CHAPTER IV

RESULTS

4.1 Sample collection and isolation of cultures

Freshwater bodies where samples were collected for the isolation of micro-algae including data of temperature and pH were shown in Table 4.1.

Table 4.1 Locations of freshwater bodies where samples were collected for the isolation of micro-algae including list of the isolates.

Collection dates In 2005	Places	Temp. (°C)	pH	Isolates
November,17	Ban Paew, Samut Sakhon	27	7.5	SS1
November,17	Ban Paew, Samut Sakhon	27	7.5	SS2
November,17	Ban Paew, Samut Sakhon	27	7.5	SS3
November,17	Ban Paew, Samut Sakhon	27	7.5	SS4
November,17	Ban Paew, Samut Sakhon	27	7.5	SS5
November,19	Wax Museum, Samut Sakhon	25	7.0	SS6
November,19	Wax Museum, Samut Sakhon	25	7.0	SS7
December,16	Microbiology Department, Chulalongkorn University	18	7.5	SS8
December,01	Lard Prao, Bangkok	25	7.2	SS9
December,01	Lard Prao, Bangkok	25	7.2	SS10
December,01	Lard Prao, Bangkok	25	7.2	SS11
November,17	Ban Paew, Samut Sakhon	27	7.5	SS12

4.2 Identification of micro-algae based on cell morphology and PCR fingerprints

Chlorella spp.

Based on morphology of cells grown in BBM medium at 25°C under 3,000 Lux continuous light intensity, the following isolates SS1, SS2, SS3, SS6 and SS7 may be the same *Chlorella* sp. strain designated as *Chlorella* sp. strain SS1. Representative morphology of these isolates was shown in Figure 4.1

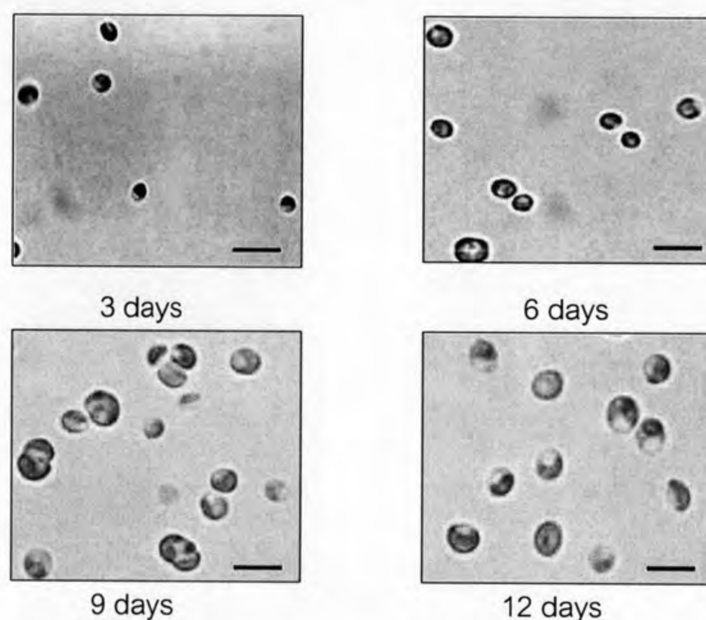

Figure 4.1 Representative morphology of *Chlorella* sp. strain SS1 grown in BBM medium under 3,000 lux light intensity for different time periods. Bar indicates 30 μm.

PCR fingerprints shown in Figure 4.2 revealed that the five isolates were the same strain.

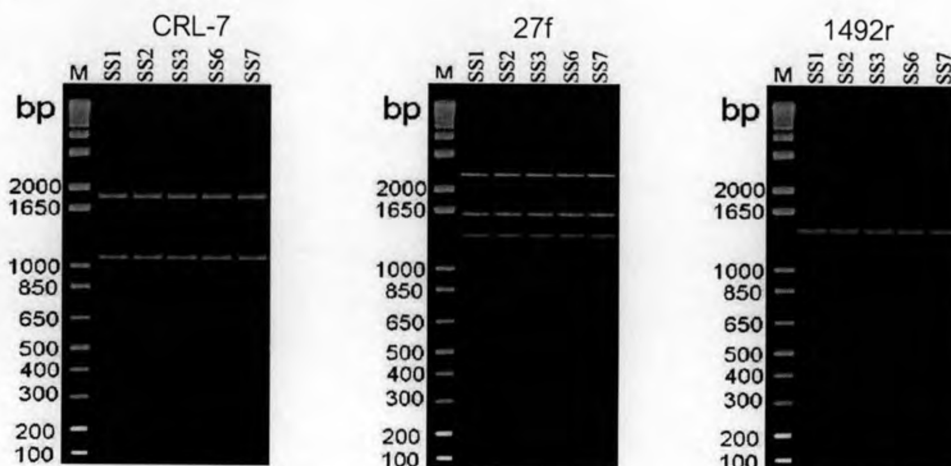

Figure 4.2 PCR fingerprints of isolates SS1, SS2, SS3, SS6 and SS7 when CRL-7 or 27f or 1492r was used as the primer. Lanes M were molecular size markers.

Based on morphology of cells grown in BBM medium at 25°C under 3,000 Lux continuous light intensity, the isolate SS 8 is a different strain of *Chlorella* sp. designated as *Chlorella* sp. strain SS8. Morphology of these strain was shown in Figure 4.3

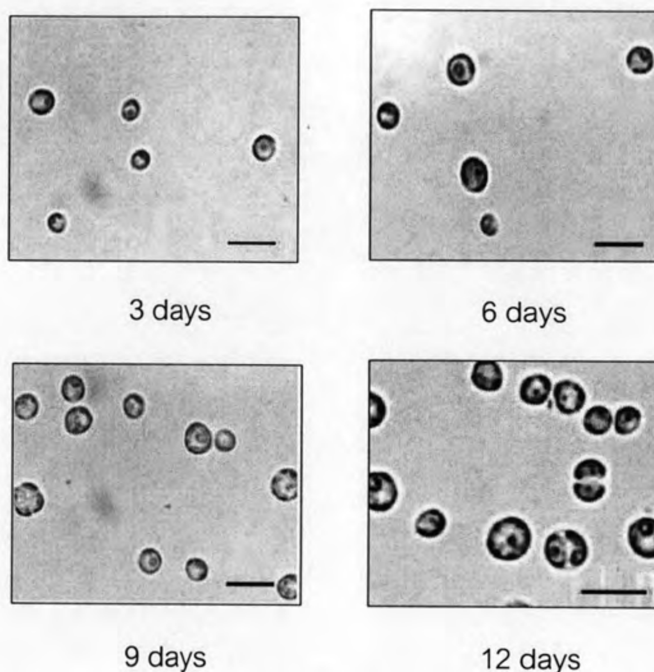

Figure 4.3 Morphology of *Chlorella* sp. strain SS8 grown in BBM medium under 3,000 lux light intensity for different time periods. Bar indicates 30 μm.

PCR fingerprints of *Chlorella* sp. strain SS8 were shown in Figure 4.4

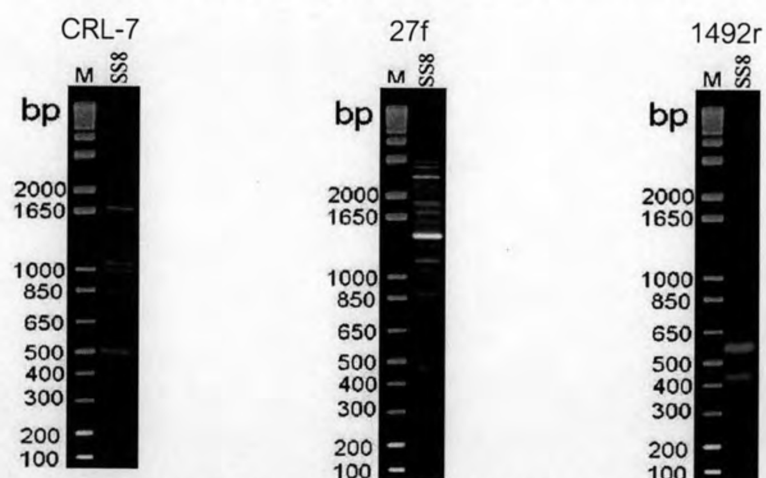

Figure 4.4 PCR fingerprints of *Chlorella* sp. strain SS8 when CRL-7 or 27f or 1492r was used as the primer. Lanes M were molecular size markers.

Scenedesmus spp.

Based on morphology of cells grown in BBM medium at 25°C under 3,000 Lux continuous light intensity, the isolate SS4 is a distinct strain of *Scenedesmus* sp. designated as *Scenedesmus* sp. strain SS4. Morphology of the isolate was shown in Figure 4.5.

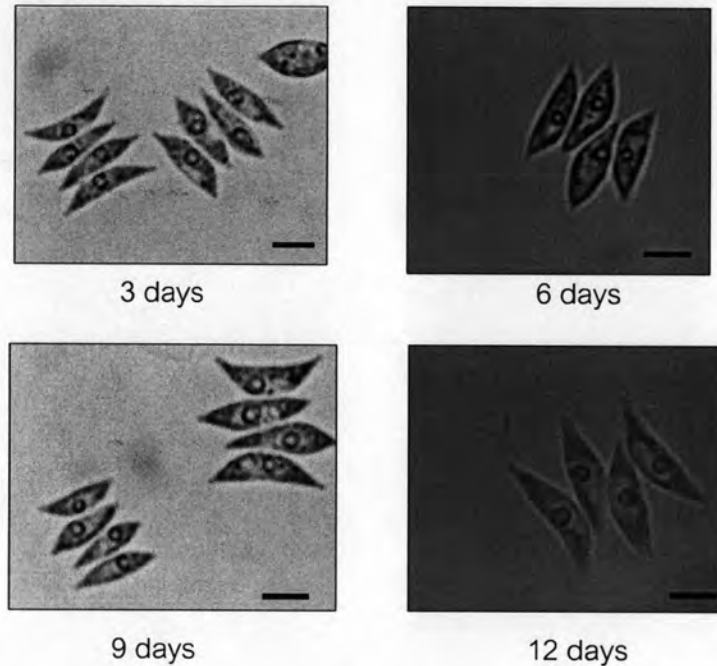

Figure 4.5 Morphology of *Scenedesmus* sp. strain SS4 grown in BBM medium under 3,000 lux light intensity for different time periods. Bar indicates 30 µm.

PCR fingerprints of *Scenedesmus* sp. strain SS4 were shown in Figure 4.6

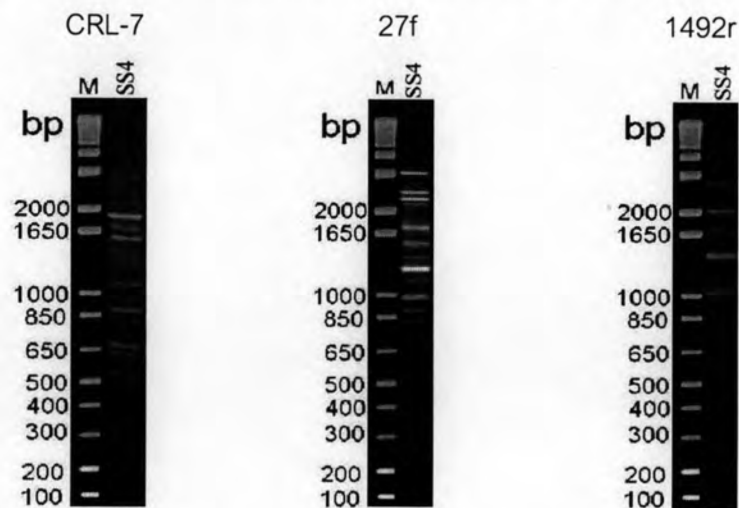

Figure 4.6 PCR fingerprints of *Scenedesmus* sp. strain SS4 when CRL-7 or 27f or 1492r was used as the primer. Lanes M were molecular size markers.

Based on morphology of cells grown in BBM medium at 25°C under 3,000 Lux continuous light intensity, the following isolates SS5 and SS12 may be the same *Scenedesmus* sp. designated as *Scenedesmus* sp. strain SS5. Representative morphology of these isolates was shown in Figure 4.7

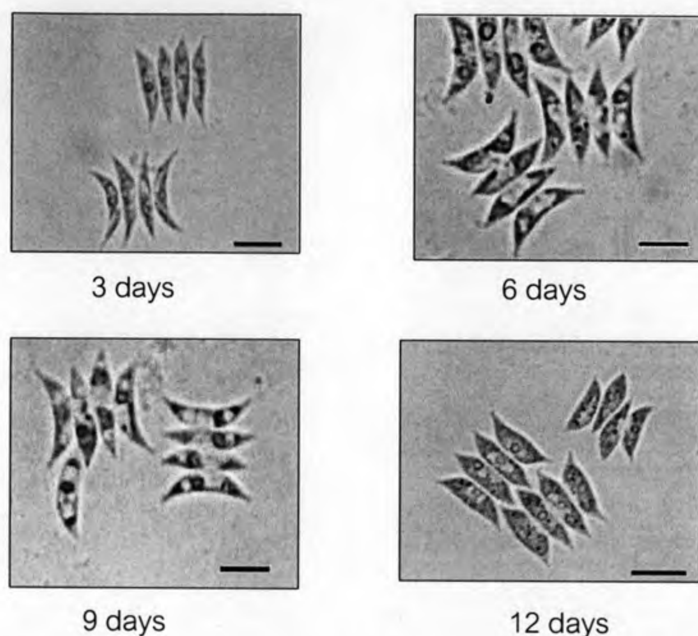

Figure 4.7 Representative morphology of *Scenedesmus* sp. strain SS5 grown in BBM medium under 3,000 lux light intensity for different time periods. Bar indicates 30 µm.

PCR fingerprints of *Scenedesmus* sp. strain SS5 were shown in Figure 4.8

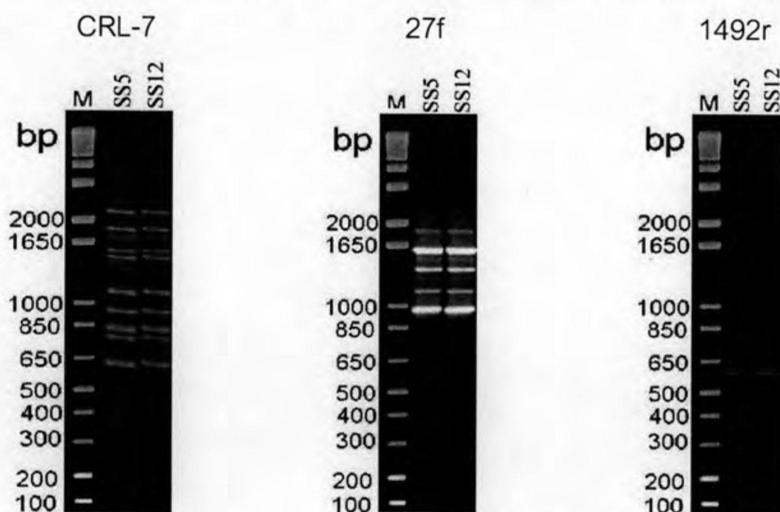

Figure 4.8 PCR fingerprints of *Scenedesmus* sp. strain SS5 when CRL-7 or 27f or 1492r was used as the primer. Lanes M were molecular size markers.

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Based on morphology of cells grown in BBM medium at 25°C under 3,000 Lux continuous light intensity, isolates SS9, SS10, and SS11 may be the same *Scenedesmus* sp. designated as *Scenedesmus* sp. strain SS9. Representative morphology of these isolates was shown in Figure 4.9

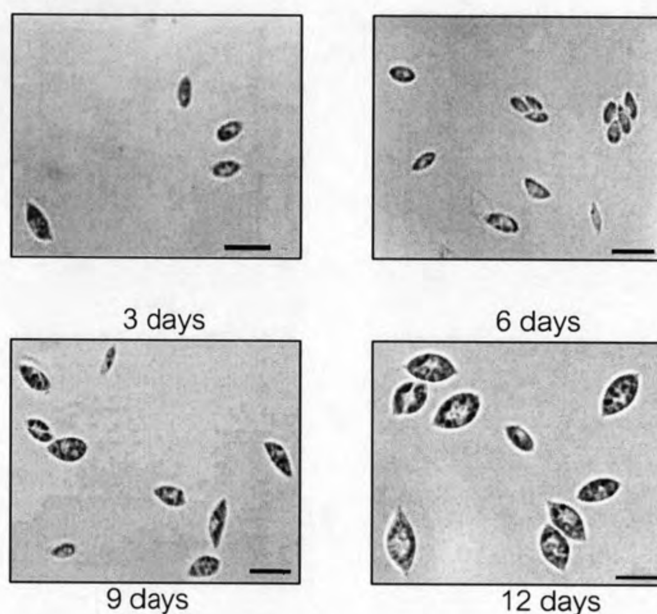

Figure 4.9 Representative morphology of *Scenedesmus* sp. strain SS9 grown in BBM medium under 3,000 lux light intensity for different time periods. Bar indicates 30 μm.

PCR fingerprints of *Scenedesmus* sp. strain SS9 were shown in Figure 4.10

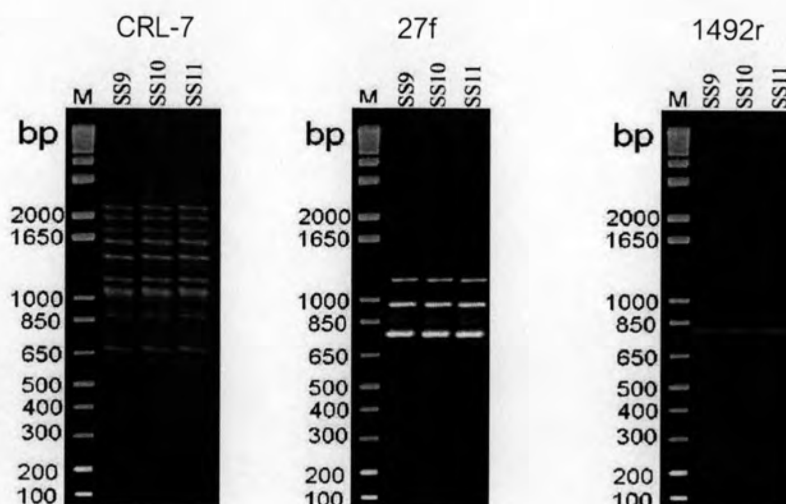

Figure 4.10 PCR fingerprints of *Scenedesmus* sp. strain SS9 when CRL-7 or 27f or 1492r was used as the primer. Lanes M were molecular size markers.

The composite RAPD-PCR fingerprints of the five isolated strains of green micro-algae as shown in Figure 4.11 indicated that the isolated *Chlorella* spp. and *Scenedesmus* spp. were different strains.

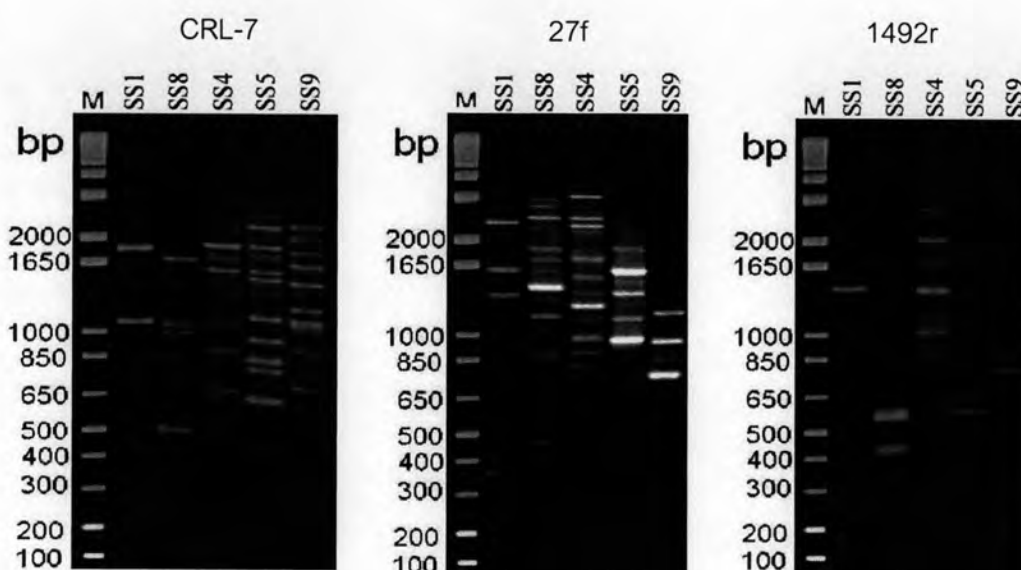

Figure 4.11 Summary of PCR fingerprints of 5 strains of isolated *Chlorella* spp. and *Scenedesmus* spp. when CRL-7 or 27f or 1492r were used as the primer. Lanes M were molecular size markers.

4.3 Scanning Electron Microscope

Scenedesmus spp. SS4, SS5 and SS9 were grown on agar plates containing BBM medium, incubated at 25 ^{0}C for 12 days. The samples were observed under a scanning electron microscope. Bar indicates 5 μm as shown in Figure 4.12. Longitudinal ridges were observed in strains SS5 and SS9.

Figure 4.12 Scanning electron micrographs of *Scenedesmus* spp. SS4, SS5 and SS9.

4.4 β-carotene contents in mid-log phase cells of isolated *Chlorella* spp. and *Scenedesmus* spp.

Figure 4.13 showed an external standard curve for the determination of β-carotene concentrations. The graph was used to determine concentration of β-carotene standards remaining after extraction. Figure 4.14 showed a standard curve for the determination of β-carotene remaining after extraction. The average recovery of β-carotene standard was approximately 50%. Figure 4.15 was used to determine the actual concentration of β-carotene taken into account the amounts lost during extraction and evaporation process.

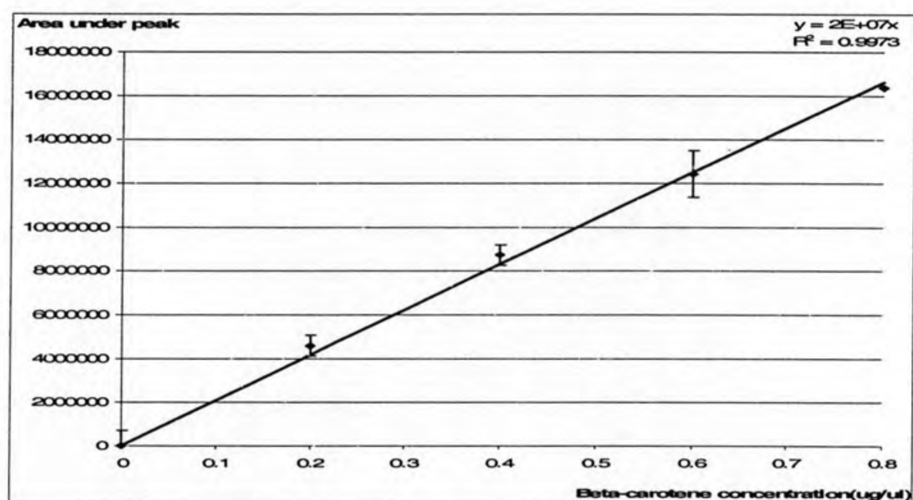

Figure 4.13 An external standard curve for the determination of β-carotene concentrations. Bars indicate means ± standard deviation. The graph was obtained by direct injection of β-carotene standard into reversed-phase HPLC system. Each data point was an average of three replicates.

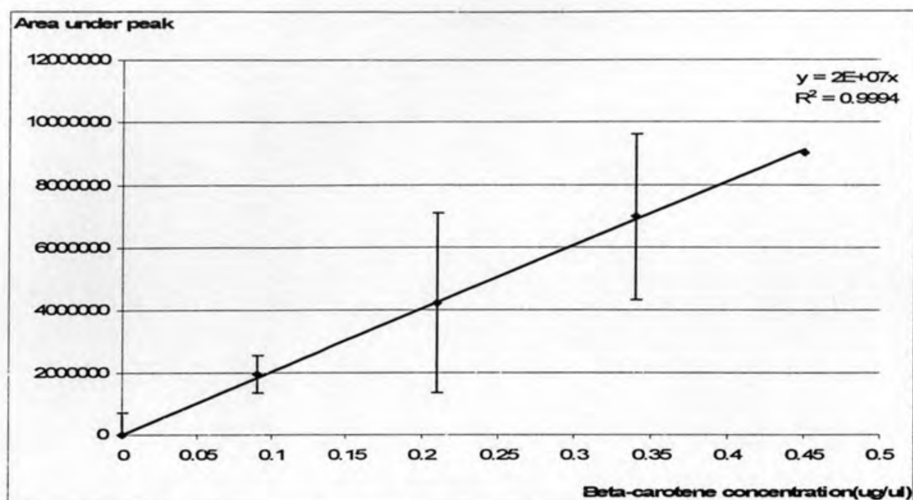

Figure 4.14 A standard curve for the determination of β-carotene concentrations after extraction. Bars indicate means ± standard deviation. The graph was obtained by injecting of β-carotene standard after extraction into reversed-phase HPLC system. The areas under peaks obtained were used to determine β-carotene concentrations using the external standard curve. Each data point was an average of three replicates. Recovery of the standards was found to be in the range of 50 %.

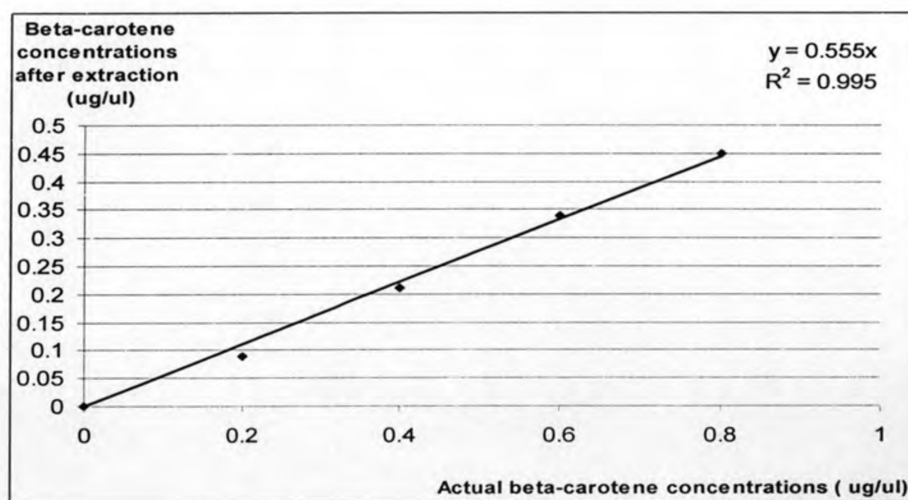

Figure 4.15 A standard curve for the determination of actual β-carotene concentrations.

Table 4.2 β-carotene contents in mid-log phase cells of 5 strains of *Chlorella* spp. and *Scenedesmus* spp. as determined by spectroscopy and by reversed-phase HPLC. All samples were extracted and analyzed in triplicates. Procedures are described in Materials and Methods.

Algal strains	β -carotene contents in mid-log phase cells							
	Spectroscopic determinations ($\mu g.mg^{-1}$ dry weight)				HPLC determinations ($\mu g.mg^{-1}$ dry weight)			
	1	2	3	Average	1	2	3	Average
Chlorella spp.								
SS1	118.44	114.38	107.90	113.57±5.31	4.61	4.19	4.99	4.59±0.4
SS8	120.07	129.85	161.16	137.02±21.46	2.93	2.93	2.41	2.75±0.3
Scenedesmus spp.								
SS4	52.73	56.67	65.30	58.23±6.42	0.32	0.32	0.23	0.29±0.05
SS5	33.05	33.14	34.07	33.42±0.50	0.27	0.18	0.5	0.31±0.45
SS9	29.71	29.71	51.35	36.92±12.49	0.23	0.46	0.68	0.45±0.22

The limit of detection in β-carotene was 0.035 μg/μl. (Data not shown).

Table 4.2, Figures 4.16 and 4.17 showed β-carotene contents in mid-log phase cells of the five isolated micro-algal strains as determined by reversed-phase HPLC and by spectrophotometry. The results showed that spectrophotometric determinations of β-carotene contents yielded for more β-carotene compared to reversed-phase HPLC determinations. One reason might be because of the presence of more carotenoids in addition to β-carotene in the spectrophotometric determinations. There were also conflicting results regarding the contents of β-carotene in *Chlorella* sp. SS1 and SS8 determined by the two methods. However, both methods showed the two *Chlorella* spp. strains contained more β-carotene than the three *Scenedesmus* spp. strains.

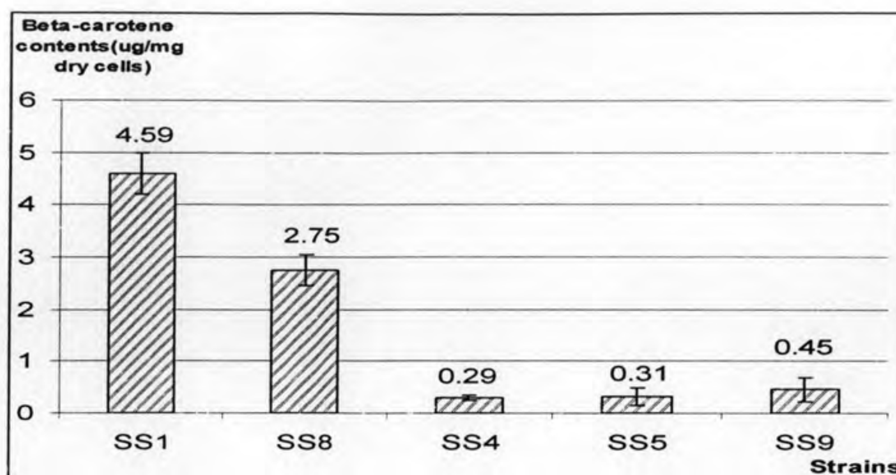

Figure 4.16 β-carotene contents in mid-log phase cells of 5 strains of *Chlorella* spp. and *Scenedesmus* spp. as determined by reversed-phase HPLC.

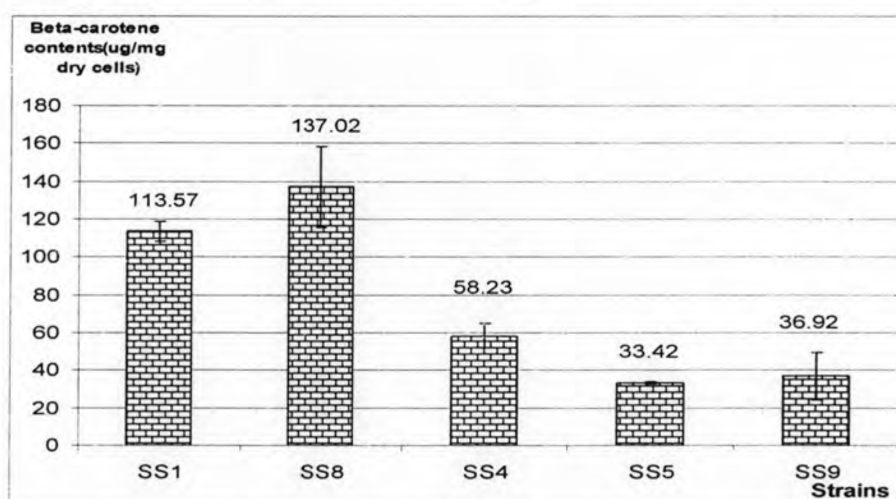

Figure 4.17 β-carotene contents in mid-log phase cells of 5 strains of *Chlorella* spp. and *Scenedesmus* spp. as determined by spectrophotometry.

4.5 Quercetin contents in mid-log phase cells of *Chlorella* spp. and *Scenedesmus* spp.

Figure 4.18 showed a standard curve for the determination of Quercetin concentrations. Since Quercetin might bind to a glycosidic moiety in nature, Quercetin-3-β-D-glycoside was hydrolysed before qualitative and quantitative reversed-phase HPLC analysis. Figure 4.19 showed a standard curve for the determination of Quercetin concentration after hydrolysis to remove the glycosidic moiety (if any). Figure 4.20

showed reversed-phase HPLC chromatograms of Quercetin, Quercetin-3-β-D-glycoside, and Quercetin-3-β-D-glycoside after hydrolysis. The results indicated that after hydrolysis the sugar moiety was removed from Quercetin-3-β-D-glycoside. The retention times of Quercetin and of Quercetin-3-β-D-glycoside were found to be 5.2 min and 1.6 min respectively.

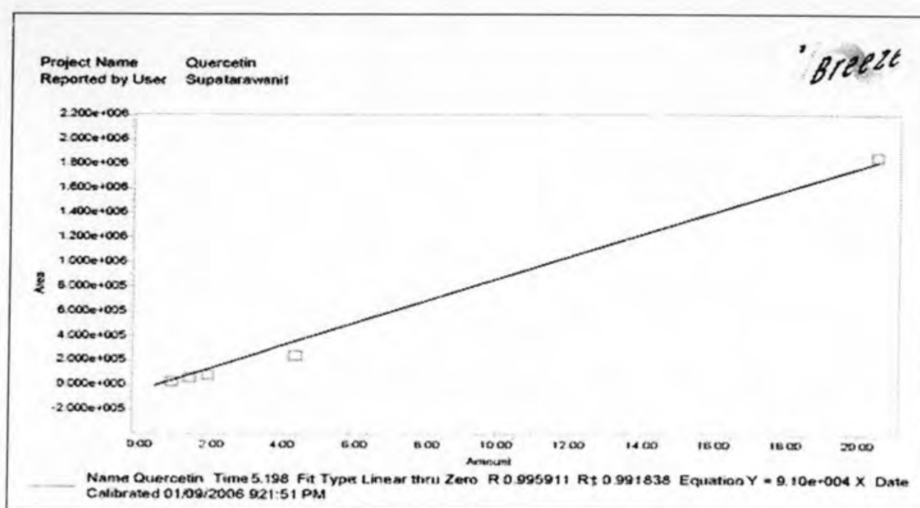

Figure 4.18 A standard curve for the determination of Quercetin concentrations.

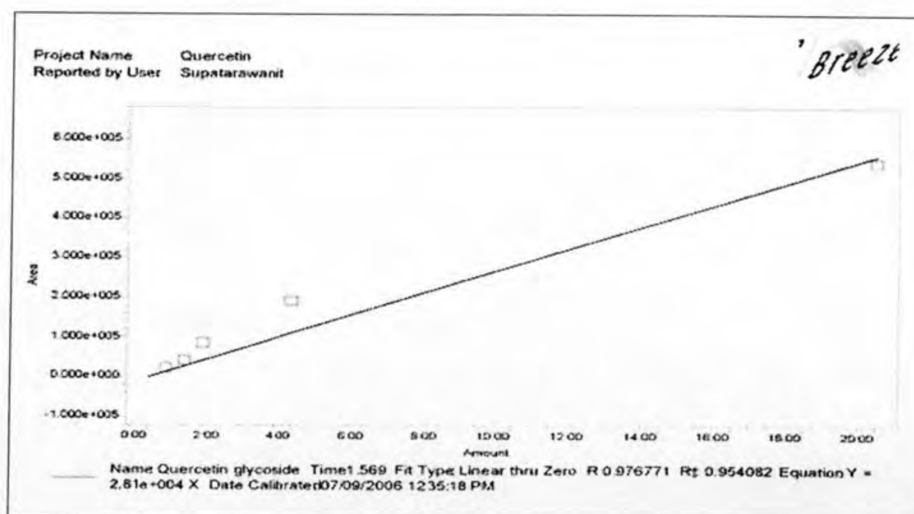

Figure 4.19 Standard curve for the determination of Quercetin concentration after hydrolysis of Quercetin-3-β-D-glycoside. The concentrations of Quercetin-3-β-D-glycoside before hydrolysis were 0.5, 1.0, 1.5, 4.0 and 20.0 μg/ml.

Table 4.3 Quercetin contents in mid-log phase cells of 5 strains of *Chlorella* spp. and *Scenedesmus* spp. and tea leaves as a positive control (Lipton Tea) as determined by HPLC. All samples were extracted and analyzed in triplicates. Procedures as described in Materials and Methods.

Algal strains	Quercetin contents in mid-log phase by HPLC determinations ($\mu g.mg^{-1}$ dry weight)
Chlorella spp.	
SS1	nd*
SS8	nd*
Scenedesmus spp.	
SS4	nd*
SS5	nd*
SS9	nd*
Tea leaves*	63.31±1.50

nd* = Not detected in at least triplicates.

Tea leaves* as a positive control

The limit of detection in Quercetin was 0.050 μg/ml. (Data not shown).