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Research article

Rhodocista sp. Strain SAIYAI: A Natural Source of Spirilloxanthin and Feed Attractability in Pacific White Shrimp (*Litopenaeus vannamei*)

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Abstract: This study investigated the potential of isolated photosynthetic bacteria (PSB) as carotenoid producers and assessed their efficacy as a low-cost functional feed additive for Pacific white shrimp (*Litopenaeus vannamei*). Fifteen PSB isolates representing 3 color groups, red, orange, and yellow, were evaluated for total carotenoid content (TCC), and the highest TCC-producing PSB were RP22-4-DPM with 0.82 ± 0.07 mg/g of DW, RP22-OR with 0.38 ± 0.03 mg/g of DW, and RS22-YB with 0.47 ± 0.07 mg/g of DW, respectively. Isolate RP22-4-DPM, further designated as PSB strain SAIYAI, exhibited the highest DPPH scavenging activity (72.65 ± 4.60%) ($p<0.05$); meanwhile, its ABTS scavenging activity (70.7 ± 2.73%) showed no significant difference compared to other strains ($p>0.05$). The predominant pigment and the major carotenoid of this strain were identified as spirilloxanthin by UV-Vis spectrophotometry and APCI LC/MS. Additionally, the attractability was evaluated by coating soybean meal with the lyophilized cell and culture media of the SAIYAI strain at 0.5%, 1%, and 1.5% to Pacific white shrimp. All concentrations significantly increased feed attractability compared to non-coated soybean meal ($p<0.05$), while 0.5% and 1.0% exhibited similar attractability ($p>0.05$). Molecular identification through 16S rRNA sequence analysis and phylogenetic tree construction suggested that strain SAIYAI belongs to the genus *Rhodocista*, which is closely related to *Rhodocista xerospirillum* and *Rhodospirillum centenum*. Accordingly, this study provides evidence that *Rhodocista* sp. strain SAIYAI is a potent spirilloxanthin producer with antioxidant properties and potential as a multifunctional, cost-effective attractant in shrimp feed.

Keywords: *Rhodocista* sp.; photosynthetic bacteria (PSB); spirilloxanthin; attractability; Pacific white shrimp (*Litopenaeus vannamei*)

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important crustaceans and a widely farmed species in several countries, including Thailand. Even though shrimp aquaculture is economically significant, the industry faces major hurdles, including disease outbreaks, environmental damage, and rising production costs. As a result, many shrimp ponds have been abandoned due to unsuccessful cultivation, particularly in the Pak Phanang Basin, which contains a high density of shrimp farms [1]. The condition of these abandoned ponds is typically hypereutrophic, fueled by an abundance of nutrients from the accumulation of particulate organic matter, primarily derived from biological production [2]. This environment enhances the presence of photosynthetic bacteria (PSB), which are considered major decomposers of organic matter, convert organic acids to H_2 and CO_2, and participate in the anoxic carbon cycle [3, 4].

PSB are prokaryotes belonging to the group of phototrophic bacteria that utilize light to generate energy. This group of bacteria is widely distributed in nature, not only in abandoned ponds but also in other environments such as soil, mangrove forests, lakes, reservoirs, and oceans [4]. PSB can be categorized into four primary groups: purple sulfur bacteria (PB), purple non-sulfur bacteria (PNSB), green sulfur bacteria (GSB), and green non-sulfur bacteria (GNB) [5]. The characteristics of each group are distinguished by their bacteriochlorophyll and carotenoid pigments, photosynthetic electron donors, and the composition of their photosynthetic machinery [6]. Carotenoids are natural biomolecules belonging to the isoprenoid subfamily with colors ranging from red, yellow, to orange [7]. Around 100 different carotenoids have been identified in PSB, including spirilloxanthin, lycopene, spheroidenone, β-carotene, zeaxanthin, canthaxanthin, and lutein, each with unique properties [8, 9].

Unlike other common carotenoids such as β-carotene or astaxanthin, spirilloxanthin is uniquely characterized by its long conjugated double-bond system and terminal methoxy groups, which contribute to its robust capacity. It has been demonstrated that spirilloxanthin extracted from *Bacillus licheniformis* RT4M10 exhibits antioxidant scavenging properties [10]. The effects of antioxidant substances have been highlighted across diverse research fields, including aquaculture, where they function in stress reduction or anti-stress, growth stimulation, and immunity improvement [11, 12]. The major bacteria that produce spirilloxanthin are members of the PNSB group, such as *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* [8, 13]. According to Cahoon *et al*. [14], PSB, especially PNSB species, are capable of synthesizing the spirilloxanthin series and exhibit consistent antioxidant properties. In addition, PSB have a shorter antioxidant life cycle and the capability to produce different types of carotenoids with various color shades and desirable biological properties [15]. Therefore, PSB that produces carotenoids with antioxidant activity is a promising candidate for dietary supplementation due to its cost-effectiveness and ease of handling.

PSB, particularly PNSB, has also been studied for its potential as a protein source and growth promoter in shrimp diets and feed additives. Supplementation of these bacteria can enhance growth rates, feed conversion ratios (FCR), survival rates, immune responses, and tolerance to stress [16-19]. Despite these benefits, there is a significant lack of data regarding the attractability effects of PSB in shrimp feed. Attractability in shrimp farming is crucial because it affects feed intake and overall growth performance, which directly influence economic viability [20, 21]. Fish, like other animals, have preferences for certain foods based on their taste, smell, and past experiences. Nutrients and chemosensory cues influence these preferences, the palatability of the food, and the fish's current dietary needs. To improve the appeal of aquafeed, the use of attractants and stimulants has been explored [22, 23]. Addressing attractability issues by incorporating effective attractants is a key consideration for optimizing shrimp farming practices and ensuring sustainable production. PSB, such as marine bacteria, have been demonstrated to play a crucial role in aquaculture by serving as feed attractants for aquatic animals. These bacteria are not only beneficial for their nutritional value but also for their ability to enhance feed palatability and stimulate feeding behavior in aquatic species [24, 25]. Therefore, PSB has shown promising effects on feed intake and is a candidate with high attractability to aquatic animals.

Only limited information about spirilloxanthin utilization and PSB attractability in aquaculture has been demonstrated. As a result, bioprospecting for PSB strains producing high-value substances has garnered considerable attention and is performing better in many fields, especially aquaculture. PSB holds promise as a future multifunctional feed additive for shrimp aquaculture, with potential benefits beyond antioxidant and feed attractant effects. The identification of novel bacterial strains with multifunctional properties represents a significant opportunity for developing integrated approaches to sustainable aquaculture that simultaneously address multiple production challenges. These phototrophic bacteria have been the focus of recent research because they can utilize elements in wastewater for protein biomass and grow under anaerobic conditions or under anoxygenic photosynthesis [17, 18].

Beyond its antioxidant properties, the metabolic profile of certain PSB, particularly those rich in carotenoids such as spirilloxanthin, is hypothesized to influence chemosensory responses in aquatic animals. In *L. vannamei*, feed attractants are crucial for improving consumption rates and reducing waste in intensive systems. However, the specific role of *Rhodocista* ap. and it's metabolites as dietary attractant remains poorly understood. Therefore, this study was designed to bridge the gap between pigment production and functional feed application. Our objectives were to characterize the spirilloxanthin-producing *Rhodocista* sp. strain SAIYAI isolated from the Pak Phanang Basin and to evaluate its efficacy as both a natural antioxidant source and a feed attractant for Pacific white shrimp. The multi-functional properties of this novel strain could provide a significant contribution to the development of sustainable, cost-effective biological feed additives for the shrimp aquaculture industry.

2. Materials and Methods

2.1 Sampling location and PSB screening

PSB were isolated from water and sediment collected from the coastal area of the Pak Phanang Basin, Nakhon Si Thammarat, Thailand. Five sampling locations included those in coordinate of mangroves: Station (1) 8°31'8.63"/ N 99°58'34.37"/E and Station (2) 8°32'36.62"/ N 99°59'49.18"/E, while Station (3) 8°24'22.63"/N 100°13'36.02"/E, Station (4) 8°17'15.11"/ N 100°15'59.41"/E, and Station (5) 8°19'55.79" N 100°12'0.85"/E represent abandoned shrimp ponds (Figure 1). Water and sediment samples used for bacterial isolation were aseptically collected following the procedures of Higuchi-Takeuchi et al. [26] and Nithya and Pandian [27], with slight modifications. Water samples were taken at a depth of 30 cm above the sediment, while sediment was collected at a depth of 30 cm below the sediment surface to avoid contamination. The samples were collected in sterile tubes and kept cool until transported to the laboratory for further processing. Bacterial isolation was conducted using the basal medium G5 (g/L) containing: peptone, 5.0; yeast extract, 5.0; L-glutamic acid, 4.0; malic acid, 3.5; KH_2PO_4, 0.12; and K_2HPO_4, 0.18. The medium pH was adjusted to 7.0 with 5 M NaOH. For the preparation of solid media G5 agar, 1.5% agar was added to the medium. 1 g of sediment sample was transferred to a sterile test tube with 1 mL autoclaved 1.5% NaCl, vigorously mixed, and then spread onto agar plates. The sediment suspension and water samples were diluted 10-fold in series and spread onto an agar plate in triplicate. All plates were incubated at 37°C for 2-10 days, and color colonies were transferred to a new plate to establish a pure culture. The purified isolates were preserved in Tryptic Soy Broth (TSB, Difco) containing 1.5% NaCl (TSB^+) and 20% glycerol, and kept at -80°C. Selected strains were characterized based on colony and cell morphology. Finally, 5 strains exhibiting distinct characteristics within each color group were chosen for evaluation of total carotenoid content (TCC).

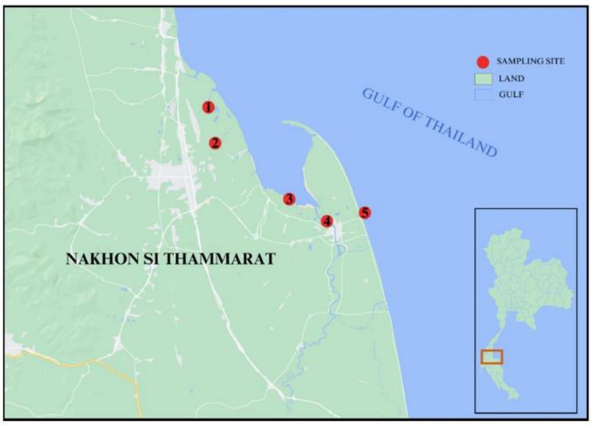

Figure 1. Sampling sites in the Pak Phanang Basin, Nakhon Si Thammarat Province, Thailand. Stations 1 and 2 are in mangrove coordinates, while stations 3, 4, and 5 represent abandoned shrimp ponds.

2.2 Bacteria cultivation

Bacterial cultivation for the downstream studies was performed using a modified method of Sibero *et al.* [28]. Each bacterial isolate was cultured in TSB^+ and shaken at 150 rpm for 48 h at room temperature to serve as a starter for further cultivation. For the assays of pigment profile, antioxidant activity, and attractability, the bacterial production was prepared by adding 100 mL of the starter to the freshly prepared TSB^+ in an Erlenmeyer flask and then culturing under the same conditions as for the starter preparation mentioned above. For the TCC and antioxidant analyses, cells were harvested after shaking for 48 h by centrifugation at 8,000 x g for 15 min and washed twice with 1.5% NaCl. For attractiveness testing, the bacterial cells and culture medium were lyophilized and stored at -80 °C.

2.3 Total carotenoid content (TCC) determination

TCC, from a total of 15 strains (5 strains from each color group: red, orange, and yellow), was determined following the method of Britton *et al*. [29] and Kiriratnikom [30]. Briefly, the cultured bacterial cell was separated into 2 parts for carotenoid quantification and cell mass in terms of dry weight. The first part was extracted with a methanol:acetone solution (2:3 v/v), centrifuged, and the supernatant was collected. The bacterial cell was re-extracted several times until a clear solution was obtained. The volume of extraction liquid was adjusted to achieve appropriate optical densities at OD480 and OD770 for calculating the carotenoid content. For cell mass, the remaining cells from each strain were centrifuged in glass tubes at 8,000 x g at 4 °C for 10 min, washed 3 times with 3% NaCl solution, and dried at 105 °C for 24 h. The dried cell was weighed, and the dry cell mass was calculated as mg dry weight/mL of culture medium. The total carotenoid content formula is:

$$\text{Total carotenoid content (TCC)} = \left(\frac{\text{OD480-0.1OD770}}{\text{DCW (g/L)}}\right) \text{x } 3.85 \text{ x} \frac{\text{B (mL)}}{\text{A (mL)}}$$

DCW: Dry cell weight (mg/g), A: Volume before extraction (mL), B: Volume after extraction (mL).

2.4 In vitro antioxidant activity

The strain with the highest TCC in each color group was further analyzed for antioxidant activity. The pigments were extracted from the bacterial cells by using 99% methanol. Then, the methanolic extract of pigments was centrifuged at 8000 x g for 15 min to separate the residual cells and pigment until the solution was colorless. The antioxidant activity was investigated using the DPPH and ABTS assays.

DPPH free radical scavenging assay was conducted following a modified method of Mukherjee *et al.* [31]. Briefly, 1 mL of the pigment extract was added to 1 mL of a 0.1 mM methanolic solution of DPPH, shaken, and kept in the dark for 30 min, and then measured at 517 nm using a UV/vis spectrophotometer (BioDrop). Methanol was used as a blank solution. The DPPH scavenging effect was determined according to the equation.

$$\text{DPPH scavenging activity (\%)} = \left(\frac{\text{A517 of control- A517 of sample}}{\text{A517 of control}}\right) \text{x } 100\%$$

The ABTS assay was also performed according to the method of Wang *et al.* [24]. ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical-scavenging activity was used to evaluate the antioxidant activity of PSB-extracted pigments. ABTS solution (7 mM) was prepared with 2.45 mM potassium persulfate and was incubated in the dark for 16 h to generate ABTS radicals. The obtained solution was adjusted with 0.01 M PBS buffer (pH 6.8) to achieve an absorbance of approximately 0.7 ± 0.2 at 734 nm. The prepared ABTS solution (600 μL) was added to 50 μL of the sample, and the mixture was incubated in the dark at room temperature for 6 min. The absorbance of the mixture was monitored at 734 nm, and the percentage of ABTS scavenging activity was calculated using the provided equation.

$$\text{ABTS scavenging activity (\%)} = 1 - \left(\frac{\text{Asample}}{\text{Acontrol}}\right) \text{x } 100\%$$

The PSB strain with the highest scavenging activity was selected for further analyses, including determination of the pigment profile, attractability, and bacterial identification.

2.5 Pigment profile

2.5.1 Color extraction and Thin Layer Chromatography analysis

Crude pigment was extracted from the selected PSB strain exhibiting the highest scavenging activity following the modified method of Fang *et al.* [32] by using acetone until the cell pellet was colorless. The color was separated by adding diethyl ether at a 1:1 ratio to the obtained color solution. After the ether phase containing the pigments was obtained, the pigments were separated by Thin Layer Chromatography (TLC) on a silica gel plate (20 cm × 20 cm) using a mixture of diethyl ether and petroleum ether as the mobile phase. The mobile phase ratio was screened until a clear separation of color was observed. The major pigment, expressed as a bold band, was purified by scraping, eluted with diethyl ether, and then dried under N_2 gas to obtain a powder, which was kept at -20 °C until use for profile analysis.

2.5.2 Major carotenoid characterization by mass spectrum analysis

The purified major carotenoid collected from the TLC method was scanned for UV–Vis absorbance spectrum with the wavelength of 400-600 nm by using a spectrophotometer (Biodrop). Moreover, the collected carotenoid powder was eluted with methanol for mass spectrometry (MS) analysis following the method of Ranga *et al*. (2009). The carotenoids were identified using LC-QTOF MS (Agilent Technologies). In brief, the APCI instrument parameters followed the machine protocol, including gas temperature at 350 °C, vaporizer temperature at 400°C, gas flow at 5 L/min, and nebulizer at 60 psig. Scan source parameter followed by VCap 3500, corona positive 4 kv, fragmentor 140, and OctopoleRFPeak at 750. The spectrometer was calibrated in the positive mode, and $[M+H]^+$ ions were recorded. Mass spectra of carotenoids were acquired over an m/z 100-1000 scan range using a diode-array detector and confirmed with respective standards.

2.6 Ethical statement

The Animal Ethics Committee approved the experimental procedures and animal care in this study, Rajamangala University of Technology Srivijaya (Ethics Record No. IAC 01-01-2024), following the guidelines of the Institute of Animal for Scientific Purposes Development, National Research Council, Thailand.

2.7 Attractability assessment

The attractiveness of the aforementioned bacterial strain with the highest scavenging activity was evaluated. Bacterial powder was obtained by freeze-drying the bacterial culture using the modified method of Kim *et al*. [33]. Four g of soybean meal were coated with 0%, 0.5%, 1%, and 1.5% of lyophilized powder and used as trial feeds for Pacific white shrimp. The bacteria-coated soybean meal at all PSB concentrations was air-dried and kept at -20 °C until used. The attractability assessment was conducted following the methods of Suresh et al. [34] and Chirapongsatonkul et al. [35], with slight modifications. Rectangular glass tanks (60 × 50 × 22 cm), with acclimatization and feeding chambers, were used. The tank contained an acclimatization chamber at one end and four feeding chambers at the other, separated by movable shutters (Figure 2). Each feeding chamber was equipped with an aperture to allow shrimp attempting to feed to enter the chamber. Twenty shrimp (1-2 g) were placed in the acclimatization chamber for a 60 min period. Five min before the time period, the trial feed, packed in a sterilized stainless steel mesh tea ball, was placed in the chamber at 3 cm above the bottom. After removing the shutter, shrimp approached the trial feeds in the feeding chambers. A video camera (SONY Zeiss) was set up and recorded for 15 min to collect data throughout the entire experimental duration. Each feed trial underwent random testing 4 times within one of the four designated feeding chambers. The assessment was calculated based on the percentage of shrimp approaching the feeding chamber (%Turn) and the time interval in the chamber (Time), as previously described [35].

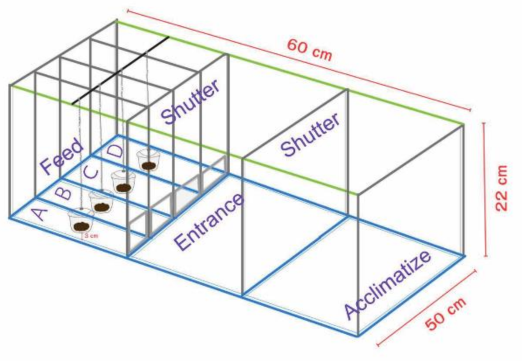

Figure 2. Schematic diagram of the glass tank and its components used for the feed attractability assessment.

2.8 Bacteria identification

The selected strain exhibiting high carotenoid content and the greatest attractability was molecularly identified using conserved 16S rRNA sequencing following the modified method of U-taynapun *et al*. [36]. Bacterial DNA was extracted using the Bacterial DNA Isolation Kit (Geneaid) according to the manufacturer's instructions. DNA concentration was measured by absorbance, and DNA purity was assessed by the 260/280 and 260/230 ratios, with values within 1.8-2.0, indicative of high-quality DNA. The DNA was stored at -80 °C until use. The obtained DNA served as a template for 16S rRNA amplification, conducted according to the manufacturer's instructions for Accustart™ II PCR Supermix (Quanta). The primer pairs used for 16S rRNA amplifications were 20F: 5′-AGAGTTTGATCATGGCTCAG-3′ and 1500R: 5′-CGGTTACCTTGTTACGACTT-3′. Bacterial DNA was amplified using a PCR program that included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. The PCR product was analyzed by agarose gel electrophoresis at 1.5%. Purification of the PCR product was performed using the Gel/PCR DNA Fragments Kit (Geneaid) according to the manufacturer's instructions. Purified DNA fragment with a size of approximately 1,500 bp was subsequently cloned into the pGEM-T® Easy Vector (Promega), and sequenced. The 16S rRNA gene was deposited in NCBI (National Center for Biotechnology Information). To identify and retrieve homologous sequences, the obtained sequences were subjected to the NCBI BLAST tool. Alignment was manually checked using BioEdit, and the ClustalW algorithm in MEGA 11 was employed for sequence alignment. The measurement (%) of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates), and MEGA 11 software was used to generate a phylogenetic tree using the Maximum Likelihood [37].

2.9 Statistical analysis

The statistical differences in TCC, antioxidant activity, and attractability were analyzed using one-way analysis of variance (ANOVA) in SPSS Statistics Software version 20.0 (SPSS Inc.). The variance and significant differences among treatments were analyzed using Duncan's Multiple Range Test (DMRT) with a significance level of $p<0.05$.

3. Results and Discussion

3.1 Screening, isolation, and characterization of PSB

Following the isolation of PSB from 5 sampling areas, 30 isolates were obtained on G5 agar plates. The morphology, Gram staining characteristics, and colony appearance were recorded for each isolate. Categorization by colony color: 10, 11, and 9 isolates for red, orange, and yellow, respectively. The majority of bacteria collected in this study were Gram-negative, rod-shaped. Details of all isolates, including sampling area, colony color, characters, and morphology, are summarized in Table 1. Afterwards, these 30 isolates were screened for pigment production based on color shade and morphology. In previous studies, PSB groups have been shown to be carotenoid producers [38-40]. Therefore, a total of 15 isolates, 5 from each color group, were selected for TCC quantification.

3.2 TCC analysis

The TCC from 15 PSB isolates was analyzed and compared within their respective color groups. The results showed that the highest TCC in the red, orange, and yellow groups was found in strains RP22-4-DPM (0.82 ± 0.07 mg/g of DW), RP22-OR (0.38 ± 0.03 mg/g of DW), and RS22-YB (0.47 ± 0.07 mg/g of DW), respectively (Figure 3). Carotenoids produced by PSB are diverse, and most isolates contain high levels of total carotenoids. Previous studies have reported that the biomass production by PNSB varies depending on the species, growth stage, and culture medium used [41]. According to Soon *et al.* [42], bacterial isolates from exposed habitats induce higher dry cell weight (g/L) and total carotenoids production (mg/g dry cell weight) than those from shaded habitats. Three isolates with significantly higher TCC levels ($p<0.05$) than other isolates within the same color group were classified as the highest TCC producers. Among the primary compounds responsible for antioxidant activity are carotenoids, which serve as vital secondary metabolites in microorganisms. These compounds have been demonstrated to play roles in regulating light absorption and preventing photodamage and oxidative stress. due to their antioxidant properties [43]. Consequently, the 3 TCC producers, RP22-4-DPM, RP22-OR, and RS22-YB, were measured for their *in vitro* antioxidant activity.

3.3 Antioxidant assay

Carotenoids exhibit antioxidant potential by neutralizing free radicals through electron transfer, donating hydrogen atoms, or attaching to radicals. The antioxidant activity of carotenoids is significantly influenced by their oxidation potential, which is closely related to the conjugation length and donor/acceptor substituents within the molecule. Shorter conjugation lengths and the incorporation of electron-withdrawing groups result in higher oxidation potentials [44]. In this study, the antioxidant capacity of the crude pigment extracted from the 3 PSB isolates with the highest TCC was evaluated using DPPH and ABTS assays. The results were expressed as the percentage free radical scavenging activity. As shown in Figure 4, each isolate demonstrated different antioxidant levels. Significant differences in DPPH scavenging activity were observed among these 3 isolates ($p<0.05$). Specifically, RP22-4-DPM exhibited the greatest DPPH scavenging activity (72.65 ± 4.60%), followed by GNew4-OC (60 ± 6.94%) and RS22-YB (42.08 ± 4.13%), respectively. In the ABTS assay, although GNew4-OC exhibited the highest scavenging activity (74.16 ± 2.17%), it did not statistically differ from that of RP22-4-DPM (70.7 ± 2.73 %) ($p>0.05$). RS22-YB exhibited the lowest ABTS activity (67.07 ± 2.35%). Based on these findings, the red-pigmented isolate RP22-4-DPM demonstrated the most robust overall antioxidant performance. Given its highest DPPH scavenging capacity and significantly higher TCC compared to the other two strains, RP22-4-DPM was designated as the PSB strain SAIYAI and selected for further investigations.

Table 1. Sample origin, color, character, and morphology of PSB isolated from Pak Phanang Basin.

Number	Isolates	Location	Colony Color	Character		Colony Morphology				
				Gram	Shape	Shape/Form	Margin	Surface	Elevation	Size
1	R3	Station 1	Red	-	Rod	Circular	Entire	Smooth	Flat	Small
2	R4	Station 1	Red	-	Rod	Circular	Filamentous	Wrinkled	Flat	Medium
3	O3	Station 1	Orange	-	Rod	Circular	Entire	Smooth	Flat	Small
4	GNEW4-OC	Station 1	Orange	+	Rod	Circular	Serrate	Concentric	Umbonate	Medium
5	GNEW3-BO	Station 1	Orange	-	Rod	Circular	Entire	Smooth	Raised	Medium
6	Y2	Station 1	Yellow	-	Rod	Circular	Entire	Smooth	Raised	Medium
7	RP22-4-DPM	Station 2	Red	-	Rod	Circular	Entire	Smooth	Flat	Small
8	RP22-4-RM	Station 2	Red	-	Filamentous	Circular	Entire	Smooth	Flat	Small
9	RP22-OR	Station 2	Orange	-	Rod	Circular	Entire	Smooth	Raised	Small
10	RP22-OT	Station 2	Orange	-	Rod	Circular	Entire	Smooth	Flat	Small
11	O1	Station 2	Orange	-	Rod	Circular	Entire	Smooth	Flat	Small
12	RS13-Y	Station 2	Yellow	-	Rod	Circular	Entire	Smooth	Flat	Medium
13	R2	Station 3	Red	-	Rod	Circular	Entire	Smooth	Flat	Small
14	R6	Station 3	Red	-	Rod	Circular	Entire	Smooth	Umbonate	Medium
15	GNEW2-O	Station 3	Orange	-	Rod	Circular	Entire	Smooth	Raised	Small
16	GY22-OB	Station 3	Orange	-	Rod	Circular	Entire	Smooth	Raised	Medium
17	GNEW-BY	Station 3	Yellow	-	Rod	Circular	Entire	Smooth	Flat	Small
18	GNEW2-Y	Station 3	Yellow	-	Rod	Circular	Entire	Smooth	Flat	Small
19	R3B	Station 4	Red	-	Rod	Circular	Entire	Smooth	Raised	Small
20	GNew-OF	Station 4	Orange	-	Rod	Undulate	Concentric	Flat	Undulate	Medium
21	O5	Station 4	Orange	-	Rod	Circular	Entire	Smooth	Umbonate	Medium
22	X1	Station 4	Yellow	-	Rod	Circular	Wavy	Smooth	Flat	Medium
23	RG-DY	Station 4	Yellow	+	Rod	Circular	Entire	Smooth	Flat	Small
24	RG-YB	Station 4	Yellow	-	Rod	Circular	Entire	Smooth	Flat	Small
25	R15	Station 5	Red	-	Rod	Undulate	Concentric	Flat	Undulate	Medium
26	R16	Station 5	Red	-	Rod	Circular	Entire	Smooth	Flat	Small
27	R17	Station 5	Red	-	Rod	Circular	Entire	Smooth	Umbonate	Medium
28	RS22-BO1	Station 5	Orange	-	Rod	Circular	Entire	Smooth	Flat	Large
29	RS4-Y	Station 5	Yellow	-	Rod	Circular	Entire	Smooth	Flat	Small
30	RS22-YB	Station 5	Yellow	+	Rod	Circular	Entire	Smooth	Flat	Small

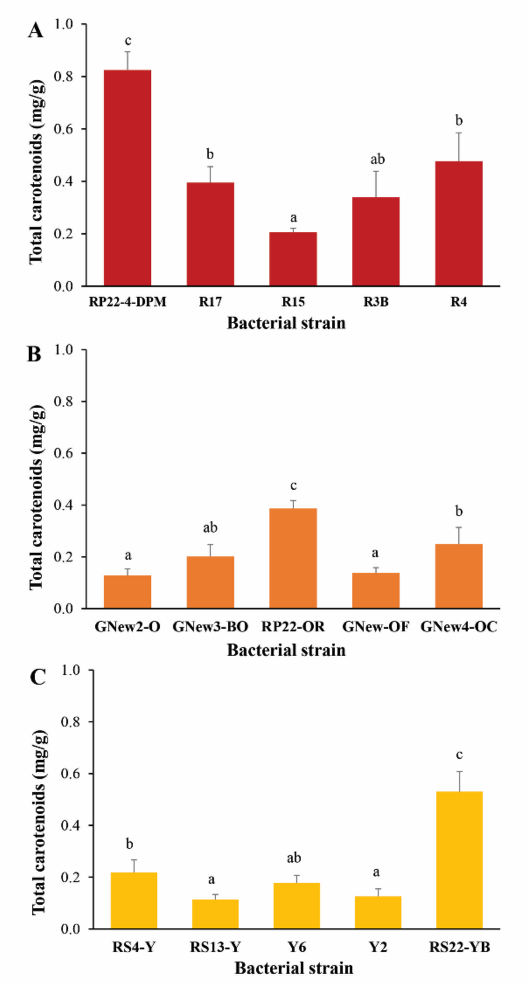

Figure 3. Total carotenoid content (TCC) produced by the selected PSB strains. (A) Red color group, (B) Orange color group, and (C) Yellow color group. Values are means ± SD. Different letters indicate statistically significant differences ($p<0.05$).

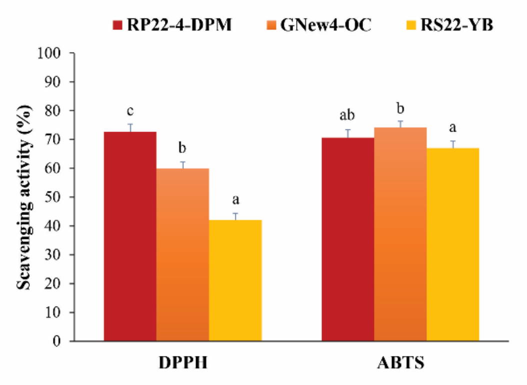

Figure 4. *In vitro* antioxidant activities analyzed by DPPH and ABTS assays of the three PSB strains exhibiting the highest TCC level selected from each colony color group, including RP22-4-DPM (red), GNew4-OC (orange), and RS22-YB (yellow). Values are means ± SD. Different letters indicate statistically significant differences ($p<0.05$).

3.4 Pigment profile

PSB strain SAIYAI, exhibiting a red colony (Figure 5A), was subcultured weekly and maintained on TSA+ agar. Since pigment production occurs during the stationary phase of bacterial growth, pigment was extracted from PSB SAIYAI cells on the fourth day of cultivation. Acetone extraction followed by ether partitioning yielded a deep red solution (Figure 5B). The carotenoid components were separated by TLC using a mixture of diethyl ether and petroleum ether as the mobile phase, varying the ratio until complete separation. A prominent pink band was identified as the major carotenoid (Figure 5C), which was further purified and characterized. The UV-Visible spectrum (400-600 nm) of the purified pigment from the PSB strain SAIYAI showed 3 peaks at 460, 480, and 520 nm, corresponding to the major carotenoid (Figure 6). To elucidate the carotenoid structure, APCI LC/MS was performed. The mass spectrum obtained by the APCI LC/MS revealed a peak at *m/z* 597.4662 $[M + H]^+$, with a molecular formula of $C_{42}H_{60}O_2$ (Figure 6). The mass spectrum and predicted structure is presented in Figure 6. Altogether, these data strongly indicate that the major carotenoid from SAIYAI isolate is spirilloxanthin. It has been reported that it is one of the carotenoids most commonly found in PNSB, with the chemical structure $C_{42}H_{60}O_2$ [13]. Indeed, Imhoff [45] reported that carotenoids in the spirilloxanthin series and bacteriochlorophyll a are key photosynthetic pigments of PNSB, including the genus *Rhodocista*. A previous study demonstrated that spirilloxanthin exhibits potent antioxidant properties, consistent with its molecular structure: a relatively long backbone of 13 conjugated double bonds and very weakly polar terminal methoxy groups [14]. Two antioxidant capacity assays have suggested that spirilloxanthin is similar but not superior to lycopene and β-carotene in this regard. In addition, it has been revealed that spirilloxanthin has antioxidant properties and can be applied as a neuroprotective agent, as tested in rats [46]. However, specific applications of spirilloxanthin in aquaculture have not yet been documented. Further antioxidant testing would be necessary for developing spirilloxanthin as a commercial antioxidant product.

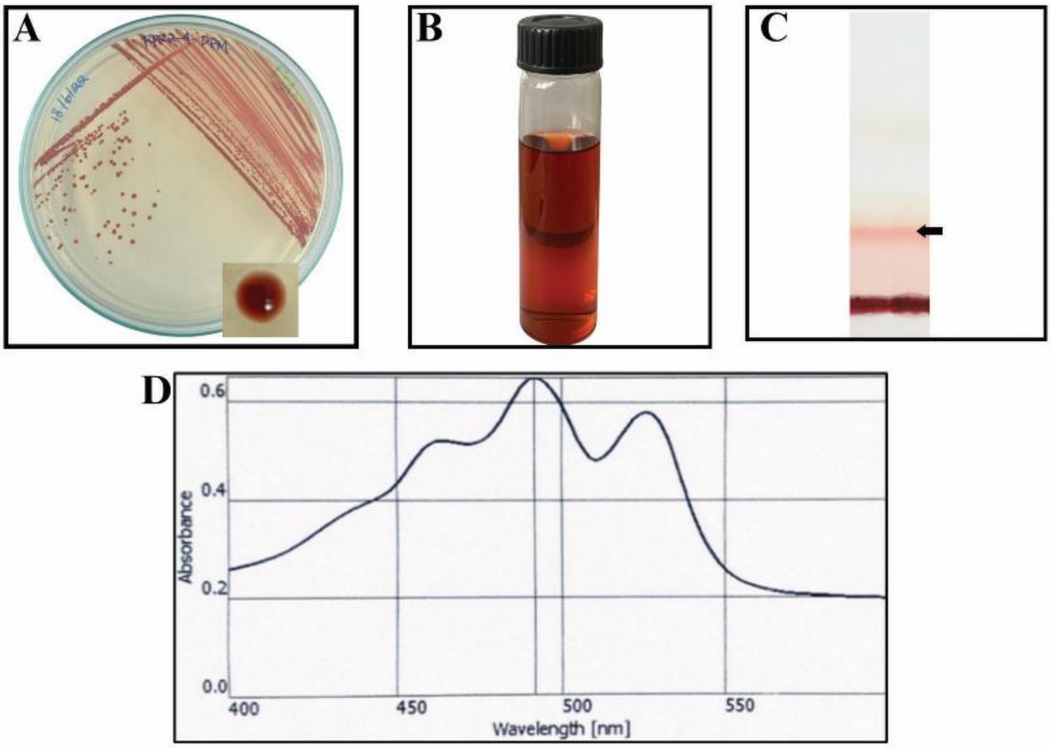

Figure 5. Colony morphology of PSB strain SAIYAI and its produced carotenoids. **(A)** Bacterial colony, **(B)** Crude pigment extracted from the bacterial cells, **(C)** Thin Layer Chromatography (TLC) separation, and **(D)** UV-Vis absorption spectrum of the purified major carotenoid.

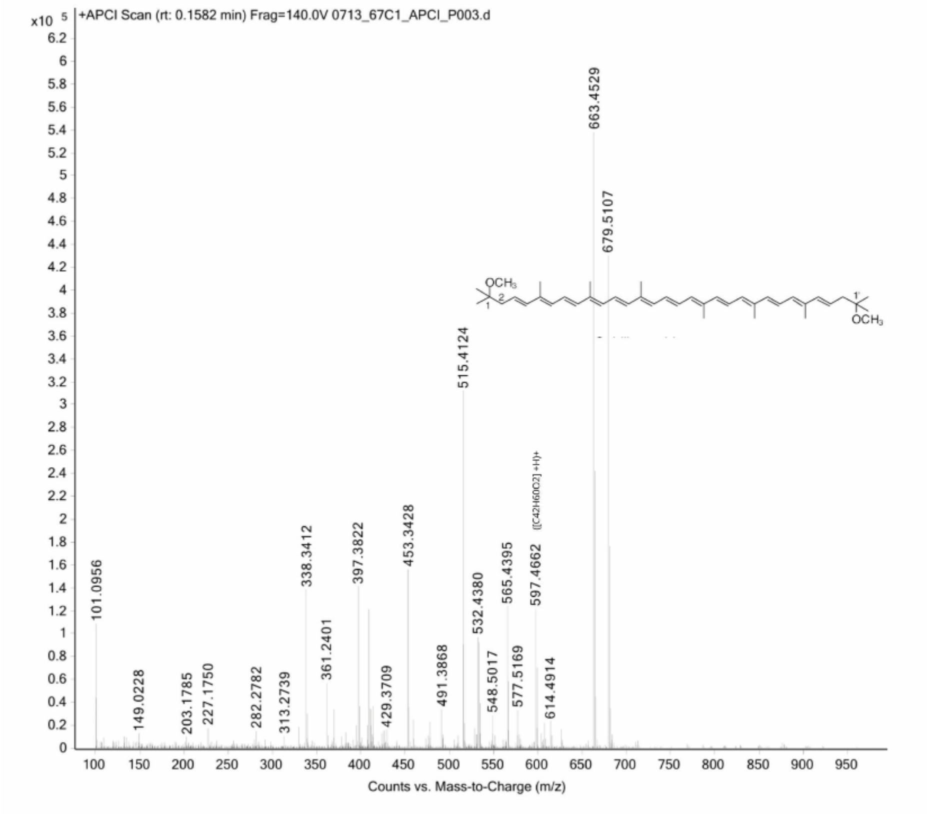

Figure 6. APCI LS/MS profile and the putative structure, spirilloxanthin with the molecular formula of $C_{42}H_{60}O_2$, of the major carotenoids extracted from PSB strain SAIYAI**.**

3.5 Attractability assessment of PSB strain SAIYAI to Pacific white shrimp

The attractability of the trial feeds coated with the PSB SAIYAI strain at 0.5%, 1.0%, and 1.5% showed a significant difference ($p<0.05$) compared to the control (Figure 7). Moreover, 1% SAIYAI-coated soybean meal exhibited the highest attractability in terms of shrimp turning (38% ± 7.83) compared with other concentrations and the control (Figure 7A). Furthermore, the duration shrimp remained in and approached the chamber for the 0.5% and 1% concentrations showed a significant difference ($p<0.05$) compared to the control and 1.5% groups (Figure 7B). These findings suggested that PSB strain SAIYAI can enhance the attractability of soybean meal, which is typically less attractive to Pacific white shrimp. Soybean meal is well known for its lower attractiveness to aquatic organisms, including shrimp [47]. Animals rely on their sensory systems to discriminate between foods that deliver pleasant or unpleasant feelings associated with eating [23]. To improve feed intake, attractants/stimulants have gained significant attention as feed additives. Bardera *et al*. [48] explained that when shrimp exhibit higher attraction to feed, they spend more time and stay longer in the feeding chamber than in the control. This corresponds with a reduction in overall locomotion, evidenced by decreased travel distance and velocity. The shrimp exhibit fewer transitions away from the feeding area, maintaining a closer average proximity to the food source. Similar to our results, PSB strain SAIYAI-coated soybean meal showed a significant, dose-dependent increase in attractiveness compared to the control (non-coated soybean meal). The best concentration of SAIYAI strain-coated soybean meal is 1% with 38% ± 7.83 of shrimp turning and 71 ± 10.06 s of residency time. Shrimp are primarily attracted to dietary components such as amino acids. Low molecular weight and high water solubility allow amino acids to act as efficient attractants, whereas shrimp chemoreceptors are better at detecting soluble compounds and small molecules. These amino acids can trigger a feeding response, which can detect an attractive food source, involving responses such as increased animal activity as the animal explores the area near the food source. Lim *et al*. [49]

have provided evidence of the efficiency of amino acids as chemoattractants for crustaceans, due to reduced food searching and feeding responses. Some amino acids have been demonstrated to act as chemoattractants for crustaceans, helping them reduce food-searching and feeding responses. Alanine, arginine, glycine, histidine, leucine, serine, taurine, and betaine could stimulate swimming crab (*Portunus pelagicus*) to feed, while alanine, serine, and betaine have been reported to have chemoreceptive properties in fiddler crab (*Uca pugilator*) [50]. Some PSB groups have a strong odor, which may also serve as an attraction factor. However, the specific amino acid profiles and odor pathways involved in PSB attractability in Pacific white shrimp require further investigation. To the best of our knowledge, this is the first report regarding the attractiveness of PSB in *L. vannamei*.

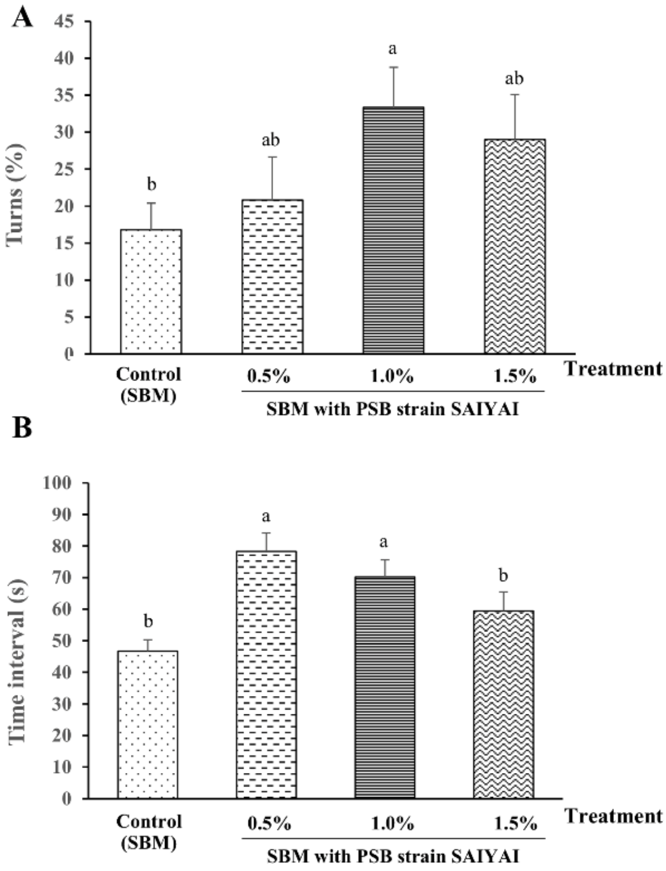

Figure 7. Attraction of soybean meal (SBM) coated with different concentrations of PSB strain SAIYAI to *L. vannamei*. **(A)** Shrimp turning (%) and **(B)** Residency time in the feeding chamber are represented as Time interval. Values are means ± SD. Different letters indicate statistically significant differences ($p<0.05$).

3.6 Bacteria identification by 16S rRNA-based analysis

To identify the PSB strain SAIYAI, molecular characterization was performed using the 16S rRNA sequence. The obtained sequence, 1 ,393 bp long, and its GenBank Accession Number (PQ143229) were compared to the GenBank database. The sequences of 16S rRNA genes from representative isolates and their closest species were used to construct a phylogenetic tree using the Maximum Likelihood method in MEGA 11. The combined dataset comprised 63 taxa (including our strain) representing 12 genera, including 40 species in the family Rhodospirillaceae, 13 species in Rhodobacteraceae, and 7 species in Nitrobacteraceae, which are affiliated with the Alphaproteobacteria class. *Escherichia coli, Aeromonas hydrophila,* and *Pseudomonas aeruginosa*, all from the Gammaproteobacteria class, were used as the outgroup. Bootstrap values, expressed as a percentage of 1000 replicates, are shown at branch points; GenBank Accession Numbers are shown in parentheses. The phylogenetic analysis demonstrated that the PSB strain SAIYAI was most closely related to

Rhodocista xerospirillum, which was clustered within the genus *Rhodocista* in the Alphaproteobacteria class (Figure 8). However, the bootstrap value for the node between them is 73%, indicating that the existing data does not well support this classification. Based on 16S rRNA analysis and phylogenetic tree construction, our target PSB was identified as *Rhodocista* sp. strain SAIYAI, which can be grouped into PNSB. This bacterial group has recently gained increasing focus as multifunctional feed additives and protein sources in aquaculture. Certain PNSB strains, such as *Rhodobacter sphaeroides* SS15 and *Afifella marina* STW181 [16], *Rhodopseudomonas faecalis* PA2 [51], *Rhodopseudomonas palustris,* and *Rhodobacter capsulatus* [17], have proper nutrient content and provide essential nutrients, including amino acids, vitamins, and carotenoids.

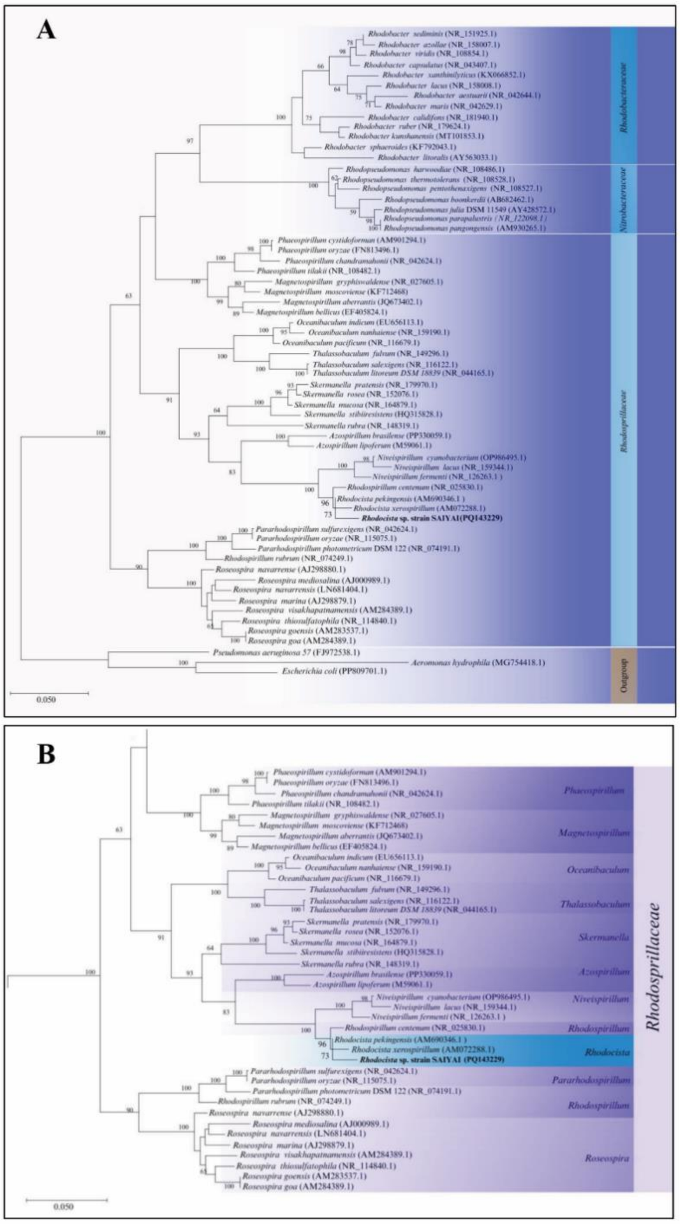

Figure 8. Phylogenetic tree based on 16S rRNA gene sequence of *Rhodocista* sp. strain SAIYAI. **(A)** The respective taxa within the Alphaproteobacteria class and **(B)** Related genera of the Rhodospirillaceae family and the members of genus *Rhodocista.* The phylogenetic tree was constructed using the Maximum Likelihood method with 1,000 bootstrap replicates in MEGA 11. Labels indicate bacterial names and GenBank Accession Numbers.

4. Conclusions

This study demonstrated that *Rhodocista* sp. strain SAIYAI possesses significant potential as a carotenoid producer, a source of spirilloxanthin, and an effective feed attractant for Pacific white shrimp. Spirilloxanthin, the prominent carotenoid produced by this bacterium, exhibits antioxidant efficiency; however, its specific mechanisms of action in aquatic systems warrant further investigation. As a feed additive, this strain has also been considered a low-cost, attractive product and valuable. Future research should explore the multifaceted impacts of *Rhodocista* sp. strain SAIYAI on the growth performance of *L. vannamei* and various quality parameters, including carotenoid-enhanced pigmentation, flesh amino acid composition, and taste. Moreover, investigating the effects of this bacterium on immune responses and gut microbiota would provide valuable insights into its application in sustainable aquaculture practices.

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