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Evaluation of Five *Nannocfhloropsis* Sp. Strains for Biodiesel and Poly-Unsaturated Fatty Acids (PUFAs) Production

Hu Qunju[1,2], Xiang Wenzhou[1]*, Yang Fangfang[1], Li Tao[1], Wang Guanghua[1], Dai Shikun[1], Wu Hualian[1] and Fan Jiewei[1]

[1]*Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China*

[2]*University of Chinese Academy of Sciences, Beijing 100049, China*

Abstract

In this study, five *Nannochloropsis* sp. strains recently isolated from China's coastal waters were evaluated for their biodiesel and poly-unsaturated fatty acids (PUFAs) production potential. The results shown, *Nannochloropsis* sp. 628 achieved the highest biomass accumulation of 7.05 ± 0.21 g L^{-1}, and *Nannochloropsis* sp. 1049 was the most promising lipid producer, with lipid content of 61.16 ± 0.03% dry cell weight (DCW) and lipid productivity of 235.89 ± 7.58 mg L^{-1} d^{-1}. For all the strains, neutral lipid was the predominant component of total lipid which counted for percentages ranging from 69.25 ± 1.06% to 87.31 ± 0.26% total lipid. The favorable fatty acids (FAs) of saturated FAs, monounsaturated FAs and C16-C18 components of total FAs of the strains were all preferable attained. Suitable biodiesel properties within the prescribed limits were also revealed. High production ability of PUFAs (89.27 ± 7.79 mg L^{-1} to 134.94 ± 9.84 mg L^{-1}) meant PUFAs could be considered as co-products of microalgae biodiesel production. The present study shown the *Nannochloropsis* sp. strains could be consider as valuable feedstock candidates for biodiesel production.

Keywords: *Nannochloropsis* sp; Lipid productivity; Neutral lipid content; Fatty acids profile; Biodiesel properties; PUFAs production

Introduction

Continuously usage of petroleum sourced fuels is now widely recognized as unsustainable and environmental unfriendly because of its depleting supplies and the emission of carbon dioxide into the atmosphere, which contributes to the worldwide greenhouse effect and global climate change [1,2]. Methods to convert biomass to competitive biofuels increasingly attract researchers' attention in recent past decades, and microalgae used as the most promising feedstock source for the third-generation biodiesel production interest biofuel researchers since they can produce and accumulate large amount of lipids and fix the greenhouse gas (CO_2) by photosynthesis at the same time [3]. Hence, development and utilization of microalgae as feedstock candidates for biodiesel production appears to be a cost effective, renewable, carbon neutral and environmentally friendly way forward and offers great opportunities in the longer term [4,5]. However, inadequate microalgae species and relatively absence of information on detailed FAs compositional profiles have limited the development of microalgae bio-resources [6]. Determination and analysis of microalgae strains with high biomass concentration, high cellular lipid content, appropriate lipid distribution, suitable FAs compositional profiles and proper biodiesel properties under specific culture conditions are of great importance to promote microalgae biodiesel production [7]. Thus isolation and evaluation of autochthonic appropriate microalgae species and exploitation of them in algal biotechnology for bio-resource usage is urgently needed [8].

In evaluation of suitable microalgae strains for biodiesel production, quantitative data of not only high lipid productivity, but also suitable FAs composition are required. That are because FAs compositional features, especially their chain length and degree of unsaturation could significantly influence the physical and chemical properties of the biodiesel which is transformed from the microalgae oil, including kinematic viscosity (KV), specific gravity (SG), cetane number (CN), cloud point (CP), iodine value (IV) and higher heating value (HHV) [6,9,10]. Beside lipid productivity and FAs composition, TAGs proportion of total lipid is another important consideration in microalgae selection for biodiesel production. Which is because only TAGs, rather than any other extracted types of microalgae lipids, could be easily transesterified into biodiesel by traditional methods [11]. Thus, neutral lipid content (mainly in form of TAGs) in total lipid could also significantly influence the efficiency of microalgae biodiesel production [2]. In addition to lipids, microalgae biomass offers opportunities for obtaining additional commercial materials which could act as alternative routes, for example, co-products, to make microalgae biodiesel production more predictable and sustainable economical and bulk markets for the co-products are potentially available [12,13].

Nannochloropsis is a marine unicellular microalgae belonging to the Eustigmatophyceae family. Traditionally, they were well appreciated in aquaculture for their high nutritional value, and has been identified as one of the most promising photoautotrophic producers of eicosapentaenoic acid (EPA) [14,15]. Eicosapentaenoic acid (EPA, C20:5), as a number of the long-chain ω-3 polyunsaturated fatty acids (PUFAs), plays essential nutritional role in human health [16]. And microalgae might be considered as the most promising source of EPA, because they are the primary peoducers of EPA and docosahexaenoic acid (DHA, C22:6) [15]. In recent past years, *Nannochloropsis* have been used as a potential feedstock for biodiesel production due to their tolerance of broad environmental and culture conditions while growing rapidly and accumulating large amount of TAGs [17]. Furthermore, high value products (e.g. PUFAs and pigments) could be used as co-products to enhance the economical value of *Nannochloropsis* cultivation, which is considered as a strategy to reduce costs of microalgae (*Nannochloropsis*)

***Corresponding author:** Xiang Wenzhou, Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China, Tel: +86-020-89023223; E-mail: xwz@scsio.ac.cn

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biodiesel to compete with low price fossil fuels [18].

In this study, five *Nannochloropsis* sp. strains newly isolated from China's coastal waters were evaluated for their potential of biodiesel production through the detailed measurement of growth characteristics, biomass accumulation, lipid yield properties, lipid productivity, lipid distribution and fatty acid profiles. Furthermore, significant biodiesel properties, such as kinematic viscosity (KV), specific gravity (SG), cetane number (CN), cloud point (CP), iodine value (IV) and higher heating value (HHV) were calculated due to their relationships between them and the FAs profiles, and then were analyzed and compared with the reported specifications. Finally, PUFAs production potential, especially the production potential of Arachidonic acid (ARA, C20:4) and EPA of the strains were estimated. The ultimately aim of this work was to evaluate the potential and feasible of the five *Nannochloropsis* sp. strains for biodiesel and PUFAs production.

Materials and Methods

Nannochloropsis sp. strains

The five new *Nannochloropsis* sp. strains were originally isolated and preserved by Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences. The names and the geographical location (origin sampling spot, habitat, and latitude and longitude) of the strains were all listed in Table 1.

These *Nannochloropsis* sp. strains were maintained both on agar plate and liquid medium containing f/2 medium. Algae colonies picked from the agar plate were firstly cultured in 25 mL liquid f/2 medium in Erlenmeyer flasks till logarithmic growth phase and then scaled large enough as it needed to inoculated in photobioreactors. The algae inoculum were centrifuged (4,000 rpm × 6 min at room temperature), and the deposited algal cells were washed twice with the final culture medium. The inoculum were then re-suspended in 1000 mL final culture medium and inoculated into each tube.

Cultivation conditions

All the five strains were cultivated in laboratory scale in 0.4-L glass bubbled tubes (3.0 cm in diameter) to investigate the induction of microalgae growth and lipid synthesis features on nutrition deprivation conditions. The final culture medium was modified Guillard's F medium [19], which composed of the following components: $NaNO_3$, 0.75 g L^{-1}; $NaH_2PO_4 \bullet 2H_2O$, 0.05 g L^{-1}; EDTA-Fe3+, 1 mL L^{-1}; Vitamins 1 mL L^{-1}; Trace elements 1mL L^{-1}. The 0.4-L tubes, which contained 0.33 L culture medium each, were bubbled with sterilized air/CO_2 mixture gas (95/5, v/v) to support growth and maintain pH of the culture medium within the desired range (7.8 ± 0.2). The tubes were installed in a room with an air conditioner, controlling the temperature at 25 ± 1°C. Continuous artificial illumination (250~300 umol photons $m^{-2}s^{-1}$) was provided by daylight fluorescent tubes on one side of the bubbled tubes. Each strain was cultivated in a triple batch and the overall cultivation time was 14 days.

Strain	Sampling Spot	Habitat	Latitude and Longitude
Nannochloropsis sp. 105	The Bohai Sea	Inshore sea surface	E123°28′48″, N31°49′12″
Nannochloropsis sp. 206	South China Sea	Sea-surface	E122°09′55″, N21°00′02″
Nannochloropsis sp. 590	Guangxi Beihai	Salt-field Ditch	E109°16′16″, N21°27′58″
Nannochloropsis sp. 628	Guangxi Beihai	Open ponds for *S. platensis* cultivation	E109°16′5.4″, N21°21′51.2″
Nannochloropsis sp. 1049	Shenzhen Daya Bay	Inshore sea surface	E114°32′54.28″, N22°35′34.57″

Table 1: Origin sampling spot, habitat, latitude and longitude of the five *Nannochloropsis* sp. strains.

Growth properties of the strains

Cell concentration of the strains were measured by optical density (OD) method using a Thermo UV-vis spectrophotometer at the absorbance of 750 nm, and the initial cell density of the five strains were all about 0.5 at OD 750. Growth rate were measured every other day at OD 750.

Specific growth rate (k) of each strain was calculated from the slope of the linear regression of time and nature log OD 750 in exponential growth phase:

$$k = \frac{\ln N_1 - \ln N_0}{t_1 - t_0} \quad (1)$$

Where k (d-1) was specific growth rate in the exponential growth phase, N0 was OD 750 at the beginning of the exponential phase (t0) and N1 represents the OD 750 at time (t1) of the exponential phase.

Doubling time (T) could be calculated based on the specific growth rate:

$$T = \frac{1}{k} \quad (2)$$

Finally, dry cell weight (DCW) of the strains were measured at the end of the cultivation time. Then the cells were harvested by centrifugation (5000 rpm × 6 min) and washed twice with distilled water (5000 rpm × 5 min). The wet microalgae pellets were freeze-dried immediately and stored under -20°C until further analyze in 10 days.

Lipid extraction and lipid distribution

The total intracellular lipid of the strains was extracted by the optimized method previously reported by Khozin-Goldberg [20]. According to which a mixture of methanol-dimethyl sulphoxide (9:1, v/v), diethyl ether and hexane (1:1:1, v/v/v) were used as solvents to extract the lipid according to priority. Then the same volume of distilled water was added into the organic solvent which loaded with lipid after the microalgae debris was removed, forming a liquid-liquid separation state. Finally, the upper layer, composed of diethyl ether and lipid, was transferred into a pre-weighed vial. Keep repeating these procedures until most of the lipid was extracted and the lipid was then dried by a stream of N_2. Each *Nannochloropsis* sp. strain was extracted for three times. Calculated the lipid content on dry cell weight. The extracted lipids were stored at 4°C until further analysis.

To distribute lipid classes of the strains, silica gel column chromatography method previously described by Christie was adopted [21]. With which neutral lipid, glycolipid and phospholipid were successively eluted by chloroform, acetone and methanol, respectively. After the elution, each lipid class was dried by a stream of N_2 and weighed, presented as percentage total lipid. Each strain was analyzed for three times.

Fatty acid composition analysis

The microalgae dry biomass samples were pretreated with the following procedures to be operated: (i) 10 mg aliquots of the samples were suspended in 1 mL saturated sodium hydroxide methanol in a glass Falcon tube and the mixture was sufficient blended immediately; (ii) The Falcon tubes were put into a 75°C water bath to saponify for 10 min; (iii) Then the tubes were cooled down to room temperature and added 2 mL BF_3-CH_3OH and immediately mixed; (iv) The tubes were

put into the 75°C water bath and methyl esterificated for 10 min; (v) Cooled down the tubes and injected 3 mL n-hexane and 1 mL deionized water, sufficiently mixed; (vi) The tubes were let stood and layered and then centrifuged at 2000 g for 3 min, the hexane layer was separated and collected; (vii) The hexane layer was dewatered with anhydrous sodium sulphite ($NaSO_4$) and filtered by a microfiltration membrane (diameter 0.22 um), and the fatty acid methyl esters (FAME) samples of the strains were obtained at last [22].

FAME profiles of the five *Nannochloropsis* sp. strains were determined using a GC-MS chromatographic instrument. With which an Omega wax 250 polyethylene glycol capillary column (length, 30 m; diameter, 0.25 mm; 0.25 μm film thickness) was adopted according to the method reported by Khozin-Glodberg [20]. One microliter of the FAs samples were injected into the capillary column with a split ratio of 5:1. Helium was employed as the carrier gas with a flow rate of 1.5 mL/min. Temperatures of both the injector and detector were maintained at 250°C, and the oven temperature was programmed as the following program: initial temperature at 130°C for 1min, temperature rose from 130°C to 250°C (5°C min^{-1}), and maintained at 250°C for 5 min.

FAs component content was determined using the area normalization method. To measure FAs quantification in percentage of microalgae DCW, an internal standard of heptadecanoic acid (C17:0) (Sigma, USA) was added to the microalgae biomass samples before methylation.

Estimation of important biodiesel properties based on FAME profiles

To measure the critical physical and chemical properties of the lipids extracted from the five *Nannochloropsis* sp. strains, predictive models based on FAs composition were used to evaluate the potential of the five strains for feedstock candidates of biodiesel production. In all the equations been built based on FAs compositional profiles to predict biodiesel properties, equations of Hoekman et al. [6,23] were adopted to predict the biodiesel properties of the microalgae oil in this study, which were shown as follows:

$$ADU = \sum M \times Y_i \quad (3)$$

$$KV = -0.6316 \times ADU + 5.2065 \quad (4)$$

$$SG = 0.0055 \times ADU + 0.8726 \quad (5)$$

$$CP = -13.356 \times ADU + 19.994 \quad (6)$$

$$CN = -6.6684 \times ADU + 62.876 \quad (7)$$

$$IV = 74.373 \times ADU + 12.71 \quad (8)$$

$$HHV = 1.7601 \times ADU + 38.534 \quad (9)$$

where ADU was average degree of unsaturation of the microalgae oil; Yi was the mass fraction of each FA constituent; M was the number of carbon–carbon double bonds in each FA constituent; KV was the kinematic viscosity of microalgae lipid; SG was specific gravity; CP was cloud point; CN was cetane number; IV was iodine value and HHV was higher heating value.

Statistical analysis

Data were presented as the mean ± standard deviation of the means of samples. Significant differences between means were determined by analysis of one-way covariance (ANCOVA), and SPSS statistical package (version 13.0; SPSS Inc., Chicago, IL, USA) with a significance level of P=0.05 was established in advance to sufficiently demonstrate a statistically significant difference unless otherwise noted.

Results and Discussion

Microalgae growth of the five strains

As clearly displayed in Figure 1, rapidly growth of strains were observed under the cultivation conditions in bubbled tubes. And all the five *Nannochloropsis* sp. strains exhibited the lag phase (0 to 3 days) and exponential phase after day 3 till to day 9. There were no significant differences (P<0.05) among the patterns of optical density (OD 750) in the five strains during the initial 9 days. Then the growth rates of the strains slowed down and expressed the stationary phase with time extension due to the nutrient depletion and wastes accumulation in the culture medium. The differences of growth among the five strains were then revealed. Remarkably, the highest and the lowest average OD 750 value were obtained by *Nannochloropsis* sp. 628 and *Nannochloropsis* sp. 1049, with the values of 16.65 ± 0.01 and 12.88 ± 0.20, respectively. In this study, decrease of growth were not recorded, and visible changes in the color of the culture medium were observed. And the color change differed among the strains, with *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 206 and *Nannochloropsis* sp. 590 changed from green to red, *Nannochloropsis* sp. 105 from green to yellow brownish, and *Nannochloropsis* sp. 628 from green to green brownish. This result was consistent with previous observations [24].

To further analyze growth properties of the microalgae strains, specific growth rate (k) and doubling time (T) were calculated and shown in Table 2. Finally, biomass accumulation and productivity were also measured and shown in Table 2. The results shown that *Nannochloropsis* sp. 206 exhibited the highest specific growth rate (0.33 ± 0.01 d^{-1}), followed by *Nannochloropsis* sp. 590 (0.30 ± 0.008 d^{-1}), *Nannochloropsis* sp. (0.28 ± 0.003 d^{-1}) and *Nannochloropsis* sp. 1049 (0.28 ± 0.01 d^{-1}), and *Nannochloropsis* sp. 628 obtained the lowest specific growth rate among the five strains with the value of 0.26 ± 0.003 d^{-1}. The double time (T) illustrated the similar tendency with the specific growth rate (k) (Table 2).

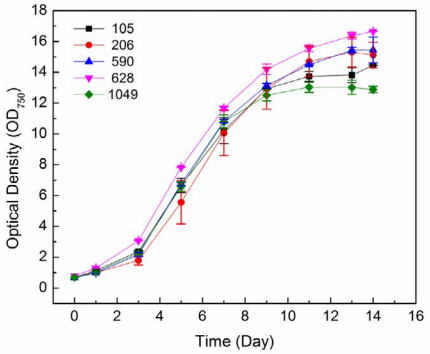

Figure 1: Optical density.

	Nannochloropsis ***sp.* 1049**	*Nannochloropsis* ***sp.* 105**	*Nannochloropsis* ***sp.* 206**	*Nannochloropsis* ***sp.* 590**	*Nannochloropsis* ***sp.* 628**
k	0.28 ± 0.01	0.28 ± 0.003	0.33 ± 0.01	0.30 ± 0.008	0.26 ± 0.003
T	3.52 ± 0.14	3.54 ± 0.04	3.03 ± 0.10	3.33 ± 0.09	3.92 ± 0.04
BA	5.40 ± 0.17	4.45 ± 0.21	5.30 ± 0.28	5.90 ± 0.28	7.05 ± 0.21
BP	385.71 ± 12.37	317.86 ± 15.15	378.57 ± 20.20	421.43 ± 20.20	503.57 ± 15.15

Table 2: Specific growth rate, doubling time, biomass accumulation and biomass productivity of the five strains. Data were given as means ± S.D., n=3 k, specific growth rate (d^{-1}); T, doubling time (d); BA, biomass accumulation (g L^{-1}); BP, biomass productivity (mg $L^{-1}d^{-1}$).

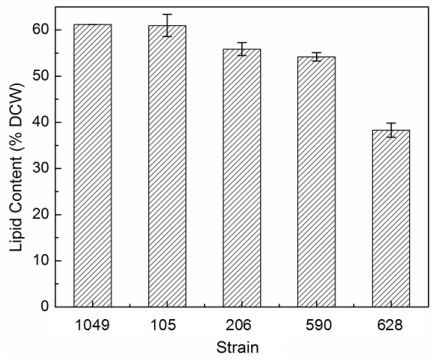

Figure 2: Lipid content.

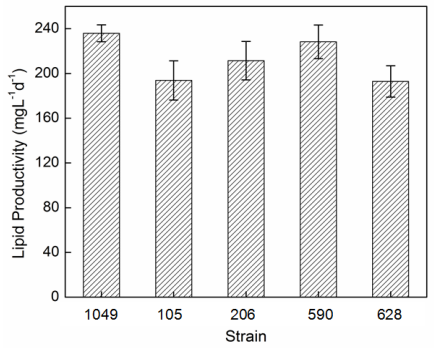

Figure 3: Lipid productivity.

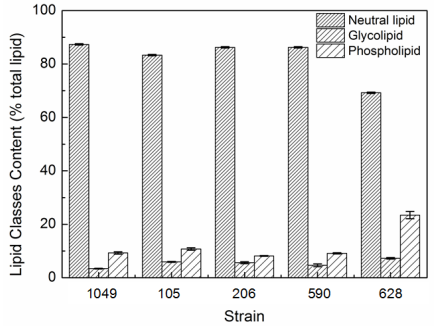

Figure 4: Lipid classes content.

Variable biomass accumulation of the strains were displayed due to the differences of the growth properties and the size of the cells. The results were shown in Table 2, which greatly distinguished from what of the growth curves or specific growth rate (k) shown. For example, *Nannochloropsis* sp. 206 attained the highest k and T, but biomass accumulation of it was the last but one among the five strains. *Nannochloropsis* sp. 628 received the lowest k value, while it achieved the highest biomass accumulation as high as 7.05 ± 0.21 g L^{-1}. Biomass productivity was computed by the cultivation time and final biomass at the end of cultivation. To be calculated from Table 2, *Nannochloropsis* sp. 628 was the most promising biomass producer (7.05 ± 0.21 g L^{-1}, 503.57 ± 15.15 mg L^{-1} d^{-1}) in the five strains. *Nannochloropsis* sp. 105 attained the lowest biomass accumulation (4.45 ± 0.21 g L^{-1}).

Lipid content and productivity of the five strains

In the present study, biodiesel production potential of the five *Nannochloropsis* sp. strains were elementary measured by evaluating their lipid content and lipid productivity in this study. Lipid content of the five strains were analyzed at the end of the 14-day cultivation and reported as % mass fraction of DCW, and the results were illustrated by Figure 2. Lipid contents of the five strains varied among the strains, and they were always higher than 50% DCW except *Nannochloropsis* sp. 628, which obtained the lipid content of 38.30 ± 1.53% DCW. For *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206 and *Nannochloropsis* sp. 590 the lipid content values were up to 61.16 ± 0.03%, 60.96 ± 2.39%, 55.85 ± 1.40% and 54.18 ± 0.90% DCW, respectively. The colors of the extracted total lipids were red-brownish or brownish and this phenomenon was consistent with the previous report.

Generally, high intracellular lipid content was one of the key criteria in evaluation of whether a microalga strain was the potential feedstock for biodiesel production [25]. However, lipid content alone was not an appropriate measure for yield, because suitable candidates also acquired high growth rate and dry weight biomass production. Current studies started to concentrate more on lipid productivity for biodiesel production [26].

Lipid productivity was not only in connection with lipid content, but also biomass productivity. Thus comprehensive considered lipid content and biomass productivity, lipid productivity of the 5 *Nannochloropsis* sp. strains was calculated, and the results were shown in Figure 3. It could be concluded that the top biomass producers in the present study did not correspond to the top lipid producers. For example, *Nannochloropsis* sp. 628 showed the highest biomass productivity, while its lipid content was the lowest. And lipid productivity of this strain obtained a value of 192.86 ± 13.99 mg L^{-1} d^{-1}, which was also the lowest of the five strains. The phenomenon was consistent with the previous report. Naturally, a microalga strain which has higher biomass productivity may manifest in relative lower lipid content and vice versa [8]. As shown in Figure 3, lipid productivity of the other four strains was also declared. *Nannochloropsis* sp. 1049 attained the highest lipid productivity (235.89 ± 7.58 mg L^{-1} d^{-1}), which was followed by *Nannochloropsis* sp. 590 (228.34 ± 15.11 mg L^{-1} d^{-1}). *Nannochloropsis* sp. 206 indicated a value of 211 ± 17.15 mg L^{-1} d^{-1}, as the third highest lipid productivity strain in the five strains, and lipid productivity of *Nannochloropsis* sp. 105 achieved 193.78 ± 16.56 mg L^{-1} d^{-1}.

Based on the lipid productivity, and compared with that reported by Ma et al. [10], lipid productivity of the five strains evaluated in this study achieved much higher levels, and all the five *Nannochloropsis* sp. strains could be consider as potential feedstock for microalgae biodiesel production. The comparison rate of the overall lipid productivity differed among the strains, which were interacted by lipid content and biomass accumulation of the strains. And it could be concluded that in screening of suitable microalgae strains for biodiesel production, the pursuing of high lipid content and high algal biomass production were of equal importance [2,8].

Lipid distribution of the five strains

In distribution of the total lipids of the five *Nannochloropsis* sp. strains, lipid classes (neutral lipid, glycolipid, phospholipid) were determined and presented as percentage total lipid, with which the results were shown in Figure 4. It could be concluded that for all the five strains, neutral lipid were the primary component of the total lipid, the percentages of phospholipid were higher than that of glycolipid, and acted as the secondary content component of total lipid. And the real ratios of these components differed among the five strains.

To be illustrated in Figure 4, *Nannochloropsis* sp. 1049 achieved the highest neutral lipid content at 87.31 ± 0.26% total lipid, while

Fatty acids	*Nannochloropsis* **sp. 1049**	*Nannochloropsis* **sp. 105**	*Nannochloropsis* **sp. 206**	*Nannochloropsis* **sp. 590**	*Nannochloropsis* **sp. 628**
C14:0	1.27 ± 0.05	3.28 ± 0.12	1.21 ± 0.06	1.05 ± 0.01	1.31 ± 0.03
C16:0	21.50 ± 0.70	20.49 ± 1.06	20.74 ± 1.43	19.51 ± 0.45	19.30 ± 0.75
C16:1	15.46 ± 0.52	14.78 ± 1.10	14.46 ± 1.05	13.97 ± 0.37	13.85 ± 0.47
C18:0	0.71 ± 0.02	0.49 ± 0.06	0.49 ± 0.04	0.67 ± 0.02	0.47 ± 0.04
C18:1	5.30 ± 0.07	6.83 ± 0.31	5.40 ± 0.38	3.96 ± 0.05	6.21 ± 0.28
C18:2	—	0.35 ± 0.02	—	—	—
C20:4	0.73 ± 0.02	0.67 ± 0.01	0.73 ± 0.07	0.94 ± 0.03	0.59 ± 0.03
C20:5	0.92 ± 0.06	1.97 ± 0.08	1.11 ± 0.14	1.19 ± 0.04	1.32 ± 0.06
SFAs	23.49 ± 0.10	24.25 ± 1.24	22.44 ± 1.53	21.23 ± 0.49	21.09 ± 0.82
MUFAs	20.76 ± 0.12	21.61 ± 1.41	19.87 ± 1.43	17.63 ± 0.41	20.07 ± 0.75
PUFAs	1.65 ± 0.07	2.99 ± 0.12	1.84 ± 0.21	2.08 ± 0.07	1.91 ± 0.09
C16+C18	42.98 ± 0.17	42.93 ± 2.55	41.10 ± 2.91	37.80 ± 0.89	39.84 ± 1.55
FAME	45.90 ± 0.29	48.86 ± 2.77	44.16 ± 3.18	40.93 ± 0.98	43.07 ± 1.67
SFAs*	51.17 ± 0.11	49.64 ± 0.27	50.83 ± 0.19	51.86 ± 0.05	48.97 ± 0.02
MUFAs*	45.23 ± 0.03	44.22 ± 0.37	44.99 ± 0.00	43.07 ± 0.00	46.59 ± 0.05
PUFAs*	3.60 ± 0.14	6.14 ± 0.11	4.18 ± 0.19	5.07 ± 0.06	4.44 ± 0.03
C16+C18*	93.63 ± 0.23	87.86 ± 0.24	93.08 ± 0.12	92.35 ± 0.03	92.51 ± 0.01

SFA: Percentage of saturated fatty acids (% DCW); MUFA: Percentage of monounsaturated fatty acids (% DCW); PUFA: Percentage of polyunsaturated fatty acids (% DCW); FAME: Percentage of fatty acids (% DCW); C16+C18: FAs with chain lengths ranging from C16 to C18 (% DCW); SFAs*: percentage of saturated fatty acids (% Total FAs); MUFAs*: Percentage of monounsaturated fatty acids (% Total FAs); PUFAs*: Percentage of polyunsaturated fatty acids (% Total FAs); C16+C18*: FAs with chain lengths ranging from C16 to C18 (% Total FAs).

Table 3: FAs composition and ratios of DCW and total FAs of the five strains. Data were given as means ± S.D., n=3.

Nannochloropsis sp. 628 displayed the lowest neutral lipid content level of 69.25 ± 1.06%, with which the trend was similar to that of total lipid content. *Nannochloropsis* sp. 590 also attained a high neutral lipid content value of 86.23 ± 0.73% total lipid, followed by *Nannochloropsis* sp. 206 and *Nannochloropsis* sp. 105, which obtained neutral lipid content values of 86.21 ± 0.54% and 83.33 ± 0.65% total lipid, respectively. As compared with lipid productivity of the five strains, neutral lipid content was somewhat in connection with lipid productivity.

Phospholipid was the secondary high content component of total lipid in all the five strains, and values of 9.27 ± 0.42%, 10.72 ± 0.46%, 8.16 ± 0.16%, 9.12 ± 0.21% and 23.46 ± 1.35% were revealed by *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206, *Nannochloropsis* sp. 590 and *Nannochloropsis* sp. 628, respectively. All the strains accumulated low levels of glycolipid, *Nannochloropsis* sp. 1049 and *Nannochloropsis* sp. 628 attained the lowest and the highest glycolipid content of 3.4 ± 0.16% and 7.29 ± 0.29% total lipid, respectively. And for *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206 and *Nannochloropsis* sp. 590, the values of glycolipid counted to 5.94 ± 0.19%, 5.64 ± 0.38% and 4.65 ± 0.53% total lipid, respectively.

Unlike the glycolipids which are mainly found in membranes, neutral lipids are usually in form of TAGs and do not perform a structural role of the microalgae cells, they serve primarily as a storage form of carbon and energy instead. Under unfavorable environmental or nutrition stress conditions for growth, many microalgae alter their lipid biosynthetic pathways toward the formation and accumulation of neutral lipids [2], which can be easily transesterified into biodiesel by traditional methods [11]. Thus, neutral lipid content in the total lipid could significantly influence the production efficiency and quality of microalgae biodiesel production [2, 27].

According to the content of neutral lipid, four in five of the strains, including *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206 and *Nannochloropsis* sp. 590 could be considered as suitable feedstock candidates for microalgae biodiesel production.

Fatty acids profiles of the five strains

Considerable information about FAs composition of the five *Nannochloropsis* sp. strains was available in this study. Based on the GC-MS analysis, all main FAs components of the strains were completely separated and identified, and the results were shown in Table 3. The total FAs contents of the five strains were at percentages of about 40% DCW under the cultivation conditions in this study and represented more than 80% of the total lipid. The most abundant long-chain FAs of the five strains were C16:0, C16:1 and C18:1, Tetradecanoic acid (C14:0), Octadecanoic acid (C18:0), eicosatetraenoic acid (C20:4) and eicosapentaenoic acid (C20:5) were also important components of the total FAs.

FAs composition and ratios of the five *Nannochloropsis* sp. strains on DCW and total FAs under the cultivation conditions in bubbled tubes were shown in Table 3. C16:0 revealed as the predominant component of the total lipid and attained percentages ranging from 19.30 ± 0.75 to 21.50 ± 0.70 DCW in the strains, C16:1 counted for percentages ranging from 13.85 ± 0.47% to 15.46 ± 0.52% DCW, lower than that of C16:0 in each strain. C18:1 appeared to be the third commonly existing FAs in the five strains, and counted for percentages ranging from 3.96 ± 0.05% to 6.83 ± 0.31% DCW. C14:0 acted as the fourth high content component of the total FAs for most of the strains, which accounted for 1.05 ± 0.01% to 3.28 ± 0.12% DCW. Dis-unsaturated fatty acid, only C18:2 under the cultivation conditions in this study, presented at a very low proportion in *Nannochloropsis* sp. 105, and lacked in the other four strains. For all the five strains, FAs with four or more double bonds, comprised of eicosatetraenoic acid (arachidonic acids, C20:4) and eicosapentaenoic acid (EPA, C20:5) in the five strains were not much high in this work, with C20:4 counted for 0.73 ± 0.02%, 0.67 ± 0.01%, 0.73 ± 0.07%, 0.94 ± 0.03% and 0.59 ± 0.03% DCW, EPA (C20:5) achieved 0.92 ± 0.06%, 1.97 ± 0.08%, 1.11 ± 0.14%, 1.19 ± 0.04% and 1.32 ± 0.06% DCW in *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206, *Nannochloropsis* sp. 590 and *Nannochloropsis* sp. 628, respectively.

To further analyze FAs characteristics of the strains, a useful comparison of the fatty acids of the five *Nannochloropsis* sp. strains, which were evaluated with respect to saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and FAs with chain lengths between C16 to C18, were measured and the results were revealed in Table 3. SFAs, which comprised of C14:0, C16:0 and C18:0, were the major components of FAs in the five strains, and accounted for percentages of 23.49 ± 0.10, 24.25 ± 1.24, 22.44 ± 1.53, 21.23 ± 0.49 and 21.09 ± 0.82 DCW in *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206, *Nannochloropsis* sp. 590 and *Nannochloropsis* sp. 628, respectively. In the meantime, SFAs took up more than a half the total lipid at values of 51.17 ± 0.11%, 49.64 ± 0.27%, 50.83 ± 0.19%, 51.86 ± 0.05% and 48.97 ± 0.02 total FAs in *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206, *Nannochloropsis* sp. 590 and *Nannochloropsis* sp. 628, respectively. MUFAs, including C16:1 and C18:1 in this study, revealed content almost the same to that of SFAs, and accounted for 20.76 ± 0.12%, 21.61 ± 1.41%, 19.87 ± 1.43%, 17.63 ± 0.41% and 20.07 ± 0.75% DCW and 45.23 ± 0.03%, 44.22 ± 0.37%, 44.99 ± 0.00%, 43.07 ± 0.00% and 46.59 ± 0.05% total FAs in *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206, *Nannochloropsis* sp. 590 and *Nannochloropsis* sp. 628, respectively. PUFAs content of the

strains ranged from 1.65 ± 0.07% to 2.99 ± 0.12% DCW and 3.60 ± 0.14% to 6.14 ± 0.11% total FAs.

To be indicated from the data in Table 3, FAs with chain lengths between C16 to C18 possessed considerable amounts in all the five strains, which were comprised of 16:0, 18:0, 18:1 and 18:2. And 18:2 presented a very low proportion in *Nannochloropsis* sp. 105, and it was lacked in the other four strains. C16–C18 fatty acids received very high proportions in all the strains, which counted for 42.98 ± 0.17%, 42.93 ± 2.55%, 41.10 ± 2.91%, 37.80 ± 0.89% and 39.84 ± 1.55% DCW and 93.63 ± 0.23%, 87.86 ± 0.24%, 93.08 ± 0.12%, 92.35 ± 0.03% and 92.51 ± 0.01% total FAs in *Nannochloropsis* sp. 1049, *Nannochloropsis* sp. 105, *Nannochloropsis* sp. 206, *Nannochloropsis* sp. 590 and *Nannochloropsis* sp. 628, respectively.

The most commonly synthesized FAs in microalgae cells have chain lengths ranging from C16 to C18, similar to that of higher plants [28]. In evaluation of appropriate microalgae strains as feedstock candidates for biodiesel production, not only high neutral lipid production but also appropriate FAs compositional profiles were acquired to be measured. Generally, saturated and mono-unsaturated fatty acids are predominant in most examined miroalgae, and the major FAs of the Eustigmatophyte are palmitic acid (C16:0), palmitoleic acid (C16:1) and oleic acid (C18:1) [29]. The total FAs content and the FAs percentages on total lipid content of the strains matched well with that previously reported by Guschina and Harwood [30]. The most abundant long-chain FAs of the five strains were also similar to that reported by Cobelas and Lechado [29,31]. And it could be calculated that the ratios of the FAs on DCW varied among the strains and were affected by culture conditions.

As previous studies revealed that in screening of favorable biodiesel candidates, low levels of PUFAs to increase oxidative stability and low levels of SFAs to avoid cold flow problems were extremely important evaluation criteria of the biodiesel quality transformed from the microgae lipids. Therefore, MUFAs were considered as the capable component to give the finest compromise between oxidative stability and cold flow [6,10]. Investigations also shown a common consensus that the most common feedstock candidates suitable for biodiesel production were enriched with the following five most common C16-C18 fatty acids [9].

Taken together, combining with the low percentage of PUFAs, high content of MUFAs and perfect compositions of C16-C18 fatty acids, it could be indicated that all the five strains were suitable feedstock candidates for biodiesel production.

Physical/chemical properties of biodiesel converted from lipids of the five strains

According to the diversity and conflicting impacts of certain FAME compositional features on fuel properties, it was difficult to clearly describe whether a kind of microalgae lipid was suitable for biodiesel production only base on FAs profiles. Hence, a further comprehensive analysis on FAs was measured to evaluate the most important properties of the biodiesel transformed from lipids of the five strains in this study [32,5]. The results were shown in Table 4. As revealed in Table 4, seven important biodiesel properties, including ADU, KV, SG, CP, CN, IV and HHV, of the five microalgae oil candidates were investigated. ADU was a computed fuel property with particularly importance in evaluation of biodiesel production candidates besides chain length and chain branching. ADU of the lipids was also a structural feature that influenced the physical and chemical properties of the fatty acid ester molecule [9], and has been proved to have obvious correlationship with the other properties of the lipids. It influenced the oxidative stability performance of the biodiesel converted from the microalgae lipids. For example, higher ADU value led to lower CN and poorer oxidation stability, but it improved low temperature performance. Six other important biodiesel properties were estimated based on the relationships between ADU and them, which were built across a range of realistic biodiesel types [6,5,10,23].

The physical and chemical properties of biodiesel are largely dictated by the specific compositional profile of the FAME material converted from the microalgae lipids. According to the three most common biodiesel quality standards of biodiesel, ASTM D6751 in the US, EN 14214 in Europe, and the ranges of qualities occurring in common biodiesel feedstocks [6,23], ADU of the five strains were during the middle ranges of common biodiesel feedstocks, and resulted in suitable values of KV, SG, CN, IV and HHV of these candidates, which satisfied almost all the specifications. There were no definite specifications of CP were installed, due to the different climate conditions in the United States and Europe [33]. For all the five strains, they shown favorable biodiesel qualities within the prescribed limits, so they could be considered as the potential microalgae strains for biodiesel production only with suitable fuel properties.

PUFAs production of the five strains

With regard to FAs contents and profiles obtained for the five *Nannochloropsis* sp. strains, PUFAs production and productivity of the five strains were calculated and the results were given in Table 5. Overall, all the strains shown good potential for C20:4 and EPA production. *Nannochloropsis* sp. 590 was declared for the best C20:4 producer (55.25

Biodiesel Properties	*Nannochloropsis sp.* 1049		*Nannochloropsis sp.* 105		*Nannochloropsis sp.* 206		*Nannochloropsis sp.* 590		*Nannochloropsis sp.* 628		Biodiesel, ASTM biodiesel, and EN biodiesel standard		
	Value	SD	Value	SD	Value	SD	Value	SD	Value	SD	Biodiesel	US (ASTMD6751-08)	Europe (EN14214)
ADU	0.62	0.01	0.71	0.00	0.64	0.01	0.66	0.00	0.67	0.00	0.6-1.6	-	-
KV	4.82	0.00	4.76	0.00	4.80	0.01	4.79	0.00	4.78	0.00	4-5	1.9-6.0	3.5-5.5
SG	0.876	0.000	0.877	0.000	0.876	0.000	0.876	0.000	0.876	0.000	0.87-0.89	0.85-0.90	-
CP	11.76	0.08	10.46	0.02	11.42	0.12	11.15	0.03	10.99	0.01	-	report	-
CN	58.77	0.04	58.12	0.01	58.59	0.06	58.46	0.02	58.38	0.01	45-55	Min 47	Min 51
IV	58.55	0.47	65.79	0.09	60.46	0.67	61.93	0.19	62.87	0.07	-	-	Max 120
HHV	39.44	0.01	39.61	0.00	39.49	0.02	39.52	0.00	39.54	0.00	38-41	-	-

ADU: average degree of unsaturation; KV: kinematic viscosity 40˚C ($mm^2 s^{-1}$); SG: specific gravity (kgL^{-1}); CP: cloud point (˚C); CN: cetane number; IV: iodine value ($gI_2/100$ g); HHV: higher heating value (MJ/kg). The data about biodiesel were taken from published literature as indicated in the text. Average degree of unsaturation (ADU) computed from compositional profiles in Table 3. KV, SG, CP, CN, IV and HHV computed from relationships between biodiesel ADU and the properties in Section 2.6. Bold values indicated that all the properties of the five strains oil satisfied the specifications of biodiesel, ASTM and EN standard.

Table 4: Comparison of seven biodiesel properties transformed from lipids of the five *Nannochloropsis* sp. strains, and biodiesel, ASTM biodiesel, and EN biodiesel standards. Data were given as means ± S.D., n=3.

Strain	C20:4 production (mg L^{-1})	EPA production (mg L^{-1})	PUFAs production (mg L^{-1})	PUFAs productivity (mg $L^{-1}d^{-1}$)
Nannochloropsis sp. 1049	39.47 ± 2.58	49.81 ± 5.21	89.27 ± 7.79	6.38 ± 0.56
Nannochloropsis sp. 105	30.00 ± 2.47	87.72 ± 9.77	133.36 ± 14.41	9.53 ± 1.03
Nannochloropsis sp. 206	38.89 ± 6.36	58.99 ± 11.53	97.87 ± 17.89	6.99 ± 1.28
Nannochloropsis sp. 590	55.25 ± 3.92	70.28 ± 5.20	122.49 ± 9.01	8.75 ± 0.64
Nannochloropsis sp. 628	41.55 ± 3.43	93.38 ± 6.41	134.94 ± 9.84	9.64 ± 0.70

Table 5: C20:4, EPA and PUFAs production and productivity of the five strains. Data were given as means ± S.D., n=3.

± 3.92 mg L^{-1}), while *Nannochloropsis* sp. 628 revealed that it was the most promising EPA producer in the five strains with EPA production of 93.38 ± 6.41 mgL^{-1}. *Nannochloropsis* sp. 105 and *Nannochloropsis* sp. 628 shown a similar PUFAs production value of 133.36 ± 14.41 mgL^{-1} and 134.94 ± 9.84 mgL^{-1}. But because of *Nannochloropsis* sp. 105 contained extra proportion of C18:2 under the cultivation conditions in this study, *Nannochloropsis* sp. 628 could be considered as the best producer of PUFAs. *Nannochloropsis* sp. 1049 attained the lowest value of PUFAs production (89.27 ± 7.79 mgL^{-1}) and productivity (6.38 ± 0.56 $mgL^{-1}d^{-1}$). Compare to fish oil, microalgae yield PUFAs is sustainable and avoid the limitation such as stability problems, high purification cost and contamination with pesticides and heavy metals, and they can carry the vegetarian label [15,34,35]. The quality of microalgae EPA is superior to that of fish oil products and the accepted cost is competitive to fish oil [36]. PUFAs content, especially EPA content of the five strains achieved high value in this strains [37]. And the high PUFAs production of the strains demonstrated the potential of PUFAs been developed as co-products of biodiesel production to enhance the economical value of the *Nannochloropsis* sp. strains, contributing to the costs reduction of biodiesel production by these microalgae strains. However, the commercially viable of PUFAs producing as co-products of biofuel production is a key current issue, finding applicable pretreatments, separating PUFAs from the extracted lipids, purifying the PUFAs and commercializing of the systems and procedures are the emphasis for future variable PUFAs co-production technologies. More work need to be done to develop this strategy to its full potential.

Conclusion

Nannochloropsis has inherent advantages that make it commercial feasible as promising feedstock candidates for biodiesel production. In this study, the 5 newly isolated *Nannochloropsis* sp. strains attained high lipid productivity and neutral lipid content, with *Nannochloropsis* sp. 1049 obtained the highest values of 235.89 ± 7.58mg L^{-1} d^{-1} and 87.31 ± 0.26% total lipid. [38] Favorable FAs profiles of high MUFAs content, low PUFAs content, perfect C16-C18 FAs percentages and suitable biodiesel properties displayed them appreciate feedstock candidates for biodiesel production. For all the five strains, PUFAs could be developed as co-products to enhance economical value of the strains, increasing their efficient and commercial feasible for biodiesel production.

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