CHAPTER 3

MATRIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Acetone

Alkaline-iodide azide reagent

Aluminium sulfate [$Al_2 (SO_4)_3$]

Ammonium chloride (NH_4Cl)

Ammonium molybdate reagent [$(NH_4)_6 MO_7 .O_{24} .4H_2O$]

Ammonium persulfate [$(NH_4)_2S_2O_8$]

Antifoam

Chloroform

Cleaning solution

EDTA reagent (Na_2EDTA)

Ethanol

Feroin indicator

Ferrous ammonium sulfate [$Fe (NH_4)_2 (SO_4)_2.6H_2O$]

Glycerol

Hexane

Manganous sulfate reagent ($MnSO_4$)

Mercuric sulfate ($HgSO_4$)

Methanol

Methyl red indicator

Nessler reagent

Nitric acid (HNO_3)

Petroleum ether

Phenoldisulfonic acid solution

Phenolphthalein indicator

Phenol

Potassium dichromate ($K_2Cr_2O_7$)

Potassium hydroxide (KOH)

Potassium iodide (KI)

Potassium nitrate (KNO_3)

Selenium

Silver sulfate (Ag_2SO_4)

Sodium azide (NaN_3)

Sodium hydroxide (NaOH)

Sodium iodide (NaI)

Sodium thiosulfate ($Na_2S_2O_3$)

Stannous chloride reagent ($SnCl_2$. $2H_2O$)

Starch solution

Sulfuric acid (H_2SO_4)

Zinc sulfate ($ZnSO_4$. $7H_2O$)

3.1.2 Equipments

Air pump

Autocave (Becthai Hirayama, model HA-300D)

Centrifuge (Kubota, model 5100)

Compound microscope

Crude fiber apparatus

Desiccators

Spectronic (Programmable Microplate Readers, model DV 990 BV4)

Spectronic (HACH, model DR 2000)

Electrical balance

Gas chromatography, GC (Agilent Technologies, model 8600)

Modified Kjeldahl (Gerhardt vapodest, model 20)

Hot air oven

High performance liquid chromatography, HPLC (Agilent

Familiarization manual, model 1100)

Incubator

Muffle furnace

pH meter (Horibra, model D21)

Suction pump

Transfer chamber

Thermometer

3.1.3 Apparatus

Beaker

BOD bottle

Burette

Cylinder

Erlenmeyer flask

Pipette

Titration set

Volumetric flask

Petridish

Test tube

Dropper

Millipore AA filter paper

Whatman filter paper

3.2 METHOD

3.2.1. Preparation of *S. platensis* stock culture

3.2.1.1 *S. platensis* cultured in Modified Zarrouk's medium (Stock Zm)

Modified Zm were composed of commercial grade chemicals (Promya, 2000): 2 g L^{-1} of $NaHCO_3$ (Qingdao Co., LTD, China), 1 g L^{-1} of NaCl (Purity Salt Industry, Co., LTD, Thailand), 1 g L^{-1} of $MgSO_4$ (UTIDS Enterprise Co., LTD, Thailand), 0.5 g L^{-1} of $NaNO_3$ (Qingdao Co., LTD, China) and 1 g L^{-1} of N: P: K (16: 16: 16, YARA International ASA Co., Ltd, Norway), adjusted to pH 10 $\pm$ 0.5 using NaOH. Stock Zm (Figure 6a): *S. platensis* was cultured in modified Zm in a 5-L bottle and allowed to grow for 2 weeks until the optical density (OD) at 560 nm reached 1 (OD_{560nm}=1).

3.2.1.2. *S. platensis* cultured in kitchen wastewater (Kw)

One hundred liters of Kw, one day of food preparation and dish washing processes at the cafeteria, Department of Biology, Faculty of Science, Chiang Mai University, was collected and allowed to ferment for 3 weeks (Figure 6b) for microorganisms to break down the solid organic wastes (Traichaiyaporn, 1994). Fermented Kw was then filtered through an 80 micron plankton net filter. The filtrate was analyzed for pH, total - N (TN) and total - P (TP) using methods in 3.2.4a.

Stock Kw (Figure 7a): *S. platensis* from stock Zm was cultured in fermented Kw in 50-L glass tanks and allowed to grow for 2 weeks until the OD at 560 nm reached 1 ($OD_{560\ nm = 1}$). Stock Kw would then be used as "raw *S. platensis*" for the experiments in 3.2.2.1, 3.2.2.2 and 3.2.2.3.

3.2.2 Cultivation of *S. platensis*

3.2.2.1 Laboratory cultures of *S. platensis*

Modified Zm, Kw and oil-extracted soybean water fermented (Sw) were used as culture media. Sw was oil-extracted soybean meal mixed with tap water (ratio = 1 : 9) Figure 6c. The soybean suspension was left for 3 weeks to ferment and then filtered through an 80 micron plankton net filter. The filtrate, together with modified Zm and Kw, were analyzed for pH, TN and TP using methods in (3.2.4a). A complete randomized design (CRD) was carried out using 22 treatments with 3 replication: modified Zm as control, 10 dilutions of Kw and 11 dilutions of Sw. All treatments were adjusted the N : P ratio to be about 6-8 : 1 (Reynolds, 1986). The dilutions were filtered again and analysed for physico-chemical water quality of pH, TN and TP using methods in (3.2.4a). *S. platensis* from stock Kw were cultured in modified Zm, Kw

and Sw in 5-L bottles (Figure 7 b). Each bottle had the initial OD of 0.30 (OD_{560nm} = 0.30) before adding the inoculum of *S. platensis*. All cultures were continuous aerated and left for 15 days. Samples (100 mL of each media) were collected every 5 days from each bottle to determine algal biomass production and analyzed for their pH, TN and TP using methods in 3.2.4a. Algal biomass production (dry weight, g L^{-1}) was also determined by filtration through a 120 μm plankton net (Figure 8 a). The media and dilution producing the best result would be selected for further experiments in 3.2.2.2.

Figure 6 *S. platensis* (a) Stock Zm (b) 3-week fermented Kw (▽) and (c) Sw (⊗)

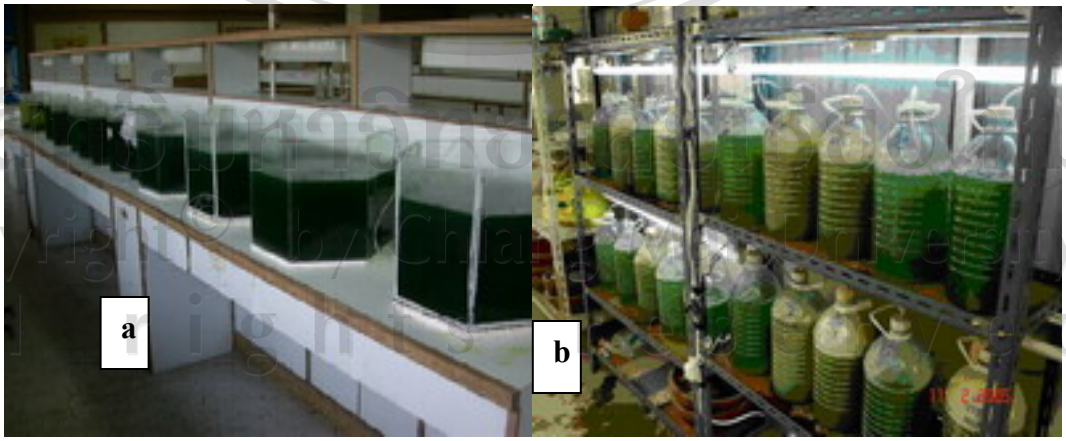


Figure 7 (a) *S. platensis* (Stock Kw) and (b) *S. platensis* cultured in laboratory (3.2.2.1)

3.2.2.2 Outdoor mass culture of *S. platensis* in experimental tank

S. platensis from stock Kw were cultured in the 5 media selected from 3.2.2.1. They were modified Zm, 90% and 100%Kw, 5% and 10%Sw. A completely randomized design (CRD) was carried out using 5 treatments and each of 3 replications Figure 8b.

Experimental tank: The control treatment consisted of triplicate preparations of raw *S. platensis* cultured in 100-L tanks containing modified Zm (modified Zm; R_1- R_3). The kitchen wastewater treatments consisted 100% Kw (100%Kw; R_1- R_3) and 90%Kw (90%Kw; R_1- R_3) treatments. The oil-extracted soybean fermented water (Sw) treatments consisted 10%Sw (10%Sw; R_1- R_3) and 5% Sw (5%Sw; R_1- R_3) treatment. The initial OD of each treatment was 0.30. All experimental tanks were cultured for 15 days with continuous aeration. Samples (1 L of each) were collected every 5 days from each tank to determine algal biomass production and analyzed for their physico-chemical parameters: water temperature, pH, DO, BOD, COD, NH_3-N, NO_3-N, NO_2-N, organic nitrogen (ON), total Kjeldahl nitrogen (TKN), total oxidized nitrogen (TON), TN and TP using methods in 3.2.4a.

Algal biomass production was determined by filtration through a 120 μm plankton net (Figure 9a) and was tray-dried at 70 ^{o}C for 5 hours. Dried *S. platensis* was then ground into powder. Samples of dried *S. platensis* powder (Figure 9b) were analyzed for production variable cost (see Appendix A 19) and their nutritional contents: protein, fat, nitrogen free extract (NFE: carbohydrate), ash, fiber, moisture, β-carotene ,C-phycocyanin and γ- linoleic acid as shown in 3.2.4b.

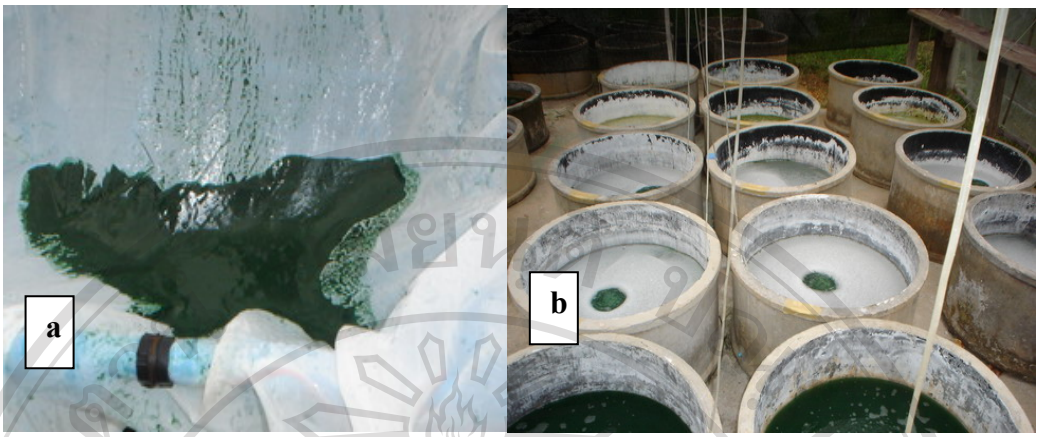


Figure 8 (a) Production of raw *S. platensis* (3.2.2.1) (b) Outdoor culture of *S. platensis* (3.2.2.2)

Figure 9 (a) *S. platensis* filtration through plankton net (b) Dried *S. platensis* and powder

3.2.2.3 Mass cultures of *S. platensis* in cement raceway ponds and earthen raceway ponds

Oil-extracted soybean fermented water (10%Sw) was the medium selected from the experiments in 3.2.2.2 because it gave the most preferable results in all parameters analyzed. The medium was then diluted with tap water (Sw: tap water = 1 : 9 (10% Sw)) and 0.1 g L^{-1} of $NaHCO_3$ was added. A two-sample t-test was carried

out using 2 treatments and 3 replicates: cement raceway ponds 10%Sw (R_1-R_3) as shown in Fig. 10a and earthen raceway ponds 10%Sw (R_1-R_3) in Figure 10b.

Experimental ponds: *S. platensis* from stock Kw was used for inoculation. Filtering 500 L stock Kw gave 8 g L^{-1} raw *S. platensis.* The raw *S. platensis* were added to a 4,000-L cement raceway ponds and an earthen raceway ponds until the OD of each pond was 0.30. All experimental ponds were left for 15 days with continuous aeration. Samples (1 L of each) were collected every 5 days from each pond to determine algal biomass production and analyzed for their physico-chemical parameters: water temperature, pH, DO, BOD, COD, NH_3-N, NO_3-N, NO_2-N and TP using methods in 3.2.4a.

Algal biomass production was determined by filtration through a 120 μm plankton net (Figure 11) and the product was tray-dried at 70 ^{o}C for 5 hours. Dried *S. platensis* was then ground into powder. Samples of dried *S. platensis* powder were analyzed for production variable cost (see Appendix A19) and their nutritional contents: protein, fat, nitrogen free extract (NFE: carbohydrate), ash, fiber, moisture using methods in 3.2.4b.

Figure 10 (a) Cement raceway pond (size 2.3x 15x .25 m^3) (b) Earthen raceway pond (size 2.3 x 15 x .25 m^3)

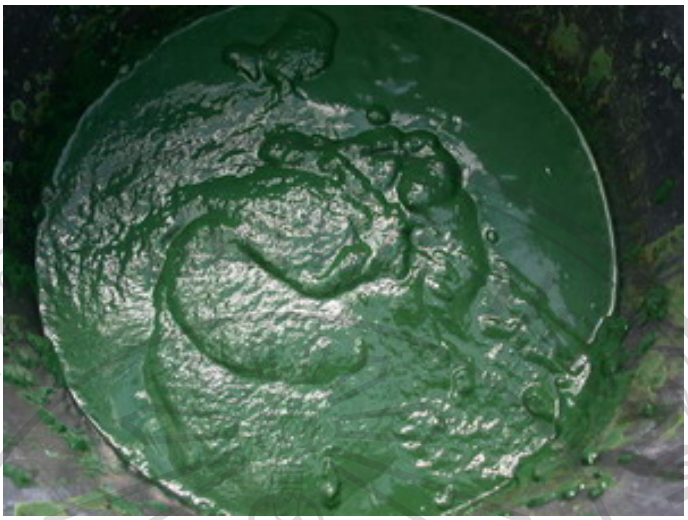


Figure 11 Biomass production of raw *S. platensis*

3.2.3 Tuptim tilapia culture

3.2.3.1 Nursing larval Tuptim tilapia in an earthen pond using raw *S. platensis*

3.2.3.1.1 The preparation of earthen pond

The earthen ponds sized 6 x 8 x 1 m^3 each (Figure 12) were located at the Fisheries Technology and Aquatic Resources, Maejo University, Chiang Mai, Thailand. The earthen ponds were cleaned and dried then $CaCO_3$ 30- 60 g/ m^2 was added and left for 7 days. The water from a reservoir was flowed into the earthen ponds to the level of 0.80 meter and manure added was (150 g/ m^2) and left for 3 days to induce plankton bloom for natural fish feed.

3.2.3.1.2 Fish preparation

The average size of larval Tuptim tilapia prepared was 0.50 to 0.60 cm and the body weight 0.02 to 0.03 gm (fry stage) Figure 13a obtained from the Fisheries Technology and Aquatic Resources. Larvael were used in each earthen pond to density of 100 fishes per 1 m^2, or 4,800 fish in each pond (Wiwuchaiset, 2001).

3.2.3.1.3 The preparation of fish feeds and analyses of their nutritional values before nursing larval Tuptim tilapia

A completely randomized design (CRD) was carried out using 4 treatments and 3 replicates:

T_1 (R_{1-3}) Commercial diet (CD) using the quantity of 5% / fish body weight / day

T_2 (R_{1-3}) 1% Raw *S. platensis* (1%RS) using the quantity of 1% of fish body weight/ day (as dry weight)

T_3 (R_{1-3}) 3%RS using the quantity of 3% of fish body weight/ day (as dry weight)

T_4 (R_{1-3}) 5%RS using the quantity of 5% of fish body weight/ day (as dry weight)

CD was purchased from a nearly animal-feed shop and RS from 3.2.2.3 (Figure 13b). The nutritional values of CD and RS were analysed (using methods in 3.2.4b) before feeding as shown in Table. 1.

Table 1 **Nutritional values of fish feeds (% of dry weight)**

Fish feed	Protein (%)	NFE (%)	Fat (%)	Fiber (%)	Ash (%)	Moisture (%)
CD	36.00	27.60	6.00	3.50	15.10	7.80
RS	48.49	-	2.10	2.15	6.86	77.50

3.2.3.1.4 The experimental planning and data collection

All experimental ponds were continuously aerated. The larval were fed 3 times / day (8.00 am, 1 pm. and 5 pm). Samples (20 fishes and 1 L water of each pond) were collected every 15 days for 90 days. Water was analyzed for physico-chemical parameters: water temperature, pH, alkalinity, total dissolved solid (TDS), conductivity, DO, PO_4-P and NH_3-N using methods in 3.2.4a. Fishs (Figure 14) were analyzed for growth performance, lysozyme activity, red blood cell (erythrocyte) and white blood cell (leucocyte) counts (Appendix A. 17, 18 and 20).

Figure 12 Earthen ponds to nurse larval Tuptim tilapia (size 6 x 8 x 1 m^3)

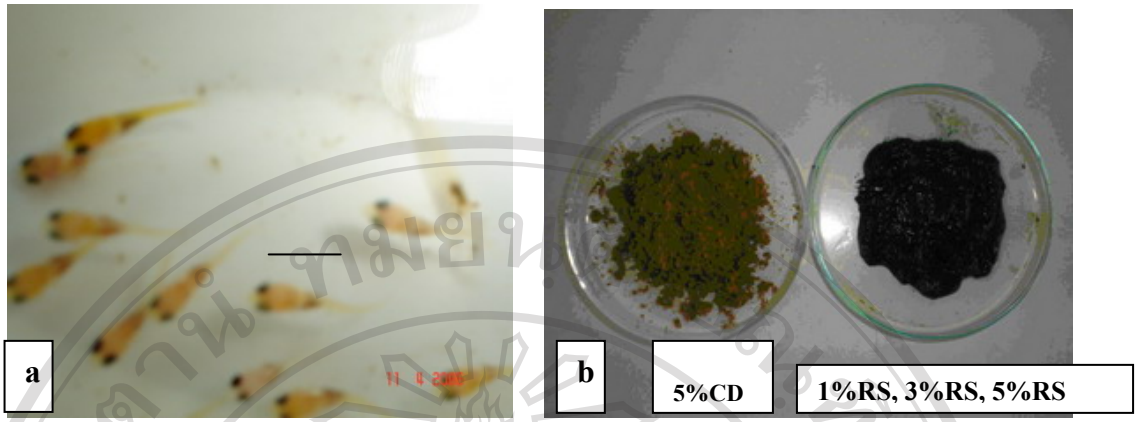


Figure 13 (a) 7 days old fry Tuptim tilapia (scale bar 1 cm) (b) Larval feed (CD = commercial diet, RS = raw *S. pltensis*)

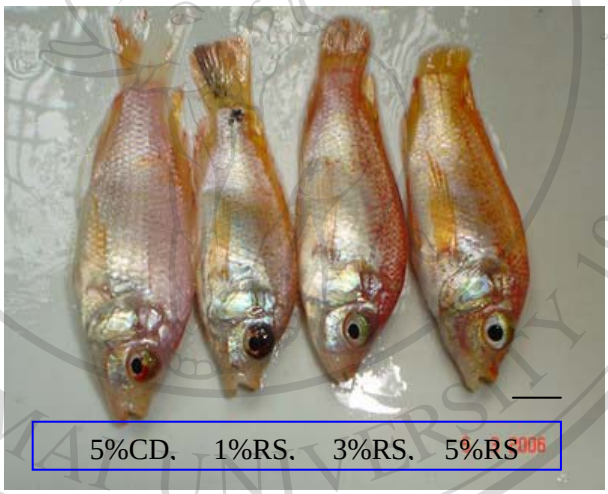


Figure 14 Production of larval Tuptim tilapia each treatment (scale bar = 2 cm)

3.2.3.2 Culturing male and female juvenile Tuptim tilapia in an earthen pond

3.2.3.2.1 The preparation earthen ponds: Similar to the method in 3.2.3.1.1.

3.2.3.2.2 Fish preparation: The juvenile Tuptim tilapias, 3,600 fishes with the body weight 9 to 10 gm each as shown in Figure 15a, were obtained from the nursing experiments (in 3.2.3.1). They were kept in floating nets (5 x 6 x 0.80 m^3) as shown in Figure 15b and fed with commercial diets for 1 month to acclimate them to the new environment.

3.2.3.2.3 Feed preparation: Results of 3.2.3 showed that the protein content in RS was high up to 48.49 % dry weight (Table 1) which was suitable as feed for fast growing larval Tuptim tilapia. However "it has been found that" (Mahajan and Kamat, 1995) at the juvenile stage the fish used less protein (only 20-30%) for fast growth rate. Therefore to reduce the feed cost with appropriate protein content rice polish (RP) was then mixed with RS in 3 combinations, all of which gave the value of protein at about 30% (Table 2). The mixtures of RS and RP were used as feed together with CD in this experiment. The commercial diet were purchased from a near by animal feed shop. Raw *S. platensis* was obtained from 3.2.2.3. A completely randomized design (CRD) was carried out using 4 treatments and 3 replicates:

T_1 (R_1- R_3): CD (0%RS) using the quantity of 3-5% of fish weight

T_2 (R_1- R_3): 45%RS+ 55%RP (by weight) using the quantity of 3-5% of fish weight

T_3 (R_1- R_3): 50%RS+ 50%RP (by weight) using the quantity of 3-5 % of fish weight

T_4 (R_1- R_3): 55%RS+ 45%RP (by weight) using the quantity of 3-5% of fish weight

Treatment feeds were made as pellets using a pellet feed machine (Figure 16a). The samples of feed pellets (Figure 16b) were analyzed before feeding for their

nutritional contents: protein, fat, ash, fiber, moisture, nitrogen free extract (NFE) = (moisture + protein + fat + ash + fiber) using methods in 3.2.4b and data obtained were shown in Table 2. Production variable cost (Appendix A.19), β–carotene, C-phycocyanin and linoleic acid were also analyzed using methods in 3.2.4b and the results are shown in Table 3.

3.2.3.2.4 Experimental planning and data collection

A completely randomized design (CRD) was carried out using 4 treatments and 3 replicates (see detail in 3.2.3.2.3). Male and female fishes (age 120 days and body weight 18.33 to 27.67 gm; Figure 17) were cultured separately in each pond (size 4 x 5 x 1 m^3) Fig. 18, 100 fishes / pond (treatment 1-4) at the quantity of 5% / day in the first 2 months, 4% / day during 2 months later and 3% / day in the last 2 month. Samples were collected every 30 days for 150 days and analyzed for their growth performance and GSI of male and female juvenile tubtim tilapia (see Appendix A 20), lysozyme activity, red blood cell (erythrocyte) and white blood cell (leucocyte) counts (Figure 19). Physico-chemical parameters of water in cultured ponds: water temperature, pH, alkalinity, TDS, conductivity, DO, PO_4-P and NH_3-N were also analyzed.

At the end of the experiment, at day 150, meat and egg (Figure 20 and 21) qualities were analysed for phycocyanin, β-carotene and γ-linoleic acid production variable cost and nutritional values; protein, fat, carbohydrate, ash, fiber, moisture and GSI (see Appendix A 19 and 20).

Table 2 Statistical summary (mean ± SD) of nutritional values in fish feed before experiment (dry weight)

Fish feeds	Protein (%)	NFE (%)	Fat (%)	Fiber (%)	Ash (%)	Moisture (%)
T_1: CD	30.14±0.16	37.73± 0.04	6.10±0.10	3.60 ± 0.10	15.11± 0.01	7.64 ± 0.35
T_2: RS + RP (45: 55% by wt.)	30.64± 0.21	32.26±0.04	8.18 ±0.01	5.52 ± 0.02	13.86± 0.01	10.54± 0.03
T_3: RS + RP (50: 50% by wt.)	30.52± 0.29	33.08±0.05	7.37± 0.06	4.53 ± 0.03	14.65± 0.03	9.85 ±0.03
T_4: RS + RP (55: 45% by wt.)	30.53± 0.32	35.03±0.05	7.53 ±0.04	4.63 ± 0.02	12.15± 0.04	10.13± 0.03

Table 3 Statistical summary (mean ± SD) of production variable cost, β-carotene, C-phycocyanin and γ- linoleic acid contents in fish (dry weight)

Fish feeds	feed cost (baht kg^{-1} feed)	β-carotene content (mg g^{-1} feed)	Phycocyanin content (mg g^{-1} feed	γ- linoleic acid (mg g^{-1} feed)
T_1: CD	22.50 ± 2.35	0.09± 0.006	4.1± 1.00	0.06± 0.012
T_2: RS + RP (45: 55% by wt.)	18.77± 1.25	0.13 ± 0.00	11.62± 2.08	0.09± 0.013
T_3: RS + RP (50: 50% by wt.)	20.85± 1.03	0.15± 0.001	12.9± 2.65	0.10± 0.011
T_4: RS + RP (55: 45% by wt.)	22.94± 1.45	0.17± 0.002	14.02± 2.51	0.11± 0.096

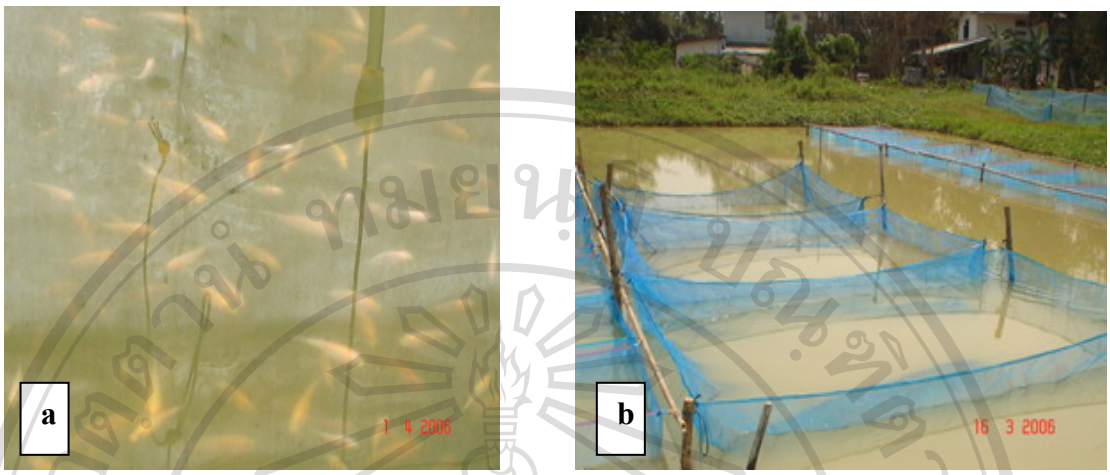


Figure 15 (a) juvenile of Tuptim tilapia being kept in floating net and (b) Floating nets

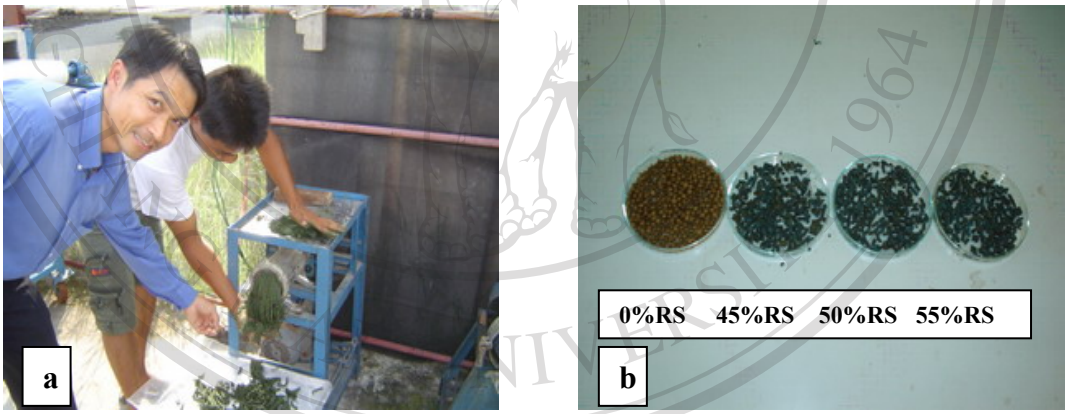


Figure 16 (a) The preparation of feed pellets (b) The samples of fish feed pellet (from left, t_{1-4})

Figure 17 120 days old juvenile Tuptim tilapia

(body weight 25.02 to 27.13 gm) scale bar = 2 cm

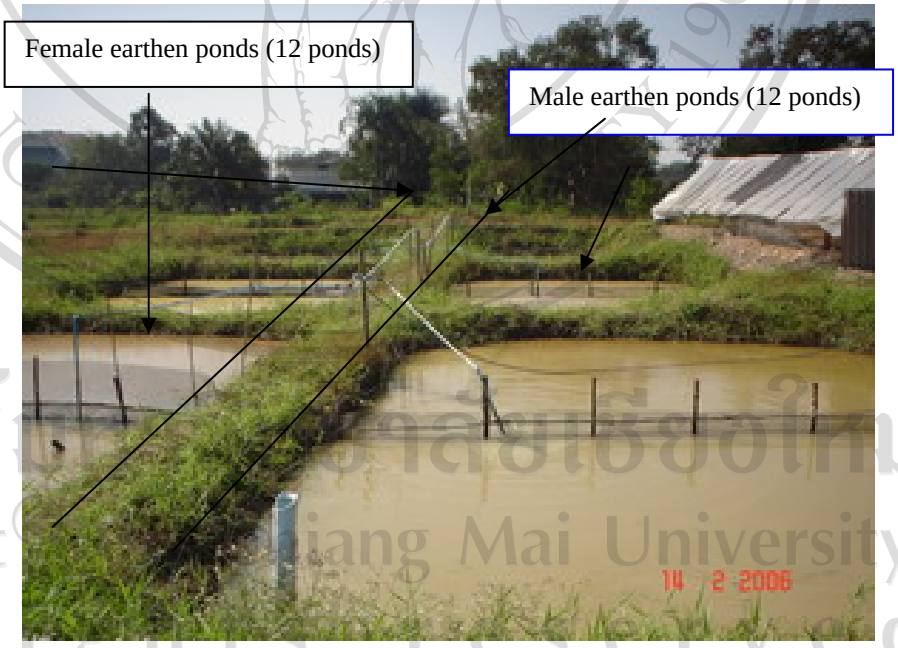
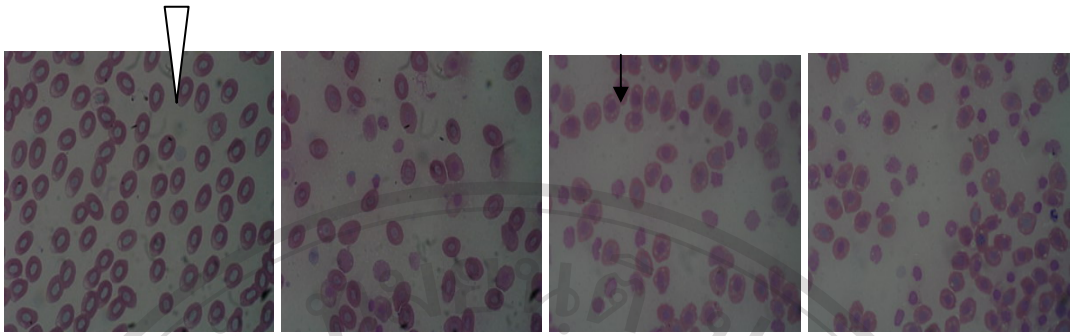


Figure 18 Earthen ponds for male and female juvenile Tuptim tilapia cultures



0%RS (T_1) 45%RS (T_2) 50%RS (T_3) 55%RS (T_4)

Figure 19 Red ( ) and white ( ) blood of juvenile tubtim tilapia

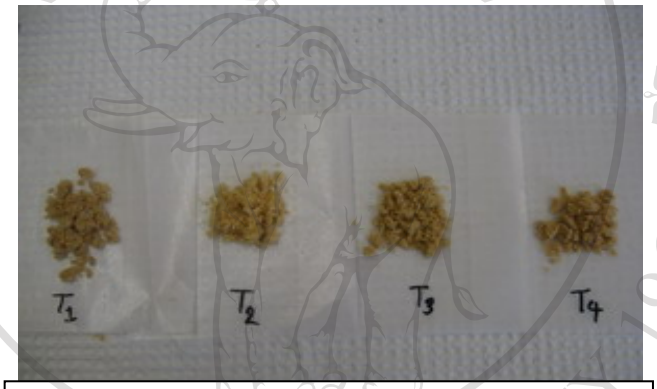


Figure 20 Flesh of juvenile Tuptim tilapia of each treatment

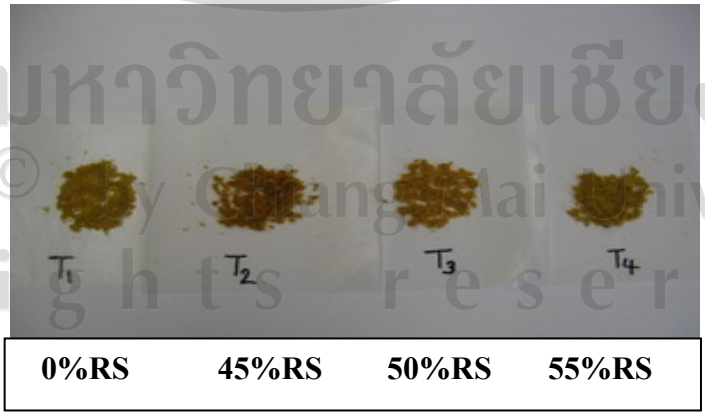


Figure 21 Eggs of juvenile Tuptim tilapia of each treatment

3.2.4 Physico-chemical water quality and nutrition value analyses

A. Laboratory investigated parameters and methods **(APHA and WPCF, 1998; Jackson, 1993; Traichaiyaporn, 2000)**

Parameter	Method
Air and water temperature	Thermometer
pH	pH meter
TDS	TDS meter
Conductivity	Conductivity meter
DO	Azide modification (Appendix A. 1)
BOD	Azide modification (Appendix A. 1)
COD	Closed reflux (Appendix A. 2)
Total phosphorus (TP)	Persulfate digestion followed by Stannous chloride (Appendix A. 3)
PO_4-P	Stannous chloride (Appendix A. 4)
NH_3-N	Nesslerization (Appendix A. 5)
NO_3-N	Phenoldisulphonic acid method B (Appendix A. 6)
NO_2-N	Diazotization and coupling method (Appendix A. 7)
Total Kjeldahl nitrogen (TKN)	Macro Kjeldahl method followed by titration (Appendix A. 8)
Organic nitrogen (ON)	TKN- NH_3-N
Total oxidized nitrogen (TON)	NO_3-N+ NO_2-N
Total nitrogen (TN)	ON + NO_3-N+ NO_2-N+ NH_3-N

B. Laboratory investigated parameters and methods / tools ((Berns *et al*., 1963; Christie, 1982; AOAC, 1990; Simonne *et al*., 1996)

Parameter	Method / Tool
β-carotene	HPLC method (Appendix A. 9)
C-phycocyanin	HPLC method (Appendix A. 10)
γ- Linoleic acid	GC method (Appendix A. 11)
Crude protein	Modified Kjeldahl (Appendix A. 12)
Crude fat	Ether extract method (Appendix A. 13)
Nitrogen free extract (NFE: Carbohydrate)	NFE = 100 - (moisture + protein + fat + ash + fiber)
Ash	Furnace preheated to 450 ^{o}C method (Appendix A. 14)
Fiber	Acid detergent method (Appendix A. 15)
Moisture	Drying method (Appendix A. 16)

3.2.5 Statistical analysis

Data were presented as mean values ± standard deviation. Comparison of mean values were made by one way-analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at a significance level of $p<0.05$ (Appendix B).