Scientific Reports

https://doi.org/10.1038/s41598-026-42679-6

Article in Press

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Received: 28 December 2025

Accepted: 26 February 2026

Published online: 05 March 2026

Cite this article as: Nguyen T.N., Ly T.C., Tran N. *et al.* Cost-effective plant-based medium for enhanced spore production of *B. amyloliquefaciens* CN12 for biofertilizer application. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-42679-6

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Cost-effective plant-based medium for enhanced spore production of *B. amyloliquefaciens* CN12 for biofertilizer application

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Abstract

In this study, endospore-forming bacteria were isolated, screened, and characterized from samples collected in Tan Phu protection forest for potential use in biofertilizer application. Twelve endospore-forming bacteria showed the ability to produce indole-3-acetic acid were evaluated. Based on the relative enzyme activity, siderophore production, phosphate and potassium solubilization, strain CN12 was selected. Strain CN12 was identified as *Bacillus amyloliquefaciens* based on 16S rRNA gene sequencing. The liquid culture medium using inexpensive substrates was optimized for increasing its endospore production. The medium consisted of 10% mung bean sprouts extract solution, 0.87% molasses, 0.12% urea, and 0.06% $MgSO_4$ produced the spore yield of 5.53 ± 3.5 ($\times 10^8$ CFU/mL) for strain CN12. Malabar spinach plants treated with strain CN12 showed significantly increases of 39.4 ± 8.9 to 77.6 ± 11.8 g in fresh weight, 17.1 ± 3.0 to 65.8 ± 17.2 cm in plant height, and 2.9 ± 0.9 to 6.8 ± 2.3 g in root weight compared to the control. These observations revealed that strain CN12 is a promising candidate for biofertilizer production.

Keywords: Biofertilizers, bacteria, soil, *Bacillus*, optimization

Introduction

Nowadays, nutrient depletion in soil has been widely concerned due to its impact on agricultural sustainability, food security, and human health [1-4]. Fertilizers are essential in agriculture because they provide important nutrients for plant [1]. However, the excessive and indiscriminate use of the chemical fertilizers can lead to environmental impacts, the loss of biodiversity, and unforeseen health hazards. Therefore, it is important to find alternative strategies that protect soil health and biodiversity while ensure competitive yield and productivity. Biofertilizer is considering as the potential solution for replenishing soil nutrients and increasing crop productivity [2,3].

It is reported that soil microbes play an essential role in improving nutrient cycling and supporting plant growth and development [5]. Plant growth-promoting bacteria (PGPB) are considered as the most promising candidates because of the capable of stimulating and enhancing plant growth or crop production [5]. Different types of mechanisms are responsible for the enhancement of plant growth by PGPB. The phosphate and potassium solubilizing strains directly promoted plant growth by acting as macronutrients, in which the potassium solubilizing bacteria can mineralize organic potassium compounds and convert inorganic potassium to a more available form to the plant [6]. The phosphate solubilizing strains were effective in releasing phosphate from inorganic and insoluble pools of total soil phosphate through solubilization [7,8]. The synthesis of phytohormones such as indole-3-acetic acid (IAA) accelerated root growth at low concentrations. IAA was reported to play a key role in the cell enlargement and the root cell division's initiation and often used as an initial indicator for screening PGPB [5,9]. In addition, the increase of IAA in root surface area could improve the access of soil nutrients through the root. Whereas, other lytic enzymes namely, cellulase, pectinase, amylase, etc., were produced by microorganisms and could indirectly support the plant growth by improving soil structure and nutrient available [2].

For several decades, studies have mainly focused on isolation, screening, and characterization of PGPB [10-14]. Various species of plant-associated bacteria from genus *Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella*, *Pseudomonas,* and *Serratia* revealed the ability to enhance plant growth [11-14]. The estimation of enzymatic activities and related biochemical traits is essential for evaluating the potential of microbial isolates as biofertilizer candidates. These parameters collectively reflect the capacity of microorganisms to promote nutrient availability and plant growth under soil conditions. Much attention has been given to cellulase, pectinase, amylase, siderophore production, and solubility of phosphate and potassium from microbes because of their potential in fertilizer industry [15,16]. For biofertilizer application, microbes must be capable of enduring the harsh conditions during production, storage, and application in diverse agricultural environments. The formation of spores is the most common mechanism to protect microbes against ecological degrading agents. Therefore, endospore-forming bacteria are especially attractive in the development of biofertilizers [17-19]. Forest soils were chosen as sampling sites due to their high microbial diversity and minimal anthropogenic disturbance, making them a rich source of resilient PGPB [20]. Tanphu protection forest in Southeast Vietnam is home to numerous endangered, rare, and valuable tree species, suggesting a unique and diverse microbial community [21]. In this study, soil and water samples collected from the forest were used to isolate endospore-forming bacteria. The selected bacterial strain was further investigated the multi-purpose potential for agricultural crops such as plant growth promotion, siderophore production, phosphate and potassium solubilization activities. Plant-based media formulated with low-cost, renewable ingredients were also tested as sustainable alternatives to conventional synthetic media for the selected microbe.

Materials and methods

Chemicals and media

Mung bean sprouts, potato, rice, corn, and soybean were obtained from a local market in Ho Chi Minh City, Vietnam. Malabar spinach (*Basella alba* L.) seeds were purchased from Phudiseed Company. Nutrient medium (g/L) consisted of peptone 10, NaCl 10, and yeast-extract 5. Nutrient agar

medium was prepared by adding 2% (w/v) bacteriological agar into nutrient medium [22]. Plant-based extract was prepared by boiling 20% (w/v) each of mung bean sprouts, potato, rice, corn, or soybean in distilled water for an hour. The extract was then filtered and restored to its original volume by adding distilled water. The extracts were used as broth media including mung bean sprouts extract medium (MEM), potato extract medium (PEM), rice extract medium (REM), corn extract medium (CEM), and soybean extract medium (SEM). For solid plant-based media, 2% (w/v) bacteriological agar was added to the broth medium. All plates were incubated at 30°C. All media were sterilized in an autoclave prior to use at 121°C for 20 min.

Sampling, isolation of endospore-forming bacteria

Soil samples were collected from forest site (11°06'12"N, 107°24'30"E) in Baunuocsoi, Tanphu protection forest, Dongnai Province, Vietnam to isolate PGPB from undisturbed environments with high microbial diversity. Water samples were collected from hot spring (11°06'12"N, 107°24'28"E) located within forest areas to enhance the ability to isolate spore-forming bacteria which may exhibit enhanced survival, stability, and functional traits desirable for biofertilizer application. Soil and water samples were stored in Zip-lock plastic bag and pre-sterilized bottle, respectively in August 2020. Water and soil samples were collected at a depth of 20 cm. Ten surficial soil samples were collected with 5 cm of diameter steel rings. For water sampling, the plastic bottles were rinsed with the hot spring water thrice and three water samples were collected. In the field site, the *in-situ* parameters of temperature, pH, and electrical conductivity which are key factors affecting microbial community structure, were measured by the multi-parameter handheld device (Eutech™ PD 650 Meter, Thermo Fisher Scientific, United States). The soil pH and electrical conductivity were measured in a soil-to-water mass ratio of 1:2.5 (w/v). The samples were immediately transferred to the laboratory and stored at 4°C. Soil water content was measured gravimetrically by oven drying moist soil samples (105°C, 24 h). Each collected sample of 1 mL water or 1 g soil were suspended in 9 mL of sterile distilled water and prepared stock. 1 mL of stock sample was serially diluted in 9 mL of sterile distilled water up to 10^{-7} dilution. In order to isolate endospore-forming bacterial strains, the diluted suspension was treated at 80°C for 15 minutes. 100 µL of sample from 10^{-3} to 10^{-7} diluted suspension was then used to spread on the nutrient agar medium separately [23,24]. Morphologically different colonies were selected and used for further study.

Screening of IAA-producing bacteria

IAA-producing bacteria were screened using the Salkowski colorimetric assay according to Lebrazi *et al.* (2020) [25], in which Salkowski reagent consists of 3 mL of 0.5M $FeCl_3$, 60 mL of 98% H_2SO_4, and 100 mL distilled water [26]. Briefly, the bacterial strains were cultured in 5 mL nutrient medium supplemented with 1 g/L L-tryptophan in test tube at 30°C for 48 h and 180 rpm. The bacterial cultures were then collected and centrifuged at 10,000 rpm for 10 min at room temperature. 1 mL of each supernatant was used to mix with 2 mL of Salkowski reagent and then incubated for 30 min in the dark at room temperature. Afterwards, the absorbance was measured at 530 nm (Spectrophotometer, Jasco v730). By using a standard IAA curve, the concentration of IAA produced was estimated. Nutrient medium supplemented with 1 g/L L-tryptophan was used as control.

Evaluation for hydrolytic enzyme activity

The extracellular enzyme production was primarily evaluated through a plate-based screening assay. In which, cellulase, amylase, and pectinase activities were tested by adding 50 µl broth culture into a 7 mm well in nutrient agar medium containing 1% carboxymethyl cellulose, starch, or pectin, respectively, followed by incubation for 24 h. The cellulase, amylase, and pectinase activity was confirmed by adding Lugol's solution (iodine 1%) for 5 minutes [15,27,28]. The relative enzyme activity was calculated as the ratio of diameter of zone of clearance divided by the diameter of the bacterial colony in millimeters.

Potassium and phosphate solubilization activity

To evaluate the solubilization ability of potassium and phosphate, the solid Aleksandrov medium and NBRIP medium were used and incubated for 5 days, respectively. Aleksandrov medium

(g/L) consisted of glucose 5, $MgSO_4$ 0.005, $FeCl_3$ 0.01, $CaCO_3$ 2, potassium feldspar powder 3, calcium phosphate 2, and agar 20 [29]. National Botanical Research Institute's Phosphate growth medium (NBRIP) (g/L) consisted of glucose 10, $MgCl_2$ 5, $MgSO_4$ 0.25, $Ca_3(PO_4)_2$ 5, KCl 0.2, $(NH_4)_2SO_4$ 0.1, and agar 20 [30]. For potassium solubility, phenol red solution was flooded with dropper for 5 minutes [31]. Whereas phosphate solubilization was evaluated without staining [30]. The clear zone around the growth of the bacteria indicates a positive result.

Estimation of siderophore formation

CAS agar plates were used to estimate the siderophore formation. The chrome azurol S - hexadecyl trimethyl ammonium bromide (CAS-HDTMA) reagent was prepared by mixing chrome azurol S solution and hexadecyl trimethyl ammonium bromide solution, as previous described by Hu and Xu (2011) [32]. CAS agar plates were prepared by mixing 10 ml CAS-HDTMA in 90 ml sterilized nutrient agar medium. After inoculation, plates were incubated for 5 days and observed for the formation of orange zone around the growth of the bacteria [33].

Identification and phylogenetic tree

The selected isolate was identified using morphological and biochemical characteristics. Pure culture obtained as above was subjected to microscopic examination for shape and size. Gram staining, oxidase, catalase, nitrate reduction, citrate utilization, gelatin hydrolysis, motility, Voges–Proskauer reaction, and tests for indole, methyl red, salt tolerance test, and urease were performed as previous described [22]. For further identifying bacterium, total genomic DNA was extracted using procedure by Ausubel *et al*. (2002) [34]. Concentration and purity of the DNA were measured in a NanoDrop 8000 (Thermo Scientific, USA). For the polymerase chain reaction (PCR) amplification, the 50 µl of PCR mixture consisted of 0.2 mM of each of the four dNTPs, 20 pmol of each primer, 10 ng of extracted DNA and 1.25 unit of Taq DNA polymerase (TOYOBO Co. Ltd., Japan) with the appropriate reaction buffer. Amplification was performed in a Program Temp. Control System, PC-808 (Astec Co. Ltd.). Two universal bacterial primers, 27F (5'- AGA GTT TGA TCM TGG CTC AG -3') and 1492R (5'- CGG TTA CCT TGT TAC GAC TT -3') were used to amplify the partial 16S rRNA gene [35]. The amplicon was then sequenced by the Sanger sequencing method in Nam Khoa Biotek company. The sequence was then analyzed by using the BLAST tool from the GenBank database (http://www.ncbi.nlm.nih.gov). For phylogenetic tree, the neighbor-joining method was used as previous described by Nguyen *et al.* (2021) [23]. Briefly, the nucleotide sequences of the 16S rRNA genes from our isolate and the published type strains were used to construct the phylogenetic tree using Clustal X version 2.0.3 via the multiple alignment algorithm.

Measurement of total spore density

The plate count method was used to evaluate the total spore density. Briefly, the cell suspensions were treated at 80°C for 15 min to eliminate vegetative cells and harvest the spores [36]. Then, the heat-treated cell suspensions were diluted and 100 µL of the 10^{-5} and 10^{-7} dilutions were used to spread on nutrient agar plate. These plates were incubated for 24 h. The spore concentration was calculated and expressed as colony-forming units per milliliter (CFU/mL).

Evaluation of carbon, nitrogen, and metal ions source adding to plant-based medium for spore formation

In this study, five plant-based media with concentration of 10% were tested to determine the optimal medium. Then, the effect of carbon, nitrogen, and metal ions sources adding to the optimal plant-based medium for spore formation were conducted in 18 mL shake tubes filled with 5 mL of medium. The bacterial pre-culture medium (5%, v/v) was inoculated in the tubes and incubated for 48 h at 30°C and 180 rpm. Different carbon sources (sugarcane molasses, glucose, soluble starch or fructose) with a concentration of 10 g/L were used. Then, based on the optimal carbon source, nine concentrations of 2, 4, 6, 8, 10, 12, 14, 16, and 18 g/L were used to evaluate the optimal concentration. For nitrogen source screening, different nitrogen sources (NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$ or urea) with a concentration of 1 g/L were added to the plant-based medium containing 10 g/L sugarcane molasses. Nine concentrations of 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, and 0.18 g/L of the optimal nitrogen

source were used to determine the optimal concentration. For metal ions screening, different inorganic salts ($MgSO_4$, $MnSO_4$, $FeSO_4$ or $ZnSO_4$) with a concentration of 1 g/L were added to the optimal plant-based medium containing 10 g/L molasses and 1 g/L urea. After that, five concentrations (0.1, 0.5, 1, 1.5, and 3 g/L) of the best inorganic salt were used to evaluate the optimal concentration. The optimal plant-based medium was used as control.

Optimization of culture condition for selected strain by Response Surface Methodology

Based on the results obtained from the single factor optimizations, the combination of the optimal carbon source, nitrogen source, and inorganic salts in the optimal plant-based medium was further investigated by the Box-Behnken design (BBD) of Response Surface Methodology (RSM). The ranges of the variables were chosen as follows: molasses dosage was 0.4-1.2%, urea was 0.06-0.14%, and $MgSO_4$ was 0.01-0.09%. To design response surface experiments and perform the regression and graphic analysis, Minitab software version 16.2.4 was used.

The response variable (Y) was fitted to a second-order model containing the independent variables as below:

$$Y = \beta_0 + \sum \beta_i X_i + \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (1)$$

Where Y is the predicted response, X_i and X_j are input variables that influence the response variable Y, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the linear-by-linear interaction between the X_i and X_j regression coefficients.

Formulation process and storage stability study

Bacterium was grown in the optimal plant-based medium supplemented the optimal carbon, nitrogen, and inorganic salt sources for 48 h at 30°C and 180 rpm. The resulting broth culture was then treated at 80°C for 10 minutes for further investigation. The liquid formulation was stored at 4°C and room temperature (30 ± 2°C) in plastic vials tubes. Viability expressed as colony-forming units was evaluated at the beginning of the study (time zero) and every 2 months for up to twelve months of storage at two temperature levels. Briefly, 9 mL of sterile distilled water was added to 1 mL of the liquid formulation. Thereafter, this suspension was diluted to 10^{-5}, 10^{-6}, and 10^{-7} with water (v/v) and 100 µL of the dilutions were used to spread on nutrient agar plate.

Pot experiment

In this study, Malabar spinach was selected for the pot experiment due to its fast growth, high nutritional value, and sensitivity to soil and nutrient conditions. The soil used in this study was collected from the experimental garden, then air-dried, crushed, and thoroughly mixed to ensure uniformity. The soil was sterilized in an autoclave at 121°C for 20 min to eliminate contaminants. Malabar spinach seeds were prepared by soaking in warm water at a ratio of 3 boiling: 2 cold and left overnight. Then, the seeds were drained and sown in four plastic pots (40 × 60 cm) containing sterile soil. Each pot contained six plants and was maintained under controlled greenhouse conditions, with temperatures at 30°C during the day and 25°C at night and irrigated once daily. After 10 days of growth, Malabar spinach plants had reached the early seedling stage with a well-established root system. At this time, 5 mL of microbial inoculants (10^6 CFU/mL) was applied to two experimental pots by gently pouring the solution onto the soil surface adjacent to the base of each plant, ensuring direct contact with the root zone. At 21 days of growth, the effects of microbial isolate on Malabar spinach were evaluated during the vegetative growth stage.

Accession number

Sequence data has been deposited in the DDBJ/EMBL/GenBank databases with the primary accession code OR492434.

Statistical analysis

Each experiment was performed in triplicates. The data obtained from the experiments were analyzed and expressed as mean and standard deviation. The differences between the groups were examined by one-way ANOVA with Tukey's post-hoc test using Minitab software version 16.2.4. A *p*-value of <0.05 was considered statistically significant.

Results

Isolation of bacteria with potential for biofertilizer application

In this study, fifty-three spore-forming bacterial strains were isolated from three water and ten soil samples, in which twelve potential bacteria revealed the ability to produce IAA in the presence of tryptophan (**Table 1**). Strain CN12 revealed the highest IAA yield of 73 µg/mL and the one-way ANOVA test has shown a significant difference in IAA production for strain CN12 compared to all other isolates (p-value < 0.05). CN5 and CN10 had no significant difference in IAA production and revealed the second highest IAA production.

Enzyme activity by isolates

Based on plate assays, eleven out of twelve selected bacterial isolates produced halo zones on the medium containing carboxymethyl cellulose or pectin, nine isolates produced halo zones on the medium containing starch (**Fig. 1a-c**). For siderophore production, only strain CN12 produced halo zones on CAS agar plate (**Fig. 1d)**. For phosphate and potassium solubility, ten isolates produced halo zones on the Aleksandrov medium, whereas nine isolates produced halo zones on the NBRIP medium, respectively (**Fig. 1e-f**). The relative enzyme activity of selected isolates by plate assay was presented in **Supplemental Table 1**. Most strains exhibited cellulase and pectinase activities, with cellulase indices generally ranging from 2.14 to 2.43, except for CN4, which showed no detectable cellulase activity. Pectinase activity was widely distributed across strains, with values clustered around 2.01-2.17. However, CN9 showed no detectable pectinase activity. Amylase activity was detected in several strains (CN2, CN3, CN4, CN5, CN8, CN9, CN10, CN11, and CN12), with CN3 showing the highest amylase activity (1.79), while multiple strains (CN1, CN6, and CN7) showed no detectable activity. Regarding solubilization, phosphate solubilization was observed in most strains, with CN12 exhibiting the highest value (2.07), followed by CN5 (1.74). In contrast, some strains (CN4 and CN7) did not show phosphate-solubilizing ability. Potassium solubilization varied widely, with CN5 (3.29), CN12 (3.00), and CN6 (2.57) showing strong activity, while several strains showed no detectable activity. Siderophore production was detected only in CN12, which exhibited a notably high value (3.71), whereas all other strains showed no detectable siderophore activity.

Characterization of potential isolate for biofertilizer application

In this study, strain CN12 demonstrated the broadest and strongest functional profile, exhibiting high enzymatic activities, efficient phosphate and potassium solubilization, and exclusive siderophore production among the tested strains. Therefore, it was selected for physiological and biochemical tests (**Supplemental Table 2**). Strain CN12 formed typical Bacillus-like colonies, and microscopic observation revealed rod-shaped cells with endospore formation. CN12 was Gram-positive and capable of aerobic growth. Biochemical characterization showed that strain was oxidase- and catalase-positive. The strain utilized citrate, as evidenced by a positive Simmons citrate test, and produced acetoin, confirmed by a positive Voges–Proskauer test. In contrast, CN12 tested negative for indole production, methyl red reaction, and urease activity. The strain demonstrated gelatin hydrolysis, indicating proteolytic activity, and was motile. Additionally, CN12 exhibited tolerance to high salinity, as shown by its ability to grow in the presence of 10% NaCl.

To clarify the taxonomic status of the isolate, 16S rRNA gene sequences of selected strain was analyzed using BlastN analysis through NCBI GenBank. 16S rRNA gene sequence of CN12 (1324 nucleotides) had the closest relative (99.85%) to the sequence of *B. amyloliquefaciens* strain GZY63 (CP126696). In this study, a phylogenetic tree that depicted the selected bacterium and 52 type strains was shown in **Fig. 2**. In which, fifty-two strains were classified into six groups including clade 1 (4 strains), clade 2 (11 strains), clade 3 (8 strains), clade 4 (3 strains), clade 5 (10 strains) and clade 6 (12 strains). Interestingly, strain CN12 was belonged to *B. amyloliquefaciens* group with high bootstrap value of 98%. Considering their multiple ability of plant cell wall degrading enzymes, endospore formation, IAA production, siderophore production, phosphate and potassium solubilization, CN12 revealed the great potential to be used as inoculants in biofertilizer.

Effect of plant-based extract medium, carbon, nitrogen, and inorganic metals source on cell growth and spore formation

In this study, five plant-based media with concentration of 10% were tested. The results demonstrate notable differences in endospore production by CN12 across five tested media. Strain showed the greatest sporulation in MEM (6.13×10^6 CFU/mL), with decreasing production in SEM, REM, PEM, and CEM. The results revealed that the bacterial growth on MEM produced the highest spore density compared to its growth on SEM, REM, PEM, and CEM medium (**Supplemental Fig. 1**).

To enhance the spore formation in mung bean sprouts extract medium, four different carbon sources (molasses, glucose, soluble starch or fructose) were added. The total spore density of molasses was the highest, followed by those of glucose, starch, and fructose (**Supplemental Fig. 2A**). Different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8%) of molasses were then used and revealed that the spore density was the highest value of 1.2×10^8 CFU/mL at a concentration of 0.8% of molasses in strain CN12 (**Supplemental Fig. 2B**).

As shown in **Supplemental Fig. 3A**, among four different nitrogen sources (NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, and urea), the highest spore density was observed in medium supplemented urea, followed by $(NH_4)_2SO_4$, and NH_4Cl in strain CN12. Then, different concentrations (0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, and 0.18%) of urea were tested to evaluate the spore formation. The highest value of spore density (2.6×10^8 CFU/mL) appeared at the concentration of 0.1% (**Supplemental Fig. 3B**).

In this study, the most obvious improvement in spore formation was observed when $MgSO_4$ was used (**Supplemental Fig. 4A**). The different concentrations (0.01, 0.05, 0.1, 0.15, and 0.3%) of $MgSO_4$ were tested. The maximum value appeared with the total spore density of 5.4×10^8 CFU/mL at the concentration of 0.05% (**Supplemental Fig. 4B**).

The best culture condition for selected strain by Response Surface Methodology

The molasses, urea, and $MgSO_4$ were studied at three levels (−1, 0, +1). The experimental design and responses of spore production were reported (**Table 2**) and use for making the fitting polynomial equation (**1**).

$$Y = 55 + 6.38X_1 + 6.25X_2 + 2.38X_3 - 17.5X_1^2 - 7.25X_2^2 - 5X_3^2 - 0.25X_1X_2 - 0.25X_2X_3 \quad (2)$$

In strain CN12, the highest spore production of 5.72 ($\times 10^8$ CFU/mL) was predicted under the following conditions: Molasse (X_1) = 0.87%; Urea (X_2) = 0.12%; $MgSO_4$ (X_3) = 0.06% (**Fig. 3**). The observed spore production by the optimized condition was 5.53 ± 3.5 ($\times 10^8$ CFU/mL), which also agrees with the predicted model.

The growth-promoting effect of strain CN12 on Malabar spinach

The impact of *B. amyloliquefaciens* CN12 on the growth of Malabar spinach (*Basella alba* L.) was tested. The result showed that the plant height, root weight, and fresh weight of Malabar spinach treated by microbial suspension were increased (**Fig. 4**). Compared with the control, the fresh plant weight, height, and root weight of Malabar spinach treated with strain CN12 increased by 39.4 ± 8.9 to 77.6 ± 11.8 g, 17.1 ± 3.0 to 65.8 ± 17.2 cm, and 2.9 ± 0.9 to 6.8 ± 2.3 g, respectively (**Fig. 4a-c**). These indicated that the isolated bacterium could be applied to Malabar spinach.

Effect of storage conditions on the viable cells in formulation

The survival of *Bacillus* spores over time in the formulation after storage was calculated (**Fig. 5**). During the 12-month period, samples stored at 4 °C showed greater stability than those kept at room temperature, with both conditions starting at 100% at time zero but declining progressively over time. The decrease was consistently slower at 4 °C, with values remaining above 90% up to 8 months and reaching approximately 83.5% at 12 months, whereas room temperature samples declined more rapidly, falling to about 83.4% by 8 months and to 73.4% by the end of the study.

Discussion

For the development of biofertilizer that can be used in crop field, isolation and selection of efficient bacterial strains play an important role. PGPBs are frequently used for increasing crop yields by promoting plant growth via various direct or indirect mechanisms. In PGPBs, the species of the genus *Bacillus* have attracted much attention because of their ability to form endospore that can survive under unfavorable conditions such as extreme pH, high salinity, and high temperatures in the field [19]. In this study, fifty-three spore-forming bacteria were isolated from water and soil samples collected from Tanphu protection forest, reflecting the diversity of spore-forming bacteria in this ecosystem. Twelve out of fifty-three bacteria revealed the ability to produce IAA in the presence of tryptophan (**Table 1**), suggesting that forest ecosystems can serve as valuable reservoirs of plant growth-promoting bacteria. It is reported that IAA production from *Bacillus* sp. ranged from 1 - 40 µg/mL [2,37,38]. The IAA yield of strain CN12 (73 µg/mL) was notably higher than those reported in earlier studies of related bacterial species, highlighting its strong plant growth-promoting potential. Interestingly, some strains belonged to *Bacillus* or *Rhizobium* can produce the high amount of IAA (around 100 µg/mL) under optimal condition [25,39]. Therefore, optimization of IAA production for these selected strains is necessary to get better amount of IAA. Strain CN12 also revealed multiple plant growth-promoting properties such as such as siderophore production and the solubilization of phosphorus and potassium (**Fig. 1 and Supplemental Table 1**). To clarify the taxonomic status of the isolate CN12, its 16S rRNA gene sequence was analyzed and identified as *B. amyloliquefaciens*. *B. amyloliquefaciens* is widely known as non-pathogenic microorganism of the genus *Bacillus* [40].

Five plant-based media using low-cost, renewable ingredients were tested as sustainable alternatives to conventional synthetic media in the present study. Our results show that the bacterial growth on MEM produced the highest spore density compared to its growth on other plant-based medium (**Supplemental Fig. 1**). It is reported that the mung bean sprout is highly nutritious with carbohydrate and protein, approximately 60 and 30% of dry weight, respectively [41]. Because of highly nutritious and cheap price, the mung bean sprout has been used as alternative medium for growing microorganism [41]. The effect of carbon and nitrogen source on spore production was also tested using mung bean sprouts extract medium. The total cell density and spore density of molasses and urea was the highest (**Supplemental Fig. 2&3**). Molasses, an economical source, is considered as a suitable candidate for *Bacillus* growth [42,43]. It is reported that the spore formation can be influenced by inorganic salts [17,18]. In this study, the most obvious improvement in spore formation was observed when $MgSO_4$ was used (**Supplemental Fig. 4**). This result agrees with previous studies [17,18]. It is reasonable because Mg^{2+} can stabilize the ribosome complex. In addition, Mg^{2+} can change the sensitivity of bacteria to antibiotics [18]. The optimum levels of the significant factors and their interactions were then evaluated by the BBD of RSM at three levels (−1, 0, +1). The fitted model was statistically valid with model F-values of 18.0 and *p*-values of 0.003. The coefficient values indicate that the molasse dosage ($p = 0.004$) and $MgSO_4$ dosage ($p = 0.113$) had the strongest and weakest influence on the spore production in strain CN12. The observed spore production by the optimized condition was 5.53 ± 3.5 ($\times 10^8$ CFU/mL). When compared with previously reported studies, the spore yield obtained in this work is within a comparable range, despite the use of a simpler and more cost-effective medium [17,36]. For instance, Ren et al. reported that *Bacillus amyloliquefaciens* BS-20 achieved a higher spore concentration of 8.05 ± 0.7 ($\times 10^9$ CFU/mL) when cultivated in a complex optimized medium containing defined amounts of glucose, beef extract, corn meal, soybean meal, and supplemented with Mn^{2+}, Fe^{2+}, and Ca^{2+} ions [17].

The impact of *B. amyloliquefaciens* CN12 on the growth of Malabar spinach (*Basella alba* L.) was tested. Compared to the control, fresh weight, height, and root weight increased significantly in the experimental group (**Fig. 4a-c**). In future study, conducting the experiment under garden or field conditions would provide highly informative and would help to validate the observed growth effects under more natural conditions. In addition, an experiment involving seed inoculation would add further value to the study by providing additional insights into the effectiveness of the treatment at early

growth stages and its potential applicability under practical agricultural condition. Regarding biomass assessment, dry weight can be used to provide a more robust and reliable measure of plant biomass, as water content can vary due to factors unrelated to bacterial effects [44]. The survival of strain CN12 over time in the formulation during storage is a key factor for its industrial application in fertilizers. After a year of storage, strain CN12 maintained a survival percentage above 70% across both temperature conditions tested (**Fig. 5**). This suggests that the formulation provided effective protection for strains preserving their relative abundance and viability over time. Conducting an additional inoculation experiment with Malabar spinach using the stored one-year stored formulation and comparing it with pot experiments using a freshly prepared solution would provide a better understanding of its suitability and effectiveness. The decrease in strain CN12 during storage may be due to the presence of chemical agent in the formulation. Our results also indicated that the greatest reduction of strain CN12 was found at room temperature (30°C) compared to its at 4°C. During storage at room temperature, some metabolisms still occurred and produced some compounds that can affect to the spore survival. Therefore, to maintain a stable level of spore viability, *Bacillus* spores were cleaned to remove the rest of medium or frequently frozen in a freezer or freeze dryer [45].

Conclusions

This study identified *Bacillus amyloliquefaciens* CN12 as a promising endospore-forming plant growth-promoting bacterium with traits suitable for biofertilizer development. Notably, high indole-3-acetic acid production was observed in strain CN12, indicating a strong potential to enhance plant growth. A cost-effective, plant-based culture medium was optimized for spore production, consisting of 10% mung bean sprouts extract solution, 0.87% molasse, 0.12% urea, and 0.06% $MgSO_4$, which is essential for large-scale biofertilizer manufacturing. Malabar spinach treated with strain CN12 showed the improvement of growth compared to the control, demonstrating its functional effectiveness. In addition, the high survival rate of spores after twelve months of storage at room temperature highlights the strain's stability and practicality for long-term use. These findings support the potential of *B. amyloliquefaciens* CN12 as a viable candidate for sustainable biofertilizer formulations and provide a foundation for further field evaluation and product developments.

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Funding

This research was funded by PetroVietnam Fertilizer and Chemicals Corporation under grant number 138-24/PVFCCo/KHCN-KHVLUD/D_UreVSV.

Author contributions

Tuan Ngoc Nguyen has designed the study. Tu Cam Ly, Nghi Tran, Nhung Hong Nguyen, Thu Thien Pham, Hien My Tran, Thuan Phuoc Dinh, Hung Phi Nguyen, Tuan Ngoc Nguyen performed the experiments. Tuan Ngoc Nguyen conducted the statistical analysis. Tuan Ngoc Nguyen and Rudolf Kiefer wrote the main manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Table 1. Physiochemical properties of the sources and IAA production of endospore-forming bacteria

Sample	Location	Sample properties				Strain	IAA (µg/mL)
		Temperature (°C)	pH	Electrical conductivity (mS/cm)	Moistrure content (%)		
Water 2	11°06'12.5"N, 107°24'28.3"E	52	6.2	1.2	ND	CN1	$12^{e,f} \pm 1$
						CN2	$25^{c,d,e} \pm 4$
Water 3	11°06'12.1"N, 107°24'28.6"E	52	6.2	1.3	ND	CN3	$16^{e,f} \pm 2$
Soil 1	11°06'11.3"N, 107°24'27.9"E	32	5.2	0.46	26	CN4	$32^{b,c,d} \pm 5$
						CN5	$45^{b} \pm 4$
Soil 2	11°06'12.2"N, 107°24'29.2"E	32	5.2	0.48	26	CN6	$35^{b,c} \pm 5$
Soil 3	11°06'12.0"N, 107°24'30.7"E	32	5.4	0.48	25	CN7	$12^{e,f} \pm 1$
						CN8	$17^{d,e,f} \pm 3$
Soil 6	11°06'11.1"N, 107°24'31.7"E	32	5.3	0.62	27	CN9	$8^{f} \pm 1$
Soil 7	11°06'13.1"N, 107°24'31.5"E	32	5.4	0.63	29	CN10	$47^{b} \pm 7$
Soil 9	11°06'14.4"N, 107°24'30.5"E	32	5.4	0.51	28	CN11	$21^{c,d,e,f} \pm 3$
Soil 10	11°06'13.2"N, 107°24'29.5"E	32	5.4	0.52	27	CN12	$73^{a} \pm 13$

IAA: Indole-3-acetic acid. Each test for IAA production was performed in triplicate. Results were presented as mean ± standard deviation. $^{a-f}$: Different letters in the same column indicate significant statistical differences ($p<0.05$, Tukey's test).

Table 2. Box–Behnken design for optimization of spore production in *B. amyloliquefaciens* CN12

Std. Order	Factor			Spore density (×10^7 CFU/mL)	
	X_1	X_2	X_3	Y	
	Molasses (%)	Urea (%)	$MgSO_4$ (%)	Actual value	Predicted value
1	0.4	0.06	0.05	15	17.4
2	1.2	0.06	0.05	31	30.6
3	0.4	0.14	0.05	30	30.4
4	1.2	0.14	0.05	45	42.6
5	0.4	0.1	0.01	25	23.8
6	1.2	0.1	0.01	35	36.5
7	0.4	0.1	0.09	30	28.5
8	1.2	0.1	0.09	40	41.3
9	0.8	0.06	0.01	35	33.9
10	0.8	0.14	0.01	46	46.9
11	0.8	0.06	0.09	40	39.1
12	0.8	0.14	0.09	50	51.1
13	0.8	0.1	0.05	53	55.0
14	0.8	0.1	0.05	60	55.0
15	0.8	0.1	0.05	52	55.0

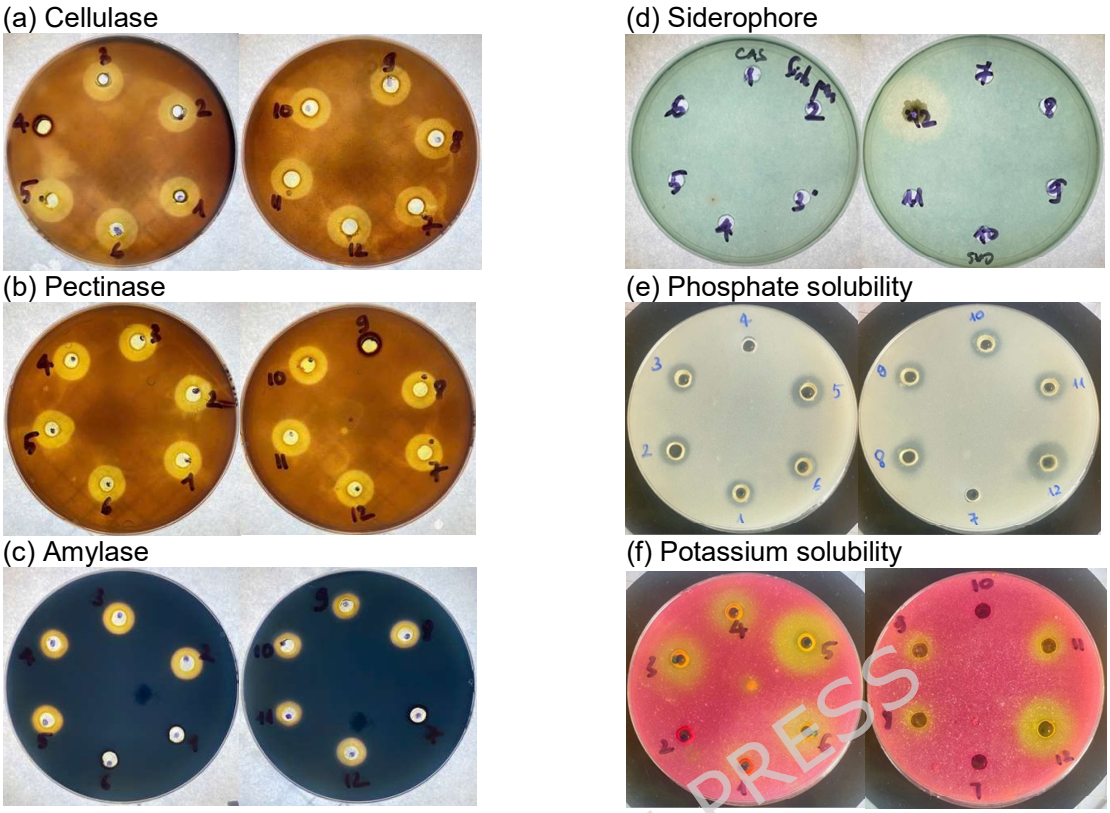

Figure 1. Plant growth-promoting traits evaluation. (a-c) Bacterial isolates with clear halo zones for cellulase, pectinase, and amylase in nutrient agar medium with Lugol. (d) Siderophores production in CAS agar plates. (e) Phosphate solubilization by bacteria in NBRIP agar medium. (f) Bacterial isolates presenting potassium solubilization in Aleksandrov medium with Phenol red. Numbers 1 to 12 indicates the strain CN1 to CN12, respectively.

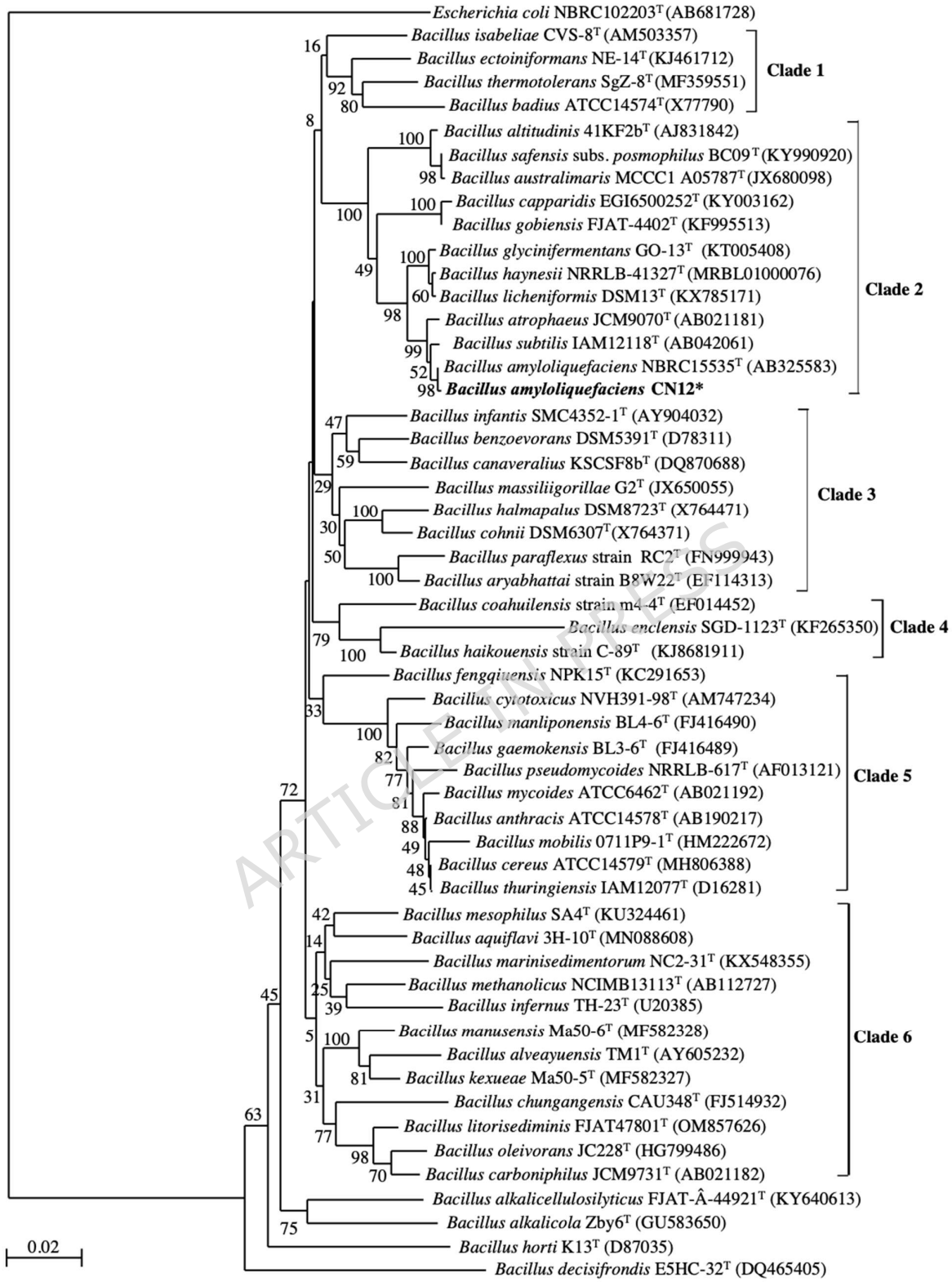

Figure 2. Phylogenetic NJ tree based on the nucleotide sequences of 16S rDNA genes. Our isolated (indicated by star), 52 type *Bacillus* strains from NCBI Genbank were used. Bar indicates 2% sequence divergence. Bootstrap values (at the nodes) were obtained from 1000 bootstrap replicates and are shown as percentages.

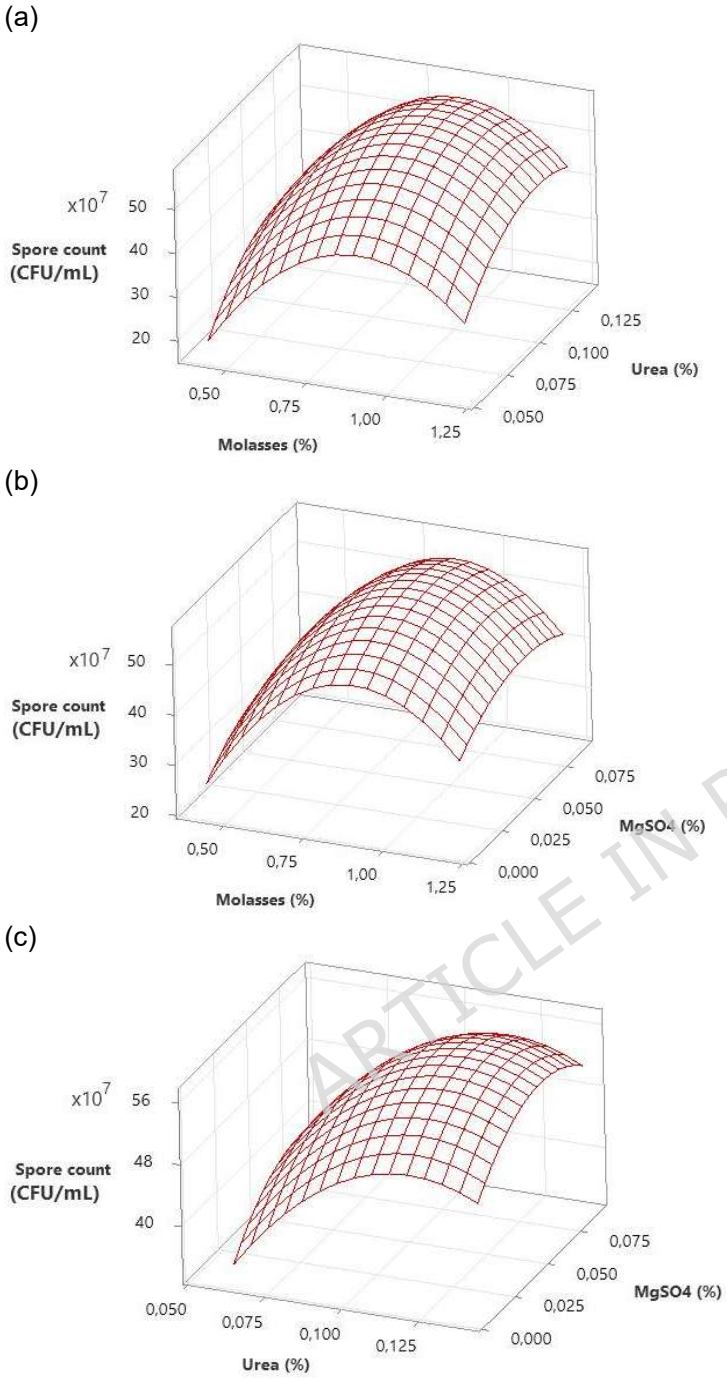

Figure 3. Response surface plots for spore production by *B. amyloliquefaciens* CN12. Effects of molasses and urea on spore formation (a); Effects of molasses and $MgSO_4$ on spore formation (b); Effects of urea and $MgSO_4$ on spore formation (c).

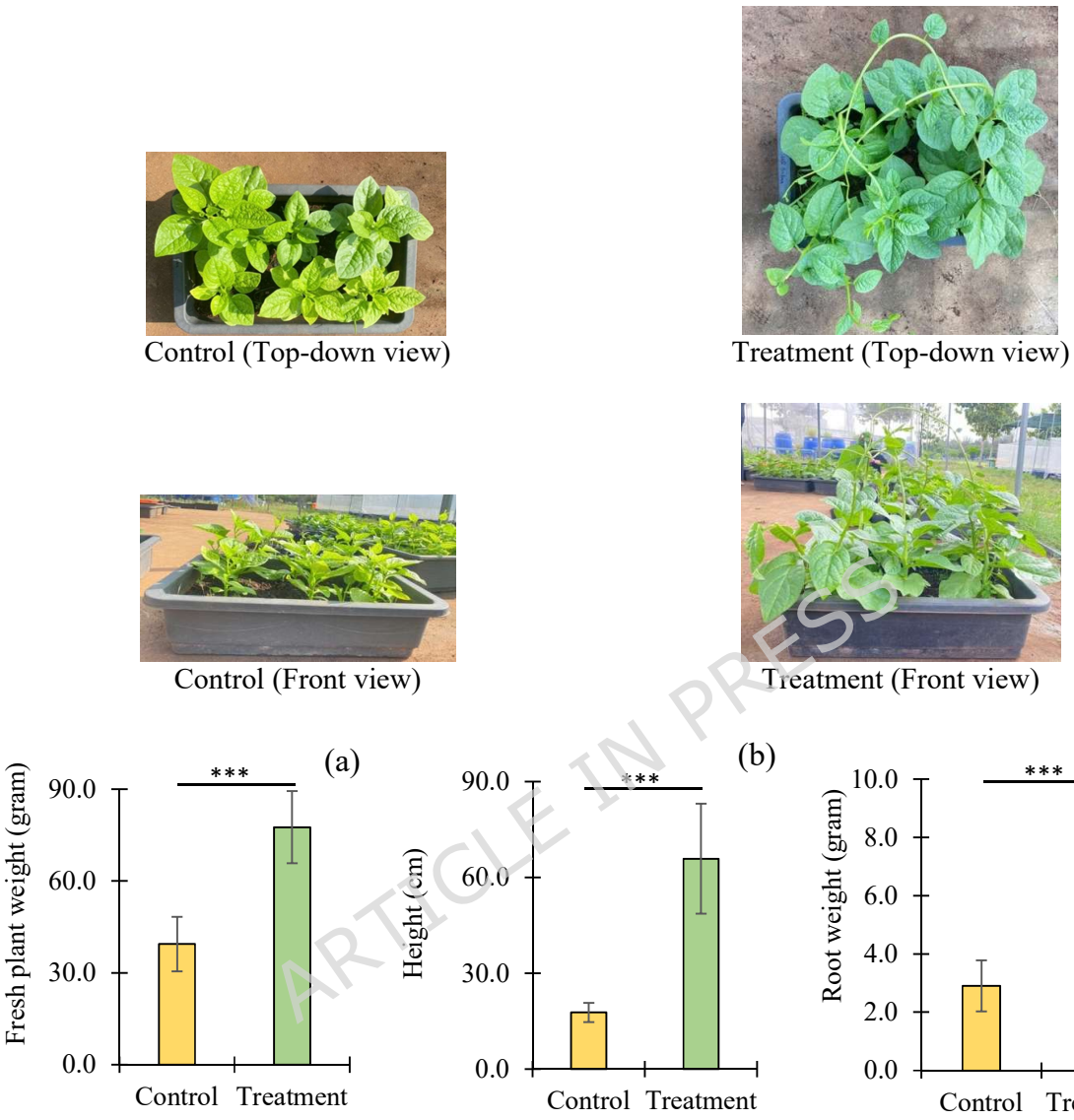

Figure 4. Growth-promoting effect of *B. amyloliquefaciens* CN12 on Malabar spinach (*Basella alba* L.). (a–c) Agronomic traits of Malabar spinach in experimental group and control group were determined, including plant fresh weight, height, and root weight. In the experimental group, 5 mL bacterial suspension (10^8 CFU/mL) was applied to each pot (40 x 60 cm) with temperatures at 30°C during the day and 25°C at night and irrigated once daily. ***$p < 0.001$

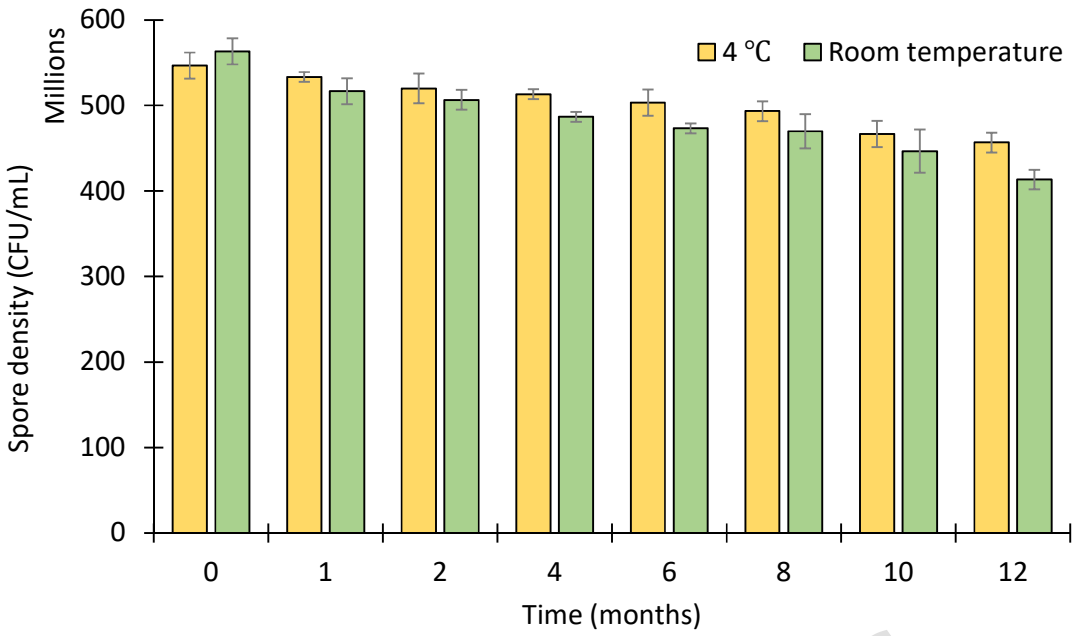

Figure 5. Survival of *Bacillus* spore in formulation during 12 months of storage at 4 °C and room temperature (30 ± 2 °C)