TẠP CHÍ KHOA HỌC
TRƯỜNG ĐẠI HỌC SƯ PHẠM TP HỒ CHÍ MINH
Tập 18, Số 9 (2021): 1359-1367
ISSN: 2734-9918
HO CHI MINH CITY UNIVERSITY OF EDUCATION
JOURNAL OF SCIENCE
Vol. 18, No. 9 (2021): 1359-1367
Website: *http://journal.hcmue.edu.vn*

Research Article

RESEARCH ON USING MICROALGAE *HAEMATOCOCCUS PLUVIALIS* POWDER EXTRACTED ASTAXANTHIN AS SUPPLEMENT FOOD FOR JAPANESE KOI FISH

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Received: December 16, 2020; Revised: March 04, 2021; Accepted: September 01, 2021

ABSTRACT

After extraction of astaxanthin with viscozyme and absolute alcohol, the nutritional composition of Haematococcus pluvialis residue remained 35.5 % protein, 14.9% fat, 36.8 % glucide, 4.48 % crude fiber and 0,69 % astaxanthin. Algal residue treated by mechanical grinding combined with 0.1M NaOH showed the highest extraction efficiency of astaxanthin (72.16 %) when compared to other methods: mechanical grinding combined with 0.1M HCl (54.75 %), olive oil solvent combined with autoclave (59.26 %) and water solvent combined with autoclave (30.54 %). The addition of astaxanthin-containing microalgae residue in the feed at concentrations of 50mg astaxanthin/kg, 100mg astaxanthin/kg and 150mg astaxanthin/kg has the effect of enhancing red pigmentation for Japanese Koi fish after 14 days of investigation.

Keywords: Astaxanthin*; Cyprinus carpio; Haematococcus pluvialis*; Koi fish

1. Introduction

The Koi carp (*Cyprinus carpio*) is one of the many favorite ornamental fish species that has been domesticated and bred for ornamental purposes in Vietnam as well as in many countries around the world. However, in the process of raising Koi fish, if the food lacks of natural carotenoid compounds, the colour of the fish becomes pale, loss of aesthetics. Research by Jagruthi et al. shows that adding astaxanthin in Koi fish feed may improve colour and increase immunity (Jagruthi et al., 2014).

Cite this article as: Do Thanh Tri, Tran Thi Phuong Dung, Do Vu Quoc Anh, Nguyen Hoang Tam, Nguyen Thanh Cong, & Ong Binh Nguyen (2021). Using creative methodology to explore factors influencing teacher educator identity. *Ho Chi Minh City University of Education Journal of Science, 18*(9), 1359-1367.

Astaxanthin is a natural compound with high antioxidant activity, stimulating growth, giving attractive colours. Astaxanthin was synthesised and accumulated the most in strains of microalgae *H. pluvialis* (Kim et al., 2015). The diet of fish supplemented with algae biomass showed that the meat and skin of the fish contain a large amount of astaxanthin, which enhances the flesh colour and colour of the fish. In addition, it also has the ability to strengthen the antioxidant system, support growth and reduce mortality at the fry stage in some species such as salmon, sea bream and rainbow trout (Sheikhzadeh et al., 2012), ornamental fish species (Amar et al., 2002) and shrimp (Parisenti et al., 2011). Currently, synthetic astaxanthin is mainly used for colour improvement in fish (about 95% of market demand) (Jin et al., 2006). It gives a distinctive pink colour to certain aquatic animals such as salmon, sea bream, and shrimp. Astaxanthin is priced at about 2500 USD/kg with sales estimated at 200 million USD/year. In the aquaculture industry, the cost of adding astaxanthin to animal feed accounts for 10-20 % of the production cost. Some recent studies have shown that artificially synthesized astaxanthin has lower biological activity than naturally derived astaxanthin (Li et al., 2014). The price of dry biomass powder for *H. pluvialis* on the market is relatively high, ranging from 200-300 USD/kg. However, astaxanthin has a ratio of only about 3% in dry biomass, with the remaining 97% being other ingredients (Tran et al., 2019). The biomass remaining after astaxanthin extraction will have a very large yield. This microalgae residue also contains low-level astaxanthin along with many other valuable nutrients such as protein, lipid... Therefore, it is really necessary to research to utilise this biomass source for breeding purposes. In this study, *H. pluvialis* residue was studied for supplementation to Japanese Koi carp feed.

2. Materials and methods

2.1. The source of the microalgae residue has been extracted astaxanthin

Biomass of *H. pluvialis* was cultured in biofilms in a Twin-layer porous substrate photobioreactor (Tran et al., 2019) and harvested. Then, astaxanthin in the algal biomass was extracted by the viscozyme and 96° ethanol method (Huynh et al., 2019). The residue of algae after the extraction process was recovered with sufficient amount for all experiments of this study. Algal residue was homogenized by mixing well to limit errors in the process of analysis.

2.2. Design of experiments

a. Analysing the nutrient content of H. pluvialis residue: After astaxanthin extraction, the algal residue sample was dried in the dark condition and sent for analysis of the content of lipid (according to TCVN 4331:2001), glucide (according to TCVN 4594:1988), protein (according to CASE.NS.0039 (Ref. AOAC 990.03)), and crude fiber (according to CASE.NS.0022 (Ref. ANKOM Technology mnethod 10)) at Center of Analytical Services and Experimentation of HCM City (CASE).

b. Experiment for choosing a method of treating H. pluvialis residue: Astaxanthin extracted algae residues still have many intact cells (under microscopic observation), so they need to be treated to break the cell walls before adding to fish feed to increase the efficiency of nutrient absorption. The methods of treating algal residue investigated in this experiment include: (1) high temperature and pressure for 30 minutes in aqueous solvent, (2) high temperature and pressure for 30 minutes in olive oil solvent, (3) mechanical grinding with 0.1M HCl and (4) mechanical grinding with 0.1M NaOH. For the high temperature and pressure treatments, a quantity of algal residue was weighed (0.01g), put into a centrifuge tube and added 3 ml olive oil or 0.1 ml distilled water, then steamed at temperature of 121 °C in the autoclave. For the mechanical and chemical treatments, five millimeter diameter glass beads were added to 0.01 g of algal residue together with 0.1 ml of 0.1M HCl or 0.1M NaOH. The mixture was shaken vigorously at 200 rpm, shaking amplitude 20 mm for 30 minutes. The efficiency of cell breakdown and internal nutrient release was determined based on the main criterion of the amount of astaxanthin obtained and the observation of cell morphology under a microscope compared with the control method. The control was the method of grinding the algal residue in acetone.

c. Method of mixing H. pluvialis residue into Koi fish feed: Based on the results of the experiment of choosing the microalgal residue treatment method, the amount of dried algae residue was determined to be added to the commercial feed to reach astaxanthin concentrations of 50, 100 and 150 mg/kg of feed. In addition, based on the results of analysing the nutritional value in algal residue, other nutrients were added to the feed to achieve nutritional composition equivalent to those available on the market as well as nutritional requirement of Japanese Koi fish.

d. Effects of food supplemented with H. pluvialis residue on the growth and colour of Japanese Koi fish: Eight-week-old Japanese Koi had been kept stably for 2 weeks in 6 tanks with a volume of 250 liters with a density of 10 individuals/tank. The selected fish were of equal length and weight between treatments (mean length was 5.6 cm, average weight was 3.5 g). This experiment consisted of 6 treatments: treatment (1), (2) and (3) with fish fed with *H. pluvialis* residue supplementation with astaxanthin contents of 50, 100 and 150 mg/kg, respectively; (4) fish were fed with diet without supplement of algal residue (negative control); (5) fish were fed with NRD 3/5 industrial feed (Aquaculture Inc., Thailand), which is specialized in colouring Koi fish (positive control 1); and (6) fish were fed with standard astaxanthin (Sigma-Aldrich, Germany) supplementation with the amount of 50 mg/kg feed (positive control 2). The daily feed intake was calculated to reach 7-10% of the fish's weight. Fish were fed two times in the morning and afternoon. The parameters of pH (7 - 8), water temperature (20 - 27 °C) and dissolved oxygen content (> 2.5 mg/l) were monitored and adjusted regularly. The duration of the experiment was 2

weeks and fish in the tank were collected for colour comparison using the colour bar comparison method of Boonyaratpalin and Unprasert (Lai & Chau, 2019).

2.3. Methods for determining the content of astaxanthin

a. Quantification of the remaining astaxanthin in the H. pluvialis residue: The dried algal residues was weighed (0.01 g) in a 2 ml centrifuge tube. Then, one ml of 90% acetone and 5 mm diameter glass beads were added. The mixture was shaken at 200 rpm with a shaking amplitude of 20 mm for 4 h. Algae suspension was then centrifuged at 1000 *g* for 5 min. The supernatant was collected and one ml of 90% acetone was added to the pellet. Those previous steps were repeated until the colour of the algae residue is white (astaxanthin free). The procedure was performed under low diffuse light. The entire supernatant was collected and added 90% acetone to reach a volume of 5 ml. The astaxanthin extracts were measured for optical density at a wavelength of 530 nm (Y. Li et al., 2012). The astaxanthin concentration was calculated using a calibration curve equation established with standard astaxanthin (Sigma-Aldrich, Germany) dissolved in 90% acetone (Fig. 1). The equation is y = 16.67x+0.05, where y is the concentration of astaxanthin (µg/ml) and x is the OD value. The astaxanthin solution was diluted so that the OD value was between 0.1 and 0.9.

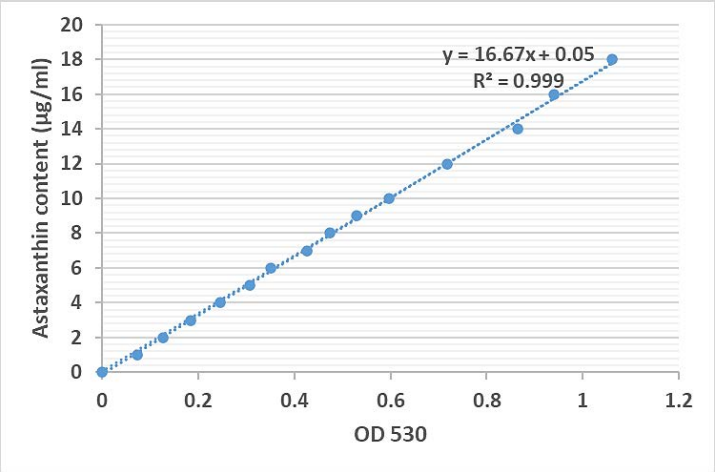

Fig. 1. *Calibration curve equation established with standard astaxanthin in 90% acetone*

b. Determination of astaxanthin content after the experiment for choosing a method of treating H. pluvialis residue: The amount of astanxathin released after the algal cell wall break experiment needs to be determined. It is the basis for the selection of an effective method of cell wall destruction prior to addition to fish feed. For the cell wall break treatments with 0.1 ml of aqueous solvent (including those treated with HCl and NaOH), the mixture was added with 0.9 ml of 100% acetone and shaken at 200 rpm for 10 min to dissolve the astaxanthin. The mixture was then centrifuged at 1000 *g* for 5 min, obtained the supernatant and added 1 ml of 90% acetone to the pellet. The above steps were

repeated until the supernatant was colourless. The extract was supplemented with 90% acetone to reach a volume of 5 ml and measured the optical density at 530 nm.

For the treatment of algal residue in olive oil, the mixture was centrifuged, collected olive oil with astaxanthin and measured OD 530 nm. The standard curve equation established with astaxanthin (Sigma -Aldrich) in olive oil is y=66.628x+0.4603 (Fig. 2), where y is the concentration of astaxanthin (µg/ml) and x is the OD value. Astaxanthin was quantified three times in each experimental treatment.

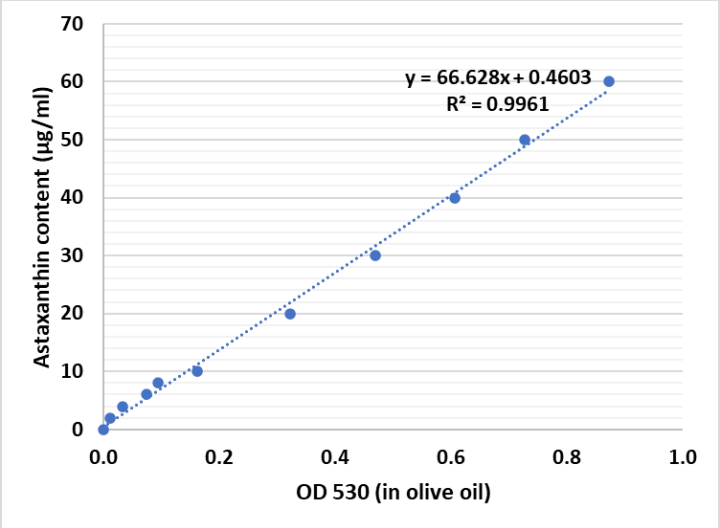

Fig. 2. *Calibration curve equation established with standard astaxanthin in olive oil*

c. Method of testing astaxanthin content in food: To ensure that the supplemented astaxanthin at concentrations of 50, 100 and 150 mg/kg, 0.1 g of feed (with standard astaxanthin or *H. pluvialis* residue added) was weighed and ground in 5 ml of 90% acetone. The mixture was centrifuged at 1000 *g* for 5 min, collected the supernatant and continued to add 5 ml of 90% acetone to the pellet. The above steps were repeated until the supernatant was colourless. Then, the entire supernatant was collected and added 90% acetone to a volume of 20 ml. Extracts were measured optical density at a wavelength of 530 nm.

2.4. Data analysis

The statistical analysis and graphing were done with R software version 3.4.2. Values are presented as mean ± standard deviation (SD) of at least three replicates of the treatment.

3. Results and discussion

3.1. The result of analysing the nutrient content of H. pluvialis residue

The protein content of *H. pluvialis* residue is 35.5% (Table 1), higher than the protein content of other foods such as chicken egg (13%), fish meat (22%), and cornstarch (10-15%). However, the protein content of algal residue is lower than that of soybeans (36%)

and meat and bone meal (49-52%). During fry stage, the dietary protein ratio of the fish should reach 50%. That ratio in fingerling stage and large fish should reach 40% and 35% respectively (Vu, 2006). Therefore, algal residue is enough to supply the protein needs of mature fish. Carp need about 12-15% lipid in the diet. With a lipid content of 14.9%, *H. pluvialis* residue shows to fully meet the needs of the fish. The proportion of fiber in the diet of fish and shrimp is recommended from 8 to 10% while the need for glucide in carp is about 40-45% (Vu, 2006). Therefore, the glucide and fiber content of algae residue does not meet the nutritional needs of carp. The quantitative results show that the remaining astaxanthin in algae residue is about 0.69% of the dry weight (Fig. 3). Therefore, algae residue can be used as a supplement to fish food along with other ingredients.

Table 1. ***Main nutritional composition of H. pluvialis residue***

Nutrient	Unit	Result	Analytical method
Lipid	%	14.9 (hydrolysed)	TCVN 4331:2001
Glucide	%	36.8	TCVN 4594:1988
Protein	%	35.5 (total nitrogen × 6.26)	CASE.NS.0039 (Ref.AOAC 990.03)
Crude fiber	%	4.48	CASE.NS.0022 (Ref.ANKOM Technology Method 10)
Astaxanthin	%	0.69	Extracted in 90% acetone and measured OD 530nm

3.2. Results of choosing the treatment method for H. pluvialis residue

Algal residue treated with 0.1 M NaOH results in the highest released astaxanthin, 0.5% dry biomass (Fig. 3). The efficiency of releasing astaxanthin from algae of this method can reach 72.16% when compares to the control (method of grinding algae in 90% acetone used to determine the exact amount of astaxanthin in algae). Meanwhile, the amount of extracted astaxanthin is the lowest (only 0.12%) when using water and high temperature autoclaving. The difference is statistically significant ($p < 0.05$, $n = 3$). The research results of Mendes-Pinto showed that the method of treating algae cells with NaOH was less effective than other methods (Mendes-Pinto et al., 2001). However, the treatment was done on intact algae biomass. In this experiment, treatments were carried out on astaxanthin extracted algae residue, which have been pretreated with viscozyme. The cell wall of *H. pluvialis* is mainly composed of cellulose, so alkaline NaOH solution can swell cellulose fibers of microalgae, which contributes to cell rupture and releases of substances inside (Zhang et al., 2002).

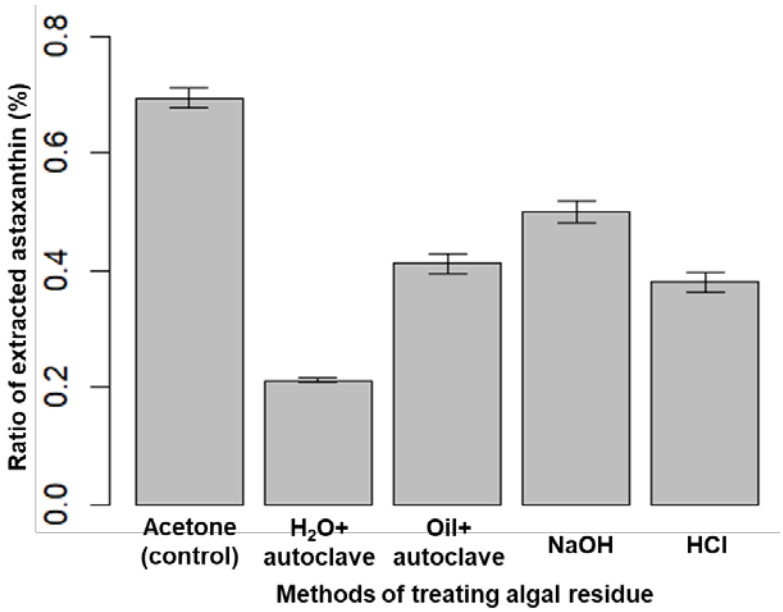

Fig. 3. *Astaxanthin content obtained in different treatment methods of algal residue*

Therefore, algae residue is treated with NaOH and neutralized with HCl to supplement to fish feed in the next experiments.

3.3. Results of mixing H. pluvialis residue into Japanese Koi fish feed

Based on the results of nutrient composition analysis and algal residue treatment methods to release astaxanthin, the formula for mixing Koi fish feed are presented in Table 2.

Table 2. *Nutritional composition of Koi fish feed in six different treatments*

Nutritional composition	Control -	Control +1	Control +2	Treatment 1	Treatment 2	Treatment 3
Protein (%)	~64	>55	~64	~64	~64	~64
Humidity (%)	~9,2	<8	~9,2	~9,2	~9,2	~9,2
Lipid (%)	~4,8	>9	~4,8	~4,8	~4,8	~4,8
Carbohydrate (%)	~4,8	<1,9	~4,8	~4,8	~4,8	~4,8
Astaxanthin (mg/kg)	no additions	unidentified	50	50	100	150

After mixing and drying, the feeds of the astaxanthin-supplemented treatments were rechecked for their astaxanthin content (Fig. 4). The astaxanthin supplementations were found at 48.9 µg (control +2), 49.1 µg (treatment 1), 100.1 µg (treatment 2), and 149.1 µg (treatment 3) per gram of feed.

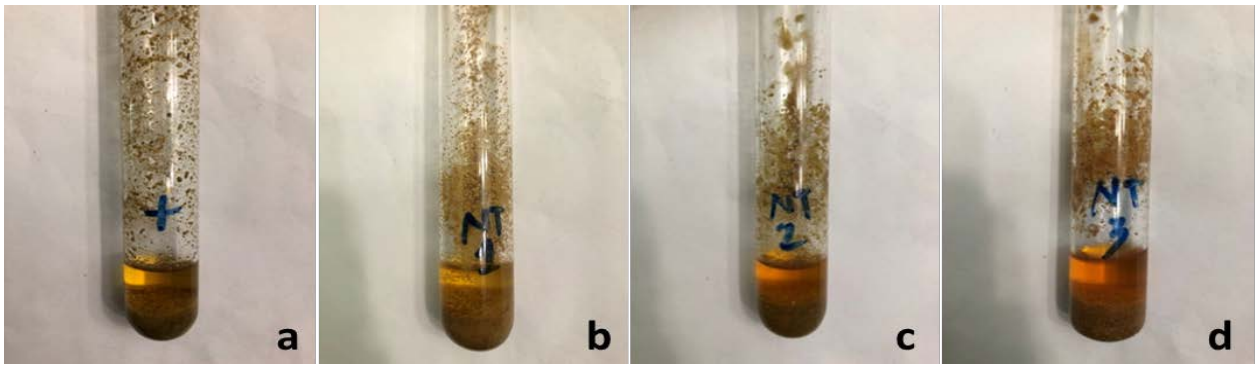

Fig. 4. *Test of astaxanthin content in feed of control +2 (a), treatment 1 (b), 2 (c) and 3 (d)*

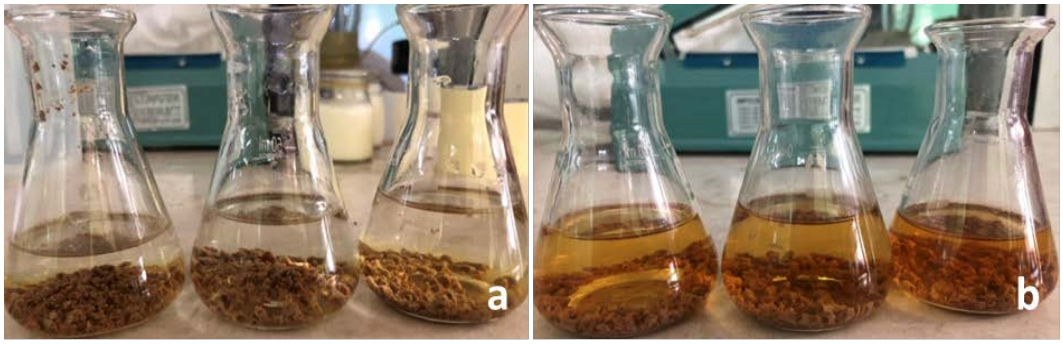

Fig. 5. *Astaxanthin-supplemented foods were soaked in water at first (a) and after 5 h (b)*

The durability of food was tested by soaking in water. The results showed that the food durability of all treatments reached 5 h. After 5 hours, the food colour was faded away and the water colour changed (Fig. 5). Therefore, to avoid affecting water quality, fish are fed with a sufficient amount of feed and leftovers are removed 30 minutes after eating.

3.4. Effects of food supplemented with H. pluvialis residue on the growth and colour of Koi fish

After 14 d, the survival rate of fish was 100% in all treatments. Fish were sampled to measure the increase in length and weight. The average fish length is from 6.5 to 6.8 cm, the average weight is 4.3-4.6 g (Fig. 6). However, fish length and weight do not differ significantly between treatments ($p>0.05$). Therefore, the supplementation of algae residue and astaxanthin do not affect the survival rate and growth of Koi fish in comparison to other treatments.

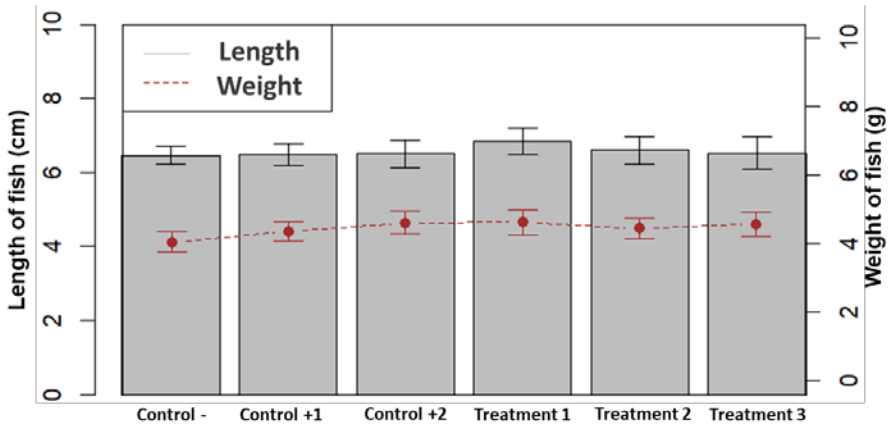

Fig. 6. *Length and weight of Koi fish after 14 d*

Results of colour comparison showed that the colour of fish of treatment 1, 2 and 3 reached 8-9, and had a significant change in comparison to the time before starting the experiment. Meanwhile, the colour of fish in control -, control +1 and control +2 only reached 7-8 (Fig. 7). Therefore, the supplementation of astaxanthin at concentrations of 50, 100 and 150 mg/kg improves fish colour after 14 d of feed, but the difference between treatments is negligible. The fish colour of control +2 was only 7-8 because the standard astaxanthin added in the diet of this treatment was in pure and free form, so it could be oxidized and deactivated. Astaxanthin in treatment 1, 2 and 3 exists in ester form, so the ability to be oxidized during storage is significantly reduced (Miao et al., 2006).

Previous studies have shown that a dietary supplement of 40-65 mg/ kg astaxanthin can maintain and enhance the colour of rainbow trout (Nguyen & Nguyen, 2013). Research by Trinh (2010) showed that astaxanthin content at 78 mg/kg feed had good results in improving fish colour. In addition, the study of Torrissen et al. (1990) also suggested that an astaxanthin content of about 50 mg/kg is reasonable. However, for swordfish, the optimal astaxanthin content is quite high, at 200 mg/kg feed (Song et al., 2017).

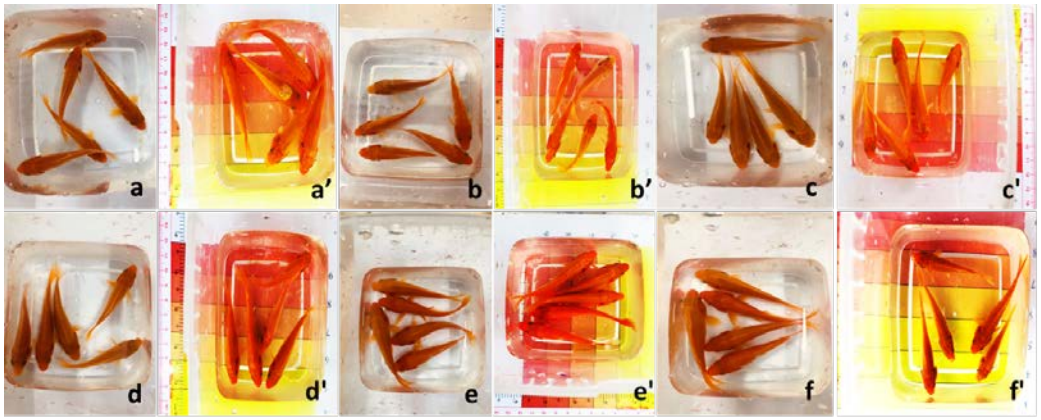

Fig. 7. *Koi fish before experiment (a, b, c, d, e, f) and after 14 d of feeding in all treatments: control -(a'), control +1 (b'), control +2 (c'), treatment 1 (d'), 2(e'), and 3(f')*

Astaxanthin content at 36-37 mg/kg is optimal for fish colour during 4 weeks of feeding (Paripatananont et al., 1999). However, our experimental results showed no significant difference between the three treatments supplemented with astaxanthin. That may be due to the short experimental period, so the pigmentation of fish is not enough to see a clear difference. Initial results show that the amount of astaxanthin supplemented to Koi fish feed at 50mg/kg is appropriate and economical.

4. Conclusions

The nutritional composition of *H.pluvialis* residue contains 35.5% protein, 14.9% lipid, 36.8% glucide, 4.48% crude fiber and 0.69% astaxanthin. The method of treating algal residue with 0.1M NaOH shows the highest extraction efficiency, reaching 72.16% of the remaining astaxanthin in the algae. The astaxanthin content in the algal residue supplemented to feed at 50 mg/kg can enhance and maintain the color of Koi fish. With the nutritional composition and remaining astaxanthin content, the *H. pluvialis* residue can be completely applied in food production for Koi fish.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

❖ ***Acknowledgement:*** ***T****his research is funded by Ho Chi Minh City University of Education, under grant number: CS.2019.19.28.*

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NGHIÊN CỨU SỬ DỤNG BỘT TẢO *HAEMATOCOCCUS PLUVIALIS* NUÔI TRONG BIOFILM ĐÃ BỊ LI TRÍCH ASTAXANTHIN LÀM THỨC ĂN BỔ SUNG CHO CÁ KOI NHẬT

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Ngày nhận bài: 16-12-2020; ngày nhận bài sửa: 04-3-2021; ngày duyệt đăng: 01-9-2021

TÓM TẮT

Thành phần dinh dưỡng của bã tảo H. pluvialis sau khi tách chiết astaxanthin bằng phương pháp xử lí bằng viscozyme và chiết cồn tuyệt đối còn lại 35,5 % protein, 14,9 % béo, 36,8 % glucide, 4,48 % xơ thô và 0,69% astaxanthin. Bã vi tảo được xử lí bằng phương pháp nghiền cơ học kết hợp dung dịch NaOH 0,1M cho thấy hiệu suất tách astaxanthin cao nhất (72,16 %) khi so với các phương pháp khác như: nghiền cơ học kết hợp dung dịch HCl 0,1M (54,75 %), phương pháp dùng dung môi dầu olive kết hợp hấp tiệt trùng (59,26 %), và phương pháp dùng dung môi nước và hấp tiệt trùng (30,54 %). Sự bổ sung bã vi tảo chứa astaxanthin vào trong thức ăn ở các nồng độ astaxanthin 50mg/kg, 100 mg/kg và 150 mg/kg đều có tác dụng tăng cường tạo sắc tố đỏ cho cá Koi Nhật sau 14 ngày khảo sát.

Từ khóa: Astaxanthin*; Cyprinus carpio; Haematococcus pluvialis*; cá Koi