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ORIGINAL RESEARCH

Isolation of freshwater and marine indigenous microalgae species from Terengganu water bodies for potential uses as live feeds in aquaculture industry

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Abstract Microalgae are considered as a promising resource for high-value substances. In this study, indigenous microalgae were isolated from various habitats and the optimum growth parameters for high lipid production were determined. Species identification was done by using universal 18S rDNA. Water quality was recorded during the sampling to determine the basic physical-chemical parameters for the growth studies. The screening for microalgae growth was done using plate reader with a focus on lipid-rich isolates by Nile Red fluorescent dye at different growth phase for 15 days. Results showed that ten microalgae species have been successfully cultivated from Terengganu water bodies. Analysis from genus *Chlorella* showed five isolates (TRG1-A01, TRG1-C01, TRG2-E01, TRG5-A01, and TRG3-B01) were clustered together, while two isolates (TRG2-A01, TRG3-A01) from the genus *Nannochloropsis* were *N. oceania.* Another two isolates (TRG1-D01, TRG1-E01) were closely clustered with *Desmodesmus subspicatus* while TRG4-C01 was clustered with *Dicloster aquatus.* The strain TRG2-A01 showed the highest fluorescent intensity indicating its potential to produce the highest intracellular lipid content. Thus from this study, we can identify which environmental conditions contribute to the most favourable conditions for algal growth to maximize lipid production which later could be used as live feeds in aquaculture industry.

Keywords Species identification . Microalgae . Nile red . 18s rDNA . Aquaculture

Introduction

Since 1930's, Malaysian phycology has been introduced to the discovery of algal resources and their practices. The inventory checklists of Malaysian algae including ecological studies and applied phycology publications have been produced since 1970 until 1990 (Phang et al. 2015). The development of gene technology encouraged the improvement of algal systematics and phylogenetic (Vello et al. 2014). In addition, several publications on the use of algae for food, medicine, biofuel production, energy and bioremediation, as well as their development and commercialisation have also been published (Borowitza

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2013; Chisti 2007; Mustafa et al. 2012). Great profits could be achieved from integrating the use of algae in environmental and other higher value applications (Mazlan et al. 2005) from increasing algal expertise to a better scope in the academic side as well as in industry.

Terengganu has an extensive coastline surrounded by numerous islands, consisting of a variety of ideal habitats, feasible for mass algae cultivation. The abundance and diversity of microalgae species in Terengganu water bodies are mostly depending on several environment factors such as light, temperature, pH, salinity, and nutrient availability (Araújo and Garcia 2005; Alonso-Rodriguez and Páez-Osuna 2003). In particular, microalgae with high potential value could be selected as new algae strains to be used in aquaculture industry as live feeds based on their biomass and lipid contents (Eryalçın et al. 2015). Since the phycology research in Malaysia particularly on the application of microalgae as live feeds is still limited, there is necessity for conducting more research on microalgae related to economic issues. Therefore, the selection of new microalgae strains from both freshwater and marine water environment are needed for pure isolation and identification of microalgae species.

Aquaculture is an upward industry, and therefore, the industry of culturing microalgae is consequently increasing. The main applications of microalgae in aquaculture could relate directly or indirectly to the nutrition impacts on various species of aquatic farmed organisms such as crustacean and fish. Moreover, microalgae can be used directly, for instance, as food for larvae of some gastropods or as food for some fish species in their earliest growth stages (Brown and Robert 2002). Microalgae could also be used indirectly as food for feeding zooplankton such as rotifers, which are essential food for some fish larvae (Welladsen et al. 2012). In aquaculture industry, microalgae were found to be applicable in a so called “green water” feeding technique, where microalgae are added as a suspension to the organisms’ environment grown simultaneously in tanks with larvae (Neori 2011). Microalgae are also recognized as the next generation sustainable feedstock (Pérez-López et al. 2014) especially due to their beneficial biochemical compounds such as lipids and carotenoids (Patil et al. 2007).

In recent years, there has been increased interest in the searching of lipid-rich microalgae for biofuel production (Vello et al. 2014) and aquaculture industry (Shah et al. 2018). The ability to reproduce fast, have high lipid and carbohydrate productivities are the characteristics of algal strains selection that have been in demand for biofuel production and aquaculture feed (Shah et al. 2018; Thi et al. 2011; Hempel et al. 2012). The interference of anthropogenic activities in biogeochemical cycles and the extensive use of fossil fuels are the primary cause of the imminent global climate change. This scenario is expected to further deteriorate in the coming years, since the human population is expected to increase up to 9 billion by 2050 (Godfray et al. 2010). Goli et al. (2016) reported that 87% of the global CO_2 emitted by anthropogenic activities result from the usage of fossil resources, with coal, oil and natural gas contributing 43%, 36% and 20%, respectively. Over the last decade, biomass has drawn increasing attention in our society as a source of bioenergy and bio-products. Various biomass sources including first, second, third and fourth generation feedstock such as edible and non-edible energy crops, wood, agricultural residues and algae have been exploited extensively for the development of bioenergy and a more sustainable bio-based products.

Aquatic animals have used microalgae as food for thousands of years (Milledge 2011). Microalgae can convert solar energy to chemical energy by fixing CO_2, and its’ efficiency is ten times greater than terrestrial plants. Approximately 5,000 tons/year of dry matter of microalgae are produced commercially (Raja et al. 2008). There are approximately 110 commercial producers of microalgae present in the Asia-Pacific region, with capacities ranging from 3 to 500 tons/year. About nine-tenths of algal cultivation is located in Asia. Very few species of microalgae have commercial importance, but those species found include *Spirulina, Chlorella, Haematococcus, Dunaliella, Botryococcus, Phaeodactylum, Porphyridium, Chaetoceros, Crypthecodinium, Isochrysis, Nannochloropsis, Nitzschia, Schizochytrium, Tetraselmis,* and *Skeletonema*. Furthermore, much of the microalgal biomass has been an attractive source for producing a wide range of highly valuable products, including polyunsaturated fatty acids (PUFA), carotenoids, phycobiliproteins, polysaccharides and phycotoxin.

Up to date, studies on identification of microalgae, commercial potential of microalgae, characterization of green microalgae, prospect of algae and their environmental applications in Malaysia are varied but there were no research on information of microalgae species from both freshwater and marine water throughout Terengganu water bodies (Phang and Chu 2004). The biodiversity of microalgal species, comprising over several thousand species with 11 divisions (Tomaselli 2004), represents a source that is practically

unexplored. Since Terengganu has a wide diversity of microalgae which potentially utilized diversely in aquaculture, their main applications related to nutritional value is critically important to be explored. Most microalgae species play a crucial nutritional role for both freshwater and marine animals, which consequently benefited to aquaculture industry. Isolation and screening of microalgae from natural habitat in the Midwestern of United States proved that selected strains demonstrated showed rapid growth in their whole life cycle (Lee et al., 2014). For instance, *Scenedesmus* sp. species had high average lipid content and have a greatest potential as source of biomass and lipids. Thus, isolation and identification of indigenous microalgae from Terengganu water bodies is needed for future potential discovery and development in application of microalgae for aquaculture purposes.

Materials and methods

Isolation of microalgae from Terengganu water bodies

Sampling of indigenous freshwater and marine microalgae species were carried out at five different locations as shown in Table 1. Seven litres of water sample was filtered through a phytoplankton net (60 µm) and the water sample containing the concentrated algae was stored in a screw-cap plastic bottle. The samples were divided into two sub-samples; one fixed with 4% formalin for microscopic examination and the second sub-sample was incubated on illuminated shelves designed for growing algal cultures, for isolation of dominant strains.

Species identification of isolated microalgae

For molecular identification, the nearly full-length 18s rDNA gene was PCR amplified using the universal eukaryotic primer set (Forward primer 5'à3': AACCTGGTTGATCCTGCC AGT; Reverse primer 5'à3': TGATCCTTCTGCAGGTTCAC CTAC) (Medlin et al. 1988). The PCR reactions included the following: 5 µL of 10 x Pfu buffer [200 mM Tris-HCl (pH 8.8 at 25 °C), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1 mg/mL BSA, 1% (v/v) Triton X-100, 20 mM $MgSO_4$], 2 unit of Pfu DNA polymerase (0.8 µL), 1 µL of 10 mM dNTPs mix, 2.5 µL of each primers (10 pmol/µL), DNA and H_2O to a final volume of 50 µL. PCR reactions were run on a Bio-Rad T100 Gradient Thermal Cycler (Bio-Rad, Malaysia) using the following conditions: Initial denaturation at 95 °C, 5 min; denaturation at 95 °C, 45 s; annealing at 55 °C, 30 s; and extension at 72 °C, 2.5 min. The denaturation, annealing and extension were repeated for 40 cycles followed by final extension at 72 °C for 10 min. The amplified PCR products were sent for sequencing and database searches were carried out using BLASTX or BLASTN program (Altschul et al. 1990) in the non-redundant protein and nucleotide sequence database at NCBI (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment were constructed using the CLUSTAL Omega program (http://www.ebi.ac.uk/Tools/ clustalw2/index.html), followed by manual optimization. Phylogenetic analyses were conducted using CLUSTALX (1.81) with Bootstrap Neighbor-Joining method (Saitou and Nei 1987; Tamura et al. 2004).

For morphology identification using light microscope, glass slides containing 5 µL of fresh microalgae culture were prepared and observed under light microscope (Leica DM LB2). Morphological characteristics of all microalgae cells were examined using 100 x oil immersion lens and images were acquired with Leica Application Suite (LAS) V4.0 software.

Table 1 Sampling location of indigenous microalgae from Terengganu water bodies.

Location	Code	Type of water	Coordinates
Kenyir Lake	TRG1	Freshwater	N05° 00' E102° 48'
Redang Island	TRG2	Marine	N05° 47' E103° 00'
Kapas Island	TRG3	Marine	N05°20' E103° 06'
Bidong Island	TRG4	Marine	N05° 37' E103° 04'
Perhentian Island	TRG5	Marine	N05° 55' E104° 44'

Water quality analyses of sampling sites

Water quality analyses were carried out at the site of algal sample collection in Setiu Wetland, Kenyir Lake, islands of Redang, Kapas, Perhentian, and Bidong (habitats). The water samples were collected in 1.5 to 25.0 L plastic bottles and kept in the cold room temperature at 18ºC. Analyses were done to determine the basic physical-chemical characteristics at the collection site. The results from these analyses were used as the baseline parameters for the growth studies. YSI meter was used to measure all the environmental parameters such as pH, dissolved oxygen, salinity, conductivity, total suspended solid, and water temperature which were taken on-site during sample collection. In the laboratory, pH of the water samples was measured again using a pH meter (Delta 320 Mettler Toledo, Mettlerr-Toledo Group, Shanghai China).

The nutrients such as BOD, NH3-N, NO3-N and PO4(3-), content in water samples were determined using the Method 8155, Method 8048, Method 8171 and Method 8000, respectively as shown in the Hach HandBook Odyssey DR/2500 Spectrophotometer Procedure Manual (Hach Company, USA 2001). The BOD content of the seawater sample was determined by the 8000 method (APHA 1998). An amber color solution will develop if the BOD is present and the absorbance of the color was read at 620 nm using Hach spectrophotometer (Hach Odyssey DR/2500, Hach Company, USA). Based on BOD value, the carbon (C) content can be calculated using the formula given by Edwards et al. (1980): Carbon (C) in mg/L = BOD (mg/L) X 12 The NH3–N content of the seawater sample was determined by the salicylate method [Method 8155] (APHA 1998), A green color solution will develop if the ammoniacal-nitrogen is present and the absorbance of the color was read at 665 nm using Hach spectrophotometer (Hach Odyssey DR/2500, Hach Company, USA). The nitrate content of the seawater sample was determined by the cadmium reduction method [Method 8171] (APHA 1998). An amber colour solution is developed if the nitrate is present and the absorbance of the color was read at 507 nm using the Hach spectrophotometer (Hach Odyssey DR/2500, Hach Company, USA). Orthophosphate is referred to as phosphate that responds to colorimetric tests without prior hydrolysis or oxidative digestion of the sample (APHA 1998). The assay employed was the ascorbic acid method [Method 8048], based on the molybdenum blue colour development from phosphomolybdic acid and the absorbance of the color was read at 880 nm using Hach spectrophotometer (Hach Odyssey DR/2500, Hach Company, USA).

Screening of high growth microalgae

All pure isolates were screened using plate reader for high throughput, associated with their natural environment. Two different growth media were used which represented marine (f/2 and Conway media) and freshwater (Bold's Basal Media, WC media), respectively. Using a 48-well plate, 1 x 10^5 cells.mL^{-1} of pure microalgae cells were added into each well. The plate were then incubated at 25°C, 300-500 µmol photons.m^{-2}.s^{-1}, with 12h light: 12h dark. On a daily basis, the microalgae samples were shaken using plate reader (Thermo, Varioskan LUX). The absorbance spectrum was measured every 5 minutes at 750 nm using the plate reader for 15 days to observe the growth of the pure algal isolates. The growth media were measured as blank. The growth rate were measured using absorbance of 750 nm towards all the pure algal isolates (Griffiths et al. 2011). The selected strains with high growth rate were then used for screening of lipid-rich microalgae.

Screening of lipid-rich microalgae with Nile Red fluorescent dye

In every 0-4 day (lag and exponential phase), 8 day (early stationary phase) and 15 day (late stationary phase), 200 µL of pure microalgal samples were taken and placed in a black 96-well plate after homogenously shaken from 48-well plate. To avoid contamination, 2 µL of Nile Red (NR) solution (1 mg/mL in DMSO) was prepared freshly in every 2 weeks and preserved in air-tight tubes in the dark and at 4°C (Greenspan and Fowler 1985; Greenspan et al. 1985; Rumin et al. 2015). NR solution was added into each well of 200 µL of transferred microalgae samples, resulting in final concentration of 10 µg NR/mL and shaken in plate reader. The fluorescent emission wavelength at 620 nm and fluorescent excitation at 488 nm were measured in every 5 minutes for a total of 30 minutes under dark condition at 25°C using plate reader. The plate was shaken prior measurement of the fluorescence. The peak fluorescence signal over this 30 minutes time

period was used as the reported signal. Fluorescence of 620 nm was then correlated with the amount of polar lipid (Alonzo and Mayzaud 1999; Johnson et al. 2017)

Results and discussion

Molecular identification of microalgae species

A total of 10 microalgae have been successfully isolated from Terengganu water bodies. In order to classify the microalgae, uniclonal and axenic culture of these microalgae samples were observed under light microscope and their morphological characteristics are summarized in Table 2. In order to confirm the microalgae species, nearly full length 18S rDNA gene was amplified from these microalgae samples using a universal eukaryotic primer set (Medlin et al., 1988). All samples exhibited similar sized products of about 1500 bp.

Phylogenetic analysis of the 18S rDNA sequences from the genus *Chlorella* showed that TRG1-A01, TRG1-C01, TRG2-E01, TRG3-B01 and TRG5-A01 were closely clustered together (Figure 1). Meanwhile, the isolates TRG2-A01 and TRG3-A01 from the genus *Nannochloropsis* were *N. oceanica* (Table 2; Figure 1) while TRG1-D01 and TRG1-E01 were closely clustered with *Desmodesmus subspicatus* (Figure 1). Another isolate, TRG4-C01 which initially identified as *Chlorella* based on morphological characteristic (Table 2) was clustered with *Dicloster aquatus* (Figure 1) based on molecular results while TRG3-B01 that

Table 2 Classification of microalgae samples according to morphological characteristics.

Genus	Code	Morphological description
Chlorella	TRG1-A01 TRG1-C01 TRG2-E01 TRG4-C01 TRG5-A01	Spherical in shape, small size of about 5-10 µm in diameter, green in colour, mostly occurred as a single cell, without flagella
Nannochloropsis	TRG2-A01 TRG3-A01 TRG3-B01	Spherical in shape, smaller size than *Chlorella* of about 2-3 µm in diameter, green in colour, mostly occurred as a single cell, without flagella
Desmodesmus	TRG1-D01 TRG1-E01	Ovoid in shape, cells are rarely solitary, mostly formed colonies of 2-4 cells, size about 5-10 µm in length, green yellowish in colour, with spines at both ends

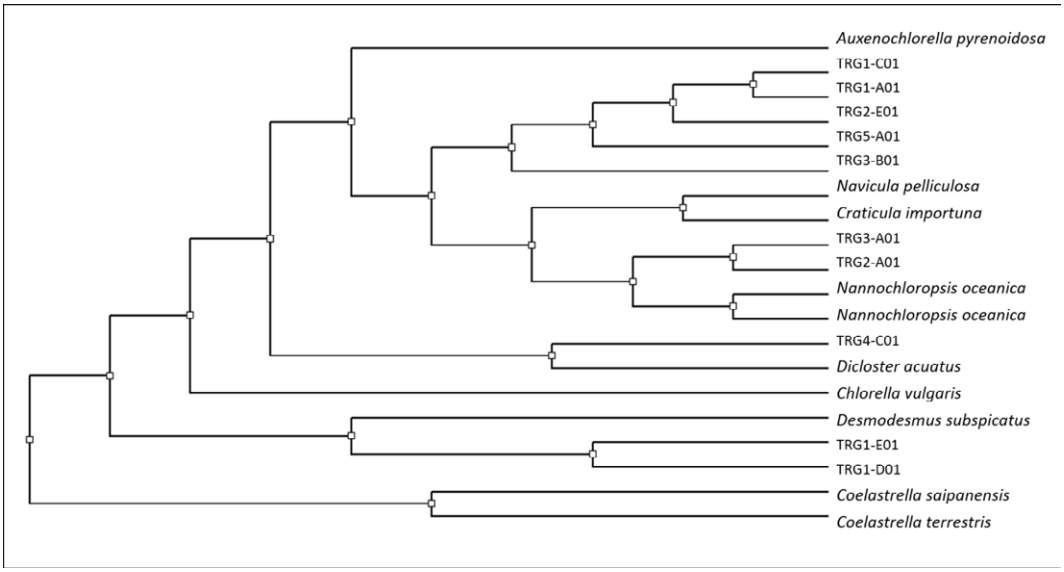

Fig. 1 Molecular phylogenetic analysis of the ten isolates based on 18S rDNA sequence comparisons. The other closely related species were retrieved from GenBank: *Auxenochlorella pyrenoidosa* isolate HIT9 (MF040792.1), *Chlorella vulgaris* culture-collection KMMCC:C-105 (GQ122343.1), *Coelastrella saipanensis* (HG328355.1), *Coelastrella terrestris* strain KZ-5-4-9 (MK231276.1), *Craticula importuna* strain AT-70Gel14a (AM501978.1), *Desmodesmus subspicatus* strain GB6 (KX389312.1), *Dicloster acuatus* strain Xmm25W2 (KY054950.1), *Nannochloropsis oceanica* strain CS-179 (KT031995.1), and *Navicula pelliculosa* strain CCMP543 (AY485454.1).

was identified as *Nannochloropsis* was later clustered together with TRG1-A01, TRG1-C01, TRG2-E01, and TRG5-A01.

During the past 20 years, molecular studies of SSU rRNA/rDNA have successfully elucidated the diversity of a number of eukaryotic microorganisms (Diez et al. 2001; Moon-van der Staay et al. 2001; Pace 1997; Stoeck and Epstein, 2003). Cloning and sequencing of the 18S SSU rRNA/rDNA gene have proven to be valuable approaches to differentiate closely related species that are extremely conserved in their SSU rRNA/rDNA sequences. More recently, SSU rRNA/rDNA sequencing together with ultra-structural phenotypic characterization were not only able to resolve several phylogenetic relationship within the genus, but also revealed novel genus within the same class (Luo et al. 2006; Stoeck et al. 2005).

In this study, only two of the isolates that were initially identified as *Chlorella* (TRG4-C01) and *Nannochloropsis* (TRG3-B01) based on morphology observation under light microscope that were later renamed based on molecular identification. This discrepancy is most likely due to the different in cell size of *Chlorella* and *Nannochloropsis* as both genus have similar morphology of green, spherical-shaped cell.

Selection of high growth microalgae

Figure 2 showed growth pattern of ten microalgae species isolated from Terengganu water bodies, following 15 days incubation periods. The growth curve was divided into four phases including lag phase, exponential phase or log phase, stationary phase and death phase.

During the lag phase (0-1 day), there was a slow increment of the cell number due to the adaptation process of the cell into new environment. At this phase, the cell was only started to grow. The slow growth of cell was influenced by the adaptation of cell metabolism to grow such as increased in the level of enzymes and metabolites involved in cell division and carbon fixation (Barsanti and Gualtieri 2014). The growth of microalgae cells started to increase rapidly at day 2 (early stationary phase). At this stage, exponential phase was begun. The cells actively proliferated and the growth rate keep on increased constantly. The cultivation parameter plays important role during this phase such as light intensity, temperature and nutrient availability.

Exponential phase continued until day 8 where the number of cell starts to plateau. This phase was called as stationary phase. The number of cell at day 9 up to day 15 were observed at similar pattern. The

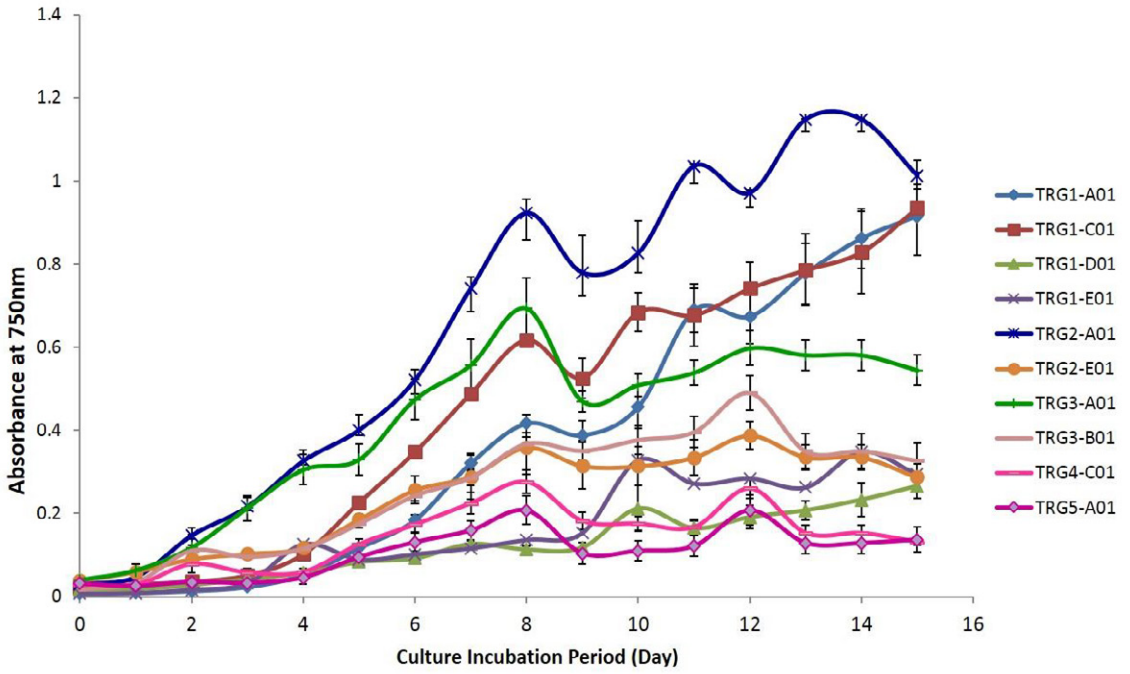

Fig. 2 Growth curve of freshwater and marine indigenous microalgae species isolated from Terengganu water bodies throughout 15 days cultivation periods. Data were indicated as mean ± standard deviation from three replicates.

number of death cell and living cell were equal owing to limited main growth factor which was nutrient content of growth media (Juneja et al. 2013). As the cell actively proliferated at the exponential phase, the nutrient content start to decrease and caused the number of cell growth and cell death to be equal.
At day 15 (late stationary phase), the growth rate started to decrease. The limited nutrient content started to give the impact where the cell started to die and the number of cell that live decreased. However, the decrease in cell growth is not only caused by the nutrient availability but also caused by some other reasons such as oxygen deficiency, overheating, pH disturbance or contamination (Barsanti and Gualtieri 2014).

Assessment of lipid-rich microalgae

Assessment of lipid-rich microalgae species from Terengganu water bodies were conducted using Nile Red fluorescent dye. The presence of lipid-rich microalgae were determine using correlation with the amount of polar lipid under fluorescence of 620 nm. Among all microalgae isolates, strain of TRG2-A01 which was isolated from Redang Island showed the highest fluorescence intensity (Figure 3). It was also noted that almost all microalgae strains showed their highest fluorescence intensity at 8 day of cultivation periods. This result dictated that almost all isolated microalgae strains, especially TRG2-A01 has the potential to produce high intracellular lipid content, which will later could be used as nutritional live feeds for aquaculture production (Doan et al. 2011; Katayama et al. 2019).

Effects of water quality and lipid production

The results indicated that the algae cultivated through the samples obtained from Redang Island (TRG2-A01) contains the highest lipid values based on the fluorescence intensity (Figure 3). In this study, the microalgae were cultured based on the optimal environmental conditions following the water quality parameters obtained from Redang Island (TRG2-A01). The water quality parameters of the cultures were closely monitored and maintained throughout the experiment to avoid large changes in the algal biochemical compositions. The cultures were maintained at the water quality parameters following the natural habitat; temperature ranged from 28ºC to 30ºC; pH ranged from 8 to 9; Marine microalgae were maintained at salinity levels ranged from 35 ppt to 36. ppt while freshwater microalgae were maintained at 0ppt; dissolved oxygen ranged from

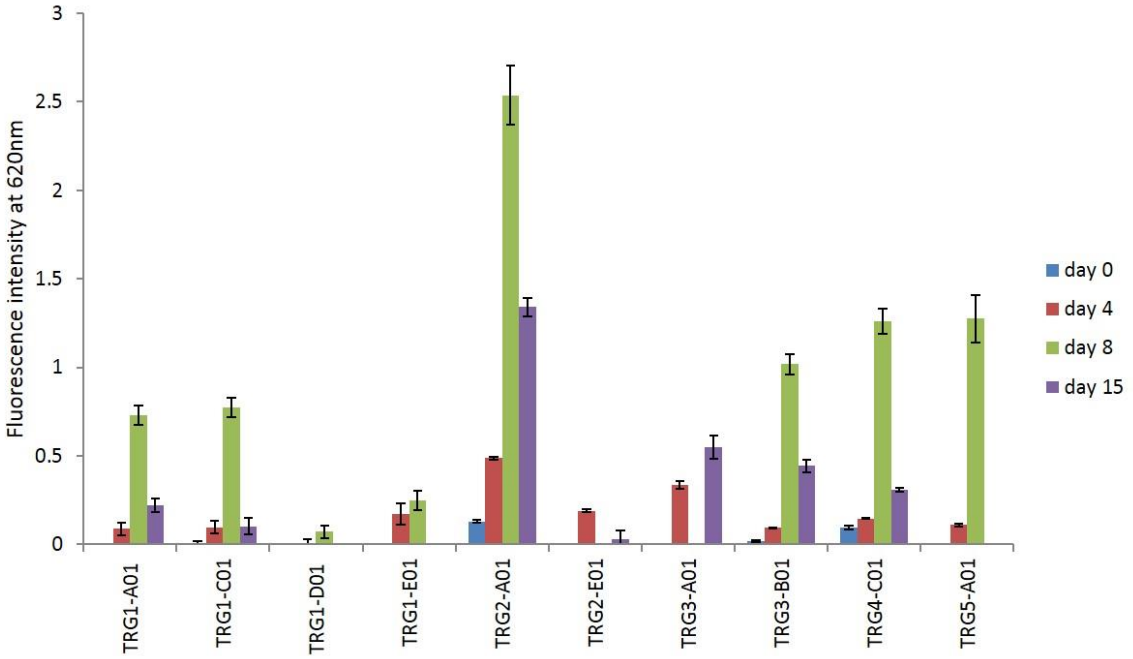

Fig. 3 Fluorescence intensity of isolated freshwater and marine indigenous microalgae species during 0, 4, 8 and 15 days of cultivation periods. Data were indicated as mean ± standard deviation from three replicates.

Table 3 Water quality parameters of the sampling area

Sampling Location	T (ºC)	pH	Salinity (ppt)	DO (mg/L)	TSS (g/L)	Nitrate (µg/L)	Orthophosphate (mg/L)	Nitrogen (mg/L)	BOD (mg/L)
Kenyir Lake	26.51-27.31	6.41-7.08	0.01	6.61-8.6	0.025	0.081	0.157	0.031	1.887
Redang Island	28.78-29.95	8.90-8.99	35.40-36.20	6.15-6.99	0.017	0.022	0.771	0.037	1.577
Kapas Island	28.20-28.40	7.59-7.94	29.68-32.75	5.74-6.03	0.345	0.010	0.005	0.044	1.817
Bidong Island	30.70-30.93	8.06-8.53	34.49	6.53-7.22	0.013	0.031	0.008	0.046	3.053
Perhentian Island	30.02-30.24	8.69-8.89	33.43-35.27	6.45-9.90	0.013	0.023	0.765	0.023	3.270

6 mg/L to 7 mg/L and the total suspended solid, TSS at 0.017g/L; Nitrate 0.022µg/L, Orthophosphate 0.771 mg/L; total Ammoniacal Nitrogen up to 0.037mg/L; and biological oxygen demand at 1.577mg/L.

The lipid content determination is achieved by comparing the resulting fluorescence values to a certain standard curve, in which the wavelength of excitation and emission may be different. Nevertheless, the lipid contents measured by this method are usually interfered by the environmental factors and other components in the cell cytoplasm, and the fluorescence intensity varies between samples. Thus, the optimal spectra and reaction conditions should be determined for each type of sample prior to the fluorescent measurement (Chen et al. 2018).

The water quality of the water sample has been analysed and the explanations are outlined in Table 3. The water quality measurement has been observed in order to identify the variety of water situations for each algal species habitat. The temperature, salinity, pH, dissolved oxygen, total suspended solid (TSS), nitrate, orthophosphate, ammonical nitrogen and biological oxygen demand has been measured and record in the results. Organisms are constantly subject to variation in their environment, and many of these varying factors act as stressors, displacing the organisms from their fundamental niches (Steinberg 2011). Besides natural stressors, human-induced environmental changes—among which, the increasing chemical pollution of ecosystems—represent important sources of stress in the environment (Suhett et al. 2015). Thus, from this study, we can identified which environmental conditions contributes to the most favourable conditions for algal growth to maximize lipid production.

Conclusion

Microalgae can achieve their highest growth in laboratory environment when they were provided with appropriate nutrients and environmental conditions similar to their natural habitat. The manipulation of these nutrients and parameters will influence their adaptation to the new lab environment to grow by increasing the level of their enzymes and metabolites activity involve in cell division and carbon fixation. Apart from the type of media culture, the present of nutrients will determine the growth pattern of microalgae and their average lipid content. Microalgae isolated from Redang Island which is known as tourist destination in Terengganu showed the highest potentials to produce high intracellular lipid content. Thus, this microalgae is selected and will be used later as a candidate for life feed diets for aquaculture production.

Conflict of interest The authors declare no conflicts of interest.

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