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Flocculation efficiency and quality of flocculated algae with chitosan at different pH values

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ABSTRACT

This study was conducted to determine the effects of different pH values on the flocculation efficiency of Chaetoceros calcitrans and using flocculated algae as diet for nursing sweet snail (Babylonia areolata) larvae. In the 1st experiment, C. calcitrans were flocculated by chitosan at three pH levels as follows: (1) control (maintaining at pH=9.0); (2) adjusted to 7.0 and (3) adjusted to 5.0. In the 2nd experiment, flocculated algae were stored at temperature of 4°C in 15 days to determine the viability and ability to grow after re-inoculation. In the 3rd experiment, flocculated algae were used for feeding sweet snail larvae in 20 days. Algae flocculation reached the highest efficiency (91%) after 4 hours at pH 5.0. At control pH treatment (pH 9.0) C. calcitrans obtained highest cell viability (52.23%). After re-inoculation at initial density of 500,000 cells/mL, the highest cell density was obtained at day 15 in pH 7 (8,750,000 cells/mL). After 20 days feeding with different flocculated C. calcitrans, the sweet snail larvae in control pH obtained not only highest survival rate (72.52%) but also the highest shell length and height (1938.89 and 1041.67µm, respectively). The findings indicated that it was necessary to remain pH value from 7.0-9.0 in order to obtain the high algal cell viability after flocculation with chitosan and effectiveness for using as diet for sweet snail larvae.

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1 INTRODUCTION

Microalgae are widely used in aquaculture as live feeds for several aquaculture species such as molluscs, fish, Penaeid prawn larvae and rotifers (Brown and Robert, 2002). One of the problems in large scale production of microalgae is to select low cost and suitable technique. In practices, there are a lot of methods to harvest microalgae such as centrifugation and filtration, but flocculation was preferred for harvesting large cells like microalgae due to its low cost compared to other methods (Bilanovic *et al*., 1988).

Up to now, there are many studies conducted to evaluate different chemicals to flocculate algae and utilization for feeding molluscs (Lubian, 1989; Brown, 2002; Liu *et al*., 2013). In those, chitosan was suggested as a chemical which had high flocculation ability and non-toxic to aquatic organisms (Lubian, 1989). The flocculation efficiency of chitosan depends on many factors such as temperature, salinity, light, the concentration of algae etc. pH is also important factor which affects directly to dissolubility and flocculation efficiency of chitosan. Flocculation efficiencies of freshwater micro-algal cells were studied in terms of pH varia-

tions. The micro-algal cells began to coagulate when the pH decreased from pH 6.7, 5.0, 4.5 to about 4.0 and lower than 2.0. The optimum flocculation efficiency was achieved at pH 4.0 (Liu *et al.,* 2013) for freshwater micro algae. However, there are limited studies about the effect of pH on the dissolubility and algae flocculation of chitosan. It is also necessary to know the flocculation efficiency of chitosan at different pH levels, which contribute to flocculation in short time and avoid algal clusters. In addition, it is necessary to evaluate the potential use of flocculated algae at different pH values as diet for filter feeder molluscs in the hatchery. This study is aimed to evaluate effects of pH on algae flocculation of chitosan and utilization to feed larvae of sweet snail *Babylonia areolata.*

2 METHODS

2.1 Effects of different pH values on flocculating *Chaetoceros calcitrans algae* with chitosan and quality of flocculated algae during preservation

2.1.1 Flocculating algae by chitosan at different pH values

Microalgae *C. calcitrans* were cultured at the density of 500×10^3 cells/mL in air-conditioned room at temperature of 26°C under continuous illumination by white fluorescent (4500-5000 lux), and aeration was provided continuously by air pump. Algae were harvested at the density of 5.10^6-6.10^6 cell/mL after 5-7 days of cultivation, and then used for flocculation experiment. Subsequently, algae were flocculated by chitosan at the concentration of 40 mg/L at three pH levels (treatments) with three replicates per each as follows: (1) control (non-adjusted and remained pH=9.0 after flocculation), (2) adjusted pH= 7.0 and (3) adjusted pH= 5.0. pH in flocculated medium was adjusted by adding HCl 0.1N until the required pH value. The flocculation process was observed, and samples were taken after every hour from algal medium to evaluate the flocculation efficiency. Flocculation efficiency of *C. calcitrans* (%) was evaluated by comparing the remained cell density in the super natant (upper part of algae bottle) with the initial density (i.e. before adding chitosan).

2.1.2 Evaluating the quality of flocculation algae in the preservation process

After siphoning to harvest, flocculated algae at different pH levels were stored in 40 mL Falcon tubes and located in the refrigerator at 4°C during 15 days. Algal quality was evaluated by 2 parameters as follows:

Algae density (cells/mL)

Samples were taken 3 days intervals for a period of 15 days. Then, the algal density was counted under microscope by using Improved Neubauer counter.

Algae viability (%)

Evan's Blue was used to stain the algae cells. Live cells (intact cells) were observed and counted with Improved Neubauer counter under light microscope (magnification of 40). Intact algae cells possess un-break cell wall and non-faded cytoplasmic.

2.1.3 Determining viability of flocculated algae by re-inoculation after preservation

After 15 days of preservation, flocculated algae from different pH values were re-inoculated at initial stocking density of 500×10^3 cells/mL in air conditioned room at 26°C, and Walne solution was supplied as the nutrient medium. Growth performance of algae was examined by sampling and determining the density at day 1, 3, 6, 9, 12 and 15 during culture period by using Improved Neubauer counter.

2.2 Effects of flocculated algae at different pH values on growth and survival rate of sweet snail (*Babylonia areolata)* larvae

2.2.1 Experiment design and management

Flocculated algae with chitosan at different pH as conducted in the 1st experiment were kept in the fridge at 4°C, and then used to feed to sweet snail larvae. This experiment included three feeding treatments with three replicates each as following: (1) flocculated algae at non-adjusted pH (Control, pH=9.0), (2) flocculated algae at adjusted pH to 7.0 (T1) and (3) flocculated algae at adjusted pH to 5.0 (T2). *Babylonia areolata* larvae were cultured in 100-liter plastic tanks at the density of 100 larvae/L. The salinity was maintained at 30ppt, and aeration was supplied continuously. Nursing period was lasted for 20 days and sweet snail larvae were fed 2 times per day (at 8:00 AM and 6:00 PM) with flocculated *Chaetoceros* algae at the density of 10×10^3 cells/mL.

2.2.2 Sampling methods

Daily water temperature was recorded twice a day at 7:00 and 14:00 using a thermometer. pH was measured weekly by pH meter (HANA). The concentration of NH_4^+/NH_3, NO_2^- and alkalinity (mg/L $CaCO_3$) was monitored weekly using test-kit (Sera, Germany).

Survival rate of sweet snail larvae was determined at day 10 and 20 of culture period. The number of snail larvae was counted to calculate the survival rate. Metamorphosis rate (%) of snail larvae was

also determined at the end of experiment by counting the numbers of fresh snail juveniles in each tank. At the same time, 10 snail larvae in each tank were randomly collected to measure the length and height by micro-ruler installed in binocular. The density of algae was determined 2 times a week to assess the filtration rate of snail larvae before and after feeding in all treatments.

2.2.3 Data analysis

The data were analyzed for mean values, standard deviation by using Excel software, and Duncan test (one-way ANOVA analysis) was applied to compare the significant difference of collected parameters among treatments at $p<0.05$ using SPSS program version 16.0.

3 RESULTS AND DISCUSSION

3.1 Effects of different pH values on flocculating *C. calcitrans* algae with chitosan and quality of flocculated algae during preservation

Flocculation efficiency

The flocculation efficiency of *C. calcitrans* with pH adjustments was significant difference ($p<0.05$) among treatments after 3 hours of flocculation (Table 1). The flocculation efficiency was drastically increased with adjustment pH from 9 to 5. Flocculation efficiency obtained at pH 9 was only 57% and increased to 88% at pH 5 after 4 hours of flocculation process. This result was in accordance with Heasman *et al*. (2001) that using chitosan 80mg/L and then pH adjustment at 5.27 led to the best results of flocculation efficiency. The flocculation efficiency reaching >80% after 4 hours of observation with pH 5 and 20 mg/L of chitosan was recorded.

Table 1: Flocculation efficiency (%) of *C. calcitrans* algae with chitosan at different pH

Duration (hours)	Control (pH 9)	pH 7	pH 5
1	20 ± 7.76^a	40 ± 8.75^b	54 ± 1.97^c
2	34 ± 3.63^a	53 ± 9.34^b	64 ± 2.05^c
3	41 ± 7.98^a	56 ± 11.19^a	79 ± 4.74^b
4	57 ± 7.36^a	60 ± 7.86^a	88 ± 5.36^b
5	63 ± 4.70^a	64 ± 9.77^a	-
6	69 ± 3.66^a	75 ± 5.50^a	-
7	81 ± 2.91^a	85 ± 0.72^b	-

The values in the same row with different letters indicating significant difference ($p<0.05$)

Quality of C. calcitrans during preservation period

The density of *C. calcitrans* flocculated with 40 mg/L of chitosan at three different pH values was reduced after 15 days of preservation at 4ºC (Fig. 1). The algae density in pH 5 decreased slowly at the beginning and was statistically different from other treatments ($p<0.05$). In addition, algae cells in pH 5 were observed with abnormal shape and greenish color due to excretion of pigments from the cells.

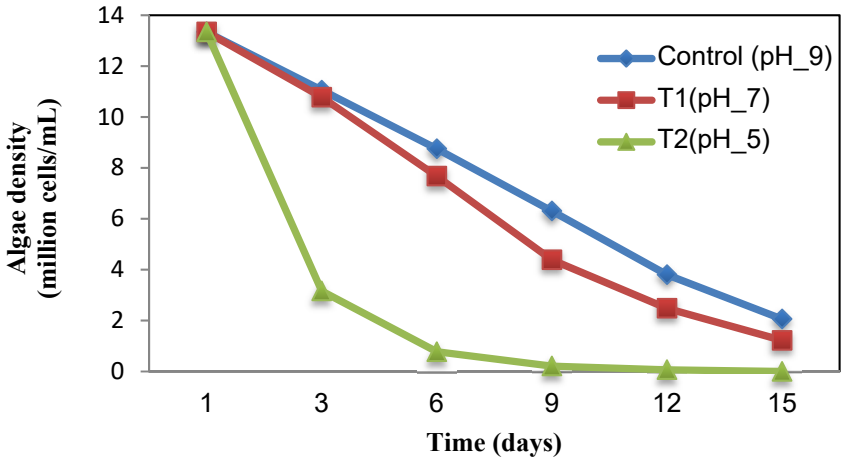

Fig. 1: Algae density (million cells/mL) during 15 days of preservation period

The percentage of viable cells (%) of *C. calcitrans* during 15 days of preservation was illustrated in Fig. 2. The highest cell viability ($52.23 \pm 4.50\%$) was obtained at control treatment and significantly different ($p<0.05$) compared to T1 and T2 ($39.95 \pm 5.94\%$ and 0%, respectively). The results were in accordance with the study of Harith *et al*. (2009) that after preservation at 4ºC, cell viability was significantly changed with reduced pH, and the highest viability was obtained at pH 8 (81%).

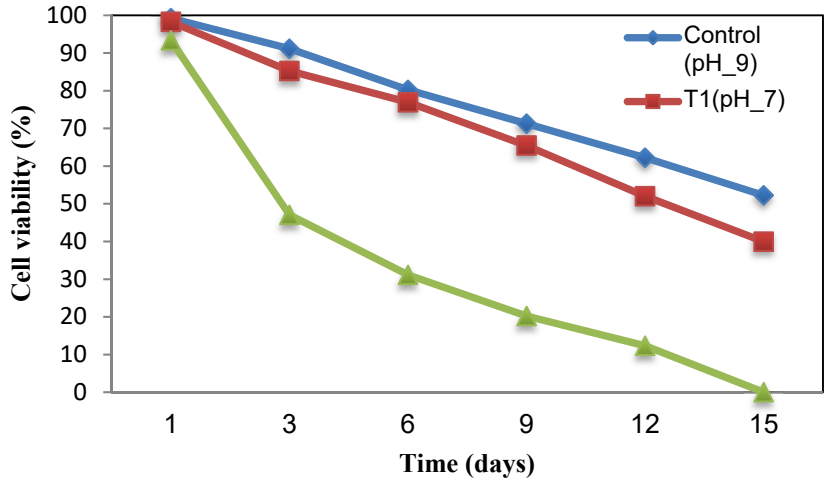

Fig. 2: Percentage of viable cells (%) during 15 days of preservation period

The density of algae by re-inoculation after 15 days of preservation

After 15 days of preservation, the flocculated algae were re-inoculated and observed the growth performance. The best results were in control treatment (8,570,000 cells/mL) at day 12 which was significantly higher than in T1 and T2 ($P<0.05$). Algae *C. calcitrans* could not grow in T2 after re-inoculation (Fig. 3). In this treatment, viable cells were not observed at day 15 of preservation. At pH 5, algae cells might have problems with the osmo-regulation, causing the cell to shrink, and could not grow as normal.

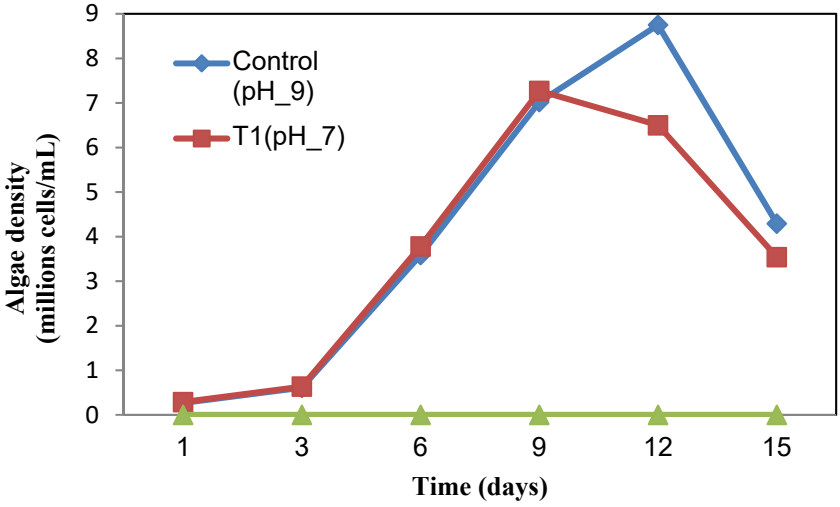

Fig. 3: The density of algae by re-inoculation after 15 days of preservation

3.2 Effects of flocculated algae at different pH values on growth and survival rate of sweet snail larvae

3.2.1 Water quality parameters

Average temperature ranged from 24.9 to 28.4 °C during the experiment. There was no significant difference of temperature among treatments. In the morning, temperature in control treatment (25.9 ± 0.08°C) was similar to T1 (25.8 ± 0.09°C) and T2 (25.8 ± 0.10°C). In the afternoon, temperature was around 27.7°C and not significantly different among treatments (Table 2). The results from the study of Jinfeng *et al*. (2005) on rearing *Babylonia areolata* larvae indicated that the optimum temperature range for larval growth was 25-30°C.

pH values in all treatments ranged from 8.4-8.5; the highest pH presented in control treatment (8.5 ± 0.05) and T1 (8.5 ± 0.03) were not significantly different from T2 (8.4 ± 0.06). pH variation was in an acceptable range for growth of sweet snail larvae as Nguyen Thi Xuan Thu (2006) suggested that the suitable pH range for rearing sweet snail larvae from 6-9.

Concentration of NH_4^+/NH_3 varied from 0.3 ± 0.14 mg/L to 0.4 ± 0.15 mg/L, and that was not significantly different among treatments ($p>0.05$). In the study of Alonso and Camargo (2009), three ammonia concentrations (0.02, 0.07, and 0.13 mg N–NH_3/L, respectively) were used to test the activity of snails (as mean time to start normal movement), and immobility were recorded for each treatment after 0, 10, 20, 30, and 40 days of continuous exposure. These authors found that *P. antipodarum* presented a high tolerance to lethal long-term effects of nonionized ammonia, as no animal died during the bioassay. However, the behavioral activity of snail *P. antipodarum* was very sensitive at the range of ammonia concentrations from 0.07 to 0.13 mg N-NH_3/L. The results of present study showed that NH_4^+/NH_3 concentrations in all treatments were higher than in previous study and it might be stressful to snail larvae.

Nitrite concentrations in all treatments fluctuated and increased gradually at the end of the experiment. Alonso *et al*. (2006) has estimated water quality criteria, NO_2^- in the range of 0.08-0.35 mg/L may be adequate to protect sensitive aquatic animals. In present study, nitrite concentration ranged from 2.6 ± 0.2 mg/L to 3.0 ± 0.2 mg/L. The level of nitrite became higher during nursing period that came from uneaten flocculated algae or feces of snail larvae. Water was renewed 50% in volume every 2 days in each rearing tank, and probably the effect of nitrite might occur in short period.

Table 2: Water quality parameters during nursing sweet snail larvae

Parameters		Treatments		
		Control	T1	T2
Temperature (°C)	Morning	25.9±0.08[a]	25.8±0.09[a]	25.8 ±0.10[a]
	Afternoon	27.7±0.06[a]	27.6±0.06[a]	27.5±0.10[a]
pH		8.5±0.05[a]	8.5±0.03[a]	8.4±0.06[a]
Alkalinity (mg $CaCO_3$/L)		84.9±1.39[a]	84.8±2.00[a]	85.3±1.15[a]
NH_4^+/NH_3 (mg/L)		0.4±0.15[a]	0.3±0.14[a]	0.4±0.13[a]
NO_2^- (mg/L)		2.6±0.20[a]	2.9±0.20[ab]	3.0±0.20[b]

The values in the same row with different letters indicating significant difference ($p<0.05$)

3.2.2 Filtration rate of sweet snail larvae

The filtration rate of snail larvae was from 52.1 ± 1.33% to 84.8 ± 0.40% (Table 3) and significantly different among treatments ($p<0.05$). The highest average filtration rate was observed in T1 (84.8 ± 0.40%). However, it was not significantly different compared to the Control (83.4 ± 0.75%). The lowest filtration rate was obtained in T2 (52.1 ± 1.33%); in this treatment, the algae cells with large clusters might be difficult for larvae to filter effectively. Besides that, the total NO_2^- (3.0 ± 0.20 mg/L) and NH_4^+/NH_3 (0.4 ± 0.13 mg/L) were rather high in T2, so it could be stressful for snail larvae and probably to reduce the growth and survival rate of larvae. In the study of Sutthinon *et al*. (2007), *Babylonia areolata* were reared from veliger stage until 60 days, and the authors pointed out that the lowest growth rate was obtained in treatment with highest concentrations of total ammonia and nitrite. Snail larvae were trendy to metamorphose in all treatments at day 12 after hatching, at that time they started settling. As soon as snail larvae metamorphosed (within day 12 to day 20), the filtration rate of snail larvae reduced quickly in all treatments (Table 3).

Table 3: Filtration rate of sweet snail larvae (%) during rearing period

Cultured days	Control	T1	T2
1	99.1 ± 0.90[b]	99.1 ±0.50[b]	92.4 ±0.90[a]
3	95.4 ± 0.61[b]	94.6 ±1.51[b]	78.0 ±1.67[a]
6	94.2 ± 0.36[b]	92.9 ±0.36[b]	64.2 ±6.05[a]
9	91.1 ±0.61[c]	85.0 ±1.35[b]	45.2 ±2.60[a]
12*	80.8 ±0.78[b]	81.0 ±0.37[b]	37.9 ±3.63[a]
15	65.3 ±0.85[c]	78.5 ±1.43[b]	31.1 ±8.03[a]
18	59.1 ±3.38[b]	62.5 ±2.05[b]	15.9 ±3.80[a]
Mean±SE	83.4 ±0.46[b]	84.8 ±0.40[b]	52.1 ±1.33[a]

The values in the same row with different letters indicating significant difference ($p<0.05$). (*) Snail larvae started metamorphosis

3.2.3 Survival and growth rate of sweet snail larvae

Survival rate of sweet snail larvae (%)

Survival of sweet snail larvae decreased gradually during 20 days in all treatments (Fig. 6). The highest survival rate presented in control treatment (72.52 ± 3.10%), and that was significant difference ($p<0.05$) with T1 (55.1 ± 9.03%) and T2 (19.70 ± 0.65%). Survival rate of larvae snail in this study was also higher than previous study by Sutthinon *et al*. (2007) when nursing of *Babylonia areolata* from veliger stage to 60 days juveniles (21.58%). These differences might be due to the different culture protocol and experiment time. Srimukda *et al*. (2005) nursed sweet snail larvae from veliger to 60 days early juvenile, fed *C. calcitrans* and *Tetraselmis sp*. with a ratio of 1:1 at a density of 20,000 cells/mL twice a day and obtained very low survival rate (9.82%). The authors nursed veliger snail at a higher density (920 larvae/L) compared to the present study (200 larvae/L). Competition for food and living habitat, and also the poor water quality might be the reasons to cause low survival rate of larvae snail at high density. In the present study, most of water quality parameters were in suitable conditions for snail growth except total ammonia and nitrite concentrations. Water exchange was done daily at the ratio of 30-50% in volume at the first stage and increased to 80-100% at the second stage; however, the toxic nitrogen compounds such as ammonia and nitrite presented in nursing tanks might be harmful for larvae in short term, especially in the feeding treatments with flocculated algae at low pH.

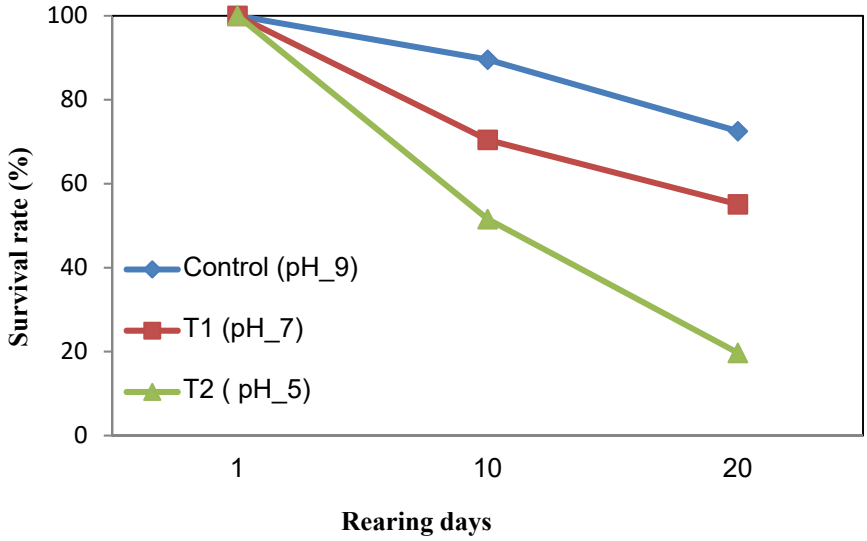

Fig. 6: Survival rate of sweet snail larvae during experiment period

Growth of sweet snail larvae

The average shell length of snail larvae was 440.91 Æm at the beginning. At day 12, larvae in control treatment began metamorphosis when the length reached 1185.65 µm (Fig. 7). At day 20, snail larvae in control treatment reached the highest length (1938.89 µm), following by T1 (1730.65 µm) and T2 (1350.17 µm) with significant difference among treatments ($p<0.05$). Nguyen Thi Xuan Thu *et al.* (2006) reported that snail larvae metamorphosed after 18-20 days when they started settling on the bottom of the nursing tanks. The results from this study also showed higher shell length than those from the previous studies of Sutthinon *et al*. (2007) or Chaitanawisuti *et al.* (2004) that shell length was low (1099 µm and 1520 µm/ respectively). These results might be due to the different management techniques, for example Chaitanawisuti *et al.* (2004) cultured larvae in 500 liter cylindrical rearing tanks with very high stocking density (1000 larvae/L) and larvae was fed with mixed microalgae consisting of *Chaetoceros calcitrans* and *Tetraselmis* sp. at a ratio of 1:1. On the contrary, in the present study, larvae were nursed at lower stocking density of 200 larvae/L and fed with only flocculated *Chaetoceros calcitrans* but obtaining the better results.

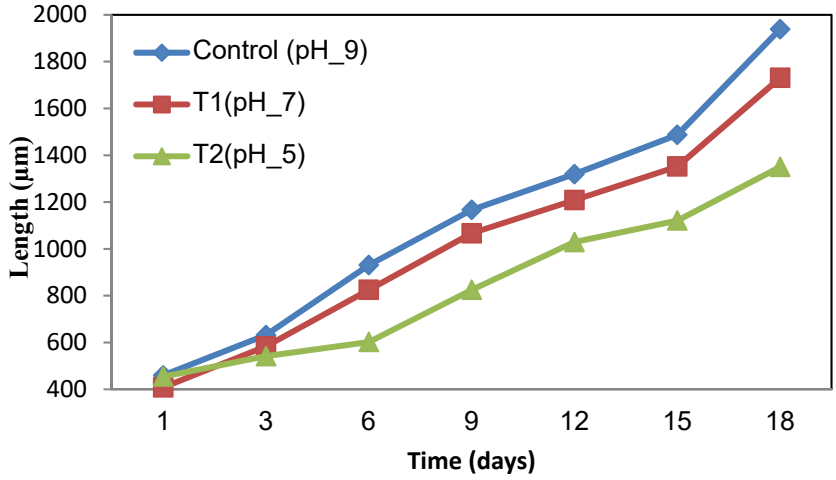

Fig. 7: Shell length of sweet snail larvae during the culture period

The height of larvae was 354.17µm at day 1. After 12 days, they started to metamorphose at the average height of 879.86 µm in the control treatment. At day 20, snail larvae in control treatment reached the highest shell height (1,041.67µm) which was significant difference ($p<0.05$) from the larvae in T2 (895.83µm). The results from the control treatment were similar to the study of Chaitanwisuti (1997), within 18 days after hatching, the height of snail larvae was 1160µm. In addition, the result of this study also showed slower growth in height than the previously reported by Jinfeng *et al.* (2006). These authors nursed veliger sweet snail at a lower density (150 larvae/L) and fed algae mixture including *Platymonas subcordiformis* and *Chaetoceros* sp. In that, sweet snail larvae metamorphosed when the larval height reached 1323.9µm after 18 days of rearing. Perhaps, the shortened nursing period (12 days), higher stocking density (200 larvae/L) and feeding with only algae species (*C. calcitrans*) could be the main reasons to explain for the lower shell height of snail larvae in the present study.

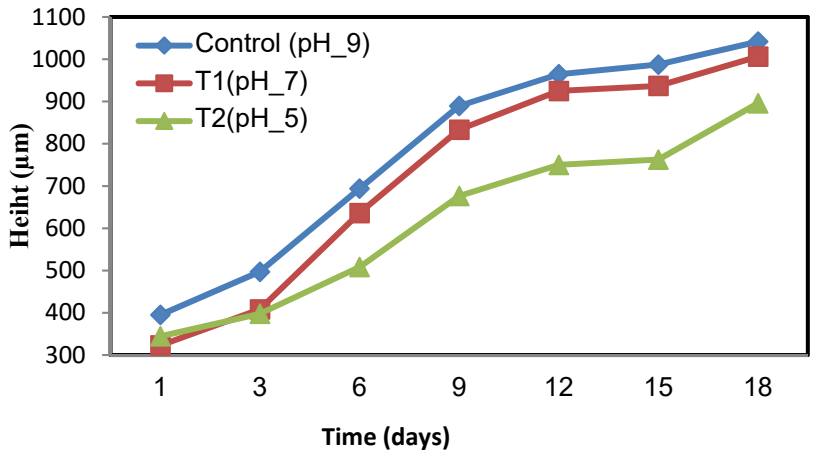

Fig. 8: Snail larvae height during the culture period

Metamorphosis rate of sweet snail larvae (%)

From day 12, larvae in all treatments started metamorphosis to become early snail juvenile with highest rate was recorded in the control treatment (36.07 ± 3.07%), followed by T1 (21.59 ± 3.77%) and very low in T2 (0.12 ± 0.05%). Until day 20, metamorphosis rate in control treatment was highest (72.52 ± 3.10%) and significant difference ($p<0.05$) from T1 (55.11 ± 9.03%) and T2 (19.70 ± 0.64%).

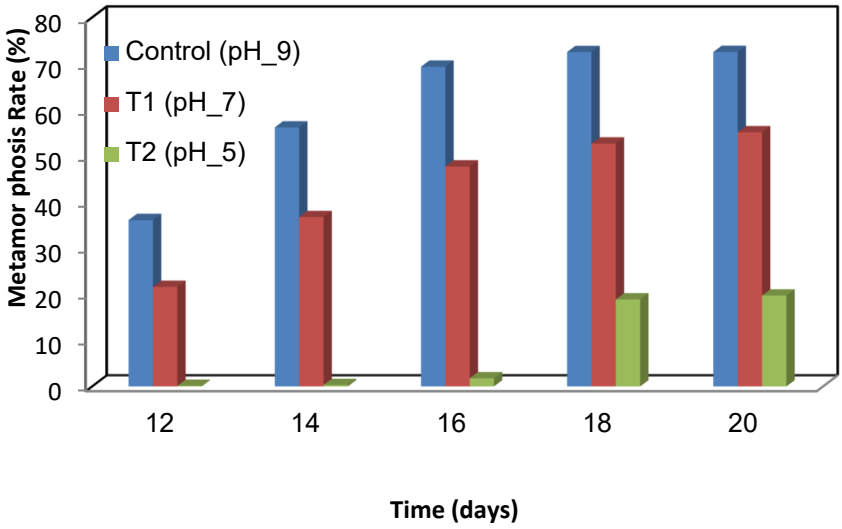

Fig. 9: Metamorphosis rate of snail larvae during 20 days

The results from this study showed that, microalgae *C. calcitrans* were flocculated with chitosan at low pH (<7.0) presented the low quality and low growth ability after flocculation and preservation. The algal cell mortality increased during preservation time will lead to the decomposition; therefore, it would reduce the nutritional values of flocculated algae at low pH. Subsequently, when using those flocculated algae as food source for rearing, it will result in the low growth, metamorphosis and survival rate of sweet snail larvae. Algae flocculated with chitosan at low pH did not affect pH levels in snail larvae rearing tanks; however, the decomposition and settling of uneaten algae might cause the lack of food and also the higher concentration of toxic dissolved nitrogen compounds such as ammonia and nitrites. These toxic compounds were harmful for respiration and metabolism of sweet snail larvae even in short term. The results from this study illustrated that it is necessary to maintain pH value in the range from 7.0-9.0 when flocculating algae *C. calcitrans* with chitosan because the algae quality was better and after feeding it showed the positive effects on the growth performance, survival and metamorphosis of sweet snail larvae. It could be helpful in mollusc hatchery practices to manage the culture and harvest of algae for feeding.

4 CONCLUSIONS AND RECOMMENDATIONS

Chaetoceros calcitrans were flocculated with chitosan at pH 7 and pH 9 showing higher viable cell and growth after re-inoculation.

Sweet snail larvae obtained higher survival and growth rate when feeding with flocculated algae at pH 9.

It is necessary to conduct further studies to evaluate the effects of feeding flocculated algae on different molluscan species.

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