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Short Communication

Effects of different culture media on the growth and proximate composition of *Dunaliella* sp. at different growth phase

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A B S T R A C T

Nutrient content in different culture media significantly influence the growth and proximate content of microalgae. Therefore, in the present study *Dunaliella* sp. has been selected to determine its growth and proximate composition in different growth phase in Conway and F2 media. For growth determination, data were collected in terms of cell density and optical density and harvested in every growth phases to determine its proximate composition. Results concluded that, *Dunaliella* sp. showed higher cell density and earlier stationary phase in F2 medium with 7.8 x 10^6 cells mL^{-1} compared to the Conway media with 4.1 x 10^6 cells mL^{-1}. Significantly higher ($p>0.05$) amount of protein content was recorded from Conway media in log phase with 67.58 % dry weight followed by lag and stationary phase with 57.53 % and 51.1 % dry weight. On the other hand, *Dunaliella* sp. showed no significant different ($p>0.05$) in carbohydrate content at different growth phase but followed an increasing pattern with the growth of microalgae. Stationary phase showed the highest amount of carbohydrate compared with other phases with 19.78 % in Conway media and 23.93 % in F2 medium. Conway media resulted significantly higher ($p>0.05$) lipid content compared to F2 medium. In lag, log and stationary phase 12.90 %, 9.80 % and 9.05 % of lipid were found in Conway media. This information might contribute to boost up the microalgal production by using the suitable culture media and can harvest the microalgae at different growth phases at their highest specific nutrient.

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1. INTRODUCTION

Microalgae are regarded as the primary producer for majority life on the planet (Wilkie et al., 2011) and accountable for manifestation of aerobic organisms together with humans by using CO_2 from the primitive atmosphere and liberated O_2 which is about 70% of atmospheric O_2 (Walker, 1980). Microalgae are capable to synthesis a number of metabolites such as proteins, lipids, chlorophyll, carotenoids and vitamins for health, food and feed additives, cosmetics and for energy production (Priyadarshani and Rath, 2012). Marine microalgae have ecological and economic significance worldwide as they ecologically

contribute to at least 30% of annual CO_2 fixation worldwide and therefore largely influence global biogeochemical cycles (Platt et al., 2003). Economically, various marine microalgae are employed or have the possibility to be utilize as nutraceuticals, for the creation of pharmaceuticals (Borowitzka, 1995), cosmetics (Kim et al., 2008), for bioremediation (Cardinale, 2011), and for biofuels (Waltz, 2009) production. *Dunaliella* sp. is free living organisms and unicellular algae. *Dunaliella* sp. is hypersaline organisms which can withstand wide range of salinity concentration. Reproduce by fusion or longitudinal division of two motile cells to form zygote. *Dunaliella* sp. presence or lack of rigid cell wall, nucleus wall and cell sheath. The cell elongated or spherical in shape and each of the cells has an ovoid space that surrounded by cell wall. It moves by using its two anterior flagella. The cell color is bright green and the color change to greenish yellow on the sixth day of the algae culture (Zainuri et al., 2008). *Dunaliella* sp. produces high ß-carotene and it is usually used as the live feed for aquaria organisms (Garcia et al., 2007).

Marine microalgae that can grow in saline-hypersaline media are especially interesting as they are more sustainable and economical to grow compared to freshwater microalgae (Indrayani et al., 2018) that can be grown in non-arable lands, thus will not compete for agricultural land (BorowitzkaandMoheimani 2013). Moreover, hypersaline microalgae are less susceptible to contaminations by other microorganisms like protozoas and bacteria as not much organisms could able to withstand high salt concentration (Indrayani et al., 2018). More so, the biochemical composition of microalgae differs depends on some factors like species variations, culture environment, and the nutrient content of culture media (Khatoon et al., 2017). It has been established that various autotrophic media can highly influence the growth rate of different microalgae species (Scott et al., 2010), for this reason, multiple growth media have been established to culture microalgae. Determining better culture media and growth conditions of microalgae are considered as an integral part towards achieving mass production of any microalgae. Therefore, two culture media such as Conway and F2 media were used in this study to evaluate the growth rate and the proximate composition of the *Dunaliella* sp. at different growth phase.

2. MATERIALS AND METHODS

Microalgae sample collection

Dunaliella sp. was obtained from the previously isolated and preserved samples at Aqua-Industry laboratory, Faculty of Fisheries, Universiti Malaysia Terengganu (UMT), Malaysia. The pure sample of *Dunaliella* sp. was cultured in an Erlenmeyer flask using Conway medium.

Media preparation

Conway Medium and Guillard's F2 media were prepared according to Tompkins et al., 1995 and Smith 1993. Conway medium includes macronutrients (solution A), trace metal solution (solution B), and vitamin (solution C). 1 mL of solution A, 0.5 mL of solution B, and 0.1 mL of solution C was added into 1000 mL of filtered and sterilized sea water (Table 1). Whereas in case of F2 medium 1 mL of nitrate, phosphate, silicate, trace metal and vitamins solutions was added into 1000 mL of filtered and sterilized sea water (Table 2).

Determination of growth curve

Dunaliella sp. was cultivated using the Conway medium and Guillard's F2 medium. Microalgae were grown in a sterile 500 mL borosilicate Erlenmeyer flask at 25±1°C temperature and the culture volume was 350 mL with three replicates where 2% pure culture stocks were added in each flask. Microalgae cultures were maintained at 24 h light condition at 2000 Lux intensity. The experiment was continued until the death phase and finally completed the growth curve using cell density (cells.ml^{-1}) and optical density (absorbance).

Determination of optical density (OD)

Optical density was measured every day for the growth curve analysis. Conway medium and Guillard's F2 medium without any microalgae cell was used as blank. Maximum absorbance was measured at the wavelength of 670 nm. Maximum absorbance value was used to perform the growth curve by optical density.

Table 1. Composition and preparation Conway Medium (Tompkins et al., 1995)

Constituents	**Quantities**
Solution A-CHEMICAL	
Sodium nitrate ($NaNO_3$)	20 g
Ferric chloride ($FeCl_3$)	1.3 g
Boric acid (H_3BO_3)	33.4 g
EDTA[(b)], di-sodium salt	45 g
Manganous chloride ($MnCl_2$, $4H_2O$)	0.36 g
Sodium di-hydrogen orthophosphate (NaH_2PO_4, $2H_2O$)	20 g
Distilled water	1000 mL
Solution B- trace metal	
Zinc chloride ($ZnCl_2$)	4.2 g
Cobaltous chloride ($CoCl_2$,6 H_2O)	4.0 g
Ammonium molybdate ($(NH_4)_6Mo_7O_{24}$, $4H_2O$)	1.8 g
Cupric sulphate ($CuSO_4$, $5H_2O$)	4.0 g
Distilled water	1000 mL
Acidify with HCl to obtain a clear solution	
Solution-C Vitamins	
Vitamin B_1	200 mg
Vitamin B12	10 mg
Distilled water	1000 mL

Table 2. Composition and preparation of Guillard's F2 medium (modified from Smith et al., 1993).

Nutrients	Final concentration (mg/L seawater)	Stock solution preparations
$NaNO_3$	75	Nitrate/Phosphate solution Working Stock : add 75 g $NaNO_3$ + 5 g NaH_2PO_4 to 1 liter distilled water (DW)
$NaH_2PO_4.H_2O$	5	
$NaSiO_3.9H_2O$	30	Silicate Solution Working Stock : add 30 g Na_2SiO_3 to 1 liter DW
$Na_2C_{10}H_{14}O_8N_2.H_2O$($Na_2EDTA$)	4.36	Trace Metal/EDTA Solution Primary stocks : make 5 separate 1-liter stocks of ($g.I^{-1}$ DW) 10.0 g $CoCl^2$, 9.8 g $CuSO_4$, 180 g $MnCl_2$, 6.3 g Na_2MoO_4, 22.0 g $ZnSO_4$
$CoCl_2.6H_2O$	0.01	
$CuSO_4.5H_2O$	0.01	
$Fecl_3.6H_2O$	3.15	
$MnCl_2.4H_2O$	0.18	Working stock : add 1 mL of each primary stock solution + 4.35 g $Na_2C_{10}H_{14}O_8N_2$ + 3.15 g $FeCl_3$ to 1 liter DW
$Na_2MoO_4.2H_2O$	0.006	
$ZnSO_4.7H_2O$	0.022	
Thiamine HCI	0.1	Vitamin Solution Primary stock : add 20 g thiamin HCl + 0.1 g biotin + 0.1 g B_{12} to 1 liter DW
Biotin	0.0005	
B_{12}	0.0005	Working stock : add 5 mL primary stock to 1 liter DW

Determination of cell density

Microalgae cell count was carried out every day by using a Neubauerhemacytometer (0.0025 mm^2, 0.1 mm deep chambers, Assistent, Germany). The cells were counted by following the procedure and formulae of Lavens and Sorgeloos, 1996.

Experimental design for proximate composition determination

For proximate composition determination 700 mL of culture media was taken in 1L Erlenmeyer flask with three replicates for each media culture. In this experiment, the culture was doing in different media Conway and F2 for every growth phase. There were nine replicates, three replicate for each growth phase that is lag phase, log phase and stationary phase. Both treatments were run at the same time and in the same environmental condition. The initial cell density of *Dunaliella* sp. is $1x10^4$ cells ml^{-1} for each treatment. Proximate compositions of protein, lipid and carbohydrate were analyzed during biochemical test after culture harvested for every growth phase. Cells were harvested at different growth phases by centrifuge the sample at 6000 rpm for 10 min followed by washing twice with sterilize distilled water.

Determination of proximate composition

Protein and carbohydrate content were determined by following the procedure of Lowry et al. (1951) and Dubois et al. (1956), respectively by using 5–6 mg dried microalgal culture. Lipid was analyzed by following the method of Bligh & Dyer, (1959) and Folch et al. (1959) where 50 mg of each dried sample was used.

Statistical analysis

In case of growth curve, both cell density and optical density data were calculated by using MS excel. Statistical analyses of proximate composition (protein, lipid and carbohydrate) in F2 and Conway media was performed by one-way analysis of variance (ANOVA) using the IBM SPSS (v. 26.0) software. Significant differences in proximate composition of F2 and Conway media was analyzed using Duncan Multiple Range Test at 95% confidence interval.

3. RESULTS

Growth phases of Dunaliella sp. in Conway and F2 medium

Result showed that, *Dunaliella* sp. showed difference in growth phases when culture in Conway and F2 medium represented in Figure 1. Cell density of *Dunaliella* sp. was comparatively higher in F2 medium than Conway media. Together with this, *Dunaliella* sp. takes longer time in Conway medium to reach at death phase compared to F2 medium. In F2 medium, *Dunaliella* sp. showed 1 to 3 days of lag phase, 4 to 6 days of exponential phase, 7 to 8 days of stationary phase and slight decline of cell density was recorded on day 9. On the other hand, in Conway media, *Dunaliella* sp. showed similar lag phase like F2 medium. The exponential phase reached at 3^{rd} – 7^{th} days of culture. The cell reached at their stationary phase on the 8^{th} – 10^{th} days of culture and death phase was recorded at day 11.

Dunaliella sp. showed higher cell density when culture in F2 medium compared in Conway media. The highest cell density of *Dunaliella* sp. is 7.8 x 10^6 cells mL^{-1} in F2 medium and 4.1 x 10^6 cells mL^{-1} in Conway media. Also, *Dunaliella* sp. takes short time to reach at their highest cell density in F2 medium than Conway media. *Dunaliella* sp. takes only 7 days to reach at highest cell density in F2 medium and 9 days in Conway media.

Proximate composition of Dunaliella sp. in Conway and F2 medium

Proximate composition of *Dunaliella* sp. at its different growth phase in both F2 and Conway media were represented in figure 2. *Dunaliella* sp. showed the highest amount of protein content in Conway compared to F2 medium.
In Conway media, significantly higher ($p<0.05$) amount of protein content was recorded in log phase with 67.58 % of dry weight followed by lag (57.53 % dry weight) and stationary phase (51.1 % dry weight). In F2 medium, log phase (33.23 % dry weight) also showed significantly higher ($p<0.05$) amount of protein than the stationary and lag phase with 30.56 % and 16.06 % dry weight, respectively.

Carbohydrate contents of *Dunaliella* sp. in both media showed an increase pattern at every phase shown in figure 2. Result showed that, there was significant different ($p>0.05$) in carbohydrate content of *Dunaliella* sp. among the growth phases in both media. Significantly higher ($p<0.05$) amount of carbohydrate content was recorded in stationary phase with 19.78 % dry weight in Conway media and 23.93 % dry weight in F2 medium. This followed by log and lag phase with 9.48 % dry weight and 9.00 % dry weight in Conway media and 12.80 % dry weight and 6.40 % dry weight in F2 medium.
Between F2 and Conway media, *Dunaliella* sp. resulted higher lipid content in Conway medium compared to F2 medium. In Conway media, there was no significant ($p>0.05$) difference in lipid content among different growth phases

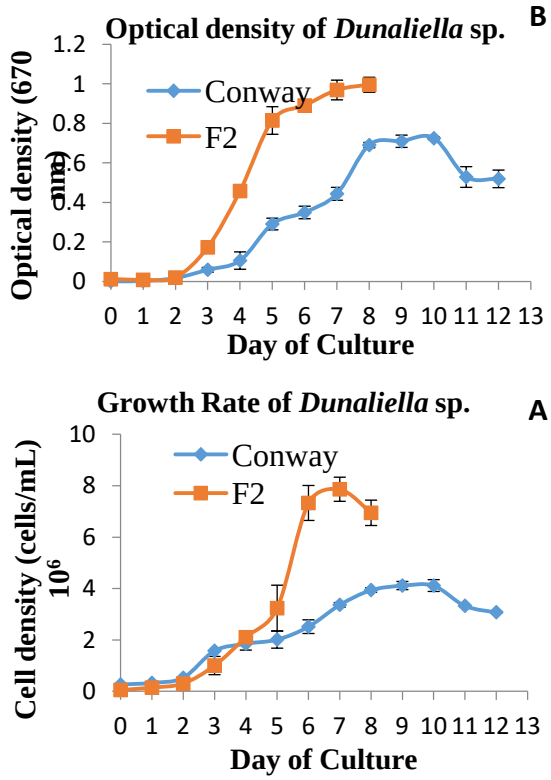

Figure 1: Cell density of *Dunaliella*sp. in F2 and Conway media (A) and optical density of *Dunaliella*sp. in F2 and Conway media (B).

where highest lipid content was detected in lag phase with 12.90 % dry weight followed by log and stationary phase with 9.80 % dry weight and 9.05 % dry weight. Whereas in F2 medium, Significantly higher ($p<0.05$) lipid content was recorded in stationary phase with 10.30 % dry weight followed by lag and log phase with 6.30 % dry weight and 4.15 % dry weight, respectively. The finding showed that Conway media resulted in higher lipid content of *Dunaliella* sp. at every growth phase compared to F2 medium.

4. DISCUSSION

Growth phases of Dunaliella sp. in Conway and F2 medium

Adequate supplement of nutrient is an important factor for the growth of microalgae (Xin et al., 2010) where most significant growth parameter is cell density as high cell density would facilitate the mass production of microalgae for biodiesel and pharmaceutical industry (Quinn et al., 2012). However, microalgae with fast growth rate are most preferably used in commercial and industrial field (Lananan et al., 2013). Sufficient nutrient from media culture will determine the best growth performance of microalgae (Xin et al., 2010). In a previous study, Lananan et al. (2013) showed that *Dunaliella* sp. had the lower cell density when culture in F2 medium compared to Conway media. From this experiment, Conway media was observed to have lower cell density compared to cell density of *Dunaliella* sp. in F2 medium. Nutrient depletion in cultivation used would cause stunted the microalgae growth (Sun & Li, 2012). According to Lourenço (2006) nitrate ($NO3^-$) is the most stable form of nitrogen present in seawater and also most assimilated nutrient by microalgae. In this study, *Dunaliella* sp. performed best in terms of cell density and earlier stationary phase in F2 compared to Conway media asnitrate reduction in the Guillard f/2 culture medium was statistically faster than observed in Conway medium (Coêlho et al., 2013). Together with this, *Dunaliella* sp. prefers nitrate instead of ammonium for growth (Chen et al., 2011), for this reason, nitrate in F2 medium, stimulate the growth of *Dunaliella* sp. and result in the higher cell density earlier stationary phase in the media culture.

Proximate composition of Dunaliella sp. in Conway and F2 medium

The proximate value of any microalgae species depends on its size, biochemical composition

and others. In this experiment, F2 and Conway media has shown the highest protein content compared to lipid and carbohydrate at each growth phase as protein is the major nutrient content followed by lipid and lastly carbohydrate (FAO, 1996). In the present study, there as a significant difference in protein content between Conway media and F2 medium, because the properties and the composition of microalgae can be varied when cultured under different conditions or when the microalgae are harvested at different growth phase (Brown et al., 1997). Together with this, log phase has the highest protein content than lag and stationary phase in both media, as the cell density of *Dunaliella* sp. was increased rapidly in this phase. The protein content was decreased both media when it reached stationary phase due to the depletion of nitrogen sources, because, microalgae generate both lipid and proteins when the carbon: nitrogen ratio is balanced, when there is a lot of nitrogen, that increases the net nitrogen consumption (Araujo et al., 2020). The amount of lipid content in microalgae are varies from 1-85 % of the dry weight (Rodolfi et al., 2009). Nutritional composition of the medium culture also affects the lipid content in microalgae. On the other hand, lipid content in Conway media is comparatively higher than in F2 medium, lower amount of nitrogen sources in Conway media than F2 medium can be reason for the observed differences herein. On the contrary, lipid content in F2 medium increased in the later stages of growth phases because when nitrogen is scarce, microalgae produce fewer nitrogen-containing molecules and store lipids, resulting in a reduction in nitrogen consumption (Araujo et al., 2020). Almost similar findings were claimed by Mohan et al. (2012) who stated that, accumulation of lipid in the microalgae is usually at the early or late end of the growth phase. Carbohydrate levels increase in growth phase as the nutrient in culture media become limiting factor for the algae growth (Brown & Jeffrey 1992). The similar result was found in this study where the carbohydrate content showed an increasing pattern starting from lag, log and stationary growth for F2 and Conway media. The decline in nutrient in media culture stimulates the production of carbohydrate content. From the findings of this study it can be said that, Conway media is the best choice for the production of protein and lipid content in *Dunaliella* sp. compared to F2 medium. The production of protein content at log phase and lipid content at stationary phase was high in Conway media. This information might help the aquaculture people to boost the production of protein, lipid and carbohydrate in microalgae by using the suitable culture media and can harvest the microalgae at different growth phase at their highest specific nutrient.

5. CONCLUSION

To utilize the microalgae it is important to know about their growth rate and biochemical composition. *Dunaliella* sp. showed higher cell density and earlier stationary phase in F2 medium compared to the Conway media. But in case of proximate composition, *Dunaliella* sp. resulted higher protein and lipid content in Conway media in every growth phases. Therefore it can be concluded that, even though Conway media possess low cell density of *Dunaliella* sp., it is the best media for higher production of protein and lipid content in *Dunaliella* sp.

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