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Isolation and Adaptation of Diatoms in a New Formulated Enriched Medium

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ABSTRACT

This study describes the adaptations of diatoms, *Cylindrotheca fusiformis* and other marine diatoms, in a new formulated enriched medium Tris-phosphate seawater (TP-SW). The medium was designed to maintain long-term cultures of wide-range marine diatoms in laboratory that produces high biomass of cultures. The diatoms were adapted and cultivated in the medium for 15 days and the number of cells was recorded daily. It was found that the number of cells declined after two weeks indicating death phase of the cells. This indicates that the TP-SW medium has supported the growth of diatoms during the period and can be used to cultivate diatoms *in vitro.* Studies on the TP-SW medium must be done to obtain optimal medium that can provide not only a conducive environment for the survival of diatoms but also high biomass production.

Keywords: Adaptation, diatoms, enriched medium, growth, nutrient

INTRODUCTION

Diatoms contribute to about one fifth of the world's photosynthesis for 200,000 different species in the sea (Armbrust, 2009). Diatoms are useful in various fields including biofuel production (Chaffin, Mishra, Kuhaneck, Heckathorn, & Bridgeman, 2011) paleoclimate research (Morley, Leng, & Sloane, 2005) forensic investigation (Zimmerman & Wallace, 2008), water quality monitoring (Tan, Sheldon, Bunn, & Zhang, 2012) marine culture (Pahl, Lewis, King, & Chen, 2012) and also biological materials production (Fan, Chow, & Zhang, 2009).

Diatoms can be cultivated in laboratory using artificial and enriched growth media. Artificial media is a defined media where exact composition of every element in it needs

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to be controlled. Enriched media are prepared by adding nutrients to natural seawater or lake, thus these types of media are not defined (Andersen, 2005). For growth of marine diatoms, natural seawater is sufficient to provide enough nutrients, with incorporations of micro and macro elements. The f/2 medium is a widely used enriched medium to cultivate various species of coastal microalgae especially diatoms (Guillard & Ryther, 1962; Guillard, 1975). Though this medium can ensure long term growth of diatoms, it does not produce high biomass of cultures. Thus, the TP-SW medium is developed as a new enriched medium that can increase the biomass and growth rate of the cultures. The TP-SW medium also facilitates the study of Malaysian diatoms *in vitro*.

In this study, diatom samples were collected from Pantai Remis, Kuala Selangor, and another diatom of known specie, *Cylindrotheca fusiformis*, was obtained from University of Texas (UTEX) in which both were used in the adaptation of the new enriched medium. *C. fusiformis* is widely studied for its lipid content and fatty acids. According to a study, *C. fusiformis* is a potential source of acid or also known as EPA Omega-3 fatty acid (Kiran, 2012). In aquaculture, *C. fusiformis* and other species of diatoms can be used to produce therapeutic proteins (Fischer, Robl, Sumper, & Kroger, 1999). Different strains of diatoms can be well adapted to different environments with different nutrient requirements. Therefore, development of a medium that can support the growth of a wide range of diatoms is beneficial economically.

MATERIALS AND METHOD

Preparation and Composition of TP-SW Medium

The composition of the media is shown in Table 1. Master stocks of all nutrients were prepared, and required volumes were dispensed aseptically into 1L working stock, the final solution which was used in the cultivation in this study. Filter sterilised vitamins were added after the final solution was autoclaved.

Diatoms Sample Collection

Water samples were collected by filling approximately half of 50 mL Falcon tube. Early detection of diatoms was made by observing the fresh water samples under light microscope (Leica, DM2500). *C. fusiformis* and the water samples collected from Pantai Remis, Kuala Selangor (N03° 12.145' E101° 18.329') were then centrifuged (NF800) at 4000 rpm for 10 minutes. The supernatant was discarded, and the pellets were transferred into a 50 mL conical flask containing 30 mL of the commercial f/2 medium which was purchased from UTEX.

Table 1
The nutrient composition of TP-SW and f/2 media

Nutrient Category	Nutrient in TP-SW	Concentration in working solution (mM)	Nutrient in f/2	Concentration in working solution (mM)
Nitrogen	NH_4Cl	15.0	$NaNO_3$	0.882
Phosphate	KH_2PO_4	0.89	NaH_2PO_4	0.036
Macro elements	$CaCl_2.2H_2O$	0.44	-	
	$MgSO_4.7H_2O$	0.42	-	
	$Na_2SiO_3.5H_2O$	0.47	$Na_2SiO_3.9H_2O$	0.0528
Micro elements	H_2BO_3	0.18	-	
	$(NH_4)6MO_7O_{24}.4H_2O$	0.00089	$Na_2MoO_4.2H_2O$	0.000026
	$CoCl_2.6H_2O$	0.012	$CoCl_2.6H_2O$	0.000042
	Na_2SeO_3	0.0001	-	
	$VOSO_4.XH_2O$	0.000012	-	
	$ZnSO_4.7H_2O$	0.079	$Zn_SO_4.7H_2O$	0.0000765
	$MnCl_2.4H_2O$	0.026	$MnCl_2.4H_2O$	0.00091
	$CuSO_4.5H_2O$	0.011	$CuSO_4.5H_2O$	0.0000393
	$Fe_2(SO4)_3.7H_2O$	0.018	$FeCl_3.\ 6H_2O$	0.0117
Chelating agent	Na_2EDTA, pH 8.0	0.55	$Na_2EDTA.2H_2O$	0.0117
Buffer	Tris-HCL, pH 7.4	10.0		
Vitamins	Thiamine, B1	0.23	Thiamine, B1	0.000296
	Cyanocobalamin, B12	0.0005	Cyanocobalamin, B12	0.000000369

Cultivation of Diatoms in TP-SW Medium

Diatoms cells were allowed to replicate and after two weeks of cultivation, they were observed under Scanning Electron Microscope (ESEM Fei Quanta 450 Feg) at the Imaging Centre in Universiti Teknologi MARA to identify the diatoms species. The cultures were centrifuged, and diatoms cells were harvested. These cells were then transferred into a sterile 50 mL conical flask containing fresh 30 mL TP-SW medium for early adaptation in the new formulated medium. The medium was added periodically until a total volume of 400 mL was reached. The experiment was conducted in triplicate. All flasks were left under continuous fluorescence light with intensity of 65 µmol m^{-2} s^{-1} and were agitated on an orbital shaker at 95 rpm. Cell counts were taken daily using haemocytometer (Neubauer, Marienfeld, Germany) to record the growth of the cells in each medium.

RESULTS AND DISCUSSION

Isolation and identification of Diatoms

Seven diatoms species from four different genera were isolated from Pantai Remis. The diatoms were morphologically identified based on the observation obtained from SEM. Figure 1 shows the SEM images from the isolated diatoms. Morphological identification was performed as described in the following references: (Hilaludin, 2011; Naz, Burhan, & Ahmad Siddiqui, 2012; Shamsudin, 1990)

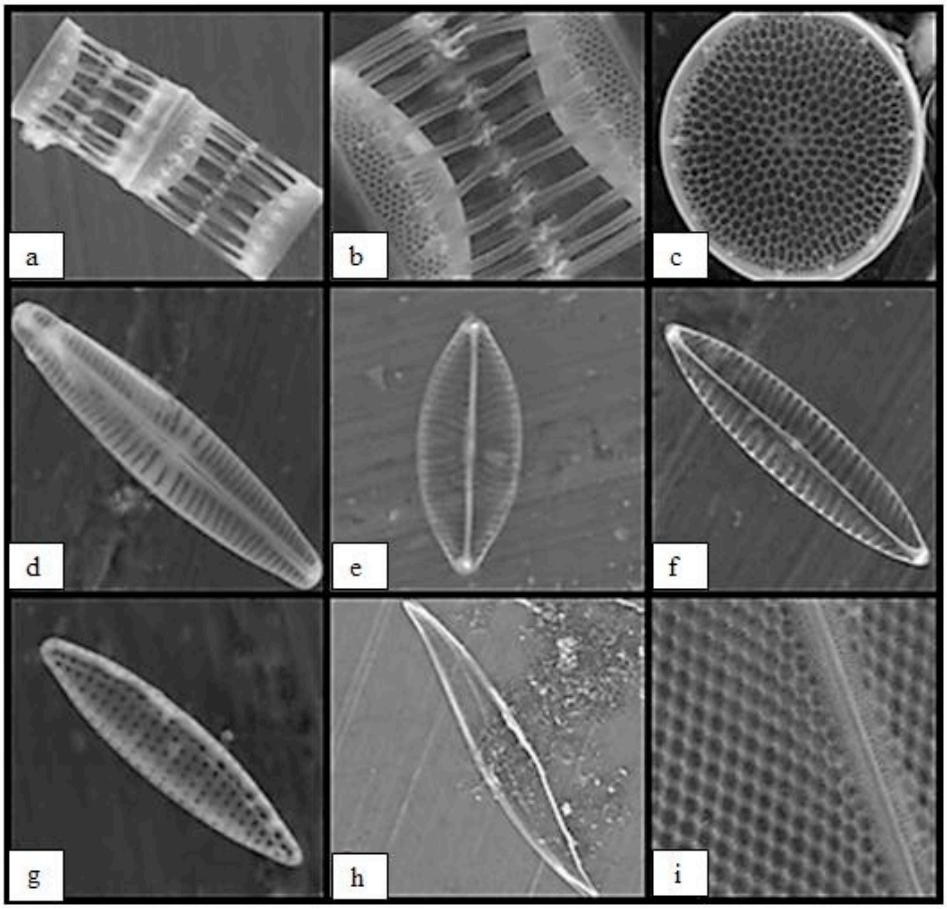

Figure 1. SEM images of isolated diatoms from Pantai Remis: (a-b) *Skeletonema* sp.; (c) *Thalassiosira* sp.; (d-g), *Navicula* sp.; and (h-i) *Pleurosigma* sp.

Adaptation of Diatoms in TP-SW Medium

Table 2 depicts the concentration of few selected nutrients from both media for comparison. Almost all elements in the TP-SW medium are significantly higher in concentration compared with the f/2 medium. For instance, nitrogen in the TP-SW is nearly 15 times higher than the concentration in the f/2. Nitrogen is found in higher concentration in the TP-SW to increase its bioavailability to prevent it from becoming the limiting nutrient. Nitrogen is an indispensable nutrient for all organisms including diatoms as it is needed for the biosynthesis of macromolecules, for example nucleic acid, proteins and chlorophylls (Hockin, Mock, Mulholland, Kopriva, & Malin, 2012)

Table 2
Comparison of selected nutrients in f/2 and TP-SW media

Elements	Concentration in working solution (mM)	
	TP-SW	f/2
Nitrogen	15.005	0.882
Phosphate	0.89	0.0036
Silica	0.47	0.0528
Magnesium	0.42	Undefined
Calcium	0.44	Undefined
Iron	0.036	0.0117

Based on a study on phosphate concentration (Katiyar, Lall, & Singh, 2010), diatoms increased in population with the increase in concentration of phosphate in the cultivation medium. Based on the study, the optimum concentration of phosphate was 0.05 mM. In this study, the concentration of phosphate in the TP-SW medium is above the recommended concentration, which is 0.89 mM. Thus, excess phosphate may exert an effect on depressing the diatoms growth as shown by diatoms from Pantai Remis (see Figure 2).

In TP-SW medium, magnesium and calcium were added at a concentration of 0.42 mM and 0.44 mM respectively. While in the f/2 medium, these elements were not incorporated but may be readily available in the seawater in lower amounts. Calcium can be found inside the chloroplast while magnesium can be found in chlorophyll. Magnesium also acts as co-factor of enzyme (Radzun et al., 2015).

The TP-SW medium has higher concentration of iron than the f/2 medium. Since diatoms are photosynthetic algae, the iron acts as one of the growth-limiting element in the media. Iron is an important component of the photosynthetic apparatus and mitochondrial electron transport chain (Kustka, Allen, & Morel, 2007). Iron limitation may cause a reduction in the cells' production of chlorophyll which reduces the efficiency of photosynthesis (Lewandowska & Kosakowska, 2004). Silica is especially important to diatoms as it is needed in the production of their siliceous cell wall called frustules. The formation of frustules depends significantly on the availability of silicic acid, which is the precursor of silica (Hildebrand & Lerch, 2015).

Diatoms from Pantai Remis and *C. fusiformis* were adapted in the TP-SW, and the cell densities of cultures in the media were recorded daily. The results are shown in Figures 2 and 3. Since diatoms cells were only few at the beginning of the experiment, it is best to grow them in small volume of the new enriched medium so that the cells will reach exponential phase at a faster rate. Thus, the cells were first inoculated into 30 mL of the medium in a 50-mL conical flask for early adaptation. Large surface area to volume ratio allows gas diffusion into the medium, thus facilitating cell growth (Andersen, 2005). Having a small volume of cultivation medium for early adaptation of a small number of cells is an advantage. Theoretically, it will provide lesser gap between the cells inside the flask, thus promoting cell communication and sexual/asexual reproduction which in turn increases the number of cells (Bates & Davidovich, 2002).

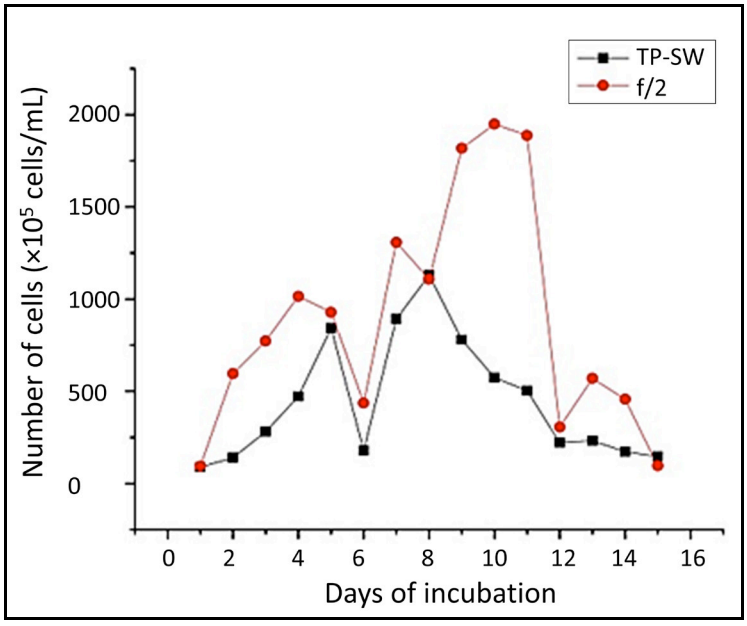

Figure 2. Cell density of diatoms from Pantai Remis against days of incubation

Growth of of diatoms cells isolated from Pantai Remis in both media was measured and recorded from day 1 to day 15 as shown in Figure 2. Number of cells in the f/2 medium was found to increase from day 1 to day 4, while in the TP-SW medium, the number of cells increased from day 1 to day 5. On day 6, cell density in both media dipped to a value as diatoms lifespan generally lasted less than a week, usually around six days (Maldonado, Riesgo, Bucci, & Ru, 2010) depending on the species. However, cell density in both media increased in the following day (day 6 to day 8) until the next six days where the number of cells again decreased. Cell density in both media continued to decline from day 14 as the nutrients started to deplete. The cells cultivated in the f/2 medium showed highest cell density at day 10 with 2.0 x 10^8 cells/mL while in the TP-SW medium, the highest cell density was achieved at day 8 with 1.0 x 10^8 cells/mL. Figure 2 shows the cell density in the TP-SW medium is lower than that in the f/2 medium.

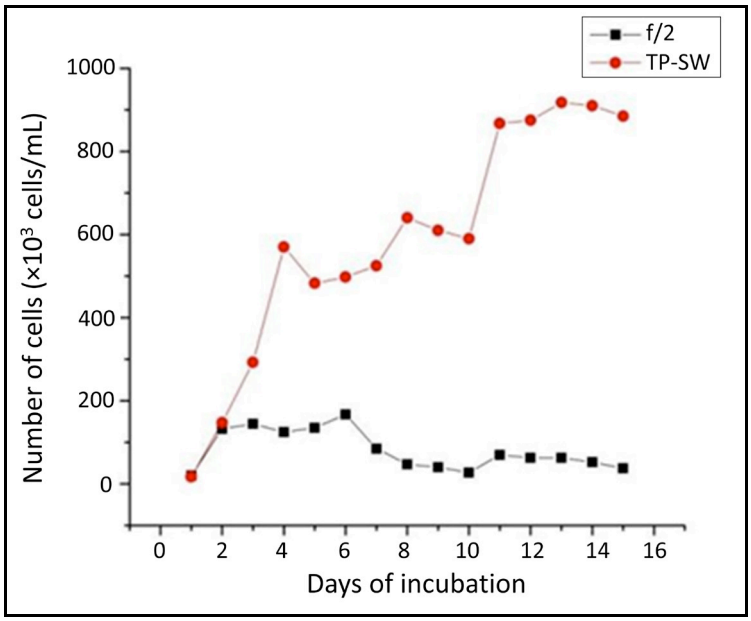

Figure 3. Cell density of C. fusiformis against days of incubation

In Figure 3, diatoms *C. fusiformis* showed an excellent adaptation in the TP-SW medium in terms of cells density. The number of cells recorded shot up from day 2 to day 4. After day 4, the cell density started to fluctuate but still showed increasing number of cells until day 14. Meanwhile, the cell growth performance in the f/2 medium was progressing very slowly compared to the cells in the TP-SW medium starting from day one of cultivation. After a week, the number of cells of *C. fusiformis* in the f/2 medium started to decline most probably due to the decrease in nutrients. Nevertheless, diatoms *C. fusiformis* adapted in the TP-SW medium achieved the highest cell density on day 13 of cultivation with 9.0 x 10^7 cells/mL which was higher than diatoms in the f/2 with 2.0 x 10^7 cells/mL on day 6.

Besides nutrients, another factor that contributed to the cells' response towards the cultivation media is turbulence or agitation. Diatoms cells in the present study were agitated by continuous mixing on an orbital shaker at 95 rpm. The advantage of allowing algal suspension in continuous movement is to prevent sedimentation of the cells (Stengel, 1970), while stimulating nutrient uptake by maintaining active contact of nutrients with the algal cells surface (Schumacher & Whitford, 1965). This will lead to stimulation of metabolic activities which will help the cells to adapt to a new environment (Müntz, 1965).

This study showed the life span of diatoms varies among species, and it depends greatly on a number of factors such as nutrient availability, other species-specific requirements as well as environmental conditions (e.g., temperature) as these factors affect their survival and cell replication. Diatoms adapted in the TP-SW and f/2 media showed significant difference in growth performance in terms of cell numbers. Nonetheless, the fact that the cells were able to survive in the TP-SW proved that the medium was suitable to cultivate diatoms. Even though the diatoms were first introduced to the f/2 medium in the early adaptation, they have proven

that they can survive in different growth medium. This is because diatoms are able to adapt to continuously changing nutrient conditions. In the ocean, this is displayed by the efficiency of growth-limiting nutrients (e.g., silicon, iron and nitrogen) uptake of diatoms in upwelling environments (Bromke et al., 2013).

CONCLUSION

The adaptation of diatoms in a new cultivation medium varies among species in which from the experiment, the growth performance of diatoms from Pantai Remis in the TP-SW differs greatly compared to the growth performance of diatoms *C. fusiformis* in similar medium. Nevertheless, TP-SW is an enriched medium that is suitable as a growth medium for diatoms for *in vitro* studies. Future nutrient optimisation of the TP-SW is highly recommended in order to improve the cell growth performance in terms of biomass and growth rate to cultivate a wide range of diatom species.

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