CHAPTER II

ISOLATION AND SCREENING FOR THE HIGHLY CAROTENOID PRODUCTION OF PHOTOSYNTHETIC BACTERIA

Introduction

Photosynthetic bacteria can be found in various kinds of habitats such fresh water, sea water, sulfur-containing hot water springs, clay and sediment (Pfennig, 1967; Imhoff, 1988). Moreover, some strains have been found in the seafood-processing wastewater (Prasertsan *et al.*, 1993) and photosynthetic sludge in the recirculating aquaculture system (Kim *et al.*, 1999). Several applications of the photosynthetic bacteria have been reported such as the single- cell protein for animal feed (Vrati, 1984), hydrogen production from waste water for the renewable and clean energy sources (Yigit *et al.*, 1999) and industrial waste water treatment (Prasertsan *et al.*, 1997; Watanabe *et al.*, 1998). Moreover some strains of photosynthetic bacteria were used in the treatment of waste water in the aquaculture system (Kim *et al.*, 1999).

Microorganisms containing high carotenoids levels have been used as natural sources of carotenoids in aquatic animal feed (Latscha, 1991). However, the efficiency of the substance depends on the type of carotenoids (Yamada *et al.*, 1990), digestion and absorption of aquatic animal (Genteles and Haard, 1991) and composition of the diet (Nickell and Bromage, 1998). Photosynthetic bacteria almost contained high levels of total carotenoids which is possible to use as a carotenoid source in aquatic animal feed. They are rich in protein (50%-70% w/w), with balance of the essential amino acids, and contain a relatively high content of vitamin B_{12}, ubiquinone and carotenoids (Vrati, 1984; Noparatnaraporn and Nagai, 1986). Moreover, the lipopolysaccharide from the Gram negative

bacteria have been reported as the immunostimulants in many crustacea (Smith and Soderhall, 1984). Thus, the photosynthetic bacteria can be used as either a nutritional carotenoid source and/or an immunological enhancer in aquatic feed.

Materials and methods

Culture medium

The synthetic medium (G5) used for enrichment and isolation of photosynthetic bacterial strains contained (g/l) : 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; (Kohlmiler and Gest, 1951). The initial pH of the culture medium was adjusted to 7.0 by using 5 M NaOH. For preparation of solid media G5 agar, 1.5 % agar was added to the medium.

The sulfide medium contained (g/l) : Na_2S 0.1, Na_2HCO_3 0.02, $(NH_4)_2SO_4$ 0.132, basal medium (nicotinic acid 1.0 mg / l, ρ-aminobanzoic acid 1.0 mg / l, thiamin 1.0 mg / l and biotin 0.001 mg / l) and distilled water 100 ml, pH was adjusted to 6.8 by using 5 M NaOH (Watanabe *et al*., 1981).

Thoisulfate medium contained (g/l) : $Na_2S_2O_3$ 0.1, $NaHCO_3$ 0.2, $(NH_4)_2SO_4$ 0.132, basal medium and distilled water 100 ml. pH is adjusted to 6.8 by using 5 M NaOH (Watanabe *et al*., 1981).

Isolation of photosynthetic bacteria

Water from shrimp ponds were collected from Nakornsrithamarat, Songkhla, Satun and Pattani Provinces, Thailand. The method used for the isolation of bacterial strains was modified from Prasertsan *et al.* (1993). Growth of the photosynthetic bacteria was enhanced by incubation under

anaerobic conditions with the illumination of 1,000-1,500 lux at room temperature (28-30 ^{0}C) for 7-12 days, followed by enrichment on a G5 agar plate. A loopful of pinkish, brownish or reddish culture broth was streaked onto G5 agar and incubated under the same conditions for 7 days. After the incubation, each colony was transferred onto new plates and subsequently cultured until a pure culture was obtained.

The isolated strains were stored using a method modified from Watanabe *et al*. (1998). The bacterial strains were cultured in agar (G5 medium with 3 % NaCl) overlying with sterile liquid paraffin under anaerobic light conditions after cultivation for 2-3 days at 30-32 ^{0}C. The stock cultures can be stored at 4^{0}C for 2-3 months.

Screening for the highly carotenoid production strain

Pre-cultures of each photosynthetic bacteria were grown in both aerobic dark and anaerobic light conditions with G5 broth media. Each bacterial strain which was grown in the basal medium (G5) in anaerobic light conditions for 3 days was inoculated into screw cap tubes containing 50 ml of G5 medium. The inoculum size of pre-culture was 5 % (v/v) of the medium. Anaerobic conditions were maintained by covering the medium surface with a 1-cm thick layer of liquid paraffin. The tubes containing bacteria were incubated at 30 $\pm$ 2 ^{0}C in the presence of light from a tungsten lamp with an intensity of 1500 lux. The aerobic condition was carried out by inoculating the pre-culture, which was grown on the G5 medium in aerobic dark conditions for 3 days, into 50 ml of G5 medium in a 125 ml flask with an inoculum size of 5%. The aerobic conditions were maintained by placing the cultivation flasks on a shaker at a speed of 150 rev/min at 30 $\pm$ 2 ^{0}C in the dark. Each strain was tested for total carotenoids content in duplicate by transfering 5% of the inoculum into the 50 ml of G5 broth in both anaerobic light and aerobic dark conditions. For anaerobic conditions the culture system was placed in a 50 ml screw cap test tube overalying with

1 cm of sterile liquid paraffin as described by Prasertsan *et al.* (1993). The tubes were incubated at 30 $\pm$ 2 ^{0}C in the presence of light from a tungsten lamp with an intensity of 1,500 lux for 3 days. Aerobic culture was carried out by inoculating the pre-culture grown in aerobic dark conditions for 3 days into 50 ml of G5 medium in an 125 ml flask. The culture flasks were placed on a shaker at a speed of 150 rev/min at 30 $\pm$ 2 ^{0}C and left in the dark for 3 days.

After incubation periods, 5-10 ml of culture broth from each testing system were transferred to determine total carotenoids content following the method modified from Hirayama (1968). After centrifugation at 10,000 rpm for 15 min, the cell pellet was washed with 1.5 % NaCl and then extracted with methanol : acetone solution (2 : 3 v/v), centrifuged and the supernatant was collected from each strain. Re-extraction of the cell was repeated until the pigment was not observed in the supernatant. The volume of extraction liquid was adjusted to have an appropriate optical density at OD 480 and OD 770 for calculating the caroteniods content.

The cells from each testing system were harvested to determined the cell mass in culture medium by the method adapted from Kim *et al.* (1999). Dry cells were obtained by centrifugation at 10,000 rpm for 10 min at 4 ^{0}C in the glass centrifuge tubes, washed 3 times with 3 % NaCl solution, and dried at 105^{0}C for 24 h, weighing the dry cell mass in each tube and calculation in mg dry weight / ml of culture medium.

$$\text{Total carotenoid content (mg/g dry weight)} = \frac{(OD_{480} - 0.1\ OD_{770}) \times 3.85 \times B}{Z} \quad \overline{A}$$

A = Volume of liquid (culture medium) before extraction (ml)

B = Volume of liquid after extraction (ml)

Z = Dry cell weight of photosynthetic bacteria (g/liter)

The pigment production of each strain from each condition was compared and selected for further studies.

Identification of the selected strain

Biochemical identification

For family identification, the selected strains (from 3) were cultured for 24 h in sulfide medium (Watanabe *et al.*, 1981) under anaerobic light (3,000 lux) at room temperature. The purple non-sulfur bacteria (Family Rhodospirillaceae) cannot grow on sulfide and thiosulfate medium (Staley *et al.*, 1989). The identification of genus and species of bacteria followed *Bergey's Manual of Determinative Bacteriology* (Buchanun and Gibbon, 1974) and biochemical tests were made according to *Biochemical Tests for Identification of Medical Bacteria* (MacFeddin, 1980), particularly:-cell shape, Gram stain, the growth of cells under anaerobic light and aerobic dark and the slime formation determined in G5 medium.

- bacteriochlorophyll was analyzed by scanning spectroscopy of the living cell measured at 300-900 nm.
- nutrient requirements were determined by media containing: acetate, propionate, lactate, citrate, glutamate, glucose, glycerol, ethanol and thiosulfate under anaerobic light after cultivation for 24, 48, 72 h and then measured for the growth at OD_{660} nm.

16S r DNA sequencing and phylogenic analysis

16S r DNA sequencing and phylogenic analysis were performed by the method modified from Amann *et al.* (1995). The 16S rRNA gene was PCR amplified from genomic DNA isolated from pure bacterial colonies. Primers used for the

amplification correspond to *E. coli* positions 5-531. Amplification products were purified from excess primers and dNTPs using Micon 100 (Millipore, USA) molecular weight cut-off membranes and checked for quality and quantity by running a portion of the products on an agarose gel. Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labelled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under vacuum and frozen at -20 ^{0} C until ready to load. Sample were resuspended in a solution of formamide/blue dextran/EDTA and denatured prior to loading. The samples were electrophoresed on an ABI Prism 377 DNA sequencer. Data was analyzed using Applied Biosystems DNA editing and assembly software and sequence comparison are obtained using MicroSeq software (Pitulle *et al.*, 1999 ; Kolbert *et al.*, 1999 ; Patal *et al.*, 2000).

Carotenoid analysis

Lyophilized cell of each strain of photosynthetic bacteria were prepared from fresh cell which cultured in the anaerobic light condition for 72 hr. The crude carotenoids were extracted from dried material by the method described by Britton *et al.* (1995). Using redistilled acetone as the first extraction solvent, and diethyl ether as secondary extraction solvent after washing with distilled water the crude carotenoids in etherial phase were condensed by means of nitrogen gas. Purification and identification of each carotenoids followed the procedure as discribed by Connor (1991). Small silica column chromatography were used for the preliminary separation and then each carotenoid compound were separated by 4:1 petrolium ether : diethyl ether on silica glass plate thin layer chromatography (TLC). Each compound were scrached from silica layer, eluted with diethyl ether, and made up to volumn with ethanol for VIS spectrum observation at the interval of 400-

600 nm. Two milligrams of dried carotenoid compound were dissolved in HPLC grade ethyl acetate and transferred to the mass spectrometric analysis (MS) by means of electron impact technique (EI-MS). The VIS spectrum and electrom impact mass spectrum of each carotenoid was compared and identified using the data provided by Britton *et al.* (2004)

Results

Isolation and screening for the highest carotenoids contents of photosynthetic bacterial isolates

Forty four of photosynthetic bacteria with the capability for growth in either aerobic-dark and anaerobic light conditions were isolated from various marine habitats, 8 isolates from Nakorn Sri Thamarat, 9 isolates from Pattani, 15 isolates from Satun and 12 isolates from Songkhla as shown in Table 2-1. Total carotenoids content of each strain after incubation for 24-96 hrs in aerobic-dark and anaerobic-light system were shown in Table 2-2 and 2-3. After 72 hrs of incubation period under anaerobic-light condition, the highest total carotenoids content were found in 4 isolated, i.e. TM3, TN5, SV2 and TM11B.

Identification of the selected isolates

The bacterial isolates which shown the highest total carotenoids content under anaerobic-light condition, i.e. TM3, TN5, SV2 and TM11B were selected for the identification of genus. The physiological characteristics of each isolates are given in table 2-4. The scanning spectrums of the living cells were shown all isolates have similar absorption spectra at 375, 590, 805 and 863 nm, which identical of bacteriochlorophyll a (Pellerin and Gest, 1983).

Table 2-1 Photosynthetic bacterial isolates with the capability for growth in either aerobic-dark and anaerobic light conditions used in the studies.

Isolates	**Habitat**	**Habitat condition**	**Province**
JRR PTL	Water, shrimp farm	Growout pond 2 months	Nakorn Sri Thamarat
KN Sed	Sediment, shrimp farm	Growout pond 1months	Nakorn Sri Thamarat
WAB R	Water, shrimp farm	Growout pond 2.5 months	Nakorn Sri Thamarat
KN TMT	Water, shrimp farm	Growout pond 2 months	Nakorn Sri Thamarat
WAB Y	Water, shrimp farm	Growout pond 3 months	Nakorn Sri Thamarat
WAB F	Water, shrimp farm	Growout pond 1 months	Nakorn Sri Thamarat
KR 12	Water, shrimp farm	Growout pond 0.5 months	Nakorn Sri Thamarat
KR 7	Water, shrimp farm	Growout pond 2 months	Nakorn Sri Thamarat
BR	Water, shrimp farm	Growout pond 2.5 months	Pattani
KS SN	Water, shrimp farm	Growout pond 3 months	Pattani
STP Sed 5	Sediment, shrimp farm	Effluent pond	Pattani
STP Sed	Sediment, shrimp farm	Effluent pond	Pattani

PJ	Water, shrimp farm	Growout pond 4 months	Pattani
PJ 6	Water, shrimp farm	Growout pond 0.5 months	Pattani
PJ se	Sediment, shrimp farm	Growout pond 3 months	Pattani
BR S	Sediment, shrimp farm	Growout pond 2.5 months	Pattani
KS SE	Water, shrimp farm	Growout pond 1 months	Pattani
TM 13B	Water, shrimp farm	Growout pond 1.5 months	Satun
APS RTN 47	Water, shrimp farm	Growout pond 1.5 months	Satun
TM 9T	Water, shrimp farm	Growout pond 2.5 months	Satun

Table 2-1 (continued)

Isolates	**Habitat**	**Habitat condition**	**Province**
TM 5, 2T	Water, shrimp farm	Growout pond 1.5 months	Satun
TM 13B	Water, shrimp farm	Growout pond 2.5 months	Satun
TN 13	Water, shrimp farm	Growout pond 2.5 months	Satun
TN 5	Water, shrimp farm	Growout pond 2.5 months	Satun
TM 3T	Water, shrimp farm	Growout pond 1 months	Satun
TM 6T	Water, shrimp farm	Growout pond 2 months	Satun
TM 11T	Water, shrimp farm	Growout pond 1.5 months	Satun
TM 11B	Water, shrimp	Growout pond 1.5	Satun

	farm	months	
TMT 4T	Water, shrimp farm	Growout pond 1 months	Satun
APS 3	Water, shrimp farm	Growout pond 2 months	Satun
APS 9	Water, shrimp farm	Growout pond 3 months	Satun
APS 11	Water, shrimp farm	Growout pond 3 months	Satun
M HY	Water, shrimp farm	Growout pond 2.5 months	Songkhla
SB 91	Water, shrimp farm	Growout pond 0.5 months	Songkhla
SB PTL	Water, shrimp farm	Growout pond 0.5 months	Songkhla
SV 1	Water, shrimp farm	Growout pond 3.5 months	Songkhla
CHONG JN	Water, shrimp farm	Growout pond 2.5 months	Songkhla
SV 4	Sediment, shrimp farm	Effluent pond	Songkhla
SV 9T	Water, shrimp farm	Growout pond 1.5 months	Songkhla
SV 2	Water, shrimp farm	Growout pond 2.5 months	Songkhla
RTN CB	Water, shrimp farm	Growout pond 3 months	Songkhla
SP Sed 5	Sediment, shrimp farm	Effluent pond	Songkhla
RTN 3	Water, shrimp farm	Growout pond 3.5 months	Songkhla
RTN 12	Water, shrimp farm	Growout pond 1.5 months	Songkhla

Table 2-2 Total carotenoids (mg/g dry weight) of each isolates during 24-96 hrs. of the incubation periods under aerobic dark condition.

Isolates	**24 hr**	**48 hr**	**72 hr**	**96 hr**
JRR PTL	0.22 ± 0.06	0.31 ± 0.08	0.36 ± 0.09	0.35 ± 0.08
KN Sed	0.66 ± 0.20	0.85 ± 0.09	0.95 ± 0.11	0.96 ± 0.04
WAB R	0.62 ± 0.14	0.85 ± 0.13	0.91 ± 0.14	0.86 ± 0.20
KN TMT	0.15 ± 0.02	0.31 ± 0.07	0.37 ± 0.08	0.36 ± 0.06
WAB Y	1.65 ± 0.02	1.75 ± 0.00	2.00 ± 0.17	1.90 ± 0.20
WAB F	0.32 ± 0.04	0.38 ± 0.01	0.47 ± 0.01	0.45 ± 0.02
KR 12	0.51 ± 0.11	0.63 ± 0.09	0.74 ± 0.11	0.70 ± 0.08
KR 7	0.37 ± 0.07	0.43 ± 0.09	0.52 ± 0.06	0.47 ± 0.07
BR	0.63 ± 0.05	0.71 ± 0.13	0.81 ± 0.10	0.75 ± 0.19
KS SN	0.41 ± 0.08	0.54 ± 0.10	0.58 ± 0.07	0.58 ± 0.09
STP Sed 5	0.34 ± 0.04	0.43 ± 0.08	0.49 ± 0.04	0.48 ± 0.04
STP Sed	0.37 ± 0.17	0.46 ± 0.17	0.51 ± 0.19	0.51 ± 0.20
PJ	0.33 ± 0.03	0.42 ± 0.07	0.50 ± 0.09	0.48 ± 0.04
PJ 6	0.37 ± 0.09	0.45 ± 0.09	0.51 ± 0.09	0.50 ± 0.08
PJ se	0.40 ± 0.15	0.51 ± 0.16	0.53 ± 0.15	0.52 ± 0.15
BR S	0.51 ± 0.08	0.63 ± 0.15	0.66 ± 0.13	0.62 ± 0.14
KS SE	0.46 ± 0.02	0.56 ± 0.06	0.62 ± 0.11	0.60 ± 0.12
TM 13B	0.44 ± 0.01	0.49 ± 0.00	0.55 ± 0.08	0.53 ± 0.06
APS RTN 47	0.08 ± 0.07	0.09 ± 0.07	0.10 ± 0.07	0.09 ± 0.06
TM 9T	0.49 ± 0.06	0.66 ± 0.07	0.74 ± 0.02	0.72 ± 0.03
TM 5, 2T	0.40 ± 0.13	0.61 ± 0.18	0.66 ± 0.13	0.63 ± 0.15
TM 13B	0.39 ± 0.13	0.54 ± 0.08	0.61 ± 0.01	0.59 ± 0.04
TN 13	0.49 ± 0.08	0.65 ± 0.10	0.70 ± 0.09	0.69 ± 0.10
TN 5	0.35 ± 0.07	0.61 ± 0.15	0.64 ± 0.17	0.62 ± 0.16
TM 3T	1.20 ± 0.09	1.34 ± 0.09	1.47 ± 0.11	1.43 ± 0.03

TM 6T	1.14 ± 0.17	1.33 ± 0.14	1.43 ± 0.25	1.37 ± 0.15
TM 11T	0.71 ± 0.06	0.87 ± 0.07	0.98 ± 0.14	0.91 ± 0.14
TM 11B	1.64 ± 0.16	1.90 ± 0.18	2.05 ± 0.13	1.98 ± 0.02

Table 2-2 (continued)

Isolates	**24 hr**	**48 hr**	**72 hr**	**96 hr**
TMT 4T	1.22 ± 0.06	1.40 ± 0.07	1.48 ± 0.05	1.41 ± 0.02
APS 3	0.76 ± 0.06	0.84 ± 0.14	0.91 ± 0.14	0.88 ± 0.17
APS 9	0.35 ± 0.06	0.54 ± 0.09	0.59 ± 0.07	0.58 ± 0.07
APS 11	0.71 ± 0.07	0.89 ± 0.06	0.98 ± 0.08	0.96 ± 0.10
M HY	0.29 ± 0.06	0.41 ± 0.06	0.46 ± 0.09	0.45 ± 0.09
SB 91	0.03 ± 0.03	0.06 ± 0.07	0.08 ± 0.05	0.10 ± 0.04
SB PTL	0.38 ± 0.10	0.53 ± 0.12	0.61 ± 0.04	0.60 ± 0.05
SV 1	0.45 ± 0.01	0.56 ± 0.02	0.61 ± 0.05	0.58 ± 0.11
CHONG JN	0.64 ± 0.11	0.72 ± 0.02	0.76 ± 0.05	0.67 ± 0.08
SV 4	1.19 ± 0.08	1.38 ± 0.17	1.59 ± 0.20	1.47 ± 0.30
SV 9T	1.27 ± 0.02	1.50 ± 0.03	1.64 ± 0.17	1.56 ± 0.23
SV 2	1.27 ± 0.04	1.48 ± 0.05	1.58 ± 0.05	1.50 ± 0.07
RTN CB	0.44 ± 0.17	0.63 ± 0.10	0.73 ± 0.13	0.67 ± 0.10
SP Sed 5	0.38 ± 0.08	0.49 ± 0.02	0.60 ± 0.01	0.57 ± 0.05
RTN 3	0.54 ± 0.14	0.66 ± 0.12	0.71 ± 0.07	0.68 ± 0.08
RTN 12	0.22 ± 0.05	0.31 ± 0.08	0.34 ± 0.06	0.34 ± 0.04

Table 2-3 Total carotenoids (mg/g dry weight) of each isolates during 24-96 hrs. of the incubation periods under anaerobic light condition.

Isolates	**24 hr**	**48 hr**	**72 hr**	**96 hr**
JRR PTL	1.12 ± 0.08	1.48 ± 0.10	1.83 ± 0.10	1.87 ± 0.03
KN Sed	1.59 ± 0.06	1.95 ± 0.07	2.23 ± 0.06	2.22 ± 0.07
WAB R	0.93 ± 0.18	1.12 ± 0.08	1.36 ± 0.09	1.32 ± 0.08
KN TMT	0.97 ± 0.04	1.09 ± 0.05	1.19 ± 0.10	1.26 ± 0.19
WAB Y	1.34 ± 0.11	1.60 ± 0.11	2.18 ± 0.38	2.18 ± 0.42
WAB F	1.04 ± 0.13	1.22 ± 0.06	1.44 ± 0.11	1.46 ± 0.02
KR 12	0.80 ± 0.06	0.97 ± 0.06	1.19 ± 0.07	1.20 ± 0.04
KR 7	1.07 ± 0.22	1.21 ± 0.21	1.40 ± 0.17	1.39 ± 0.22
BR	1.40 ± 0.18	1.64 ± 0.16	1.85 ±0.14	1.84 ± 0.29
KS SN	1.36 ± 0.12	1.56 ± 0.06	1.80 ± 0.07	1.84 ± 0.29
STP Sed 5	0.93 ± 0.11	1.13 ± 0.07	1.21 ± 0.15	1.22 ± 0.05
STP Sed	0.82 ± 0.06	0.96 ± 0.07	1.12 ± 0.15	1.20 ± 0.09
PJ	0.99 ± 0.09	1.13 ± 0.07	1.32 ± 0.16	1.26 ± 0.00
PJ 6	0.97 ± 0.09	1.16 ± 0.03	1.32 ± 0.04	1.39 ± 0.03
PJ se	0.84 ± 0.11	0.96 ± 0.07	1.14 ± 0.07	1.06 ± 0.07
BR S	0.87 ± 0.07	0.99 ± 0.02	1.12 ± 0.05	1.09 ± 0.02
KS SE	0.90 ± 0.02	1.00 ± 0.01	1.11 ± 0.11	1.01 ± 0.13
TM 13B	1.48 ± 0.05	1.72 ± 0.05	2.19 ± 0.23	2.20 ± 0.40
APS RTN 47	1.89 ± 0.20	2.27 ± 0.05	2.34 ± 0.02	2.34 ± 0.22
TM 9T	1.65 ± 0.18	1.96 ± 0.08	2.06 ± 0.09	2.14 ± 0.04
TM 5, 2T	2.07 ± 0.03	2.27 ± 0.05	2.45 ± 0.05	2.46 ± 0.23
TM 13B	1.12 ± 0.15	1.35 ± 0.04	1.45 ± 0.09	1.83 ± 0.24
TN 13	1.19 ± 0.10	1.50 ± 0.08	1.59 ± 0.06	1.82 ± 0.07
TN 5	2.51 ± 0.15	2.91 ± 0.33	3.06 ± 0.49	3.00 ± 0.39
TM 3T	2.84 ± 0.17	3.15 ± 0.23	3.41 ± 0.08	3.42 ± 0.06
TM 6T	1.89 ± 0.37	2.08 ± 0.42	2.59 ± 0.12	2.58 ± 0.24
TM 11T	1.81 ± 0.09	2.32 ± 0.22	2.61 ± 0.08	2.61 ± 0.19

Table 2-3 (continued)

Isolates	24 hr	48 hr	72 hr	96 hr
TM 11B	2.86 ± 0.19	3.27 ± 0.35	3.54 ± 0.05	3.52 ± 0.09
TMT 4T	2.06 ± 0.08	2.33 ± 0.09	2.44 ± 0.11	2.48 ± 0.11
APS 3	1.43 ± 0.10	1.69 ± 0.09	1.82 ± 0.10	1.90 ± 0.04
APS 9	0.93 ± 0.17	1.05 ± 0.17	1.24 ± 0.02	1.30 ± 0.06
APS 11	0.97 ± 0.10	1.11 ± 0.09	1.24 ± 0.03	1.32 ± 0.07
M HY	0.85 ± 0.06	0.98 ± 0.10	1.21 ± 0.04	1.24 ± 0.03
SB 91	1.24 ± 0.02	1.49 ± 0.05	1.71 ± 0.04	1.61 ± 0.09
SB PTL	1.67 ± 0.17	1.96 ± 0.28	2.21 ± 0.07	2.19 ± 0.10
SV 1	0.71 ± 0.20	1.00 ± 0.11	1.21 ± 0.02	1.22 ± 0.10
CHONG JN	1.22 ± 0.06	1.50 ± 0.07	1.57 ± 0.09	1.71 ± 0.06
SV 4	1.70 ± 0.21	1.91 ± 0.08	1.94 ± 0.05	1.95 ± 0.07
SV 9T	1.72 ± 0.45	1.96 ± 0.29	2.10 ± 0.18	2.08 ± 0.27
SV 2	2.55 ± 0.13	2.86 ± 0.01	3.03 ± 0.11	3.09 ± 0.25
RTN CB	1.01 ± 0.06	1.06 ± 0.07	1.12 ± 0.09	1.19 ±0.11
SP Sed 5	0.84 ± 0.03	0.99 ± 0.10	1.10 ± 0.06	0.99 ± 0.09
RTN 3	0.60 ± 0.06	0.88 ± 0.03	1.05 ± 0.02	1.04 ± 0.14
RTN 12	0.95 ± 0.09	1.17 ± 0.14	1.32 ± 0.09	1.34 ± 0.12

16S rDNA sequencing and phylogenic analysis

The first 500bp DNA sequence of the 16S rDNA from each isolates were shown in Figures 2-1 to 2-4, The phylogenic tree of each isolate are given in Figure 2-5 to Figure 2-8. From the results it was found that the isolate TM 3 exhibited 94.06 % sequence similarity to *Rhodobacter* sp. The isolate TM 11 shown 94.06 % sequence similarity to *Rhodobacter* sp. The isolate SV 2 exhibited 99.79 % sequence similarity to *Rhodobacter sphaeroides* and the isolateTN 5 exhibited 99.79 % sequence similarity to *Rhodobacter sphaeroides*.

Table 2-4 Physiological characteristics of cultures TM3, TN5, SV2 and TM11B.

Characteristic	**TM 3**	**TN 5**	**SV 2**	**TM 11 B**
Cell shape	Ovoid to rod	Ovoid to rod	Ovoid to rod	Ovoid to rod
Cell motility	Motile	Motile	Motile	Motile
Spore formation	Absent	Absent	Absent	Absent
Gram stain	Negative	Negative	Negative	Negative
Type of bacteriochlophyll	Bacteriochlophyll a	Bacteriochlophyll a	Bacteriochlophyll a	Bacteriochlophyll a
Utilization of carbon sources :				
Lactate	+	+	+	+
Glucose	+	+	+	+
Ethanol	-	+	+	-
Citrate	+	+	+	+
Acetate	+	+	+	+
Glycerol	-	+	+	-
Glutamate	+	+	+	+
Propionate	+	+	+	+

TGGAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGG
CAGGCCTAACACATGCAAGTCGAGCGAACCCTTCGGG
GTTAGCGGCGGACGGGTGAGTAACGCGTGGGAACGTG
CCCTTCTCTGCGGAATAGGCTCGGGAAACTGGGTTTAA
TACCGCATACGCCCTTCGGGGGAAAGATTTATCGGAGA
AGGATCGGCCCGCGTTAGATTAGGTAGTTGGTGGGGTA
ATGGCCTACCAAGCCTACGATCTATAGCTGGTTTGAGA
GGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTGAGGAATCTTGGACAA
TGGGGGAAACCCTGATCCAGCCATGCCGCGTGAGCGAT
GAAGGCCTTAGGGTTGTAAAGCTCTTTCAGyCGTGAAG
ATAATGACGGTAGCGACAGAAGAAGCCCCGGCTAACT
CCGTGCCAGCAGCCGCGGTA

Figure 2-1 The first 500bp DNA sequence of the 16S rDNA from strain TM3

TGGAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGG
CAGGCCTAACACATGCAAGTCGAGCGAACCCTTCGGG
GTTAGCGGCGGACGGGTGAGTAACGCGTGGGAACGTG
CCCTTCTCTGCGGAATAGGCTCGGGAAACTGGGTTTAA
TACCGCATACGCCCTTCGGGGGAAAGATTTATCGGAGA
AGGATCGGCCCGCGTTAGATTAGGTAGTTGGTGGGGTA
ATGGCCTACCAAGCCTACGATCTATAGCTGGTTTGAGA
GGATGATCAGCCACACTGGGACTGAGACACGGCCCAG

ACTCCTACGGGAGGCAGCAGTGAGGAATCTTGGACAA
TGGGGGAAACCCTGATCCAGCCATGCCGCGTGAGCGAT
GAAGGCCTTAGGGTTGTAAAGCTCTTTCAGTCGTGAAG
ATAATGACGGTAGCGACAGAAGAAGCCCCGGCTAACT
CCGTGCCAGCAGCCGCGGTA

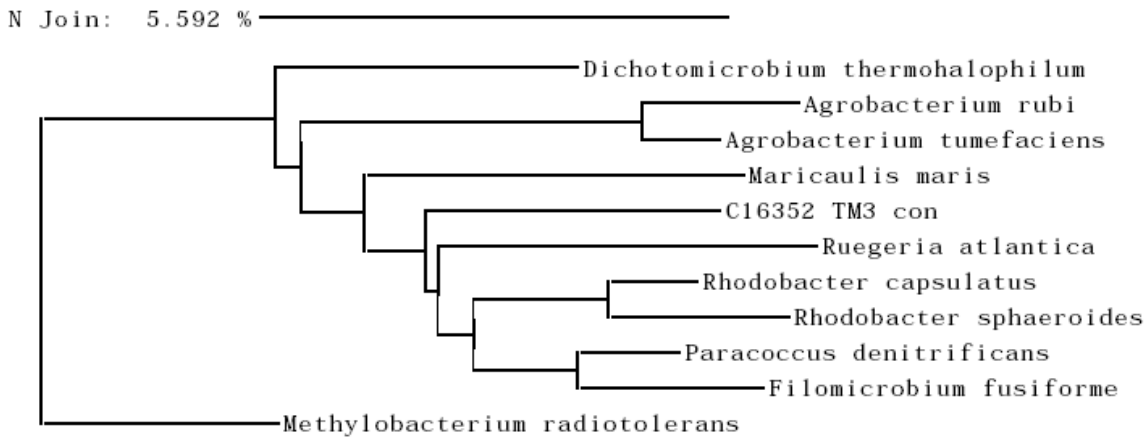
Figure 2-2 The first 500bp DNA sequence of the 16S rDNA from strain TM11 B

TGGAGAGTTTGATCCTGGCTCAGAATGAACGCTGGCGG
CAGGCCTAACACATGCAAGTCGAGCGAAGTCTTCGGAC
TTAGCGGCGGACGGGTGAGTAACGCGTGGGAACGTGC
CCTTTGCTTCGGAATAGCCCCGGGAAACTGGGAGTAAT
ACCGAATGTGCCCTATGGGGGAAAGATTTATCGGCAAA
GGATCGGCCCGCGTTGGATTAGGTAGTTGGTGGGGTAA
TGGCCTACCAAGCCGACGATCCATAGCTGGTTTGAGAG
GATGATCAGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAAT
GGGCGCAAGCCTGATCTAGCCATGCCGCGTGATCGATG
AAGGCCTTAGGGTTGTAAAGATCTTTCAGGTGGGAAGA
TAATGACGGTACCACCAGAAGAAGCCCCGGCTAACTCC
GTGCCAGCAGCCGCGGTA

Figure 2-3 The first 500bp DNA sequence of the 16S rDNA from strain SV2

TGGAGAGTTTGATCCTGGCTCAGAATGAACGCTGGCGG
CAGGCCTAACACATGCAAGTCGAGCGAAGTCTTCGGAC
TTAGCGGCGGACGGGTGAGTAACGCGTGGGAACGTGC
CCTTTGCTTCGGAATAGCCCCGGGAAACTGGGAGTAAT
ACCGAATGTGCCCTATGGGGGAAAGATTTATCGGCAAA
GGATCGGCCCGCGTTGGATTAGGTAGTTGGTGGGGTAA
TGGCCTACCAAGCCGACGATCCATAGCTGGTTTGAGAG
GATGATCAGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATCTTAGACAAT
GGGCGCAAGCCTGATCTAGCCATGCCGCGTGATCGATG
AAGGCCTTAGGGTTGTAAAGATCTTTCAGGTGGGAAGA
TAATGACGGTACCACCAGAAGAAGCCCCGGCTAACTCC
GTGCCAGCAGCCGCGGTA

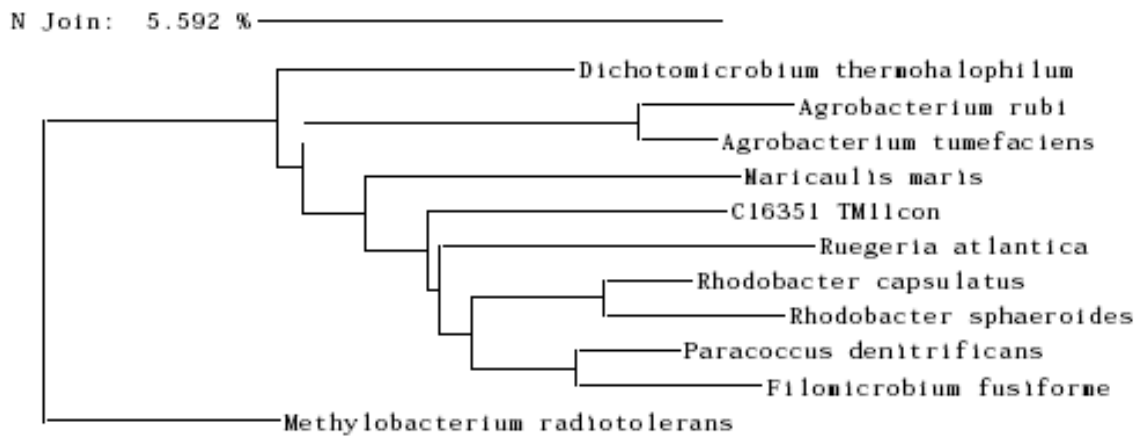
Figure 2-4 The first 500bp DNA sequence of the 16S rDNA from strain TN5



Alignment: 471 TM3

5.94 % 471 *Rhodobacter capsulatus*
7.32 % 471 *Paracoccus denitrificans*
7.43 % 471 *Rhodobacter sphaeroides*
7.78 % 469 *Filomicrobium fusiforme*
8.21 % 469 *Ruegeria atlantica*
8.81 % 471 *Maricaulis maris*
9.91 % 469 *Agrobacterium rubi*
9.91 % 469 *Dichotomicrobium thermohalophilum*
9.91 % 469 *Agrobacterium tumefaciens*
10.72 % 471 *Methylobacterium radiotolerans*

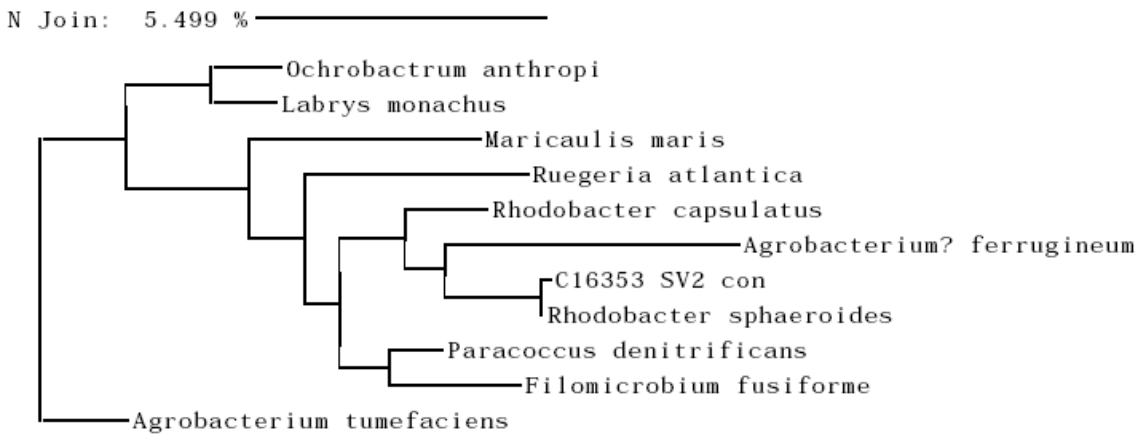
Figure 2-5 The phylogenic tree of strain TM 3



Alignment: 471 TM11 B

5.94 % 471 *Rhodobacter capsulatus*
7.43 % 471 *Paracoccus denitrificans*
7.43 % 471 *Rhodobacter sphaeroides*
7.89 % 469 *Filomicrobium fusiforme*
8.32 % 469 *Ruegeria atlantica*
8.92 % 471 *Maricaulis maris*
10.02 % 469 *Agrobacterium rubi*
10.02 % 469 *Dichotomicrobium thermohalophilum*
10.02 % 469 *Agrobacterium tumefaciens*
10.83 % 471 *Methylobacterium radiotolerans*

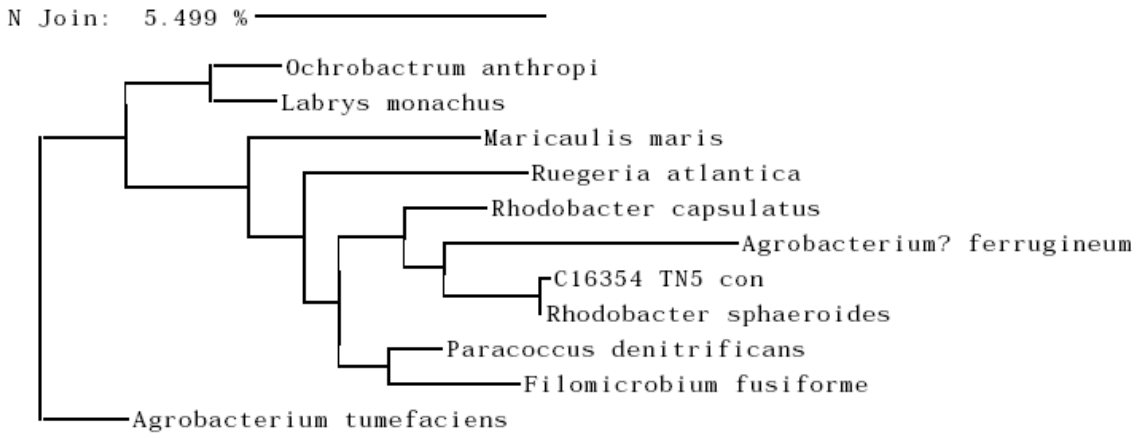
Figure 2-6 The phylogenic tree of strain TM 11 B



Alignment: 471 SV2

0.21 % 471 *Rhodobacter sphaeroides*
3.40 % 471 *Rhodobacter capsulatus*
6.16 % 471 *Paracoccus denitrificans*
7.43 % 486 *Agrobacterium ferrugineum*
7.68 % 469 *Filomicrobium fusiforme*
9.13 % 471 *Maricaulis maris*
9.81 % 469 *Ruegeria atlantica*
10.87 % 469 *Oschrobactrum anthropi*
11.09 % 469 *Labrys monachus*
11.09 % 469 *Agrobacterium tumefaciens*

Figure 2-7 The phylogenic tree of strain SV 2



Alignment: 471 TN5
0.21 % 471 *Rhodobacter sphaeroides*

3.40 % 471 *Rhodobacter capsulatus*
6.16 % 471 *Paracoccus denitrificans*
7.43 % 486 *Agrobacterium ferrugineum*
7.68 % 469 *Filomicrobium fusiforme*
9.13 % 471 *Maricaulis maris*
9.81 % 469 *Ruegeria atlantica*
10.87 % 469 *Oschrobactrum anthropi*
11.09 % 469 *Labrys monachus*
11.09 % 469 *Agrobacterium tumefaciens*

Figure 2-8 The phylogenic tree of strain TN 5

Carotenoids analysis

Thin layer chromatogram of pigments extracted from all strains of photosynthetic bacteria are similar as given in Figure 2-9, VIS spectrum and mass spectrum of each separated pigment shown in Figure 2-10 to 2-14. In strain TM3 and TM11 B the pigments were separated into 5 different compounds, band A showed a VIS spectrum and mass spectrum corresponded to neurosporene. Band B, C, D and E showed their VIS spectrum and mass spectrum corresponded to spheroidene, spheroidenone, demethylsphroidene and hydroxyspheroidene respectively. In strain TN5 and SV2, the pigments were separated into 2 different compounds, band B showed a VIS spectrum and mass spectrum corresponded to spheroidene and band C showed a VIS spectrum and mass spectrum corresponded to spheroidenone.

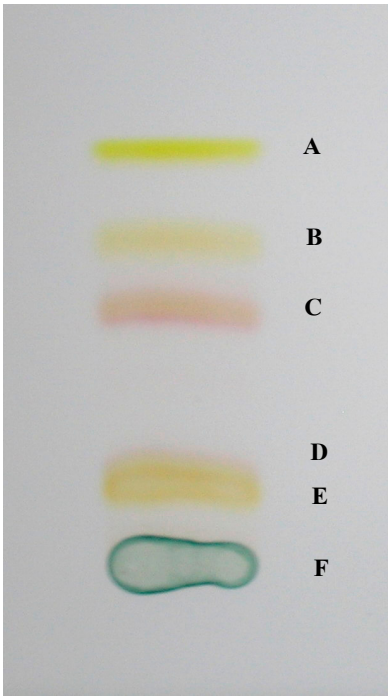
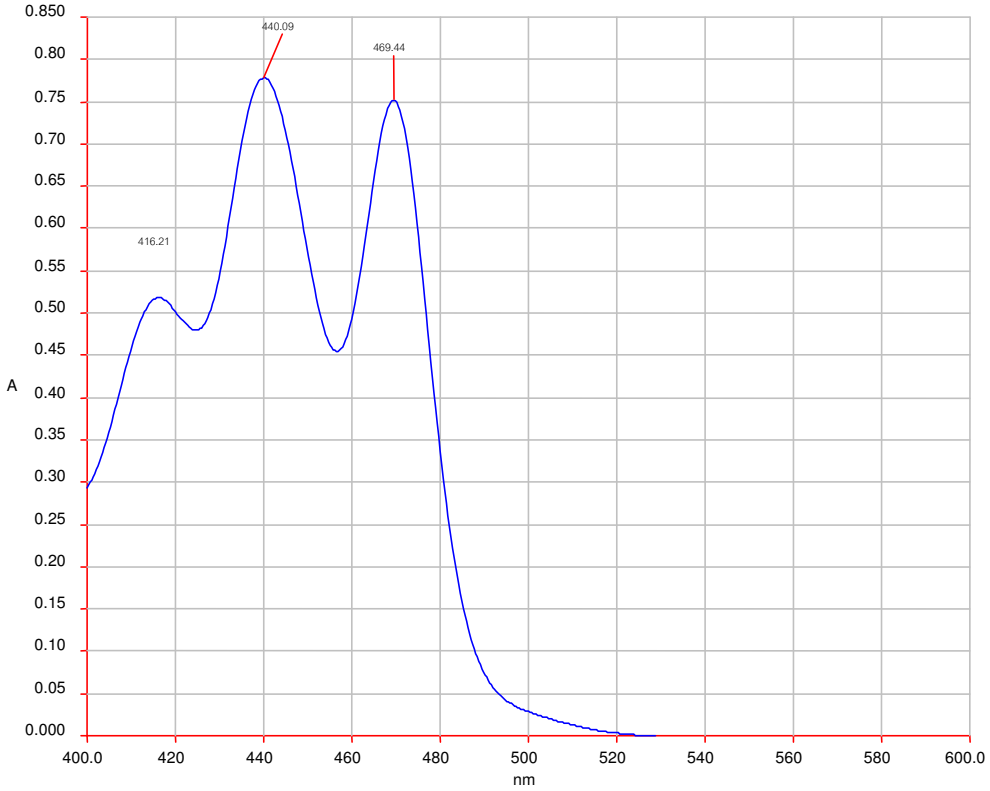


Figure 2-9 Thin layer chromatogram of pigments extracted from photosynthetic bacteria, siliga plate, 4:1 petroleum ether : diethyl ether. (A= neurosporene, B = spheroidene, C= spheroidenone, D = demethylspheroidene, E = hydroxyspheroidene, F = bacteriochlorophyll a)



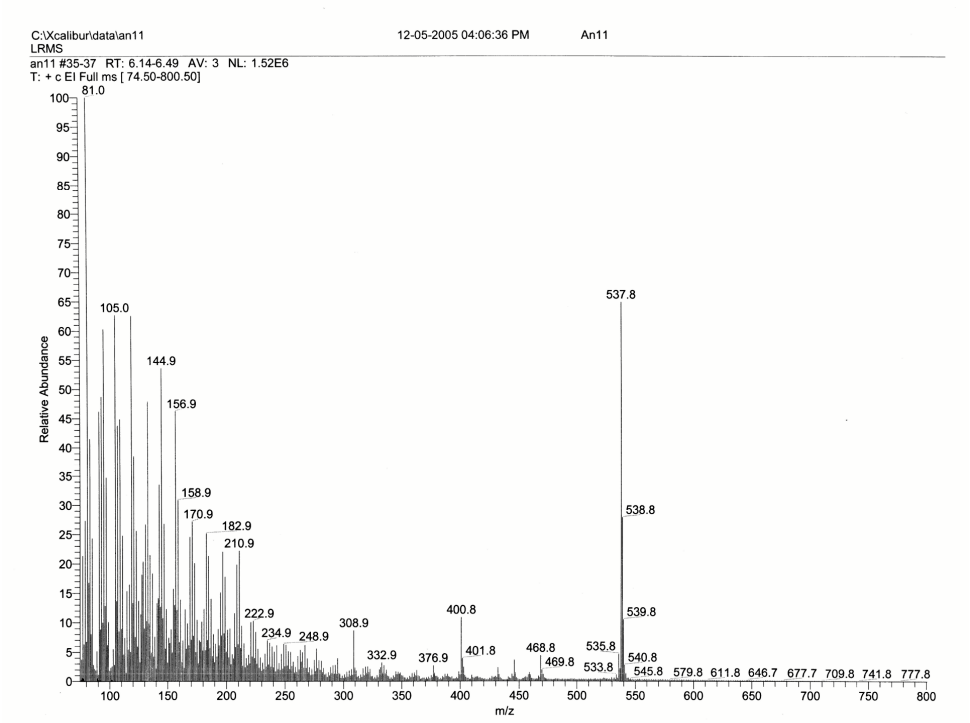


Figure 2-10 VIS spectrum (upper) and mass spectrum (lower) of band A which

corresponds to neurosporene

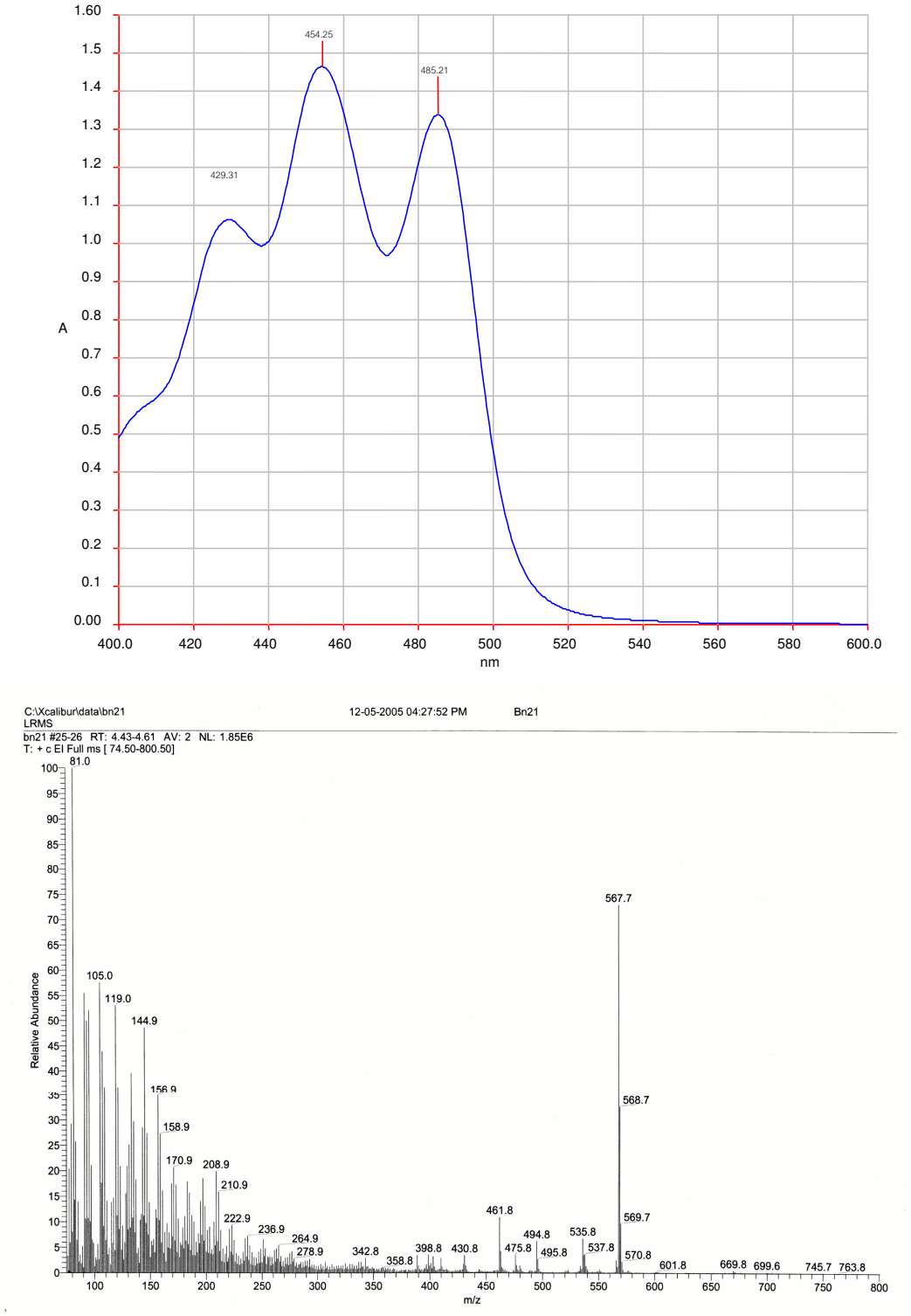


Figure 2-11 VIS spectrum (upper) and mass spectrum (lower) of band B which

corresponds to spheroidene

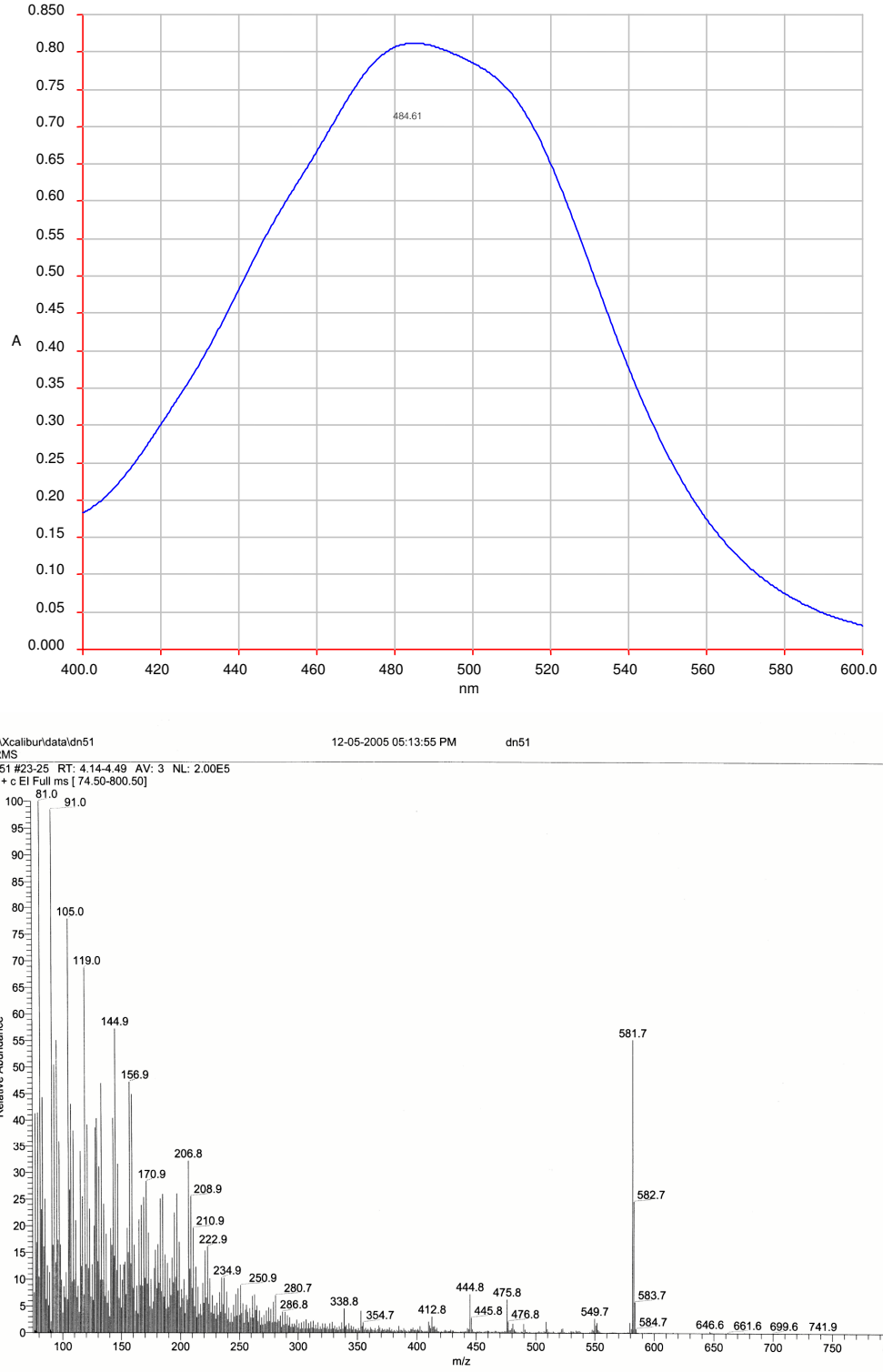


Figure 2-12 VIS spectrum (upper) and mass spectrum (lower) of band C which

corresponds to spheroidenone

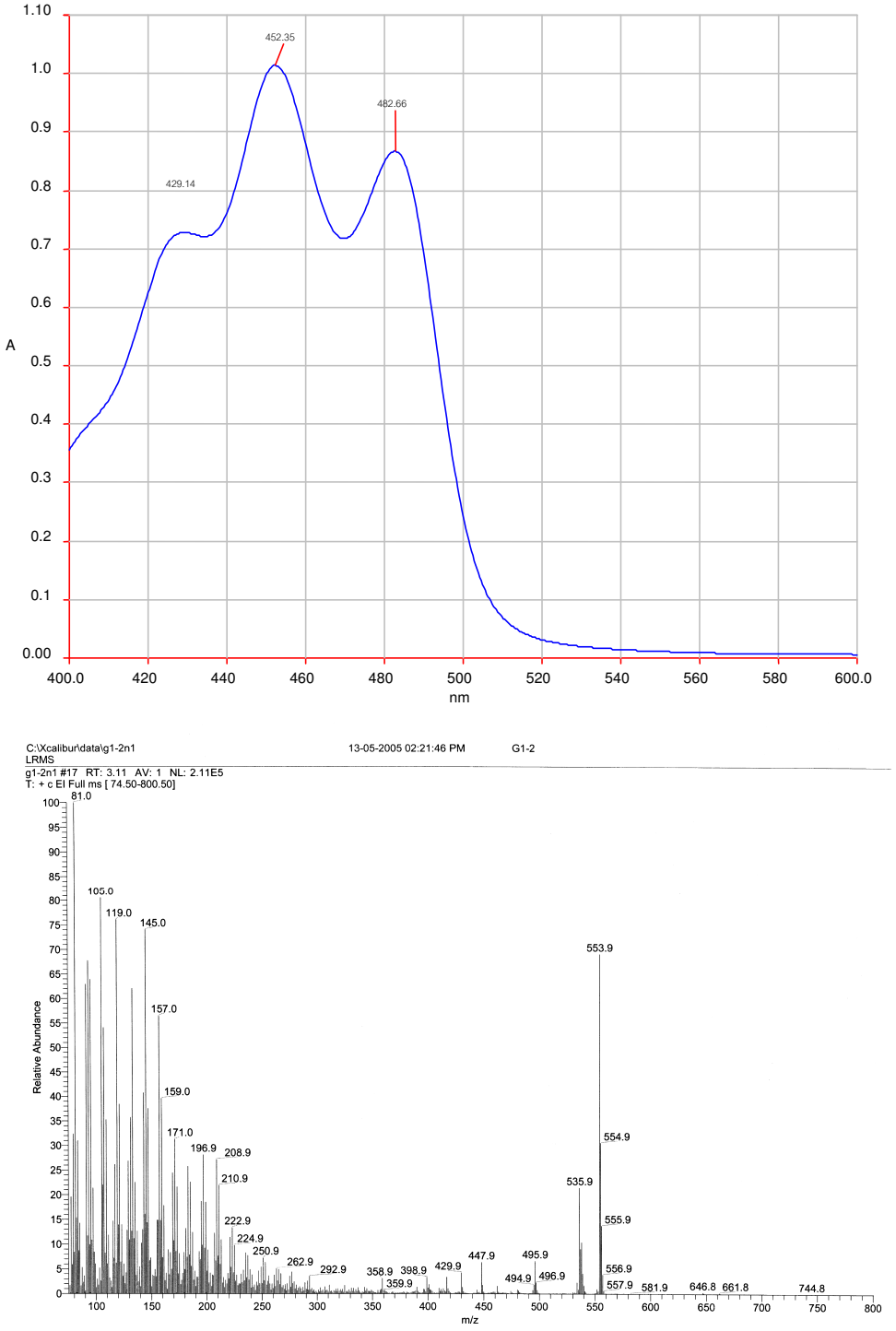


Figure 2-13 VIS spectrum (upper) and mass spectrum (lower) of band D which

corresponds to demethylspheroidene

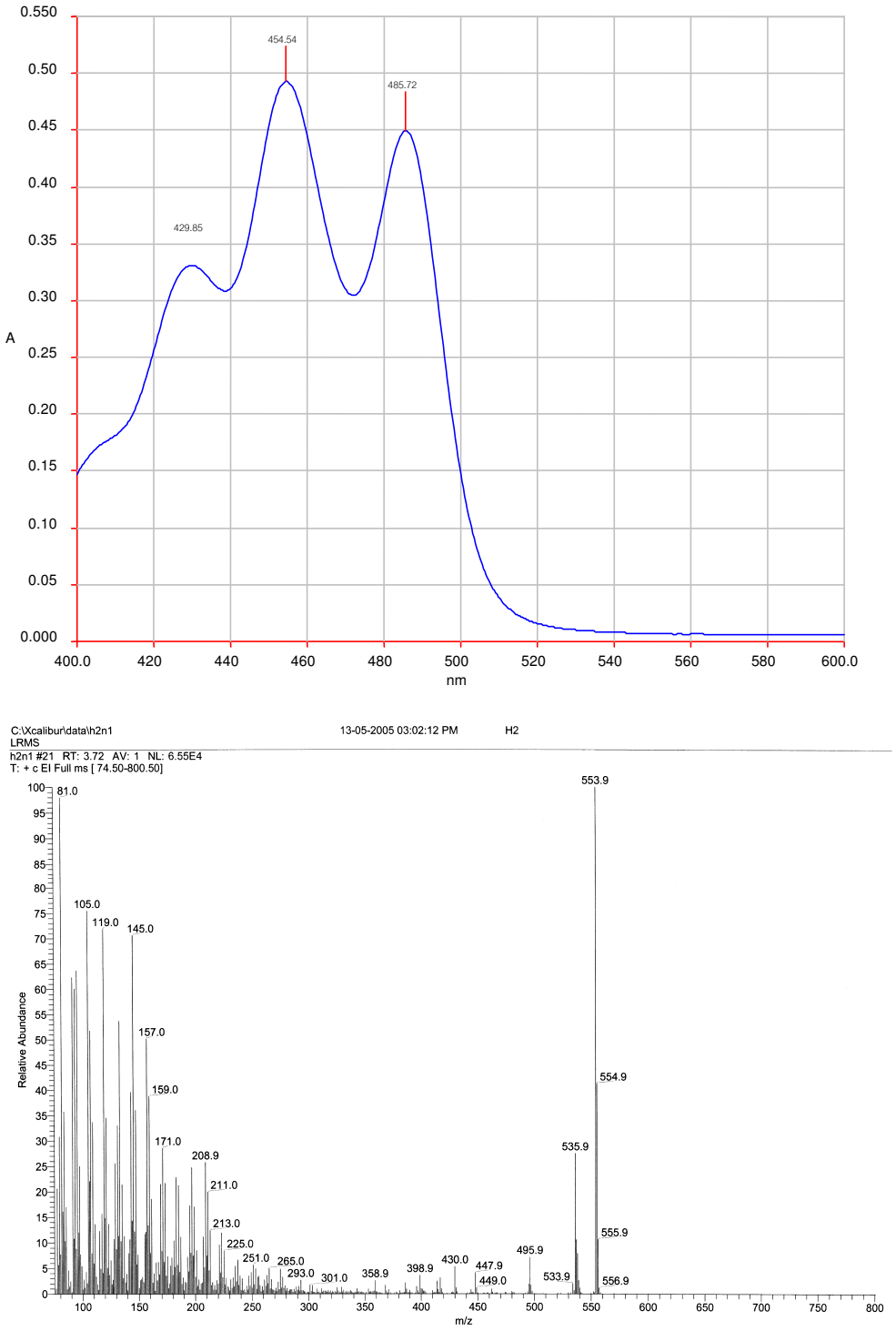


Figure 2-14 VIS spectrum (upper) and mass spectrum (lower) of band E which corresponds to hydroxyspheroidene.

Discussion

The carotenoids produced by the photosynthetic bacteria in the aerobic dark condition after the incubation period of 72 hr were ranging from 0.10-2.05 mg/g dry cell. Higher concentration found in anaerobic light condition which ranging from 1.05- 3.54 mg/g dry cell weight. The highest carotenoids production of the photosynthetic bacteria isolated from this study were found in the anaerobic light condition as in agreement with the study on isolation and growth condition of photosynthetic bacteria which found in seafood processing wastewater by Prasertsan *et al.* (1993 a). In the anaerobic-light condition the photosynthetic bacteria produce large amounts of pigments to store light energy for photosynthesis (Shipman *et al.*, 1977). The carotenoids in purple non-sulfur photosynthetic bacteria are generally spheroidene and spheroidenone which were produced from isoprenoid pathway (Takaichi, 2001 ; Armstrong, 1994). But, in the aerobic condition, oxygen affects and inhibits the pigment synthesis and acts as bleaching agent (Cohen-Bazire *et al.*, 1957), and lead to the reduction of carotenoids content.

Four isolates (TM3, TN5, TM11B and SV2) of the photosynthetic bacteria which having the highest carotenoids production have the average carotenoids content of 3.03-3.54 mg/g dry cell weight, these concentrations were higher than the carotenoid content of *Rhodocyclus gelatinosus* R7 which cultured under anaerobic light condition in tuna condensate diluted with shrimp-bleaching water (0.98 mg/g dry cell weight) (Prasertsan *et al.*, 1993 b). Since all of the isolates were Gram-negative, rod shaped and can grow in both aerobic and anaerobic condition. All of isolates contained bacteriochlorophyll a , so all of them are belong to the member of purple bacteria. Each of them could not grow in sulfide or thiosulphate media, they were identified as members of the purple-non sulfur family *Rhodospirillaceae* (Staley *et al.*, 1989). According to “Key to the

genera of the purple nonsulfur bacteria" (Imhoff and Truper, 1989) all isolates characterized as ovoid to rod shape morphology, 0.5-1.2 um wide, cell division by binary fission, containing carotenoids of the spheroidene series, slime production and chain formation which belong to the genus Rhodobacter. When study on the carbon source utilization of all isolates, it was found that the Rhodobacter can grow in numbers of carbon sources, especially citrate which can be used by only *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, but only strain TN5 and SV2 can grow in the glycerol and ethanol, which is the typical characteristic of *Rhodobacter sphaeroides.* From the sequencing of 16s rDNA and phylogenic analysis, it was found that the isolates SV2 and TN5 showed very high percent similarity to the *Rhodobacter sphaeroides* and the isolates TM3 and TM11B are closed to the 16s rDNA sequences of *Rhodobacter capsulatus*. The carotenoids analysis data showed that all isolates produced carotenoids in the spheroidene series, which is the typical carotenoid found in only 4 genera, *Rhodobacter*, *Rhodoferax*, *Rubrivibrix* and *Rhodovulum* (Takaishi, 2001). From this study, 4 isolates of photosynthetic bacteria (*Rhodobacter* sp. TM3, *Rhodobacter* sp. TM11B, *Rhodobacter sphaeroides* TN5 and *Rhodobacter sphaeroides* SV2) were selected for the further study in the application of these bacterial isolates as feed supplement in the black tiger shrimp.

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