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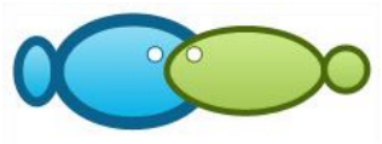

Outdoor cultivation of newly isolated tropical marine microalgae *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in raceway ponds for biodiesel feedstock production

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Abstract. The microalgae *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 are newly isolated marine microalgae from coastal areas in Kendari, Southeast Sulawesi, Indonesia. Indoor studies of the microalgae showed potential as biodiesel feedstock due to their high growth rates, high biomass and lipid productivity. However, for commercial uses, especially for biodiesel feedstock, the species should have the ability to perform well under real outdoor conditions. This study aimed to determine the growth, biomass, and lipid productivity of the *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in outdoor raceway ponds. The species were cultured using 1 m^2 raceway ponds at 30 cm depth. The two species were cultured using water-based sea media enriched with f/2 nutrients for *Skeletonema* sp. and Walne media for *Nannochloropsis* sp. Cultures were initially operated in batch mode until they reached the stationary phase before operating in semi-continuous mode. The culture duration was about three months. Cell counting was done every two days, while sampling for biomass (dry weight - DW, and ash-free dry weight - AFDW) and lipids were done every four days. The results showed that the two species of microalgae could grow well in outdoor raceway ponds. The specific growth rate of the *Nannochloropsis* sp. UHO3 ranged from 0.105 to 0.447 d^{-1} while the specific growth rate of *Skeletonema* sp. UHO29 ranged from 0.127 to 0.457 d^{-1}. *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 have high lipid content ranging from 15 to 44% AFDW and from 14 to 53% AFDW, respectively. The biomass productivity of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 range between 10-122 g m^{-2} d^{-1} and 13-103 g m^{-2} d^{-1} respectively, while the lipid productivity value for *Nannochloropsis* sp. UHO3 ranges from 4 to 31 g m^{-2} d^{-1} and for *Skeletonema* sp. UHO29 range from 5 to 25 g m^{-2} d^{-1}. The results of this study indicate that the two microalgae species are potentially developed as biodiesel feedstocks due to their fast growth rate, high lipid content, high biomass, and lipid productivity under outdoor conditions.
Key Words: biodiesel feedstock, marine microalgae, *Nannochloropsis* sp., raceway ponds, *Skeletonema* sp.

Introduction. Algae cultures growing in outdoors are exposed to varying environmental conditions during the day and with seasons (Borowitzka 2005). The ability of microalgae to tolerate wide variation of outdoor environmental conditions is a prerequisite characteristic of microalgae to be successfully cultured outdoors (Indrayani 2017; Indrayani et al 2019). Microalgae that are successfully produced commercially such as *Dunaliella salina*, *Spirulina* sp., *Haematococcus pluvialis*, and *Chlorella* sp. have the ability to tolerate variations in environmental conditions under outdoor conditions (Béchet et al 2013; Belay 2013; Borowitzka 2016). In addition, the variation of environmental conditions not only affects the growth of algae but also their biochemical composition including lipid. Lipids are of interest due to their various potential applications including

as biodiesel feedstock (Mata et al 2010; Ndimba et al 2013). Growth and lipid content are two important parameters used to assess the feasibility of microalgae as feedstock for biodiesel (Parmar et al 2011; Indrayani et al 2020a).

Studies on evaluation of microalgal species for biodiesel feedstocks are enormous but the feasibility studies for outdoor culture of microalgae potential as biodiesel feedstocks in an open system such as raceway ponds are limited specifically in Indonesia. In this study, two newly isolated tropical marine microalgal species *Nannochloropsis* sp. and *Skeleonema* sp. were assessed for their ability to grow in open raceway ponds. From the indoor studies, it is known that the two species of microalgae have the potential to be developed as biodiesel feedstocks due to rapid growth, a broad salinity tolerance (2-7% NaCl), high biomass and lipid productivities (Indrayani et al 2020b, 2021). However, it is unknown whether the microalgae can grow well under outdoor conditions where the environmental conditions are different from the controlled laboratory conditions. Therefore this study aims to determine the growth, biomass and lipid productivities of two newly isolated tropical marine microalgae *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 under outdoor conditions using 1 m^2 paddle-wheel driven raceway ponds.

Material and Method

Microalgae species. The microalgae species used in this study are *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 isolated from Kendari waters, Southeast Sulawesi, Indonesia, in June 2017 (Indrayani et al 2018). The strains were isolated using agar plating technique (Andersen & Kawachi 2005) in f/2 medium (Guillard & Ryther 1962). Briefly, 0.5 mL water samples are spotted in the middle of the agar f/2 medium (2%) and spread evenly on the agar surfaces using glass rod. Pure colonies were obtained after repeated streaking on the fresh agar f/2 medium.

Inoculum preparation. To obtain sufficient inoculum for mass culture in outdoor raceway ponds, both microalgae species were scaled up indoors. The scale-up culture was carried out in stages starting from a volume of 2 L to 50 L. Scale-up culture used f/2+Si medium for *Skeletonema* sp. and Walne media for *Nannochloropsis* sp. The cultures were incubated at ambient room temperature (26-32°C), 12 hours light and 12 hours dark cycles, light intensity ranging from 60 to 150 µmol photons m^{-2} s^{-1} and bubbled with air to facilitate mixing the cultures.

Outdoor culture condition. The outdoor cultivation was conducted at the Shrimp Seed Center, Marine and Fisheries Service, Purirano District, Kendari, Southeast Sulawesi, Indonesia using 1 m^2 tarpaulin paddle wheel-driven raceway ponds at 30 cm depth. The cultures were intermittently mixed using paddle wheel (mixed during the day and unmixed during night time). The cultures used filtered seawater based medium enriched with f/2+Si medium for *Skeletonema* sp. and Walne media for *Nannochloropsis* sp. at 3.2% salinity (w/v NaCl). The cultures were initially operated in batch mode until reached stationary phase before initiating semi-continuous regime by periodically harvesting a certain amount of the culture and replacing the harvested volume with the same amount of fresh medium. The outdoor cultures were conducted for 3 months (July-September 2019).

Cell counting was carried out every two days, whereas dry weight (DW), ash-free dry weight (AFDW), and lipid were measured before harvesting.

Analytical methods. The growth of the cultures was monitored by counting the numbers of microalgae cells every two days using a Neubauer haemocytometer (Moheimani et al 2013).

The specific growth rate (µ) was calculated using the following equation:

$$\mu = (Ln\left(\frac{N2}{N1}\right))/(t2 - t1)$$

where: *N1* and *N2* are the cell density at time 1 (*t1*) and 2 (*t2*) within the exponential phase.

For DW determination, five mL of culture was filtered through pre-weighed and pre-combusted Whatman GF/C, 25 mm filter paper using a Millipore filter apparatus. The filters were dried in an oven at 75°C for 5 hours and then weighted (Moheimani et al 2013). Determination of DW used the following formula:

$$Dry\ Weight\ (gram\ per\ liter) = weight\ of\ filter\ plus\ algae - weight\ of\ filter$$

For AFDW determination, the DW filters were transferred to a furnace at 450°C, ashed for 5 hours, and then weighted after cooling. The following equation is used for AFDW calculation:

$$Ash\text{-}Free\ Dry\ Weight\ (gram\ per\ liter) = DW - weight\ of\ ash$$

The biomass productivity was calculated through the equation below:

$$Biomass\ productivity\ (gram\ per\ liter\ per\ day) = \mu \times Yield\ (gramAFDWperLiter)$$

Total lipid determination was conducted by the method of Bligh & Dyer (1959) as modified by Kates & Volcani (1966). Briefly, 5 mL of culture was filtered through Whatman GF/C, 25 mm filters. The filters containing cells were crushed with a glass rod until a smooth green paste of about 0.5 mL was obtained. One mL of solvent mixture (methanol: chloroform: DI water in the ratio of 2:1:0.8 v/v/v) was added and mixed well with the glass rod and then transferred into a plastic centrifuge tube with screw cap. Another 1 mL of the solvent mixture was added into the glass tube to wash and clean all the remaining cells debris then transferred in the centrifuge tube. An extra 3.7 mL of the solvent mixture was added. The sample was centrifuged at 1107xg for 10 minutes. After centrifugation, the supernatant was transferred to a 20 mL glass tube with screw cap. For the second extraction, 5.7 mL of the solvent mixture were added to pellet in the centrifuge tube, vortexed to re-suspend the pellet and then centrifuged again at 1107xg for 10 minutes. The supernatants were combined in the 20 mL glass tube. Three mL of DI water and 3 mL of chloroform were added to the 20 mL tubes and mixed well by vortexing. The samples were then stored in the fridge undisturbed for 24 h for complete phase separation. After phase separation the upper layer was removed and then evaporated under a stream of pure N2 gas on heating plates at 38°C until complete dryness. After complete evaporation, the vials containing lipids were carefully weighed using analytical balance. Weight of lipids was calculated by subtracting the weight of vials containing lipids with the weight of the vials.

Lipid productivity was calculated using the following equation:

$$Lipid\ Productivity\ (gram\ per\ liter\ per\ day) = \mu \times lipid\ yield$$

Results. The growth curves of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 during the culture period under semi-continuous regimes in outdoor raceway ponds can be seen in Figures 1. Both species can grow well under outdoor conditions. Initially, the cultures were run in batch mode until they reached early stationary phase before initiating semi-continuous regimes. The initial cell density of *Nannochloropsis* sp. UHO3 culture was about 150 x 10^4 cells mL^{-1} and the culture achieved the highest cell density at about 5367 x 10^4 cells mL^{-1} on day 14^{th}. The *Nannochloropsis* sp. culture experienced the lag phase in the first two days before entering the exponential phase until day 8^{th} day. On day 14^{th}, the culture began to run semi-continuously by harvesting part of the culture (30-50%) and adding fresh culture media as much as the volume of harvested culture. The cultures were then allowed to grow until they reached maximum cell density (about four days) for further harvesting and so on during the culture period. For the *Skeletonema* sp. UHO29 culture, the initial cell density was about 50 x 10^4 cell mL^{-1} reaching the maximum cell density of about 523 x 104 cells mL^{-1} on day 14 (stationary phase). After day 14^{th}, the cultures were operated under semi-continuous regime by partial harvesting at interval 4 days.

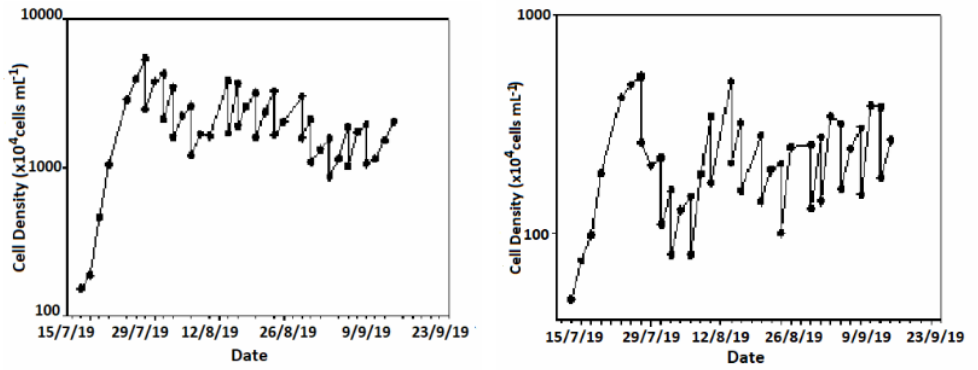

Figure 1. Growth curve of *Nannochloropsis* sp. UHO3 (left) and *Skeletonema* sp. UHO29 (right) under semi-continuous regime in outdoor raceway ponds.

The specific growth rate of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 mass culture in the raceway pool outside can be seen in Figure 2. The specific growth rate of *Nannochloropsis* sp. UHO3 ranged from 0.105 to 0.447 d^{-1} (0.202±0.133 d^{-1}) while the specific growth rate of *Skeletonema* sp. UHO29 ranged from 0.127 to 0.457 d^{-1} (0.317± 0.129 d^{-1}).

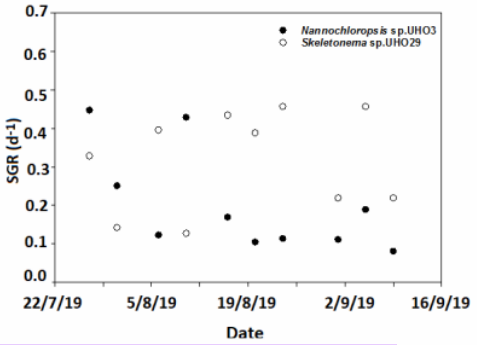

Figure 2. Specific growth rate (d^{-1}) of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in outdoor raceway ponds.

Biomass yield of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 mass culture in outdoor raceway ponds for 3 months can be seen in Figure 3. The average biomass yield of *Nannochloropsis* sp. UHO3 was about 0.614±0.146 g L^{-1} and the biomass yield of *Skeletonema* sp. UHO29 was about 0.515±0.219 g L^{-1}.

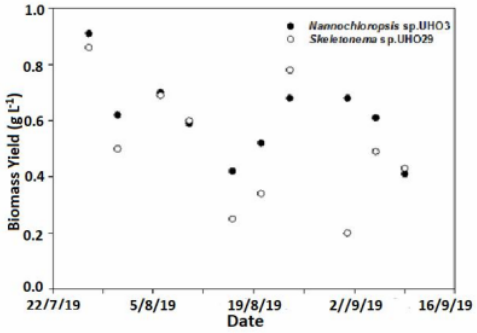

Figure 3. Biomass yield (g L^{-1}) of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in outdoor raceway ponds.

Biomass productivity of the two microalgae species mass-cultured in outdoor raceway ponds can be seen in Figure 4. The average biomass productivity of *Nannochloropsis* sp. UHO3 per volume was 0.133±0.115 g L^{-1} d^{-1} and per area was 39.89±34.46 g m^{-2} d^{-1}. While *Skeletonema* sp. UHO29 has an average biomass productivity per volume of about 0.166±0.109 g L^{-1} d^{-1} and per area of about 49.85±32.57 g m^{-2} d^{-1}.

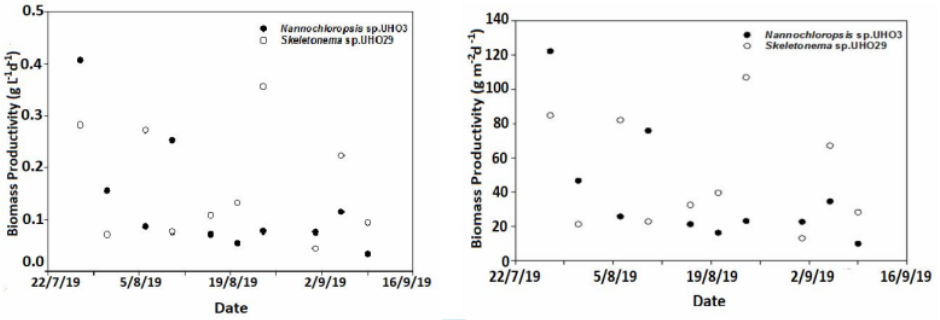

Figure 4. Biomass productivity of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in outdoor raceway ponds in g L^{-1} d^{-1} (left) and g m^{-2} d^{-1} (right).

The lipid yield of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 during the culture period in outdoor raceway ponds can be seen in Figure 5. The *Nannochloropsis* sp. UHO3 culture has a lipid yield of 0.18±0.063 g L^{-1} whereas the *Skeletonema* sp. UHO29 has a lipid yield of about 0.138±0.043 g L^{-1}. The average lipid content of *Nannochloropsis* was 30.47±11.06% biomass while *Skeletonema* has a lipid content of 30.05±11.64% biomass weight.

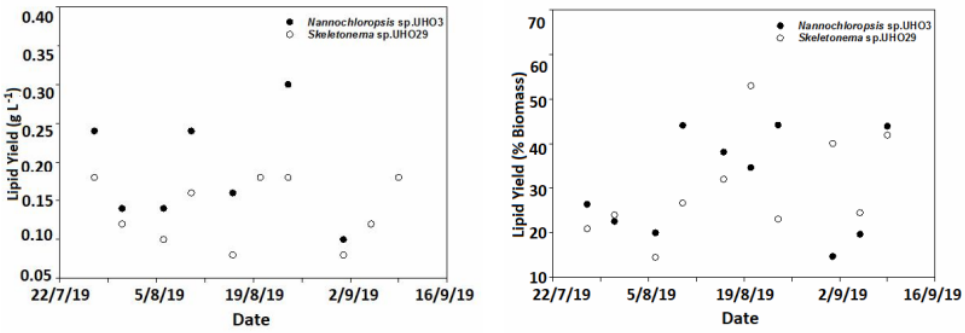

Figure 5. Lipid yield (g L^{-1}) (left) and lipid content (% biomass) (right) of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in outdoor raceway ponds.

The lipid productivity of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in outdoor raceway ponds during culture period can be seen in Figure 6. The average lipid productivity of *Nannochloropsis* sp. UHO3 per volume and per area was 0.039±0.036 g L^{-1} d^{-1} and 11.733±10.699 g m^{-2} d^{-1}, respectively. While the *Skeletonema* sp. UHO29 has an average lipid productivity per volume about 0.044±0.023 g L^{-1} d^{-1} and per area 13.043±6.767 g m^{-2} d^{-1}.

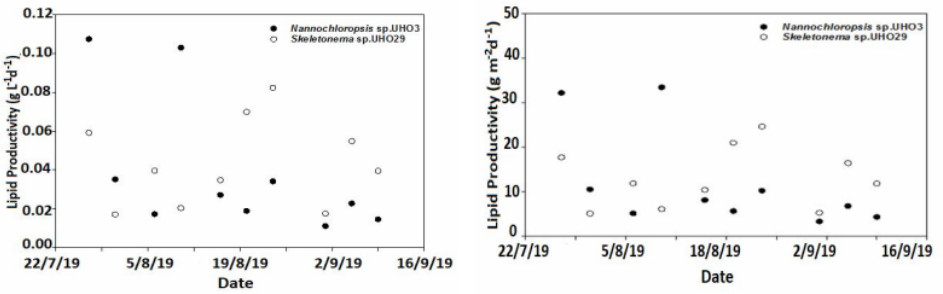

Figure 6. Lipid productivity of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 in outdoor raceway ponds in g L^{-1} d^{-1} (left) and in g m^{-2} d^{-1} (right).

Discussion. There are two systems of mass-scale microalgae culture, namely an open system and a closed system (Borowitzka & Moheimani 2013; Zittelli et al 2013). This study used an open pond system namely paddle-wheel driven raceway ponds as this is a low cost and ease of operation culture system widely applied by microalgal industries (Borowitzka & Moheimani 2013). This system is used for the production of *Spirulina* / *Arthrospira* by Earthrise Nutritionals, LLC (California, USA) and Hainan DIC Microalgae (China) and to produce astaxanthin from *Haematococcus pluvialis* by Cyanotech Co. (Hawaii, USA) and Parry Agro Industries Ltd. (India) (Zittelli et al 2013). Unlike other commonly used raceway ponds which are made of concrete or fiberglass, the raceway ponds used in this study used white tarpaulin with metal frame due to low cost and ease of mobilization/assembly.

The success of commercial production of microalgae in outdoor system is highly dependent on many factors, including microalgae's ability to tolerate environmental changes encountered in outdoor such as changes in temperature, light intensity, and salinity (Borowitzka 2005; Indrayani 2017; Indrayani et al 2019). From this study, it is known that the newly isolated marine tropical microalgae *Nannochlropsis* sp. UHO3 and *Skeletonema* sp. UHO29 can grow well in outdoor raceway ponds during the culture period. This shows that both microalgae can tolerate fluctuations in environmental parameters in outdoor, including temperature, salinity, and light intensity. The specific growth rate of *Nannochloropsis* sp. UHO3 ranged from 0.105 to 0.447 d^{-1} while the specific growth rate of *Skeletonema* sp. UHO29 ranged from 0.127 to 0.457 d^{-1}. The specific growth rates obtained in this study were slightly lower than the specific growth rates obtained under indoor/laboratory conditions (Indrayani et al 2020a, 2021). The higher specific growth rate obtained under laboratory conditions was due to the stable and optimum growing condition maintained in the laboratory compared to the fluctuation of the environmental condition occurred under real outdoor conditions. However, the specific growth rates of the microalgae in this study are comparable with other studies (Moheimani & Borowitzka 2006; Vadiveloo & Moheimani 2018; Indrayani et al 2019, 2020b).

The *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 cultured in outdoor raceway ponds had high lipid content, ranging from 15 to 44% AFDW and from 14 to 53% AFDW, respectively. Compared to the value of the lipid content when cultured in the laboratory, the lipid content in the outdoor raceway pond is higher (Indrayani et al 2020b, 2021). The content of microalgae lipids in this study was higher than the research conducted by Crowe et al (2012), which obtained lipid content of *Nannochloropsis salina* cultured in raceway ponds Tucson, USA rangeing from 15 to 25% AFDW. Research conducted by Benavides et al (2013) reported that the lipid content of diatom *Phaeodactylum tricornutum* cultured in open ponds ranged from 25 to 27.5% dry weight.

Research on microalgae mass culture in outdoor raceway ponds specifically in Indonesia is limited. In this study, the productivity values of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 were obtained, ranging from 10 to 122 g m^{-2} d^{-1} (average 39.87±34.46 g m^{-2} d^{-1}) and from 13 to 107 g m^{-2} d^{-1} (average 49.85±32.57 g m^{-2} d^{-1}) respectively. The biomass productivity value of these two microalgae is higher than other

species of microalgae cultured in outdoor raceway ponds. For example, *Nannochloropsis salina* cultured in raceway ponds in Israel at 12 cm depth had biomass productivity of 24.5 g m^{-2} d^{-1} (Boussiba et al 1987). *Nannochloropsis gaditana* cultured in raceway ponds in Spain had biomass productivity of 22.4 g m^{-2} d^{-1} (San Pedro et al 2015). *Cyclotella* sp. had biomass productivity of 12 g m^{-2} d^{-1} (Huaseman et al 2009), *Scenedesmuss obliquus* with average annual productivity of 15 g m^{-2} d^{-1} (Payer et al 1978), *Pleurochrysis carterae* with the productivity of 33.68 g m^{-2} d^{-1} (Moheimani & Borowitzka 2006), *Phaeodactylum tricornutum* with the productivity of 11.7 g m^{-2} d^{-1} (Benavides et al 2013), *Tetraselmis* with 5-40 g m^{-2} d^{-1} (Matsumoto et al 1995), *Amphora* sp. MUR258 with the highest biomass productivity of 24 g m^{-2} d^{-1} (Indrayani 2017; Indrayani et al 2019). The higher biomass productivity obtained in this study can be due to several factors. First, the two microalgae species used in this study have fast growth and high biomass yields under outdoor conditions. Second, the depth of the cultures in the raceway ponds was also different. In this study, cultures were operated at 30 cm depth, while other studies operated at a 15-25 cm depth. So that the value of biomass productivity per area will be higher at higher depths even though the growth and yield are the same or slightly lower.

In this study, the lipid productivity of *Nannochloropsis* sp. UHO3 ranged from 4 to 31 g m^{-2} d^{-1} (average 11.73±10.69 g m^{-2} d^{-1}) while *Skeletonema* sp. UHO29 had lipid productivity ranging from 5 to 25 g m^{-2} d^{-1} (average 13.043±6.767 g m^{-2} d^{-1}). Compared to the previous studies, the two microalgae species used in this study had higher lipid productivity. A study conducted by Indrayani et al (2019) obtained the maximum value of lipid productivity from the diatom *Amphora* sp. MUR258 cultured in an outdoor raceway pond in Perth of 6.8 g m^{-2} d^{-1}. *Graesiella* sp. WBG-1 cultured in raceway ponds had lipid productivity ranging from 2 to 2.9 g m^{-2} d^{-1} (Wen et al 2016). High lipid productivity value is closely related to high specific growth rates and high lipid yield of the species.

Conclusions. The *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 could grow well in outdoor raceway ponds. The specific growth rate of the *Nannochloropsis* sp. UHO3 ranged from 0.105 to 0.447 d^{-1} whereas the specific growth rate of *Skeletonema* sp. UHO29 ranged from 0.127 to 0.457 d^{-1}. The lipid content of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 ranged from 15 to 44% AFDW and 14 to 53% AFDW, respectively. The biomass productivity of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 ranged between 10-122 g m^{-2} d^{-1} and 13-107 g m^{-2} d^{-1} respectively. The lipid productivity of *Nannochloropsis* sp. UHO3 and *Skeletonema* sp. UHO29 ranged from 4 to 31 g m^{-2} d^{-1} and 5 to 25 g m^{-2} d^{-1} respectively. This study indicate that the two microalgae species are promising species as biodiesel feedstocks due to their high growth rate, high lipid content, high biomass and lipid productivities when grown in outdoor raceway ponds.

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Conflict of interest. The authors declare that there is no conflict of interest.

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