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Biomass Production and Extraction of Secondary Metabolites from a Fresh Water Microalga *Haematococcus pluvialis* KUDBT18 under Photoautotrophic Cultivation

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ABSTRACT: Objective: In this investigation, we have focused work on isolation, identification, biomass production, synthesis and evaluation of metabolites of economic importance from a native microalgal strain *Haematococcus pluvialis* KUDBT18. The study includes assessment of cultivation parameters, nutritional requirements for growth, observation of morphological and physiological changes with response to nutrient stress. Materials and methods: The isolate was identified by using microscopy and molecular techniques and cultivated photoautotrophically by using Bold Basal Media (BBM). Inoculum from seed culture 10% was added to 2000ml culture media. The physiological stress was induced by adding 0.1mM NaCl to 200ml media for synthesis of secondary metabolites with response to saline stress. Results and conclusion: The fresh water microalga isolated from Karnataka University Campus (KUC) is identified and named as *Haematococcus pluvialis* KUDBT18. The partial sequence 18S rRNA ribosomal gene was submitted to NCBI with Gene bank accession ID MH201223.1. Upon photoautotrophic cultivation of the strain with response to stress, high amount of a red color carotenoid pigment in aplanospore stage was produced quantified 1.2% of the total dry weight of cell. The biomass yield reached to a maximum of 2.7mg of biomass/2000ml growth media. The evaluated bioactive properties of extracted pigments shown to have economic importance. Process parameters developed for growth and cultivation of species generates useful data for large scale cultivation of species in open systems.

Keywords: Bold Basal Media, Carotenoids , *Haematococcus pluvialis*,, Photoautotrophic culture, Nutrient stress.

Abbreviations: BBM, Bold Basal Media; KUC, Karnatak University Campus.

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INTRODUCTION

Microalgae are one of the promising sources of natural compounds which can be effectively utilized as substitute to the synthetic and chemical dietary supplements (Herrero *et al.,* 2015). In present scenario due to synthesis of unique metabolites like pigments and fatty acids algae and algal based food products are gaining much interest in food, pharmacy and cosmeceutical sector (Shah *et al.,* 2016). Among such microalgae the green microalga *Haematococcus pluvialis* reported for accumulating red colored pigment known as Astaxanthin is one of the most promising natural antioxidant compound (Igielska–Kalwat *et al.,* 2015). At present industrial development, *Haematococcus pluvialis* is extensively used as potential fresh water microalgae candidate for algae based biorefinary industries (Liu *et al.,* 2017). Though production and extraction of Astaxanthin and other important fatty acids from different strains of *Haematococcus* sp. is being popularized yet overcoming the issues related to strain improvement and techno economic development is a challenging task (Khan *et al.,* (2018). Hence sustainable biorefinary industries can be established by exploring promising native microalgal strain, development of reproducible experimental background suitable for large scale cultivation and assessment of the production cost (Lee *et al.,* 2014; Panis and Carreon 2016).

Present study aimed at isolation and cultivation of native freshwater microalgal strain of *Haematococcus pluvialis* for synthesis of industrially important secondary metabolites. This experimental work is carried out at Department of Biotechnology, Karnatak University Dharwad, Karnataka.

Evaluation of morphological, molecular and physiological characters of isolated strain KUDBT18 studied significantly. The isolated strain belongs to class Chlorophyceae, strain follows traditional life cycle also grows differently in two stages green vegetative stage and red cyst stage (Kim *et al.*, 2015; Sun *et al.*, 2015).

Photoautotrophic cultivation attempted by using glass aquarium tank aerated with filtered ambient air and culture illuminated using cool white fluorescent lamp (42.35µmol $m^{-2}S^{-1}$ photon) light intensity. Assessment of stress condition for pigment induction was mainly studied and changes in morphological features were examined using microscopy (Simak *et al.*, 2015).

MATERIALS AND METHODS

A. Isolation of fresh water microalga Haematococcus pluvialis KUDBT18

Initially microalgal samples collected on random basis from three different temperate fresh water ponds located at Karnatak University Campus (KUC), Dharwad. Morphological stages observed using optical microscope Olympus, CX23 (Fig. 1). Unialgal cultures from collected samples isolated by using serial dilution and aseptic streak plate technique (Cullen and Maclntyre 2016). Different species of microalgae from class Chlorophyceae, Bacillariophyceae and Trebouxiophyceae are observed and isolated from the collected samples along with species of *Haematococcus*. Axenic culture of *Haematococcus* sp. Isolated by streak plate technique using basal growth medium - Modified Bold Basal media supplemented with vitamins, soil extract and trace elements (Kim *et al.*, 2015). Pure cultures in both agar slant and liquid media maintained at pH 6.8, temperature 25±2°C, with 12:14h of photoperiod. Stock culture maintained by sub-culturing the cells from old stock to agar slant after every week and stored at temperature 80°C in refrigerator.

B. Scanning Electron microscopic analysis

For scanning electron microscopic analysis algal cells were primarily using glutaraldehyde followed by secondary fixation using osmium tetroxide, followed by serial dehydration with ethanol. Finally dried algal cells mounted on carbon tape fixed to a metal stab then sputter coating done in gold sputter coater, coated sample fixed on a holder and field emission was observed in JSM – IT 500 scanning electron microscope (Begum *et al*., 2016).

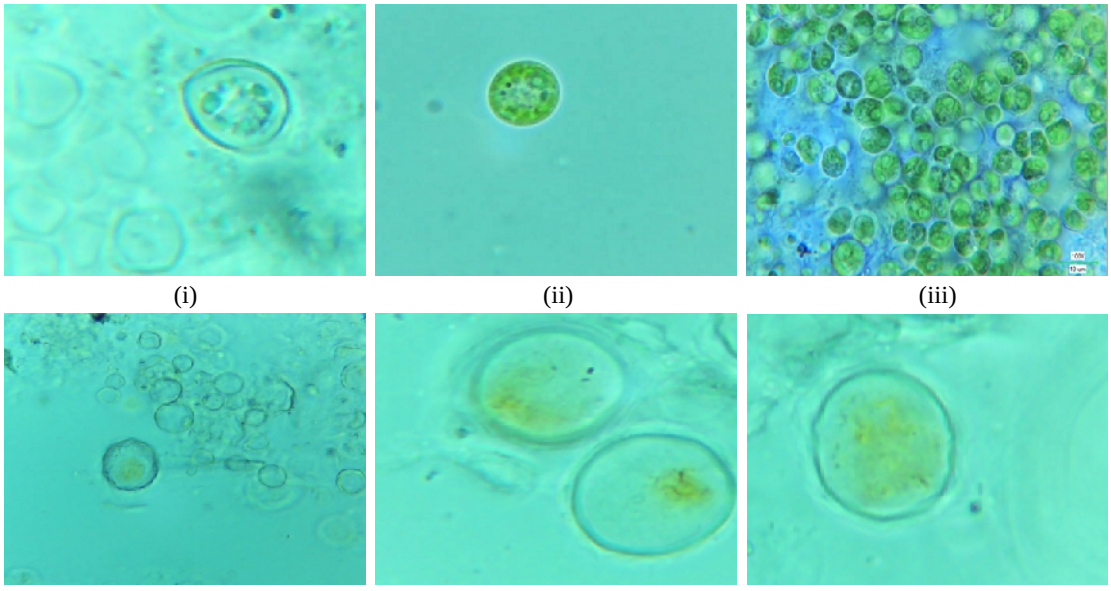

Fig. 1. Light microscopic view at 60X and 100X oil immersion magnification using L.M.CDX23 , images (i)-(vi) describing morphological changes in different stages of growth in *Haematococcus pluvialis* KUDBT18 Scale bar represents size 10µm. (i) biflagellate motile cell of *Haematococcus pluvialis* KUDBT18. (ii) - (iii) unialgal vegetative cell at 2-3days old culture. (iv) - (vi) cell turned to cyst form forming aplanospore increase in size and accumulating red color pigment inside the cyst, cells growing 8th day onward in BBM media with physiological stress.

C. Molecular characterization

Isolated unialgal axenic culture of *Haematococcus* sp. subjected for extraction of genomic DNA. Quality of isolated DNA then evaluated on 1.2% Agarose gel and single band of high molecular weight DNA observed. Isolated DNA was amplified with 18S rRNA specific primer (1F and 4R) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band of 900bp was observed. Enzymatically purified PCR amplicon subjected to Sanger sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 1F and 4R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 915bp of 18S gene in SSU region was generated from forward and reverse sequence data using aligner software.

The 18S gene in SSU region sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA5. Partial sequence deposited at NCBI data repository with Gene Bank accession ID MH201223.1 (Bhambri and Gupta 2012).

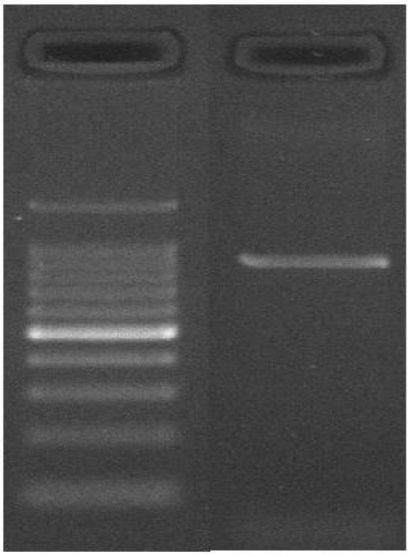

Fig. 2. 1.2% Agarose gel image showing 900bp amplicon (SSU region) of *18S* rDNA. Lane 1: 1000bp DNA Ladder and Lane 2: 900bp amplicon (SSU region) of *18S* rDNA.

D. Photoautotrophic cultivation and saline stress for pigment induction

The isolate *Haematococcus pluvialis* KUDBT18 grown photoautotrophically. Cultures were grown in glass aquarium tank containing growth media inoculated with 10% previously grown seed culture of cell density 4×10^5/ml. Cultivation parameters maintained at 23±2°C. Culture illuminated with light intensity of 42.035µmol $m^{-2}s^{-1}$, with 14:16h light: dark photoperiod and culture media aerated continuously with filtered ambient air. Cell density determined after every one day interval till 14th day by cell count using haemocytometer (Lee *et al.,* 2014). Through the phase of phototrophic cultivation, 6th day onwards NaCl concentration in the medium was increased gradually at concentration 0.1mM to 1mM/100 ml of growth medium. With response to physiological saline stress vegetative cells turned into cyst stage in aplanospore stage and the cells accumulated red color pigment thus confirmed by microscopy (Gao *et al*., 2015). Biomass harvested on 14th day and dry weight of biomass was determined by cell drying method (Sun *et al*., 2015).

E. Carotenoid estimation

To carry out carotenoid extraction, cells were first lysed mechanically by sonication and extracted with methanol. Estimation of carotenoid was done by using colorimetric assay based on Beer-Lambert's law by measuring absorbance at 480nm (Shivani and Chandresh 2013; Liang *et al*., 2015; Doria *et al*., 2018).

RESULTS AND DISCUSSION

The results of phylogenetic analysis reveals that isolated strain of *Haematococcus* KC986379.1 found closely related to *Haematococcus pluvialis* species synonymously called *Haematococcus lacustris* KC196724.1, (Fig. 3). Hence it is confirmed that genera *Haematococcus* and designated with strain number KUDBT18 (Kim *et al.,* 2016). The KUDBT18 successfully cultivated on artificial growth media BBM supplemented with vitamins and micronutrients (Kavitha *et al.,* 2015). The isolated green microalga identified as native species to the Dharwad and throughout the year it grows predominantly into temperate ponds in the KUC region. Microscopic examination discovered KUDBT18 follows traditional growth pattern - four phases of growth as vegetative form, encystment, maturation and germination (Fig. 1). Evaluating with previous reports on physiological stress induced encystment in *Haematococcus* isolated strain also observed to form aplanospore in the cyst stage with response to NaCl stress and accumulates red color pigment (Gao *et al.,* 2015; Xi *et al*., 2016). Synthesis of carotenoids in *Haematococcus* sp. can be correlated with synthesis of the fatty acid esters (Shah *et al*., 2016). *Haematococcus pluvialis* KUDBT18 accumulates ketocarotenoids along with fatty acid esters (Chen *et al*., 2015).

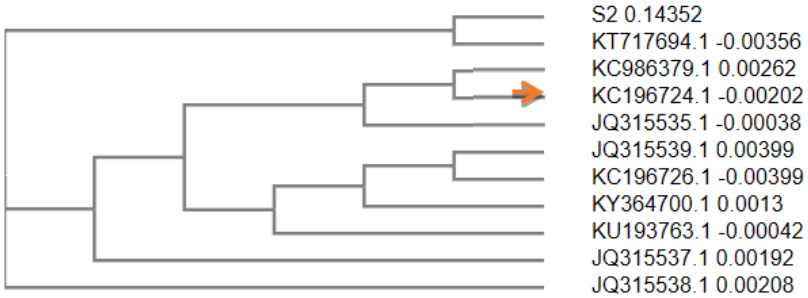

Fig. 3. Phylogenetic tree showing evolutionary relationship of isolated strain of *Haematococcus pluvialis* KUDBT18 with other species of *Haematococcus*. The species KC986379.1 is closely related to species of *Haematococcus* KC196724.1. Analysis conducted using MEGA5 software.

Photoautotrophic cultivation system turned favorable for grown and biomass production by isolate, the strain withstand extreme environmental conditions throughout the process and yields high amount of pigments 1.2% of total dry weight of cell (Prochazkova *et al*., 2014). It also produces efficient quantity of biomass 2.7mg biomass/2000 ml of growth media and biomass yield can be improved using strain improvement and media optimization procedures (Gomez *et al*., 2016). In present experimental analysis cellular morphology during pigment synthesis also observed through scanning electron microscopy (Fig. 4). Earlier reports on pigments synthesized by other species of *Haematococccus* have bioactive properties as antioxidant, tumor inhibition, neuroprotective and cardio protective activity; in this context isolated strain KUDBT18 holds great economic importance and may serve as one of the best neutraceutical and dietary natural supplement (Begum *et al*., 2016; Koutra *et al*., 2018). Strains collected from culture collections mainly forms bottlenecks in large scale set up of biorefinary industries as they fail to compete in natural environment (Zhang *et al*., 2016; Nahidian *et al*., 2018; Khoo *et al*., 2019). In present study indigenous strain isolated from local environment and its cultivation parameters and potential for synthesis of biomass is studied which generates useful data for large scale cultivation of isolated strain in open system with respect to the biorefinary approach (Chew *et al*., 2017; Khoo *et al*., 2019).

Future study objectives are focused on strain improvement strategies for high yield of the biomass; identification and purification of pigments and evaluation of bioactivities of the pigment (Abomohra *et al*., 2016).

This investigation also deals with discovery of important cultural characteristics of the isolate KUDBT18 as the organism being isolated is unique. We aim for economic assessment of process parameters in order to achieve sustainable large scale industrial production of listed important natural compounds.

CONCLUSION

Molecular identification of *Haematococcus pluvialis* KUDBT18 strain confirmed by sequencing of 18S gene from 18S rRNA subunit. Partial sequence deposited to NCBI data repository with Gene bank accession number *MH201223.1*, hence the organism authenticated. The culture of *Haematococcus* grown on previously described growth media with addition of one or more combination of vitamins found to enhance the growth. Saline stress induction was responsible for encystment and production of secondary carotenoids. *Haematococcus pluvialis* is one of the industrially important organisms in trend it is reported from very few places in India as its existence is sporadic thus isolation, identification and maintenance of pure unialgal axenic culture is a challenging task. Hence in present study, experimental techniques developed for isolation and maintenance of pure cultures of KUDBT18 aiming its future use as promising neutraceutical compound. Techno economic assessment of the experimental design may help to develop improved and sustainable large scale production for important secondary metabolites.

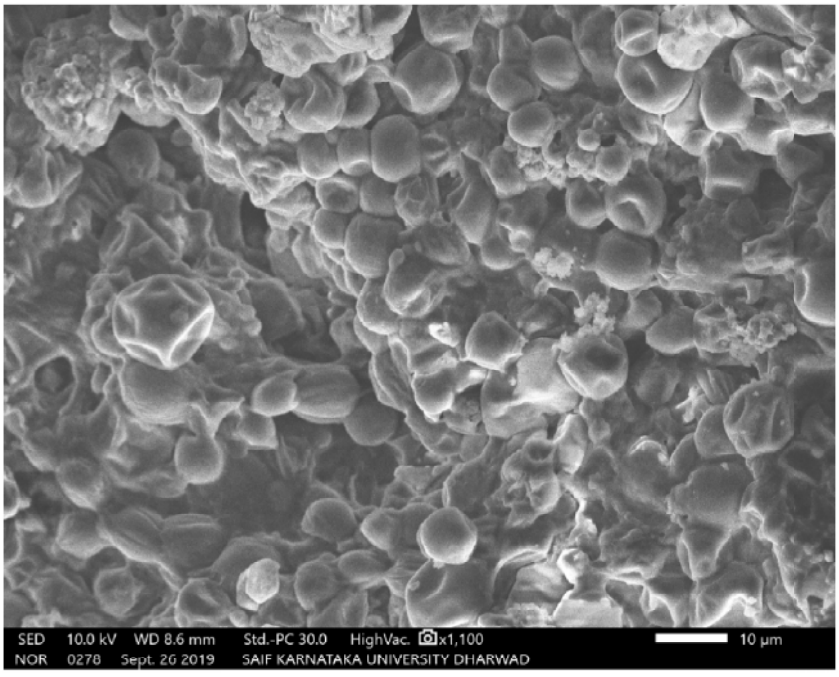

Fig. 4. SEM image *of Haematococcus pluvialis* KUDBT18, morphology changes with response to stress as a result cells turn from vegetative flagellated motile form to non-motile cyst forming aplanospore cyst.

FUTURE SCOPE

Future scope of present study aims towards extensive use of isolated green microalgal strain KUDBT18 for microalgae based biorefinary applications. Structural elucidation and evaluation of the bio active neutraceutical formulation, using carotenoid pigment extract of *Haematococcus pluvialis* KUDBT18 holds great economic importance toward commercializing secondary metabolites production from local algal strain.

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Conflict of interest. Authors declare no Conflict of Interest.

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