment of gaps and terminal ends of the alignment was

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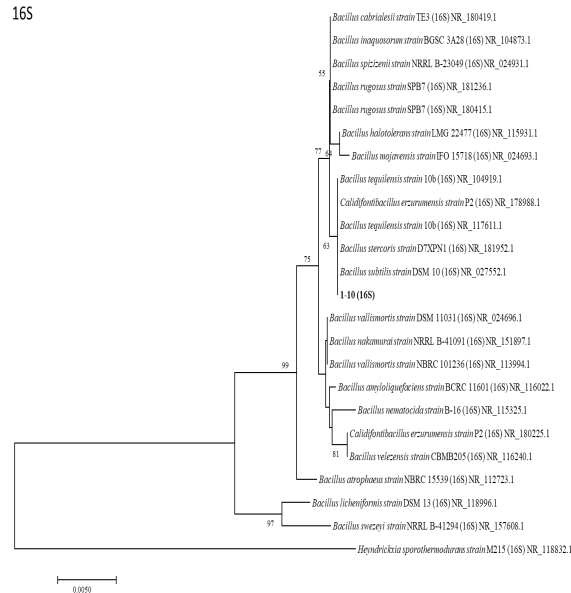


Figure 3. A neighbor-joining phylogenetic tree constructed from 16S rRNA gene sequences illustrates the evolutionary relationships of strain S1-10 and related members of the *Bacillaceae* family. Nodes with bootstrap support values exceeding 50% are marked. The strain that was isolated is highlighted in bold. *Heyndrickxia sporothermodurans* M215 served as the outgroup. The scale bar represents 0.0050 substitutions per nucleotide position.

performed with BioEdit version 5.0.6 to ensure accuracy and completeness of the gene sequences. Identification of the phylogenetically closest sequences and calculation of pairwise sequence similarity values were conducted using the EzBioCloud server and BLAST searches targeting the 16S rRNA gene.

Sequences of the 16S rRNA gene from related taxa were retrieved from GenBank, and multiple alignments were carried out using the CLUSTALW software [17]. A phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA 7 software [18]. Evolutionary distances were calculated using Kimura's two-parameter model, and the reliability of the tree topology was assessed through bootstrap analysis with 1,000 replicates.

Results and Discussions

Analysis of Morphology and Physiology

Colonies of the S1-10 strain grown on nutrient agar supplemented with 3% NaCl appeared flat, white, and were approximately 3–5 mm in diameter after three days of incubation at 30 °C (**Figure 1**). Significant growth was observed on nutrient agar, marine agar, TSA, and LB agar. The S1-10 strain demonstrated the ability to grow in NaCl concentrations ranging from 1% to 10%, within a pH range of 4 to 10, and at temperatures between 15 °C and 45 °C. Optimal growth conditions were determined to be 3% NaCl, pH 7, and 30 °C. In this research, a new strain of *Bacillus subtilis*, named S1-10, was extracted from

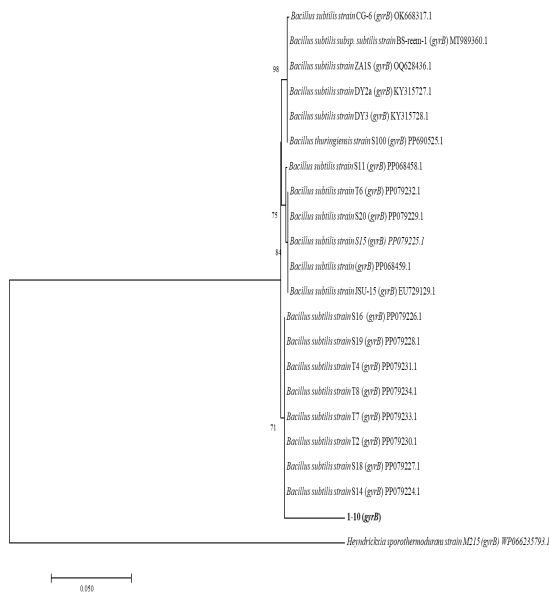


Figure 4. Neighbor-joining phylogenetic tree constructed from *gyrB* gene sequences, illustrating the phylogenetic placements of strain S1–10 alongside the *Bacillus subtilis* group. Bootstrap support values exceeding 70% are shown at the nodes. The isolate is indicated in bold. *Heynadriskxia sporothermodurans* M215 served as an outgroup. Bar, 0.050 replacements for each nucleotide position.

marine sediment in Myanmar and evaluated for its antagonistic characteristics against several phytopathogenic fungi. The S1-10 strain of *B. subtilis* showed notable antagonistic effects on *Sclerotinia sclerotium*, *Botrytis cinerea*, *Fusarium tricinctum*, *Rosellinia necatrix*, *Cytospora* sp., *Colletotrichum aenigma*, and *Colletotrichum fructicola*. It demonstrated minimal inhibition of the plant pathogenic fungus *Diaporthe eres* and showed no inhibitory effect on the development of *Sclerotium rolfsii* and *Botryosphaeria sinensis*. These findings are consistent with earlier research by [19], which reported growth suppression in various phytopathogenic fungi, with the exception of *Sclerotium rolfsii* and *Botryosphaeria sinensis*. Moreover, [20] noted that the effect of *Bacillus subtilis* BS-1 cell-free supernatant (CFS) on *Botryosphaeria dothidea* was due to the stimulation of oxidative damage in the mycelial cells (Figure S1).

The degradation of cellulose by the cellulase enzyme is vital, as many agricultural byproducts include cellulose, which is a key element of plant cell walls. This cellulose can be transformed into glucose, which acts as a key raw ingredient for alcohol manufacturing [21]. The fermentation process has been employed for a long time, as it increases nutritional value while minimizing anti-nutritional elements, toxins, and levels of contamination [22]. A successful method for waste management includes the enzymatic hydrolysis of lignocellulosic waste, where fermentation produces reducing sugars that can be utilized for creating desired

metabolites or biofuels [23]. In this research, the peak activity of cellulase enzyme production and the optimum growth conditions for the *Bacillus subtilis* isolate were observed after four days of incubation (Figure 2).

Phylogenetic Analysis

The 16S rRNA gene of strain S1-10 is 793 base pairs long. Its sequence shows a 100% match with several species, including *Bacillus subtilis*, *Bacillus halotolerans*, *Bacillus amyloliquefaciens*, *Bacillus safensis*, *Bacillus velezensis*, *Bacillus mojavensis*, and *Bacillus stercoris*. A phylogenetic tree built using the Neighbor-Joining method indicates that strain S1-10 is grouped on the same branch as *Bacillus subtilis*, *Bacillus stercoris*, *Bacillus tequilensis*, and *Colidifontibacillus erzurumensis* (Figure 3).

Therefore, the *gyrB* gene, which encodes the B subunit of DNA gyrase, was selected as an alternative marker for phylogenetic analysis and species identification within the *Bacillus* group. The NCBI BLAST results showed that the isolate shares 100% identity with *Bacillus subtilis*. A phylogenetic tree constructed using the Neighbor-Joining method again places strain S1-10 alongside *Bacillus subtilis* strains. Similar clustering patterns were also observed in trees generated using the Maximum Likelihood and Maximum Parsimony methods (Figure 4).

Conclusions

The indigenous *Bacillus* strain found in the marine sediment of Myanmar was classified as *Bacillus subtilis* through gene analysis of 16S rRNA and *gyrB*. The isolated strain exhibited antagonistic effects on the mycelial growth of the plant pathogenic fungi *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Fusarium tricinctum*, *Rosellinia necatrix*, *Cytospora* sp., *Colletotrichum aenigma*, and *Colletotrichum fructicola*. It demonstrated weak inhibition against the plant pathogenic fungus *Diaporthe eres* and showed no inhibition on the growth of *Sclerotium rolfsii* and *Botryosphaeria sinensis*. The *Bacillus subtilis* S1-10 isolate may be used as an effective strain for composting agricultural waste due to its cellulolytic and antagonistic properties.

Supplementary

Figure S1: Screening the antifungal activity of isolate (S1-10) by showing fungal growth inhibition compared with the control and treatment plates. Red box indicates the isolate (S1-10).

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