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Research Article

Influence of Culture Trophic Conditions on Growth Performance and Microanatomy Changes of Microalgae, *Tetraselmis suecica*

Mojtaba Azma[1], Joo Shun Tan[2], Sahar Abbasiliasi[3], Mohd Shamzi Mohamed[1], Raha Abdul Rahim[4] and Arbakariya B Ariff[1,5,]*

[1]*Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, Malaysia*

[2]*Bioprocess Technology, School of Industrial Technology, University Sains Malaysia, Malaysia*

[3]*Halal Products Research Institute, University Putra Malaysia, Malaysia*

[4]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, Malaysia*

[5]*Bioprocessing and Biomanufacturing Research Centre, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, Malaysia*

Abstract

This study aimed to evaluate the influence of photo- and hetero-trophic culture conditions on growth performance and microanatomy changes of microalga, *Tetraselmis suecica* shake flask and stirred tank bioreactor. The changes in cell composition such as lipid, protein and carbohydrate content were determined and the kinetic parameters and microanatomy of T. suecica under heterotro- and photo-autotrophic conditions were studied. Results revealed that lipid content in the heterotrophic cells was about two times higher when compared to that of photoautotrophic cells. The final cell concentration obtained at the end of exponential phase in heterotrophic cultivation (74 $g.L^{-1}$) was higher than that obtained in photoautotrophic cultivation (13.7 $g.L^{-1}$), in photobioreactor. The shape of photoautotrophic *T. suecica* cells was oval (12.5 µm long and 7.5 µm wide) with a volume of about 552 $µm^3$. In heterotrophic cultivation, the cell was changed to a spherical shape with a diameter of approximately 3.41 µm, giving a cell volume of about 20.6 $µm^3$.

Keywords: ***Tetraselmis*; Cell composition; Kinetics; Microanatomy; Trophic status; Microalgae**

Introduction

Although some reports have been published on influence of medium composition, illumination technique and photobioreactor design on growth and photosynthetic rates of microalgae, however the information regarding to the metabolism of growing of microalgae under different illumination conditions is scarce. Carbon and energy metabolism in microalgal cells need to be studied for improvement of culture performance. Changing in carbon nature and energy sources leads to elucidation of metabolic status of the cells especially the effect of light on carbon and energy metabolism. Furthermore, growth kinetic should be determined due to the remarkable changes which occur in the algal biochemical composition at different age of culture. These changes in biochemical composition could affect to the nutritious components in microalgae especially during consumption as aquatic animal feed [1]. There are profound differences in microalgae in terms of cell organization, growth modes and manipulation of metabolism [2].

Main photosynthetic pigment found in all algae contain chlorophyll a. In green algae only chlorophyll a is active, and the activation excited by the light absorbed by chlorophyll b. Therefore, chlorophyll a and b are the more important photosynthesis pigments in green algae [3]. Photosynthesis and oxidative glucose metabolism exist simultaneously for algae grown under mixotrophic conditions, such as *P. subcordiformis* cells, which may immediately utilize glucose in the medium for their metabolism [4]. At the same time, the function of photosynthesis is weakening, which may be attributed at least in part to the reduction in the number of chloroplasts. Many algae can grow over wide range of combination of culture conditions such as light, temperature and nutrient concentrations which resulted from limitation for elemental compositions.

Green microalgae, *Tetraselmis suecica*, are able to grow phototrophically or heterotrophically under different culture conditions. The effect of medium composition, illumination technique and various designs of photobioreactor on growth and photosynthetic rates of microalgae have been studied extensively [5]. However, little is known about the metabolism, cell composition and kinetic of microalgae cells, especially in heterotrophic cultivation. Therefore, this study evaluated growth performance of a green microalgae, *T. suecica*, cultivated under photo- and hetero-trophic conditions and studied the changes in cell composition and microanatomy of *T. suecica* at different growth phases at different illumination times and medium formulations in shake flask and stirred tank bioreactor.

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***Corresponding author:** Arbakariya B. Ariff, Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia, Tel: 603 9467591; Fax: +603 89467593; E-mail: arbarif@upm.edu.my

Materials and Methods

Microalga strain, maintenance and inoculum preparation

The axenic culture of green microalgae, *T. suecica* (Kylin) Butcher (Chlorophyta, Prasinophyceae), was used throughout this study. The photoautotrophic cell of this microalgae strain was obtained from the Institute of Bioscience, University Putra Malaysia, Serdang, Selangor, Malaysia. For preparation of heterotrophic cells, microalgae were streaked on bacteriological agar (12 g/L) with the addition of glucose (5.78 g/L), peptone (9 g/L), yeast extract (4.48 g/L), and meat extract (3.01 g/L), and incubated in an oven at 30° C for 10 days. The cultures were stored at 4°C for subsequent use in the experiments. Inoculum will be prepared by inoculating a 50 uL of aliquot into 25 ml of medium which incubated at 30° C, and agitated at 130 rpm using an orbital shake incubator (TS-560, Germany). After 120 h which the culture reached to mid log phase, 5 ml of seed culture was propagate in 100 ml of the same medium until the cell concentration reached 32 × 10^6 cell/ml. This culture was used throughout the study.

Photoautotrophic and heterotrophic cultivations in shake flasks

Walne medium was used as the basal medium for photoautotrophic cell cultivation [6]. For heterotrophic cultivation, glucose (6 g/L), peptone (9 g/L), yeast extract (5 g/L), and meat extract (3 g/L) were added to the Walne medium. To start the cultivation, the medium was inoculated with a standard inoculum (5% v/v) into 250 ml conical flask containing 100 ml medium. A circular cool white fluorescent lamp (Philips 32W) with light intensity of 32 µmol photons/m^2.s) which stand around the flask was used as the energy source. The phototrophic cells were grown at different periodic illumination times; (24 h light; 8 h light and 16 h dark; 4 h light and 20 h dark); and heterotrophic cells at 24 h dark condition. The cells were grown in a 250 ml flask in an orbital shaker at 30° C and, agitated at 130 rpm for 958 h under autotrophic conditions and for 155 h under heterotrophic conditions. All cultivations were carried out in triplicates. Culture samples were withdrawn from each culture at exponential and late exponential growth phase for biochemical analyses.

Cultivation in stirred tank photobioreactor

A 2 L stirred tank bioreactor (Biostat B, B. Braun Biotech, Germany) which illuminated continuously at intensities of 26 (µmol quanta.m^{-2}.s^{-1}) by circular cool white 32W fluorescent lamp (Philips) was used for cultivation. The photobioreactor consisted of stainless steel top plate and double jacketed glass vessel, which was equipped with 6 bladed-Rushton turbine impeller (diameter = 0.052 m).

For phototrophic cultivation, aeration was performed by sparging air through the ring air sparger, located below the impeller, at a flow rate of 0.5 L.min^{-1} and the impeller speed was fixed at 150 rpm. For heterotrophic cultivation, the photobioreactor was operated as bubble-free-aeration systems. Silicone tubing (2 m) was wrapped around a stainless steel support and placed inside the bioreactor vessel. Air was pumped through this gas permeable thin tubing at a pressure of 2 bars. The impeller speed was fixed at 270 rpm, which was optimized in a preliminary study. To initiate fermentation, the photobioreactor was seeded with 10% (v/v) of inoculum. The temperature within the vessel was maintained at 28° C ± 0.5 throughout the fermentation. Culture samples were withdrawn at time interval for analysis.

Analytical methods

Cell density was measured as suggested by Garoma and Jand a [7]. An aliquot was collected from each culture at the exponential and latex exponential growth phases for biochemical analyses. The cells were harvested by centrifugation at 5000 × g for 10 min. The pellet was washed with 100 ml of 0.5 M ammonium format to remove residual salts from the seawater medium and centrifuged at 5000 × g to separate the cells. The cell pellets were prepared separately for protein, carbohydrate, and total lipid assay.

For determination of the protein content, samples of known volume were centrifuged at 5000 × g and 4° C for 15 min. The supernatant was replaced with 10ml of sodium phosphate buffer containing 1% (w/v) Sodium Dodecyl Sulphate (SDS) and sonicated on ice (3 cycles, for 1 min) [8]. The sonicated solution was centrifuged to remove the pellet. The amount of protein in the cells was determined using the method proposed by Bradford [9].

Extraction of Lipids was performed as suggested by Chen [10]. Carbohydrate analysed using the phenol-sulfuric acid method [11]. The cellular ultrastructure was observed via Transmission Electron Microscopy (TEM) examination as described by Li [12].

Kinetic parameters assessment and statistical analyses

The doublings time (t_d) and overall productivity (g.L^{-1}.h^{-1}) was calculated using Equations (1) and (2), respectively:

$$T_d\ (h) = \ln 2 / \mu \qquad (1)$$

$$P\ (g.L^{-1}.h^{-1}) = (X_T - X_0) / (t_T - t_0) \qquad (2)$$

Where, X_0 and X_t are initial and final cell concentration (g.L^{-1}); t_0 and t_t are the initial and final time (h) of the cultivation. Data were analyzed by the Analysis Of Variance (ANOVA) method, with microalgae protein, lipid, and carbohydrate composition representing the sources of variances. The growth kinetics of microalga, *T. suecica* was made according to the Baranyi-Robert microbial propagation model [12] by following Equation:

$$X(t) = X_0 + \mu_{max} B(t) - h\left(1 + \frac{\exp^{\mu_{max} \cdot B(t)} - 1}{\exp^{(X_{max} - X_0)}}\right) \qquad (3)$$

Where the coefficient, B:

$$B(t) = t + \frac{1}{\mu_{max}} h\left(\exp^{(-\mu_{max} \cdot t)} + \exp^{(-\mu_{max} \cdot \lambda)} - \exp^{(-\mu_{max}(t+\lambda))}\right)$$

X_0: Initial cell Concentration; X: Cell Concentration; t: Cultivation Time; μ_{max}: Maximum Specific Growth Rate and λ: Time During Lag Phase

Results

Effect of illumination times on growth of *T. suecica* in shake flask

The growth performance of *T. suecica* cultivated under heterotrophic culture conditions (24 h dark) and photoautotrophic culture conditions at different illumination times (24 h, 8 h and 4 h illumination) in shake flask are shown in Table 1. The cultivation time to reach maximum cell concentration (8.4 g.L^{-1}) was 408 h in photoautotrophic condition with 24 h light, and the cultivation time increased with reduced illumination time. The phototrophic cultivation time for 8 h light was 548 h, and increased to 958 h with decreased the illumination time to 4 h. On the other hand, final cell concentration was gradually decreased with decreased in illumination time. The maximum cell concentration of illumination of 8 h light (6.4 g.L^{-1}) and 4 h light (4.5 g.L^{-1}) reduced at 24% and 30% as compared to 24

h light. Cultivation time (142 h) in heterotrophic culture was markedly reduced compared to that of photoautotrophic. In addition, the final cell concentration obtained in heterotrophic cultivation (24.3 $g.L^{-1}$) was about 3 times higher than that obtained in fully photoautotrophic cultivation (24 h light). The μ_{max} (0.25 h^{-1}) for heterotrophic cultivation was higher than that exhibited by the phototrophic cultivation. Moreover, the productivity obtained in heterotrophic cultivation (0.17 $g.L^{-1}.h^{-1}$) increased more than eight times as compared to the highest productivity obtained in photoautotrophic cultivation (0.02 $g.L^{-1}.h^{-1}$). The doubling time of heterotrophic cultivation (7.7 h) was 3.3-fold higher than photoautotrophic cultivation (25.5 h). The result indicated that the growth kinetics of the phototrophic cultivation of *T. suecica* was markedly influenced by the different illumination times.

Effect of heterotrophic and photoautotrophic culture conditions on cell composition in shake flask

Table 2 presents the biochemical composition expressed as percentage of dry cell weight and organic weight for *T. suecica* cultivated in shake flask under heterotrophic culture conditions (24 h dark) and photoautotrophic culture conditions at different illumination times (24 h, 8 h and 4 h illumination). Protein content ranging from 21% to 46% was obtained in the cells of all photoautotrophic cultures of *T. suecica*, irrespective of illumination time or growth phase (exponential and late-exponential). During the exponential phase, protein was apparently richest in cells grown at the 24 h illumination time (45.7%). At late exponential phase, the percentage of protein was decreased to 37.2%.

The carbohydrate content had the lowest percentage of cell composition over the different photoautotrophic illumination times, ranging from 7% to 16.3% of cell dry weight. The cells had one-quarter to one-third of the carbohydrate content of cells grown under 24 h light during both the logarithmic and stationary phases. The carbohydrate content of all cells increased with onset of the late exponential phase. The percentage of lipid showed some differentiations in all cells during the exponential phase, ranging from 18.1% to 27.6%. Lipid content increased during the stationary phase and the highest increment (24.5% to 27.6%) was obtained after 24 h illumination.

Total protein, carbohydrate, and lipid supply/were 55.5% to 77.6% of the total dry cell weight. This is done the relative amounts of the organic nutrients was calculated by recalculating the results from Table 1, as a percentage of organic weight (where organic weight is defined as the total protein, carbohydrate, and lipid). The percentage of organic weight for carbohydrate showed similar trends across the exponential and late exponential phases, expressed as carbohydrate percentage of dry weight. Cells after 24 h illumination had one-third to one-half of the carbohydrate content as compared to those of 8 h and 4 h light conditions in both the exponential and late exponential phases. The carbohydrate percentage/level increased with onset of the late exponential phase. Protein percentage was highest in cells after 24 h illumination time (58.9% organic weight), but this percentage decreased in cells of all cultures during the late exponential phase. Lipid percentage in the exponential phase of autotrophic cultures increased from 31.5% to 33.8% of organic weight at different illumination times and increased slightly during the late exponential phase. Lipid content in heterotrophic cells was about 2 times higher as compared with photoautotrophic cells. However, the protein content in heterotrophic cells was 3 times lower than that of photoautotrophic cells.

Influence of photoautotrophic cultivation on size of organelles and cell division of *T. suecica* in shake flask

Influence of different illumination times on size of organelles and organic cell composition as shown in Figure 1. The pyrenoid when the cells were grown under 24 h illumination time was larger as compared to that of 8 h and 4 h illumination times, while the amount of starch was slightly higher after 4 h illumination time than the cells cultured

Table 1: Cultivation performances and growth kinetics of *T. suecica* cultivated in different photoautotrophic and heterotrophic conditions in shake flask.

Performance/growth kinetics	24 h light	8 h light	4 h light	24 h dark
Cultivation time to reach maximum cell concentration; (h)	408 ± 34^{b}	546 ± 28^{b}	958 ± 17^{c}	142 ± 8^{a}
Final Cell concentration; ($g.L^{-1}$), Actual	8.4 ± 0.71^{b}	6.4 ± 0.42^{c}	4.5 ± 0.14^{d}	24.3 ± 0.28^{a}
Final Cell concentration; ($g.L^{-1}$), Model	8.2 ± 0.14	6.3 ± 0.10	4.1 ± 0.16	23.54 ± 0.39
Maximum specific growth rate; (h^{-1})	0.03 ± 0.01^{b}	0.02 ± 0.02^{c}	0.01 ± 0.00^{d}	0.25 ± 0.02^{a}
Productivity; ($g.L^{-1}.h^{-1}$)	0.021 ± 0.01^{b}	0.012 ± 0.0^{c}	0.005 ± 0.0^{d}	0.170 ± 0.02^{a}
Doubling time; (h)	25.5 ± 5^{b}	26.0 ± 1^{b}	55.0 ± 9^{c}	7.7 ± 1.5^{a}

Values are mean ± STDEV (*n=3*).

[a-d] subscript with the letter in the same row are not significantly different at P<0.05.

Table 2: Biochemical composition of *T. suecica* cultivated under photoautotrophic conditions at different illumination times and heterotrophic conditions.

Illumination time(h)	*Sampling phase	Protein		Total lipid		Carbohydrate	
		Dry weight (%)	Organic weight (%)	Dry weight (%)	Organic weight (%)	Dry weight (%)	Organic weight (%)
Photoautotrophic							
24 h light	Exp	45.7 ± 2.3	58.9 ± 3.1	24.5 ± 2.8	31.5 ± 3.0	7.4 ± 1.8	9.6 ± 2.1
	Lexp	37.2 ± 2.8	49.2 ± 3.6	27.6 ± 1.5	36.6 ± 2.7	10.6 ± 0.9	14.1 ± 0.6
8 h light and 16 h dark	Exp	34 ± 1.2	49.2 ± 3.7	23 ± 1.8	33.8 ± 2.4	11.7 ± 1.4	16.9 ± 0.9
	Lexp	29.7 ± 2.9	43.6 ± 2.4	25.5 ± 1.2	37.3 ± 1.5	12.9 ± 1.1	19 ± 5.7
4 h light and 20 h dark	Exp	22.3 ± 1.1	40.3 ± 3.9	18.1 ± 0.8	32.6 ± 2.4	14.8 ± 0.9	26.9 ± 4.7
	Lexp	21.2 ± 1.9	36.6 ± 1.9	20.5 ± 1.9	35.3 ± 1.3	16.3 ± 1.2	28 ± 1.7
Heterotrophic							
24 h dark	Exp	10.5 ± 0.9	13.6 ± 0.8	51.9 ± 4.3	67.2 ± 0.7	14.8 ± 0.8	19.1 ± 3.1
	Lexp	10.3 ± 1.5	12.9 ± 1.5	53.8 ± 3.3	68.1 ± 2.7	15.2 ± 1.0	19.2 ± 3.1

Values are mean ± STDEV (n=3).

*Exp – during exponential phase; LExp – during late exponential phase

under 24 h and 8 h. The cup-shape and regulation of chloroplast were more typical in the cells grown under 24 h illumination time as compared to cells cultured at 8 h and 4 h illumination times.

General arrangement of organelles in *T. suecica* under photoautotrophic condition under TEM is as shown in Figure *T. suecica*, is quite small vegetative oval shape with 12.5 ± 0.5 µm length, 7.5 ± 0.3 µm width, and an average volume of 552 μm^3 (Figure 2A). The cell appears rectangular in longitudinal section, and quadriflagellate arise from the anterior end (Figure 2B). The flagella emanat from the base of an anterior invagination, the flagella pit, and the flagellar membrane is covered with scale. The Golgi bodies were observed in the cell (Figure 2C), adjacent to the nucleus. The structure of the flagella and basal bodies were as shown in Figure 3. The prominent fibers were connected to a pair of basal bodies (Figure 3B). The flagellar root system consisted of fibrous and microtubular roots, which elongated from the proximal end of the basal bodies to the chloroplast inner surface. The Golgi body was located in the anterior central part of the cell (Figure 3G). C is ternae of the Golgi body were arranged obliquely on the side facing the nucleus.

Figure 4 shows micrographs of *T. suecica* during cell division. Cell division orientation was vertical. The flagella were autotomized prior to the cells settling (Figure 4A). At the same time, the cell was covered by an extra cell wall (Figure 4B). All organelles, like Golgi body, started to multiply, followed by the division of cell organelles. The cell wall was thickened prior to dividing into two cells (Figure 4C - I). The extra cell wall was digested (Figure 4J), and cells swam to be separated far from each other (Figure 4K and 4L). Cell wall covered by scales; I: Flagella covered by scales and microtubules with two central microtubules; J: Flagella with two central microtubules; K: Pit fiber and flagella; L: Layers of cell wall.

Growth kinetic of phototrophic cultivation and heterotrophic cultivation in stirred tank photobioreactor

The growth performance of *T. suecica* cultivated in stirred tank photobioreactor under phototrophic 24 h light and heterotrophic 24 h dark conditions are as summarized in Table 3. The final cell concentration obtained at the end of exponential phase in heterotrophic cultivation (74 $g.L^{-1}$) was higher than that obtained in photoautotrophic cultivation 13.7 ($g.L^{-1}$). The cell concentration for heterotrophic cultivation (74 g/L) in stirred tank photobioreactor was about three and eight times higher as compared to heterotrophic culture (24.3 g/L), and photoautotrophic culture (8.4 g/L) in shake flask culture, respectively. The maximum specific growth rate (μ_{max}) for heterotrophic cultivation (0.59 h^{-1}) was higher than that observed in photoautotrophic cultivation (0.05 h^{-1}). In addition, the productivity for heterotrophic cultivation (0.76 $g.L^{-1}.h^{-1}$) was higher than that obtained photoautotrophic cultivation (0.07 $g.L^{-1}.h^{-1}$). On the other hand, the minimum doubling time for heterotrophic cultivation (9.9 h) was lower than phototrophic condition (12.4 h).

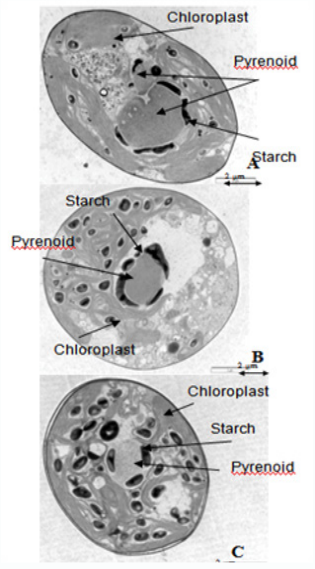

Figure 1: Transmission electron micrographs of *T. suecica* grown under photoautotrophic conditions with different illumination times; 24 h light (A), 8 h light and 16 h dark (B), and 4 h light and 20 h dark (C) conditions.

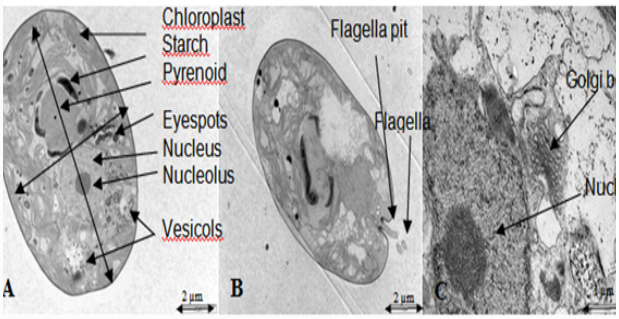

Figure 2: Transmission electron micrographs of *T.suecica* cultured under photoautotrophic conditions showing the organelles general arrangement; A: Longitudinal section of *T. suecica*; B: Flagella pit and flagella; C: Golgi body.

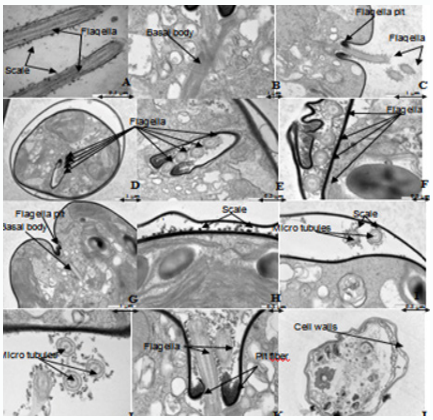

Figure 3: Transmission electron micrographs of *T. suecica* cultured under photoautotrophic conditions showing the organelles general arrangement; A: The flagella covered by scales; B: Basal body; C: Flagella and flagella pit; D, E and F: Flagella; G: Flagella pit and basal body; H: Cell wall covered by scales; I: Flagella covered by scales and microtubules with two central microtubules; J: Flagella with two central microtubules; K: Pit fiber and flagella; L: Layers of cell wall.

Discussion

Carbon content showed to be a significant constituent of *D. salina* mixotrophic growth media as it supports rapid growth with high cell growth rate. Held [13] reported that the contribution of energy in microalgae growth was largely depended on medium composition rather than light. Toro [14] claimed that the growth performance of *C. gracilis* and *Isochrysisgalbana* cultivated under limited light (12 h) was similar to that obtained in cultivation with continuous illumination, where the energy source from light was doubled. Our results also show

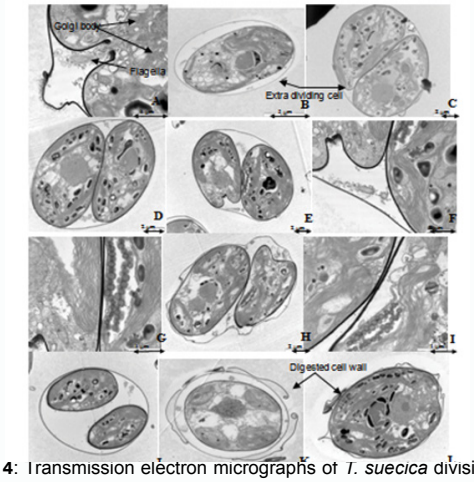

Figure 4: Transmission electron micrographs of *T. suecica* division cultured under photoautotrophic conditions; A: Flagella and Golgi body before cell division; B: Extra dividing cell wall before cell division; C, D, E, F, G, H and I: Different stages of cell dividing and improvement of the cell wall prior to separation of sister cells; J: Sister cells surrounded by common cell wall; K: Digesting of extra cell wall after cell division; L: Digestion of outer cell wall after the separation of sister cells.

Table 3: Cultivation performance and growth kinetics of *T. suecica* cultivated in 2L stirred tank photobioreactor under phototrophic and heterotrophic conditions.

Performance/Growth kinetics	Photoautotrophic	Heterotrophic
Cultivation time to reach a maximum cell concentration; (h)	348 ± 41	139 ± 19
Final cell concentration; ($g.L^{-1}$), Actual	13.7 ± 0.6	74.0 ± 0.5
Final cell concentration; ($g.L^{-1}$), Model	13.4 ± 0.2	76.2 ± 1.3
Maximum specific growth; rate (h^{-1})	0.05 ± 0.00	0.59 ± 0.00
Maximum productivity; ($g.L^{-1}.h^{-1}$)	0.07 ± 0.02	0.76 ± 0.1
Minimum doubling time; (h)	12.4 ± 0.7	9.9 ± 1.5

Values are mean ± STDEV (n=3).

that the cells of *T. suecica* were capable to grow under fully darkness (24 h dark) as heterotrophic cells, using glucose as the energy source. Cultivation time for heterotrophic cultivation was reduced by about 2.5 times as compared to phototrophic cultivation, suggesting that the organic carbon source produced more energy as compared to light for the growth of *T. suecica*. Although heterotrophic cultivation improve the cultivation performance of microalgae, in term of final cell concentration and productivity, it can be used for efficient production metabolites favourable for bio-oil production or lipids.

It is difficult to clearly identify which factor exactly had effect on enhancement of cell growth in heterotrophic cultivation as compared to photoautotrophic. Heterotrophic cultivation offers the possibility of improving the final microalgae cell concentration by efficient utilization of organic carbon source as energy. Uptake of energy source and nutrition in heterotrophic cultivation are easier as compared to photoautotrophic cultivation. Nitrogen deficiency was identified as one of the factors that induce an increment in lipid content in microalgae [15]. In a nitrogen starvation, there was no growth on the microalgae. However, the lipid contents of four strains (TRG, KB, SK, and PSU) identified as *Botryococcus spp.* Increased [16]. Under limitation of nitrogen in the medium resulted in a significant change in cell composition such as decrease in protein content in *Spirulina platensis* [17]. The availability of nitrogen allowed the microalgae performed protein synthesis during the exponential phase [18]. As algal culture age increased, carbohydrates accumulated in response to nitrogen depletion [19], and metabolism shifts from protein formation to energy (carbon) storage.

The comparison of organic weight percentage ratio to total cell dry weight in photoautotrophic and heterotrophic cultivation showed that the amount of organic weight in heterotrophic culture (79.3%) was higher than that obtained in photoautotrophic culture (75.4%), indicating that the amounts of other cell components, like ash, were reduced in heterotrophic cells. Langdon [20] suggested that microalgae as a food must supply both energy and essential nutrients. Although little is known about the specific nutrient requirements for lipid, carbohydrate, and protein, these nutrients remain the major dietary source of energy for growth and development. Conditions under which microalgae grow dictate biochemical composition and can affect energy and nutrient value.

The results from this study demonstrated that the green microalgae *T. suecica* can be grown under different culture conditions, both photoautotrophically and heterotrophically at different performances. The biochemical composition of *T. suecica* varied with the culture age, illumination times and energy source. The genus of *Tetraselmis* (Platymonas) includes flattened, unicellular green algae, which possess four scaly or in some species hair-covered flagella and a rigid extracellular wall that covers the protoplast surface, including the characteristic apical depression, the pit [21]. Cell growth can be divided into two phases which are earlier cell proliferation and later enlargement in cell volume, both of which directly contribute to algal biomass accumulation. Therefore, it is expected that rapid division and enlargement in cell volume after division in photoautotrophic. When the source of energy was changed from light to organic carbon, it was necessary for cells to adapt and survive under the new environmental conditions. During the adaptation, heterotrophic cells lost their flagella, and the cell walls digested from inside to outside, which clearly shown there was no enlargement in cell volume. The proliferation rate of the cells increased greatly as the heterotrophic cells easily consumed the organic molecule from environment for metabolic pathway and biomass production.

Marin [22] reported that some strains of *Tetraselmis* spp. have hairs on flagella surfaces but cells and flagella surfaces of *T. suecica* in this study were covered with small square scales without any hair. Moreover the heterotrophic cells surfaces had no scale or hair. After cell division, Golgi apparatus activated and Golgi bodies placed around the daughter nuclei. Longitudinally-sectioned of the cell component under autotrophic conditions showed that the Golgi bodies were close to the nuclei.

Cell damage in agitated bioreactor which is due to the shear stress and bubble break-up is the main problem in cultivation of microalgae [23]. In this study, bubble free system has been used for the heterotrophic cultivation of microalgae, where the effect of shear stress on cell viability could be minimized. In addition, excessive foaming problems which normally accounted in air sparged bioreactor could also be avoided with bubble free system. Very few quantitative studies have been conducted in relation to the hydrodynamic stress created in gas-sparged photobioreactor to the growth characteristics of microalgae. The growth rates of some microalgae increase initially with increasing turbulence, which could be due to the improved supply of light or CO_2. However, at an optimum level of turbulence, the growth decreased with further increase in the superficial-gas velocity, due to cell damage. Cell damage is due to bubble formation, bubble rising or bubble break-up and mainly bubble bursting at the liquid surface.

Conclusions

Results from this study revealed that heterotrophic growth could be performed in stirred tank bioreactors, which may improve the yield and reduce the cost of microalgal biomass and oil production. This is important, since a low cost is desirable for commercial application in food industries and also biodiesel production. Development of microalgae with high lipid content would be a new and promising means for biodiesel production in the future.

Acknowledgments

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