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TRẦN QUANG VINH

STUDY ON CULTIVATION AND EXTRACTION OF ASTAXANTHIN FROM *HAEMATOCOCCUS PLUVIALIS* AND *RHODOSPORIDIUM* SP., AND TESTING SOME BIOACTIVES

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Advisor 1: Assoc. Prof. Dr. Ngo Dai Nghiep

Advisor 2: Prof. Dr. Hoang Nghia Son

Reviewer 1: …

Reviewer 2: …

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INTRODUCTION

1. The urgency of the thesis

Nowadays, natural-derived antioxidant compounds are a top concern. Among them, astaxanthin is being researched and applied by many scientists around the world, because it has been found to have antioxidant activity. Stronger than β-carotene, lycopene, lutein or vitamin E, astaxanthin can prevent the growth of cancer cells, lower blood cholesterol, protect the skin from ultraviolet rays, prevent skin aging and degeneration gold spots, ...

Currently, most commercial astaxanthin is either chemically synthesized products or carotenoids. However, due to the rapidly increasing demand for natural products and the high cost of artificial products, the search and exploitation of natural sources of astaxanthin are of particular interest.

Therefore, this thesis studies the culture, stress to accumulate high astaxanthin in *Haematococcus pluvialis* cells. In addition, the yeast *Rhodosporidium toruloides* is a new for science. It was isolated and identified in the study with the ability to produce synthesized astaxanthin from nature, under Vietnamese conditions. Also, it be used to research, obtain and extract astaxanthin for testing some biological activities for application to the pharmaceutical, cosmetic, and aquatic industries.

2. Objective of the thesis

Optimizing the culture process of *H. pluvialis* and *Rhodosporidium toruloides* yeast that biosynthesize high astaxanthin.

Extraction of astaxanthin from *H. pluvialis* and yeast *R. toruloides*; Evaluation of some biological activities of astaxanthin, in order to orient the application for pharmaceutical, cosmetic, animal husbandry and aquaculture industries.

3. Research contents

Research, optimize and upgrade the culture process of *H. pluvialis* to obtain astaxanthin; Stress experiments with monochromatic light and nitrogen content to obtain high astaxanthin content.

Research and optimize the process of culturing yeast *R. toruloides* to obtain high astaxanthin content; Upgrade the culture process at the scale of 10 liters to extract and obtain astaxanthin.

Extraction of astaxanthin from biomass of *H. Pluvialis* and yeast *R. toruloides*.

Testing some biological activities of astaxanthin obtained from the above two subjects such as: reducing, oxidizing, antibacterial and pigment enhancing ability on red discus *Symphysodon* sp.

CHAPTER 1. OVERVIEW

1.1. Research of astaxanthin on Haematococcus pluvialis

1.1.1. Research in the world

According to the study of Esra Imamoglu et al. (2009), the initial process of proliferation of *H. pluvialis* cells was carried out in BG11 medium until the cells reached the highest density, then transferred to RM medium with changes on nutritional composition and light intensity to induce stress. The results obtained were that in the RM medium without the presence of nitrogen under the illumination intensity of 546 μmol photons/m2/s, the accumulated astaxanthin content was highest (30.07 mg/g) on day 13 (Ye et al., 2012).

Meanwhile, *H. pluvialis* cells in BBM lip (control) started to accumulate astaxanthin on day 9, and the maximum astaxanthin content was 4.17 mg/g. Li et al. (2011) evaluated the economic

efficiency of astaxanthin production from large-scale cultivation of *Haematococcus* in China with a two-stage culture of this microalgae.

1.1.2. Research in Vietnam.

Research results of Dang Diem Hong et al. (2012) showed that when the nitrate concentration in the culture medium increased by 4 times, the maximum cell density increased by 25%, reaching 0.95×10^6 TB. /ml. Simultaneously, culturing *H. pluvialis* in a medium with high nitrate concentration and in combination with adjusting the lighting regime and refreshing the medium has been shown to be an effective method to achieve high cell densities. The maximum cell density of *H. pluvialis* reached 3.2×10^6 TB/ml after 22 days of culture in RM - 4X medium ($NaNO_3$ concentration is 1,200 mg/l), combined with white light and UV irradiation with high intensity. the irradiance is 4.3 klux and 1.4 klux respectively, the light-dark cycle is 16:8 hours, of which 10 hours of white light and 6 hours of white light combined with UV are 6 hours.

Le Thi Thom et al. (2013) studied the effect of nitrate concentration in the culture medium on the growth of *H. pluvialis* Flotow at the level of 250 mL conical flask. The experimental nitrate concentrations were: 219 mg/L, 438 mg/L, 876 mg/L, 1,314 mg/L, 1,752 mg/L and 2,190 mg/L, respectively, in which the nitrate concentration was 876 mg/L (nitrate concentration 4 times higher than basal RM), was determined to be the most suitable for the growth of this microalgae. At the above appropriate nitrate concentration, the highest cell density, chlorophyll a and astaxanthin content were 1.74×10^6 TB/mL, 2,081 μg/L, 1,053 μg/L, respectively.

Research by Trinh Thi Lan Chi (2010), on the trial of adding astaxanthin and canthaxanthin pigments to food for Japanese carp (koi carp - *Cyprinus carpio*), the results showed that: With the supplement content > 25 mg/ kg of feed, astaxanthin had a positive effect on improving color in Japanese carp, in which the most effective content was 78.22 ± 5.84 mg/kg feed.

1.2. Research of astaxanthin from *Rhodosporidium toruloides*

1.2.1. Research in the world

Yang et al. (2011) used cassava residues - industrial by-products, used for the fermentation of *Phaffia rhodozyma* strain to obtain astaxanthin. The appropriate factors are as follows: total sugar content in cassava residue is 40 g/l, KH_2PO_4 is 1.5 g/l, $MgSO_4$ is 0.5 g/l. Yeast extract and $(NH_4)2SO_4$ with a ratio of 3:2 (w:w) are believed to be the best nitrogen source for the growth of yeast *Phaffia rhodozyma*. The obtained astaxanthin content can be up to 2.98 mg/l.

Ferrao and Garg (2012) also focused on investigating the two main influencing factors, carbon and nitrogen, on the acquisition of β-carotene in *Rhodotorula graminis* strain. The study showed that mannitol (as a carbon source) had a positive effect on biomass accumulation and β-carotene content, with mannitol content ranging from 10 to 20 g/l and nitrogen source being selected as yeast extract with the content ranging from 9.5 to 10 g/l, the maximum dry weight achieved was 3.8 - 4.3 g/l and the maximum β-carotene content was obtained. the maximum is 190 - 220 mg/l.

Anfeng Xiao et al. (2015) studied the relationship between intracellular metabolites and astaxanthin accumulation during fermentation of 4 mutant strains of *Phaffia rhodozyma*. The results

showed that the accumulation of ethanol, intracellular proteins, and fatty acids competitively affects astaxanthin synthesis. In which, strains of *P. rhodozyma* JMU-VDL668 and JMU-7B12 achieved lower yields (1.7 and 1.2 mg/L) than the two strains JMU-MVP14 and JMU-17W (20.4 and 3.9 mg/L).

Carla Dias et al. 2016 studied the effect of culture medium pH (3.5-6.0) on carotenoids and lipids (such as fatty acids) produced by the yeast *Rhodosporidium toruloides* NCYC 921, the results showed that Yeast biomass and lipid concentrations were maximum at pH 4.0 (5.90 g/L and 21.85% w/w, respectively), while carotenoid content was maximum (63.37 mg/g) at pH 5.0. The reported results may contribute to the optimization of fermentation on *Rhodosporidium toruloides.*

1.2.2. Research in our country

Vo Thi Hong Trieu (2015) investigated different carbon sources used to culture *Rhodosporodium toruloides* strains including molasses, glucose, saccharose and glycerol to obtain the highest carotenoid content. The results showed that when rearing the strain *R. toruloides* with molasses as the carbon source, the highest carotenoid content was obtained (2.5 ml/L of culture solution).

CHAPTER 2. MATERIALS AND METHODOLOGY

2.1. Study on environment and culture conditions for astaxanthin collection on *H. pluvialis*

The algae strain *H. pluvialis* capable of astaxanthin biosynthesis was provided by the Aquaculture Research Institute II.

Select the medium, establish the conditions for the multiplication of *H. pluvialis.*

Investigation of the effect of light on the growth cycle of *H. pluvialis.*

Table 2.1. Nutritional composition of the medium C, RM, F/2 and B

Components of culture medium	Medium (mg/l)			
	C	**RM**	**F/2**	**B**
$NaNO_3$	-	300	75	250
$NaH_2PO_4.H_2O$	-	-	5	-
$Na_2SiO_3.9H_2O$	-	-	30	-
Na_2EDTA	2,71	-	4,36	-
$CoCl_2.6H_2O$	0,012	-	0,01	-
$CuSO_4.5H_2O$	-	0,08	0,01	0,06
$FeCl_3.6H_2O$	5,888	17	3,15	-
$MnCl_2.4H_2O$	0,108	-	0,18	-
$Na_2MoO_4.2H_2O$	0,0075	-	0,006	-
$ZnSO_4.7H_2O$	0,066	0,1	0,022	0,2
HCl Thiamin	0,01	-	0,1	-
Biotin	0,0001	-	0,0005	-
B_{12}	0,0001	-	0,0005	-
K_2HPO_4	-	80	-	75
KH_2PO_4	-	20	-	175
$CaCl_2$	-	-	-	25
NaCl	-	20	-	25
$MgSO_4.7H_2O$	40	10	-	75
$FeCl_3$	-	-	-	0,3
$MnSO_4.4H_2O$	-	-	-	0,3
H_3BO_3	-	0,3	-	0,2
$Ca(NO_3)_2$	150	-	-	-
KNO_3	100	-	-	-
β – Na_2glycerolphosphate	50	-	-	-
$CaCl_2$. 2 H_2O	-	58,5	-	-
EDTA	-	7,5	-	-
$MnSO_4$. H_2O	-	1,5	-	-
$(NH_4)_6Mo_7O_{24}$. 4 H_2O	-	0,3	-	-
$Co(NO_3)$. 6 H_2O	-	0,26	-	-

Trisaminomethiane	500	-	-	-

2.2. Study of stress by monochromatic light and nitrogen content to obtain astaxanthin

Investigate the influence of light mode on the astaxanthin accumulation efficiency of *H. pluvialis.*

Investigate the influence of nitrogen sources and suitable nitrogen concentrations for the growth of *H. Pluvialis*.

2.3. Study on conditions of culture medium for astaxanthin acquisition on yeast *Rhodosporidium toruloides*

The yeast strain *Rhodosporidium toruloides* (sequencing rDNA-ITS with a primer pair ITS1-ITS4-5.8S rDNA at Nam Khoa BioTek) was able to biosynthesize astaxanthin.

2.4. Optimization of the culture process of *Rhodosporidium toruloides* yeast for astaxanthin

Process optimization was performed by Box-Behnken surface response method and Design Expert software to obtain high astaxanthin-containing carotenoids.

2.5. Improvement of the process of culturing, extracting and obtaining astaxanthin from the biomass of *H. Pluvialis* and *Rhodosporidium toruloides*

Upgrade at 20 liter scale on *H. Pluvialis* and 10 liter scale on yeast *Rhodosporidium toruloides.*

Extraction was carried out by mechanical agent, solvent, hydrochloric acid and enzyme to obtain the highest astaxanthin.

2.6. Evaluation of some biological activities of astaxanthin obtained from *H. Pluvialis* and *Rhodosporidium toruloides*

Evaluation of reducing, oxidizing, antibacterial and pigment enhancing properties in red discus *Symphysodon* sp.,

CHAPTER 3. RESULTS AND DISCUSSIONS

3.1. Extraction of astaxanthin from *H. pluvialis*

The life cycle of the microalgae *H. pluvialis* consists of four stages, each with different morphology, size, and pigment content. In this study, we observed four basic growth stages of the algae: the vegetative cell (TB) stage, cystogenic stage, complete follicular cell stage, and germination stage.

The RM medium yielded the highest cell density, astaxanthin content, and dry weight of algae, with respective values of $5.13x10^5$ TB/ml, 487 μg/l, and 2.32 g/l. The worst algae growth was observed in the F2 medium.

During the first 14 days of culture, the MTB increased gradually and reached a maximum on the 14th day, after which the density gradually decreased. In contrast, the dry weight of the algae tended to increase gradually over time. This is because when the microalgae enter the stable phase (day 14 of culture), the algal cells decrease in division, and the algae move from the vegetative cell stage to the follicular phase, causing the cell size of this stage to start changing. As the head increases, the dry weight of the algae also increases. After the 18th day of culture, the medium becomes deficient in nutrients suitable for the accumulation of astaxanthin in algal cells.

H. pluvialis algae grew and thrived best in white light (maximum cell density was $5.53x10^5$ TB/ml, astaxanthin content was 469.33 μg/l, and dry weight was 2.35 g/l). The lowest was red light (maximum cell

density was $2.6x10^5$ TB/ml, astaxanthin content was 66.67 µg/l, and dry weight was 0.62 g/l).

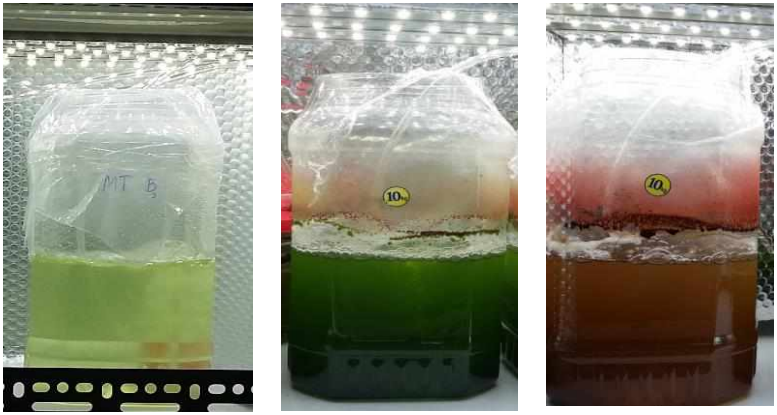


Figure 3. 1: Color change of *Haematococcus pluvialis* (10 liters culture scale)

3.2. Stress by light intensity on astaxanthin accumulation efficiency of *H. pluvialis*

When using the stress agent of high light intensity during phase II, the astaxanthin content increased dramatically, and there was a statistically significant difference between the control group and the experimental group. After four days of stress, the treatment with 120 µmol/m2/s light intensity had the highest astaxanthin content of 4618.67 µg/l, while the control treatment with 90 µmol/m2/s illumination had an astaxanthin content of 1073.6 µg/l. However, in the treatment with a light intensity of 180 µmol/m2/s, the astaxanthin content decreased rapidly during the second culture phase. This treatment had highly fluctuating light intensity, which caused most of the algae to sink and gradually die.

A light intensity of 120 µmol/m2/s and a temperature of 35°C were found to be suitable for astaxanthin accumulation. When algae were stressed at 120 µmol/m2/s for four days during phase II, the highest

astaxanthin content obtained was 4,618.67 µg/l, and the dry weight obtained was 4.44 g/l.

Table 3. 1: Astaxanthin content of *H. pluvialis* when shocked by different light intensities

Day	Experiment		
	Control (µmol/m^2/s)	120 (µmol/m^2/s)	180 (µmol/m^2/s)
14	324,8 ± 0,00^a	324,8 ± 0,00^a	324,8 ± 0,00^a
1	558,93 ± 26,06^b	724,27 ± 16,03^a	752,53 ± 33,57^a
2	628,27 ± 16,11^b	942,4 ± 31,96^a	360 ± 54,19^c
3	968,53 ± 54,19^b	1891,2 ± 3,2^a	96 ± 135,1^c
4	1073,6 ± 66,7^b	4618,67 ± 12,1^a	0,00 ± 0,00^c

The data presented in the table are the mean ± standard deviation of each treatment, the data in the same row with different letters represent the statistically significant difference (P < 0.05).

3.3. Stress by nitrogen content during culture of *H. pluvialis* and acquisition of astaxanthin

During the growth of *H. Pluvialis* algae, it consumes a large amount of nitrogen. Specifically, in all four experimental plots, the nitrogen content on the first day of culture in the 2nd and 3rd batches was 112.14 mg/L and 112.42 mg/L, while in batches 1 and 4, the nitrogen content was 111.07 mg/L. After switching to growing H. Pluvialis in phase 2, these values gradually decreased until the H. Pluvialis cells switched from the cyst to the complete cyst state, which accumulated a large amount of astaxanthin. At this time, the nitrogen content in the 2nd batch decreased to 63.38 mg/L, while batches 1 and 3 were 63.05 mg/L and batch 4 had a nitrogen content of 60.23 mg/L.

Based on the analysis results, the astaxanthin content after extraction ranged from 5.144 µg/L to 7,535.8 µg/L, corresponding to 2.34% to 2.61% of the starting material.

Table 3. 2: Astaxanthin content after extraction

Parameter	lô 1	lô 2	lô 3	lô 4

Dry biomass (g/L)	0,25	0,3	0,22	0,3
Astaxanthin content (μg/L)	6.530,5	7.520	5.144	7.535,8
Astaxanthin in dry biomass (%)	2,61	2,51	2,34	2,51

3.4. Testing the reducing and oxidizing activity and the ability to enhance red pigment in discus of astaxanthin from microalgae H. *pluvialis*

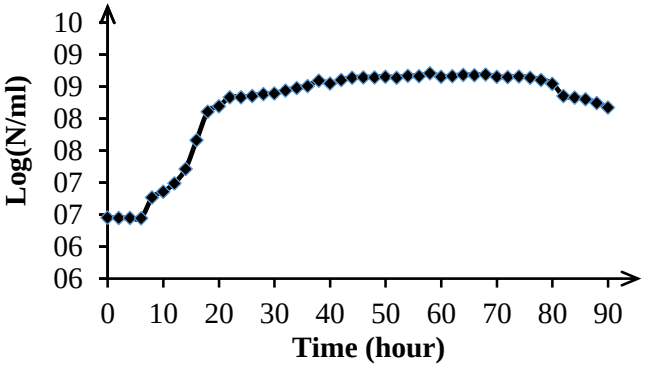
The astaxanthin extract obtained from the algae *H. Pluvialis* has antioxidant activity and higher antioxidant capacity than the positive control BHA, resulting in 1.17 times stronger reducing capacity than BHA and strong ABTS+ radical scavenging ability, which was 1.53 times higher than the BHA sample. The research results on color enhancement in discus with a dose of 75 mg/kg of astaxanthin obtained from Haematococcus pluvialis algae resulted in enhanced red pigmentation in discus.

3.5. Acquisition of astaxanthin from yeast *Rhodosporidium toruloides*

3.5.1. Determination of some components in molasses before and after treatment required for the cultivation of yeast *Rhodosporidium toruloides* to obtain astaxanthin.

The results showed that the mineral composition added to the molasses medium such as $MgSO_4.7H_2O$ 3 g/l, urea 0.5 g/l, and KH_2PO_4 1 g/l, with a similarity ratio of 10% compared to the culture medium, increased the cell density from 8 to $10x10^6$ cells/ml. The total sugar content in molasses was 25 g/l. The time taken to acquire astaxanthin from *Rhodosporodium toruloides* was 82 hours.

3.5.2. The results of building the growth curve of *Rhodosporidium* sp. on the molasses environment and investigating the appropriate factors have been obtained.



To calculate the cell density in 1 ml of log medium (N/ml), the yeast cell counts were determined based on OD610 nm values from 0.1, 0.2, 0.3, 0.4, and 0.5. The research results showed a linear correlation between cell density and OD610 nm values. Based on this correlation, the growth curve of *Rhodosporidium toruloides* strain was constructed in the investigated molasses medium. The yeast strain showed different growth stages, including the latent phase around the first 6 hours, the exponential growth phase between the 8th and the 30th hours, the steady phase from the 32nd hour to the 80th hour, and the decay phase from the 82nd hour onwards.

To optimize the environmental composition for obtaining the highest astaxanthin in the yeast strain *R. toruloides*, we used Design Expert software. The optimal media composition was predicted and is shown in Table 3.3. We then cultured the yeast strains on the predicted medium and obtained the results shown in the following table.

Table 3.3: Experimental results and prediction of astaxanthin content in the optimal medium.

	Total sugar content (g/l)	**Urea (g/l)**	**$MgSO_4.7H_2O$ (g/l)**	**KH_2PO_4 (g/l)**	**pH**	**Carotenoids content (µg/g)**
Preddiction	26,04	1,04	3,0	2,0	5,96	183,162
Experiment	26,04	1,04	3,0	2,0	5,96	**186,48**

The results obtained after optimizing the composition of the medium showed that when rearing the yeast strain *Rhodosporidium toruloides* in molasses medium with a total sugar content of 26.04 g/l, urea content of 1.04 g/l, $MgSO_4.7H_2O$ of 3 g/l, KH_2PO_4 of 2 g/l, pH of 5.96, and seed rate of 10% for a culture time of 82 hours (v/v), the highest carotenoid content of 186.48 µg/g was obtained.

3.6. Upgrading and surveying culture conditions for astaxanthin collection in a 10 liters fermentation system.

Dry biomass weight and astaxanthin content were measured in a 10-liter culture system with a 10% seed ratio of *Rhodosporodium toruloides* on optimal molasses medium. We investigated the suitable factors for *Rhodosporodium toruloides* yeast culture (volume 100 ml) to obtain the most effective astaxanthin with a total sugar content of 25 g/l, industrial urea of 0.5 g/l, industrial $MgSO_4.7H_2O$ of 3 g/l, KH_2PO_4 of 0 g/l, and a similarity rate of 10% compared to the volume

of culture broth cultured for 80 hours. The astaxanthin content was 1,186.88 µg/l, and the dry biomass weight was 6,3312 g/l.

Table 3. 4: Astaxanthin content obtained in a culture volume of 100 ml

Weight of dry biomass obtained (g/l)	**Astaxanthin content**	
	(µg/g dry biomass)	**(µg/l culture fluid)**
6.3312 ± 0.0507	187.666 ± 3.695	**1186.88 13.638**

Based on the above results, we can conclude that when culturing the yeast strain *Rhodosporodium toruloides* on upgraded molasses medium in a 10-liter system, the dry biomass obtained was 6,3682 (g/l), and the corresponding astaxanthin content per body was 1932.21 (µg/l).

3.7. Extraction of astaxanthin by acid HCl/DMSO/enzyme cellulase

The astaxanthin content obtained through the extraction methods varied. DMSO yielded the highest amount of astaxanthin (287,159 µg/g), followed by extraction with HCl that gave a lower amount of astaxanthin (133,428 µg/g). Extraction with cellulase enzyme yielded the lowest amount (82.319 µg/g).

DMSO extraction resulted in the highest astaxanthin content because DMSO can hydrophobically bind to the phospholipid layer of the cell membrane, causing membrane dilation and even cell swelling. Additionally, DMSO can disrupt lipid and protein interactions, which affects the function of cell membranes. Some water-soluble solvents such as acetone, ethanol, and methanol can easily penetrate the outer

water layer and effectively extract astaxanthin from pink yeast. Acetone is the solvent for extracting astaxanthin, and this effect is consistent with the study of Hui et al. (Hui Ni, 2008).

3.8. Test of the reducing and oxidizing activity and the red pigment enhancement ability in discus fish of astaxanthin from yeast Rhodosporodium toruloides.

The antioxidant and antibacterial activities of astaxanthin extract obtained by DMSO were evaluated, and the astaxanthin content was determined by HPLC/MS to be 5.09 µg/mg. The results of antioxidant activity were evaluated through ABTS+ and DPPH free radical scavenging capacity. The DPPH free radical scavenging ability of astaxanthin extract was found to be 2.62 times and 1.93 times lower than that of vitamin C and BHA, respectively. The ABTS+ free radical scavenging activity of astaxanthin extract had an IC50 value of 14.83 µg/ml, which was equivalent to vitamin C and BHA having IC50 values of 14.33 µg/ml and 14.43 µg/ml, respectively. The reducing power of astaxanthin extract at 25 µg/ml was 1.28 times higher than the control, and vitamin C had a reducing power 1.16 times higher than BHA. Therefore, astaxanthin is a substance with high antioxidant activity, which is higher than the standard substances vitamin C and BHA.

Astaxanthin also showed high antibacterial ability with an antibacterial diameter (mm) of about 16 to 18 mm at an astaxanthin concentration of 100 µg/ml against Bacillus subtilis, E. coli, Salmonella typhi, Pseudomonas aeruginosa, and Staphylococcus aureus strains (depending on the type of bacteria). The antibiotic chloramphenicol, used as a positive control, had the highest

antibacterial diameter (mm) ranging from 20 mm to 35 mm, depending on the strain.

Astaxanthin extract obtained from the pink yeast Rhodosporidium toruloides was capable of inhibiting the growth curve of bacteria such as Bacillus subtilis, E. coli, Pseudomonas aeruginosa, Salmonella typhi, and Staphylococcus aureus right from the first concentration of 12.5 µg. Thus, the minimum inhibitory concentration of astaxanthin against the five bacterial strains studied (MIC) was 12.5 µg/ml.

The study on color enhancement in discus fish using a dose of 90 mg/kg of astaxanthin obtained from the yeast Rhodosporidium toruloides resulted in enhanced red pigmentation in discus fish.

3.9. Comparison of results on *H. pluvialis* and yeast *Rhodosporidium toruloides*

The results of comparing and contrasting the results of astaxanthin obtained from *Haematococcus pluvialis* and *Rhodosporidium toruloide*s yeast, as well as testing some biological activities are shown in Table 3.5.

Table 3.5: Comparison of results on the acquisition and biological activity of astaxanthin extracted from *Haematococcus pluvialis* and *Rhodosporidium toruloides* yeast

Contents	**Microalgae *Haematococcus pluvialis***	**Yeast Rhodosporidium toruloides**
Cultivation of astaxanthin	Life cycle of *Haematococcus pluvialis*: During the first 12 to 14 days of culture, the algae take on a globular, blue	The life cycle of yeast *Rhodosporidium toruloides* on molasses media is divided into 4 phases. The latent phase lasts about 6 hours, followed by the exponential growth phase

	appearance, lose two flagella, and become immobile. From days 14 to 20 of culture, the algae enter the follicular phase, and the endoplasm within the cells turns brown as the algae begin to accumulate astaxanthin. The maximum cell density, astaxanthin content, and dry weight of algae cultured in RM medium were $5.13x10^5$ TB/ml, 487 µg/l, and 2.32 g/l, respectively. Algae *H. pluvialis* thrived best under white light, with a maximum cell density of $5.53x10^5$ TB/ml, astaxanthin content of 469.33 µg/l, and dry weight of 2.35 g/l. The lowest growth was observed under red light, with a maximum cell density of $2.6x10^5$ TB/ml, astaxanthin	which lasts from 8 to 30 hours. The stable phase lasts from 32 to 80 hours, and then the decay phase begins at the 82nd hour. To the molasses medium, mineral components such as $MgSO_4.7H_2O$ (3 g/l), urea (0.5 g/l), and KH_2PO_4 (1 g/l) were added, resulting in a similarity ratio to the culture medium of 10% (cell density from 8 to 10x106 cells/ml). The total sugar content in molasses is 25 g/l. Astaxanthin was acquired from *Rhodosporodium toruloides* after 82 hours. Optimal conditions for obtaining carotenoids from the yeast strain *Rhodosporidium toruloides* include a molasses medium with total sugar content of 26.04 g/l, urea content of 1.04 g/l, $MgSO_4.7H_2O$ content of 3 g/l, KH_2PO_4 content of 2 g/l, pH of 5.96, and seed rate of 10% (v/v). The culture time was 80 hours, resulting in the highest carotenoid content of 186.48 µg/g.

	content of 66.67 µg/l, and dry weight of 0.62 g/l.	
Extraction	Samples extracted with DMSO had a higher astaxanthin content than those extracted with glass	Extraction with DMSO yielded the highest astaxanthin content (287,159 µg/g), followed by HCl (133,428 µg/g), while the lowest was obtained through enzymatic extraction at 82,319 ± 4,4823 µg/g.
Bioactivity test	The astaxanthin extract obtained from *H. pluvialis* algae exhibited antioxidant activity, as evidenced by a 1.53 times stronger ABTS+ radical reduction capacity compared to BHA samples. Additionally, the astaxanthin sample had a 1.17 times stronger reducing capacity than BHA. Moreover, research findings demonstrated that the administration of a 75 mg/kg dose of feed to discuss resulted in an	The astaxanthin extract from yeast *Rhodosporodium toruloides* showed antioxidant activity 2.62 times stronger than that of the control and 1.93 times stronger in DPPH assay. The reducing power of the astaxanthin extract at 25 µg/ml was 1.28 times higher than that of the control vitamin C and 1.16 times higher than that of BHA. The extract inhibited the growth curve of bacteria, including Bacillus subtilis, E. coli, Pseudomonas aeroginosa, Salmonella typhi, and Staphylococcus aureus, starting from the first concentration of 12.5 µg. The research showed enhanced red pigmentation

	enhanced red pigment in the fish.	in discus with a dose of 90 mg/kg feed.

CHAPTER IV. CONCLUSIONS AND RECOMMENDATIONS

For the microalgae Haematococcus pluvialis:

- The Algae RM medium resulted in the highest cell density, astaxanthin content, and dry weight, with respective values of 5.13x105 TB/ml, 487 µg/l, and 2.32 g/l. The F2 medium, on the other hand, resulted in the worst algae growth.

- H. pluvialis thrived best in white light with a maximum cell density of 5.53x105 TB/ml, astaxanthin content of 469.33 µg/l, and dry weight of 2.35 g/l. In contrast, red light resulted in the lowest values with a maximum cell density of 2.6x105 TB/ml, astaxanthin content of 66.67 µg/l, and dry weight of 0.62 g/l.

- When stressed with a light intensity of 120 µmol/m2/s for 4 days (phase 2) at a temperature of 35°C, the corresponding astaxanthin content was 4,618.67 µg/l, and the dry weight obtained was the highest at 4.44 g/l. When the stress was increased by 24 hours of light, the astaxanthin content was 3,076.8 µg/l, and the dry weight obtained was 3.72 g/l at a high temperature of 33°C.

- A 20-liter process for cultivating *H. pluvialis* algae biomass has been developed using a 2-phase culture process: Phase I uses RM liquid medium, an initial algae density of 105 TB/ml, white LED light with an intensity of 90 µmol/m^2/s, lighting time of 12 hours, continuous aeration, and CO_2 with a content of 18 ppm for photosynthesis. Phase 2 uses a lighting intensity of 120 µmol/m^2/s and a lighting time of 12 hours. The

astaxanthin content after extraction ranged from 5,144 to 7,535.8 µg/L, accounting for 2.34 - 2.61% of SKK (defined as a unit of dry weight of H. pluvialis biomass).

The astaxanthin extract obtained from the algae *H. Pluvialis* exhibits antioxidant activity and higher antioxidant capacity than the positive control BHA. Specifically, it has a 1.17 times stronger reducing ability than BHA and a 1.53 times stronger ABTS+ radical scavenging ability. Additionally, when administered at a dose of 75 mg/kg of feed, the astaxanthin extract from *H. Pluvialis* enhances the red pigment in discus fish.

For Yeast *Rhodosporodium toruloides*

- Mineral ingredients, including $MgSO_4.7H_2O$ (3 g/l), urea (0.5 g/l), and KH_2PO_4 (1 g/l), were added to the molasses medium. The seed to culture medium ratio was 10% (cell density from 8 to $10x10^6$ cells/ml), and the total sugar content in the molasses was 25 g/l. Astaxanthin from Rhodosporodium toruloides was acquired after 82 hours.

- Rhodosporodium toruloides was cultivated on a 10-liter molasses medium, resulting in a dry biomass of 6.3682 g/l and an astaxanthin content per volume of culture solution of 1.243 µg/l.

- The medium composition was optimized to increase astaxanthin yield from Rhodosporidium toruloides. The optimized medium included a total sugar content of 26.04 g/l, urea content of 1.04 g/l, $MgSO_4.7H_2O$ (3 g/l), KH_2PO_4 (2 g/l), pH 5.96, and seed rate of 10% (v/v). The culture time was 80 hours, and the highest astaxanthin content obtained was 186.48 µg/g. Comparison of the experimental results with the predicted results showed that the carotenoid content in the experimental optimal

medium was 186.48 µg/g, which was higher than the predicted carotenoid content of 3,318 µg/g.

- Astaxanthin was extracted from Rhodosporidium toruloides using DMSO solvent, 0.4N HCl, and cellulase enzyme. The highest yield was obtained using DMSO (287,159 µg/g), followed by 0.4N HCl (133,428 µg/g) and the lowest was obtained using enzyme (82,319 ± 4,4823 µg/g).

- To evaluate the antioxidant and antibacterial activities of the astaxanthin extract obtained by DMSO, the astaxanthin content was determined by HPLC/MS to be 5.09 µg/mg. The results showed that the astaxanthin extract had high antioxidant activity as determined by ABTS+ and DPPH free radical scavenging capacity. The IC50 values for DPPH free radical scavenging capacity were 2.62 times and 1.93 times higher for vitamin C and BHA, respectively, compared to the astaxanthin extract. The IC50 value for ABTS+ free radical scavenging capacity of the astaxanthin extract was 14.83 µg/ml, which was equivalent to the values for vitamin C and BHA of 14.33 µg/ml and 14.43 µg/ml, respectively. Additionally, the reducing power of the astaxanthin extract at 25 µg/ml was 1.28 times higher than the control vitamin C and 1.16 times higher than BHA. These results indicate that astaxanthin is a substance with high antioxidant activity and is superior to standard substances such as vitamin C and BHA.

- Astaxanthin also showed high antibacterial ability. At a concentration of 100 µg/ml, the antibacterial diameter (mm) for Bacillus subtilis, E. coli, Salmonella typhi, Pseudogonas aeroginasa and Staphylococcus aureus strains was about 16 to 18 mm, respectively (depending on the extraction method). The antibiotic chloramphenicol was used as a positive control and showed the highest antibacterial

diameter (mm) ranging from 20 mm to 35 mm depending on the strain.

- The astaxanthin extract obtained from the pink yeast *Rhodosporidium* sp. could inhibit the growth curve of bacteria, including Bacillus subtilis, E. coli, Pseudomonas aeroginosa, Salmonella typhi, and Staphylococcus aureus, right from the first concentration of 12.5 µg. Thus, the minimum inhibitory concentration (MIC) of astaxanthin on the five bacterial strains that were studied was 12.5 µg/ml.

- In addition, the astaxanthin extracted from yeast biomass was found to improve the color of red discus *Symphysodon* sp. at a dose of 90 mg/kg of feed after feeding the fish for three months.

4.2. Recommendations:

Further studies are necessary to investigate the antineoplastic activity of astaxanthin on cell lines. It is also recommended to research creating nano-astaxanthin to increase the water solubility of astaxanthin for pharmaceutical and aquaculture applications.

NEW CONTRIBUTIONS OF THE THESIS

(1) The thesis provides a comprehensive study of the process and mechanism of astaxanthin biosynthesis in *Haematococcus pluvialis* microalgae cells. The research includes the application of monochromatic light technology (LEDs) and the investigation of the effect of nitrogen on growth and astaxanthin accumulation in microalgae *Haematococcus pluvialis*. The study was carried out in the lab and also upgraded to a 20-liter Pilot Scale.

(2) The thesis presents a newly isolated and identified yeast strain, Rhodosoridium toruloides, from Vietnam. The study involves an in-

depth investigation of the utilization of molasses by-products as a culture medium, as well as optimization of the culture process to achieve high astaxanthin production. The research was conducted at a scale of 10 liters and aimed to increase biomass and extract astaxanthin for practical applications.

(3) A comparative study was conducted to evaluate the biological activities of astaxanthin obtained from natural sources (algae and yeast Rhodosporidium turoloides) and chemically synthesized astaxanthin. The study includes the analysis of DPPH antioxidant activity, oxidation with ABTS+ and free radical scavenging ability, reducing capacity, as well as the antibacterial properties for pharmaceutical and cosmetic industries. Additionally, the research evaluated the color enhancement effect of astaxanthin on ornamental fish and aimed to provide useful insights for aquaculture in general and ornamental fish farming in particular.

LIST OF PUBLICATIONS

1. **Quang-Vinh Tran**, Quoc-Cuong Duong, Dang-Khoa Tran, Dai-Nghiep Ngo,*Rhodosporidium sp. Growth in molasses medium and extraction of its astaxanthin by using hcl*, Journal of Scienceand Technology, 2017-55 (1A) (2017) 8-18.

2. Tuyet Nhung Tran, **Quang-Vinh Tran,** Hao Thanh Huynh, Nghia-Son Hoang, Hoang Chinh Nguyen, Dai-Nghiep Ngo, *Astaxanthin Production by Newly Isolated Rhodosporidium toruloides: Optimization of Medium Compositions by Response Surface Methodology*,Not Bot Horti Agrobo, 2019, 47(2):320-327.

3. **Tran Quang Vinh**, Nguyen Thi Kim Lien, Ngo Đai Nghiep, 2020, *Studying the effect of supplementing with astaxanthin and β-glucan extracted from yeast biomass Rhodospridium sp. in the food for red discus Symphysodon sp., (translation)* Journal of Fisheries Science and Technology, 3/2020, ISSN:1859 – 2252.

4. **Tran Quang Vinh**, Hoang Nghia Son, Ngo Đai Nghiep, *DPPH-rating activities and differentation of astaxanthin Isolated from Rhodosporidium sp. by cellulase, Journal of Genetics and Applications (Translation),* Journal of Genetics and Applications, 2020, ISSN:0866-8566.

5. **Tran Quang Vinh**, Duong Quoc Cương, Ngo Đai Nghiep, Hoang Nghia Son. *Research for Isolation and research of antioxidant, anti-background activities of astaxanthin extract from Rhodosporidium sp. by DMSO (translation),* Tay Nguyen Journal of Science, 47/2021, ISSN 1859-4611.