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Isolation and screening of carotenoid-producing *Bacillus* spp. from seashore saline soil and seawater at Hon Son islet

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ABSTRACT

Carotenoids are natural pigment compounds with a variety of colors. They are antioxidants and boost the immune system. It is interesting to examine strains of bacteria capable of producing carotenoids applied in the food industry, animal feed, and aquaculture. Rach Gia bay of Kien Giang province has a good ecosystem, and the carotenoid-producing microorganisms in these regions are very plentiful. However, there are fewer studies about them in this region. This study was conducted to isolate and select carotenoids-producing *Bacillus* from Hon Son islet of Rach Gia bay. Seashore saline soil and seawater samples were shocked at 65°C in 1 hour and plated on Difco sporulation medium. Colored colonies of bacteria were selected and incubated at 37°C on Difco sporulation medium. Pigments from these isolates were extracted in the mixture of methanol and chloroform (with the ratio 1:2 of v/v). The extracted solutions were scanned absorbance by spectrophotometer at the wavelength range from 400 to 600 nm. Bacterial isolates were identified by 16S rRNA sequencing. Twenty carotenoid-producing bacterial species were isolated, including five colony colors such as pale yellow, yellow, yellow-orange, pale pink, and pink. They were Gram-positive, positive - catalase test, motile, and endospore-forming. The results of extracted solutions from 20 bacterial species indicate that all the extracts have absorbance from 400-600 nm, this is the popular absorbance of carotenoids. Two high-carotenoid-producing species (HS3-7 and HS8-3) were analyzed the 16S rRNA gene sequence. They were showed high similarity with *Bacillus vietnamensis* species Marseille-P799 and *Bacillus infantis* species NRRL B-14911, respectively.

1. INTRODUCTION

Carotenoids are one of the largest and most diverse groups of biologically active substances, which are responsible for the colors of yellow, orange, and red in many plants, microorganisms, and animals. These

natural products can protect cells against harmful oxygen radicals or serve as a precursor of vitamin A, a membrane stabilizer (Asker et al., 2012). Therefore, these natural pigments have received considerable attention for product development applied in biotechnology, food industry, animal

feed, aquaculture, and pharmaceutical industry (Fiedor & Burda, 2014; Vilchez et al., 2011). However, commercial carotenoids are mainly produced by chemical synthesis methods. The microbiological pigments can replace the synthetic colors because they are completely natural, high productivity, and diversity of molecules (Indra Arulselvi et al., 2014).

The bacterial spores are well-documented about their survivability in harsh conditions such as high temperature, toxic chemical, or UV radiation (Nicholson et al., 2000; Riesenman & Nicholson, 2000). Carotenoids are secreted to help the bacterial spores withstand UV damage. *Bacillus subtilis* spores have melamine in their coats to help them survive under solar radiation. Furthermore, a wide range of carotenoid pigments is also found in the membrane of *Bacillus* spp. that can scavenge reactive oxygen species (ROS) that originated from UV radiation (Khaneja et al., 2010). For examples, *B. aquimaris* and *B. marisflavis* produce yellow pigments (Yoon et al., 2003), *B. indicus* and *B. clarkii* with yellow and orange pigments (Duc et al., 2006; Nielsen et al., 1995), *B. firmus* with pink pigment (Khaneja et al., 2010) and *B. megaterium* with red pigment (Mitchell et al., 1986). Carotenoid production in spore-forming *Bacillus* species has attracted interest from both academia and industries because of their potential to act as a biological source for mass-production of these natural compounds with improved stability and solubility for the feed and food industry (Abdel-Fattah et al., 2012). In this present study, carotenoid-producing *Bacillus* spp. with high productivity were isolated, screened, and identified from seashore saline soil samples and seawater samples at Hon Son islet, Kien Hai district, Kien Giang province.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Sample collection

Seven saline soil samples from the seashores and two seawater samples were collected from Hon Son islet, Kien Hai district, Kien Giang province at a depth of 2-5 cm from the soil and seawater surface, respectively. Soil (500 g) and seawater (500 mL) samples were kept in plastic bags and sterile bottles, respectively, and transferred to the laboratory for isolation.

2.1.2. Chemicals

Difco sporulation medium (DSM) and Luria-Bertani medium (LB) were purchased from Himedia, India. Methanol 99.5%, phosphate-buffered saline (pH 7.4), and chloroform 99.5% were purchased from Merck, Germany. F-primer f27, R-primer r1492, DNA master mix, and PCR water were purchased from Phusa biochem LTD. Company.

2.2. Methods

2.2.1. Isolation of carotenoids-producing *Bacillus* spp.

The isolation was referred to the method described in the study of Duc *et al.* (2006). Each sample was diluted (1:10) by phosphate-buffered saline (PBS, pH 7.4) in the flask. For sample preparation, 1 g of seashore saline soil was diluted by phosphate-buffered saline (PBS, pH 7.4) with the ratio 1:10 (w/v), and the seawater sample (1 mL) was diluted in PBS buffer with the ratio 1:10 (v/v). The mixture was heated at 65°C for 1 hour to remove all vegetative cells. Next, serial dilutions (from 10⁻¹ to 10⁻⁷) were made by using PBS, and 100 µL of each diluted concentration was plated on DSM Petri dishes. After three days of incubation at 37°C, colored colonies (from red to yellow) were selected and then transferred to new DSM Petri dishes to isolate single bacterial species. Each isolate was incubated at 37°C and the morphology was described based on Holt et al. (1994).

For the taxonomy identification, all species were confirmed by morphology and a series of biochemically tests including gram staining, catalase synthesis, mobility, and spore formation ability (Amin et al., 2012). The gram staining test was based on the standard protocol by using the Nam Khoa gram staining kit (Nam Khoa company, Viet Nam). The detailed procedures of catalase synthesis, mobility, and spore formation ability were described below.

Catalase synthesis: one bacterial colony was picked and put on the clean surface of the microscope slide, and one drop of hydroxyl peroxide (3%) was dropped on the slide and allowed to contact the bacterial colony. The presence of bubbles caused by the hydrogen peroxide degradation reaction indicated the presence of catalase in the sample (positive result) while the negative results were represented by the disappearance of bubbles (Abdulkadir & Waliyu, 2012).

Mobility: Luria-Bertani semi-agar (0,5%) was used for this assay. Inoculation was performed by the stab method with a straight needle. The sample was incubated at 37°C for 24 hours. The positive result was confirmed by a diffusion zone of microorganisms spreading from the inoculation line (Tittler & Sandholzer, 1936).

Spore formation ability: the bacterial smear was prepared and fixed on the clean surface of the microscope slide. Malachite green 5% was added to the fixed bacteria smear and then heated for a few minutes, which can soften the hard outer of the spore for sticking dye to the spore. After that, the sample was washed with distilled water to remove excess dye. Then, safranin dye 0,5% was added to samples. The green color indicated spores, and the pink color indicated vegetative cells (Oktari et al., 2017).

2.2.2. Screening of carotenoids-producing *Bacillus* spp.

Carotenoids compounds were extracted based on the method of Indra Arulselvi et al. (2014). One colony of each strain was pre-cultured in 10 mL Luria-Bertani medium (LB), incubated at 37°C and shaken at 200 rpm by a shaker in 24 hours. Next, two mL of pre-inoculum was transferred into a 250 mL flask containing 100 mL of LB, incubated at 37°C, and shaken at 200 rpm in 48 hours. Cells were harvested by centrifugation at 5,000 rpm for 30 minutes at 4°C, and then the pellet was washed with distilled water. For carotenoid extraction, six mL of methanol: chloroform (1:2, v/v) was mixed with 0,2 grams of fresh pellet, and the suspension was sonicated at 200 W at 4°C for 10 minutes, which was following by adding 6 mL of distilled water into the tube. The suspension was separated by centrifugation at 12,000 rpm for 15 minutes at 4°C. The lower phase having carotenoids was collected. Extracted liquid was monitored at 400 to 600 nm by UV/VIS spectrophotometer (GENESYS™ 10S UV-Vis Spectrophotometer - Thermo Fisher). The total carotenoid content was calculated by the method of Liaaen-Jensen and Jensen (1971) with the following formula:

$$c = \frac{D \times v \times f \times 10}{2,500}$$

Where, c is total carotenoid content (mg); D is the maximum absorbance; v is the volume (mL); f is dilution coefficient, 10 is the conversion factor to mg,

and 2,500 is the specific extinction coefficient of carotenoid.

2.2.3. Identification of carotenoid producing strains by 16S rRNA analysis

High carotenoid-producing species were identified by 16S rRNA analysis. The primers used for 16S rRNA PCR were F-primer f27 (5'-AGAGTTT-GATCCTGGCTCAG-3') and R-primer r1492 (5'-GGTTACCTTGTTACGACTT-3') (Nugraheni et al., 2010). The PCR reaction was initiated by the denaturation step at 94°C for 2 minutes, which followed by 45 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minute), and extension (72°C for 2 minutes), and the final extension step was 72°C for 10 minutes (Nugraheni et al., 2010). The electrophoresis of PCR products was performed by 2% agarose. The amplified products were then sequenced and subjected to DNA data bank by NCBI web-based BLAST program, and the closest species was identified by the percentage of identity. The sequences were aligned, and the phylogenetic tree was constructed by the MEGA X program.

2.3. Statistical analysis

The results of carotenoid extraction were expressed by mean of three replications. All data were processed by SPSS 20.0 software to determine statistical significance. The level of significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Isolation of *Bacillus* spp. producing carotenoids

There were 20 bacterial species isolated from seven seashore saline soil samples and two seawater samples at Hon Son islet. Four bacterial species were isolated from seawater samples (HS1-1, HS1-3, HS1-4 and HS2-1), and 16 species were isolated from seashore saline soil samples (HS3-3, HS3-4, HS3-7, HS3-8, HS4-1, HS5-1, HS5-2, HS5-3, HS6-1, HS7-1, HS8-3, HS8-6, HS8-7, HS8-8, HS8-9 and HS9-1).

On DSM Petri dishes, colonies of 20 bacterial species showed different colors: pale yellow, yellow, yellow-orange, pale pink, and pink. Bacterial colonies were all flat, circular, and entire or irregular and undulated, diameter range from 2-5 mm. Eight bacterial species were having circular and entire colonies, and 12 bacterial species having irregular and undulate colonies (Figure 1 & Table 1).

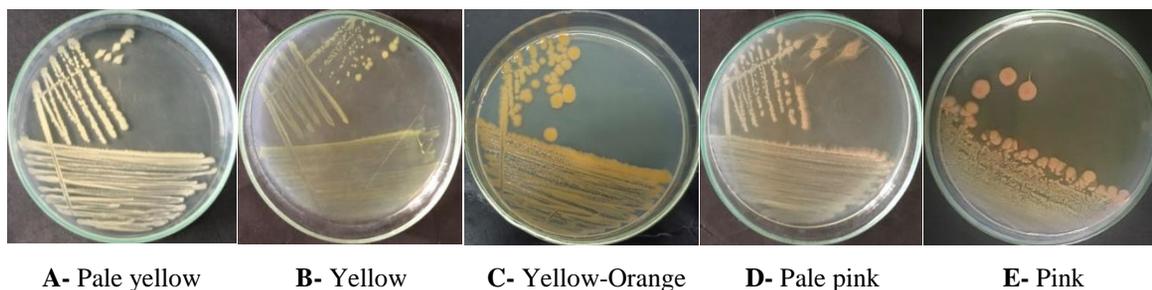


Figure 1. Colony color of HS1-1 (A), HS5-1 (B), HS3-7 (C), HS3-8 (D) and HS8-3 (E)

Table 1. The morphological characteristics of the colony of the isolated bacterial species

Order	Bacterial strains	Colony color	Colony shape	Colony edge	Colony surface	Colony diameter (mm)
1	HS1-1	Pale yellow	Irregular	Undulate	Flat	3.0
2	HS1-3	Pale yellow	Irregular	Undulate	Flat	3.0
3	HS1-4	Pale yellow	Circular	Entire	Flat	2.0
4	HS2-1	Pale yellow	Irregular	Undulate	Flat	3.0
5	HS3-3	Pale pink	Circular	Entire	Flat	2.0
6	HS3-4	Pale yellow	Irregular	Undulate	Flat	3.0
7	HS3-7	Yellow-orange	Irregular	Undulate	Flat	4.0
8	HS3-8	Pale pink	Circular	Entire	Flat	3.0
9	HS4-1	Pale yellow	Irregular	Undulate	Flat	3.0
10	HS5-1	Yellow	Circular	Entire	Flat	2.0
11	HS5-2	Pink	Circular	Entire	Flat	4.5
12	HS5-3	Yellow	Circular	Entire	Flat	2.5
13	HS6-1	Pale yellow	Irregular	Undulate	Flat	3.0
14	HS7-1	Yellow	Irregular	Undulate	Flat	2.5
15	HS8-3	Pink	Irregular	Undulate	Flat	5.0
16	HS8-6	Pale yellow	Irregular	Undulate	Flat	2.0
17	HS8-7	Pale yellow	Irregular	Undulate	Flat	3.0
18	HS8-8	Pale yellow	Irregular	Undulate	Flat	5.0
19	HS8-9	Yellow	Circular	Entire	Flat	2.5
20	HS9-1	Pale pink	Circular	Entire	Flat	2.0

Under the microscope at 1000X magnification, 20 isolates were rod-shaped cells with (2-4 μm) in length and 1 μm in width. All bacterial species were gram-positive, motile, able to form endospore, and synthesize catalase (Table 2). Based on the morphological and biochemical properties, 20 isolated

species were identified as *Bacillus* spp. according to the key Bergey’s manual of determinative bacteriology (Amin et al., 2012; Holt et al., 1994).

Table 2. The morphological and biochemical characteristics of the isolated bacterial species

Order	Bacterial strains	Shape	Size (μm)	Gram	Catalase	Motile	Endospore
1	HS1-1	Rod	2.5 x 1	+	+	+	+
2	HS1-3	Rod	2.0 x 1	+	+	+	+
3	HS1-4	Rod	4.0 x 1	+	+	+	+
4	HS2-1	Rod	4.0 x 1	+	+	+	+
5	HS3-3	Rod	3.0 x 1	+	+	+	+
6	HS3-4	Rod	2.5 x 1	+	+	+	+
7	HS3-7	Rod	3.0 x 1	+	+	+	+
8	HS3-8	Rod	3.0 x 1	+	+	+	+
9	HS4-1	Rod	2.0 x 1	+	+	+	+
10	HS5-1	Rod	2.0 x 1	+	+	+	+
11	HS5-2	Rod	3.5 x 1	+	+	+	+
12	HS5-3	Rod	2.0 x 1	+	+	+	+
13	HS6-1	Rod	4.0 x 1	+	+	+	+
14	HS7-1	Rod	2.5 x 1	+	+	+	+
15	HS8-3	Rod	3.5 x 1	+	+	+	+
16	HS8-6	Rod	3.5 x 1	+	+	+	+
17	HS8-7	Rod	2.5 x 1	+	+	+	+
18	HS8-8	Rod	2.0 x 1	+	+	+	+
19	HS8-9	Rod	2.0 x 1	+	+	+	+
20	HS9-1	Rod	2.5 x 1	+	+	+	+

*(+): Gram-positive, Positive catalase test, Motile, and Form endospore.

3.2. Selection of high carotenoid producing strains

The extract colors of 20 *Bacillus* species were identified with the colony's color of each bacterial species. The carotenoid extracts of the bacterial species had 5 different colors, namely pale yellow, yellow, yellow-orange, light pink, and pink that have the highest absorbance at the wavelength of 400, 448, 460, 495, and 500 nm, respectively (Table 3).

The yellow-orange pigment of the extract can be derived from ϕ,ϕ -Carotene, β -Carotene, and Zeaxanthin because these compounds have the highest absorbance at 460 nm (Table 3) (Britton et al., 2004; Butnariu, 2016). The total carotenoid extract of HS3-7 (yellow-orange) was estimated at 0.00287 mg/mL and showed the highest carotenoid content among that of other species. On the other hand, the pale-yellow pigments had the maximum wavelength at 433 nm, which could be α -Carotene and Zeaxanthin, while the yellow pigments were γ -Carotene

due to the maximum wavelength was 448 nm (Britton et al., 2004). The carotenoid extracted from HS5-2 and HS8-3 species had pink color, which reached a maximum wavelength at 500 nm, while that of HS3-3, HS3-8, and HS9-1 species had pale pink color and had a maximum wavelength at 495 nm (Britton et al., 2004). The explanation for these results was that the pale pink pigment could be the mixture of Zeaxanthin and ϕ,ϕ -Carotene, while the pink pigment was the δ -Carotene (Britton et al., 2004). The total carotenoid content of HS8-3 reached the second-highest one with 0.00250 mg/mL to compare with that of other species, but this extract had pink color, so it might δ -Carotene while the extract from HS3-7 (yellow-orange pigment) lacked this carotenoid (Britton et al., 2004; Butnariu, 2016). In short, two species of *Bacillus* spp. (HS3-7 and HS8-3) producing the highest content of total carotenoid were selected for identification by using the 16sRNA sequencing method.

Table 3. The highest absorbance and total carotenoid content of extract solutions from 20 isolates

Bacterial strains	The color of extracts	Total carotenoid content (mg/mL)	The highest absorbance wavelength (nm)	Predicted carotenoid compounds
HS1-1	Pale yellow	0.000741 ^j ± 0.025	433	α-Carotene, Zeaxanthin
HS1-3	Pale yellow	0.000691 ^k ± 0.021	433	
HS1-4	Pale yellow	0.000536 ^o ± 0.018	433	
HS2-1	Pale yellow	0.000581 ⁿ ± 0.013	434	
HS3-4	Pale yellow	0.000611 ^m ± 0.016	434	
HS4-1	Pale yellow	0.000640 ^l ± 0.010	433	
HS6-1	Pale yellow	0.000476 ^q ± 0.012	433	
HS8-6	Pale yellow	0.000504 ^p ± 0.012	433	
HS8-7	Pale yellow	0.000405 ^s ± 0.016	433	
HS8-8	Pale yellow	0.000439 ^r ± 0.012	434	
HS5-1	Yellow	0.00124 ^f ± 0.032	448	γ-Carotene
HS5-3	Yellow	0.00130 ^e ± 0.015	448	
HS7-1	Yellow	0.00155 ^c ± 0.016	448	
HS8-9	Yellow	0.00137 ^d ± 0.010	448	
HS3-7	Yellow-orange	0.00287 ^a ± 0.018	460	φ,φ-Carotene, β-Carotene, Zeaxanthin
HS3-3	Pale pink	0.000823 ⁱ ± 0.016	495	Zeaxanthin, φ,φ-Carotene
HS3-8	Pale pink	0.000872 ^h ± 0.014	495	
HS9-1	Pale pink	0.000912 ^g ± 0.018	495	
HS5-2	Pink	0.00157 ^c ± 0.018	500	δ-Carotene
HS8-3	Pink	0.00250 ^b ± 0.016	500	

*Values in the column with the same letters are not significantly different ($p < 0.05$) by Duncan post hoc test. Values were shown in the table as mean ± SD of triplicate.

3.3. Identification of carotenoid producing strains

The 16sRNA fragments of bacterial isolate (HS3-7 and HS8-3) were amplified by PCR and electrophoresed on 2% agarose gel for qualification. The results showed positive results, which indicated the presence of bacterial DNA with a base length of approximately 1500 bp (Figure 2).

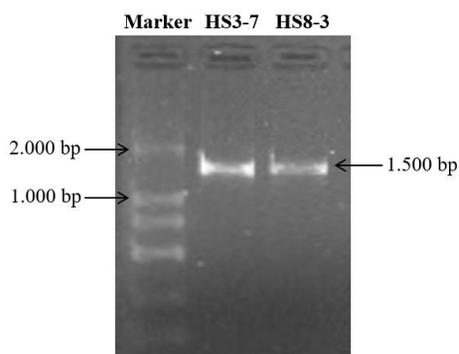


Figure 2. Electrophoresis of bands of HS3-7 and HS8-3

The 16S rRNA fragments of two isolates (HS3-7 and HS8-3) obtained from partial sequencing were 519 bp and 532 bp, respectively (Figure 3 & Figure 4). The obtained 16s rRNA sequences of two isolated strains were aligned to the bacterial 16s RNA sequences recorded in the gene database (NCBI) by using BLAST software. The results showed that the 16S rRNA gene sequence of HS3-7 had high similarity (99.81%) with the gene sequence of *Bacillus vietnamensis* (strain Marseille-P799, accession no. LT558810), and the gene sequence of HS8-3 was 100% similarity with *Bacillus infantis* (strain NRRL B-14911, accession no. CP006643) (Table 4). From morphology, biochemical properties, and 16sRNA identification, it can be concluded that the bacterial strains, HS3-7 and HS8-3, were *Bacillus vietnamensis* and *Bacillus infantis*, respectively.

TCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGATTGATGGGAGCT
 TGCTCCCTGATATCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACT
 GGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACTCATTTCCTCGCATGAGGAAAT
 GTTGAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCATTAGCTAGTTGG
 TGAGGTAACGGCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
 ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
 ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTTCGGATCGTAAA
 GCTCTGTTGTTAGGGAAGAACAAGTGCCGTTTGAATAGGGCGGCACCTTGACGGTACCTA
 ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCC GCGGTAA

Figure 3. The 16S rRNA gene sequence of HS3-7 bacterial strain

GAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAG
 CGGACGGATGGGAGCTTGCTCCCTGAAGTCAGCGGCGGACGGGTGAGTAACACGTGGGC
 AACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATGCAT
 AACCTCTCATGAGGCTATGCTGAAAGATGGTTTTTCGGCTATCACTTACAGATGGGCCCGCG
 GCGCATTAGCTAGTTGGTGAGGTAACGGCTACCAAGGCAACGATGCGTAGCCGACCTG
 AGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
 GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAA
 GGTTTTTCGGATCGTAAA ACTCTGTTGTCAGGGAAGAACAAGTGCCGGAGTAACTGCCGGC
 ACCTTGACGGTACCTGACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCC GCGGTAA

Figure 4. The 16S rRNA gene sequence of HS8-3 bacterial strain

Table 4. Comparison 16S rRNA of HS3-7 and HS8-3 with Gen bank on NCBI

No.	Bacterial strains	Species	Query Cover	Percent Identity	Accession
1	HS3-7	<i>Bacillus vietnamensis</i> strain Marseille-P799	100%	99.81%	LT558810
2	HS8-3	<i>Bacillus infantis</i> strain NRRL B-14911	100%	100%	CP006643

Thirty-nine sequences obtained from aligning the sequences of strain HS3-7 and HS8-3 with the genebank were used for building the phylogenetic tree (Figure 5). The phylogram was constructed using the UPGMA method (Sneath and Sokal, 1973). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The differences in

the composition bias among sequences were considered in evolutionary comparisons (Tamura & Kumar, 2002). This analysis involved 41 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 447 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The phylogram showed that the species HS7-3 and *Bacillus vietnamensis* were closely related and the bootstrap index of this branch (HS7-3) is 77%, and the HS8-3 was also closely related to *Bacillus infantis* (bootstrap index of 100%).

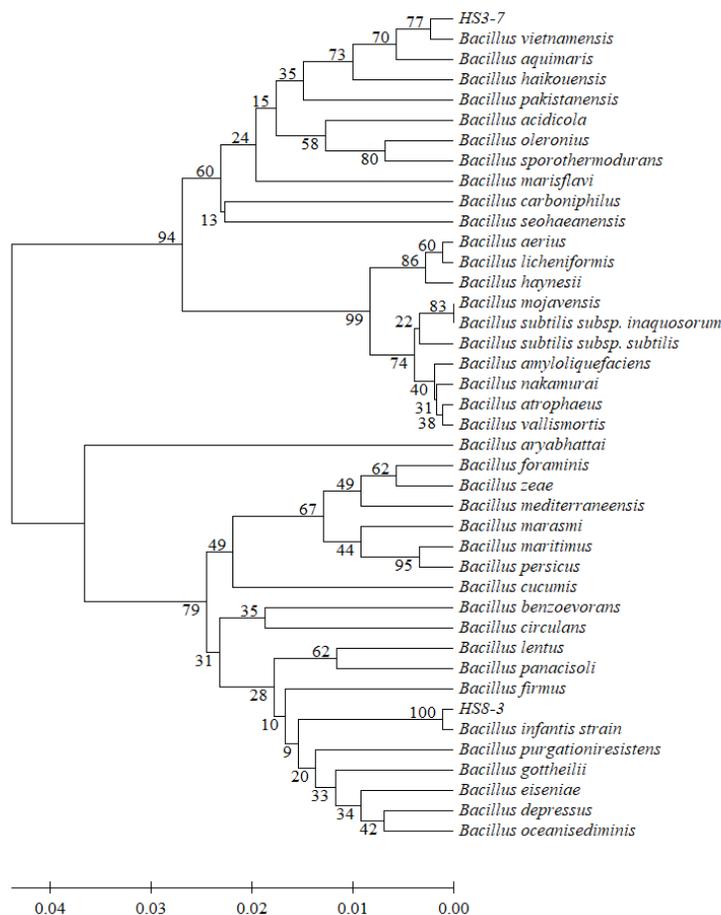


Figure 5. Phylogenetic tree generated by MEGA X of HS 3-7 and HS8-3 isolates

The scale bar indicates genetic distance and bootstrap percentages are represented on nodes.

4. CONCLUSIONS

Twenty pigment-producing bacterial species were discovered and isolated from seashore saline soils and seawater in Hon Son islet, Kien Hai district, Kien Giang province. There were five types of colony colors, namely pale yellow, yellow, yellow-orange, pale pink, and pink. All bacterial cells were rod-shaped, gram-positive, positive catalase test, motile, and able to form endospore, which is similar to the taxonomy characteristic of *Bacillus* spp. Carotenoids extracted from 20 isolates had the highest absorbance at wavelengths from 400 to 600 nm. The total carotenoids extracted from HS3-7 strain (yellow-orange) and HS8-3 (pink) were the highest among 20 species. These two species were identified as *Bacillus vietnamensis* and *Bacillus infantis* based on morphology, biochemical properties, and 16sRNA partial sequencing data. It can be

concluded that the marine bacteria isolated from Vietnam coastal area can be used as the biological source for producing carotenoid natural pigments.

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