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ISOLATION AND IDENTIFICATION OF BIOFLOCCULANT-PRODUCING BACTERIA FROM CASSAVA CULTIVATED SOIL IN VIET NAM

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Abstract. Microorganisms are found everywhere in the environment and play a leading role in countless natural processes, and amongst them, soil microorganisms play important roles in plant performance by improving mineral nutrition. Soil bacteria possess different biological activities; for example, producing bioactive compounds such as bioflocculant which makes soil smoother and more breathable, as plant growth stimulants or compounds with antibiotic activity. Beside this, the same soil bacteria could also be able to fix atmospheric nitrogen, converting it into the form that plants can use. In order to make a microbial formula from local bacteria capable of producing bioflocculant as well as indole-3-acetic acid (IAA) and fixing nitrogen towards microbial organic fertilizer production for application in cassava cultivation, the bacteria were isolated from cassava cultivated soils and screened for above mentioned activities. From 37 cassava root and cassava cultivated soil samples collected at different provinces in the North, Central and South regions of Viet Nam, 80 strains of bacteria with bioflocculant activity over 50 % were screened. In which 3 strains (DQT1 Po6, DTAN6 Po2 and DSBV Po1) possess not only high bioflocculant and IAA producing capacities, but also the ability to fix atmospheric nitrogen. The flocculant rate of these strains was 80 ÷ 90 % and can produce 6 ÷ 11 µg/mL IAA. The selective strains were identified by having an analysis of 16S rRNA gene sequence. The obtained results showed that the 16S rRNA sequence of DQT1 Po6 was 100 % identifiable to corresponding sequence of *Bacillus aryabhattai* FORT 21 (Ass.No MG561348.1); and the 16S rRNA sequences of DTAN6 Po2 and DSBV Po1 had 100 % homology with those of *Enterobacter* sp. (Ass.No KJ184880.1) and *Bacillus subtilis* SAN1.5 (Ass.No KX098457.1), respectively. These strains are potential candidates as perfect inoculant for biofertilizer.

Keywords: bioflocculant producing-bacteria, flocculating rate, *Bacillus aryabhattai*, *Enterobacter* sp., *Bacillus subtilis*.

Classification numbers: 1.3.1; 1.3.2.

1. INTRODUCTION

Cassava is one of the crops of the 21st century and a "food for the poor", becoming a versatile crop meeting the priorities of developing countries, including Viet Nam. But the mass

production of cassava also carries great risks, including pest and disease outbreaks, and depletion of soil nutrients. For cassava as well as all other crops, soil quality is an important factor determining the yield and quality of the product. Many biotic and abiotic factors affect soil quality, including microbiota present in the soil. Bacteria and fungi together influence the structure of the soil because they produce humus, the most important component of the soil, which affects the surface permeability and the richness of organic compounds, to its fineness and soil aeration by the generation of mucus particles [1]. Auxin-producing, nitrogen-fixing species of *Bacillus* genus have been reported to have a positive effect on the growth of some plants [2].

Bioflocculant is an extracellular polymer produced during the growth of microorganisms or from metabolites of microorganisms, resulting in the formation of stable aggregates of flocks and it has different composition such as protein flocculants, polysaccharide flocculants, glycoprotein flocculants and poly (amino acid) flocculants [3 - 6]. In recent years, many studies have shown that microbial flocculants is safe, non-toxic to human and the environment. Many flocculant-producing microorganisms have since been isolated from sludge, soil, sediments, river, seawater, etc. [7]. Common microorganisms that can produce flocculants include gram positive bacteria (*Bacillus pumilus*, *Bacillus mojavensis*, *Bacillus* sp., *Rhodococcus* sp., etc.), Gram negative bacteria (*Enterobacter* sp., *Alcaligenes latus*, etc.), and other microorganisms (*Agrobacterium*, *Colletotrichum dematium*, *Acinetobacter*, *Aspergillus*, *Paecilomyces*, etc.) [5 - 10].

The polysaccharides are the most studied components of bioflocculant. The investigation of bioflocculant from numerous strains of microorganisms has demonstrated that the polysaccharides in these biopolymers vary immensely in composition and structure. Bioflocculants have been successfully used in various fields including treatment of waste water and industrial effluents for turbidity and organic pollutant removal, dye decolorization, and heavy metal removal. Another function of bioflocculant mostly related to protection. The matrix produced by bioflocculant around microbial cells has the capability of shielding them against antimicrobial compounds and heavy metals; bioflocculant can also retain water, protecting microbes and the environment against drought. In addition, other functions, such as antioxidant, adhesion, aggregation, carbon storage, and entrapment of nutrients have also been reported [11 - 13].

One of the roles of the bioflocculant that has been explored for decades is the capacity to aggregate soil particles, a function that is important for soil structure, health, and fertility. Since bioflocculant has a slimy texture and ionic charges, it can act like a glue, getting attached to clay and ions, holding solid particles together. According to some authors, it is possible to study and apply bioflocculant to soils for improvement of soil structure, fertility, and quality [11]. In this study, the bioflocculant producing bacteria from cassava cultivated soils in Viet Nam were isolated and screened potential strains for application in micro-biofertilizer production. The selected strains were also assessed for their ability of indole-3-acetic acid (IAA) synthesis and Nitrogen fixation.

2. MATERIALS AND METHODS

2.1. Materials

37 soil and cassava root samples (25 soil samples and 12 root samples) were collected from cassava growing provinces in the North (Ha Noi city, Phu Tho and Hoa Binh provinces), Central (Quang Tri province), and the South (Tay Ninh province) of Viet Nam. The samples were

collected from each site at about 5 - 30 cm depth. Samples were collected in the dry season (April - May 2019) and rainy season (July - December 2019). The samples were transferred into sterile sampling bags to avoid loss of moisture and any outside contamination, and kept at 4 ^{o}C until processing in the laboratory.

The composition of the medium for screening was as follows: 10 g/L glucose, 2 g/L KH_2PO_4, 5 g/L K_2HPO_4, 0.2 g/L $MgSO_4.7H_2O$, 0.1 g/L NaCl, 0.5 g/L carbamide, 0.5 g/L yeast extract was used for isolation of polysaccharide bioflocculant producing bacteria (Poly medium). Initial pH of the medium was set at 7.0, then was autoclaved at 121 ^{o}C for 20 minutes [14].

Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, and distilled water 1.000 mL) with L-tryptophan (5 mM) was used for qualitative assay of IAA production [15].

Burk's N-free medium is used for cultivation of nitrogen fixing bacteria comprising: 10 g/L glucose, 0.41 g/L KH_2PO_4, 0.52 g/L K_2HPO_4, 0.05 g/L Na_2SO_4, 0.2 g/L $CaCl_2$, 0.1 g/L $MgSO_4.7H_2O$, 0.005 g/L $FeSO_4.7H_2O$, 0.0025 g/L $Na_2MoO_4.2H_2O$. The pH of the medium was adjusted to 7.0 ± 0.1 before autoclaving at 121 ^{o}C for 15 min [16].

2.2. Methods

Isolation of bioflocculant-producing bacteria from cassava cultivated soil and cassava root samples

For soil samples: 10 g of each sample (after removing root or leaves in soil) were mixed with 90 mL sterile saline and homogenised (NB-205QF, N-Biotek, Korea) for 10 min. For root samples: 1 g soil tightly adhering to the cassava root was put in 50 mL flask, 9 mL sterile saline was added and homogenized (NB-205QF, N-Biotek, Korea) for 5 min. Next, 1 mL of solution of each sample was serially diluted up to 10^{-7}, then 0.1 mL of diluted solution was spread on sterile production Poly-agar medium and incubated for 2 - 3 days at 30 ^{o}C. Single colonies were picked up and streaked on fresh Poly-agar medium plates to get pure culture and observe the morphological characterization [17].

Bioflocculants production and determination of the flocculating activity

All experiments were performed in triplicate. Flocculating rate was used as a measurement of the flocculating activity of the bioflocculants. Pure bacterial strain was inoculated into a flask containing 50 mL of Poly medium and incubated on a shaker at 150 rpm for 4 days at room temperature for bioflocculant production. The flocculating rate was determined by absorbent measurement of a mixture of 90 mL Kaolin solution (5 g/L), 10 mL of 1 % $CaCl_2$ solution (pH 7.0), 100 µL of cultured bacteria solution (cell density 10^9 CFU/mL). The solution was gently and thoroughly mixed at room temperature and left for standing for 5 minutes. The formation of visible aggregates was observed and its absorbance was measured at wavelength of 550 nm [6, 9]. The control sample was prepared similarly, but without cultured bacteria. The flocculating rate was defined and calculated as follows:

$$\text{Flocculating rate} = [(A - B)/A] \times 100\ \%,$$

where: A = OD_{550} of control sample, B = OD_{550} of bacterial sample.

Biochemical characterization and identification of the strains

Bacterial strains with high bioflocculant production ability Gram stained (according to Manual of Gram Staining Kit, Mecrk, Germany), morphological characteristics were observed and some biochemical tests were performed, such as: mobility, indole test, methyl red (MR) test, catalase test, starch hydrolysis and casein hydrolysis, growth in 7 % NaCl, growth at 50 ^{o}C and 65 ^{o}C, lecithinase production, Voges-Proskauer (VP) test, citrate fermentation reaction [18].

Identification of bacteria by analysis of 16S rRNA sequence: The total DNA of selected strain was isolated according to DNeasy Power Lyzer Microbial Kit (Qiagen, Germany) and the 16S rRNA gene was amplified using universal primers: 16SF (5′-AGA GTT TGA TCA TGG CTC A-3′) and 16SR (5′-AAG GAG GTG ATC CAG CC-3′) (Invitrogen, USA). The PCR cycle parameters: an initial denaturation at 94 ^{o}C for 3 minutes, followed by 30 cycles of denaturation at 94 ^{o}C for 1 minute, annealing at 50 ^{o}C for 1 minutes, amplification at 72 ^{o}C for 1.3 minutes and final extension at 72 ^{o}C for 7 minutes. The 16S rRNA genes were sequenced by DNA Analyzer (ABI PRISM 3100, Applied Bioscience). 16S rRNA sequences were matched with sequences in the GenBank database using the Blast search programme (http://www.ncbi.nlm.nih.gov/). A phylogenetic tree based on the 16S rRNA sequences of the strains was generated using MegAlign via the Clustal W algorithm available in DNA Star Lasergene v.8.0 with 1000 bootstrap replicates.

Other biological activities screening

Bacterial strains with high bioflocculant production capacity were selected for the evaluation of phosphate solubilizing activity, IAA biosynthesis and nitrogen fixation ability.

Assessment of phosphate solubilizing activity

Bacteria were inoculated on the sterile Pikovskaya agar medium [19] containing 10 g of tricalcium phosphate (TCP) as sole phosphorus source for selectively screening P-solubilizing bacteria. The reaction was considered positive when a clear halo surrounding the bacterial colonies was observed after 3-7 days of incubation at 30 ^{o}C. The experiment was performed in triplicate.

Qualitative assay of IAA production

Bacteria were inoculated in the LB broth medium and incubated at 30 ºC on rotary shaker (150 rpm) for 24 hrs. Each bacterial inoculum was further transferred into 10 mL LB fresh broth medium amended with 1 % of 5 mM L-tryptophan, and then incubated at 30 ºC on rotary shaker (120 rpm) for 96 hrs. IAA production assay was conducted using Salkowski's reagent. One mL of the culture was centrifuged at 13,000 rpm for 5 min. Supernatant was further transferred into tubes amended with 1 mL Salkowski's reagent. The tube was incubated at 30 ºC for 30 min. Positive reaction was indicated by a change in the color of the medium into pink or violaceous. The generated IAA was measured at 530 nm using spectrophotometer [15].

Qualitative assay of nitrogen fixing

Solutions for testing of nitrogen fixation include: (1) phenol-alcohol solution: 11.1 mL liquidified phenol (89 %) was mixed with ethyl alcohol 95 % (v/v) to a final volume of 100 mL; (2) sodium nitroprusside 0.5 %: dissolve 0.5 g sodium nitroprusside in 100 mL deionized water; (3) The alkaline citrate solution was prepared by dissolving 100 g trisodium citrate and 5 g sodium hydroxide in deionized water and diluting to a final volume of 500 mL. Sodium

hypochlorite was diluted to 5 %. 100 mL of alkaline citrate solution was mixed with 25 mL sodium hypochlorite to prepare the oxidizing solution. NH_4Cl standard solution (1 mg/L). All analyses were performed in the same day [39].

Construction of standard curve $NH4^+$(Cl) (mL):

Tube's number	0	1	2	3	4	5
Deionized H_2O	4.00	3.96	3.92	3.88	3.84	3.80
NH_4^+(Cl) 100 ppm	0.00	0.04	0.08	0.12	0.16	0.20
Phenol-alcohol solution	0.00	0.04	0.08	0.12	0.16	0.20
Phenol-alcohol solution	0.16	0.16	0.16	0.16	0.16	0.16
Sodium nitroprusside	0.16	0.16	0.16	0.16	0.16	0.16
Oxidizing solution	0.40	0.40	0.40	0.40	0.40	0.40
Standard road concentration (ppm)	0.00	1.00	2.00	3.00	4.00	5.00

Handling cultures and quantification:

Bacterial strain was inoculated in the N-free Burk's broth medium and incubated at 28 - 30 ºC on rotary shaker (150 rpm) for 48 hrs. Then culture solution was centrifuged at 12,000 rpm for 5 minutes. 1 mL supernatant was transferred into a test tube containing 3.5 mL of demineralized water, then 0.16 mL phenol solution, 0.16 mL sodium nitroprusside solution and 0.40 mL oxidizing solution were added with thorough mixing after each addition. The samples were covered with plastic wrap or Parafilm and kept in the dark at room temperature (22 to 25 ^oC) for 15 - 20 minutes. The absorbance was measured at 640 nm [20].

Effects of cultural conditions on the flocculating activity

Influence of cultural conditions (carbon and nitrogen sources, pH, temperature, incubation time) on flocculating activity was determined by "one factor at a time" method. The bacteria were incubated in production medium supplemented with 1 % different carbon sources (glucose, lactose, mannitol, sucrose) and 0.5 % different nitrogen sources (beef extract, yeast extract, peptone and tryptone); varying pH medium (5.0, 6.0, 7.0 and 8.0); different cultivation temperatures (25, 30, 37 and 40 ^oC), and different incubation periods (24 hrs, 48 hrs, 72 hrs and 120 hrs), then the flocculating activity of strains was measured.

3. RESULTS AND DISCUSSION

3.1. Isolation of bioflocculant-producing bacteria

Mucus producing bacteria were isolated from 37 soil and root samples collected in cassava growing fields of Viet Nam. The total amount of bacteria isolated on the Poly-agar medium was in the range of 10^5 - 10^6 CFU/g. The slimy colonies obtained from soil samples were more than from root samples.

Most colonies on production medium were round, white, had a slippery, sticky, and wet surface. Out of total 599 isolates, there were 80 strains that exhibited more than 50 % of flocculating activity.

Three strains (DQT1 Po6, DTAN6 Po2 and DSBV Po1) with the highest flocculant rate (98.01 %; 85.07 % and 80.65 %, respectively) were selected for further studies. The Gram

staining showed that, cells of selected bacterial isolates were rod shape. Morphological characteristics of these selected 3 strains were described in Figure 1 and Table 1.

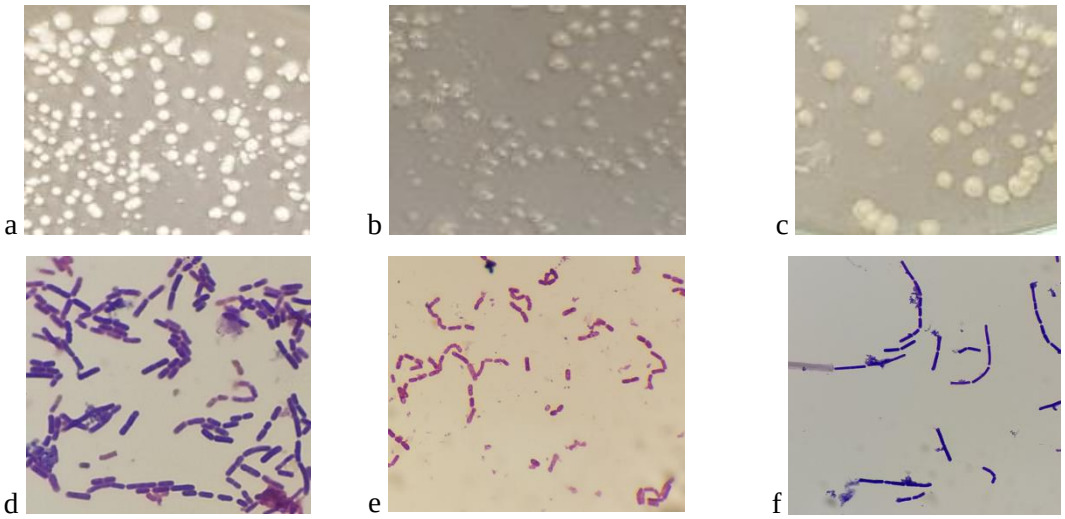


Figure 1. Colonies of 3 selected strains on Poly-agar medium and gram staining results {(a, d) DQT1 Po6; (b, e) DTAN6 Po2 and (c, f) DSBV Po1}.

Table 1. Morphological characteristics of 3 selected strains.

Morphological characteristics	Strains		
	DQT1 Po6	DTAN6 Po2	DSBV Po1
Gram staining	Positive	Negative	Positive
Colony	Irregular, circular, white, convex and viscous	Irregular, circular, off-white, viscous	Irregular, circular, opaque, white and convex.
Morphology of cell	Average rods, single or in double, small	Short rods, single or in double, small	Long rods, single or in double, small

3.2. Biochemical characterization and identification of the 3 selected strains

The biochemical tests showed that the 3 selected strains can produce catalase, hydrolyze starch and casein and utilize citrate as a source of energy. The strains were not able to convert tryptophan to indole and do not possess lecithinase activity. Besides, 3 selected strains showed positive motility through diffuse zone of growth extending out from the line of inoculation in semisolid LB agar medium. Concerning the capacity of growing at high temperature, all 3 selected strains were able to grow at 50 ^{o}C, but not at 65 ^{o}C. The VP test of the DTAN6 Po2 isolate was negative, but for the remaining 2 strains (DQT1 Po6, DSBV Po1) this assay was positive (Table 2).

For identification of the strains by analyzing 16S rRNA sequences, the total DNA of 3 selected strains was isolated and verified for purity by ultraviolet absorption spectra and 1 % agarose gel electrophoresis (Fig. 2a). The A_{260}/A_{280} ratio of isolated DNA was in the range 1.8 - 1.9, indicating that the DNA met the requirements of PCR. The PCR was carried out according

to the conditions described above. The PCR product of 16S rRNA gene was about 1.5 kb on electrophoresis image, corresponding to the theoretical calculation (Fig. 2b). PCR products were purified using DNA purification kit (Invitrogen) and sequenced (ABI PRISM 3100, Applied Bioscience). Obtained sequences were analyzed using Bioedit programme and aligned with correlative sequences in GenBank by online software BLAST (www.ncbi.nlm.nih.gov/BLAST/). The obtained result showed that nucleotide sequence of 16S rRNA gene of DQT1 Po6 strain having 100 % homology with 16S rRNA gene *Bacillus aryabhattai* FORT21 (Ass.No MG561348.1); the 16S sequence of the strain DTAN6 Po2 showed 100 % sequence homology with those of *Enterobacter* sp. (Ass.No KJ184880.1) and the 16S sequence of the isolate DSBV Po1 exhibited 100 % homology with this gene of *Bacillus subtilis* SAN1.5 (Ass.No KX098457.1). Constructed phylogenetic tree and biochemical characteristics of these strains also confirmed that DQT1 Po6, DTAN6 Po2, DSBV Po1 were close related to *Bacillus aryabhattai*, *Enterobacter* sp. and *Bacillus subtilis*, respectively (Fig. 3).

Table 2. Some biochemical tests of the 3 selected strains.

Name of the tests	Response of the strains		
	DQT1 Po6	DTAN6 Po2	DSBV Po1
Motility	+	+	+
Indole production	-	-	-
Methyl Red reaction	+	-	+
Catalase	+	+	+
Starch hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Growth in 7 % NaCl	+	+	+
Growth at 50 ^{o}C	+	+	+
Growth at 65 ^{o}C	-	-	-
Lecithinase production	-	-	-
Voges-Proskauer reaction	+	-	+
Citrate Utilization	+	+	+

(+): positive; (-) : negative

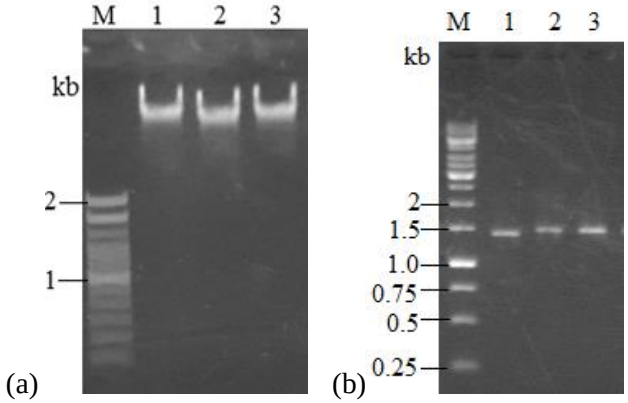


Figure 2. Total DNA (a) and PCR product (b) of 16S rRNA gene on 1 % agarose gel.

M: Marker DNA 100 bp (a) and 1 kb (b) (Invitrogen). 1: DQT1 Po6; 2: DTAN6 Po2 and 3: DSBV Po1.

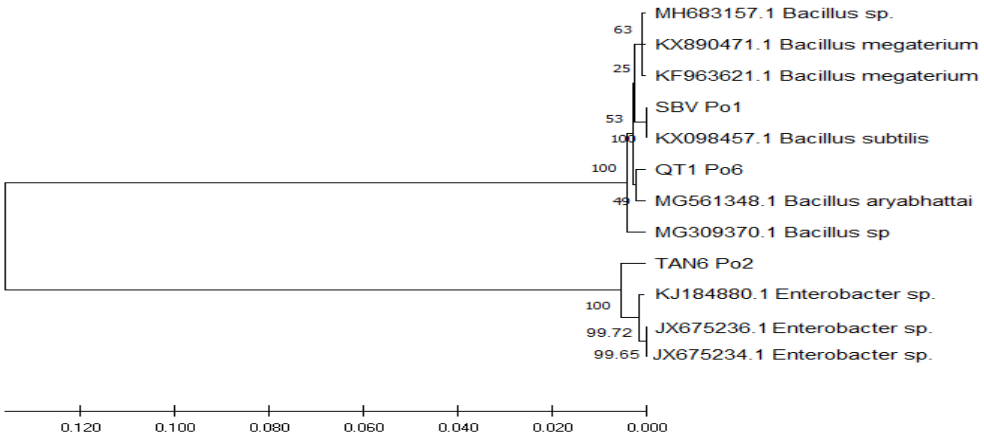
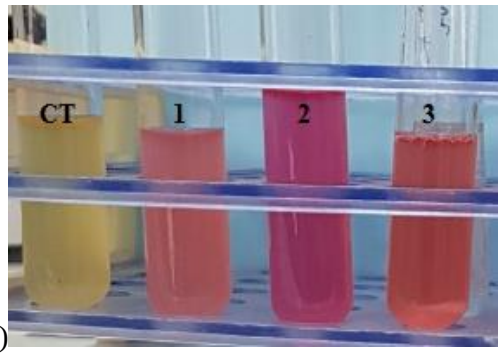


Figure 3. Phylogenetic tree of flocculant producing strains.

3.3. Evaluation of other bioactivities of the selected strains

Three studied strains (*B. aryabhattai* DQT1 Po6, *Enterobacter* sp. DTAN6 Po2 and *B. subtilis* DSBV Po1) were evaluated for phosphate solubilization, biosynthesis of IAA and nitrogen fixation as described in the method section. Phosphorus hydrolyzing capacity of the strains was negative because after 3 - 7 days of incubation on Pikovskaya's agar plate, no clear zone was observed around colonies of them. Thus, all 3 strains are unable to dissolve inorganic insoluble phosphorus compounds to soluble P form. Concerning the qualitative IAA production analysis, all 3 investigated strains could be able to metabolize L-tryptophan (TRP) into IAA or some analogous compounds of IAA and this was indicated by changing of the medium's color into pink (Fig. 4a).

(a) 

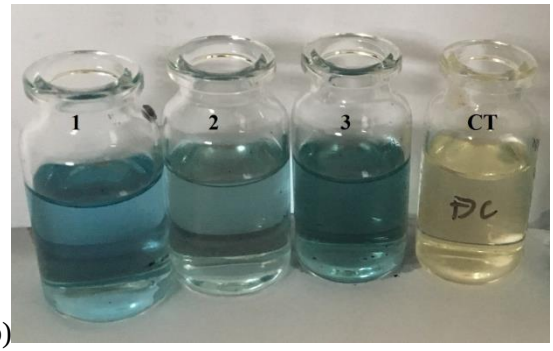
(b) 

Figure 4. Qualitative screening of IAA production (a) and nitrogen fixation (b).

(a): CT: Negative control, 1: *B. aryabhattai* DQT1 Po6, 2: *Enterobacter* sp. DTAN6 Po2, 3: *B. subtilis* DSBV Po1,

(b): CT: Negative control, 1: *B. aryabhattai* DQT1 Po6, 2: *Enterobacter* sp. DTAN6 Po2, 3: *B. subtilis* DSBV Po1.

This phenomenon occurrs due to the reaction of Salkowski's reagent with IAA, forming tris-(indole-3-acetato)-iron(III) complex having a pink coloration. In the quantitative assay, the intensity of pink color determined the robustness of IAA amount produced by bacterial strains.

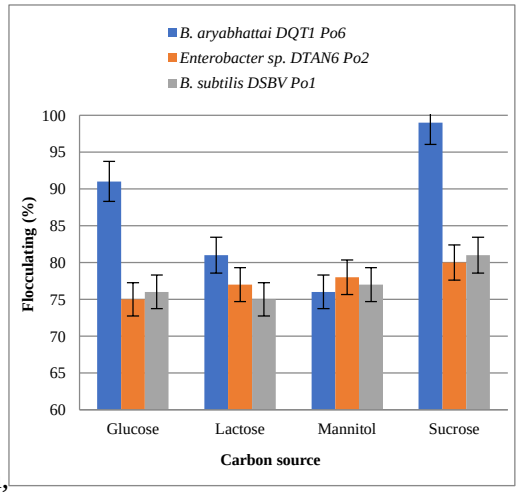
Based on this parameter, all 3 studied strains had produced a quite high amount of IAA (Fig. 4a). Qualifying test pointed out that the amount of IAA produced by the strain *B. subtilis* DSBV Po1 and *Enterobacter* sp. DTAN6 Po2 was higher than that of *B. aryabhattai* DQT1 Po6, about 10.12 µg/mL, 9.92 µg/mL and 6.33 µg/mL, respectively.

The nitrogen fixing ability of the bacterial strains was demonstrated by the spectral absorption at 640 nm of the samples after treatment. The darker the green samples, the greater the spectral absorption and the higher the nitrogen synthesis capacity (Fig. 4b). Quantification of nitrogen fixation ability of 3 studied bacterial strains after 2 days cultured in N-free Burk's broth medium showed that the amount of ammonium produced ranged from 1.5 mg/L to 5.3 mg/L. The strain *B. subtilis* DSBV Po1 had displayed the highest ability to synthesize (5.3 mg/L), followed by *Enterobacte*r sp. DTAN6 Po2 (4.3 mg/L) and strain *B. aryabhattai* DQT1 Po6 has the lowest ability to synthesize the 1.5 mg/L.

3.4. Effects of cultural conditions on the bioflocculant-producing ability

In this study, sucrose was the most favorable carbon source for bioflocculant production by *B. aryabhattai* DQT1 Po6, *Enterobacter* sp. DTAN6 Po2, and *B. subtilis* DSBV Po1, with 98.3 %, 80.0 % and 80.7 % maximum flocculant activity (MFA) after 48 hrs, respectively (Fig. 5a).

a,



b,

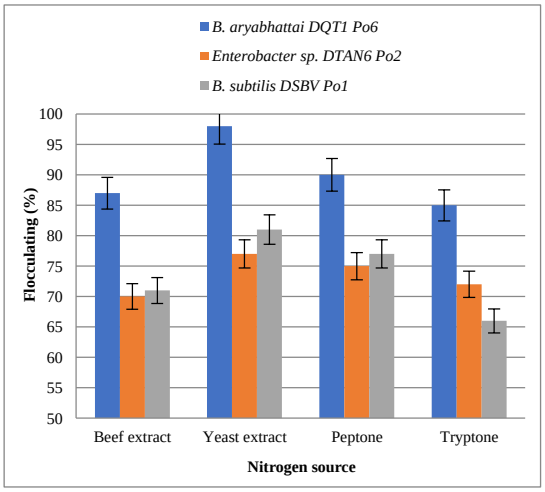


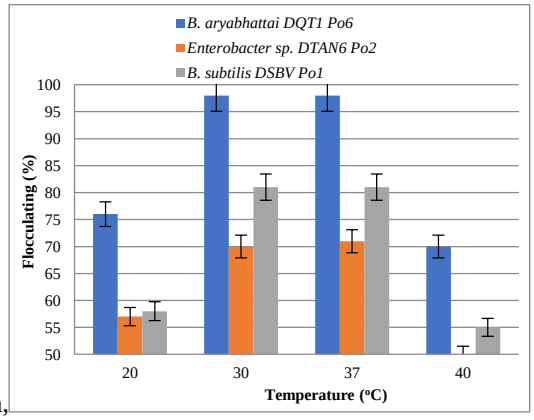
Figure 5. Effect of carbon sources (a) and nitrogen source (b) on flocculating activity

Nitrogen source was also a key factor affecting flocculating activity. Among the nitrogen sources surveyed, the yeast extract was the best one for enhancing flocculating activity of *B. aryabhattai* DQT1 Po6, *Enterobacter* sp. DTAN6 Po2, and *B. subtilis* DSBV Po1, with 98 %, 77 % and 81 % MFA after 48 hrs, respectively (Fig. 5b). Regarding the temperature, MFA of the selected strains activated in a temperature range between 30 ^{o}C and 37 ^{o}C (Fig. 6a).

Bacillus aryabhattai DQT1 Po6 and *Bacillus subtilis* DSBV Po1 had a high and stable flocculating rate at pH from 5.0 to 7.0, with 98 % and 81 % MFA after 48 hrs, respectively; while strain *Enterobacter* sp. DTAN6 Po2 had a high rate of flocculating and was stable in the range of pH 5.0 - 6.0 (MFA was 80 % after 48 hours of incubation) (Fig. 6b). Depending on strains and incubation time, flocculating activity varied greatly. This was supported by the fact that the flocculating rates increased rapidly during the logarithmic growth period (from 24 hrs to

72 hrs), flocculating rate of *B. aryabhattai* DQT1 Po6 and *B. subtilis* DSBV Po1 was reaching maximum after 48 hrs incubation, with 98 % and 81 % MFA, respectively; while strain *Enterobacter* sp. DTAN6 Po2 reaching maximum after 72 hrs incubation (MFA is 85 %) (Fig. 7). The viscosity of the culture broth increased with increasing cultivation time. The increase in viscosity of the culture broth was caused by high molecular weight polymer produced by strains during the growth period. The study results also showed that the pH of the culture broth was decreased from 7.0 to 6.0 with the increase in cultivation time from 0 to 72 hrs, which suggested that some organic acids were produced and released into the medium by strains during growth.

a,



b,

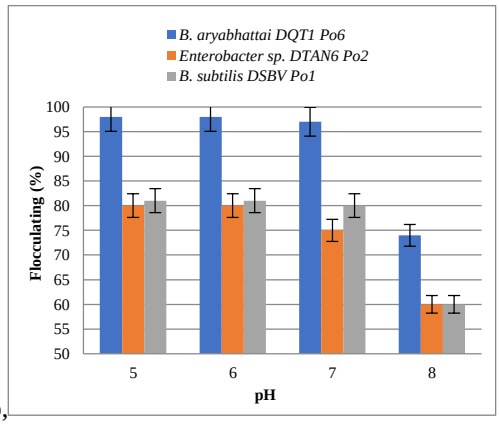


Figure 6. Effect of temperature (a) and pH (b) on flocculating activity.

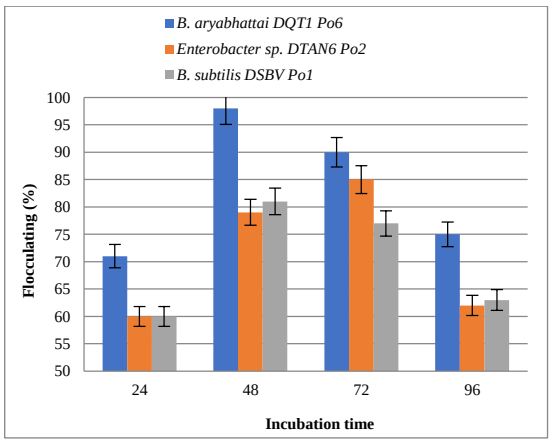


Figure 7. Effect of incubation time on flocculating activity.

3.5. Discussion

Indigenous microorganisms are a group of innate microbial consortium that inhabit the soil and the surfaces of all living things inside and have different potential bioactivities such as biodegradation, nitrogen fixation, improving soil fertility, phosphate solubility and plant growth promoters. Indigenous microorganisms play an important role by protecting hosts from invasion by pathogens. These microorganisms increase the availability of nutrients to host plants and increase the water-holding capacity, improves the aeration to the plant roots such that exchange of gases takes place effectively and prevents soil erosion [21].

In this study, 3 strains with high rate of flocculating belonging to 2 genera: *Bacillus* and *Enterobacter* were screened. These two genera have also been identified as components of slime flora in earlier studies. Different bacteria may produce different type of bioflocculant. For example, *Bacillus subtilis* IFO3335, *Bacillus mucilaginosus*, *Bacillus subtilis* NCIM 2063, *Klebsiella* sp. and *Bacillus aryabhattai* KG12S and others were able to produce polysaccharide bioflocculant; while *Bacillus licheniformis*, *Pacilomyces* sp. and *Nocardia amarae* YK1 were able to produce protein bioflocculants; and *Arcuadendron* sp. TS-4 and *Arathrobacter* sp. were able to produce glycoprotein bioflocculant [9, 17, 22 - 24].

The results of our study showed that B. *aryabhattai*, *B. subtilis* and *Enterobacter* sp. were 3 strains with the highest rate of flocculating among the isolates. Other studies had also proven that, the *B. aryabhattai*, *B. subtilis* and *Enterobacter* sp. could be used for water and wastewater treatment due to their bioflocculant activity. Bacterial bioflocculants are able to remove easily pollutants organic or inorganic, turbidity, colour, even pathogens [9, 23, 25, 26].

Microbial growth and bioflocculant production had been reported to be influenced by various factors. Obtained results in Figure 5a showed that carbon sources significantly influenced the flocculating activity of three studied strains. Of the 4 types of examined sugars (glucose, lactose, mannitol and sucrose), the highest flocculating activity was achieved for all 3 studied strains when sucrose was used as a carbon source, followed by glucose and the other 2 sugars. Selecting the most favorite carbon source for bacterial flocculating activity was reported in previous studies. Production of bioflocculant has been carried out on medium containing glucose and sucrose as the main carbon source. Both of these sugars appeared favorable for cell growth as well as for bioflocculant production of the *Bacillus* genus [6]. Bragadeeswaran *et al*. indicated that sucrose was the best carbon source for exopolysaccharides produced by *Bacillus cereus* GU 812900 [27]. Similar findings were reported by Makapela *et al*. (2016) with a strain of *B. pumilus* isolated from Thyume River in Alice, Eastern Cape (South Africa). For this strain, maltose and sucrose were the favorable carbon sources for the production of bioflocculants (respectively 71.7 % and 69.8 % of activities), after 120 hrs incubation [10]. Gouveia *et al*. also reported that sucrose was the most favorable carbon source for bioflocculant production by *B. megaterium* LBPMA-APFSG3Isox, and *B. toyonensis* LBPMA-ACOPR1.Isox, respectively, with 41 % and 39 % bioflocculating activity after 24 hrs. For *B. pumilus* LBPMA-BLD07 and *B. thuringiensis* LBPMA-EFIII, maltose was the best source of carbon to stimulate this bioflocculant activity (13 % and 25 %, respectively) [28].

In our research, yeast extract was the best nitrogen source for enhancing flocculating activity of all 3 studied strains (Fig. 5b). According to Sekelwa *et al*., a complex nitrogen source consisting of urea, yeast extract and $(NH_4)_2SO_4$ supported optimum bioflocculant production by *Virgibacillus* sp. [29]. Deng *et al*. [30] reported that peptone combined with sodium nitrate is the most suitable source of nitrogen for bioflocculant production by the fungus *Aspergillus parasiticus*, but when it was combined with $(NH_4)_2SO_4$ bioflocculant was not produced.

Experimental results also showed that cultural temperature had a major influence on flocculating activity of 3 studied strains (Fig. 6a). In this case, all 3 strains showed higher flocculating activity in culture temperature range from 30 ^oC to 37 ^oC. Reports of other studies indicated that temperature was the physical factor influencing on flocculating activity and flocculant production. Cultural temperature for maximum extracellular polysaccharide of *Halobacillus trueperi* AJSK [31] and *Bacillus subtilis* [32] was 35 ^oC, while the optimal flocculant production temperature for *Citrobacter koseri* has been reported was 30 ^oC [6]. According to Salehizadeh and Shojaosadati [33] and Zhang *et al*. [34] the enzymes which were responsible for bioflocculant production were activated in a temperature range between 25 ^oC

and 37 ^{o}C. The study of Gouveia *et al.* [28] also showed that 37 ^{o}C was the optimal temperature for the growth as well as for the bioflocculant production of the *B. pumilus* LBPMA-BLD07, *B. thuringiensis* LBPMA-EFIII, *B. toyonensis* LBPMA-ACOPR1 and *B. megaterium* LBPMA-APFSG3.

This study showed that there was a significant effect of pH on flocculation activity (Fig 6b). *B. aryabhattai* DQT1 Po6 and *B. subtilis* DSBV Po1 achieved high flocculating activity in a range pH of 5.0 - 7.0, while flocculating activity of *Enterobacter* sp. DTAN6 Po2 was stable at a range pH of 5.0 - 6.0. Most reports indicated that pH affects the flocculating efficiency of bioflocculant. According to Prasertsan *et al*. [35] bioflocculant activity of *Enterobacter cloacae* WD7 was optimal at pH 6.0. Li *et al*. [36] verified that *Agrobacterium* sp. M-503 produced bioflocculant with high activity at neutral to alkaline pH (7.0 - 12.0). Wang *et al.* [13] observed that the MAF of the CBF-F26 bioflocculant (mixed culture of *Rhizobium radiobacter* F2 and *B. sphaericus* F6) occurred in neutral and weak alkaline conditions, while Xiong *et al*. [37] detected MFA of *B. licheniformis* CGMCC 2876 at pH 7.5. The three investigated strains in this study gave high flocculating activity at pH near to neutral.

Depending on strains and incubation time, flocculating activity varied greatly. Xia *et al*. [38] observed that the maximum flocculating activity (MFA) was reached in the beginning of the stationary phase. Incubation time for maximum extracellular polysaccharide of *Halobacillus trueperi* AJSK [31], *Bacillus subtilis* [32] and *Cobetia* sp. L222 [39] was 72 hrs. In the case of *Bacillus firmus*, bioflocculant peaked after 33 hrs culture [33], while bioflocculant produced by *Agrobacterium* sp. M-503 reached a peak after 48 hrs [36], and for *Vagococcus* sp. W31, flocculant production was maximum after 60 hrs cultured [40]. Okaiyeto *et al*. (2016) registered MFA of 85.8 % after 72 hrs of cultivation for *Bacillus* sp. AEMREG7 [25]. Likewise, Gouveia *et al*. (2019) also reported that *B. thuringiensis* LBPMA-EFIII, *B. toyonensis* LBPMA-ACOPR1 Isox and *B. megaterium* LBPMA-APFSG3 Isox achieved their MFA 33 %, 21 % and 34 %, respectively, after 24 hrs of incubation, while *B. pumilus* LBPMA-BLD07 achieved MFA was 57 % after 72 hrs of incubation [28]. The results of our study showed that the strain of *Enterobacter* sp. DTAN6 Po2 produced maximum of bioflocculant after 70 hrs cultured at 150 rpm, while flocculating activity of *B. aryabhattai* DQT1 Po6 and *B. subtilis* DSBV Po1 achieved maximum after 48 hrs (Fig. 7).

From the evaluating analysis, 3 bacterial isolates were selected for their high flocculating activity. Beside this, the selected strains exhibited high capacity for IAA biosynthesis and nitrogen fixation. There for, these strains (*Bacillus aryabhattai* DQT1 Po6 (isolated from cassava cultivated soil in Central Viet Nam), *Enterobacter* sp. DTAN6 Po2 (isolated from cassava cultivated soil in the South) and *Bacillus subtilis* DSBV Po1 (isolated from cassava cultivated soil in the North) have potential to use as biofertilizer.

4. CONCLUSIONS

In this research, bioflocculant producing-bacteria were isolated from cassava cultivated soils and cassava roots in the North, Central and South regions of Viet Nam. Three strains with high bioflocculant activity (flocculating rate about 80 - 98 %) were selected and their other activities, such as phosphate solubilizing, IAA production and nitrogen fixation ability were evaluated. The results showed that, in addition, bioflocculant producing capacity, the selected strains exhibited high capacity of IAA production (from 6.33 µg/mL to 10.12 µg/mL) and can grow on medium without nitrogen. After 16S rRNA gene analysis, the strains were assigned to *Bacillus aryabahattai* DQT1 Po6, *Enterobacter* sp. DTAN6 Po2 and *Bacillus subtilis* DSBV

Po1. Influence of carbon and nitrogen sources, as well as cultural conditions such as temperature, pH and incubation time on flocculating activity of these strains have also been investigated. Obtained results revealed that selected isolates preferred yeast extract as a nitrogen source and sucrose as a carbon source for maximum flocculating activity. The suitable culture temperature and pH for their flocculant activity ranged from 30 - 37 ^{o}C and from light acidity to light alkalinity, respectively. The incubation time for highest flocculating activity of the strains varied from 48 hrs (*B. aryabhattai* DQT1 Po6 and *B. subtilis* DSBV po1) to 72 hrs (*Enterobacter* sp. DTAN6 Po2). These isolates are potential candidates as perfect inoculant for biofertilizer production.

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