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Isolation and Characterization of Purple Non-Sulfur Bacteria, *Afifella marina*, Producing Large Amount of Carotenoids from Mangrove Microhabitats

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This study determined the effect of light intensity and photoperiod on the dry cell weight and total amount of carotenoids in four isolates of purple non-sulfur bacteria obtained from shaded and exposed microhabitats of a mangrove ecosystem in Kota Kinabalu, Sabah, Malaysia. The initial isolation of the bacteria was carried out using synthetic 112 medium under anaerobic conditions (2.5 klx) at 30 ± 2°C. On the basis of colony appearance, cell morphology, gram staining, motility test, and 16S rRNA gene sequencing analyses, all four bacteria were identified as *Afifella marina*. One of the bacterial isolates, designated as *Af. marina* strain ME, which was extracted from an exposed mud habitat within the mangrove ecosystem, showed the highest yield in dry cell weight (4.32 ± 0.03 g/l) as well as total carotenoids (0.783 ± 0.002 mg/g dry cell weight). These values were significantly higher than those for dry cell weight (3.77 ± 0.02 g/l) and total carotenoid content (0.706 ± 0.008 mg/g) produced by the isolates from shaded habitats. Further analysis of the effect of 10 levels of light intensity on the growth characteristics of *Af. marina* strain ME showed that the optimum production of dry cell weight and total carotenoids was achieved at different light intensities and incubation periods. The bacterium produced the highest dry cell weight of 4.98 g/l at 3 klx in 72 h incubation, but the carotenoid production of 0.783 mg/g was achieved at 2.5 klx in 48 h incubation. Subsequent analysis of the effect of photoperiod on the production of dry cell weight and total carotenoids at optimum light intensities (3 and 2.5 klx, respectively) revealed that 18 and 24 h were the optimum photoperiods for the production of dry cell weight and total carotenoids, respectively. The unique growth characteristics of the *Af. marina* strain ME can be exploited for biotechnology applications.

Keywords: *Afifella marina*, carotenoids, dry cell weight, light intensity, mangrove microhabitats, purple non-sulfur bacteria

Introduction

Purple non-sulfur bacteria (PNSB) are distributed widely in natural habitats, particularly in those with large amounts of soluble organic matter, such as mangrove swamps, wastewater ponds, coastal lagoons, and waste lagoons [12]. They can tolerate a wide range of habitats and possess the ability to grow under photoautotrophic, photoheterotrophic, and chemoheterotrophic conditions [20]. In the marine environment, most commonly isolated PNSB strains include *Afifella* sp. [39], *Rhodobium* sp. [17], *Rhodovulum* sp. [24], *Rhodovibrio* sp. [27], *Roseospira* sp. [23], and *Rhodothalassium* sp. [13]. These groups of PNSB are known to have various biotechnological applications, which include bioremediation of sardine waste water [6], aquaculture feed additive of post larvae of tiger shrimp [5], production of biofuel [39], production of antiviral substances [18], production of enzymes [9], bioremediation of dissolved heavy metals [40], production of single cell protein [29], and production of vitamins and ubiquinone Q10 [30].

The growth characteristics in terms of dry cell weight (g/l) and total carotenoids production in PNSB are generally influenced by pH [25], temperature, dissolved oxygen, nutrients [10], and light intensity [3]. However, light intensity seems to play a major role in regulating the growth characteristics of PNSB. Generally, PNSB biomass reaches a maximal value at higher light intensity, but the highest concentration of the pigments is usually achieved under dimmer light [3]. The highest biomass (5.6 g/l) of *Rhodocyclus gelatinosus* was obtained when the cells were grown in medium containing glutamate-malate (GMM) at a light intensity of 3 klx [29], whereas the highest carotenoids production was reported at 2 klx [1]. Generally, continuous light seems to favor the growth and production of total carotenoids in PNSB [14]. However, reduction in photoperiod was also not found to significantly decrease the production of dry cell weight and total carotenoids in PNSB. For example, a dry cell biomass of 39 mg/l and carotenoids production of 0.55 g/l dry cell weight of *Rhodovulum sphaeroides* incubated under continuous light (24L/0D) did not differ from the dry cell biomass (41 mg/l) and total carotenoids (0.50 g/l dry cell weight) recorded when the bacterium was exposed to 12 h of light [14].

Many of the previous studies have not considered the relationship between the growth characteristics of PNSB and the microenvironments from which they were isolated. Hence, this paper reports the ability of PNSB isolated from different microhabitats within a mangrove ecosystem to produce dry cell weight and total carotenoids at different light intensities and photoperiods.

Materials and Methods

Isolation of Purple Non-Sulfur Bacteria

PNSB were isolated from mud and water samples collected in shaded and exposed areas of the mangrove intertidal zone of Kingfisher Park (06.02° N, 116.12° E), Sabah, Malaysia. Briefly, a spoonful of mud (~5 g) and 5 ml water samples were inoculated aseptically into McCartney bottles containing 25 ml of sterile 112 medium according to a previously described method [11]. Then, all bottles inoculated with samples were incubated under anaerobic-light conditions (2.5 klx illumination with tungsten bulbs) at 30 ± 2°C for five days. After the incubation, a drop of inoculum from each bottle was aseptically streaked onto a freshly prepared 112 agar plate supplemented with 1.2% agar (w/v). Subsequently, the petri dish was incubated in an anaerobic jar under light. The anaerobic condition in the jar was maintained by using a gas generating kit (Oxoid, UK). Then, on the 7th day of incubation, the petri dishes were examined and the largest colored single colony of bacteria from each agar plate was aseptically picked and serially subcultured onto freshly prepared 112 medium agar plates. The subculturing was repeated until single and pure bacterial colonies were obtained.

Morphological Characterization of Bacterial Isolates

Morphological characterization of the bacterial isolates was done according to the colony appearance on solid medium, gram staining, motility, and cell size and shape [35]. In addition, the salt tolerance of each of the bacterial isolates was tested using 112 medium with and without 3% (w/v) sodium chloride supplementation.

DNA Extraction

Genomic DNA from the bacterial isolates was extracted using the proteinase K extraction method [15]. The bacterial isolates were grown in liquid 112 medium and incubated for 48 h under anaerobic-light conditions. Subsequently, bacterial cells were harvested by centrifugation at 12,000 rpm for 1 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 600 µl of 1× TE buffer, 30 µl of 20% SDS, and 5 µl of 20 mg/ml proteinase K (Sigma, St. Louis, MO, USA). The mixture was briefly vortexed, and then incubated at 37°C for 1 h. Then, 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl were added and the mixture was further incubated at 65°C for 10 min. Thereafter, the mixture was combined with 700 µl of chloroform:isoamyl alcohol (24:1) and centrifuged at 9,000 rpm for 10 min. A volume of 500 µl of the upper aqueous layer was transferred to a new microcentrifuge tube that contained an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged again at 8,000 rpm for 5 min. Then, 450 µl of aqueous phase (upper layer) was transferred to a new microcentrifuge tube and combined with an equal volume of isopropanol. Later on, the DNA was pelleted by centrifugation at 13,000 rpm for 15 min. Finally, the DNA was briefly air dried before dissolving in 50−100 µl of 1× TE buffer.

PCR Amplification and DNA Cloning

The DNA from each bacterial isolate was subjected to polymerase chain reaction (PCR) amplification using 16SJR33F (5′-GAACGC TGGCGGCAGGCCTAA-3′) and 16SJR1449R (5′-ACTCCCATG GTGTGACGGGCGG-3′) primers. Briefly, the DNA was initially denatured at 94°C for 2 min, followed by 30 cycles of denaturation at 95°C for 60 sec, annealing at 55°C for 60 sec, and extension at 72°C for 60 sec. Finally, the PCR amplification was extended for 5 min at 72°C. Then, the PCR products were purified using the DNA-Spin plasmid DNA purification kit (iNtRON, Korea) according to the manufacturer's instruction. Subsequently, 2 µl of the PCR product was cloned into the pGEM-T Easy cloning vector (Promega) and transformed into *E. coli* JM109 according to the manufacturer's instructions. Subsequently, plasmids from the transformant *E. coli* JM1009 were extracted using the Purelink Quick Plasmid Miniprep Kit (Invitogen) and sent to AITbiotech Pte Ltd, Singapore for DNA sequencing.

Table 1. Reference bacterial strains obtained from GenBank.

Bacterial group		Name	16S rRNA gene accession number
Purple non-sulfur bacteria	Alpha-proteobacteria	*Afifella* sp.	EU445270 GU370095 FR 733717
		Rhodobaca sp.	NR_044285 NR_025089
		Rhodobacter sp.	FR 821196
		Rhodovulum sp.	FR828479 NR_042645
		Rhodoblastus sp.	JN408838 NR_042408
		Rhodopseudomonas sp.	HQ154127 NR_028641
		Rhodomicrobium sp.	FN995218 HE863941
		Roseospirillum sp.	NR_041955 AJ011919
		Rhodospira	NR_036971 AJ001276
		Rhodovibrio sp.	NR_043397 JN196522
		Roseospira sp.	JN697474 JN697481
		Rhodopila sp.	NR_037120 D86531
	Beta-proteobacteria	*Rhodoferax* sp.	DQ664242 JQ087036
		Rubrivivax sp.	NR_042385 EU704902
Green sulfur bacteria		*Chlorobium* sp.	HM448355
Purple sulfur bacteria		*Chromatium* sp.	NR_025315
Green non-sulfur bacteria		*Chloroflexus* sp.	AJ 308501

Sequence Analysis

The 16S rRNA gene sequences from the PNSB isolates in this study were subjected to BLAST [2] and aligned against the gene sequences from other marine PNSB (Table 1) using Clustal W (DNASTAR ver. 7). The nucleotide sequence similarity was calculated and the phylogenetic tree was constructed based on the 10.00 (gap penalty), 0.20 (gap length penalty), 30 (delay divergence seq (%)), and 0.5 (DNA translation weight) multiple alignment using Clustal W in DNASTAR (ver. 7).

Growth Characterization of the Bacterial Isolates

A single colony of each bacterial isolate was incubated anaerobically in a McCartney bottle containing 25 ml of sterilized 112 medium under 2.5 klx light intensity at 30 ± 2°C for 7 days. Thereafter, 1 ml of the culture was transferred to a new McCartney bottle containing 24 ml of freshly prepared sterile 112 medium and incubated as described above. A 48 h culture was used as the inoculum, where an inoculum size of 10% (v/v) was used in the study of growth characterization. The dry cell weight (mg/l) and total carotenoids (mg/g dry cell weight) of the bacterial isolates were analyzed in triplicate at 0, 24, 48, 72, 96, and 120 h of light exposure (2.5 klx) at 30 ± 2°C according to the respective previously described method [31, 21].

Dry Cell Weight

The bacterial cells from 10 ml of overnight culture were harvested by centrifugation at 4,400 rpm for 20 min at 4°C. Then, the bacterial pellet was resuspended in sterile distilled water, followed by centrifugation as described above. Subsequently, the bacterial cells were dried in an oven at 105°C until a constant weight was achieved. The dry cell weight (g/l) was then calculated according to the formula described by Sawada *et al.* [31].

Total Carotenoids

The total caroteniod content was determined using the acetone-methanol extraction method [21]. The concentration of carotenoids was determined spectrophotometrically against acetone methanol as the blank. The total carotenoids were expressed in mg/g dry cell weight according to the following equation:

$$C = \frac{D.V.f\left(\frac{10}{2,500}\right)}{\text{Dry cell weight (g)}}$$

where

C = total carotenoids (mg/g dry cell)
D = absorbance at 480 nm
V = total volume of sample used (ml)
f = dilution factor of sample (if absorbance greater than 0.8)

Growth Characteristics of *Af. marina* Strain ME at Different Light Intensities

The growth characteristics of the selected bacterial isolate (*Af. marina* strain ME) under anaerobic conditions exposed to various light intensities (0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 klx) at different incubation periods (0, 24, 48, 72, 96, and 120 h) were determined in triplicate at 30 ± 2°C. The light intensity was measured using a mini light meter CENTER 337. The *Af. marina* strain ME was selected for the study based on its exhibiting the largest dry cell weight and production of total carotenoids compared with the other isolates.

Growth Characteristics of *Af. marina* Strain ME Under Different Photoperiods

The dry cell weight and the total carotenoid production in *Af. marina* strain ME exposed to different photoperiods (24, 18, 12, 6,

and 0 h) under anaerobic culture condition were determined at 30 ± 2°C under two light intensities (3 and 2.5 klx).

Statistical Analysis

The mean values and standard deviation (mean ± SD) of dry cell weight (g/l) and total carotenoids (mg/g dry cell weight) recorded at different light intensities and photoperiods were analyzed using one-way ANOVA (SPSS Windows Statistical Package ver. 17.0) at $p < 0.05$ significance level.

Results

Isolation of Purple Non-Sulfur Bacteria from Mangrove Microhabitats

A total of four bacterial isolates were obtained from specific microhabitats within the mangrove environment. Isolates designated as ME, WE, MS, and WS were from exposed mangrove mud, exposed mangrove water, shaded mangrove mud, and shaded mangrove water habitats, respectively. All the bacterial isolates exhibited reddish bloom when grown in 112 liquid medium supplemented with 3% salt under anaerobic-light conditions. Interestingly, no color was exhibited when the bacterial isolates were grown in 112 liquid medium without salt supplementation. Colonies of bacterial isolates on solid media were circular with convex elevation. Gram staining and microscopic observation indicated that all bacteria isolates were gram negative, rod shaped, and motile. The estimated cell size ranged from 0.8 to 0.9 μm (width) and 1.8 to 2.0 μm (length) (Table 2).

Phylogenetic Analysis

Partial 16S rRNA gene sequences of the bacterial isolates were compared with the gene sequences of members of alpha-proteobacteria and beta-proteobacteria. The results revealed that the bacterial isolates (ME, WE, MS, and WS) were more closely related to the representatives of alpha-proteobacteria than to the beta-proteobacteria. The nucleotide sequence similarity of the bacterial isolates to alpha-proteobacteria was observed to range from 86.6% to 99.9%. In contrast, the isolates showed only 79.1% to 80.7% nucleotide similarity to beta-proteobacteria.

Specifically, the bacterial isolates ME, WE, MS and WS exhibited the highest level of nucleotide sequence similarity of 99.9%, 99.9%, 99.9%, and 99.7%, respectively to *Af. marina* strain EU445270. Moreover, the phylogenetic tree also showed that all the isolates were clustered within the class alpha-proteobacteria and were most closely related to the type species, *Af. marina* (Fig. 1). On the basis of DNA sequence similarity and the phylogenetic tree, the four bacterial isolates in this study were identified as *Af. marina* strains ME, WE, MS, and WS, respectively.

Growth Characteristics of Bacterial Isolates

The growth curve of the bacterial isolates grown under anaerobic-light condition (2.5k lux) in liquid 112 medium supplemented with 3% salt was shown to have a negligible lag phase, followed by an exponential phase until 72 h and a phase of decline after 72 h (Fig. 2A). The maximum dry cell weight and maximum production of carotenoids of *Af. marina* strains (ME and WE) isolated from exposed habitats were 12.8% and 24.2% higher than the corresponding values recorded for *Af. marina* strains (MS and WS) isolated from the shaded habitats ($p < 0.05$). However, no significant differences ($p > 0.05$) in both maximum dry cell weight (0.6%) and maximum carotenoid production (5.7%) were recorded between *Af. marina* isolates from mangrove mud and water, respectively (Fig. 2B). In this study, *Af. marina* strain ME exhibited the highest dry cell weight of 4.34 g/l

Table 2. Phenotypic characteristics of PNSB isolated from exposed mangrove mud (ME), exposed mangrove water (WE), shaded mangrove mud (MS), and shaded mangrove water (WS).

Characteristics	Isolates			
	ME	WE	MS	WS
Gram-staining	Negative	Negative	Negative	Negative
Motility	Positive	Positive	Positive	Positive
Shape	Rod	Ovoid rod	Rod	Ovoid rod
Cell width (μm)	0.8–0.9	0.7–0.9	0.8–0.9	0.7–0.8
Cell length (μm)	1.8–2.5	1.5–2.0	1.8–2.5	1.5–2.0
Color in liquid medium	Dark red	Dark red	Dark pink	Light Pink
Color in agar medium	Dark red	Dark red	Brick red	Brick red
Salt response (3% salt)	Positive	Positive	Positive	Positive

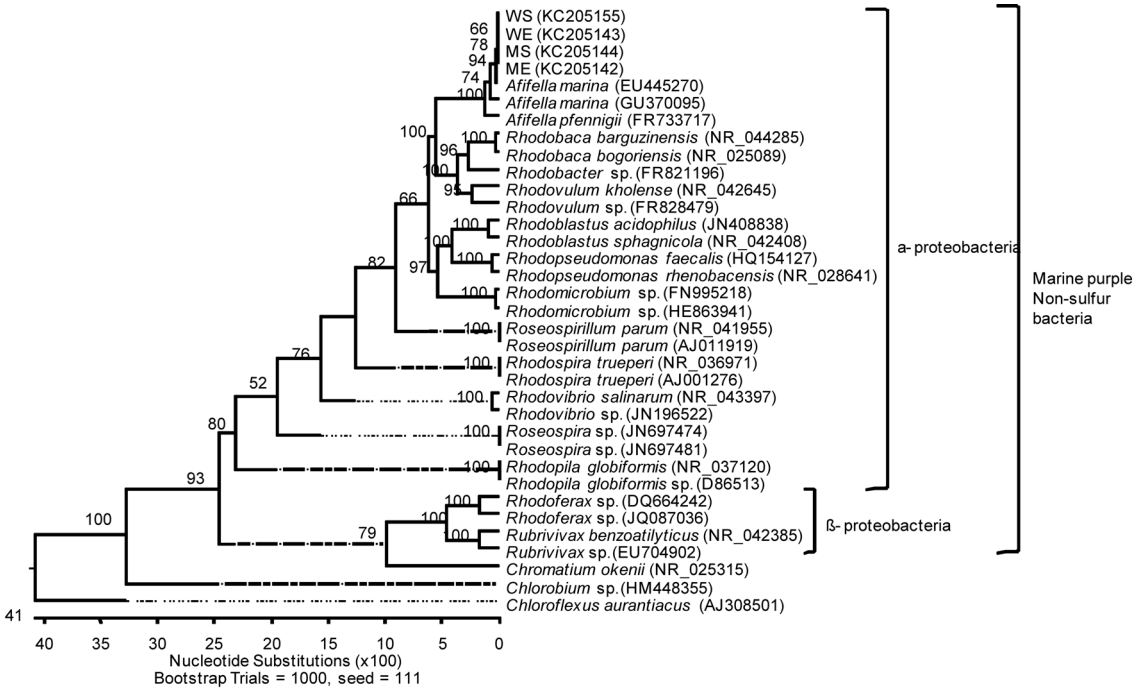

Fig. 1. Phylogenetic tree of 16S rRNA gene sequences of isolates and strains from representative marine PNSB genera.
The tree is rooted with *Chloroflexus aurantiacus* strain DSM 637T (AJ308501), *Chlorobium* sp. (HM448355), and *Chromatiumokenii* (NR_025315) as an out-group. Bootstrap values are given at branch points.

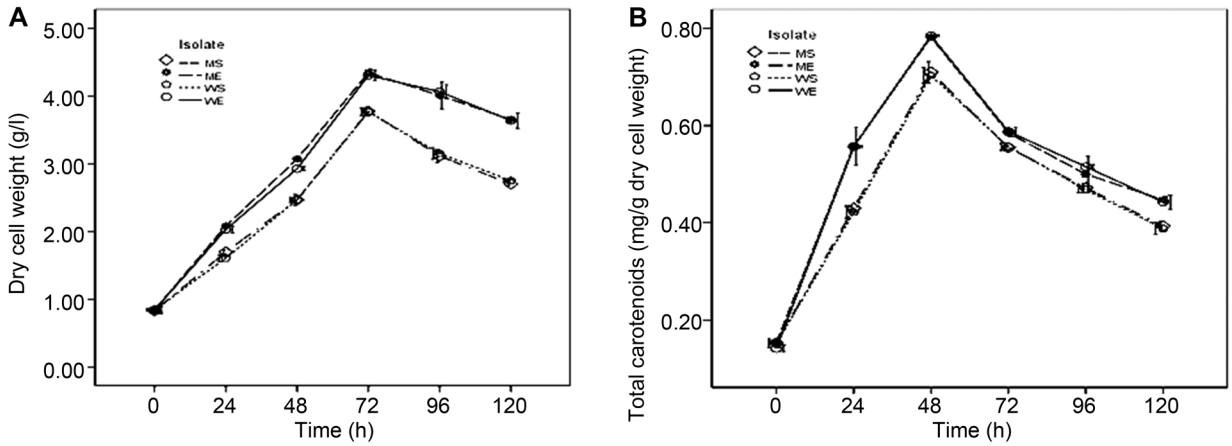

Fig. 2. Changes in (**A**) dry cell weight (g/l) and (**B**) total carotenoids (mg/g) of isolates grown under anaerobic-light (2.5 klx) conditions at 30 ± 2°C, over a period of 120 h.

and total carotenoids of 0.97 mg/g dry cell weight at 72 h and 48 h culture, respectively. Accordingly, *Af. marina* strain ME was selected for further analysis.

Growth Characteristics of *Af. marina* Strain ME Under Different Light Intensities

Af. marina strain ME was found to grow both under anaerobic-light and anaerobic-dark conditions. However, its growth was inhibited in the dark and under low light conditions (1 and 1.5 klx), where cells exhibited a low specific growth rate of 0.009 to 0.020 per hour and low dry cell weight of 1.57 to 3.24 g/l. Nevertheless, cell growth was evident in moderate light intensities (2, 2.5, 3, and 3.5 klx) with a higher specific growth rate of 0.021 to 0.050

Table 3. Growth characteristics of *Afifella marina* strain ME in 112 medium under different light intensities for a 5-day culture period (mean ± SD)

Treatment (klx)	X_{max}	μ_{max}
0.0	1.57 ± 0.05	0.009 ± 0.001
1.0	2.97 ± 0.03	0.014 ± 0.002
1.5	3.24 ± 0.04	0.020 ± 0.001
2.0	3.55 ± 0.04	0.021 ± 0.001
2.5	4.34 ± 0.02	0.038 ± 0.002
3.0	4.97 ± 0.01	0.050 ± 0.002
3.5	4.67 ± 0.05	0.049 ± 0.001
4.0	3.20 ± 0.03	0.033 ± 0.001
4.5	3.19 ± 0.03	0.025 ± 0.001
5.0	3.32 ± 0.04	0.022 ± 0.003

X_{max}, maximum dry cell weight (g/l); μ_{max}, specific growth rate (per hour).

per hour and also a higher dry cell weight of 3.55 to 4.97 g/l (Table 3). At light intensities beyond 3.5 klx, the growth rate of isolates was found to negatively correlate with the light intensity.

The maximum dry cell weight and total carotenoids at all light intensities were achieved at 72 and 48 h of incubation, respectively (Fig. 3). The highest dry cell weight of 4.97 g/l was obtained in a 72 h bacterial culture grown under 3 klx illumination at 30 ± 2°C. This value was significantly ($p < 0.05$) higher than the maximum dry cell weight obtained from the cultures grown under other light intensities.

The production of total carotenoids in *Af. marina* increased with elevation in light intensity from 0 to 2.5 klx. Low carotenoid production of 0.145 to 0.348 mg/g dry cell weight was found to occur under dim light conditions (≤ 2 klx illumination intensity). The highest total carotenoid production of 0.783 mg/g dry cell weight was recorded at 2.5 klx illumination. The production of carotenoids then decreased gradually as the light intensity increased beyond the optimum level of 2.5 klx. Interestingly, the total carotenoid production by *Af. marina* strain ME rebounded when it was exposed to 4.5 and 5 klx light intensities.

Growth Characteristics of *Af. marina* Strain ME at Different Photoperiods

The dry cell weight in *Af. marina* strain ME was observed to increase with increase in the photoperiod (Fig. 4). The highest dry cell weight of 4.97 g/l was recorded under continuous light conditions (24L/0D). However, there was no significant difference ($p > 0.05$) between the dry cell weight obtained with photoperiods of 24 h (4.97 g/l) and 18 h (4.85 g/l). Higher production of total carotenoids (0.782 mg/g dry cell weight) was also observed with a longer photoperiod (24L/0D). This concentration was

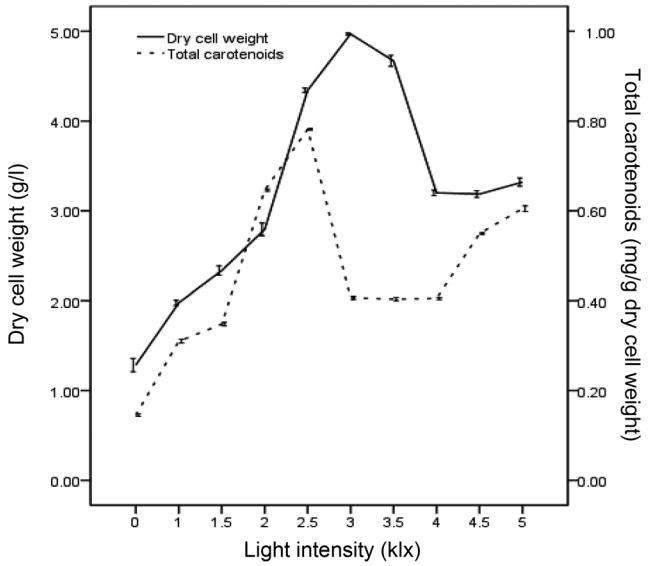

Fig. 3. Effects of light intensity on the maximum dry cell weight (g/l) and total carotenoids (mg/g dry cell weight) production in *Af. marina* stain ME.

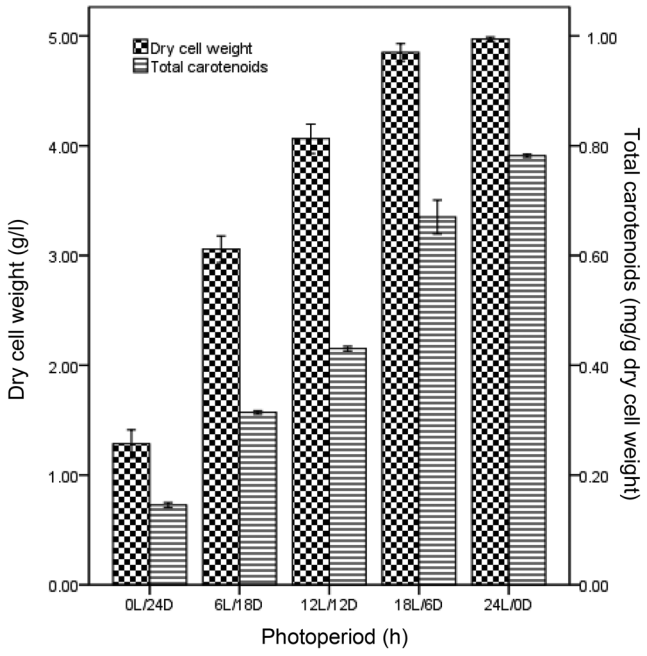

Fig. 4. Effect of photoperiod on the maximum dry cell weight (g/l) and total carotenoids (mg/g dry cell weight) production in *Af. marina* strain ME.

significantly higher ($p < 0.05$) than the concentration of carotenoids produced during other photoperiods.

Discussion

Photosynthetic Bacteria from Mangrove Microhabitats

The change in color of the bacterial culture in 112 medium with 3% salt, from golden to reddish, suggests that all the bacterial isolates belonged to the marine PNSB. Pink to reddish coloration of the bacterial culture under anaerobic-light conditions is one of the important characteristics of PNSB [26]. This coloration is the result of biosynthesis of reddish pigments of the spirilloxanthin series (bacteriochlorophyll *a* and carotenoids) by *Af. marina* strain C3 [39], *Rhodobium gokarnense* strain JA173 [36], and *Af. marina* strain MB303 [17] grown in various culture media under anaerobic-light conditions.

The phenotypic characteristics of all the bacterial isolates (ME, MS, WE, and WS) were comparable to those of marine PNSB, especially *Afifella* sp. [17] and *Rhodovulum* sp. [16]. Cells of *Afifella* sp. are ovoid to rod shaped, 0.7 to 0.9 µm wide, and 1.0 to 2.5 µm long. The living cells are motile and exhibit optimum growth in the presence of 1% to 5% of salt [17,19]. On the other hand, *Rhodovulum* sp. is a gram-negative rod with a cell size ranging from 0.5 to 0.9 µm in width, and 0.9 to 2.0 µm in length. The living cells of *Rhodovulum* sp. are also motile and require salt for optimal growth [16]. Both genera usually form a uniformly distributed pink to reddish bloom under anaerobic-light conditions. The size, shape, and other morphometric characteristics of the bacterial isolates suggest that they could be members of *Afifella* sp. or *Rhodovulum* sp.

Phylogenetic Analysis

The phylogenetic analysis of the bacterial isolates showed that they are clustered within the PNSB group and most closely related to *Af. marina*. The nucleotide sequence similarity between all the isolates (ME, WE, MS, and WS) and *Af. marina* was in the range of 99.7% to 99.9%, and this is in agreement with a previous study, which reported that *Af. marina* shared 99.8% nucleotide sequence similarity among themselves [39].

The present study also suggests that *Af. marina* can be easily isolated from a wide range of habitats, including shaded and exposed areas of mangrove swamps. *Af. marina* and similar organisms were previously isolated mainly from saline ponds [39], coastal seawater [20], sediment [36], and mud from the bottom of a tidal pool [17]. Therefore, it is reasonable to believe that *Af. marina* is able to tolerate a wide range of natural marine microhabitats.

Growth Characteristics of Isolates

All the bacterial isolates demonstrated a similar growth pattern under anaerobic-light conditions at 30 ± 2°C and 2.5 klx. An increase in dry cell weight was associated with an increase in carotenoid production for the first 48 h of incubation. However, carotenoid production decreased by 17.9% to 27.8% at 72 h of incubation, despite an increase in dry cell weight. The decrease in total carotenoid production after 48 h of incubation indicates that the cells may have entered the stationary phase or died. However, complete degradation of the dead cells may take some time and, therefore, this could contribute to an increase in dry cell weight up to 72 h of incubation [1]. The optimum incubation periods for achieving maximum dry cell weight and total carotenoid production of the isolates were 72 and 48 h, respectively. The observation was comparable to the results of previous studies where the optimum incubation time for dry cell weight and carotenoid production in PNSB was recorded within 48 to 72 h of incubation [1, 6, 33].

The range of dry cell weight (3.77 to 4.34 g/l) and total carotenoids (0.71 to 0.78 mg/g dry cell weight) from 72 and 48 h cultures was in agreement with the results of other studies. The dry cell weight (4.8 g/l dry cell weight) and carotenoids (1.04 mg/g dry cell weight) of *Rv. sulfidophilum*, grown in glutamate-malate media (GMM) under continuous anaerobic-light conditions (2.5 klx), was higher than that seen in the current study [6]. The bacterial isolates exhibited a much higher dry cell weight (2.75 to 3.5 g/l) and total carotenoids (~0.5 mg/g dry cell weight) than those reported for *Rhodopseudomonas acidophila* [34], *Rhodomicrobium vannielii* [1], and *Rhodobium marinum* [22] cultured in modified aSy medium, GMM, and GL medium, respectively. This suggests that the *Af. marina* strains in this study were fast growing and perhaps can be biotechnologically manipulated for large-scale production of carotenoids.

The bacterial isolates from exposed habitat exhibited a relatively high dry cell weight (g/l) and total carotenoid production (mg/g dry cell weight) than the isolates from shaded habitats. This result can be explained by the fact that light stimulates the photoheterotrophic growth of PNSB, thereby accelerating their growth in the presence of readily usable organic compounds such as malate or pyruvate, and ammonia, which are abundant in the mangrove ecosystem [34]. Under dark conditions, however, the growth of PNSB is retarded because it causes them to

undergo fermentation or anaerobic respiration. This explains why exposed habitats are favorable to PNSB [13]. In many species of PNSB such as *R. capsulate*, *R. sphaeroides*, and *R. rubrum*, synthesis of the photosynthetic apparatus occurs predominantly under low oxygen tension, due to the high-level anerobic expression of the *puh*, *puf*, and *puc* operons that encode proteins for the light-harvesting and reaction center complexes [7]. However, the current study showed no significant difference ($p > 0.05$) in dry cell weight and total carotenoid production among the isolates. This result may be due to the shallow and active mixing of water in the sampling sites, which allowed the same microflora to be well distributed within the habitats.

Growth Characteristics of *Af. marina* Strain ME Under Different Light Intensities

Af. marina strain ME was able to grow under both anaerobic-light and dark conditions. However, the dry cell weight (1.28 g/l) at anaerobic-dark conditions was lower than the dry cell weight (1.98 to 4.97 g/l) achieved under anaerobic-light conditions. *Af. marina* is known to exhibit fermentative growth under anaerobic-dark conditions [17]. The utilization of organic carbon sources by PNSB under anaerobic-dark conditions is associated with slow oxidation through the TCA cycle [37]. The biosynthesis of carotenoids was also inhibited under anaerobic-dark conditions. It is likely that the photosynthetic bacterium totally relies on fermentative growth in the dark; therefore, the biosynthesis of carotenoids is not necessary [32]. However, light seems to favor the production of total carotenoids in PNSB under anaerobic conditions [4, 8].

The dry cell weight was also strongly influenced by the light intensity. An increase in light intensity from dark conditions to 3 klx was shown to enhance the production of dry cell weight in *Af. marina*. Interestingly, the dry cell weight decreased drastically when the light intensity increased beyond 3.5 klx. When *Rhodomicrobium vanielii* was grown in GM medium and exposed to 4 klx under anaerobic conditions, the growth decreased and no production of carotenoids was detected [1]. This result may be due to the heat generated during the exposure to high light intensities (4 to 5 klx), inhibiting the growth of the bacterial isolates in the culture medium. In the current study, 3 klx seemed to be the optimal light intensity for dry cell weight production (4.97 g/l) in tropical *Af. marina*. A similar observation was reported in the case of *Rhodocyclus gelatinosus* [29]. It was also evident that the dry cell weight generated at 96 h was not significantly different from that produced at 72 h incubation. Therefore, from an economical point of view, an incubation period of 72 h is sufficient to obtain the highest dry cell weight as it lowers the production cost.

The optimum light intensity for total carotenoid production (0.97 mg/g dry cell weight) was recorded at 2.5 klx. However, it was inhibited at higher light intensities, particularly from 3 to 4 klx. This was not a surprising finding, since a similar observation was also reported for *R. capsula* and *R. sphaeroides* [1]. Such a phenomenon was proposed to be due to the diminishing level of *puf*, *puh*, and *puc* transcripts [41]. However, the lower carotenoids production in dim light (2 klx) conditions, and high production under high light intensities (4.5 and 5 klx) are unexpected. This finding may suggest that the salt-tolerant *Af. marina* strain ME may have an unusual light-induced repression mechanism that accelerates the expression of genes controlling the production of carotenoid pigment.

Growth Characteristics of *Af. marina* Strain ME Under Different Photoperiods

Generally, an increase in photoperiod has been shown to increase the maximum dry cell weight and production of total carotenoids. However, the dry cell weight yield and the total carotenoid production during the 24 h light exposure did not significantly differ from those obtained during 18 h. In another study, *Rhodobacter sphaeroides* exposed to 12 h light (12L/12D) yielded a similar amount (0.55 g/l) of dry cell weight as under the continuous light condition (24LD) [14]. Although the photoperiod can be shortened without affecting the production of dry cell weight and total carotenoid in PNSB, the *Af. marina* strain ME in this study was shown to require 18 to 24 h light exposure in order to produce the optimum dry cell weight and carotenoids.

In conclusion, the purple non-sulfur bacterium *Af. marina* can tolerate a wide range of mangrove microhabitats. *Af. marina* isolated from mangrove exposed habitats had better growth characteristics in terms of dry cell weight and production of carotenoids than those strains isolated from mangrove shaded habitats. The optimum total carotenoid production in *Af. marina* was achieved when the bacteria were exposed to a light intensity of 2.5 klx and incubated for 48 h. However, the optimal production of dry cell weight could only be achieved when the bacteria were exposed to 3 klx light intensity at a longer incubation period of 72 h. For optimal production of dry cell weight and total carotenoids, the bacteria have to be exposed to at least 18 to 24 h photoperiods.

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