

โปรตีนโพรไฟลีน *Chlorella* spp., *Desmodesmus* spp., และ *Scenedesmus* spp. ซึ่งเลี้ยงที่  
อุณหภูมิช่วง 28°C - 40°C และการวิเคราะห์หลายพิมพ์ดีเอ็นเอ  
และปริมาณบีตาแคโรทีน



นางสาวปาริชาติ กิตติมาสกุล

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

PROTEIN PROFILES IN *Chlorella* spp., *Desmodesmus* spp., AND *Scenedesmus* spp.  
GROWN AT TEMPERATURE RANGE 28°C - 40°C AND ANALYSIS OF DNA  
FINGERPRINTS AND  $\beta$ -CAROTENE CONTENTS



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ปาริชาติ กิตติมาสกุล : โปรตีนโพรไฟล์ใน *Chlorella* spp., *Desmodesmus* spp., และ *Scenedesmus* spp. ซึ่งเลี้ยงที่อุณหภูมิช่วง 28°C - 40°C และการวิเคราะห์หลายพิมพีดีเอ็นเอและปริมาณบีตาแคโรทีน (PROTEIN PROFILES IN *Chlorella* spp., *Desmodesmus* spp., AND *Scenedesmus* spp. GROWN AT TEMPERATURE RANGE 28°C - 40°C AND ANALYSIS OF DNA FINGERPRINTS AND  $\beta$ -CAROTENE CONTENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รองศาสตราจารย์ ดร.กาญจนา ชาญสง่าเวช, 109 หน้า.

สาหร่ายสีเขียวขนาดเล็กรวมทั้ง *Chlorella* spp., *Desmodesmus* spp., และ *Scenedesmus* spp. เป็นผู้ผลิตขั้นต้นในระบบนิเวศน้ำ ผลร้ายแรงต่อผู้ผลิตขั้นต้นย่อมส่งผลร้ายแรงต่อผู้บริโภครวมถึงมนุษย์ซึ่งอยู่ในห่วงโซ่อาหาร นอกจากนั้นสาหร่ายสีเขียวขนาดเล็กเหล่านี้ผลิตสารที่มีประโยชน์ในระดับห้องปฏิบัติการซึ่งสามารถขยายส่วนเป็นระดับนำร่องและระดับอุตสาหกรรม เนื่องจากสภาวะโลกอุ่นส่งผลต่อผู้ผลิตขั้นต้นรวมทั้งสาหร่ายสีเขียวขนาดเล็ก และสาหร่ายสีเขียวขนาดเล็กมีความสำคัญทางอุตสาหกรรมดังกล่าวข้างต้นวัตถุประสงค์ประการแรกของงานวิจัยนี้เพื่อคัดเลือกสาหร่ายสีเขียวขนาดเล็กสายพันธุ์ที่เพิ่มจำนวนเร็วและทนร้อน โดยคัดเลือกจากสาหร่ายสีเขียวขนาดเล็ก *Chlorella* spp., *Desmodesmus* spp., และ *Scenedesmus* spp. ชนิดละ 5 สายพันธุ์ รวมเป็น 15 สายพันธุ์ วัตถุประสงค์ประการที่สองเพื่อรวบรวมผลการทดลองเบื้องต้นที่จะใช้อธิบายกลไกการทนร้อนในสาหร่ายสีเขียวขนาดเล็กสายพันธุ์ที่คัดเลือกได้ ในแง่ของปริมาณบีตาแคโรทีนที่มีอยู่ในเซลล์ที่เลี้ยงที่ 25°C และโปรตีนโพรไฟล์หลังจากเลี้ยงเซลล์  $5.5 \times 10^6$  โคโลนีต่อมิลลิลิตรของแต่ละสายพันธุ์ที่ 28°C -32°C 16 ชั่วโมงสลับกับเลี้ยงที่ 40°C 8 ชั่วโมงในอ่างน้ำควบคุมอุณหภูมิที่วางในเรือนเพาะปลูกที่ขาดการรดน้ำตลอดการทดลอง ผลการหาลายพิมพีดีเอ็นเอของสาหร่ายสีเขียวขนาดเล็กทั้ง 15 สายพันธุ์โดยวิธี RADP-PCR พบว่าเป็นสายพันธุ์ต่างกัน ผลการเลี้ยงที่ 25°C แสดงให้เห็นว่าสาหร่ายสีเขียวขนาดเล็กเซลล์เดียว *Chlorella* spp. เพิ่มจำนวนเร็วกว่าสาหร่ายสีเขียวขนาดเล็กหลายเซลล์ *Desmodesmus* spp. และ *Scenedesmus* spp. ผลการเลี้ยงที่ 28°C-32°C และที่ 28°C-32°C 16 ชั่วโมงสลับกับ 40°C 8 ชั่วโมง พบ *Chlorella* spp. สายพันธุ์ PK37 และ SS1 ทนร้อน และพบ *Desmodesmus* sp. สายพันธุ์ NJ40 เพิ่มจำนวนได้ดีที่อุณหภูมิสูง ผลการทดลองไม่พบความสัมพันธ์ระหว่างความสามารถในการทนร้อนหรือการเพิ่มจำนวนได้ดีที่อุณหภูมิสูง กับปริมาณบีตาแคโรทีนและปริมาณพอลิเปปไทด์ที่มีขนาดโมเลกุลเท่ากับโปรตีนที่สร้างขึ้นเมื่อได้รับความร้อนกระทันหัน

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PARICHART KITTIMASAKUN : PROTEIN PROFILES IN *Chlorella* spp., *Desmodesmus* spp., AND *Scenedesmus* spp. GROWN AT TEMPERATURE RANGE 28°C - 40°C AND ANALYSIS OF DNA FINGERPRINTS AND  $\beta$ -CAROTENE CONTENTS. THESIS ADVISOR : ASSOC. PROF. KANJANA CHANSA-NGAVEJ, Ph.D., 109 pp.

Green microalgae including *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. are primary producers in the aquatic ecological systems. Deleterious effects on the primary producers undoubtedly lead to harmful effects on the consumers including human along the food chains. In addition, these microalgae have been found to be microorganisms for the production of useful products at laboratory scale which, with further research, could potentially be scaled-up to pilot and industrial scales. With the imminent global warming and its effects on primary producers including green microalgae and with the industrial importance of selecting fast-growing and heat-tolerant green microalgae for industries, this research was initiated with the aims of selecting fast-growing and heat-tolerant strains from 5 strains each of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. The second aim of the research is to gather preliminary results on mechanisms of heat-tolerance in terms of existing  $\beta$ -carotene contents at 25°C and SDS-PAGE protein profiles when  $5.5 \times 10^6$  CFU/ml of each strain were grown at 28°C-32°C in a temperature-controlled greenhouse and at 28°C-32°C for 16 h alternated with growth at 40°C for 8 h in a temperature - controlled water bath placed in the greenhouse throughout the experiments. Results of RAPD-PCR fingerprints of the 15 green microalgal strains showed they were different strains. Growth at 25°C showed unicellular green microalgae *Chlorella* spp. grew more rapidly than multicellular microalgae *Desmodesmus* spp. and *Scenedesmus* spp. Growth at 28°C-32°C and at 28°C-32°C for 16 h alternated with 40°C for 8 h showed *Chlorella* spp. strains PK37 and SS1 were heat-tolerant while *Desmodesmus* spp. strains NJ40 and SS5 were mesophilic. No correlation was obtained between heat tolerance or mesophilicity properties and contents of  $\beta$ -carotene and polypeptides which had similar molecular weights to those of heat-shock proteins.

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## CHAPTER I

### INTRODUCTION

Green microalgae including *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. are primary producers in the aquatic ecological systems. Deleterious effects on the primary producers undoubtedly lead to harmful effects on the consumers including human along the food chains. In addition, these microalgae have been found to be microorganisms for the production of useful products at laboratory scale which, with further research, could potentially be scaled-up to pilot and industrial scales. The best known green algae that have been commercially produced as supplementary food are freshwater *Chlorella* spp. while the best known marine green alga commercially used for the production of  $\beta$ -carotene is *Dunaliella salina*. Mass cultivation of these microalgae began in 1950s in USA, Japan, and Israel (Masojédek and Torzillo, 2008; Schlipalius, 1991). It has been 50 years since the beginning of the mass cultivation of micro green algae for supplementary food, feed, and some biologically-active compounds such as  $\beta$ -carotene. According to Borowitzka (1999) there are several problems facing the commercialization of green algae. The problems range from the algal slow growth rates to the cost-effective design of illuminated fermenters for closed indoor cultivation and the efficiency of outdoor cultivation in open ponds.

In the following research, 5 strains each of *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. were chosen as oxygenic photosynthetic micro-organisms for selection of fast-growing and mesophilic or heat-tolerant strains for further basic and applied research on mechanisms for rapid cell division and heat tolerance which will pave the way for strain improvement through molecular biology manipulations including development of reporter gene, gene transformation by electroporation, and particle gun bombardment.

The aim of this research is to obtain an overall result on the growth characteristics at 25°C, 28°C - 32°C in a greenhouse, and 28°C-32°C for 16h in a greenhouse alternated with 8 h incubation at 40°C in a temperature-controlled water

bath placed in the greenhouse. RAPD-PCR fingerprints of all the 15 strains, SDS-PAGE protein profiles of these algae grown at 28°C - 32°C alternated with 40°C, as well as the algal existing  $\beta$ -carotene contents will be determined. The ultimate aim of the research is a compilation of growth characteristics, SDS-PAGE profiles of the green algae grown at 28°C-32°C, and at 28°C-32°C /40°C as well as their existing  $\beta$ -carotene contents to determine if it is possible to explain the mechanisms for rapid cell division and heat tolerance by means of the above-mentioned properties. Rapid growth rates of the green microalgae lead to reduction in time and investment costs for cost-effective industrial applications. Heat tolerance leads to growth at high temperature under the global warming phenomenon. In addition, the molecular data obtained will be used to either support or argue against the current controversy on the transfer of the spiny forms of *Scenedesmus* spp. to a new genus, *Desmodesmus* (An et al., 1999).



## CHAPTER II

### LITERATURE SURVEY

#### 2.1 Records of *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. in Thailand for genetic conservation and industrial applications

Table 2.1 showed unpublished records of *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. found in Thailand. The records were a small part of the unpublished compilation of Algae in Thailand which was an initiative of the Ministry of Natural Resources and Environment and were compiled by a group of Thai phycologists in 2009. Part of the records consisted of surveys of the three genera of green microalgae mainly conducted by graduate students at the Department of Biology, Chiangmai University, under the supervision of Dr Yuwadee Peerapornpisal. The records are presented in this thesis for public and academic uses in genetic conservation and industrial applications.

All of the species recorded in Table 2.1 were identified by morphology. With the advent of molecular techniques including RAPD-PCR fingerprinting where only one arbitrary primer is used in the amplification of target DNA (Williams et al., 1990), and multi-locus sequencing analysis of genes and intergenic spacer regions such as the ITS-2 region which lies between the 5.8S rDNA and 26S rDNA in the *rrn* operon, many taxonomic changes have been proposed. In 1999, An et al., constructed a consensus unrooted phylogenetic tree with three methods for phylogenetic tree construction, namely, the maximum likelihood method, the maximum parsimony method, and the neighbor-joining method as shown in Figure 2.1 to show that the spiny forms of *Scenedesmus* spp. were clearly separated from the non-spiny forms of *Scenedesmus* spp.

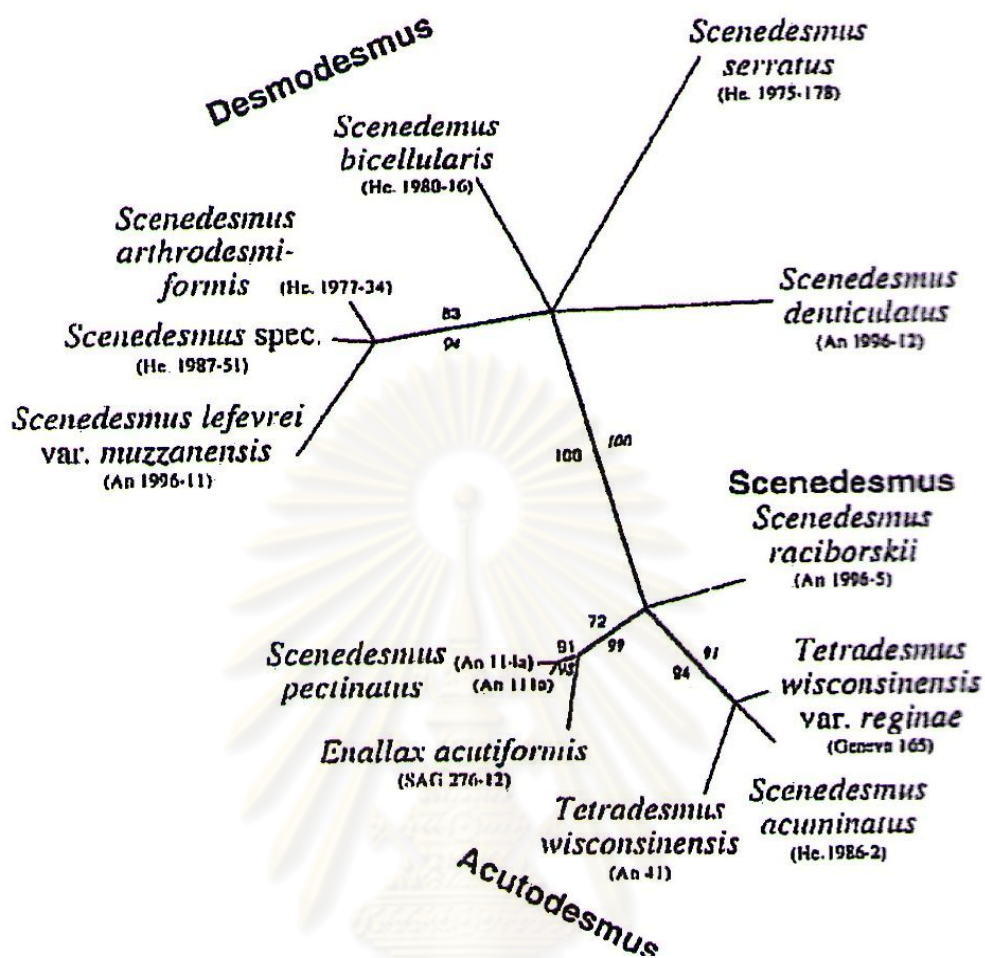


Figure 2.1 Consensus unrooted phylogenetic tree obtained from ITS-2 sequences of *Scenedesmus* and *Scenedesmus*-like taxa using three phylogenetic tree construction methods: the maximum likelihood method, the maximum parsimony method, and the neighbor-joining method. Bootstrap values were computed for 500 resamplings using the neighbor-joining and the maximum parsimony methods (An et al., 1999).

The core ITS-2 sequences for taxa of *Scenedesmus* and *Acutodesmus/Scenedesmus* clusters shown in Figure 2.1 were superimposed on the secondary structure of *S. acuminatus* strain Hegewald 1986-2 as shown in Figure 2.2.



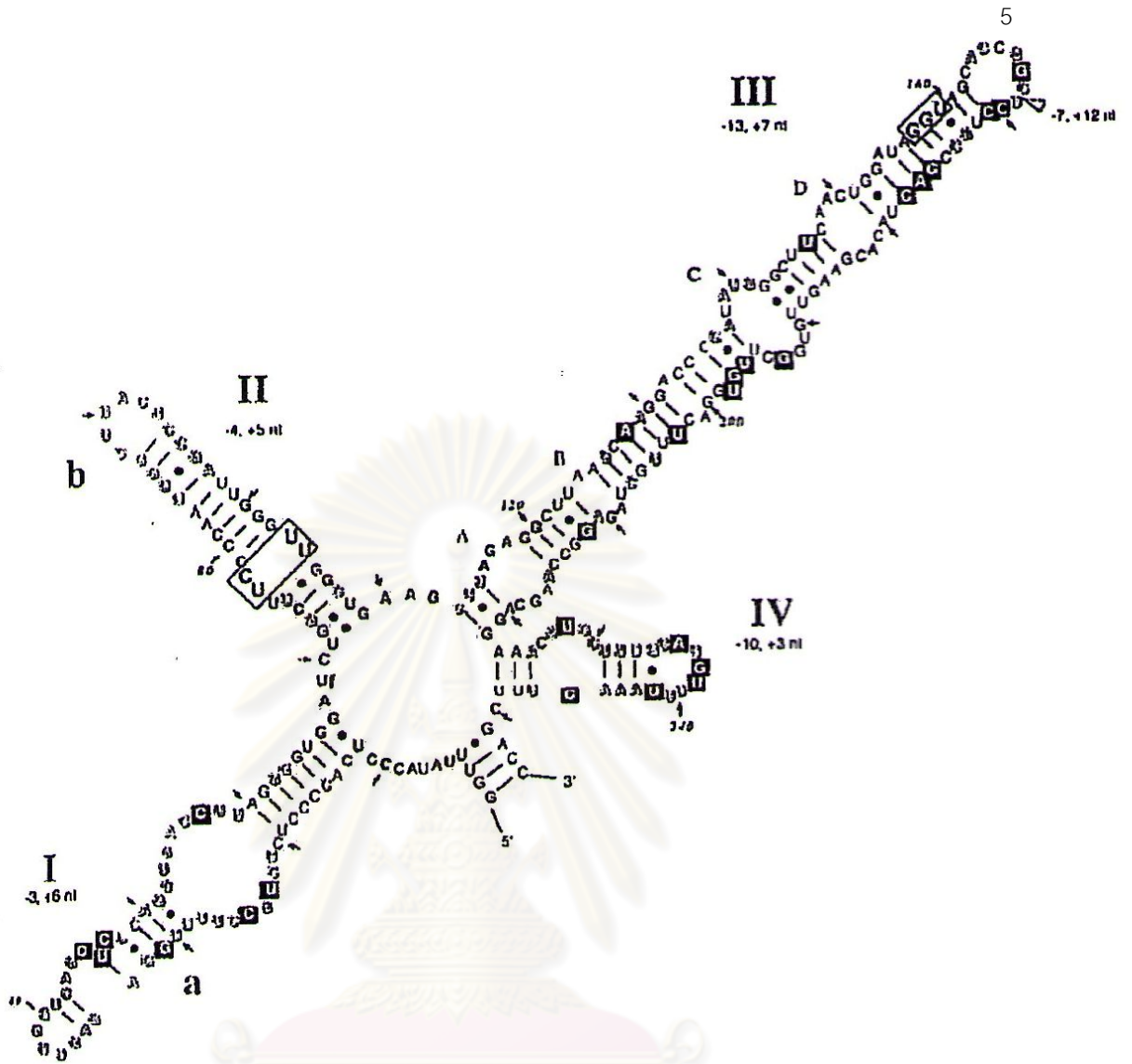


Figure 2.2 Core ITS-2 sequences for taxa of *Scenedesmus* and *Acutodesmus/Scenedesmus* clusters shown in Figure 2.1 were superimposed on the secondary structure of *S. acuminatus* strain Hegewald 1986-2. There were 4 stem-and-loop regions I-IV. Nucleotides were numbered for every 40 nucleotides with small arrows indicating every 10 nucleotides. Nucleotides with black background indicated the 26 positions that distinguished the *Desmodesmus* and *Acutodesmus/Scenedesmus* clusters in Figure 2.1. The universally conserved pyrimidine-pyrimidine juxtaposition in stem-and-loop II and the universally conserved GGU near the apex of stem-and-loop III were boxed (An et al., 1999).

In addition to the phylogenetic tree and the secondary structure of the core ITS-2 structure as shown in Figures 2.1 and 2.2 respectively, An et al. (1999) also reported that most species of *Desmodesmus* had one or several spines on each cell while such structures were absent in *Scenedesmus*. In some *Scenedesmus* spp. rib-like wall structures which might be formed by the hemicellulosic cell wall layers were observed. From the combined results of ITS-2 sequences and morphology (presence of absence of spines and rib-like wall structures), An et al. (1999) proposed that spiny forms of *Scenedesmus* spp. be transferred to a new genus *Desmodesmus*. However, this new genus is still not unanimously accepted by phycologists (John et al., 2002). For the completeness of the records of *Scenedesmus* spp. in Thailand, Dr Kanjana Chansangavej had added in Table 2.1 *Desmodesmus* spp. for spiny forms of *Scenedesmus* spp. as well as the algal synonyms as found in the Algaebase at [www.algaebase.org](http://www.algaebase.org)

Other taxonomic changes that have been made with the advent of molecular biology techniques are the identification of microorganisms by polyphasic taxonomy which employs morphological, biochemical, physiological, and molecular characteristics in the identification process, and the concept of cryptic species which refer to species which are phenotypically similar but genotypically different. So far there is only one research paper on polyphasic taxonomy of the green microalgae *Chlorella* spp., *Desmodesmus/Scenedesmus* spp., and *Scenedesmus* spp. in Thailand (Sawangdee et al., 2007). The paper highlighted previous findings by Muller et al. (2005), Lewis and Flechtner (2004), and Vanormalingen et al. (2007) that there were cryptic species in *Chlorella vulgaris*, *Scenedesmus* spp., and *Desmodesmus costato-granulatus*, respectively.

Table 2.1 Unpublished compilation of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. found in Thailand. The records were a small part of the compilation of Algae in Thailand which was an initiative of the Ministry of Natural Resources and Environment.

| Division/Class/Order/Family  | Scientific name  | Locality   | Bibliography                                       |
|--|--|------------|--|
| Class Trebouxiophyceae<br>Order Chlorellales<br>Family Chlorellaceae | <i>Chlorella ellipsoidea</i><br>Gerneck (Syn.= <i>Chlorella</i><br><i>saccharophila</i> var. | Chiang Mai | Somdee (1998),<br>Mulsin (1999),<br>Sompong (2001) |

|  |   |   |  |
|--|---|---|--|
|  | <i>ellipsoidea</i> (Gerneck) Fott et Nováková)  |   |  |
|  | <i>Chlorella vulgaris</i> Beijerinck (Syn.= <i>Chlorella pyrenoidosa</i> var. <i>duplex</i> (Kützing) West , <i>Pleurococcus beijerinckii</i> Artari, <i>Chlorella pyrenoidosa</i> Chick, <i>Chlorella communis</i> Artari, <i>Chlorella vulgaris</i> var. <i>viridis</i> Chodat, <i>Chlorella terricola</i> Gollerbach, <i>Chlorella candida</i> Shihira & Krauss) | Chiang Mai, Lop Buri, Pathum Thani, Phra Nakhon Sri Ayutthaya, Yala | Somdee (1998), Mulsin (1999), Ariyadej et al. (2004), Pongswat et al. (2004), TISTR (2009) |
|  | <i>Chlorella vulgaris</i> var. <i>vulgaris</i> Beyerinck  | Bangkok   | TISTR (2009)   |

| Division/Class/Order/Family | Scientific name   | Locality  | Bibliography  |
|-----------------------------|---|---|---|
| Family Scenedesmaceae       |   |   |   |
|                             | <i>Scenedesmus acuminatus</i> (Lagerheim) Chodat (Syn.= <i>Scenedesmus acuminatus</i> var. <i>elongatus</i> G.M. Smith) | Bangkok, Chiang Mai, Chon Buri, Khon Kaen, Lop Buri, Nakhon Sri Thammarat | Mulsin (1997), Somdee (1998), Poarlai (1999), Kiatpradub (2003), Prommana (2006), Thonkamdee (2007), TISTR (2009) |
|                             | <i>Scenedesmus acuminatus</i> (Lagerheim) Chodat var. <i>acuminatus</i>   | Bangkok, Pathum Thani, Phang-Nga, Phuket, Krabi,                          | TISTR (2009), Pongswat et al. (2004)  |

|  |   |  |  |
|--|---|--|--|
|  |   | Ranong   |  |
|  | <i>Scenedesmus acutus</i><br>Meyen (Syn.=<br><i>Scenedesmus crassus</i><br>Chodat, <i>Scenedesmus</i><br><i>dimorphus</i> f. <i>granulatus</i><br>Isabella et R.J. Patel.,<br><i>Scenedesmus</i><br><i>scenedesmoides</i> Chodat,<br><i>Arthrodesmus acutus</i><br>Ehrenberg et Ralfs | Bangkok,<br>Nakhon<br>Pathom,<br>Nonthaburi,<br>Yala | Ariyadej et al.<br>(2004), TISTR<br>(2009) |
|  | <i>Scenedesmus acutiformis</i><br>Schröder var. <i>spinuliferum</i><br>West & G.S. West   | Nakhon<br>Pathom,<br>Pathum Thani,<br>Trat           | TISTR (2009)                               |
|  | <i>Scenedesmus acunae</i>   | Yala   | Ariyadej et al.<br>(2004)                  |
|  | <i>Scenedesmus arcuatus</i><br>Lemmermann (Syn.=<br><i>Scenedesmus bijugus</i> var.<br><i>arcuatus</i> Lemmermann,<br><i>Scenedesmus bijugus</i> f.<br><i>arcuatus</i> (Lemmermann) W.<br>West et G.S. West   | Chiang Mai,<br>Lampang,<br>Surat Thani               | TISTR (2009)                               |
|  | <i>Scenedesmus arcuatus</i><br>Lemmermann var.<br><i>platydiscus</i> G.M. Smith<br>(Currently accepted name<br>= <i>Scenedesmus</i><br><i>platydiscus</i> (G.M. smith)<br>Chodat  | Krabi, Nakhon<br>Sawan,<br>Nonthaburi,<br>Phuket     | TISTR (2009),                              |
|  | <i>Scenedesmus armatus</i> var.<br><i>longispina</i> (Chodat)<br>E.Hegewald   | Chonburi   | Ekchai (1997)                              |
|  | <i>Scenedesmus bernardii</i>  | Bangkok,   | Wongshomphu                                |

|  |  |  |   |
|--|--|--|---|
|  | G.M. Smith (Syn.=<br><i>Scenedesmus acuminatus</i><br>var. <i>bernardii</i> (G.M. Smith)<br>Dedusenko            | Chonburi, Lop<br>Buri  | (1996), TISTR<br>(2009)   |
|  | <i>Scenedesmus brasiliensis</i><br>Bohlin (Basionym of<br><i>Desmodesmus brasiliensis</i><br>(Bohlin) E.Hegewald | Krabi, Khon<br>Kaen, Nakhon<br>Sri<br>Thammarat,<br>Phang-Nga,<br>Phuket,<br>Ranong, Satul   |   |
|  | <i>Scenedesmus bijugatus</i><br>(Turpin) Kützing   | Chiang Mai,<br>Chonburi,<br>Phitsanulok,<br>Trat   | Paweenawat<br>(1987)<br>Wongshomphu<br>(1996)                             |
|  | <i>Scenedesmus bujuga</i><br>(Turpin) Lagerheim  | Bangkok,<br>Chaiyaphum,<br>Chiang Mai,<br>Khon Kaen,<br>Krabi,<br>Lampang,<br>Lamphun,<br>Lop Buri,<br>Nonthaburi,<br>Phuket, Samut<br>Prakan, Satul,<br>Tak, Yala | Ariyadej et al.<br>(2004), TISTR<br>(2009)                                |
|  | <i>Scenedesmus acuminatus</i><br>var. <i>minor</i> G.M. Smith  | Nakorn<br>Ratchasima   | Panuvanitchakorn<br>(2003)  |
|  | <i>Scenedesmus acuminatus</i><br>var. <i>tetradesmoides</i> Smith  | Chiang Mai,<br>Sakon Nakorn  | Prommana (2006)   |
|  | <i>Scenedesmus armatus</i><br>Chodat (Syn.=<br><i>Scenedesmus columnatus</i><br>Hortobágyi, <i>Scenedesmus</i>   | Bangkok,<br>Chiang Mai,<br>Khon Kaen,<br>Krabi, Nakorn   | Kraibut (1996),<br>Somdee (1998),<br>Pooarlai (1999),<br>Panuvanitchakorn |



|  |   |   |   |
|--|---|---|---|
|  | <i>helveticus</i> Chodat  | Ratchasima,<br>Nonthaburi,<br>Pathum Thani,<br>Udon Thani                 | (2003), Pongswat<br>et al. (2004), TISTR<br>(2009)  |
|  | <i>Scenedesmus bicaudatus</i><br>Dedusenko  | Chiang Mai,<br>Chonburi,<br>Khon Kaen                                     | Wongshomphu<br>(1996), Prommana<br>(2006),<br>Thonkamdee<br>(2007)  |
|  | <i>Scenedesmus calyptratus</i><br>Lemmermann  | Chiang Mai,<br>Chonburi,<br>Khon Kaen,<br>Nakorn<br>Ratchasima,<br>Phayao | Wongshomphu<br>(1996), Mulsin<br>(1997), Chorum<br>(1998), Somdee<br>(1998), Pooarlai<br>(1999), Pekkoh<br>(2002), Kiatpradub<br>(2003),<br>Panuvanitchakorn<br>(2003), Seekhao<br>(2006), Prommana<br>(2006), Chompusri<br>(2006),<br>Prommana (2006), |
|  | <i>Scenedesmus communis</i><br>E.Hegewald<br>(Syn.= <i>Scenedesmus</i><br><i>quadricauda</i> (Turpin)<br>Br  b,<br>(Basionym of<br><i>Desmodesmus communis</i><br>(E.Hegewald) E.Hegewald | Chiang Mai,<br>Khon Kaen  | Pooarlai (1999),<br>Dhitisudh (2006)  |
|  | <i>Scenedesmus bicaudatus</i><br>Dedusenko  | Chiang Mai  | Prommana (2006)   |
|  | <i>Scenedesmus denticulatus</i><br>Lagerheim (Basionym of   | Chiang Mai,<br>Chiang Rai,  | Waiyaka (1996)  |

|  |  |  |  |
|--|--|--|--|
|  | <i>Desmodemus denticulatus</i><br>(Lagerheim) An, Friedl et E.<br>Hegewald   | Lop Buri   |  |
|  | <i>Scenedesmus dimorphus</i><br>(Turpin) Lagerheim (Syn.=<br><i>Scenedesmus acutus</i><br>Meyen, <i>Scenedesmus</i><br><i>antennatus</i> Bréb.,<br><i>Scenedesmus obliquus</i> var.<br><i>dimorphus</i> (Turpin)<br>Hansgirg, <i>Scenedesmus</i><br><i>costulatus</i> Chodat,<br><i>Scenedesmus acutus</i><br>(Turpin) Rabenhorst,<br><i>Scenedesmus arcuatus</i><br>var. <i>obliquus</i> Rabenhorst | Bangkok,<br>Chiang Mai,<br>Chonburi,<br>Lampang,<br>Lamphun, Lop<br>Buri | Wongshomphu<br>(1996), Pooarlai<br>(1999), Dhitisudh<br>(2006), Seekhao<br>(2006), Chompusri<br>(2006) |
|  | <i>Scenedesmus falcatus</i><br>Chodat  | Bangkok,<br>Chiang Mai   | Somdee (1998)  |
|  | <i>Scenedesmus</i><br><i>incrassulatus</i> Bohlin  | Bangkok,<br>Lampang,<br>Yala   | Ariyadej et al.<br>(2004)  |
|  | <i>Scenedesmus indicus</i> Hort.   | Chonburi   | Wongshomphu<br>(1996)  |
|  | <i>Scenedesmus obliquus</i><br>(Turpin) Kützing (Syn.=<br><i>Achnanthes bijuga</i> Turpin,<br><i>Scenedesmus bijuga</i><br>Kützing., <i>Scenedesmus</i><br><i>chlorelloides</i> Chodat,<br><i>Scenedesmus</i><br><i>dactylococcoides</i> Chodat  | Bangkok,<br>Chonburi,<br>Kalasin,<br>Lampang,<br>Phuket, Satul           | Wongshomphu<br>(1996), TISTR<br>(2009)   |
|  | <i>Scenedesmus opoliensis</i><br>Richter (Syn.=<br><i>Scenedesmus opoliensis</i><br>var. <i>setosus</i> Dedusenko  | Bangkok,<br>Chiang Mai,<br>Nakorn<br>Ratchasima,                         | Pooarlai (1999),<br>Kunpradid (2000),<br>Panuvanitchakorn<br>(2003), Poonswan                          |

|  |   |  |  |
|--|---|--|--|
|  |   | Pathum Thani   | (2005), Dhitisudh (2006), Kraibut (2006), Pongswat et al. (2004) |
|  | <i>Scenedesmus perforatus</i><br>Lemmermann   | Chiang Mai,<br>Krabi, Nakhon<br>Sri<br>Thammarat,<br>Satul   | Pooarlai (1999)  |
|  | <i>Scenedesmus quadricauda</i><br>(Turpin) de Bréb.   | Bangkok,<br>Chiang Mai,<br>Khon Kean,<br>Lop Buri,<br>Nakhon<br>Pathom,<br>Nonthaburi,<br>Samut Prakan,<br>Satul, Trat,<br>Ubon<br>Ratchathani | Kunpradid (2000),<br>Dhitisudh (2006),<br>TISTR (2009)           |
|  | <i>Scenedesmus perforatus</i><br>Lemmermann (Basionym of<br><i>Desmodesmus perforatus</i><br>(Lemmermann)<br>E.Hegewald | Chiang Mai,<br>Chon Buri   | Sompong (1998),<br>Kiatpradub (2003)                             |
|  | <i>Scenedesmus perforatus</i><br>Lemmermann var.<br><i>perforatus</i>   | Phayao, Satul  | Prommana (2002)  |
|  | <i>Scenedesmus quadrispina</i><br>Chodat  | Chiang Mai   | Seekhao (2006)   |
|  | <i>Scenedesmus pannonicus</i><br>Hortob var. <i>pannicu</i>   | Chiang Mai   | Vijaranakorn<br>(2003)   |
|  | <i>Scenedesmus javanensis</i><br>Chodat   | Chiang Mai,<br>Khon Kaen,<br>Krabi, Lop  | Pekthong (1998),<br>Somdee (1998),<br>Pooarlai (1999)            |

|  |   |   |                                  |
|--|---|---|----------------------------------|
|  |   | Buri, Phang-Nga, Phuket, Ranong, Udon Thani |                                  |
|  | <i>Scenedesmus incus</i> Phillip  | Chiang Mai                                  | Poarlai (1999)                   |
|  | <i>Scenedesmus smithii</i><br>Teiling   | Chiang Mai,<br>Lop Buri,<br>Udon Thani      | Seekhao (2006)                   |
|  | <i>Scenedesmus spinosus</i><br>Chodat (Syn.=<br><i>Scenedesmus breviaculeolatus</i> Chodat)<br>(Basionym of<br><i>Desmodesmus spinosus</i><br>(Chodat) E.Hegewald | Khon Kaen,<br>Nonthaburi                    | TISTR (2009)                     |
|  | <i>Scenedesmus spinulatus</i><br>Biswas (Basionym of<br><i>Desmodesmus spinulatus</i><br>(Biswas) E.Hegewald  | Pathum Thani                                | TISTR (2009)                     |
|  | <i>Scenedesmus regularis</i><br>Swir.   | Chiang Mai,<br>Nakorn<br>Ratchasima         | Panuvanitchakorn<br>(2003)       |
|  | <i>Scenedesmus ovalternus</i><br>Chodat<br>(Syn.= <i>Scenedesmus obtusus</i> Meyen  | Bangkok,<br>Chiang Mai,<br>Krabi            | Poarlai (1999),<br>TISTR (2009), |
|  | <i>Scenedesmus lunatus</i> (W<br>et G.S. West) Chodat   | Chiang Mai                                  | Dhitisudh (2006)                 |
|  | <i>Scenedesmus obtusicalus</i><br>Chodat  | Chiang Mai                                  | Poarlai (1999)                   |
|  | <i>Scenedesmus opoliensis</i> P.<br>Richt var. <i>mononesis</i>   | Chiang Mai                                  | Chompusri (2006)                 |
|  | <i>Scenedesmus pretervisus</i><br>Chodat  | Nakhon<br>Pathom                            | TISTR (2009)                     |

|  |   |                                     |   |
|--|---|-------------------------------------|---|
|  | <i>Scenedesmus tropicus</i><br>Crow (Basionym of<br><i>Desmodesmus tropicus</i><br>(Crow) E.Hegewald                                | Chiang Mai,<br>Nakhon<br>Sawan      | Pooarlai (1999),<br>Chittapalapong et<br>al. (2008) |
|  | <i>Scenedesmus clathratus</i><br>(Biswas) comb. Nov.  | Chiang Mai                          | Dhitisudh (2006)                                    |
|  | <i>Scenedesmus longispina</i><br>Chodat (Basionym of<br><i>Desmodesmus armatus</i><br>var. <i>longispina</i> (Chodat)<br>E.Hegewald | Chiang Mai                          | Pooarlai (1999)                                     |
|  | <i>Scenedesmus perisiensis</i><br>Chodat  | Chiang Mai                          | Waiyaka (1996),<br>Pooarlai (1999)                  |
|  | <i>Scenedesmus intermedius</i><br>Chodat (Basionym of<br><i>Desmodesmus intermedius</i><br>(Chodat) E.Hegewald                      | Chiang Mai,<br>Phang-Nga,<br>Ranong | Waiyaka (1996),<br>Kraibut (1996),                  |

## 2.2 Heat shock proteins

Heat shock proteins are a set of proteins some of which are constitutively expressed when cells are grown under normal physiological temperatures. However, upon transferring cells to a relatively higher temperature, or under heat shock, these proteins are increased in quantity in order for cells to deal with the temperature stress conditions. Gene expression for other heat shock proteins are induced upon heat shock (Narberhaus, 2002).

The first category of heat shock proteins which are constitutively expressed when cells are grown under normal physiological temperatures but increased in quantity upon heat shock are molecular chaperones which assist in protein folding (Hartl, 1996; Narberhaus, 2002). Molecular chaperones are cellular proteins with central hollow sphere where protein folding takes place. There are many kinds of molecular chaperones, some of which include GroESL (Figure 2.3) which contains a central hollow sphere where proteins are folded as shown in Figure 2.4.



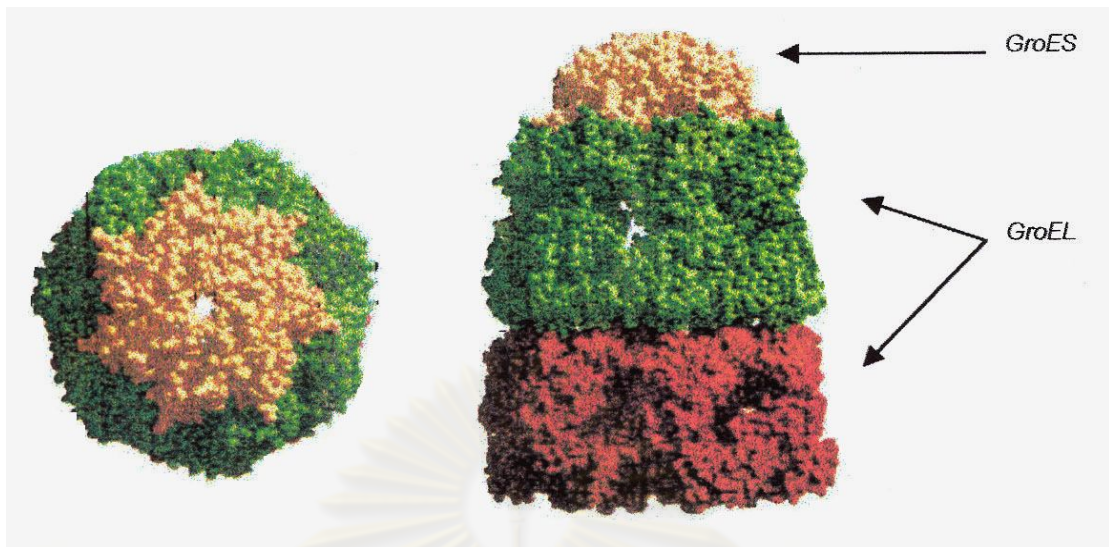


Figure 2.3 Diagrammatic representation of molecular chaperone GroESL, top view (left), and side view (right). GroES consists of 7 identical 10 kDa subunits. GroEL consists of 2 stacks of 7 identical 60 kDa subunits. The whole structure of GroESL contains a central hollow sphere where protein folding takes place (Voet and Voet, 1995).

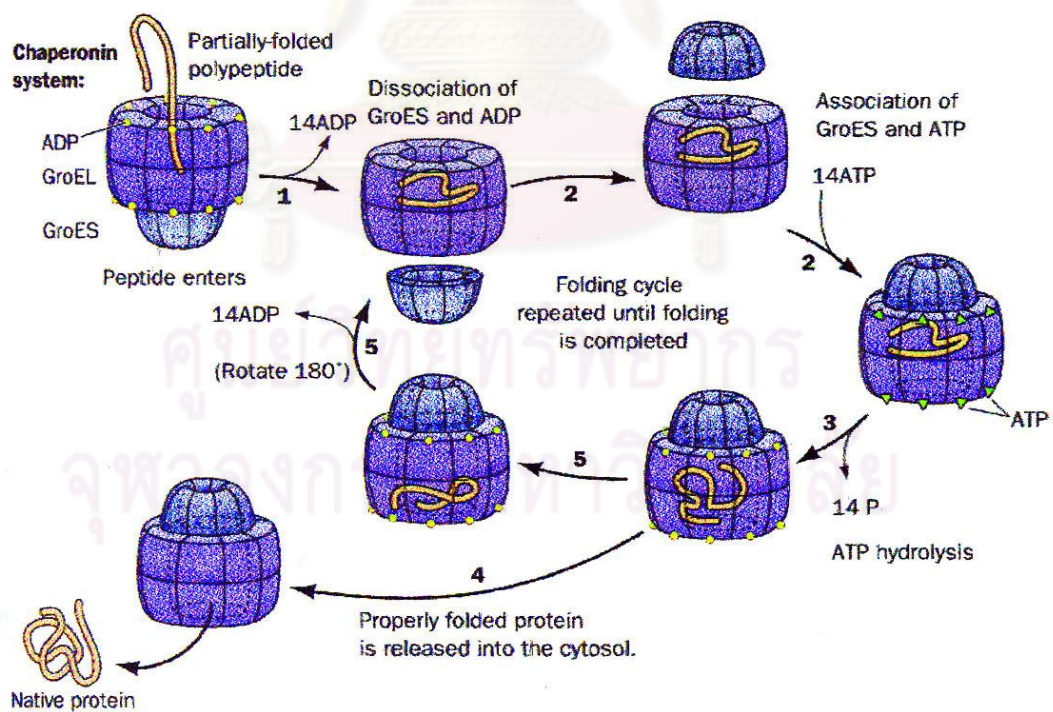


Figure 2.4 Diagrammatic representation of protein folding in GroESL which requires energy from hydrolysis of ATP (Voet and Voet, 1995).



Literature survey conducted for this thesis has found no report on GroESL in *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. However, in 2009 Balczun et al. reported the presence of chloroplast heat shock protein Cpn60 in the green microalga *Chlamydomonas reinhardtii*.

The second kind of molecular chaperones is called small heat shock proteins (sHSPs) whose function is to bind unfolding intermediates or partially denatured proteins, upon heat shock, to prevent aggregate formation. When heat is removed from the system, the molecular chaperones dissociate from the partially denatured proteins to enable the proteins to be folded into active conformation in GroESL. This type of molecular chaperones which, in some organisms, is induced upon heat shock is known as small heat shock proteins (sHSP) which have molecular weight in the range of 12-43 kDa (Narberhaus, 2002). Small heat shock proteins are also oligomers with a central hollow sphere. The first small heat shock protein that was crystallized was obtained from the bacterium *Methanococcus jannaschii* (Kim et al., 1998).

The second category of heat shock proteins are ATP dependent intracellular proteases which are encoded by the *clp* gene family (Clark, 1999). This category of heat shock proteins perform two functions as molecular chaperones and proteases. ClpP is a double ring tetra-decameric protease whose active site lies in the inner surface of the ring-like structure. ClpA or ClpX is a hexameric ATPase which associates with the apical domain of ClpP (Beuron et al, 1998; Gribun et al., 2005). Since each ClpP ring consists of 7 subunits and both ClpA and ClpX consist of 6 subunits, the association of ClpAP or ClpXP results in a "gate" at the apex of ClpAP or ClpXP complex. Thus, the only way for a partially unfolded or a partially denatured polypeptide to get to the inner surface of the complex is to be unfolded and thread through the "gate" to be digested in the inner chamber of the ClpAP or ClpXP complex (Wang et al., 1997). The ClpXP protease is substrate specific, since it degrades casein (known to be degraded by ClpAP) and DnaK slowly or not at all. These results suggest that ClpX protein directs ClpP protease to specific substrates (Wojtkowiak et al., 1993). Literature survey conducted for this thesis has found no report on Clp proteins in *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. However, in 2005 Majeran et al. reported *Chlamydomonas reinhardtii* contained chloroplast ClpP complex with an unusual high

molecular mass (approximately 540 kDa which is about 200 kDa higher than ClpP complexes in higher plant chloroplasts, mitochondria or bacteria).

### 2.3 Synthesis of $\beta$ carotene in green microalgae

$\beta$  carotene is a member of the carotenoid pigments which are made up of 5-membered carbon isoprenoid (IPP) units. In 1966, Schwender et al. labeled *Scenedesmus obliquus* culture with  $[1-^{13}\text{C}]$ glucose and followed the fate of the radioactive carbon in the synthesis of isoprenoid units. The researchers proposed the pathway for isoprenoid synthesis in *Scenedesmus obliquus* as shown in Figure 2.5 .

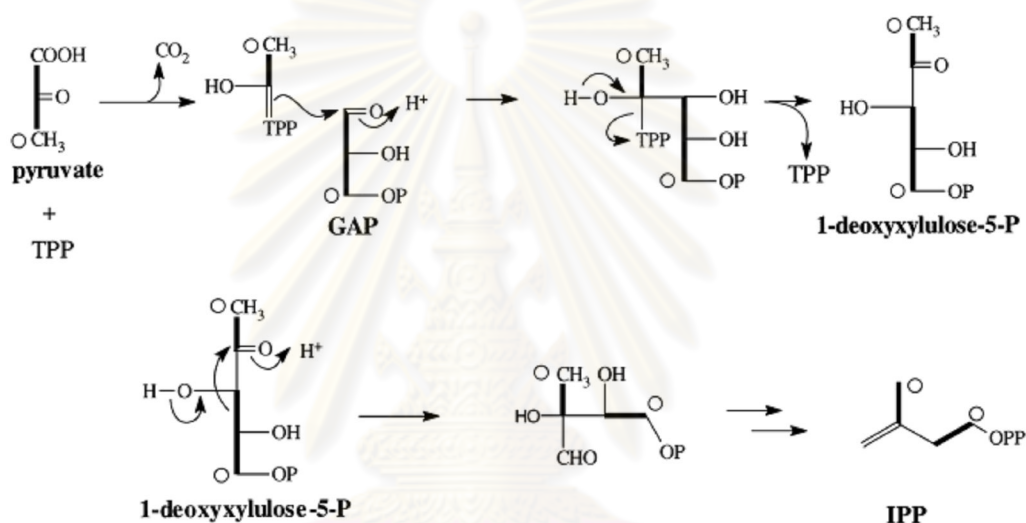


Figure 2.5 Biosynthesis of isoprenoid (IPP) from pyruvate and glyceraldehyde-3-phosphate (GAP) in *Scenedesmus obliquus* (Schwender et al., 1966).

According to Eonseon et al. (2003),  $\beta$  carotene synthesis in microalgae proceeds through the joining of 4 isoprenoid units to form the 20-carbon geranylgeranyl pyrophosphate (GGPP). Two molecules of geranylgeranyl pyrophosphate combine to form phytoene which is desaturated to lycopene and cyclized to form  $\beta$  carotene as shown in Figure 2.6.

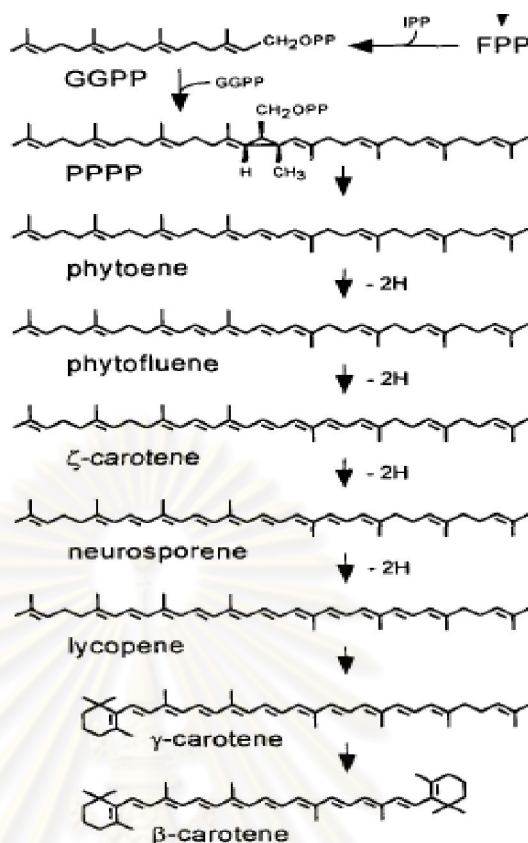
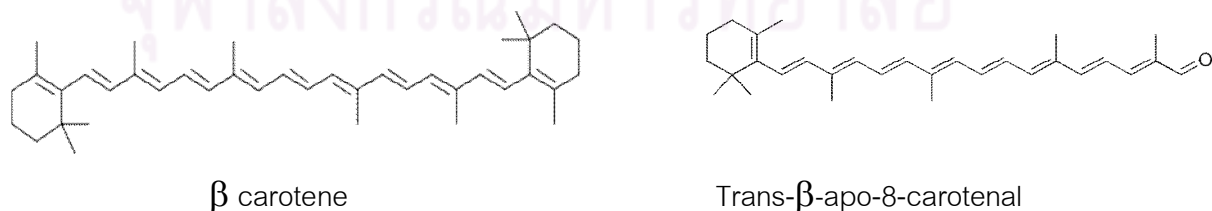


Figure 2.6  $\beta$  carotene synthesis in microalgae proceeds through the joining of 4 isoprenoid units to form the 20-carbon geranylgeranyl pyrophosphate (GGPP). Two molecules of geranylgeranyl pyrophosphate combine to form phytoene which is desaturated to lycopene and cyclized to form  $\beta$  carotene (Eonseon et al., 2003).

In HPLC determination of  $\beta$  carotene, trans- $\beta$ -apo-8-carotenal is often used as an internal standard (Inbaraj et al., 2006). The structures of  $\beta$  carotene, trans- $\beta$ -apo-8-carotenal are shown below:



## CHAPTER III

### MATERIALS AND METHODS

#### 3.1 Microalgal strains

Out of the 15 green microalgal strains used in this research, 9 strains were previously recorded and 6 strains were newly isolated by the author in January 2006. Surface-water samples collected were centrifuged at 3,000 rpm for 5 mins. The green microalgal strains were isolated and purified on petri dishes containing Bold's Basal Agar (BBM) medium incubated at 25°C under continuous light intensity of approximately 3,000 lux. Details of the isolation sites were outlined in Table 3.1. All the 15 strains were stored in BBM slants at 4°C with subculturing every 6 months. All newly-isolated strains were deposited for public access with the Culture Collection of the Thailand Institute for Scientific and Technological Research (TISTR) with the TISTR accession numbers as given in Table 3.2.

Table 3.1 Isolation sites of the newly-isolated green microalgal strains.

| Genus                               | Isolation sites  |
|-------------------------------------|--|
| <i>Chlorella</i> sp. strain PK10    | A pond next to the football field at Kasetsart University, Bangkok campus                                |
| <i>Chlorella</i> sp. strain PK30    | Soil in Nam Muap subdistrict, Nan province   |
| <i>Chlorella</i> sp. strain PK37    | A pond next to the football field at Kasetsart University, Bangkok campus                                |
| <i>Chlorella</i> sp. strain PK38    | Soil in Na Lueang subdistrict, Nan province  |
| <i>Desmodesmus</i> sp. strain TA008 | A road side canal on Rama II road  |
| <i>Scenedesmus</i> sp. strain PK25  | A channel that carries water away from the milk processing plant at Kasetsart University, Bangkok campus |
| <i>Scenedesmus</i> sp. strain PK92  | A pond next to the football field at Kasetsart University, Bangkok campus                                |

Table 3.2 Green microalgal strains used in this study.

| Genus              | Strains | TISTR accession numbers | References        |
|--------------------|---------|-------------------------|-------------------|
| <i>Chlorella</i>   | PK10    | TISTR 8270              | This study        |
|                    | PK30    | TISTR 8269              | This study        |
|                    | PK37    | TISTR 8271              | This study        |
|                    | PK38    | TISTR 8272              | This study        |
|                    | SS1     | TISTR 8877              | Sawaengdee, 2006  |
| <i>Desmodesmus</i> | NJ14    | TISTR 8863              | Jamkangwan, 2004  |
|                    | NJ23    | TISTR 8864              | Jamkangwan, 2004  |
|                    | NJ40    | TISTR 8865              | Jamkangwan, 2004  |
|                    | NJ45    | TISTR 8866              | Jamkangwan, 2004  |
|                    | TA008   | TISTR 8275              | Klinkumouan, 2005 |
| <i>Scenedesmus</i> | PK25    | TISTR 8273              | This study        |
|                    | PK92    | TISTR 8274              | This study        |
|                    | SS4     | TISTR 8879              | Sawaengdee, 2006  |
|                    | SS5     | TISTR 8880              | Sawaengdee, 2006  |
|                    | NJ42    | TISTR 8860              | Jamkangwan, 2004  |

### 3.2 Observation of cells under light microscope

Cells of each strain were grown in 3 ml BBM medium at 25°C in an illuminated growth chamber with continuous light intensity of approximately 3,000 lux for 3, 6, 9 and 12 days before observing the cells under light microscope (Nikon OPTIPHOT-2) equipped with a camera (Nikon FDX-35).

### 3.3 Scanning electron microscope of *Desmodesmus* spp. and *Scenedesmus* spp.

Cells of each strain of *Desmodesmus* spp. and *Scenedesmus* spp. were streaked on petri dishes containing BBM, incubated at 25°C in an illuminated growth chamber with approximately 3,000 lux continuous light intensity for 7-10 days. Cells were fixed for observation with a scanning electron microscope (JOEL, JSM 5410LV) at the Research and Equipment Center at Chulalongkorn University. Cells washed with 0.1

M phosphate buffer, pH 7.2, were filtered on 0.45  $\mu\text{m}$  cellulose membrane and fixed with 2.5% Glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for one hour. Fixed cells were washed twice with 0.1 M phosphate buffer, pH 7.2. The samples were then gradually dehydrated with 30%, 50%, 70%, 90%, and 100% Ethanol. Samples were dried with a critical point drier (Balzers, CPD020), covered with a thin layer of gold with an ion sputter (Balzers, CPD020) before observation under the scanning electron microscope.

### 3.4 RAPD-PCR fingerprinting

In order to show that the green microalgae used in the experiment were different strains, RAPD-PCR fingerprinting was carried out with CRL-7 as the primer (Mathis and McMillen, 1996). Sequence of the primer was 5'GCCCGCCGCC3'. There were two parts in the fingerprinting experiment.

#### 3.4.1 Chromosomal DNA isolation

One loop of each microalgal strain from BBM slant was streaked into BBM plate and incubated at 25°C for 7-10 days. One loop from the plate was placed in sterilized eppendorf tube. Cells were broken by vortexing with sterilized acid-washed glass beads. (Sigma cat, # G1277) in 400  $\mu\text{l}$  TE buffer, 10% SDS, 40 seconds, 2 times. Each homogenate was centrifuged at 10,000 rpm, 4°C, for 10 min. DNA precipitate was obtained by precipitation with 500  $\mu\text{l}$  ice cold ethanol, left at -80°C for 15 min and spun down at 12,000 rpm for 10 min. DNA pellet was washed with 70% ethanol, dried in the air before adding 20  $\mu\text{l}$  sterilized distilled water. Quality of DNA was checked by 1.25% agarose gel electrophoresis and OD ratios of  $\text{OD}_{260}/\text{OD}_{280}$  by standard methods. Quantities of DNA were determined by using  $\text{OD}_{260}$  of 1.00 equals to 50  $\mu\text{g}/\text{ml}$  DNA (Sambrook et al., 1984)

#### 3.4.2 RAPD-PCR fingerprinting

RAPD-PCR fingerprints were obtained with the composition of PCR mixtures as follows : 10x PCR buffer (with 50 mM  $\text{MgCl}_2$ ) 2.0 $\mu\text{l}$ , 10 mM dNTPs, 0.2  $\mu\text{l}$ , primer CRL-7 (100 pmole/ $\mu\text{l}$ ) 0.2  $\mu\text{l}$ , *Taq* polymerase (5 U. $\text{ml}^{-1}$ ) 0.2  $\mu\text{l}$ , distilled water to 20  $\mu\text{l}$ . PCR program was as follows : 95°C 15 sec, 55°C 30 sec, 72°C 90 sec, (5 cycles) ; 95°C 15 sec, 60°C 30 sec, 72°C 90 sec (30 cycles) ; 72°C 10min. PCR products were separated



by 0.8% agarose gel electrophoresis before recording pictures of gels with the Fuji polaroid film (F3000B) with a camera under UV-transilluminator (Bio-rad).

### 3.5 Growth at 28°C–32°C and at 28°C–32°C alternated with 40°C and separation of proteins by SDS-PAGE

#### 3.5.1 Growth of seed culture

Seed culture of each strain was obtained by incubating one loop of cells in 50 ml BBM in a 250 ml Erhenmeyer flask, incubated in an incubator shaker at 25°C , 200 rpm, approximately 3,000 lux continuous light intensity for 21 days. The numbers of days for growing cells to mid-log phase were determined from the constructed growth curves. Specific growth rates were determined from growth curves and the following standard

formula:  $N_t = N_0 e^{\mu t}$

when  $N_t$  = colony forming unit (CFU.ml<sup>-1</sup>) at time t

$N_0$  = initial CFU.ml<sup>-1</sup>

$\mu$  = specific growth rate (day<sup>-1</sup>)

T = incubation time (days)

Duncan's multiple range test was carried out with SPSS program version 15.0 for Windows.

#### 3.5.2 Growth at 28°C–32°C and 28°C–32°C alternated with 40°C

Equal number of cells ( $5.5 \times 10^6$  CFU/ml) were inoculated in 150 ml BBM medium in each 500 ml Erhenmeyer flask. The flasks were incubated in a temperature- controlled greenhouse at 28°C – 32°C or at 28°C – 32°C for 16 h alternated with incubation in a 40°C temperature-controlled waterbath for 8 h. throughout the experiments. One ml of each cell culture was obtained on a daily interval for plating and counting of colony forming units (CFU). Growth curves were constructed to obtain the numbers of days needed to grow cells to mid-log phase for use in the isolation of proteins for SDS-PAGE as described in the following section.

#### 3.5.3 Separation of proteins by SDS-PAGE

Cells of each strain were grown at 28°C-32°C or 25°C-32°C for 16 h alternated with 40°C for 8 h throughout the experiments as described in section 3.5.2. Mid-log phase cells were broken by vortexing with glass beads (Sigma cat.# G1277) for 40 seconds 10 times in a cold room. Soluble proteins were obtained by centrifugation in a

refrigerated microcentrifuge (Eppendorf) at 13,000 rpm, 4°C, for 30 min. Concentrations of soluble proteins were determined by Bradford method (Bradford, 1996) with 0.1 ml sample volume and 5 ml of Bradford reagent (Bio-rad Protein Assay Reagent). The protein mixture was left standing for 5 min before measurements of optical density at 595 nm. A standard curve for the determination of protein concentrations was obtained by using bovine serum albumin (5 mg/ml stock solution) (Sigma). 5 µg proteins were loaded onto each well in the polyacrylamide gel. Protein separation with SDS-PAGE was performed with a vertical minigel SDS-PAGE apparatus (ATTO model AE-6531) by the method of Laemmli (1970) with 12.5% polyacrylamide separate gel and 4% polyacrylamide stacking gel. SDS-PAGE Molecular Weight standards, low range (Bio-rad) were used as molecular weight standards according to the manufacturer's instruction. Gels were stained with Silver-stain plus (Bio-rad) according to the manufacturer's instruction.

### **3.6 Determination of $\beta$ carotene contents**

#### **3.6.1 Growth of seed culture**

Growth of seed culture for the determination of  $\beta$ -carotene contents was obtained as previously described in section 3.5.1.

#### **3.6.2 Growth of cells for the determination of $\beta$ -carotene contents**

150 ml mid-log phase seed culture of each strain were added to 1,350 ml of BBM medium in a 2-litre flask. The flasks were incubated at 25°C in a rotary incubator shaker at 200 rpm, 25°C, for 21 days. The numbers of days in order to grow cells to mid-log phase for each green microalgae strain were determined from the constructed growth curves. Mid-log phase cells were collected by centrifugation at 10,000 rpm, 4°C 10 min. Cells were lyophilized before use in the determination of  $\beta$ -carotene contents.

#### **3.6.3 Determination of $\beta$ -carotene contents by reversed –phase HPLC**

2 ug of the internal standard trans-beta-apo-8'-carotenal (Sigma) were added to 60 mg of lyophilized cells of each strain in a 30 ml polyallomer centrifuge tube. Polyallomer centrifuge tubes were used because the material withstands solvents including ethanol and KOH which were used in the extraction process. Cells were broken by two methods. In the first method, cell breakage was by a combination of heat (50°C) and solvents (5 ml Ethanol and 500 µl 60% KOH). Each cell suspension in the

polyallomer tube was occasionally swirled in a 50°C temperature-controlled waterbath for 5 min or 10 min in order to determine the optimum time for complete cell extraction. In the second method, cells were broken by vortexing 30 mg dried cells in an eppendorf tube with acid-washed glass beads (Sigma cat.# G1277) 40 seconds each time for ten times. The eppendorf tubes were placed on an ice bath after each vortexing to dissipate heat. 30 mg lyophilized cells were put in an eppendorf tube for two tubes which contained a total of 60 mg lyophilized cells. Cell debris after centrifugation at 8,000 rpm, 25°C, for 5 min were observed under the light microscope to determine the extent of cell breakage. The supernatant was poured into an aluminium foil-covered separating funnel. Three ml diethyl ether and three ml 9% NaCl were added into each separating funnel which was shaken on a separating funnel shaker (HSIANGTA) to extract carotenoids. The separating funnels were left on a stand in the dark fumehood until the mixture was separated into two layers of green coloration and yellow coloration. The bottom green layer of chlorophylls was collected and discarded. Three ml 9% NaCl were added into the yellow layer and the extraction was repeated once until the mixture was separated into yellow coloration and clear solution. The carotenoid layer was combined in a 125 ml Ehrenmeyer flask and some Na<sub>2</sub>SO<sub>4</sub> was put into the flask to absorb water. The β-carotene extract was evaporated to dryness by incubating in a 50°C heat block. One ml of the mobile phase for reversed-phase HPLC determination of β-carotene contents (Acetonitrile : Dichloromethane : Methanol 70:20:10,) was added into the residue before filtration with a 0.45 μm filter syringe and injected into the reversed-phase HPLC (Waters). The conditions for HPLC were as follows : Steel column Zorbax C<sub>18</sub>, length 150 mm, I.D. 3.9 mm. Mobile phase : Acetonitrile : Dichloromethane : Methanol (70 : 20 : 10). Flow rate 0.8 ml/min, detection was by optical density at 450 nm, Temperature 25°C. Sample loop volume 20 ul; Injection volume 60 ul; Run time 10 min. External standard curves for the determination of β-carotene and trans-beta-apo-8'-carotenal were obtained with β-carotene and trans-beta-apo-8'-carotenal standards (Sigma).

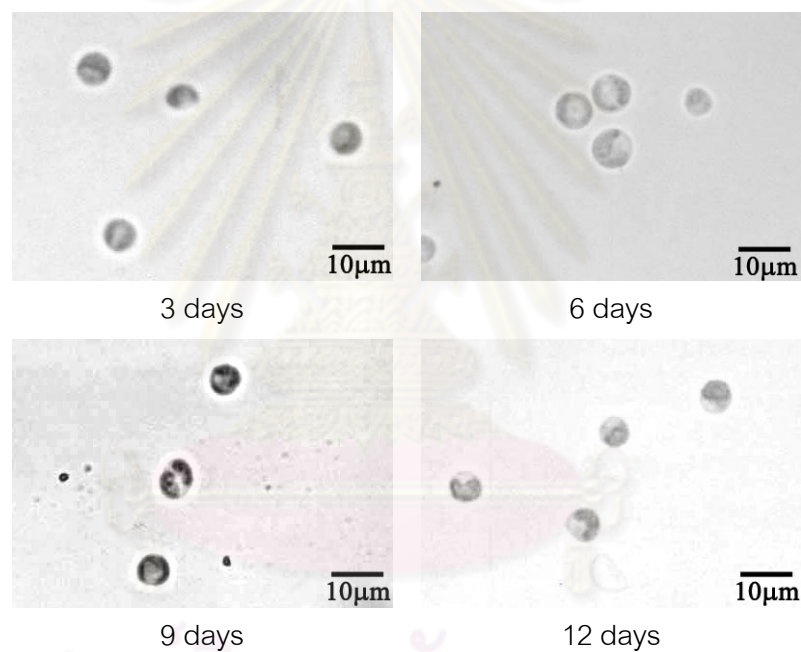
## CHAPTER IV

## RESULTS

## 4.1 Cell morphology under light and scanning electron microscopes

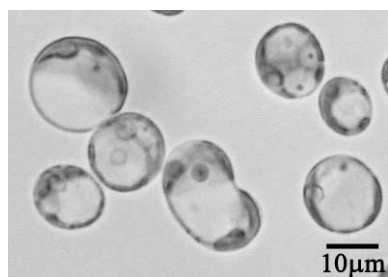
Figure 4.1 showed cell morphology under light microscope of 15 strains of green microalgae used in the experiments. The cells were grown in BBM medium at 25°C under continuous light intensity of approximately 3,000 lux, for 3, 6, 9, and 12 days.

*Chlorella* sp. strain PK10 (TISTR 8270)

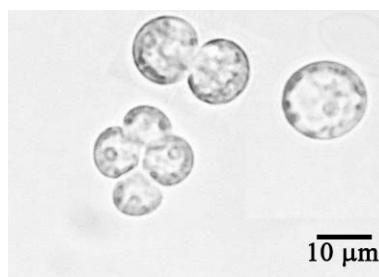


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จุฬาลงกรณ์มหาวิทยาลัย

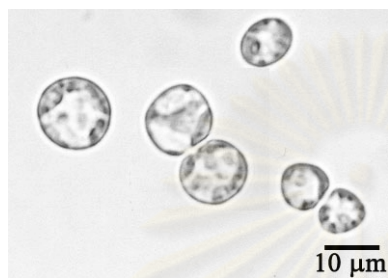
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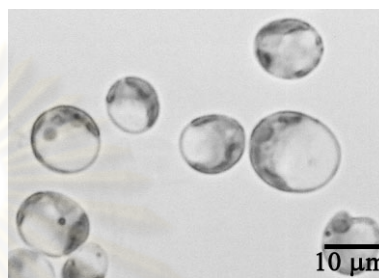
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6 days

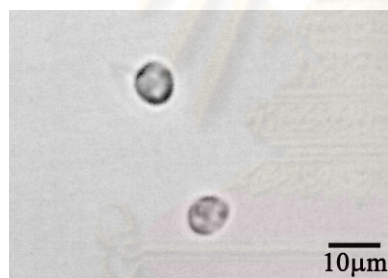


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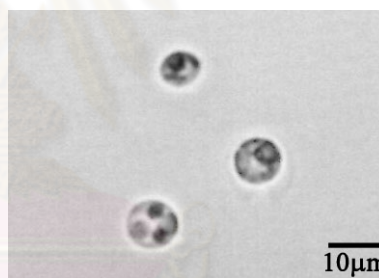


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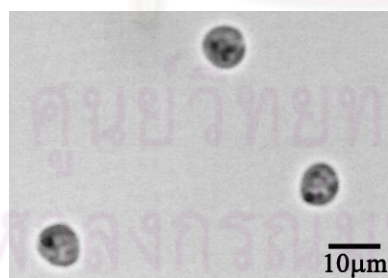
*Chlorella* sp. strain PK37 (TISTR 8271)



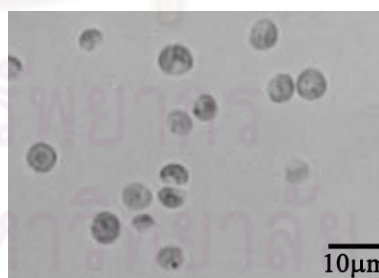
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6 days



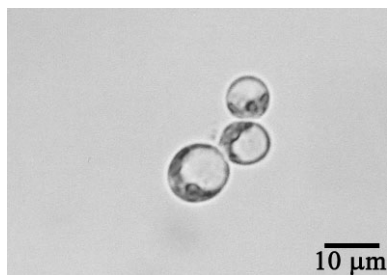
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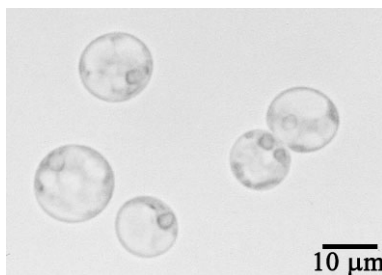
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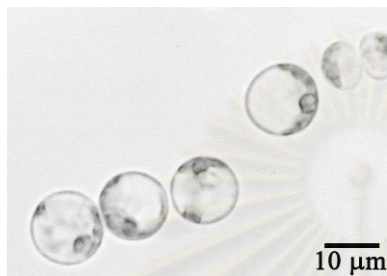
*Chlorella* sp. strain PK38 (TISTR 8272)



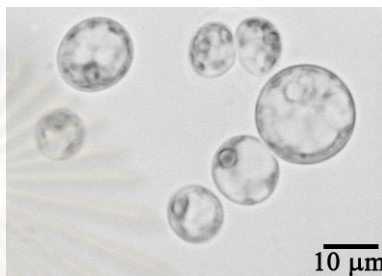
3 days



6 days

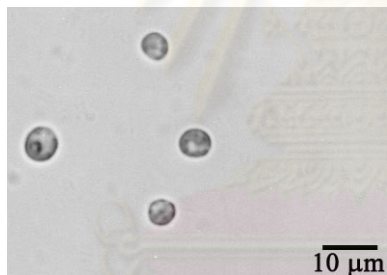


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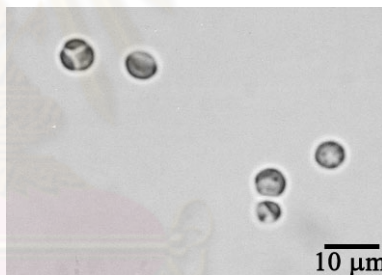


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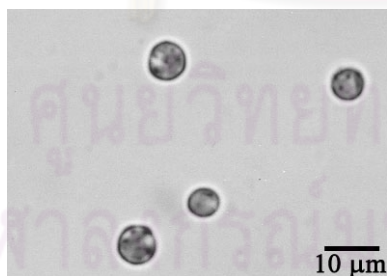
*Chlorella* sp. strain SS1 (TISTR 8877)



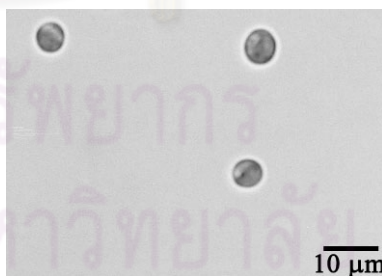
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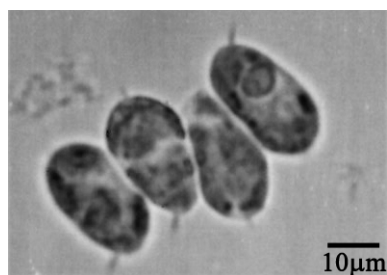
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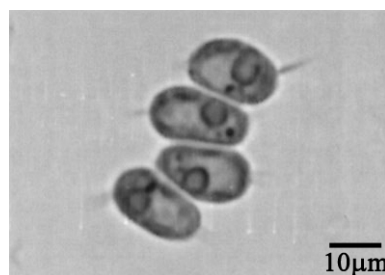
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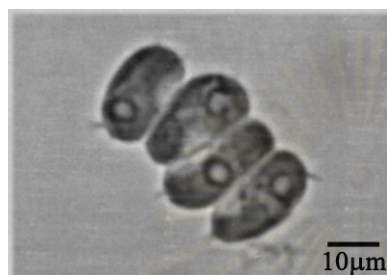
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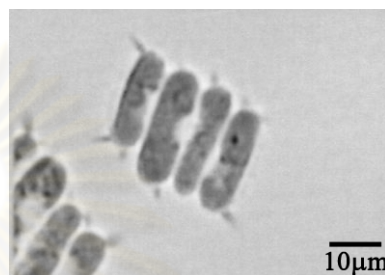
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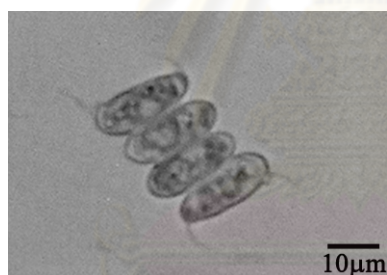


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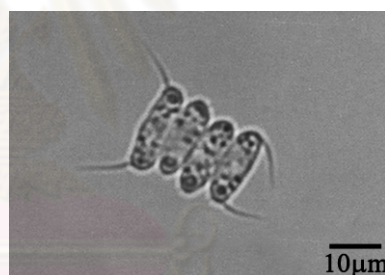


12 days

*Desmodesmus* sp. strain NJ23 (TISTR 8864)



3 days



6 days

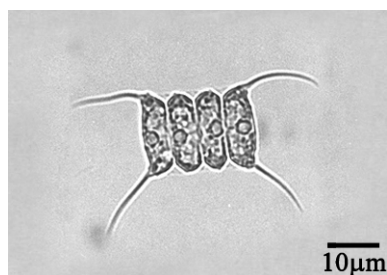


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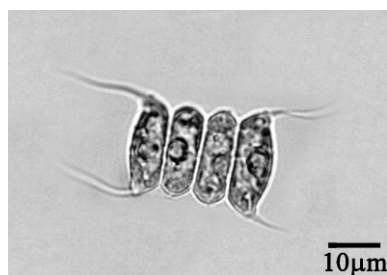


12 days

*Desmodesmus* sp. strain NJ40 (TISTR 8865)



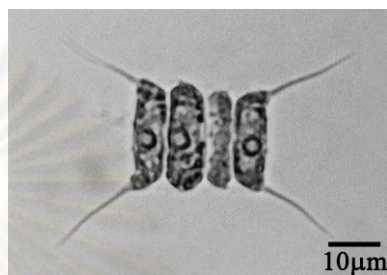
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6 days



9 days

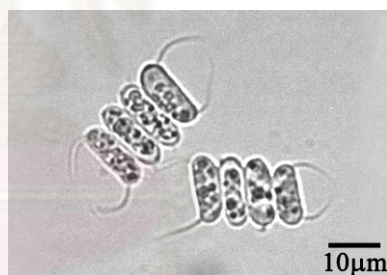


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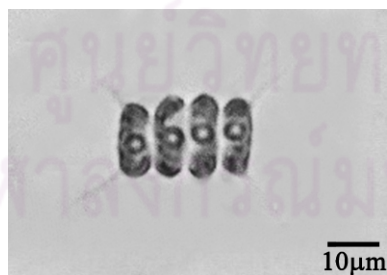
*Desmodesmus* sp. strain NJ45 (TISTR 8866)



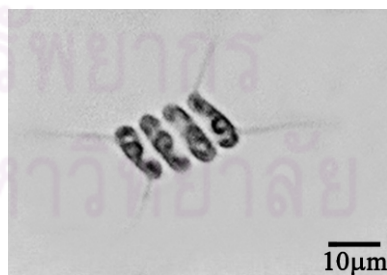
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6 days

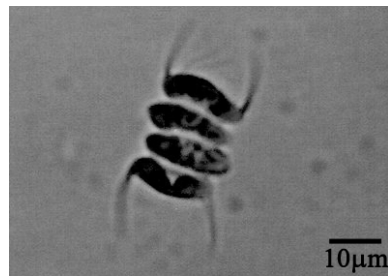


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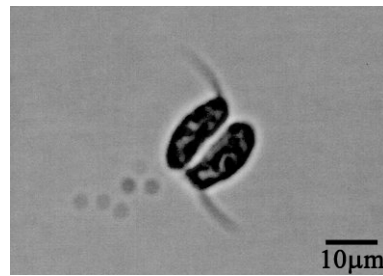


12 days

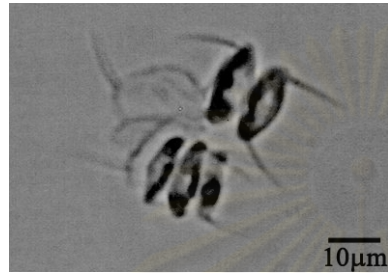
*Desmodesmus* sp. strain TA008 (TISTR 8275)



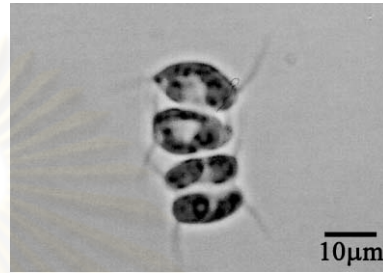
3 days



6 days



9 days



12 days

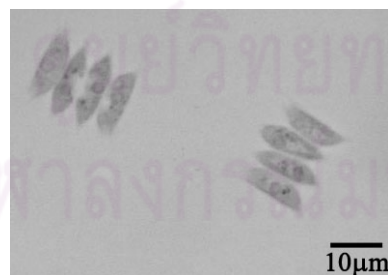
*Scenedesmus* sp. strain PK25 (TISTR 8273)



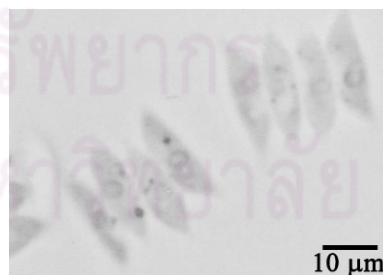
3 days



6 days

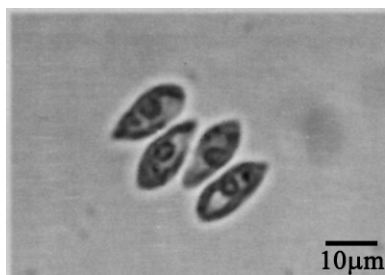


9 days

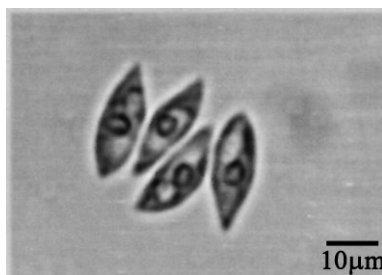


12 days

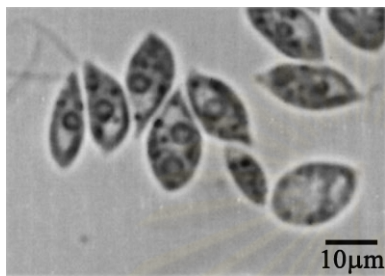
*Scenedesmus* sp. strain PK92 (TISTR 8274)



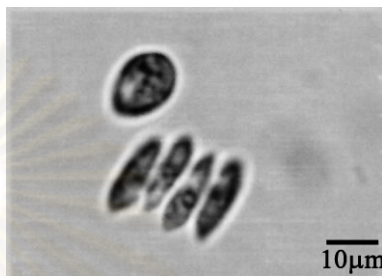
3 days



6 days



9 days

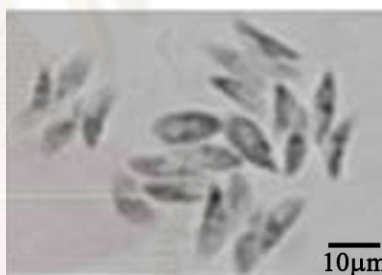


12 days

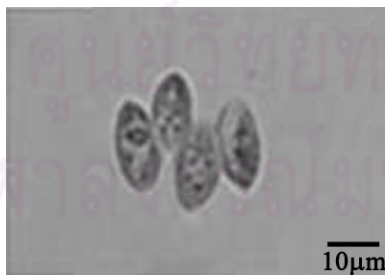
*Scenedesmus* sp. strain NJ42 (TISTR 8860)



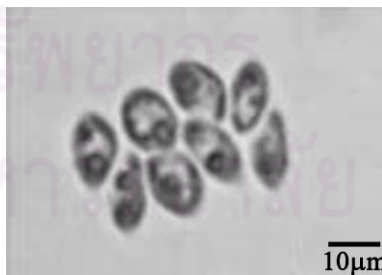
3 days



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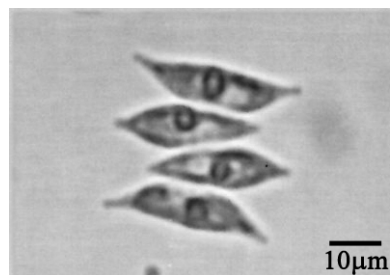
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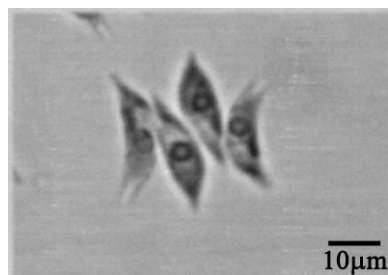
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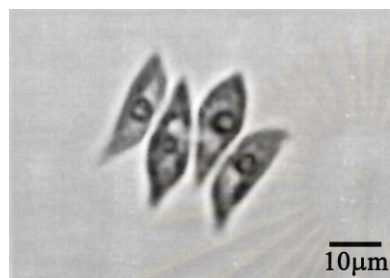
*Scenedesmus* sp. strain SS4 (TISTR 8879)



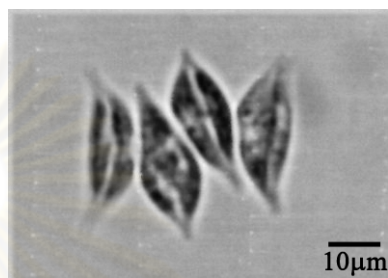
3 days



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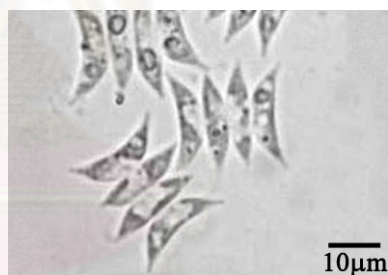


12 days

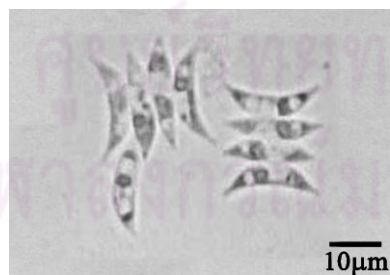
*Scenedesmus* sp. strain SS5 (TISTR 8880)



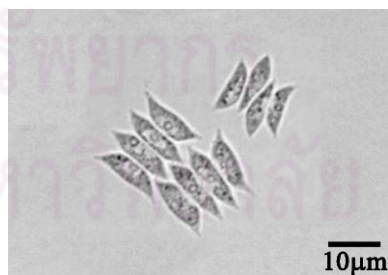
3 days



6 days



9 days



12 days

*Chlorella* spp. cells are unicellular with one cup-shaped chloroplast containing a prominent pyrenoid where starch is stored (Ikeda and Takeda, 1995). Diameters of the isolated *Chlorella* spp. ranged from 10-25  $\mu\text{m}$  (strains PK30 and PK38) to 2-5  $\mu\text{m}$  (strains PK10, PK37, and SS1). Two of the newly-isolated *Chlorella* spp. strains used in this study were collected from soil samples in soybean cultivation areas in Nam Moup (strain PK30) and Na Lueang (strain PK38) subdistricts in Wiangsa district, Nan province. It is noted that the soil *Chlorella* spp. isolates were two to five times larger in diameter when compared with the freshwater strains. The finding that the two newly-isolated soil *Chlorella* spp. strains were much larger in diameter than the freshwater strains may be either a coincidence or the finding may have further implications of the distribution and ecology of *Chlorella* spp. The soil *Chlorella* spp. strains appeared in large patches of green coloration in the soybean cultivation areas which have been planted with soybeans in rotation with rice as seen in the typical agricultural land in Nan and Phitsanulok in the northern and the upper central parts of Thailand. The relatively large diameters of the soil *Chlorella* spp. strains probably were a result of unintentional fertilization through residual chemical fertilizers used in the fields (Figure 4.2). A larger scale survey of *Chlorella* spp. in agricultural areas as well as in freshwater bodies will shed light on whether *Chlorella* spp. strains obtained from the fertile agricultural land are in fact relatively larger in diameters when compared with freshwater *Chlorella* spp. species. Large-scale surveys of soil and freshwater *Chlorella* spp. strains will provide information on the biodiversity of *Chlorella* spp. in Thailand for basic research on cryptic species of *Chlorella* spp. which have similar phenotypes but different genetic materials (Sawangdee et al., 2007). The biodiversity data will also provide *Chlorella* spp. strains for applied research on the industrial utilization of *Chlorella* spp.

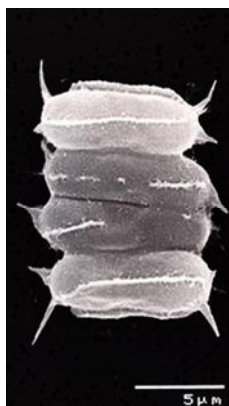




Figure 4.2 Green patch area in a field at Bueng Kok subdistrict, Bang Rakam district, Phitsanulok province in August 2008 showing agricultural land where soybeans were planted in rotation with rice and green patches of *Chlorella* sp. similar to the patches of land in Nam Moup and Na Lueng subdistricts, Wiangsa district, Nan province, where the large *Chlorella* spp. strains PK30 and PK38 were isolated in January 2006.

Diameters of *Chlorella* spp. cells were measured from cells under light microscope. Cells of *Chlorella* spp. strains PK30 and PK38 were found to have large average diameters of 10 - 25  $\mu\text{m}$ . Average diameters of the other 3 *Chlorella* strains (2-5  $\mu\text{m}$ ) were found to be much smaller than those of *Chlorella* spp. strains PK30 and PK38.

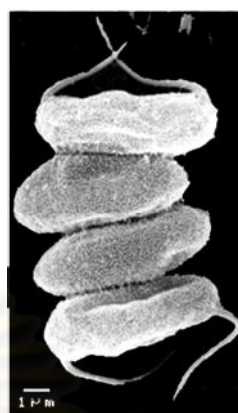
Figure 4.3 showed scanning electron micrographs of 4 *Desmodesmus* strains and 5 *Scenedesmus* strains.

*Desmodesmus*

NJ14



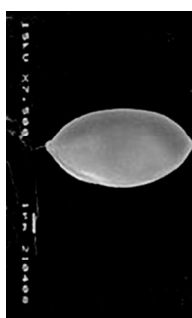
NJ23



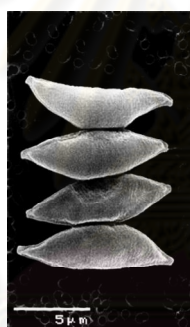
NJ40



NJ45

*Scenedesmus*

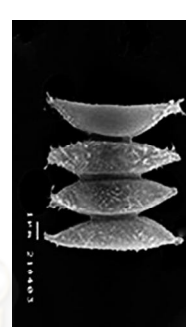
NJ42



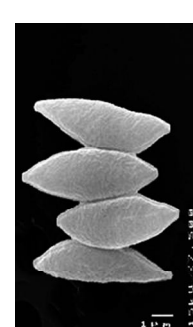
SS4



SS5



PK25



PK92

Figure 4.3 SEM micrographs of *Desmodesmus* spp. and *Scenedesmus* spp.

Table 4.1 Dimensions of *Desmodesmus* spp. and *Scenedesmus* spp.

| Genus              | Strain | Average cell width ( $\mu\text{m}$ ) | Average cell length ( $\mu\text{m}$ ) | Average spine length ( $\mu\text{m}$ ) |
|--------------------|--------|--------------------------------------|---------------------------------------|--|
| <i>Desmodesmus</i> | NJ14   | 2.93                                 | 8.62                                  | 2.10                                   |
|                    | NJ23   | 3.90                                 | 11.9                                  | 8.75                                   |
|                    | NJ40   | 2.56                                 | 7.13                                  | 2.52                                   |
|                    | NJ45   | 2.80                                 | 6.70                                  | 2.10                                   |
| <i>Scenedesmus</i> | NJ42   | 3.50                                 | 7.00                                  | No spines                              |
|                    | SS4    | 3.15                                 | 9.82                                  | No spines                              |
|                    | SS5    | 3.70                                 | 13.00                                 | No spines                              |
|                    | PK25   | 2.17                                 | 8.08                                  | No spines                              |
|                    | PK92   | 2.31                                 | 5.94                                  | No spines                              |

SEM micrographs of *Desmodesmus* spp. as shown in Figure 4.3 and their sizes as given in Table 4.1 showed the 4 strains contained spines with different lengths and the spines were either straight (NJ14 and NJ23) or curved (NJ40 and NJ45). In addition, rough surface with mid-ridges were observed in strains NJ14, NJ23, and NJ40. Pores in the middle lateral lines were found in strains NJ23 and NJ45. Strain NJ45 was found to have relatively spiny cells with short and sharp protuberances. The overall surface morphology indicated the 4 *Desmodesmus* spp. were different strains which could be used in this thesis as 4 representative species of the genus.

SEM micrographs and sizes of the 5 strains of *Scenedesmus* spp. as shown in Figure 4.3 and Table 4.1 revealed interesting findings. Cells of the 5 *Scenedesmus* strains were of different sizes with different cell organization and surface topology. Dimensions of the largest to the smallest strains as shown in Table 4.1 were as follows: SS5 > SS4 > NJ42 > PK92 > PK25. The 5 strains were found to have different types of cell surface and cell organization. Strains SS4, SS5, and PK25 were coenobia of 4 cells. Strain SS5's surface was slightly reticulated with a middle ridge while strain SS4 cells were densely reticulated with no mid-ridge. The surface of strain PK25 was covered with short protuberances. Cells of strain PK92 were arranged alternately. The cell surface was fairly reticulated with no mid-ridge. In BBM culture, cells of strain NJ42 were sometimes found to be unicellular with no organization into 4-celled coenobia. The cell surface of strain NJ42 was found to be relatively smooth.

#### 4.2 RAPD-PCR DNA fingerprints of 15 strains of *Chlorella* spp. , *Desmodesmus* spp. and *Scenedesmus* spp.

Figure 4.4 showed RAPD-PCR DNA fingerprints of the 15 strains of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. used in the experiments. The results showed the green microalgae had different DNA fingerprints. Therefore, they were different strains. The results also showed all morphologically similar *Chlorella* isolates (the large *Chlorella* strains PK30 and PK38 and the small *Chlorella* strains PK10, PK37, and SS1) have different RAPD-PCR DNA fingerprints. Therefore, cryptic species with similar morphology but different genetic composition exist in *Chlorella* spp. Results on morphology of *Desmodesmus* spp. and *Scenedesmus* spp as shown in the scanning electron micrographs in Figure 4.3 and their RAPD-PCR DNA fingerprints as shown in Figure 4.4 did not reveal any cryptic species in the 10 *Desmodesmus* spp. and *Scenedesmus* spp. used in the study.



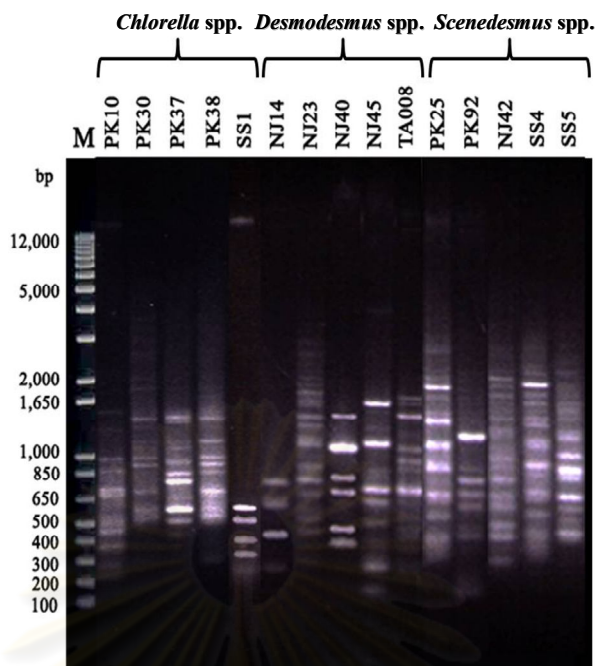


Figure 4.4 RAPD-PCR fingerprints of the 15 strains of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp.

#### 4.3 Growth at 25<sup>0</sup>C of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp.

Figures 4.5 – 4.7 showed growth patterns in 50 ml Bold's Basal Medium at 25<sup>0</sup>C under continuous light intensity of approximately 3,000 lux of 5 strains of *Chlorella* spp., 5 strains of *Desmodesmus* spp. and 5 strains of *Scenedesmus* spp. respectively.

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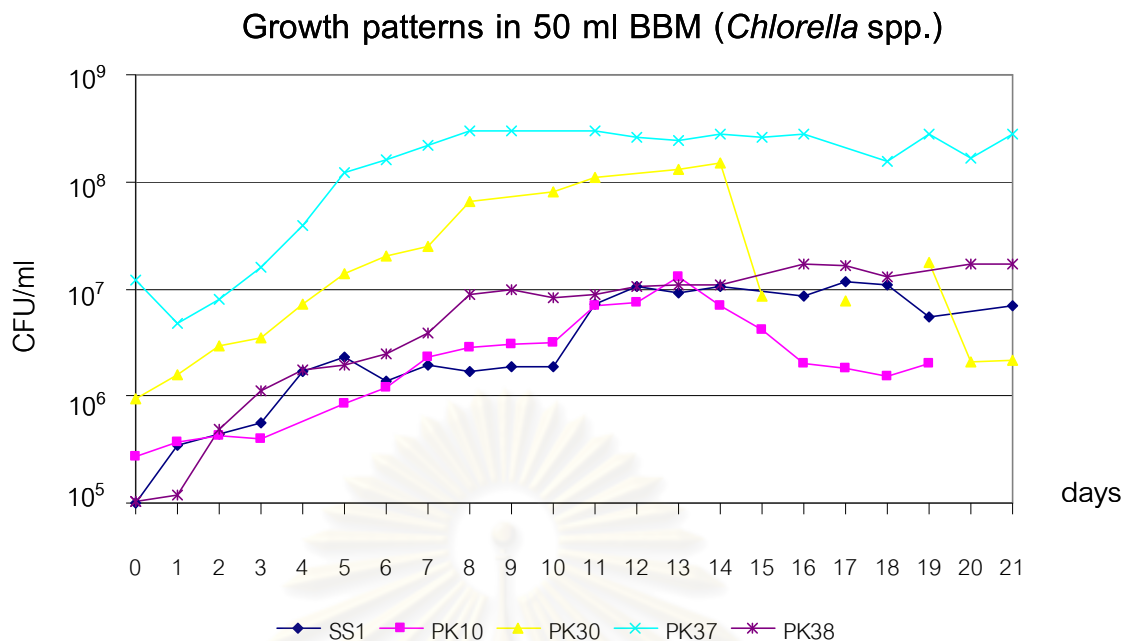


Figure 4.5 Growth of 5 strains of *Chlorella* spp. in 50 ml Bold's Basal Medium (BBM) at 200 rpm, 25°C under continuous light intensity of approximately 3,000 lux. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

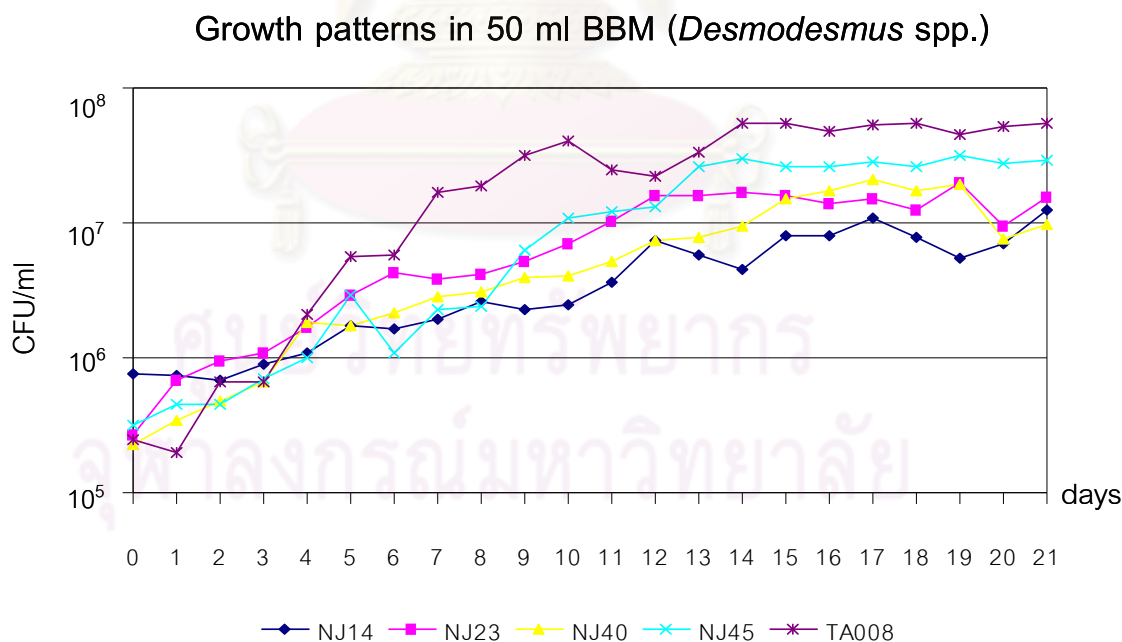


Figure 4.6 Growth of 5 strains of *Desmodesmus* spp. in 50 ml Bold's Basal Medium at 200 rpm, 25°C under continuous light intensity of approximately 3,000 lux. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.



### Growth patterns in 50 ml BBM (*Scenedesmus* spp.)

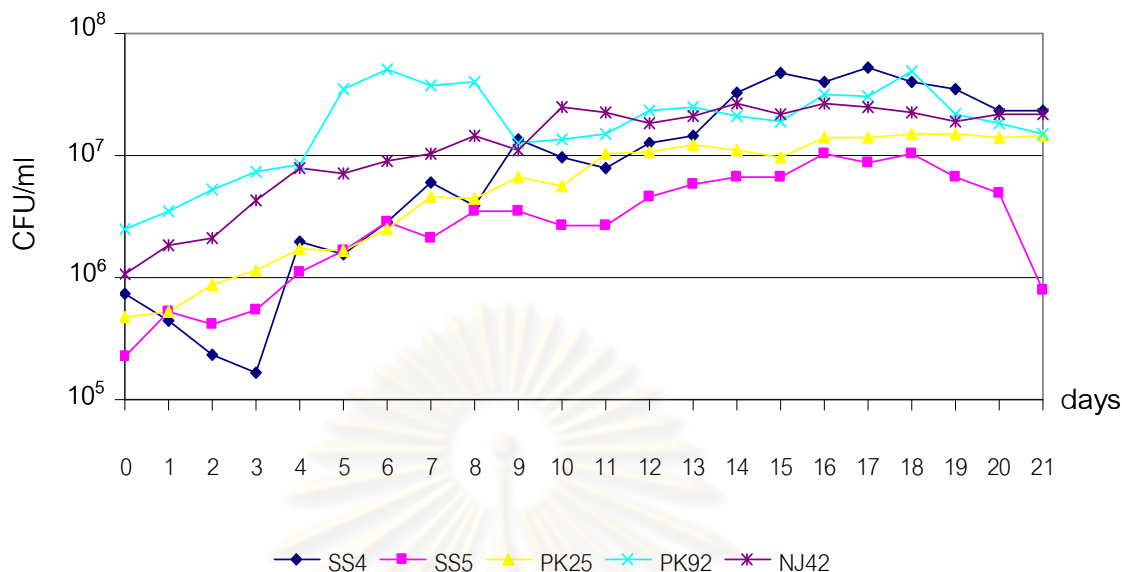


Figure 4.7 Growth of 5 strains of *Scenedesmus* spp. in 50 ml Bold's Basal Medium at 200 rpm, 25°C under continuous light intensity of approximately 3,000 lux. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

The results as shown in Figures 4.5 – 4.7 showed *Chlorella* sp. strain PK37 had the highest growth up to approximately  $5 \times 10^8$  CFU/ml in 8 days followed by *Chlorella* sp. strain PK30 which reached  $10^8$  CFU/ml in 10 days. *Scenedesmus* sp. strain PK92 was found to reach approximately  $7 \times 10^7$  CFU/ml in 6 days. The remaining strains of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. were found to have less growth. Cells were found to reach a maximum of only  $10^7$  CFU/ml in 8-13 days. Cells of *Desmodesmus* sp. strain NJ14 and *Scenedesmus* sp. strain SS5 were found to have the least growth which reached approximately  $10^7$  CFU/ml in 18 days. The specific growth rates as shown in Table 4.2 reflected the relative extent of growth as described above. The results showed that growth (CFU/ml) and specific growth rates at 25°C of the 10 strains of *Desmodesmus* spp. and *Scenedesmus* spp. were in the same range therefore the growth properties cannot be used to support the transfer of the spiny forms to the new genus *Desmodesmus*.

Table 4.2 Specific growth rates and doubling time of *Chlorella* spp. , *Desmodesmus* spp. and *Scenedesmus* spp. grown in 50 ml Bold's Basal Medium at 200 rpm, 25°C under continuous light intensity of approximately 3,000 lux. The same superscripts (a,b,c, etc.) indicated no significant differences ( $p < 0.05$ ) as determined by the Duncan's multiple range test.

| Strains                 | Specific growth rates (days <sup>-1</sup> ) | Double time (days) |
|-------------------------|---|--------------------|
| <i>Chlorella</i> spp.   |   |                    |
| PK10                    | 0.348 <sup>e</sup>                          | 2.0                |
| PK30                    | 0.531 <sup>b</sup>                          | 1.3                |
| PK37                    | 0.594 <sup>a</sup>                          | 1.2                |
| PK38                    | 0.517 <sup>c</sup>                          | 1.3                |
| SS1                     | 0.386 <sup>d</sup>                          | 1.8                |
| <i>Desmodesmus</i> spp. |   |                    |
| NJ14                    | 0.238 <sup>i</sup>                          | 2.9                |
| NJ23                    | 0.341 <sup>f</sup>                          | 2.0                |
| NJ40                    | 0.297 <sup>h</sup>                          | 2.3                |
| NJ45                    | 0.338 <sup>f</sup>                          | 2.1                |
| TA008                   | 0.390 <sup>d</sup>                          | 1.8                |
| <i>Scenedesmus</i> spp. |   |                    |
| PK25                    | 0.186 <sup>k</sup>                          | 3.7                |
| PK92                    | 0.316 <sup>g</sup>                          | 2.2                |
| NJ42                    | 0.300 <sup>h</sup>                          | 2.3                |
| SS4                     | 0.276 <sup>i</sup>                          | 2.5                |
| SS5                     | 0.162 <sup>l</sup>                          | 4.3                |

Since growth in microorganisms is obtained via an increase in cell numbers, the relatively fast growth rates observed for *Chlorella* spp. could be explained in terms of the organization of cells. *Chlorella* spp. might have more rapid growth rates when compared with *Desmodesmus* spp. and *Scenedesmus* spp because *Chlorella* cells are

relatively small (2-25  $\mu\text{m}$  in diameter) and unicellular. Cells are not organized into coenobia as found in *Desmodesmus* spp. and *Scenedesmus* spp. Reproduction in *Chlorella* spp. is through a formation of autospores inside a mother cell. According to Agrawal (2007), protoplast inside a *Chlorella* mother cell divided into 4 to 8 autospores which were released to grow to the size of vegetative cells of *Chlorella*. Figure 4.8 showed reproduction in *Chlorella* sp. strain PK37, *Desmodesmus* sp. strain TA008, and *Scenedesmus* sp. strain SS4. The micrographs showed released autospores in the *Chlorella* sp. strain PK37, released daughter coenobia with smaller size in *Scenedesmus* sp. strain SS4, and daughter coenobia just left the empty mother cells of *Desmodesmus* sp. strain TA008. It can be pictured from the micrographs that it is relatively faster to obtain daughter cells in unicellular *Chlorella* spp. since mother cells do not have to form coenobia before the release of daughter coenobia. Hence, unicellularity could be used to explain the relatively large number of CFU/ml and the relatively high specific growth rates observed in *Chlorella* spp. when compared with *Desmodesmus* spp. and *Scenedesmus* spp. as shown in Figures 4.5 – 4.7 and Table 4.2.

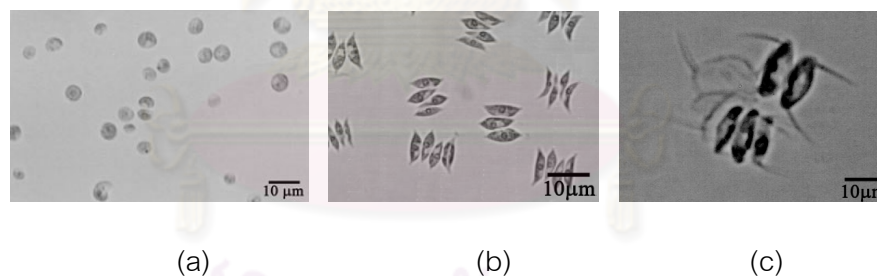


Figure 4.8 Growth of (a) *Chlorella* sp. strain PK37 (b) *Desmodesmus* strain SS4 and (c) Daughter coenobia just left the empty mother cells of *Desmodesmus* sp. strain TA008.

The results showed that, in terms of rapid and good growth for further industrial applications, unicellular green microalgae *Chlorella* spp. should be chosen over the multi-cellular *Desmodesmus* spp. and *Scenedesmus* spp. However, with the imminent global warming phenomenon, another characteristics of the green microalgae to be considered is the heat tolerance property.

#### 4.4 Growth at high temperature of *Chlorella* spp. , *Desmodesmus* spp. and *Scenedesmus* spp.

Figures 4.9 – 4.23 showed growth patterns of 5 strains of *Chlorella* spp., 5 strains of *Desmodesmus* spp. and 5 strains of *Scenedesmus* spp. in 150 ml Bold's Basal Medium at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C for 16/8 h in a 40°C temperature-controlled water bath placed in the greenhouse throughout the experiments.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Chlorella* PK10

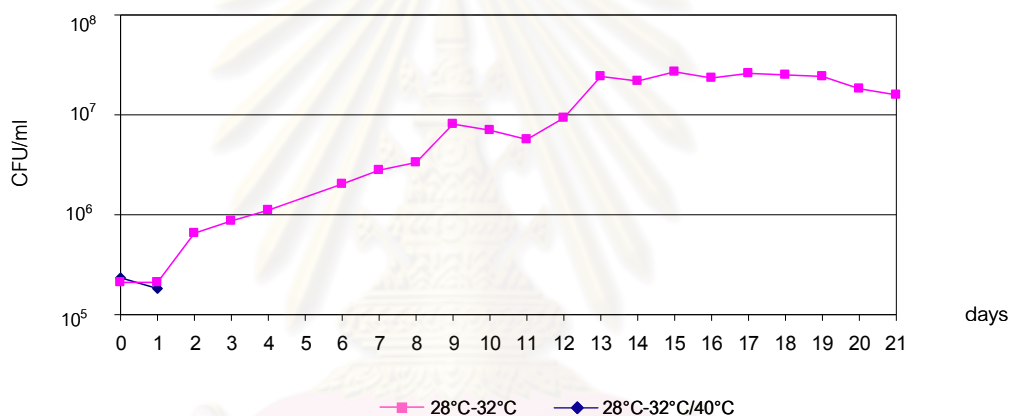


Figure 4.9 Growth patterns of *Chlorella* sp. strain PK10 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Strain PK10 was found to be heat-sensitive and died after culture at 28°C-32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h for one day. For the rest of the cultures, 5 ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Chlorella* PK30

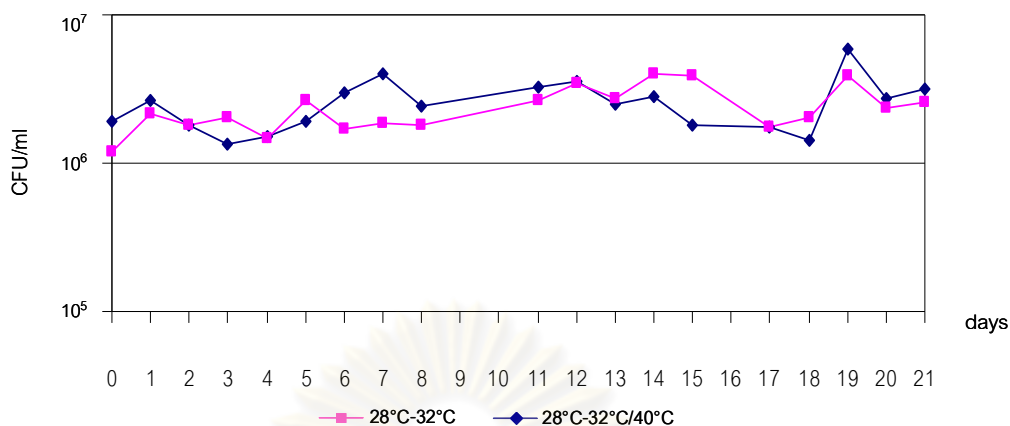


Figure 4.10 Growth patterns of *Chlorella* sp. strain PK30 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Chlorella* PK37

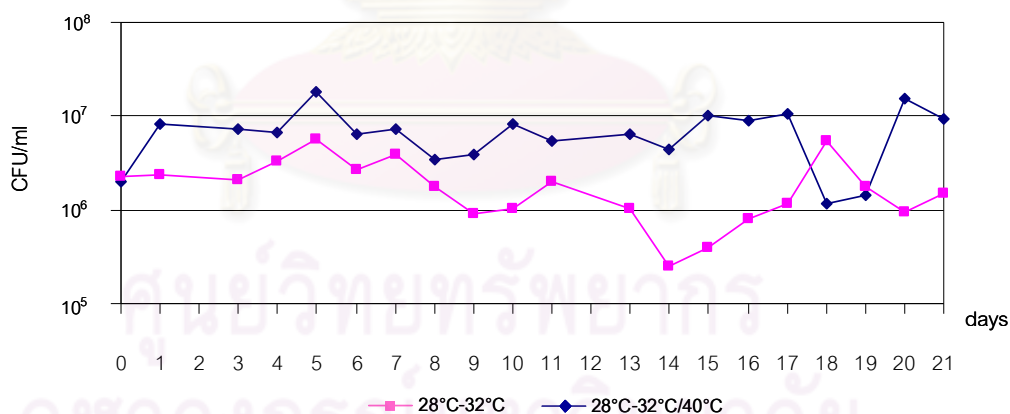


Figure 4.11 Growth patterns of *Chlorella* sp. strain PK37 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.



Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Chlorella* PK38

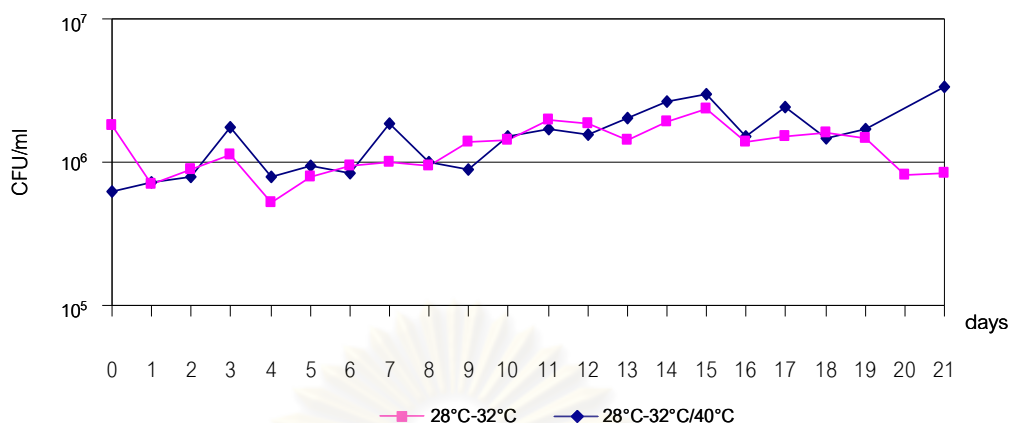


Figure 4.12 Growth patterns of *Chlorella* sp. strain PK38 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Chlorella* SS1

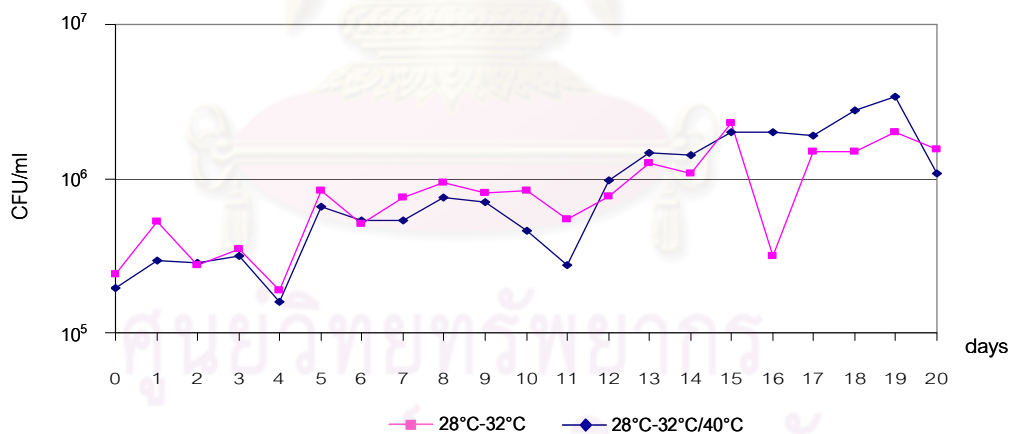


Figure 4.13 Growth patterns of *Chlorella* sp. strain SS1 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°/40°C *Desmodesmus* NJ14

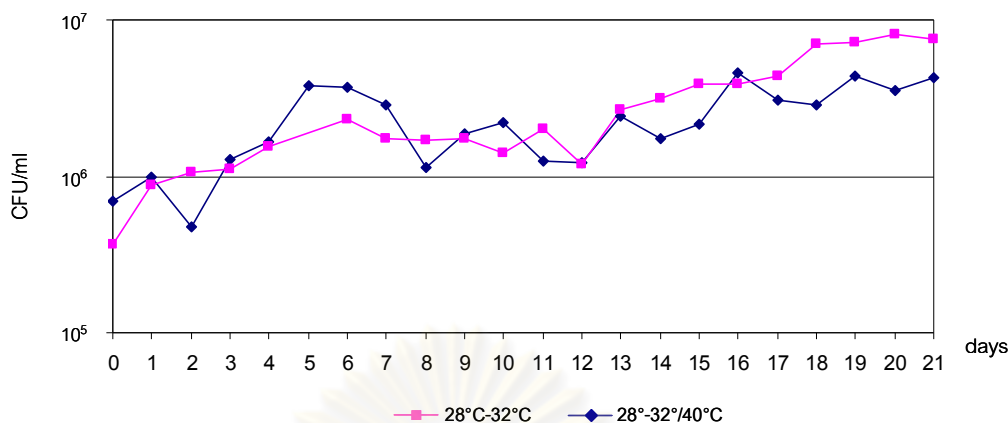


Figure 4.14 Growth patterns of *Desmodesmus* sp. strain NJ14 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C /40°C *Desmodesmus* NJ23

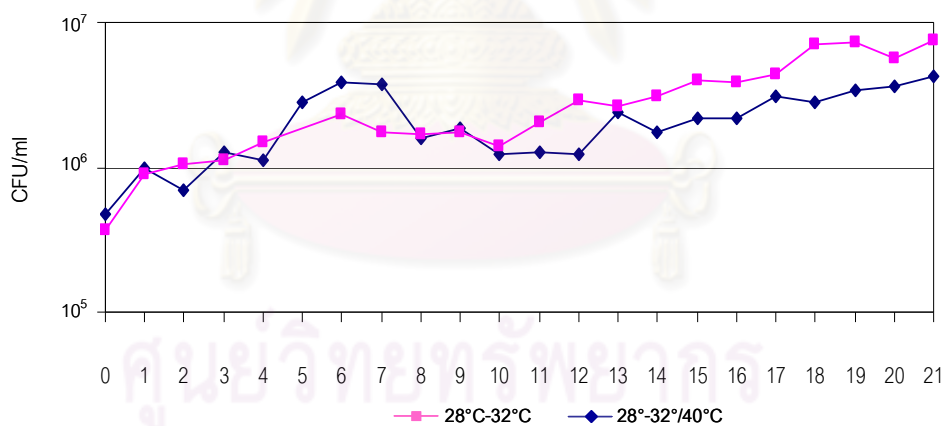


Figure 4.15 Growth patterns of *Desmodesmus* sp. strain NJ23 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Desmodesmus* NJ40

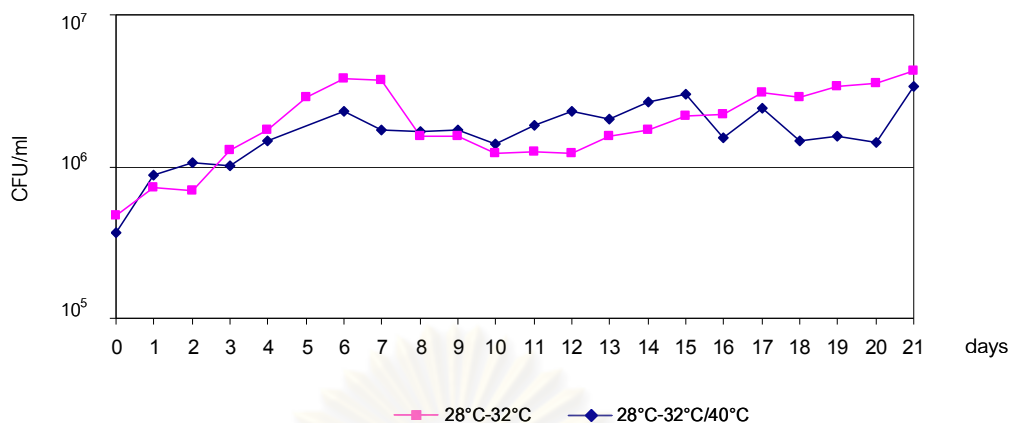


Figure 4.16 Growth patterns of *Desmodesmus* sp. strain NJ40 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Desmodesmus* NJ45

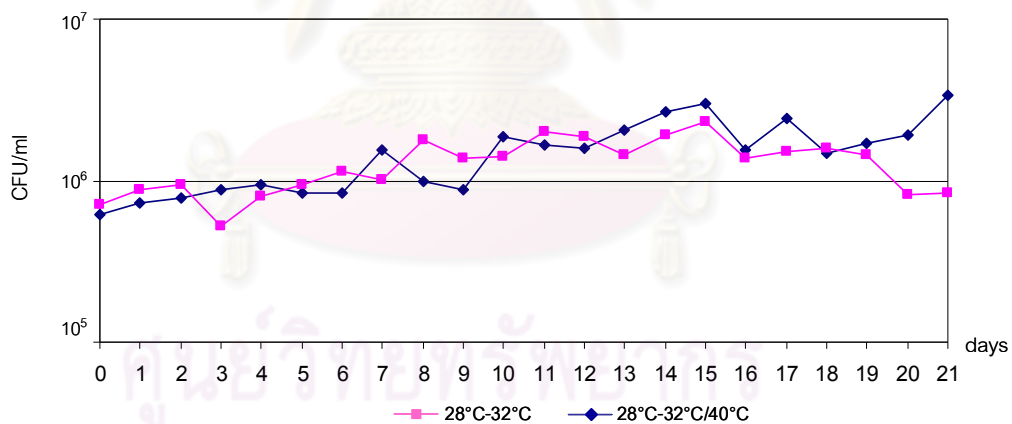


Figure 4.17 Growth patterns of *Desmodesmus* sp. strain NJ45 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Desmodesmus* TA008

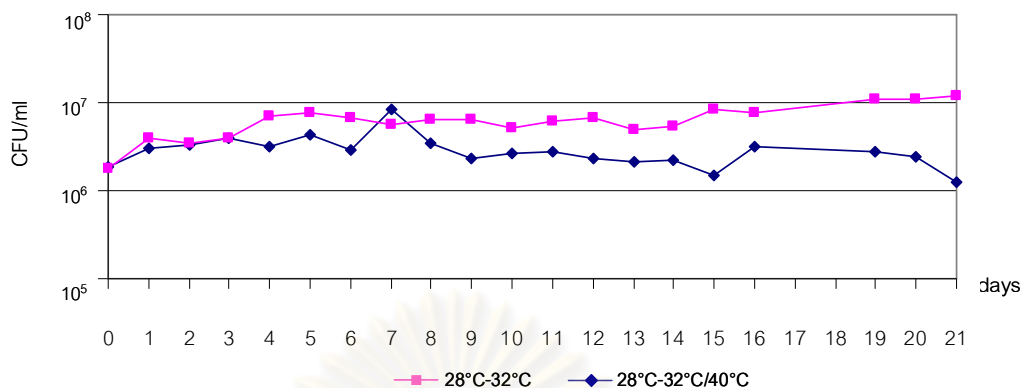


Figure 4.18 Growth patterns of *Desmodesmus* sp. strain TA008 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Scenedesmus* PK25

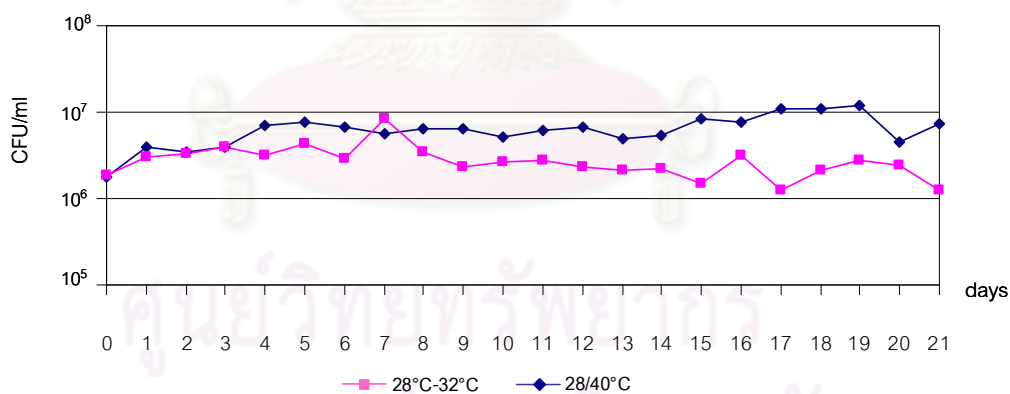


Figure 4.19 Growth patterns of *Scenedesmus* sp. strain PK25 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C and 28°C-32°C/40°C *Scenedesmus* PK92

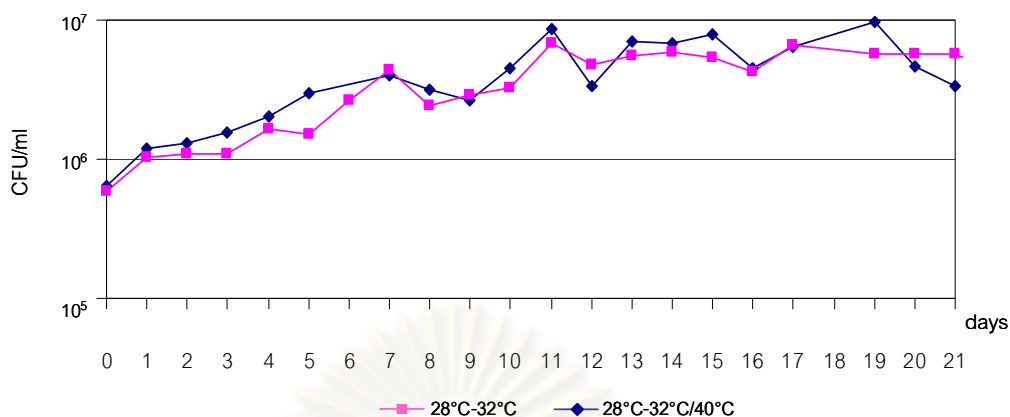


Figure 4.20 Growth patterns of *Scenedesmus* sp. strain PK92 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Scenedesmus* NJ42

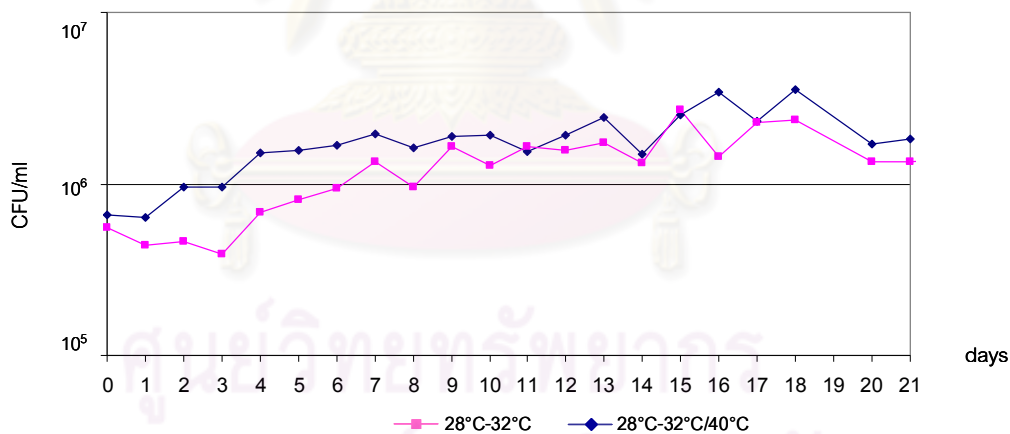


Figure 4.21 Growth patterns of *Scenedesmus* sp. strain NJ42 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.



Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Scenedesmus* SS4

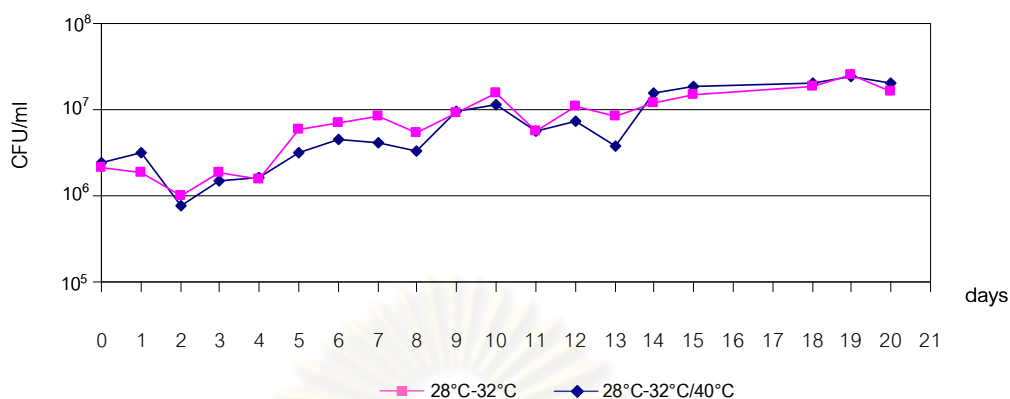


Figure 4.22 Growth patterns of *Scenedesmus* sp. strain SS4 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

Growth patterns in 150 ml BBM at 28°C-32°C and 28°C-32°C/40°C *Scenedesmus* SS5

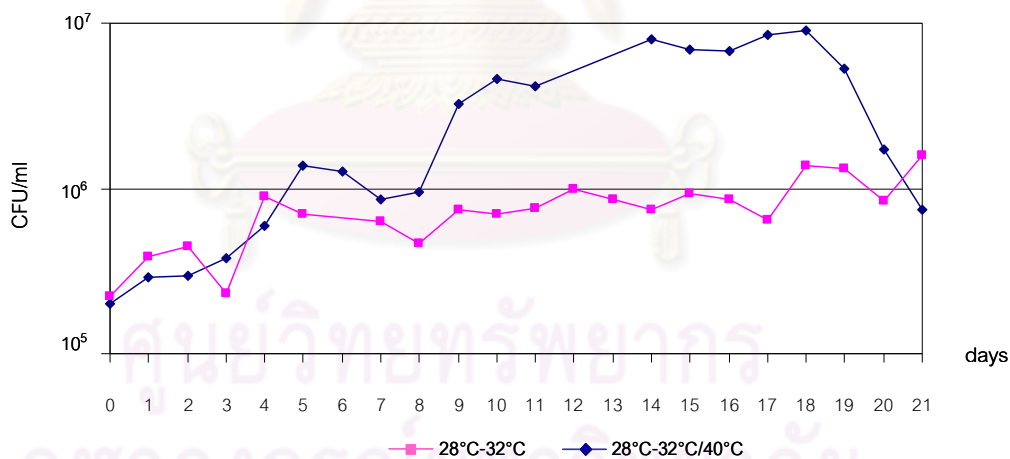


Figure 4.23 Growth patterns of *Scenedesmus* sp. strain SS5 grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments. Five ml of fresh BBM medium were added to the cultures at the end of the 10<sup>th</sup> day incubation. Each data point was an average of three replications.

The results of growth of the 15 green microalgal strains grown in 150 ml BBM at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C in a temperature controlled water bath placed in the greenhouse for 16/8 h throughout the experiments as shown in Figures 4.9–4.23 and Table 4.3 showed that *Desmodesmus* sp. strain NJ40 and *Scenedesmus* sp. strains SS4 and SS5 were mildly mesophilic with relatively high specific growth rates when grown at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments when compared with specific growth rates when grown at 28°C - 32°C. *Chlorella* spp. strains PK37 and SS1 were found to be heat – tolerant with higher specific growth rates when grown at 28°C - 32°C alternated with 40°C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments compared with growth at 28°C - 32°C. The following strains were found to have high specific growth rates when grown at 28°C - 32°C in the following decreasing order: *Desmodesmus* sp. strain NJ14 > *Chlorella* sp. strain PK37 > *Chlorella* sp. strain SS1 > *Chlorella* sp. strain PK10 > *Desmodesmus* sp. strain NJ23 . The results seemed to indicate that *Chlorella* spp. strains PK37 and SS1 were the green microalgae of choice when high productivity of biomass was desired at relatively high temperature (28°C - 32°C alternated with 40°C) while *Desmodesmus* sp. strain NJ40 and *Scenedesmus* sp. strains SS4 and SS5 could be used in comparative studies of mechanism(s) for mesophily in *Desmodesmus* spp. and *Scenedesmus* spp. The mechanism(s) of mesophily obtained could be used to either support or argue against the transfer of the spiny forms of what used to be named *Scenedesmus* spp. to the new genus *Desmodesmus*. Table 4.3 showed specific growth rates when grown at 28°C - 32°C and at 28°C - 32°C alternated with 40°C in a temperature controlled water bath placed in the greenhouse for 16/8 h throughout the experiments of the 15 green microalgal strains which reflected the findings of growth as described above.

Table 4.3 Specific growth rates and doubling time of 14 strains of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. grown in 150 ml Bold's Basal Medium at 200 rpm, at 28°C - 32°C and at 28°C - 32°C alternated with 40°C in a temperature controlled water bath placed in the greenhouse for 16/8 h throughout the experiments under continuous light intensity of approximately 3,000 lux. The same superscripts (a,b,c,etc.) indicate no significant difference at  $P < 0.05$  as determined by the Duncan's multiple range test.

| Strain code        | Specific growth rates (days <sup>-1</sup> ) |                     | Doubling time (days) |                 |
|--------------------|---|---------------------|----------------------|-----------------|
|                    | 28°C-32°C                                   | 28°C-32°C /40°C     | 28°C-32°C            | 28°C-32°C /40°C |
| <i>Chlorella</i>   |   |                     |                      |                 |
| PK10               | 0.455 <sup>cd</sup>                         | No growth           | 1.5                  | No growth       |
| PK30               | 0.272 <sup>e</sup>                          | 0.065 <sup>f</sup>  | 2.6                  | 10.7            |
| PK37               | 0.543 <sup>b</sup>                          | 0.287 <sup>c</sup>  | 1.3                  | 2.4             |
| PK38               | 0.122 <sup>g</sup>                          | 0.085 <sup>f</sup>  | 5.7                  | 8.2             |
| SS1                | 0.494 <sup>bc</sup>                         | 0.402 <sup>ab</sup> | 1.4                  | 1.7             |
| <i>Desmodesmus</i> |   |                     |                      |                 |
| NJ14               | 0.692 <sup>a</sup>                          | 0.118 <sup>ef</sup> | 1.0                  | 5.9             |
| NJ23               | 0.425 <sup>d</sup>                          | 0.191 <sup>d</sup>  | 1.6                  | 3.6             |
| NJ40               | 0.196 <sup>f</sup>                          | 0.425 <sup>a</sup>  | 3.5                  | 1.6             |
| NJ45               | 0.108 <sup>g</sup>                          | 0.106 <sup>ef</sup> | 6.4                  | 6.5             |
| TA008              | 0.168 <sup>fg</sup>                         | 0.169 <sup>de</sup> | 4.1                  | 4.1             |
| <i>Scenedesmus</i> |   |                     |                      |                 |
| PK25               | 0.169 <sup>fg</sup>                         | 0.168 <sup>de</sup> | 4.1                  | 4.1             |
| PK92               | 0.197 <sup>f</sup>                          | 0.187 <sup>d</sup>  | 3.5                  | 3.7             |
| NJ42               | 0.105 <sup>g</sup>                          | 0.161 <sup>de</sup> | 6.6                  | 4.3             |
| SS4                | 0.156 <sup>fg</sup>                         | 0.203 <sup>d</sup>  | 4.4                  | 3.4             |
| SS5                | 0.275 <sup>e</sup>                          | 0.352 <sup>b</sup>  | 2.5                  | 2.0             |

#### 4.5 SDS-PAGE separation of proteins in cells grown at different temperatures

Figure 4.24 showed SDS-PAGE separation of soluble proteins extracted from cells of 4 strains of *Chlorella* spp. (*Chlorella* sp. strain PK10 died when grown at 28°C - 32°C alternated with 40°C) , 5 strains of *Desmodesmus* spp. and 5 strains of *Scenedesmus* spp. in 150 ml Bold's Basal Medium at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C for 16/8 h in a 40°C temperature-controlled water bath placed in the greenhouse throughout the experiments.

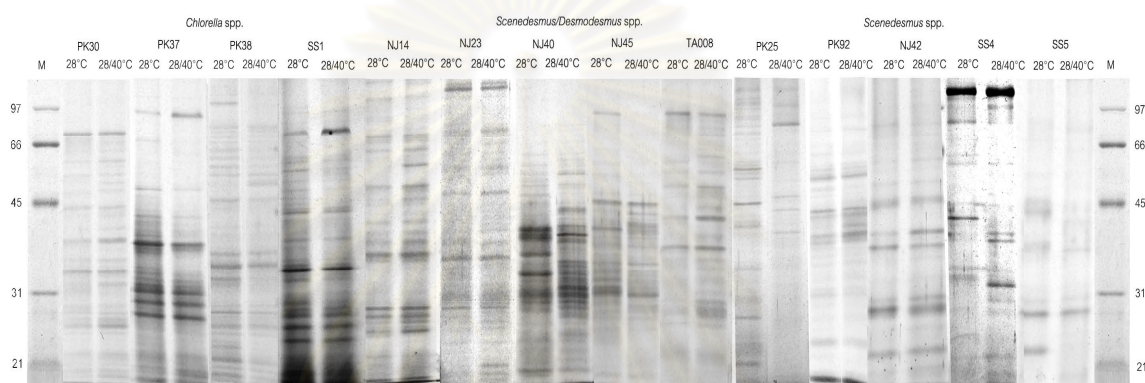
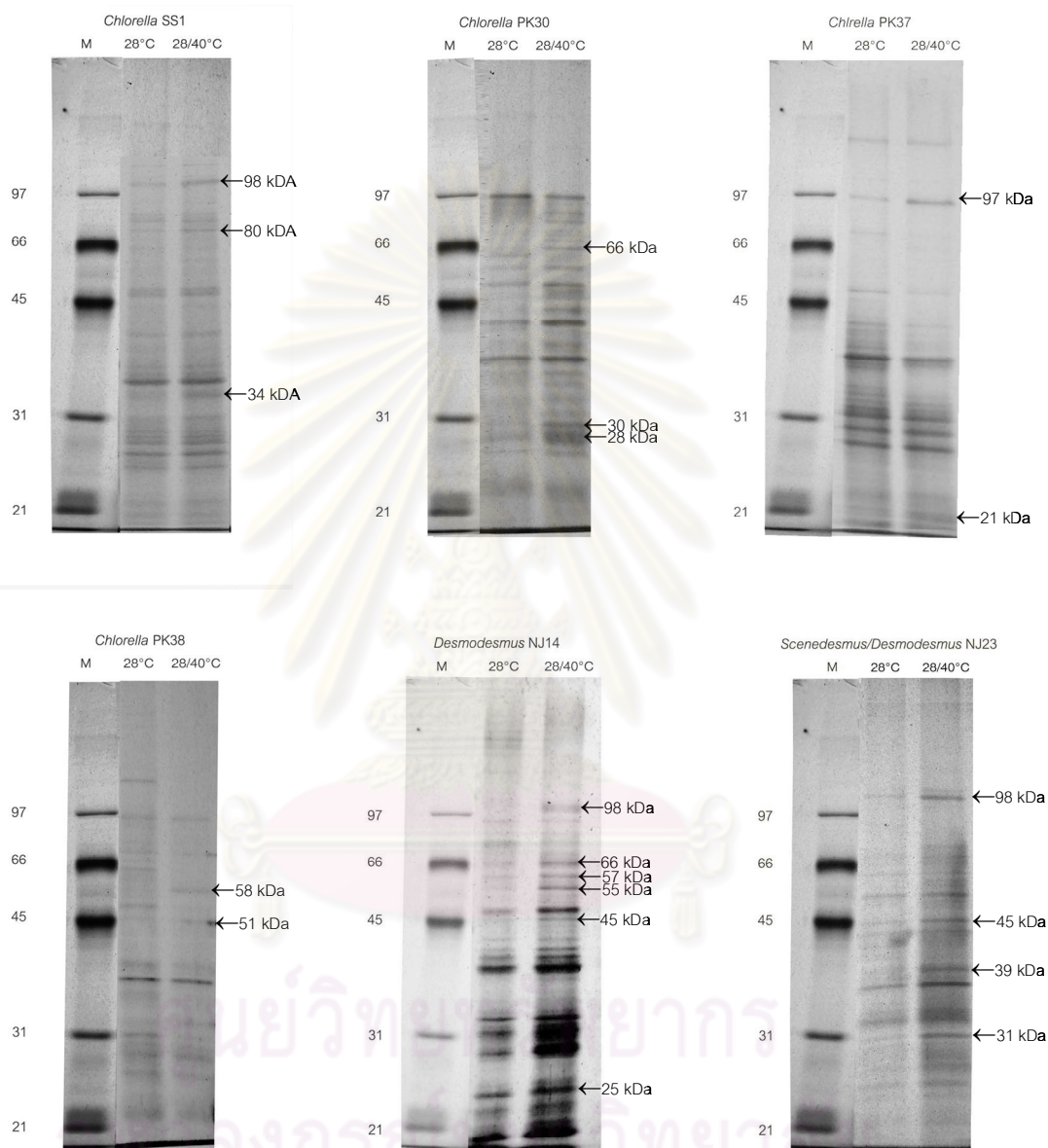


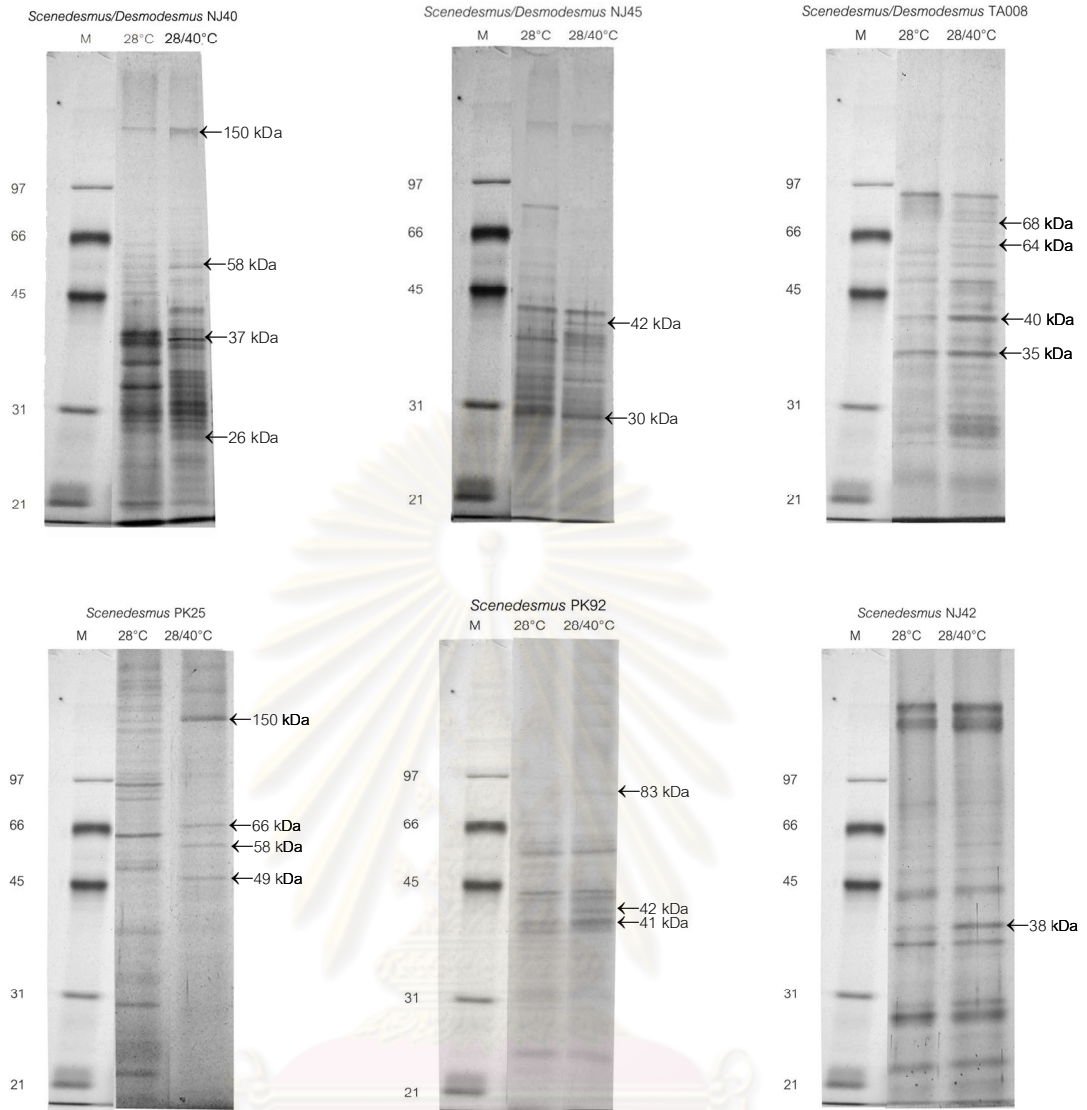
Figure 4.24 SDS-PAGE separation of soluble proteins in 14 strains of *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. grown in 150 ml Bold's Basal Medium at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C for 16/8 h in a 40°C temperature-controlled waterbath placed in the greenhouse throughout the experiments. Each well was loaded with 2 µg proteins as determined by the Bradford method.

SDS-PAGE profiles shown in Figure 4.24 were unsatisfactory and the experiments should have been repeated. The amounts of proteins loaded should have been adjusted to give the same silver staining intensity. Some bands were hardly observed. Thus, it was not possible to satisfactorily compare qualitative and quantitative differences in soluble protein profiles of the 14 green microalgal strains. However, the overall results of all the protein profiles showed that the profiles were different for strains within the same genus and in strains belonging to the different genera. Although it was not possible to obtain any meaningful results on comparative protein profiles of all the 14 green microalgal strains used in the experiments, it was possible to compare

quantitative differences in protein profiles of the same strain grown under different temperature regimes as shown in Figure 4.25.







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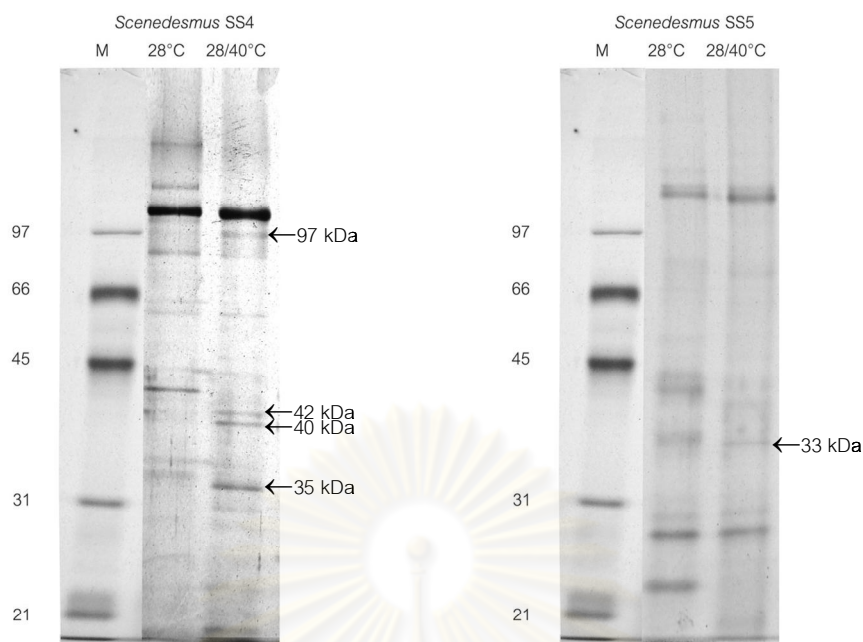


Figure 4.25 Quantitative differences in SDS-PAGE protein profiles of 4 *Chlorella* spp., 5 *Desmodesmus* spp. and 5 *Scenedesmus* spp. strains grown in 150 ml Bold's Basal Medium at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C for 16/8 h in a 40°C temperature-controlled water bath placed in the greenhouse throughout the experiments.

The results as shown in Figure 4.25 indicated that at 28°C - 32°C cells of *Scenedesmus* spp. strains NJ40 and SS5 were found to contain more of the polypeptides with molecular weight in the range of 30-45 kDa. All the rest of the 14 strains were found to contain more of some polypeptides when grown at 28°C - 32°C alternated with 40°C for 16/8 h in a 40°C temperature-controlled water bath placed in the greenhouse throughout the experiments as indicated in Figure 4.25 and as shown in Table 4.4.

Table 4.4 Molecular weights of some polypeptides found to be present more in cells of 4 *Chlorella* spp., 5 *Desmodesmus* spp. and 5 *Scenedesmus* spp. strains grown in 150 ml Bold's Basal Medium at 28°C - 32°C in a greenhouse and at 28°C - 32°C alternated with 40°C for 16/8 h in a 40°C temperature-controlled water bath placed in the greenhouse throughout the experiments.

| Strains                           | Molecular weight of polypeptides (kDa) |
|-----------------------------------|--|
| <i>Chlorella</i> spp. strain PK30 | 66, 30, 28                             |
| PK37                              | 97, 21                                 |
| PK38                              | 58, 51                                 |
| SS1                               | 98, 80, 34                             |
| <i>Desmodesmus</i> strain NJ14    | 98, 66, 57, 55, 45, 25                 |
| NJ23                              | 98, 45, 39, 31                         |
| NJ40                              | 150, 58, 37, 26                        |
| NJ45                              | 42, 30                                 |
| TA008                             | 68, 64, 40, 35                         |
| <i>Scenedesmus</i> strain PK25    | 150, 68, 66, 49                        |
| PK92                              | 83, 42, 41                             |
| NJ42                              | 38                                     |
| SS4                               | 97, 42, 40, 35                         |
| SS5                               | 33                                     |

### High molecular weight polypeptides

The results in Figures 4.24, 4.25 and Table 4.4 indicated that the heat-tolerant *Chlorella* spp. strains PK37 and SS1 grown at 28°C - 32°C alternated with 40°C contained more of the polypeptides in the high molecular weight range of 97-80 kDa when cells were grown at 28°C - 32°C alternated with 40°C for 16/8 h in a 40°C temperature-controlled water bath placed in the greenhouse throughout the experiments. In addition, the mesophilic *Desmodesmus* sp. strain NJ40 was found to contain high molecular weight 150 kDa when grown under the high temperature regime. However, the heat-sensitive *Scenedesmus* sp. strain PK25 was also found to contain more of the high molecular weight 150 kDa polypeptide, the heat-sensitive *Desmodesmus* spp. strains NJ14 and NJ23 were found to contain more of the 98 kDa polypeptide and the heat-sensitive *Scenedesmus* sp. strain PK92 were found to contain more of the high molecular weight 83 kDa polypeptide when grown under the high temperature regime. Therefore, no conclusive evidence was obtained for the exclusive presence of the high molecular weight polypeptides in heat-tolerant *Chlorella* spp. strains PK37 and SS1 and in mesophilic *Desmodesmus* sp. strain NJ40. Moreover, the protein staining intensity in SDS-PAGE protein profiles of the mesophilic *Scenedesmus* spp. strains SS4 and SS5 as shown in Figure 4.25 was so weak that the experiments needed to be repeated to find out if there were any differences in the quantities of high molecular weight polypeptides when cells of these two mesophilic *Scenedesmus* spp. strains were grown under the high temperature regime.

### Medium to Low molecular weight polypeptides

The results in Figures 4.24, 4.25 and Table 4.4 indicated that, under the 28°C - 32°C alternated with 40°C regime, the heat-tolerant *Chlorella* spp. strains PK37 and SS1 contained more of the low molecular weight polypeptides 21 kDa and 34 kDa respectively. In addition, the mesophilic *Desmodesmus* sp. strain NJ40 and the mesophilic *Scenedesmus* spp. strains SS4 and SS5 were found to contain more of medium and low molecular weight 58 kDa, 37 kDa, 26 kDa (*Desmodesmus* sp. strain NJ40), 42 kDa, 40 kDa, 35 kDa (*Scenedesmus* sp. strain SS4) and 33 kDa

(*Scenedesmus* sp. strain SS4) respectively. However, medium and low molecular polypeptides were also detected in heat-sensitive cells of *Chlorella* spp. strains PK30 and PK38, *Desmodesmus* spp. strains NJ14, NJ23, NJ45 and TA008, and *Scenedesmus* spp. strains grown under the 28°C - 32°C alternated with 40°C regime. The 66-68 kDa polypeptides are in the same molecular weight range as the 70 kDa heat shock protein while the low molecular weight polypeptides with molecular weights 55-58 kDa are in the same range as the 60 kDa GroEL. Further experiments on Western blotting will reveal if the observed increased polypeptides detected in cells were grown under the 28°C - 32°C alternated with 40°C regime were heat shock proteins.

#### 4.6 Determination of $\beta$ -carotene contents

##### 4.6.1 Growth patterns in 1.5 liters BBM medium at 25°C

Figure 4.26 showed growth patterns in 1.5 liters BBM medium of 11 strains of green microalgae at 25°C, 200 rpm, in an illuminated incubator shaker with continuous light intensity of approximately 3,000 lux. Table 4.5 showed their specific growth rates and doubling time.



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## Growth patterns in 1.5 liters BBM

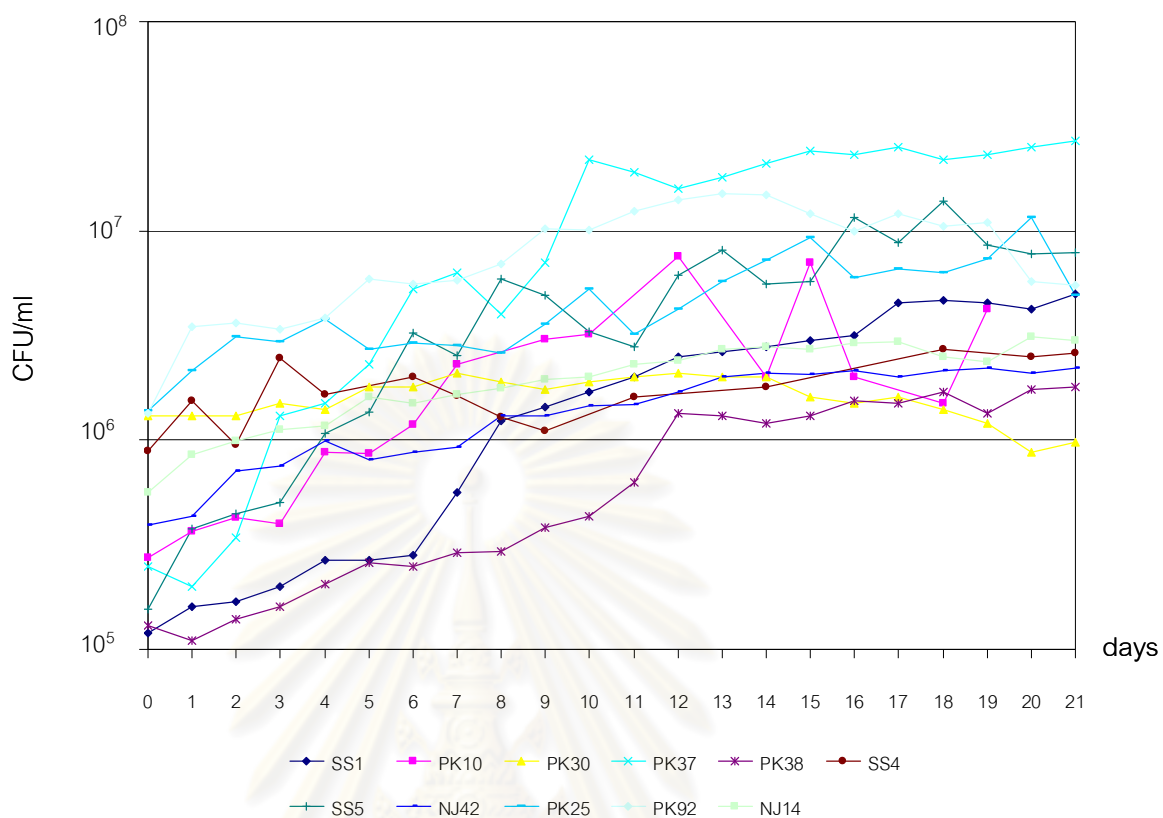


Fig. 4.26 Growth patterns of each of the 11 strains of *Chlorella* spp. strains SS1, PK10, PK30, PK37, PK38, *Scenedesmus* spp. strains SS4, SS5, NJ42, PK25, PK92, and *Desmodesmus* sp. strain NJ14 grown in 1.5 liters BBM medium at 25°C, 200 rpm, in an illuminated incubator shaker with continuous light intensity of approximately 3,000 lux. Each data point was an average of three replications.

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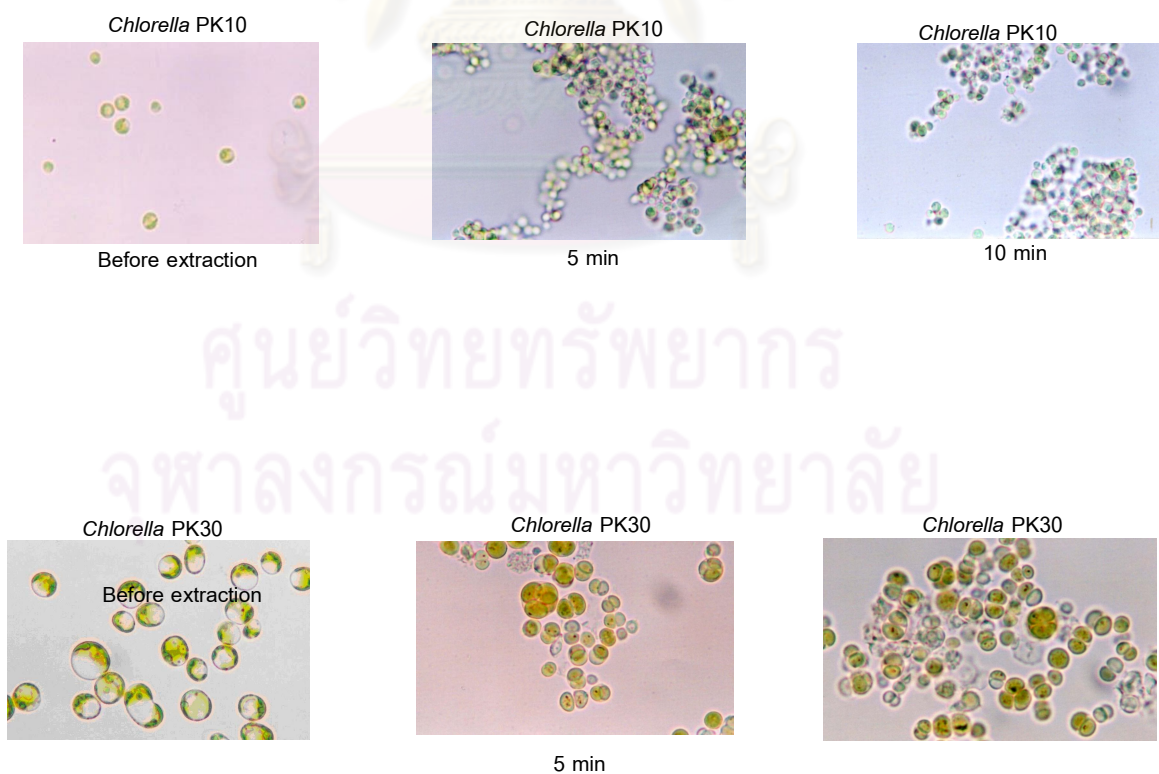
Table 4.5 Specific growth rates and doubling time of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. grown in 1.5 liters Bold's Basal Medium at 25°C, 200 rpm, in an illuminated incubator shaker with continuous light intensity of approximately 3,000 lux. The same superscripts (a,b,c, etc.) indicated no significant differences ( $P < 0.05$ ) as determined by the Duncan's Multiple Range Test.

| Strain code        | Specific growth rates (days <sup>-1</sup> ) | Doubling time (days) |
|--------------------|---|----------------------|
| <i>Chlorella</i>   |   |                      |
| PK10               | 0.287 <sup>c</sup>                          | 2.4                  |
| PK30               | 0.080 <sup>f</sup>                          | 8.7                  |
| PK37               | 0.318 <sup>bc</sup>                         | 2.2                  |
| PK38               | 0.227 <sup>d</sup>                          | 3.1                  |
| SS1                | 0.365 <sup>b</sup>                          | 1.9                  |
| <i>Desmodesmus</i> |   |                      |
| NJ14               | 0.088 <sup>f</sup>                          | 7.9                  |
| <i>Scenedesmus</i> |   |                      |
| PK25               | 0.182 <sup>de</sup>                         | 3.8                  |
| PK92               | 0.161 <sup>e</sup>                          | 4.3                  |
| NJ42               | 0.093 <sup>f</sup>                          | 7.5                  |
| SS4                | 0.135 <sup>ef</sup>                         | 5.1                  |
| SS5                | 0.490 <sup>a</sup>                          | 1.4                  |

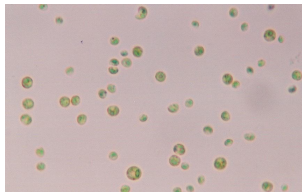
The results as shown in Table 4.5 showed *Chlorella* spp. strains PK37 and SS1 still increased in number rapidly as obtained in 50 ml BBM medium (Table 4.2). However, *Scenedesmus* sp. strain SS5 showed the highest specific growth rate when the medium was increased from 50 ml to 1.5 liters under the same experimental conditions. All strains of *Chlorella* spp. except strain PK30 showed good growth at 25°C, 200 rpm, in an illuminated incubator shaker with continuous light intensity of approximately 3,000 lux. Mid-log phase cells of 5 strains (*Chlorella* spp. strains PK30, PK38, *Desmodesmus* sp. strain NJ14, and *Scenedesmus* spp. strains PK25 and PK92) were extracted for the determination of  $\beta$ -carotene contents present in the cells at 25°C, 200 rpm, in an illuminated incubator shaker with continuous light intensity of approximately 3,000 lux.

#### 4.6.1 Determination of $\beta$ -carotene contents in mid-log phase cells

Figure 4.27 showed micrographs of *Chlorella* spp. strains PK10, PK30, PK37, *Desmodesmus* sp. strain NJ14, and *Scenedesmus* spp. strains PK25, PK92, NJ42, and SS4 broken by incubating lyophilized cells in Ethanol and 60% KOH at 50°C for 5 min and 10 min. Although *Chlorella* spp. strains PK10 and PK30 cells were found to be partially broken even with the 10 min incubation period, the 10 min incubation period was chosen for use in breaking cells to release intracellular contents because the conditions were found to provide satisfactory cell breakage for the other strains as shown in Figure 4.27. The method of breaking cells by incubating in Ethanol and 60% KOH at 50°C for 10 min was chosen over the breakage method using vortexing cells in an extraction buffer with acid-washed glass beads because the latter method yielded a lesser extent of cell breakage (results not shown).

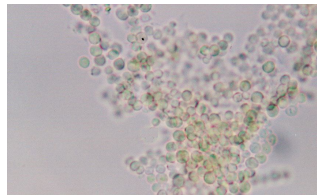


*Chlorella* PK37



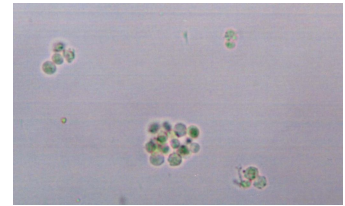
Before extraction

*Chlorella* PK37



5 min

*Chlorella* PK37



10 min

*Desmodesmus* NJ14



Before extraction

*Desmodesmus* NJ14



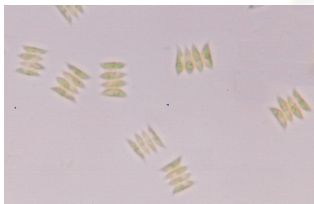
5 min

*Desmodesmus* NJ14



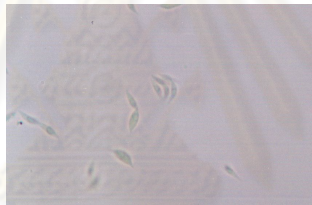
10 min

*Scenedesmus* PK25



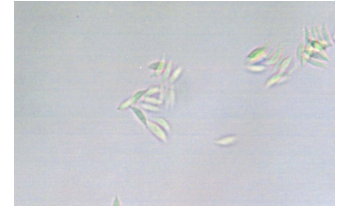
Before extraction

*Scenedesmus* PK25



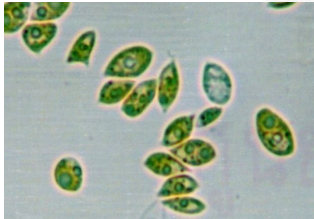
5 min

*Scenedesmus* PK25



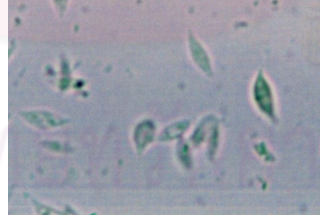
10 min

*Scenedesmus* PK92



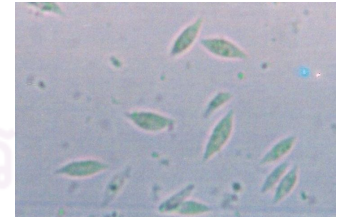
Before extraction

*Scenedesmus* PK92



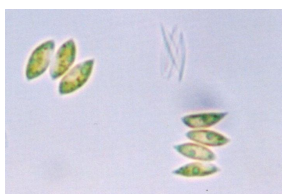
5 min

*Scenedesmus* PK92



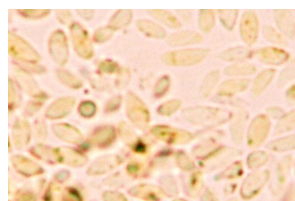
10 min

*Scenedesmus* NJ42



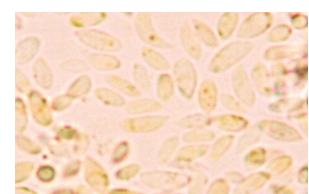
Before extraction

*Scenedesmus* NJ42



5 min

*Scenedesmus* NJ42



10 min

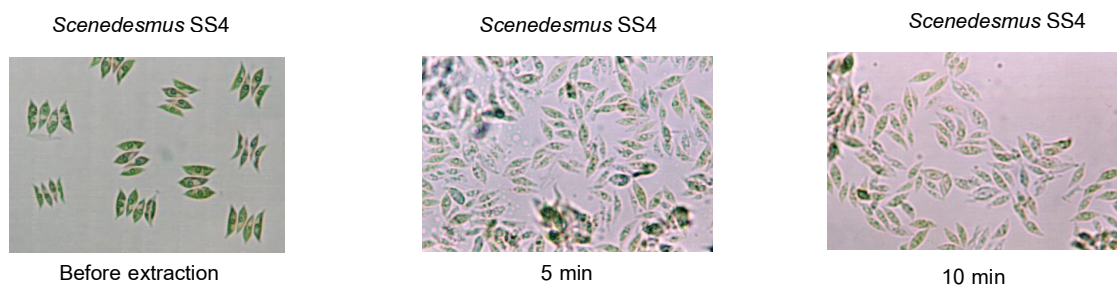


Figure 4.27 Micrographs of cells of some green microalgae before and after cell breakage in Ethanol and 60% KOH at 50°C for 5 min and 10 min.

Table 4.6  $\beta$ -carotene contents in mid-log phase cells of some green microalgae grown in 1.5 liters Bold's Basal Medium at 25°C, 200 rpm, in an illuminated incubator shaker with continuous light intensity of approximately 3,000 lux.

| Strains | Area under peak                    | $\beta$ -carotene (ug/20ul injection volume) | $\beta$ -carotene (ug/ 500 ul or 60mg) | $\beta$ -carotene in 1 mg dried cells | Percent recovery | $\beta$ -carotene (ug/mg dried cells) |
|---------|------------------------------------|--|--|---------------------------------------|------------------|---------------------------------------|
| PK92    | 215331                             | 0.960  | 23.990                                 | 0.400                                 | 6.6              | 6.039                                 |
| SS1     | 969216                             | 4.319  | 107.978                                | 1.800                                 | 36.2             | 4.971                                 |
| SS5     | 37332                              | 0.166  | 4.159                                  | 0.069                                 | 7.7              | 0.897                                 |
| PK38    | 118541                             | 0.528  | 13.206                                 | 0.220                                 | 47.0             | 0.468                                 |
| NJ42    | 181223                             | 0.808  | 20.190                                 | 0.336                                 | 90.0             | 0.374                                 |
| PK30    | 33155                              | 0.148  | 3.694                                  | 0.062                                 | 32.9             | 0.187                                 |
| SS4     | 17071                              | 0.076  | 1.902                                  | 0.032                                 | 17.8             | 0.179                                 |
| PK37    | 29974                              | 0.134  | 3.339                                  | 0.056                                 | 31.7             | 0.176                                 |
| NJ14    | 22132                              | 0.099  | 2.466                                  | 0.041                                 | 32.8             | 0.125                                 |
| PK10    | 1680                               | 0.007  | 0.187                                  | 0.003                                 | 21.9             | 0.014                                 |
| PK25    | -----No peak of beta carotene----- |  |  |                                       | 13.4             | Can not calculate                     |

Table 4.6 showed results of  $\beta$ -carotene contents in mid-log phase cells of the mesophilic *Scenedesmus* sp. strain SS5, heat-tolerant *Chlorella* spp. strains PK37, SS1 and heat-sensitive *Chlorella* spp. strains PK10, PK30, PK38, *Desmodesmus* sp. strain NJ14, *Scenedesmus* spp. strains PK92, NJ42, and SS4 grown at 25°C, 200 rpm, in an illuminated incubator shaker with continuous light intensity of approximately 3,000 lux. No  $\beta$ -carotene was extracted from *Scenedesmus* sp. strain



PK25 even though the extraction and determination of  $\beta$ -carotene contents were repeated three times. The results showed a maximum recovery of 90% for *Scenedesmus* sp. strain NJ42. The high  $\beta$ -carotene found in this strain was probably due to a combination of the relatively high specific growth rate ( $0.227 \text{ days}^{-1}$ , Table 4.5) and the high percentage of recovery. The second best percentage of recovery of 33% was obtained for the majority of the strains used in the study including *Desmodesmus* sp. strain NJ14 and *Chlorella* sp. strain PK30. Since cells of *Chlorella* sp. strain PK30 were found to be partially broken as shown in Figure 4.27,  $\beta$ -carotene content in this strain should be more than the reported value. The high  $\beta$ -carotene content obtained in *Scenedesmus* sp. strain PK92 was probably due to its larger cells which were completely broken to release the  $\beta$ -carotene content. No correlation between  $\beta$ -carotene content and mesophily was found in this study.



## CHAPTER V

### DISCUSSION

#### 5.1 Selection of fast-growing and heat-tolerant green microalgal strains

*Chlorella* spp. strains PK37 and SS1, *Desmodesmus* sp. strain NJ40, and *Scenedesmus* sp. strain SS5 were found to have better growth when grown at 28<sup>o</sup>C - 32<sup>o</sup>C alternated with 40<sup>o</sup>C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments compared with growth at 28<sup>o</sup>C - 32<sup>o</sup>C in the greenhouse. The reason cells had to be grown at 28<sup>o</sup>C - 32<sup>o</sup>C alternated with 40<sup>o</sup>C in a temperature-controlled water bath placed in the greenhouse for 16/8 h throughout the experiments was because the green microalgal strains might not have survived had they been grown continuously at 40<sup>o</sup>C. The culture of green microalgae at relatively low temperature alternated with high temperature is a normal procedure in green algal physiological studies.

The growth characteristics in an illuminated incubator shaker at 25<sup>o</sup>C, approximately 3000 lux continuous light intensity and growth characteristics at 28<sup>o</sup>C – 32<sup>o</sup>C and 28<sup>o</sup>C – 32<sup>o</sup>C (16h) alternated with growth at 40<sup>o</sup>C in a temperature-controlled waterbath with light intensity in a greenhouse of the 15 strains of *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. provide data for the selection of relatively fast-growing strains of the three genera under heat stress (40<sup>o</sup>C for 8 h for 21 days) for further industrial exploitation. As an example, with the global warming phenomenon, the earth temperature is expected to rise. The selection of fast-growing, heat-tolerant green microalgal strains could be useful in algal biotechnology. With the trend towards moving away from the use of food crops such as corn and cassava for the production of biofuels there is a trend towards the use of microalgae as a source of biofuels (Gouveia and Oliveira, 2009; Mandal and Mallick, 2009; Xu et al., 2006).

*Chlorella* spp. strains PK37 and SS1 were found to be heat-tolerant while *Desmodesmus* sp. strain NJ40 and *Scenedesmus* sp. strain SS5 were found to be mesophilic (Table 4.3). With the results on the growth, heat-tolerance and mesophilic growth characteristics of the 15 strains of green microalgae obtained in this thesis,



strains could be selected for further research on mechanisms for heat tolerance and mesophilicity in *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. The mechanisms for heat tolerance and mesophilic growth characteristics in *Desmodesmus* spp. and *Scenedesmus* spp. could be used to either support or argue against the transfer of group formerly known *Scenedesmus* spp. with spines into a new genus *Desmodesmus* (An et al., 1999). The RAPD-PCR fingerprints could be used for quality control in the uses of the microalgae for industrial applications. Upon prolonged use of the algae there may be alterations including deletion, insertion or gene duplication which may reflect in changes in the RAPD-PCR fingerprints. Once changes in RAPD-PCR fingerprints are detected, the quality control personnel of an algal factory can get rid of the currently-used culture and get a new culture of the same strain which has been kept, for example, as a long-term preservation under 10% glycerol at -80°C.

In this thesis, *Chlorella* spp. strains PK30 and PK38 were isolated from soils in Nam Muap and Na Lueang subdistricts in Nan province respectively. Other researchers also isolated *Chlorella* spp. from soil samples. In 2009, Chader et al. isolated *Chlorella sorokiniana* strain Ce, *Chlorella salina* strain Mt, and *Chlorella* sp. strain Pt6, from soil in the Algerian Sahara. Lien et al. (1979) isolated *Chlorella* sp. from soils contaminated with sodium fluoroacetate or "Compound 1080" which is extensively used for the control of opossum and rabbits in the South Island of New Zealand. Megharaj et al. (1992) isolated *Chlorella vulgaris* and *Scenedesmus bijugatus* from soil and used immobilized cells to remove nitrogen and phosphorus from waste water.

Micrographs were taken of the 15 strains of green microalgae grown at 25°C under a continuous light intensity of approximately 3,000 lux for 3, 6, 9, and 12 days. The rationale for presenting the micrographs of different-day-old cells was to illustrate that at different times during the life cycle of the microalgae, cells of different sizes and in some cases, different shapes, were obtained depending of the stage of growth of the autospores in *Chlorella* spp. or daughter coenobia in the case of *Desmodesmus* spp. and *Scenedesmus* spp. as illustrated in Figure 4.1 for all the *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. strains. Different cell morphology at different times of incubation was observed for *Desmodesmus* sp. strain NJ14 and *Scenedesmus* spp. strains PK92 and NJ42. Changes in cell morphology during

different stages of growth in the cell cycle in *Desmodesmus* spp. and *Scenedesmus* spp. have been well-documented as phenotypic plasticity in *Desmodesmus* spp. and *Scenedesmus* spp. (Lurling, 2003).

The 15 green microalgal strains have been deposited at the algal culture collection of the Thailand Institute for Scientific and Technological Research (TISTR) as part of an effort to establish a world-class culture collection of microalgae as normally done in other countries such as The American Type Culture Collection and the *Scenedesmus* strains of the culture collection of the University of Texas at Austin (UTEX) in USA (Hegewald, 1989), the NIES culture collection in Japan and in Ukraine where more than 430 species of halophilic and freshwater green microalgae including 50 strains of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. have been kept. Most (150 strains) of the collection are halotolerant *Dunaliella* spp. for biotechnological applications including production of  $\beta$ -carotene (Borisova and Tsarenko, 2004). The algae in the culture collection serve as starting culture materials for basic and applied research for industrial applications.

## 5.2 Protein profiles and $\beta$ -carotene contents in *Chlorella* spp. *Desmodesmus* spp. and *Scenedesmus* spp.

Table 4.4 showed different sets of polypeptides of different sizes with molecular weight ranging from 98 kDa to 21 kDa. were found to increase when 4 different strains of *Chlorella* spp. were grown under the high temperature regime. Each *Chlorella* strain seemed to have increased amounts of different polypeptides. In 1987 Valliammai et al. reported increased amounts of yet another set of polypeptides of molecular weight 74, 72, 70, 64, 57, and 54 kDa in *Chlorella protothecoides*. Table 4.4 also showed that the 5 strains of *Desmodesmus* spp. produced more of polypeptides with molecular weight 150, 98, 68, 66, and 58-30 kDa. In 2010, Tukaj and Tukaj reported that pretreatment of *Desmodesmus subspicalus* at 40°C for 1 hour resulted in the induction of two Hsp70, one being the cytoplasmic heat shock protein and the other was chloroplast stroma protein as determined by western blotting.

The  $\beta$ -carotene contents obtained for *Chlorella* spp. in this research were in the same range as those reported in literature. Inbaraj et al. (2006) reported that *Chlorella pyrenoidosa* contained *cis* isomer  $\beta$ -carotene 2,159.3  $\mu\text{g}$  per gram dried cells and *trans*

isomer  $\beta$ -carotene 2,155.0  $\mu\text{g}$  per gram dried cells. In addition, Mutsukawa et al. (2000) reported that *Chlorella sorokiniana* contained  $\beta$ -carotene 600  $\mu\text{g}$  per gram dried cells. According to Dr Pongstorn Sungpuag (personal communication) the variable percent recovery as shown in Table 4.6 was probably due to less solubility of the internal standard, Trans-8-apo-carotenal, in the mobile phase (acetonitrile: dichloromethane: methanol 70:20:10) which was used to dissolve the residue before HPLC analyses. It was recommended that a few drops of dichloromethane which is more non-polar than the mobile phase be used to dissolve the residue more completely before making up the volume of the samples to 500  $\mu\text{l}$  for HPLC analyses.

### 5.3 Recommended future research on biodiversity, protein profiles, and $\beta$ -carotene contents of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp.

It is recommended that further research be conducted on large-scale surveys of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. in Thailand in order to select fast-growing and heat-tolerant or mesophilic strains for use in the determination of microalgal products which may be of interest commercially. In addition, the strains obtained should be used for further basic research on cryptic species and mechanism(s) for heat tolerance and/or mesophilicity in *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. SDS-PAGE of protein profiles of all the 15 green microalgal strains used in this thesis should be repeated and western-blotting be conducted in order to record the presence of heat shock proteins in *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. Finally, preliminary results obtained for the determination of  $\beta$ -carotene contents indicated that two variables are important in order to get complete cell breakage to release  $\beta$ -carotene contents. The variables are the incubation time for breaking cells in ethanol and 60% KOH and the temperature of incubation. To this end, it is envisaged that the response surface methodology (Mead and Pike, 1975) could be used to determine the optimum incubation time and incubation temperature for complete cell breakage for the determination of  $\beta$ -carotene contents as indicated in Figure 5.1.

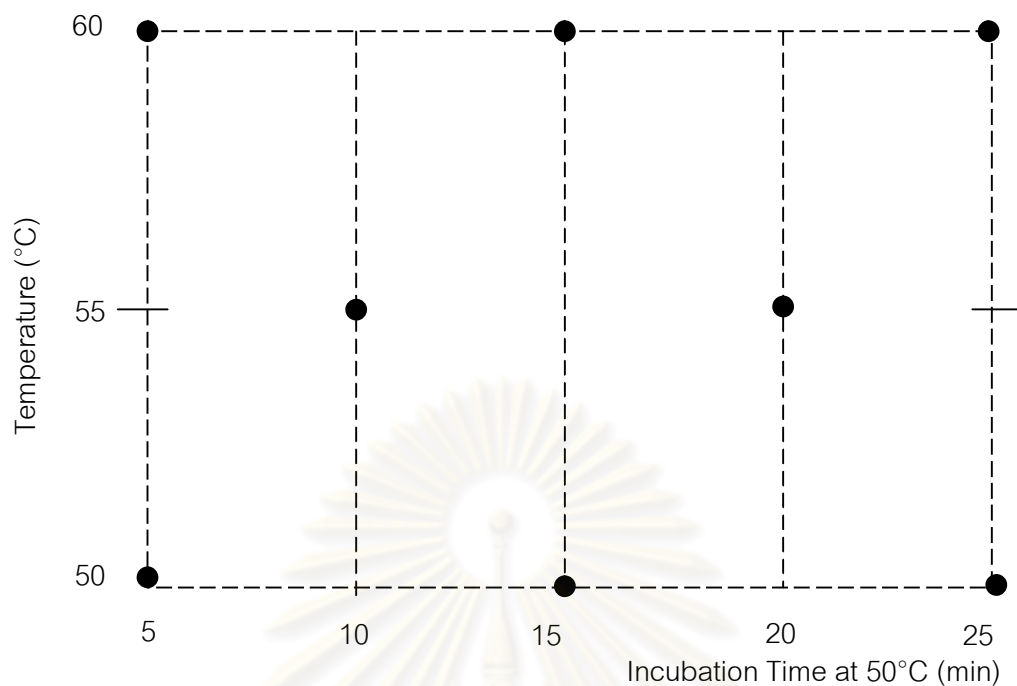


Figure 5.1 Grid showing variations of incubation time and incubation temperature for complete cell breakage of *Chlorella* spp., *Desmodesmus* spp., and *Scenedesmus* spp. in future experiments on the determination of  $\beta$ -carotene contents.

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## CHAPTER VI

### CONCLUSION

Freshwater green microalgae *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. have been used as model oxygenic photosynthetic micro-organisms in physiological studies including the effects of temperatures on growth and soluble protein profiles. RAPD-PCR fingerprinting refers to the use of one primer which randomly anneals to the target DNA in PCR reactions to obtain DNA fingerprints. In this experiment, an arbitrary GC-rich primer CRL-7(5'GCCCCGCCGCC3') was used to obtain RAPD-PCR fingerprints of 15 strains of *Chlorella* spp. strains PK10, PK30, PK37, PK38, and SS1, *Desmodesmus* spp. strains NJ14, NJ23, NJ40, NJ45, and TA008 and *Scenedesmus* spp. strains PK25, PK92, NJ42, SS4, and SS5. The results showed all strains have different DNA fingerprints. Cryptic species with similar morphology but different genetic composition were found to exist in *Chlorella* spp. One aim of the research is to obtain fast-growing strains of the microalgae at 25<sup>0</sup>C for further biotechnological applications. In addition, with the increasing extent of global warming, the research aims to determine if there is a correlation between existing  $\beta$ -carotene contents and heat tolerance at 40<sup>0</sup>C. The third aim of the project is to find out if SDS-PAGE protein profiles of soluble proteins of cells grown at 28<sup>0</sup>C - 32<sup>0</sup>C, and 28<sup>0</sup>C - 32<sup>0</sup>C alternated with 40<sup>0</sup>C for 16/8 h for 21 days could be used to account for heat tolerance in some strains of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. Each of *Chlorella* spp. strains PK10, PK30, PK37, SS1, and *Desmodesmus* sp. strain NJ14, *Scenedesmus* spp. strains NJ42, PK25, and SS4 was grown in Bold's Basal Medium, incubated at 25<sup>0</sup>C under continuous illumination at 200 rpm. Plate counts showed at 25<sup>0</sup>C unicellular green microalgae *Chlorella* spp. grew more rapidly than multicellular microalgae *Desmodesmus* spp. and *Scenedesmus* spp. SDS-PAGE soluble protein profiles of mid-log phase cells of *Chlorella* spp., *Desmodesmus* spp. and *Scenedesmus* spp. showed the amounts of different sets of polypeptides were increased under the high temperature regime. Growth at 28<sup>0</sup>C-32<sup>0</sup>C and at 28<sup>0</sup>C-32<sup>0</sup>C for 16 h alternated with growth at 40<sup>0</sup>C for 8 h showed *Chlorella* spp. strains PK37 and SS1 were heat-tolerant

while *Desmodesmus* spp. strains NJ40 and SS5 were mesophilic. No correlation was obtained between heat tolerance or mesophilicity property and contents of  $\beta$ -carotene and polypeptides which had similar molecular weights to those of heat-shock proteins.



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## REFERENCES

- Agrawal, S.C. 2007. Growth, survival and reproduction in *Chlorella vulgaris* and *C. variegata* with respect to culture age and under different chemical factors. *Folia Microbiol.* 52(4): 399-406.
- An, S.S., Friedl, T., and Hegewald, E. 1999. Phylogenetic relationships of *Scenedesmus* and *Scenedesmus*-like coccoid green algae as inferred from ITS-2 r-DNA sequence comparisons. *Plant Biol.* 1: 418-429.
- Armstrong, G.A. 1994. Eubacteria show their true colors: Genetics of carotenoid pigment biosynthesis from microbes to plants. *J. Bacteriol.* 176(16): 4795-4802.
- Ariyadej, C., Tansakul, R., Tansakul, P. and Angsupanich, S. 2004. Phytoplankton diversity and its relationships to the physico-chemical environments in the Banglang Reservoir, Yala Province. *Songklanakarin J. Sci. Technol.* 26(5):595-607.
- Balczun, C., Bunse, A., Schwarz, C., Piotrowski, M., and Kück, U. 2009. Chloroplast heat shock protein Cpn60 from *Chlamydomonas reinhardtii* exhibits a novel function as a group II intron-specific RNA binding protein. *FEBS Letts.* 580(18): 4527-4532.
- Beuron, F., Maurizi, M.R., Belnap, D.M., Kocsis, E., Booy, F.P., Kessel, M., and Steven, A.C. 1998. At sixes and sevens: Characteriation of the symmetry mismatch of the ClpAP chaperone-assisted protease. *J. Struct. Biol.* 123(3): 248-259.
- Borisisova, E.V. and Tsarenko, P.M. 2004. Microalgae culture collection of Ukraine (IBASU-A). *Nova Hedwigia.* 79: 127-134
- Borowitzka, M. A. 1999. Commercial production of microalgae: ponds, tanks, and fermenters. *Progress in Industrial Microbiol.* 35: 313-321.
- Bradford, M. M. 1966. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72 : 248-254.
- Chader, S., Hacene, H., and Agathos, S.N. 2009. Study of hydrogen production by three strains of *Chlorella* isolated from soil in the Algerian Sahara. *Int. J. Hydrogen Energy.* 34: 4941-4945.

- Chorum, M. 1998. *Biological analysis of water quality using phytoplankton and coliform-bacteria in Ang Kaew reservoir, Chiang Mai University 1996-1997*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 117 pp. (in Thai).
- Chompusri, W. 2006. *Seasonal effect on water quality and phytoplankton community in Doi Tao reservoir, Chiang Mai Province, Year 2004-2005*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 80 pp. (in Thai).
- Clarke, A.K. 1999. ATP-dependent Clp proteases in photosynthetic organisms- A cut above the rest! *Annals of Botany* 83:593-599.
- Dhitisudh, L. 2006. *Diversity, vertical distribution and population ecology of plankton for water quality monitoring in Doi Tao reservoir, Chiang Mai province*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 158 pp. (in Thai).
- Eonseon, J., Polle, J.E.W., Lee, H.K., Hyun, S.M., and Chang, M. 2003. Xanthophylls in microalgae: From biosynthesis to biotechnological mass production and application. *J. Microbiol. Biotechnol.* 13(2): 165-174.
- Gouveia, L., and Oliveira, A.C. 2009. Microalgae as a raw material for biofuels production. *J. Ind. Microbiol. Biotechnol.* 36: 269-274.
- Gribun, A., Kimber, M.S., Ching, R., Sprangers, R., Fiebig, K.M. and Houry, W.A. 2005. The ClpP double-ring tetra-decameric protease exhibits plastic ring-ring interactions, and the N-termini of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation. *J. Biol. Chem.* 280: 16185-16196.
- Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. *Nature* 318 : 571-580.
- Hegewald, E. 1989. The *Scenedesmus* strains of the culture collection of the University of Texas at Austin (UTEX). *Algol. Stud.* 55: 153-189.
- Ikeda, T. and Takeda, H. 1995. Species-specific differences of pyrenoids in *Chlorella* (Chlorophyta). *J. Phycol.* 31(5): 813-818.

- Inbaraj, B.S., Chien, J.T., and Chen, B.H. 2006. Improved high performance liquid chromatography method for determination of carotenoids in the microalgae *Chlorella pyrenoidosa*. *J. Chromato. A* 1102:193-199.
- Jamkangwan, N. 2004. *Molecular characterization of cyanobacteria Synechococcus sp. and micro-algae Chlorella spp. and Scenedesmus spp. isolated in Thailand*. Master's thesis, Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok. 130p.
- John, D.M.,Whitton,B.A.,and Brook, A. J. 2005. *The Freshwater Algal Flora of the British Isles; An identification Guide to Freshwater and Terrestrial Algae*. London: Cambridge University Press. 702 pp.
- Kiatpradub, S. 2003. *Diversity of toxic blue green algae and water quality in Bang Phra reservoir, Chon Buri province in the year 2000-2001*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 111 pp. (in Thai).
- Kim, K.K., Kim, R., and Kim, S.H. 1998. Crystal structure of a small heat-shock protein. *Nature*. 394: 595-599.
- Klinkhamouan, S. 2005. RAPD-DNA fingerprinting of microalgae *Chlorella spp.*, *Scenedesmus spp.* and *Ankistrodesmus sp.* B.Sc. Senior Project report. Faculty of Science. Chulalongkorn University. 25 pp. (in Thai).
- Kraibut, H. 1996. *Water quality and phytoplankton distribution in Ang Kaew Reservoir, Chiang Mai*. Master's thesis , Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 76 pp. (in Thai).
- Kunpradid, T. 2000. *Diversity of phytoplankton and macroalgae in Mae San Stream, Doi Suthep-Pui national park, Chiang Mai*. Master's thesis , Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai.129 pp. (in Thai).
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 : 680-685.
- Lewis, L. A. and Flechtner, V.R. 2004. Cryptic species of *Scenedesmus* (Chlorophyta) from desert soil communities of western north America. *J. Phycol.* 40: 1127-1137.

- Lien, B.C., Cole, A.L.J., and Walker, J.R.L. 1979. Effect of sodium fluoroacetate ("Compound 1080") on the soil microflora. *Soil Biol. Biochem.* 11: 13-18.
- Lurling, M. 2003. Review paper: Phenotypic plasticity in the green algae *Desmodesmus* and *Scenedesmus* with special reference to the induction of defensive morphology. *Ann. Limnol. – Int. J. Lim.* 39(2): 85-101.
- Majeran, W., Friso, G., Wijk, K.J., and Vallon, O. 2005. The chloroplast ClpP complex in *Chlamydomonas reinhardtii* contains an unusual high molecular mass subunit with a large apical domain. *FEBS J.* 272: 5558-5571.
- Mandal, S., and Mallick, N. 2009. Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. *Appl. Microbiol. and Biotechnol.* 84(2): 281-291.
- Masojédék, J. and Torzillo, G. 2008. Mass cultivation of freshwater microalgae. *Encyclopedia of Ecology.* p. 2226-2235.
- Mead, R., and Pike, D.J. 1975. A review of response surface methodology from a biometric viewpoint. *Biometrics.* 32: 803-851.
- Megharaj, M., Pearson, H.W., and Venkateswarlu, K. 1992. Removal of nitrogen and phosphorus by immobilized cells of *Chlorella vulgaris* and *Scenedesmus bijugatus* isolated from soil. *Enzyme Microb. Technol.* 14: 656-658.
- Muller, J., Friedl, T., Hepperle, D., Lorenz, M. and Day, J.G. 2005. Distinction between multiple isolates of *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae) and testing for conspecificity using amplified fragment length polymorphism and its rDNA sequences. *J. Phycol.* 41(6):1236-1247.
- Mulsin, P. 1997. Phytoplankton biovolume and water quality in reservoir of Mae Kuang Dam, Chiang Mai. M.S. thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 110 pp. (in Thai).
- Mutsukawa, R., Hotta, M., Masuda, Y., Chihara, M., and Karube, I. 2000. Antioxidants from carbon dioxide fixing *Chlorella sorokianina*. *J. Appl. Phycol.* 12: 263-266.
- Narberhaus, F. 2002.  $\square$ -crystalline-type heat shock proteins: Socializing minichaperones in the context of a multichaperone network. *Microbiol. Mol. Biol. Rev.* 66(1): 64-93.

- Pannuvanitchakorn, N. 2003. *Distribution of diatoms in Joud and Jae Sun Peat Land, Thalang district, Phuket province, in the year 2002*. Master's Independent study Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 77 pp. (in Thai).
- Pekkon, J. 2002. *Distribution of toxic algae and water quality in the reservoir of Mae Kuang Udomtara Dam, Chiang Mai province in 1999-2000*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 103 pp. (in Thai).
- Pekkon, J. 2008. *Diversity and cyanotoxins of cyanobacteria in some water resources of Thailand*. Ph.D. thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 146 pp. (in Thai).
- Pongsawat, S., Thammathaworn, S., Peerapornpisal, Y., Thanee, N. and Somsiri, C. 2004. Diversity of phytoplankton in the RAMA IX Lake, A Man-Made Lake, Pathumthani Province, Thailand. *ScienceAsia* 30(2004): 261-267.
- Prommana, R. 2002. *Diversity of toxic algae and water quality in Kwan Phayao, Phayao Province in 1999-2000*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 113 pp. (in Thai).
- Prommana, R. 2006. *Correlations between some parameters of cyanobacterial growth, microcystin quantity, water and sediment qualities in some Thai water resources*. Ph.D. thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 188 pp. (in Thai).
- Pooarlai, P. 1999. *Analysis of water quality using phytoplankton and coliform bacteria as indicator in Huai Tung Thao reservoir, Chiang Mai province*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 85 pp. (in Thai).
- Sawangdee S. 2006. *DNA fingerprints and  $\beta$ -carotene and quercetin contents in green micro-algae *Chlorella* spp. and *Scenedesmus* spp.* Master's thesis, Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok. 68pp.
- Sawangdee, S., Jamkangwan, N., Prateepasen, R., Kittimasakun, P. and Chansangavej, K. 2007. Polyphasic taxonomy in fresh water *Chlorella* and



- Scenedesmus/Desmodesmus*. *J. Sci. Res. Chulalongkorn University* (Section T) 1: 43-54.
- Schlipalius, L. 1991. The extensive commercial cultivation of *Dunaliella salina*. *Bioresource Technol.* 38: 241-243.
- Schwender. J., Seemann, M., Lichtenthaler, H.K., and Rohmer, M. 1966. Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde-3-phosphate non-mevalonate pathway in the green alga *Scenedesmus obliquus*. *Biochem J.* 316:73-80.
- Seekhao, I. 2006. *Monitoring of microcystins from toxic blue-green algae and water quality in Mae Kuang Udomtara reservoir, Chiang Mai province, Year 2004-2005*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 91 pp. (in Thai).
- Somdee, T. 1998. *Distribution of Microcystis aeruginosa Kütz. in the reservoir of Mae Kuang Udomtara Dam, 1996-1997*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 102 pp. (in Thai).
- Sompong, U. 2001. *Biodiversity of algae in some hot spring areas in the upper part of northern Thailand*. Master's thesis , Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 119 pp. (in Thai).
- Thonkamdee, T. 2007. *Water quality in some water resource of northeastern and central areas by using phytoplankton as bioindicator*. B.Sc. project, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 55 pp. (in Thai).
- TISTR. 2009. List of microalgae. *Thailand Institute of Scientific and Technological Research*. Bangkok, Thailand.
- Tukaj, S., and Tukaj, Z. 2010. Distinct chemical contaminants induce the synthesis of Hsp70 proteins in green microalgae *Desmodesmus subspicalus*: Heat pretreatment increases cadmium resistance. *J. Thermal Biol.* 35(5): 239-244.
- Valliammai, T., Gnanam, A., and Mannan, R. M. 1987. Heat shock response of *Chlorella protothecoides* during greening. *J. Biosci.* 12(3): 219-228.



- Vanormelingen, P., Hegewald, E., Braband, A., Kitschke, M., Friedl, T., Sabbe, K., and Vyverman, W. 2007. The systematics of a small spineless *Desmodesmus* species, *D. costato-granulatus* (Sphaeropleales, Chlorophyceae), based on ITS2 rDNA sequence analyses and cell wall morphology. *J. Phycol.* 43: 378-396.
- Vijaranakorn, T. 2003. *Distribution of Microcystis aeruginosa Kütz. and water quality in Mae Kuang Udomtara reservoir, Chiang Mai province*. Master's thesis, Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 88 pp. (in Thai).
- Voet, D. and Voet J.G. 1995. *Biochemistry*. Second edition. New York : John Wiley and Sons, p. 191-214.
- Waiyaka, P. 1996. *Biological water quality and phytoplankton fluctuation in the reservoir of the Office of Agriculture and Cooperatives, Chiang Mai province*. B.Sc. project Biology Department, Faculty of Science, Chiang Mai University, Chiang Mai. 57 pp. (in Thai).
- Wang, J., Hartling, J.A., and Flanagan, J.M. 1997. The structure of ClpP at 2.3 Å resolution suggests a model for ATP-dependent proteolysis. *Cell* 91: 447-456.
- Wojtkowiak, D., Georgopoulos, C., and Zylicz, M. 1993. Isolation and characterization of ClpX, a new ATP-dependent specificity component of the Clp protease of *Escherichia coli*. *J. Biol. Chem.* 268: 22609-22617.
- Xu, H., Miao, X., and Wu, Q. 2006. High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. *J. Biotechnol.* 126(4): 499-507.



APPENDICES

ศูนย์วิทยทรัพยากร  
จุฬาลงกรณ์มหาวิทยาลัย

## Appendix A

### MICRO-ALGAL GROWTH MEDIA

#### Bold's Basal Medium (BBM) (Stein, 1973)

|  |                |                  |        |
|--|----------------|------------------|--------|
| 1. $\text{KH}_2\text{PO}_4$  | stock solution | 8.75 g/500 ml    | 10 ml  |
| 2. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$                                     |                | 1.25 g/500 ml    | 10 ml  |
| 3. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$                                     |                | 3.75 g/500 ml    | 10 ml  |
| 4. $\text{NaNO}_3$   |                | 12.5 g/500 ml    | 10 ml  |
| 5. $\text{K}_2\text{HPO}_4$  |                | 3.75 g/500 ml    | 10 ml  |
| 6. $\text{NaCl}$   |                | 1.25 g/500 ml    | 10 ml  |
| 7. $\text{Na}_2\text{EDTA-KOH}$  |                | 10 g/L/ 6.2 g/L  | 1 ml   |
| 8. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} \cdot \text{H}_2\text{SO}_4$ (conc.) |                | 4.98 g/L/ 1 ml/L | 1 ml   |
| 9. Trace Metal Solution  | See below*     |                  | 1 ml   |
| 10. $\text{H}_3\text{BO}_3$  |                | 5.75 g/500 ml    | 0.7 ml |

#### \*Trace Metal Solution: (g/l)

|   |          |
|---|----------|
| 1. $\text{H}_3\text{BO}_3$                              | 2.86 g   |
| 2. $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$            | 1.81 g   |
| 3. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$            | 0.222 g  |
| 4. $\text{Na MoO}_4 \cdot 5\text{H}_2\text{O}$          | 0.390 g  |
| 5. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$            | 0.079 g  |
| 6. $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ | 0.0494 g |

Deionized water 1 liter

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

## Appendix B

### CHEMICALS AND SOLUTIONS

#### 1. Solutions for DNA extraction

**TE buffer** (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

0.12 g Tris-HCl, and 0.037 g EDTA were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

**10% SDS**

10 g SDS were dissolved in 90 ml distilled water with gentle stirring and brought to 100 ml with distilled water.

**3 M Sodium acetate**

24.61 g Sodium acetate were added to 100 ml distilled water.

**Absolute ethanol**

**70% Ethanol**

#### 2. Solutions for $\beta$ -carotene extraction

**60% KOH**

60 g KOH were added to distilled water. The final volume was made to 100 ml.

**95% Ethanol**

**9 % NaCl**

9 g NaCl were added to distilled water. The final volume was made to 100 ml.

**Diethyl ether**

**Na<sub>2</sub>SO<sub>4</sub>**

**$\beta$ -carotene (Merck)**

**Mobile phase**

Acetonitrile: Dichloromethane: Methanol = 70: 20: 10

### 3. Solutions for SDS-PAGE (Bio-rad)

#### Stock solutions

##### A. Acrylamide/bis

acrylamide (29.2 g/100 ml)

N'N'-bis-methylene-acrylamide (0.8 g/100 ml)

Make to 300 ml with distilled water. Filter and store at 4°C in the dark.

For a maximum of 30 days.

##### B. 1.5 M Tris-HCl, pH 8.8

Tris base (18.15 g/100 ml)

80 ml deionized water

Adjust to pH 8.8 with 6N HCl. Make volume up to 100 ml with distilled water and store at 4°C

##### C. 0.5 M Tris-HCl, pH 6.8

6 g Tris base

60 ml deionized water

Adjust to pH 6.8 with 6N HCl. Make volume up to 100 ml with distilled water and store at 4°C

##### D. 10% SDS

Dissolve 10 g SDS in 90 ml distilled water with gentle stirring and bring to 100 ml with distilled water.

##### E. Sample buffer (SDS reducing buffer) (store at room temperature)

Distilled water 3.8 ml

0.5 M Tris-HCl, pH 6.8 1.0 ml

Glycerol 0.8 ml

10% (w/v) SDS 1.6 ml

2-mercaptoethanol 0.4 ml

1 % (w/v) bromophenol blue 0.4 ml

Dilute the sample at least 1:4 with sample buffer, and heat at 95°C for 4 minutes

## F. 5X running buffer, pH 8.3

|           |        |          |
|-----------|--------|----------|
| Tris base | 9.0 g  | (15 g/l) |
| Glycine   | 43.2 g | (72 g/l) |
| SDS       | 3.0 g  | (5 g/l)  |

Make to 600 ml with distilled water.

Store at 4°C. Warm to room temperature before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml distilled water for one electrophoretic run.

## G. 10% Ammonium persulphate

One milliliter of aqueous 10% (w/v) Ammonium persulphate stock solution was prepared and stored at 4°C. Ammonium persulphate decomposes slowly, and fresh solutions were prepared weekly.



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## Appendix C

REPRESENTATIVE CHROMATOGRAMS FOR THE CONSTRUCTION OF  
AN EXTERNAL STANDARD CURVE FOR THE DETERMINATION  
OF  $\beta$ -CAROTENE CONTENTS

Figure C.1 showed an external standard curve for the determination of  $\beta$ -carotene contents. Representative chromatograms of  $\beta$ -carotene standard as determined by reversed phase HPLC were shown in Figures C.2-C.6. The average retention time of  $\beta$ -carotene standard was found to be  $10.662 \pm 0.030$  minutes.

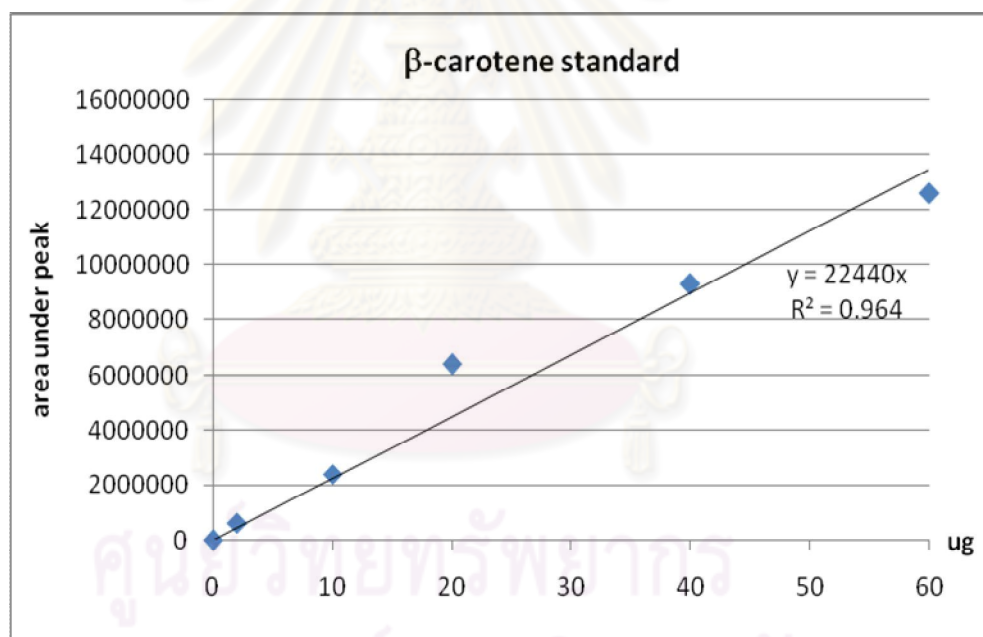


Figure C.1 An external standard curve for the determination of  $\beta$ - carotene contents.

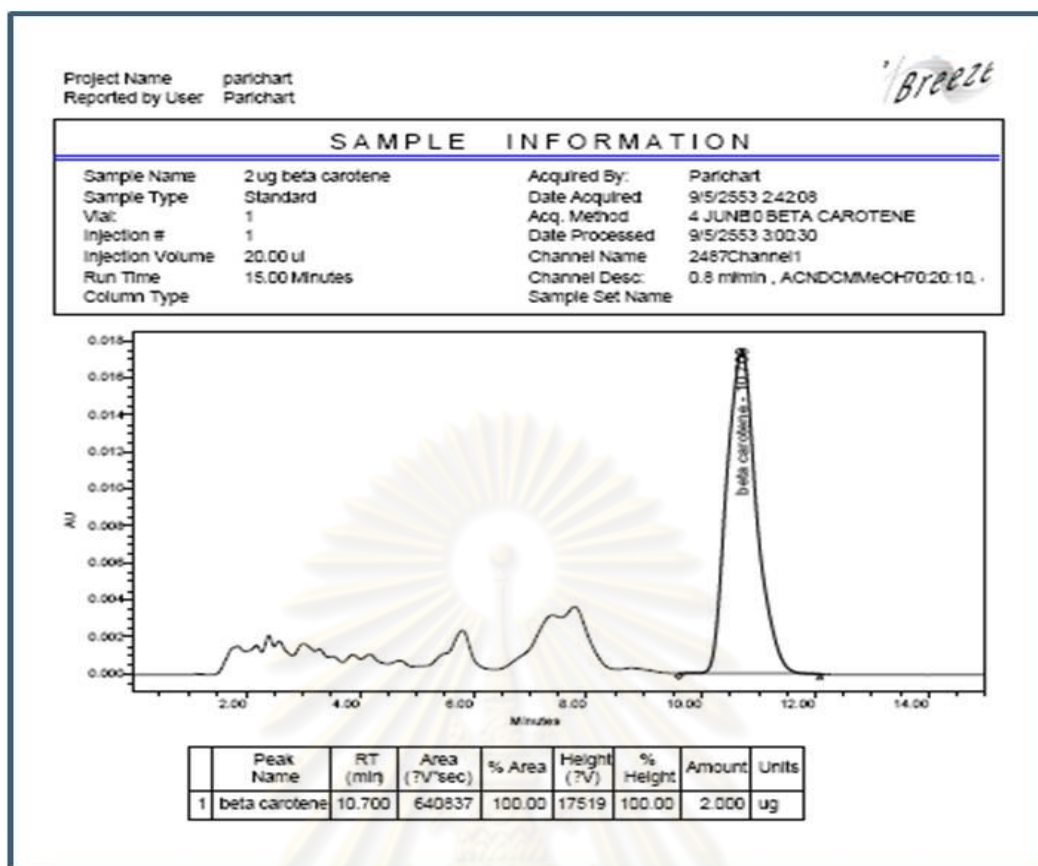


Figure C.2 Representative chromatogram of 2  $\mu\text{g}$   $\beta$ -carotene standard as determined by reversed phase HPLC.

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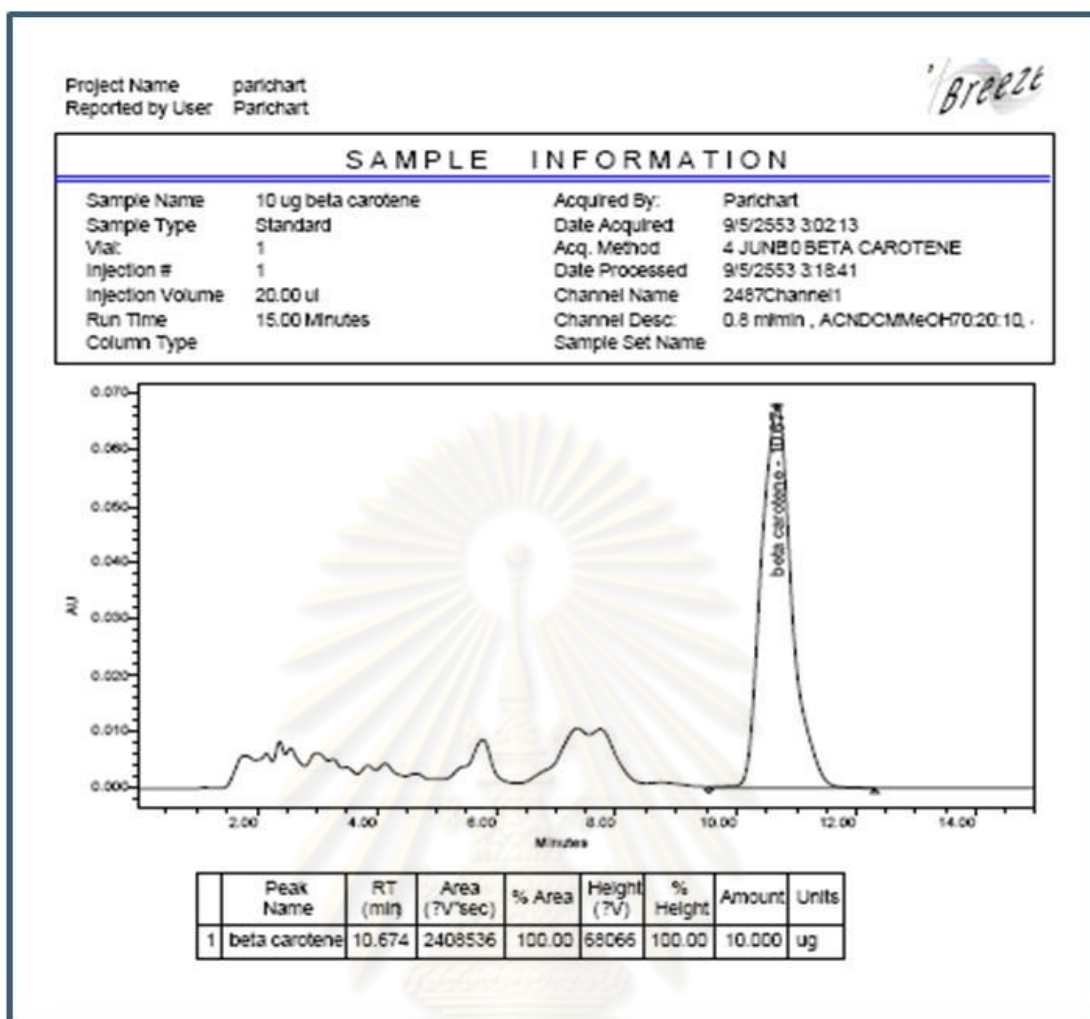


Figure C.3 Representative chromatogram of 10  $\mu\text{g}$   $\beta$ -carotene standard as determined by reversed phase HPLC.

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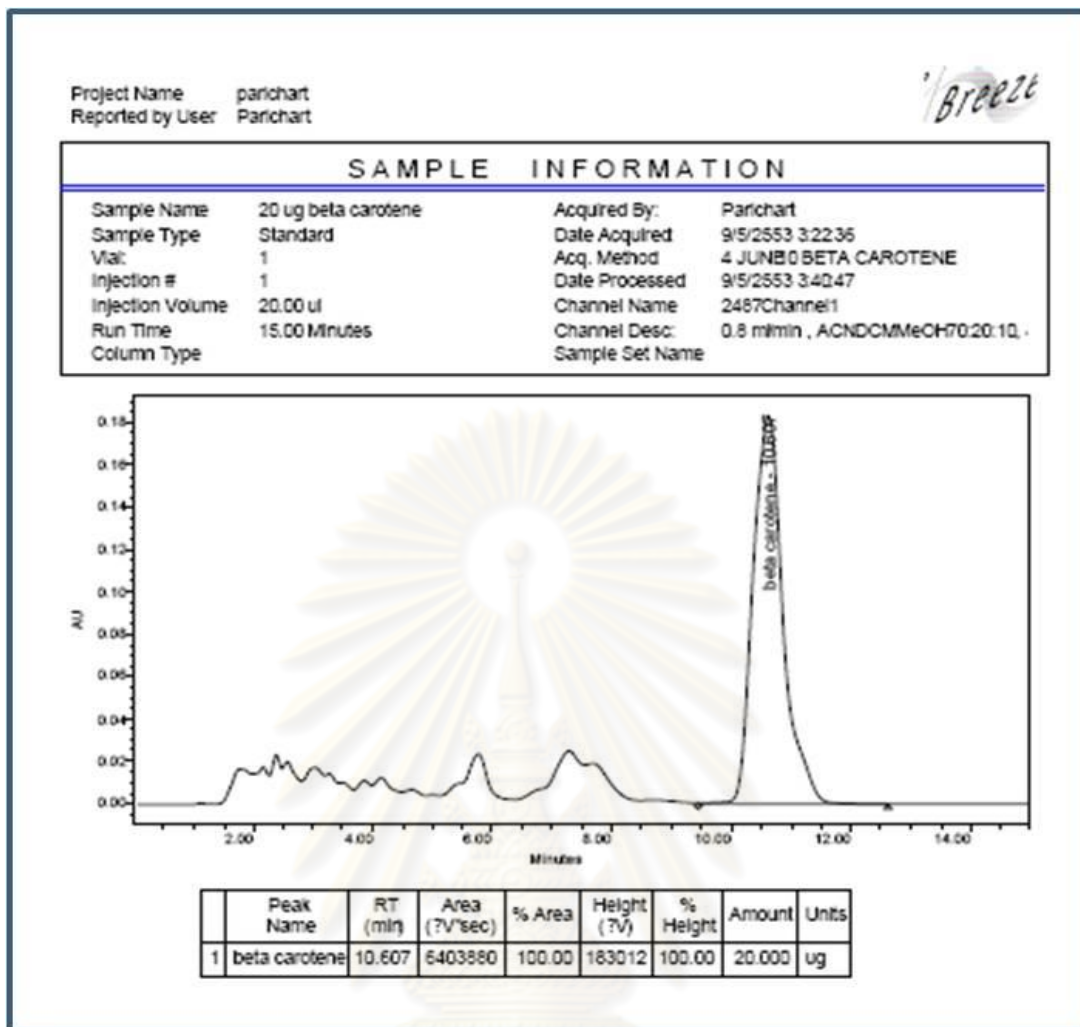


Figure C.4 Representative chromatogram of 20  $\mu\text{g}$   $\beta$ -carotene standard as determined by reversed phase HPLC.

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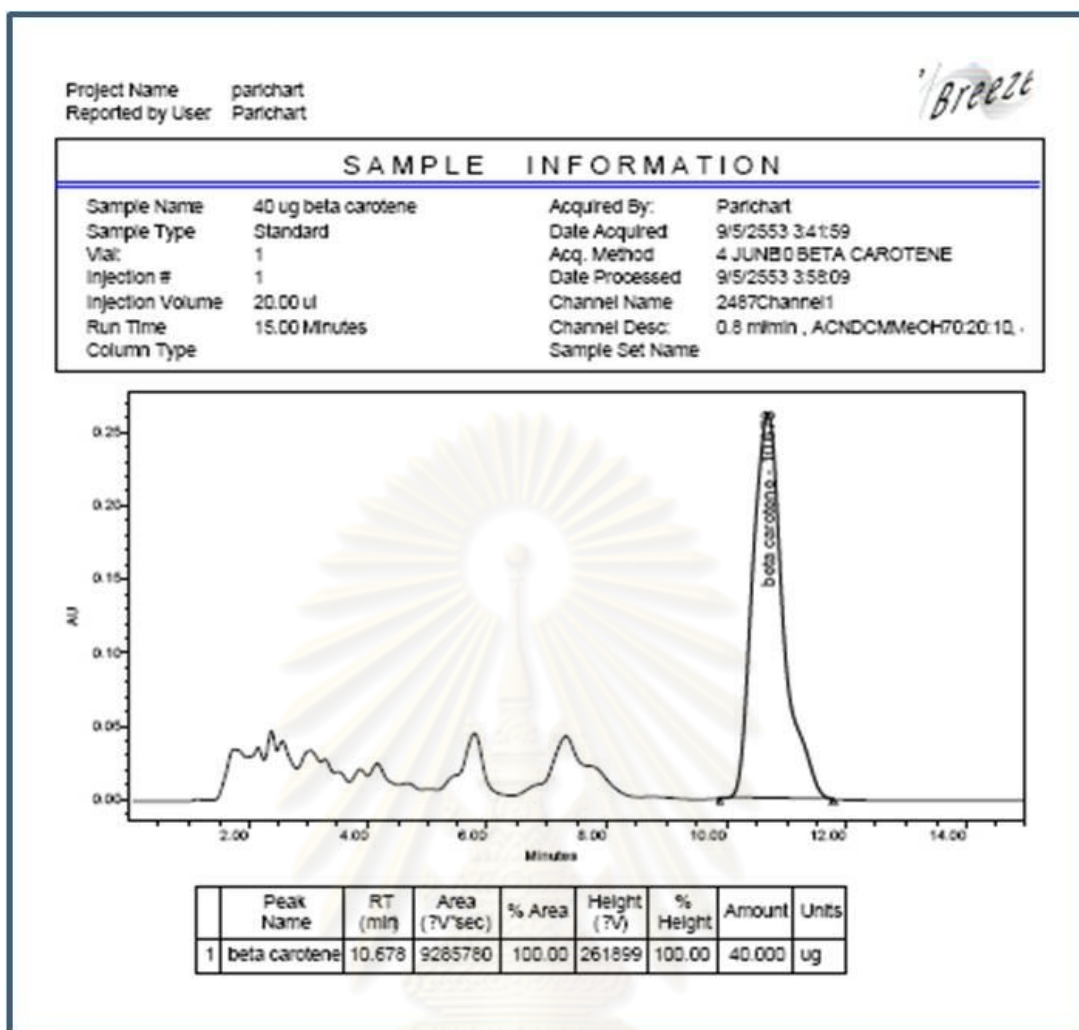


Figure C.5 Representative chromatogram of 40 $\mu$ g  $\beta$ -carotene standard as determined by reversed phase HPLC.

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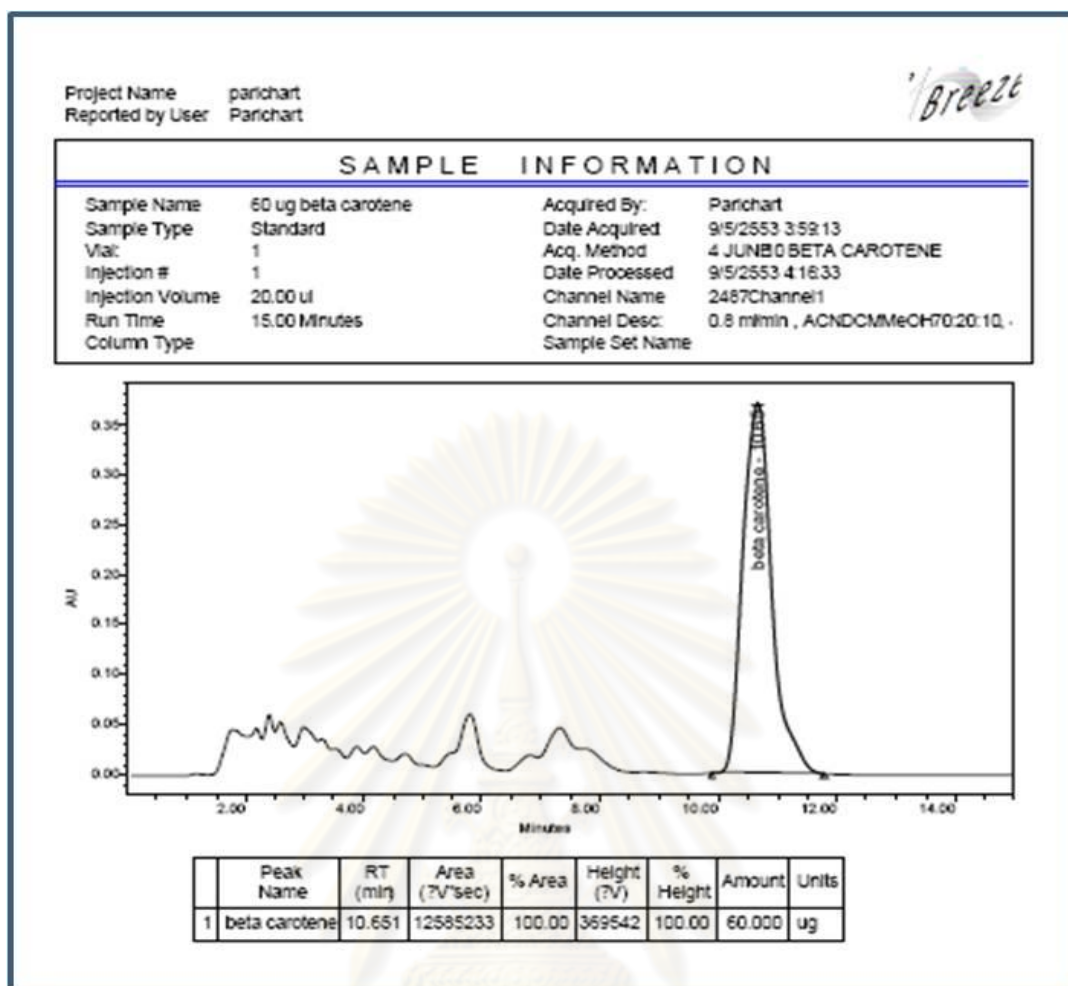


Figure C.6 Representative chromatogram of 60  $\mu\text{g}$   $\beta$ -carotene standard as determined by reversed phase HPLC.

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## Appendix D

REPRESENTATIVE CHROMATOGRAMS FOR THE CONSTRUCTION OF  
AN EXTERNAL STANDARD CURVE FOR THE DETERMINATION  
OF TRANS-APO-8-CAROTENAL CONCENTRATIONS

Figure D.1 showed an external standard curve for the determination of trans-apo-8-carotenal concentrations. Representative chromatograms of trans-apo-8-carotenal standard as determined by reversed phase HPLC were shown in Figures D.2-D.7. The average retention time of trans-apo-8-carotenal was found to be  $3.670 \pm 0.014$  minutes.

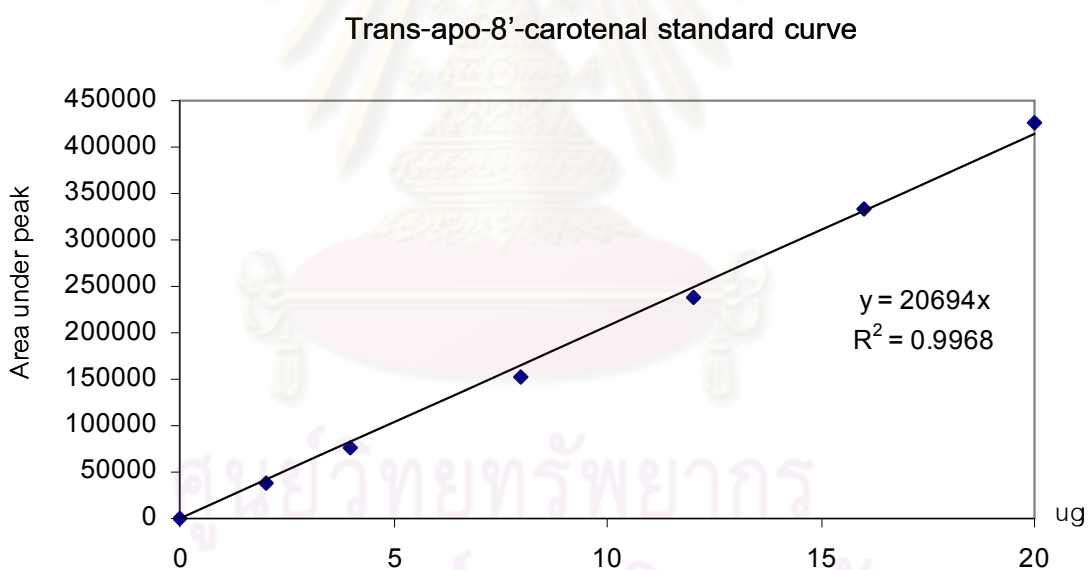


Figure D.1 An external standard curve for the determination of trans-apo-8-carotenal concentrations in 20  $\mu$ l injection volume.

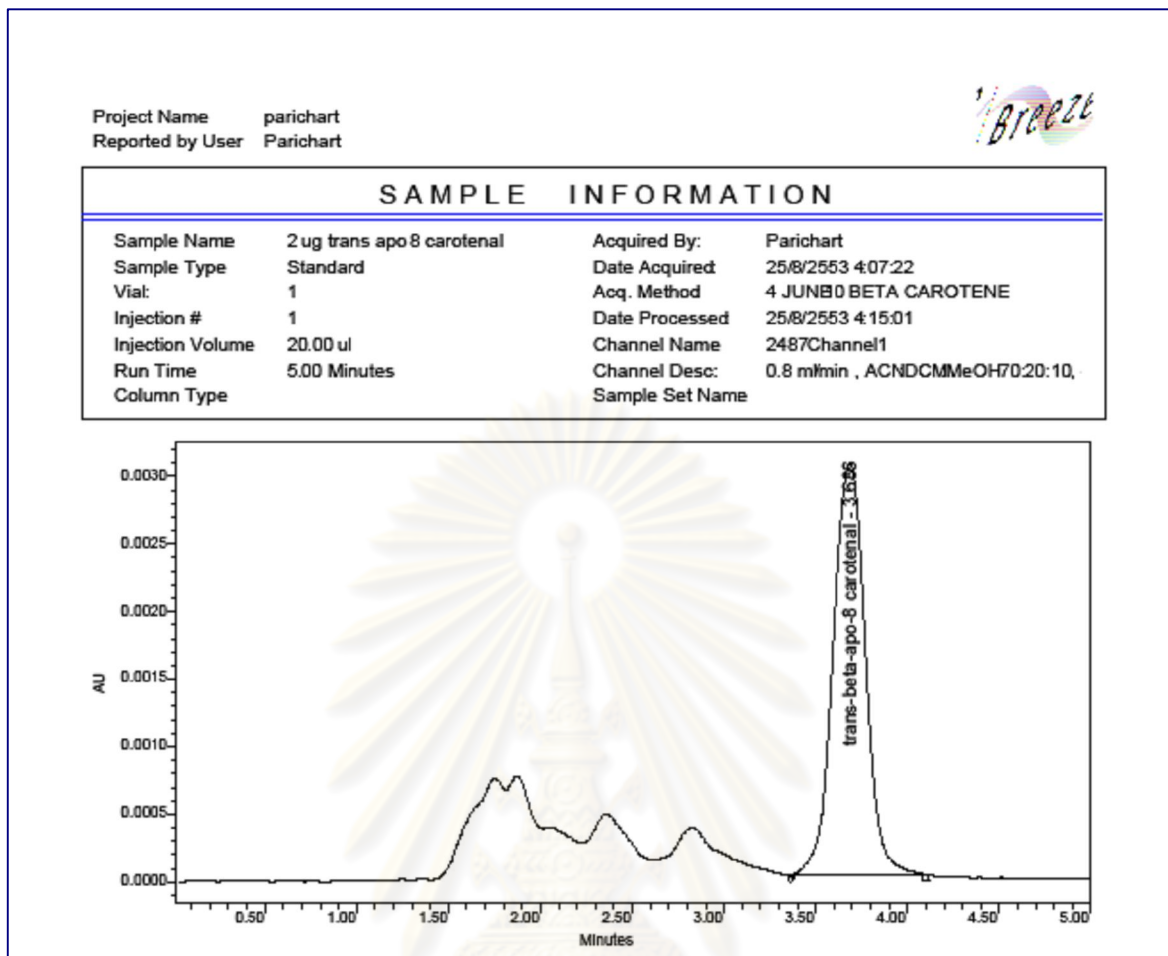


Figure D.2 Representative chromatogram of 2  $\mu\text{g}$  trans-apo-8-carotenal as determined by reversed phase HPLC.

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Project Name parichart  
Reported by User Parichart



### SAMPLE INFORMATION

|                  |                           |                 |                                 |
|------------------|---------------------------|-----------------|---------------------------------|
| Sample Name      | 4 ug trans apo8 carotenal | Acquired By:    | Parichart                       |
| Sample Type      | Standard                  | Date Acquired   | 25/8/2553 4:21:05               |
| Vial:            | 1                         | Acq. Method     | 4 JUN80 BETA CAROTENE           |
| Injection #      | 1                         | Date Processed  | 25/8/2553 4:28:15               |
| Injection Volume | 20.00 ul                  | Channel Name    | 2487Channel1                    |
| Run Time         | 5.00 Minutes              | Channel Desc:   | 0.8 ml/min , ACNDCMMeOH70:20:10 |
| Column Type      |                           | Sample Set Name |                                 |

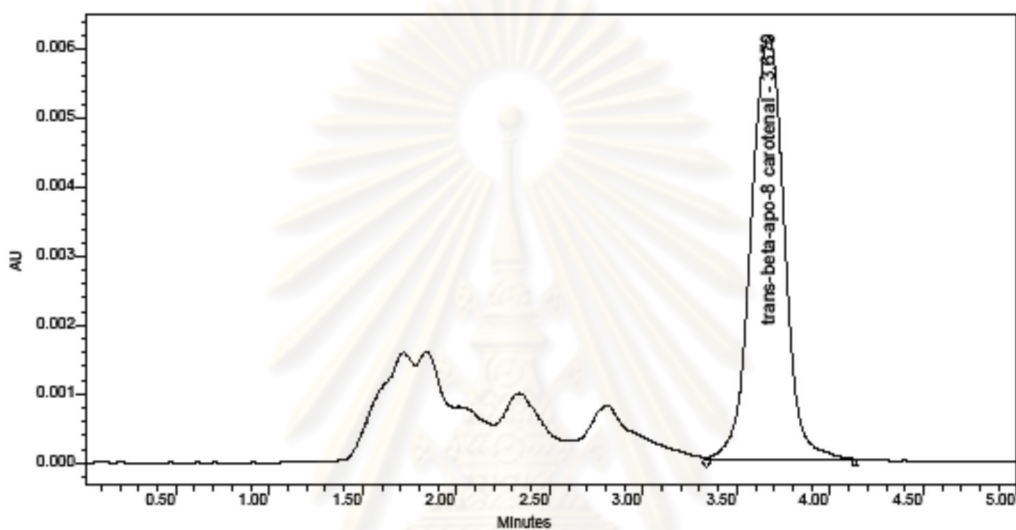


Figure D.3 Representative chromatogram of 4  $\mu\text{g}$  trans-apo-8-carotenal as determined by reversed phase HPLC.

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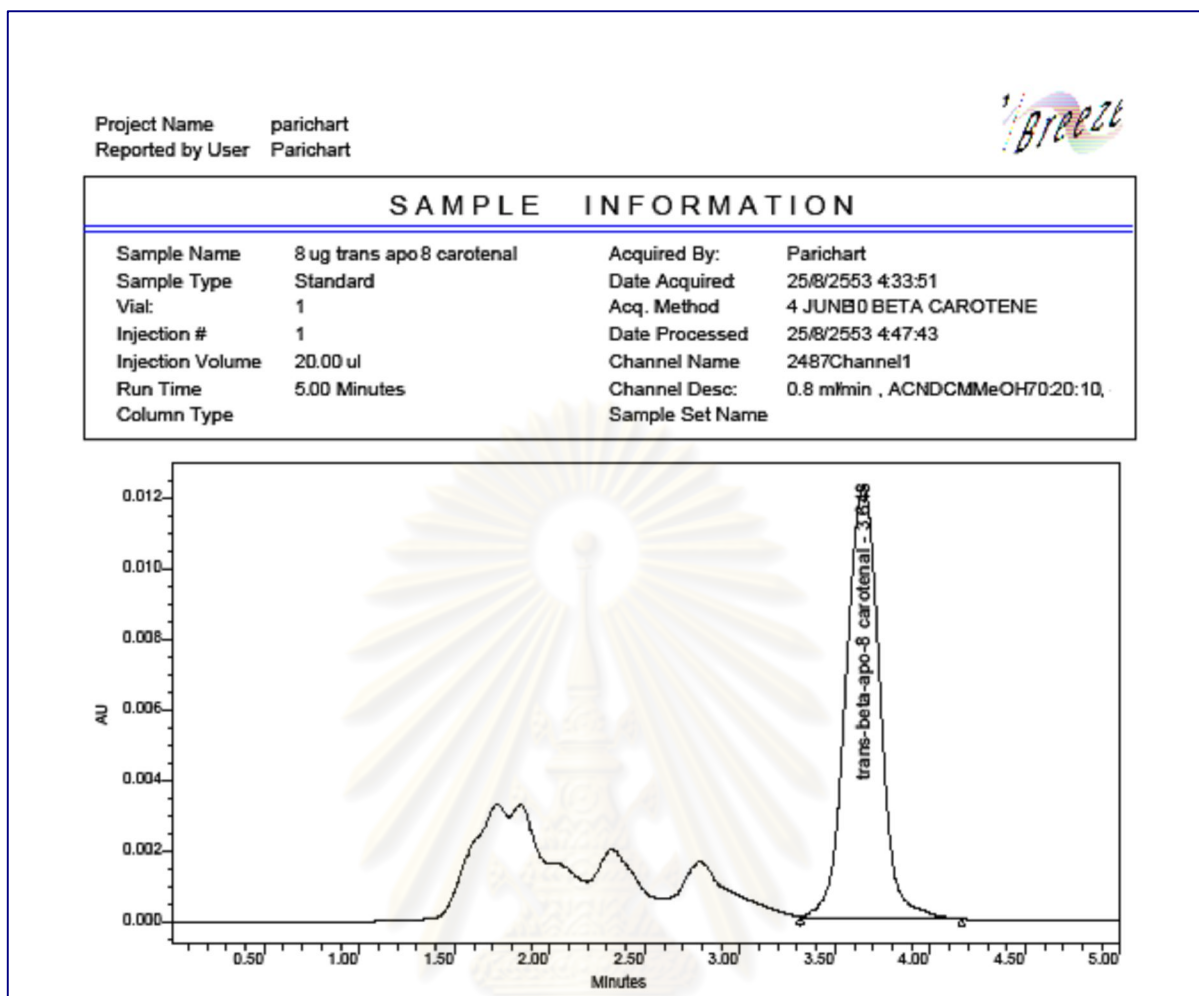


Figure D.4 Representative chromatogram of 8  $\mu\text{g}$  trans-apo-8-carotenal as determined by reversed phase HPLC.

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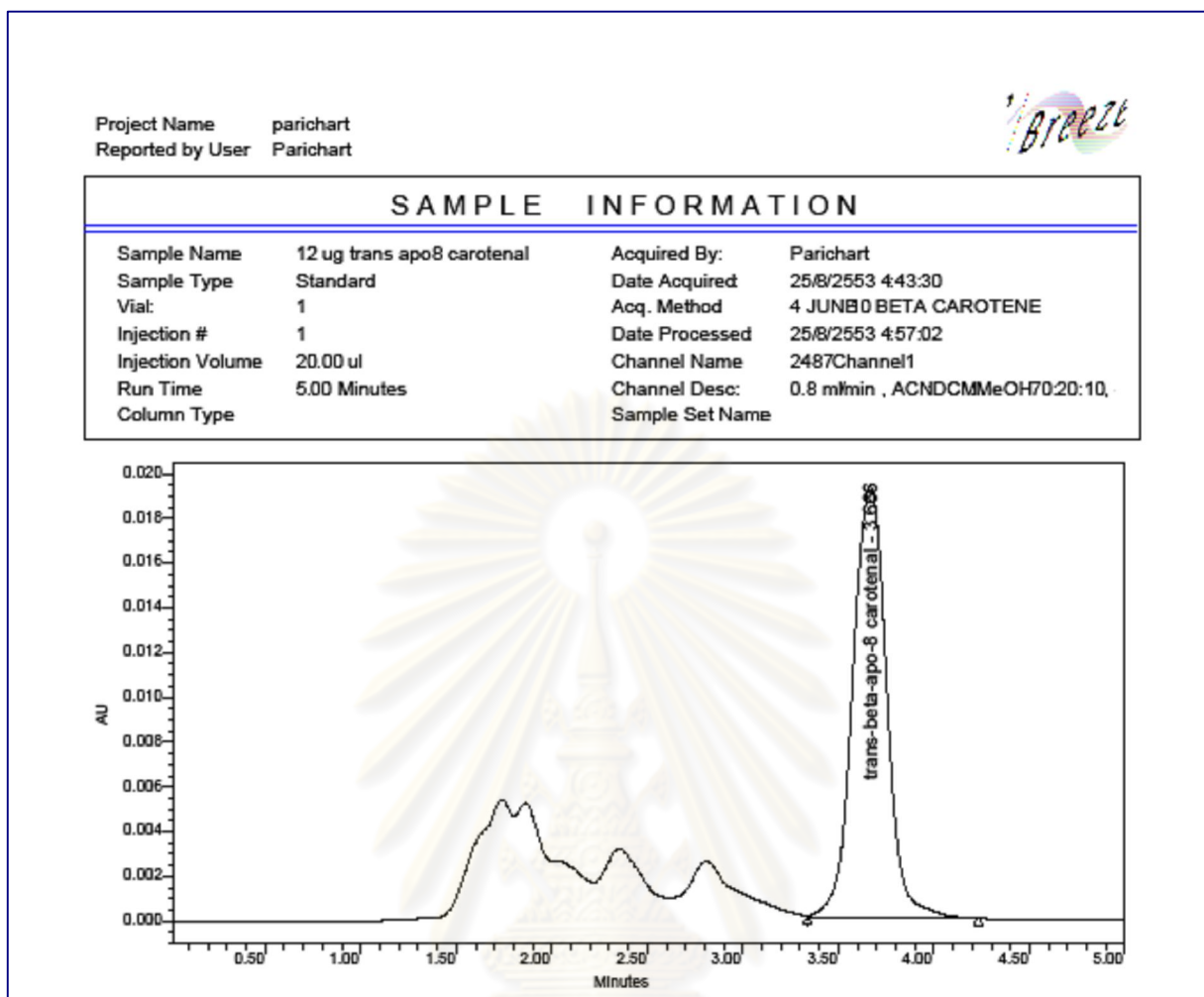


Figure D.5 Representative chromatogram of 12  $\mu\text{g}$  trans-apo-8-carotenal as determined by reversed phase HPLC.

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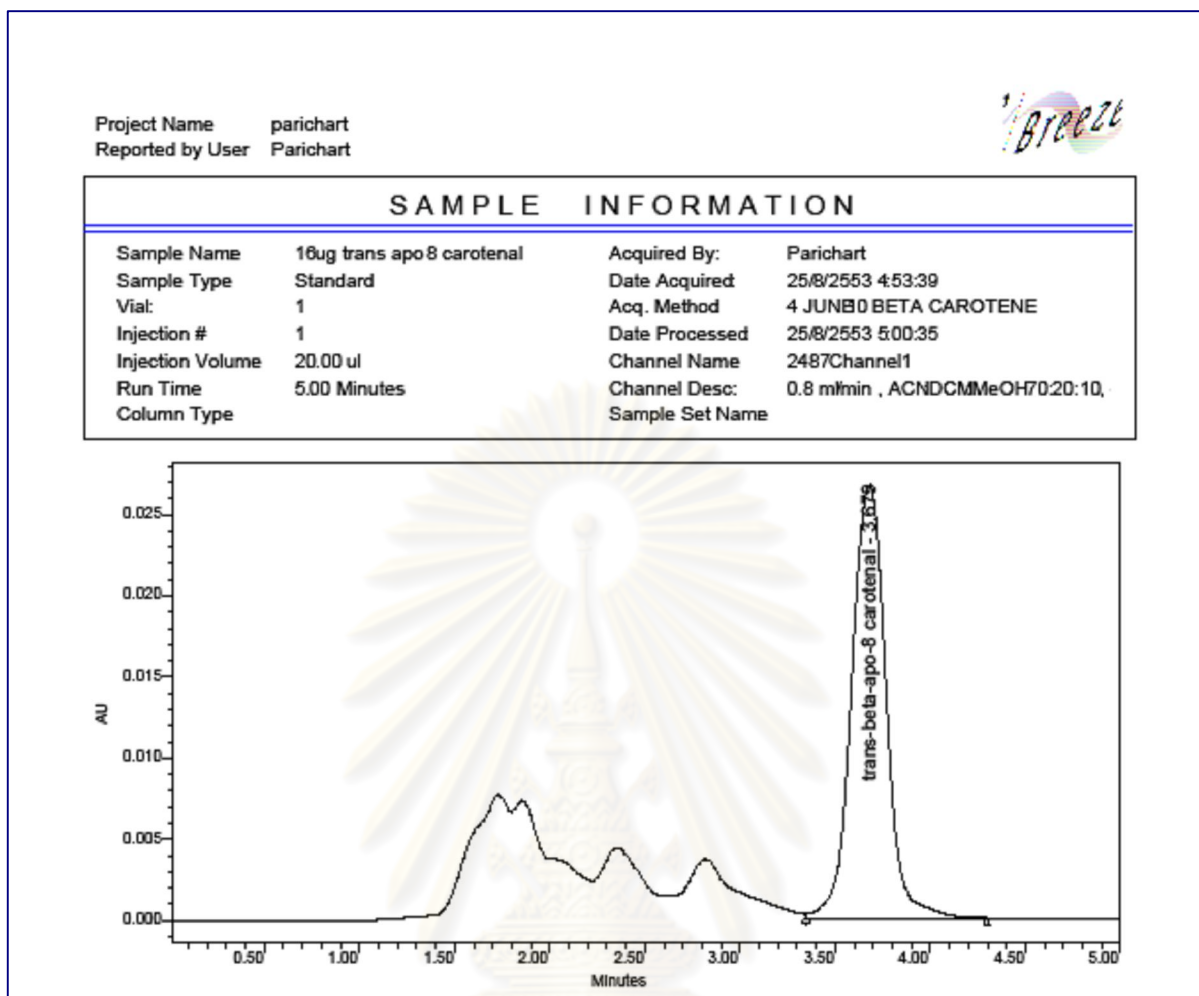


Figure D.6 Representative chromatogram of 16  $\mu\text{g}$  trans-apo-8-carotenal as determined by reversed phase HPLC.

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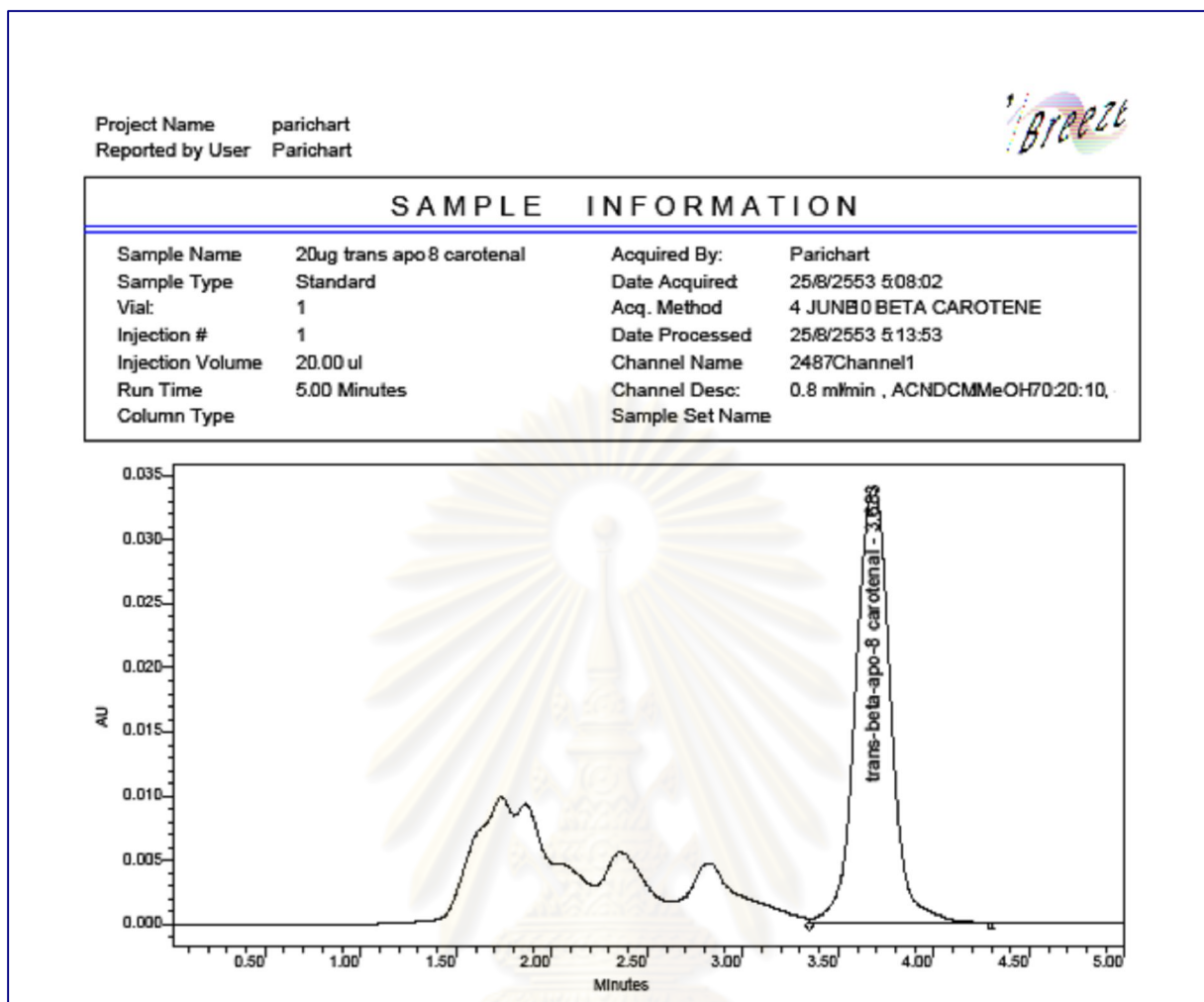


Figure D.7 Representative chromatogram of 20  $\mu\text{g}$  trans-apo-8-carotenal as determined by reversed phase HPLC.

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## Appendix E

## CHROMATOGRAMS FOR DETERMINATION OF $\beta$ -CAROTENE CONTENTS AND PERCENTAGE OF RECOVERY

Chromatograms of pigment profiles and the internal standard trans-8-apo-carotenal for the determination of  $\beta$ -carotene contents in mid-log phase cells of 11 strains of *Chlorella* spp. strains PK10, PK30, PK37, PK38 and SS1, *Desmodesmus* sp. strain NJ14 and *Scenedesmus* spp. strains PK25, PK92, NJ42, SS4 and SS5 as well as the percentages of recovery were shown in Figures E.1-E.11, respectively.

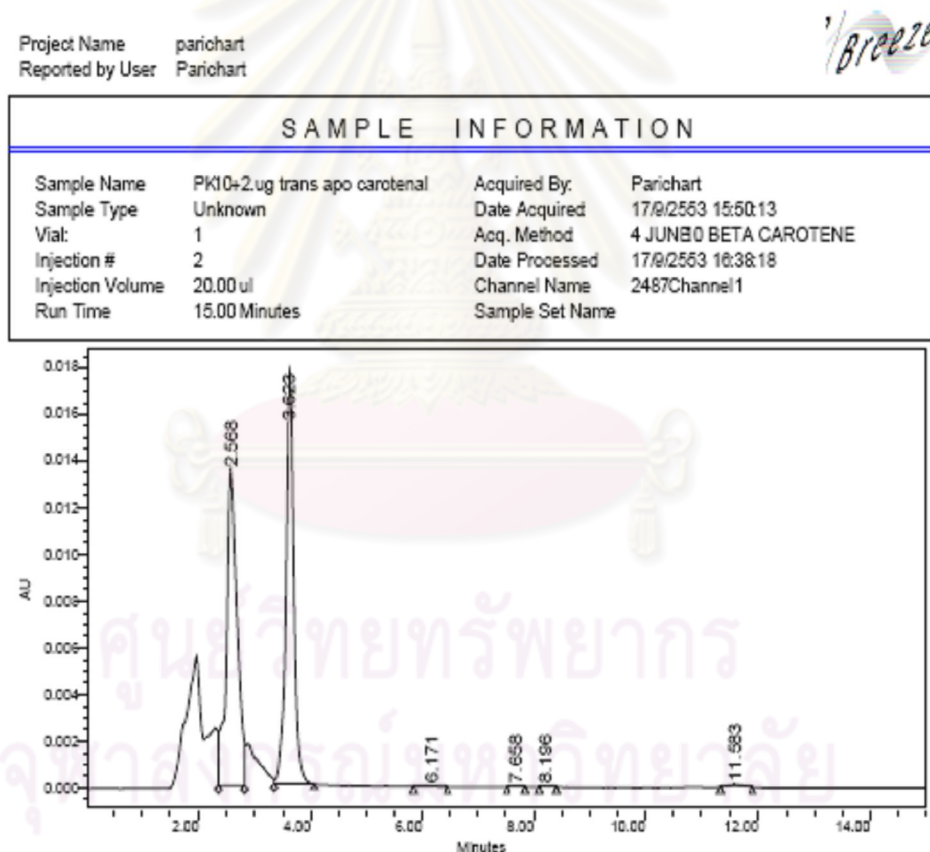


Figure E.1 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Chlorella* sp. strain PK10 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |               |                 |                                 |
|--------------------|---------------|-----------------|---------------------------------|
| Sample Name        | PK30          | Acquired By:    | Parichart                       |
| Sample Type        | Unknown       | Date Acquired:  | 1/8/2553 3:42:25                |
| Vial:              | 9             | Acq. Method     | 4 JUN80 BETA CAROTENE           |
| Injection #        | 1             | Date Processed  | 1/8/2553 10:18:17               |
| Injection Volume   | 20.00 ul      | Channel Name    | 2487Channel1                    |
| Run Time           | 15.00 Minutes | Channel Desc:   | 0.8 ml/min , ACNDCMMeOH70:20:10 |
| Column Type        |               | Sample Set Name |                                 |

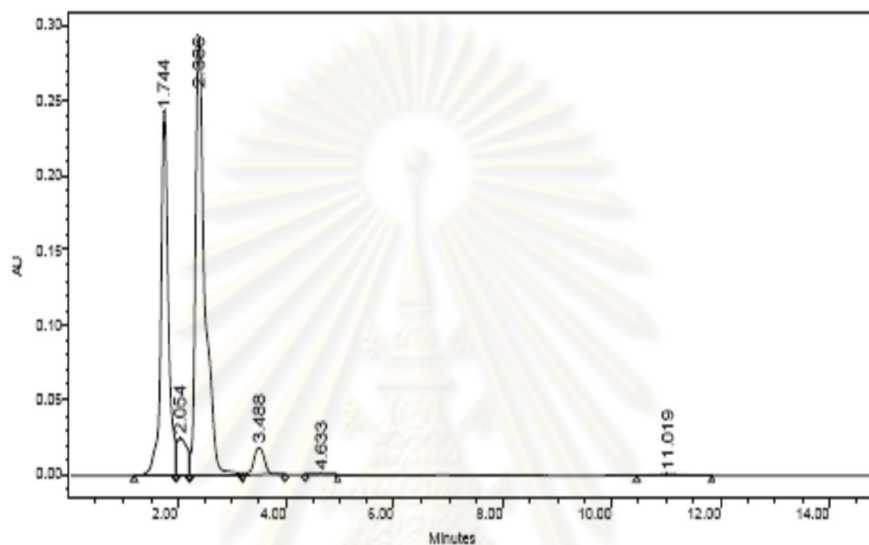


Figure E.2 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Chlorella* sp. strain PK30 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |                                  |                 |                                 |
|--------------------|----------------------------------|-----------------|---------------------------------|
| Sample Name        | PK37+2ug trans apo b 8 carotenal | Acquired By:    | Parichart                       |
| Sample Type        | Unknown                          | Date Acquired:  | 14/9/2553 21:45:49              |
| Vial:              | 1                                | Acq. Method     | 4 JUNB0 BETA CAROTENE           |
| Injection #        | 1                                | Date Processed  | 14/9/2553 22:17:39              |
| Injection Volume   | 20.00 ul                         | Channel Name    | 2487Channel1                    |
| Run Time           | 15.00 Minutes                    | Channel Desc:   | 0.8 ml/min , ACNDCNMeOH70:20:10 |
| Column Type        |                                  | Sample Set Name |                                 |

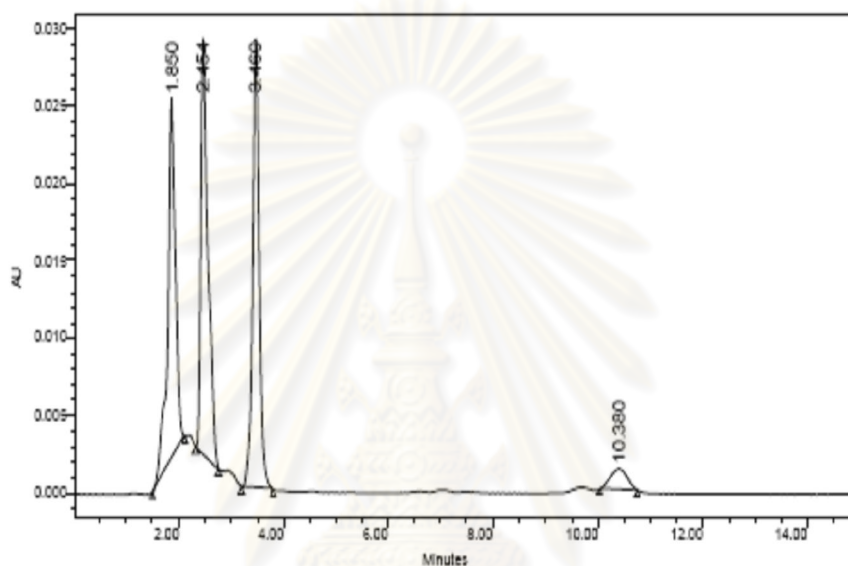


Figure E.3 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Chlorella* sp. strain PK37 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |               |                 |                                 |
|--------------------|---------------|-----------------|---------------------------------|
| Sample Name        | PK38          | Acquired By:    | Parichart                       |
| Sample Type        | Unknown       | Date Acquired:  | 1/8/2553 3:21:24                |
| Vial               | 8             | Acq. Method     | 4 JUNB0 BETA CAROTENE           |
| Injection #        | 1             | Date Processed  | 1/8/2553 10:16:06               |
| Injection Volume   | 20.00 ul      | Channel Name    | 2487Channel1                    |
| Run Time           | 15.00 Minutes | Channel Desc:   | 0.8 ml/min , ACNDCNMeOH70:20:10 |
| Column Type        |               | Sample Set Name |                                 |

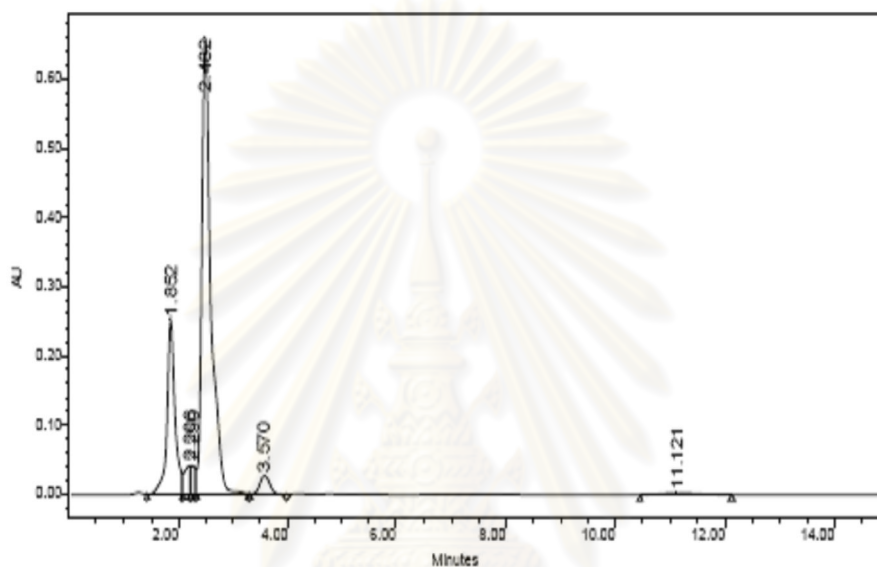


Figure E.4 Chromatogram of pigment profile and trans-*apo*-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Chlorella* sp. strain PK38 and the percentage of recovery. The retention times for trans-*apo*-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |               |                 |                       |
|--------------------|---------------|-----------------|-----------------------|
| Sample Name        | NJ14          | Acquired By:    | Parichart             |
| Sample Type        | Unknown       | Date Acquired   | 5/9/2553 23:21:15     |
| Vial:              | 1             | Acq. Method     | 4 JUN00 BETA CAROTENE |
| Injection #        | 1             | Date Processed  | 5/9/2553 23:40:26     |
| Injection Volume   | 20.00 ul      | Channel Name    | 2487Channel1          |
| Run Time           | 15.00 Minutes | Sample Set Name |                       |

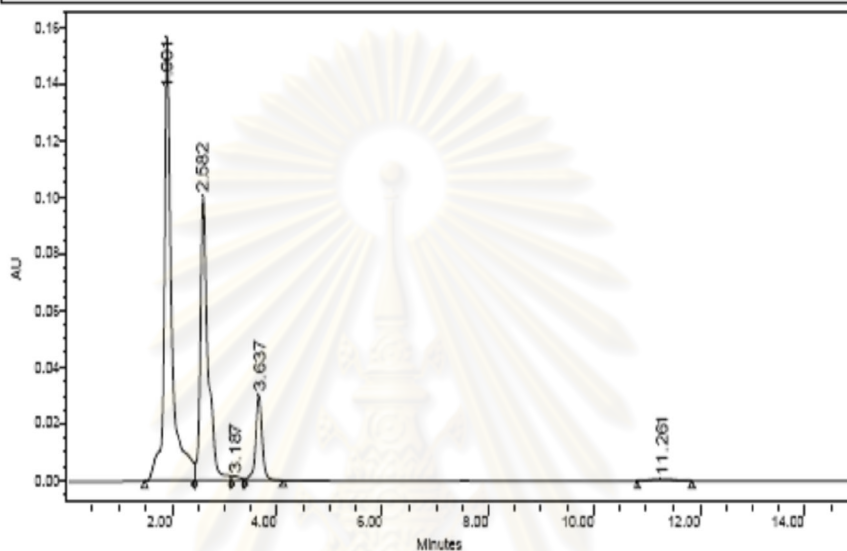


Figure E.5 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Desmodemus* sp. strain NJ14 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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จุฬาลงกรณ์มหาวิทยาลัย



Project Name parichart  
 Reported by User Parichart



| SAMPLE INFORMATION |               |                 |                                 |
|--------------------|---------------|-----------------|---------------------------------|
| Sample Name        | SS1           | Acquired By:    | Parichart                       |
| Sample Type        | Unknown       | Date Acquired:  | 1/8/2553 11:15:49               |
| Vial:              | 10            | Acq. Method:    | 4 JUNBO BETA CAROTENE           |
| Injection #        | 1             | Date Processed: | 1/8/2553 11:38:21               |
| Injection Volume   | 20.00 ul      | Channel Name    | 2487Channel1                    |
| Run Time           | 15.00 Minutes | Channel Desc:   | 0.8 ml/min , ACNDCMMeOH70:20:10 |
| Column Type        |               | Sample Set Name |                                 |

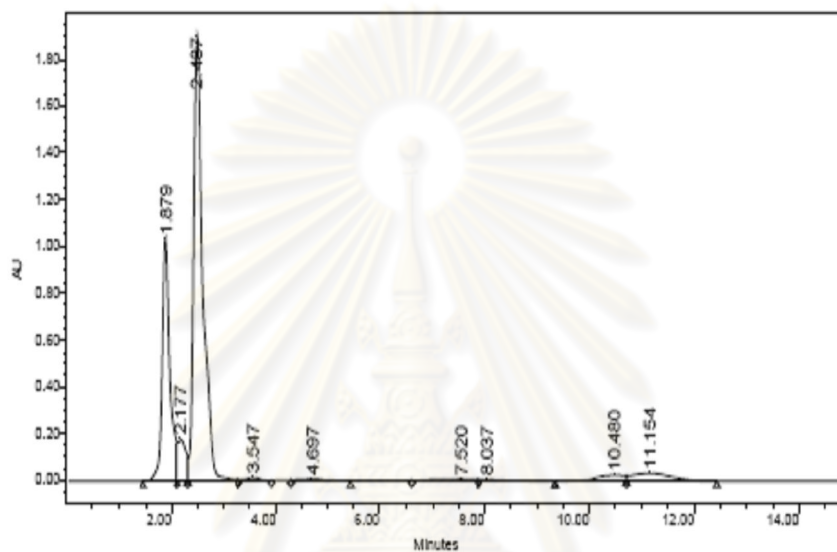


Figure E.6 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Chlorella* sp. strain SS1 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

ศูนย์วิทยทรัพยากร  
 จุฬาลงกรณ์มหาวิทยาลัย

Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |               |                 |                       |
|--------------------|---------------|-----------------|-----------------------|
| Sample Name        | PK25          | Acquired By:    | Parichart             |
| Sample Type        | Unknown       | Date Acquired   | 5/9/2553 23:49:32     |
| Vial:              | 1             | Acq. Method     | 4 JUN80 BETA CAROTENE |
| Injection #        | 1             | Date Processed  | 6/9/2553 0:11:47      |
| Injection Volume   | 20.00 ul      | Channel Name    | 2487Channel1          |
| Run Time           | 15.00 Minutes | Sample Set Name |                       |

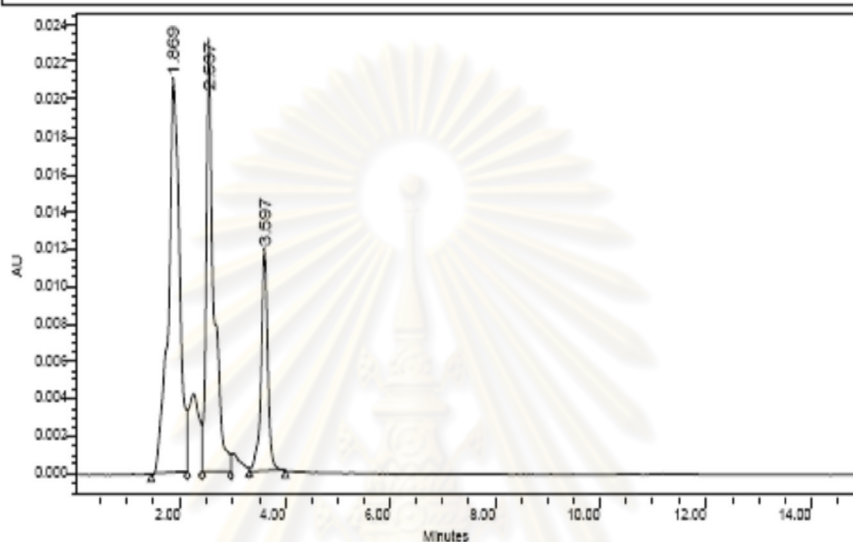


Figure E.7 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Scenedesmus* sp. strain PK25 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |               |                 |                                 |
|--------------------|---------------|-----------------|---------------------------------|
| Sample Name        | PK92          | Acquired By:    | Parichart                       |
| Sample Type        | Unknown       | Date Acquired:  | 1/8/2553 11:39:34               |
| Vial:              | 11            | Acq. Method     | 4 JUN80 BETA CAROTENE           |
| Injection #        | 1             | Date Processed  | 20/9/2553 12:16:48              |
| Injection Volume   | 20.00 ul      | Channel Name    | 2487Channel1                    |
| Run Time           | 15.00 Minutes | Channel Desc:   | 0.8 ml/min , ACNDCMMeOH70:20:10 |
| Column Type        |               | Sample Set Name |                                 |

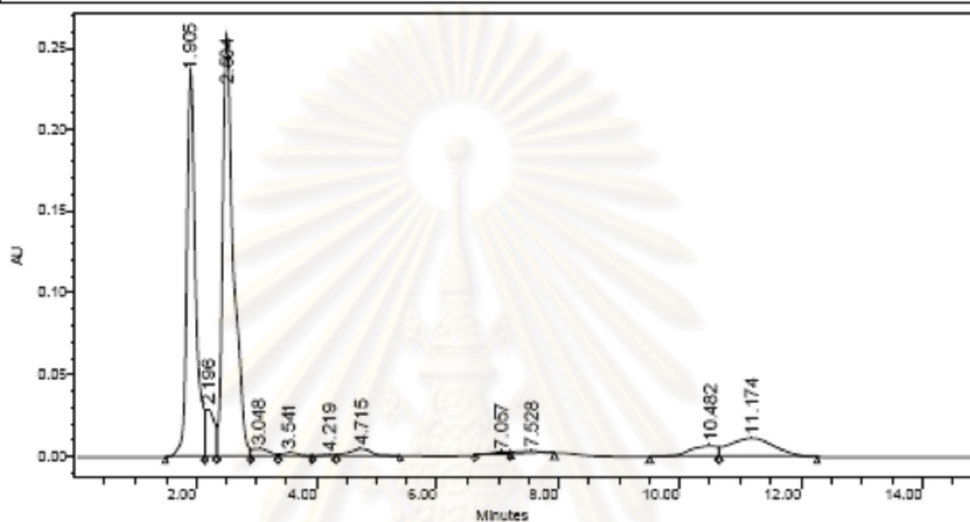


Figure E.8 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Scenedesmus* sp. strain PK92 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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จุฬาลงกรณ์มหาวิทยาลัย

Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |                                 |                 |                                 |
|--------------------|---------------------------------|-----------------|---------------------------------|
| Sample Name        | NJ42+2ug trans apo b8 carotenal | Acquired By:    | Parichart                       |
| Sample Type        | Unknown                         | Date Acquired:  | 14/9/2553 2212:03               |
| Vial:              | 2                               | Acq. Method     | 4 JUN80 BETA CAROTENE           |
| Injection #        | 1                               | Date Processed: | 14/9/2553 2233:08               |
| Injection Volume   | 20.00 ul                        | Channel Name    | 2487Channel1                    |
| Run Time           | 15.00 Minutes                   | Channel Desc:   | 0.8 ml/min , ACNDCMMeOH70:20:10 |
| Column Type        |                                 | Sample Set Name |                                 |

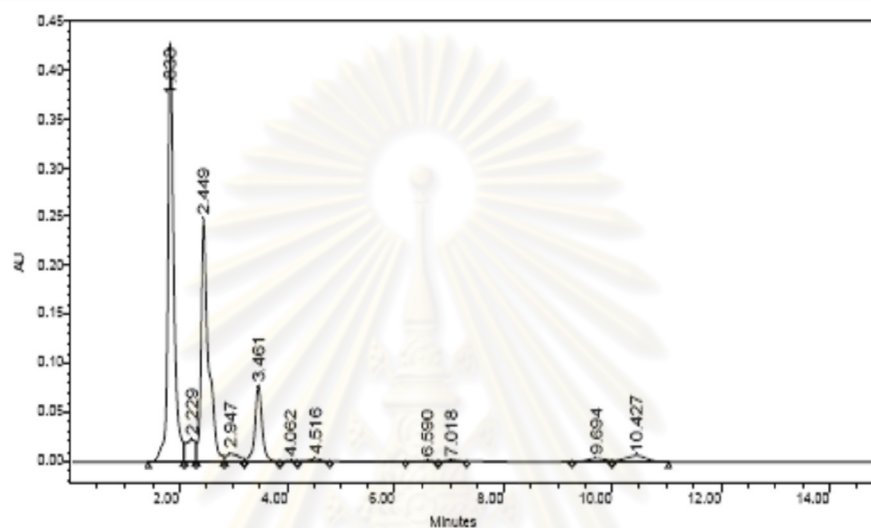


Figure E.9 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Scenedesmus* sp. strain NJ42 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |                                |                 |                       |
|--------------------|--------------------------------|-----------------|-----------------------|
| Sample Name        | SS4+2ug trans apo b8 carotenal | Acquired By:    | Parichart             |
| Sample Type        | Unknown                        | Date Acquired   | 15/02/53 13:58:17     |
| Vial:              | 3                              | Acq. Method     | 4 JUNB0 BETA CAROTENE |
| Injection #        | 1                              | Date Processed  | 15/02/53 14:25:59     |
| Injection Volume   | 20.00 ul                       | Channel Name    | 2487Channel1          |
| Run Time           | 15.00 Minutes                  | Sample Set Name |                       |

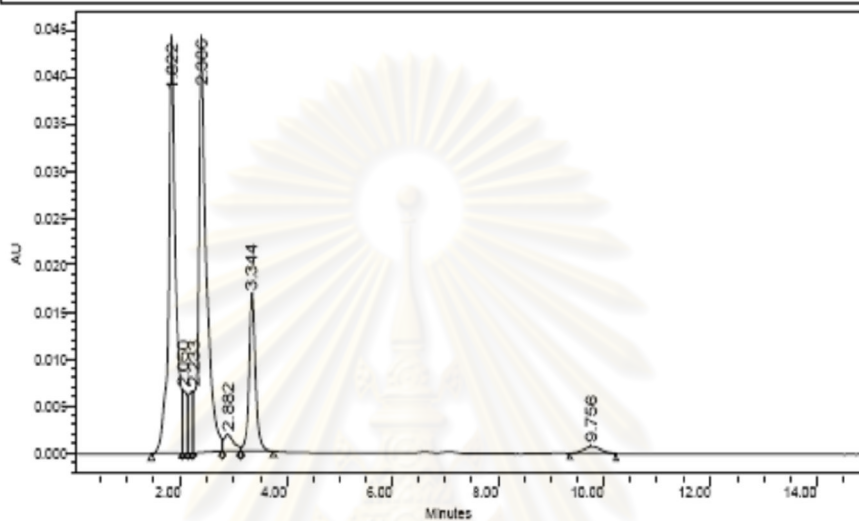


Figure E.10 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Scenedesmus* sp. strain SS4 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

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Project Name parichart  
Reported by User Parichart



| SAMPLE INFORMATION |                             |                 |                       |
|--------------------|-----------------------------|-----------------|-----------------------|
| Sample Name        | SS5+2ugtrans apo8 carotenal | Acquired By:    | Parichart             |
| Sample Type        | Unknown                     | Date Acquired   | 19/9/2553 14:24:35    |
| Vial:              | 2                           | Acq. Method     | 4 JUN90 BETA CAROTENE |
| Injection #        | 1                           | Date Processed  | 19/9/2553 14:40:19    |
| Injection Volume   | 20.00 ul                    | Channel Name    | 2487Channel1          |
| Run Time           | 15.00 Minutes               | Sample Set Name |                       |

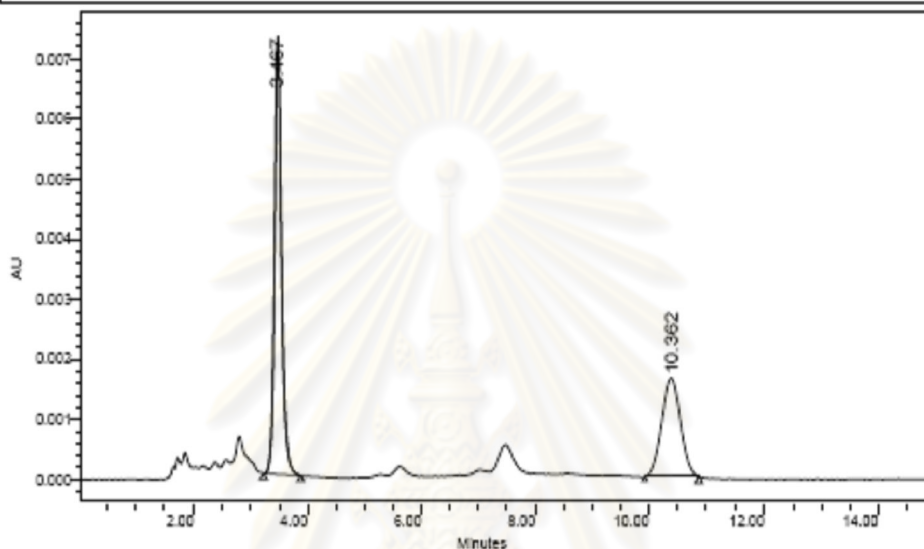


Figure E.11 Chromatogram of pigment profile and trans-apo-8-carotenal for the determination of  $\beta$ -carotene content in mid-log phase cells of *Scenedesmus* sp. strain SS5 and the percentage of recovery. The retention times for trans-apo-8-carotenal and  $\beta$ -carotene were  $3.670 \pm 0.014$  min and  $10.662 \pm 0.030$  min respectively.

ศูนย์วิทยทรัพยากร  
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## BIOGRAPHY

Miss Parichart Kittimasakun was born on September 28, 1982. She obtained a Bachelor of Science Degree in Biology (Microbiology) from Kasetsart University, Bangkok, Thailand, in 2005.

### Publication

1. สุภัทรวณิช แสงวงดี นนทิตา แจ่มกั้งवाल รุจิพร ประทีปเสน **ปาริชาติ กิตติมาสกุล** และ กาญจนา ชานูสง่าเวช. 2007. อนุกรมวิธานแบบพอลิฟาสสิกในสาหร่ายน้ำจืด *Chlorella* spp. และ *Scenedesmus/Desmodesmus* spp. วารสารวิจัยวิทยาศาสตร์(Section T) 6 (ฉบับพิเศษ 1) : 43-54.

### Presentation at Scientific Conferences

- 1) **Parichart Kittimasakun**, Panita Dusitsuttirat and Kanjana Chansa-ngavej. 2006. Isolation of cyanobacteria and micro-algae from soybean cultivation areas in Wiangsa district, Nan province. Abstract Book., The 2007 Annual Meeting : Steps Towards the 10<sup>th</sup> Decade of the Faculty of Science, March 15-16, 2007. Chulalongkorn University. Bangkok, Thailand. p. 62-63.
- 2) **Parichart Kittimasakun** and Kanjana Chansa-ngavej. 2007. Isolation and DNA-fingerprinting of green micro-algae in the genus *Chlorella* from soil samples. Abstract Book. The 12<sup>th</sup> Biological Science Graduate Congress 2007, December 17-19, 2007. University of Malaya. Kuala Lumpur , Malaysia. p. 331.
- 3) **Parichart Kittimasakun** and Kanjana Chansa-ngavej. 2008. Effects of high temperatures on growth and pigments of *Chlorella* spp. strains PK37, PK30 and PK10. Abstract Book. The Science Forum 2008. March 13-14, 2008. Chulalongkorn University. p. 28.
- 4) **Parichart Kittimasakun** and Kanjana Chansa-ngavej. 2008. Use of RAPD-PCR fingerprints to differentiate among freshwater green algal *Chlorella* and *Scenedesmus* isolates. The 13<sup>th</sup> Biological Science Graduate Congress, December 15-17, 2008. National University of Singapore, Singapore. p. 102.
- 5) **Parichart Kittimasakun** and Kanjana Chansa-ngavej. 2009. Growth and protein profiles of *Chlorella* spp., *Desmodesmus* sp. and *Scenedesmus* spp. at different temperatures. Abstract Book. The 14<sup>th</sup> Biological Science Graduate Congress, December 10-12, 2009. Bangkok, Thailand, p. 156.