Thesis for the Degree of Master of Fisheries Science

Symbiotic relationship between a microalga, *Pavlova viridis* and associated bacteria

by

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KOICA-PKNU International Graduate Program of Fisheries Science

The Graduate School

Pukyong National University

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미세조류 파블로바 비리디스와 표면 부착 세균의 공생관계

Advisor: Professor Tae Jin Choi

by

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Abstract

The symbiotic relationship between the flagellate microalga *Pavlova viridis* and its associated bacteria was investigated. The axenic culture of *P. viridis* was obtained by repeated treatment of the microalga with antibiotic cocktail. Axenic status was confirmed after sub culturing three times in sterile f/2 medium without antibiotic. The axenic alga was then co-inoculated with bacteria isolated from the alga to test the growth promotion of the algae. All bacterial isolates promoted the growth of *P. viridis* and

bacterial isolate I3 was the most effective among the five tested bacteria. The cell numbers of microalga was significantly higher than that of control culture. The I3 isolate was further characterized and sequence analysis of the 16S rRNA of I3 isolate revealed 97% nucleotide sequence similarity with that of *Arthrobacter* sp. The optimum growth condition of *Arthrobacter* sp. was 37˚C, pH 7 and NaCl 1%. The growth of I3 isolate was also increased effectively by co-culturing with *P. viridis*, showing a symbiotic relationship between microalga and its associated bacterium. The association between microalga and bacterium was confirmed by scanning electron microscopy.

Key words: Symbiosis, *Pavlova viridis*, axenic status, co-culture, *Arthrobacter* sp.

Introduction

Microalgae are microscopic and unicellular organism typically found in marine and fresh waters and usually coexist with bacteria (Jones, 1982). They are primary producers and affect the nutrients cycles in aquaculture ecosystems (Cole, 1982).

Microalgae are indispensable natural food sources in aquaculture for all growth stages of bivalve, crustaceans and fish species in aquaculture and they are also serving as food for zooplankton to support continuously the food webs. They provide energy and nutrients to marine organisms essential for growth and development. Microalgae possess well balanced nutrient content so that, it is used to improve the nutritional conditions of feed. They can play a significant role for most aquatic animals (Volkman et al., 1989; Brown and Farmer, 1994; Brown et al., 1997).

Microalgae are used for various other purposes. They are good sources of highly valuable bioactive compounds, polyunsaturated fatty acid, antioxidant and pharmaceuticals (Gouveia et al., 2008). Algae are well known by their conspicuous color pigments. They produce not only chlorophyll, the photosynthetic pigment but also phycobiliproteins and

carotenoids that are used for the protection of skin damage from sunlight, also used as natural food colorant, application in cosmetics etc. (Spolaore et al., 2006; Hallmann, 2007).

The marine microalga *Pavlova viridis* can grow fast, having high amount of polyunsaturated fatty acids, EPA and DHA are used as food for raring larval fish and prawn (Lu and Lin, 2001). They have the ability to accumulate essential nutrients for culturing marine animals (Chen et al., 1998).

Established culture of microalgae is often accompanied by bacterial contamination. The axenic algal cultures are essential because of physiological, chemical, molecular or taxonomic studies and determination of zooplankton food preference and algal histories (Stanier et al., 1971). Some aquatic bacteria can inhibit the algal growth or cause lyse of algal cells (Cole, 1982), and to protect the algae from such algicidal action is important reason for the establishment of axenic algal culture.

Axenic culture of alga is important for many purposes. In fact, there are a few methods of axenic cultures of microalgae have so far been reported; actually it is an arduous job to obtain an axenic culture from a

highly contaminated algal culture. Treatment with antibiotics is the most common way to attain the axenicity of microalgae from bacteria (Connell and Cattolico, 1996; Cho et al., 2002).

Many bacteria live on the cell surface of microalgae and they have diverse effects on the growth of host algae. The relationships between microalgae and bacteria are quite variable, depending on the species and environmental conditions (Jones, 1982).

Bacteria associated with microalgae may have positive or negative impacts on the growth of microalgae. Many reports have demonstrated that aquatic bacteria can cause algal cells to lyse or inhibitory effects on the growth of micro algal because of detrimental chemicals produced by the bacteria (Barker and Herson, 1974; Jones, 1982; Fukami et al., 1997). Apart from these suppressive actions, influencing or stimulating relationship between bacteria and microalgae has also been narrated (Riquelme et al., 1988; Suminto and Hirayama, 1996; Ferrier et al., 2002).

Watanabe et al. (2005) reported high influential growth of microalga *Chlorella sorokiniana* co-culture with bacteria *Microbacterium trichotecenolyticum*. Moreover, Riquelme et al. (1988) found the highest growth of alga *Asterionella gracialis* with *Pseudomonas* sp. They assumed that the bacteria produce some chemicals like vitamins, and lipoprotein that promote the growth of microalgae.

It has also been observed that eight bacterial isolates separated from *Chlorella ellipsoidea* had growth promoting effect in the co-culture with the microalga (Park et al., 2008).

The objective of the present study was to isolate and characterize of bacteria associated with *Pavlova viridis* and study their interactions by co-culturing of axenic alga and the isolated bacteria or bacterial free culture filtrate as well as finally find out their best combination for the best growth.

Materials and Methods

Experimental design

First of all, axenic culture of the microalga was obtained by repeated treatment of the contaminated alga with antibiotic cocktail. Single colony of bacterial isolates was established from the contaminated microalga through spreading and streaking. The isolated bacteria were then identified by cloning and sequencing of the 16S rRNA. Finally the axenic microalga was mixed with the isolated bacterial isolates, and its growth performance was compared to that of single culture of alga and bacteria (Fig. 1).

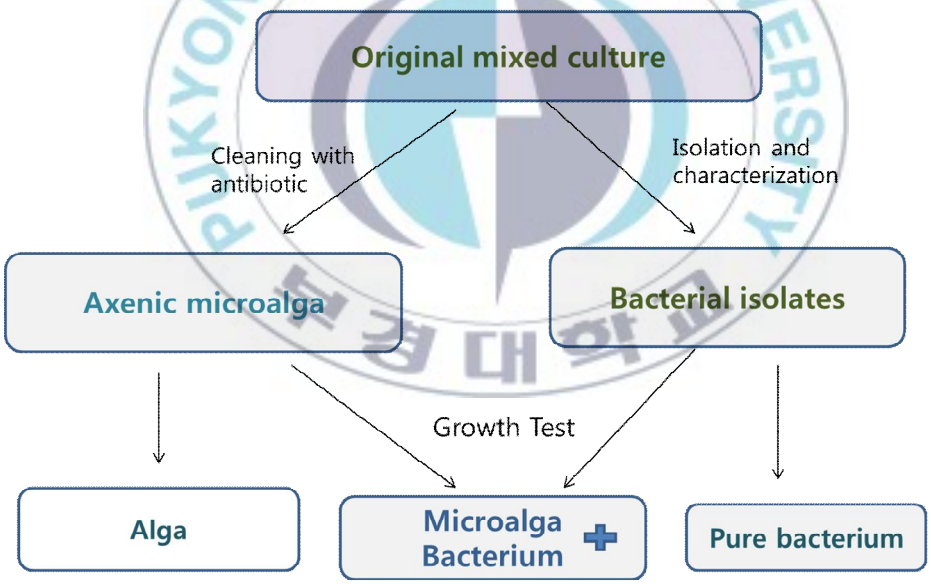

Fig. 1. Flow diagram of experimental design

Microalga culture

The marine microalga *Pavlova viridis* was obtained from the Korea Marine Microalgae Culture Center, Pukyong National University, Busan, Korea. Alga was cultured in f/2 medium (Guillard and Ryther, 1962; Guillard, 1975) at 20˚C under a 16/8-h light-dark cycle with a light intensity of 30 μmol $m^{-2}s^{-1}$ (Agrawal and Sarma, 1982).

Fig. 2. Culture of alga in laboratory. Alga was cultured in test tube, 100ml flask or 250ml culture flask.

Isolation of bacteria

In order to obtain bacterial isolates from the original algal culture, 100 μL algal cultures of *P. viridis* was spreaded on LB agar plate and incubated at 37˚C for 72 h. Colonies showing different morphology and color were streaked again on LB plates and incubated at 37˚C for 72 h. After three passages on LB plate, glycerol stocks of single colonies were prepared in LB broth containing 20% glycerol and then kept at -80˚C for further study.

Antibiotic resistance

To determine the optimal concentration of antibiotic treatment to *P. viridis* for cleaning, five different antibiotics were tested against the isolated bacteria. LB agar plates with different concentration of antibiotic were prepared. Single colonies were streaked into LB plates and incubated at 37˚C for 72 h. Antibiotic susceptibility was identified by the growth of bacterial colonies. Antibiotic cocktail was prepared based upon the antibiotic resistance.

Axenic culture of *Pavlova viridis*

An axenic culture *of P. viridis* was established by following the method described by Hong et al. (2010), which is shown on Fig. 3. Well-grown alga in f/2 medium was transferred to f/2 medium with antibiotic cocktail. After 8-10 days, alga was again transferred to fresh f/2 medium with the same antibiotic cocktail. Axenic status was checked through spreading on LB agar plates incubated at 37˚C for 7 days and repeated until there is no bacterial growth.

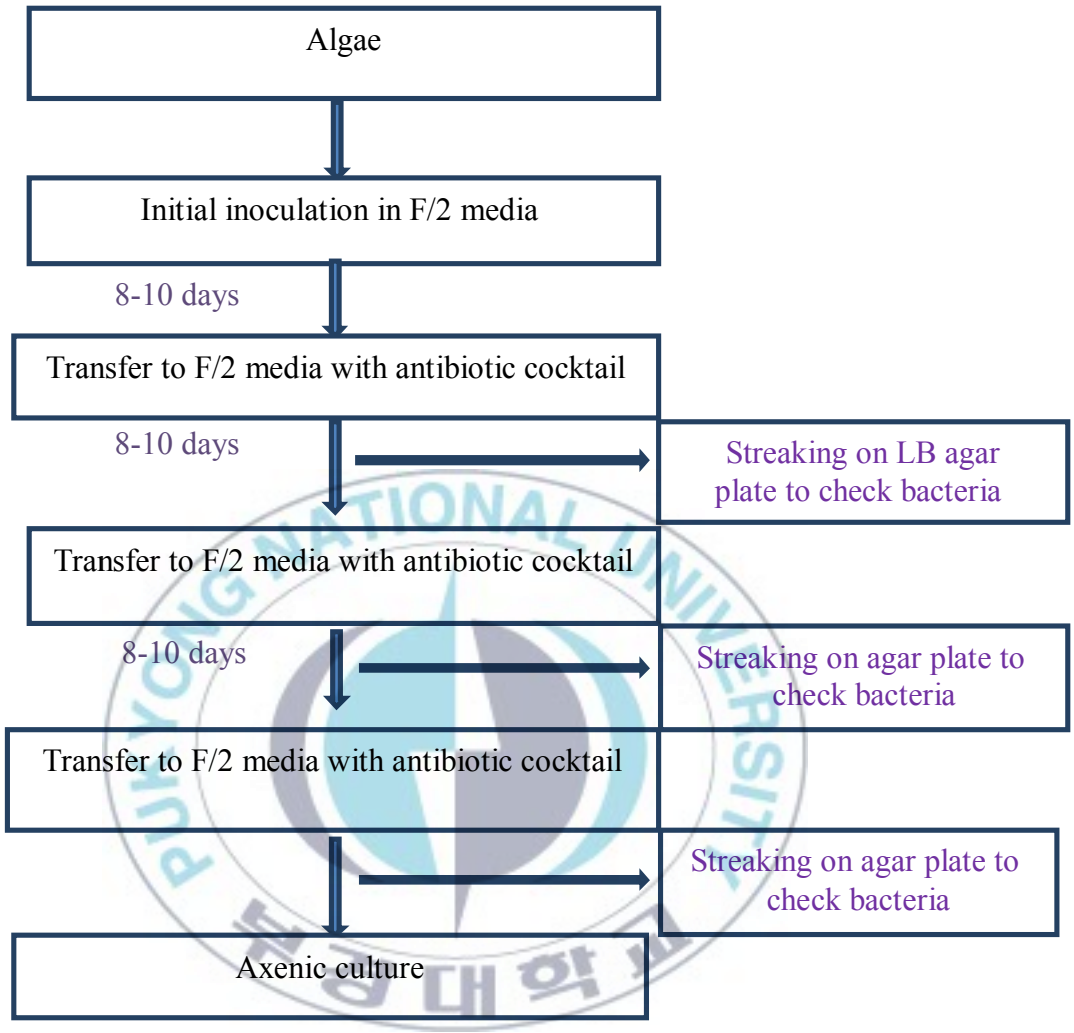

Fig. 3. Schematic of obtaining axenic culture of *P. viridis*.

Identification of bacterial isolates

The genetic identification of isolated bacteria was performed by sequence analysis of the 16S rRNA. Overnight culture of 3-5 ml was centrifuged at 13,000 rpm for 1 minute, after which pellets were suspended in 500 µl buffer S solution. Fifty microliters of 20% SDS and 500 µl of phenol: chloroform: isoamylalcohol (25:24:1) was added to the suspensions. The mixture was vortexed for 1 minute and then spun down at 13,000 rpm for 5 minutes. Supernatant was transferred to a new tube. 3M sodium acetate of 0.1 volume and 0.6 volume of isopropanol were added to the tube and mixed gently by inverting and then centrifuged at 13,000 rpm for 20 minutes. Finally, dried DNA was dissolved in 50 µl dH_2O. The 16S rRNA of bacteria was amplified by PCR (HS Prime Taq premix 2X, Genet Bio, Korea) using primers 27F and 1492R (James, 2010). The PCR products were visualized in 1% agarose gel. The amplified DNA fragments were purified from agarose gels using a Gel SV kit (Gene All, Seoul, Korea) The PCR product was sequenced using 16s rRNA primers (27F/1492R) (G&C Bio, Daejon, Korea). Homology analysis was carried out using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Identification of host algae

For the genetic identification of host alga, genomic DNA was isolated according to previously described method (http://www.gbiogene.com). The 18S rRNA of alga was amplified by PCR (HS Prime Taq premix 2X, Genet Bio, Korea) using primers 512F and 978R (Zimmermann et al., 2011). The PCR product was visualized in 1% agarose gel. The amplified DNA fragments were purified from agarose gels using a Gel SV kit (Gene All, Seoul, Korea). The PCR product was sequenced using 18s rRNA primers (512F/978R) (G&C Bio, Daejon, Korea). Homology analysis was carried out using the BLAST. (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Table 1. Oligonucleotide primers used for the amplification

Primer name	sequence	Remarks
27F	5′ AGA GTT TGA TCC TGG CTC AG 3′	Used for 16S rRNA
1492R	5′ GGT TAC CTT GTT ACG ACT T 3′	Used for 16S rRNA
512F	5′ ATT CCA GCT CCA ATA GCG 3′	Used for 18S rRNA
978R	5′ GAC TAC GAT GGT ATC TAA TC 3′	Used for 18S rRNA

Table 2. PCR profile for the amplification of bacterial 16S rRNA

Steps	Temperature(˚C)	No. of cycle
Initial denaturation	95˚C for 3 min	1 cycle
Denaturation	95˚C for 30 sec	
Primer annealing	50˚C for 1 min	35 cycles
Extension	72˚C for 1 min	
Final extension	72˚C for 10 min	1 cycle

Table 3. PCR profile for the amplification of algal 18S rRNA

Steps	Temperature(˚C)	No. of cycle
Initial denaturation	94˚C for 2 min	1 cycle
Denaturation	94˚C for 45 sec	
Primer annealing	52˚C for 45 sec	35 cycles
Extension	72˚C for 1 min	
Final extension	72˚C for 10 min	1 cycle

Culture of bacterial isolate I3

The suitable growth condition for bacterial isolate I3 was tested in with different temperature, NaCl concentrations and pH. The measurement of temperature for optimum growth of bacterial isolate I3 was determined as follows: A 40 µl aliquot of bacterial isolate I3 culture freshly grown at 37˚C overnight in LB broth was inoculated to 4 ml of LB broth prepared as described above and kept at 4, 20, 25, 30, 37 or 45˚C with shaking at 150 rpm for 24 h. Three replications were made for each temperature. Bacterial growth on each temperature was monitored after 24 h by measuring the absorbance at 600 nm using a spectrophotometer (MBA 2000; Perkin Elmer, Wellesley, MA, USA). Means were determined from three replications.

For measurement of NaCl concentration for the bacterial growth, LB broth with 0, 1, 2, 3, 4, 5, 7 and 10 % NaCl concentration were prepared first then a 40 µl aliquot of bacterial isolate I3 culture freshly grown at 37˚C overnight in LB broth was inoculated to each of 4 ml LB broth and incubated with shaking at 150 rpm for 24 h at 37˚C. Three replications were made for each NaCl concentration. Bacterial growth of each NaCl concentration was monitored after 24 h by measuring the absorbance at 600

nm using a spectrophotometer (MBA 2000; Perkin Elmer, Wellesley, MA, USA). Means were determined from three replications.

For the determination of optimum pH, LB with 1% NaCl concentration was made and pH was adjusted to 3, 4, 5, 6, 7, 8, 9, or 10. After that a 40 μl aliquot of bacterial isolate I3 culture freshly grown at 37˚C overnight in LB broth was inoculated to each of 4 ml LB broth and incubated at 37˚C with shaking at 150 rpm for 24 h. Three replications were made for each pH. Bacterial growth of each pH was monitored after 24 h by measuring the absorbance at 600 nm using a spectrophotometer (MBA 2000; Perkin Elmer, Wellesley, MA, USA). Means were determined from three replications.

Algal growth assays with five bacterial isolates

To determine the growth promoting effect of the bacteria, 100 ml of f/2 medium was inoculated with axenic *P. viridis* and cultured for 7 days at 20˚C under a 16/8-h light-dark cycle with a light intensity of 30 μmol $m^{-2}s^{-1}$, five bacterial isolates were inoculated separately, each in 4 ml LB broth cultured overnight at 37˚C and used as inoculum sources.

A aliquots of 100 ml of f/2 medium each containing 1 ml axenic culture of alga having 7.08 × 10^4 cells/ml was inoculated with 1 ml of bacterial suspension maintaining optical density of 0.5 each of five bacterial isolates as a treatment or without bacterial isolate as control. Co-culture was prepared triplicate for each bacterial isolate and three control cultures of the alga without bacterium were also prepared. Each culture was incubated at 20˚C for 10 days, and the growth of *P. viridis* was monitored daily by counting of the cells using a hemocytometer.

Algal growth assays with bacteria-free filtrates

In order to analyze the growth promoting activity of bacteria-free filtrate, firstly 25 ml of bacterial culture in LB broth was obtained by shaking at 150 rpm at 37˚C for 3 days. The cultured was centrifuged at 4,000 ×g for 10 min, and filtered twice through 0.2 μm polycarbonate filters (Minisart,Goettingen,Germany). Then, a 1-ml aliquot of this bacteria-free filtrate was added to 100 ml of f/2 medium containing 7.02 $\times 10^4$ algal cells/ml prepared as like before, and the mixture was incubated at 20˚C for 8 days. Each culture was prepared triplicate for each bacteria free filtrate and three control cultures of alga without bacteria free filtrate were prepared. The algal cells were measured daily for up to 8 days by counting using a hemocytometer.

For comparative assays of algal growth with different amount of filtrates, 1 ml, 2.5 ml, 5.0 ml and 10 ml of bacteria free-filtrates were added to 98 ml, 96.5 ml, 94 ml and 89 ml of f/2 medium, respectively containing 1 ml of alga cells. The same amounts of LB broth were used as controls without bacteria-free filtrate. Then the samples were cultured in 20˚C and

numbers of cells were measured after five days of inoculation using hemocytometer.

Growth promotion of alga and bacteria in co-culture

For the analysis of symbiotic relationship between bacterium and microalga, 100 ml of f/2 medium was inoculated with axenic *P. viridis* and cultured for 7 days at 20˚C under a 16/8-h light-dark cycle with a light intensity of 30 μmol $m^{-2}s^{-1}$ and bacterial isolate I3 was cultured in 4 ml LB broth over night at 37˚C. These were used as inoculum sources.

One milliliter of bacterial suspension of the optical density 0.5 and 1 ml of axenic *P. viridis* bearing initial cells of 7.06×10^4 prepared as described above were inoculated in 100 ml of fresh f/2 medium as co-culture treatment. One milliliter of either bacteria or alga with same conditions was separately inoculated in 100 ml of fresh f/2 medium as controls. All cultures were prepared in triplicate. Three replicates of 100 μl samples were taken every day from the *P. viridis* single culture and bacteria-alga co-culture. Samples were serially diluted and algal cells were counted using a hemocytometer. Colony forming unit (CFU) of bacteria co-

cultivated with the alga was used to measure the bacterial growth. One hundreds μl samples were taken each day from the bacterial single culture and bacteria-algal co-culture. These were serially diluted to 10^4-10^5 times using f/2 medium and 100 μl of each bacterial dilution was spread on the LB agar plates and incubated at 37˚C for 3 days. Bacterial colonies on each plate were counted.

Scanning electron microscopy (SEM)

The association of the bacteria and algae in co-culture was verified by SEM observation. To 14 ml of overnight culture, 1 ml of 2% osmium tetroxide was added for fixation and then kept in 4˚C overnight. The cells were collected by low-speed centrifugation (700 rpm) for 20 mins and the supernatant was discarded. The cells on the surface of the filter membrane were then rinsed twice with the distilled water and suspended with 1 ml distilled water, and filtrated through 0.2 μm polycarbonate filter. The cells were sequentially dehydrated twice (30 mins for each) with 10%, 20%, 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol. Finally, cells dehydrated with 100% ethanol for overnight and the ethanol was replaced with isoamyl alcohol twice. The samples were dried at critical point, coated with gold-palladium and observed under SEM (JEOL JSM-6490LV, Japan).

Results

Isolation and identification of bacteria

Five bacterial isolates of different color and/or colony morphology were obtained from the microalga *Pavlova viridis* and named as I1, I2, I3, I4, and I5. Genomic DNA was prepared of each bacterial isolate for the amplification of the 16S rRNA and their sequences were determined and compared with sequences in the Gene Bank by BLAST (Fig 4). The 16S rRNA sequences of bacterial isolate I1 demonstrated 100 % sequence identity to that of the bacteria *Kocuria marina* (Fig 5, Table 4). The 16S rRNA sequence of bacterial isolate I2 showed 100 % sequence identity to that of the bacteria *Staphylococcus cohnii* (Fig 6, Table 5). The 16S rRNA sequence of bacterial isolate I3 revealed 97 % sequence identity to that of the bacteria *Arthrobacter* sp. (Fig 7, Table 6). The 16S rRNA sequence of bacterial isolate I4 showed 99 % sequence identity to that of the bacteria *Micrococcus leuteus* (Fig 8, Table 7). The 16S rRNA sequence of bacterial isolate I5 exhibited 100 % sequence similarity to that of the bacteria *Staphylococcus* sp. (Fig 9, Table 8).

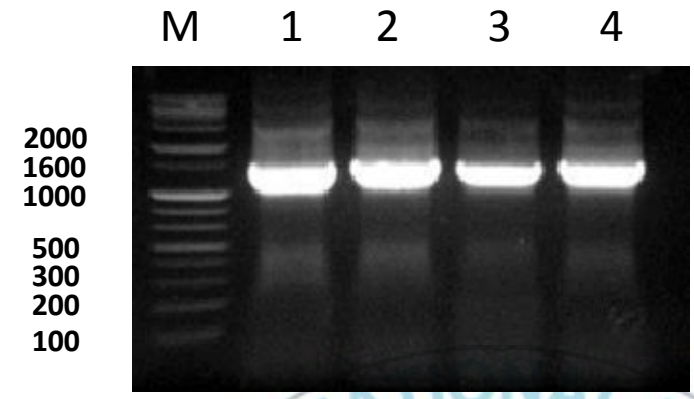

Fig. 4. Amplification of 16S rRNA of bacterial isolates; Lane 1, 2, 3, and 4 corresponds to the DNA amplified from isolates I3, I2, I4 and I5 respectively. M: 100 bp DNA marker.

AGGCGTCACCTTCGACGGCTCCCCCCACAAGGGTTAGGCCACCGGCTTCGGGTGT
TACCAACTTTCGTGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC
CGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCACGTGGTCGAGTTG
CAGACCACGATCCGAACTGAGACCAGCTTTTTGGGATTAGCTCCACCTCACGGTA
TCGCAACCCATTGTACTGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGGG
CATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCT
ATGAGTCCCCACCATCACGTGCTGGCAACATAGAACGAGGGTTGCGCTCGTTGCG
GGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTA
CACCAGCCCCACAAGGAGGAAAACCCATCTCTGAGCCGATCCGGTGTATGTCAAG
CCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTGT
GCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCG
GGGCACTTAATGCGTTAGCTACGGCGCGGAAAACGTGGAATGTCCCCCACACCTA
GTGCCCAACGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCA
TGCTTTCGCTCCTCAGCGTCAGTAACAGCCCAGAGACCTGCCTTCGCCATCGGTG
TTCTTCCTGATATCTGCGCATTTCACCGCTACACCAGGAGTTCCAGTCTCCCCTA
CTGCACTCAAGTCTGCCCGTACCCACTGCACACCCGGGGTTAAGCCCCGGGCTTT
CACAGCAGACGCGACAAACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACA
ACGCTTGCGCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTC
TTCTGCACGTACCGTCACTTTCGCTTCTTCCGTGCTGAAAGAGGTTTACAACCCG
AAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAA
TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTG
GCCGGTCACCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTAGGCCATTACCCC
ACCAACAAGCTGATAGGCCGTGAGCCCATCCACAACCAGTACAAACCCTTTCCAC
CCCCCACCATGCGACAGGAGGTCATATCCAGTATTAGACCCAGTTTCCCAGGCTT
ATCCCAGAGTCAAGGGCAGGTTACTCACGTATTACTCACCCGTTCGCCACTCATC
CACCCAGTGCAAGCACCAAGCTTCAGCGTTCGACTGCATGGTAGCTGCGCATCAC

Fig. 5. 16S rRNA sequencing results of bacterial isolate I1.

Table 4. Alignments results of BLAST using 16S rRNA sequences of bacterial isolate I1

Test isolate	Accession No.	Closest described species represented by the isolate	Sequence Similarity (%)
I1	AB648996.1	*Kocuria marina* gene for 16S rRNA, partial sequence, strain: S32326	100
	FJ789660.1	*Kocuria marina* strain Inje LM M07-0128 16S ribosomal RNA gene, partial sequence	100
	FM872994.1	Uncultured bacterium partial 16S rRNA gene, clone FB02F06	100
	AM990819.1	*Kocuria* sp. MOLA 44 partial 16S rRNA gene, culture collection MOLA:44	100
	JN644586.1	*Kocuria marina* strain LEH5_4B 16S ribosomal RNA gene, partial sequence	99
	EU536424.1	Uncultured bacterium clone nbt213f06 16S ribosomal RNA gene, partial sequence	99
	AJ717363.1	*Kocuria* sp. CV36 16S rRNA gene, isolate CV36	99
	EU073966.1	*Kocuria marina* strain CMGS2 16S ribosomal RNA gene, partial sequence	99
	FJ789661.1	*Kocuria marina* strain Inje LM M07-1336 16S ribosomal RNA gene, partial sequence	99
	FM873454.1	Uncultured bacterium partial 16S rRNA gene, clone FD03H06	99

AGAATGCGCAGCTATAATGCAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGA
CGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGAAT
AACTCCGGGAAACCGGGGCTAATGCCGGATAACATTTAGAACCGCATGGTTCTAA
AGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGT
TGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGG
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GGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGT
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CAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAG
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GGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTT
CTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAA
TCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCG
TCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGGAGC
TAGCCGTCGAAGTGACAATG

Fig. 6. 16S rRNA sequencing results of bacterial isolate I2.

Table 5. Alignment results of BLAST using 16S rRNA sequences of bacterial isolate I2

Test isolate	Accession No	Closest described species represented by the isolate	Sequence Similarity (%)
I2	JN128237.1	*Staphylococcus cohnii* strain HNS003 16S ribosomal RNA gene, partial sequence	100
	JF799887.1	*Staphylococcus cohnii* strain CIFRI D-TSB-9-RS 16S ribosomal RNA gene, partial sequence	100
	JF784040.1	*Staphylococcus cohnii* strain CIFRI P-TSB-54 16S ribosomal RNA gene, partial sequence	100
	HQ538680.1	*Staphylococcus* sp. bk_9 16S ribosomal RNA gene, partial sequence	100
	HM218041.1	*Staphylococcus cohnii* strain NM7-1 16S ribosomal RNA gene, partial sequence	100
	HM217963.1	*Staphylococcus cohnii* strain G65-5 16S ribosomal RNA gene, partial sequence	100
	HM217947.1	*Staphylococcus cohnii* strain G28-4 16S ribosomal RNA gene, partial sequence	100
	GQ169062.1	*Staphylococcus* sp. CTDB5 16S ribosomal RNA gene, partial sequence	100

ACGGATGTCACTTCGACGGCTCCCCCCACAAGGGTTAGGCCACCGGCTTCGGGTG
TTACCAACTTTCGTGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCA
CCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTT
GCAGACCCCAATCCGAACTGAGACCGGCTTTTTGGGATTAGCTCCACCTCACAGT
ATCGCAACCCATTGTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACATAAGGG
GCATGATGATTTGACGTCGTCCTCACCTTCCTCCGAGTTGACCCCGGCAGTCTCC
CATGAGTCCCCACCATTACGTGCTGGCAACATGGAACGAGGGTTGCGCTCGTTGC
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ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGG
CCGGTCACCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTGAGCCATTACCTCA
CCAACAAGCTGATAGGCCGCGAGTCCATCCAAAACCGATAAATCTTTCCAACACC
CACCATGCGGTGGACGCTCCTATCCGGTATTAGACCCAGTTTCCCAGGCTTATCC
CAGAGTTAAGGGCAGGTTACTCACGTGTTACTCACCCGTTCGCCACTAATCCACC
CAGCAAGCTGGGCTTCATCGTTCGACTTGCATGGTAGCAGCGCATGCCA

Fig. 7. 16S rRNA sequencing results of bacterial isolate I3

Table 6. Alignment results of BLAST using 16S rRNA sequences of bacterial isolate I3

Test isolate	Accession No	Closest described species represented by the isolate	Sequence Similarity (%)
I3	NR_026193.1	*Arthrobacter* sp. strain DSM 20546 16S ribosomal RNA, partial sequence	97
	NR_026191.1	*Arthrobacter pascens* strain DSM 20545 16S ribosomal RNA, partial sequence	97
	NR_025771.1	*Citricoccus alkalitolerans* strain YIM 70010 16S ribosomal RNA, partial sequence	96
	NR_041545.1	*Arthrobacter oryzae* strain KV-651 16S ribosomal RNA, partial sequence	96
	NR_025488.1	*Citricoccus muralis* strain 4-0 16S ribosomal RNA, partial sequence	96
	NR_026190.1	*Arthrobacter nicotianae* strain DSM 20123 16S ribosomal RNA, partial sequence	96
	NR_027226.1	*Arthrobacter psychrophenolicus* strain AG31 16S ribosomal RNA, partial sequence	96
	NR_044338.1	*Arthrobacter soli* strain SYB2 16S ribosomal RNA, partial sequence	96

AGCAGTGCGCTCTTACCATGCAAGTCGAACGATGAAGCCCAGCTTGCTGGGTGGA
TTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAA
GCCTGGGAAACTGGGTCTAATACCGGATAGGAGCGCCGACCGCATGGTGGGTGTT
GGAAAGATTTATCGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGT
AATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
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TGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGC
ACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTATCC
GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGTCGTGAAAGT
CCGGGGCTTAACCCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGCAGTAGGG
GAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCG
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AGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGT
GTGGGGACCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAGTGCCCCGCCT
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CGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGA
CATGTTCTCGATCGCCGTAGAGATACGGTTTCCCCTTTGGGGCGGGTTCACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTCGTTCCATGTTGCCAGCACGTAATGGTGGGGACTCATGGGAGAC
TGCCGGGGTCAACTCGGAGGAAGGTGAGGACGACGTCAAATCATCATGCCCCTTA
TGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTCGCGATACTGTG
AGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCG
ACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTAACACCCG
AAGCCGGTGGCCTAACCCTTGTGGAGGAGCCGTCGAAGTGACATCCGT

Fig. 8. 16S rRNA sequencing results of the bacterial isolate I4

Table 7. Alignment results of BLAST using 16S rRNA sequences of bacterial isolate I4

Test isolate	Accession No.	Closest described species represented by the isolate	Sequence Similarity (%)
I4	FHM584259.1	*Micrococcus luteus* strain CJ-G-TSA7 16 S ribosomal RNA gene, partial sequence	99
	AB188213.1	*Micrococcus* sp. TUT1210 gene for 16S rRNA, partial sequence	99
	FJ357605.1	*Micrococcus* sp. BBN3L-04d 16S riboso mal RNA gene, partial sequence	99
	HM629400.1	*Micrococcus* sp. B-D-TSA2 16S ribosomal RNA gene, partial sequence	99
	AB617561.1	*Micrococcus luteus* gene for 16S rRNA, partial sequence, isolate: D1-2T	99
	GU085223.1	*Micrococcus* sp. BD-15 16S ribosomal RNA gene, partial sequence	99
	FN551251.1	*Micrococcus* sp. RMB40 partial 16S rRNA gene, strain RMB40	99
	GQ143751.1	*Micrococcus* sp. SP4 16S ribosomal RNA gene, partial sequence	99
	AM403126.2	*Micrococcus* sp. V7 partial 16S rRNA gene, strain V7	99
	AM990780.1	*Micrococcus* sp. MOLA 4 partial 16S rR NA gene, culture collection MOLA:4	99

GAGATGCGCATGCTATAATGCAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTG
ACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGA
TAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTCGAACCGCATGGTTCGA
TAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAG
TTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGG
GAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACATACGTGTAAGTAACTGT
GCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCG
TAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCAT
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TGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTG
TAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTG
CTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAA
GCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGA
GCTTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGC
CATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAA
TGGACAATACAAAGGGCAGCGAAACCGCGAGGTCAAGCAAATCCCATAAAGTTGT
TCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTA
ATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCC
GTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTGGAGCT
AGCCGTCGAAGGTGACA

Fig. 9. 16S rRNA sequencing results of the bacterial isolate I5

Table 8. Alignment results of BLAST using 16S rRNA sequences of bacterial isolate I5

Test isolate	Accession No.	Closest described species represented by the isolate	Sequence Similarity (%)
I5	AB680516.1	*Staphylococcus* sp. NBRC 13889 gene for 16S rRNA, partial sequence	100
	HM352415.1	*Staphylococcus* sp. SeLB4 16S ribosomal RNA gene, partial sequence	100
	HM352390.1	*Staphylococcus* sp. PrNA7 16S ribosomal RNA gene, partial sequence	100
	HM566083.1	*Staphylococcus* sp. 08EPH15 16S ribosomal RNA gene, partial sequence	100
	EU867340.1	*Staphylococcus* haemolyticus strain CCGE3068 16S ribosomal RNA gene, partial sequence	100
	EU867334.1	*Staphylococcus* haemolyticus strain CCGE2295 16S ribosomal RNA gene, partial sequence	100
	FM873418.1	Uncultured bacterium partial 16S rRNA gene, clone FD03C10	100
	EU379304.1	*Staphylococcus haemolyticus* strain 6R-J-5 16S ribosomal RNA gene, partial sequence	100
	EF188282.1	*Staphylococcus* sp. GYZ 16S ribosomal RNA gene, partial sequence	100
	EU071616.1	*Staphylococcus haemolyticus* strain EHFS1_AU1Ha 16S ribosomal RNA gene, partial sequence	99

Antibiotic resistance test

Five antibiotics, ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline were tested to remove the bacteria from alga. Each antibiotic was tested separately at final concentrations of 60, 75, 30, 30, and 30 μg/ml. Bacterial isolates I2, I3, and I4 were found to be susceptible to all antibiotics with above concentrations. Bacterial isolate I1 demonstrated susceptibility to all above antibiotics except resistance in ampicillin at the concentration of 60 μg/ml and bacterial isolate I5 showed susceptible to all antibiotic but resistance in streptomycin at 30 μg/ml (Table 9). The mixtures of these antibiotics were used to clean the microalga at the concentrations of 60, 75, 30, 30 and 30 μg/ml correspondingly. Mixtures of antibiotics were found more active than individual antibiotic for cleaning of alga.

Table 9. Antibiotic sensitivity of bacterial isolates

Alga name	Isolate name	color of colony	Name of antibiotic				
			Ampicillin (60 μg/ml)	Chloramphe. (75μg/ml)	Kanamycin (30μg/ml)	Streptomycin (30 μg/ml)	Tetracycline (30 μg/ml)
P. viridis	I-1	grey	R	S	S	S	S
	I-2	white	S	S	S	S	S
	I-3	yellow	S	S	S	S	S
	I-4	yellow	S	S	S	S	S
	I-5	grey	S	S	S	R	S

S- Susceptible

R- Resistant

Cleaning of microalga

Cleaning of microalga from bacteria was carried out by using a mixture of five antibiotics. The antibiotic cocktail was prepared with ampicillin 2 mg/ml, chloramphenicol 2.5 mg/ml, kanamycin 1 mg/ml, streptomycin 1 mg/ml and tetracycline 1 mg/ml. The mixtures of these antibiotics were used to clean the microalga at final concentrations of 60, 75, 30, 30 and 30 μg/ml respectively.

The bacterial flora in the microalga before antibiotic treatment was 8.1×10^5 cells/ml. The entire bacterial floras were removed from the alga after first treatment with antibiotic cocktail. However, antibiotic treatment was repeated two more times to confirm axenicity of the microalga (Fig.10).

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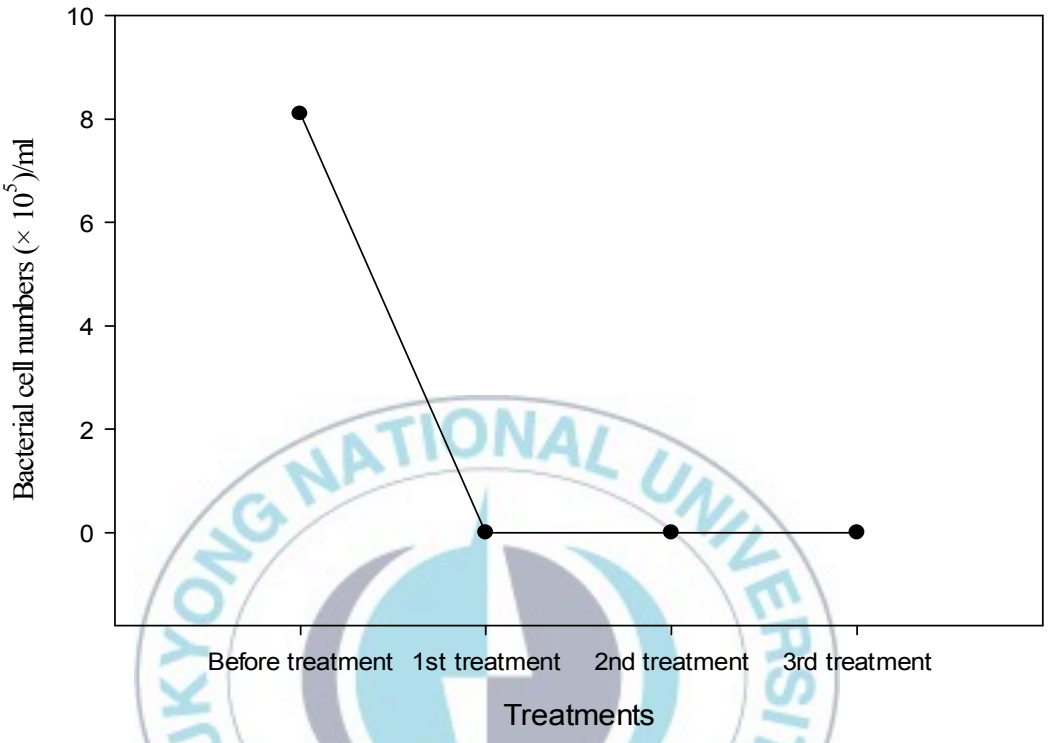


Fig. 10. Cleaning of microalga by antibiotic cocktail treatment. After each treatment, 100 µl of algal culture was spreaded on LB plate to measure the number of bacterial contaminations.

Identification of the host

For identification of the host alga, genomic DNA was prepared from the pure culture of the microalga and used for PCR amplification. A DNA fragment of 490 base pairs was obtained whose sequence was determined without cloning. Homology analysis was carried out from Gene Bank database using the BLAST. The BLAST search of sequence in the Gene Bank showed 99% nucleotide sequence identity (Fig. 11, Table. 10) to that of the 18S rRNA sequence of f *P. viridis* (Gene Bank accession number HQ877913.1).

AAATGTTGAGTTATGCTCGTAGTCGGATTTCGGGCCGATCGCTCCGGTCTGCCGT
TGGGTATGCACTGGTAGTGTTTGGTCTTTTTGCGTGAGGCCTTATGCGTCGTTGA
TTCGTCGTGTTTGGTGCTCTGCACGTTTACTTTGAGAAAATCAGAGTGTTCAAAG
CAGGCCTTTGCCATTGTATGTGTTAGCATGGGATAATGGAATAGGACCTTGGTGC
TATTTTGTTGGTTTCGAACGCCGAGGTAATGATTAATAGGGATAGTCAGGGGCAC
TCGTATTCCGTAGAGAGAGGTGAAATTCTTAGACCCACGGAAGACGCACTACTGC
GAAAGCATTTGCCAGGGATGTTTTCACTGATCAAGAACGAAAGTTAGGGGATCGA
AGATGATTAGATACCCTCGTAGTCCACATCTTCGATCCCTTACTTTTGTTTTTTT
TTTAATGAAAACATCCCGGGCAAAGCTTTCGAGGAGGGGGTCTTCCTGGG

Fig. 11. 18S rRNA sequencing results of host alga

Table 10. Alignment results of BLAST using 18S rRNA sequences of the host.

Test isolate	Accession No.	Closest described species represented by the strain	Sequence Similarity (%)
I	HQ877913.1	*Pavlova viridis* strain KMMCC H-13 18S ribosomal RNA gene, partial sequence	99
	DQ075201.1	*Pavlova viridis* 18S ribosomal RNA gene, partial sequence	99
	HQ877919.1	*Pavlova* sp. KMMCC H-21 18S ribosomal RNA gene, partial sequence	99
	HQ877916.1	*Pavlova* sp. CCMP 620 18S ribosomal RNA gene, partial sequence	99
	AF106056.1	*Diacronema vlkianum* 18S ribosomal RNA gene, partial sequence	97
	JF714242.1	*Pavlova ennorea* strain AC253 18S ribosomal RNA gene, partial sequence	96
	JF714239.1	*Diacronema* sp. AC247 18S ribosomal RNA gene, partial sequence	96

Test for growth promotion of *Pavlova viridis* by isolated bacteria

Each of the five bacterial isolates was inoculated with *P. viridis* containing 7.08 $\times 10^4$ cells/ml in f/2 medium. The growth of microalga was observed for 10 days. All bacterial isolates significantly increased the growth of *P. viridis* over 10 days but stimulatory effect varies among the isolates. Bacterial isolate I3 showed the highest growth promotion actively among the five isolates (Fig.12). The growth of *P. viridis* was increased approximately two times than that of the control culture by the best effective bacterial isolate. The other bacterial isolates increased the growth of the microalga almost 0.5-1.5 times than that of the control culture. The weak enhancing bacterial isolate was I4.

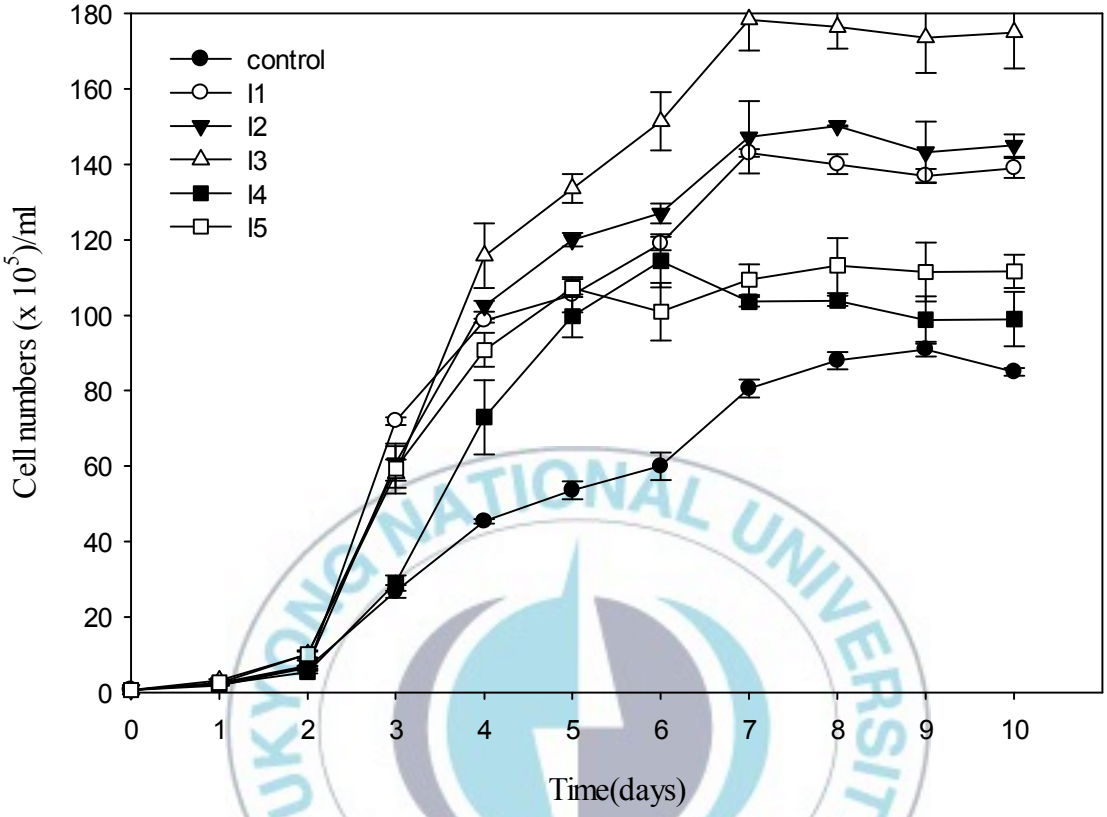


Fig. 12. Growth promotion of *Pavlova viridis* by five bacterial isolates. Data are the means ± SD of three replicates

Growth and molecular characteristics of the bacterium I3

The culture and molecular characteristics of bacterial isolate I3 was investigated. The bacterial colony on LB was yellow and rough in shape. The effects of temperature, pH, and different concentrations of NaCl on the growth of bacterial isolate I3 was observed. The bacterium showed the highest growth at 37˚C, pH 7 and 1 % NaCl.

The 16S rRNA sequence of the bacterial isolate I3 was analyzed for the molecular identification of this bacterium. Firstly, genomic DNA of the isolate I3 was purified after inoculating on LB broth culture for overnight. Then PCR amplification was performed by using 27F and 1492R primers. The size of the DNA band was about 1.5 kbp on 1 % agarose gel. After preparing PCR product, 16S rRNA sequences were executed. Sequences with homology were searched from Gene Bank database using the BLAST. The 16S rRNA sequences of the bacterial isolate I3 showed 97 % similarity with that of *Arthrobacter* sp. (Gene Bank accession number NR_026193.1).

Effects of temperature on the growth of *Arthrobacter* sp.

The effect of temperature on the growth of *Arthrobacter* sp. was observed. The growth of the bacterium gradually increased with the increase of temperature. The bacterial isolate could grow even at 4˚C but growth rate was much higher when temperature was increased from 25˚C to 30˚C and the highest growth was recorded at 37˚C. After that, the growth was slow down drastically with increase of temperature (Fig. 13).

Effects of NaCl concentration on the growth of *Arthrobacter* sp.

The effect of NaCl concentration on the growth of *Arthrobacter* sp. was measured. Considerably high growth was observed at 0% NaCl concentration and the best performance was observed at 1% NaCl concentration. Then the growth of the bacterial isolate was gradually slow down with the increase of NaCl concentrations and a very little growth was recorded at 10% NaCl concentrations (Fig. 14).

Effect of pH on the growth of *Arthrobacter* sp.

The effect of pH of the culture medium on the growth of *Arthrobacter* sp. was also measured. Little growth was found at pH 3 then progressively increased with the increase of pH and the highest growth was recorded at pH 7. Then the growth of the bacterium retarded slowly with the increase of pH but sudden decrease of growth was observed at pH 10 (Fig. 15).

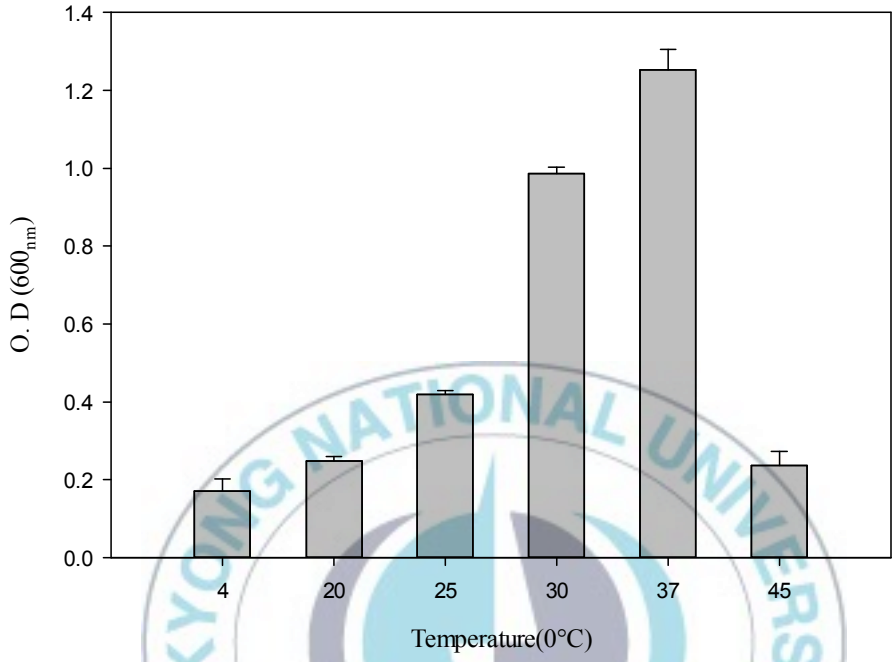


Fig. 13. Effects of temperature on the growth of *Arthrobacter* sp. Data are the means ± SD of three replicates.

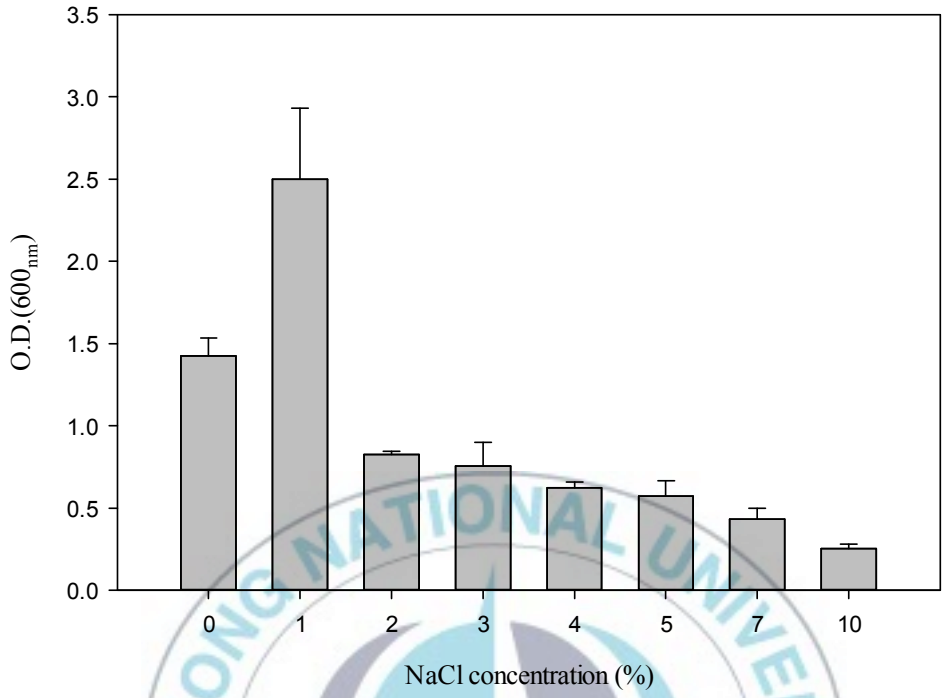


Fig. 14. Effects of NaCl concentration on the growth of *Arthrobacter* sp. Data are the means ± SD of three replicates.

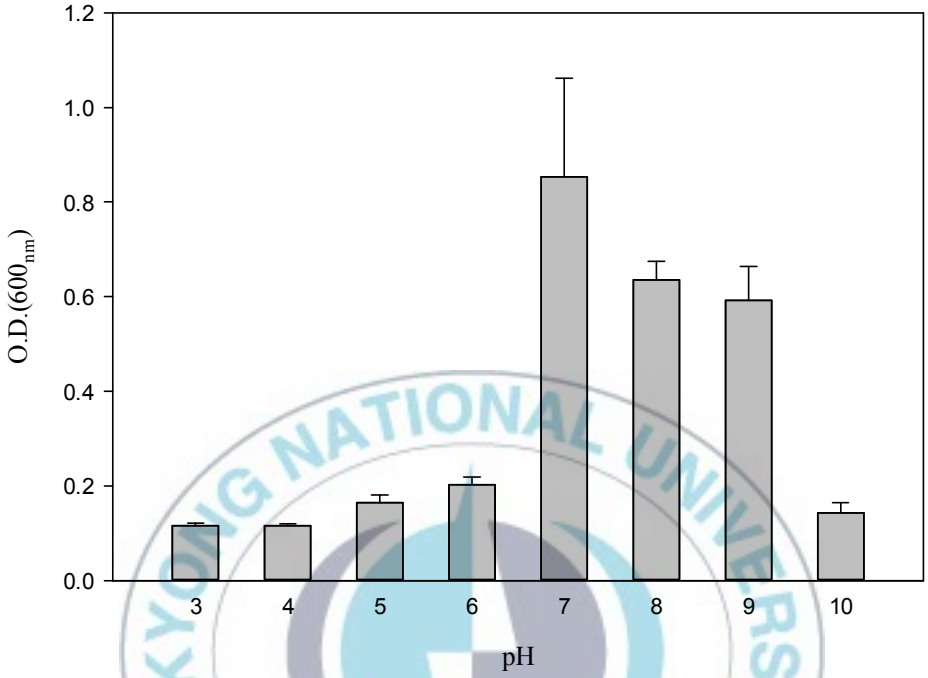


Fig. 15. Effects of pH of the culture medium on the growth of *Arthrobacter* sp. Data are the means ± SD of three replicates.

Growth promotion of bacteria and alga in co-culture

The co-culture of microalga and bacterial isolates were investigated by mixing the axenic culture of *P. viridis* and isolate I3 together to observe the growth status of both in co-culture and in single culture. It was found high influential growth of bacteria and microalga. Initially, the lag phase of bacterial growth in co-culture and in single culture was almost same. But at the end of the culture period, the growth of bacteria in co-culture was much higher than that in single culture (Fig.16). The exponential growth phase was also longer than that in single culture. Significant difference was found between the two conditions ($p<0.05$). In case of microalga, bacterial isolate markedly promoted the growth of *P. viridis* in co-culture ($p<0.05$). The exponential growth phase of *P. viridis* in co-culture was higher than in single culture.

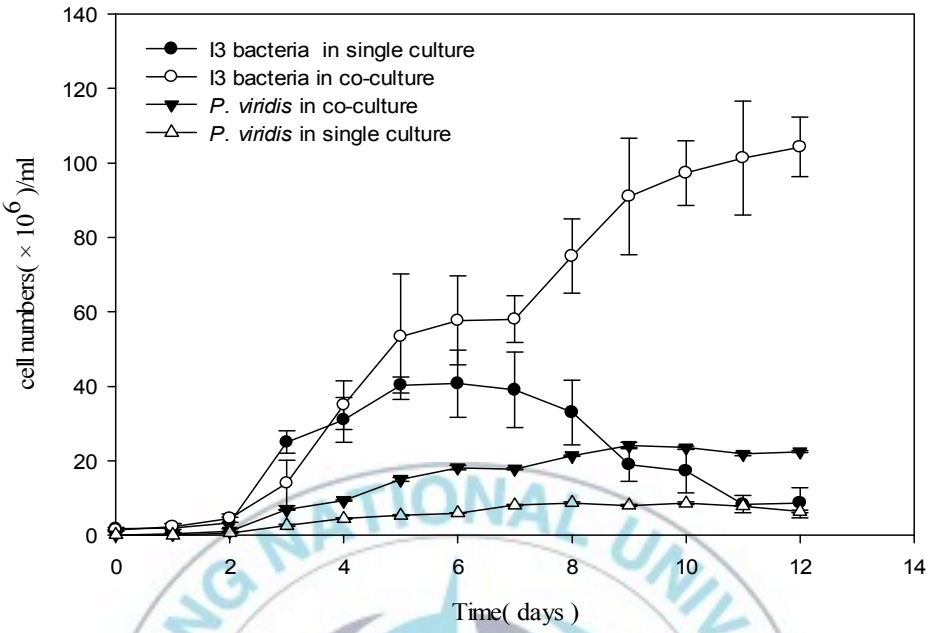


Fig. 16. Growth promotion of the bacterium and microalga in co-culture. Data are the means ± SD of three replicates.

Growth promotion of alga with bacteria-free filtrate

The bacteria-free filtrates of bacteria I3 were mixed with pure culture of *P. viridis* and the effect on algal growth was investigated for eight days. Significant growth enhancement was observed in the algal culture containing bacterial free filtrate compared to alga in pure f/2 medium culture. At the beginning, there was no difference in growth between the two. However, after two days, microalga with filtrate started to increase continuously up to the end of the culture period. The exponential growth phase of *P. viridis* together with filtrate was longer twice than that of microalga in single culture (Fig. 17).

The growth promotion effect of bacterial filtrate was dose dependent. When the filtrate was added at the final concentration of 1%, 2.5%, 5%, and 10% of the f/2 medium, more growth of *P. viridis* was observed in culture with 5% and 10% culture filtrate than 1% or 2.5%. There was no significant growth in 5% or 10% bacterial culture filtrate. Although pure LB promoted the growth of *P. viridis*, it was not significant compared to pure f/2 but significantly less than bacteria-free culture filtrate (Fig. 18).

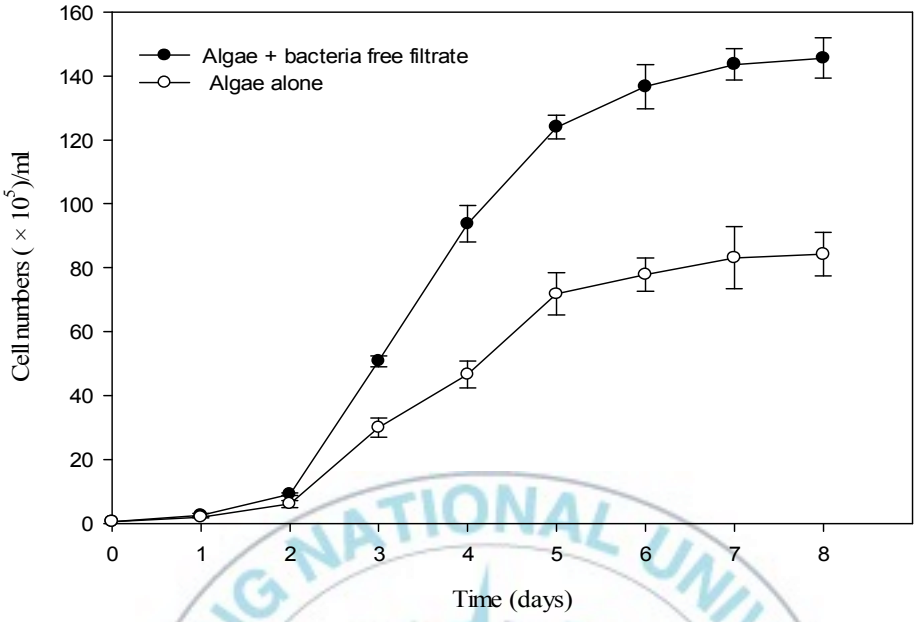


Fig. 17. Growth promotion of microalga by bacteria-free filtrates. Data are the means ± SD of three replicates.

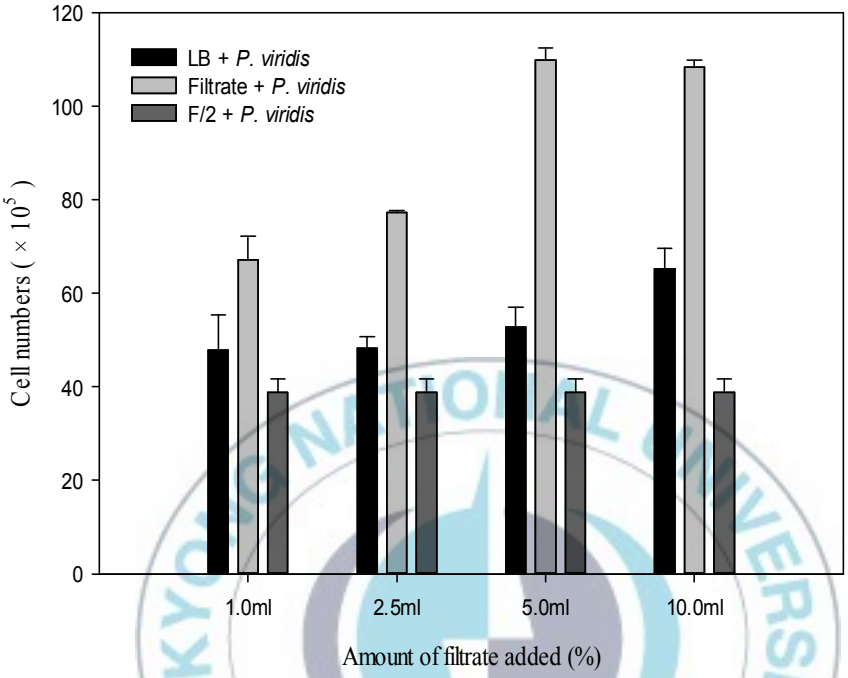
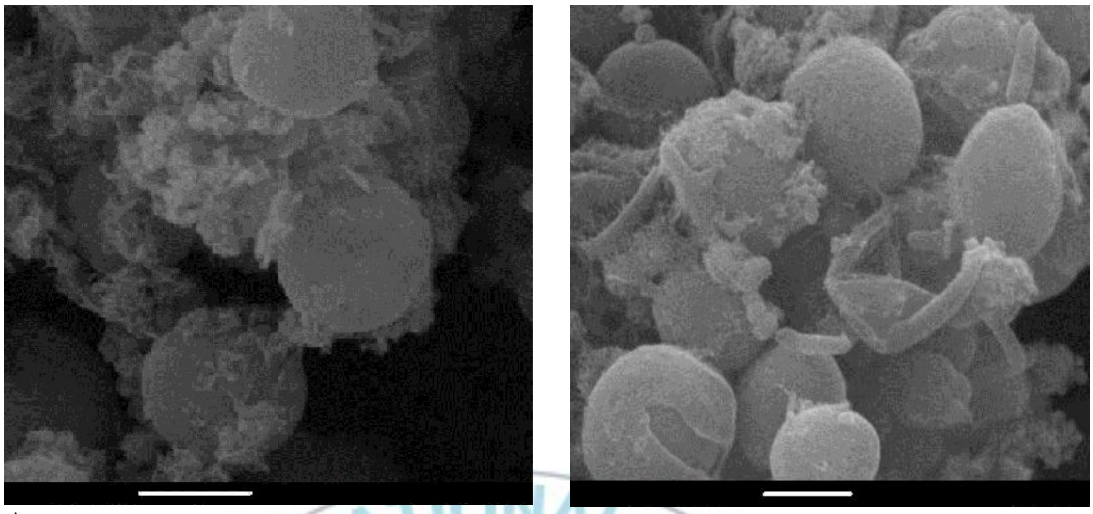


Fig.18. Growth promotion of microalga with different amount of bacteria-free filtrates. Data are the means ± SD of the three replicates.

Observation by SEM method

The intimate relationship between bacterial isolate I3 and *P. viridis* was observed under SEM. The average size of the algal in two different culture conditions was similar with average diameter of 3.5 μm. Bacterial isolate I3 showed direct adhesion to the surface of alga cell (Fig.19).The average size of the bacteria on the algal surface was about 2.7 μm X 0.5 μm. Other than the presence of bacteria on the surface of the alga, there was no morphological change of alga in the co-culture.



A **B**

Fig. 19. Scanning electron microscope pictures of the *P.viridis* culture either without isolate I3 (A) or with bacterial isolate I3 (B). The bar at the bottom is representing 2 μm in size.

Discussion

Microalgae and bacteria are most influential organisms with respect to numbers in all aquatic environments and control the nutrients cycling (Cole, 1982). Many bacteria associated with microalgae and they may have progressive or suppressive effects on the growth of host algae. The interactions between microalgae and bacteria are completely variable, depending on the species and environmental conditions (Jones, 1982; Riquelme et al., 1988).

Riquelme et al. (1988) reported that bacterial isolate *Flavobacterium* sp. NAST inoculated in the axenic culture of marine diatom *Asterionella gracialis* successfully promoted the growth of microalga. They also reported that naturally occurring bacterial isolate *Peudomonas* sp. promoted the growth of microalga whereas *Vibrio* sp. did not affect the algal growth. Several bacterial isolates isolated from benthic diatom *Nitzschia* sp. were co-cultured with the diatom, and it was found that among many bacterial isolates only *Alcaligenes* sp. had significant growth promoting effects on *Nitzschia* sp.(Fukami et al., 1997).

Furthermore, marine bacterium *Flavbacterium* sp. increased the growth of diatom *Chaetoceros gracilis* but was algicidal to the microalga *Gymnodinium mikimotoi*. Surprisingly, this bacterium has no obvious effects on the growth of *Isochrysis galbana* and *Pavlova lutheri* (Suminto and Hirayama, 1997). All these results implied that the interactions between bacteria and microalgae are species-specific (Jones, 1982; Fukami et al., 1997).

In the present study, five bacterial isolates were separated from the culture of *Pavlova viridis* and were co-cultured with the microalga. All bacterial isolates promoted the growth of *P. viridis,* which exhibited the positive relationships of *P. viridis* with its associated bacteria.

Park et al. (2008) investigated that eight bacterial isolates isolated from the non-axenic culture of *Chlorella ellipsoidea* and mixed with the microalga. All bacterial isolates were found to stimulate the growth of *C. ellipsoidea*.

Bacteria-free filtrate of the most effective bacterial isolate *Arthrobacter* sp. was applied with pure *P. viridis*, and revealed the stimulating effect on

the growth of microalga. Moreover, promotive effects on the growth of microalga were also found by increasing amount of bacteria-free filtrates.

These results suggested that in bacteria-algae association, bacteria might produce some metabolites, vitamins which play significant role on the growth of microalga (Riquelme et al., 1988). However, nutrients are always regenerated by heterotrophic activity through microorganisms in the environment. Heterotrophic demineralization supply continuous nutrients and algae also produce some organic compounds to run the nutrients cycle smoothly. This phenomenon play important role making relationship to each other's (Cole, 1982; Watanabe et al., 2006).

Five bacterial isolates were obtained from the microalga. These results suggested that there were different microorganisms on the surface of the microalga. More characterization was done of the best bacterial isolate that stimulated the highest growth of *P. viridis*. The suitable environmental conditions for the growth of bacterial isolate I3 were measured. The favorable environmental condition of the bacterium was identified as temperature 37˚C, pH 7 and 1 % NaCl concentrations. The 16S rRNA of the bacterial isolate showed 97 % similarity with *Arthrobacter* sp.

Direct adhesion of bacterial isolate to the surface of microalga *P. viridis* was found in SEM observation. Bacterial isolate could be attached to the microalga either in direct attachment of the surface of the cell or indirect attachment on the sheath produces by the microalga (Watanabe et al., 2005).

Croft et al. (2005) separated *Halomonas* sp. a vitamin B_{12} producing bacterium from a non-axenic culture of *Amphidinium operculatum* and co-cultured with two auxotrophic vitamin B_{12} requiring algae *Porphyridium purpureum* and *A. operculatum* in a mineral medium without organic carbon sources. It was found that vitamin B_{12} was supplied by *Halomonas* sp. for the algae in exchange for the products of photosynthesis and the type of this interaction is symbiotic.

Many microalgae require extra nutrients such as biotin, thiamine, and cobalamin as growth factors (Croft et al., 2005). A survey has shown that 171 out of 326 algal species require exogenous vitamin B12 for their growth. Microalgae collect this vitamin B12 from associated bacteria implying symbiotic interactions (Croft et al., 2005).

Close relationship between alga and fungi also has been reported. Watanabe et al. (2005) has investigated the symbiotic association in chlorella culture. They isolated one fungal isolate and four bacterial isolates from *Chlorella sorokiniana*. The fungal isolate and one bacterial isolate were found to promote the growth of algal cells significantly while others two inhibited the growth of microalga. They assumed that fungal and the bacterial isolate demonstrated symbiotic relationship with the alga, exchanging nutrients from the microalga that promoted their growth.

In co-culture of *P. viridis* and *Arthrobacter* sp, promotive growth of both microalga and bacterial isolate were found. Surprisingly, the growth of bacterial isolate in co-culture was much higher than that of in single culture at the end of the culture period. The growth of *P. viridis* in co-culture was also increased. Bacteria-free filtrate of bacterial isolate was also found to have the stimulating effect on the growth of microalga. These results might suggest that alga produce some organic compounds that increase the growth of bacterium, in contrast, bacterial isolate produce some metabolites that promote the growth of microalga indicating these two organisms have developed the symbiotic relationship.

Conclusion

Microalgae are ubiquitous in aquatic environment. They are used for various purposes. They can serve as feed for aquaculture, as food for human and animals, in pigment production, bio-removal of heavy metals and in agriculture (Wilde and Benemann, 1993; Hallmann, 2007). The marine microalga *Pavlova viridis* is commonly used in marine aquaculture as live feed due to high content of EPA and DHA. To increase the volume of microalgae is getting much more attention now a days.

Symbiotic relationship between marine microalga *P. viridis* and its consortium have been found. Among five bacteria isolated from *P. viridis*, bacterium identified as *Arthrobacter* sp. was the most effective in microalgae growth promotion. Both the growth of algae and bacteria was promoted in co-culture and the association between the two species was confirmed by SEM. Bacteria-free filtrate of bacterial isolate promoted the algal growth but the specific factors needed to be elucidated.

So, the using the bacterial isolate in the mass culture of *P. viridis* could be an efficient tool for increasing the micro algal population for potential use in aquaculture.

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