Malays. Appl. Biol. (2020) 49(4): 149–156

ISOLATION AND NILE RED SCREENING OF INDIGENOUS MICROALGAE SPECIES FROM PAHANG LAKES AS POTENTIAL LIPID SOURCE IN AQUACULTURE FEED

MUHAMMAD FAIZ ZAKARIA[1], NOORAZILAH HARIS[2], MOHD EFFENDY ABD WAHID[2,3,4], TOMOYO KATAYAMA[5] and MALINNA JUSOH[1,4*]

[1]Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Malaysia
[2]Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Malaysia
[3]Faculty of Fisheries and Food Sciences, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Malaysia
[4]Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Malaysia
[5]Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo, Tokyo, 113-8657 Japan
[*]E-mail: malinna@umt.edu.my

Accepted 18 November 2020, Published online 25 December 2020

ABSTRACT

The high nutritional composition of microalgae that includes lipids is considered as one of the promising alternative lipid sources for animal feeds enrichment. Since microalgae have higher photosynthetic efficiencies than terrestrial plants, highly adaptable to environmental changes, and do not compete with conventional agriculture for resources, the inclusion of lipid sources from microalgae in feed could help the sustainability of livestock production systems. This study has reported the indigenous microalgae strains from Pahang lakes and determined lipid-rich strains that have the potential for alternative lipid sources. Pure isolated strains were identified using an 18S rDNA marker. The microalgae strain biomass was determined for 15 days. Meanwhile, the screening for high-rich lipid contents in microalgae strains was carried out using Nile Red fluorescent dye. A total of 11 strains were successfully isolated that consist of 8 different species (*Carteria radiosa*, *Spongiosaccinopsis terrestris*, *Desmodesmus* sp., *Desmodesmus abundans*, *Dendodesmus brasiliensis*, *Chlamydomonas reinhardtii*, *Mychonastes timauensis,* and *Mychonastes ovahimbae*). Results showed that PHG C01, PHG C02, and PHG F03 have the highest biomass production among all strains. Meanwhile, for lipid production, PHG B01 and PHG F03 showed the highest results. Thus, PHG B01 and PHG F03 strains were chosen as potential candidates to be used as an alternative lipid source in animal feed feedstock.

Key words: Indigenous microalgae, species identification, 18s rDNA, nile red, feedstock, animal feed

INTRODUCTION

Studies on plant-based lipid sources including microalgae have attracted lots of attention due to their broad impact on the environment and aquaculture (Jia-Yi *et al*., 2019). In aquaculture, microalgae have been used as a potential source and energy for aquaculture feeds due to their photosynthetic efficiency and high nutritional value (Ju *et al*., 2012). At present, more than 40 species of microalgae have been isolated and cultured to be used in aquaculture feedstock (Sen *et al*., 2005). To sustain the aquaculture source demands, these microalgae are used as a food source for the aquatic organism and the main live feed due to the presence of lipids (Mehdi *et al*., 2015). Lipid sources in the diet of aquatic animals are significant for growth and production for sustainable aquaculture. High demand for lipid sources from fisheries resources has led to the overexploitation of certain wild fish and the increasing price of fish stock (Adissin *et al.*, 2019). Hence, to maintain aquaculture feed supply,

* To whom correspondence should be addressed.

a study on plant-based protein and oil that includes microalgae as an alternative source of protein and lipids from fish stock has been conducted (Adissin *et al*., 2019).

Bera Lake is the largest swam-lake system in Malaysia that consists of freshwater swamp forest, peat swamp forest, and secondary swamp forest (Fahmi-Ahmad *et al*., 2015). In 1994, Bera Lake has been gazetted as Ramsar Site as Bera Lake is home for more than 200 species of birds, 50 mammals and 94 species of fish (Chong *et al*., 2007). Since 2008, the Bera Lake ecosystem experience deforestation of wetland and river pollutions that reduced the lake diversity (Gharibreza *et al*., 2013). Meanwhile, Chini Lake is the second largest natural inland lake in Malaysia and has been reported to sustain high biodiversity of terrestrial and aquatics resources. The area consists of 138 species of terrestrial plant, 304 species of terrestrial vertebrate, and 84 species of fish (Abdullah *et al*., 2018). Due to the unique endemic habitat that can only be found in this area, Chini Lake has been gazetted as the UNESCO Biosphere Reserves status site in 2009 (Abdullah *et al*., 2018). The rich diversity in both lakes has led to more scientific research, and this includes isolation and identification of new microalgae species that inhabit both lakes. Since the utilization of indigenous freshwater microalgae for lipid sources in aquaculture feeds are still limited, the selection of potential species with high biomass and rich in lipid from Pahang Lakes could be used as alternative lipid sources for commercial uses.

Each microalgae species has a different composition of lipid content, biomass yield, and geographical distribution (Liu *et al*., 2011). Isolation and identification of potential microalgae strain with high lipid content from the natural habitat of Pahang Lakes crucial to discover the potential of local strains for the production of lipid. Therefore, this study aims to isolate indigenous microalgae species and to determine the highest biomass and cellular lipid production found in these indigenous microalgae.

MATERIALS AND METHODS

Sample collection and isolation of microalgae

The freshwater samples were collected from Bera Lake and Chini Lake in October 2017 (Table 1). Samples were collected 1 m from the surface using Van Dorn water sampler and stored in 1 L bottles. The freshwater sample was inoculated into Bold's Basal Media (BBM; Kanz & Bold *et al*., 1969), BBM+ NH_4Cl, WC (Guillard & Lorenzen *et al*., 1972), and WC + NH_4Cl for culture incubation. The samples were incubated at 25°C, light intensity of 550 µmol m^{-2} s^{-1} with 12:12 light: dark cycle. Then, microalgae were isolated via streaking plate and capillary pipetting method. To ensure the purity of the microalgae cultures, repeated streaking on an agar plate and routine microscopic observation were conducted.

Molecular identification of microalgae stains

Genomic DNA was extracted using GeneMTRIX Series Soil DNA Purification Kit (EURX, Poland). 18s rDNA molecular markers were used to identified microalgae species which were Euk-A (forward primer; 5'-AACCTGGTTGATCCTGCCAGT-3') and Euk-B (reverse primer; 5'TGATCCTTCTGC AGGTTCACCTAC-3'). A PCR mixture containing 2.5 µL of 10X Easy Taq Buffer; 1 µL of 10 µmol each primer; 0.2 µL of Taq DNA polymerase (Vivantis Technologies, Malaysia); 1 µL of 10 µM dNTP and 2 µL of 10 ng DNA sample were prepared into a 25 µL reaction. The cycling profile began with the initial step of 5 min at 95°C, followed by 30 cycles of denaturation (at 95°C for 45 sec), annealing (at 55°C for 30 sec) and extension (at

Table 1. Sampling location of indigenous microalgae from Pahang lakes

Location	Code	Coordinate	Condition
Bera Lake	PHG A	N 03°07.202' E 102°36.182'	Pristine area Surrounded by *Pandanus* sp.
	PHG B	N 03°07.324' E 102°36.222'	Pristine area Surrounded by *Pandanus* sp.
	PHG C	N 03°07.438' E 102°36.236'	Aquaculture site
	PHG D	N 03°07.544' E 102°36.201'	Jetty area
	PHG E	N 03°08.062' E 102°36.260'	Pristine area Lake Downstream
Chini Lake	PHG F	N 03°25.022' E 102°54.393'	Pristine area Surrounded by *Pandanus* sp.

72°C for 3 min) and finally subjected to the final extension (at 72°C for 10 min). The amplifications were done in a Thermal Cycler (Bio-Rad, Malaysia). PCR products were then sent for sequencing at First Base Laboratories Sdn. Bhd. The result of nucleotide sequences was identified using BLAST at the National Center of Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Determination of growth curve, growth rate, and doubling time

The concentration of 1 x 10^5 cells/ml isolated strains were cultured in the 48-well microplate for 15 days before placement into a photo-incubator set at 25 ± 1°C, a light intensity of 500 µmol photon/m^2/s and a 12:12 h dark: light photoperiods. The growth absorbance was determined every day according to an optical density at 750 nm wavelength ($OD_{680\ nm}$) using Thermo (Varioskan LUX) spectrophotometer (Kasan *et al*., 2020). The specific growth rates and doubling time of all strains were calculated according to Wood *et al*. (2005) and Mujizat *et al*. (2015), respectively.

Nile red fluorescence of microalgae

Lipid screening was done in lag and exponential phase (0 – 4 days), early stationary phase (8 days), and late stationary phase (15 days). The pure strains of 200 µL from the 48-well plate were transferred into a 96-well plate and 2 µL Nile red solution was added into each well. The wavelength of excitation and emission was measured at 480 nm and 575 nm for neutral lipid, and 488 nm and 620 nm for polar lipid using Thermo (Varioskan LUX) spectrophotometer, and the Nile red fluorescence were measured by normalized the reading of strains with the reading of strain without Nile red.

RESULTS AND DISCUSSION

Eleven microalgae strains were isolated from Pahang Lakes

Eleven microalgae strains were isolated from Pahang lakes (Figure 1; Table 2). Eight strains were identified from Bera Lake consist of *Carteria radiosa*, *Spongiosarcinopsis terrestris*, *Desmodesmus* sp., *Chlamydomonas reinhardtii*, *Desmodesmus brasiliensis*, *Mychonastes timauensis,* and *Desmodesmus abundans*. Two strains were identified from Chini Lake which is *Spongiosarcinopsis terrestris* and *Myhonastes ovahimbae*. Most of the species found are different between Bera Lake and Chini Lake except for *Spongiosarcinopsis terrestris* that was found in both lakes. Also, *Mychonastes* genus was found in both freshwater lakes with different species which are *Mychonastes timauensis* (Bera Lake) and *Mychonastes ovahimbae* (Chini Lake). All isolated microalgae belonged to Chlorophyceae or "green algae" group as described by Guiry (2020) on the AlgaeBase website. This algae is green in color due to the presence of chlorophyll b, which is also found in land plants.

Three microalgae strains showing high growth

The highest growth absorbance observed in PHG C02 (*Desmodesmus brariliensis*) with 0.43 d^{-1} specific growth rate and 1.60 day doubling time, PHG F03 (*Spongiosarcinopsis terrestris*; 0.32 d^{-1} specific growth rate; 2.11 day doubling time) and PHG C01 (*Chlamydomonas reinhardtii*; 0.28 d^{-1} specific growth rate; 2.44 day doubling time) (Figure 2, Table 3). The highest growth absorbance, PHG C02 was achieved stationary phase at day 13 with the highest bloom strains and suggested to be the best culture. Meanwhile, PHG F03 and PHG C01 achieved a stationary phase on day 12 where the growth absorbance was lower than PHG C02.

Strains that showed lower absorbance are PHG A01 (*Carteria radiosa*; 1.15 d^{-1} specific growth rate; 0.60 day doubling time), PHG D01 (*Carteria radiosa*; 1.14 d^{-1} specific growth rate; 0.60 day doubling time), PHG E01 (*Carteria radiosa;* 0.70 d^{-1} specific growth rate; 1.00 day doubling time), PHG E03 (*Desmodesmus abundans*; 0.27 d^{-1} specific growth rate; 2.56 day doubling time), where all of these strains achieved stationary phase at day 13. Meanwhile, PHG B02 (*Desmodesmus* sp.; 0.74 d^{-1} specific growth rate; 0.93 day doubling time) didn't grow well during the experiment where the absorbance reading remained constant along the cultivation day of 15 days (Fig. 2), this situation might be due to the unfavorable condition such as unsuitable media for this species. Previous studies showed that most freshwater microalgae can grow in BBM or WC media, however, some studies have used specific mineral Bristol's medium (Grabski *et al*., 2016) and BG-11 medium (Solovchenko *et al*., 2013) for culturing *Desmodesmus* sp.

Nile red fluorescence of microalgae

The fluorescent stain Nile Red has been used for the quantification of lipids in microalgae as this stain is economically saving and sensitive to use for screening purposes (Johnson *et al*., 2017). Microalgae strain with high intracellular lipid contents is important for downstream application such as animal feedstock production (Doan *et al*., 2011). The lipid composition of microalgae consists of neutral and polar lipids (Xuxiong *et al*., 2013). Polar lipid exists in the form of energy reserve, glycol- and phospholipids (Doan *et al*., 2011). Neutral lipids consist of triacylglycerols (TAGs) and wax esters where TAGs are the dominant structure of neutral lipids (Guschina & Harwood *et al*., 2009). Based on Figure 3 and Figure 4, the maximum yield

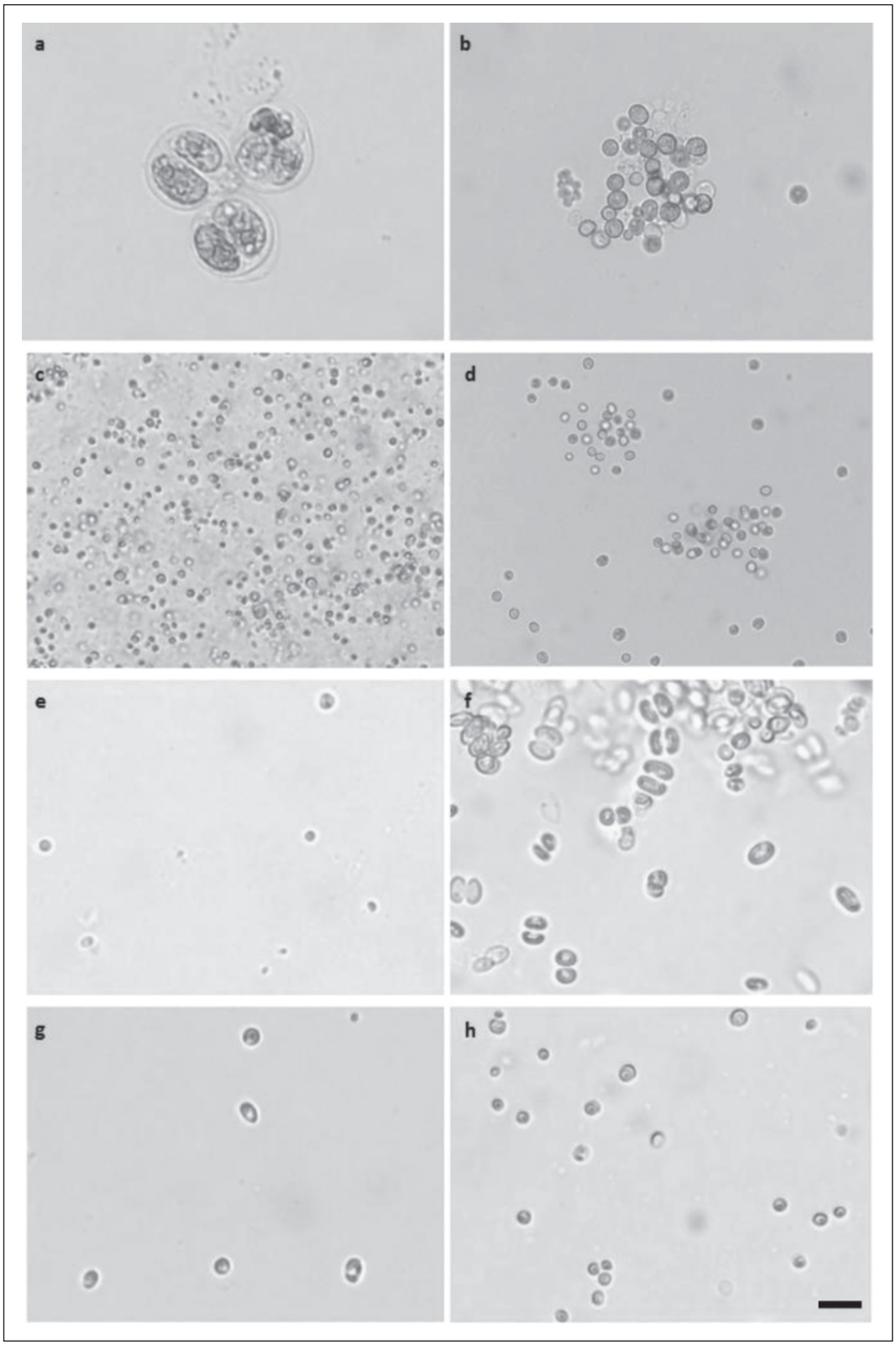

Fig. 1. Photos of the isolated microalgae species from Pahang lakes. (a) *Carteria radiosa*; (b) *Spongiosarcinopsis terrestris*; (c) *Mychonastes ovahimbae*; (d) *Chlamydomonas reinhardtii*; (e) *Mychonastes timauensis*; (f) *Desmodesmus brasiliensis*; (g) *Desmodesmus abundans*; (h) *Desmodesmus* sp. The bar represents 10 μM.

Table 2. Molecular identification using 18S rDNA gene

Sources of collection	Strain	Genera/ Species	Identity (%)	Accession number
Bera Lake	PHG A01	*Carteria radiosa*	86.48	D86500.1
	PHG B01	*Spongiosarcinopsis terrestris*	91.10	MF687231.1
	PHG B02	*Desmodesmus* sp.	98.67	KX550420.1
	PHG C01	*Chlamydomonas reinhardtii*	96.20	FR865586.1
	PHG C02	*Desmodesmus brasiliensis*	75.08	AB917106.1
	PHG D01	*Carteria radiosa*	80.64	D86500.1
	PHG E01	*Carteria radiosa*	79.38	D86500.1
	PHG E02	*Mychonastes timauensis*	99.18	GQ477055.1
	PHG E03	*Desmodesmus abundans*	94.52	MH307943.1
Chini Lake	PHG F03	*Spongiosarcinopsis terrestris*	91.03	MF687231.1
	PHG F04	*Mychonastes ovahimbae*	99.48	GQ477052.1

Table 3. The growth rate of indigenous microalgae strains isolated from Pahang lakes

Strain	Genera/ Species	Specific growth rate (d^{-1})	Doubling time (d)
PHG A01	*Carteria radiosa*	1.15	0.60
PHG B01	*Spongiosarcinopsis terrestris*	0.63	1.10
PHG B02	*Desmodesmus* sp.	0.74	0.93
PHG C01	*Chlamydomonas reinhardtii*	0.28	2.44
PHG C02	*Desmodesmus brasiliensis*	0.43	1.60
PHG D01	*Carteria radiosa*	1.14	0.60
PHG E01	*Carteria radiosa*	0.70	1.00
PHG E02	*Mychonastes timauensis*	0.44	1.56
PHG E03	*Desmodesmus abundans*	0.27	2.56
PHG F03	*Spongiosarcinopsis terrestris*	0.32	2.11
PHG F04	*Mychonastes ovahimbae*	0.79	0.88

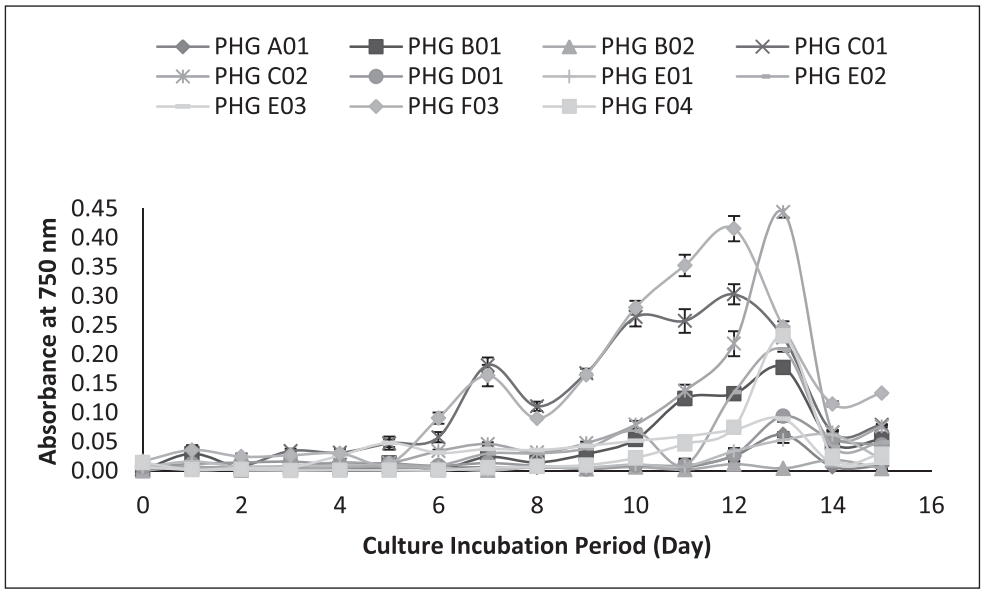

Fig. 2. Growth curve of indigenous microalgae strains isolated from Pahang lakes. Data were indicated as the mean ± standard deviation from three replicates.

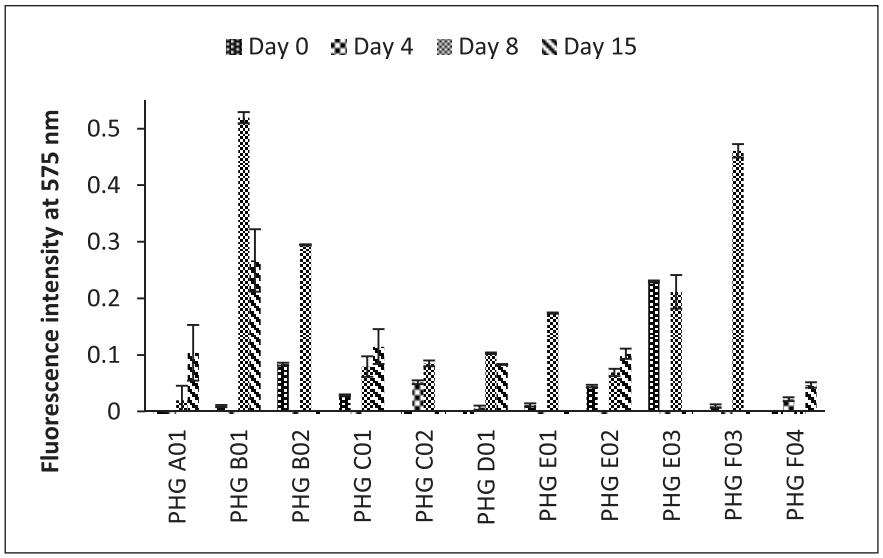

Fig. 3. Neutral lipids fluorescence intensity using Nile Red screening of microalgae strains isolated from Pahang lakes. Data were indicated as the mean ± standard deviation from three replicates.

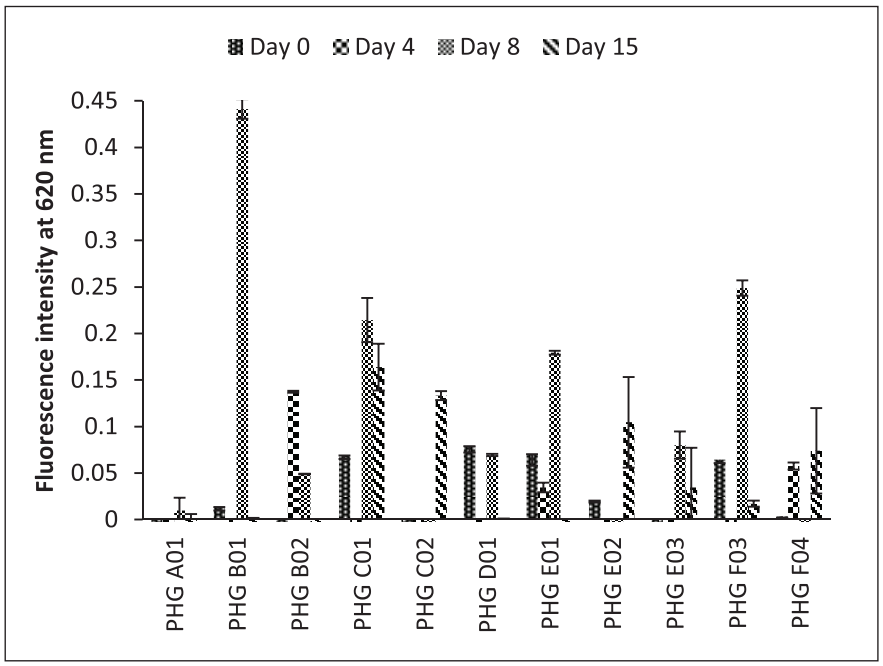

Fig. 4. Polar lipid fluorescence intensity using Nile Red screening of microalgae strains isolated from Pahang lakes. Data were indicated as the mean ± standard deviation from three replicates.

of cellular lipid is in the stationary phase (8 days) which is similar to the study by Doan *et al*. (2011). Among all strains, the production of neutral lipid was highest in PHG B01 (*Spongiosarcinopsis terrestris*), PHG F03 (*Spongiosarcinopsis terrestris*), and PHG B02 (*Desmodesmus* sp.). Meanwhile, the production of polar lipid was highest in PHG B01 (*Spongiosarcinopsis terrestris*), PHG F03 (*Spongiosarcinopsis terrestris*), and PHG C01 (*Chlamydomonas reinhardtii*). *Spongiosarcinopsis terrestris* from Bera Lake (PHG B01) and Chini Lake (PHG F03) show high neutral and polar lipid yield among all 11 strains tested.

CONCLUSIONS

Eleven indigenous microalgae were isolated and identified from Pahang lakes. The best strain concerning growth rate and lipid accumulation is PHG B01 (*Spongiosarcinopsis terrestris*) strain. Results from this study suggested that PHG B01 (*Spongiosarcinopsis terrestris*) strain has the potential to be used as a source of lipid for animal feedstock.

ACKNOWLEDGEMENTS

This research was supported by Japan Science and Technology Agency (JST)/Japan International Cooperation Agency (JICA), Science and Technology Research Partnership for Sustainable Development (SATREPS) through the project for Continuous Operation System for Microalgae Production Optimized for Sustainable Tropical Aquaculture (COSMOS) and the SATREPS-COSMOS Matching Fund from the Ministry of Higher Education Malaysia (MOHE). The authors would like to thank Jabatan Perhilitan, The National University of Malaysia, and the research assistant for technical assistance during the sampling.

REFERENCES

Abdullah, M., Mamat, M.P., Emang, D., Hussain, F.N.T. & Fui, L.H. 2018. Economic valuation of Tasik Chini Biosphere Reserve, Pahang, Malaysia. *ASM Science Journal,* **11**: 74-78.

Adissin, T.O.O., Manabu, I., Shunsuke, K., Saichiro, Y., Amina, S.M. & Dossou, S. 2019. Nutritional evaluation of *Nannochloropsis* powder and lipid as alternative to fish oil for kuruma shrimp, *Marsupenaeus japonicas*. *Aquaculture*, **504**: 427-436.

Chong, G. 2007. Tasek Bera: past, present and future, in: Colloquium on Lakes and Reservoir Management, Status and Issues. Putrajaya, Malaysia. 2-3August 2007.

Doan, T.T.Y., Sivaloganathan, B. & Jeffrey, P.O. 2011. Screening of marine microalgae for biodiesel feedstock. *Biomass & Energy*, **35(7)**: 2534-2544.

Fahmi-Ahmad, M., Rizal, S.A. & Amirrudin, B.A. 2015. Ichthyofaunal diversity of Tasek Bera Ramsar Site, Pahang, Peninsular Malaysia. *Journal of Wildlife and Parks*, **30**: 27-43.

Gharibreza, M., Raj, J.K., Yusoff, I., Ashraf, M.A., Othman, Z., Wan, M.T. & Wan, Z. 2013. Effects of agricultural projects on nutrient levels in Lake Bera (Tasek Bera), Peninsular Malaysia. *Agriculture, Ecosystem, and Environment*, **165**: 19-27.

Grabski, K., Baranowski, N., Skórko-Glonek, J. & Tukaj, Z. 2016. Chlorophyll catabolites in conditioned media of green microalga *Desmodesmus subspicatus*. *Journal of Applied Phycology*, **28**: 889-896.

Guillard, R.L. & Lorenzen, C.J. 1972. Yellow-green algae with chlorophyllide. *Journal of Phycology*, **8**: 10-14.

Guiry in Guiry, M.D. & Guiry, G.M. 2020. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway [WWW Document]. URL http://www.algaebase.org (accessed 4.16.20)

Guschina, I.A. & Harwood, J.L. 2009. Algal lipids and effect of the environmental on their biochemistry. In: *Lipids in Aquatic Ecosystem*. M.T. Arts, M.T. Brett and K.J Kainz. (Eds.). Springer, Dordrecht. pp. 1-24.

Jia-Yi, C., Zhou-Yan, K., Meng-Wei, Y., Yu-Fan, Z., Ji-Lin, X., Cheng-Xu, Z., Kai, L. & Xiao-Jun, Y. 2019. Metabolomic and transcriptomic analyses reveal the effects of ultraviolet radiation deprivation on *Isochrysis galbana* at high temperature. *Algal Research*, **38**: 101424.

Johnson, Z.I., Bidigare, R.R., Blinebry, S.K., Brown, S.L., Cullen, J.J., Loftus, S.E., Redalje, D.G., Swink, C. & Van, M.B.A.S. 2017. Screening for lipids from marine microalgae using Nile Red. In: Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Production of Fuels and Chemicals, Handbook of Hydrocarbons and Lipid Microbiology. S.Y. Lee (Ed.). Spinger International Publishing AG, USA. pp. 88-96.

Ju, Z.Y., Deng, D.F. & Dominy, W. 2012. A defatted microalgae (*Haematococcus pluvialis*) meal as a protein ingredient to partially replace fishmeal in diets of Pacific white srimp (*Litopenaeus vannamei*, Boone, 1931). *Aquaculture,* **354(355)**: 50-55.

Kanz, T. & Bold, H.C. 1969. Physiological studies, morphological and taxonomical investigation of *Nostoc* and *Anabaena* in culture (Ph.D). University of Texas.

Kasan, N.A., Hashim, F.H., Haris, N., Zakaria, M.F., Mohamed, N.N., Rasdi, N.W., Wahid, M.E.A., Katayama, T., Takahashi, K. & Jusoh, M. 2020. Isolation of freshwater and marine indigenous microalgae species from Terengganu water bodies for potential uses as live feeds in aquaculture industry. *International Aquatic Research*, **12**: 74-83.

Liu, J., Chen, F. & Huang, J. 2011. Microalgae as feedstock for biodiesel production. In: Biodiesel–Feedstocks and Processing Technologies, M. Stoytcheva, and G. Montero (Eds.). INTECH Open Access Publisher. pp. 133-160.

Mehdi, M., Najmeh, K. & Mehran, J.B. 2015. Fatty acid composition of the marine micro alga *Tetraselmis chuii* Butcher in response to culture conditions. *Journal of Algal Biomass Utilization*, **6(2)**: 49-55.

Mujizat, K., Junkwon, H., Dina, A. & Hary, A. 2015. Comparison of density, specific growth rate, biomass weight, and doubling time of microalgae *Nannochloropsis* sp. cultivated in open raceway pond and photobioreactor. *Aquaculture, Aquarium, Conservation & Legislation – International Journal of the Bioflux Society*, **8**: 5.

Sen, B., Alp, M.T. & Kocer, M.A.T. 2005. Studies on growth of marine microalgae in batch cultures: I. *Chlorella vulgaris* (Chlorophyta). *Asian Journal of Plant Sciences*, **4**: 636-638.

Solovchenko, A.E., Chivkunova, O.B., Semenova, L.R., Selyakh, I.O., Shcherbakov, P.N., Karpova, E.A. & Lobakova, E.S. 2013. Stress induced changes in pigment and fatty acid content in the microalga *Desmodesmus* sp. isolated from a White Sea Hydroid. *Russian Journal of Plant Physiology*, **60(3)**: 313-321.

Wood, A.M., Everroad, R.C. & Wingard, L.M. 2005. Measuring growth rate in microalgal culture. In: *Algal Culturing Techniques*. R.A. Anderson (Ed.). Academic Press, USA. pp. 269-85.

Xuxiong, H., Zhengheng, H., Wen, W. & Jiaqi, Y. 2013. Effects of nitrogen supplementation of the culture medium on the growth, total lipid content and fatty acid profiles of three microalgae (*Tetraselmis subcordiformis*, *Nannochloropsis oculata* and *Pavlova viridis*.). *Journal of Applied Phycology*, **25**: 129-137.