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ASSESSMENT OF CULTURE CONDITIONS OF *Bacillus* sp. DTAN1-M5 STRAIN FOR HIGH PRODUCTION OF INDOLE-3-ACETIC ACID

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Abstract. The bacteria capable of producing indole-3-acetic acid (IAA) were isolated from roots of cassava growing in the Tay Ninh region (Southern of Vietnam). The isolate producing the highest amount of IAA was chosen for the identification and optimization of culture conditions for IAA production. By analyzing the 16S rRNA sequence, the selected isolate was identified as *Bacillus* sp. DTAN-M5. The influence of different parameters on IAA production of these strains was evaluated by the "one factor at a time" method and the obtained results showed that the best conditions for IAA production of DTAN1-M5 were a medium with the following compositions: 1 % peptone, 0.5 % sucrose, 0.6 % yeast extract, 0.5 % NaCl, pH 7.1, supplemented with 0.2 mg/mL L-tryptophan, which was incubated in a shaker at 150 rpm and 30 ^{o}C for 84 hours. The maximum amount of IAA was 84.13 µg/mL.

Keywords: Indole-3-acetic acid, *Bacillus*, PGPB, roots of cassava.

Classification numbers: 3.7.2

1. INTRODUCTION

The plant rhizosphere acts as a unique ecological niche for each plant species and the beneficial bacteria associated with the plant are known as the plant growth-promoting bacteria (PGPB). The microbial consortium in the rhizosphere of plants influences their development and growth, both beneficially and detrimentally. Various soil microorganisms that exert beneficial effects on plants and antagonism on soil-borne pathogens have potential uses in agriculture for improving crop yield and quality. Some of them may be able to improve plant growth by producing phytohormones, increasing seed germination, seedling emergence, protecting plants from external stress factors and pathogens. Plant growth-promoting rhizobacteria can increase nutrient uptake by plants due to their produced plant growth regulators, which stimulate root growth, especially secondary roots, and improve water and nutrient absorption from the subsoil [1, 2].

Indole-3-acetic acid (IAA) is the most abundant naturally occurring auxin, which plays an important role in plant physiology. IAA regulates many plant development processes including differentiation of vascular tissues, elongation growth, apical dominance, lateral root initiation,

fruit setting, and ripening. Besides this, IAA involves in bacteria-plant interaction and plays an important role in microbial physiology, for example, acting as a signaling molecule [2, 3]. IAA is synthesized by soil microorganisms such as bacteria, fungi, and algae through a number of related pathways and the best understood is tryptophan dependent pathway. Plant associated microorganisms synthesize and release IAA as a secondary metabolite using substrates exuded from the plant roots. Members of genera *Rhizobium, Dendrobium, Microbacterium, and Mycobacterium* have been reported to be among the most active IAA producers. Other genera as *Pantoea, Rahnella, Pseudomonas* associated with pear trees also exhibit IAA production ability. Actinomycetes and fungi such as *Streptomyces* sp., *Aspergillus* sp., *Fusarium* sp. and *Paecilomyces* sp. also can synthesize IAA [4]. The capacity of bacteria to synthesize IAA has been studied and confirmed primarily by experiments and in silico analysis of individual genomes mainly affiliated to Proteobacteria or Actinobacteria [3]. Some studies evaluated the effect of IAA-producing bacteria on plant growth [5], isolated IAA-producing bacteria from soil samples and assessed the effect of IAA producers on seed germination by pot experiments. The result showed that isolates F1 and G2 were found to be efficient for plant growth promotion in desired aspects. Some isolates capable of producing IAA were selected to determine their growth-promoting activities of maize, betel, and Kabuli chickpea plants. Among treatments, P7 isolate in a talc-based carrier which produced about 100 ppm IAA could enhance root formation significantly and also keeps its high bacterial population in the talc-based carrier to 7×10^4 CFU/g for up to four months [6]. As a result, the use of biofertilizers with potential biostimulant activity (IAA activity) in agriculture may help enhance plant rooting and productivity. In this study, IAA-producing bacterium was isolated from cassava cultivated soil (cassava root sample) and identified, and growth parameters affecting IAA production of the selected strains were optimized.

2. MATERIALS AND METHODS

2.1. Isolation of bacteria from five cassava root samples

Five cassava root samples in the Tay Ninh region (Southern of Viet Nam) were collected at the center and 4 corners in the same orchard, placed in sterile plastic bags and transported to the laboratory under cold conditions. The standard ten-fold serial dilution method was used for bacterial isolation from the roots. After removing adherent soil, the root samples were put into an autoclave containing 10 mL of distilled water, vortexed, and 1 ml of the solution from each autoclave was passed to a tube and subsequently, a dilution range from 10^{-1} to10^{-7} was prepared. 100 µL of the aliquot from each diluted solution was spread on nutrient agar plates and incubated at 37 ^oC for 2 days. Well-isolated colonies and morphologically distinguishable colonies were picked and purified by streaking on nutrient LB agar plate.

2.2. IAA production assay

IAA-producing isolates were selected by growing them in an IAA production medium as described by Devi *et al*. [7] with some modifications. Briefly, 10 mL of nutrient broth amended with 0.1 mg/mL of L-tryptophan was inoculated with freshly grown cultures and kept at 30 ^oC and 150 rpm for 72 h in an incubating shaker. One mL of the culture was centrifuged at 13.000 rpm for 5 min and 1 mL of supernatant was mixed with 2 mL of Salkowski reagent (50 mL of 35 % perchloric acid mixed with 1 mL of 0.5 M ferric chloride [$FeCl_3$]), followed by 60 minutes of incubation in dark. The optical density (OD) of the solution was measured at 530 nm

(spectrophotometer, Helios) and the amount of generated IAA was calculated by comparing it with the standard curve prepared with pure IAA.

2.3. Identification of selected isolate

The isolate producing the highest amount of IAA was characterized by the 16S rRNA sequence analysis. The universal primer set 16F (5' AGA GTT TGA TCC TGG CTG 3') and 16R (5' GGT TAC CTT GTT ACG ACT 3') (Thermo Fisher) were used for amplification of the 16S rRNA gene. Amplification was performed in a thermocycler with the following PCR conditions: 30 cycles of 95 ^{o}C for 1 min, 50 ^{o}C for 1 min, and 72 ^{o}C for 1 min with an initial denaturation at 95 ^{o}C for 3 min and a final extension at 72 ^{o}C for 7 min.

2.4. The optimal method for IAA production

The process was carried out by "one factor at a time" method. The influence of different parameters such as concentration of L-tryptophan, initial pH medium, different carbon sources, and culture conditions (static or shaking, incubation time) was evaluated. For assessment of the influence of shaking and static culture on IAA production, fresh culture was inoculated in an IAA production medium supplemented with 0.1 mg/mL tryptophan at 30 ^{o}C, shaked at 150 rpm, or kept in static condition. The concentration of L-tryptophan amended in the culture medium was varied from 0.05 mg/mL to 0.25 mg/mL; the pH was adjusted to the desired value using 1 N HCl or 1 N NaOH and four different sugars (sucrose, glucose, lactose, and fructose) were used in the fermentation medium at a concentration of 0.5 % for analyzing their effect on IAA production. The effect of different incubation time (in an interval from 12 h to 120 h) was studied in NB medium supplemented with 0.1 g/L tryptophan, shaking at 150 rpm, 30 ^{o}C, neutral pH.

2.5. Methods of statistical analysis

The statistical analyses of the data were performed using one-way analysis of variance (ANOVA) and graphically represented as the mean ± standard deviation (n=3).

2.6. Determination of nitrogen fixation ability of *Bacillus* sp. DTAN1-M5 strain

The *Bacillus* sp. DTAN1-M5 strain was cultured in liquid Burk's medium (sucrose 20.0 g, K_2HPO_4 0.64 g, KH_2PO_4 0.16 g, $MgSO_4.7H_2O$ 0.20 g, NaCl 0.20 g, $CaSO_4.2H_2O$ 0.05 g, $Ca_2MoO_4.2H_2O$ (0.05 %) 5.0 mL, $FeSO_4.7H_2O$ (0.3 %) 5.0 mL up to 1000 mL H_2O), shaking at 150 rpm, 30 °C with pH ranging from 7.0 to 7.2, collecting samples after 6 days. The concentration of ammonia was determined colorimetrically with Nessler's reagent at the wavelength of 630 nm.

2.7. Agar disk-diffusion method

Agar disk-diffusion testing method was developed in 1940 [8]. In this procedure, agar plates are inoculated with fungal. Then, filter paper discs (about 6 mm in diameter), containing 100 µL of the test compound at the desired concentration, are placed on the agar surface. The Petri dishes are incubated at 28 ^{o}C under suitable conditions. Generally, antifungal agent diffuses into the agar and inhibits germination and growth of the test fungus *P.he* strain and then the diameters of inhibition growth zones are measured.

3. RESULTS AND DISCUSSION

3.1. Isolation and identification of selected isolate

Nine isolates appeared on nutrient agar plates and after obtaining pure isolates, their IAA production ability was evaluated. The isolate DTAN1-M5, which exhibited the highest IAA production capacity of 46.15 ± 1.03 µg/mL after 72 h of incubation in NB medium supplemented with 1 mg/mL L-tryptophan, shaking at 150 rpm and 30 ^{o}C, was selected for further investigation. The isolate was identified by analysis of 16S rRNA sequence and was assigned to *Bacillus* sp. due to the fact that its 16S rRNA sequence was 100 % similar to the corresponding sequence of *Bacillus* sp. strain SG3 in NCBI (As.No MN173963) (Fig. 1).

Before selecting suitable culture conditions for the synthesis of IAA of the selected strain, the bacteria were cultured in NB medium with L-tryptophan for the examination of the IAA production pathway. Salkowski's reaction showed a significant amount of IAA in tryptophan-supplemented medium (changing medium color to pink), whereas no medium color change was observed for NB medium without L-tryptophan. The result confirmed the IAA production pathway of this bacteria was tryptophan-dependent.

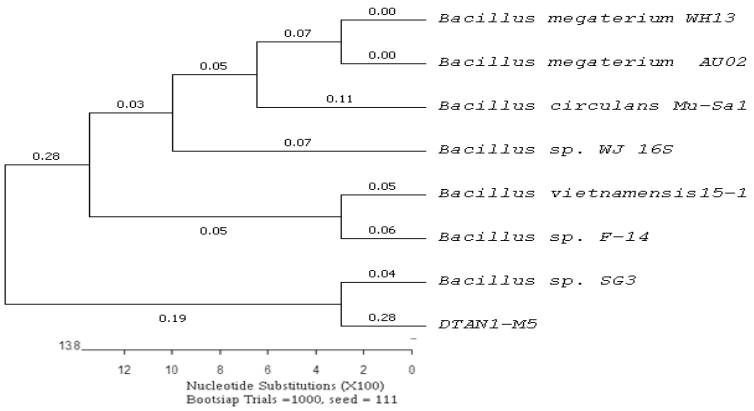

Figure 1. Phylogenetic tree of the isolate *Bacillus* sp. DTAN1-M5.

3.2. Effect of incubation time on IAA production

Analysis of the effect of incubation time on IAA production of the *Bacillus* sp. DTAN1-M5 revealed that the isolates had a significant increase in IAA production with an increase in the incubation period and maximum IAA production was observed at 84 h of culture (56.8 ± 1.35 µg/mL) (Fig. 2).

Studies on the production of IAA by *Aspergillusniger* for 5 - 15 days revealed an exponential increase in IAA production with increasing the incubation period [9]. But the increasing yield of 11.43 µg/mL of IAA production in *Cyanobacteria* was reported on the 21st day [10]. The bacterium *A.diazotrophicus* L1 started to produce IAA at the beginning of its growth and reached the maximum at the starting of the stationary phase, i.e., after 6 days of incubation [11]. Endophytic bacterial isolates were found to have a maximum yield of IAA for 15 days of incubation [12]. Mishra and Kumar [13] stated that the highest accumulation of IAA was observed after 96 h by *B. subtilis* WR-W2. Similarly, Harikrishnan *et al*. [14] also reported the optimum IAA production after 96 h in strain *Streptomyces* sp. VSMGT1014 isolated from rice rhizosphere. The immobilized *A. agilis* A17 cells gradually increased IAA production from

6 to 24 h and peaked (490 mg/L) at 24 h of fermentation, but significantly decreased beyond this time [15]. The strains *Enterobacter* sp. DMKU-RP206 and *B. subtilis* DR2 started to produce IAA after 24 h of incubation. The maximum amount of IAA (448.5 mg/L) was observed at 10 days of incubation (for *Enterobacter* sp.) and 96 h (for *B. subtilis* DR2) and then declined gradually [16, 17]. Our studied strains need only 84 h of incubation for maximum IAA production.

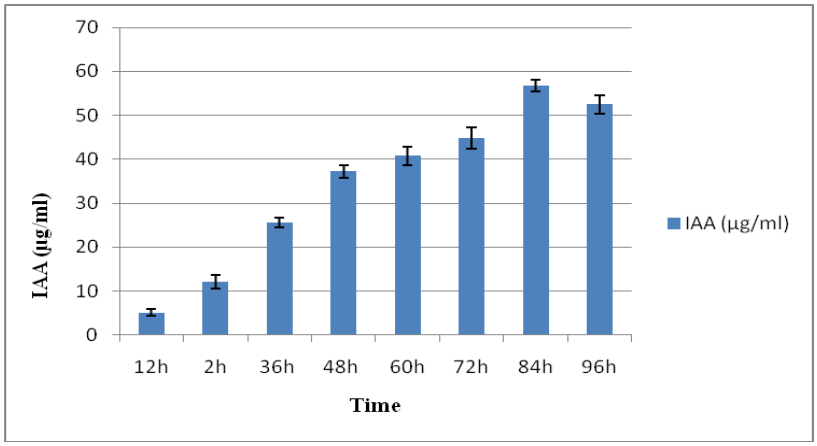

Figure 2. Effect of incubation time on IAA production.

3.3. Effect of L-tryptophan on IAA production

The tryptophan-dependent IAA synthesis in bacteria includes four pathways, in which tryptophan is converted into IAA via different intermediates. The amino acid L-tryptophan serves as a physiological precursor for the biosynthesis of auxins in microbes, so its concentration in the culture medium has a certain effect on the IAA synthesis. As IAA production of *Bacillus* sp. DTAN1-M5 occurs in a tryptophan-dependent manner, the effect of tryptophan on IAA production is very significant. Different concentrations of L-tryptophan in the range from 0.05 to 0.25 mg/mL were applied in the IAA production medium for assessment of its affection. The spectrophotometric analysis showed a gradual increase in the IAA production with increasing L-tryptophan concentration up to 0.2 mg/mL, reaching 61.16 ± 2.2 IAA µg/mL, followed by a decrease when the concentration of L-tryptophan in the medium was 0.25 mg/mL (Fig. 3).

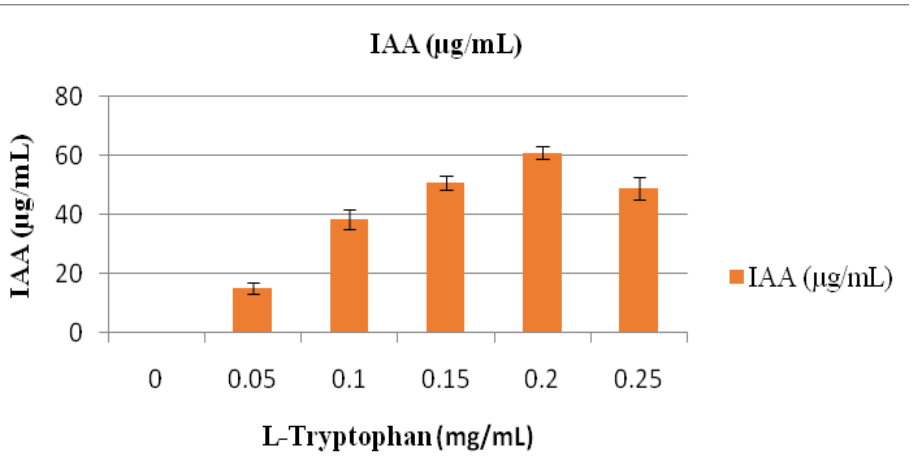

Figure 3. Effect of L-tryptophan concentration on IAA production.

Many studies reported the effect of L-Tryptophan concentration on IAA production. For *Acetobacter diazotrophicus*, the best concentration of tryptophan for IAA production was 1.0 g/L, and up to 1.2 g/L when remaining static, and then declined with increasing tryptophan concentration in the medium [10]. About 1.0 % concentration of L-tryptophan was found to be optimum for IAA production by strains *Serratia marcescens* AL2-16 and then decreased at a higher concentration of tryptophan [7].

Diazotrophic *B. subtilis* DR2, isolated from the rhizosphere of *Eragrostis cynosuroides* produced maximum IAA (168.09 µg/mL) at tryptophan concentration of 1.2 g/L [16]. The report of Napitupulu *et al*. [17]. showed that the IAA production of *Trichoderma harzianum* InaCC F88 in LB medium reached a maximum (9.22 µg /mL) when 1.0% L-tryptophan concentration was amended in the medium. But the presence of the precursor (tryptophan) with a concentration of over 1.0 % decreased the production of IAA. Many existing reports have revealed that among microorganisms, the optimum L-tryptophan concentration for the production of IAA is varied. Plant growth-promoting Actinobacteria *Streptomyces* sp. isolated from rice rhizosphere showed maximum production of IAA (15.96 µg/mL) in the presence of 05 % L-tryptophan, but at concentrations over 0.5 % the production of IAA tended to decrease [14]. In another case, for white-rot fungus *Pleurotus ostreatus* under fermentation condition of Jatropha seedcake in basal salt medium, the maximum concentration of IAA (362.53 µg/mL) was obtained at a concentration of only 0.1 % L-tryptophan [18]. The effect of L-tryptophan on IAA production of marine bacteria *Marinobacter pelagius* B7 was checked to employ the King B medium and the optimum concentration for IAA production was 2 g/L (191 ± 1.52 µg/mL IAA), and the growth of the isolate C7 gradually decreased in proportional with the increase of the L-tryptophan concentration (179 µg/mL IAA). Although the production of IAA of investigated microbes is dependent on L-tryptophan presence and concentration, the concentration of L-tryptophan in the medium excessed optimum concentration decreases IAA production. Our results showed that concentrations of the precursor above 0.25 mg/mL decreased IAA production.

3.4. Effect of agitation on IAA production

For increasing the cell density and improving process productivity, currently, there is an accepted practice to use oxygen-enriched air to support the aerobic growth of both prokaryotic and eukaryotic cells in bioreactors. The effect of agitation on IAA production of *Bacillus* sp. DTAN1-M5 was studied by incubating one set of cultures in a shaking incubator at 150 rpm and the other set was incubated at 30 ^{o}C without shaking. Analysis after the incubation period showed that agitation enhanced IAA production of the bacteria, as IAA produced in agitated samples was much higher (63.4 ± 2.56 µg/mL) than that in the unagitated samples (41.6 ± 1.72 µg/mL) (Fig. 4).

The effect of agitation on IAA production of endophytic bacterial isolates (*Klebsiella pneumonia* PnB 8, *Enterobacter cloacae* PnB 9, *Enterobacter* sp. PnB10, *Klebsiella* sp. PnB11, and *P. agglomerans* Pn B 12) from *P. nigrum* was evaluated and it was found that agitation was a favorite condition for IAA production as unagitated samples showed a significant reduction in the IAA yield. For example, the isolate PnB 8 showed a maximum IAA yield of 295.667 µg/mL under agitation, but the yield of the same under still condition was only 41.66 ± 4.72 µg/mL. Agitation might have favored increased circulation of nutrients and higher availability of oxygen that might have directly affected more microbial mass and enzymatic transformations of tryptophan to auxins [12]. The report of Bharucha *et al.* [19] and this study support the effect of shaking conditions compared to static conditions on increased IAA production.

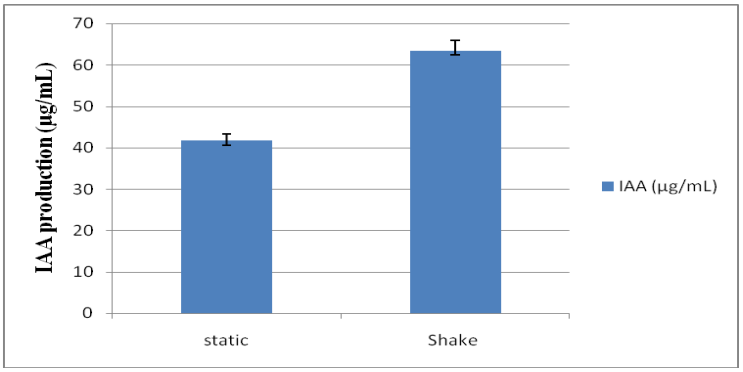

Figure 4. Effect of agitation on IAA production.

3.5. Effect of pH on IAA production

Physicochemical conditions of the media used are always specific for the organisms to biosynthesize the products. One of the most important parameters for each growth of microorganisms, including IAA producing organisms and their metabolic activity is the pH of the production media [8]. The impact of pH in the range of 6-8 on IAA production was checked. The obtained result showed that IAA production of DTAN1-M5 increased with increasing pH from 6 to 7.1 and reached the maximum value (76.67 ± 2.15 µg/mL) at this pH. The IAA production then decreased stepwise with further increasing pH (Fig. 5).

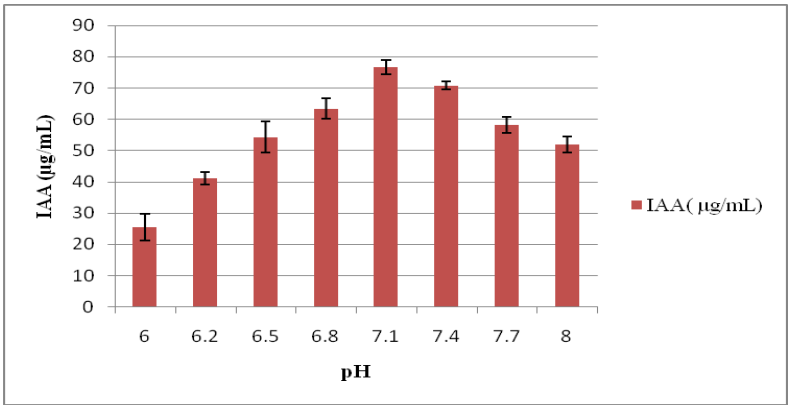

Figure 5. Effect of pH on IAA production.

Mandal *et al.* [20] reported that pH 7.2 elaborated high levels of IAA production in *Rhizobium* strain VMA 301. There's maximum IAA production at pH 6 by *A. diazotrophicus* L1, high acidic and alkaline pH was not suitable for IAA production of these strains [11]. pH 7 has also been reported to be optimum for maximum IAA production by *Pantoea agglomerans* PVM [21]. The best IAA formation by *Streptomyces viridis* CMU-H009 was obtained at pH 7 [22]. Synthesis of the highest IAA level was determined in cultures (*Bacillus megaterium*, B. *subtilis*, *Lactobacillus casei*) cultivated in an alkaline medium at pH 8 [23]. Maximum IAA concentration (370 mg/L) was obtained at an initial pH of 8.0, but no statistically significant difference between pH 8.0 and pH 9.0 of immobilized *Arthrobacter agilis* A17 cells [15]. In the investigation of Kumari *et al.* [16] maximum (158.79 µg/mL) indole production was observed at pH7 in Trp+ media and high acidic and alkaline pH was not suitable for IAA production.

Interestingly, endophytic bacteria isolated from *P.nigrum* had the maximum production of IAA at pH 4, and IAA production by these selected isolates reduced dramatically with increasing pH to alkaline level. Maybe these bacteria were isolated from the slightly acidic pH of the stem tissue of the *P.nigrum*, so they might have adapted for maximal IAA production at the acidic pH [11]. Moreover, according to Duca *et al.* [24] the pH and temperature can affect the activity of enzymes involved in the biosynthesis of IAA. From our obtained result and other reports, bacteria can be expected to have a preference to produce IAA at a particular pH, based on the source of isolation.

3.6. Effect of carbon source on IAA production

The carbon sources provide energy and improve co-factor recycling in the cells, and are used in the production of secondary metabolites, so they have a profound effect on the overall efficiency of biosynthesis. Thus, carbon sources contribute to the overall efficiency of IAA biosynthesis by microorganisms [16, 1]. In this study, four different sugars (sucrose, fructose, lactose, and glucose) were added externally to the production medium in order to evaluate their effect on IAA production of DTAN1-M5 strain. Glucose at a concentration of 0.5 % was found to be the best carbon source, producing 84.13 ± 1.36 µg/mL of IAA, followed by sucrose and lactose (82.7 ± 2.84 and 66.4 ± 1.01 µg/mL, respectively). Contrary, fructose exhibited a negative effect on IAA production of DTAN1-M5, producing only 55.13 ± 2.78 µg/mL of IAA, much lower than that from the control sample (63.73± 1.9 µg/mL) (Fig. 6).

The report of Patil *et al.* [15] showed that sucrose is the best carbon source for the growth of *Acetobacter diazotrophicus*, and maximum IAA production occurred at 12 % (w/v) of sucrose. The *Enterobacter* sp. DMKU-RP206 utilized various carbon sources for IAA production and among the 17 carbon sources tested, lactose was found to be the best choice for IAA production (559.6 mg/L), followed by mannitol and starch [17]. But IAA-producing bacterial isolates CA1001, CA2003, and CA2004 from plant root preferred dextrose for their IAA production, with 104 µg/mL of IAA for CA1001 and 50 µg/mL of IAA for CA2003 [1]. For B. *subtilis* DR2 using the mannitol carbon source in the synthetic culture medium, the maximum yield was achieved at 160.85 µg/mL [16]. Other studies also optimized IAA production by different carbon sources as well as their combinations, such as mannitol in *B.subtilis*-W2 [15, 16] and *Arthrobacter agilis* [25]. The strains *Bacillus* sp. DTAN1-M5 also preferred sucrose over other sugars.

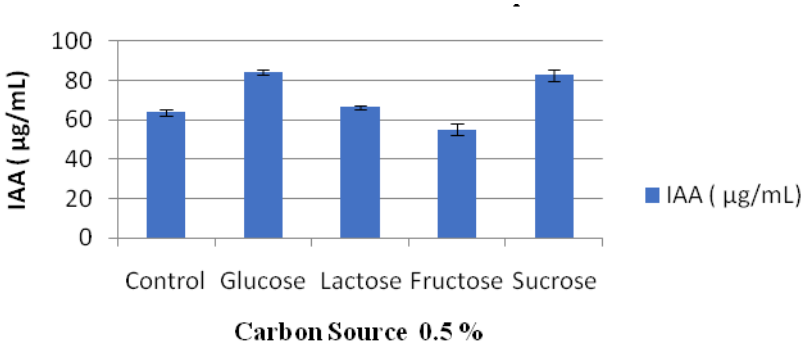

Figure 6. Effect of carbon source on IAA production.

3.7. Determination of nitrogen fixation ability of *Bacillus* sp. DTAN1-M5 strain

Many studies show that the *Bacillus* sp. strains beyond the ability to synthesize IAA also have the ability to fix nitrogen. Our survey showed that *Bacillus* sp. DTAN1-M5 strain has a high nitrogen fixation ability up to 4.82 µg/mL compared to the report Nguyen Hai Van & Nguyen Thi Minh [3]. *Bacillus* sp. DTAN1-M5 antagonist we isolated on roots of diseased cassava tubers, after 1 day, has a diameter of 22 mm of inhibition ring. Many studies have reported that the *Bacillus* sp. strains beyond the ability to synthesize IAA also have the ability to fix nitrogen.

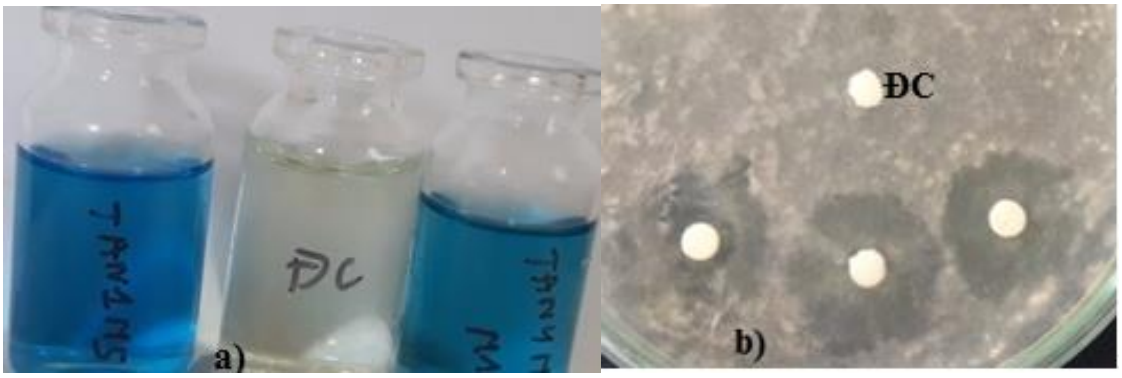

Figure 7. a) Nitrogen fixation ability of *Bacillus* sp. DTAN1-M5 strain.
b) Antifungal *P.he* activity of *Bacillus* sp. DTAN1-M5 strain.

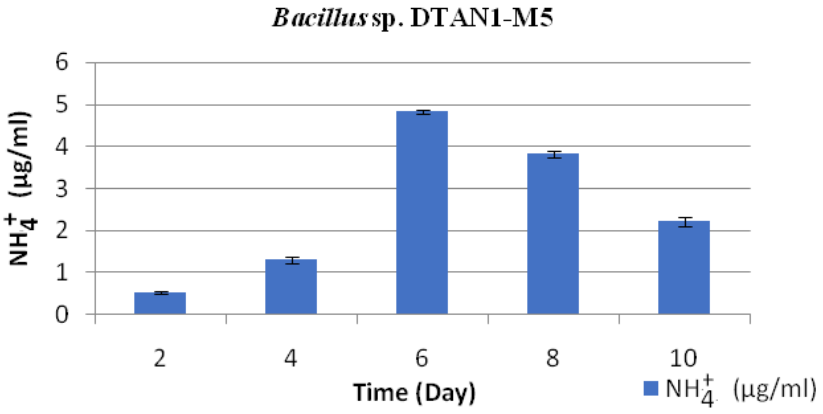

Figure 8. Nitrogen fixation ability of *Bacillus* sp. DTAN1-M5 strain.

Bacillus sp. DTAN-M5 is a strain isolated from an infected cassava soil sample in Tay Ninh province. Our study showed that the strain of *Bacillus* sp. DTAN1-M5 has many good biological activities and high ability to synthesize IAA, and can fix nitrogen supply for cassava. The study also demonstrated that *Bacillus* sp. DTAN1-M5 is an inhibitor for pathogenic fungi isolated from pathogenic tapioca root samples, we denote *P.he*.

P.he is a fungus isolated from pathogen samples of cassava tubers and roots. Soil samples collected in the same area also have this fungus. When we reinfected on 1 month old fresh cassava roots and saplings, the symptoms of the disease were similar to the samples of diseased

cassava roots and tubers collected in Tay Ninh province.

4. CONCLUSIONS

The strains *Bacillus* sp. DTAN-M5 isolated from cassava roots can produce IAA in a tryptophan dependent manner, and suitable conditions for IAA production of this isolate were determined as follows: a medium containing 1 % peptone; 0.5 % sucrose, 0.6 % yeast extract, 0.2 mg/mL L-tryptophan, 0.5 % NaCl, pH 7.1 was prepared, followed by the culturing process in a shaking incubator at 150 rpm and 30 ^{o}C for 84 hours. The DTAN-M5 has a great potential for improving cassava growth and yield. *Bacillus* sp. DTAN1-M5 strain has a high nitrogen fixation ability up to 4.82 µg/mL. We isolated *Bacillus* sp. DTAN1-M5 on the antifungal tapioca roots with an inhibitory ring diameter of 22 mm on the pathogenic fungi isolated from the diseased cassava.

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CRediT authorship contribution statement. Pham Viet Cuong: conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft. Nguyen Phuong Hoa: conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Declaration of competing interest. The authors declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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