CHAPTER 4

CULTIVATION DIATOM, *ACHNANTHIDIUM EXIGUUM* AARL D025-2, FOR LIPID PRODUCTION

4.1 Introduction

Biotechnological uses of diatoms are still being developed. Encourage advancement at the mechanical scale will rely on upon streamlining of the way of life process with the point of lessening expenses. Given the photoautotrophic status of the larger part of diatoms, microalgal cultures experience the unpleasant effects of the constraint of light dissemination, which requires the improvement of appropriate photobioreactors. In this manner, hereditarily built microalgae that might be developed under heterotrophic conditions. Other constraining variables, for example, supplements (phosphate, nitrogen, silicon), pH, temperature, bioturbation and numerous more should be considered. More often than not, metabolic anxiety conditions prompt an overproduction of the results of enthusiasm, with a reduction in biomass creation as an outcome. Outside societies in open lakes are committed to aquaculture for the nourishing of shrimps and bivalve molluscs (business generation), while shut axenic indoor/outdoor photobioreactors are utilized for biotechnological mixes of homogeneous synthesis (still at the research facility scale). Nevertheless, the ideal culture conditions that must be considered for photobioreactor plan, the localization of delivered metabolites (intra-or extracellular) may likewise be considered while picking the outline. Microalgal cell immobilization might be an appropriate procedure for application to benthic diatoms, which are touchy to bioturbation and additionally metabolites which might be overexpressed (Lebeau and Robert 2003).

Over the previous decade, diatoms have been perceived as an essential sort of algal biomass that can be a source of lipids for biodiesel feedstock (Popovich *et al.* 2012). Also, some diatoms have more oil content compared with other microalgae (Table 4.3). Some diatom strains have been distinguished as oleaginous species including *Cyclotella*

and *Aulacoseira* (Graham *et al.* 2011). Additionally, diatoms are heat tolerant species because of their silica frustules and because they can exist in extreme environments such as hot springs. Thus, a greater understanding of this thermotolerant property may be a significant advantage regarding the cultivation of diatoms in high-temperature arable areas that exist without temperature control such as in deserts. However, the growth process of hot spring diatoms has not yet been investigated. Thus, it has become even more important to acquire vital information about the factors involved in the growth and lipid production of each certain species of diatoms.

Achnanthidium exiguum (Grunow) Czarnecki 1994 is a benthic and pennate diatom that is usually present in freshwater as well as estuarine water bodies (Cleve and Grunow 1880). Naturally, *A. exiguum* exhibits at temperature gradients in the range of 39 to 45°C and a pH value of 7 in the sediment. However, initially, isolation using Bold Basal's Medium (BBM) was not achieved at 40°C. Moreover, the diatom could only be grown at 30°C. Hence, it should be noted that certain factors have a significant influence on the physiology of the diatom with regard to suppressing its thermostability. Environmental conditions such as light, temperature and nutrient availability have been found to influence algal growth and lipid accumulation (Guschina and Harwood 2006). However, a factor that has not yet been given much consideration is that of pH value. The pH value can be considered of significance with regard to the growing conditions and physiology of algal cells, for instance, the pH value is known to increase to higher levels as the structural lipids decrease in numbers (Spilling *et al.* 2013). Nevertheless, the effect of pH on the thermotolerance of diatom cultivation has not yet been investigated. A specific range of pH is one of the factors of efficiency for diatom growth and diatom biochemical composition. The aim of this research study was to focus on the effects of pH on the thermotolerance and lipid production of *A. exiguum* AARL D025-2 at various temperatures. In addition, the fatty acid methyl ester (FAME) of this diatom was also appraised for its potential to resolve concerns over the culture diatom's ability to achieve thermostability. Moreover, the FAME profile could be employed to evaluate the potential of this diatom for use as a raw material for biodiesel production. Additionally, the cultivation and application of this diatom will provide vital basic information for both the academic and industrial sectors.

4.2 Materials and Methods

The methods used to study hot spring diatoms cultivation for lipid production is presented in Figure 4.1

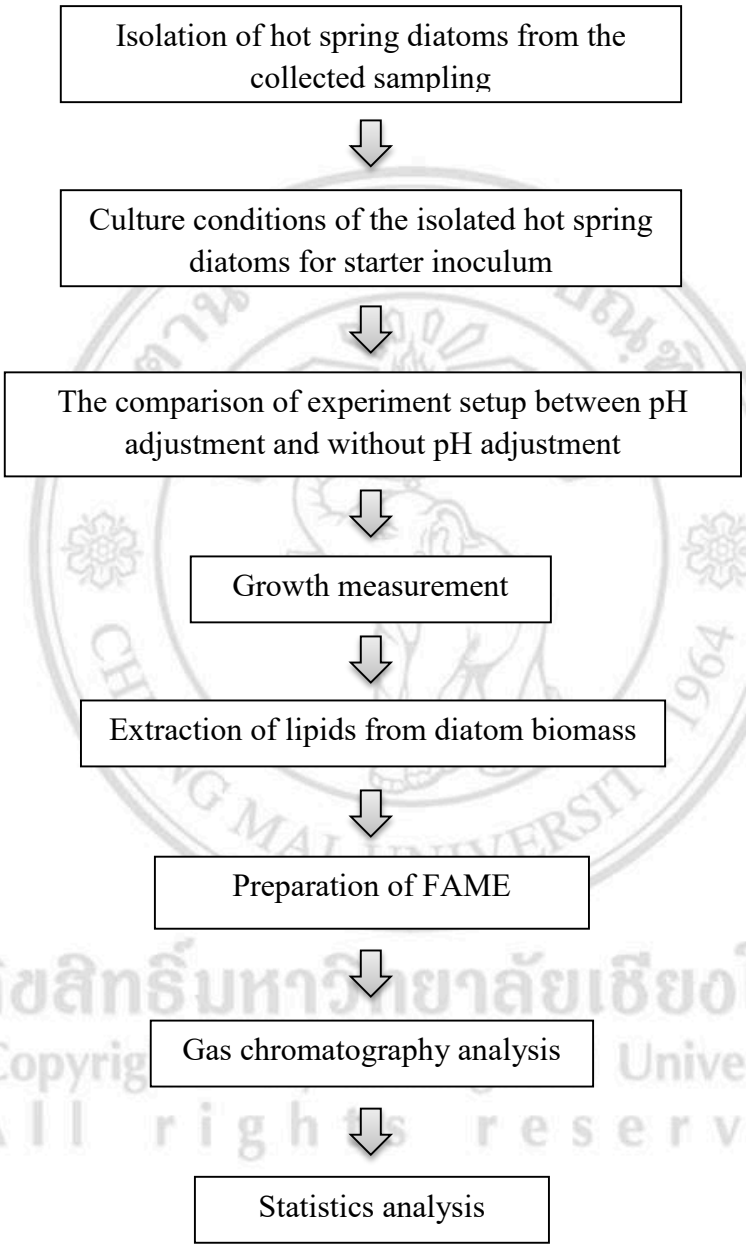

Figure 4.1 Flowchart diagram of hot spring diatoms cultivation for lipid production

4.2.1 Culture conditions

All the sampled diatom species were isolated for selectivity of culturing. The common species *Achnanthidium exiguum* AARL D025-2, that the fastest growing diatoms, was obtained from the culture collection of the Applied Algal Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University. This diatom was initially isolated from the Tha Pai Hot Spring, Mae Hong Son Province, Thailand. It was grown in Bold Basal's Medium (BBM) without pH adjustment (initial pH value of 6.8) at 30°C (Bischoff and Bold 1963). The light was constantly delivered by a light emitting diode (LED) with a light intensity of 70 µmol photons·m^{-2}·s^{-1}. The isolated diatom was inoculated in fresh medium for use as an inoculum for further studies.

4.2.2 Experimental setup

This study investigated the cultivation of the hot spring diatom *A. exiguum* AARL D025-2 at different temperatures and pH levels. The experiments were performed in triplicate in 500-mL Erlenmeyer flasks with 250 mL of the BBM. The initial culture was adjusted to be approximately 4 × 10^6 cells·mL^{-1} (OD_{450} of 0.4). The cultures were manually shaken every two days. Different pH values were achieved by the small addition of amounts of HCl and NaOH into the medium. The pH was adjusted at the beginning of the culturing process and maintained by adjusting each pH medium every two days, after the sampling. Cells were exposed to continuous light illumination at 70 µmol·photons·m^{-2}·s^{-1}. The first experiment involved independently growing diatoms at 30, 40 and 50°C over 14 days. This process was compared with diatom cultivation in BBM at a pH of 7.0 and a temperature of 30°C. The pH levels (pH 7, 8, 9 and 10) and various temperatures (30, 40 and 50°C) were investigated under the same conditions in the first experiment to define the effect of pH on diatom heat tolerance (Figure 4.2).

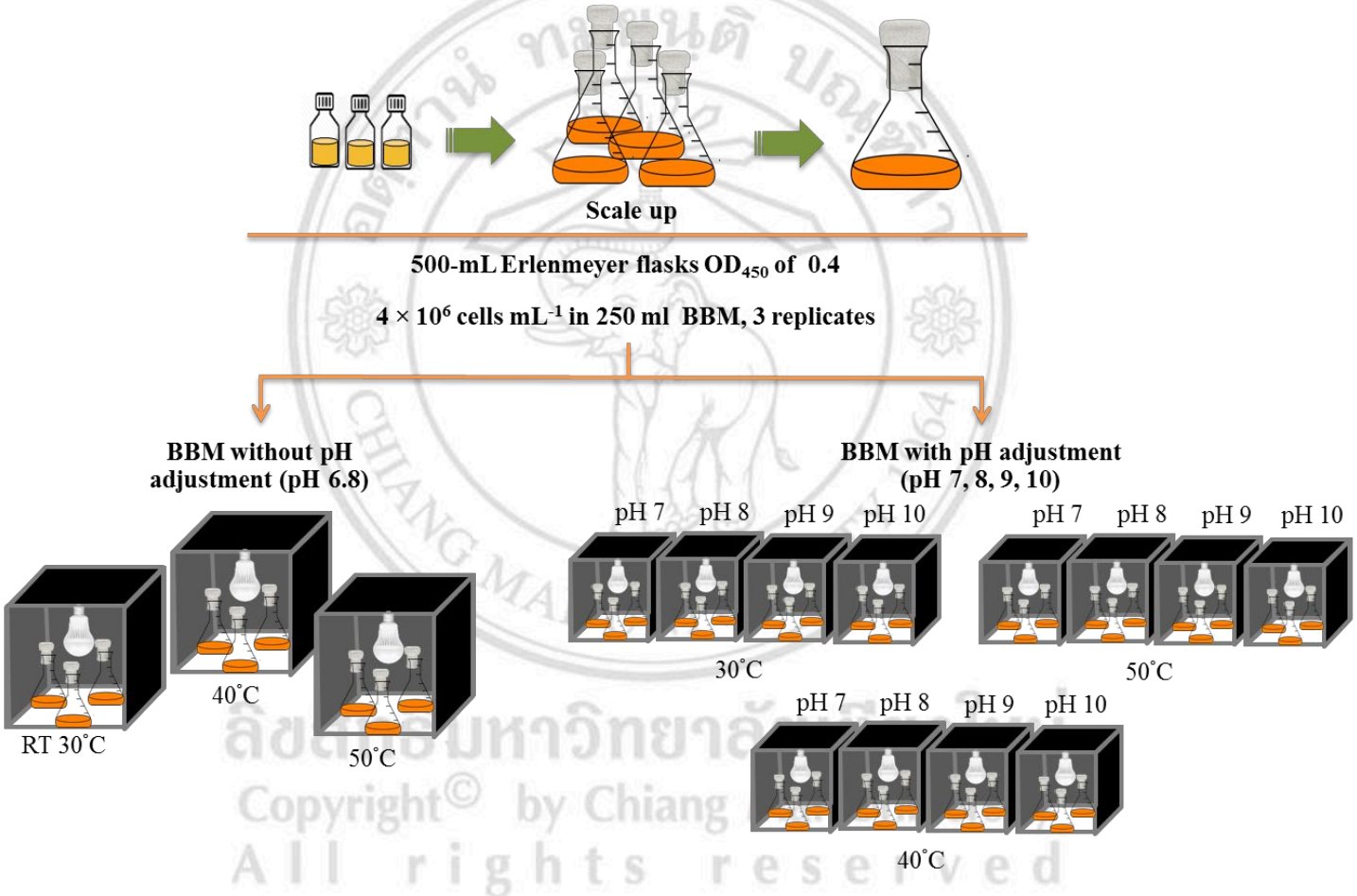

Figure 4.2 The experiment set up of species *Achnanthidium exiguum* AARL D025-2

4.2.3 Growth measurement

Cell numbers were observed every 2 days by counting the cells using a light compound microscope with a hemocytometer by loading 10 µL of culture onto the slide. The specific growth rate (μ) was estimated from the Eq. (1) (Krzemińska *et al*. 2013):

$$\mu\ (\text{day}^{-1}) = \frac{\ln(N_2/N_1)}{t_2 - t_1} \qquad (1)$$

where, N_2 and N_1 represent the concentrations of cell numbers at times t_2 and t_1, respectively.

4.2.4 Extraction of lipids from diatom biomass

A certain volume of diatom suspension was transferred to the preweighted-centrifuge tube and centrifuged at 2400×g for 5 min. The cell pellets were rinsed with acidified water, centrifuged, and then the cell pellets were dried at 60°C for 48 h, left in a vacuum dryer for cooling and weighed in order to measure dry cell-weight. The dry weight and biomass production were calculated using the ensuing equations (Eq. (2) and (3), respectively) (Moheimani *et al*. 2013):

$$\text{Dry weight (g)} = \text{W2} - \text{W1} \qquad (2)$$

Where W1 is weight of centrifuge tube (g), and W2 is weight of centrifuge tube plus dried diatom pellet (g)

$$\text{Biomass production (g L}^{-1}) = \frac{\text{DW } x\ 1000}{\text{V}} \qquad (3)$$

Where DW is the dry weight (g), V is the certain volume of the diatom suspension (mL).

Total lipids were extracted according to the method of (Cequier-SáNchez *et al*. 2008). The dried biomass was suspended in 5 mL of dichloromethane/methanol (2:1, v/v). The cell suspensions were exposed to sonication for 1 h (with 10sec on, 15sec off intervals on ice) at 40% amplitude using a probe sonicator (Vibra-Cell™ VC505, 350 W output with a 20 kHz converter). The solvent was collected and removed by evaporation at room temperature. Lipids were quantified gravimetrically, and the lipid content was expressed as a percent on a dry-weight basis. The percent value of lipid content was calculated using the ensuing equation (Eq. (4)), and the lipid production ($mg \cdot L^{-1}$) was obtained using the ensuing equation (Eq. (5)): (Moheimani *et al*. 2013):

$$\text{Lipid percentage } (\%) = \frac{\text{LW } x\ 100}{\text{DW}} \qquad (4)$$

Where LW is the dry lipid weight (g), DW stands for the dry biomass weight (g).

$$\text{Lipid production } (\text{mg} \cdot \text{L}^{-1}) = \frac{\%\ \text{LP} \times \text{BP} \times 1000}{100} \qquad (5)$$

Where % LP denotes lipid percentage, BP denotes biomass production ($g \cdot L^{-1}$).

4.2.5 Preparation of fatty acid methyl ester (FAME)

Lipids were saponified by being boiled with 1 mL of saponification reagent (15 g NaOH in 100 mL of 1:1 methanol: water) for 30 minutes in a water bath at 100°C. The samples were vortexed every 10 minutes while 2 mL of the boiling methylation reagent (1:1.18 methanol: 6 N HCl) was added. After that, the samples were then boiled at 80°C for 20 minutes. Afterward, the samples were cooled, and 1 mL of extraction solvent (1:1 distilled hexane: anhydrous diethyl ether) was added, and the samples were blended carefully. Subsequently, the remaining upper phase was washed with 3 mL of base-wash solution (1.2% NaOH w/v) (Miller and Berger 1985). The percentages of FAME content and FAME production were calculated in the same manner as the lipids.

4.2.6 Gas chromatography analysis

Fatty acids were analyzed using a gas chromatograph that was equipped with an HP-5ms capillary column (30 m x 0.25 mm GC 6890, Agilent Technology, USA) with flame ionization detector (FID). All operations were conducted under low light conditions and with the protection of helium. The temperature program was employed as follows. The initial temperature was recorded at 45°C. The lipids were placed in an oven that was set at 190°C (5 min) and increased to 250°C at 4°C min^{-1}. The experiment was held for 10 min; carrier gas: helium, 20 cm s^{-1}, detector temperature 250°C, and a split ratio of 50:1 was employed. A single sample run time was 57 minutes. FAME was recognized by chromatographic comparison with library entries in NIST/EPA/NIH Mass Spectral Library 2002 and the authentic chemical standards (Cat. No. 18920-1AMP, Sigma Chemical Co., St. Louis, MO).

4.2.7 Statistics investigation

Data were revealed as the mean ± standard deviation (SD). The differences between biomass, lipid and FAME production at the different temperatures and pH levels of the cultures were equaled statistically by one-way analysis of variance (ANOVA) and post-hoc Duncan tests using SPSS version 14.0. *P*- values of less than 0.05 were considered significantly different.

4.3 Results

In the first experiments, *A. exiguum* AARL D025-2 was cultivated under 30, 40 and 50°C in the original Bold Basal Medium without pH adjustment. The initial pH value of the medium was pH 6.8. The results revealed that *A. exiguum* AARL D025-2 could only be grown at 30°C and the highest number of diatom cells was recorded at 1.153 x 10^6 cells·mL^{-1} on the 14th day of the cultivation protocol (16 days). Consequently, when the pH of the medium was adjusted to pH 7, better growth was observed (Figure 4.3). The growth curve steadily increased after the 2nd day of cultivation. The cell number rapidly increased and reached 2.85 x 10^6 cell·mL^{-1} on day 14 of the experiment with a specific growth rate (μ) of 0.142 day^{-1} (Table 4.1). Additionally, the growth rate and pH level of

Achnanthidium exiguum AARL D025-2 when compared to other diatoms were found a higher growth in high pH level than others (Table 4.2).

Table 4.1 Maximum specific growth rate and division rate of *Achnanthidium exiguum* AARL D025-2, cultivated at various pH values and temperatures

Not adjudged pH	Maximum specific growth rate (day^{-1})	Adjudged pH condition	Maximum specific growth rate (day^{-1})
T30 pH 6.8	0.072	T30 pH7	0.143
T40 pH 6.8	-0.061	T30 pH8	0.072
T50 pH 6.8	-0.650	T30 pH9	0.152
T30 pH 7	0.142	T30 pH10	0.081
T40 pH 7	0.004	T40 pH7	0.092
		T40 pH8	0.119
		T40 pH9	0.119
		T40 pH10	0.092

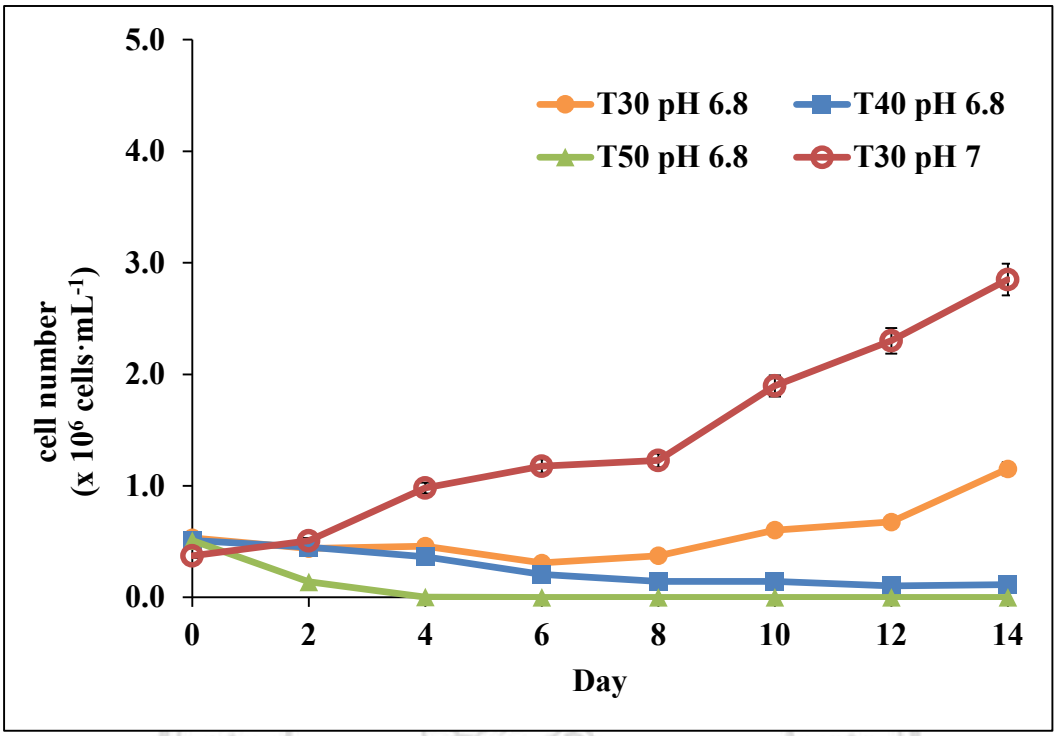

Figure 4.3 Growth of *Achnanthidium exiguum* AARL D025-2 in BBM medium without pH adjustment at different temperatures and compared with pH 7 adjusted-BBM medium at 30ºC. The data are expressed as mean ± standard deviation (SD), n = 3

By these results, the diatom growth appeared to be sensitive to the pH level. The diatoms were further cultured at various levels of pH and temperatures of 30, 40 and 50°C. The results revealed that a maximum growth at 30°C was obtained at a pH value of 7 and a 14-day interval (Figure 4.4), while the highest level of growth at 40°C was achieved at a pH value of 9 (Figure 4.5). However, at 50°C, the diatoms could not survive at any pH value (Figure 4.6).

Table 4.2 The comparison of pH level and growth rate from diatoms cultivated in different conditions.

Microalgae	Type	pH	μ (day^{-1})	Refs.
Skeletonema costatum	Marine diatom	9.4	0.4	(Taraldsvik and Myklestad, 2000)
Chaetoceros muelleri	Marine diatom	8.2	0.25	(Thornton, 2009)
Phaeodactylum tricornutum and *Amphiprora* sp.	Marine diatom	7.5	0.3	(Spilling *et al.*, 2013)
Fragilariopsis cylindrus	Polar diatom	8.0	0.9	(Pancic *et al.*, 2015)
Achnanthidium exiguum AARL D025-2	Hot spring diatom	9.1	1.1	This study

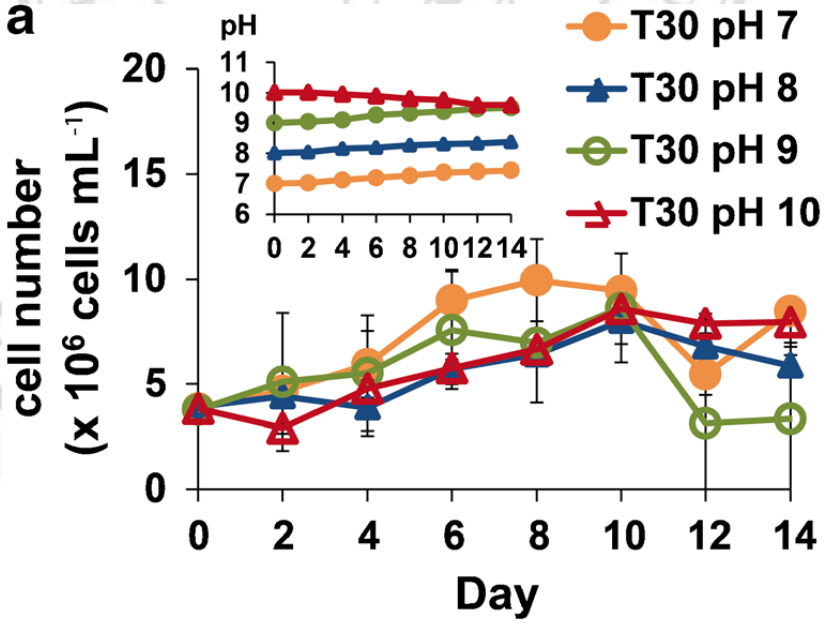

Figure 4.4 Growth of *Achnanthidium exiguum* AARL D025–2 at 30 °C and pH values over 14 days. The data are expressed as mean ± standard deviation (SD), n = 3

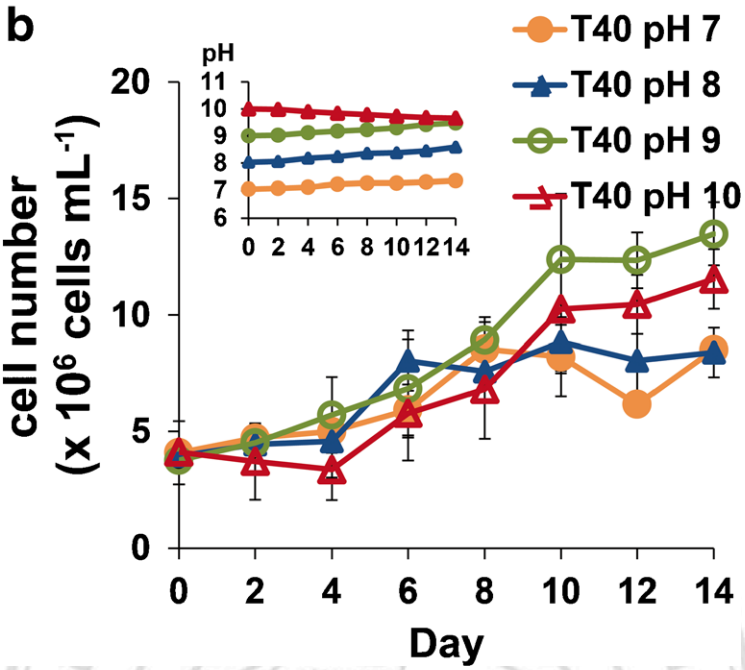

Figure 4.5 Growth of *Achnanthidium exiguum* AARL D025–2 at 40 °C and pH values over 14 days. The data are expressed as mean ± standard deviation (SD), n = 3

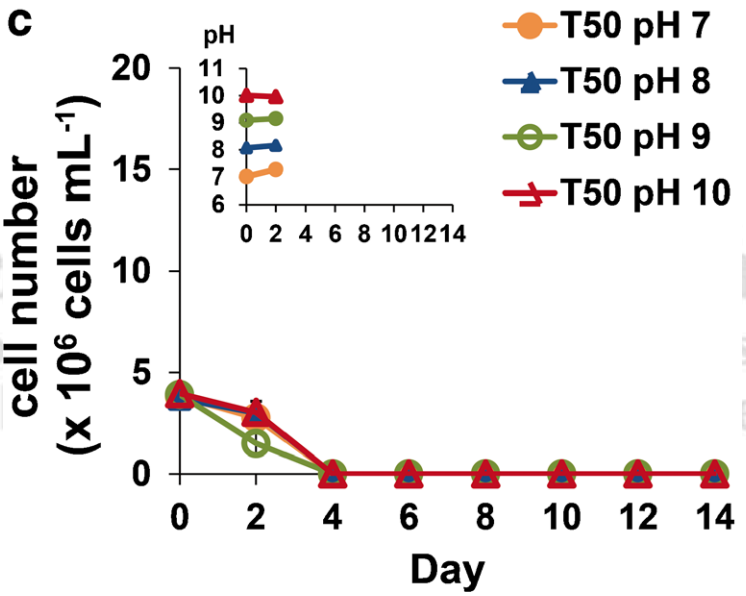

Figure 4.6 Growth of *Achnanthidium exiguum* AARL D025–2 at 50 °C and pH values over 14 days. The data are expressed as mean ± standard deviation (SD), n = 3

Table 4.3 Oil content of some microalgae (Chisti, 2007)

Microalgae	**Oil content (% dry weight)**
Ankistrodesmus sp.	24-31
Botryococcus braunii	25-75
Chaetoceros muelleri	33.6
Chlorella vulgaris	5-58
Chlorella sp.	28-32
Cylindrotheca sp.	16-37
Dunaliella tertiolecta	16.7–71
Dunaliella sp.	17.5-67
Nannochloropsis sp.	31-68
Nitzschia sp.	45-47
Pavlova salina	30.9
Schizochytrium sp.	50-77
Skeletonema sp.	13.3–31.8

Thus, the results of this investigate indicate that the tolerant ability of this species tends to be influenced by the pH level. In both temperature, the biomass production recorded from alkaline pH-medium (pH 10) was statistically significantly greater than that of the specimens exposed to the neutral-weak alkaline pH-medium (pH 7-8) (Table 4.4). The maximum level of biomass production was recorded with cultivation at a pH value of 10 at both temperatures. In contrast, the factor affecting lipid production was found to be temperature, while pH level did not display a significant effect. From this result, the average level of lipid production at 40°C was found to be significantly higher than at 30°C.

Table 4.4 Biomass Lipid and FAME production of *Achnanthidium exiguum* AARL D025-2 cultivated at various pH values and temperatures

	Biomass production ($g \cdot L^{-1}$)	% Lipid	Lipid production ($g \cdot L^{-1}$)	% FAME	FAME production ($mg \cdot L^{-1}$)
T30 pH7	5.74 ± 0.41a	2.90	167.5 ± 45.96a	0.76	43.80 ± 5.30
T30 pH8	5.69 ± 0.65a	2.10	91.25 ± 28.28a	0.63	35.80 ± 3.02
T30 pH9	5.43 ± 0.68a	5.12	268.12 ± 128.16a	0.43	23.30 ± 0.71
T30 pH10	7.23 ± 0.13c	2.48	245.62 ± 45.06a	1.40	101.30 ±4.83
T40 pH7	5.61 ± 0.41a	3.18	183.75 ± 190.92a	0.75	42.10 ± 2.73
T40 pH8	6.21 ± 0.64ab	9.68	682.50 ± 291.68b	0.64	40.00 ± 2.91
T40 pH9	6.72 ± 0.19bc	17.72	1,211.88 ± 25.63c	0.74	50.00 ± 4.94
T40 pH10	7.34 ± 0.35c	1.26	89.38 ± 38.01a	0.34	25.00 ± 1.42
F value	7.431		27.793		0.794
P value	0.001		0.000		0.604

The data were expressed as the mean ± standard deviation (SD) of the triplicate. Letters of the English alphabet designate statistical significance at $p < 0.05$ using one-way ANOVA test with post-hoc Duncan's multiple-range test

Although FAME production was neither significantly affected by temperature nor pH, these two factors did reveal an influence on FAME profiles (Table 4.5). Some saturated fatty acids identified at a pH range of 7-8 for both temperatures were higher than those in the pH range of 9-10, while the amounts of unsaturated fatty acids were in contrast. However, the level of saturated fatty acids recorded at a pH range of 9-10 and 40°C was elevated higher than it was at 30°C. The main FAMEs produced from all treatments were palmitic acid methyl ester (C16:0) and Cis-10-Heptadecenoic acid (C17:1n-1cis). The major FAMEs in all treatments were initiated at a range of C16-C18, but a concentration of C16-C18 was not found to be effective with a pH of the cultivation. The percentage of C16-C18 from the pH value of 10 was lower than those of the other pH values.

Table 4.5 FAME profile of *Achnanthidium exiguum* AARL D025-2

FAME	% Chemical composition							
	T30 pH7	T30 pH8	T30 pH9	T30 pH10	T40 pH7	T40 pH8	T40 pH9	T40 pH10
Decanoic acid (C10:0)	-	-	-	-	-	0.45	-	-
Tridecanoic acid (C13:0)	-	-	0.43	-	-	0.32	-	-
12-Tridecenoic acid (C13:1n-1)	-	-	-	0.38	-	-	-	-
Myristoleic acid (C14:1n-5)	-	-	-	-	-	-	-	2.80
Pentadecanoic acid methyl ester (C15:0)	3.33	3.15	3.71	3.56	4.10	4.37	4.83	5.12
Palmitic acid methyl ester (C16:0)	47.05	42.75	34.81	33.37	36.03	37.53	35.47	32.50
Cis-10-Heptadecenoic acid	32.62	33.47	38.71	36.64	32.76	35.97	36.23	31.62
Stearic acid methyl ester (C18:0)	8.06	9.74	5.01	5.36	9.39	7.67	6.05	6.50
Oleic acid methyl ester (C18:1n9c)	-	-	1.80	1.13	-	0.87	0.98	-
Arachidonic acid (C20:4ω-6)	-	-	-	-	-	-	-	2.24
Others	8.94	10.88	15.56	19.57	17.76	12.82	16.43	19.23
Saturated FA	58.44	55.65	43.94	42.29	49.48	50.34	46.36	46.35
Unsaturated FA	32.62	33.47	40.50	38.15	32.76	36.84	37.21	34.42
C16-18 FA	87.73	85.96	80.33	76.50	78.18	82.04	78.73	70.62

4.4 Discussion

This study found that abnormal conditions affected the growth and ability of diatoms to withstand the high temperatures of *A. exiguum* AARL D025-2. This result agrees with a previous study which found that diatom production could occur as a result of environmental conditions caused by changes in relative growth factor levels. These signals are often reflected by physical changes that are related to the stability of a thermocline, exposure to turbulence, nutrient or thermal conditions, or changes in pH (Bradbury *et al*. 2002). Even though this diatom species is a hot spring diatom which originally thrives in a range of temperatures from 37-49.5°C, this diatom could not be grown in BBM without pH adjustment at 40°C and 50°C. According to the fact that the natural environment of this diatom species is approximately pH 7, the pH level of the BBM was adjusted to 7.0, and consequently, a high growth rate was observed.

From the study of diatom cultivation at various temperatures and pH levels, the diatom *A. exiguum* AARL D025-2 could be effectively grown at 40 °C under a pH range of 9-10, for which the cell concentration was found to be higher than that of 30 °C. This phenomenon was validated by another study that found that a significant interaction between temperature and pH affected the growth of the diatom *Fragilariopsis cylindrus* (Grunow) Helmcke & Krieger (Pancic *et al*. 2015). Furthermore, the findings of the optimal growth conditions for this thermotolerant diatom to be grown at high temperatures and high pH levels should indicate that it would be advantageous for open-pond cultivation without contamination by other microorganisms.

Temperature also influenced the lipid production of the diatom *A. exiguum* AARL D025-2. The amount of growth observed at 40°C significantly increased lipid production when compared to the amount of growth observed at 30°C. Also, in the high alkaline medium (pH 9-10), the percentage of saturated fatty acids (SFAs) at 40°C significantly increased when compared with those of 30°C. The previous study of Johansen *et al*. (1990) found that the fatty acid composition of the marine diatom *Chaetoceros muelleri* Lemmermann changed in response to the stress conditions of temperature, ionic composition, and conductivity on growth rates related to nutrient deficiencies. However, the processes connected with fatty acid alteration of the diatom *A. exiguum* AARL D025-

2 may differ from those associated with a range of stresses. This finding was similar to that of the previous study of Rousch *et al.* (2003), which suggested that the rising temperature point (24°C to 40°C) is responsible for changes in fatty acid composition, especially about the degree of SFAs of the thermo-tolerant diatom *C. muelleri* Lemmermann. However, the alteration of fatty acid components of this diatom did not involve heat stress. It may be distinctive in that these organisms can be influenced by alkaline culture conditions, which was the case with *A. exiguum* AARL D025-2. However, the cellular mechanism of this phenomenon is still unclear, which may be revealed by the transcriptome analysis in future studies.

From the SFAs accumulation during the culture temperature escalation, the saturated fatty acid ratio may play a major role in the thermotolerant properties. Temperature could affect membrane fluidity under either hyperthermic or hypothermic condition. The increase in membrane fluidity from 30 °C to 45 °C of a temperature upshift has been referred to as superfluidity (Mejía *et al*. 1999). Renaud *et al*. (1995) found that microalgae tended to maintain high levels of PUFAs at low temperatures because fatty acids with a high degree of unsaturation are essential for the maintenance of membrane fluidity at low temperatures. Thus, on the other hand, the accumulation of fatty acids at greater levels of saturation under hyperthermic conditions may occur for contrary motives.

The highest level of lipid production was found in the cultivation experiment at 40°C at a pH value of 9. However, FAME production was recorded at 30°C at a pH value of 10. This may be because of the non-specific factors of the solvent extraction process. Dichloromethane: methanol 2:1 was used as the solvent in this study which may effectively extract other compounds in addition to lipids. The most imperative finding related to the utilization of FAME in this diatom was specified in a range of C16-C18 with a relatively high proportion of palmitic acid (C16:0) and Cis-10-Heptadecenoic acid (C17:1n-1cis). This result was by those of previous studies. Prestegard *et al.* (2015) found that the highest level of the fatty acid of *Phaeodactylum tricornutum* Bohlin strain cultivation was palmitoleic acid (16:1 n-7). Additionally, the cultures of the diatoms *Skeletonema costatum* (Greville) Cleve and *Navicula gregaria* Dokin revealed neutral lipids, including myristic, palmitic, palmitoleic and oleic acids, as the main fractions of

total lipids in both species (Popovich *et al*. 2012). These palmitic and stearic acids are considered preferable fatty acids for biodiesel production (Knothe 2008). Thus, the FAME of this diatom may be used for biodiesel production. However, a somewhat different fatty acid profile may impact on the parameters of the physical properties of the oils (Martínez-Force *et al*. 2009).

4.5 Conclusion

Our data support the hypothesis that pH value affects the growth and heat tolerance of *A. exiguum* AARL D025-2. Cell growth obtained at 40°C at a pH value of 9 revealed the highest levels of biomass and lipid production. This study was primarily conducted to assess culture conditions and FAME profiles of hot spring diatoms. FAME in this diatom was specified in a range of C16-C18 with a relatively high proportion of palmitic acid (C16:0) and Cis-10-Heptadecenoic acid (C17:1n-1cis). The highest FAME production was recorded at 30°C at a pH value of 10.