WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 7, 726-739. Research Article ISSN 2277 – 7105

TAPPING THE ANTIOXIDANT POTENTIAL OF A NOVEL ISOLATE - *CHLORELLA EMERSONII*

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Article Received on
13 July 2014,
Revised on 11 August 2014,
Accepted on 29 August 2014

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ABSTRACT

There is a quest for alternative and economical antioxidants, to be substituted for the chemical ones used in personal and health care sectors. These alternative sources are usually of plant or algal origin. With a known potential antioxidant activity of freshwater microalga, and the obvious advantage of scaling up in comparison to seaweeds/macroalgae, this study isolates and evaluates the antioxidant principles from a so far an unexplored species of *Chlorella*. **Materials and Methods:** *Chlorella* was enriched from a fresh water pond in Chu's medium No.10 at 28±3^{0}C and was identified as *Chlorella emersonii* morphologically as well as by 18s rDNA sequencing (NCBI accession number-KJ725233). *C. emersonii* was cultivated in BG-11 medium at different temperatures, so as to optimize its temperature for biomass production. The mass obtained was then dried and three different solvents: water, methanol and hexane were used to extract the antioxidants. The antioxidant activity and the total phenolic content were quantified by FRAP and Folin- Ciocalteau methods respectively. **Results**: Highest activity of 15.348±0.0908 and 17.975±0.436 mg ascorbic acid g^{-1}; 9.963±0.3667 and 18.23±0.525 mg ascorbic acid g^{-1} for aqueous and methanolic extract respectively. The antioxidant potential of the hexane fraction was found to be the lowest 0.257±0.024 and 0.380±0.10 mg ascorbic acid g^{-1}. **Conclusion:** The correlation coefficients for antioxidant potential and phenolic content of aqueous fractions was found to be R^2 = 0.9999 indicating significant contribution of the phenolic compounds to the antioxidant activity for *Chlorella emersonii* KJ725233.

Keywords: Microalgae, *Chlorella emersonii*KJ725233, Antioxidant activity, Phenolic content.

INTRODUCTION

Cells are continuously exposed to stress due to intrinsic as well as extrinsic factors. The effects of this stress, especially induced by reactive oxygen species (ROS) range from chronic diseases to cancer and ageing. To combat these, there is a wide interest in finding newer and safer antioxidants. Plant materials have been used as potential sources and replacement for chemical antioxidants, however, with levels of active principles fluctuating with seasons, coupled with presence of herbicides, heavy metals and pesticides in plant extracts, a need for an alternative reliable source of antioxidants is highlighted [1].

Photoautotrophic microorganisms like microalgae represent one of such untapped resource of antioxidants [2].

The absence of the photo oxidative damage in microalgae is indicative of the presence of protective mechanisms to combat the ROS and free radicals [3]. However, not all groups of microalgae can serve as potential sources due to the limitations in cultivations, variability in growth rate and induction of target products [4]. *Chlorella* strains are now regarded as competent candidates due to their highest chlorophyll content amongst all the microalgae [5] , fast growth rate and distribution all over the regions including the polar areas [6-9]. In addition to the ease of cultivation and a reservoir of bioactive metabolites like flavonoids, phenolics, carotenoids, terpenoids, glycosides and polysaccharides it is needless to say that this species finds its applications in health food, pharmaceuticals and cosmetic industries [10-11].

Chlorella is reported to have anticarcinogenic and skin lightening effect [12]. It is also reported to possess an ability to lower cholesterol, preventive against arteriosclerosis and antitumor activity [13]. 'Chlorella Growth Factor' a water soluble extract containing amino acids, vitamins, proteins, peptides, sugars and nucleic acids, is given as a product to increase the growth of lactic acid bacteria that are established probiotic agents [14].

Different species of this genus with varied temperature tolerance exist in freshwater, sea as well as soil [15-16]. One such being *Chlorella emersonii* also known as *Graesiella emersonii,* is an inhabitant of fresh water bodies [6]. The species is known for its high lipid content of upto 63% followed by *Chlorella minutissima* with 57%, *Chlorella vulgaris* 14-40/56 %, *Chlorella protothecoides*55% and *Chlorella sorokianiana* i.e 22% [17]. Due to the high lipid content, the alga appears to be a potential source of good quality biodiesel (high heating value and viscosity) [18].

Microalgal components also find wide applications in cosmetics as thickening agents, water binding agents and antioxidants. The microalgae have been represented as sun protectors in hair and skin care products possibily due to the presence of UV absorbing compounds like sporopollenin, scytonemin and mycosporine-like-amino acids. Microalgal pigments have also been used as natural colorants in food and cosmetics [19]. The carotenoid antioxidants like astaxanthin and β-carotene from *Haematococcus* and *Dunaliella* respectively have been used in food and feed industries [20].

The present work reports the growth characteristics of a novel isolate of *Chlorella emersonii* and taps its antioxidant potential by shake flasks and sonication techniques. We have also checked the solubility of the active components in aqueous as well as non-aqueous solvents like hexane, methanol and acetone. The antioxidant extracted in the solvents were determined using standard methods like Ferric reducing antioxidant potential and total phenol content expressed as tannic acid equivalent [21,22]. The work finds significance since it is one of the first reports on identification, isolation and quantification of antioxidant compounds from *Chlorella emersonii* (Accession No.KJ725233).

MATERIALS AND METHODS

Materials and methods: All the reagents and solvents were purchased from Sdfine, HIMEDIA Co (India). unless otherwise mentioned. All chemicals used were of analytical grade.

Isolation of *Chlorella emersonii* (KJ725233): A freshwater pond on the campus of the University of Mumbai, India served as a source of an algal consortia including *Chlorella emersonii* (KJ725233). 1 litre of this water sample was collected in a sterile glass jar and centrifuged at 10000 rpm for 5 minutes. 20 ml of this supernatant was then transferred to 250 ml of Chu's medium No.10 ($Ca(NO_3)$ 40 mgL^{-1}, $MgSO_4.7H_2O$ 25 mgL^{-1}, K_2HPO_4 5 mgL^{-1}, Na_2CO_3 20 mgL^{-1}, Na_2O_3Si 25 mgL^{-1} and $FeCl_3$ 8 mgL^{-1}) and incubated at 28 +/- 2 C for 15 days. The growth obtained was subcultured in the same media after every 7 days to enrich *Chlorella* species. The flasks were kept under sunlight with a 12 hour photoperiod and 12 hours of aeration. The green coccoid microalga *Chlorella* obtained by enrichment was taken up for identification by morphological as well as molecular methodsusing18s rDNA sequencing.

DNA isolation for confirmation of *Chlorella* species: Algal genomic DNA was isolated using geneO-spin Microbial DNA isolation kit (geneOmbio technologies, Pune; India). The 18S rDNA was amplified using following 18S typing primers (S Rattana et al., 2012).

forward sequence 5' – CTGCGAATGGCTCATTAAATC – 3'
reverse sequence 5' – AAGGCCAGGGACGTAATCAA – 3'.
Amplification was carried out using a GeneAmp PCR System (Applied Biosystems, USA) with the following PCR conditions : 95 °C, 10 minute (initial denaturation) ; 94 °C, 1 minute (denaturation); 60°C, 1 minute (annealing); 72 °C, 1 minute (elongation); steps from denaturation to elongation were repeated for 30 cycles; 72 °C, 10 minutes (final elongation) ; and 4 °C hold.

The products were purified using a geneO-spin PCR product Purification kit (geneOmbio technologies, Pune; India), visualized using Gel Doc XR documentation system (Bio-Rad) and sequenced using an ABI PRISM Big Dye Terminator V3.1 kit (Applied Biosystems, USA).End sequences were trimmed and only high quality sequence of length greater than 1000 bp was generated using sequence assembly tool available in Chromas Pro software version 1.34. The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at BlastN site at NCBI server.

Mass cultivation of *Chlorella emersonii* (KJ725233): In order to obtain sufficient algal biomass, the microalga was grown in 10 litres of the BG-11 medium (Stock solution A - $NaNO_3$ 1.5 gm L^{-1}, K_2HPO_4 40 mg L^{-1}, $MgSO_4$. $7H_2O$ 75 mg L^{-1}, $CaCl_2.2H_2O$36 mg L^{-1}, $C_6H_8O_7$ 6 mg L^{-1}, $C_6H_8FeNO_7$ 6 mg L^{-1}, Na_2EDTA 1 mg L^{-1}, Na_2CO_3 20 mg L^{-1}; for a litre of the media to the stock solution A add 1ml of Trace Metal Solution comprising of H_3BO_3 2.86 g L^{-1}, $MnCl_2$. $4H_2O$ 1.81 g L^{-1}, $ZnSO_4.7H_2O$ 0.22 g L^{-1}, Na_2MoO_4. $2H_2O$ 0.39 g L^{-1}, $CuSO_4.5H_2O$ 0.079 g L^{-1}, $Co(NO_3)_2.6H_2O$ 0.05 g L^{-1} was added) at 38^0 C [24,25].

The microalgae was grown at three different temperatures (28, 33 and 38^0C) to assess the optimal temperature for biomass production. After 48 hours of incubation at the respective temperatures with a 12 hour photoperiod and12 hours aeration, two ml of the sample was drawn from the tank and the growth monitored by cell count using lugol's iodine [26] in addition to assessing the cell density at 684 nm. Both the Chu's media No.10 and BG-11 were prepared in dechlorinated water.

Preparation of *Chlorella* extracts: After determining the growth optima of the culture, the microalga was centrifuged at 5000 rpm for 20 minutes to harvest the biomass. The biomass of microalga obtained was then kept at 50^0 C till a dry powder was obtained. This was subsequently suspended in solvents such as water, hexane and methanol at a concentration of 0.1 gm/ml. For extraction of bioactives, cell suspensions were sonicated (Digital ultrasonic cleaner LMUC series- LABMAN, India) for 15 and 40minutes in ice. 10% of the cell suspensions in the respective solventswere also put on shaker (NEOLABS, India) at 200rpm from 20minutes [27] as well as 48hours [28,29]. These extracts were then centrifuged at 5000 rpm for 10 minutes at 4^0C and the supernatants used for further studies.

Antioxidant capacity assays: The antioxidant potential of *chlorella emersonii* (KJ725233)was determined by Ferric reducing antioxidant potential (FRAP) as well as Total phenolic content (TPC).

Ferric reducing antioxidant potential: Reducing power was determined by following the method as described by Anantharaman P et al.,2013 [2] modified to suit analysis using 96-well microtitre plate. The absorbance was read at 700 nm in Tecan 200. For this, to 125 microlitres of 1% potassium ferricyanide, 50 microlitres of the extract was addedand incubated at 50^0 C for 20 minutes. After incubation, 125 microlitres of 10% Trichloroacetic acid was added to the reaction.100 microlitres of this reaction mixture was transferred to fresh wells with 100 microlitres of distilled water and 20 microlitres of 0.1% ferric chloride. Ferric reducing antioxidant potential was expressed as ascorbic acid equivalents.

Determination of total phenol content: The total phenol content was determined by modifying the method as described by Wu et al., 2005 [30] using Folin- Ciocalteau reagent and 0.1mg/ml tannic acid as a standard. Absorbance was measured at 760 nm in Tecan 200. For this, to 50 microlitres of the extract, 25 microlitres of the 50% Folin-Ciocalteau reagent was added and allowed to stand for 5 minutes at room temperature after which 125 ul of 2% sodium carbonate was added. The reaction was incubated in the dark at room temperature for 40 minutes. Distilled water was used as a blank. The total phenol content was expressed as tannic acid equivalents.

Statistical analysis : All experiments for estimation of antioxidant potential were performed in triplicates and the data are reported as mean +/- standard deviation.

RESULTS

Enrichment and identification of chlorella

The lake water was found to be rich in algal species belonging to the genus *chlorella, spirulina (arthrospira), scenedesmus, oedogonium, volvoxetc* as identified by their morphology (Fig 1a, 1b). However with the use of Chu's medium No.10, 12 hour aeration, 12 hour photoperiod, and incubation temperature of 28 +/- 3^0 C the chlorella population outgrew the rest of the algae (Fig 1c). The pure culture of chlorella obtained was then maintained in BG-11 media and characterized for confirmation of its identification upto species level.

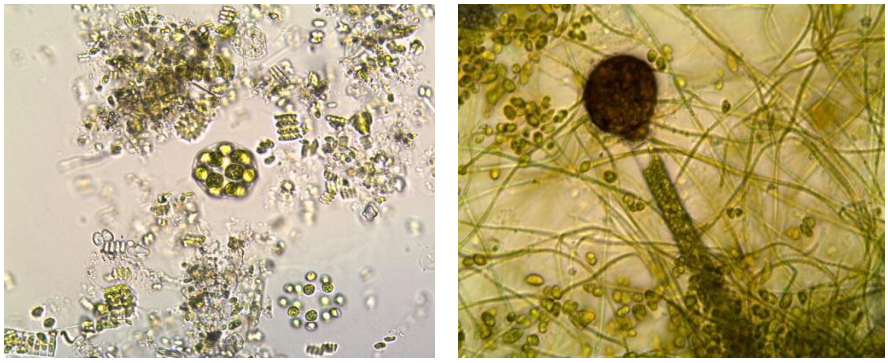

Figure 1 a. Mixed culture from pond water (400x)

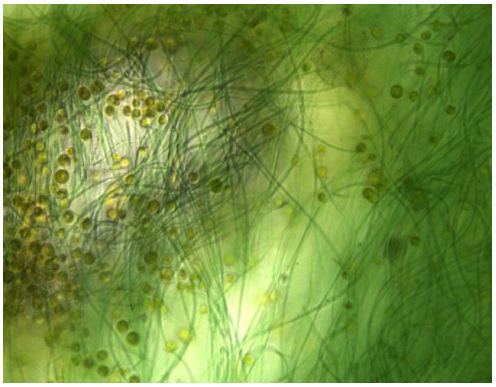

Figure 1 b. First subculture : *Chlorella – Spirulina* (400x)

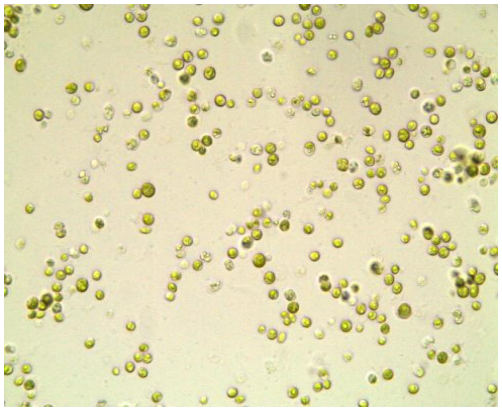

Figure 1 c. *Chlorella emersonii* KJ725233- Pure culture(400x)

When grown at temperatures from 28, 33 to 38^{0}C, the isolate was found to have a growth optima at 38^{0}C producing the highest biomass as compared to the other incubation temperatures (Table 1).

Table 1 Biomass yield of *Chlorella emersonii* KJ725233 after incubation for 10 days at the different temperatures.

Temperature (^{0}C)	Dried Biomass g/litre
28	0.35
33	0.25
38	1.09

Staining with lugol's iodine for morphological identification revealed characteristic features of *Chlorella* species as reported in literature [31] (Fig.1c).The amplification (Fig.2) and sequencing of the 18s rDNA confirmed the identity of the alga as *Chlorella emersonii* KJ725233.

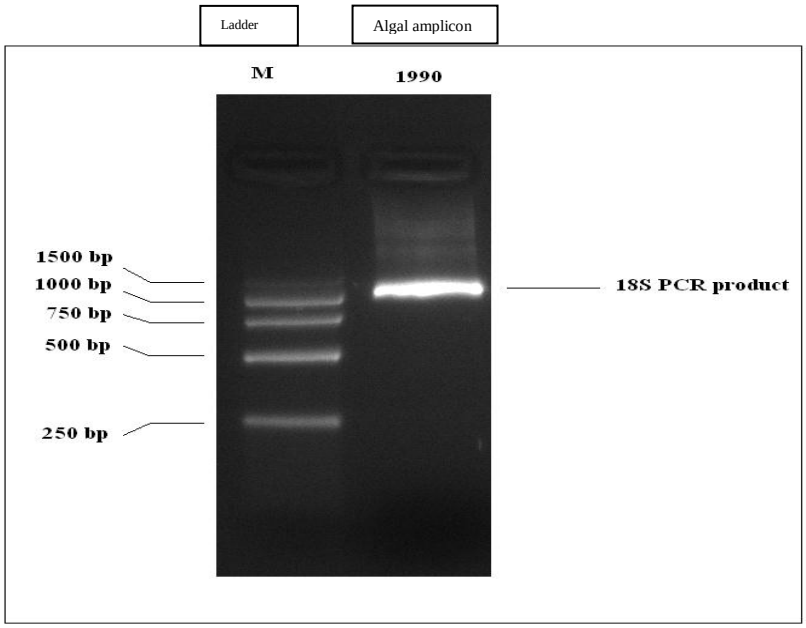

Figure 2. PCR amplicon on 2% (w/v) agarose gel with DNA standard ladder and 18s rDNA gene amplicon.

Table 2 Sequence of the 18s PCR product – *Chlorella emersonii* KJ725233

ATTCTAGAGCTAATACGTGCGTAAATCCCGACTTTTGGAAGGGACGTATATATTA
GATAAAAGGCCGACCGGGCTTTGCCCGACCCGCGGTGAATCATGATATCTTCACG
AAGCGCATGGCCTTGCGCCGGCGCTGTTCCATTCAAATTTCTGCCCTATCAACTTT
CGATGGTAGGATAGAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGT
TCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCA
GGCGCGCAAAATTACCCAATCCTGATACGGGGAGGTAGTGACAATAAATAACAA
TACCGGGCATTTAATGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACG
AGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA

ATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGGTGG
GTTCTAGCGGTCCGCCTATGGTGAGTACTGCTATGGCCTTCCTTTCTGTCGGGGAC
GGGCTTCTGGGCTTAATTGTCCGGGACTCGGAGTCGACGTGGTTACTTTGAGTAA
ATTAGAGTGTTCAAAGCAGGCTTACGCCCTGAATACTTTAGCATGGAATAACACG
ATAGGACTCTGGGCCTATCTTGTTGGTCTGTAGGACTGGAGTAATGATTTAAGAG
GGACAGTCGGGGGCATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATG
AAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTTCATTAATCAAGAAC
GAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCGTAGTTTCAACCATAAACG
ATGCCGACTAGGGATTGGCGAATGTTTTTTTAATGACTTCGCCAGCACCTTATGA
GAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAA
AGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTC
AA

Antioxidant assays: Two standard methods (FRAP and TPC) were used to assay the antioxidant potential of the isolate. The FRAP assay showed a higher antioxidant activity in the aqueous extract in comparison to methanol and hexane. The antioxidant activity as well as the phenol content was calculated and reported in terms of mg of the standard compound per gram of dried algal biomass.

Antioxidant extraction with sonication and shake flask methods: For shake flask methods wherein the biomass was shaken with the respective solvents for 20 minutes and 48 hours resulted in almost comparable yield (+/- 0.25-1.0) of the actives in case of methanol as well as water, however the yield dropped when hexane was used as a solvent (Fig 3).

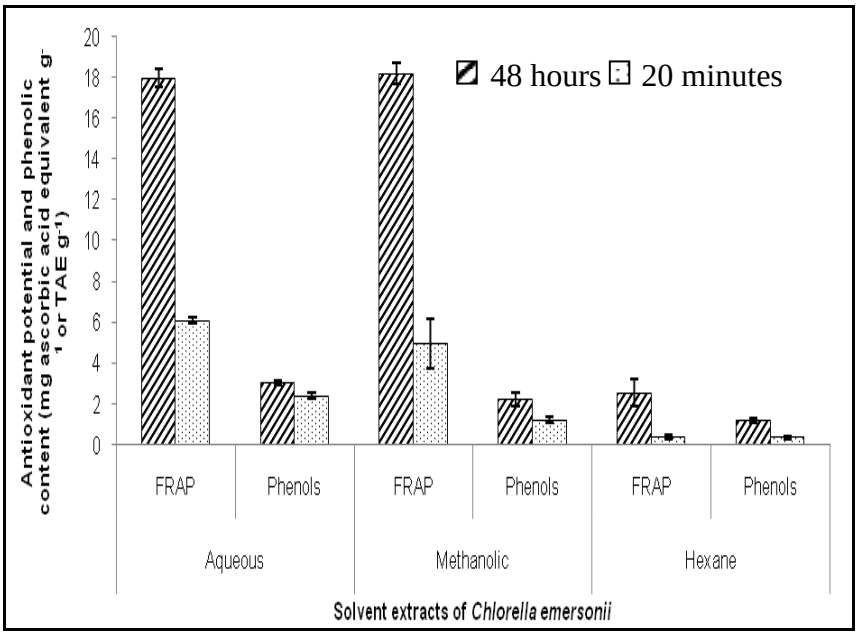

Figure 3. Antioxidant potential and phenolic content of different extracts of *Chlorella emersonii* KJ725233 after ◪48 hours and ⊡20 minutes of shaking. Bars indicate mean ± SD (n=3).

Table 3 Correlation of the FRAP and phenols of different extracts of *Chlorella emersonii* after 48 hours and 20 minutes of shaking. FRAP and phenols expressed as mg ascorbic acid and tannic acid equivalent respectively g^{-1} dried algal biomass.

	48 hours			20 minutes		
	FRAP	**Phenols**	**Correlation R^2**	**FRAP**	**Phenols**	**Correlation R^2**
Aqueous	17.975±0.436	3.062 ±0.115	0.9852	6.086±0.160	2.401±0.168	0.9935
Methanol	18.23 ± 0.525	2.203±0.321	0.9039	4.956±1.229	1.214±0.159	0.9107
Hexane	2.2 ± 0.661	1.18±0.126	0.9827	0.380±0.10	0.374±0.076	0.7377

Extraction of antioxidants by was also carried out for 20 and 40 minutes sonication. Higher antioxidant activity as well as total phenol content was observed for all the solvents sonicated, with the cells for 40 minutes as compared to 20 minutes sonication. Hexane however proved to be a poor solvent, except for phenols, irrespective of the time of sonication.

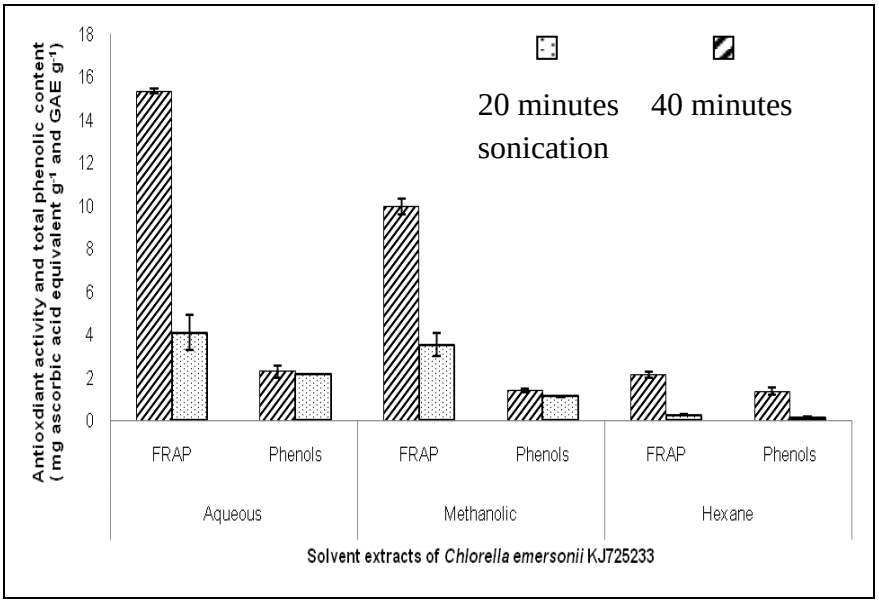

Figure 4. Antioxidant capacity and phenolic content of different solvent extracts of *Chlorella emersonii* KJ725233 after ⊡ 20 minutes and ◪ 40 minutes of sonicating the cells. Bars indicate mean ± SD (n=3).

Table 4 Correlation of the FRAP and phenols of different extracts of *Chlorella emersonii* after 40 minutes and 20 minutes of sonication. FRAP and phenols expressed as mg ascorbic acid and tannic acid equivalent respectively g^{-1} dried algal biomass.

	40 minutes			20 minutes		
	FRAP	**Phenols**	**Correlation R^2**	**FRAP**	**Phenols**	**Correlation R^2**
Aqueous	15.348±0.0908	2.288±0.303	0.8971	4.103±0.812	2.160±0.0001	0.9999
Methanol	9.963±0.3667	1.405±0.065	0.8319	3.538±0.518	1.123±0.012	0.9921
Hexane	2.144±0.143	1.364±0.157	0.3859	0.257±0.024	0.138±0.025	0.7222

40 minutes sonication resulted in higher antioxidant activity with water followed by methanol and then hexane. When sonicated for 40 minutes the antioxidant extraction was found to be 15.348 mg ascorbic acid equivalent g^{-1} in case of water, followed by the methanolic extract i.e 9.963 mg ascorbic acid equivalent g^{-1}. Minimal extraction was found in hexane with 2.144 mg ascorbic acid equivalent g^{-1}. The total phenol content in methanol as well as water ranged from 1.4 -2.3 mg/g however in case of hexane of the total antioxidant activity, 1.364 mg/g could be attributed to its phenolic content.

DISCUSSIONS

The study reported here is one such similar effort to isolate, enrich and tap the antioxidant potential of one of the *Chlorella* strains found in the western region of India. The samples collected from a freshwater lake on the campus of the University of Mumbai, Maharashtra India were studied for algal diversity and subsequently enriched so as to obtain a strain of *Chlorella* native to this habitat. Of the five different medias (data not represented) used to enrich the microalgal population, Chu's medium no.10 was found to selectively enrich *Chlorella* species at 28 +/- 3 C. The species identification carried out by morphological as well as molecular tools indicated the presence of a novel strain of *Chlorella emersonii* now catalogued under the NCBI accession no.KJ725233.

The strain was found to grow at temperature ranging from 25-42^0 C, however the growth optimization studies revealed maximum biomass at 38^0 C. In order to tap its total antioxidant potential, FRAP (Ferric reducing Antioxidant potential) assay was carried out. FRAP value is based on reduction of the ferric ion by the antioxidants, as they are capable of donating a single electron or hydrogen atom for reduction. Higher FRAP values thus, relate to higher antioxidant capacity [32]. Also in order to determine the contribution of phenolic compounds to the antioxidant potential total phenolic content was also determined. It is well known that the induction and yield of the active components depends on several factors like growth conditions and duration and irradiation intensity [33] while the chemical extraction of these components varies with the polarities of the solvents used, extraction time as well as the chemical composition of the sample [34] Our results for solvent optimization corroborate well with Choochote W et al 2014 [35] who reported higher antioxidant activity of *Chlorella* species in hot water as compared to 70% ethanol.

To study the extracellular as well as freely soluble antioxidant components the *Chlorella* biomass was subjected to shaking with the solvents for extraction. The antioxidant potential

of the aqueous as well as methanolic fraction was found to outperform that of hexane fraction indicating presence of polar compounds serving as potential antioxidants. Since cell sonication methods are known to release intracellular as well as membrane bound active components the isolates were subjected to a maximum sonication maximum time of 40 minutes. With an increase in sonication time from 20 to 40 minutes the antioxidant yield was also found to increase three to four times in methanolic as well as aqueous fractions. The solubility of the antioxidant compounds in hexane was evidently lower irrespective of the time of sonication.

CONCLUSIONS

Chlorella is a marine as well as fresh water microalga which exhibits highest photosynthetic activity, is enabled to grow at varied temperature range from 25 to 40^0C. So far chlorella has been identified as a good option for biodiesel production. This microalga is known to be an oil producing genus found in various environment including soil, humid rock, fresh water and oceans. Though most "true chlorella species" grow at temperatures 26-32^0C, heat resistant species *C. sorokiniana* is reported to exist with an upper limit of growth at 32-48^0C while Antarctica *chlorella* strains are known to grow at temperatures 5-30^0C [6]. *Chlorella* species has a higher light energy utilization rate (10-20%) for photosynthesis as compared to common plants [36,37]. According to Belasco (1997) [38] and Zelitch (1971) [39] *Chlorella* genus contains a number of actives with a good capacity for scavenging radicals as well as reducing blood lipids. Thus it is not surprising that over the 20^{th} century numerous studies focused on developing cultivation methods for the production of this microalga [40].

The study thus opens up a possible potential use of an easy growing comparatively sturdy strain of *Chlorella* for induction, isolation and application of water soluble antioxidants as a substitute to the chemical antioxidants used in food, cosmetics and pharma industry.

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