

Effects of Temperature and Nitrogen Limitation on Growth and Lipid Production of Oleaginous Microalgae from Hot Springs

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Abstract

Oleaginous microalgae have gained increasing attention as an alternative feedstock for biodiesel production due to the increasing demand of fuel, climate change, and global warming. This study aimed to isolate and screen robust microalgal strains from hot springs for cultivation in subtropical and tropical areas. The newly isolated oleaginous microalgae were cultivated at 30, 35 and 40 °C. Among mesophilic and thermophilic strains tested, *Chlamydomonas* sp. HP59 is considered the most robust strain as it showed high cell growth in a broad range of temperatures (30 - 40 °C), with the maximum dried cell weight at 40 °C. Un-optimal temperatures for cell growth did improve lipid content by 2 - 4 folds. To increase lipid production, the 2-stage cultivation, in which nitrogen-rich was applied to promote cell growth in the 1st stage and nitrogen-limitation was applied to stimulate lipid accumulation in the 2nd stage, was performed. The high temperature combined with nitrogen-limitation did improve lipid production by all microalgae. With this strategy, *Chlamydomonas* sp. HP59 showed the highest dried cell weight of 4.33 g/L and lipid production of 1.72 g/L. This study has shown the potential use of the newly isolated oleaginous microalgae from hot springs to be cultivated at high temperatures and under nitrogen-limited conditions for the production of biodiesel feedstocks.

Keywords: Lipids, Hot spring microalgae, Nitrogen starvation, Two-stage cultivation

Introduction

Microalgae are photosynthetic microorganisms that can accumulate carbohydrates or lipids at high levels depending on their species. This makes them promising feedstocks for the production of biofuels, especially oleaginous species, which offer alternatives to plant and crop oils [1]. In addition, some strains of algae also contain valuable components such as pigments and proteins those could be used to improve the quality of food supplements, cosmetics, and drugs because of their beneficial antioxidant as well as other activities [2,3]. When compared to conventional plants and crops, microalgae have more

advantageous characteristics such as the superior ability to fix carbon dioxide through their higher photosynthetic efficiencies, higher biomass and oil productivities and also no arable land required for their cultivation [1]. Furthermore, as microalgae also have great tolerance to environmental stress, they can grow in variety of environments, including freshwater, saltwater, and even wastewater. The distribution and diversity of algae vary widely and is influenced by environmental factors such as nutrient availability, temperature, and light intensity [4]. Hot springs are gaining more attention these days since their special characteristics may be crucial for determining their biotechnological potential [5]. Similarly, isolating native strains of microalgae from these harsh environments is a promising strategy because several of these species might have specific features and commercially viable [6]. But in order to industrialize and market microalgae-based renewable bioenergy, 2 factors need to be taken into account. One issue is the selection of microalgal strains that can accumulate high lipid content and can tolerate high temperature in tropical and subtropical areas, and another issue is the strategies to increase the lipid content.

The lipid content in the microalgal cells diverges from species to species ranging from 10 to 60 % depending on the strains and culture conditions [7]. Nutritional stresses, especially nitrogen limitation, have been reported as effective in improving the accumulation of lipids in oleaginous microalgae species [8-10]. It has been reported that under nitrogen limitation, the cells change cellular carbon flux from protein synthesis to lipid synthesis and accumulate as lipid globules [9,11]. Unfortunately, the nitrogen limitation did limit cell growth and result in low biomass production. Thus, although microalgae accumulate a high content of lipids, their low biomass production results in low overall lipid production [12-15]. One strategy to solve this problem is a 2-stage cultivation, in which nitrogen-rich was applied to promote cell growth in the 1st stage and nitrogen limitation was applied to stimulate lipid accumulation in the 2nd stage. As a result, the 2-stage culture strategy can be an option for enhancing lipid productivity [8,9]. This study aimed to screen oleaginous microalgae isolated from hot spring water in southern Thailand and evaluate their growth and lipid production under ambient and high temperatures. The effect of nitrogen limitation combined with high temperature was also evaluated in 2-stage cultivations.

Materials and methods

Isolation of microalgae strains from hot springs

The microalgal strains (including *Scenedesmus*, *Chlamydomonas* and *Chlorella*) were isolated based on their morphological species from Tha Sathon hot spring and Krut hot spring in Surat Thani province, Thailand. The water samples were collected using plankton net (10 - 12×7 - 9 µm in size). The water in hot springs was clear and had a neutral pH of 6 - 7. The microalgal cells were isolated by using the capillary glass method [15]. The isolated microalgae were subsequently purified by serial dilution and cultured in modified Chu 13 medium [15]. The medium is composed of Fe citrate, 0.01 g; NaHCO₃, 0.036 g; K₂HPO₄, 0.04 g; CaCl₂•2H₂O, 0.054 g; citric acid, 0.1 g; MgSO₄•7H₂O, 0.1 g; KNO₃, 0.2 g; and 1 mL of microelement solution per liter. The microelement solution consisted of Na₂MoO₄•2H₂O, 0.05 g; ZnSO₄•7H₂O, 0.02 g; CoCl₂•6H₂O, 0.08 g; CuSO₄•5H₂O, 0.08 g; MnCl₂•4H₂O, 1.8 g; H₃BO₃, 2.85 g per liter, pH 6.8 [13]. To confirm the purity of the microalgae, the morphology was observed under a

microscope, and the species were tentatively identified according to their morphology [16]. The microalgal cells were cultivated under light intensity of $64 \mu\text{mol. photon.m}^{-2} \text{ s}^{-1}$ with light:dark cycles at 16:8 h.

Cultivation of oleaginous microalgae under ambient and high temperatures

The isolated microalgae from hot springs were 10 % inoculated in 200 mL modified Chu 13 medium and incubated at 30, 35 and 40 °C under light intensity of $64 \mu\text{mol.photon.m}^{-2} \text{ s}^{-1}$ with light:dark cycles at 16:8 h and aerated using filter-sterilized 5 % CO_2 in air at 0.01 air volume per medium volume per min (vvm) for 7 days. *Chlorella* sp. which was isolated from freshwater pond at ambient temperature (30 ± 2 °C) was also used in this study. The optical density (OD), dried cell weight and lipid content were analyzed.

Growth and lipid production of microalgae under 2-stage cultivation strategy

The microalgae were cultivated under 2-stage cultivation. In the 1st stage, the microalgae were cultivated in nitrogen-rich medium for 7 days before being transferred to the 2nd stage using modified Chu 13 medium without nitrogen source (without KNO_3) and cultivated for 3 more days. The cultures were performed at different temperatures at 30, 35 and 40 °C under light intensity of $64 \mu\text{mol. photon.m}^{-2} \text{ s}^{-1}$ with light:dark cycles of 16:8 h. The temperature was the same for 2 stages. The biomass and lipid contents of all microalgae under two-stage cultivation were compared.

Analytical methods

The optical density (OD) was measured at 660 nm. To determine dried cell weight, the microalgal biomass was harvested from culture medium by centrifuging at 10,000 rpm and 4 °C for 20 min. The microalgal biomass were washed with deionized water twice, and then dried at 60 °C until a constant dry weight was obtained [14]. The lipids were extracted from the dried microalgal biomass (0.1 g) using mixed solvents of chloroform: Methanol (2:1 v/v) at solvents to solid ratio of 10:1 and sonicated for 30 min before being centrifuged at 10,000 rpm for 20 min. The lipid extraction step was repeated for 2 cycles. The solvent fraction was collected and the solvent was evaporated under vacuum condition to obtain the microalgal lipids. Eqs. (1) and (2) were used to calculate the lipid content and lipid production, respectively [15];

$$\text{Lipid content (\%)} = \text{Extracted lipids/Dried biomass} \times 100 \quad (1)$$

$$\text{Lipid production (g/L)} = \text{Lipid content (\%)} \times \text{Dried biomass (g/L)} \quad (2)$$

Statistical analysis

The experiments were carried out at least in triplicate. The experimental data were presented as means and standard deviations. The data were statistically analyzed through a one-way analysis of variance and Duncan's test as a post hoc comparison test ($p < 0.05$).

Results and discussion

Isolation and purification of microalgae from hot springs

Table 1 shows the microalgae isolated from Tha Sathon hot spring and Krut hot spring in Surat Thani province, Thailand. The pH of hot spring water was neutral at 6 - 7, and the water temperature was 28, 56 and 59 °C. The microscopic observation was used to confirm the purity of the microalgae. Based on preliminary screening of their morphology as oleaginous species, 4 strains were selected. These include *Scenedesmus* sp. HP28, *Chlamydomonas* sp. HP59, *Chlorella* sp. HP56, and *Scenedesmus* sp. HP56 strains (**Table 1**). The capillary method has been proven to be an effective method to isolate target strains based on morphology. The isolation of oleaginous microalgae from a local lake in Thailand and obtained 6 groups of oleaginous species, including *Botryococcus*, *Chlamydomonas*, *Chlorella*, *Haematococcus*, *Nannochloropsis*, and *Scenedesmus*, based on the morphological characters [15]. Similarly, Dani *et al.* [18] also isolated native microalgal strains from the sludge of Mellat Park Lake in Tehran, Iran and obtained *Chlorella vulgaris* ISC23 strain that showed rapid growth and high lipid productivity.

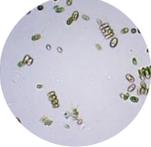
Effect of temperature on cell growth

Temperature is an important factor affecting growth rate and carbon fixation rate. The effect of temperature on cell growth of all microalgae was tested at 30, 35 and 40 °C, which are the temperature range in Thailand (**Figure 1**). It is obvious that all microalgae are thermotolerant and can grow at temperatures up to 40 °C. Two microalgae isolated from water bodies at 28 - 30 °C, *Scenedesmus* sp. HP28 and *Chlorella* sp. grew similarly at 30 and 35 °C. But at 40 °C, both microalgae grew much slower. This property indicates their mesophilic nature. *Chlamydomonas* sp. HP59 and *Scenedesmus* sp. HP56 grew better at 35 °C. *Chlamydomonas* sp. HP59 also grew well at 40 °C and *Chlorella* sp. HP56 grew best at 40 °C, indicating their thermophilic properties and potential use in tropical areas during the summer. Regarding microscopic images of 5 microalgae cultivated at different temperatures, there was no difference in size or shape of the microalgal cells (data not shown). Among the 5 strains tested, *Chlamydomonas* sp. HP59 is considered the most robust strain as it showed high cell growth in a broad range of temperature (30 - 40 °C). These results have confirmed that it is possible to obtain thermotolerant and thermophilic microalgae from hot springs.

Figure 2 shows the effect of temperature on the dried cell weight of 5 microalgae. At 30 °C, there was no significant difference between the dried cell weight of 3 microalgae, including *Scenedesmus* sp. HP28, *Scenedesmus* sp. HP56, and *Chlorella* sp., and those at 35 °C. *Chlorella* sp. HP56 and *Scenedesmus* sp. HP56 grew better at 35 °C and therefore gave higher biomass at this temperature. At 40 °C, only *Chlorella* sp. HP56 and *Chlamydomonas* sp. HP59 gave comparable dried cell weight. *Chlorella* sp. HP56 gave a higher dried cell weight at 35 °C when compared to that at 40°C, while *Chlamydomonas* sp. HP59 gave a higher biomass at 40 °C. Several researches have shown that *Scenedesmus* spp. and *Chlorella* spp. are the most widely studied for being used as biofuel feedstocks due to their great tolerance, fast growth rate, and high content of lipids/starch [8,17,18]. It has been reported that at temperature higher than optimum level, the microalgae might response to the heat stress by changing the activities of essential enzymes in photosynthesis, i.e. ribulose1,5-bisphosphate carboxylase/oxygenase (Rubisco) as well as cell

metabolisms [19]. Among most of the available microalgal species, *Chlamydomonas* spp. are known as thermotolerant or thermophiles, fast growing rates, high lipid/carbohydrate contents, and able to grow in saline water and wastewater [20].

Table 1 Sources and microalgae strains isolated from hot spring water.

Source	Temperature	pH	Microalgae strains	Name
Tha Sathon hot spring, Phunphin, Surat Thani, Thailand	28 °C	6.4	 <i>Scenedesmus</i> sp. HP28	SHP28
	59 °C	5.8	 <i>Chlamydomonas</i> sp. HP59	CHP59
Krut hot spring, Kanchanadit, Surat Thani, Thailand	56 °C	7.2	 <i>Scenedesmus</i> sp. HP56	SHP56
			 <i>Chlorella</i> sp. HP56	CHP56

The magnification of the microscope was $\times 40$.

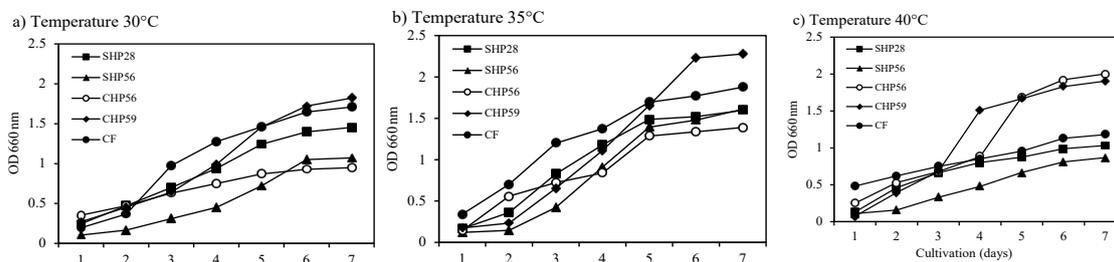


Figure 1 Effect of cultivation temperature on cell growth of microalgae under light intensity of $64 \mu\text{mol}\cdot\text{photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with light:dark cycles at 16:8 h and aerated with 5 % CO_2 in air at 0.01 vvm. SHP28: *Scenedesmus* sp. HP28, SHP56: *Scenedesmus* sp. HP56, CHP56: *Chlorella* sp. HP56, CHP59: *Chlamydomonas* sp. HP59, CF: *Chlorella* sp.

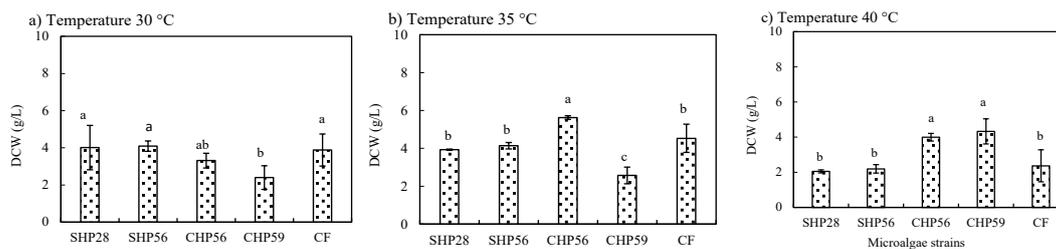


Figure 2 Effect of temperature on dried cell weight (DCW) of microalgae cultivated under light intensity of $64 \mu\text{mol}\cdot\text{photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with light:dark cycles at 16:8 h and aerated with 5 % CO_2 in air at 0.01 vvm for 7 days. SHP28: *Scenedesmus* sp. HP28, SHP56: *Scenedesmus* sp. HP56, CHP56: *Chlorella* sp. HP56, CHP59: *Chlamydomonas* sp. HP59, CF: *Chlorella* sp. Different small letters on the bars indicate significant differences between strains.

Effect of temperature on lipid content and lipid production

Environmental stresses such as nutrient limitation stress (nitrogen and phosphorus), salt stress, high temperature stress, and high light intensity stress are crucial strategies to enhance lipids and/or carbohydrates, or high-value products in marine and freshwater microalgae [22,23]. Nitrogen limitation has negative effect on protein synthesis and reduces photosynthetic rates, resulting in a metabolic flux changed toward lipid biosynthesis [11]. All microalgae could grow well under nitrogen-rich condition during 7 days but the lipid contents were relatively low in the range of 5 - 13 %. The microalgae responded to high temperature differently. The lipid content of mesophilic *Scenedesmus* sp. HP28 and *Chlorella* sp. slightly increased when the temperature was increased up to 40 °C. While the lipid content of thermophilic *Chlamydomonas* sp. HP59 at 30 °C was higher than that at 40 °C. It was possible that the temperature at 30 °C was stress for this thermophilic strain. **Figure 4** shows the effect of temperature on lipid production of microalgae strains. The lipid production depends on both dried cell weight and lipid content. Even though the microalgae gave high dried cell weight but if the lipid content was low, this will lead to the low lipid production. On the other hand, even though the lipid content increased with increasing temperature up to 40 °C but with the lower dried cell weight the lipid production became lower than those at low temperature. Therefore, not only the cell growth and lipid content should be concerned but the lipid production should be considered to select the suitable strain for lipid production.

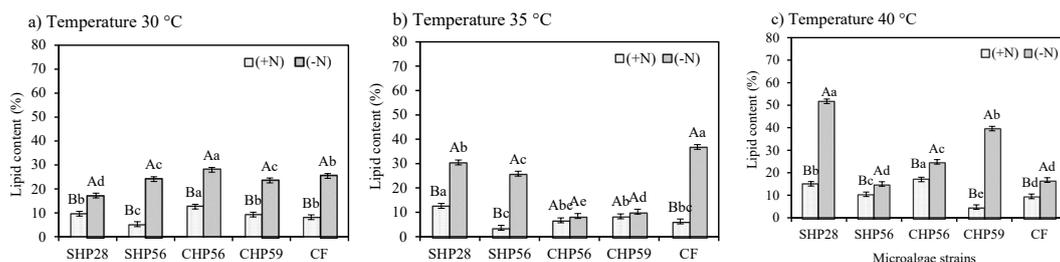


Figure 3 Effect of cultivation temperature on lipid content of microalgae under nitrogen-rich (+N) for 7 days and nitrogen-limited (-N) conditions. The microalgae were cultivated in nitrogen-rich medium for 7 days before being transferred to medium without nitrogen source and cultivated for 3 more days under light intensity of $64 \mu\text{mol. photon.m}^{-2} \text{s}^{-1}$ with light:dark cycles of 16:8 h. SHP28: *Scenedesmus* sp. HP28, SHP56: *Scenedesmus* sp. HP56, CHP56: *Chlorella* sp. HP56, CHP59: *Chlamydomonas* sp. HP59, CF: *Chlorella* sp. Different capital letters on the bars indicate significant differences between treatments. Different small letters on the bars indicate significant differences between strains.

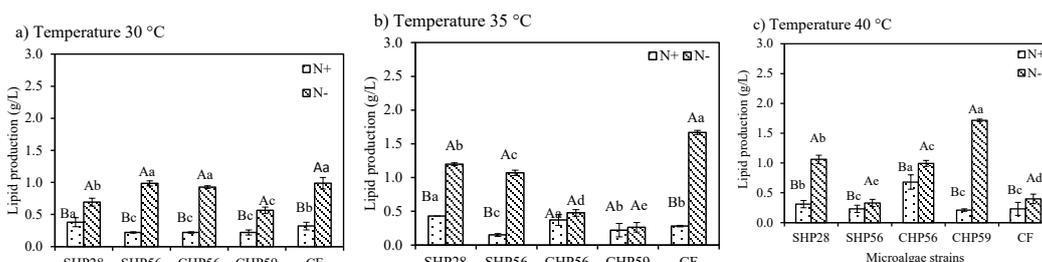


Figure 4 Effect of temperature on lipid production of microalgae under nitrogen-rich (N+) and nitrogen-limited (N-) conditions. The microalgae were cultivated in nitrogen-rich medium for 7 days before being transferred to medium without nitrogen source and cultivated for 3 more days under light intensity of $64 \mu\text{mol. photon.m}^{-2} \text{s}^{-1}$ with light:dark cycles of 16:8 h. SHP28: *Scenedesmus* sp. HP28, SHP56: *Scenedesmus* sp. HP56, CHP56: *Chlorella* sp. HP56, CHP59: *Chlamydomonas* sp. HP59, CF: *Chlorella* sp. Different capital letters on the bars indicate significant differences between treatments. Different small letters on the bars indicate significant differences between strains.

Among the strains tested under nitrogen rich condition, *Chlorella* sp. HP56 gave the maximum lipid production at 40 °C despite this strain gave lower dried cell weight. This was because the higher lipid content of this strain. It has been reported that the default pathway for microalgae under stresses would divert internal resources that would have gone into growth, to synthesize lipids in triacylglycerol form and accumulate them in cytosolic lipid bodies [19]. However, it should be noted that the heat stresses also affect photosystem II activity and both cell growth and lipid production might decrease with increasing temperature much over the optimal level [26]. Similarly, the cell growth, lipids and lutein production of *Chlamydomonas reinhardtii* under meso-thermophilic conditions, found that this strain gave the highest

biomass of 2.56 g/L and the lipids and lutein obtained were 893 and 23.5 mg/L, respectively at 35 °C [20]. In contrast, *Chaetoceros* sp. FIKU035 which could grow at a broad temperature range of 25 - 40 °C, accumulated a lower content of lipids at higher temperature [27]. Therefore, it can be concluded that the impact of temperature on the cell growth, lipid content, and lipid productivity of microalgae varies depending on the species.

Effect of temperature combined with nitrogen limitation

Nutrients stress and alterations of cultivation conditions are commonly used as lipid enhancement strategies [7,8]. As nitrogen limitation is one of the important factors in boosting lipid content in microalgae but gives low biomass production, 2-stage cultivation was performed. In the 1st stage, nitrogen-rich was applied to promote cell growth for 7 days, in the 2nd stage nitrogen-limitation was used to stimulate lipid accumulation for 3 days, (**Figure 3**). It should be noted that no obvious growth was observed during the nitrogen limitation, but the lipid contents of all microalgae were 2 - 4 folds boosted. When increasing temperature up to 40 °C coupled with nitrogen limitation, mesophilic *Scenedesmus* spp. SHP28 accumulated more lipids, likely due to the combined stresses [13]. It has been reported that the combined stresses might intensify the accumulation of stress-induced reactive oxygen species (ROS) [28], and microalgae have integral defensive mechanisms against the accumulation of ROS. These include accumulation of high lipid content [15]. While *Chlorella* sp. showed increased lipid content with increasing temperature up to 35 °C. The effect of temperature at 40 °C seemed to be deteriorating for *Chlorella* sp. and led to lower lipid content. Interestingly, *Chlamydomonas* sp. HP59, which could grow well at 40 °C, showed a positive response to nitrogen limitation. It has been reported that during nitrogen limitation, the cells stopped division and began to store lipids and reverse energy source. With this nitrogen limitation, the lipid content of microalgae could be enhanced by 2 to 3 folds [18]. However, to maximize microalgal lipid production, both cell growth and lipid content should be considered. The lipid production under various temperatures and nitrogen limitations is shown in **Figure 4**. It was found that a temperature at 35 °C combined with nitrogen limitation was suitable for lipid production by mesophilic microalgae like *Scenedesmus* sp. HP28 and *Chlorella* sp. At 40 °C, *Chlamydomonas* sp. HP59 showed the highest lipid production, likely due to its high dried cell weight and high lipid content. Two-stage cultivation of *Chlorella* sp. HS2 using nitrogen-replete and nitrogen-deplete methods led to the highest lipid content of 36.7 % [8]. These results have confirmed that extreme temperatures are preferred for attaining higher lipid production [29-31].

Conclusions

This study has shown that it is possible to isolate mesophilic and thermophilic oleaginous microalgae from hot springs. Mesophilic microalgae grew best at moderate temperature of 30 - 35 °C but accumulated high lipid content at high temperature of 40 °C, while thermophilic microalgae grew best at high temperatures but showed high lipid content at low temperatures. The nitrogen limitation could boost lipid content by 2 - 4 folds. The combined effect of high temperature and nitrogen limitation effectively increased

the lipid content of the mesophilic microalgae. The microalgae cultivated at high temperatures using a 2-stage cultivation strategy of nitrogen-rich and nitrogen-limited conditions gave the maximum dried cell weight and lipid content and led to the maximum lipid production. Among the strains tested, *Chlamydomonas* sp. HP59 was the thermophilic microalga that gave the maximum dried cell weight of 4.33 g/L and lipid production of 1.72 g/L at 40 °C. Thus, nitrogen limitation combined with high temperature was an effective strategy to increase lipid production. These strategies may contribute greatly to the development of biofuels and valuable bioproducts from microalgae.

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