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Airlift Photobioreactor Cultivation of a New Strain of *Haematococcus pluvialis* Collected from High Altitude Regions of Himalayas

Infant Santhose. B[1], Elumalai. S[2], Rajesh Kanna. G[3]

[1, 2, 3]Department of Plant Biology & Plant Biotechnology, Presidency College (Autonomous), Chennai -05, Tamil Nadu, India

Abstract: ***A new strain of Haematococcus pluvialis was isolated from the fresh water samples of Pithorogarh district of Uttarkhand in India. The optimized BBM medium was used in Airlift photobioreactor (3 l) cultivation and biomass was obtained in the green stage of the alga Haematococcus pluvialis. Using modified BBM media with nitrate stress the alga was again cultured in the Airlift photobioreactor (3 l) for induction of astaxan thin accumulation and the carotenoids was extracted with 0.2 N HCl which showed 85% extraction. From the extracted carotenoid pigments free astaxanthin, astaxanthin monoesters and astaxanthin diesters were obtained using Chromatographic techniques.***

Keywords: Haematococcus pluvialis; Pithoragarh; Carotenoids; Airlift photobioreactor; Chromatography

1. Introduction

Photosynthetic microalgae are the primary producer's organic matter in aquatic environments. Clinically and medicinally important biochemicals from microalgae have been attracted the interest of the new modern world because they are not obtained by artificial chemical synthesis. The fresh-water unicellular green microalga *Haematococcus pluvialis* (Chlorophyceae) has been studied due its orange-red pigment astaxanthin (3, 3o-dihydroxy-b; b-carotene-4,4o-dione) a pharmaceutically and commercially most important compound [1]. Astaxanthin has high antioxidant activity approximately 10 times and 500 times greater than other carotenoids (zeaxanthin, lutein, canthaxanthin and β-carotene) and α-tocopherol respectively. And includes major functions of cancer prevention, immune response enhancement and free radical quenching; anticancer agent through singlet oxygen quenching and immune-modulator in pharmaceutical industry [2]. Artificial synthetic antioxidants are not considered for human health because of their negative effects like carcinogenicity and toxicity [3], which led to the attraction towards natural antioxidants [4].

Astaxanthin being a strong antioxidant and its commercial production from microalga *Haematococcus pluvialis* has been increasing attention nowadays and considered as a possible natural source of astaxanthin [5]. Accumulation of astaxanthin in Haematococcus pluvialis related to the palmella and aplanospore stage formation in its life cycle and usually induced by stress conditions [6]. Various factors takes place in astaxanthin formation as stress including nitrogen starvation, high light intensity and with agents prevent cell division [7]; [8]. Even though astaxanthin can be induced and produced in high mass from *H. pluvialis,* it is not recommended for human food because of the presence of thick impermeable cell walls. Thus, the most efficient extraction method of astaxanthin is necessary. The modern method of extraction known as Supercritical fluid extraction was followed in case of pharmaceutical and food processing industries [9]. In this study *Haematococcus pluvialis* a new strain collected from high altitude region of Himalayas for astaxanthin production. Vegetative growth conditions such pH, different photoperiod of light and different concentrations of $NaNO_3$, K_2HPO_4 and $NaHCO_3$ were analyzed and optimized for air lift photobioreactor cultivation. Vegetative stage converted to cyst stage (aplanospore) for astaxanthin production. Extraction of astaxanthin using different solvents and HCl were optimized and extractability percentage calculated. Thin layer chromatography and column chromatographic separation were done.

2. Materials and methods

2.1 Isolation of *Haematococcus pluvialis* from fresh water samples

Haematococcus pluvialis a Green alga belongs to the order Volvocales and family Haematococcaceae occurs in isolated high altitude regions at relatively low temperature. Uttarkhand state found in the northern parts of India at low Himalayan ranges, which is highly rich in Floral and Faunal diversity. Pithoragarh district of Uttarkhand is 7, 110 km^2 in geographical area at an average altitude of 1635 m ASL. The average temperature and average rainfall is 5.5-8.0ºC and 36.7 cm respectively. The seasonal sequence starts from cold winter from December to March, summer from March to June, rainy from mid-June to mid-September and retreating monsoon from September to November.

Water samples from various fresh water resources were collected randomly using plastic vials from different regions of Pithoragarh region in Pithoragarh district of Uttarkhand in India. And instant streaks were made in Petri-plates contains BBM and BG11 media at the time of water sample collection. Collected water samples were subjected to streak in the above mentioned solid media in our laboratory (In-vitro). Petri-plates were incubated in 120 µ Photons ms^{-1} S^{-1} for 12:12 hrs L/D (Light and Dark) conditions at 20ºC-22ºC. After 13-15 days of incubation the microalgal cultures were analyzed and checked every day under Compound light microscope (OLYMPUS CH20i) and photographed. Mixed

microalgal colonies were isolated and pure cultures were maintained in slants. All the isolated pure microalgal cultures were identified morphologically by Dr. V. Krishnamurthy, Krishnamurthy Institute of Algology (KIA), Chennai.

2.2 Airlift Photobioreactor culture of *Haematococcus pluvialis*

The optimized culture conditions were maintained in Airlift photobioreactor to grow *Haematococcus pluvialis*. Overall volume of the photobioreactor is 3 *l* in which 2.5 *l* of modified and experimentally optimized medium was prepared and autoclaved at 15 psi for 15 min. using in-made sterilization unit inside the photobioreactor. Pure culture of *Haematococcus pluvialis* was inoculated in to the medium using Peristatic pump. Other optimized conditions such as pH and photoperiod of light were also maintained, 1 N HCl and 1 N NaOH were used to control the exact optimized pH and optimized light condition was given externally by light up to 30, 000 lux. Filter sterilized air was given in to the medium using external air pump at the rate of 4.0 LPH.

Well grown *Haematococcus pluvialis* photobioreactor culture was centrifuged to yield green coloured biomass and then the whole pellet was inoculated in to the modified BBM media specifically to convert it into red stage. Different stresses such as continuous and high light intensity (30,000 lux), sodium acetate (0.2 %) and nitrogen starvation using low amount of sodium nitrate (50 mg L^{-1}) in special media were given to induce astaxanthin accumulation from biomass and red stage was acquired after incubation for 15 to 20 days.

2.3 Extraction of Carotenoid from biomass

The red stage *Haematococcus pluvialis* biomass was centrifuged and the pellet was air dried and about 2 g of biomass was estimated. Twenty milligrams of biomass was homogenized using mortar and pestle and different extraction methods were optimized such as acetone, methanol and 1-10 N HCl and 2N formic acid, citric acid, acetic acid and tartaric acid [10]. Different wavelengths of 470, 480, 645 and 661 nm were used to observe the absorbance from the extracts using UV-visible spectrophotometer Hitachi U-2900. Astaxanthin presence was analyzed and extractability in terms of percentage was calculated for all the samples by the formula given below.

Extractability (%) = free astaxanthin (µg mL-1) / total astaxanthin (µg mL-1) × 100

2.4 Thin Layer Chromatographic (TLC) separation of Astaxanthin

Different solvents were used and optimized to extract free astaxanthin and separation it from different carotenoids mixed with it. Acetone, Petroleum ether and Chloroform of different ratios were used to optimize successive separation of astaxanthin. TLC Silica gel 60 F_{254} from MERCK was employed to perform thin layer chromatography.

2.5 Column Chromatographic separation of Astaxanthin

Silica gel 60-120 mesh from Qualigens was used to run column chromatography. Gel was staked by wet method using acetone.

3. Results

3.1 Isolation of *Haematococcus pluvialis*

Isolated microalgae were identified morphologically and the presence of *Haematococcus pluvialis* from the collected microalgae was confirmed (Fig. 1).

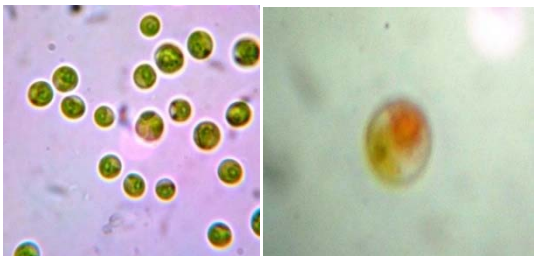

Figure 1: Microscopical view of *Haematococcus pluvialis*

3.2 Airlift Photobioreactor culture of *Haematococcus pluvialis*

All the optimized parameters including pH-7.0, 12:12 hrs of photoperiod of light and dark, 0.3 M concentration $NaNO_3$, 0.08 M concentration of K_2HPO_4 and 0.08 M concentration of $NaHCO_3$ were followed for cultivation of *Haematococcus pluvialis* in Photobioreactor. The three ingredients including NaNO3, K2HPO4 and NaHCO3 were given in the medium and other condition factors such as pH and light were set inside the medium and maintained using 1 N NaOH and 1 N HCl using inbuilt pH meter inside the Photobioreactor and external light was provide outside the photobioreactor set up of 15, 000 lux (Fig. 2). Green coloured vegetative stage was obtained after 26th days of incubation. Environmental stresses especially light (30, 000 lux) and specialized medium were used in case of astaxanthin induction in Airlift Photobioreactor cultivation of *Haematococcus pluvialis*. Red coloured stage was initially seen at 15th day of incubation and whole biomass was harvested on 20th day of incubation and approximately 3 g of dried biomass from 3 *l* airlift photobioreactor cultivation was harvested centrifugically.

Figure 2: *Haematococcus pluvialis* cultivation and red stage (Aplanospore) in Photobioreactor

3.3 Extraction and TLC separation of Astaxanthin

Among all the experimented extraction methods, 0.2 N HCl treated method has shown increased extractability percentage of astaxanthin and about 85 % of astaxanthin was recovered using HCl extraction method. Thin layer chromatography was used to separate and check the purity of separation of carotenoid and astaxanthin separated from it. The running solvent optimized and used was petroleum ether, acetone and chloroform in the ratio of 2:2:1. The TLC sheet shows three distinct bands including free Astaxanthin (at bottom), Astaxanthin Monoesters (middle) and Astaxanthin Diesters (Top) which has a range of Relative front (R*f*) value of 0.63, 0.77 and 0.82 respectively (Fig. 3).

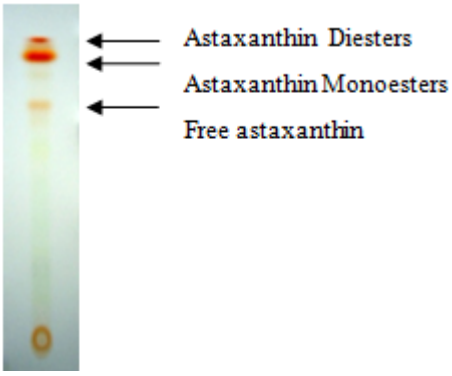

Figure 3: TLC sheet showing Astaxanthin and their derivatives

3.4 Column chromatographic separation of Astaxanthin

The concentrated reddish orange content from the extract was carefully eluted through the column and collected during elution from column used and checked spectrophotometrically. Different concentrated and diluted elutions were collected in which dark red and concentrated elution was found to be rich in Astaxanthin.

4. Discussion

Astaxanthin can be synthesized naturally by various different microalgae including *Botryococcus braunii* (0.1 % by dry weight) [11], *Chlamydocapsa* spp. (0.04 % by dry weight) [12], *Chlorella zofingiensis* (0.7 % by dry weight) [13]; [14], *Chlorococcum* sp. (0.7 % by dry weight) [15], *Chloromonas nivalis* (0.004 % by dry weight) [12], *Eremosphera viridis* (16), *Neochloris wimmeri* (1.9 5 by dry weight) [14], *Protosiphon botryoides* (1.4 % dry weight) [14], *Scenedesmus* sp. (0.3 % by dry weight) [14], *Scotiellopsis oocystformis* (1.1 % by dry weight) [14], *Trachelomonas valvocina* [17] and *Haematococcus pluvialis* (4 % by dry weight) [18]. Among all the species, the green microalga *H. pluvialis* found to be the promising synthesizer of natural astaxanthin to accumulate in large amounts under stress conditions [19]. Our major task in this study is to find a new strain of *Haemaotcoccus pluvialis* and to module it growth for high yield of astaxanthin production. Fresh water sample collected from different high altitude regions of Himalayas in Pithoragarh district of Uttarkhand has yielded us a new strain of *Haematococcus pluvialis*.

From this present study, our new strain requires 12:12 hrs of light and dark for high vegetative growth (1.095 OD at 690 nm), biomass, and pigment production including carotenoid (125.7 mg L^{-1}), Chl. a (10.39 µg mL^{-1}), Chl. b (15.49 µg mL^{-1}). *H. pluvialis* growing in red light with normal intensity and sudden change of red to blue light with high light intensity can induce high level of astaxanthin [20]; [21].

In *H. pluvialis,* nitrate concentration played a most important role in cell division (growth) and in accumulation of secondary carotenoids [22]. Nitrogen supplement is required for astaxanthin synthesis and likely to reflect the needs of protein synthesis in order to massive accumulation of pigment. Thus, N_2 starvation is an effective way to induce and enhance astaxanthin accumulation in *H. pluvialis* and the optimum concentration of nitrate to accumulate astaxanthin and to avoid cessation of cell division was 0.15 gL^{-1} $NaNO_3$ [23].

Our new strain of *H. pluvialis* grown well in the airlift photobioreactor cultivation and looks greenish (1.204 OD at 690 nm) in growth rate and increase in pigment content such as carotenoid (145.2 mg L^{-1}), chl. a (13.37 µg mL^{-1}) and chl. b (19.35 µg mL-1) in 25 days of cultivation. In the Double-layered photobioreactor cultivation of *H. pluvialis,* reaching high cell density of 4.3 × 10^5 cells ml^{-1} in the inner core region and 5.79 % of astaxanthin by dry cell weight in the outer jacket region and it is feasible and commercially potent for astaxanthn [24].

Hydrochloric acid (HCl of 0.2 N) treatment was chosen to extract astaxanthin from the cells of *H. pluvialis*, because 85 % of astaxanthin can be recovered. About 87.42 % of astaxanthin was yielded from *H. pluvialis* using the supercritical fluid extraction method in pharmaceutical and food processing industries [25].

Three distinct bands occurs including Free astaxanthin, Astaxanthin Monoesters and Astaxanthin Diesters were clearly occurs in our study at R*f* value of 0.63, 0.77 and 0.82 respectively. The R*f* values of astaxanthin in TLC from H. pluvialis was 0.37, 0.58 and 0.73 for free astaxanthin, astaxanthin monoesters and astaxanthin diesters respectively [26]. Thus, isolated *H. pluvialis* can be efficient producer of astaxanthin of commercial and economical value.

5. Conclusion

From this overall study, it is concluded that new strains of *Haematococcus pluvialis* from different high altitude regions can be able to pave the way for efficient astaxanthin production of commercial value. Various stress factors are available but chemical stress and abiotic stresses are of high impact and cheap in case of induction of astaxanthin. In our airlift photobioreactor study, biomass production seems to be limited when compare to the novel double-layered photobioreactor. When compare the extraction methods, supercritical fluid extraction is considered to be an efficient method but, HCl extraction can also extract almost similar amount of astaxanthin compared to the former method and it is of cheap cost. From our study column chromatographic separation does not assure the quality of free astaxanthin.

And it is a new report that the isolation of *H. pluvialis* from high altitude regions of Himalayas.

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Author Profile

Infant Santhose .B received B.Sc, M.Sc and M.Phill degree in Department of Plant Biology and Biotechnology from Loyola College (Autonomous), Chennai. Presently perusing Ph.D in Department of Plant Biology and Biotechnology, Presidency College (Autonomous), Chennai.