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IDENTIFICATION AND CHARACTERIZATION OF A PURPLE NONSULFUR BACTERIUM ISOLATED FROM COASTAL AREA OF HAI PHONG FOR USING IN PRODUCTION OF UNSATURATED FATTY ACID (OMEGA 6, 7, 9)

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Abstract. Purple nonsulfur bacteria are a group of diverse biotechnological applications, particularly in producing functional food rich in unsaturated fatty acids. In this study, a purple nonsulfur bacterium strain HPB.6 was chosen based on its strong growth, high lipid production and synthesis of unsaturated fatty acid (omega 6, 7, 9). The studying on biological characteristics showed that strain HPB.6 comprises ovoid-rod shaped cells with diameter of 0.8 - 1.0 µm, none motile, Gram negative stained. The cells divide by binary fission and contain bacteriochlorophyll a (*Bchl a*). This bacterium grew well on medium with carbon and nitrogen sources such as acetate, succinate, pyruvate, butyrate, glutamate, arginine, leucine, tyrosine, alanine, methionine, threonine, glutamine, yeast extract and NH_4Cl. This selected strain grew well on medium with salt concentrations from 1.5 - 6.0 % (optimum 3 %), pH from 5.0 to 8.0 (optimum at pH 6.5) and could withstand Na_2S at 0.4 - 5.2 mM. All the data and particularly 16S rRNA analysis received demonstrated that HPB.6 strain belongs to the species *Rhodovulum sulfidophilum*.

Keywords: *Rhodovulum sulfidophilum*, PNSB, characterization, identification, omega.

Classification numbers: 1.3.2, 1.4.1, 2.7.1.

1. INTRODUCTION

Purple nonsulfur bacteria (PNSB) are either alpha or beta-proteobacteria that are able to carry out photosynthesis without oxygen production such as cyanobacteria, algae and green plants [1]. Under micro aerobic conditions in the light, all species grow as photoheterotrophs with various organic substrates or as photoautotrophs with either molecular hydrogen or in some

species, sulfide, thiosulfate or elemental sulfur as electron donor and CO_2 as sole carbon sources [2].

Purple nonsulfur bacteria are the most diverse and most useful group among all anoxygenic phototrophic bacteria for various biotechnological applications such as production of single cell protein (SCP); production of valuable compounds (vitamin, ubiquinone, carotenoid, hormones, enzymes, etc.) and for wastewater treatment [3] and functional food formation [4].

Functional foods rich in unsaturated fatty acids (omega 6, 7, 9), especially omega 7 (palmitoleic acid and vaccenic acid), are beneficial to human health. Health supporting function such as antioxidant, anti-inflammatory, immune system modulator, strengthens the cardiovascular system and mucous membrane tissue regenerator have been proven [5]. Beside that vaccenic acid can prevent coronary heart disease [6], atherosclerosis [7] and inhibited growth of HT-29 cell [8]. Omega 6, 9 are abundant in animal and vegetable oils but omega 7 is rare. It is extracted from sea buckthorn berries and macadamia oil [9]. Omega 7 (vaccenic acid) is discovered in the cell of PNSB with high rate (65-82 % of total fatty acids) [10].

From 25 strains of purple nonsulfur bacteria isolated from mud and waste water samples collected at Haiphong coastal area in Vietnam, a strain named HPB.6 was selected depended on criteria as performing best growth, highest lipid synthesis and especially synthesizing unsaturated fatty acid (omega 6, 7, 9). In order to use this strain for extracting unsaturated fatty acid (omega 6, 7, 9) and for other biotechnological applications, characterization and identification of the strain is reported.

2. MATERIALS AND METHODS

2.1. Cultivation of purple nonsulfur bacteria

Growth of HPB.6 was determined by cell density at 660 nm (ΔOD_{660}) after 4 days of culture in DSMZ-27 medium [11] under micro aerobic conditions (cultivated in 13 ml tube contained 10 ml medium) with intensity illuminate about 5.000 lux, the temperature was 28 - 30 ^oC. The DSMZ-27 (pH 7.0) contains the following components per liter of distilled water: 0.3 g yeast extract; 0.5 ml ethanol; 1 g succinate; 0.5 g acetate; 5 ml ferric citrate from 0.1 % (w/v) stock; 0.5 g KH_2PO_4; 0.4 g $MgSO_4$.$7H_2O$; 0.05 g $CaCl_2$. $2H_2O$; 0.4 g NH_4Cl; 25 g NaCl; trace element solution SL6; and 1 ml vitamin B_{12} solution (filter sterilized). Trace element solution SL6 contains (l^{-1}) 1.8 g $FeCl_2.4H_2O$; 0.25 g $CoCl_2.6H_2O$; 0.01 g $NiCl.6H_2O$; 0.01 g $CuCl_2.5H_2O$, 0.07 g $MnCl_2.4H_2O$; 0.1 g $ZnCl_2$; 0.5 g H_3BO_3; 0.01 g $Na_2SiO_3.5H_2O$; 0.03 g $Na_2MoO_4.2H_2O$. Vitamin B_{12} solution was added after autoclaving.

2.2. Analysis of photosynthesis pigments

To determine the presence of photosynthetic pigments such as *bacteriochlorophyll* a and b (*Bchl* a, *Bchl* b), the whole cell suspensions spectra were recorded using UV-Vis spectroscopy within a range of 380-1.000 nm [12]. In this range, *Bchl* a with absorption peak at 800-900 nm wide spread and *Bchl* b with absorption peak at 1000-1030 nm.

2.3. Morphological characterization

The pigmentation of the PNSB ranges from brown, red to red-purple (Munsell Color Chart®). The cell size and shape was determined using optical microscope OLYMPUS Model

CH-S (Japan) and Scanning Electron Microscopy (SEM) JEOL-5410LV (Japan).

2.4. Physiological characterization

Carbon and nitrogen requirement

Strain HPB.6 was cultivated in DSMZ-27 medium in micro aerobic conditions with intensity illuminate about 5.000 lux, the temperature was 28 - 30 ºC. In the experiment with different carbon sources, to the DSMZ-27 medium without organic carbon different carbon sources were added (1 g/l). In the experiment with nitrogen sources, to the DSMZ-27 medium without nitrogen, different defined nitrogen sources were added (10 mM), and the undefined nitrogen sources such as yeast extract, was added at 1 g/l.

Effect of sulfide and NaCl on the growth

Strain HPB.6 was cultured in liquid DSMZ-27 medium containing Na_2S (from 0 to 5.2 mM) and NaCl (0 - 10 %) in micro aerobic conditions, at temperature: 28 - 30 ºC, intensity illuminate: 5.000 lux.

Effect of initial pH on the growth

Strain HPB.6 was cultured in liquid DSMZ-27 medium with pH range from 4.5 - 10 and incubated under micro aerobic conditions with intensity illuminate about 5.000 lux, the temperature was about 28 – 30 ºC.

2.5. Molecular analysis

HPB.6 strain was cultured on DSMZ-27 at temperature 28 – 30 ^{o}C, illumination intensity about 5.000 lux at micro aerobic condition. After the cultivation of 4 days, the biomass was harvested by centrifugation at 8.000 r/min for DNA isolation. Genomic DNA was extracted and purified by using Gene JET Genomic DNA Purification Kit (Thermo). Taq DNA polymerase with standard Taq Buffer (NEB) was used for PCR. The complete length of the 16S rRNA gene sequence was obtained by amplification with two primers, F1 (5'-AGAGTTTGATCCTGGCTCAG-3'), and R1 (5'-ACGGCTACCTTGTTACGACT-3') [Positions 8–27 and 1491–1512 for F1 and R1 respectively [13]. PCR amplification was performed following Imhoff [14,15] and 16S rRNA gene sequencing was performed on 3130xl Applied Biosystems ABI prism automated DNA sequencer [16].

2.6. Phylogenetic tree construction

The 16S rRNA gene sequence of strain HPB.6 was determined on relatedness to reference type strain sequences in the Gen Bank database using the BLASTn program. Sequence was aligned with reference type strain (GenBank database) using MUSCLE alignment tool. Phylogenetic tree was constructed using the maximum likelihood method and Tamura-Nei model [17]. Evolutionary analyses were conducted in MEGA X [18].

3. RESULTS AND DISCUSSION

3.1. Determination of bacteriochlorophyll

A typical characteristic of PNSB is bacteriochlorophyll containing in the cell. For its determination was used UV-VIS spectroscopic analysis indicated that absorption peaks were from 380-900 nm. In this range, the maximum peaks absorption of bacteriochlorophyll signals were at 802 and 853 nm. This result strongly demonstrated that HPB.6 cell contains *Bch a* (Fig. 1).

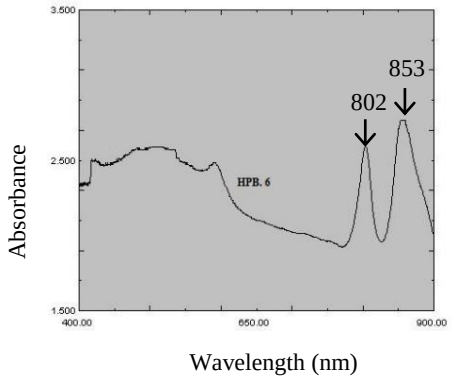

Figure 1. Whole absorption spectrum of strain HPB.6.

3.2. Morphological characteristics

Strain HPB.6 forms brown colonies with diameter of 2.3-2.7 mm on agar DSMZ-27 medium after one-week cultivation (Fig. 2a). Under scanning electron microscope (SEM), the cells were observed as ovoid-rod shape and the diameter of a single bacterium was about 0.8-1.0 µm, none motile, Gram negative bacterium, cells divided by binary fission (Fig. 2b).

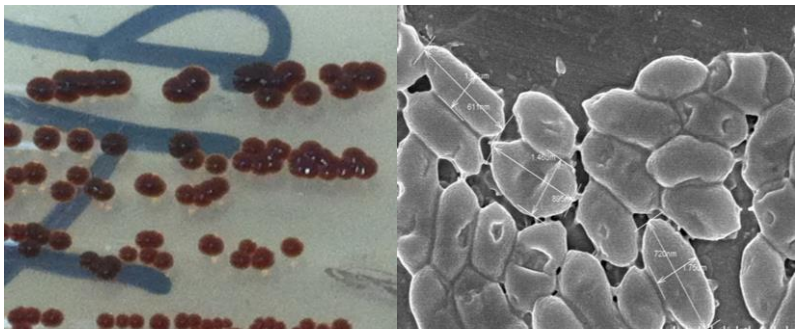

Figure 2. Morphological characteristics of strain HPB.6 (a) –The colonies under optical microscope; (b) – Cells under scanning electronic microscope (20.000 ×).

3.3. Physiological characteristics

3.3.1. Carbon and nitrogen requirement

For determination of carbon and nitrogen sources requirement for growth, strain HPB.6 was cultivated in liquid organic carbon/nitrogen free DSMZ - 27 medium, and incubated with light (5.000 lux) under limited oxygen condition (by cover with tube cap). Growth after 4 days incubation was accessed as cell density (OD_{600}), the obtained result was showed in Table 1.

Table 1. Carbon and nitrogen requirement for growth of HPB.6 in modified DSMZ-27 medium.

Carbon and nitrogen compounds		**HPB.6**
Carbon sources	Acetate	+++
	Succinate	+++
	Pyruvate	+++
	Formate	++
	Propionate	++
	Butyrate	+++
	Malate	++
	Lactate	++
	Citrate	-
	Glutamate	+++
	Glucose	+
	Fructose	-
	Glycerol	++
	Benzoate	-
	Ethanol	-
Nitrogen sources	Arginine	+++
	Leucine	+++
	Tyrosine	+++
	Alanine	+++
	Methionine	+++
	Threonine	+++
	Glutamine	+++
	Phenylanine	++
	Lysine	-
	KNO_3	++
	Urea	++
	NH_4Cl	+++
	Yeast extracted	+++

Note: Increased cell density at 660 nm after the incubation of 4 days (-): $\Delta OD_{660} < 0.1$ - no growth; (+): ΔOD660 from 0.1 - 0.5 - weak growth; (++): ΔOD_{660}, from 0.5 to 1.0 - normal growth; (+++): $\Delta OD_{660} > 1.0$ - strong growth

Data in Table 1 showed that strain HPB.6 used almost all tested carbon and nitrogen sources. The strain grew strongly on carbon sources such as acetate, succinate, pyruvate, butyrate, glutamate; normally on the media containing formate, propionate, malate, lactate, glycerol and weakly on media containing glucose and no growth in media containing citrate, fructose, benzoate and ethanol. While strain HPB.6 grew best on arginine, leucine, tyrosine, alanine, methionine, threonine, glutamine, yeast extracted and NH_4Cl; it grew normally in the media containing phenylanine, KNO_3, urea and none growth in the media containing lysine.

The above obtained data indicated that strain HPB.6 was capable of using a variety of carbon and nitrogen sources for growth. This is also common characteristic of PNSBs, giving them ability to exist in a variety of environments with different conditions [1].

Among PNSBs, species of the genus *Rhodovulum* have several phenotypic properties resemble to species of the genus *Rhodobacter*. The characteristics of strain HPB.6 obtained in this study, including colony and cell morphology, bacteriochlorophyll content, the pattern of carbon and nitrogen sources for growth indicated that the strain might belong either to the genus *Rhodobacter* or *Rhodovulum* [1]. The only difference between these two genera is salt dependent growth i.e. *Rhodobacter* spp are slight halophiles, whereas *Rhodovulum* spp. are stimulated by salt and are considered as moderate halophiles [1]. Therefore, investigation of salt-dependent growth of strain HPB.6 would give hint of identification at genus level.

3.3.2. Effect of salt (NaCl) concentration on growth

Strain HPB.6 was cultivated in liquid DSMZ-27 medium containing NaCl at concentrations of 0 – 10 % and incubated under micro aerobic conditions with intensity illuminate was about 5.000 lux, the temperature was about 28 - 30 ºC. The growth after 4 days, expressed as cell density at 600 nm (ΔOD_{660}) (Fig. 3) showed that salt stimulated growth of this strain. HPB.6 strain grew better in medium contained salt concentration range from 1.5 to 6.0 % and the optimum salt concentration was 3.0 % (ΔOD_{660} reached 1.785). At the salt concentration of more than 8.0 % and at no salt concentration, the growth of this bacterium was decreased (ΔOD_{660} reached 1.023 and 0.914 respectively). This result suggested that strain HPB.6 belonged to the genus *Rhodovulum*.

For determination which species HPB.6 strain may be belong to *Rhodovulum* genus, the effect of pH and sulfide on its growth was conducted.

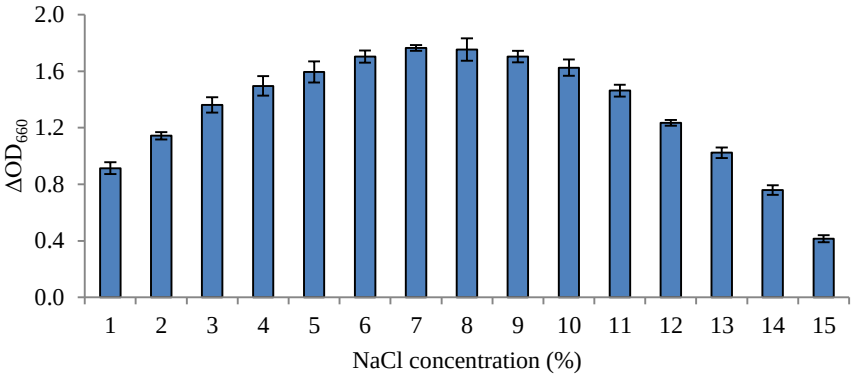

Figure 3. Growth of HPB.6 strain in liquid DSMZ-27 medium contained different NaCl concentration.

3.3.3. Effect of pH

In order to determine the pH effect on growth, HPB.6 was cultured on liquid DSMZ-27 medium with pH range from 4.5 - 10, at microaerobic conditions, illumination intensity about 5.000 lux, temperature 28 – 30 °C. After the cultivation of 4 days (96 h), the biomass accumulation (ΔOD_{660}) of the studied strains was determined, the results were shown in Fig. 4.

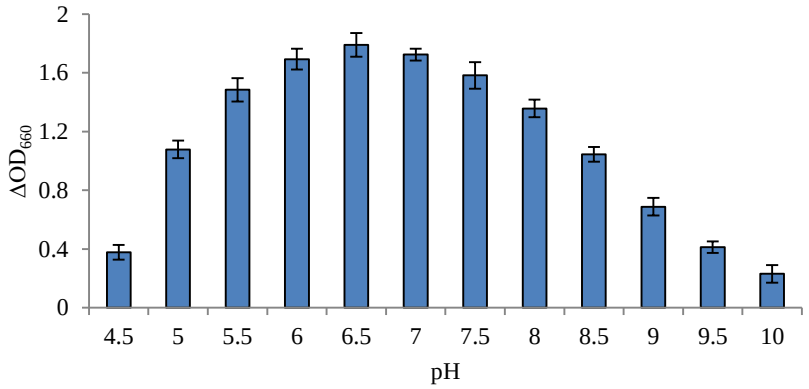

Figure 4. Effect of pH on growth of HPB.6 strain.

Figure 4 indicated the strain grew at a wide range of pH from 5.0 to 8.0 (optimum at pH 6.5). At the current time, the genus *Rhodovulum* comprises 20 published species in which 3 species named *Rhodovulum marinum, Rhodovulum sulfidophilum* (old name was *Rhodopseudomonas sulfidophila)* and *Rhodovulum mangrovi* can grow well on acidic medium (pH ≥ 5.0) [19-21]. Beside that *Rhodovulum visakhapatnamense* species can be tolerated in medium with pH 4.0 [22]. From the effect of pH on growth of the HPB.6 strain suggested that the strain may be one of these species.

3.3.4. Effect of sulfide

Strain was cultured on DSMZ-27 containing Na_2S at different concentrations (0 to 5.2 mM) under micro aerobic conditions. The biomass accumulation (ΔOD_{660}) after the cultivation of 4 days showed in Fig. 5.

Figure 5 showed that the strain could withstand Na_2S at 0.4 - 5.2 mM. The genus *Rhodovulum* have 3 species named *R. sulfidophilum; R. lacipuncei* and *R. aestuarii* can be tolerated high sulfide (from 4.0 to 7.0 mM) [20, 23, 24]. From result the ability of using sulfide, HPB.6 strain may belong to one of the three species as above mentioned.

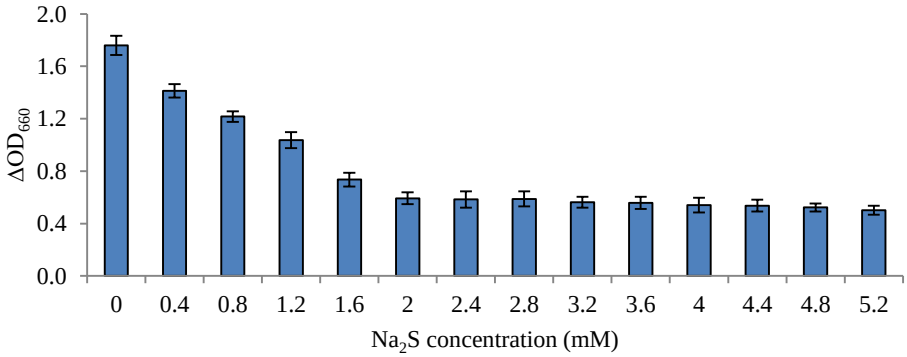

Figure 5. Effect of Na_2S on growth of HPB.6 strain.

Combination of tolerated acidic pH and Na_2S property, demonstrated HPB.6 may belong to *R. sulfidophilum*. The comparison of some basic characteristics of HPB.6 species with *R. sulfidophilum* (Hansen W^{4T}) was described in Table 2.

Table 2. Differential characteristics between strain HPB.6 and type strains of closely related species of the genus *Rhodovulum.*

Characteristic	**HPB.6**	***R. sulfidophilum (HansenW^{4T})***
Cell diameter (μm)	0.8 - 1.0	0.6-1.0
Cell shape	Ovoid - rod	Ovoid – rod
Motility	+	+
Negative staining	+	+
Color of cell suspension	Brown	Brown
Bacteriochlorophyll	a	A
NaCl range (%) (optimum)	0-9 (3)	0-10
pH range (optimum)	5.0 - 8.0 (6.5)	5.0 - 9.0
Sulfide tolerance (mM)	4.0 - 5.2	5.2 - 8.0
Carbon sources utilization		
Acetate	+	+
Succinate	+	+
Pyruvate	+	+
Formate	+	+
Propionate	+	+
Carbon sources utilization		
Butyrate	+	+
Malate	+	+
Lactate	+	+
Citrate	-	-
Glutamate	+	+
Glucose	+	+
Fructose	-	-
Glycerol	+	+
Benzoate	-	-
Ethanol	-	-

(-): no growth; (+): growth

Table 2 showed that HPB.6 strain had almost biological characteristic nearly the same *R. sulfidophilum* (Hansen W^{4T}) species. However, in order to make more accurate conclusions the 16S rRNA gene sequences of the strain was analyzed.

3.4. 16S rRNA gene analysis

After extracting by using GeneJET Genomic DNA purification Kit, genomic DNA was tested on 1 % agarose gel and the result was showed in Fig. 6. Duplication of the 16S rRNA

genome by PCR using Taq DNA polymerase and the 16S primer (shown in materials and methods). The PCR product was tested on 1 % agarose gel in TAE buffer and results was showed in Fig. 7.

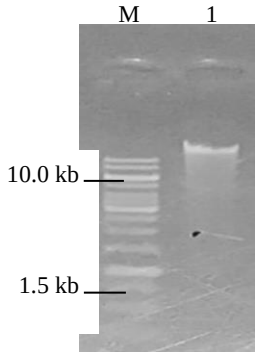

Figure 6. Agarose gel electrophoresis.
Lane M - 1kb DNA molecular weight markers;
Lane 1 - genomic DNA of HPB.6.

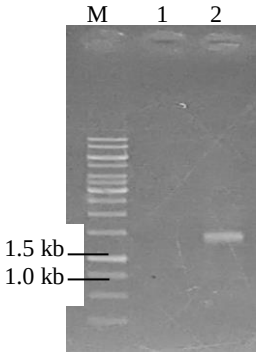

Figure 7. Agarose gel electrophoresis.
Lane M -1kb DNA molecular weight markers
Lane 1 - negative control, Lane 2 - PCR product of HPB.6.

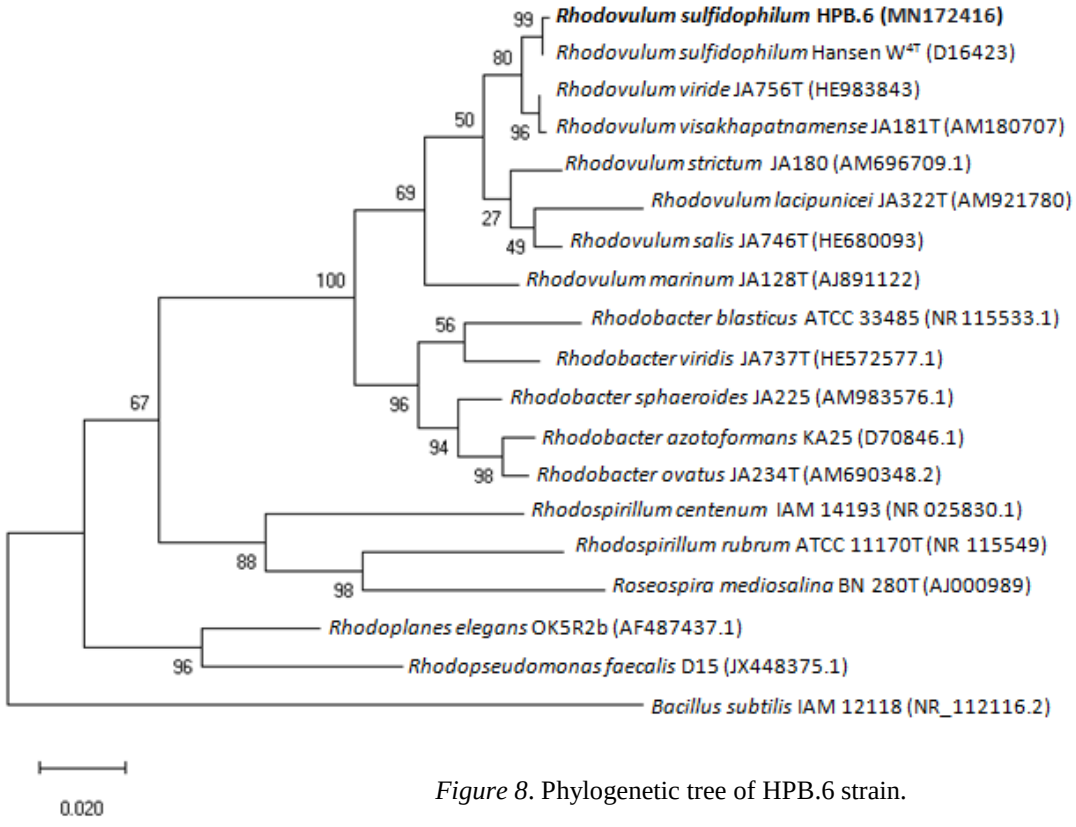

Figure 8. Phylogenetic tree of HPB.6 strain.

After sequencing, the 16S rRNA sequence were submitted to GenBank to obtain accession numbers MN172416. The sequence showed 99,7 % similarity with *R. sulfidophilum* Hansen W^{4T} (D16423) in genbank. The phylogenetic analysis was done to compare the 16S rRNA gene

sequences of HPB.6 with sequences of other members of the genus *Rhodovulum* and their relatives that belong to the genus *Rhodobacter*, *Rhodoplanes*, *Rhodopseudomonas*, *Rhodospirillum*, *Roseospira* (Fig. 8).

The phylogenetic tree showed that the *Rhodovulum* and *Rhodobacter* genus were separated into two distinct branches. Strain HPB.6 was in the same branch of the genus *Rhodovulum* and shared a high bootstrap value of 99 % with *Rhodovulum sulfidophilum* Hansen W^{4T} (D16423). The result showed that they can conveniently be grouped as the same species.

The HPB.6 strain was found to be closely related to the species *R. sulfidophilum* based on morphology, physiological properties and phylogenetic analysis of HPB.6 strain using 16S rRNA gene sequence data accordingly confirmed HPB.6 belongs to *Rhodovulum sulfidophilum.*

4. CONCLUSIONS

In this paper, identification and characterization of a PNSB (named HPB.6) isolated from coastal of Haiphong producing unsaturated fatty acid (omega 6, 7, 9) were reported. The result of morphological study showed that the cells of HPB.6 were observed as ovoid-rod shape, none motility, Gram negative staining. The diameter of single bacterium was about 0.8 - 1.0 µm. The cells divide by binary fission and has bacteriochlorophyll a (*Bchl*a). The physiological characterization indicated that this bacterium grew well on medium rich in carbon and nitrogen sources, salt concentrations from 1.5 - 6.0 % (optimum 3 %), pH from 5.0 to 8.0 (optimum at pH 6.5) and could particularly stand Na_2S at 0.4 - 5.2 mM.

Based on morphological, physiological properties and 16SrRNA analysis, the HPB.6 was identified as belongs to *Rhodovulum sulfidophilum* and it opens the possibilities to use this strain for biomass and important fatty acid (omega 6, 7, 9) production.

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