

Original Research Article

Characterization of High Carotenoid Producing *Coelastrella oocystiformis* and its Anti-Cancer Potential

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ABSTRACT

Keywords

Coelastrella oocystiformis, DNA sequencing, Anti-cancer, Carotenoids, Characterization

Microalgae *Coelastrella oocystiformis* from Mumbai, India was identified using 18s rDNA sequencing. The strain has a doubling time of 27 ± 0.5 h without CO₂ and 12 ± 1.0 h with 3% CO₂. With 3% CO₂ a biomass of 89.6 ± 2.0 mg/l/day and without CO₂ only 42.55 ± 2.0 mg/l/day was the yield. Similarly, without CO₂ the organism had 19 ± 0.5 % lipids, 22.5 ± 0.5 % proteins, 54 ± 1.0 % carbohydrates and 4% ash content; when cultured with 3% CO₂, it had 27 ± 1.0 % lipids, 24.6 ± 0.5 % proteins, 44 ± 1.0 % carbohydrates and 4% ash content. Fatty acid profile was identified using GCMS. The organism has a carotenoid content of 1.972% of its dry weight. HPTLC revealed carotenoids β -carotene, lutein-518.64 μ g/g, free astaxanthin 120.5 μ g/g, canthaxanthin 208.84 μ g/g and phytofluene 232.50 μ g/g of the dry weight. Carotenoid extract was capable of inhibiting 5% of human prostate cancer cell line DU-145.

Introduction

Green algae consists of chlorophyll-a, chlorophyll-b as the major chlorophyll pigments. Amongst the carotenes, α -carotene and β -carotene are the major entities. With respect to Xanthophylls, there can be lutein, zeaxanthin, violaxanthin, neoxanthin, astaxanthin, canthaxanthin (Smith, 1950; Del Campo *et al.*, 2004). The beneficial effects of carotenoids are thought to be due to their role as antioxidants. β -carotene may have added benefits due its ability to be converted to vitamin A. Furthermore, lutein and zeaxanthin may be protective in eye disease because they absorb damaging blue light that enters the

eye (Johnson, 2002). A number of scientific papers discuss on the anti-cancer potential of carotenoids with respect to their anti-oxidation properties. Microalgae in chlorophyta like *Chlorella zofingiensis* (Del Campo, 2004), *Haematococcus pluvialis* (García-Malea, 2005), *Dunaliella salina* (Kleinegris, *et al.*, 2010), *Euglena sanguinea* (Sae-Tan *et al.*, 2007) are a few most prominently studied organisms for their high carotenoid producing ability. Algae are one of the potential organisms for bio fuels around the world (Feinberg, 1984). Production of carotenoids in such a high content on nutrient depletion by algae may

be to protect themselves from heat and ultra-violet rays during resting phases when there is drought (Kana *et al.*, 1998).

The aim of the investigation was to identify the particular strain using DNA sequencing, estimate its carotenoid production and to test its anti-cancer activity.

Materials and Methods

BG11 media procured from HiMedia Laboratories, Mumbai (Product Code: M1541) was used to isolate & culture the strain *Coelastrella oocsyiformis* (J.W.G.Lund) E. Hegewald & N.Hanagata 2002 from Vasind (19.407512° N, 73.264773° E), near Mumbai, India. Composition of Media BG11 from HiMedia Laboratories, Mumbai - all ingredients in g/l is as follows Sodium nitrate-1.500; Dipotassium hydrogen phosphate-0.0314; Magnesium sulphate-0.036; Calcium chloride dehydrate-0.0367; Sodium carbonate-0.020; Disodium magnesium EDTA-0.001; Citric acid - 0.0056; Ferric ammonium citrate - 0.006; Final pH=7.1 after sterilization. Culture Conditions in the laboratory were maintained at Temperature: 25 degree Celsius; Light: 5000 lux; Light Cycle: 12 hours light & 12 hours dark.

DNA extraction, Polymerase Chain Reaction of 18s rDNA sequence

The culture was centrifuged at 15000 rpm for 10 min. After centrifugation minimum amount of T10E1 buffer was added to the pellet and again centrifuged for 10 min. The pellet was then treated with 10% SDS (about 50 µl), the tubes were then kept in a water bath for 20 min at 60°C. An equal volume of Tris buffer phenol was added, inverted and mixed for 15 min and again centrifuged for 1 min at 4°C at 7500 rpm. After centrifugation, the upper aqueous layer was taken with the help of micropipette and an

equal volume of phenol: chloroform: iso-amyl alcohol mixture was added. It was mixed for 15 min at room temperature and centrifuged for 5 min at 4°C at 10000 rpm. The supernatant was transferred to a new eppendorf tube and an equal volume of iso-amyl alcohol: chloroform mixture was added. It was gently mixed for 15 min at R.T. It was then centrifuged at 10000 rpm for 5 min at R.T. Again the upper aqueous layer was taken in a new eppendorf tube and 1/10th volume of 3M sodium acetate solution (pH-5.2) was added and shaken for 5 min. Twice the volume of ethanol was added and mixed gently by inversion 2-3 times. Incubated at -20°C for 20 min, centrifuged at 10000 rpm for 10 min and to the pellet again 500 µl of ethanol was added, inverted a few times and spun at 14000 rpm for 2 min at 4°C. The pellet was air dried and then 10-15 µl of T10E1 buffer was added and the genomic DNA was stored at 4°C.

PCR and DNA sequencing

The PCR amplifications was performed in 25 µl reactions with Taq master mix (HiMedia Master Mix, HiMedia laboratories Pvt. Ltd, Mumbai, India), 1 µl of each 5 µM primer, and 1 µl of template. Reactions were performed in a thermo-cyclers (Bio-Era Peltier Grade) under the following thermal profile: The PCR program consisted of one step at 95°C for 5 min (initial denaturation), followed by 35 cycles with 95°C for 30 s, 55 °C for 30 s (primer annealing) and 72°C and a final step at 72°C for 10 min (final extension) and last cooling to 4°C. The 18SF2 5'- GTCAGAGGGTGAAATTCTTG GATTTA -3' as forward primer and 18SR2 5'- AGGGCAGGGACGTAATCAACG -3' as reverse primer. The amplicon obtained was sequenced using Sanger's Dideoxy method at GeneOmbio Technologies Pvt. Ltd, Pune. The 18s rDNA sequence obtained was analyzed using NCBI-BLAST and the

following sequences were randomly selected sequence for genus *Coelastrella* for constructing a Phylogenetic tree using Unweighted Pair-Group method with Arithmetic Mean (UPGMA)

>gi|669688764|gb|KM020171.1|
Coelastrella sp. SAG 2470 18S ribosomal RNA gene, partial sequence
>gi|57864399|dbj|AB012848.2| *Coelastrella oocystiformis* gene for 18S rRNA
>gi|669688681|gb|KM020088.1|
Coelastrella oocystiformis strain SAG 277-1 18S ribosomal RNA gene, partial sequence
>gi|480327716|gb|JX513882.1| *Coelastrella terrestris* strain CCALA 476 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
>gi|12964687|dbj|AB055800.1| *Coelastrella saipanensis* gene for 18S ribosomal RNA, complete sequence

Effect of CO₂ on biomass generation

Coelastrella oocystiformis was cultured using BG11 liquid media by supplying 3% carbon – di-oxide (CO₂) daily for 1 hour. The total volume of culture was 1000 ml. The culture was then harvested on completion of 10th Day by centrifugation and dried in hot air oven at 60°C to obtain dry biomass.

This biomass for each culture was weighed using weighing balance. pH of media was monitored using pH strips. With the CO₂ supplied culture, a control culture without CO₂ was also cultured to identify the difference in characters and growth rate.

Proteins and carbohydrate estimation

Protein content was estimated quantitatively by Folin-Lowry Method (Sharma *et al.*,

2012). Phenol–Sulphuric Acid method of DuBois *et al.* (1956) was used for estimation of Carbohydrates.

Lipid Extraction, Esterification of the extracted lipids & GCMS fatty acid profiling

Lipid extraction was performed using Bligh & Dyer Method 1959. The extracted lipids were weighed gravimetrically. The lipids were profiled for their fatty acid content using Gas Chromatography Mass Spectroscopy (GC-MS) Gas Chromatography Mass Spectroscopy: Esterification reagent preparation: 0.6 N Methanolic HCl. For every 3mg of lipids, add 5ml of esterification reagent in the round bottom flask with lipids. The round bottom flask was subjected to 60°C temperature for 2 h in water bath to fasten the process of esterification. The esterified component was transferred to a test tube, to which 2 ml hexane was added. The solution was mixed thoroughly and the upper hexane layer was collected in separate glass vials and hexane was evaporated at room temperature. 1ml Chloroform was added to the remaining fraction of esterified component and was injected in Gas Chromatography Mass Spectroscopy (GC-MS) machine Shimadzu GC-MS-QP2010 Ultra. The parameters of GC for the detection were as follows: Total flow 24.5 ml/min, Column flow 3.59 ml/min, Linear velocity 70.4, Purge flow 3.0, Total run time: 50.0mins, Carrier gas: Helium. The column used was 30 meter FAMEWAX Column (USP G16) from Restek Corporation.

Extraction, Estimation and Analysis of Pigments

The pigments were extracted separately from green and orange (carotenoid) stage of *Coelastrella oocystiformis*. Dried Algal

Biomass was used for extraction and estimation of pigments. Bligh & Dyer Method 1959 was used for extraction of total carotenoids. After extraction, chloroform was allowed to evaporate and the residual carotenoids were dissolved in petroleum ether. U.V.-Vis Spectrum was recorded and analyzed. The equipment used for analysis is BIOERA made Catalogue No. BI/CI/SP/SB-S-03 - Single Beam UV-Vis Spectrophotometer with Software. Quartz cuvettes were used for measuring the optical density in Ultraviolet range 200nm-390nm & glass cuvettes were used for measuring the optical density in visible spectrum 390-700nm (Eijkelhoff and Dekker 1997). The total carotenoid content and chlorophyll content were estimated using the following formula:

Chlorophyll a (Ca) = $11.93 \times A_{664} - 1.93 \times A_{647}$

Chlorophyll b (Cb) = $20.36 \times A_{647} - 5.50 \times A_{664}$

Total Chlorophyll = Chlorophyll a + Chlorophyll b

Total Carotenoids = $1000 \times A_{470} - 2.270 \times Ca - 81.4 \times Cb / 227$

(Sükran *et al.*, 1998; Jefferey and Humphrey, 1975).

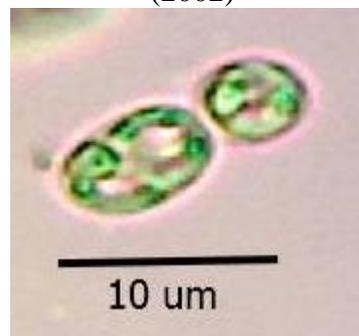
HPTLC was performed using Merck HPTLC plates silica gel 60 F 254. The solvent system used was a fresh mixture 25% acetone in hexane (Grung, D'Souza, Borowitzka and Liaaen- Jensen, 1992). The size of the plate used was 10 x 10 cm & the solvent front run was 8 cm. Sample application was performed using CAMAG Linomat IV and the results were observed and analyzed using CAMAG TLC Scanner "Scanner_211407" and winCATS Planar Chromatography Manager respectively.

Anti-Cancer Potential The extracted pigments of the red cells of *Coelastrrella oocystiformis* which consisted mainly of carotenoids was tested for its potential towards cancer cell growth inhibition using in-vitro assay at anti-cancer drug screening facility (ACDSF) at Advanced Centre for Treatment Research and Education in Cancer, Kharghar, Navi Mumbai, India. Human prostate cancer cell line DU-145 was used for the assay. Ethanol was used to dissolve the extracted pigments and was used as negative control and Adriamycin (ADR) drug was used as positive control.

Results and Discussion

DNA sequencing and Phylogenetic Tree analysis

Fig.1 *Coelastrrella oocystiformis* (J.W.G.Lund) Hegewald and Hanagata (2002)



Basionym: *Scotiella oocystiformis* J.W.G. Lund

Synonym: *Scotiellopsis oocystiformis* (J.W.G.Lund) Puncocharova and Kalina.

Description: Cells 4.5 µm wide, 7 µm long solitary lemons shaped to broadly ovoid, with pyrenoid; asexual reproduction by spindle-shaped or broadly oval autospores arranged three dimensionally in mother cell and released by fracture of mother cell wall.

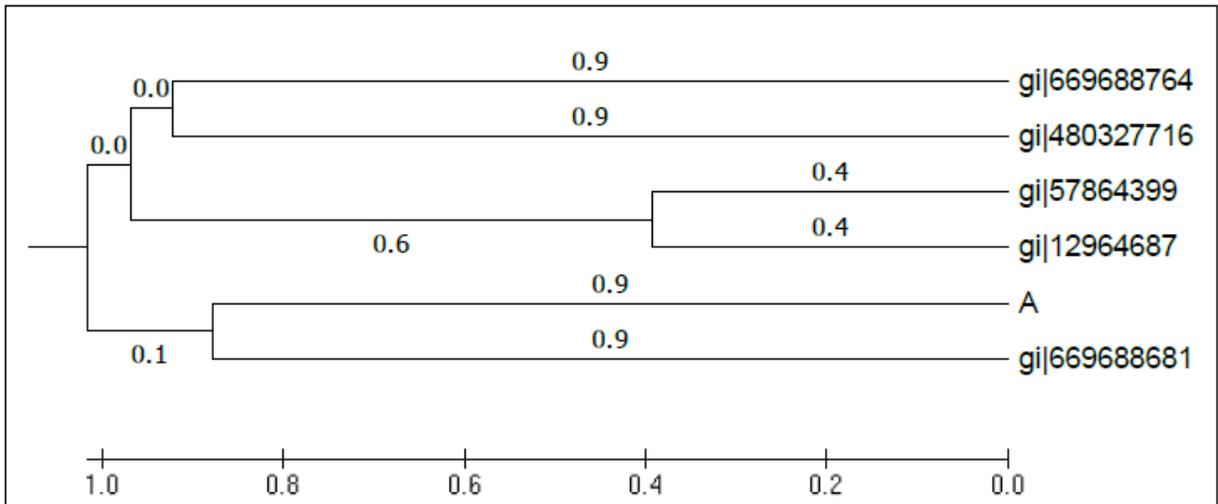
Fig.2 704 base pair 18s rDNA sequence of the amplified fragment using 18s F2 and 18s R2 primers

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1 TCCTTGACTG CGTCCGCATT TGCCAGGATG TTTTCATTAA TCAAGAACGA
AAGTTGGGGG
61 CTCGAAGACG ATTAGATACC GTCGTAGTCT CAACCATAAA CGATGCCGAC
TAGGGATTGG
121 CGAATGTTTT TTAATGACT TCGCCAGCAC CTTATGAGAA ATCAAAGTTT
TTGGGTTCGG
181GGGGGAGTAT GGTCGCAAGG CTGAAACTTA AAGGAATTGA CGGAAGGGCA
CCACCAGGCG
241 TGGAGCCTGC GGCTTAATTT GACTCAACAC GGGAAAACTT ACCAGGTCCA
GACATAGTGA
301 GGATTGACAG ATTGAGAGCT CTTTCTTGAT TCTATGGGTG GTGGTGCATG
GCCGTTCTTA
361 GTTGGTGGGT TGCCTTGTC A GGTGATTCC GGTAACGAAC GAGACCTCAG
CCTGCTAAAT
421 AGTCCTAGTT GCTTTTTGCA GCTAGCTGAC TTCTTAGAGG GACTATTGGC
GTTTAGTCAA
481 TGGAAGTATG AGGCAATAAC AGGTCTGTGA TGCCCTTAGA TGTTCGGGC
CGCACGCGCG
541 CTACACTGAT GCATTCAACA AGCCTATCCT TGACCGAAAG GTCCGGGTAA
TCTTTGAAAC
601 TGCATCGTGA TGGGGATAGA TTATTGCAAT TATTAGTCTT CAACGAGGAA
TGCCTAGTAC
661 GCACATAAAA AATCAGCTTG CGTTGATACG TCCCCTGCC CTAA
    
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The 18s rDNA sequence of the amplified fragment using 18s F2 and 18s R2 primers is provided in figure 2.

Fig.3 A Phylogenetic tree of randomly selected sequences from the results of BLAST were aligned using MEGA 6, ClustalW standard parameters. Phylogenetic tree was constructed using UPGMA in MEGA 6 where A= *Coelastrella oocystiformis*



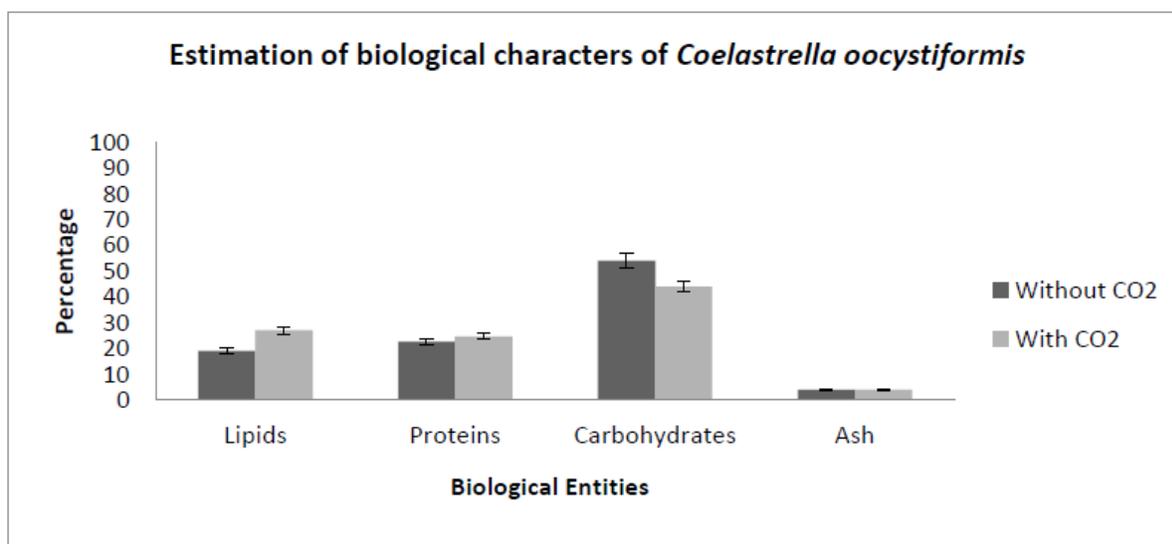
The Phylogenetic tree as observed in figure 3. *Coelastrella oocystiformis* being studied is labelled as “A” in figure 3. “A” is has maximum similarity to the sequence of >gi|669688681 and thus the two share a common ancestor. The closest sequences are >gi|57864399 & >gi|12964687 with a distance of 0.4 that separate them from a common ancestor. These organisms are further closely related to >gi|669688764 & >gi|480327716.

The common ancestors to all the above organisms is related to the common ancestor for “A” and >gi|669688681. NCBI-BLAST proved a 98% similarity to all the sequences. The overall tree helps us to understand the similarity of 18s rDNA sequence of *Coelastrella oocystiformis* “A” to other DNA sequences of *Coelastrella* in the database of NCBI and also confirms the identity of the organism being studied.

Effect of CO₂ on biomass generation and estimation of lipids, proteins, carbohydrates and ash content

The culture of *Coelastrella oocystiformis* has a doubling time of 27±0.5 h without CO₂ and 12±1.0 h with 3% CO₂. Dry biomass obtained on growth without supply of 3% carbon-di- oxide was 42.55 ± 2.0 mg/l/day and with 3% carbon-di-oxide was 89.6 ± 2.0 mg/l/day. The estimation of biological characters is provided in figure 4. Gas Chromatography Mass Spectroscopy was used to identify the fatty acid profile of this strain. The major fatty acids present in green cells were Undecanoic acid, Tricosanoic acid, Hexadecanoic acid, 9,12-Hexadecadienoic acid, 7,10,13-Hexadecatrienoic acid, 4,7,10,13 – Hexadecatetraenoic acid, 9-Octadecenoic acid, 9,12-Octadecadienoic acid, gamma-linolenic acid, 9,12,15- Octadecatrienoic acid, cis-6,9,12,15-Octadecatetraenoic acid.

Fig.4 Estimation of biological characters lipids, proteins, carbohydrates and ash. extraction, estimation and analysis of pigments



Extraction, Estimation and Analysis of Pigments

Chlorophylls and carotenoids are the major

pigments in Chlorophyta. One of the observations for this particular strain was its ability to produce high carotenoids under long time stationary phase with nutrient

depletion. This particular phenomenon may be similar to formation of aplanospores as observed in *Haematococcus pluvialis* by Grung *et al.* (1992). Table 1 represents the estimates obtained for Chlorophyll a, Chlorophyll b, total Chlorophyll, Total Carotenoids and the percentage of Chlorophyll and carotenoids present in the particular strain of *Coelastrrella oocystiformis*. *Euglena sanguinea* is one strain that is known for its high carotenoid production (Grung and Liaaen-Jensen, 1993) and the table 1 also compares the carotenoid contents of *Coelastrrella oocystiformis* and *Euglena sanguinea* Ehrenberg. Sae-Tan *et al.* (2007) reported *E. sanguinea* had an average carotenoid content of 0.5497 ± 0.0044 , 3.5906 ± 0.5147 and 4.0603 ± 1.0308 mg/g cell dry weight using the three methods. Garc'ia- Malea *et al.* (2005) have described *Euglena sanguinea* to have total carotenoid content of 1.5% of the dry weight. Thus the strain being studied here has a potential for high carotenoid production. The strain as observed under florescent microscope and light microscope

at high carotenoid produce are shown in figure 5. The images show green cells (in white light) with chlorophyll red florescence and reddish-orange cells (in white light) with less chlorophyll florescence. Apart from these two cells, the population also consisted of white or colourless cells (in white light) which fluoresced blue. Blue florescence of these cells may be due to presence of phytoene or phytofluene as Engelmann *et al.* (2011) and Erdođan *et al.* (2015) mentioned that phytoene and phytofluene act as precursors for carotenoids. This also shows that within a population of algal cells, few cells possess chlorophyll and continue their normal growth, whereas cells which had to protect themselves or form spores due to nutrient depletion will initiate the process of carotenoid formation. The carotenoid accumulation started when a highly dense culture of above 2.0 at 700 nm was kept at rest without any agitation or aeration for a period of 30–60 days. The maximum accumulation occurs being after 60th day.

Fig.5 *Coelastrrella oocystiformis* cell - a) blue florescence under florescence microscope using blue filter b) colourless under white. c&d) orange cells have faint red to orange florescence and the chlorophyll rich green cells have bright pink to red florescence under florescence microscope & white light

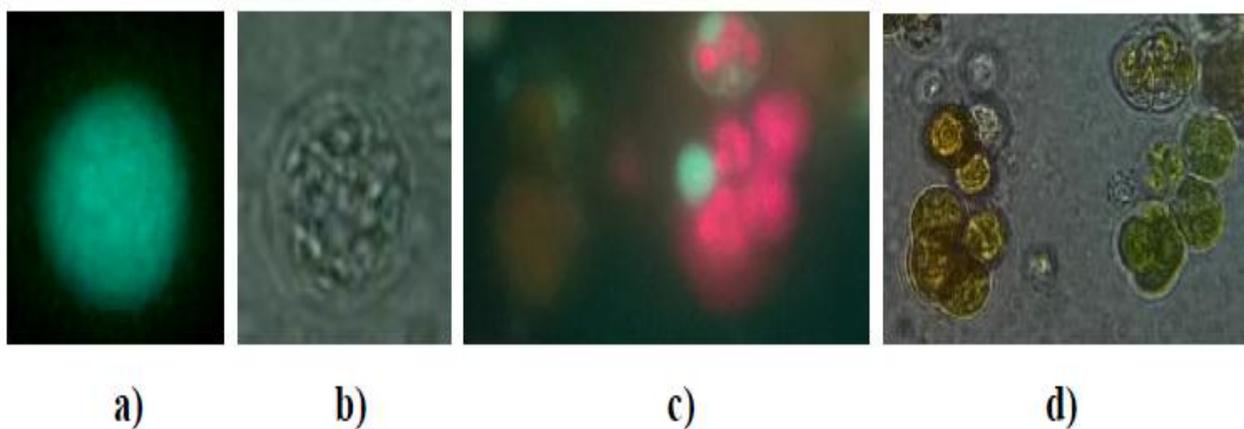
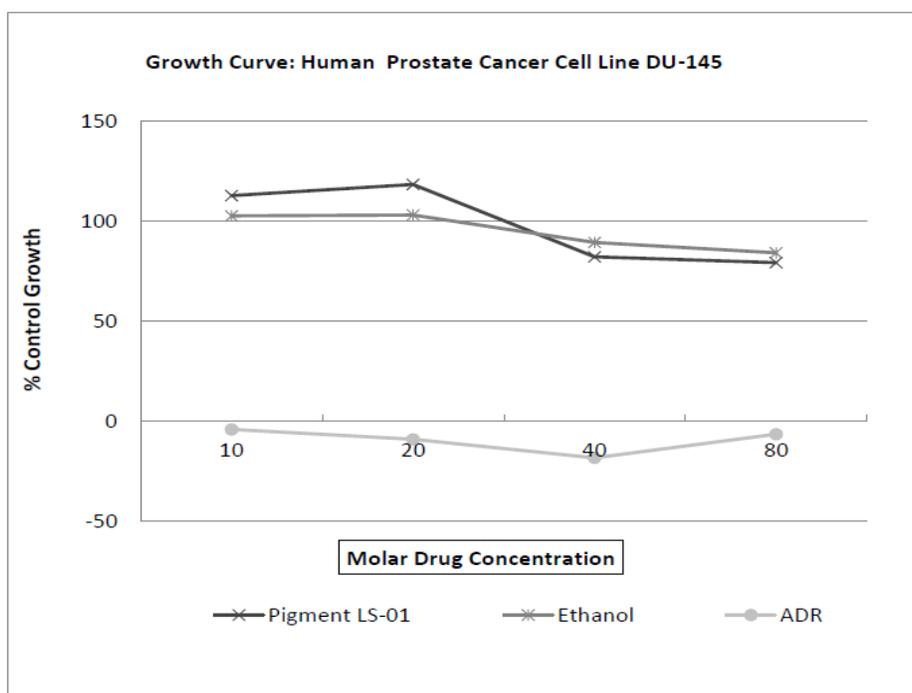


Table.1 Estimation of Pigments between green cells and red cells of *Coelastrella oocystiformis* and red cells of *Euglena sanguinea*

Contents	<i>Coelastrella oocystiformis</i>		<i>Euglena sanguinea</i>
	Red cells	Green cells	Red cells
Total Chlorophyll-a (µg / ml)	1.675	2.014	1.444
Total Chlorophyll-b (µg / ml)	0.49	1.018	0.404
Total Chlorophyll (µg / ml)	2.165	3.032	1.849
Chlorophyll (%)	0.216	0.303	0.184
Total Carotenoids (µg/g)	1972.05	129.62	1986.75
Carotenoids (%)	1.972	0.130	1.986

Fig.6 Molar drug concentration vs % control growth. Pigment LS-01 is the extracted pigment of *Coelastrella oocystiformis*



Microalgae *Chlorella vulgaris* contains 2.16% chlorophyll-a, 0.59% chlorophyll-b, and 0.385% carotenoids (Sharma *et al.*, 2012). It can have 3% of chlorophyll content in its dry biomass (Kong *et al.*, 2011).

Scenedesmus dimorphus had a growth rate of 558 mg/litre for which it contained 3 mg/litre of Chlorophyll and 1 mg/ litre of carotenoids (Velichkova *et al.*, 2013). *Scenedesmus protuberans* had 1.45 mg/g of

violaxanthin, 2.47 mg/g of all-trans-lutein, 0.15 mg/g of all-trans- α -carotene, 0.55 mg/g of all-trans- β -carotene, and 0.20 mg/g of 9 or 9'-cis- β -carotene (Erdoğan *et al.*, 2015). *Scenedesmus almeriensis* was studied by Sánchez, *et al.* (2008) where at increased salinity of media, increase in light radiance and increase in temperature at about 48°C the culture could accumulate carotenoids, where 0.53% of its dry biomass was lutein.

HPTLC of the extracted carotenoids helped in identification of compounds. The total quantity of carotenoid obtained was 1.972 μ g. The winCATS software enabled in measurement of area of each carotenoid under the band and this area to concentration ratio was used to calculate the concentration of each carotenoid. β -carotene could not be quantified as the Rf value matched with that of the solvent front, however it was present in the sample. The organism could produce 518 μ g/g of lutein (Rf-0.23), 120 μ g/g of free astaxanthin (Rf-0.36), 208 μ g/g of canthaxanthin (Rf-0.39) and 232 μ g/g of phytofluene (Rf-0.68). A total of 9 different bands were observed in the sample, which indicate presence of those many carotenoid compounds, however only 4 could be identified with help of standards.

Anti-Cancer Potential

The results of in-vitro tests are shown in figure 6. Ethanol was used as a negative control and at concentration of 40 μ g/ml 89% growth of DU-145 cells could be observed; whereas at 80 μ g/ml only 84% cell growth was observed. The extracted carotenoids dissolved in ethanol of *Coelastrella oocystiformis* showed 82% growth of DU-145 at a concentration 40 μ g/ml and 79% growth at a concentration 80 μ g/ml. The results thus suggest carotenoids were capable of 5% more cells than ethanol. 5% inhibition of cancer cell line DU-145 is

not a significant value with respect to the active drugs that are used presently, however the potential as an anti-cancer agent cannot be denied. None the less, unlike ADR the carotenoids are much less harmful to the normal cells during the treatment of cancer. It can be postulated that the carotenoids can be used as a adjunct so as to reduce the effective concentration of a cancer drug. This will cause less harm to the normal cells present besides the tumour growth.

In conclusion, the organism isolated is identified as *Coelastrella oocystiformis* using 18s rDNA sequencing. The growth rate under conditions where 3% CO₂ is supplied was determined to be twice of the growth rate when CO₂ was not provided. Also there was 40% increase in the lipid content when CO₂ was provided during growth. Under high carotenoid accumulation, the strain is capable of producing 2% carotenoids, which contain β -carotene, lutein, free astaxanthin, canthaxanthin and phytofluene. With a growth rate of approximately 90 mg/l/day, the strain is capable of producing 18 mg of lipids/l/day. Anti-cancer activity of the carotenoid fraction of this strain is minimal; however it reflects potential at higher concentrations. The study provides a detailed characterization of the strain *Coelastrella oocystiformis* and the results suggest it to be an important strain for algal industry.

Highlights

- 3% CO₂ per day increases algal biomass doubly.
- 2% Carotenoids producing algal strain *Coelastrella oocystiformis*
- Potential for development of anti-cancer agent
- HPTLC analysis of carotenoids

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