Extraction of Astaxanthin From *Haematococcus Lactustris*

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May 2018

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A THESIS SUBMITTED TO THE
NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF BACHELORS OF ENGINEERING IN
ENVIRONMENTAL ENGINEERING

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APPROVAL SHEET

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ACKNOWLEDGEMENTS

We would like to give all our credit to Allah, for without His will, this project would not have been possible and our efforts would have gone in vain.

Secondly, we offer our sincerest appreciation to Engr. Rashid Iftikhar whose constant support and knowledge was a driving force for our achievement.

We are also extremely thankful to Associate Dean, Dr. Imran Hashmi for financially helping us import our algae culture without which our project would not have been possible.

Also, we are extremely grateful for all the help and support provided by the Masters and PhD students at the microbiology lab for teaching us various procedures and always taking out the time from their busy schedules to aid us in our work.

Lastly, we are thankful to our friends and families who constantly motivated us and provided the support we needed to complete our final year project.

DEDICATION

We dedicate this work to all the hard working individuals of the society who want to see sustainable growth and strive towards it. This is for all those who think differently than the rest and struggle to get their ideas across the table. For all those who understand the needs of the society and spend their whole lives working to fulfill them. This work is for all those people who want to see the environment sustain within their nation and who want things to be economical for even the poorest of the poor to afford; to all those who want equality of the society and equal distribution of resources. We also dedicate this work to our beloved land Pakistan, which has been our home in all tough and happy times and all those who hope to bring a positive change in this nation.

ABSTRACT

Extraction of Astaxanthin From *Haematococcus Lactustris*

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Microalgae is well-known for its role as the ecosystem stabilizer however, the importance of microalgae in industrial applications remain obscure even though it can be grown to become a source of some of the highly precious industrial products. Microalgae remains a commercially viable source of carotenoids, protein pigments with antioxidant properties, that can play a major role in cancer prevention supplements manufactured by neutraceutical companies. In this study carotenoid, Astaxanthin, was extracted from a microalgae specie, Haematococcus Lacustris. The microalgae was grown autotrophically for this purpose at 25°C under the illumination of 100 µmol photons m^{-2} s^{-1} with a 14:10 hour light:dark photo period. The algae was cultured in Bold's Basal Medium for 14 days and was then subjected to stress conditions of increased light intensity at 450 µmol photons m^{-2} s^{-1} to increase the Astaxanthin contents which was then extracted by different organic solvents and analyzed through UV-visible spectrophotometer. The result showed that highest extractability of Astaxanthin is achieved by Dimethyl Sulfoxide (DMS0) and its peak comes at 490 nm under the UV-visible spectrophotometer.

Key Words: Microalgae, Carotenoids, Astaxanthin, *Haematococcus Lacustris*

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1. INTRODUCTION

1.1 Background

Microalgae has a significant role in the ecosystem with respect to its fundamental property of taking up CO_2 from the environment and giving off O_2 and the quality of uptaking nutrients from the water bodies. However, researches on microalgae has truly raised the importance of microalgae from an ecosystem stabilizer to a major source of commercially viable products. Carotenoid is one such valuable product that is abundantly present in microalgae and extracting these compounds can prove to be a breakthrough in the field of biotechnology.

1.1.1 Chemistry of Carotenoids

Carotenoids are lipid soluble pigments that have a broad range of structures hence making up more than 700 types of naturally occurring carotenoids (Lorenz and Cysewski, 2000). These pigments can be found in different crops such as spinach and carrots as well as in fish and phtyoplanktons and are responsible for giving these things the colour that they appear to have. A common structural trait that is shared by all the carotenoids is that they consist of a C40 backbone structure of isoprene units (Gong and Bassi, 2016). The structures of some of the most researched carotenoids is shown in Figure 1.

Haematococcus synthesizes a carotenoid, Astaxanthin following the same carotenoid structure however, the variance in structure comes as the monoesters of astaxanthin link to 16:0, 18:1 and 18:2 fatty acids hence making the compound fat-soluble (Lorenz and Cysewski, 2000).

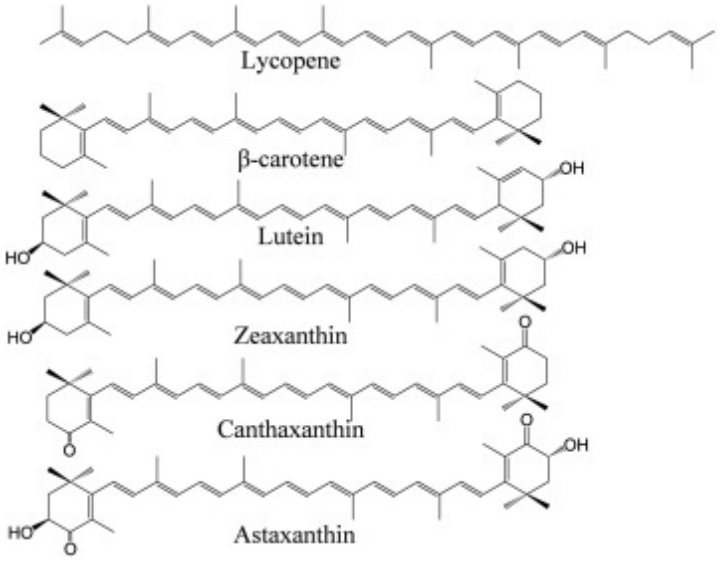


Figure 1:Structures of carotenoids (Gong and Bassi, 2016)

1.1.2 Application of Microalgae for Carotenoid Production In Nutraceutical Industry

The neutraceutical companies in the existing scenario rely heavily on food crops and chemicals to synthesize antioxidant supplements that are used to prevent cancer. However, the food crisis and economically unfeasible chemical synthesis of these supplements causes the society to not benefit completely from these products. In this situation, carotenoids from microalgae can function as a breakthrough in neutraceutical industries. The carotenoids obtained from microalgae have exceptional antioxidant properties which are linked with the prevention of formation of free radicals in human bodies. With this property, the carotenoid pigments can save the human cells from the harmful effects of free radicals which can otherwise cause cancer, AIDS, diabetes and neurodegeneration (Lorenz and Cysewski, 2000).

Microalgae have an edge over the other conventional sources of carotenoids as the algae tends to have faster production rate and is abundantly available. Moreover, microalgae can be produced rather cheaply as compared to the food crops since land requirements for its growth are not necessary and it can be grown in large amounts in a comprehensive photo substrate bioreactors (PSBR) in an industry (Podola et al., 2017). The cultivation of algae for carotenoid production in nuetraceutical industries is less labour and energy extensive and results in as higher production.

Lutein obtained from the marigold flowers goes as high as 0.3mg/g while the same carotenoid is produced in a quantity of 4mg/g in microalgae (Gong and Bassi, 2016). Hence, producing carotenoids from microalgae is highly beneficial for neutraceutical industries.

1.2 Research Motivation

- Limited research on valuable products that can be obtained from algae.
- To untap the potential of algae in order to benefit human health by producing affordable supplements.

The neutraceutical companies are playing a huge role in curbing diseases such as cancer by providing the society with supplements that contain antioxidant carotenoids. However, these chemically synthesized carotenoids are not affordable in an economically disturbed society. Moreover, the supplements produced from other food sources contribute to the food crisis. In the light of these issues, extracting valuable carotenoids from cheap algal sources can be a breakthrough for neutraceutical companies.

1.3 Objectives

The objectives of this study are:

- Cultivation and harvesting algae in suspension phase.
- Extraction of carotenoid from algae using organic solvents in order to achieve maximum extractability
- Inspection of carotenoids under UV- visible spectrophotometer.
- Comparison of obtained peaks with reference Astaxanthin.

1.4 Scope of Study

The research project was focused on extracting the cancer preventing carotenoid from microalgae in a commercially viable method. The study was carried out to grow *Haematococcus Lucastris* in two phases, optimal conditions and then stress conditions, so that maximum amount of Astaxanthin can be retrieved from the algal cells while maintaining the production cost at affordable levels.

In order to achieve this, the study concentrates on the cheap algae cultivation methods as well as the combination of organic solvents through which maximum Astaxanthin

could be extracted. The project would conclude its scope by using analytical tools to qualitatively establish the presence of Astaxanthin in the algal cells and the efficiency of organic solvents in extracting this valuable product.

1.5 Problem Statement

"Limited research on algal uses has restricted how we can fully utilize it for human benefits in terms of its health and economic feasibility."

1.6 Aim

"Develop and transform renewable biomass resources into commercially viable, high-performance bio-product through targeted research and development."

2. LITERATURE REVIEW

2.1 Production of Astaxanthin in *Haematococcus Lacutris*

Haematococcus Lacustris is the best natural source of Astaxanthin as when proper conditions are provided, the microalgae can accumulate up to 5% dry weight of the cell with Astaxanthin (Wayama et al., 2013). The algal specie goes under various changes in its lifecycle that determines the extent of accumulation of carotenoids in the cell. Primarily, the growth of *Haematococcus Lacustris* occurs in two stages, vegetative (exponential) and aplonospore(stationary) and the whole lifecycle in which these phases are observed normally last for 14 days. Under the vegetative phase, the algal cells transform from motile macrozooids cells to the resting vegetative cells, palmella. This transformation occurs under favourable conditions and exponential growth can be observed in this phase.

The aplonopores or haematocysts are formed in *H.Lacustris* under the "red nonmotile astaxanthin accumulated encysted phase". This phase is achieved under stress conditions such as high light intensity, high salinity or starvation. The macroozoids start losing the flagella under stress conditions hence forming resting vegetative cells, palmella. However, if the stress conditions continue to prevail, the vegetative cells transform into haematocysts with the development of thick trilaminar sheath and a secondary cell wall. The morphological changes observed in *Haematococcus Lacustris* are summarized in Figure 2 (Shah et al., 2016).

The production of Astaxanthin can thus be gained through two-stage cultivation where the cells are provided with favourable conditions in the first stage so that vegetative biomass increases and later on, the algal cells face extreme stress conditions so that the vegetative biomass converts into haematocysts, accumulating high amounts of Astaxanthin.

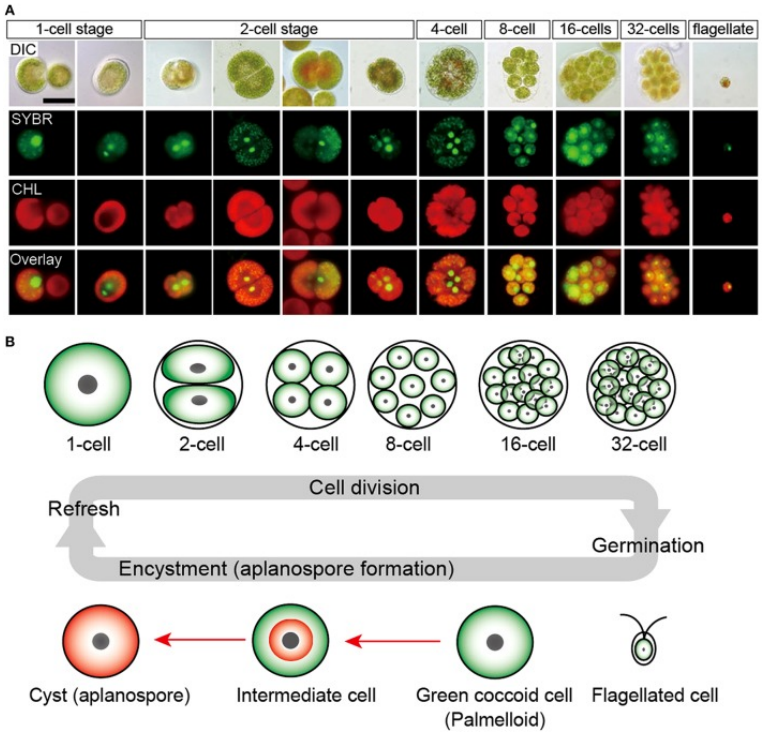


Figure 2:Morphological changes of Haematococcus Lacustris (Shah et al., 2016)

2.2 Cultivation of *Haematococcus Lacustris*

Cultivation of algal specie can be done through a variety of conventional and non-conventional methods which primarily differ in the phase of the growth of microalgae that is, in suspension or in attached phase. The most conventional method of algal cultivation is pond system however, it is often discouraged since there are higher risks of contamination. In laboratory scale, the conventional cultivation method of growth in suspension is utilized where the microalgae is placed in a covered flask, consisting of a nutrient medium.

A recent technology of PSBR allows the growth of microalgae in attached phase where the algae is placed on the twin layer of membrane through which the cells uptake nutrients. This method provides better prevention from contamination and allows much faster production of algal biomass as compared to the conventional cultivation methods (Podola et al., 2017).

2.2.1 Optimal conditions

Haematococcus Lacustris grows exponentially in vegetative stage when provided with nutrients through Bold's Basal Medium. The optimum environmental conditions were maintained by keeping the temperature at 25◦C under the illumination of 100 μ mol photons m^{-2} s^{-1} with a 14:10 hour light:dark photo period. A constant flow of sterilized air is also maintained in order to provide the algae with 0.03% carbon dioxide (Cheng et al., 2016).

2.2.2 Stress conditions

The cyst formation in *Haematococcus Lacustris* begins when the normal metabolic activity is altered due to the presence of stress conditions. The stress conditions can be applied through various means such as limiting the nutrient concentration in the culture medium. The starvation conditions can be prevailed by excluding nitrogen, phosphorus or both from the BBM. Apart from starvation, the stress conditions can also be maintained by subjecting the microalgae to high light intensities or high salinity (Imamoglu et al., 2009).

2.3 Harvesting and Cell Disruption methods

A series of pretreatment processes need to be implemented before the extraction of Astaxanthin from the algae cells can be made possible. Harvesting is carried out to separate the algal biomass from the culture medium. The biomass is retrieved from the suspension by centrifugation. This process is easy to conduct and is efficient for lab scale experiments however, in large scale projects centrifugation is not cost efficient because of the energy it requires (Barros et al., 2015).

Recent developments in the harvesting procedures has led to the growth of algae in photo substrate bio reactors (PSBR) which consists of harvesting in solid phase instead of in suspension phase. This technology allows cost and energy effective harvesting as the biomass is mechanically removed from the membrane.

Cell disruption methods primarily breakdown the thick cell wall that entails Astaxanthin within it. This helps in increasing the recovery yield of the Astaxanthin. Mechanical procedures such as such as high-pressure homogenization, grinding through mortar and pestle or glass beads and bread milling are some of the conventional

methods of cell disruption. The recent technological advances have paved way for non-mechanical cell disruption methods such as microwave, ultrasonication and pulsed electric field (Gong and Bassi, 2016).

2.4 Extraction Methods

The Astaxanthin produced in the secondary metabolic phase by *Haematococcus Lacustris* needs to be extracted first in order to carry out the analytical procedures.

The extraction is done on the basis of difference between the solubility of the pigment and the solvents since this causes the phase separation of a variety of compounds that are found in the algal cells such as chlorophyll, lipids from the Astaxanthin. Super and sub critical solvent extraction is the most recent method used for this purpose where CO_2 is used as the solvent. However, this process requires a huge amount of biomass and is not cost efficient. On the other hand, the conventional solvent extraction method is widely used because of the convenient and cost-effective procedure.

2.4.1 Organic solvents

The extraction through organic solvents is obtained on the concept of "like dissolves like" hence polarity is an important factor to take into consideration while choosing the organic solvents to carry out phase separation (Gong and Bassi, 2016). Organic solvent extraction can be conducted as single or binary systems. Dichloromethane, dimethyl sulfoxide, methanol, ethanol, hexane and acetone are some of the organic solvents that are widely used in extraction of Astaxanthin from microalgae (Sarada et al., 2006).

2.5 Analytical methods

The Astaxanthin production in the algal cells can be observed through a number of analytical tools such as LC-MS, HPLC, UV-visible spectrophotometer however, the most preferred ones to determine quantitative and qualitative analysis of these pigments are HPLC and UV-visible spectrophotometer, respectively.

2.5.1 HPLC

High performance liquid chromatography (HPLC) is widely used in order to quantify the amount of Astaxanthin produced by the microalgae. The specifications of HPLC are as follows:

- Reverse phase 25 cm×4.6 mm, 5 µm, C_{18} column
- Isocratic solvent system consisting of dichloromethane:acetonitrile:methanol (20:70:10, v/v/v)
- Solvent system run at flow rate of 1.0 ml/min.

The peak of Astaxanthin determined by HPLC is 490 nm (Ambati et al., 2009).

2.5.2 UV-visible spectrophotometer

The UV-visible spectrophotometer gives a qualitative analysis of the Astaxanthin extract from the algal specie. The absorption spectra gives a measure of the wavelength at which Astaxanthin shows peak. However, through this analytical method, peaks of Chlorophyll B as well as the total carotenoids are also prominently observed. The range of total carotenoid absorbance is observed from 478 nm to 490 nm (Ambati et al., 2009).

3. MATERIALS AND METHODS

3.1 Protocol

To ensure that the algae grew in an optimal state, there were certain protocols that were followed. All equipment was subject to cleaning in the autoclave at 121^{o}C for three hours. This included the 1000 ml flask in which the stock solution was grown. The same protocol was followed for distilled water too. This was done to ensure minimum contamination in the algal species. Furthermore, BBM was also autoclaved at 121^{o}C for three hours to eliminate the chance of any bacterial/fungal growth. Not only that, all transfers of algae or BBM or any stock solution were done in a laminar flow hood. Lastly, to ensure complete cleanliness, all smaller equipment, such as, pipette, 50 ml beakers or flasks were thoroughly washed with distilled water before use.

3.2 Growth of Algae in suspension phase

Haematococcus Lacustris, was obtained from The Norwegian Culture Collection of Algae, NORCCA, maintained and owned by the Norwegian Institute for Water Research (NIVA) and the University of Oslo (UiO). A 1 liter stock culture was prepared from the 20 ml *H.Lucastris* in 20x BBM, 25^{0}C under continuous illumination (100 mmol photons m^{-2} s^{-1}) in a 1 liter flask. The growth period lasted for 15 days before the color of the solution went from colorless to green. The sample was then observed under the microscope, which represented maturation in the cells.

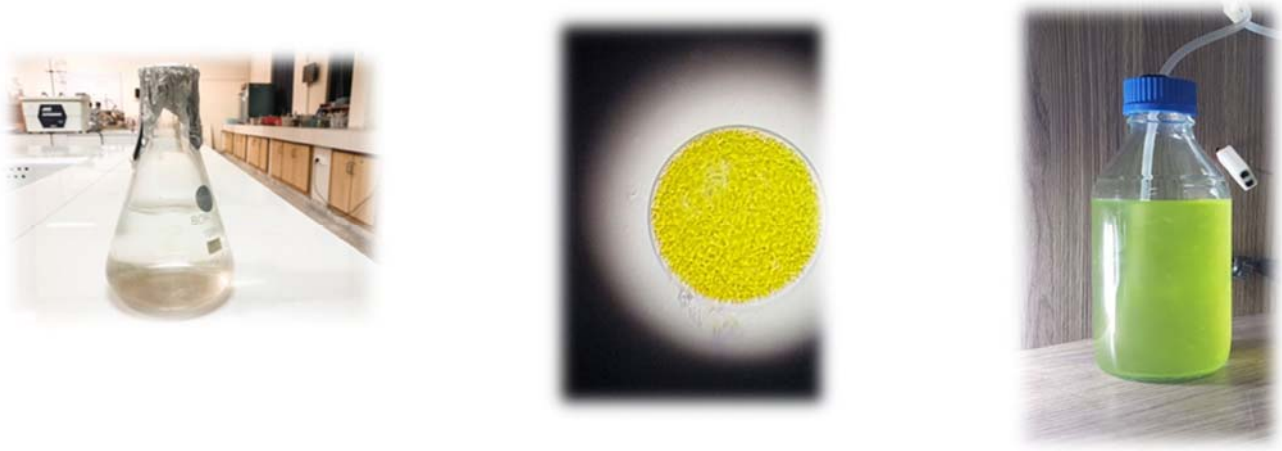


Figure 3: Growth of Algae in suspension phase

3.3 Stress conditions

After the algae grew, it was put under stress conditions i.e. high light intensity of 450 mmol photons m^{-2} s^{-1} and lowering the amount of BBM provided. This decreased the rate at which chlorophyll was produced and increased the growth of Astaxanthin within the cell.

3.4 Selection of media for growth

BBM was preferred over all other media, such as synthetic wastewater, because BBM provides the most appropriate amount of nutrients for the specific algae to grow in. This results in faster growth of algae and hence Astaxanthin. (Chadwick, 2014)

3.5 Solvents for BBM

The Bold Basal Medium was made by mixing various chemicals like Sodium Nitrate ($NaNO_3$), Sodium Chloride (NaCl), Monopotassium phosphate (K_2HPO_4), Manganese Chloride ($MnCl_2$) and other chemicals listed in the table below at room temperature and with constant stirring. The pH for the BBM was maintained at 7.

No.	Stock solution	Grams per liter of stock solution	Milliliter (ml) per liter medium
1	$NaNO_3$ $K_2HPO_4.3H_2O$ KH_2PO_4 NaCl	25 17.5 2.5	10
2	$CaCl_2.2H_2O$	2.5	10
3	$MgSO_4.7H_2O$	7.5	10
4	EDTA (Titriplex II) KOH	50 31	10
5	$FeSO_4.7H_2O$ H_2SO_4(Conc.)	4.98 1	1
6	H_3BO_3	11.42	1
7	$ZnSO_4.7H_2O$ MoO_3 $CuSO_4.5H_2O$	8.82 0.71 1.57	1

	$Co(NO_3)_2.6H_2O$ $MnCl_2.4H_2O$	0.49 1.44	

Figure 4:BBM stock solution chemicals

3.6 Solvents for extraction of Astaxanthin

Since carotenoids are soluble in apolar solvent (which include edible fats and oils), they were extracted using mild solvents to separate the carotenoid from the chlorophyll (Butnariu M, 2016). Three main solvents were used for the process of extraction. These have high polarities and allow saponification on the initial extraction procedure. The solvents are listed below:

i. Potassium Hydroxide (KOH) and Methanol
ii. Dimethyl Sulfoxide (DMSO)
iii. Hexane and Di-Chloromethane

A reference astaxanthin was also obtained from polyfine Nutra sciences for comparison of results.

3.7 Extraction procedures

The extraction procedures were carried out to separate out the required carotenoid from the solution containing cholorophyll and various sorts of nutrients. Furthermore, each extraction procedure was carried out twice, once when the algae was in its initial growth phase and one after full maturation has occurred. This was done in order to present that algae growth also results in astaxanthin growth. The extraction procedures are given as follows:

3.7.1 Mixing with KOH and Methanol

A 100 ml solution was taken out of the total stock solution and was centrifuged at 4000 rpm for three minutes. Once the solution was centrifuged and the supernatent was removed, 10 ml of 5% (v/v) KOH was measured with a pipette and added to the beaker containing the concentrated algal cells. Next, 30% (v/v) of Methanol was obtained and 10 ml of it was added into the beaker containing algae and KOH. Next, the process of homogenization was carried out. This was done via pipetting, where the solution was inserted into the pipette and expelled again and repeated. Once the solution was properly homogenized, the solution was centrifuged again at 4000 rpm for 3 minutes and the supernatent was removed again. The next process included the addition of 10

ml DMSO into the settled solid. The beaker was then placed into the waterbath, which was set at a temperature of 75^{o}C for 5 minutes.

3.7.2 Mixing with DMSO

100 ml of stock solution was obtained and was centrifuged at 4000 rpm for three minutes. Next, the supernatent was removed and the settled alga was then mixed with 10 ml DMSO. The obtained solution was placed in a water bath for 5 minutes at 75^{o}C and finally the mixture was homogenized via pipetting.

3.7.3 Mixing with Hexane and di-choloromethane

For the last method, a 100 ml stock solution was centrifuged at 4000 rpm for three minutes. After the supernatent was removed, 10 ml of dichloromethane and hexane were poured into the beaker at a ratio of 7:3 (dichloromethane: hexane). Homogenization was done via pipetting and constant stirring.

3.8 Analysis

3.8.1 Pure astaxanthin sample

The pure astaxanthin sample was used as a reference and the powder was mixed with water before observing it in a UV spectrophotometer.

3.8.2 Extracted samples

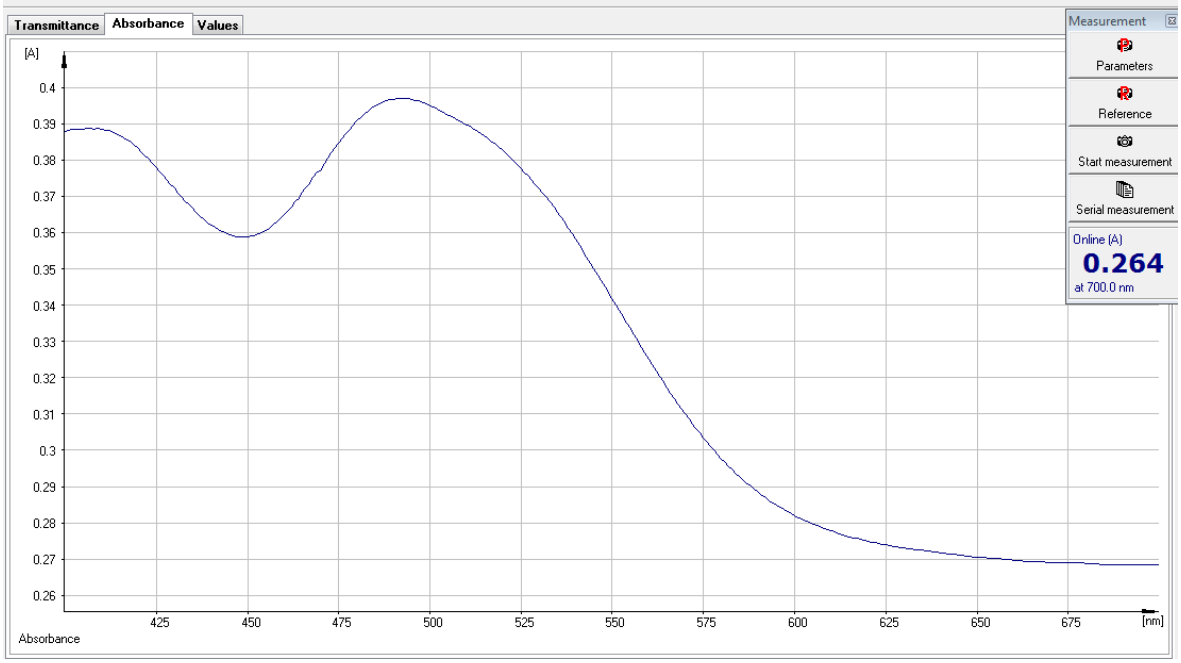
All the solutions obtained were then observed in a UV spectrophotometer at a range of 400-600 nm and the procedure was repeated twice to ensure accuracy in the readings and answers obtained.

4. RESULTS AND DISCUSSIONS

4.1 Reference astaxanthin

The reference astaxanthin obtained from nutra sciences gave a peak at 460-490 nm. All other peaks were observed in reference to that. Furthermore, peaks at 420nm depicted the presence of chlorophyll within the mixture. Any peak not corresponding to 460-490nm was assumed to be either chlorophyll or any other carotenoid present.

The peak of pure astaxanthin was obtained at 460. A peak at 410 was assumed to be some sort of impurity present within the mixture.

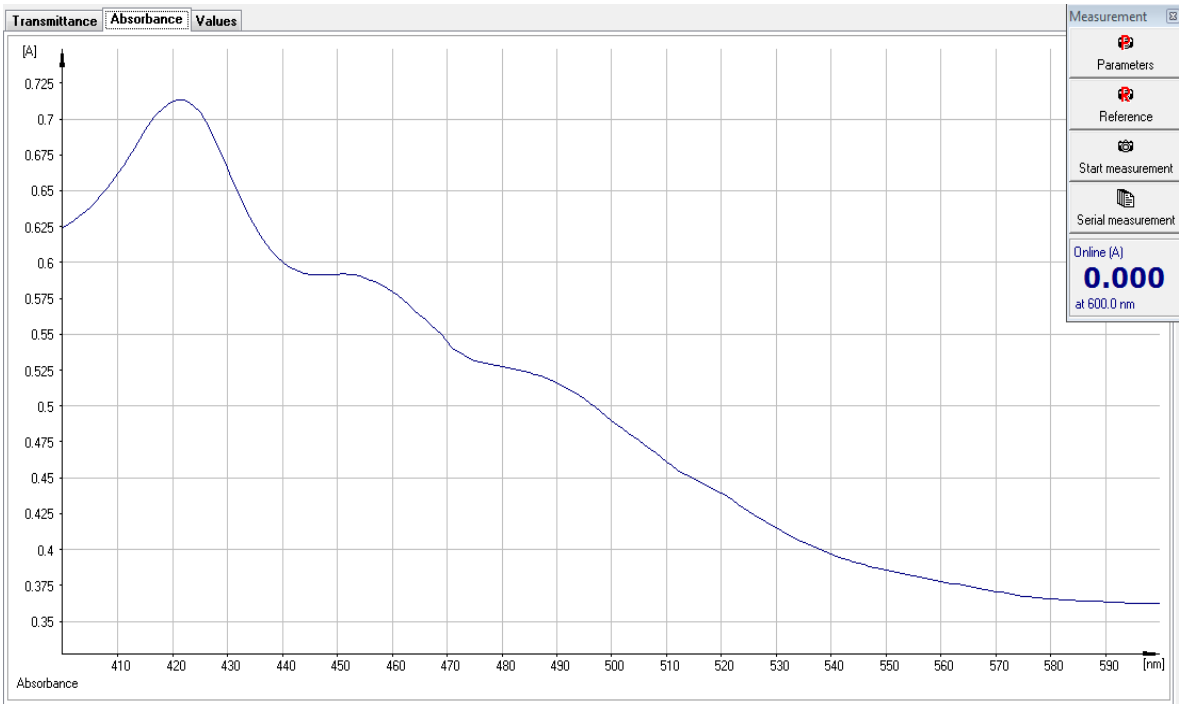


Graph 1:Peak at 490 nm of pure Astaxanthin sample in the absorption spectra

4.2 Results obtained with KOH and Methanol

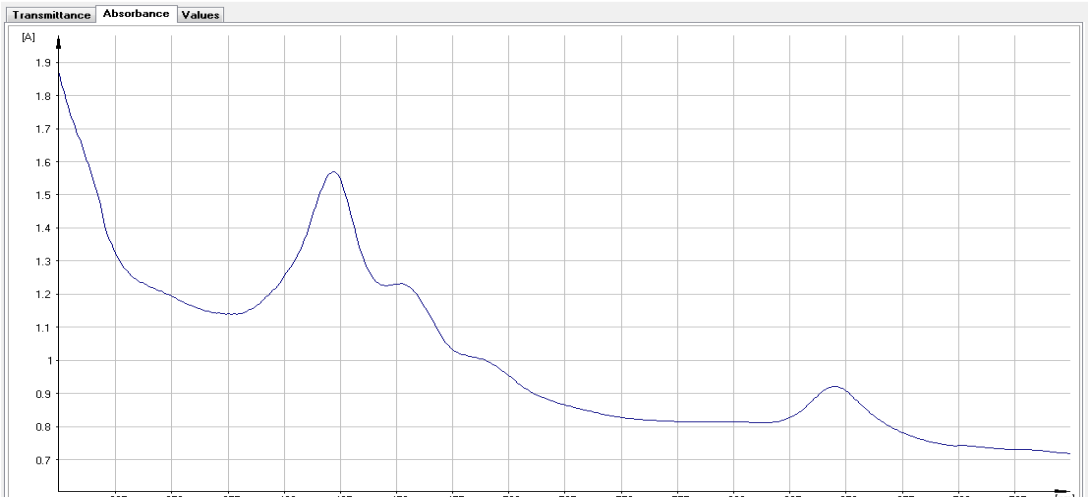
After initial growth of the algae had taken place, extraction of KOH and methanol was done and the following graph was obtained at a range of 400-600 nm from a UV spectrophotometer with the reference being distilled water. Any soft peaks obtained within the graph were assumed to be impurities present in the sample and were passed off as random error

Graph 2 presented a qualitative result regarding whether astaxanthin was present or not. Another extraction was done via KOH and methanol once the algae had reached its maturation phase. Graph 3 is a clear representation of that.



Graph 2:Peak at 420 nm for KOH and methanol after initial growth

Peaks of astaxanthin are supposed to be found at 490 nm. However, the peak in this graph is at 420 nm and a very soft peak is found at 450 nm. This means that due to the initial growing phase of algae, the astaxanthin had not matured and hence did not give a sharp peak at 490 nm as compared to the reference astaxanthin.



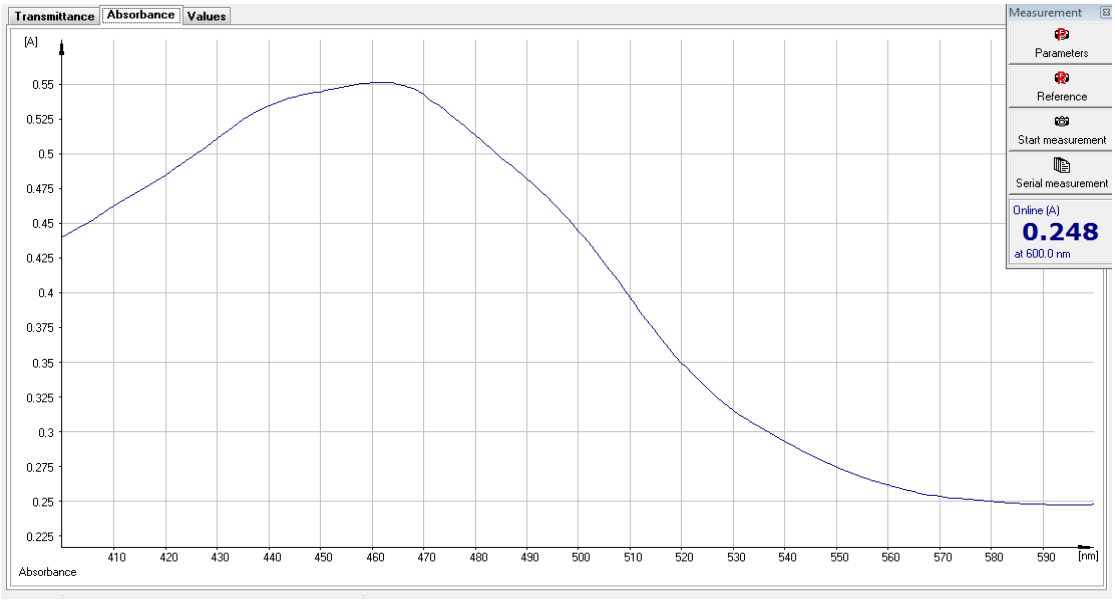
Graph 3: Peak at 460 nm for KOH and methanol after maturation

The next extraction procedure was done when algal cells had matured and the graph 3 depicts the peak obtained.

After the peak was obtained it was observed that a sharp peak was present near the 460nm-490 nm range. This meant that after maturation, astaxanthin was present and was detected by the UV-spectrophotometer. However, there were also a lot of other peaks present in the graph. This meant that KOH and methanol were not able to fully isolate and extract the astaxanthin.

4.3 Results obtained with DMSO

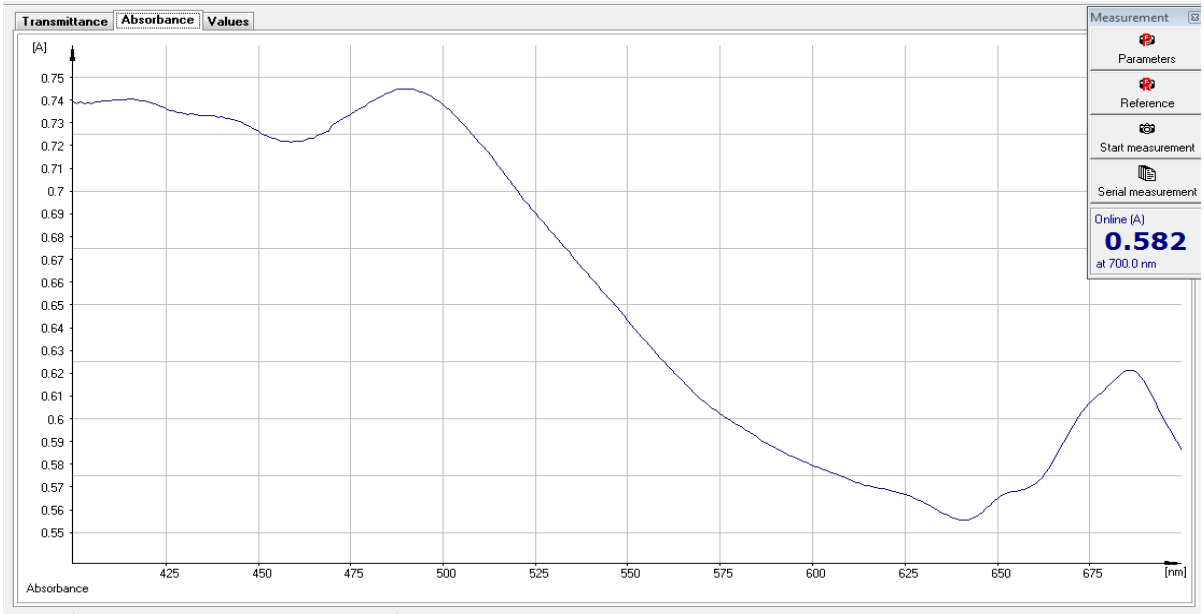
After initial growth of algae, the graph with DMSO was also obtained.



Graph 4:Peak at 460 nm DMSO after initial growth

Here, the graph shows a soft peak at 460 nm. Hence, it can be observed that small amounts of astaxanthin are present even at the initial phase of the growth process, but KOH and methanol were not able to extract it as well as only DMSO did. Furthermore, it can also be observed the the peak is completely smooth and there are no other peaks within this range. This means that there were no impurities within the mixture and the chlorophyll was completely removed from the mixture because of DMSO.

After maturation, the following graph was observed



Graph 5:Peak at 460 nm from DMSO after maturation

A sharp peak was observed in the 460nm-490nm range which indicated a presence of astaxanthin within the cells of *H.Lucastris.*

4.4 Results obtained with Hexane and Di-chloromethane

Initial extraction of astaxanthin showed the following results. These depict that even extraction with hexane and dichloromethane detected the presence of astaxanthin even at an initial stage, showing a many small peaks in the range of 460 nm-490 nm. However, these did not clearly represent the peak for astaxanthin. Neither were the other peaks recognizable. This shows that even hexane and dichloromethane did not

completely isolate the astaxanthin from other substances present within the algal species of *Haematococcus Lucastris.*

The second peak obtained after maturation is given as follows.

Graph 7: Peak at 460 nm obtained after extraction with hexane and dichloromethane after maturation

Graph 6: Multiple peaks obtained after extraction with hexane and dichloromethane after initial growth

The peak for astaxanthin observed after maturation was much smoother. However, it did not eliminate and completely isolate the astaxanthin from other substances present in the algae such as chlorophyll etc.

	Peak obtained after initial growth/nm	**Peak obtained after maturation of algal cells/nm**
Extraction with KOH and Methanol	420	460
Extraction with Dimethyl sulfoxide (DMSO)	460	490
Extraction with Hexane and Dichloromethane	Undefined	460

After all the peaks were obtained, it was found that the most smoothest and similar peaks obtained to the reference astaxanthin were gained by the extraction procudure with DMSO. The solvent showed complete removal of chlorophyll and did not present any peak with that. It also showed maximum extractibility of the carotenoid that was required after extraction. The results also depicted that the algae and the extraction procedures are prone to gaining many impurities and that is also represented in the UV Spectrophotometer. However, all methods did show a peak of astaxanthin apart from a couple of experiments and the best extractibility was obtained by DMSO.

5. CONCLUSION

This study showed the extractibility of astaxanthin from *H.Lucastris* using various solvents which dissolve lipids. This astaxanthin can then be used in the pharmaceutical and nutraceutical industry for further use. From the scope of this study, we were able to determine that algal growth is a highly unpredictable and irregular process. There are many uncertainities attached when growing algae. However, BBM proved to be the best in terms of helping the algae grow at a quick pace. This meant that BBM did prove to be the best medium for growth. Furthermore, following the correct protocol helped remove the unpredictability of algae as the best environment was provided for its growth, which also resulted in growth of Astaxanthin within the cells of the algae. All extraction procedures did not give the most appropriate results. However, extraction of Astaxanthin with DMSO did give the most smoothest peak as well as removed chlorophyll prior to observation in the UV spectrophotometer.

6. RECOMMENDATIONS

i. To increase the carotenoid yield, a porous substrate bioreactor (PSBR) can be utilized which grows algae in immobilized phase. This will result in faster and more concentrated growth of algae.

ii. To gain more comprehensive results in the future, the solution-after extraction-can be observed in a High Performance Liquid Chromatograph (HPLC) to have more quantitative results.

7. BIBLIOGRAPHY

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