Optimization of Physical Parameters of Astaxanthin Production from *Haematococcus pluvialis*

K.R. Monica Devi, S. Rubavathi, G. Ayyappadasan, N. Ruthra

Department of Biotechnology, K.S. Rangasamy College of Technology, Tiruchengode - 637 215, Namakkal, Tamil Nadu, India

Corresponding Author: S. Rubavathi

ABSTRACT

Haematococcus pluvialis, fresh water microalgae has been considered as a possible natural source for the production of astaxanthin and it has been widely studied. The general composition of *Haematococcus* algae consists of common carotenoids, fatty acids, proteins, carbohydrates, and minerals. Both of the main physical and chemical parameters, especially medium and light, directly control the growth rate of *Haematococcus pluvialis* Astaxanthin is a keto-carotenoid which is built from five carbon precursors isopentenyl diphosphate (IPP) and dimethyl allyldiphosphate. Astaxanthin has important metabolic functions in animals and humans ranging from protection against oxidation of essential polyunsaturated fatty acids, protection against UV light effects, pro vitamin A activity and vision, immune response, pigmentation and communication to reproductive behaviour and improved reproduction. The astaxanthin is the pigment that is considered as the most powerful antioxidants in nature. Due to the high production cost of synthetic astaxanthin and the market demand for natural astaxanthin, the biological sources of astaxanthin have long been widely exploited. To extract more astaxanthin from *Haematococcus pluvialis,* Growth optimization of the microalgae was done by using various medium such as BG-11, Basal, RM, BBM and TAP Medium**.** This comprehensive study on the determination of the culture medium and the light intensity was carried out to maximize the growth of *Haematococcus pluvialis* for batch cultivations. optimization was done by using different pH, temperature and light. The chlorophyll and protein content were analysed. And finally the astaxanthin was produced. The protein content of *H. Pluvialis* was found to be 338.27µg/ml.

Key words: *Haematococcus pluvialis*, fresh water microalgae, astaxanthin production

INTRODUCTION

The unicellular fresh water microalgae, *Haematococcus pluvialis* (Volvocales, Chlorophyceae) is a green-coloured, biflagellate, and motile in its vegetative stage. In its growth stages, it has both motile and non-motile forms. In the algal life cycle of *H. pluvialis*, green vegetative cells with two flagella can grow autotrophically in the light and heterotrophically on acetate in the dark. This microalga shows low growth rates and low final cell densities under optimal growth conditions. [1] In recent years the green microalgae *Haematococcus pluvialis* has been considered as a possible natural source for the production of astaxanthin and it has been widely studied. However, one of the main problems, according to the production of astaxanthin from *Haematococcus* is contamination with fast-growing unicellular green or blue-green algae due to the relative slow growth of *Haematococcus pluvialis*. *Haematococcus* cells are sensitive to high hydrodynamic stress and changes in cell morphology under various environmental conditions. No toxicity associated with *Haematococcus* has ever been reported in the literature. The general composition of *Haematococcus* algae consists of common carotenoids, fatty acids, proteins, carbohydrates, and minerals.

Astaxanthin synthesis in *H. pluvialis* is directly correlated in space and time with deposition of cellular reserves in lipid droplets under conditions of cellular stress. [2] Marine micro algae are focused in biomass production due to their ability to survive under environmental stresses like light intensity, dark, heat, UV exposure, nitrogen and also the metabolites production. [3]

During unfavourable growth conditions, *H. pluvialis* initiates carotenogenesis and undergoes morphological transformation from green vegetative cells to deep red, astaxanthin - rich, immotile aplanospores. Both the light intensity and the pH of the medium influenced the rate of growth of the microalgae. [4] The green alga that undergoes transformation to red algae after being exposed to some certain stress conditions in the carotenogenesis stage. More algal growth related with massive accumulation and production of astaxanthin

Astaxanthin is a keto-carotenoid. It belongs to a larger class of chemical compounds known asterpenes, which are built from five carbon precursors, isopentenyl diphosphate (or IPP) and dimethylallyl diphosphate. Astaxanthin is classified as a, but currently employed to describe carotenoid compounds that have oxygen-containing moities, hydroxyl (-OH) or ketone (C=O), such as zeaxanthin and canthaxanthin. Indeed, astaxanthin is a metabolite of zeaxanthin and canthaxanthin containing both hydroxyl and ketone functional groups. Like many carotenoid, astaxanthin is a colourful, lipid-soluble pigment. Astaxanthin has a wide range of applications in the food, feed, cosmetic, aquaculture, nutraceutical, and pharmaceutical industries because of its free radical scavenging capacity. [5] The study focussed on the production of astaxanthin in the isolate of *H.Pluvialis* since it accumulate high amount of astaxanthin. The optimization of environmental factors such as pH, temperature, light intensity, inoculum, and incubation period.

MATERIALS AND METHODS

SAMPLE COLLECTION AND INOCULUM PREPARATION

Pure culture microalgae sample of *Haematococcus pluvialis* stock culture was obtained from the company of ACME ProGen biotech (India) Pvt. Ltd., Salem. Stock culture of *H. Pluvialis* was grown in its adapted condition of photo autotrophically used BG11 medium under continuous illumination condition.

PREPARATION OF INOCULUM FOR PRODUCTION MEDIUM:

Prepared BG11 medium and culture was inoculated and kept in shaking incubator at 25ºC for 7 days. After incubation culture broth was centrifuged at 12,000rpm for 20mins. For the preparation of the inoculum, the cells from the stock culture were collected and concentrated by centrifugation (1,160 x g, 2 min) and the supernatant was removed. The collected cells were transferred, incubated aseptically in a 100 ml Erlenmeyer flask containing 80 ml of fresh BG11 medium under continuous illumination at 25°C for 4 days. Air was supplied to the culture at a flow rate of 1 L min-1 (1.25 vm). 4-day old culture (at vegetative cell growth phase) was used as inoculum at 10% volume for all experiments.

OPTIMIZATION OF ASTAXANTHIN PRODUCTION MEDIUM

The production of astaxanthin from *H. Pluvialis* sample for this studies astaxanthin production optimum medium was studied by 6 different type of medium was used such as BBM, Modified BG11, Basal medium, NPK complex, RM medium & TAB Medium. 5 different medium was prepared and sterilized, 10% of inoculum was inoculated into the medium and kept in incubator at 25ºC for 7 days under continuous illumination. After incubation 5 ml culture broth was transferred aseptically into the cuvette and take colorimetric reading at 680nm for checking culture growth rate.

DETERMINATION OF OPTIMUM PH FOR ASTAXANTHIN PRODUCTION

The growth rate of *H. Pluvialis* growing culture medium pH was studied by incubating the BBM medium at 5 different pH such as 5, 6, 7, 8, 9 and 10% of inoculum was inoculated into the medium and kept in incubator at 25ºC for 7 days under continuous illumination. After incubation 5 ml culture broth was transferred aseptically into the cuvette and take colorimetric reading at 680nm for checking culture growth rate [6]

DETERMINATION OF OPTIMUM TEMPERATURE FOR ASTAXANTHIN PRODUCTION

The growth rate of *H. Pluvialis* growing culture medium temp was studied by incubating the BBM medium at 5 different temperatures such as 23ºC, 25ºC, 30ºC, 37ºC, 40ºC and 10% of inoculum was inoculated into the medium and kept in incubated for 7 days under continuous illumination. After incubation 5 ml culture broth was transferred aseptically into the cuvette and taken colorimetric reading at 680nm for checking culture growth rate.

DETERMINATION OF OPTIMUM LIGHT INTENSITY FOR ASTAXANTHIN PRODUCTION

The growth rate of *H. Pluvialis* growing culture medium light intensity was studied by incubating the BBM medium under 3 different type of light conc. such as 12/12 light/dark, continuous light and continuous dark of inoculum was inoculated into the medium and kept in incubated for 7 days. After incubation 5 ml culture broth was transferred aseptically into the cuvette and colorimetric reading at 680nm for checking culture growth rate.

DETERMINATION OF OPTIMUM INOCULUM CONCENTRATION FOR ASTAXANTHIN PRODUCTION

The growth rate of *H. Pluvialis* growing culture medium inoculum conc. was studied by incubating the BBM medium at 5 different Conc. such as 2%, 4%, 6%, 8% & 10% of inoculum was inoculated into the medium and kept in incubated for 7days under continuous illumination. After incubation 5 ml culture broth was transferred aseptically into the cuvette and colorimetric reading was taken at 680 nm for estimation of culture growth rate.

DETERMINATION OF OPTIMUM INCUBATION PERIODS FOR ASTAXANTHIN PRODUCTION

The growth rate incubation period was studied by incubating the BBM medium at 4 different period of incubation such as 7 days, 10 days, 14 days & 20 days. The inoculum was inoculated into the medium and kept in incubated under continuous illumination. After incubation, 5 ml culture broth was transferred aseptically into the cuvette and colorimetric reading was taken at 680 nm for estimation of culture growth rate.

PRODUCTION OF ASTAXANTHIN

Based on optimization of production medium, 1000ml of BBM medium were taken and 6% of culture was inoculated and kept in incubator at 25ºC for 7 days under 12/12 light/dark condition. After incubation 5 ml culture broth was transferred aseptically into the cuvette and colorimetric reading was taken at 680nm for estimation of culture growth rate.

ESTIMATION OF CHLOROPHYLL

The chlorophyll content was determined by the method of Acetone solution. Acetone of 5 ml of uniform of the culture suspension of the culture was taken for the estimation of chl. Cells were centrifuged at 10000 rpm for 5 minutes in REMI C -24 centrifuge and to that pellet 5 ml of 80% acetone was added and left at 4ºC in dark. After 24 hours the suspension was centrifuged and the optical density of the supernatant was read at 663nm in a spectrophotometer.

Chlorophyll Content (µg/ml) = OD*12.63*Volume of acetone extract / Volume of culture

EXTRACTION OF PROTEIN:

Cells were harvested by centrifugation and washed with Tris-HCL buffer (50mM, pH 7.5). The cells were resuspended in the same buffer and

sonicated in UP200s Hielscher sonicator until the cells were completely disrupted. Cell disruption was confirmed by observing the cell free extract under a microscope. The cell free extract was centrifuged at 12000 rpm in a sigma A-15 micro centrifuge for 10 minutes and the supernatant was used for the protein estimation. Estimation of protein was done by (Lowry *et al,* 1951) using BSA- Bovine albumin serum as a standard.

RESULTS AND DISCUSSION

OPTIMIZATION OF ASTAXANTHIN PRODUCTION MEDIUM:

H. Pluvialis used for Astaxanthin production. The production medium was optimized using 5 different type of medium was used such as BBM, Modified BG11, Basal medium, NPK complex, RM medium & TAB Medium. Based on colorimetric OD value at 680 nm growth rate of *H. Pluvialis* showed BBM medium only having high growth rate than other medium. So, BBM medium used for further optimization studies.

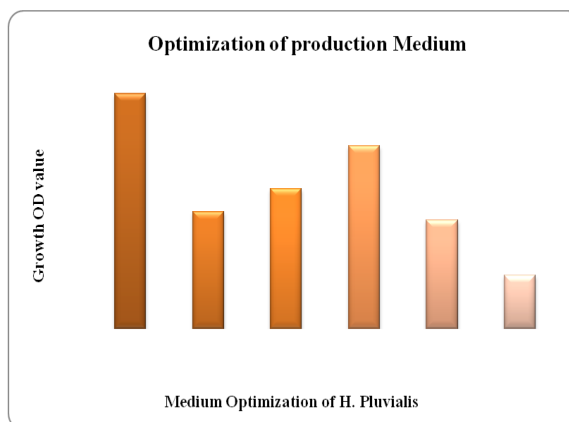


Figure 1: Optimization of production medium

DETERMINATION OF OPTIMUM pH:

The growth rate of *H. Pluvialis* for astaxanthin production medium pH was studied by incubating the BBM medium at 5 different pH such as 5, 6, 7, 8, 9. Based on colorimetric OD value at 680 nm growth rate of *H. Pluvialis*grown well in pH 7 than other pH. Nevertheless, pH less than 5 and more than 9 may has effect on the algal growth because it will become too acidic or too alkaline which can give effect to the growth.

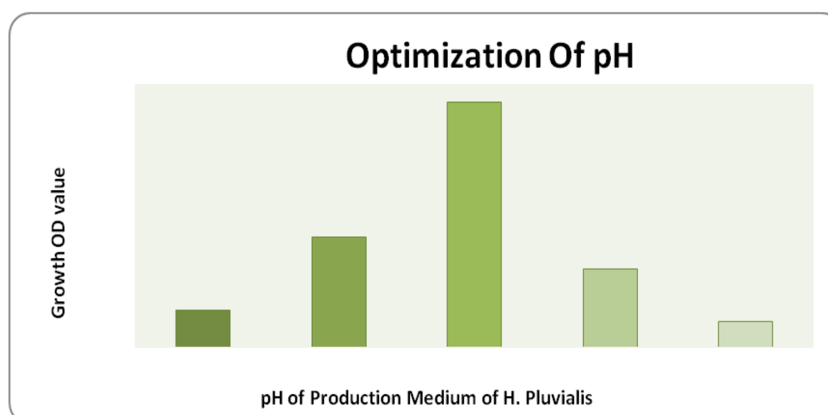


Figure 2: Optimization of pH for growth of *H. Pluvialis*

DETERMINATION OF OPTIMUM TEMPERATURE:

The growth rate of *H. Pluvialis* for astaxanthin production medium temp was studied by incubating the BBM medium at 5 different temperatures such as 23ºC, 25ºC, 30ºC, 37ºC and 40ºC**.** Based on colorimetric OD value at 680 nm growth rate of *H. Pluvialis* grown well in temp 25ºC than other temperature.

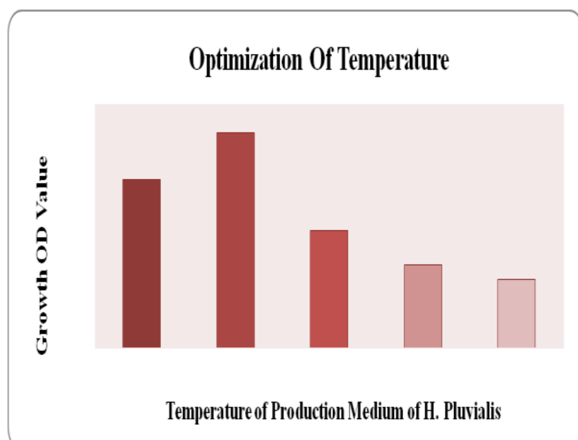


Figure 3: Optimization of Temperature for the growth of *H.pluvialis*.

DETERMINATION OF OPTIMUM LIGHT INTENSITY:

The growth rate of *H. Pluvialis* for astaxanthin production medium light source was studied by incubating the BBM medium at 3 different type of light intensity such as 12/12 light/dark, continuous light and continuous dark**.** Based on colorimetric OD value at 680 nm growth rate of *H. Pluvialis* grown well in 12/12 light/dark than other light intensity.

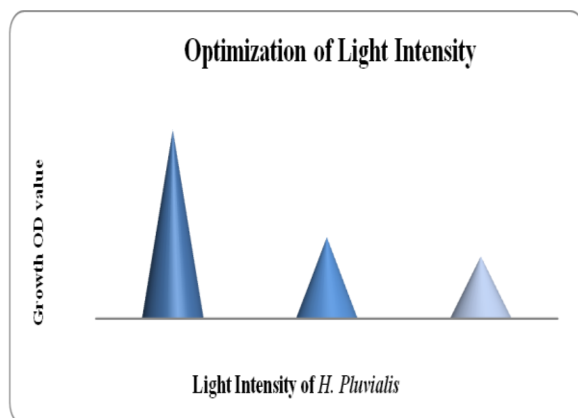


Figure 4: Optimization of Light Intensity for the Growth of *H.pluvialis*

DETERMINATION OF OPTIMUM INOCULUM CONCENTRATION:

The growth rate of *H. Pluvialis* for astaxanthin production medium inoculum concentration was studied by incubating the BBM medium at 5 different concentration of inoculum such as 2 ml, 4 ml, 6 ml, 8 ml, 10 ml. Based on colorimetric OD value at 680 nm growth rate of *H. Pluvialis* grown well in **6 ml** inoculum concentration than other.

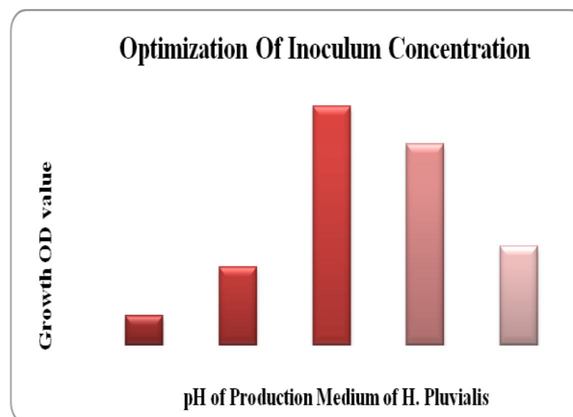


Figure 5: Optimization of Inoculum Concentration for Growth of *H. Pluvialis*

DETERMINATION OF OPTIMUM INCUBATION PERIOD:

The growth rate of *H. Pluvialis* for astaxanthin production medium incubation period was studied by incubating the BBM medium at 4 different days of incubation such as7 days, 10 days, 14 days, 20 days. Based on colorimetric OD value at 680 nm growth rate of *H. Pluvialis* grown well in **10 days** culture than other incubation periods

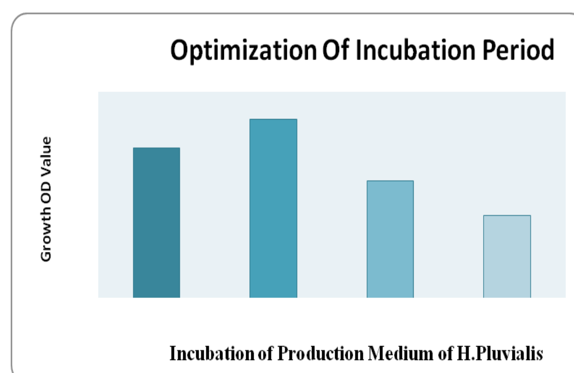


Figure 6: Optimization of Incubation period for Growth of *H. Pluvialis*

PRODUCTION OF ASTAXANTHIN

Based on physical parameter of production medium optimization results BBM medium used for astaxanthin production. The BBM medium was prepared at pH - 7, inoculum concentration 6%, temp 25ºC, light source 12/12 light/dark and 10 days of incubation. After incubation the culture broth was used for further estimation processes.

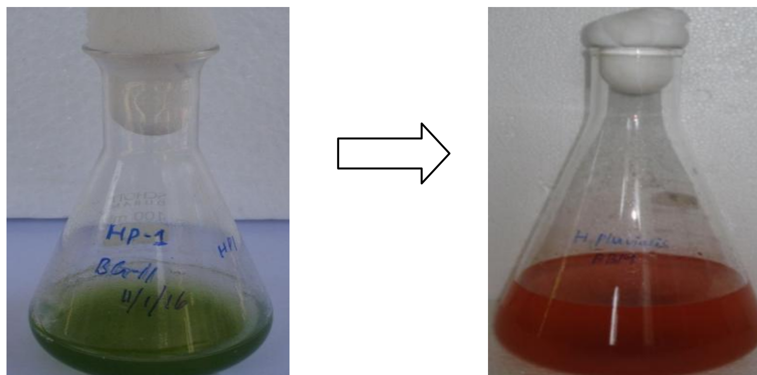


Figure 7: Astaxanthin production and confirmed by the colour change

ESTIMATION OF CHLOROPHYLL CONTENT

The processed supernatant of *H. Pluvialis* culture was used for chlorophyll estimation. The chlorophyll content of *H. Pluvialis* is 6.83µg/ml.

EXTRACTION AND ESTIMATION OF PROTEIN CONTENT

The pellet form of *H. Pluvialis was* processed and supernatant was used for protein estimation by By Lowry's et al.,1951 with BSA as standard. The protein content of *H. Pluvialis* is 338.27µg/ml. Estimated sample was subjected to 10 % SDS PAGE to identify the protein profile of *H. Pluvialis* using the maker.

SDS PAGE

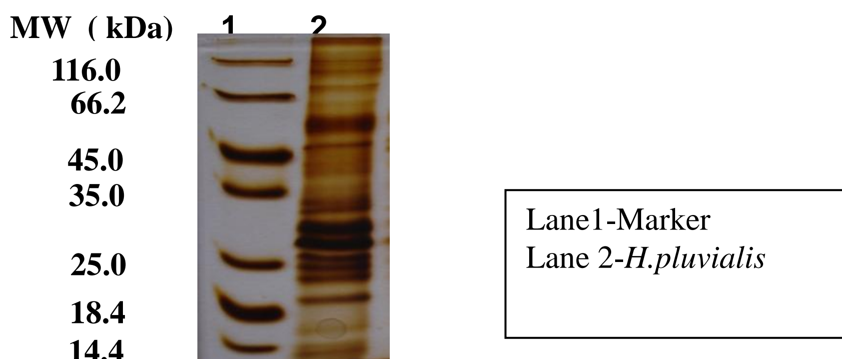
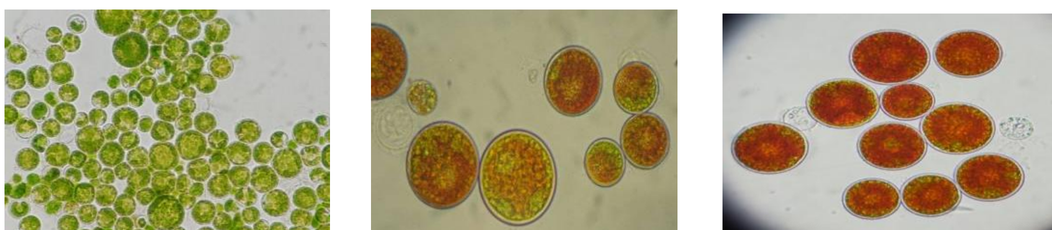


Figure 8: Protein profile of *Haematococcus pluvalis*

STAGES OF HAEMATOCOCCUS PLUVIALIS



Vegetative Cell Growth Maturation Secondary metabolite production

DISCUSSION

Temperature is another critical parameter effecting biomass production due to seasonal and diurnal fluctuations in the real world. [7] He conducted an experiment to investigated both daytime and nighttime temperature (tested at 8, 13, 18, 23, 28, and 33°C) effects on cell growth and astaxanthin accumulation of H. pluvialis during a light-dark cyclic cultivation. The results showed that the optimal daytime temperature for cell growth and astaxanthin accumulation was 28°C with highest net biomass production of 0.064 g L -1 d -1 and astaxanthin production of 2.3 mg L -1 d -1 . Besides this daily cycle, pH usually increases at high microalgal cell densities. [8] Microalgae cultivation is justified by the production of high-value fine chemicals and biofuels, essential to reduce the emissions of gases that cause global warming. The growth of microalgae *Haematococcus pluvialis* considering light conditions from 2000 to 10,000 lux, temperature 22°C and pH in the 6.5 - 12.5 range. [9] reported that *Haematococcus pluvialis* is the current better source of natural astaxanthin, a high-value carotenoid. Traditionally, the production process of astaxanthin by these algae is achieved by a two-stage system: during the first stage, vegetative "green" cells are produced and then converted, in the second stage, into cysts that accumulate astaxanthin. [1] proposed that the growth rate of *H. pluvialis* is controlled or regulated by the physical and chemical parameters. The aim of this study was to investigate and compare the effect of various culture media and light intensities on the growth of *H. pluvialis* in batch culture. The experiments were achieved by five different culture media and three different light intensities. The protein content in dry biomass ranged from 30% to 55%. In the present study, the effects of pH, light intensities, various inorganic nitrogen sources, NaCl, K_2HPO_4 and different light periods were investigated on the vegetative cells of *Haematococcus pluvialis* correspondence to maximum number of cells 6.62, 6.31, 6.31, 6.52, 6.81, 6.51 and 6.41 log10 cell number/mL respectively. [10]

The growth of this micro-alga from green vegetative stage to red sporulating stage is similar to the growth stages of any fruit especially apple of Himachal Pradesh. Therefore, we can conclude that this apple-rich producing state seems to be a promising state for the production of astaxanthin-rich *Haematococcus pluvialis*.

CONCLUSION

This study demonstrates that the production of astaxanthin by cultivation of *Haematococcus pluvialis* can be realized with current available technologies. The production of astaxanthin in the isolate of *H.Pluvialis* since it accumulate high amount of astaxanthin. The optimization of environmental factors such as pH, temperature, light intensity, inoculum, and incubation period were found to be signficant effect on the growth of the *Haematococcus pluvialis*. Light is essential for the life cycle of *H. pluvialis*. Higher light intensities can lead to photo inhibition. The effect of light intensity is dependent on the nutritional state of the cultures. The astaxanthin production, chlorophyll content and the protein was estimated in the *H. pluvialis optimized* culture. The cost might be further reduced with the advances of technologies and optimization of processes. Studies have suggested that astaxanthin, besides having antioxidant activities, also may provide anticancer, anti-inflammatory, and anti-diabetic activities, among various other health benefits

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