Improved Protocol for the Preparation of *Tetraselmis suecica* Axenic Culture and Adaptation to Heterotrophic Cultivation

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Abstract: The effectiveness of various physical and chemical methods for the removal of contaminants from the microalgae, *Tetraselmis suecica*, culture was investigated. The information obtained was used as the basis for the development of improved protocol for the preparation of axenic culture to be adapted to heterotrophic cultivation. Repeated centrifugation and rinsing effectively removed the free bacterial contaminants from the microalgae culture while sonication helped to loosen up the tightly attached bacterial contaminants on the microalgae cells. Removal of bacterial spores was accomplished using a mixture of two antibiotics, 5 mg/mL vancomycine and 10 mg/mL neomycine. Walne medium formulation with natural seawater was preferred for the enhancement of growth of *T. suecica*. Adaptation of growth from photoautotrophic to heterotrophic conditions was achieved by the repeated cultivation of photoautotrophic culture with sequential reduction in illumination time, and finally the culture was inoculated into the medium containing 10 g/L glucose, incubated in total darkness to obtain heterotrophic cells. Changes in the morphology and composition of *T. suecica* cells during the adaptation from photoautotrophic to heterotrophic condition, as examined under Transmission Electron Microscope, were also reported.

Keywords: Axenic microalgae culture, *Tetraselmis suecica*, adaptation, heterotrophic, phototrophic, microalgae cell composition.

INTRODUCTION

The growing demand for natural products to be used in various applications has increased interest in algal biotechnology over the past two decades [1]. Through algal biotechnology, various high-value compounds could be produced and isolated from numerous phototrophic and heterotrophic microalgae cultures [2]. Preparation of microalgae axenic culture is needed to obtain a viable culture of a single species, free of other species or "contaminants" [3]. The axenic culture of microalgae is required in large scale cultivation for production of various valuable products. In addition, physiological, biochemical, genetic and taxonomic studies of microalgae required axenic (bacteria-free) cultures [4]. Axenic cultures of microalgae are usually prepared by single-cell isolation and density gradient centrifugation, rinsing [5, 6], UV irradiation, filtration, treatment with antibiotics [7], and treatment with other germicidal chemicals [8].

For algal units with tenaciously attached contaminants, the single cell isolation method may not yield axenic cultures, and it may be necessary to use chemical methods. Treatment with antibiotics or lysozyme is still the most common way to remove the bacterial contaminants [8, 9, 10]. It is apparent that two paths could be applied toward purification of microalgae culture. The first path is to separate the desired organisms (quarry) from the unwanted ones (contaminants) in one or more steps. The other path is to kill the contaminants but not the quarry. If several contaminants exist, several steps using different methods may be required to eliminate the various contaminants successively. This may also be needed if the contaminants have more than one stage of its life cycle in the culture. The streak or spread plating method may yield axenic culture directly in some microalgae species, but in tenaciously attached contaminants, it may be necessary to kill or to inhibit growth of the contaminants *in situ* by chemical and physical methods.

Natural seawater is a complex medium containing more than 50 known elements and a large number of organic compounds. For efficient cultivation of microalgae, direct use of natural seawater is seldom acceptable. Without the addition of nutrients and trace metals, the cell yield is usually very low. Thus, enrichment of sea water as cultivation medium for microalgae is required, especially macronutrients such as nitrogen, phosphorous, vitamins, buffers and chelators [11].

The main objective of this study was to develop the improved protocol for the preparation of *Tetraselmis suecica* axenic culture for the adaptation to heterotrophic cultivation. Suitable media for the enhancement of growth of *T. suecica* in photoautotrophic and heterotrophic cultivation were formulated. Changes in growth morphology of microalgae during the adaptation from phototrophic to heterotrophic culture were also examined under the Transmission Electron Microscope.

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MATERIALS AND METHODS

Microalgae

The green microalgae, *Tetraselmis suecica* (Kylin) Butcher (Chlorophyta, Prasinophyceae), was used in this study. This microalgae was obtained from the Marine Culture Collection Unit, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia,

Media for Photoautotrophic Cultivation of Non-Purified *T. suecica* Culture

Natural sea water (NW) with a salinity of 32%, obtained from Port Dickson seashore, Negeri Sembilan, Malaysia, was used in this study. The natural sea water was filtered through a membrane filter with a nominal pore size of 0.7 μm using Aspirator A-3S (Eyela, Japan). The filtered seawater in 2 L glass bottle was autoclaved at 121°C for 20 min and stored in the chiller at 4˚C in the dark prior to use in the cultivation experiments.

Artificial sea salts supplied by Sigma, USA (AWS) and Aquarium Systems, USA (AWIO) were used as the "basal seawater" for the preparation of artificial seawaters. The artificial sea salt (40 g) was dissolved in 1 L distilled water to achieve the salinity of 32%. The enrichment solution components (macronutrient, trace metals, and vitamins) were added into the sterile salt solution. The quantity of each component was varied according to the requirement of each medium formulation.

Three different media (ES-enriched seawater medium, f/2 Guillard and Walne) were tested in this study for the cultivation of *T. suecica.* Method of preparation and the final composition of these media are shown in Table **1**. Three different sea waters (natural sea water and artificial sea water prepared using salts supplied by Sigma and Aquarium System) as mentioned above were used for the preparation of the media. ES-enriched seawater medium was initially formulated by Provasoli and Carlucci [12] and then modified by Berges *et al.* [13]. This medium was used as a general purpose marine medium for axenic cultures. f/2 Guillard medium was first proposed by Guillard and Ryther [14] and modified by Guillard and Keller [15]. This is a common and widely used general enriched sea water medium designed to grow coastal marine algae. The Walne medium was first formulated by Walne [16] using either natural or artificial seawater.

Different Treatments Tested for Purification of Microalgae

Several steps and treatments were investigated for the development of suitable protocol in the preparation of axenic culture of *T. suecica.* The treatments tested in this study include agar plating technique, centrifugation and rinsing, salinity, sonication, screening and filtration, acid and alkaline treatment, UV irradiation, antibiotic disc test and antibiotic treatment.

Agar Plating Technique

Agar plating technique was used for the separation of a single colony of microalgae according to the method as suggested by Lavens and Sargeloos [17]. In this method, microbiological agar (Merck, Germany) was added to Walne medium for the preparation of solid medium.

Centrifugation and Rinsing Treatment

The microalge cultures were centrifuged (Centrifuge B5, B. Braun Biotech International, Germany) at 2000 rcf for 10 min. To repeat the centrifugation, the supernatant was discarded and the cell pellet was resuspended in a fresh sterile Walne medium.

Salinity Treatment

The algal units were washed three times (15 min for each wash) using a sterile Walne medium with different salinities ranging from 0% to 300% as suggested by Guillard and Morton [18]. The salinity of Walne medium was adjusted either using instant ocean artificial sea salt or distilled water.

Sonication

Ultrasonic water bath (Bransonic 2510E-MT, USA) was used at low sonication intensity (90 K cycles.s^{-1}) as the step to physically separate contaminants from algal units [3]. In this technique, the test tube containing the algal unit was placed in ultrasonic water bath at 30^{o}C for various exposure times (1 to 30 min). The treated algal units were washed at least for five times, centrifuged and resuspended in fresh liquid between washes.

Membrane Filtration

Separation of microalgae cells from contaminants by the different in sizes was also conducted using tangential flow membrane filtration system (Pall, USA) with membrane pore size of 0.8 μm. The inlet pressure of the filter was regulated at 1 bar and fresh sterile medium was aseptically pumped through the filter chamber. A 1 L sterile medium was used for washing 100 mL of non-purified algal unit to reduce the number of bacterial cell contaminants.

Acid and Alkaline Treatment

Acid and alkaline treatments were performed using medium with various pH [18]. Sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl), acetic acid (CH_3COOH) and citric acid ($C_6H_8O_7.H_2O$) were used to adjust the pH of the medium at the required values (pH 3 to 9). The algal units were washed 3 times using the medium with similar pH.

UV Radiation

The sterile plates were filled with 10 mL of microalgae cell suspension in a respective medium (2×10^4 cell/mL) and exposed to UV light at a distance of 20 cm from the UV lamp in a laminar flow cabinet (ERLA Technology, CFM4). The viability of microalgae and bacterial cells was checked at 2 min intervals.

Adaptation from Phototrophic to Heterotrophic Culture

The single colony obtained from agar plating technique was initially cultured in phototrophic conditions (24 h light) for 10 days. This phototrophic culture was then used as an inoculum for the adaptation steps to heterotrophic culture. In the first step, the culture was grown in phototrophic conditions with reduced illumination time (12 h light: 12 h dark). The cell harvested at late logarithmic phase (400 h) was then inoculated into another phototrophic cultivation with further reduction in illumination time (8 h light: 16 h dark). Similar procedure was used to start the next phoautototrophic culti-

Table 1. Preparation and Composition of Three Different Media (ES-Enriched Seawater (ES), f/2 Guillard (f/2G) and Walne Medium (W)) Used in this Study for the Cultivation of *T. suecica*

Component	Concentration in stock solution (g/L)			Quantity used			Final concentration in the medium (M)		
	ES	f/2G	W	ES	f/2G	W	ES	f/2G	W
Major nutrient I nitrate									
$NaNO_3$	46.67	75	-	1 mL	1mL	100 g	5.5×10^{-4}	8.8×10^{-4}	1.2×10^{-3}
Major nutrient II phosphate									
$NaH_2PO_4.H_2O$	3.1	5	-	1 mL	1mL	20 g	2.2×10^{-5}	3.6×10^{-5}	1.3×10^{-4}
Metals stock I iron (1 mL/L)									
$FeCl_3.6H_2O$	1.77	-	-	1 mL	3.2 g	1.3 g	6.5×10^{-6}	1.2×10^{-5}	4.8×10^{-6}
$Na_2EDTA.2H_2O$	-	-	-	2.4 g	4.4 g	45 g	6.6×10^{-6}	1.2×10^{-5}	1.5×10^{-4}
Metals stock II trace metals									
$Na_2EDTA.2H_2O$	-	-	-	3.1 g	-	-	8.3×10^{-6}	-	-
$ZnSO_4.7H_2O$	-	22	-	0.1 g	1 mL	-	2.5×10^{-7}	7.6×10^{-8}	-
$CoSO_4.7H_2O$	-	-	-	0.2 g	-	-	5.7×10^{-8}	-	-
$MnSO_4 \cdot H_2O$	-	-	-	0.5 g	-	-	2.4×10^{-6}	-	-
$Na_2MoO_4.2H_2O$	1.48	6.3	-	1 mL	1 mL	-	6.1×10^{-9}	2.6×10^{-8}	-
Na_2SeO_3	0.17	-	-	1 mL	-	-	1.0×10^{-9}	-	-
$NiCl^2.6H_2O$	1.49	-	-	1 mL	-	-	6.3×10^{-9}	-	-
$CoCl_2.6H_2O$	-	10	-	-	1 mL	20 g	-	4.20×10^{-8}	8.4×10^{-8}
$MnCl_2.4H_2O$	-	180	-	-	1 mL	0.4 g	-	9.10×10^{-7}	1.8×10^{-6}
$CuSO_4.5H_2O$	-	9.8	-	-	1 mL	-	-	3.93×10^{-8}	-
H_3BO_3	-	-	-	-	34 g	-	-	-	5.4×10^{-4}
$ZnCl_2.6H_2O$	-	-	-	-	-	21 g	-	-	1.5×10^{-7}
$(NH_4)_5Mo_7O_{24}.4H_2O$	-	-	-	-	-	9 g	-	-	7.3×10^{-9}
Vitamin stock									
Thiamine HCl	-	-	-	0.1 g	200 mg	1 g	2.6×10^{-7}	3×10^{-7}	3×10^{-10}
Biotin	1.0	1.0	-	1 mL	1 mL	-	4.1×10^{-9}	2.1×10^{-9}	-
Cyanocobalamin	2.0	1.0	-	1 mL	1mL	50 mg	1.5×10^{-9}	3.7×10^{-10}	4×10^{-12}

vation with reduction in illumination time to only 4 h light until growth reached a late logarithmic phase (700 h). The cells harvested from this cultivation were used to investigate the efficiency of the different treatments for the purification. All cultivations for this set of experiment were conducted under phototrophic culture with minimal illumination time (4 h light: 20 h dark). The semi-purified culture obtained from this step was used as an inoculum for antibiotic treatment and heterotrophic cultivation (24 h dark). The Walne medium with the addition of 10 g/L glucose was used for the heterotrophic cultivation.

In all cultivations, 250 mL Erlenmeyer flask containing 100 mL medium were used. The flasks were incubated in an orbital shaker at 30˚C and agitated at 130 rpm.

Antibiotic Treatment

The susceptibility of microorganisms, which were contaminants to the microalgae cells, to antibiotics was performed using antibiotic disc sensitivity test [19]. The principle of this method is dependent upon the inhibition of reproduction of a microorganism on the surface of a solid medium

by the antibiotic which diffuses into the medium from a filter paper disc. The microbial colonies isolated from the algal unit were cultured in nutrient agar plates. Thus, for the bacterial contaminant which was sensitive (susceptible) to the particular antibiotic, a zone of inhibition around the disc impregnated with the antibiotic was appeared. Chemical method normally used antibiotics, singly or in combination, to kill or inhibit the growth of tenaciously attached contaminants [3]. The lethality of the antibiotic treatment is an intensity-time relationship. The intensity is the dose of antibiotics and the time is the period of exposure before the culture is transferred to antibiotic-free medium. In this study, the most effective antibiotics were chosen according to the diameter of zone of inhibition obtained from the antibiotic disc sensitivity test.

In order to determine the optimal concentration of antibiotic treatment for *T. suecica*, 5 different antibiotics (oxytetracyclin, chloramphenicol, tetracycline, neomycin and vancomycin) were tested against the viability of microoalgae and bacterial cells. The antibiotics solutions were prepared by dissolving vancomycin (5 mg/mL) (Merck) and neomycin (10 mg/mL), tetracycline (10 mg/mL) and oxytetracyclin (10 mg/mL) (Sigma-Aldrich) separately in 10 mL medium. Chloramphenicol solution was prepared by adding chloramphenicol 10 mg/mL (Sigma-Aldrich) to 1 mL of 95% ethanol. The treatment was carried out using a single and combination of antibiotic. A mixture of antibiotic was prepared by adding 10 mg/mL neomycin and 5 mg/mL vancomycin to 10 mL of fresh medium.

The required quantity of antibiotics (100 µL) were injected into 5 mL of microalgae culture in modified Walne medium using 10 g/L glucose as a carbon source. The culture contained about 2.6×10^4 microalgae cells/mL was incubated in an orbital shaker at 30°C, agitated at 50 rpm in the heterotrophic conditions (24 h dark) for 10 days. The culture was then centrifuged at 200 rpm for 10 min at every 8 h intervals. The supernatant was decanted and replaced with the required amount of fresh medium and the antibiotics to be tested.

Analytical Procedures

The microalgae cell number and cell mobility were determined using Haemocytometer by fixing the cells with methanol according to the method as described by Guillard [20]. On the other hand, microalgae cell concentration, expressed as dry cell weight per culture volume, was determined using filtration and oven dried method [21]. The culture samples of known volume and cell density were washed with 0.5 M ammonium bicarbonate to remove salts and then filtered through a known weight of dry filter paper (Whatman, No 1). The filtered cells and filter papers were dried in an oven at 80°C for at least 24 h until a constant weight was obtained.

For examination under Transmission Electron Microscope (TEM), the culture samples were centrifuged at 2000 rcf for 7 min. The resultant supernatants were discarded and the pellets were resuspended in 1 mL of 4% glutaraldehyde for the primary fixation and kept in the refrigerator at 4˚C overnight. The cells were washed 3 times with 0.1 M sodium cacodylate buffer at 10 min time interval. The cells were post fixated in 1% osmium tetroxide and kept at 4˚C for 2 h. The cells were washed again for 3 times with 0.1 M sodium cacodylate buffer at 10 min time intervals. The cells were dehydrated using acetone at a series of concentration (35%, 50%, 75% and 95%) for 15 min in each treatment. Subsequently, the cells were dehydrated using 100% acetone for 20 min and repeated 3 times.

The cells were infiltrated with the addition of a mixture of acetone and resin (agar 100 resin 10 mL; dodecenyl succinic anhydrous 5.5 mL; methyl nadic anhydride 6 mL and benzyl dime thylamine 0.5 mL). Initially, the infiltration was performed with 1 mL of acetone and a mixture of resin at a ratio of 1:1 for 1 h. Subsequently, the cells were infiltrated with 1 mL of acetone and a mixture of resin at a ratio of 1:3 for 2 h. Finally, 1 mL of 100% resin were added to the cells and kept for overnight at room temperature, followed with the addition of 100% resin for another 2 h.

The cells were embedded by placing them into beam capsules and filled up with resin. The cells were polymerized in an oven at 60˚C for 24 h. The samples were cut to thin section using glass knife and ultra microtome was used to cut the cells into section with 1 µm thick. The cells sections were then placed onto glass slide and stained with Toluidine blue, followed by drying on a hot plate. The stain was washed and the specimens were examined under a light microscope. The selected area of cell of interest was cut to ultra thin sections using Cryo-Ultramicrotome (Richert Jung FC4EN). The selected sections were picked with a grid and dried with the filter paper. The sections were stained with uranyl acetate for 10 min and washed with 50% filtered alcohol. The stain was leaded for 10 min and finally washed with double distilled water. The sections were then examined under the Transmission Electron Microscope (Philips, HMG 400).

RESULTS

Effect of Different Types of Medium on Growth of *T. suecica*

Growth of *T. suecica* was greatly influenced by the types of medium used for the cultivation in photoautotrophic conditions (Fig. **1**). In general, medium formulated in natural sea water gave significantly higher growth of *T. suecica* as compared to artifical sea water. The highest growth of *T. suecica*, with a final cell concentration of 7.4 g/L was obtained in Walne medium and this value was about 2 times higher than those obtained in Es-enrichment and f/2 Guillard medium formulated in natural sea water. Growth of *T. suecica* in different media formulated in artificial sea water was not significantly different. However, the lowest growth of *T. suecica* was observed in all media formulated with Sigma artificial sea salt. Based on this result, Walne medium formulated using natural sea water was used in the subsequent experiments to investigate the effect of the various treatments for the preparation of axenic culture.

The Effectiveness of Various Treatments for the Preparation of Axenic Culture

The number of bacterial colony was reduced from 180 to 62 colonies after centrifugation and rinsing for 5 times. The number of colony with different morphologies appeared on the agar plate was not changed before and after centrifugation and rinsing treatments. The resistance of microalgae

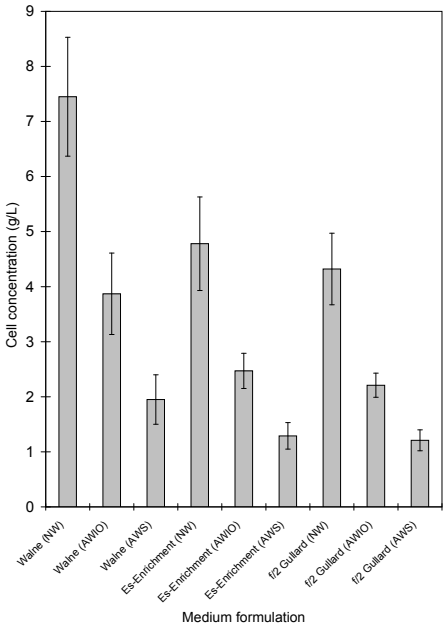

Fig. (1). Growth performance of heterotrophic *T. suecica* cultivated in different medium formulations. The media were prepared using three different sea waters [natural sea water (AW), artificial sea water of Aquarium System (AWIO) and artificial sea water of Sigma (AWS)]. Cell concentration was determined after 14 days of cultivation. Arrow bars indicate standard deviation (±) of triplicates data.

cells and the bacterial contaminants to salinity are shown in Table **2**. *T. suecica* cells were still viable up to 70% salinity and totally died when exposed to salinity of 100% and above. The viability of bacterial contaminants decreased with increasing salinity. It is important to note that the bacterial contaminants were still viable at 300% salinity. Significant changes in the morphology of the bacterial colonies before and after the treatments were not observed up to 200% salinity. Significant changes in the morphology of the bacterial colonies before and after the treatments were only observed at salinity of 300%. This result indicated that salinity treatment was not suitable to remove bacterial contaminants from the algae cell because it was only effective at high salinities where it became hazardous to the algae cells.

The effectiveness of sonication treatment on the purification of *T. suecica* cells from bacterial contaminants are shown in Table **3**. For all sonication times tested in this study, only a slight reduction in cell number of bacterial contaminants was observed after the treatment. In addition, the number of colony with different shapes and morphologies was not significantly different before and after the sonication treatment. These results indicated that sonication was not effective to reduce or to kill the bacterial contaminants from the microalgae cells. Sonication may be used to loosen up the bacterial cells that tightly attached on the surface of the algae cells. However, prolonged sonication period (30 min) killed the microalgae cells. From this study, it was found that sonication at 90 K cycles.s^{-1} for 10 to 15 min may be the most suitable to detach the bacterial contaminants from algae cells effectively without causing damage to the algae cells.

The use of tangential flow membrane filtration system reduced the number of bacterial contaminants from 170 to 23 colonies, suggesting that this technique was very effective in the removal of bacterial contaminants from the microalgae cells. However, microalgae cells were died after prolonged filtration period (> 5 min). In addition, this technique can only be applied to relatively large volume of culture.

Table **4** shows the effect of acid and alkaline treatments on the viability of *T. suecica* cell and bacterial contaminants. When sulfuric acid (H_2SO_4), hydrochloric acid (HCl) and citric acid ($C_6H_8O_7.H_2O$) were used for acid treatment, *T. suecica* cells died at pH lower than 6.5, 7 and 6.5, respectively. Microalgae cells died when treated with NaOH at pH 8.5. The number of bacterial colony with different shapes and morphologies was also significantly reduced with acid and alkaline treatments. Acid treatment, in general, reduced the number of colony with different shapes and morphologies from 5 to 3. On the other hand, alkaline treatment reduced the number of morphology of bacterial colonies from 5 to 2. It is interesting to note that *T. suecica* cells were still viable after treatment with acetic acid (CH_3COOH) at pH 4.5, while the number of shape and morphology of the bacterial colony was reduced from 5 to 3.

T. suecica cells died when exposed to UV irradiation for 10 min, while the bacterial contaminants were still viable after exposure for 20 min. However, the morphological difference of bacterial colonies, after and before UV irradiation,

Table 2. The Effect of Salinity Treatment on the Viability of *T. suecica* Cells and the Contaminants Cells in Non-Purified Culture

Salinity %	No of colony of the bacterial contaminants		Morphological difference of colony of the bacterial contaminant	Viability of *T. suecica*
	Before treatment	After treatment		
0	142±5	68±3	-	Non-Viable
50	175±3	52±4	-	Viable
70	96±6	73±7	-	Viable
100	136±5	82±3	-	Non-Viable
200	178±4	40±5	-	Non-Viable
300	157±5	48±4	+	Non-Viable

±Standard deviations for triplicate values.
+Significant changes were detected.
-Significant changes were not detected.

Table 3. The Effect of Sonication Treatment (90 K cycles.s^{-1}, 15 min) on the Viability of *T. suecica* cells and the Contaminant Cells in Non-Purified Culture

Time (min)	No of colony of the bacterial contaminants		Morphological difference of colony of the bacterial contaminant	Viability of *T. suecica*
	Before treatment	After treatment		
1	163±3	142±7	-	Viable
5	214±6	185±6	-	Viable
10	188±3	173±5	-	Viable
15	115±7	122±5	-	Viable
20	210±4	179±4	-	Viable
30	170±9	156±6	-	Non- Viable

±Standard deviations for triplicate values.
+Significant changes were detected.
-Significant changes were not detected.

Table 4. Effect of Acid and Alkaline Treatments on the Viability of *T. suecica* Cells and the Contaminant Cells in Non-Purified Culture

Acid /Alkaline	pH	No of colony of the bacterial contaminants		Morphological difference of colony of the bacterial contaminants	Viability of *T. suecica*
		Before treatment	After treatment		
Sulfuric acid (H_2SO_4)					
	6.5	192±7	28±5	+	Viable
	5.5	175±8	0	+	Viable
Sodium hydroxide (NaOH)					
	8.5	145±8	40±3	+	Viable
	9.5	168±7	0	+	Viable
Hydrochloric acid (HCl)					
	7	156±3	17±7	+	Viable
	5.5	172±5	0	+	Viable
Citric acid ($C_6H_8O_7.H_2O$)					
	6.5	218±4	37±7	+	Viable
	5.5	163±5	0	+	Viable
Acetic acid (CH_3COOH)					
	5.5	127±6	22±3	+	Non- Viable
	4.5	215±7	36±5	+	Non- Viable
	4	168±5	16±6	+	Viable

±Standard deviations for triplicate values.
+Significant changes were detected.
-Significant changes were not detected.

was remained the same. This means that this treatment is not suitable for the preparation of axenic culture of *T. suecica.*

The results of antibiotic disc sensitivity tests on 5 different bacterial colonies isolated from *T. suecica* culture are shown in Table **5**. Vancomycin had the highest growth inhibition zone (20±3 mm, 31±2 mm and 19±1 mm) on bacterial colony number 1, 2 and 5, respectively (P<0.05). On the other hand, chloramphenicol (34±2 mm), oxytetracyclin (30±3 mm), tetracycline (28±4 mm) and neomycin (29±3 mm) were more effective (P<0.05) on growth inhibition of bacterial colony number 3 compared with other bacterial colony. It is important to note that growth of bacterial colony

Table 5. The Diameter of the Inhibition Zone Measured from the Antibiotic Disc Sensitivity Test of Five Different Morphologies of the Colonies of the Bacterial Contaminants Isolated from Non-Purified Culture of *T. suecica*

Antibiotic disc	Dosage	Diameter of the inhibition zone (mm)				
		Colony No. 1	Colony No. 2	Colony No. 3	Colony No. 4	Colony No. 5
Chloramphenicol	30 µg	9±2 [bc]	17±3 [c]	34±2 [a]	46±4 [a]	9±3 [c]
Oxytetracyclin	30 µg	9±3 [c]	10±4 [d]	30±3 [a]	20±3 [c]	9±2 [d]
Tetracycline	30 µg	13±3 [bc]	11±2 [d]	28±4 [a]	31±3 [b]	9±2 [d]
Neomycin	30 µg	9±3 [bc]	10±2 [d]	29±3 [a]	32±4 [b]	10±2 [bc]
Vancomycin	30 µg	20±3[a]	31±2 [b]	11±1 [b]	10±3 [d]	19±1 [a]
Mixture of Neomycin and Vancomycin	30 µg + 30 µg	23±5[a]	39±3 [a]	35±2 [a]	34±4 [b]	22±1 [a]

±Standard deviations for triplicate values.

[a-e]Superscript with the same letter in the same column are not significantly different at $P < 0.05$.

number 4 was inhibited significantly by chloramphenicol (64±4 mm), tetracycline (31±3 mm) and neomycine (32±4 mm) (P<0.05). It is important to note that the diameter of the inhibition zone for all bacterial colonies was significantly increased when a mixture of antibiotic (vancomycin and neomycin) was used, suggesting that the use of a mixture of antibiotic was more effective than the use of individual antibiotic.

Oxytetracyclin and tetracycline reacted with certain compound in the media, which changed the color of *T. suecica* culture from light yellow to brown, suggesting that these two antibiotics were not suitable for the purification of *T. suecica* culture. In addition, *T. suecica* cells were also found non-viable after the addition of oxytetracyclin and tetracycline. On the other hand, colloidal suspension was appeared when chloramphenicol was added to *T. suecica* culture. Treatment with chloramphenicol not only killed the bacterial contaminants but also killed the microalgae cells. All the bacterial contaminants present in the culture were killed with the present of a mixture of vancomycin and neomycin, while *T. suecica* cells were still viable (data not shown). Based on these observations, a mixture of vancomycin and neomycin was chosen to be used in antibiotic treatment for the removal of bacterial contaminants, especially in the form of spore, from the microalgae cells.

Proposed Protocol for the Preparation and Adaptation of *T. suecica* Cells from Autotrophic to Heterothrophic

The observation obtained from the many experiments to investigate the effectiveness of several physical and chemical treatments on the non-purified *T. suecica* culture for the removal of bacterial contaminants were used to design the protocol for the preparation of axenic culture with simultaneous adaptation to heterotrophic conditions. The proposed protocol is shown in Fig. (**2**), where it was successfully used to obtain axenic culture of heterotrophic *T. suecica* and gave consistent results for many experiments conducted repeatedly. The main treatments involved in the protocol were centrifugation, sonication and antibiotic treatment. The non-purified *T. suecica* culture was initially treated with hydrochloric acid at pH 5.5 to reduce the number of bacterial contaminants. Centrifugation was used to rinse out the free cell contaminants from the culture while the sonication was used to detach the cell tightly attached to the algae cell surface. The detached cells contaminants were removed from the culture through rinsing by centrifugation for many times. The addition of a mixture of antibiotic (vancomycin and neomycin) was used to kill the bacterial contaminants, especially the spore forming, during the heterotrophic cultivation.

Morphology Changes During the Adaptation of *T. suecica* Cells from Photoautotrophic to Heterothrophic Culture

Changes in the morphology of *T. suecica* cells, as examined under Transmission Electron Microscope, during the adaptation from photoautotrophic to heterotrophic condition are shown in Fig. (**3**). Size of flagella of the photoautotrophic cells was reduced with decreasing illumination time and total lost of flagella was observed in heterotrophic cells, where the cells were cultivated in total darkness with medium supplemented with glucose. The cells lost their flagella and settled when the cells were adapted from photoautotrophic to heterotrophic conditions because the source of energy was changed from light to organic carbon source. Thick cell wall with several layers was also observed in photoautotrophic cells (Fig. **3F**). The shape of cell and the composition of cell organelles were changed with the changes from phototrophic to heterotrophic culture (Fig. **3K-L**). The composition of starch and lipid also significantly changed when the cells were adapted to heterotrophic from the photoautotrophic cultivation. Lipid content was increased while starch content was slightly reduced when the cells were changed from photoautotrophic to heterotrophic culture. The density of chloroplast was also reduced when the cells changed from photoautotrophic (Fig. **3K**) to heterotrophic culture (Fig. **3L**). Chloroplasts are organelles found in plant cells and other eukaryotic organisms that conduct photosynthesis. The chloroplast was widely spread in the cell surface especially close to the cell wall in the heterotrophic cells.

The thickness of cell wall was reduced when the cell changed from photoautotrophic (Fig. **3N**) to heterotrophic culture (Fig. **3O**). Five layers of cell wall were observed in phototrophic cells (Fig. **3F**) and the cell walls were digested from inside to outside with the adaptation from photoautot-

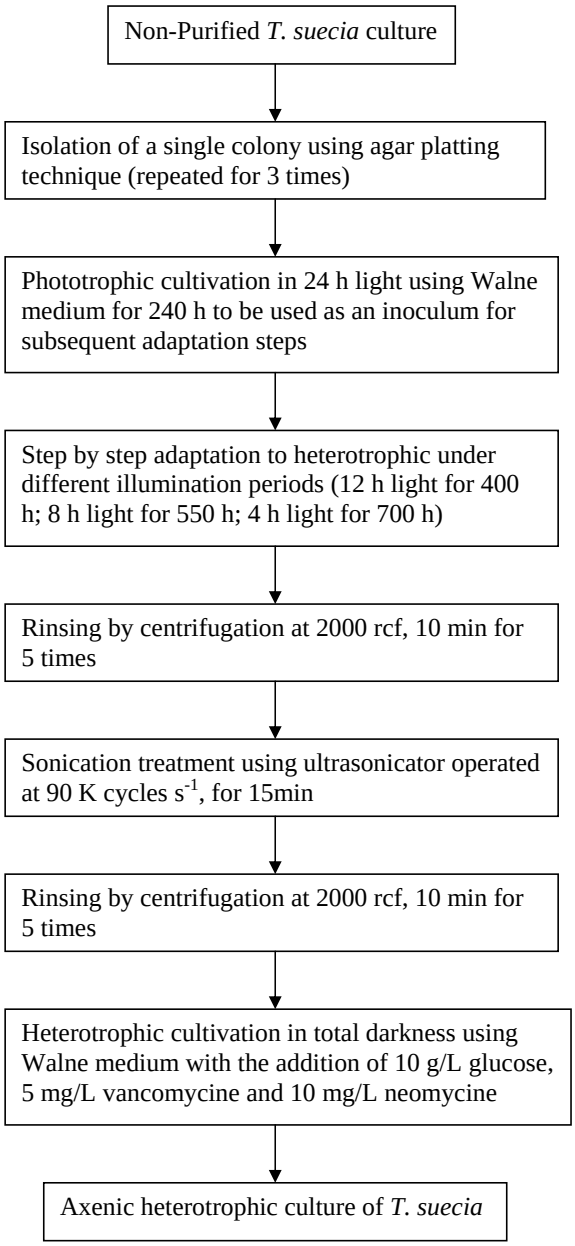

Fig. (2). The proposed protocol for the preparation of axenic culture and simultaneous adaptation from photoautotrophic to heterotrophic culture.

rophic to heterotrophic culture. In addition, the shape of the cell changed from oval for the photoautotrophic cell to spherical for the heterotrophic cell. The heterotrophic cell became spherical in shape mainly due to the lost of flagella.

DISCUSSION

From this study it was found that the formulation of medium was one of the important aspects in the development of high performance cultivation of microalgae. Walne medium using either natural or artificial sea water was superior compared to ES-enriched medium and f/2 Guillard medium in the enhancement of growth of *T. suecica*. Walne medium was also successfully used for the preparation of axenic culture of *T. suecica* and for the adaptation from photoautotrophic to heterotrophic conditions. The main component of this medium that greatly enhanced growth of *T. suecica* is difficult to evaluate due to large quantitiy of macronutrients and micronutrients present in the medium. Walne enriched natural sea water medium was designed for mass culture of marine phytoplankton for subsequent use as feed for shellfish [16]. Walne medium is supplemented with boron, which is completely unnecessary when natural sea water is used as a base [13]. The amount of vitamins in Walne medium was very low and biotin was not present. However, the concentration of inorganic phosphate, nitrate, zinc, molybdenum and manganese were higher in Walne compared to f/2 Guillard and ES-enriched natural sea water medium. Moreover EDTA concentration in Walne medium was approximately ten times higher than the usual concentration for coastal culture media. Iron concentration in Walne medium was lower than other media. Trace metals solution were added into Walne medium in the form of chlorate while sulfate form was used for f/2 Guillard and ES-enriched natural sea water medium.

Several established procedures have been suggested for the preparation of axenic culture of microalgae [4, 7-10, 18, 22, 23]. However, some of the procedures involved many laborious steps and consistent results were difficult to achieve. In addition, the procedure may be specific to certain microalgae species. Although a single technique may be used to remove the associated microorganisms or contaminants from the microalgae, purification of microalage for heterotrophic cultivation required a combination of techniques as well as the development of other new methods. In this study, a suitable protocol for the preparation of axenic culture of heterotrophic *T. suecica* was proposed. Although the protocol involved many laborious steps, it was successfully used and gave consistent results. Each step in the protocol has different functions and discussed as follows.

Agar plates is probably the oldest and the most used method of purification, but this method is effective only for algae that can grow either on the surface of agar or embedded in it [3, 24, 25]. In our study, it was observed that bacterial contaminants were tightly attached to a single colony of *T. suecica* due to large cell size and scaly cell wall. The bacterial contaminant can be easily attached on the surface or within the microalgae cell wall. Thus, a single step of agar plating was not enough to produce axenic culture. A combination of physical and chemical methods was required to remove the tightly attached bacterial contaminants on the microalgae cells.

Rinsing by centrifugation effectively reduce the number of bacterial contaminants by removing the free cell contaminants from the microalgae cells. Reardon *et al.* [26] also suggested that centrifugation is effective when used in conjunction with sonication and other methods for separating algal cells from bacterial contaminants. Appropriate centrifugal forces may separate the algae cells from the contaminants due to different in the sedimentation rates of the different sizes of cells. Free contaminants and those not tightly attached to the algae cells were eliminated by this procedure when repeated for several times. Guillard and Morton [18] claimed that the bulk of the smallest contaminants could be removed using centrifugation technique.

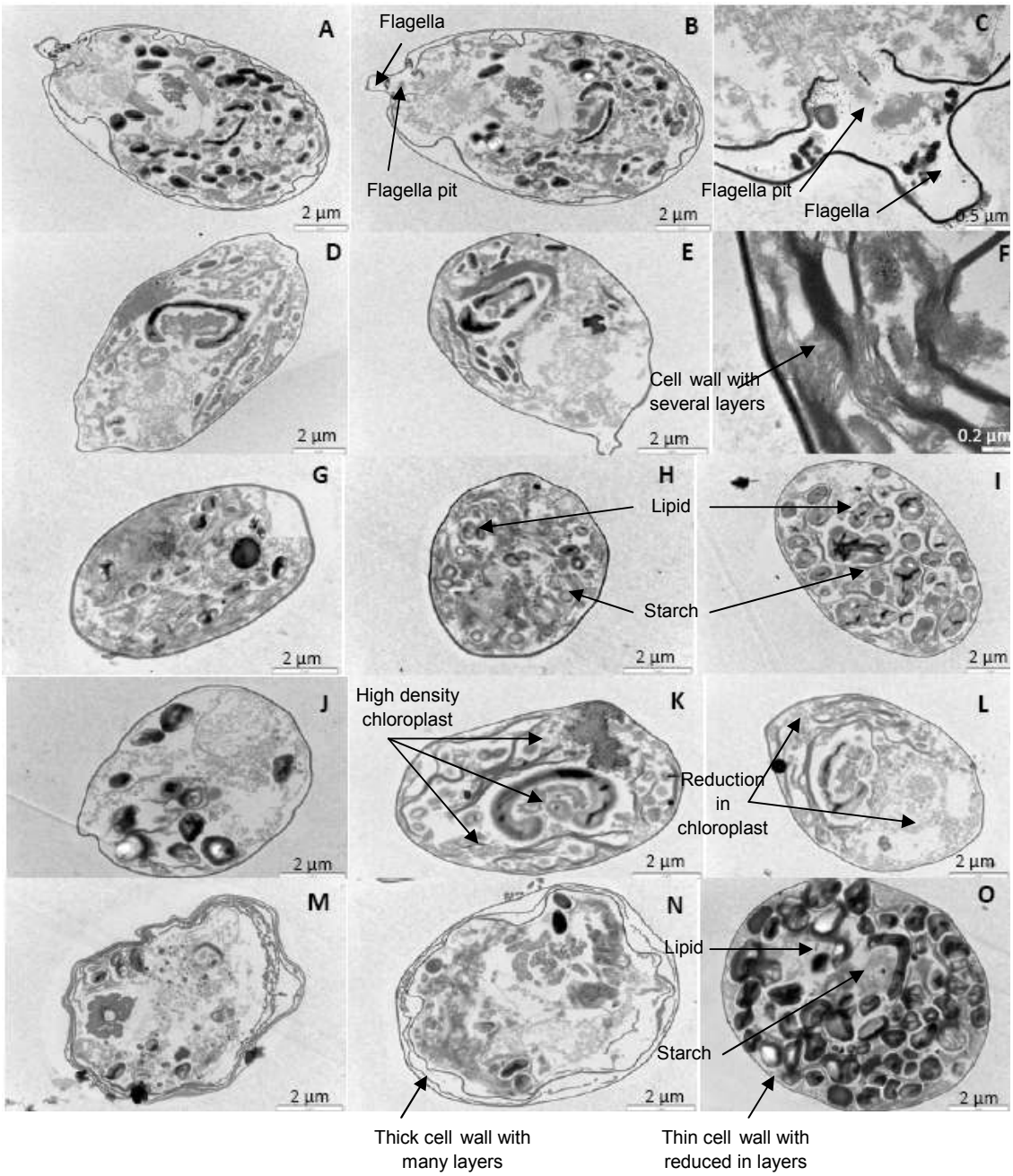

Fig. (3). Transmission electron micrographs (TEM) of *T. suecica* during the adaptation from photoautotrophic to heterotrophic culture conditions by changing the illumination time and medium composition. Heterotrophic culture was achieved in total darkness with the addition of glucose in a medium. (**A-F**) Size of flagella was reduced with decreasing illumination time and total lost of flagella was observed in heterotrophic cells; (**F**) Thick cell wall with several layers was observed in photoautotrophic cells; (**G-J**) The shape of cell and the composition of cell organelles were changed with the changes from phototrophic to heterotrophic culture; (**K-L**) The density of chloroplast was reduced when the cells change from photoautotrophic (**K**) to heterotrophic culture (**L**); (**N-O**) The thickness of cell wall was reduced when the cell changed from photoautotrophic (**N**) to heterotrophic culture (**O**).

Sonication produced shear forces that acted on the surface of algal cells to remove tightly attached bacterial contaminants [3]. The use of sonication treatment by ultrasonicator operated at 90 K cycles.s^{-1} for 5–20 min for purification of microalgae has also been reported by Brown and Bischoff [27] and Shirai [28]. Although tangential flow membrane filtration system was found superior in the separation of bacterial contaminants from microalgae cells, but the difficulty in maintaining the aseptic technique limited its usage in the preparation of axenic culture. In addition, high culture volume was also required by this technique.

One of the major difficulties in the preparation of axenic culture of *T. suecica* as observed in our study was the present of bacterial contaminants in the form of spore as examined under the microscope. Separation of these spores using physical treatments was not effective. Thus, the use of antibiotic to kill those spores became an important step in the protocols. The use of a mixture of antibiotics containing

neomycin and vancomycin was more effective than the use of a single antibiotic. For effective killing mode, the antibiotics mixture was added to the semi purified culture at every 8 h according to antibacterial LD_{50}. The lethality of the antibiotic treatment is an intensity–time relationship. The intensity is the dosage of antibiotics and the time is the period of exposure before transfer to antibiotic-free medium. Vancomycin has activity against most gram-positive organisms and penicillin-resistant *Pneumococci* [29, 30]. This type of antibiotic also has the activity against gram-negative anaerobes and mycobacteria [31] as well as bacteriostatic for enterococci and staphylococci, but bactericidal for *Streptococcus pyogenes* and *Bacteroides fragilis* [32]. Vancomycin exhibits bactericidal activity by inhibition of bacterial cell wall synthesis, and also capable of injuring protoplasts by altering the permeability of their cytoplasmic membrane and selectively inhibiting RNA synthesis [33, 34].

The effect of medium composition, illumination technique and various designs of photobioreactor on growth and photosynthetic rates of microalgae have been studied extensively [35, 36, 37]. Microalgal cells can trap light as the energy source and assimilate CO_2 as the carbon source. It is important to note that the organic substrates can also be utilized as the carbon and energy sources by some microalgae [38, 39, 40]. Changes in the proportions of individual photosynthetic pigments may occur in response to variation in the intensity of light and a species in which a particular type of pigmentation is determined genetically may become adapted to an environment [40]. There is no doubt that many algae grow over a wide range of combinations of temperature, light and nutrient concentrations and yet maintain their elemental composition within more or less narrow limits. It is therefore clear that microalgae have the mechanisms for regulating the uptake of each element and that these mechanisms serve to maintain composition and to achieve balanced growth [41]. In this preliminary study, *T. suecica* cells were successfully adapted to heterotrophic cultivation under total darkness. This means that the *T. suecica* heterotrophic cells used organic compounds as energy source instead of light, which was required by the phototrophic cells. Heterotrophic culture may be preferred for the industrial cultivation of microalgae in a large scale close bioreactor similar to microbial fermentation for the production of various biotechnology products. Our preliminary study also showed that significant changes in chemical composition of cells were occurred during adaptation from photoautotrophic to heterotrophic cultures. Thus, heterotrophic culture may also be used as an approach to enhance the production of certain cell component in the microalgae cells for industrial used. Kinetic study of heterotrophic cultivation of *T. suecica* and analysis of its chemical composition as well as optimization of medium and culture conditions for improvement of the cultivation process are being carried out in our laboratory.

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