# Screening and phylogenetic analysis of cellulolytic *Bacillus* spp. isolated from fermented brown algae (*Sargassum* sp.)

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**Abstract.** *Sargassum* sp., a type of brown algae, is rich in bioactive compounds that offer significant health benefits. However, the presence of cellulose-rich cell walls limits their availability, highlighting the need for efficient cellulolytic bacteria. Fermentation of brown algae, including *Sargassum* sp., offers a sustainable strategy to enhance the extraction of these compounds by breaking down the cellulose barrier. The present study aimed to isolate and characterize cellulase-producing bacteria from fermented *Sargassum* sp., evaluate their enzyme activity, and identify potential strains suitable for fermentation starter applications. Fresh *Sargassum* was fermented under anaerobic conditions for 12 days, both with and without pH adjustment. Bacterial isolates were obtained using CMC and MRS agar, followed by screening for cellulase activity through clear zone formation on Congo red-stained plates and quantitative assays using the DNS method. Of the 22 isolates obtained, 12 exhibited cellulase activity, with cellulase activity values ranging from 0.063 to 0.32 U/mL. Among the isolates, SF4 (*Bacillus subtilis*) exhibited the highest cellulase activity (0.32 U/mL). However, its β-hemolytic activity raises potential safety concerns, limiting its applicability in food fermentation. In contrast, isolate F6 (*Bacillus amyloliquefaciens*) demonstrated moderate cellulase activity and was non-hemolytic, making it a safer and more promising candidate for use as a microbial starter culture. These results underscore the potential of *Bacillus*-derived cellulolytic enzymes to enhance the functional properties of fermented *Sargassum*, and support the application of *B. amyloliquefaciens* F6 in the development of safe, bioactive-rich fermented food products.

## 1 Introduction

Brown seaweeds such as *Sargassum* are commonly found in tropical and subtropical coastal regions, where they grow abundantly in nutrient-rich waters. In Indonesia, which has extensive coastlines and rich marine biodiversity, *Sargassum* species are widely distributed across various regions, including along the coastal waters of Madura Island. In particular, Talango Island (Sumenep Regency) is known habitats for species such as *S. cristaefolium*, *S. cinereum*, and *S. echinocarpum, S. filipendula, S. polycystum* and *S. aquifolium* [1]. This

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natural abundance supports the growing interest in utilizing marine macroalgae, particularly brown algae (*Sargassum* spp.), as a source of functional food ingredients. *Sargassum* is rich in dietary fibers, polyphenols, and polysaccharides such as alginate, fucoidan, and laminarin, all of which are associated with antioxidant, anti-inflammatory, and blood sugar-lowering effects [2, 3]. These bioactive compounds make *Sargassum* a valuable candidate for the development of health-promoting food products and nutraceuticals.

Despite its potential, the rigid cellulose-rich cell wall structure of *Sargassum* hinders the release of bioactive compounds during conventional extraction processes. The complexity of this barrier arises from the presence of cellulose microfibrils tightly embedded within a matrix of alginate and fucoidan, which provides mechanical strength and resistance to enzymatic breakdown. This recalcitrant architecture limits the accessibility of intracellular compounds and necessitates effective pretreatment or bioconversion strategies. Conventional techniques like acid hydrolysis, solvent-based extraction, and enzymatic processes face challenges related to environmental issues, high operational costs, and deterioration of bioactive compounds that are sensitive to temperature fluctuations [4, 5]. These approaches may also generate chemical residues and are not suitable for clean-label or natural product applications in food and nutraceutical industries [6]. Moreover, certain solvents used in extraction may negatively affect the structural integrity of delicate compounds such as phlorotannins and fucoidans, leading to loss of functional activity [7, 8]. Therefore, alternative green processing technologies, particularly fermentation-based bioconversion, are being explored to increase yield, preserve compound integrity, and reduce environmental impact [9, 10].

Fermentation using *Bacillus* spp. is considered a more sustainable and cost-effective alternative for the bioconversion of brown algae biomass. *Bacillus* spp. are widely recognized as efficient producers of extracellular cellulolytic enzymes, making them important agents in the bioconversion of lignocellulosic biomass, including brown macroalgae such as *Sargassum* sp. These enzymes facilitate the breakdown of complex polysaccharides, enabling the release of valuable bioactive compounds from the algal matrix. Unlike acid or solvent-based extraction methods, *Bacillus*-mediated fermentation can enhance cellulolytic activity while maintaining the structural integrity of sensitive bioactive compounds. Previous studies have shown that *Bacillus subtilis* strain MU S1 was capable of increasing cellulase production by 3.2-fold (from 179.06 U/mL to 573 U/mL) under optimized fermentation conditions involving CMC, yeast extract, and NaCl at specific concentrations [11]. Additionally, solid-state fermentation using *Bacillus subtilis* subsp. *subtilis* A-53 produced CMCase activity as high as 196.8 U/mL in a 100-L bioreactor under optimized conditions [12]. These quantitative advantages make *Bacillus* spp. an attractive choice for eco-friendly, large-scale applications in the development of functional food products from *Sargassum*.

Fermentation offers an environmentally sustainable strategy to improve the health-promoting properties of plant-derived materials. In the case of *Sargassum sp.*, fermentation by cellulolytic bacteria facilitates hydrolysis of cellulose, enabling efficient extraction of bioactives without the use of toxic solvents [10]. The process is driven by microbial metabolism and enzymatic secretion that modifies the polysaccharide structure, often resulting in increased bioaccessibility and bioactivity of compounds [13]. Furthermore, fermentation can enrich the functional properties of algal biomass by increasing phenolic content, reducing antinutritional factors such as phytic acid, and generating novel metabolites including organic acids, peptides, and exopolysaccharides [14]. These transformations not only improve health-promoting qualities but also extend shelf-life and enhance sensory attributes, making fermented seaweed more acceptable for functional food development [15].

Cellulolytic bacteria, particularly from the genera *Bacillus*, *Paenibacillus*, and *Clostridium*, are known to produce extracellular cellulases capable of hydrolyzing cellulose into glucose [16]. These enzymes— β-glucosidase, exoglucanase, and endoglucanase —

degrade crystalline and amorphous regions of cellulose synergistically. In marine environments, cellulolytic *Bacillus* strains have adapted to salinity and produce thermostable enzymes, making them suitable for algal biomass bioprocessing [17, 18]. In addition, many *Bacillus* spp. are spore-forming and resistant to environmental stress, which supports their viability during fermentation and industrial application [19]. The cellulase production by these bacteria is also inducible by complex substrates such as carboxymethyl cellulose (CMC), which stimulates gene expression for cellulase synthesis under fermentation conditions [20]. The diversity of cellulase-producing strains offers the possibility to screen and select specific isolates with optimized activity profiles for targeted applications such as prebiotic development, bioethanol production, and nutraceutical formulation. To support such targeted selection, 16S rDNA-based phylogenetic analysis provides reliable identification at the species level and allows the assessment of genetic relatedness among isolates [21]. This information is crucial for correlating phylogenetic traits with functional enzyme profiles and ensuring biosafety in food-related applications.

Although lactic acid bacteria (LAB) such as *Lactiplantiobacillus plantarum* are not well known for high cellulase activity, they are often involved in fermentation processes and may contribute to the breakdown of complex carbohydrates [22]. LAB are widely regarded as safe (GRAS), and their use in conjunction with cellulolytic strains could create synergistic effects during the fermentation of algal biomass. This current study aimed to isolate and characterize cellulolytic bacteria from fermented *Sargassum* spp., evaluate their cellulase enzyme activity, assess their hemolytic potential to ensure safety, and identify potent strains using 16S rDNA sequencing. The integration of microbial fermentation and enzymatic bioconversion of *Sargassum* is expected to support the development of high-value functional food ingredients from underutilized marine biomass.

## 2 Materials and methods

### 2.1 Fermentation of brown algae (*Sargassum* sp.)

Fresh samples of *Sargassum* sp. derived from Talango Island, Sumenep Regency, Madura Island were meticulously rinsed using tap water and subsequently with sterile aquadest to eliminate attached debris and epiphytes. The cleaned algae were blended into a homogenate, and 10 g of this material was mixed with 0.1 g of yeast extract and 0.5 g of glucose in 100 mL of sterile distilled water. The mixture was thoroughly homogenized using a vortex mixer and subjected to anaerobic fermentation by tightly sealing the Erlenmeyer flask and incubating it under static conditions (without agitation) at room temperature for 12 days. Two fermentation conditions were established: (1) medium with no pH adjustment, and (2) medium adjusted to pH 5 to favor lactic acid bacteria growth [23].

### 2.2 Isolation of bacteria from fresh and fermented brown algae (*Sargassum* sp.)

Bacterial isolates were obtained from both fresh and fermented *Sargassum* sp. samples using spread plate techniques on two types of media: MRS agar with 1% $CaCO_3$ for lactic acid bacteria (LAB) and 1% CMC agar for cellulolytic bacteria. Ten-fold serial dilutions were prepared from 25 g of fresh *Sargassum* blended in 225 mL of 0.85% NaCl, and from 25 mL of fermented suspension in 225 mL of 0.85% NaCl. A volume of 0.1 mL from suitable dilution levels was spread onto selective media and incubated at 37°C for 48 hours. Colonies displaying unique morphological features including size, shape, edge, elevation, texture, consistency, optical properties, and pigmentation, were subsequently purified and characterized through Gram staining and catalase assays [24].

## 2.3 Semi-quantitative assay of cellulase activity

Purified bacterial isolates were evaluated semi-quantitatively for cellulase activity on carboxymethyl cellulose (CMC) agar using Congo red staining. Each isolate was cultured in 1% CMC broth (comprising 10 g CMC, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.75 g $KNO_3$, 0.5 g $K_2HPO_4$, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 2 g yeast extract, and 1 g glucose per liter of distilled water) and incubated at 37°C for 48 hours. Bacterial suspensions were standardized to an $OD_{620}$ of 0.7 and 25 µL of each suspension was spotted onto sterile paper disks placed on 1% CMC agar plates. After incubation at 37°C for 48 hours, the plates were flooded with 1% Congo red solution and allowed to stain for 15 minutes at room temperature. The excess dye was then decanted, and the plates were gently rinsed with 1 M NaCl for another 15 minutes to destain the background and enhance the visibility of hydrolysis zones. Clear halos formed around the bacterial growth indicate areas of cellulose degradation, due to enzymatic hydrolysis of β-1,4-glycosidic bonds [24]. The cellulolytic index (CI) was calculated using Formula 1 [25].

$$Cellulolytic\ index\ (CI)\text{:}\frac{Clear\ Zone\ Diameter\ - Colony\ Diameter}{Colony\ Diameter} \quad (1)$$

The results were classified into categories based on the ratio of the clear zone to colony diameter, as adapted from Choi et al. [26]:

- Negative (CI = 0): No or minimal enzyme activity
- Low (CI ≤ 1): Weak extracellular cellulase activity
- Moderate (1 < CI < 2.0): Moderate extracellular cellulase activity
- High (CI ≥ 2.0): Strong extracellular cellulase activity

## 2.4 Quantitative determination of cellulase activity

The activity of cellulase enzymes in selected isolates was quantified through the dinitrosalicylic acid (DNS) assay following the protocol outlined by Grata [27], which quantitatively measures the amount of reducing sugar released during cellulose hydrolysis. Each selected bacterial isolate was grown in 1% CMC broth medium at 37°C for 48 hours under shaking conditions. After incubation, the cultures ($OD_{620}$ = 0.7) were centrifuged at 10,000 rpm for 5 minutes at 4°C to obtain the cell-free supernatant, which served as the crude enzyme extract. The enzymatic reaction was carried out by mixing 0.5 mL of the crude enzyme extract with 0.5 mL of 1% CMC substrate dissolved in 0.05 M citrate buffer at pH 4.8. The mixture was incubated at 50°C for 30 minutes to allow the reaction to proceed. The reaction was terminated by the addition of 1.5 mL of DNS reagent, followed by boiling (100°C) the tubes in a water bath for 5 minutes to develop color. Once cooled to room temperature, 10 mL of sterile distilled water was added, and the absorbance of the reaction mixture was subsequently measured at 540 nm with a spectrophotometer. The amount of reducing sugar (glucose) released was determined using a glucose standard curve. A glucose standard curve was established by preparing a series of glucose solutions ranging from 0 to 10,000 ppm. For each concentration, 0.5 mL of the glucose solution was mixed with 1.5 mL of DNS reagent and 0.5 mL of citrate buffer in a sealed reaction tube. The mixture was homogenized and heated in a boiling water bath at 100°C for 5 minutes. After heating, each tube was diluted to a final volume of 10 mL with distilled water. The absorbance of the resulting solution was then measured at 540 nm using a spectrophotometer to construct the standard curve. One unit (U) of cellulase activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under assay conditions. The enzyme activity in U/mL was calculated using the formula 2.

$$Enzymatic\ Activity\ (EA) = \frac{G}{MW\ glucose\ .\ t} \ x\ \frac{V}{E} \qquad (2)$$

Where:
AE : Enzyme activity (U/mL)
G : Glucose concentration (ppm)
MW : Molecular weight of glucose (180 g/mol)
t : Reaction time (min)
V : Total volume of reaction mixture (mL)
E : Volume of enzyme extract used (mL)

## 2.5 Hemolysis test of cellulase-producing bacterial isolates

To assess the safety of cellulase-producing bacterial isolates, hemolysis tests were conducted using blood agar medium supplemented with 5% defibrinated sheep blood. Each bacterial isolate was streaked on the blood agar plates using a sterile inoculating loop, then incubated at 37°C for 24–48 hours under aerobic conditions. Subsequently, zones of hemolysis surrounding the bacterial growth were observed. The type of hemolysis was categorized as follows: α-hemolysis (partial lysis of red blood cells, indicated by a green or brownish discoloration), β-hemolysis (complete lysis of red blood cells, shown by a clear zone around the colony), and γ-hemolysis (no hemolysis, indicated by no change in the surrounding medium). These hemolytic patterns were used as indicators of pathogenic potential, with β-hemolysis associated with higher risk and γ-hemolysis considered non-pathogenic [28].

## 2.6 Molecular identification of selected isolates

The identification of the most promising cellulolytic bacterial isolates was conducted through molecular analysis targeting the 16S rDNA gene following Devi and Jatmiko [29] with a minor modification. Genomic DNA was extracted using the Zymo-Spin™ extraction kit following the manufacturer's instructions. Amplification of the 16S rDNA region was performed using primers 27F (5'-AGAGTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') in a total reaction volume of 50 μL. The PCR mixture consisted of My Taq® HS Red Mix 2×, 10 pmol each of forward and reverse primers, the DNA template (21.66 ng/μL for isolate SF4 and 37.93 ng/μL for isolate F6), and sterile distilled water (ddH$_2$O).

The DNA amplification using PCR was following this program: an initial denaturation of 94°C was performed for 5 minutes, followed by 35 cycles involving denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 90 seconds, followed by 5 minutes of extension at 72°C. The PCR products were validated through electrophoresis on a 1% agarose gel prepared in TBE buffer. The agarose gel was cast into a mold with combs inserted and allowed to solidify at room temperature for 30 minutes. DNA samples were mixed with Gel Red at a ratio of 1 μL dye per 5 μL of sample prior to loading. Electrophoresis was carried out at 60 V for 60 minutes to separate the PCR products. DNA bands were visualized under UV illumination, and successful amplification was indicated by clear bands corresponding to the expected amplicon size.

The amplified DNA products were purified and sent to First BASE Laboratories (Malaysia) for sequencing using the same primer sets. The resulting sequence data were edited and assembled with BioEdit software. The nucleotide sequences were aligned and compared using BLASTN to determine sequence similarity with known bacterial taxa. Phylogenetic trees were constructed using the Neighbor-Joining method and Tamura-Nei model with 1000 bootstrap replications in MEGA11 software to confirm the identity and evolutionary relationship of the isolates.

### 2.7 Data analysis

Prior to statistical analysis, the data were assessed for normality using the Kolmogorov–Smirnov test and for homogeneity of variances using Levene's test. Only data that met these assumptions were subjected to one-way analysis of variance (ANOVA) to evaluate differences in the cellulolytic index and cellulase activity among bacterial isolates. Post hoc comparisons were performed using Tukey's Honestly Significant Difference (HSD) test to identify statistically significant pairwise differences ($\alpha = 0.05$). All statistical analyses were conducted using IBM SPSS Statistics software version 27.

## 3 Results

### 3.1 Bacterial density and isolates from brown algae

Fresh and fermented brown algae were used as sources for the isolation of bacteria potentially capable of producing cellulase enzymes, using two types of media: CMC agar and MRS agar. The bacterial density in fresh brown algae was $6.8 \times 10^6$ CFU/mL on CMC agar, and no isolates were recovered on MRS agar. In fermented brown algae, bacterial densities on CMC agar and MRS agar were $2.5 \times 10^7$ CFU/mL and $6.5 \times 10^4$ CFU/mL, respectively. Fermentation of the brown algae caused an increase in pH from 8 to 11 in medium without pH adjustment, while in the pH-adjusted medium, the pH increased from 5 to 6. A total of 22 bacterial isolates were obtained, with four isolates recovered from fresh brown algae on CMC agar and no presumptive lactic acid bacteria (LAB) isolates obtained on MRS agar (Table 1). From the fermented brown algae, 18 isolates were obtained, consisting of 14 isolates on CMC agar and four presumptive LAB isolates from MRS agar (Table 2). These presumptive LAB isolates exhibited colony morphology that formed clear zones on MRS agar supplemented with 1% $CaCO_3$.

**Table 1.** Bacteria isolates obtained from fresh brown algae (*Sargassum* sp.).

| Media | Cell Density (CFU/mL) | Isolates |
|---|---|---|
| CMC agar | $6.8 \times 10^6$ | 4 isolates (SS1, SS2, SS3, SS4) |
| MRS agar | 0 | - |

Note: "–" indicates no growth

**Table 2.** Bacteria isolates obtained from fermented brown algae (*Sargassum* sp.).

| Media | Cell Density (CFU/mL) | Initial pH | Final pH | Isolates |
|---|---|---|---|---|
| CMC agar | $2.5\times10^7$ | 8 | 11 | 14 isolates (F1- F11, F13, F17, F19) |
| MRS agar | $6.5 \times 10^4$ | 5 | 6 | 4 isolates (SF1, SF2, SF3, SF4) |

Bacterial characterization was conducted via Gram staining and catalase tests to classify the isolates as LAB or non-LAB. All isolates were Gram-positive bacteria, with cell morphology varying between cocci (7 isolates) and bacilli (15 isolates). Catalase testing showed that 20 isolates were catalase-positive, while only two isolates (F7 and SF1) were catalase-negative. Among these, isolate SF1 met the criteria for LAB, as it was isolated from MRS agar, formed a clear zone, and had a rod-shaped cell morphology.

## 3.2 Cellulase activity assay on solid medium

Screening of cellulolytic bacteria on CMC agar medium was carried out for all bacterial isolates obtained from both fresh and fermented brown algae by observing the appearance of clear zones surrounding the colonies (Figure 1). The clear zones were clearly visible after adding Congo red solution to the colonies growing on CMC agar. The screening results showed that out of 22 bacterial isolates, 12 demonstrated the ability to secrete cellulase enzymes (Figure 2). These twelve isolates had cellulolytic indices ranging from 0.59 to 2.59 mm. Ten isolates were confirmed to be incapable of producing cellulase enzymes, one of which was the LAB isolate (SF1). Isolate F1 exhibited the highest ability to hydrolyze cellulose on CMC agar medium with a cellulolytic index of 2.59 mm.

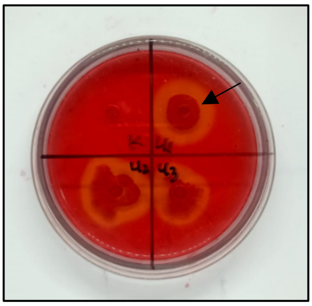

**Fig. 1**. Detection of cellulase activity on CMC agar using Congo red staining. Black arrow: Clear zone around the colony.

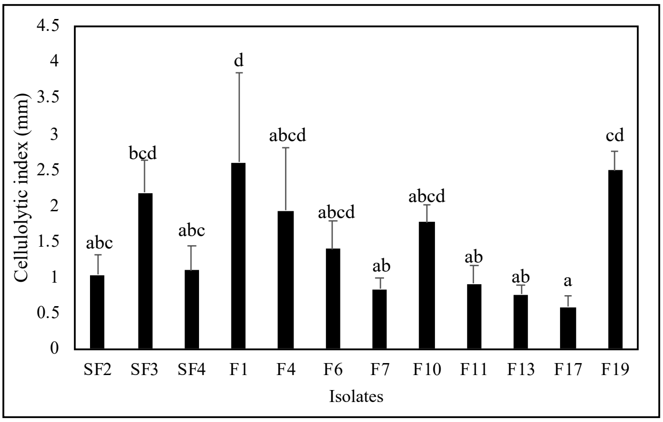

**Fig. 2.** Cellulolytic index of bacterial isolates cultured on CMC agar. Different superscript letters indicate statistically significant differences in cellulolytic index among bacterial isolates, based on Tukey's post hoc test ($p < 0.05$).

The determination of extracellular cellulase enzyme ratio (low, moderate, and high) and its viability during subculturing was used as the basis for selecting bacterial isolates to be further tested for cellulase enzyme activity. Among the 12 isolates, 3 were categorized as high, 5 as moderate, and 4 as low. Two isolates (F13 and F17) were excluded from the enzyme activity testing phase due to their low enzyme ratios and low viability in CMC medium.

### 3.3 Cellulase activity and hemolytic profiles of selected isolates

Ten selected bacterial isolates were tested for cellulase activity in liquid CMC medium. The enzyme activity test results showed varying enzyme activity values in the range of 0.063 to 0.32 U/mL. Isolate SF4 had the highest enzyme activity at 0.32 U/mL, which was significantly different from the enzyme activities of the other isolates ($p<0.05$) (Figure 3). The assessment of hemolytic activity for all isolates was conducted by observing the appearance of clear zones surrounding colonies grown on blood agar medium. The hemolysis test results showed that almost all isolates exhibited hemolytic activity on blood agar medium, including isolate SF1 which was previously suspected to be a LAB (Table 3). Nine out of ten cellulolytic isolates showed the potential to be pathogenic (exhibiting β-hemolysis activity on blood agar medium) except for isolate F6. Therefore, isolates SF4 and F6 were selected for molecular identification of their species.

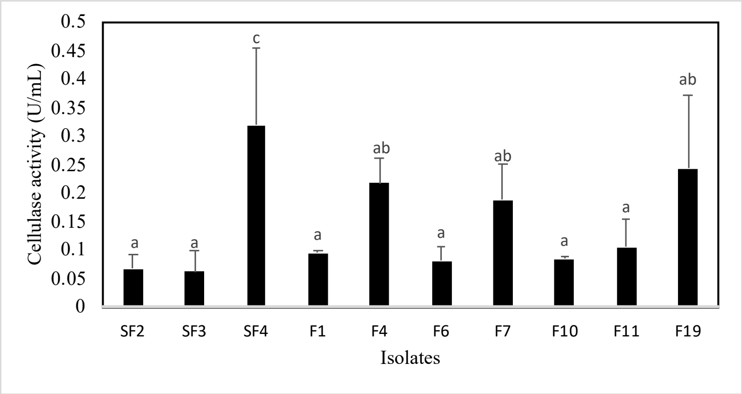

**Fig. 3.** Quantitative cellulase activity of selected bacterial isolates. Different superscript letters indicate statistically significant differences in cellulase activity among bacterial isolates, based on Tukey's post hoc test ($p < 0.05$).

**Table 3.** Hemolytic activity of selected cellulolytic bacterial isolates.

| No | Hemolytic activity | Isolates | | | |
|---|---|---|---|---|---|
| | | **Fermented *Sargassum* sp.** | | **Fresh *Sargassum* sp.** | |
| | | **LAB** | **Non-LAB** | **LAB** | **Non-LAB** |
| 1 | β-*hemolysis* | SF1 | SF2, SF3, SF4, F1, F2, F3, F4, F5, F7, F8, F9, F10, F13, F17, F19 | - | SS1, SS2, SS3, SS4 |
| 2 | γ-*hemolysis* | - | F6 | - | - |

Note: LAB = Lactic Acid Bacteria; "–" indicates no isolates observed.

### 3.4 Molecular identification of the best cellulolytic isolates

To determine the taxonomic identity of the most promising cellulolytic isolates, molecular analysis was performed using 16S rDNA sequencing. This method provides high-resolution insights into bacterial phylogeny and allows for accurate species-level identification. Phylogenetic analysis of the 16S rDNA sequences revealed that isolate SF4 shared 100% sequence similarity with *Bacillus subtilis* strain YEBN5, confirming its close relationship with this species. Meanwhile, isolate F6, a non-pathogenic strain with moderate cellulase activity, exhibited 97.89% similarity to *Bacillus amyloliquefaciens* strain TCCB001 (Figure 4).

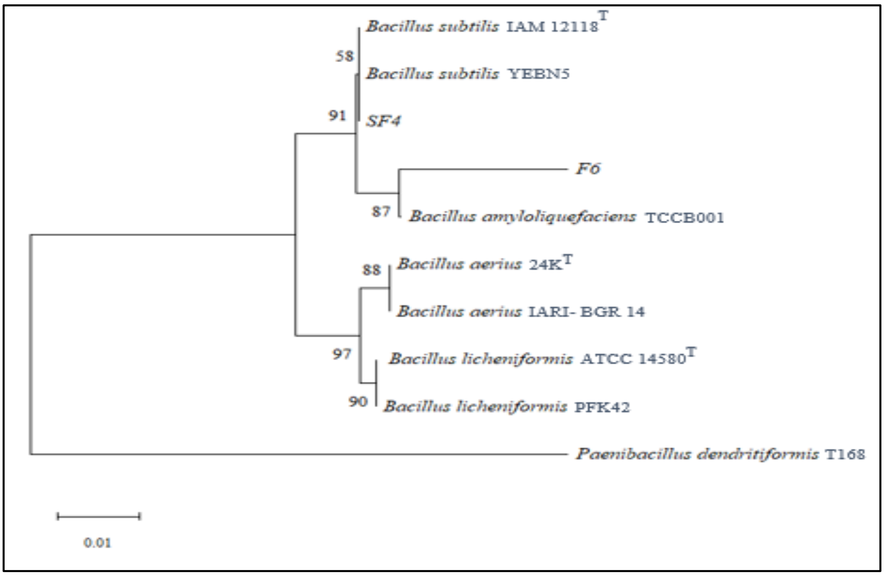

**Fig. 4.** Phylogenetic tree of SF4 and F6 isolates with reference strains based on the Neighbor-Joining method and Tamura–Nei model with 1,000 bootstraps.

## 4 Discussion

### 4.1 Potential of algae-derived bacteria and LAB in fermentation

Cellulolytic bacteria synthesize cellulase enzymes that function to break down cellulose structures embedded within the cell walls of brown algae. The use of cellulase-producing bacteria as starter cultures in the fermentation of brown algae can hydrolyze cellulose complexes, thereby enabling optimal extraction of bioactive compounds [18]. The best cellulolytic bacterial candidates can be isolated from brown algae tissue. These are considered indigenous bacteria that have naturally adapted to complex substrates [30]. Lactic acid bacteria are commonly used as fermentation agents for brown algae [31]. In this study, LAB isolates were successfully obtained from fermentation medium whose pH was adjusted to acidic conditions (pH 5). The pH adjustment functioned to optimize LAB growth [32]. The fermentation process in this study showed an increase in pH toward alkalinity, thereby increasing the potential for alkaliphilic bacteria to grow and decreasing the likelihood of obtaining LAB from fermented brown algae. The rise in pH during fermentation is attributed to ammonia production due to protein breakdown by *Bacillus* species, a phenomenon also commonly found in legume-based fermented foods like kinema, chongkukjang, douchi, natto, and doenjang, which are extensively produced and consumed across Asia and Africa [30].

The density of LAB cells in fermented brown algae was lower compared to non-LAB bacteria, which was also reflected in the number of LAB isolates obtained. The present finding is consistent with those of Kumar et al. [33] who did not detect any LAB in several samples of macroalgal surface epiphytes from intertidal waters of India. The bacteria observed had the potential to produce polymer-degrading enzymes such as xylanase, amylase, cellulase, and pectinase with frequencies of 61.3%, 59.7%, 58.8%, and 52.2%, respectively. Although some algae have been reported to act as prebiotics for LAB, the low detection of LAB may be due to the dominance of halophilic LAB groups that symbiotically associate with algae [10]. The growth characteristics of these LAB groups, which optimally grow in salt-containing media, require isolation media that support growth conditions resembling their natural environments.

## 4.2 Evaluation of isolates based on cellulase activity and safety

Screening of cellulolytic bacteria was performed to evaluate their ability to degrade cellulose using semi-quantitative analysis on CMC agar medium. CMC, an anionic polymer, serves as both a substrate and inducer for cellulase production by providing β-1,4-glycosidic bonds targeted by bacterial cellulase enzymes [19]. Congo red binds to these linkages, staining the medium red, while clear zones around bacterial colonies indicate enzymatic hydrolysis and loss of Congo red binding due to cellulose degradation [18]. The presence of clear zones reflects bacterial capability to break down cellulose into monosaccharides, thus identifying potential cellulolytic isolates.

The enzymatic activity was subsequently measured in liquid culture by assessing the amount of reducing sugars released from a 1% CMC substrate using the DNS assay, which forms 3-amino-5-nitrosalicylic acid upon reaction with reducing sugars at high temperature and alkaline conditions [27]. Although the semi-quantitative assay on solid agar showed isolate F1 with the highest cellulolytic index, quantitative analysis in liquid medium revealed isolate SF4 as the most active. This discrepancy highlights the influence of media conditions: solid media may concentrate enzymes around colonies, making clear zones more apparent, whereas in liquid culture, enzymes may be too diluted to detect easily. These differences can significantly impact gene expression and enzyme yield [34].

The cellulase activity of isolate SF4 at 0.32 U/mL is considerably higher than that reported in previous studies. *Bacillus subtilis* and *Bacillus megaterium* isolated from *Sargassum* sp. and *Turbinaria* sp. showed cellulolytic activities of 0.0099 U/mL and 0.0037 U/mL, respectively [35]. Cellulolytic bacterial isolates derived from the fermentation of fruit peel waste demonstrated the highest activity in samples from watermelon peel mixed with orange peel at 0.036 U/mL, and banana peel mixed with orange peel at 0.035 U/mL [36].

Isolate F6 was also selected as a potential cellulolytic bacterial candidate. Although its cellulase activity was relatively low (0.08 U/mL), it was classified as non-pathogenic based on the hemolysis test (γ-hemolysis). As a non-pathogenic cellulolytic bacterium, *Pediococcus acidilactici*, which is applied in the food industry, exhibits even lower cellulase activity than isolate F6 at only 0.0153 U/mL [24]. Additionally, *Citrobacter amalonaticus* has cellulase activity of 0.042 U/mL, while *Pseudomonas mendocina* has 0.049 U/mL [37]. Thus, isolates SF4 and F6 are feasible to be applied as starter culture candidates in brown algae fermentation because their enzyme activity values are higher compared to those reported in previous studies.

### 4.3 Phylogenetic insights and functional characteristics of identified isolates

Isolates SF4 and F6 were identified as belonging to the genus *Bacillus*. *Bacillus* species were capable of producing endospores, enabling them to survive under extreme conditions. *Bacillus* species have the capacity to produce extracellular enzymes (cellulase, amylase, pectinase, and xylanase) [18, 33]. Along with *Vibrio* sp., *Bacillus* sp. (12.6%) was found to dominate seven algal samples from the central west coast of India [33]. Isolate SF4 was identified as *Bacillus subtilis*. Previous studies have reported that fermentation of *Laminaria japonica* using *B. subtilis* can increase levels of anti-inflammatory compounds and inhibit nitric oxide production [38].

Although *Bacillus subtilis* SF4 exhibited strong cellulase activity, its hemolytic behavior on blood agar raises safety concerns for food-related applications. Hemolysin production in *B. subtilis* has been associated with the *spoVG* gene, which plays a multifaceted role beyond its classical involvement in sporulation. *spoVG* encodes a small, conserved regulatory protein that modulates the expression of several downstream genes, including those involved in membrane disruption and virulence factor production. Disruption of *spoVG* markedly reduced hemolytic activity in *B. subtilis*, while complementation with an intact copy of the gene restored the hemolytic phenotype, indicating its regulatory control over cytolytic pathways [39]. Surfactin, one of the most well-known biosurfactants produced by *B. subtilis*, has been shown to induce red blood cell lysis as evidenced by the formation of clear hemolytic zones on blood agar plates. The blood agar lysis method, commonly used to screen for biosurfactant-producing strains, relies on this hemolytic activity to indicate surfactin production [40].

Despite these concerns, other strains of *B. subtilis* have been documented as safe fermentation starters. For instance, *B. subtilis* SN7, used in the traditional Korean fermented food Cheonggukjang, meets the Qualified Presumption of Safety (QPS) criteria and poses no health risks to consumers. This strain produces bacteriocins and effectively inhibits *Bacillus cereus*, including its spores, during fermentation and cold storage. Furthermore, fermentation with *B. subtilis* SN7 has been shown to improve sensory qualities of the product, supporting its potential as a safe and effective starter culture [41].

Isolate F6, identified as *Bacillus amyloliquefaciens*, is another member of the *Bacillus* genus showing potential as a fermentation starter for brown algae. Fermentation of soybeans using *Bacillus amyloliquefaciens* RWL-1 significantly increased total phenolic content compared to unfermented soybeans. Fermentation also enhanced DPPH radical scavenging activity (increased by 323.39%) and ABTS radical scavenging activity (increased by 121.18%), as well as macro- and micronutrient contents in soybeans. These enhancements are likely due to bacterial involvement in metabolite breakdown and increased mineral bioavailability [42].

Previous studies have also noted that *Bacillus subtilis* and *Bacillus amyloliquefaciens* are potent cellulolytic bacteria. *Bacillus subtilis* BY-2, isolated from pig intestines, exhibited cellulase activity of 1.5 U/mL [43]. *Bacillus amyloliquefaciens*, isolated from the intestinal wall of broiler chickens, produced cellulase enzymes with activity ranging from 0.335 to 1.085 U/mL [44]. While *Bacillus subtilis* SF4 may be optimal for bio-industrial purposes due to its high enzyme yield, isolate F6 represents a safer alternative for food applications and warrants further development as a functional microbial starter. These findings reinforce that the enhancement of bioactive constituents in *Sargassum* sp. can be achieved through fermentation by cellulolytic bacteria, opening opportunities for developing functional food products based on brown algae.

Despite the promising results, this study has several limitations that should be addressed in future research. The cellulolytic activity of the isolates was assessed only on carboxymethyl cellulose (CMC) medium, which does not fully represent the complexity of natural substrates such as brown seaweed. Moreover, enzyme activity was measured under fixed laboratory conditions without optimization of key variables such as temperature, pH, and substrate concentration. The study also did not include a direct comparison of enzyme yield or performance using actual biomass or commercial benchmarks. Furthermore, the efficiency and cost-effectiveness of enzyme production from isolates SF4 and F6 could not be evaluated, as no large-scale fermentation or production trials were conducted. Lastly, while hemolytic activity was tested to assess basic safety, broader genomic or proteomic screening would be needed to comprehensively evaluate the suitability of these isolates for industrial food applications. These limitations present opportunities for future studies to optimize fermentation parameters, validate substrate specificity, and assess the scalability and economic feasibility of applying these indigenous strains.

## 5 Conclusion

This study confirmed the presence of cellulolytic bacteria in fermented *Sargassum* sp., with *Bacillus subtilis* SF4 and *Bacillus amyloliquefaciens* F6 showing the highest enzymatic activity. Among them, F6 stands out as a non-hemolytic isolate that meets basic food safety criteria and holds promise for direct application in seaweed-based fermented products. These findings suggest that *Sargassum* can serve as a habitat for beneficial cellulolytic bacteria with biotechnological potential. Further research is needed to optimize fermentation parameters, conduct laboratory-scale trials, and evaluate economic feasibility to support future industrial application. Additionally, the use of *Sargassum* in fermentation could contribute to environmental sustainability by reducing marine biomass waste through value-added bioconversion.

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