Growth and lipid production of a newly isolated microalga Nannochloropsis sp. UHO3 at increasing salinity

by Cek Turnitin

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Abstract. Salinity affects growth and biochemical composition of microalgae and the ability of microalgae to tolerate wide range of salinity is one of the important criteria for successful mass cultivation in outdoor open pond systems for any commercial application. The aim of this study was to determine the growth and lipid production of the newly isolated marine microalga *Nannochloropsis* sp. UHO3 at increasing salinity. The strain was isolated from a coastal area in Kendari, Southeast Sulawesi, Indonesia in June 2017. The strain was cultured in 500 mL Schott bottles containing 300 mL f/2 medium at increasing salinities from 2 to 7% NaCl, light intensity of about 100 µmol photon m^{-2} s^{-1}, 12:12 hours light and dark cycles at ambient room temperatures. The highest specific growth rate (0.779±0.02 d^{-1}) was achieved at 3% salinity and the lowest (0.455±0.02d^{-1}) was obtained at 7% salinity. The cultures grown at 3% salinity had the highest lipid content and lipid productivity (22.06±2.92% Ash-Free Dry Weight (AFDW) and 0.161±0.009g L^{-1} d^{-1}, respectively). This study suggests that the alga has a wide salinity tolerance (2-7% NaCl) and produce high lipid at 3-4% salinity. Hence, the species is potential for outdoor mass cultivation in saline-hypersaline media for biodiesel feedstock due to its high growth rate and lipid productivity.

Key Words: biodiesel feedstock, saline-hypersaline media, *Nannochloropsis* sp.

Introduction. Microalgae are photosynthetic microorganisms including the prokaryotic cyanobacteria and eukaryotic microalgae that use light energy and carbon dioxide for biomass production (Benneman 1997; Richmond 2004; Mata et al 2010). They are an extremely heterogeneous group of microorganisms which have a wide range of potential applications including for feed, food, cosmetics, pharmaceutical and biofuels (Olaizola 2003; Spolaore at al 2006; Borowitzka 2013a).

Microalgae have been suggested as biodiesel feedstock due to their ability to produce lipids that can be converted to biodiesel (Chisti 2007; Mata et al 2010; Parmar et al 2011). Yields of microalgal lipids are higher than terrestrial crops. Depending on the lipid content, microalgae can produce about 58,700-136,900 L oil ha^{-1} $year^{-1}$ compared to that of soybean (636 L oil ha^{-1} $year^{-1}$), jatropha (741 L oil ha^{-1} $year^{-1}$), canola (974 L oil ha^{-1} $year^{-1}$) and palm oil (5366 L oil ha^{-1} $year^{-1}$) (Ahmad et al 2011). Furthermore, microalgae are more sustainable to grow for lipid production due to their ability to grow on non-arable land and to utilise sea water so that they will not compete with food crops

for habitats and for limited source of fresh water (Borowitzka & Moheimani 2013). They also can use carbondioxide from industrial plants for biodiesel production (Sawayama et al 1995; Yun et al 1997; Chisti 2007). Given the higher growth rate and short generation time as well as high lipid content (up to 80% of dry weight) and lipid productivity, it is clear that microalgae are potential for large scale oils production (Converti et al 2009).

The lipid content in microalgae is species specific and it is influenced by environmental factors. Salinity is one of the important factors influencing the growth and biochemical composition of marine microalgae (Al-Hasan et al 1987, 1990; Aizdaicher et al 2010; Takagi et al 2006; Ranga Rao et al 2007; Zhila et al 2011; Fon Sing & Borowitzka 2016; Indrayani et al 2018). Salinity fluctuation is inevitable under outdoor conditions and when the microalgae species are cultured in outdoor open ponds, evaporation of the cultures in hot sunny days increases salt concentration in the medium. To make up for evaporation losses, freshwater is added to the culture to maintain constant salinity. Alternatively, saline water is used leading to increase in salinity over time (Borowitzka 2013b). Therefore, if the latter option is used, microalgae species with high salinity tolerance is preferred to obtain reliable cultures for long period. In addition, microalgae species capable to grow well at high salinity will potentially less prone to contamination in large-scale culture for long period, emphasizing the importance of evaluating high salinity tolerance of marine microalgae species intending to be mass cultured in outdoors.

Nannochloropsis sp. is one of the most studied microalgal species for lipid and biodiesel production (Chisti 2007; Mata et al 2010; Cheng-Wu et al 2001; Campos et al 2014; Bartley et al 2015). In addition members of the *Nannochloropsis* genus exhibit high growth rate, lipid productivity and tolerance to a wide range of environmental conditions (Richmond & Cheng-Wu 2001; Gu et al 2012a, 2012b; Fakhri et al 2015; Bartley et al 2015). The present study investigates the effect of increasing salinity on the growth and lipid production of the newly isolated microalgal strain *Nannochloropsis* sp. UHO3. This study could provide information about the salinity tolerance and the optimum salinity for growth and lipid production of the microalga *Nannochloropsis* sp. UHO3.

Material and Method

Source of algal strain. This study was conducted from March to June 2019. The algal strain used in this study is *Nannochloropsis* sp. UHO3 isolated from Kendari Waters, Southeast Sulawesi, Indonesia in June 2017. The strain was isolated using agar plating technique (Andersen & Kawachi 2005) in f/2 medium (Guillard & Ryther 1962). The strain is non-axenic and maintained in Microalgae Culture Collection at Faculty of Fisheries and Marine Science, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia.

Culture condition. The *Nannochloropsis* cultures were grown in 500 mL conical flasks containing 300 mL of f/2 medium at increasing salinity (2, 3, 4, 5, 6 and 7% NaCl (w/v). The cultures were initially grown at 2% salinity at initial cell density of about 150×10^4 cells mL^{-1} for 10 days in a batch mode and gradually increased the salinity to 7% salinity with 1% increment after 10 days of culturing. The cultures were incubated at room temperature (26-30°C), light:dark cycle 12 h:12 h, light intensity of about 100 µmol photons m^{-2} s^{-1} (in triplicates). The cultures were bubbled with air to facilitate mixing the cultures. Cell counting was carried out every two days, whereas dry weight (DW), ash free dry weight (AFDW) and lipid were measured prior to dilution.

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 (SGR) was calculated using the following equation:

$$SGR = (Ln(N2/N1)/(t2-t1)$$

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 removed from the Millipore filter apparatus, folded and patted dry with a paper towel. The filters were dried in an oven at 75°C for 5 hours and then weighted (Moheimani et al 2013). DW was determined by the following equation:

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

The filters were then transferred to a furnace at 450°C and ashed for 5 hours and weighted after cool. Organic dry weight (AFDW) was calculated by the following equation:

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

Biomass productivity was calculated by the equation:

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

Total lipid determination was conducted by the method of Bligh & Dyer (1959) as modified by Kates & Volcani (1966). Lipid productivity was calculated using the following equation:

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

Statistical analysis. Significant differences between treatments were analysed with a one-way analysis of variance (ANOVA). Pairwise multiple comparison procedure (Holm-Sidak Method) was used to precisely test differences between conditions. All statistical analysis was performed using Sigma-Plot 14 Systat Software Inc., USA.

Results

Growth of the Nannochloropsis sp. UHO3. The growth of the *Nannochloropsis* for about 60 days of culturing at increasing salinity from 2 to 7% NaCl is shown in Figure 1. The alga showed good growth over the wide range of salinity tested. The alga was initially grown at 2% salinity at initial cell density of about 150×10^4 cells mL^{-1} and cultured until reaching the stationary phase (10 days) at the cell density of about about $2478 \pm 118 \times 10^4$ cells mL^{-1}. The salinity of the cultures was increased by 1% NaCl at 10 days interval until the salinity of the cultures reached 7% NaCl. The cell density of all cultures increased up to two folds in the first two days of culturing indicating that the cells could adapt well with increasing salinity. From day 2 to day 4, all the cultures showed exponential growth before entering early stationary phase. The highest maximum cell density of about $3565 \pm 201 \times 10^4$ cells mL^{-1} was achieved at 4% salinity and the lowest maximum cell density ($2356 \pm 187 \times 10^4$ cells mL^{-1}) was obtained at the highest salinity (7% NaCl).

The specific growth rate (SGR) of the alga was significantly affected by the salinity tested (One Way ANOVA, $p < 0.001$). The SGR of the *Nannochloropsis* decreased at increasing salinity in which the highest SGR (0.779 ± 0.02 d^{-1}) was achieved when rising the salinity from 2 to 3% salinity and the lowest (0.455 ± 0.02 d^{-1}) obtained when rising the salinity from 6 to 7% salinity (Figure 2). There was a significant difference in the SGR between 2 and 7%, 3 and 7%, 4 and 7%, 5 and 7%, 2 and 6%, 3 and 6%, 4 and 6%, 3 and 5%, 4 and 5%, 3 and 2%, 4 and 2% (Holm-Sidak Method, $p < 0.05$) but no significant difference in the SGR between 2 and 5%, 3 and 4%, 5 and 6%, 6 and 7% was observed (Holm-Sidak Method, $p > 0.05$).

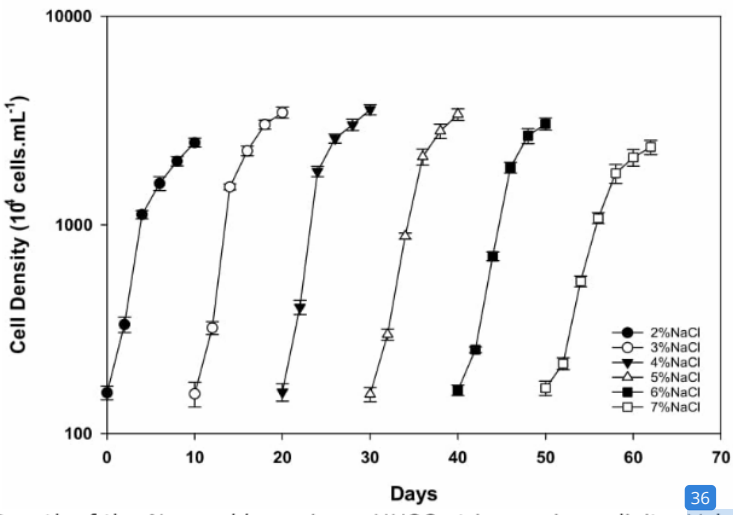

Figure 1. Growth of the *Nannochloropsis* sp. UHO3 at increasing salinity. Values represent mean±standard deviation (n = 3).

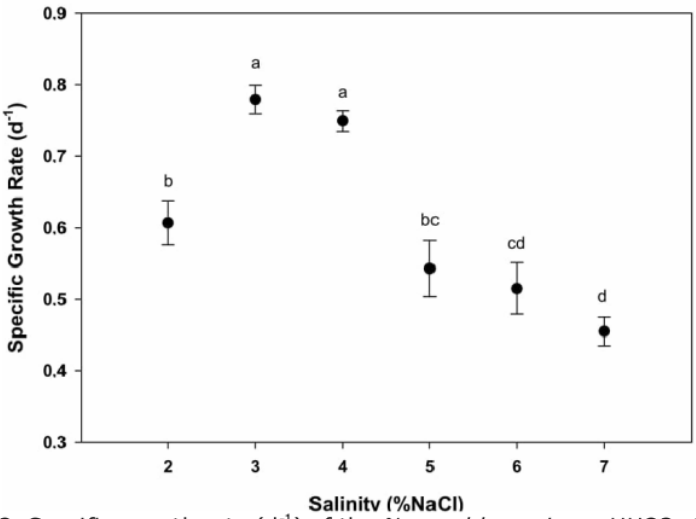

Figure 2. Specific growth rate (d^{-1}) of the *Nannochloropsis* sp. UHO3 at increasing salinity. Values represent mean±standard deviation (n = 3).

Biomass yield and productivity of the Nannochloropsis sp. UHO3. Biomass yield of the *Nannochloropsis* sp. was not affected by the salinity tested (One Way ANOVA, $p = 0.101$). However, there was a significant difference in the biomass productivity between salinity (One Way ANOVA, $F_{(5,12)} = 19.5$, $p < 0.001$). The highest biomass productivity was achieved at 4% salinity (0.814±0.036 g L^{-1} d^{-1}) and the lowest at 7% salinity (0.418±0.01g $L^{-1}d^{-1}$). Significant difference in the biomass productivity was observed between salinity 4 and 7%, 4 and 6%, 3 and 7%, 4 and 5%, 4 and 2%, 3 and 6%, 3 and 5%, 3 and 2%, 2 and 7%, 5 and 7% (Holm-Sidak Method, $p < 0.05$) but no significant difference in the biomass productivity was observed between salinity 4 and 3%, 6 and 7%, 2 and 6%, 5 and 6%, 2 and 5% (Holm-Sidak, $p > 0.05$) (Figure 3).

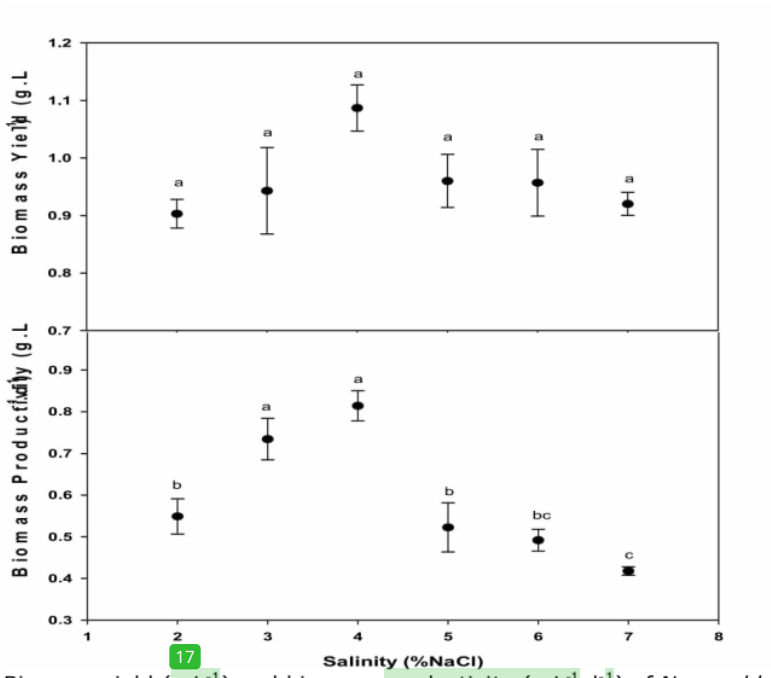

Figure 3. Biomass yield (g L^{-1}) and biomass productivity (g L^{-1} d^{-1}) of *Nannochloropsis* sp. UHO3 at increasing salinity. Values represent mean±standard deviation (n = 3).

Lipid of Nannochloropsis sp. UHO3. The lipid yield of the alga (g L^{-1}) was significantly affected by the salinity tested (One Way ANOVA, $p = 0.001$). The highest lipid yield was 0.207±0.012 g L^{-1} achieved at 3% salinity and the lowest was 0.127±0.012 g L^{-1} obtained at 7% salinity (Figure 4). The lipid content of the alga was significantly different between salinity 3 and 7%, 3 and 6%, 4 and 7%, 3 and 2% (Holm-Sidak Method, $p < 0.05$) but no significant difference was observed between salinity 5 and 7%, 4 and 6%, 4 and 2%, 3 and 5%, 5 and 6%, 2 and 7%, 5 and 2%, 3 and 4%, 4 and 5%, 6 and 7%, 2 and 6% (Holm-Sidak Method, $p > 0.05$).

The lipid content of the alga (%AFDW) was significantly affected by the salinity tested (One Way ANOVA, $p = 0.001$). The alga achieved its highest lipid content when grown at 3% salinity (22.06±2.92% AFDW) and the lowest lipid content achieved when the alga was grown at 7% salinity (13.79±1.54% AFDW). There was a significant difference in the lipid content between 3 and 7%, 3 and 6% (Holm-Sidak Method, $p < 0.05$). However, no significant difference was observed between salinity 3 and 2%, 3 and 4%, 5 and 7%, 3 and 5%, 5 and 6%, 4 and 7%, 2 and 7%, 4 and 6%, 2 and 6%, 5 and 2%, 5 and 4%, 6 and 7%, 4 and 2% (Holm-Sidak Method, $p > 0.05$) (Figure 4).

The lipid content per cell (ng) of the alga was not affected by the salinity tested One Way ANOVA, $p = 0.170$). The lipid per cell decreased as salinity increase ranging from 3.94 to 7.26 ng $cell^{-1}$ (Figure 4).

The lipid productivity of the alga was significantly affected by the salinity (One Way ANOVA, $p < 0.001$). The highest lipid productivity obtained at 3% salinity (0.161±0.009 g L^{-1} d^{-1}) and the lowest lipid productivity obtained at 7% NaCl (0.058±0.008 g L^{-1} d^{-1}). The lipid productivity was significantly different between salinity 3 and 7%, 3 and 6%, 4 and 7%, 4 and 6%, 3 and 2%, 3 and 5%, 4 and 2%, 4 and 5%, 5 and 7%, 2 and 7% (Holm-Sidak Method, $p < 0.05$) but no significant difference was observed between 5 and 6%, 2 and 6%, 3 and 4%, 6 and 7%, 5 and 2% (Holm-Sidak Method, $p > 0.05$) (Figure 4).

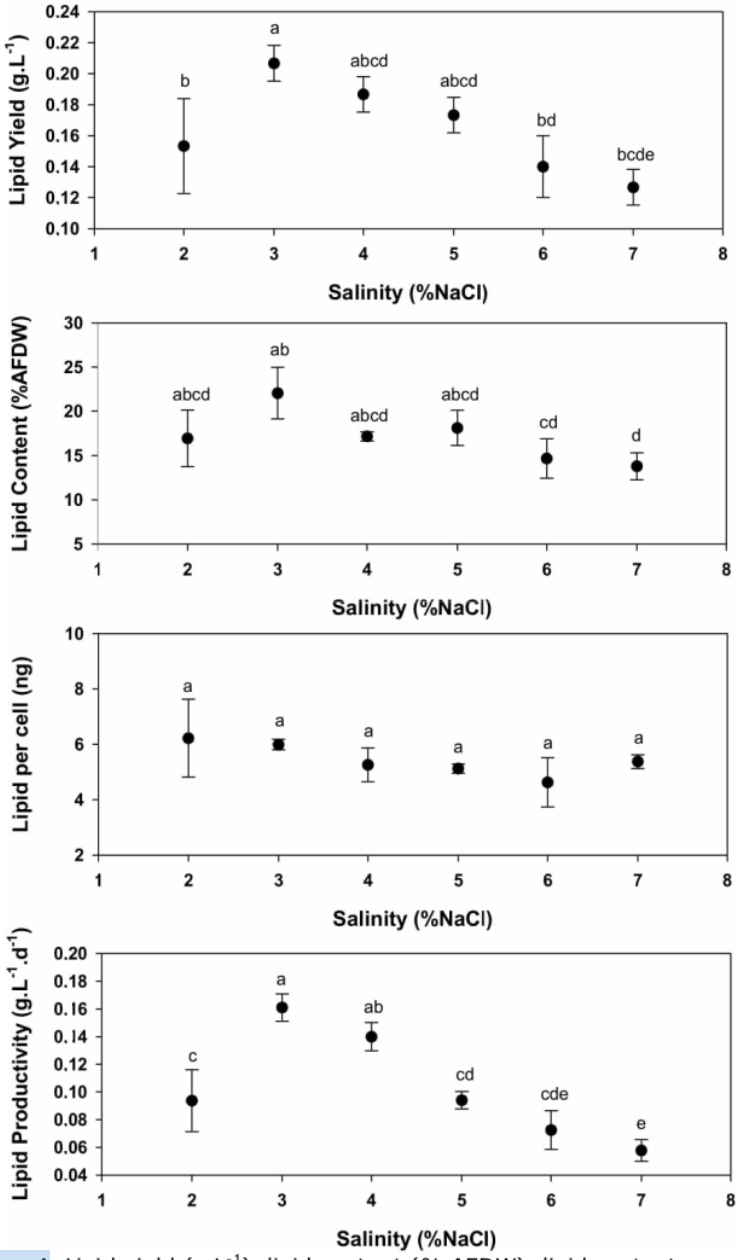

Figure 4. Lipid yield (g L^{-1}), lipid content (% AFDW), lipid content per cell (ng) and lipid productivity (g L^{-1} d^{-1}) of *Nannochloropsis* sp. UHO3 at increasing salinity. Values represent mean±standard deviation (n = 3).

Discussion. The ability of a marine microalgal species to tolerate wide range of high salinity is one of the important criteria for successful microalgal cultivation in outdoor open pond system. Therefore, it is important to determine the salinity tolerance of any microalgal species intending to be mass produced in outdoor open pond system for any

commercial application. In this study, we tested to grow the newly isolated marine *Nannochloropsis* sp. UHO3 at a wide range of salinity and found that the alga can grow very well over a wide range of high salinity tested from 2 to 7% NaCl which is up to more than two fold of the seawater salinity. This is possibly the highest salinity tolerance reported in the literature for the genus *Nannochloropsis* (Eustigmatophyceae, Monodopsidaceae). Most of the similar studies on the effect of salinity on the growth of *Nannochloropsis* examined small range of salinity from brackish to seawater salinity (< 3.5% NaCl) ignoring the higher salinity values above seawater salinity. For example, a study done by Gu et al (2012a) reported the optimum salinity for the growth of *N. oculata* under nutrient-replete conditions was 25‰ (2.5%), and it grew better at 35‰ (3.5%) under nutrient-deplete conditions. Renaud & Parry (1994) and Wilkerson (1998) reported the optimum salinity for the growth of the *N. oculata* was 22 to 25 g L^{-1} (2.2 to 2.5% NaCl). Bartley et al (2015) observed the salinity range for *N. salina* growth was 14.5-45.5 PSU. The optimum salinity for the growth of *Nannochloropsis* was 10‰ or 1% NaCl (Fakhri et al 2015). The variation of the salinity tolerance of *Nannochloropsis* sp. is species specific (Richmond 1986) and it is also related to origin (Banerjee et al 2011).

The highest SGR of the *Nannochloropsis* sp. UHO3 (0.779±0.02 d^{-1}) was achieved when rising the salinity from 2 to 3% NaCl and the lowest (0.455±0.02 d^{-1}) was obtained when increasing the salinity from 6 to 7% salinity. A decrease in the algal growth at increasing salinity would be due to energy use for maintaining the turgor pressure resulted in a decrease in productivity or reduction in growth (Kirst 1989) and also due to lower photosynthetic rate (Hart et al 1991). The SGR reported in this study was comparable with other studies. For example, the maximum growth rate of the *Nannochloropsis salina* under optimum growing condition in the laboratory was 0.030 h^{-1} or 0.72 d^{-1} corresponding to a doubling time of 23 h (Boussiba et al 1987). The highest SGR of *Nannochloropsis oculata* was about 0.282±0.017 d^{-1} obtained at salinity 35 g L^{-1} or 3.5% NaCl (Gu et al 2012b). A study done by Cho et al (2007) reported the highest SGR of the *N. oculata*was 0.46 d^{-1} obtained at salinity 10‰ or 1% NaCl. Pal et al (2011) reported the highest SGR of the *Nannochloropsis* sp. (0.81 d^{-1}) was achieved at salinity 27 g L^{-1} (2.7% NaCl) and the lowest SGR (0.55 d^{-1}) was obtained at salinity 40 g L^{-1} (4.0% NaCl). According to Garcia et al (2007), variation in growth rates of microalgae are more strain specific than species specific and differ between geographycal location (de Boer et al 2005). The optimum salinity for the growth of the *Nannochloropsis* sp. UHO3 (3% NaCl) is very close to the initial salinity condition from which the strain was collected (3.2% NaCl).

This study tested the growth of the *Nannochloropsis* sp. at wide range of high salinity up to two times of the seawater salinity (2-7% NaCl). The main reason is that the alga is going to be mass produced in outdoors for any potential applications using seawater based medium. If the seawater is used then the salinity of the culture media will increase over time (Borowitzka 2013b) and therefore with capability of the alga to tolerate wide range of high salinity, the reliable culture could possibly be maintained for long periods. In addition, microalgae growing in hypersaline media are less prone to contamination by other microrganisms including other microalgal species, protozoas and bacteria as not many organisms can tolerate high salt concentration (Mutanda et al 2011; Indrayani et al 2018).

Salinity does not only affect the growth of microalgae but also affects biochemical composition of microalgae including lipid. This study is particularly focused on the effect of the salinity on the growth and lipid productivity of the *Nannochloropsis* sp. UHO3 due to the fact that genus *Nannochloropsis* is one of the most studied microalgae genera owing to its ability to synthesize n only neutral lipids for biodiesel production but also EPA for functional food (Hoffmann et al 2010; Ma et al 2014; Ma et al 2016; Hulatt et al 2017). Neutral lipids are the dominant storage compounds in *Nannochloropsis* under nitrogen-deprivation condition whereas under nutrient-sufficient conditions, the biosynthesis of polar lipids is preferred (Ma et al 2016). Hu et al (2008) pointed out that lipids both polar membrane lipids and neutral lipids are important structural and functional parts of microalgae in which under favourable growth condition, microalgae synthesize membrane lipids of about 5-20% of the cell dry weight; under unfavourable

conditions, more neutral lipids in the form of TAGs are synthesized of about 20-50% of dry weight. In this study, we found that the highest lipid content (25.29%AFDW) and lipid productivity (0.172 g $L^{-1}d^{-1}$) of the *Nannochloropsis* sp. UHO3 were obtained at 3% salinity although no significantly difference at 4% salinity. The highest lipid yield of the alga coincides well with the highest growth rate achieved at salinity 3% NaCl resulted in the highest lipid productivity. It is interesting to note that the intracelullar lipid accumulation of the alga was relatively higher at the lowest salinity (2% NaCl) although there was no statistically significant difference between other salinities. Higher intracelluar lipid content of the alga at lower salinity could be due to the increase accumulation of neutral lipids as energy-rich stotage products produced under unfavourable salinity conditions for the growth of the alga. Therefore, the optimum salinity for higher growth rate and lipid productivity of the *Nannochloropsis* sp. UHO3 is at salinity 3-4% NaCl.

The lipid content of the *Nannochloropsis* found in this study is comparable with other studies. For example, *N. gaditana* strains can accumulate 20% of lipid (wild type) and 40-45% (mutant type) under nutrient-replete conditions (Ajjawi et al 2017). San Pedro et al (2014) studied outdoor pilot scale production of *Nannochloropsis gaditana* in tubular photobioreactors and found that the species produced maximum lipid productivity of about 0.110 g L^{-1} d^{-1}. Dianursanti et al (2018) reported the highest lipid content of *N. oculata* (20.3% dry weight) was obtained after the addition 25 ppm HCO_{3-}. The lipid content of *N. salina* ranged from 22 to 26% AFDW (Boussiba et al 1987).

The results of this study suggests that the newly isolated *Nannochloropsis* sp. UHO3 is a potential microalgal species for biodiesel feedstock due to its high growth rate, high lipid content and lipid productivity. This is in line with a study done by Doan et al (2011) who conducted a comprehensive high-throughput screening study. Out of the 96 strains screened, they recomended *Nannochloropsis* strains as the best feedstock for biodiesel due to its high lipid content ranging from 39.4 to 44.9% of dry weight biomass. Ma et al (2014) also suggested *N. oceanica* IMET1 as an excellent strain for lipid production due to its high lipid productivity of 158 mg L^{-1} d^{-1}. The ideal microalgae as an alternative biodiesel source must have high growth rate, lipid content and lipid productivity (Griffiths & Harrison 2009; Gong & Jiang 2011). In addition, microalgal species with a wide salinity tolerance is preferred for outdoors cultivation to obtain reliable cultures for long period (Indrayani 2017; Indrayani et al 2019, 2020). Microalgae with these characteristics will greatly reduce the production cost of biodiesel (Ruangsomboon et al 2013).

Conclusions. The microalga strain *Nannochloropsis* sp.UHO3 has a wide range of salinity tolerance from 2% to 7% NaCl concentration. The algal strain can yield high lipid production at salinity 3-4% NaCl. On the basis of its growth characteristics, lipid content and lipid productivity, this strain seems to be suitable for biodiesel feedstock. Research on this strain is continuing to determine other limits to growth factors to further enhance biomass, lipid productivities and fatty acids compositions for biodiesel and high value product production (i.e DHA and EPA contents) and also to determine its reliability for mass cultivation in outdoor raceway ponds.

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Conflict of interest statement. The authors have no conflicts of interest to declare.

Statement of informed consent, human/animal rights. Not applicable

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